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# From the Desk of Editor-in-Chief

On behalf of the Editorial board, it is my great pleasure to herald the release of a special issue of the journal- International Journal of Pharma and Bio Sciences (SCOPUS Indexed, ISSN: 0975-6299). Recently we have successfully organised an International Conference on Recent Advances in Biotechnology and Nanobiotechnology (Int-BIONANO-2016) at Amity University Madhya Pradesh, Gwalior, India. Papers presented during the conference were selected for the full length publication as proceedings in the special issue of the journal. There are 22 papers in this special issue, which were selected from 150 abstracts. These papers have undergone a critical review process by the experts from various fields.

The main advantage of conference is associated with the ready availability of information, the immediate possibility of opening a critical discussion and establishing networking between individuals working in the same areas. The applications of biotechnology have tremendous impacts on human welfare and society. The areas including Plant biotechnology, Bioremediation, Tissue culture, Stem cell therapy, Microbial Technology, Omics Technology, Pharmaceutical Technology etc. have a wide variety of applications. Biotechnology and Nanotechnology are two of the 21$^{st}$ century's most promising technologies. Biotechnology deals with metabolic and other physiological processes of biological subjects including microorganisms. Nanotechnology deals with developing materials, devices, or other structures possessing at least one dimension sized from 1 to 100 nanometers. Association of these two technologies, i.e. *Nanobiotechnology* can play a vital role in developing and implementing many useful tools in the study of life. The multidisciplinary field of nanobiotechnology is bringing the science of the almost incomprehensibly small device closer and closer to reality. The effects of these developments will at some point be so vast that they will probably affect virtually all fields of science and technology. Nanobiotechnology offers a wide range

of uses in medicine and surgery. Innovations such as drug delivery systems are only the beginnings of the start of something new. Many diseases that do not have cures today may be cured by nanotechnology in the future.

With the above discussion in mind, the papers are broadly divided into those describing applications of biotechnology and nanotechnology in biological sense. This Special Issue presents range of applications for biotechnology and nanobiotechnology for human health and environment. It is hoped that the reader will gain, from a reading of these papers a better appreciation and recent advances in the area.

Many individuals have contributed a great deal of time and energy towards the success of the Int-BIONANO-2016 and this special issue. We would like to thank the authors of all submitted papers, the members of the Organising Committee, as well as the International Advisory Committee for their many hours of hard work. We would like to extend our sincerely thanks to the journal- International Journal of Pharma and Bio Sciences for covering and publishing our conferences proceedings in the form of a special issue.

**Prof. (Dr.) Rajesh Singh Tomar**
Director, Amity Institute of Biotechnology &
Dean (Academics), Amity University Madhya Pradesh, Gwalior

# Index

# Synthesis, characterization of Mg $(OH)_2$ nanoparticles and its effect on photosynthetic efficiency in two cultivars of *Brassica juncea* germinated under cadmium toxicity

**ALFIYA BOHRA*, DHEERA SANADHYA, AKASH SHUKLA**

School of Life Sciences, Jaipur National University, Jaipur, Rajasthan, India

*Corresponding author email**:** alefiya7bohra@gmail.com

## ABSTRACT

The present work is focused on synthesis and characterization of Magnesium Hydroxide Nanoparticles ($Mg(OH)_2$ NPs) to study its effects on two *Brassica juncea* cultivars stressed with different toxicity levels of Cadmium (Cd). $Mg(OH)_2$ NPs were synthesized by sol gel method and characterized using X-Ray Diffraction (XRD) and Field Emission Scanning Electron Microscope (FESEM)) techniques. In present investigation the positive effects of $Mg(OH)_2$ NPs were observed in protecting *Brassica juncea* from cadmium toxicity by enhancing the chlorophyll and carotenoids content as well as increasing root-shoot length, seedling size, germination percentage and biomass accumulation . Seeds of *Brassica juncea* were primed with $Mg(OH)_2$ NPs suspensions for four hours. Then seeds were dried back to their original weight and germinated in glass petri dishes using four levels of cadmium stress (0.5mM, 1 mM, 1.5 mM, and 2 mM). Non- primed seeds were drastically influenced by Cd toxicity and showed decrease in photosynthetic parameters as well as seed and seedling parameters in both the cultivars, but reduction was maximum in RH-0406 as compared to RB-50. While, seed primed with $Mg(OH)_2$ NPs suspension (10ppm) ameliorate the Cd toxicity, by increasing above mentioned parameters in two varieties of *Brassica juncea*. The research work demonstrates the positive effect of Nanoparticle treatment for Cd toxicity.

## KEYWORDS

Magnesium Hydroxide Nanoparticles, Characterization, FESEM, XRD, Cadmium, *Brassica juncea*, Chlorophyll, Carotenoids.

## INTRODUCTION

Cadmium (Cd) being highly toxic element enters into agricultural soils from various sources such as mining, industrial and farming practices and has been ranked number seven among top twenty toxins.[1] Assimilation of Cd in plants induces several symptoms of phytotoxicity such as delayed germination, decrease in root-shoot length, reduction in biomass, chlorosis, affects nutrient uptake, alteration in the chloroplast structure, reduce photosynthesis which finally results in the growth retardation and decreased in yield of crop plants.

Photosynthesis is highly sensitive to Cd because it directly affects chlorophyll biosynthesis and cause improper development of chloroplast ultrastructure.[2,3] It has been reported that $Cd^{+2}$ ions negatively affects the Photosystem II by replacing $Mn^{+2}$ ions, thereby inhibiting the reactions of Photosystem II and cause uncoupling of electron transport in the chloroplast.[4,5] The main targets of $Cd^{+2}$ ions are the two key enzymes of $CO_2$ fixation i.e. RuBPC (ribulose1,5-biphosphate carboxylase) and PEPC (phosphoenol pyruvate carboxylase). It has been reported that $Cd^{+2}$ ions damage the ultrastructure of RuBPC by replacing $Mg^{+2}$ ions and cause an irreversible dissociation of the small and large subunits of RuBPC thus causing total retardation in RuBPC activity which results in decrease in net photosyhthetic efficiency of plants.

Engineered Nanomaterials (ENs) have gained vast impact in agriculture over past few years. Due to their Nano size they have the potential to alter the physio-chemical properties and posses greater surface area than their bulk material. This characteristic enhances their solubility and surface reactivity.[6] Nanoagriculture utilizes ENs to improve the growth of plants and for control of plant diseases. Magnesium (Mg) is a divalent cation and an important component of chlorophyll molecule which plays vital role in photosynthesis in plants. Studies suggest protective role of $Mg^{+2}$ ions was primarily due to enhance photosynthesis rate and increased antioxidants status in its presence.[7] It has been reported that elevated concentration of Mg in media improves the nutritional quality of plants and this may be applied to plants growing in Cd polluted soils, if Mg can play a protective role in overcoming Cd phytotoxicity.[8]

*Brassica juncea* is an important oil seed crop cultivated mainly for mustard oil, which has got medicinal importance and residual part of seed, is used as cattle feed and fertilizer. Since the growth of *Brassica juncea* is adversely affected by Cd toxicity, the present study deals with the synthesis, characterization of $Mg(OH)_2$ NPs and their effects on germination and photosynthetic efficiency in Two cultivars (RB-50 and RH-0406) of *Brassica juncea* grown under Cd toxicity.[9]

## MATERIALS AND METHODS

### 1) Synthesis of $Mg(OH)_2$ NPs

$Mg(OH)_2$ NPs were synthesized using sol gel method. Equal concentration of Magnesium acetate and Potassium hydroxide were mixed in methanol separately and dissolved by constant stirring, when both the salts dissolved completely in methanol, both the solutions were mixed together to obtained a milky white solution. The chemicals used were of analytical reagent grade. Stirring was continued for 30 minutes on magnetic stirrer with heating at 60°C. The solution was kept undisturbed for 24 hrs to form the precipitate. Then the solution was centrifuged at 15000 rpm for 30 minutes. The precipitate so obtained was washed thrice with deionised water at 15000 rpm for 25 minutes to remove impurities. The sample was dried at 300°C for 9 hrs. The white powder so obtained was collected and characterized.

### 2) Characterization of $Mg(OH)_2$ NPs

The synthesized sample was characterized by using following techniques, such as XRD, FESEM, and EDX. X-Ray Diffraction analysis of synthesized sample was obtained by X-pert pro diffractometer having an X'celerator detector. The measurements were taken at 25°C and value of 2ɵ ranges from 20° to 80°. The scan rate was noted per 2 sec to determine the size of crystal. Surface morphology was studied using FESEM (Field Emission Scanning Electron Microscope) model (NOVA NANOSEM 450) operated at 18KV and

elemental analysis of sample was carried out using EDX (Energy Dispersive Analysis of X-Rays) model (Bruker X Flash 6130).

**3) Preparation of $Mg(OH)_2$ NPs suspension**

The Nano $Mg(OH)_2$ with concentration 10 ppm was suspended in deionised water by using ultrasonicator (115W, 60 kHz) for 60 minutes.

**4) Seeds**

Two Cultivars of *Brassica juncea* were procured from CCSHAU Hisar. Uniformly selected seeds were surface sterilized with 5% NaoCl for 5 minutes and then washed repeatedly for two to three times with distilled water to prevent fungal/bacterial contamination. Filter papers were also sterilized in autoclave to reduce any chances of microbial growth. The seeds were primed for 4 hrs using prepared nanoparticles suspension by keeping in orbital shaker at constant temperature. Heavy metal test solutions were prepared using four different concentrations of Cadmium ($Cd^{+2}$) viz. 0.5mM, 1mM, 1.5mM and 2 mM. The chemicals used were of analytical reagent grade. Distilled water was used as control. Seeds were germinated in glass Petri dishes of 15 cm diameter lined with filter paper circles moistened with control and various concentrations of Cadmium to impose four levels of stress. Nearly 10 seeds were sown in each Petri dish and incubated in growth chamber set at 25 ± 2°C for 7 days and each treatment was replicated thrice. Occurrence of Germination was considered when roots were 2mm long; Germination percentage was recorded in every 24h, till the end of experiment. The following parameters were analyzed for the study.

**5) Seed and seedling parameters**

i) Germination percentage

A mean of 10 seeds were taken and expressed as percentage.

$$G.P\ (\%) = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds}} \times 100$$

ii) Root length and Shoot length

The length of root and shoot of seedlings were recorded at $7^{th}$ day of germination. Mean values were calculated for both root and shoot length and values were expressed in cm.

iii) Seedling Height

Heights of seedling were recorded at $7^{th}$ day of germination. Mean values were calculated and expressed in cm.

iv) Fresh Biomass

The Fresh Weight was recorded from the $7^{th}$ day old seedling. The whole seedling was surface dried with the blotting paper and their fresh weights were recorded.

v) Dry Biomass

The same seedlings were used and dried in oven for 24 h at 80°C and weighed again. This represented the dry matter.

**6) Photosynthetic parameters**

i) Chlorophyll content

Chlorophyll content was estimated according to the method given by.[10]

For chlorophyll extraction, fresh leaves were washed, blotted dry and then homogenised in 80% acetone followed by centrifugation at 10000 rpm for 15 min. The absorbance of the supernatant was read at 647 nm and 664 nm against blank containing 80% acetone.

The amount of chlorophyll a, chlorophyll b and total chlorophyll (mg $g^{-1}$ FW) were calculated according to the following formulas:

$$\text{Chlorophyll a} = (13.19 \times A_{664\text{-}} — 2.57 \times A647)\frac{V}{1000 \times W}$$

$$\text{Chlorophyll b} = \left(22.10 \times A_{647} — 5.26 \times A_{664}\right)\frac{V}{1000 \times W}$$

$$\text{Total chlorophyll} = \left(7.93 \times A_{664} + 19.53 \times A_{647}\right)\frac{V}{1000 \times W}$$

Where,

A = absorbance

V = total final volume of the extract

W = fresh weight in gram of the tissue

ii) Carotenoids content

Carotenoid content was assayed according to the method given by.[11] For carotenoid extraction, fresh leaves (0.2 g) were washed, blotted dry and then homogenised in 14 ml of 80 % acetone followed by centrifugation at 10,000 rpm for 15 min. The absorbance of the supernatant was read at 470 nm, 647 nm and 664nm against blank containing 80 % acetone.

The amount of chlorophyll a, chlorophyll b and carotenoid (xanthophyll, carotene) (mg $g^{-1}$ FW) was calculated according to the following formulae:

$$\text{Chlorophyll a} = \left(12.25 \times A_{664} — 2.79 \times A_{647}\right)\frac{V}{1000 \times W}$$

$$\text{Chlorophyll b} = \left(21.5 \times A_{647} — 5.10 \times A_{664}\right)\frac{V}{1000 \times W}$$

$$\text{Carotenoids} = \frac{\left(1000A_{470}\text{-}1.82\ C_a\text{-}85.02\ C_b\right)}{198}\frac{V}{1000 \times W}$$

Where
A = absorbance
V = total final volume of the extract
W = fresh weight in gram of the tissue

## RESULTS

### A) X-Ray diffraction analysis

The crystalline structure of $Mg(OH)_2$ NPs were determined by XRD analysis. Fig 1 shows the XRD pattern of synthesized $Mg(OH)_2$ NPS. The peak and relative intensities obtained for the $Mg(OH)_2$ match with the literature values.[12] The presence of strong and sharp diffraction peaks located at the 2□ value of 18.105°, 38.445°, 51.255° and 58.935° corresponding to (001), (011), (012) and (003) planes respectively indicating the formation of $Mg(OH)_2$ NPs (Figure-1) .The average particle size of the nanomaterial was determined using the Scherer equation and it was found to be 23.00 nm.

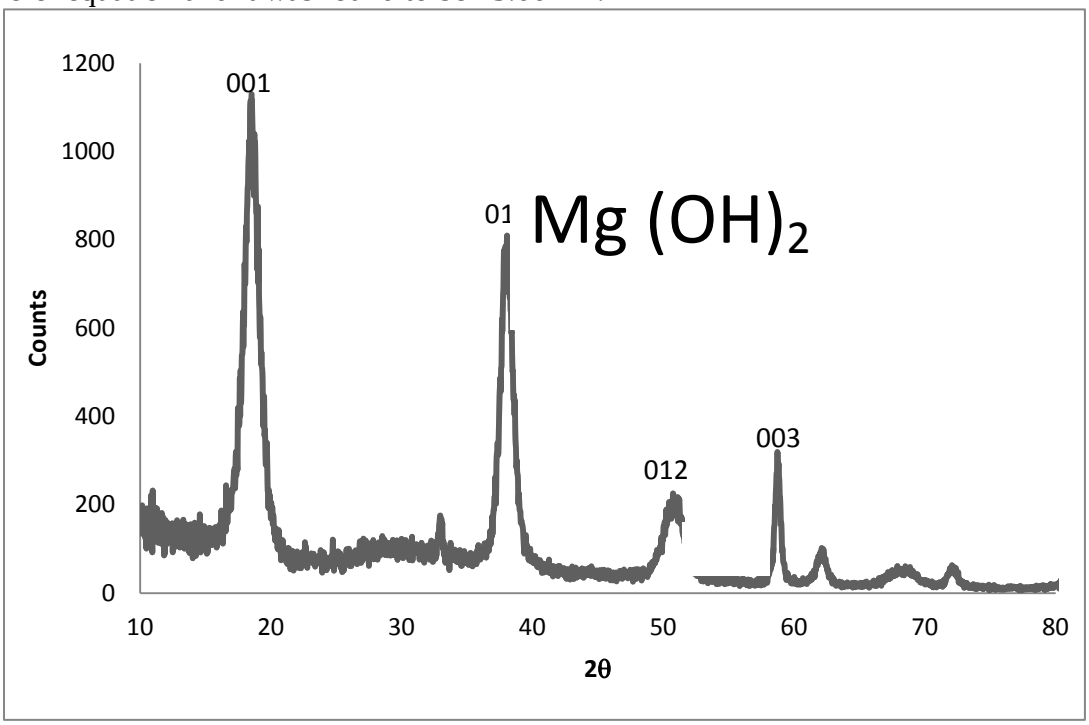


**Figure-1**. **XRD pattern of $Mg(OH)_2$ Nanoparticles**.

### B) FESEM Analysis

FESEM was used to determine the surface morphology and size distribution of $Mg(OH)_2$ NPs. FESEM images of $Mg(OH)_2$ NPs with different magnification are shown in Figure-2. The average particle size of $Mg(OH)_2$ NPs ranges from 23- 32 nm. It was observed that FESEM results are in good agreement with size distribution of $Mg(OH)_2$ NPs measured by XRD.

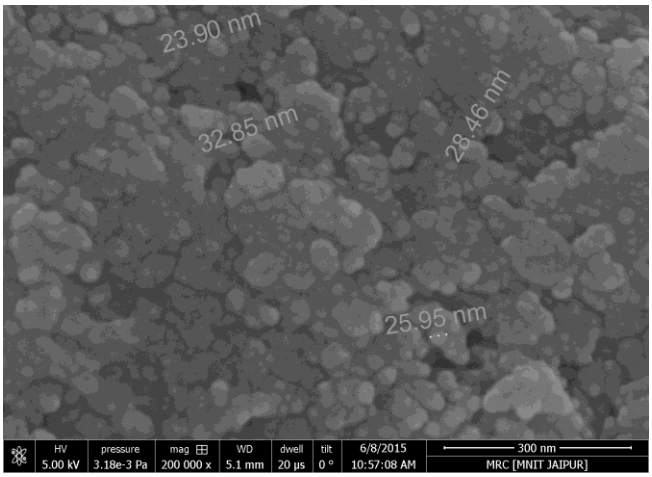


**Figure 2- FESEM images of $Mg(OH)_2$ Nanoparticles**.

**C) Effect of $Mg(OH)_2$ NPs on Root-Shoot length and Seedling size with response to Cd toxicity**

When two cultivars of *Brassica juncea* (RB-50 and RH-0406) were exposed to Cd toxicity at four stress levels (0.5mM, 1 mM, 1.5 mM, and 2mM), showed reduced root-shoot length and seedling size. Reduction in root-shoot length and seedling size increases with increasing Cd concentration. Between two cultivars RB-50 showed less reduction in root-shoot length and seedling size as compared to RH-0406, these two cultivars had been evaluated earlier by us[2] for their responses to Cd toxicity and it was observed that RB-50 was more tolerant than RH-0406. $Mg(OH)_2$ NPs (10ppm) primed seeds showed increase in root-shoot length and seedling size at all four Cd stress levels in both cultivars as compared to unprimed seeds.

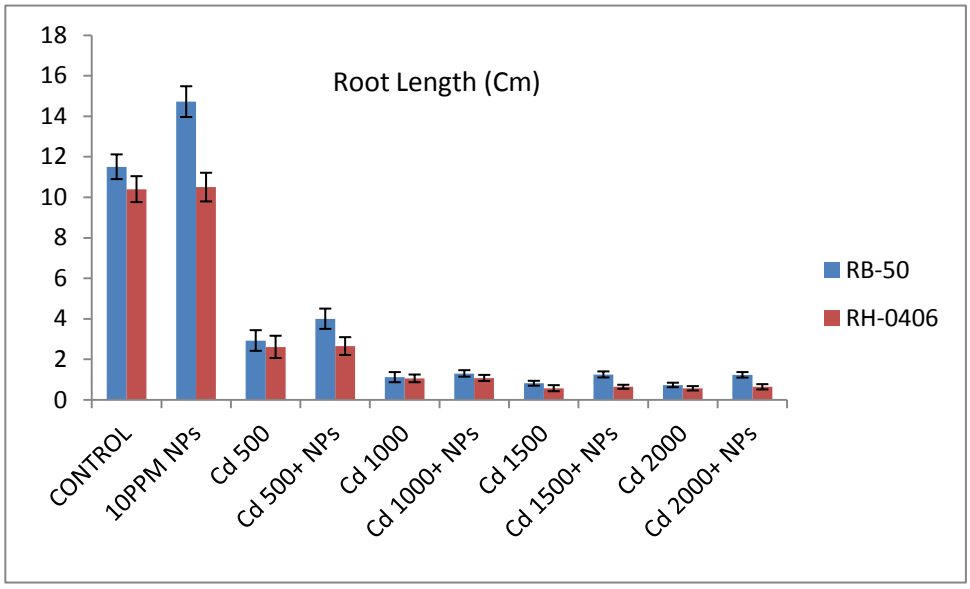


**Figure-3 Effect of different concentration of Cadmium on root length of $Mg(OH)_2$ Nanoparticles primed and unprimed seeds of two *Brassica juncea* cultivars. Data are the means of three replicates ± s.e.**

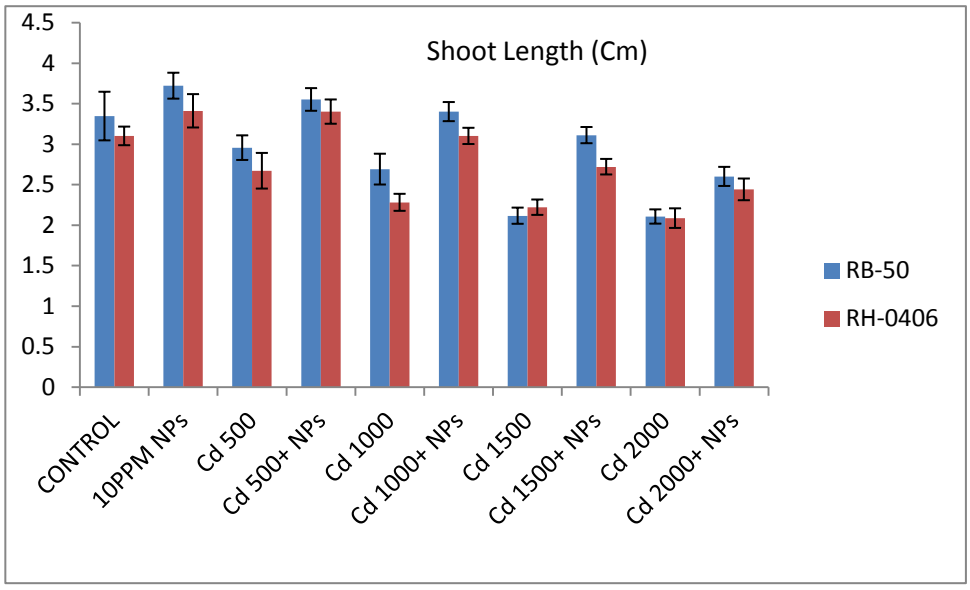


**Figure-4 Effect of different concentration of Cadmium on shoot length of $Mg(OH)_2$ Nanoparticles primed and unprimed seeds of two *Brassica juncea* Cultivars. Data are the means of three replicates ± s.e.**

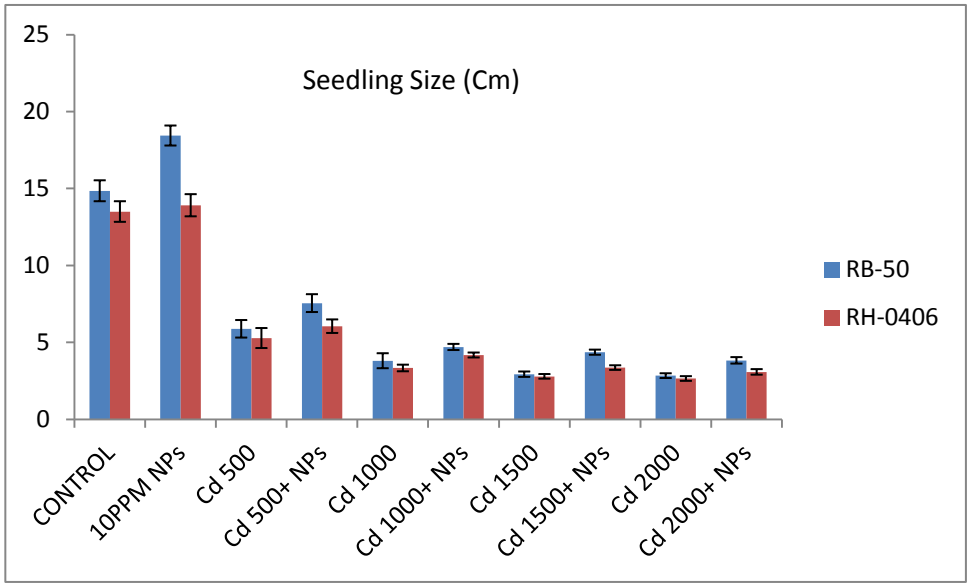


**Figure-5 Effect of different concentration of Cadmium on seedling size of $Mg(OH)_2$ Nanoparticles primed and unprimed seeds of two *Brassica juncea* Cultivars. Data are the means of three replicates ± s.e.**

D) Effect of $Mg(OH)_2$ NPs on Seed Germination Percentage

Cd treatment drastically reduced the seed germination percentage in both the cultivars at all the four stress levels. $Mg(OH)_2$ NPs (10ppm) primed seeds exhibits higher seed germination percentage as compared to unprimed seeds. Cultivar RB-50 showed higher germination percentage than Cultivar RH-0406. $Mg(OH)_2$ NPs primed seeds of RB-50 showed 100% germination, whereas control (unprimed seeds) showed 90% germination. $Mg(OH)_2$ NPs primed seeds of RB-50 at Cd concentration 0.5mM, 1 mM, 1.5 mM, and 2mM showed 86%, 84%, 80% and 80% germination respectively. Whereas unprimed seeds of RB-50 at Cd concentration 0.5mM, 1 mM, 1.5 mM, and 2mM showed 80%, 80%, 73% and 66% germination respectively.

$Mg(OH)_2$ NPs primed seeds of RH-0406 showed 96% germination, whereas control (unprimed seeds) showed 86% germination. $Mg(OH)_2$ NPs primed seeds of RH-0406 at Cd concentration 0.5mM, 1 mM, 1.5 mM, and 2mM showed 86%, 86%, 80% and 76% germination respectively. Whereas unprimed seeds of RH-0406 at Cd concentration 0.5mM, 1 mM, 1.5 mM, and 2mM showed 80%, 80%, 73% and 66% germination respectively.

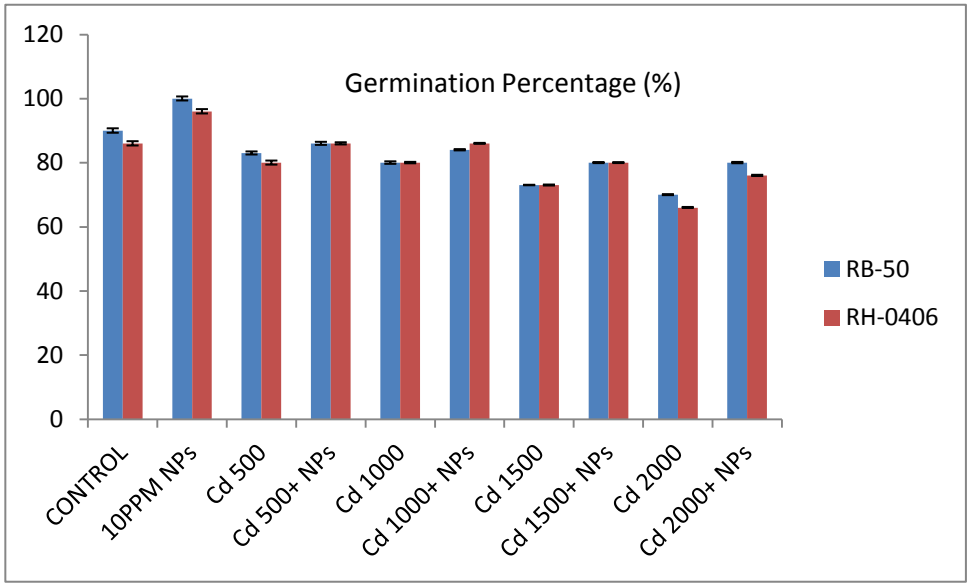


**Figure-6 Effect of different concentration of Cadmium on seed germination percentage of $Mg(OH)_2$ Nanoparticles primed and unprimed seeds of two *Brassica juncea* Cultivars. Data are the means of three replicates ± s.e.**

**E) Effect of $Mg(OH)_2$ NPs on Fresh and Dry Biomass accumulation**

Cd stressed seedlings showed reduction in wet and dry biomass as compared to control. Wet biomass was more affected than dry biomass at all four stress levels. Reduction in biomass accumulation was directly proportional to Cd concentration. Cultivar RB-50 showed less reduction in biomass as compared to Cultivar RH-0406. $Mg(OH)_2$ NPs primed seeds exhibit significantly higher biomass accumulation at all four Cd stress levels as compared to unprimed seeds in both the cultivars.

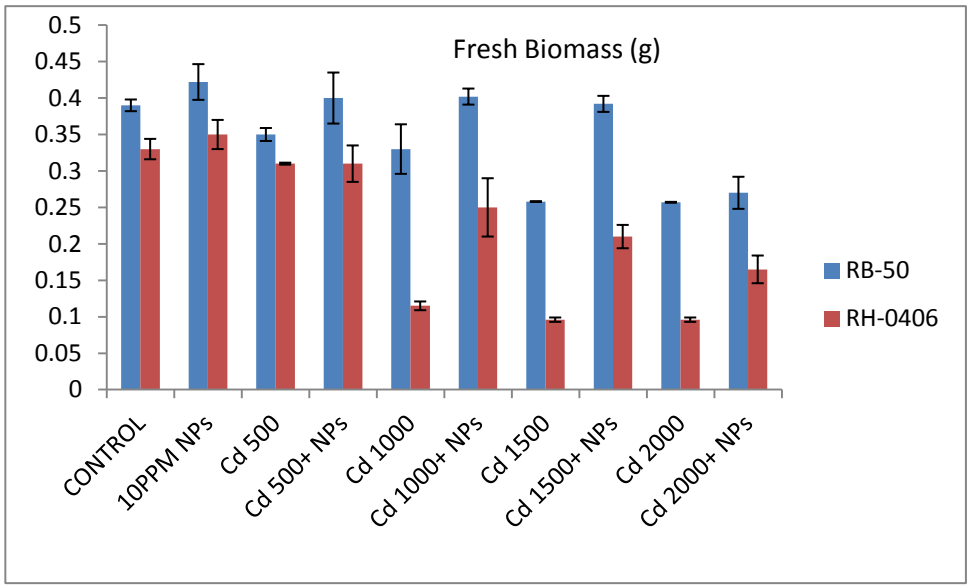


**Figure-7 Effect of different concentration of Cadmium on fresh biomass of $Mg(OH)_2$ Nanoparticles primed and unprimed seeds of two *Brassica juncea* Cultivars. Data are the means of three replicates ± s.e.**

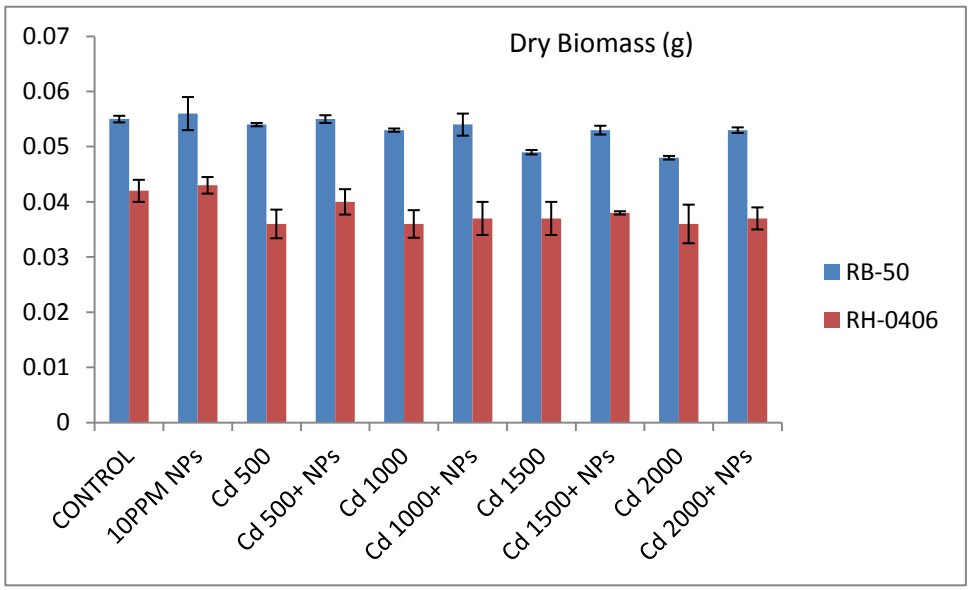


**Figure-8 Effect of different concentration of Cadmium on dry biomass of $Mg(OH)_2$ Nanoparticles primed and unprimed seeds of two *Brassica juncea* Cultivars. Data are the means of three replicates ± s.e.**

**F) Effect of $Mg(OH)_2$ NPs on Chlorophyll and total Carotenoids content of two cultivars with response to Cd toxicity**

Exposing two cultivars to different levels of Cd toxicity resulted in decrease in Chlorophyll content as well as Carotenoids content. Enhanced Cd accumulation in leaves cause reduction in Chlorophyll a, b and (a+b) contents and total caroteniods content in both the cultivars, whereas leaves of control did not show remarkable variations in both the tested parameters. Chlorophyll and Carotenoids content of both the cultivars decrease with increasing Cd concentrations. However Cultivar RB-50 showed less reduction in chlorophyll and Carotenoids content than Cultivar RH-0406. $Mg(OH)_2$ NPs primed seeds showed remarkable increase in the chlorophyll a, b and (a+b) content as well as total Carotenoids content in both the cultivars at all four Cd stress levels as compared to unprimed seeds.

**Table-1 Effect of different concentration of Cadmium on Chlorophyll (mgg-1FW) and Carotenoids contents of $Mg(OH)_2$ Nanoparticles primed and unprimed seeds of two *Brassica juncea* Cultivars. Data are the means of three replicates ± s.e.**

| | RB-50 | | | | RH-0406 | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | chl a | chl b | Total chl | Carotenoids | chl a | chl b | Total chl | Carotenoids |
| Control | 0.54 | 0.44 | 0.98 | 0.24 | 0.41 | 0.38 | 0.79 | 0.2 |
| Cd 500 | 0.4 | 0.32 | 0.72 | 0.19 | 0.32 | 0.26 | 0.58 | 0.14 |
| Cd 1000 | 0.32 | 0.26 | 0.58 | 0.16 | 0.26 | 0.2 | 0.46 | 0.11 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Cd 1500 | 0.28 | 0.16 | 0.44 | 0.14 | 0.19 | 0.11 | 0.3 | 0.08 |
| Cd 2000 | 0.17 | 0.1 | 0.27 | 0.11 | 0.11 | 0.08 | 0.19 | 0.07 |
| 10ppm NPs | 0.83 | 0.61 | 1.44 | 0.44 | 0.75 | 0.49 | 1.24 | 0.4 |
| Cd 500+ NPs | 0.75 | 0.45 | 1.2 | 0.37 | 0.65 | 0.42 | 1.07 | 0.36 |
| Cd 1000+ NPs | 0.73 | 0.36 | 1.09 | 0.32 | 0.61 | 0.3 | 0.91 | 0.29 |
| Cd 1500+ NPs | 0.66 | 0.31 | 0.97 | 0.23 | 0.56 | 0.23 | 0.79 | 0.2 |
| Cd 2000+ NPs | 0.57 | 0.25 | 0.82 | 0.19 | 0.46 | 0.2 | 0.66 | 0.15 |

## DISCUSSION

Agri food nanotechnology is emerging technology that uses nanoparticles for growth of plants under stressful conditions and for control of plant diseases.[13] Application of nanotechnology to the agriculture and food sector is relatively recent as compared to its uses in drug delivery and pharmaceuticals. Nanotechnology has the potential to protect plants against biotic and abiotic factors, detect plant and animal diseases and monitor plant growth.[14] Therefore present study was performed to analyse the effects of $Mg(OH)_2$ nanoparticles on two cultivars of *Brassica juncea* exposed to toxic Cd concentrations.

Heavy metal toxicity causes negative effects on physiological processes such as photosynthesis, germination and early seedling growth in higher plants.[15] Current research illustrated that 7 days of Cd stress in *B.juncea* seedlings showed visible symptoms of phytotoxicity and their survival rate declined greatly. In present model system *Brassica juncea* seedlings were unable to tolerate Cd concentration higher than 2mM. In general it can be said that tolerance of plant species to heavy metal stress depends on concentration of heavy metal, duration of treatment, age of plant during heavy metal exposure, genetic makeup of plant species and plant part examined.

Present results indicated that all four levels of Cd toxicity negatively affected the root-shoot length and seedling size; Roots were found to be more sensitive to Cd toxicity than shoots, as roots are the first organ to come in contact with toxic heavy metal which results in its higher accumulation.[16] Nanoparticle primed seeds of both cultivars showed significant increase in root-shoot length and seedling size as compared to unprimed seeds. $Mg(OH)_2$ nanoparticles treated seedlings displayed good growth of root as well as shoot, looked green and healthy, inferring an alleviation effect of Mg on Cd phytotoxicity. Similar results were obtained by Kashem and Kawai, 2007 who observed the alleviation of cadmium toxicity by giving bulk form of magnesium treatment in Japanese mustard spinach.

Current research showed that seed germination of both cultivars were affected adversely with Cd exposure, causing a complete cessation of seedling growth caused by inhibition of hydrolysis of carbohydrate reserves and translocation of hydrolyzed sugars.[17] $Mg(OH)_2$ nanoparticles treated seedlings of both the cultivars displayed increased seed germination percentage as compared to unprimed seeds. However cultivar RB-50 showed better results as compared to cultivar RH-0406. It may be concluded that high Mg content during seed germination could act as starter fertilizer and proves to be very effective in increasing rate of germination by overcoming harmful effects of Cd ions. The results of present investigation are in conformity with our earlier work with ZnO nanoparticles and it was observed that ZnO nanoparticles enhances the tolerance mechanism in two cultivars of *Brassica juncea* when exposed to toxic Cd concentrations.[10]

Biomass (Wet and Dry) accumulation is the important growth parameter, which is severely affected by Cd stress. Present research showed that Wet biomass of both the cultivars were found to be more affected with Cd toxicity than Dry biomass. This drastic reduction in Biomass accumulation was reimbursed with application of $Mg(OH)_2$ nanoparticles in both cultivars.

Our research illustrated that the Cd toxicity inhibited the chlorophyll and Carotenoids contents in both the cultivars. Chlorophyll content (a,b and a+b) greatly declined with increasing Cd concentrations . Similar results were obtained by John et al.[18] who reported that Cd treatments significantly reduced chlorophyll and Carotenoids content in *Brassica juncea.* This can be attributed to the disruptive action of heavy metals on chlorophyll synthesis, photosystem efficiency, and activity of photosynthetic enzymes and on chloroplast damage. Carotenoids act as light harvesting pigments, and protect chlorophyll and membrane disruption by quenching triplet chlorophyll and removing oxygen from the exited chlorophyll- oxygen complex.[19] In present work along with chlorophyll content Carotenoids content also decreased with increasing Cd concentrations. $Mg(OH)_2$ NPs primed seeds showed remarkable increase in the chlorophyll a, b and (a+b) content as well as total Carotenoids content in both the cultivars at all four Cd stress levels as compared to unprimed seeds. Current findings clearly illustrated that magnesium being central atom in chlorophyll porphyrin structure and is also intimately involved with photosynthesis in plants, plays vital role in improving growth of plants grown under Cd toxicity.

In conclusion the results of present work showed that treatment of two Cultivars of *Brassica juncea* with $Mg(OH)_2$ NPs enhance their tolerance to Cd stress especially by enhancing the rate of photosynthesis. It may be concluded that $Mg^{+2}$ ions counteracted and detoxified Cd toxicity in *Brassica juncea* seedlings. It may be assume that application of $Mg(OH)_2$ NPs on Cd contaminated soils would eliminate or reduced the Cd toxicity. However mechanism of alleviation of Cd toxicity by $Mg(OH)_2$ NPs is not clear. Future work into the mechanism of $Mg(OH)_2$ NPs in alleviating Cd toxicity needs to be conducted.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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# Role of resveratrol in hyperglycemia induced eNOS uncoupling: A review

**TARANG GAUR**

Advanced Genomic Institute and Laboratory Medicine, Guman Puri Complex, Kotla Mabarakpur, Delhi - 110001, India

*Corresponding author email: taranggaur777@gmail.com

## ABSTRACT

The nitric oxide, formed by endothelial nitric oxide synthase (eNOS) has a predominant role in maintaining homeostasis of endothelial cells under normal conditions. This vasoprotective state of eNOS is referred to as coupled state. It can convert from coupled to an uncoupled state, form resulting in superoxide production instead of NO production. Hyperglycemia plays a significant role in this uncoupling of eNOS which can redound to endothelial dysfunction. It leads to accumulation of reactive species which causes decoupling of eNOS and increases the level of eNOS inhibitor, ADMA. Moreover, it also oxidizes BH4, the cofactor of eNOS and contributes in formation of advanced glycation end products which decrease the bioavailability of NO. Resveratrol is a polyphenol commonly found in Red wine. The aim of this review to highlight the mechanism of action of Resveratrol and its pivotal role in reversing eNOS uncoupling. Furthermore, it also unravels the problem associated with Resveratrol taken as supplement.

## KEYWORDS

Endothelial Nitric Oxide Synthase; Hyperglycemia; Resveratrol; Homeostasis

## INTRODUCTION

Nitric oxide synthase (NOS) forms major 3 isoforms: The neural NOS (nNOS), inducible NOS (iNOS) and the endothelial NOS (eNOS). All 3 isoforms convert L-Arginine to L-citrulline utilizing oxygen molecule as substrate and FAD and BH4 as cofactors. Under normal conditions, the vascular NO is majorly formed by eNOS. This enzyme is present in the endothelium and its activation is initiated by shear stress or molecular mechanisms. The mechanism involved in eNOS activation includes enhancement of intracellular Calcium ions concentration, post-translational modification of the eNOS enzyme and protein-protein interactions. [1]

The nitric oxide produced by eNOS as several key roles to play endothelial homeostasis and maintenance. It is a major regulator of both EPC mobilization and function. Studies proved that eNOS-mediated NO improves mobilization capacity of EPC by cytoskeleton changes and activating vasodilator-stimulated phosphoprotein (VASP).[2] It relaxes the blood vessel and reduces the blood pressure. Furthermore, it inhibits the platelet

aggregation and prevents the plaque formation in atherosclerosis. It leads to suppression of low-density lipoprotein (LDL) oxidation and halting of vascular smooth muscle cell proliferation. The recent studies suggested it promotes the mitochondrial biogenesis along with anti aging effects and prolong lifespan induced by caloric restriction.

## eNOS UNCOUPLING

The eNOS forms the vasoprotective nitric oxide (NO) under physiological conditions. However under certain conditions the function of this protective enzyme is altered and instead of formation of NO, it produces superoxides. This alteration of function is referred as eNOS uncoupling. It leads to monomerization of enzyme. eNOS uncoupling is considered to be particularly deleterious due to simultaneous decline in NO production and rise in production of the free radical superoxide. Several mechanisms have been posited to mediate eNOS uncoupling, such as decreasing bioavailability of L-arginine, accumulation of asymmetric dimethyl L-arginine (ADMA) the endogenous eNOS inhibitor, oxidation of the eNOS cofactor tetrahydrobiopterin (BH4), and S-glutathionylation of cysteine residues in eNOS. These factors disrupt electron flow in eNOS leading to formation of reactive species.[3]

## ROLE OF HYPERGLACEMIA IN eNOS UNCOUPLING

Glucose is stored in form of glycogen in body. However failure of conversion of glucose to glycogen leads to presence of excessive amount of sugar in blood and the condition is called Hyperglycaemia. Decrease in insulin concentration and reduce glucose utilization leads to chronic hyperglycemia. This chronic form of hyperglycemia contributes to Diabetes Mellitus. Perpetuate exposure to hyperglycemia is known to be the as a major factor in augmenting the pathogenesis of complications in diabetes, including atherosclerosis.[5]

The hyperglycemia promotes accumulation of reactive oxygen species and increases oxidative stress. The superoxide formed react rapidly with the NO and forms a reactive species of nitrogen, called peroxynitrite, that causes damages in the cellular DNA as well as initiates the decoupling of eNOS, which redound to a higher production of superoxide promoting the endothelial damage.

ADMA, an inhibitor of eNOS is degraded by dimethylarginine diethylaminohydrolase (DDAHs), which can cleave ADMA into citrulline and dimethylamine. Oxidative stress by hyperglycemia reduces renal DDAH function and accumulation that further increases the level of eNOS inhibitor, ADMA.

Further studies suggested hyperglycemia induced oxidative stress oxidized BH4, the cofactor of eNOS into BH2 and other biopterin species. This would lead to insufficiency of BH4 that leads to eNOS uncoupling.

Elevated glucose level can cause glycation of proteins, promoting formation of advanced glycation end products or AGEs, protein cross-linking, and ROS formation. Formation of AGEs changes the functional property of matrix components. Glycation modifies the structure of the molecules that further disturbs their function and receptor recognition properties. AGEs leads to decreases in NO bioavailability and eNOS expression.[6]

Protein kinase C, belongs to a class of enzymes which are involved in directing the function of other proteins. Hyperglycemia activates the isoforms β and δ of PKC in culture of vascular cells. High activity of PKC has been observed in the reduced NO production in case of smooth-muscle cell and this has been shown to halt insulin-initiated expression of endothelial NO synthase (eNOS) in endothelial cell culture. [4]

## ROLE OF RESVERATROL IN eNOS UNCOUPLING

Resveratrol or (3,5,4'-trihydroxy-trans-stilbene) is a polyphenol found in grapes, red wine, peanuts, and some berries. It is classified as phytoalexin or plant toxin. It is produced in response to *Bothrytis cinerea* infection [14]. The grapes cultivated in cooler climates have a higher concentration of this compound as the fungal infections are very common in such climates. The Resveratrol content in any wine depends upon the time taken by the grape skins to undergo fermentation. Therefore the concentration of Resveratrol content is much higher in red wine as compared to white wine as in white wine the skins of fermented grapes are removed earlier during the process of production. Resveratrol is also found in market as supplement pills and liquids.

### MECHANISM OF ACTION

The exact mode of action of Resveratrol is not yet known. However it is claimed to act according to following pathways:

(i). Sir 1 dependent activation

Sirtuin 1 is a protein encoded by SIR1 gene which stands for silent mating type information regulation 2 homolog. It is an enzyme that basically deacetylase those proteins which contribute to cellular regulation such as aging. One of the main activator of Sir1 is ligand Lamin A. According to studies Resveratrol is involved in indirect activation of Sir1. Resveratrol causes SIRT1 to initiates the migration of FOXO transcription factors into the nucleus. The FOXO3a transcriptional activity is further shown to elevate the sirtuin-catalyzed deacetylation of FOXO3a.The MnSOD which is a cellular antioxidant enzyme, known to be a target of FOXO3a, causes over expression of FOXO3a. Also it is proposed that Resveratrol increases the binding of Lamin A ligand.

(ii). Inactivation of phosphodiesterases

According to research in 2012, it was found Resveratrol inactivates several phosphodiesterases such as PDE4 which increases the concentration of several secondary messengers such as cAMPs. PDE4 hydrolyzes cyclic adenosine monophosphate (cAMP) which inactivates adenosine monophosphate (AMP). As cAMP hydrolysis blocks due to inhibition of PDE4, the concentration of cAMP increases within cells. This leads to increases in mitochondrial biogenesis.

(iii). Binding to estrogen receptors

Estrogen receptors have two forms alpha (ESR1) and beeta (ESR2). They forms dimer when binds with estrogen. Upon binding the DNA binding domain enters nucleus and binds to estrogen responsive elements present in nucleus itself. Similarly Resveratrol activates gene transcription by binding to estrogen receptors.

## ROLE OF RESVERATROL IN Enos UNCOUPLING

(i). Supplementation of BH4

Copious mechanisms have been posited to define the role of Resveratrol in eNOS uncoupling. As mentioned before, BH4 unavailability is a major reason of eNOS uncoupling under pathological conditions. The oxidative stress by hyperglycemia plays a significant role in BH4 depletion. The BH4 supplementation could help to redress the eNOS dysfunction in different types of pathophysiology.7. One of the core mechanisms of action of Resveratrol is improving deacetylase activity of Sir-1. The activated Sir-1 deacetylase FOXO genes migrate to nucleus and upregulates GTP cyclohydrolase 1 (GCH1) enzyme. GCH1 is a rate limiting enzyme

in BH4 synthesis from GTP. As a result BH4 synthesis gets enhanced. The study of Xia at al supports these findings. The ApoE-KO mice are considered as mouse model of atherosclerosis. Resveratrol treatment elevates the expression of GCH1 (SIRT1-dependently) and BH4 biosynthesis. Further, treatment of ApoE-KO mice with resveratrol resulted in marked decrease of cardiac superoxide formation. [1]

(ii). Increase in enzymatic activity of eNOS

The studies have shown that treatment of human endothelial cells with Resveratrol led to a concentration-dependent up regulation of eNOS expression by enhancing the activity of human eNOS promoter fragments indicating the utility of this region in Resveratrol mediated up regulation of eNOS activity. [8] Also they found Resveratrol augment the activity of FOXO transcription factors which are involved in Resveratrol-initiated eNOS transcriptional activation.

(iii). Phosphorylation of eNOS

Residue sites-serine, threonine and tyrosine are phosphorylated by eNOS. The studies have proved that enhancing phosphorylation of eNOS by inhibiting the activity of serine/threonine phosphatases, increased eNOS activity.[9] Resveratrol and initiates signal cascade that involves kinase leading to phosphorylation of eNOS. The data demonstrated that Resveratrol binds with estrogen receptors and induces signal cascade. Caveolin-1 (Cav-1) is an eNOS interacting protein that down regulates eNOS activity. The Resveratrol on attachment with ER receptors decreases the activity of Cav-1. [10]

AMPK can also phosphorylate eNOS at serine. As shown in studies on cultured endothelial cells, Resveratrol at concentrations of 10–100 μM activates AMPK, which has connotation with eNOS phosphorylation and enhanced NO production. [11]

(iv). Endogeneous eNOS inhibitor

As mentioned before, ADMA (Asymmetrical dimethylarginine) is the inhibitor of eNOS. It is degraded by DDAH (dimethylarginine dimethylaminohydrolase). However the activity of DDAH is decreased by oxidative stress associated with hyperglycemia.[12] Resveratrol up regulates the activity of DDAH that could decrease the formation of ADMA.

(v). Upregulation of antioxidant enzymes

The hyperglycemia increases the level of reactive oxygen species. Resveratrol augments this accumulated ROS detoxification by enhancing the level of antioxidant enzymes in tissues. Nrf2 is a basic leucine zipper (bZIP) protein. It provides protection against oxidative damage by regulating the levels of antioxidant proteins. The findings proved that Resveratrol can activate Nrf2 that can further increase the level of antioxidant enzymes in body. [13]

## DISCUSSION

Studies on bioavailability of Resveratrol in humans have shown low bioavailability of Resveratrol due to rapid metabolization in intestine and liver due to first pass effect. This is due to instantaneous conjugation through sulphation and glucuronidation by P450 enzymes that reduce the amount of free Resveratrol. As a result only small portion of Resveratrol reaches bloodstream unchanged. [15]

One of the major problems associated with Resveratrol is dosage recommendation. The dosages in Resveratrol supplements are typically lower than the recommended amounts that have been shown beneficial effects in research studies. Even the Red Wine contains very less amount of Resveratrol. Most supplements contain 250 to 500 mg of Resveratrol. For getting the equivalent dose required in animal studies, people

would have to consume 2 grams of Resveratrol (2,000 mg) or more a day. Since the product is not approved by FDA so there is lot of apprehension among people to consume large dose.
So far, studies have not yet found any major side effects, even though Resveratrol is consumed in large doses. However, Resveratrol supplements might react with blood thinners such as warfarin and non steroidal anti-inflammatory drugs such as aspirin and ibuprofen which can increase the risk for bleeding.
In year 2012, new research at Washington University School of Medicine in St. Louis indicated no health benefits of Resveratrol when a research was conducted on 15 post-menopausal women to check the insulin sensitivity when administered with different doses of Resveratrol. However this does not eliminate the possibility that Resveratrol could have a synergistic effect when combined with other compounds in red wine.

## CONCLUSION

Thus we can conclude that Resveratrol helps in reversing eNOS uncoupling by number of mechanisms including supplementation of BH4, increasing enzymatic activity and Phosphorylation of eNOS, downregulation of eNOS inhibitor and up regulation of antioxidant enzymes. In short, Resveratrol can mitigate the effects of hyperglycemia induced oxidative stress contributing to eNOS uncoupling. But there are several problems associated with the use of Resveratrol in vivo system. However, with more advancement in technology that could structurally modify synthetic Resveratrol, it could prove to be an important daily supplement. Further clinical trials are required to test the efficacy of Resveratrol on humans along with the correct dosage recommendation.

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# Role of free radicals in DNA damage with special reference to different stages of breast cancer patients

**NEHA SHARMA*[1] NALINI SHRIVASTAVA[2] AND MEENU RAI[3]**

[1]Department of Biotechnology**,** Kamla Raja Girls P.G. (Auto.) College, Gwalior (M P) India
[2]Professor, Department of Biochemistry, Jiwaji University Gwalior, (M P) India
[3]Principal, College of life sciences, Cancer Hospital and Research Institute, Gwalior (M P) India

*Corresponding author email drneha16may@gmail.com

## ABSTRACT

Scientists have shown an increased interest in role of free radicals in the development of breast cancer. Reactive oxygen and nitrogen species are highly reactive and produced during normal cellular metabolism into living organisms, and can damage major cell molecules such as nucleic acids, lipid and proteins and also change their physiological functions and structure. Free radicals are high unstable and reactive due to the presence of an odd number of electrons in the outermost orbit of their atoms. Reactive oxygen and nitrogen species are the types of free radicals, which are principal component in the initiation and progression of breast cancer and increase their metastatic power. In fact, free radicals are also called as hallmark of cancer. It is proof that oxidative DNA damage is responsible for cancer development. This study was planned to observe the effect of free radical in breast cancer. The activity of free radicals had shown significant change in DNA damage in patients of different stages of breast cancer.

## KEY WORDS

Free radicals, chemotherapy, DNA damage, breast cancer.

## INTRODUCTION

Free radicals play an important role in the biogenesis of different diseases such as cancer, has been widely recorded.[1] They have specific cell functions, as well as toxic for the cells that produce them or for neighboring cells in a tissue recorded by many scientist. In the case of oxygen which is a highly stable molecule and can change into different reactive species, such as free radicals. In cell metabolism functions these free radicals constitute the product or are involve performing important cell functions, especially when the reactivity of molecular oxygen is insufficient.[2]

The cells produce free radicals as well as degrade, that is strictly necessary to avoid the damage generates from an uncontrolled cellular metabolism. However, various intrinsic and extrinsic factors and the

biochemical activity of the cell can make it lose control over the formation and management of free radicals. This imbalance between the formation and use of free radicals in cell is called "oxidative stress". Oxidative stress results from a disturbance between the formation of reactive oxygen species (ROS) and the defense which is provided by cell antioxidants. [3] The treatment of chemotherapy in cancer treatments can also favor oxidative stress.[4]

Free radicals may cause breaks and damage in the DNA molecule, as a consequence, it producing mutation and cancer. The main source of mutations in living organisms is DNA damage by oxidation. The estimated frequency of DNA damage is $10^4$ lesions/cell/day in human cells .[5]

Oxidative damage to cell molecules especially DNA is a result of interaction with ROS or RNS. Free radicals such as $^{\bullet}OH$, and $H^{\bullet}$ react with nucleic acid (DNA) by addition to bases or removal of hydrogen atoms from the sugar moiety. In case of pyrimidine C4-C5 double bond is particularly sensitive to attack by $^{\bullet}OH$, generating a broad range of oxidative pyrimidine damage products such as thymine glycol, uracil glycol, urea residue, 5-hydroxydeoxyuridine, 5-hydroxydeoxycytidine and others. Similarly, interaction of $^{\bullet}OH$ with purines will generate 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxy deoxyadenosine, formamidopyrimidines and other less characterized purine oxidative products. In cell there are many repair pathways which repair DNA damage, but 8-OHdG has been involve in carcinogenesis and is considered as a reliable marker for oxidative DNA damage by many researcher. Nucleic acids are polymer of pentose-phosphate that can undergo reactions with hydroxyl radical like those depicted for hyaluronic acid. In addition there are several important examples of modifications to the base portion of the polymer. These types of base modifications may be responsible for genetic disorders produced by oxidative stress. Recently, 8-hydroxy guanosine has considered as a product of hydroxyl radicals attack on DNA that can be used to estimate DNA damage in humans suffering from cancer.[6]

### MATERIALS AND METHODS

97 healthy subjects and 157 confirmed cases were considered for the study of DNA damage in lymphocytes of breast cancer. All these samples were histopathologically positive proved by Cancer Hospital & Research Institute; Gwalior (M.P.). The blood samples of healthy subjects and patients were collected for the study of DNA damage in breast cancer.

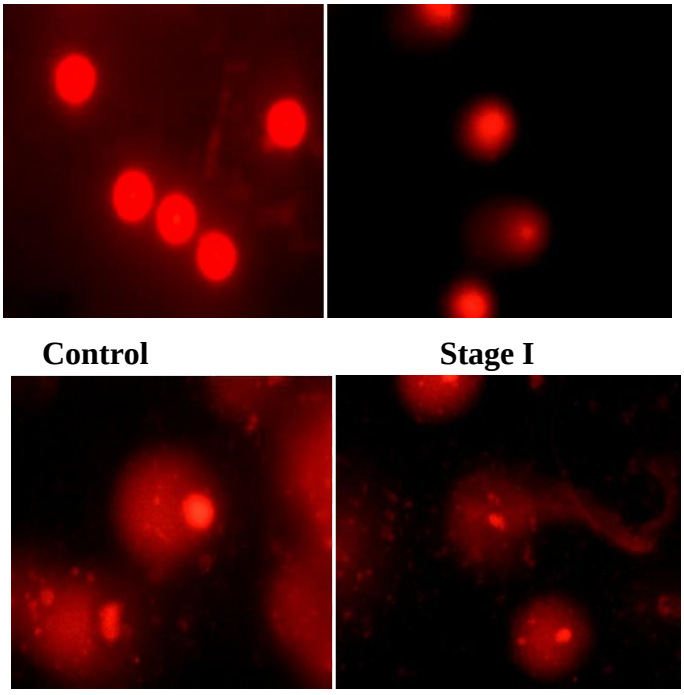
The subjects were divided into following groups on the basis of disorder:

Normal healthy subjects
Stage I subjects
Stage II subjects
Stage III subjects

DNA damage was assayed by the method of Sasaki *et al* (1997) with minor modification.[7]

## RESULTS

Lymphocytes of breast cancer patients showed significant DNA damage. Comet assay is used to study DNA damage in breast cancer patients. Quantization of DNA damage can be done by measurement of comet tail length. The result pointed out that comet tail was significantly increased in different stages of breast cancer when compared with control. The comet tail length is larger in patients of stage third cancer, which showed excessive DNA damage in breast cancer patients.




## DISCUSSION

Free radicals possess an odd number of electrons in the outermost orbit of their atoms so they are highly reactive. Their aggressive action due to attempts of gain "balance" by binding with electrons of neighboring atoms, giving rise to chain reactions.[6, 8]

Many cellular metabolic systems frequently produce free radicals from oxygen as their normal cell metabolism. 80% of molecular oxygen is consumed by mitochondria, and 5% of this is transformed into superoxide radicals and hydroxyl radicals. Smooth endoplasmic reticulum is involve in metabolism of many endogenous (prostaglandins, fatty acids, etc.) and exogenous (drugs, colorants, flavorings, antioxidants, etc.) substrate and consuming 15% of molecular oxygen, of which 20-30% is reported to be transformed into free radicals, especially $^{\bullet}OH$ was reported by many scientist. Macrophages and leucocytes involve in defense mechanisms against bacteria and virus, also involve to the formation of free radicals. Free radicals are worked in synthesis of hormone such as prostaglandin, cholesterol and steroidal as well as biosynthesis of collegen require the participation of free radicals which cause the hydroxylation of lysine and proline amino acids and change into hydroxylysine and hydroxyproline, respectively.[9] Hence, In normal cellular metabolism of cells, free radicals have an essential functions. However, their presence show a risk, especially for large molecules, e.g., nucleic acids (DNA), proteins, polymerized carbohydrates (polysaccharides), and lipids, which are highly susceptible for damaged by oxygenated free radicals.[9, 10, 11]

During aerobic metabolism in body, oxygen free radicals such as super oxide, hydroxyl radicals, hydrogen peroxide, hypochloric acid and nitrogen free radicals like nitric oxide and proxy nitrite are produced. In living cells, mitochondria involve in oxidative phosphorylation, leukocytes involve in oxidative burst, peroxisomes have done degradation of fatty acids and cytochromep450 system may contribute to the production of reactive oxygen species (ROS) occurring in vivo can cause oxidative damage of amino acids, lipids, proteins and DNA. Besides these *in vivo* activity of ROS, DNA can also be damaged through exogenous ROS sources including cigarette smoking, UV and ionizing radiation. Free radicals play dual role in the body. They play as stimulators of signal transduction (e.g. Ca2+ signaling and protein phosphorylation) and regulatory molecules at physiologic levels as well as they are highly cytotoxic oxidants at pathologic levels in body. DNA strand breaks, base modification and chromosomal aberration cause by excess ROS. Available evidence has shown that DNA damage can result from free radical attacks if not repaired, the damage may lead to deteriorated gene expression, development of a number of diseases such as cancer, diabetes, neurodegenerative and vascular diseases and also aging. Especially in development of atherosclerosis, ROS have important roles including promoting cell proliferation, hypertrophy, growth arrest, apoptosis and oxidation of LDL. [12]
Oxidative mechanisms of DNA damage have been demonstrated a potential role in the initiation, promotion and malignant conversion (progression) stages of carcinogenesis. It also reported that cumulative cancer risk increases with the age and is associated with an accumulation of DNA damage, oxidative DNA damage has been investigated in cancer. Lesions such as 8-OH-dG are established biomarkers of oxidative stress; coupled with their potential mutagenicity in mammalian cells, this has led to their proposed potential as intermediate markers of a disease endpoint—for example, cancer. Many studies have attempted to establish a relationship between levels of oxidative DNA damage and cancer.

It has been reported by many researcher that at least some tumor cell lines can produce significant levels of $H_2O_2$, without exogenous stimulation, perhaps accounting for the elevated levels of oxidative DNA damage seen. Transcription factors and their corresponding genes are permanently activated as a result of increasing ROS, which coupled with increased DNA damage, creates a selection pressure on malignant phenotype seen in cancer. Although such studies have furthered the hypothesis that oxidative DNA damage may be an important risk factor for carcinogenesis, it has been argued that the presence of 8-OH-dG in DNA is sufficient or necessary to cause tumor formation or directly involve in breast cancer.[13]

In our present study lymphocytes of cancer patients showed significant DNA damage. DNA damage in breast cancer patients was studied by comet assay. The result pointed out that comet tail was significantly increased in different stages of breast cancer when compared with control. The comet tail length is larger in patients of stage third cancer, which showed excessive DNA damage in breast cancer patients. Previous studies have suggested that elevated DNA damage levels may be associated with breast cancer risk.[14,15]

## CONFLICT OF INTEREST

Conflict of interest declared none.

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# Effect of *Trichoderma* fused silver nano-particles on fungal phyto-pathogens

**BINA PANI GUPTA[1*], VEENA RAINA[2], RASHMI JAIN[3] AND ABHISHEK MATHUR[2]**

[1]Dept of Microbiology, Himalayan University, Arunachal Pradesh, India.
[2]National Centre of Fungal Taxonomy (NCFT), New Delhi; Institute of Transgene Life Sciences (ITLS), Dehradun, Uttarakhand, India.
[3]National Institute of Biologicals, Noida (U.P), India

*Corresponding author e-mail: binapani.gupta@gmail.com

## ABSTRACT

The genus, *Trichoderma* and its metabolites are meant for antimicrobial activity against the microbial strain and thus it is considered to be natural antimicrobial agents. Silver is also known as an antimicrobial agent and is utilized in several antimicrobials and medications. In the present investigation, the nano-particles were prepared both of *Trichoderma* and $Ag^+$ separately and further fused *Trichoderma* and $Ag^+$ nanoparticles were also prepared. *Trichoderma harzianum* secretes secondary metabolites which act as a capping and reducing agent. The biosynthesized silver nanoparticles (AgNPs) were characterized by UV–Vis spectroscopy and Transmission electron microscopy (TEM). UV–Vis spectra of silver and *Trichoderma* nano-particles showed absorption spectra at 450 nm and 430 nm respectively while fused nanoparticles showed absorption spectra at 415 nm corresponding to the surface plasmon resonance of silver nanoparticles. The size and morphology of the fused nanoparticles was determined by TEM, which shows the formation of spherical nanoparticles in the size range of 8–24 nm. The antifungal activity of biosynthesized AgNPs, *Trichoderma* nanoparticles and fused nanoparticles was evaluated by measuring the diameter of zone of inhibition against pathogenic fungal phyto-pathogens viz. *Aspergillus niger*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Fusarium monoliforme* and *Sclerotenia sclerotiorum*. It was also observed that, fused nanoparticles of Trichoderma-$Ag^0$ were having prominent antifungal activity in comparison to individual nanoparticles.

## KEYWORDS

Nano particles, *Trichoderma*, Ag+, TR-Ag fused nanoparticles (TR-Ag Nps), antifungal activity, phyto-pathogens.

## INTRODUCTION

The anamorphic fungal genus *Trichoderma* (*Hypocreales*, *Ascomycota*) is cosmopolitan in soils and on decaying wood and other forms of plant organic matter. [1] *Trichoderma* species are among the most widely

distributed and common fungi in nature and exist in climates ranging from the tundra to the tropics. This may be attributable to their diverse metabolic capability and aggressively competitive nature.[2,3] Rapid growth rates in culture and the production of numerous spores (conidia) that are mostly varying shades of green characterize fungi in this genus. A growing number of teleomorphs in *Hypocrea* have been linked to commonly occurring *Trichoderma* anamorphs, but most strains of *Trichoderma* are classified as imperfect fungi because they have not been associated with a sexual state. Interestingly, *Hypocrea/Trichoderma* spp. are even able to induce systemic resistance, which is characterized by the occurrence of disease control in the plant at a site distant from the location of *Hypocrea/Trichoderma*. They stimulate the production of low-molecular weight compounds that have antimicrobial activity like e.g. phytoalexins which are normally produced by plants in response to an attack by pathogens. A large area of interest in biocontrol is the reduction of plant diseases caused by soil-borne and foliar plant pathogenic fungi. Roughly 70% of all the major crop diseases are caused by fungi, or the fungus-like Oomycota. Notorious examples are species belonging to the genera *Rhizoctonia*, *Botrytis*, *Phytophthora*, *Pythium*, *Sclerotinia* and *Fusarium*. Most of the formulations of commercially available biocontrol products against plant pathogenic fungi contain the bacteria *Pseudomonas* and *Bacillus* or fungi belonging to the genus *Hypocrea/Trichoderma. Hypocrea/Trichoderma* spp. produces a wide range of enzymes for degradation of homo- and hetero-polysaccharides, which are designative for their broad spectrum of substrate utilization and their ubiquitous occurrence in nature. Furthermore they possess a wide spectrum of proteases which help them in the defense of their habitats and the competition for nutrients with other microorganisms. Biological synthesis of metal nanoparticles involving the use of microbes is easy, cost-effective and eco-friendly technique. These nano-particles are effective, bio-compatible and bio-degradable.[4] Silver compounds have been used to treat burns, wounds and infections. Various salts of silver and their derivatives are used as antimicrobial agents. [5,6]. Recent studies have reported that nanosized silver particles exhibit antimicrobial properties. [7,8] Since Trichoderma as a natural source and Ag+ as synthetic source both have antimicrobial effect against the variety of pathogens. Thus the present study was focused on the preparation of Trichoderma-Ag fused nanoparticles (TR-Ag Nps) for determination of inhibitory effect on fungal phyto-pathogens.

## MATERIALS AND METHODS

### Isolation of Trichoderma species from soil samples

To isolate *Trichoderma* from soil, a *Trichoderma* selective medium will be prepared (Mohammad Akrami *et al.* 2011). The basal medium consisted of 0.2 g MgSO4 (7H2O), 0.9 g K2HPO4, 0.15 g KCl, 1.0 g NH4NO3, 3.0 g D-glucose anhydrous, 0.15 g rose bengal and 20g agar. These constituents will be added to 950 ml of distilled water and autoclaved at 121°C for 30 minutes. The biocidal ingredients, 0.25g tetracycline will be mixed in 50 ml of sterilized distilled water and added to the autoclaved basal medium where it cooled to 40 to 50°C. 10 grams of soil were suspended in 50 ml of sterile distilled water and agitated for 30 min at 200 rpm in a rotary shaker. Serial dilutions were made and 0.1 ml of each was spread on the *Trichoderma* selective medium plates with a glass rod. Three plates of each sample were prepared and incubated for 5 days at 30°C. *Trichoderma* isolates will be collected and transferred onto potato dextrose agar (PDA) plates for maintaining pure culture. [9] **(Figure 1)**.

## Cultivation and culture conditions

*Trichoderma* cultures obtained will be cultivated and maintained on slants of potato dextrose agar for 5 days at 28°C. Three hundred milliliters flasks were incubated for 14 days at 28°C on a laboratory incubator. The fungal biomass was collected for further use.

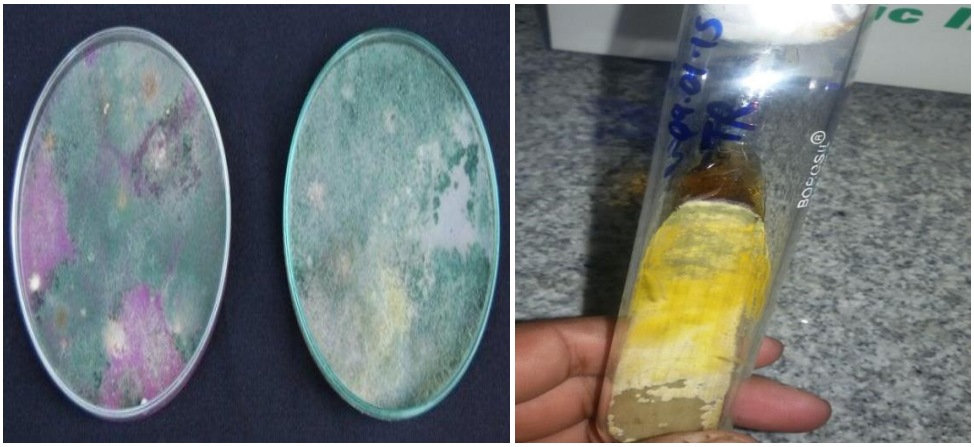


**Figure 1: Isolated Trichoderma cultures**

## Preparation of silver nanoparticles

For the preparation of silver nanoparticles two stabilizing agents, sodium dodecyl sulphate (SDS) and sodium citrate will be used. For the synthesis of silver nanoparticles, silver nitrate solution (from 1.0 mM to 6.0 mM) and 8% (w/w) sodium dodecyl sulphate (SDS) will be used as a metal salt precursor and a stabilizing agent, respectively. Hydrazine hydrate solution with a concentrate ranging from 2.0 mM to 12 mM and sodium citrate (1. 0 mM to 2.0 mM) will be used as reducing agents. Citrate of sodium will be used as stabilizing agent at room temperature. The transparent colorless solution will be converted to the characteristic pale yellow and pale red colour, when citrate of sodium will be used as stabilizing agent. The occurrence of colour will be indicated by the formation of silver nanoparticles. The silver nanoparticles will be purified by centrifugation. To remove excess silver ions, the silver colloids will be washed at least three times with deionized water under nitrogen stream. A dried powder of the nanosize silver will be obtained by freeze-drying. [10-14]

## Preparation of Trichoderma fused Silver nanoparticles

The silver nitrate (1 mM) solution was prepared in 50 ml deionised water. Fungal biomass (5 g) was brought in contact with the silver nitrate solution in a 200 ml Erlenmeyer flask. The solution was then kept in dark condition at 29±1 $^{0}$C under continuous shaking at 200 rpm for 72 h. After 72 h of reaction time the colour change was observed. [15-18]

**Characterization of prepared nanoparticles via UV- absorption spectra and Transmission electron microscopy (TEM)**

The formation of AgNPs and TR-AgNPs by the bioreduction of Ag+ to $Ag^0$ will be easily monitored using UV–Vis spectroscopy. The scanning will be performed in the range of 200–700 nm. The morphology and size will be determined by TEM. [19-23]

**Determination of antifungal activity of Trichoderma-Ag* fused nanoparticles against the fungal phyto-pathogens**

Plant pathogenic fungal cultures viz. *Fusarium oxysporum*, *Rhizoctonia solani*, *Fusarium monoliforme* and *Sclerotenia sclerotiorum* were grown separately in 20 ml potato/dextrose/broth (PDB) in 250 ml flasks. The culture flasks were incubated on an orbital shaker at 150 rpm at 30 $^0$C. Further the cultures were poured along with PDA in sterilized petriplates. Sterilized paper discs soaked with fused nanoparticles in different concentrations mentioned [viz. 5 (a), 10 (b), 15 (c), 20 (d), 25 (e) ppm] were placed on the culture plates at the peripheral region of the culture plate. The plates were further kept for 48-72 h for observation of growth inhibition if any. The inhibition of the radial growth was observed and recorded. [24-29]

## RESULTS

In the present investigation, Trichoderma fused silver nanoparticles (TR-Ag Nps) were produced **(Figure 1 and 2).** The particle size was determined by SEM and their absorption spectrum was determined at 200-700 nm. It was observed that the TR-Ag Nps produced were of very fine shape and size having optimal 20 nm size. These nano particles were having slightly rough spherical structures which were observed in free and interconnected form **(Figure 3)**. The UV absorption spectra of the fused nanoparticles recorded the maximum wavelength at 415 nm.

**Figure 1: Preparation of silver nanoparticles**


**Figure 2: Preparation of Trichoderma fused silver nanoparticles (TR-Ag Nps)**

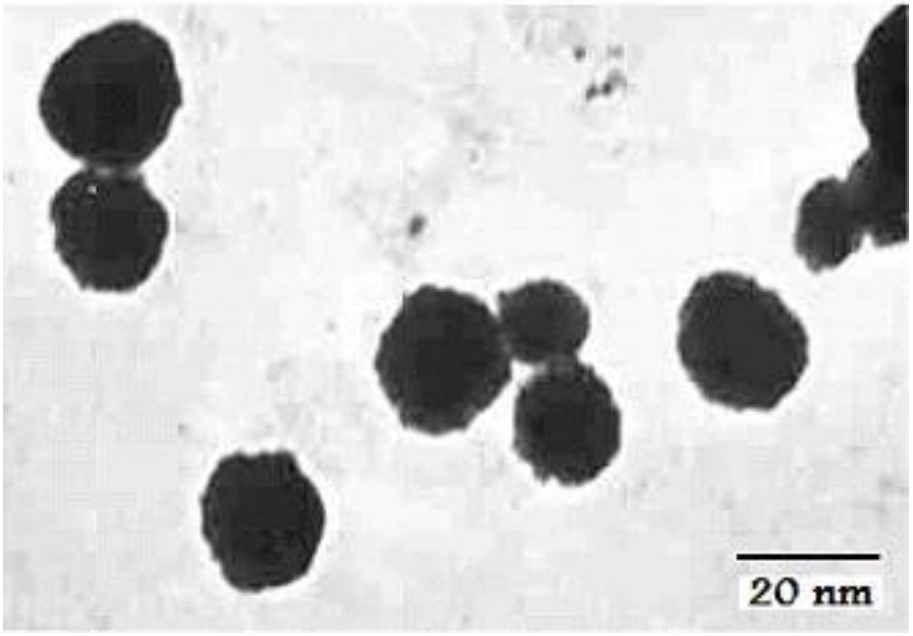



**Figure 3: Observation of Trichoderma fused silver nanoparticles (TR-Ag Nps) as determined by SEM**

The antifungal activity of the fused nanoparticles was evaluated against the plant pathogenic fungal cultures viz. *Fusarium oxysporum*, *Rhizoctonia solani*, *Fusarium monoliforme* and *Sclerotenia sclerotiorum* at different doses varying from 5 -25 ppm. It was observed that the fused nanoparticles had strong antifungal activity against the concerned pathogens. It was also observed that, by varying the concentration of doses of

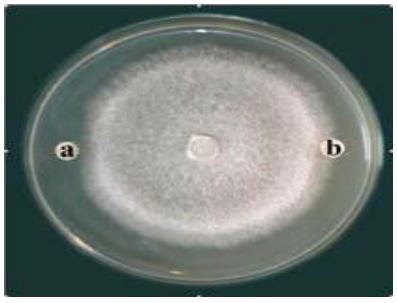
the fused nanoparticles, the radial axis of the fungal was getting decreased in more effective manner. The results are shown in **Table 1** and **Figure 4**.

**Table 1:** Anti-fungal activity of Trichoderma fused silver nanoparticles (TR-Ag Nps) against the fungal phyto-pathogens

| **Labeled dose amount on paper discs as-** | **Dose quantity (ppm)** | **Diameter of inhibition of radial axis of the fungal cultures against the fungal phyto-pathogens (mm)** | | | |
|---|---|---|---|---|---|
| | | ***Fusarium oxysporum*** | ***Rhizoctonia solani*** | ***Fusarium monoliforme*** | ***Sclerotenia sclerotiorum*** |
| **a.** | **5.0** | 3.0 | 10.0 | 3.0 | 5.0 |
| **b.** | **10.0** | 7.0 | 15.0 | 8.0 | 10.0 |
| **c.** | **15.0** | 12.0 | NT | NT | NT |
| **d.** | **20.0** | 18.0 | NT | NT | NT |
| **e.** | **25.0** | 25.0 | NT | NT | NT |

*NT-Not tested


**(A)** **(B)** **(C)**




**(D)**

**Figure 4: Anti-fungal activity of Trichoderma fused silver nanoparticles (TR-Ag Nps) against the fungal phyto-pathogens**

## DISCUSSION AND CONCLUSION

*Trichoderma* have been known since at least the1920s for their ability to act as biocontrol agents against phytopathogenic fungi. The classical mechanisms of control have included antibiosis, mycoparasitism, and competition for nutrients. *Trichoderma* strains inhibit or kill plant-pathogenic fungi through production of antifungal antibiotics and/or hydrolytic enzymes. [30-34] The significant combination of Trichoderma with synthetic salts/metallic ions can be utilized in treatment of different infections within the plants and animals both. These can be utilized as alternatives to different antibiotics etc. The present study suggests that, Trichoderma secretes some potent secondary metabolites responsible for antifungal activity against dreadful fungal phyto-pathogens which causes different diseases within the plants and damages the crops. Silver is a potent antimicrobial agent against different pathogens. The combination of secondary metabolites and Ag ions in terms of fused nanoparticles can results in preparation of potent antimicrobial agents. The study may thus lead to the isolation and identification of significant antimicrobial molecule (s) from Trichoderma which can be utilized in preparation of different types of nanoparticles. These nanoparticles can thus be utilized as a potent fungicide against different phyto-pathogens.

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# Combined effect of stress and indecorous lipid profiles on the cytokine expression in type 2 diabetes mellitus

**HIMANI CHATURVEDI, MAYURI KHARE, RAHI SHARMA, VINOD SINGH***

Department of Microbiology, Barkatullah University, Bhopal, India

* Corresponding author email: vsingh3@rediffmail.com

## ABSTRACT

Stress has been shown to be a potential contributor to chronic hyperglycemia in type 2 diabetes. The effect of stress and cholesterol levels was studied in type 2 diabetes patients. 50 subjects of both sex and varying age groups were divided into two categories-25 patients (11 males and 14 females) and 25 age and sex matched healthy controls. Blood samples were analyzed for pro-inflammatory cytokines (interleukin-6, tumor necrosis factor-α, interferon-γ), anti-inflammatory cytokines (interleukin-4, interleukin 10), total cholesterol and cortisol. Stress was evaluated by scoring for stress Performa filled by subjects. Stress was found to have a positive and negative correlation with pro-inflammatory and anti-inflammatory cytokines respectively. Cortisol and total cholesterol levels showed a positive correlation with stress. Results evince that stress and abnormal lipoprotein levels can be a major factor for deteriorating the condition of patients of type 2 diabetes whereas can pose a risk of diabetes in healthy individuals.

## KEYWORDS

Stress, type 2 diabetes mellitus, lipid profile, cortisol, pro- and anti-inflammatory cytokines

## INTRODUCTION

Type 2 diabetes mellitus (T2DM) is characterized by insufficient synthesis of insulin and its secretion, secondary to insulin resistance. It is normally diagnosed after the fourth decade of life, and accounts for approximately 90% of all diabetes worldwide. The incidence and prevalence of T2DM are found to increase with age.[1] Body loses the ability to maintain homeostasis as in to regulate the blood sugar levels in the body. This high blood sugar disturbs the functioning of either the pancreas or the cells which respond to insulin) causing symptoms of polyuria, polydipsia and polyphagia.[2, 3]

Regulation of the levels of glucose in the blood is based on a negative feedback loop and acts via the release of insulin and glucagon. High blood glucose levels trigger β cells of the islet of langerhans in the pancreas to release insulin.[4] Insulin lowers blood glucose levels by facilitating glucose uptake mainly into skeletal muscle and fat tissue and by inhibiting endogenous glucose production by the liver. In insulin resistant states, these organs do not properly respond to insulin, thereby causing hyperglycemia and a reactive increase in insulin secretion by the pancreatic β cells.[5, 6] More than required amount of insulin is produced in the cells due to inadequate strength of insulin signaling from the insulin receptor downstream to the final substrates of insulin action involved in multiple metabolic and mitogenic aspects of cellular function. The elevated insulin levels can compensate for the poor insulin response only for a limited period, but on the other hand impair insulin resistance.[7, 8]

Severe stress and prolonged inflammation may be a risk factor for diabetes. They make body produce increased amount of cortisol which can result in elevated blood glucose levels and increased levels of circulatory acute response proteins and cytokines. Although this is of adaptive importance in a healthy organism, in diabetes, as a result of the relative or absolute lack of insulin, stress-induced increases in glucose cannot be metabolized properly.[9, 10]

T2DM is closely correlated with chronic inflammation, with increased levels of circulatory acute response proteins and cytokines. Many pro-inflammatory cytokines like interleukin-6(IL-6), tumor necrosis factor- α (TNF-α) and interferon-γ (IFN-γ) play a central role in inflammatory reactions and were shown to increase the risk of T2DM. These pro-inflammatory cytokines can enhance insulin resistance directly in adipocytes, muscle and hepatic cells, leading to systemic disruption of insulin sensitivity and impaired glucose homeostasis. The anti-inflammatory cytokines are a series of immune-regulatory molecules that control the pro-inflammatory cytokine response. Interleukin-4(IL-4) and interleukin-10(IL-10) have been shown to increase glucose tolerance and insulin sensitivity.[11-13] All these cytokines play different roles in increasing the risk to develop diabetes mellitus.

Hyperlipidemia is also a common problem in patients of T2DM. The levels of cholesterol and triglycerides rise in the patients. There are several reasons responsible for this. Insulin plays a major role in the regulation of lipid metabolism and fluctuations in the same produce variable effects in the lipid levels of the person.[14, 15]

Stress within a certain limit is beneficial for the body but prolonged or permanent stress makes the body prone to various diseases including obesity and T2DM.

## MATERIALS AND METHODS

### I. Subject Selection

A group of 50 adult patients were considered. 25 subjects were in the category of type 2 diabetes mellitus with increased sugar levels and 25 were age and sex matched normal healthy patients. Blood samples were obtained from patients diagnosed with T2DM in National Hospital Critical Care Centre, Bhopal. Measures were taken to ensure that patients were not suffering from any other disease. A complete diagnosis chart was filled for each patient and healthy control. Patients and normal controls were of similar age and sex. Ethical clearance and consent from has been filled by normal healthy individual and patients.

## II. Collection of blood samples and Isolation of serum

A total of 20 ml of peripheral blood was collected from each subject, diabetic patients as well as controls. Blood sugar levels (fasting and random) were collected from the responsible physician. Blood was allowed to stand at room temperature and allowed to clot. Samples were centrifuged for 10 minutes at 5000 rpm. The serum was collected using sterile pipettes. Serum vials were then stored at $-20^0$ C.

## III. Evaluation of IL-4 , IFN-γ and TNF-α

IL-4, IFN-γ and TNF-α levels in serum samples were determined by ELISA(Enzyme linked immunosorbent assay) method. Ready to use kits from KRISHGEN BioSystems, India were used.100 µl/well of standards and samples was added to the plate. Plate was sealed and incubated for 2 hours at room temperature. The content of the wells was emptied and washed four times with wash buffer. 100 µl of biotin conjugated detection antibody was added to each well and plate was incubated at room temperature for 2 hours. The plate was again washed four times with wash buffer. 100µl of diluted streptavidin- HRP (horse radish peroxidase) was added to each well and plate was incubated for 30 minutes at room temperature. The plate was washed four times. 100µl TMB (3, 3', 5, 5'-tetramethylbenzidine) substrate was added and the plate was incubated in dark for 15 minutes. 100µl of stop solution was added to each well. Absorbance was measured at 450 nm using ELISA reader. A standard curve was plotted.

## IV. Evaluation of IL-6 and IL-10

IL-6 and IL-10 levels in the serum samples were determined by ELISA method using kits from Orgenium laboratories, Finland were used. 50 µl/well of standards and samples were added to the plate followed by 50µl of green colored biotin antibody was added to each well and incubation at room temperature for 1 hour 30 minutes. The plate was washed five times with wash buffer. 100µl of streptavidin- HRP was added to ach well and plate was incubated for 30 minutes at room temperature. The plate was washed five times. 50µl TMB substrate was added and the plate was incubated in dark for 20 minutes. 25µl of stop solution was added to each well. Absorbance was measured at 450 nm using ELISA reader. Values were obtained by plotting a standard curve.

## V. Evaluation of Cortisol

ELISA was used to determine cortisol levels. EIA-STEROID-CORTISOL kit was used to determine the values. 25 µl of the appropriate Calibrators, Control serum and patient samples were pipette into the assigned well.100 µl of conjugate was added to all wells. Microplate was swirled gently for 30 seconds and then covered with a lid or a protective film and incubated for 60 minutes at room temperature (20-$25^0$C). The wells were washed 5 times with 300 µl of working washing solution per well.100 µl of TMB-Substrate was pipette into each well at timed intervals. The plate was incubated for 25 minutes at room temperature in the dark.150 µl of stopping reagent was added into each well and mixed for 5-10 seconds. The absorbance was taken on the micro plate reader at 450 nm within 20 minutes after stopping reaction. Then a standard curve was plotted.

### VI. Evaluation of total cholesterol

Levels of total cholesterol were determined spectrophotometrically using Cholesterol Kit from Agappe. 1000 µl of reagent 1 was added to all the tubes followed by10 µl of reagent 2. Then 10 µl of samples were added to sample tubes and were incubated at 37$^{o}$C for 10 minutes. Absorbance was measured at 505nm.

### VII. Evaluation of stress

All subjects were asked to fill a stress Performa and then scoring was done. Scores above 20 were considered as indicators of high stress levels. They were asked to give the marking from zero to five for irritability, anxiety etc. Questions were related to problems they faced in handling their emotions according to the situations of their life in the past few months.

## RESULTS

Table I and table II show comparisons of different parameters as observed in male and female patients and controls respectively. Statistically significant increase in levels of IL-6, TNF-α and IFN-γ were found in T2DM male patients as compared to the healthy controls ($p<0.05$). The levels of IL-4 and IL-10 were found to be decreased in T2DM male patients as compared to healthy controls ($p<0.05$). Total cholesterol levels of patients were found to be slightly increased as compared to healthy controls ($p>0.05$). Cortisol and stress levels were found to be similar ($p>0.05$).

**Table I-Comparisons of Male T2DM Patients and Healthy Controls**

| CHARACTERSTICS | MEAN ± STANDARD DEVIATION | | p value |
|---|---|---|---|
| | PATIENT | NORMAL | |
| **IL-4(pg/ml)** | 86.36±22.99 | 151.35±31 | 0.000000197 |
| **TNF-α(pg/ml)** | 1145.45±313.5 | 436.25±216.19 | 0.000000320 |
| **IFN-γ(pg/ml)** | 561.81±340.58 | 450.5±172.16 | 0.000000169 |
| **IL-6(pg/ml)** | 177.18±33.45 | 70.92±14.13 | 0.000000119 |
| **IL-10(pg/ml)** | 17.45±7.03 | 79.14±12.84 | 0.000000413 |
| **Cortisol(nmol/l)** | 656±604.77 | 476.35±514.87 | 0.220 |
| **Total Cholesterol(mg/dl)** | 206.09±58.73 | 224.69±99.76 | 0.283 |
| **Stress Score** | 18.72±5.29 | 17±5.4 | 0.215 |

**Table II-Comparisons of Female T2DM Patients and Healthy Normals**

| CHARACTERSTICS | MEAN ± STANDARD DEVIATION | | P value |
|---|---|---|---|
| | PATIENT | NORMAL | |
| **IL-4(pg/ml)** | 85.95±31.32 | 157.72±29.73 | 0.000000597 |
| **TNF-α(pg/ml)** | 1231.90±417.17 | 524.27±160.30 | 0.000000103 |
| **IFN-γ(pg/ml)** | 645.23±451.38 | 533.18±388.83 | 0.000000185 |
| **IL-6(pg/ml)** | 177±43.4 | 83.72±15.83 | 0.000000158 |
| **IL-10(pg/ml)** | 14.62±6.52 | 76.90±13.99 | 0.000000786 |
| **Cortisol(nmol/l)** | 544.19±460.46 | 408.72±352.07 | 0.454 |
| **Total Cholesterol(mg/dl)** | 227.88±60.65 | 178.18±104.38 | 0.086 |
| **Stress Score** | 19.57±2.40 | 18±7.2 | 0.260 |

As shown in table III IL-4 and IL-10 showed negative correlation with stress (r is negative) whereas IL-6, TNF-α, IFN-γ and cortisol showed positive correlation with stress (r is positive).

**Table III Correlation of Different Parameters with Stress**

| PARAMETERS | CORRELATION COEFFICIENT (r) | | | |
|---|---|---|---|---|
| | Male patients | Male controls | Female patients | Female controls |
| **Stress- IL-4** | **-0.24** | **-0.31** | **-0.61** | **-0.60** |
| **Stress- IL-6** | **0.37** | **0.25** | **0.59** | **0.51** |
| **Stress- IL-10** | **-0.23** | **-0.36** | **-0.54** | **-0.53** |
| **Stress- TNF-α** | **0.61** | **0.51** | **0.28** | **0.17** |
| **Stress- IFN-γ** | **0.56** | **0.54** | **0.35** | **0.72** |
| **Stress- cortisol** | **0.84** | **0.81** | **0.54** | **0.94** |

As shown in table IV IL-4 and IL-10 showed negative correlation with cholesterol levels (r is negative) whereas IL-6, TNF-α, IFN-γ and cortisol showed positive correlation with cholesterol levels (r is positive).

**Table IV-Correlation of Different Parameters with Lipid Profile**

| PARAMETERS | CORRELATION COEFFICIENT (r) | | | |
|---|---|---|---|---|
| | Male patients | Male controls | Female patients | Female controls |
| **Total cholesterol- IL-4** | **-0.27** | **-0.26** | **-0.57** | **-0.54** |
| **Total cholesterol- IL-6** | **0.41** | **0.43** | **0.54** | **0.47** |
| **Total cholesterol- IL-10** | **-0.19** | **-0.41** | **-0.47** | **-0.44** |
| **Total cholesterol-TNF-α** | **0.54** | **0.47** | **0.21** | **0.11** |
| **Total cholesterol- IFN-γ** | **0.65** | **0.66** | **0.41** | **0.65** |
| **Total cholesterol-cortisol** | **0.89** | **0.75** | **0.49** | **0.42** |

## DISCUSSION

T2DM is an independent risk factor for coronary artery disease and the risk of coronary artery disease is increased by three-four folds in T2DM patients as compared to non-diabetic ones. Hyperlipidemia and altered lipid metabolism is commonly seen in diabetes. Results of this study establish a positive correlation between cholesterol and levels of pro-inflammatory cytokines which in turn pose a risk to development of diabetes.[15, 16]

Stress is a potential contributor to chronic hyperglycemia in diabetes and has long been shown to have major effects on metabolic activity. Energy mobilization is a primary result of the fight or flight response. Stress stimulates the release of various hormones, which can result in elevated blood glucose levels.[11] In diabetes, as a result of the relative or absolute lack of insulin, stress-induced increases in glucose cannot be metabolized properly.

Cortisol is a steroid hormone released in response to stress which increases blood sugar through gluconeogenesis, suppress the immune system, and aid with fat, protein, and carbohydrate metabolisms. The relationship between cortisol and insulin is important to energy balance, and can become dysregulated with chronic stress which can lead to inhibition of insulin secretion from pancreatic β-cells leading to insulin resistance.[17-20, 23]

In this study increased levels of cortisol along with the stress score (in stress Performa filled) of patients and controls were used to demonstrate the increased levels of stress in a particular individual as cortisol levels showed a high level of correlation with stress irrespective of the person being a patient or a healthy control.

It has been observed earlier that that psychological stress may affect the production of pro-inflammatory and immune-regulatory cytokines. Psychological stress significantly increases the stimulated production of TNF-α, IL- 6 and IFN-γ. A lower production of the negative immuno-regulatory cytokines, IL-10 and IL-4 has also been observed under stress.[21, 22]

The results obtained show positive correlation of stress with IL-6, TNF-α and IFN-γ whereas negative correlation with IL-4 and IL-10 in both patients and healthy controls.

**Ethical approval**: "All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."
**Informed consent**: "Informed consent was obtained from all individual participants included in the study."

## CONCLUSION

Diabetes mellitus type 2 is one of the fastest growing diseases globally because of the factors that contribute to the path-physiology of this disease. Stress being a major contributor of this disease has become a part of everyday life. People of every age and group are suffering from one or the other kind of psychological stress. Fast moving life also contributes to stress. Today, the eating habits of people are changing. It is an era of fast food. People do not have time to exercise and burn the calories they have taken in the form of fast food. The combined effect of all these contributes to the increased risk of various diseases or making the conditions even more worse for those who are already suffering from the disease.
It can be concluded from the results that stress plays a major role in T2DM. Stress hormones like cortisol are increased in response to stress and then they regulate the levels of pro-inflammatory as well as anti-inflammatory cytokines. Stress further worsens the condition because the regulatory mechanisms of these inflammatory cytokines are found to be disrupted. Healthy persons with increased levels of stress and stress hormones can be considered to be at increased risk for T2DM.
Eating habits and exercise contribute a lot to the lipid profiles of a person. Under stress it has been observed that people tend to eat more due to increased appetite and cravings for high calorie carbohydrates. This leads to increased BMI and consequently increased cholesterol levels, which are potent factors for increased risk of T2DM.

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## CONFLICT OF INTEREST

Conflict of interest declared none.

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# Haematological alterations due to *Crataeva nurvala* leaf extract on albino rats

**SHIVKUMAR PATEL*, VINITA AHIRWAR, SAPNA RANI AND KUSUM SINGH**

Department of Zoology, Bundelkhand University, Jhansi (UP), India.

* Corresponding author email: shivzoology@gmail.com

## ABSTRACT

Medicinal plants have been used as traditional treatment for numerous human diseases for thousands of years in many parts of the world. In rural areas of developing countries, herbal materials continue to be used as the primary source of medicines. About 80% of the people in developing countries use traditional medicines for their primary health care. At least 122 compounds, 80% of which were used for the same or related ethnomedical purposes, have been derived from 94 species of plants. There are approximately 5,00,000 plant species occurring worldwide, but only 1% has been phytochemically investigated. There is great potential for discovering novel bioactive compounds from the rest of the plant kingdom.*Crataeva nurvala* commonly known as 'varuna' is considered valuable in treating vata (blood flow, waste elimination and breathing), pitta (fever and metabolic disorders), and kapha (joint lubrication, skin moisture, wound healing, strength and vigor, memory loss, heart and lung weakness and weak immune systems). In the Unani system of medicine, the bark of the plant is used to promote appetite and to decrease the secretion of bile and phlegm. Presently, herbal medicine practitioners primarily use varuna to treat kidney and prostate problems. The bark of the tree is particularly used to treat infections of the urinary tract, kidney stones as well as benign prostatic hypertrophy (BPH). Researches undertaken by scientists have demonstrated that this particular herb's actions support the cardiovascular system by properly maintaining the suppleness as well as openness of the arteries.
Present study is designed to evaluate haematological alterations due to *Crataeva nurvala* on albino rats at a dose of 250 mg/kg once as well as daily for 7, 14 and 21 days. It was noticed that RBC count, Hb% and Blood sugar was decreased due to administration of crude extract however level of SGPT, SGOT and WBC count has increased significantly. The extract does not seem to be toxic since the results show recoupment after the dose was withdrawn.

## KEYWORDS

*Crataeva nurvala***,** Blood Sugar, SGPT and SGOT

## INTRODUCTION

India has a glorious tradition of health care system based on plants, which dates back to *Vedic Era*. In *RigVeda* which is the oldest known repository of human knowledge and wisdom (4500-2500 BC), mention about hundred medicinal plants used by the Aryans while in *Charak Samhita* (1000 BC), records the use of

over 340 drugs of vegetable origin.Medicinal plants have curative properties due to the presence of various complex chemical substance of different composition, which are found as secondary plant metabolites in one or more parts of these plants. These plant metabolites, according to their composition, grouped as alkaloids, glycosides, corticosteroids, essential oils etc.

The World Health Organization has estimated that 80% of the populations of developing countries, being unable to afford pharmaceutical drugs, rely on traditional medicines, mainly plant-based, to sustain primary health care needs. India, one of the most medicoculturally diverse countries of the world, has a respected, time-honored tradition of using medicinal plants in health care. The main traditional systems of medicine in India include Ayurveda, Unani, and Siddha. Literally translated, Ayurveda means the science of life. Among ancient civilizations, India has been known to be rich repository of medicinal plants. The forests of India are the principle repository of large number of medicinal plants and aromatic plants. In 5000 BC RigVeda records 67 medicinal plants, Ayurveda has identified 81 species in 4500-2500 BC, and about 2900 species were reported by Atharveda were as Charak Samhita (700 BC) and Sushreet Samhita in 200 BC had described properties and use of 1100 and 1270 species respectively.

The discovery of a novel chemical component from medicinal plants may form the basis of development of various therapeutic agents with better activity. More than 500 medicinal plants have been reported to possess medicinal properties in Bangladesh and *Crataeva nurvala* (family: Capparidaceae) is one of the most common species among them. The plant *Crataeva nurvala* belonging to the family Capparidaceae is a leafy small to medium sized soft wooded tree with fragrant white flowers and oblong to rounded hard fruits, grows on the banks of canals, rivers, lakes etc[1]. The evergreen tree grows widely in all parts of Bangladesh, Pakistan, India, Philippine, South America, China, and Africa. The common names are Borun or Bonna Pithagola (Bengali), Barna (Hindi), Varuna (Sanskrit), three leaved caper (English)[2]. The bark of the tree is an important drug for problem affecting the kidneys and bladder. It is especially effective in the urinary complaints, kidney and bladder stones (inhibit the formation of stones), fever, vomiting and gastric irritation. It also acts as contraceptive and oxytocic; juice of bark is given to women after childbirth. Root and bark are also laxative and lithontriptic. They increase appetite and bilary secretion[3]. Leaves are externally rubefacient and used in rheumatism; internally they are given as febrifuge and tonic. In the Ayurvedic system of medicine, the general health and well-being of an individual is governed by a balance of the five major elements of nature: space, air, fire, water, and earth. All body processes are regulated by equilibrium among the three doshas viz ; vata, pitta and kapha.

*Crataeva nurvala* is considered valuable in treating vata (blood flow, waste elimination and breathing), pitta (fever and metabolic disorders), and kapha (joint lubrication, skin moisture, wound healing, strength and vigor, memory loss, heart and lung weakness and weak immune systems). In the Unani system of medicine, the bark of the plant is used to promote appetite and to decrease the secretion of bile and phlegm.

Phytochemical studies showed that stem bark of the plant contains saponins, flavonoids, sterols and glucosilinates and ceryl alcohol, friedelin, cadabicine diacetate, lupeol, betulinic acid and diosgenin[4]. Fruits contain glucocapparin, beta-sitosterol, triacontane, triacontanol, cetyl and ceryl alcohol. Leaves contain L-stachydrine, dodecanoic anhydride, methyl pentacosanoate, kaemferol-0-α-D-glucoside and quercitin-3-0-α-D-glucoside[5]. Root bark contains rutin, quercitin, lupeol, varunol and β- sitosterol. Presence of alkaloids has been reported in bark and stems.

Varuna is available in several varieties and may also be used as an ingredient in other remedial products. The use of varuna or any medication prepared with it is generally considered to be safe and without any adverse after effects provided the herbal medication is taken according to the dose recommended by physician or

healthcare provider. It is important to bear in mind that this particular herb is not meant to be an alternate or a replacement for knowledgeable medical recommendation or care. However, the safety of the herb's use in children, pregnant women, nursing mothers as well as individuals suffering from acute kidney or liver problems is yet to be ascertained. Varuna may be taken internally in various forms - tea or even as a tincture. This herb is promptly and easily available with any Ayurvedic practitioner and, hence, should always be taken as per the instructions or directions of the physician or healthcare provider who is recommending its use.

## MATERIALS AND METHODS

**Plant Materials:** *Crataeva nurvala*

**Collection of plant material and preparation of extract:**

The leaves and small twigs were collected from Narayan bagh, Shivajinagar Colony and adjacent areas and dried in shade till total moisture is removed from the plant. Then further it is extracted with Ethanol by maceration process. The plant is air dried and grounded to powered form. The powered plant material is extracted with ethanol in a soxhlet extractor to drying yielding a semisolid mass.

**Animals Used**

The present study was carried out at Department of Zoology, Institute of Basic Science, Bundelkhand University Campus, Jhansi (UP). The study was conducted on sexually mature, Albino rats of Wistar strain (150 ± 10gm/kg b.wt), purchased from DRDE (Defense Research Development Establishment) Gwalior, Government of India. Prior to study, the ethical clearance was obtained from the animal Ethical Committee, Proposal No. ICMR, BU/Pharm/IAEC/11/051. After purchasing the animals were housed in animal house having standard conditions, at a temperature of $25^0$C to $30^0$C and 12 hours light and 12 hours darkness. They were fed with rat pelleted diet and water *ad-libtum*.
For experimentation animals were randomly distributed into two groups. One serves as experimental and other as control. The rats were weighing about 150 ± 10gm were used for study. Both groups were having equal number of rats. Experimental group will receive test material and control will receive vehicle only. Autopsy of both groups will also be performed on the same day, which is done by chloroform if rat is to be sacrificed and by diethyl ether if to keep alive.

**Preparation and administration of dose:**

Doses were prepared in Gum Acacia in distilled water. The dose of the extract of *Crataeva nurvala* plant was prepared according to 250 mg/kg body wt. Then it is given orally to rats for different duration and their effect was studied after 7, 14, and 21 days of treatment. The dosages were given to the animals via oral route through gastric feeding needle.

**HAEMATOLOGICAL STUDIES**

After single and daily administration of dose for the intervals of 7, 14 and 21 days, Anesthesia is given to animals by using diethyl ether. The blood samples were collected from each rat by puncturing the optical vein of rat eye i.e. Retro orbital Plexus with the help of capillaries. The collected blood samples were analyzed for haematological parameters. Following hematological studies were done on the day of autopsy.

**1- Haematocellular Components**

**(a)** Red Blood Cell Count (Naubaur's Chamber)
**(b)** White Blood Cell Count (Naubaur's Chamber)
**(c)** Haemoglobin Percentage (Sahli's Apparatus)
**(d)** Erythrocyte Sedimentation Rate (Wintrobe and Landsberg, 1935)
**(e)** Packed Cell Volume (Wintrobe and Landsberg, 1935)

**2- Clinical Chemistry**

**(a)** Blood Sugar (Asatoor and King Method, 1969)
**(b)** SGOT and SGPT (Reitman and Frankel 1957)

## Statistical analysis

The results obtained were expressed as Mean ± S.E. Significance of differences compared to the control groups was determined using Students t- test.

## RESULTS

In the present study effect of *Crataeva nurvala* at a dose level of 250 mg/kg was studied for 7, 14 and 21 days on various haematocellular components and clinical parameters.

### (A) Haematocellular components:

The RBC count showed significant decrease at an earlier duration when the dose was administered once only. However due to daily administration of dose the changes were significant at 21 days duration with respect to control (Graph-1). Similarly results were shown by Haemoglobin percent due to administration of crude extract singly or daily (Graph-3). On the other hand the WBC count has increased due to administration of dose. The changes were more significant due to daily administration of dose for 14 and 21 days with respect to control (Graph-2). The ESR and PCV have also decreased at an earlier duration when dose was given once only, whereas the changes were more significant at longer duration (14 and 21 days) when dose was given daily.(Graph 4 and 5).

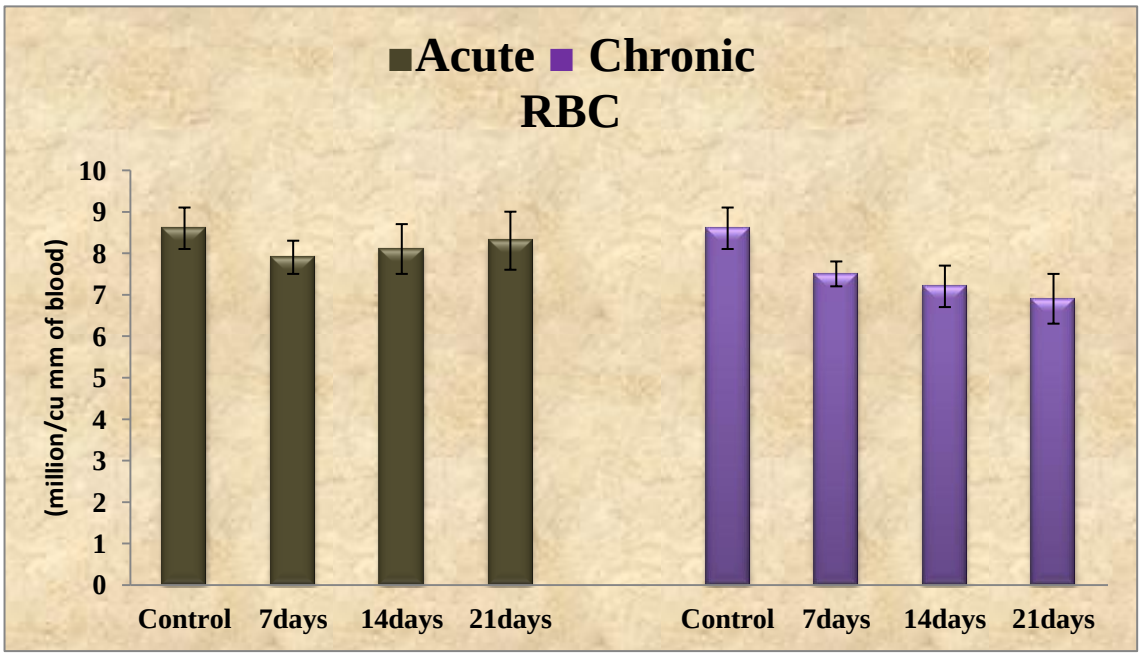
**Table-1 Showing the effect of single and daily administration of *Crataeva nurvala* on haematocellular components of albino rats. Values are Mean ± S.E. and n =6.**

**(* P Vs respective control, P < 0.05)**

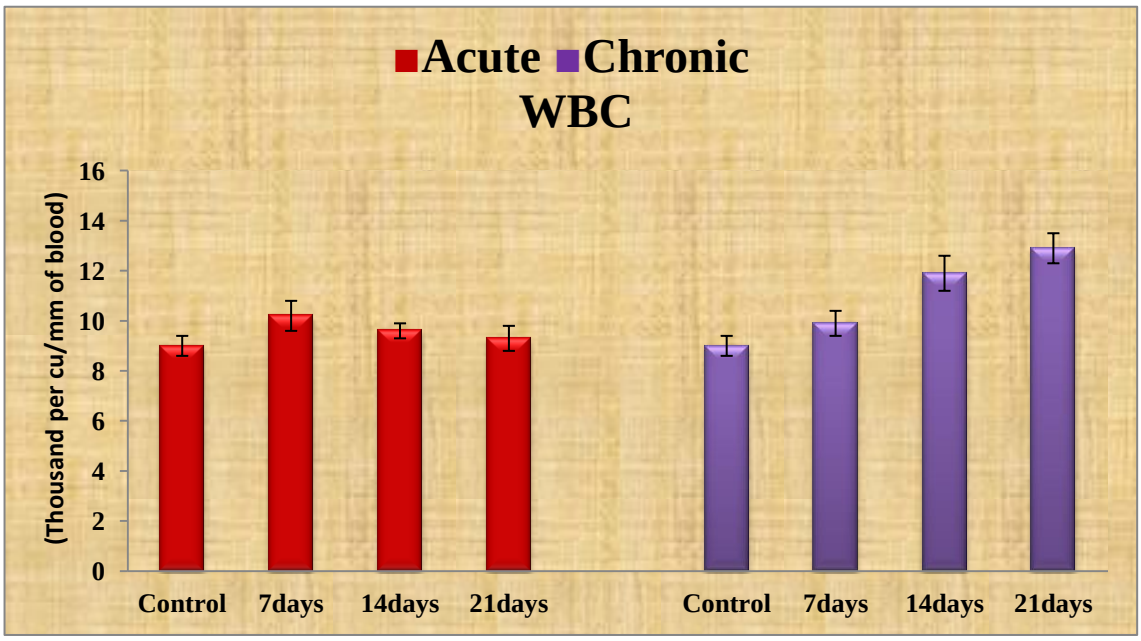
| RBC COUNT (×$10^6$ µl ) Count at the dose level of 250 mg/kg b.wt | | | |
|---|---|---|---|
| **S.No.** | **Duration** | **Single administration** | **Daily administration** |
| **1.** | Control | 8.6±0.5 | 8.6±0.5 |
| **2.** | 7- Days | 7.9±0.4 | 7.5±0.3 |
| **3.** | 14- Days | 8.1±0.6 | 7.2±0.8 |
| **4.** | 21-Days | 8.3±0.7 | 6.9±0.7* |

| WBC COUNT (×$10^3$ µl ) Count at the dose level of 250 mg/kg b.wt | | | |
|---|---|---|---|
| **S.No.** | **Duration** | **Single administration** | **Daily administration** |
| **1.** | Control | 9.0±0.4 | 9.0±0.4 |
| **2.** | 7- Days | 10.2±0.6 | 9.9±0.5 |
| **3.** | 14- Days | 9.6±0.3 | 11.9±0.7 |
| **4.** | 21-Days | 9.3±0.3 | 12.9±0.6* |
| **Hb Percent (g/dl ) at the dose level of 250 mg/kg b.wt** | | | |
| **S.No.** | **Duration** | **Single administration** | **Daily administration** |
| **1.** | Control | 13.2±0.4 | 13.2±0.4 |
| **2.** | 7- Days | 11.2±0.3 | 11.0±0.3 |
| **3.** | 14- Days | 11.6±0.2 | 10.7±0.2 |
| **4.** | 21-Days | 11.6±0.2 | 9.8±0.2* |
| **ESR (mm/hr) at the dose level of 250 mg/kg b.wt** | | | |
| **S.No.** | **Duration** | **Single administration** | **Daily administration** |
| **1.** | Control | 1.7±0.1 | 1.6±0.1 |
| **2.** | 7- Days | 1.4±0.2 | 1.3±0.1 |
| **3.** | 14- Days | 1.5±0.1 | 1.1±0.2 |
| **4.** | 21-Days | 1.6±0.1 | 0.9±0.1* |
| **PCV (%) at the dose level of 250 mg/kg b.wt** | | | |
| **S.No.** | **Duration** | **Single administration** | **Daily administration** |
| **1.** | Control | 41.0±0.9 | 41.0±0.9 |

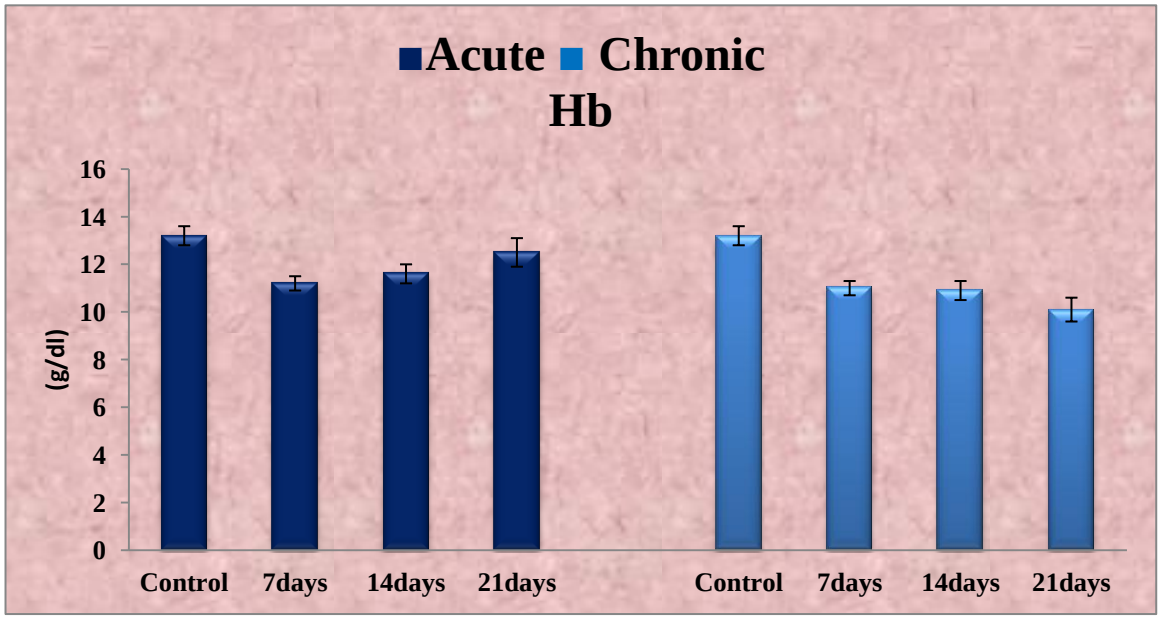
| | | | |
|---|---|---|---|
| **2.** | 7- Days | 38.9±0.6 | 37.4±0.4 |
| **3.** | 14- Days | 39.5±0.7 | 35.8±0.5 |
| **4.** | 21-Days | 40.3±0.5 | 33.9±0.7* |



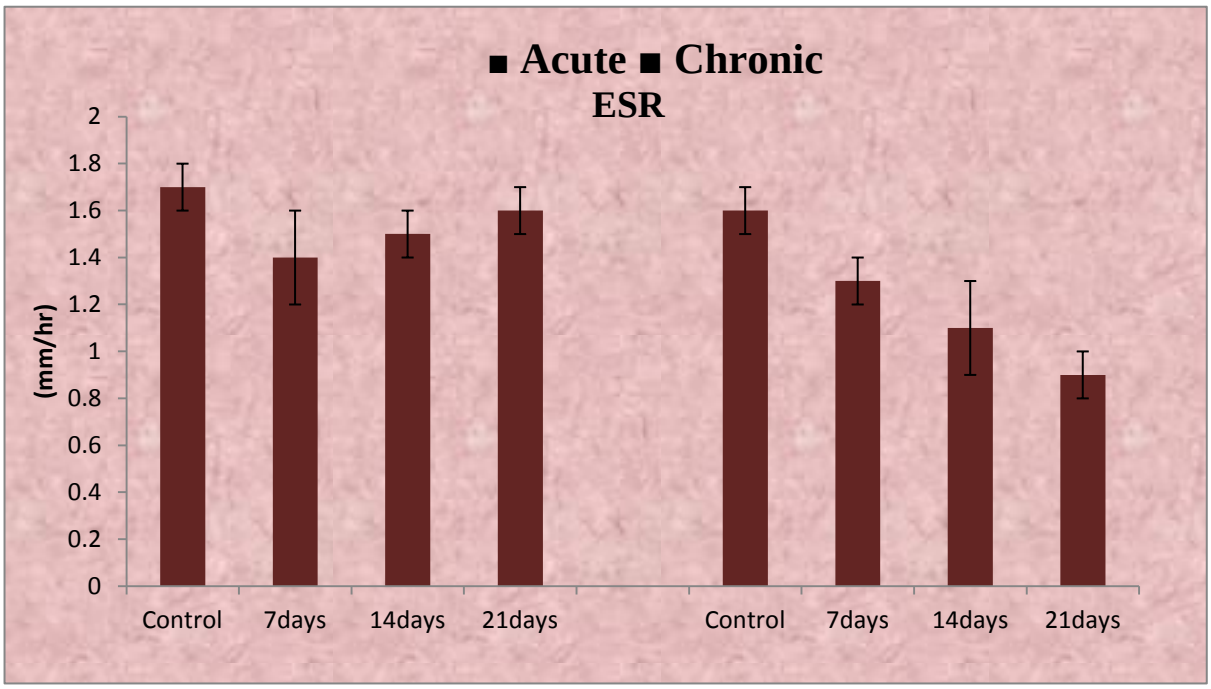

**Graph 1**



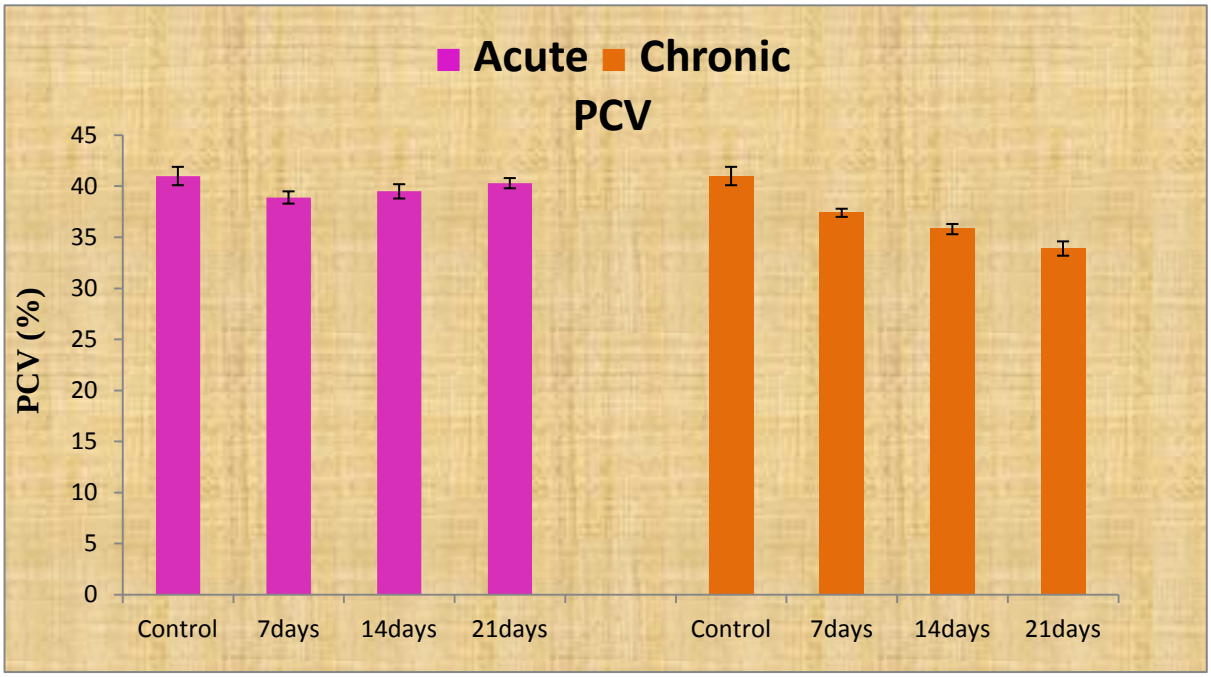

**Graph 2**




**Graph 3**




**Graph 4**



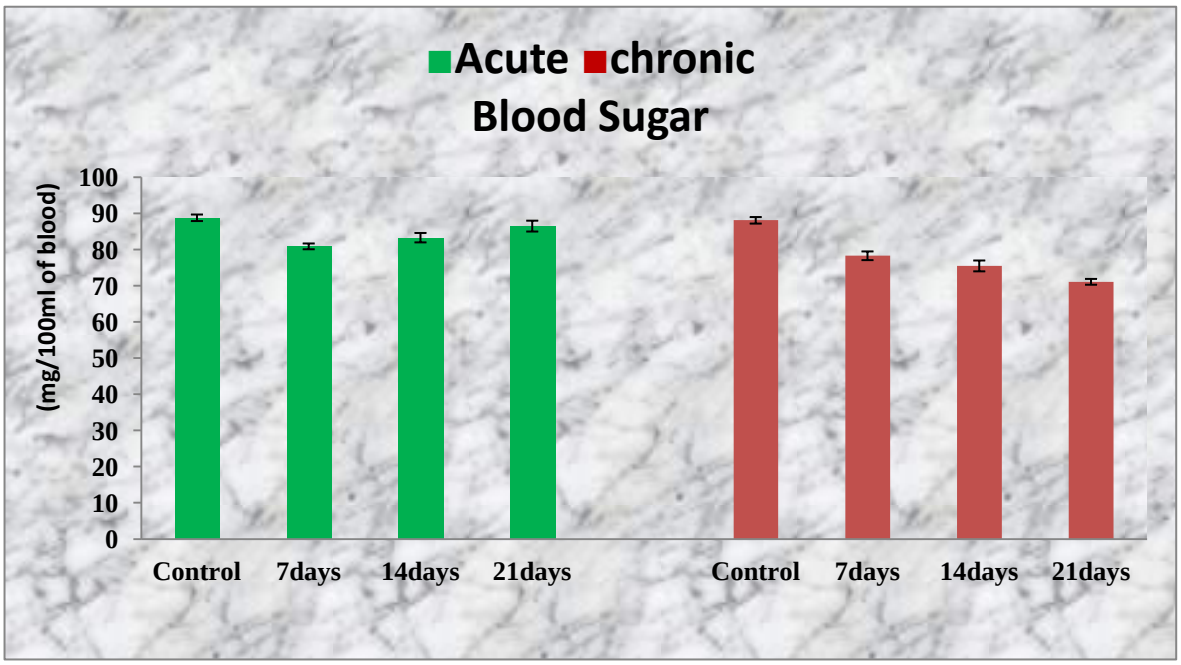

**Graph 5**

## (B) Clinical parameters

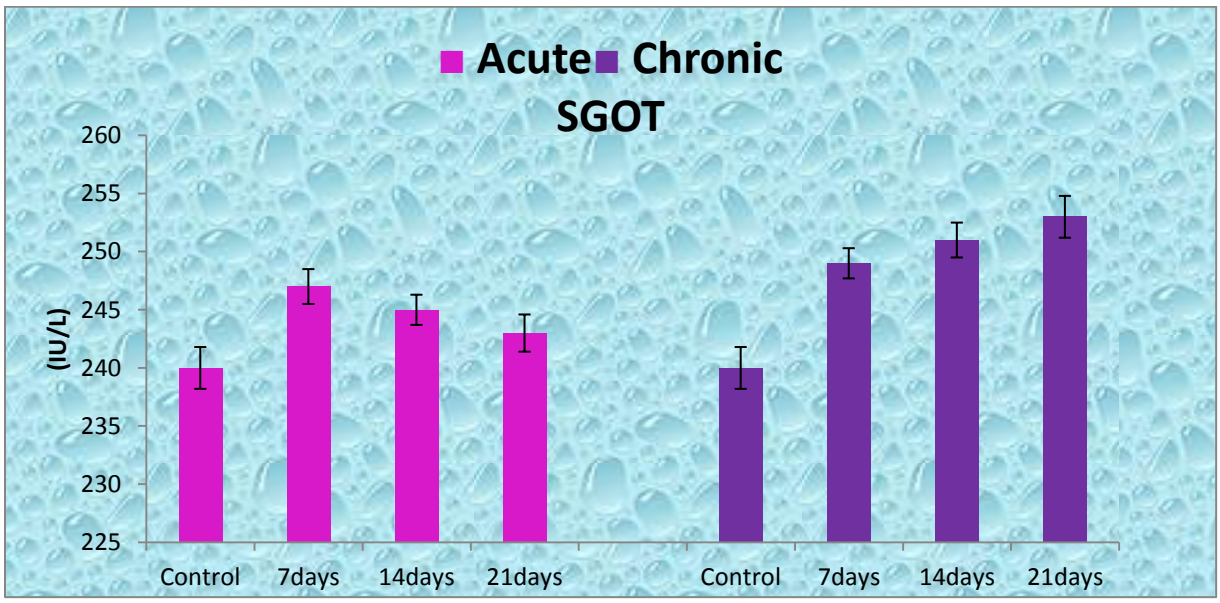
The Blood sugar level, SGOT and SGPT were also studied at same dose and for similar durations. It was noticed that the level of blood sugar was decreased significantly after 21 days of daily administration of crude extract (Graph-6). However the level of SGOT and SGPT has shown significant increase with respect to control at longer duration and due to daily administration of crude extract (Graph-7 and 8) for 14 and 21 days of durations.

**Table-2 Showing the effect of single and daily administration of *Crataeva nurvala* on clinical components of albino rats. Values are expressed in Mean** ± S.E Where n=6
**(P Vs respective control, P < 0.05)**

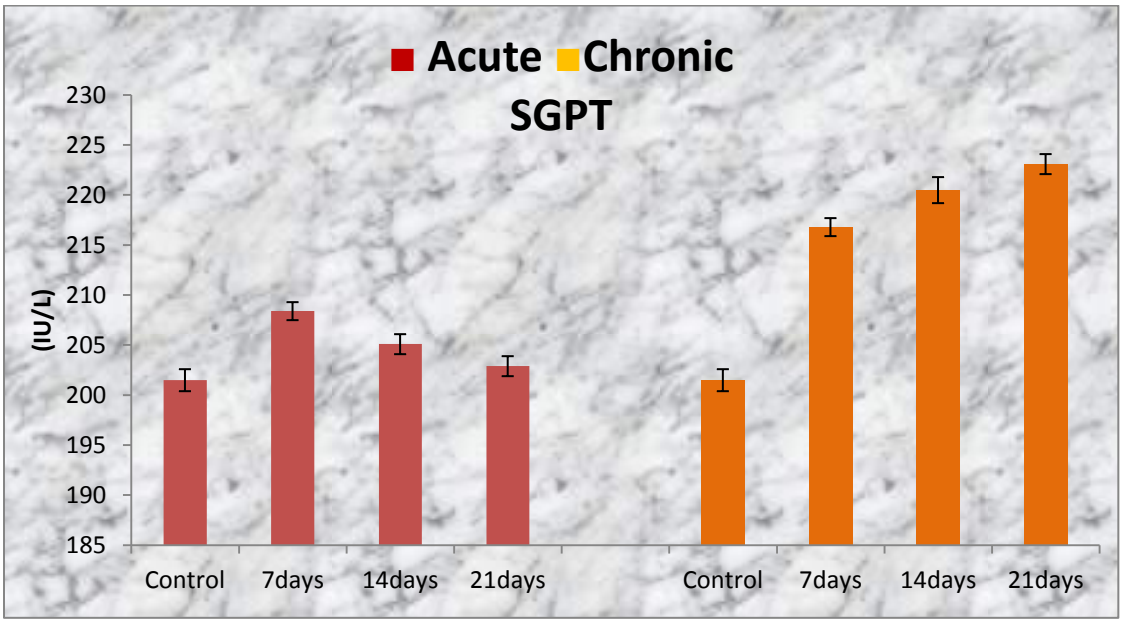
| **BLOOD SUGAR (mg/100ml) Count at the dose level of 250 mg/kg b.wt** | | | |
|---|---|---|---|
| **S.No.** | **Duration** | **Single administration** | **Daily administration** |
| **1.** | Control | 88.8±0.9 | 88.1±0.9 |
| **2.** | 7- Days | 80.9±0.5 | 78.3±0.5 |
| **3.** | 14- Days | 83.3±0.3 | 75.5±0.7 |
| **4.** | 21-Days | 86.5±0.8 | 71.1±0.6* |
| **SGOT (IU/L) Count at the dose level of 250 mg/kg b.wt** | | | |
| **S.No.** | **Duration** | **Single administration** | **Daily administration** |
| **1.** | Control | 240±1.8 | 240±1.8 |
| **2.** | 7- Days | 247±1.5 | 249±1.3 |
| **3.** | 14- Days | 245±1.3 | 251±1.5* |
| **4.** | 21-Days | 243±1.6 | 253±1.0* |
| **SGPT (IU/L) at the dose level of 250 mg/kg b.wt** | | | |
| **S.No.** | **Duration** | **Single administration** | **Daily administration** |
| **1.** | Control | 201.5±1.1 | 201.2±1.1 |
| **2.** | 7- Days | 208.4±0.9 | 216.8±0.9 |
| **3.** | 14- Days | 205.1±1.0 | 220.5±1.3* |
| **4.** | 21-Days | 202.9±1.1 | 223.1±1.0* |




**Graph 6**




**Graph 7**




**Graph 8**

## DISCUSSION

The cellular components viz: RBC, Hb, PCV and ESR) showed significant decrease due to administration of dose at 250 mg/kg daily for 7, 14 and 21 days. Which may cause anaemia of normocystic, normochromic haemolytic type of anaemia[6]. Similar types of results were also reported by various other scientists[7,8,9.] The decrease in haemoglobin percent and then recoupment may be due to the presence of various chemical constituents like flavanoids which act as antioxidants[8]. The herbal plants are also reported to contain glycosides which are known to cause hemolysis of RBC, which may be the cause of decrease in Hb, ESR and PCV [10]. The blood sugar level has decreased significantly due to daily administration of crude extract similar trend in results were reported due to aqueous and alcoholic extract of *Crataeva nurvala* in alloxan induced diabetic rats[11]. A number of other herbal plants are also reported to lower blood sugar[12,13,14] the decrease in blood sugar may be antidiabetic activity of herbs[15,16] as *Crataeva nurvala* was traditionally used as antidiabetic agent. In living organism the hazardious effect of toxicant are well judged through alterations in serum enzymes especially transeaminase (SGOT and SGPT). In Oral administration resulted in gradual normalization of activities of AST and ALT. The values recouped to normal when administration of dose was withdrawn. This showed improved functional integrity of liver. The increase may be due to saponins as one of the chemical constituent[17]. Increase in the activity of SGOT and SGPT was also reported due to various plants product like neem oil, *Ecalipta alba* [18,19].

## CONCLUSION

On the basis of present study it is established that due to oral administration of crude extract of Crataeva nurvala, the blood parameters show significant changes but they recouped to normal when the dose was withdrawn. This is due to various phytochemicals present in the extract.

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# Immune and oxidative stress variation in the patients of hypothyroidism on the basis of hormonal changes

**VINOD SINGH[*1], MAYURI KHARE[1], POONAM SINGH[2]**

[1]Department of Microbiology, Barkatullah University, Bhopal, (M.P), India
[2]Clinical Psychologist, Bhopal, (M.P), India

[*]Corresponding author email: vsingh3@rediffmail.com

## ABSTRACT

Hypothyroidism is most common autoimmune disorders generally caused by an attack on the thyroid gland it results in inflammation and damage of the thyroid cells, linked to hormonal, human immune system under pathophysiology and psychological stress. The study focuses on the level of thyroid and stress hormone and its impact on the immune system, and oxidative stress. Two hundred human subjects were randomly selected. Hormonal analysis was conducted of both thyroid and stress hormones and level of cytokines, both pro and anti-inflammatory and oxidative stress was performed using serum samples. Statistical analysis was performed of all the parameters. The study depicts significant increase in TSH and decrease in T3 and T4 hormones. Also rise in stress hormones was observed. The rise in the hormones resulted in significant increase in the level of pro and decrease in anti-inflammatory cytokines. Increase in oxidative stress have lead to increase in normal process of the body, the production of free radicals, the reactive oxygen species (ROS), GSH, GPx, LPx which naturally created stress in the body. The level of superoxide dismutase (SOD), repaired cells and reduced the damage done by superoxide. It also acts as anti-inflammatory, neutralizing the free radicals maintaining the levels of hormones which proved to be same in hypothyroidism when compared with the normal groups. Apart from the physical and emotional point of view, our study focused on the immunological changes in the body in correlation with other metabolic processes in patients of hypothyroidism caused due to stress.

**KEYWORDS**

Autoimmunity, cytokines, Hashimoto's thyroiditis, oxidative stress, psychological-physiological stress

## INTRODUCTION

All living organisms maintain a complex dynamic equilibrium, or homeostasis, which is constantly challenged by internal or external adverse effects. Stress is defined as a state in which homeostasis is actually threatened or perceived to be so.[1] Homeostasis is reestablished by a complex repertoire of behavioral and physiological adaptive responses of the organism.[2]

Stress may cause immunodepression but may also exert an immunoenhancing effect on cell numbers same stressor may have a positive effect rather than a negative one, depending on its duration or intensity.[3] According to investigations, stress has been classified as acute laboratory stress and natural stress. Stressor induced neurosensory signals are processed in the paraventricular nucleus (PVN) of the hypothalamus. In response to stressors, hypothalamus secrets corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). CRH-containing neurons have different pathways and projections to noradrenergic centers in the brain stem and spinal cord.[4] CRH activates hypothalamic pituitary axis (HPA), leading to release of peptides from the pituitary and adrenocorticotropic hormone, enkephalins, and endrophins. Adrenocorticotropic hormone induces release of glucocorticoids from the adrenal cortex and CRH and central nervous system (CNS) together stimulate noradrenergic neurons resulting in secretion of norepinephrine (NE) by peripheral sympathetical nervous system (SNS) and release of epinephrine (EPI) from the adrenal medulla. The activation of these two neurochemical pathways and release of hormones and transmitters have profound effects of immune function.[5]

Stress hormones influence numerous physiologic processes; they regulate inflammatory diseases, their effects maintaining balance between cell-mediate and humoral immunity and on neurogenic inflammation in peripheral tissues. The hypothalamic-pituitary-adrenal (HPA) axis and SNS represent the peripheral stress system, its activation occurs in CNS in response to distinct blood-borne, neurosensory signals.[6, 7] Homeostasis within the immune system is largely dependent on cytokines, the chemical messengers between immune cells, which play crucial roles in mediating inflammatory and immune responses, for instance, immune challenges such as blood-borne stressor of infections with bacteria release bacterial lipopolysaccharides (LPS), which induce the nuclear factor (NF) mediated secretion of IL-1 and IL-6 and activates HPA axis and stimulates the hypothalamic stress response.[8] The HPA axis regulates a wide variety of immune functions affecting cell trafficking, migration, maturation and differentiation; this regulation is the result of several neuroendocrine pathways including hormones.

Thyroid dysfunction is an important cause of depression.[9] Hypothyroidism is considered a potentially reversible cause of depression, and both disorders have symptoms that may complicate studies attempting to clarify the relationship between them.[10, 11] The changes occur due to hormone involving both stress and thyroid hormones, among these cortisol, prolactin, thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) are the major.[12]

The normal process of the body is the production of free radicals, the reactive oxygen species (ROS) and reactive nitrogen species (RNS) which naturally creates stress in the body, different enzymes act as antioxidant which helps in maintaining the oxidative stress.[13] The level of superoxide dismutase (SOD), one of the antioxidant, repairs cells and reduces the damage done by superoxide. It also acts as anti-inflammatory, neutralizing the free radicals maintaining the levels of hormones which proved to be same in hypothyroidism when compared with the normal groups.

Cytokines encompasses all the immunomodulating agents that trigger inflammation and respond to infections. It includes two classes pro-inflammatory and anti-inflammatory. Stress hormones changes stress system activity through modulating pro or anti-inflammatory cytokines, TNF-α, IFNγ, IL-2, IL-6, IL-4 and IL-10, balance by stimulating or suppressing the progression of this autoimmune disease.[14]

Present study emphasizes on the effect caused due to stress, oxidative stress on an autoimmune hypothyroidism and changes in the immune system by the stressors, also study will be a stepping stone in understanding the thyroid–depression interaction, people suffering from hypothyroidism and the one with psychological disorder.

## MATERIALS AND METHODS

### Study Participants

200 samples, 100 as patients and 100 as normal healthy control, age ranging from 20-60 years from different outdoor patients (OPD) of hospitals of Bhopal. Clinical examination included height and body weight measurements, and body mass index (BMI). Blood pressure, medical histories, bleeding and smoking habits, were recorded, heart disease, diabetes, stroke or other neurological disorders or depression; significant medication use beta-blockers, inhaled beta agonists, hormonal contraceptives, corticosteroid use within prior three months, psychotropic medication use within prior eight weeks; psychiatric hospitalization within past year; was confirmed at the beginning of the study.

### Blood collection and sample preparation

After the Institutional Ethical committee (IEC) cleareance, 10ml blood was withdrawn in serum separation vials from selected subjects after overnight fasting with dry disposable syringe and needle by venous puncture under aseptic conditions. Serum was separated after 30 minutes by centrifuging at 3000 rpm for 10 minutes; this sample was then used for all the assays.

### Hormonal Analysis

All the tests were performed using commercially available enzyme immunoassay kits (from Krishgen Biosystems, Mumbai, India). The level of the hormones in serum sample of the subjects was determined by ELISA.

### Thyroid hormone analysis

The specific thyroid hormone (TSH, T3, and T4) enzyme linked immunosorbent assay (ELISA) applies quantitative sandwich immunoassay. The microtiter plate was pre-coated with a monoclonal antibody specific for the hormone. Standards, samples and control (25µL) were added to the microtiter plate wells and the hormone if present binds to the antibody pre-coated wells. In order to quantitatively determine the amount of hormone present in the sample, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for the hormone was added (100 µL) to each well to sandwich the hormone immobilized on the plate. The microtiter plate was incubated (60 minutes), and then the wells were thoroughly washed by working washing solution 5 times (300 µL) to remove all unbound components. TMB (3, 3', 5, 5' tetramethyl-benzidine) substrate solution was then added (100 µL) to each well. The enzyme (HRP) and substrate were allowed to react for a short incubation period in dark (20 minutes). Only those wells that contain the specific hormone and enzyme-conjugated antibody for it exhibit a change in color. The enzyme substrate reaction is terminated by the addition of the stopping reagent (150 µL) (1 N sulphuric acid solution) and the color change was measured by the ELISA reader at a wavelength of 450 nm.[15, 16]

### Stress hormone analysis:

The stress hormones (cortisol, prolactin) immunoassay was performed using competitive microplate enzyme immunoassay. Plate coated with anti-cortisol antibodies was used. Serum reference, patient specimens and control (25 µL) was first added to the microplate well. Enzyme- conjugate (100µL) was added. The conjugate binds with antibody coated microplate to form an antigen-antibody complex. Unbound conjugate was

removed by working washing solution 5 times (300 µl each time). The enzyme activity in the antibody-bound fraction is inversely proportional to the native stress hormone concentration. The enzyme activity was revealed by a color change in TMB-Substrate solution (100µL). The plate was incubated for 20-30 minutes at room temperature in the dark. Stop Reagent (150µl) was added into each well at the same timed intervals and absorbance was taken by the ELISA reader at 450nm.[17, 18]

**Cytokine Analysis:**

***Proinflammatory Cytokines:***

The procedure is an enzyme-linked immunosorbent assay for quantitative detection of human proinflammatory cytokine (TNF-α, IFN-γ) in cell culture supernatants, human plasma (EDTA, heparin and citrate), serum, cerebrospinal fluid, urine, synovial fluid or other body fluids. Two-fold serial dilution of standards (2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml 125pg/ml, 62.5pg/ml, and 31.3pg/ml) and samples (100 µl) was pipette into the wells. The plate was incubated for 2 hours at room temperature then washed with working washing solution 4 times (300µl each time) to remove unbound labeled antibodies. Detection antibody (100 µl) was pipette to the wells. The plate was incubated for 2 hours at room temperature and were again washed using same working washing solution following same procedure. Streptavidin-HRP (100 µl) was pipette to the wells. The plate was incubated for 30 minutes at room temperature and were again washed using same working washing solution. TMB substrate solution was added (100 µl) to the wells, resulting in color development proportional to the amount of specific cytokine bound. The plate was incubated for 15 minutes at room temperature in dark. The stop reagent changes the color from blue to yellow, and the intensity of the color was measured at 450 nm.

***Anti-inflammatory cytokines:***

Human anti-inflammatory cytokines (IL-4, IL-6, IL-10) ELISA assay employs an antibody specific for human anti-inflammatory cytokine coated on microtiter plate. Two-fold serial dilution of standards (2000 pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml and 31.5pg/m), samples (50 µl) and biotinylated anti-human specific cytokine (50µl) was pipette into the wells. The plate was incubated for 1 hour 30 minutes at room temperature. Cytokine present in a sample is captured by the antibody immobilized to the wells and by the biotinylated specific detection antibody wells were washed with working washing solution 5 times (300µl each time) to remove unbound labeled antibodies. HRP-conjugated streptavidin (100 µl) was pipetted to the wells. The plate was again incubated for 30 minutes at room temperature and were again washed using same working washing solution following same procedure. Following the second wash step, TMB substrate solution (50 µl) was added to the wells, resulting in color development propoportional to the amount of cytokine bound. The plate was incubated for 20 minutes at room temperature in dark. The stop solution (25µl) changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

**Oxidative stress Analysis:**

***Reduced glutathione (GSH)***

The method is based on the fact that DTNB (5,5- dithio bis (2- nitro benzoic acid) is reduced by sulfydryl group (SH), present in reduced glutathione to form one mole of 2-nitro-5- mercapto benzoic acid per mole of SH.[19] 0.2 ml of homogenate was taken. 1.8 ml of chilled Distilled Water and 3.0 ml Sulphosalicyclic acid was

added and vortexed at 2500 rpm for 15 minutes. 0.5 ml of supernatant was taken into a new tube, 1.0 ml of Phosphate buffer and 0.5 ml of DTNB was add and incubated at room temperature for five minutes. Absorbance was taken at 412 nm.

***Superoxide dismutase (SOD)***

Superoxide dismutase catalyses the breakdown of the superoxide free radical ($O_2^-$). This method is based on the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazan complex. Acetic acid in the concentration used was found to arrest the formazan formation. Colour intensity of the chromogen was measured at 560 nm. To a test tube add 1.2ml of sodium pyrophosphate, 0.3 ml of phenazine methosulphate, 0.3 ml of nitrobluetetrazolium, 0.2 ml of homogenate, 0.8 ml of distilled water and 0.2 ml of NADH solution was added. Control was also kept in which water was added in place of homogenate. Voilet coloured was observed and it was incubated at 37˚C for 90 seconds. Reaction was stopped by adding 1 ml of acetic acid. After 10 minutes absorbance was read at 560 nm.

***Glutathione peroxidase (GPx)***

Selenium-containing glutathione peroxidase present in the cytoplasm of cells, catalyzes the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) at the expense of hydrogen peroxide that is converted to water. 0.2 ml of phosphate buffer, 0.2 ml GSH, 0.1 ml distilled water, 0.1 ml of hydrogen peroxide was added in a tube containing 0.3 ml of homogenate. This was incubated at 37˚C for 15 minutes. 0.5 ml of TCA was added to terminate the reaction. Above mixture was centrifuged at 2000 rpm for 15 minutes. After centrifugation, 0.5 ml of supernatant was taken into a new tube. 0.7 ml of Na2HPO4 and 0.2 ml of DTNB. Absorbance was taken at 420nm.

***Lipid peroxide (LPx)***

Malonaldehyde (MDA), a decomposition product of lipid hydro peroxides, used as an indicator of oxidative damage to cells and tissues. Thiobarbituric acid test is one of the most frequently test for measuring the production of peroxidation of fatty acids, membranes and food products. 0.4 ml of tissue homogenate was taken and mixed with 0.1 ml of Sodium Dodecyl Sulphate and vortexed on cyclomixer. This was incubated at room temperature for 10 minutes. 0.75 ml of acetic acid and 0.75 ml of thiobarbaturic acid was added and kept in a boiling water bath for 60 minutes. After cooling 2.5 ml of butanol and pyridine was added and centrifuged at 2500 rpm for 10minutes at room temperature. Carefully absorbance of pink colour layered was taken at 532 nm against blank.

## STATISTICAL ANALYSIS

Statistical analysis were carried out by using the statistical packages for GraphPad Prism 6.0 for Windows (GraphPad Software Inc. California, CA, USA). Mean and standard deviation (SD) were calculated for continuous variables. The group size was small t-test was used to assess the differences of the variables. One tailed p values were considered statistically significant at $p<0.0001$.

## RESULTS

### Thyroid Hormones

The changes in thyroid hormones of patients are compared with the control. Data is represented in Mean ± SEM (n = 100). Values among thyroid patients are significantly higher in case of TSH (P< 0.0001) and significantly not different in case of T3 (P< 0.1116) and T4 (P< 0.0773) from control (Table 1). The values are illustrated in Fig. 1.

**Table 1. Comparative values of thyroid hormones of patients with normal healthy controls.**

| S. No. | Groups | TSH (µIU/ml) Mean ± SEM | T3 (ng/ml) Mean ± SEM | T4 (nmol/l) Mean ± SEM |
|---|---|---|---|---|
| 1. | Control | 2.11 ± 0.09 | 0.83 ± 0.02 | 83. ± 09 |
| 2. | Patients | 9.30 ± 0.83 | 0.97 ± 0.11 | 91.81 ± 5.85 |

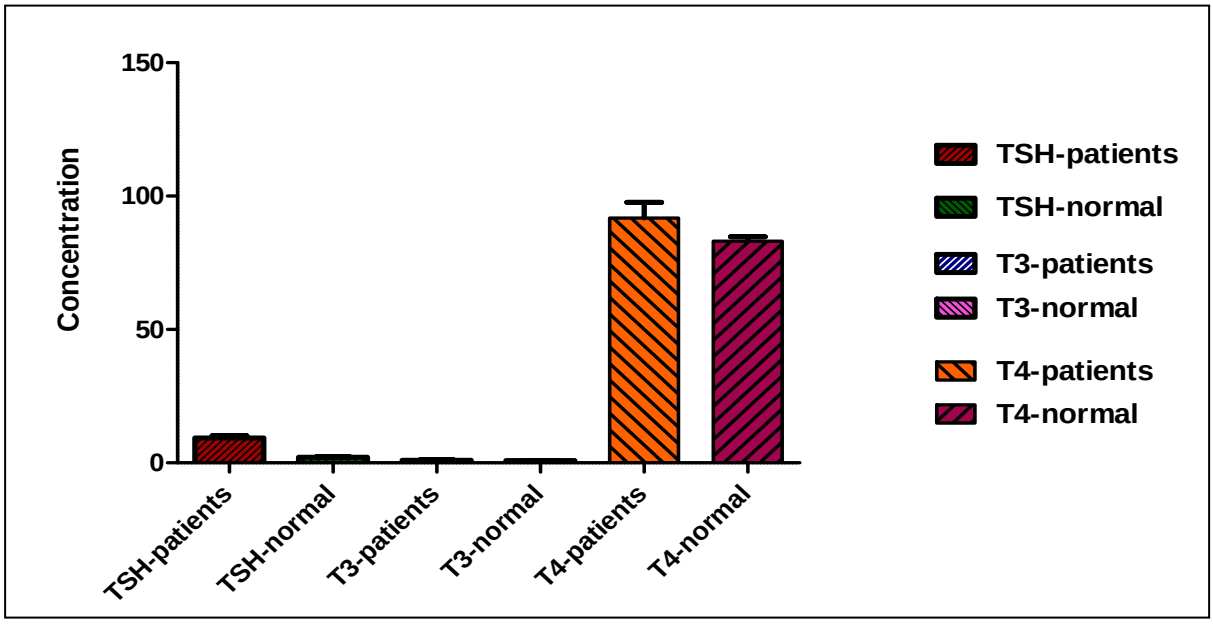


**Figure 1. Mean concentration of thyroid hormones**

**Stress Hormones**

The changes in stress hormones cortisol among patients as compared with normal healthy control in Table 2. Data is represented in Mean ± SEM (n = 100) are highly significantly higher (P< 0.0001) from normal healthy control. (Fig. 2. represents the mean ± sem of the hormone).

**Table 2. Data representing values of stress hormones**

| S. No. | Groups | Cortisol (nmol/l) Mean ± SEM | Prolactin (mIU/l) Mean ± SEM |
|---|---|---|---|
| 1. | Control | 289.30 ± 17.12 | 115.50 ± 19.69 |
| 2. | Patients | 471.4 ± 29.07 | 168.90 ± 10.51 |

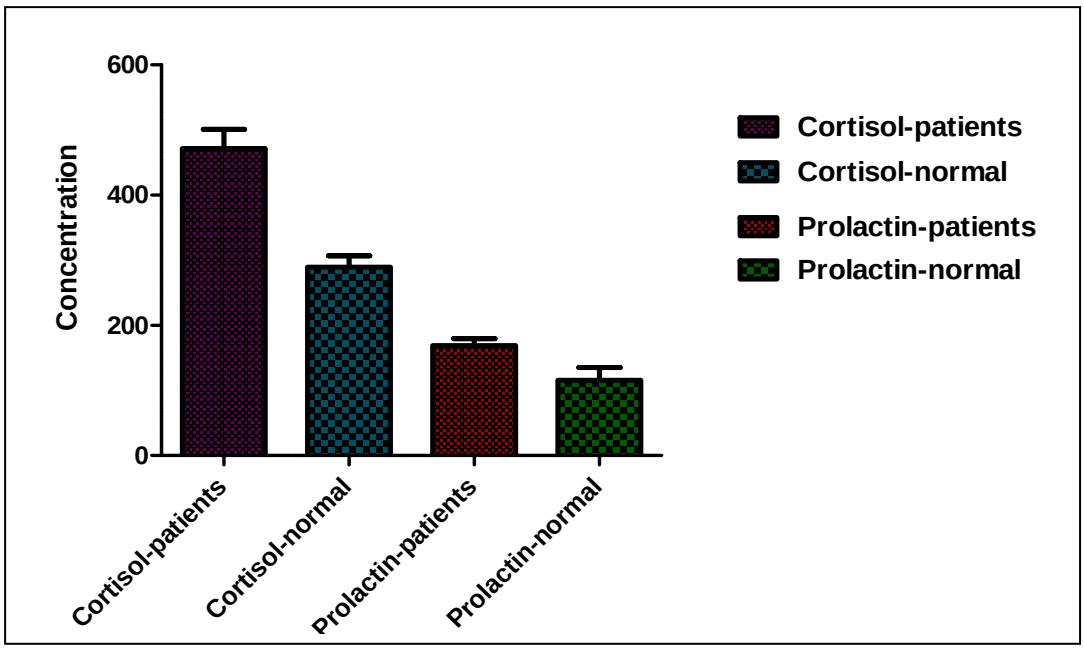


**Figure 2. Mean ± sem values of stress hormones.**

**Cytokines**

**Anti-Inflammatory Cytokines and Pro-inflammatory Cytokines**

The level of changes in cytokines of thyroid patients as compared with that of the normal healthy control. (Data represented in Mean ± SEM (n = 100)) are highly significantly different (P< 0.0001) from normal healthy control Table. 3.
It has been also observed that the level of pro-inflammatory cytokines value (TNF-α, IFN-γ) was significantly raised as comparison to anti-inflammatory cytokines (IL-4, IL-6 and IL-10) Fig. 3.

**Table 3. Values of anti-inflammatory and pro-inflammatory cytokines**

| S. No. | Groups | †IL – 4 (pg/ml) Mean ± SEM | †IL – 6 (pg/ml) Mean ± SEM | †IL – 10 (pg/ml) Mean ± SEM | *TNF – α (pg/ml) Mean ± SEM | *IFN – γ (pg/ml) Mean ± SEM |
|---|---|---|---|---|---|---|
| 1. | Control | 545.6 ± 21.64 | 147.70 ± 8.11 | 82.03 ± 3.59 | 553.9 ± 21.75 | 265.20 ± 10.4 |
| 2. | Patients | 1006 ± 36.64 | 73.78 ± 6.56 | 51.63 ± 5.62 | 1193.0 ± 32.51 | 1081.00 ± 33.8 |

† *Anti-inflammatory cytokines,* * *Pro-inflammatory cytokines*

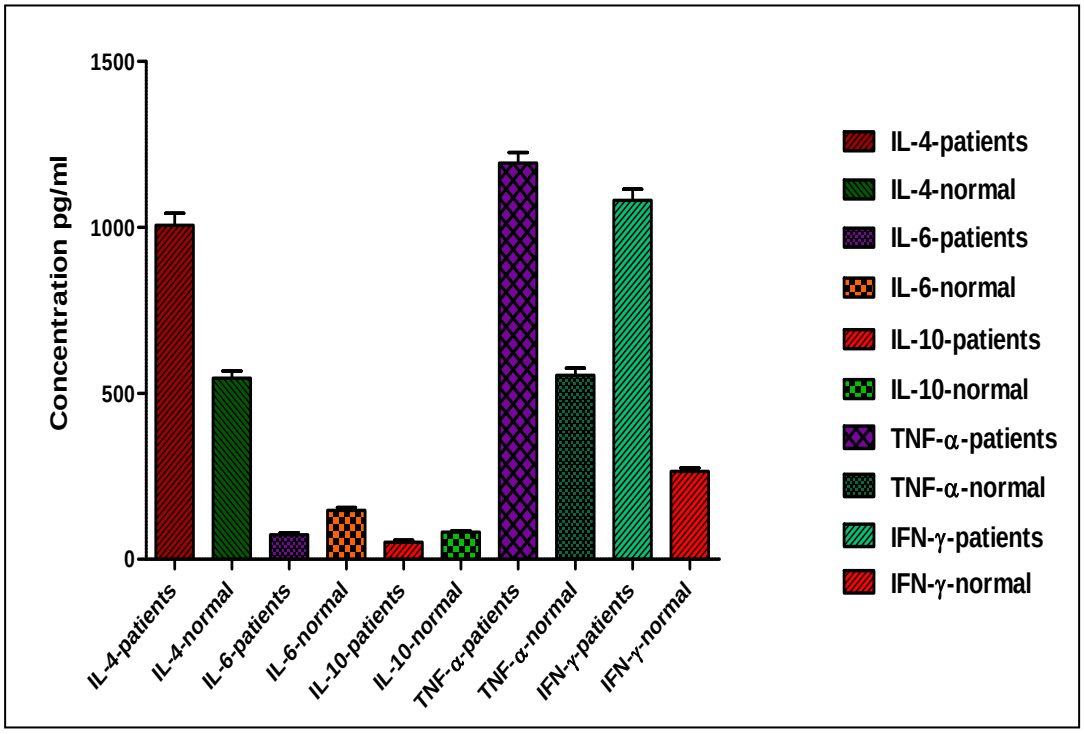


**Figure 3. Concentration of anti and pro-inflammatory cytokines**

**Oxidative Stress**

Depicts changes in oxidative stress in comparison to control. Data represented in Mean ± SEM (n = 100). Values of patients are significantly different in all (P< 0.0001) except GPx (P< 0.0040) from control. Table 4. Fig. 4

**Table 4. Values of oxidative stress compared between control and patients**

| S. No. | Groups | GSH (µmol/l) Mean ± SEM | SOD (Units/ml) Mean ± SEM | GPx (mmol/l) Mean ± SEM | LPx (nmol/l) Mean ± SEM |
|---|---|---|---|---|---|
| 1. | Control | 7.89 ± 0.28 | 174.70 ± 4.35 | 3.14 ± 0.10 | 0.89 ± 0.05 |
| 2. | Patients | 11.23 ± 0.44 | 258.30 ± 4.04 | 2.74 ± 0.10 | 2.78 ± 0.10 |

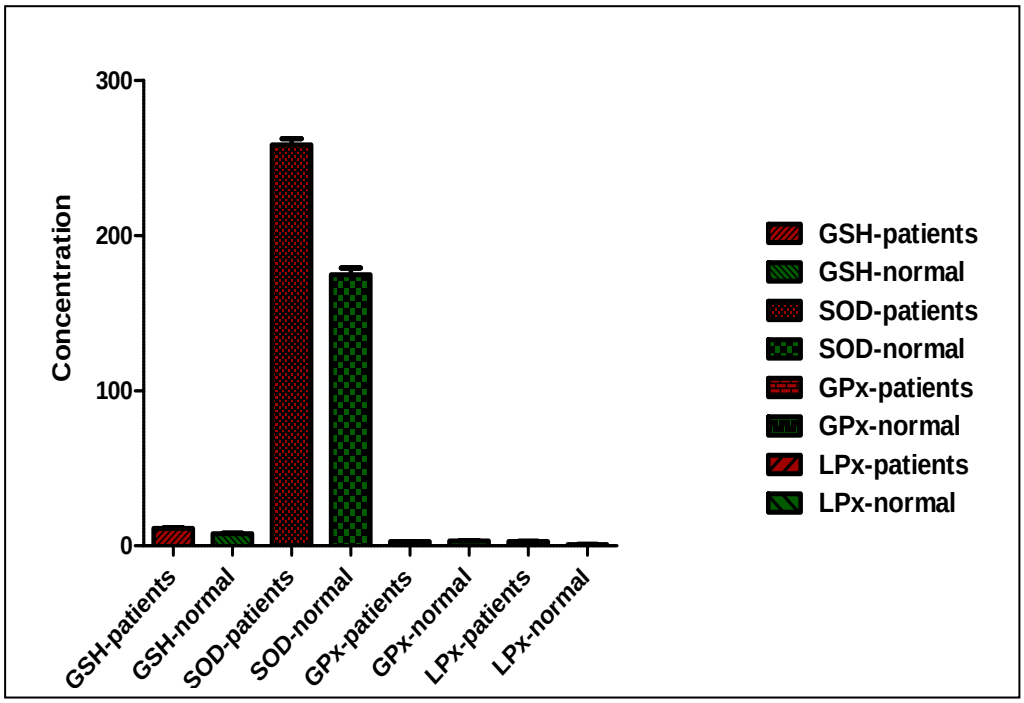


**Figure 4. Overall representation of the concentration of oxidative stress**

## DISCUSSION

The elevated basal levels of stress hormones like cortisol and prolactin have shown association with chronic stress suppress immunity by directly affecting cytokine profiles. Cytokines are communicatory molecules produced primarily by immune cells. [20] Proinflammatory cytokines mediate acute inflammatory reactions. Th1 cytokines mediate cellular immunity by stimulating natural killer cells and cytotoxic T cells, immune cells that target intracellular pathogens (e.g., viruses). Finally, Th2 cytokines mediate humoral immunity by stimulating B cells to produce antibody, which "tags" extracellular pathogens (e.g., bacteria) for removal. In a meta-analysis of over 30 years of research, intermediate that stressors in life style could promote a Th2 shift (i.e., an increase in Th2 cytokines relative to Th1 cytokines).[20] A Th2 shift has the effect of suppressing cellular immunity in favor of humoral immunity. In response to more chronic stressors (e.g., long-term caregiving for a dementia patient), proinflammatory, Th1, and Th2 cytokines become dysregulated and lead both to suppressed humoral and cellular immunity.[21] Intermediate and chronic stressors are associated with slower wound healing and recovery from surgery, poorer antibody responses to vaccination, and antiviral deficits that are believed to contribute to increased vulnerability to viral infections (e.g., reductions in natural killer cell cytotoxicity.[22]

Immune challenges such as infections with bacteria release bacterial lipopolysaccharides (LPS), which induce the nuclear factor (NF) kB mediated secretion of IL-1 and IL-6, which stimulate the hypothalamic stress response.[6, 7, 23] Immune responses are regulated by antigen presenting cells (APC), such as monocytes/macrophages, dendritic cells, and other phagocytic cells that are components of innate immunity, and by the helper T-lymphocytes subclasses Th1, Th2, and Treg that are components of adaptive immunity. Homeostatis within the immune system is largely dependent on cytokines, the chemical messengers between immune cells, which play crucial roles in mediating inflammatory and immune responses. It has also been found that increase in cytokine like (IFN)-gamma, TNF-alpha lead to cell-mediated immunity; whereas increase in IL-4, TL-10 leads to the cell stimulation enhance humoral immunity.[24, 25] Naïve T cells (Th0) are precursors of Th1 and Th2 cells, and IL-12 (produced by APCs) is the major inducer of Th1 differentiation and hence, cellular immunity. Thus, IFN-gamma inhibit Th2, whereas IL-4 and IL-10 inhibit Th1 cell activities. IL-4 and IL-10 promote humoral immunity by stimulating the growth and activation of mast cells and eosinophils, the differentiation of B cells into antibody secreting B cells, and immunoglobulin switching to IgE. Importantly, these cytokines also inhibit macrophage activation, T-cell proliferation, and the production of proinflammatory cytokines.[26, 27]

We have found the significant increase in level of TSH and decrease status in T3 and T4 hormone. As thyroid hormones makes and stores hormones that help regulate the heart rate, blood pressure, body temperature, and the rate at which food is converted into energy.[28] It achieves this by manufacturing the hormones, thyroxine (T4) and triiodothyronine (T3) and secreting them into the blood stream. Thyroid stimulating hormone (TSH) secreted by the pituitary gland stimulates the release of other thyroid hormones T4 and T3 which further enhances all the cells of the body to metabolize at a faster rate.[29] On a broad extent two types of hypothyroidism has been studied, primary hypothyroidism caused by decreased production of T4 and T3 due to thyroid dysfunction increase production of TSH; by pituitary (TSH) or hypothalamic (TRH) disease.[30, 1]

Hypothyroidism is generally caused by an attack on the thyroid gland it results in inflammation and damage of the thyroid cells.[31] We have seen changes due to hormone involving both stress and thyroid hormones, among these prolactin, cortisol, thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) are the major.[32] Hypothyroidism is routinely considered in the differential diagnosis of

depressive and anergic states, and is screened for with determinations of serum thyroxine (T4), trio dothyronine (T3), and basal thyroid-stimulating hormone (TSH).[33] Thyroid failure with its predilection for behavioral presentation is much more likely to manifest as depression or lack of energy to a psychiatrist.

Cytokines are involved in common endocrine diseases, such as diabetes mellitus and autoimmune thyroid disease (ATD). The hormones mediate the differentiation of Th0 (naïve T Helper cells) towards the Th2 humoral immune response to the detriment of the Th1 cell-mediated response. APC's secrete cytokines that mediate Th1 differentiation, however the presence of bacterial products such as LPS that bind to Toll-like Receptors induce the production of IL-1 and IL-6, which cross the blood-brain barrier and trigger the hypothalamic CRH-stress response.[35] In this manner, a blood borne stressor of infectious nature can activate the HPA axis. Th1 effects are mediated by the cytokines IL-12,18,2 and γInterferon and T cells and Macrophages. Th2 responses are mediated by IL-4,6,13 and B Cells, Eosinophils and Mast Cells. CRH: Corticotropin releasing Hormone; NE: Norepinephrin; Th0: Naïve Helper cells; APC: Antigen Presenting Cell; LPS: Lipopolysaccharide; HPA: Hypothalamic-Pituitary-Adrenal Axis.[36]

Cytokines include two classes of it pro and anti inflammatory. We have found stress hormone changes stress system activity through modulating pro or anti inflammatory cytokines. Increase in significant level of pro-inflammatrative cytokines of TNF-α and IFN-γ and significant decrease in IL-4 and IL-10. Stress hormones changes stress system activity through modulating pro or anti-inflammatory cytokines, TNF-α, IL-6, IFN-γ, IL-4 and IL-10, balance by stimulating or suppressing the progression of autoimmune diseases.

Increase in oxidative stress have lead to increase in normal process of the body is the production of free radicals, the reactive oxygen species (ROS) and reactive nitrogen species (RNS) which naturally creates stress in the body. Different enzymes act as antioxidant which helps in maintaining the oxidative stress.[13] The level of superoxide dismutase (SOD), one of the antioxidant, repairs cells and reduces the damage done by superoxide. It also acts as anti-inflammatory, neutralizing the free radicals maintaining the levels of hormones which proved to be same in hypothyroidism when compared with the normal groups

Studies from humans and animal models have revealed significant new insights into the complex role of cytokines in the pathogenesis of AITD. Modulating cytokine responses have yielded highly encouraging results and they hold considerable promise in the treatment of autoimmune diseases. Pro-inflammatory cytokines such as GM-CSF and IL1b can contribute to Foxp3 + Treg expansion, whereas a regulatory suppressor cytokine such as TGF-b can initiate a pathogenic Th17 T cell response. These observations highlight the paradoxical effects of cytokines and their critical roles in maintaining a delicate balance between health and disease. Therefore, additional studies to understand the complex interplay between different cytokines and their effects on the different components of the immune system in the context of a particular disease are essential.

## CONCLUSION

Present era is full of stress that to of environmental, psychological, physiological and physical stress and all of it compound to produce severe impact. Any of above if dominates leads to affect our body physiology directly. Surprisingly out of the above psychological stress, life style pattern and physiological interaction leads to progressive disturbance in producing disease syndrome. Study interprets a significant rise in stress hormones like cortisol and prolactin, leading to immune regulation of body homeostasis. We have found that stress hormone after recognizing the immune cells which promote the mother T and B cells and Th cells type Th1 and Th2 are the most affected. The balance of Th1 and Th2 has been disturbed leading to autoimmune process. Increase in cytokines alpha-TNF and gamma interferon attacked on thyroid cells

destruction and predominates for Th2 mediated immune response promoting antigen specific B cells. These B cells produce anti-TSH receptor antibodies leading to hypothyroidism.

The study concludes that to regulate the hypothyroidism like autoimmune disorder one has to take proper care of compound stress increasing day by day particularly in immune suppression autoimmune conditions. Supportive and suggestive combination therapy in addition to regular treatment will benefit the patient of hypothyroidism because stress, immunity and infection play a major role not only hypothyroidism but may lead to other autoimmune diseases.

## CONFLICT OF INTEREST

There are nonfinancial competing interests (political, personal, religious, ideological, academic, intellectual, commercial, or any other) to declare in relation to this manuscript.


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# The future of biomass energy in Shahdol division of Madhya Pradesh, India

**BALRAM PRAJAPATI, SMITA VERMA AND MAMTA PRAJAPATI**

Pt. S. N. S. Govt. P. G. College, Shahdol (M.P.)

* Corresponding author email: drmamtaprajapati@gmail.com

## ABSTRACT

Madhya Pradesh is a power starved country with a larger share of its population without electricity. About 85 % of Madhya Pradesh power generation is currently coal based. Though Madhya Pradesh has one of the largest reserves of coal and it can be assumed that Madhya Pradesh power sector will always be principally coal dependent, however, it is very much a fact that this reserve will not last forever. In a world with changing climate, Madhya Pradesh is certainly one of those states which will have to confront severe energy shortages. And energy security not just increases a state's vulnerability to climate change but also limits its ability to adapt to the impacts. It goes without saying that coal based energy in Madhya Pradesh will supplement global carbon emissions and moreover fossil fuel based power generation in centralized manner results in lot of losses in transmission and distribution which means wastage of precious power which is already in short supply. And on top of that, many remote villages are still not connected with centralized grids and meeting their power requirement needs decentralized and distributed power generation options. The obvious solution to all these problems is renewable energy. Given the potential that Madhya Pradesh has, sources like solar, wind, and biomass can be prospective sources of power in times to come, that too without leaving negative impact on the environment. Most common form of the renewable energy is the biomass. Biomass is the term for the living material-plants, animals, fungi, bacteria. Taken together the earth's biomass represents an enormous store of energy. The handling out facility of the solid biomass may also generate heat and the electricity. It has been estimated that just one-eighth of the total biomass produced annually would provide all humanity current demand for energy. To avoid the replenishment of the fossils fuels the much attention is required to pay on identifying suitable biomass species, which can provide higher output energy. During the process of conversion the energy is released from the biomass in Heat form carbon is re-oxidized to carbon dioxide and the plant uses this for growing .Thus it helps to reduce global carbon emissions. Shahdol division of Madhya Pradesh is a rich source of biomass along with fossil fuel-coal. About 79.89% population lives in the rural areas and their main occupation is agriculture which produces a lot of biomass. There is a strong need for the awareness about using the techniques of biomass conversion into energy instead of burning wood and coal. This paper mainly deals with the study of Shahdol division in growing interest as well as techniques for sustainable and renewable energy.

**KEYWORDS**

Biomass, Sustainable, Renewable Energy, Power Generation, Global Carbon Emission.

## INTRODUCTION

Given current trends world energy demand is expected to increase by 50 % by 2030. There are expectations that renewable resources will be able to play a significant role satisfying this future energy demand. Others have a more pessimistic view and forecast that it will not make up even 2% of the total energy mix by 2030. In 2001 global primary-energy consumption was 418 EJ (an exajoule (EJ) is $10^{18}$
joules; for comparison, 1.055 EJ is roughly equivalent to one quadrillion BTU or 172 million barrels of oil equivalent.. Of this, biomass supplied 45 EJ. This is significantly more than the 2% predicted to be used by 2030, but is probably overlooked because about 39 EJ of this was in the form of traditional uses for heating and cooking, which do not enter world trade and are mostly beyond governmental control and taxation. Global biomass production on the earth's land surface is equal to 4,560 EJ (the gross primary production) of which half is lost by autotrophic respiration and decomposition, leaving 2,280 EJ (net primary production or NPP).The currently installed renewable energy capacity of India is nearly 17,000 MW. The lion's share of renewable power generation is of wind power having a current installed capacity of about 11,807 MW3. India has also been endowed with vast solar energy potential. About 5,000 trillion kWh per year energy is incident over India's land area with most parts receiving 4-7 kWh per sq. m per day . Madhya Pradesh is one of the highest biomass producing state of India and it is estimated that the power generation potential from biomass in India is about 19000 MW. Achieving "Sustainable Development" is a formidable challenge in the present world. It concerns technologies that can help manage growth while considering economic, social, and environmental sustenance of the society. In fact there is an urgent need to solve the present problems faced by the society without creating any long term negative impact, which could become a critical issue to resolve for the future generations. Energy need is an important ingredient in the modern economy and modern energy services must be evolved and deployed in all aspects of the development process – e.g., energy and communications, energy and industry, energy and the environment, energy and agriculture, energy and education, energy and public health and safety. Biomass is one such source that can be used to provide sustainable supply of the required energy through biogas, vegetable oil, biodiesel, producer gas, and by directly burning the biomass. Notwithstanding whether the biomass is "waste" of some process or is cultivated specifically as fuel for energy generation, it is considered a "green" technology,
Since-

* The life cycle of the fuel is short (could even be less than three months).

* The net carbon-dioxide emission from the fuel is zero as the CO2 emitted is generated by burning the carbon that the plant had fixed by taking CO2 from the atmosphere and converting it to food (glucose) with the help of photosynthesis.

*The cycle can be water neutral as well. If biogas or biodiesel is produced from biomass, the left over can be put back into the field as high quality manure.

* The remaining biomass could also be used with a gasifier to make producer gas. The ash could be spread in the fields as micro-nutrient, completing its cycle as well.

Biomass, in particular wood, has historically been an important energy source for fires, ovens and stoves. During the Industrial Revolution, coal displaced biomass because of its high energy content and because it is available in large quantities at low cost. At the beginning of the 20th century, oil (which was discovered in the U.S. in 1859) supplied only 4% of the world's energy. Decades later it became the most important energy

source. Especially developed countries are highly dependent on oil, which supplies about 96% of their transportation energy. With world energy demand projected to rise by about 40% from now to 2020, it is possible that natural gas, which supplies almost 25% of the world's energy demand today, overtakes oil as the most important energy source.

**Study area**

The study is confined to the rural areas of Shahdol division which consists of Umariya and Anuppur districts. Total area available for cultivation in these districts are 12056 hectares in Shahdol,5827 hectares in Anuppur, 6970 hectares in Umariya . Waste land available in these areas are 18052 hectares, 17534 hectares and 3628 hectares respectively. The main objective of the study is to evaluate the availability and usage of degraded land/wasteland in the selected districts for production of biomass feedstock for the biomass power generation projects. It will review the techno-commercial and social viability; and the existing policy and regulatory frameworks for energy plantation on wastelands in the areas and at the central level. In addition, it is expected that the study would develop a perspective plan for at least three districts from within the selected areas for the accelerated utilization of wasteland for the production of biomass resources for power generation.

## MATERIAL AND METHODS

**Biomass Potential of Madhya Pradesh**

Madhya Pradesh is one of the highest biomass producing state of India and Shahdol and its neighbouring districts are higher biomass producing areas and it is estimated that the power generation potential from biomass in Shahdol division is about 260 MW .The details of total agricultural biomassgenerated from various crops are as follows. Soyabean stalks; wheat stalks and pod; jowar stalks, cobs and husk; paddy husk and straws; maize stalks and cobs; arhar stalks and husk; mustard husk and stalks; bajra stalks and cobs; gram stalks; guar stalks; masoor stalks; small millets stalks; urad husk and stalks; moong stalks and husk; til stalks; barley stalks and linseed stalks. Total power generation potential estimated is 833.76 MW.

Forest is the second largest land-use in Shahdol division of Madhya Pradesh after agriculture, covering 51.57% of the total land of the division. It has been known that two to three thousand people are engaged in wood fuel collection, making it the largest source of employment in the energy sector of the area. The estimated amount of harvested wood fuel from the forests, and trees growing on homestead, farmlands, and common lands was 100 thousand tons in 2011.

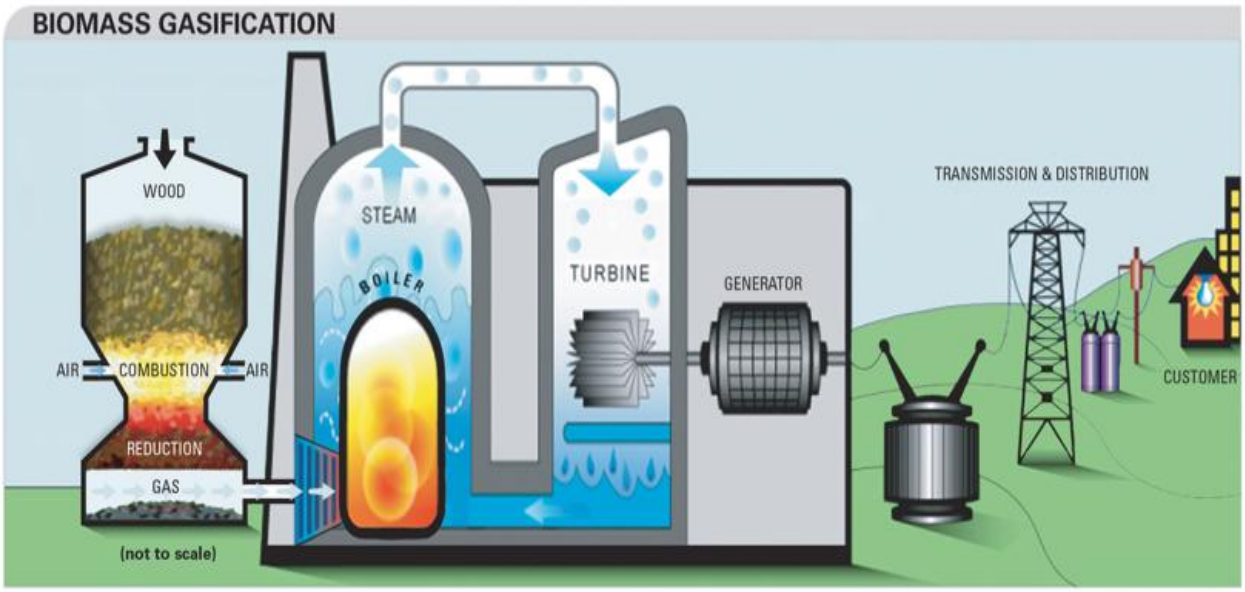
**Table 1. Structure of total available land area in the study area of Shahdol division**

| Name of District | Total Land | Cultivable area | Forest area | Non-agriculture use | Permanent Pastures | Waste land | Barren and uncultivated land |
|---|---|---|---|---|---|---|---|
| Anuppur (in hect.) | 450.3 | 105.2 | 236.7 | 50.1 | 32.8 | 16.6 | 8.7 |
| Shahdol (in hect.) | 561.0 | 231.3 | 227.8 | 44.6 | 6.5 | 40.8 | 10 |
| Umaria (in hect.) | 374.7 | 211 | 76.5 | 32.2 | 15.1 | 28.8 | 21.8 |

It can be advantageous to use biomass instead of fossil fuels for power generation, including lower greenhouse gas (GHG) emissions, energy cost savings, improved security of supply, waste management/reduction opportunities and local economic development opportunities. In order to analyse the use of biomass for power generation, it is important to consider three critical components of the process:
1.Biomass feedstocks
2.Biomass conversion
3.Power generation technologies.
Bioenergy can be converted into power through thermal-chemical processes (i.e. combustion, gasification and pyrolysis) or bio-chemical processes like anaerobic digestion. The gasification-based power generation (based on engine-driven power generation system) is the most attractive one. The advance biomass gasification proposed in this paper is being discussed.

**Gasification: most attractive method**

Gasification-based power generation in the scales of 250 kW to 2 MW can deliver a plant efficiency at 27%–30%. This is at least 30% higher than the efficiency achieved through combustion-based power generation technology in this range. Gasification-based plants are less dependent on water and offer greater flexibility in their operation. Moreover, these power plants offer an option to generate Synthesis Natural Gas (SNG) and hydrogen which by themselves are fuels for applications in cooking and in the transport sector.




This technology provides an easy and elegant solution to generate gas from biomass. Additionally, gasification-based biomass-to-power plants through gas engines provide an opportunity for extraction of additional thermal energy from flue gas as well as from engine jacket cooling. This can be usefully converted into power or using an Organic Rankine Cycle (ORC) and/or into chilled water using vapours absorption cooling thereby further boosting the overall efficiency of conversion. The heart of the technology is based on indirect gasification system. The process of indirect gasification segregates the process of combustion from pyrolysis , which is the main conversion step for biomass to gas. This is established by using a solid heat transfer medium, which acts as a barrier between these two zones. The heat transfer is carried out using solid

circulation between the combustion zone and the pyrolysis zone. The design of the fluidized-bed system allows use of different forms of biomass. The two sections of gasification allow use of two types of biomass (tough biomass and easy-to-handle biomass) and provide basic flexibility. There are a number of other important features in this technology such as use of 'olivine sand as a bed material', which, while acting as the heat transport medium, helps in reducing heavy tars by cracking them catalytically. The design envisages recycling of the tars using an innovative 'water free' tar removal system and putting them back into the combustion zone of the reactor chamber. This results in high carbon conversion efficiency in excess of 98%. The indirect nature of gasification has an important bearing on producing higher and consistent calorific value of the gas. The calorific value of the gas is in the range of 3200/kcal/Nm3 and is almost three times that of conventional down-draft fixed bed gasifiers. Hence the gas essentially is 'synthesis gas (CO+H2)' in nature and such high calorific value is an advantage in terms of handling smaller quantity of gas making the clean-up system compact and cost-effective. Further, the high calorific value gas offers itself an enabling and smooth and stable engine operation. This is a big plus for this technology as the major reason for poor reliability of the gasification based biomass-to-power plant is due to the inadequate clean-up system.

## RESULTS AND DISCUSSION

In fact, the dense forests are degrading into scrub or sparsely covered forest areas, and/or conversion into secondary plantation areas, in many parts of Madhya Pradesh. The barren and uncultivated lands are not generally suitable (due to low soil nutrients, low soil moisture, steep or terrain landscape etc.) for agricultural practice, since it involves high investment cost and low economic output. Part of these lands and degraded forest lands can be exploited for energy wood plantation through afforestation/reforestation and forest enrichment programs. In this regard, the energy wood plantation on different types of uncultivated lands, such as: marginal lands of roadsides, railway tracks, and embankments; forest lands; fallow lands; and lands belonging to the local body.Suitable tree species for energy wood plantation on government and private uncultivated lands are; *Acacia spp., Eucalyptus spp, Prosopis juliflora, Pongamia pinnata, Casuarina* spp.,*Shorea robusta, Quercus* spp ,*Melia azedarach*.

Utilization of renewable energy resources is imperative due to energy access, energy security, and energy sustainability coupled with the rising environmental concern. Madhya Pradesh is one of the large land mass state in India and amply bestowed with biomass resources. Investigations on biomass supply potential, socio-economic challenges, local people attitudes, current bio- energy markets, and technologies are prerequisite while seeking to develop sustainable energy plants. Several policies had been formulated by the Government of India aimed to increase domestic production of biomass fuels. Biomass fuel has been recognized as a local, widely accessible and renewable resource, and potentially the most suitable to alleviate the macro and micro level energy crisis. It has been considered one of the most promising resources for achieving the national energy target . However, lack of information related to the opportunities and challenges associated with the promotion of bioenergy in the perspective of environmental and socio-economic concerns has been identified as the major challenge to modernize biomass based energy in India. In addition, various challenges associated with the development of large-scale bioenergy projects are recognized as: lack of available land for energy crop plantations; lack of information on the impacts of bioenergy production on food security; lack of motivation of farmers and lack of institutional, financial, and initiatives in promotion of bioenergy marketing . A recent study revealed that lack of public acceptance, political support, available technology, and infrastructure are the major hindrances in the development of forest-based bioenergy projects in India.

## CONCLUSION

Shahdol division posseses 86.2 hectares of wasteland, both in forest and non forest areas. High-yielding plantation in such wastelands offers a good option for secured supply of biomass to decentralized biomass-based power plants and other rural energy demands on sustainable basis. Such varieties could provide enough resources for sustaining a megawatt-sized plant from just about 60 acres of land. However, the above bottlenecks are very common in the promotion of bioenergy, not only in India but also in other developed and developing countries. In the context of India, the Indian Forest Service (IFS) officers can be considered as one of the most important stakeholders in bioenergy related projects, since they are involved in implementation of forest, environment, climate, rural development strategies, and policy frameworks.

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# Molecular characterization of *Ganoderma lucidum* and analysis of few secondary metabolites use as antimicrobial activity

VINOD SINGH*, SHAILENDRA SINGH PARIHAR, GOVIND GUPTA, MAYURI KHARE

Department of Microbiology, Barkatullah University, Bhopal

*Corresponding author email: vsingh3@rediffmail.com

## ABSTRACT

*Ganoderma lucidum* is a herbal mushroom that has been used for centuries in Traditional Chinese Medicine use indifferent illness which include migiraine, headache, hypertension, arthritis, bronchitis, asthma, anorexia, gastritis, hemorrhoids, hypercholesterolemia, nephritis, dysmenorrheal, constipation, hepatitis, leucopenia, cardiovascular problems and cancer including leukemia. Isolates of *Ganoderma lucidum* were collected randomly from the various Madhya Pradesh.The samples were brought to the Lab for their DNA isolation and quantification as well amplified ITS region DNA using ITS-1, 4 primers. All PCR product sizes of the ITS region were of variable length from 636 to 650 bp among them, the PCR product analysis was used to Molecular differentiation between isolated *Ganoderma* species through Restriction fragment length Polymorphism (RFLP),five Restriction enzyme were used BamHI, EcoRI, HindIII and SmaI. Result of RFLP analysis all isolated Ganoderma samples were found the similar band patterns.Phytochemical analysis of flavonoids and phenolic compounds were analysed by TLC method. Further the powder of Ganoderma lucidum was used by antimicrobial activity was evaluated against Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus Acetone, ethanol and methanol used as extractive solvents.two flavonoids and two kinds of phenolic compounds were reported in Ganoderma lucidum. Maximum antibacterial activity shows methanol extracts of Ganoderma lucidum (17± 0.01 mm) shows high level of antibacterial activity against Staphylococcus aureus. Acetone and ethanol extracts shows good antibacterial activity respectively, against *Pseudomonas aeruginosa, Escherichia coli*.

## KEYWORDS

RFLP- Restriction fragment length Polymorphism, TLC- Thin layer Chromatography, ITS- Internal Transcribed Spacers,

## INTRODUCTION

Mushroom belongs to a separate group of organism called fungi. They lack the green matter (Chlorophyll) present in plant and grow on dead and decaying organic materials. In a broad sense a

"mushroom is a macrofungus with a distinctive fruiting body, which can be either epigeous or hypogeous and large enough to be seen with naked eye and to be picked by hand". [1] Ganodermataceae is a widespread family of wood decaying organisms reaching its greatest diversity in the tropics and subtropics where conditions are hot and humid.[2.] described *G. lucidum* species complex as medicinal fungus species belonging to the Polyporaceae family. The Polyporaceae fungi are classified as such as they have many tiny holes on the underside of the fruiting body, which are pores that contain the reproductive spores. The genus Ganoderma lucidam, however, was established in the West by a Finnish botanist, P. Karsten.[6], and more than 120 species have been reported in the world and since then a majority of them have been reported in China. For more than 4000 years *Ganoderma* as nutraceuticals are used as a remedy to treat more than 200 different illness which include migiraine, headache, hypertension, arthritis, bronchitis, asthma, anorexia, gastritis, hemorrhoids, hypercholesterolemia, nephritis, dysmenorrheal, constipation, hepatitis, leucopenia, cardiovascular problems and cancer including leukemia . *Ganoderma* works in the treatment of cancer because it helps in cleansing the body from toxins and strengthens the immune system. Anticancer agents in *Ganoderma* are the polysaccharides and Germanium, There appears to be limited information available that reports the antimicrobial properties of *Ganoderma* species. amusingly high MIC value of an aqueous *Ganoderma* extract.against*B. subtilis* (3.5mg/ml), *Bacillus* species (3.5mg/ml) have been reported. [9]

## MATERIALS AND METHODS

### Sample collection of *Ganoderma lucidum* from different area of Madhya Pradesh

The samples were collected from different areas of Madhya Pradesh, Shivpuri, Bhopal, Raisen and Hoshangabad region.

### Morphological Identification

All collected samples were identified on morphological bases on fruiting body color, size, stem color and size, and spores, and spore print.

### Isolation of fungal mycelium on Agar plate through fruiting body of *Ganoderma lucidum*

After microscopic and morphological identification, fungal mycelium have been isolated through fruiting body of *Ganoderma* on PDA/Malt extract agar plate through tissue culture method , after growing the mycelium culture on plate, it is purified on PDA slants.

### Molecular identification of collected sample

Work is going on isolation of DNA by CTAB method of all collected samples and amplifies 5.8rDNA using ITS1 and ITS 2 primer.

### Molecular diagnostic

Molecular differentiation between isolated Ganoderma species through Restriction fragment length Polymorphism (RFLP) method described.[3]

**Preparation of extract**

The freshly collected Ganoderma fruiting bodies were air-dried in the shade. Each dried Ganoderma sample will be broken into pieces placed in a heavy duty commercial blender and crushed to powdered material. Each powdered material were placed in the respective khaki paper bags and stored in the dark and dry place at room temperature till the time of extraction. To prepare *Ganoderma* extracts three different solvents were used

**Analysis of Phenolic and Flavonoid Content**

The mushroom powder was subjected for the analysis of flavonoid and phenolic content by TLC techniques. TLC was performed on the 20 × 20 cm plates precoated with microcrystalline cellulose .A volume of 1 µL of 1% methanolic solutions of standards and investigated extracts was spotted on the plates. One-dimensional TLC analysis was performed with ethyl acetate : formic acid : acetic acid : water in volume ratio 100 : 11 : 11 : 26 as mobile phase.

**Antimicrobial activity**

The extracts were individually tested against a set of human pathogenic microorganisms, including one Gram positive and two Gram negative *Staphylococcus aureus* and two Gram negative *Escherichia coli*, *Pseudomonas aeruginosa.*

**Preparation of inoculums**

The bacterial inoculums were revivify by transferring loop full of organisms from mother culture into a 250ml conical flasks containing sterilized Nutrient broth (Hi Media) .The flasks were incubated on a rotary shaker for 24 h at 37° C.

**Estimation of antibacterial activity (well diffusion method**)

Antibacterial activity was evaluated by well diffusion method by. The four wells were prepared in the medium using sterile cork borer (5mm) carefully without damaging the agar. The four wells were loaded with control using respective solvent, standard antibiotic, 100µl and 150µl of solvent extract respectively. Similarly separate plates were ready with separate wells for the solvents of acetone, methanol and ethanol for selected microorganism. The diameter of the zone of inhibition was calculated in mm using scale.

## RESULTS AND DISCUSSION

10 samples of *Ganoderma lucidum* were collected during July-October from different area of Madhya Pradesh. Cultural studies. Morphology Different isolates collections were studied in details and identified following standard description of the species as under: Fruit bodies are usually large, stipitate, dimidiate, rarely suborbicular, reddish brown, lateral and upper surfaces coated with hard shiny substance resembling with sealing wax. Pileus 2.0- 5.0 cm broad, pileal surface rough,reddish brown in colour. Stipe 1.5-4.5 cm long and 0.5- 2.0 cm thick. Pileus surface often appeared varnished. Basidiospores brown, ovate, with a rounded base and truncate to narrowly rounded apex; spore surface slightly too strongly dimpled; wall

composed of several layers.all result shown Table no 1 and Fig 1 **.**All the isolates were found to be typical of *G. Lucidum.* Pure culture of *G. lucidum* was raised on Malt Extract Medium following tissue culture method.

**Table -1 Summary of macroscopic characters of *Ganoderma* isolates.**

| S.No. | Color and size | Stem color and size | Spores | Spore print |
|---|---|---|---|---|
| GB1 | Brownish, red, yellow 20cm | Red brown 5cm | Ellipsoidal to Oval thin walled | Reddish brown |
| GB2 | Brownish, red, yellow 25cm | brown 6cm | Ellipsoidal to Oval thin walled | Reddish brown |
| GB3 | Brownish, yellow 18cm | Red brown 5cm | Ellipsoidal to Oval thin walled | Reddish brown |
| GB4 | Brownish, red, yellow 21cm | Red brown 5cm | Ellipsoidal to Oval thin walled | Reddish brown |
| GB5 | Brownish, red, yellow 26cm | Red brown 5cm | Ellipsoidal to Oval thin walled | Reddish brown |
| GB6 | Brownish, red, yellow 16cm | Red brown 5cm | Ellipsoidal to Oval thin walled | Reddish brown |
| GB7 | Reddish,yellow,22cm | Red brown 4cm | Ellipsoidal to Oval thin walled | Reddish brown |
| GB8 | Brownish, red, yellow 14cm | Red 3cm | Ellipsoidal to Oval thin walled | Reddish brown |
| GB9 | Brownish, yellow 2cm | Red brown 4cm | Ellipsoidal to Oval thin walled | Reddish brown |
| GB10 | Brownish, red, yellow 22cm | Red brown 5cm | Ellipsoidal to Oval thin walled | Reddish brown |

**Figure 1. Isolated Different *Ganoderma* samples from Different region of Madhya Pradesh**

The intact genomic DNA was isolated from 10 different isolates of *Ganoderma lucidum* by adopting CTAB DNA extraction method. The quantity of extracted genomic DNA was establish acceptable and determined by taking absorbance at 260 nm. The OD value was originated to be in range of 120-170 µg/ µl. The quality of

extracted genomic DNA was determined by doing Agarose Gel Electrophoresis and the intact genomic DNA band at 10000 bp was observed result shows in fig.1 After amplification of isolated DNA, product of ITS rDNA size was around 636-650 bps in 1.2% agarose gel which included the sequences of ITS1, 5.8S subunit and ITS2 shows result in fig.3The internal transcribed spacers region is now perhaps the most widely sequenced DNA region in fungi. They are versatile genetic markers and have been used for phylogenetic analysis, evaluation of the evolutionary process, as well as for determination of taxonomic identities [5]., the PCR product analysis was used to Molecular differentiation between isolated *Ganoderma* species through Restriction fragment length Polymorphism (RFLP),five Restriction enzyme were used BamHI, EcoRI, HindIII and SmaI. Result of RFLP analysis all isolated Ganoderma samples were found the similar band.

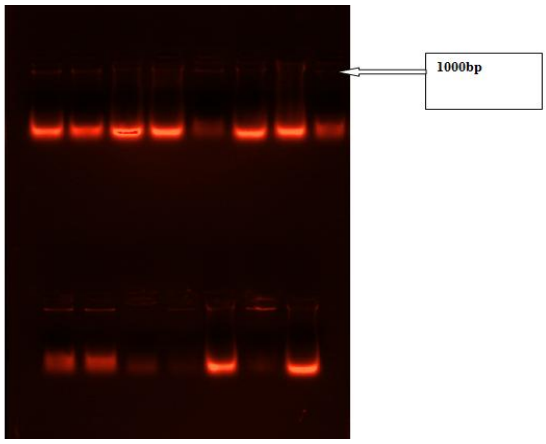


**Figure 2. Isolated *Ganoderma* DNA**

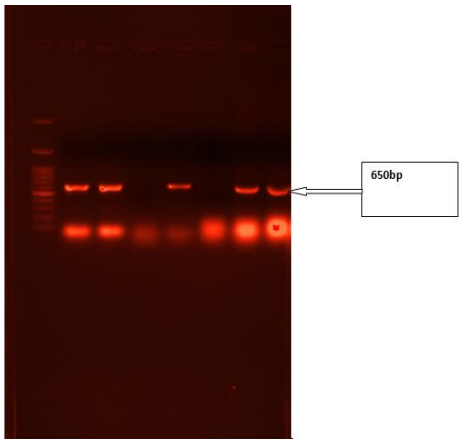


**Figure 3. Amplify *Ganoderma* DNA**

The preliminary phytochemical tests result indicates the presence of 2 phenolic compounds,and 2 flavonoids detected by TLC

The *antibacterial* potential of different solvent extracts of *G.lucidum* was carried out by agar well diffusion method and results were tabulated in Table 2. The methanol extract were found to be having significant antibacterial activity against *S.aureus* (17mm) and *E.coli* (13mm) and moderate effect on *P.aeruginosa* (12mm) and mild effect petroleum ether and aqueous extract was found to be having moderate antagonistic effect on *S.aureus* (12mm) and mild effect on *E.coli* (8mm) and negligible *effect P.aeruginosa* (7mm) The chloroform extract showed moderate effect on *S.aureus* (7mm) and not at all showed any effect on and *P.aeruginosa* . The results suggested that only methanol extract possessed a broad spectrum antimicrobial effect including potent antibacterial and antifungal activitiesThis also confirmed the suggestion.[7],[2].The chloroform extracts exhibited little or no growth inhibiting activities.

**Table 2. Showing antibacterial activity of *lucidum* against human pathogenic bacteria**

| **Test microorganisms** | **Methanol Extract** | **Pet. Ether Extract** | **Chloroform Extract** |
|---|---|---|---|
| *S.aureus* | 17mm | 12mm | 7mm |
| *E.coli* | 13mm | 8mm | 00 |
| *P.aeruginosa* | 12mm | 7mm | 00 |

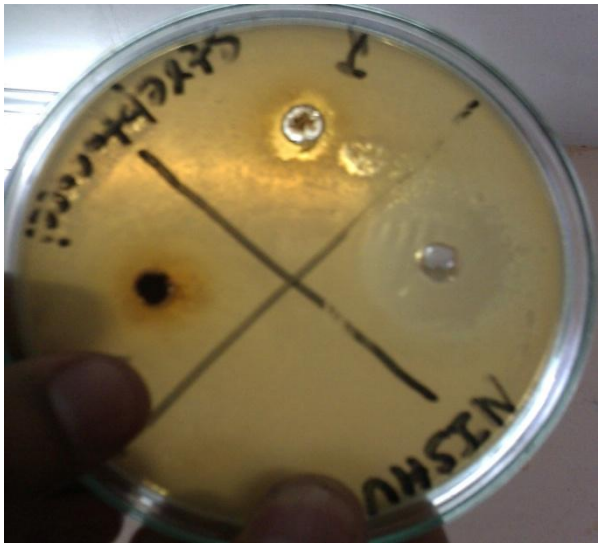

**Figure 4. Antimicrobial activity of Ganoderma lucidum methanolic extracted against *S.aureus***

## CONCLUSION

The present study focuses diversity basis morphological and genetically. Effect of antimicrobial agents against Multi drug resistant pathogens. Furthermore, these bioactive compounds of mushroom extracts were active Demonstration of broad-spectrum antimicrobial activity by various solvent extracts of *G. lucidum* may help to find out new chemical classes of antibiotic substances that could serve as discerning agents for communicable disease chemotherapy and control. The methanol takes out had exceptional antimicrobial activity against tested human pathogenic bacteria compared new antimicrobial agents


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# Intellectual Property Rights for medicinal and aromatic plants in India: an overview

****MK TRIPATHI, S.S. BIMAL, A.K.SINGH AND*ASHOK AHUJA**

Department of Plant Molecular Biology & Biotechnology,
College of Agriculture, RVSKVV, Gwalior 474002, MP India
*Ex Chief Scientist CSIR-Indian Institute of Integrative Medicine, Jammu

** Corresponding author email: drmanojtripathi64@gmail.com

## ABSTRACT

With a change in current world scenario after post WTO era there is a need to develop an efficient safeguarding strategies by developing capacity building of the people through networking with various groups who own the knowledge portfolios in the form of IPR in their interest in particular and nation as a whole. MAPs are important bio- resources providing drugs. Indian system of medicines is mostly plant based systems. Where medicinal and aromatic plants are utilized as raw material. In recent years number of cased of biopiracy have come to knowledge. Assuch several declarations, at national and international level, emphasize the need for identification, evaluation, conservation, and sustainable utilization of plant genetic resources (PGR) particularly medicinal plants. These declarations are important after post-WTO era where the TRIPS Agreement requires patent protection for at least 20 years for any invention of pharmaceutical product or process. The present overview provides information related to some of prominent Indian medicinal plants and cases where patents on various uses of traditional medicinal plants have been granted. Some of the ethical issues while patenting of Indian medicinal plants are also discussed.

**KEY WORDS**

Farmers Rights, GAT, IPR, Legal protection, Medicinal plants, TKDL, WTO

## INTRODUCTION

The Indian System of medicine *viz*:Ayurveda, Siddha, Unani and Homeopathic system are plant based where plants are largely utilized in preparations and formulations. At least 25% of drugs based on modern pharmacopeia are derived from plants and many other which are synthetic analogues are natural compounds isolated from plants. According to World Health Organization (WHO) estimates 80% of the population of developing countries depends on Herbal drugs for their primary healthcare needs. Medicinal plants being natural, non-narcotic, having no side effect, safe, cost effective, preventive and curative therapies proved to be be useful. Being efficaeous and cost effective demand for medicinal plants is increasing both in developing

and developed countries to meet the demand but 90% material is harvested from wild sources without applying scientific management hence many species are overexploited and under threat to become extinct[1,2].

Indian medicinal plants are in use since ancient times due to their medicinal properties. Number of cases of biopiracy has come to knowledge, in recent years where researchers have tried to patent known properties of Indian medicinal plants. Indian government has successfully some of these patents based on documented proof of ancient traditional knowledge. To challenge further and for patent filling Indian government took proactive steps. Traditional Knowledge Digital Library (TKDL) has been set up by Council of Scientific and Industrial Research (CSIR) to prevent biopiracy and granting of patents on Indian medicinal plants[3,4].

IPR for medicinal and aromatic plants are filled in two context: Firstly medicinal and aromatic plants those found in nature as wild and collected by wild crafting from natural stands for use and (2) newly developed varieties through plant breeding systems including genetic engineering approaches and used as cultivated MAPs. Most of the varieties developed through plant breeding systems are mostly conventional based on selection and controlled pollination. These methods have provided large number of improved better varieties in equally large number of species. The improvement so far been achieved mostly in terms of yield, intrinsic quality and to a lesser extent to resistance or tolerance against a few pest and disease. Modern plant breeding and recent genetic engineering approaches and molecular techniques to introduce new hereditary characters in to MAPs are currently in progress. More legal protection of the various process and products of molecular breeding are in demand laws and regulations are not clear provide in view of progress made in developing varieties through rDNA approaches[5,6].The present overview provide information related to a prominent Indian medicinal plants and some cases where patents on various traditional medicinal plants have been granted. Some of the ethical issues attached to patenting of Indian medicinal plants in patenting varieties developed *via* genetic engineering approaches are also discussed.

## INTELLECTUAL PROPERTY RIGHTS, IPR AND VAROUS LEGAL PROTECTION MECHANISM

IPR is grant of exclusive rights in a form of patents for a limited time in respect of a new and useful invention. The exact requirements for grant of a patent, the scope of protection it provides and its duration differs depending on national legislation. However, generally the invention must be of patentable subject matter, novel (new), non-obvious (inventive), of industrial application and sufficiently disclosed. Patents provide a wide range of legal rights, including the right to possess, use, transfer by sale or gift, and to exclude others from similar rights. Duration will be for around 20 years. These rights are generally confined to the territorial jurisdiction of the country granting the patent and thus an inventor wishing to protect his/her invention in a number of countries has to seek separate patents in each of those countries.

Most of the countries now provide some form of patent protection, but patent protection for biotechnology is by some countries only. The reasons for this may differ, but generally it has been because biotechnology has been thought inappropriate for patent protection, either because of technical or practical reasons, or for one or more ethical, religious or social concerns. In all the National Patent Offices of the various countries where patents are granted for biotechnology there is a considerable backlog of pending applications. Reason being in those countries where patent protection to biotech is provided, the type and extent of that protection is different in nearly every national system.

There are three international intellectual property treaties which are of particular importance for the protection of biotechnology: the Paris Convention for the Protection of Industrial Property (the Paris Convention); the

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purposes of Patent Procedure (the Deposit Treaty) and the Patent Cooperation Treaty (PCT).
Agriculture has become subject to IPR only after the creation of World Trade Organization (WTO) after General Agreement on Tariffs and Trade (GATT) Uruguay Round agreement. To provide protection mechanisms to harness the benefit in country's interest are:

## PROTECTION OF PLANT VARIETIES AND FARMER'S RIGHTS ACT (2001)

In order to provide for the establishment of an effective system for protection of plant varieties the rights of farmers and plant breeders and to encourage the development of new varieties of plants it has been realized to safe guard the rights of the farmers in respect of their contribution made at any time in conserving, improving and making available plant genetic resources for the development of the new plant varieties. Moreover to accelerate agricultural progress, it is necessary to protect plants breeders' rights to promote investment for further research and development for developing new plant varieties. This protection will help the growth of the seed industry and ensure the availability of high quality seeds and planting material to the farmers. India after ratifying the Agreement on Trade Related Aspects of the Intellectual Property Rights has to make provision for giving effect to Agreement. To give effect to the aforesaid objectives the Protection of Plant Varieties and Farmers' Rights Act, 2001 has been enacted in India. For the purposes of this Act, Protection of Plant Varieties and Farmers' Rights Authority has been established and is located at New Delhi.

Most of the MAPs that are in cultivation, are farmers' varieties and an instrument is available now to safe guard these varieties from piracy by registration. However, much benefit can not be achieved in MAPs by the farmers because rule states that all the extant varieties are to be registered within the three years from the date of enforcement of this Act. According to the Act, extant varieties include farmers' varieties also. Only option available now is to register the farmers' variety as new variety, since provision is available for the farmers also to register new varieties.

## BIOLOGICAL DIVERSITY ACT (2002)

The Biological Diversity Act (2002) was born out of India's attempt to realize the objectives enshrined in the United Nations Convention on Biological Diversity (CBD) 1992 which recognizes the sovereign rights of states to use their own Biological Resources. The Act aims at the conservation of biological resources and associated knowledge as well as facilitating access to them in a sustainable manner and through a just process For purposes of implementing the objects of the Act it establishes the National Biodiversity Authority in Chennai. The Biodiversity Act is a federal legislation enacted by the Parliament of India for preservation of biological diversity in India, and provides mechanism for equitable sharing of benefits arising out use of traditional biological resources and knowledge. The Act was enacted to meet the obligations under Convention on Biological Diversity (CBD) to which India is a party. The Biodiversity Act was passed by the Lok Sabha on 2$^{nd}$ December 2002 and by the Rajya Sabha on 11$^{th}$December 2002. Relevant definitions under the Act : Biodiversity has been defined under Section 2(b) of the Act as "the variability among living organisms from all sources and the ecological complexes of which they are part and includes diversity within species or between species and of ecosystems".

Biological resources as "plants, animals and micro-organisms or parts thereof, their genetic material and by-products (excluding value added products) with actual or potential use or value, but does not include human genetic material." Bio-survey and bio-utilization: means survey or collection of species, subspecies,

genes, components and extracts of biological resources for any purpose including for characterization, inventories and bioassay. Commercial utilization: means using biological resources as drugs, industrial enzymes, food flavors, fragrances, cosmetics, emulsifiers, oleoresins, colours, extracts and genes used for improving crops and livestock through genetic intervention.

Medicinal and aromatic plants constitute important biological resources and its usages have been recognized by the Act. Even after developing IPR by using the country's MAPs bio-resources through proper legal permission, one has also to take approval from the National Biodiversity Authority (NBA) for filling an application for any form of IPR. To take care of the benefit sharing due to utilization of these biological resources for commercial purpose.

## GEOGRAPHICAL INDICATIONS OF GOODS REGISTRATION AND PROTECTION ACT (1999)

Geographical indications (GI) is a *sui generis* Act of the Parliament of India for protection of indications in India. India as a member of the World Trade Organization (WTO) enacted the Act to comply with the Agreement on Trade-Related Aspects of Intellectual Property Rights[(1)]. GI tag ensures that none other than those registered as authorized users (or at least those residing inside the geographic territory are allowed to use the popular product name. Darjeeling (Tea) became the first GI tagged product in India, in 2004–05.With the Geographical Indication of Goods (Registration and Protection) Act (1999), the varieties from which the medicines prepared from certain genotypes and at a particular location having a good quality can be protected after registering it under this act with the Controller Designs and Trade Marks. There are few examples existing in medicinal plants that a product is known because its location such as Trinvelly- senna, Java- citronella, Neemach -Aswagandha, *etc*. Some of the Indian plants where patents have been filled and granted are enlisted.

## TRADITONAL KNOWLEDGE DIGITAL LIBRARY (TKDL)

TKDL is an Indian digital knowledge repository of the traditional knowledge, especially about medicinal plants and formulations used in Indian systems of medicine. Set up in 2001as a collaboration between the Council of Scientific and Industrial Research (CSIR) and the Department of Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homoeopathy (Department of AYUSH), Ministry of Health & Family Welfare, Government of India. The objective of the library is to protect the ancient and traditional knowledge of the country from exploitation through biopiracy and unethical patents by documenting it electronically and classifying it as per international patent classification systems. Apart from that the non-patent database serves to foster modern research based on traditional knowledge, as it simplifies access to this vast knowledge of remedies or practices. Till recent it had transcribed 148 books on Ayurveda, Unani, Siddha and Yoga in public domain, into 34 million pages of information, translated into five languages - English, German, French, Spanish and Japanese. Data on 80,000 formulations in Ayurveda, 1,000,000 in Unani and 12,000 in Siddha had already been put in the TKDL. Plus it has signed agreements with leading international patent offices such as European Patent Office (EPO), United Kingdom Trademark & Patent Office (UKPTO) and the United States Patent and Trademark Office to protect traditional knowledge from biopiracy by giving patent examiners at international patent office's access to the TKDL database for patent search and examination.

## PATENT PROTECTION

MAPs varieties developed using modern plant breeding techniques cannot be patented as such as per the Indian patent law. But the process developed utilizing such varieties can be protected through patents. Process developments by using MAPs and usages of MAPs for new purposes are patentable subject matter in the national law if they meet the standards of novelty, inventive steps and industrial applicability.

## BIOTECHNOLOGY PATENTING ACTIVITY IN INDIA

Though inventions in biotechnology category filed were mostly of the foreign origin but there was considerable increase in Indian applications. Most of the inventions were mostly in the area of recombinant DNA molecule, recombinant vaccine, monoclonal antibodies, recombinant therapeutic molecules, diagnostic kits, stem cells, recombinant vectors, isolated nucleic acid encoding a gene, method of preparation of recombinant hormones, DNA related inventions such as preparing plasmids, vectors *etc*, bioleaching, biotransformation, biological treatment of waste, gene and somatic cell therapy, pluripotent stem cells derived from regenerative tissue, recombinant microbes expressing chimeric HIV protein, regulation of cell mediated immune response, recombinant interleukin IL-18 inhibitors, plastid transformation vectors, waste water and sewage treatment using microorganism, conjugate vaccine against cholera and tetanus, peptide based immunotherapy for atherosclerosis, mixed cell gene therapy, bone regeneration by gene therapy and treatment of flower. Biotechnological tissue culture procedures. and media formulations have also been protected as national and international patents.

## PATENTS ON SOME OF IMPORTANT MEDICINAL & AROMATIC PLANTS

CSIR institutes viz. IIIM, Jammu, CIMAP, Lucknow, NBRI, Lucknow and IHBT, Palampur working in the areas of bioprospection of MAPs have strong IP portfolio on patents on varieties and processes relates to Medicinal and aromatic plants. CIMAP Lucknow has an IP portfolio of more than 135 foreign and Indian patents granted in major medicinal and aromatic plants including molecules and bioactive (17), improved new processes (52), new methods and techniques (13), formulations and compositions (36) plants varieties (23) and cell cultures/enzymes/strains (06) ( Table 1-3).

# CONCLUSION

India has adopted pluralistic legislative framework a combination of methods for the protection of medicinal plants. The efficacy of the system depends to integrative approach. Some legislative proposal come in to force by a Governmental in view of WTO and GATT. Still many issues are not clear which are being discussed and raised at various forum to provide protection to these bioresources of MAPs Indin being one of the Hot spots.

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13. http://www.ihbt.res.in/patents
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**Table 1 Patents Records In Some of Medicinal and Aromatic Plants***

| S.No. | Name of plants/crops | Numbers of patents |
|---|---|---|
| 1. | *Glychyrrhiza glabra* | 259 |
| 2. | *Catharanthus roseus* | 145 |
| 3. | *Gymnea sylvestris* | 94 |
| 4. | *Morinda citrifolia* | 71 |
| 5. | *Momordica charantia* | 64 |
| 6. | *Centella asiatica* | 56 |
| 7. | *Piper nigrum* | 38 |
| 8. | *Tagete serecta* | 21 |
| 9. | *Melia azardirachta* | 52 |
| 10. | *Rubia cordifolia* | 19 |

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Taneja, S, C.; Shahi, A, K.; Verma,V,; Sethi, V, K.; Andotra, S,S.; Hashmi, A.Z.; Dutt; P.; Bakshi, S. K.; Koul; Maharaj, K.; Sharma, S.N.; Chandra, S.; Qazi, G. N .(+)-1-Bisabolone isolated from *Cymbopogon flexuosus* and antibacterial activity thereof**. (US Patent No. 6787674)**

Ahuja, A.; Koul, S.; Kaul, B.L.; Verma,N.K.; Kaul, M.K.; Raina, R.K.; Qazi, G.N. Synergistic tissue culture media formulation for *in vitro* regeneration of *Swertia chirata* Ham .**(US Patent No. 6855547)**

Ahuja, A.; Koul, S.; Kaul,B.L.; Verma, N.K.; Kaul, M.K..; Raina, R.K.; Qazi, G.N. Synergistic tissue culture media formulation for *in vitro* regeneration of *Swertia chirata* Ham .**(ZA Patent No. 2004/4059)**

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# Effect of phenolic compounds on the metabolic profile of *Labeo rohita*

**VIKASH GAUR[1*], VEENA RAINA[2], RASHMI JAIN[3] AND ABHISHEK MATHUR[2]**

[1]Dept of Biochemistry, Himalayan University, Arunachal Pradesh, India.
[2]National Centre of Fungal Taxonomy (NCFT), New Delhi & Institute of Transgene Life Sciences (ITLS), Dehradun, Uttarakhand, India.
[3]National Institute of Biologicals, Noida (U.P), India.

*Corresponding author email: vikashgaur07@gmailcom

## ABSTRACT

With the advancements in growth and development of man and his interference, different environmental disasters are coming across and widespread in today's scenario Out of some known environmental disasters, water pollution is the severe problem by which not only population of animals and human beings are being affected but also a large population of aquatic life is also affected which thus changes the quality of water. The presence of toxic pollutants in aquatic ecosystems poses a serious threat to environmental health Industrialization and growth of human population have led to a progressive deterioration in the quality of the earth's environment. These harmful chemicals/xenobiotics are harming the aquatic ecosystem involving flora and fauna of the water bodies The present study was thus performed for investigating the effect of different types of phenolic compounds on biochemical parameters and enzymes involved in metabolic pathways of *Labeo rohita* (Rohu), a fresh water fish. The present study reveals the disaster and lethargic effect of phenolic compounds on the metabolic and physical parameters of *Labeo rohita* The calculated LC50 value for phenol and m-cresol exposure in *L rohita* over periods of up to 96 hours is 3212 mg $l^{-1}$ and 2957 mg $l^{-1}$ respectively. The studies showed that after exposure to phenolic compounds (treated group) of Rohu, there was a significant decreased level of serum cortisol, total carbohydrate concentration, blood glucose concentration, pyruvate concentration and different enzymes studied which are associated with the normal functioning of metabolic organs at P<005 in comparison to normal (untreated group).

## KEYWORDS

*Labeo rohita*, fresh water fish, phenolic compounds, metabolic profile, biochemical studies.

## INTRODUCTION

Water pollution is significant only when it influences living or biological systems either directly or indirectly The unique physical and chemical properties of water have allowed life to evolve in it The quote illustrates this point of view: "Life originated in water, is thriving in water, water being its solvent and medium[1] The presence of toxic pollutants in aquatic ecosystems poses a serious threat to environmental health Industrialization and growth of human population have led to a progressive deterioration in the quality of the earth's environment Urban, agricultural and industrial activities release xenobiotic compounds that may pollute the aquatic habitat About 300 million tons of synthetic compounds seep annually into water systems (rivers, lakes and sea) The potential utility of biomarkers for monitoring both environmental quality and the health of organisms inhabiting in the polluted ecosystems has received increasing attention during the last years[2-6] Phenolic compounds decomposition in water bodies can cause toxicity [7] Their inhalation and ingestion may be dangerous for human health; causing systemic damage to the nervous system[8-10] Since the interaction between toxicants and biomolecules is the first step in the generation of toxic effects (preceding cellular and systemic dysfunction), the understanding of biochemical alterations induced by the exposure to pollutants may contribute to the prediction of toxic effects that may occur later at higher levels of biological organization Moreover, the use of biochemical biomarkers may allow early interventions with the objective of protecting wild populations exposed to chemical agents.[11] The present study is thus stating the effect of phenolic compounds on biomarkers/biochemical parameters and metabolic enzymes in *Labeo rohita.*

## MATERIALS AND METHODS

### Phenolic compounds used for the study

Analar monohydric phenol ($C_6H_5OH$, MW-9411) and m-cresol ($CH_3C_6H_4OH$, MW -10814) purchased from Sisco Research Laboratories (SRL), India were used The nominal concentrations needed were prepared from fresh stock solutions

### Chemicals and Reagents used for the study

The chemicals and reagents used for the study were of Analytical Grade and were procured from Sisco Research Laboratories, Ranchem and CDH, India The diagnostic kits used in the study for biochemical testing were from Span Diagnostics Ltd Gujrat, India

### Experimental design

**(i) Collection and maintenance of test fish**

*Labeo rohita* (20-25g) were collected from the culture ponds of Yamuna river of Delhi region, India and brought to the ITLS, Dehradun in small aerated tanks In the laboratory, they were kept in large tanks where a continuous and gentle flow of tap water was maintained They were fed on a commercial diet *ad libitum* and were acclimated in tanks for a month before the experiment (Fig 1).

**(ii) Experimental design for lethal toxicity study**

$LC_{50}$ determination was carried out by following semi-static acute toxicity test For the experiment, 6 fishes were transferred to large experimental tubs, each containing 18 litres of dechlorinated tap water Eight phenol concentrations from 27 mg $l^{-1}$ (no mortality) to 34 mg $l^{-1}$ (100 % mortality) were chosen for the final 96-

hour test to determine the 50 % lethal concentration ($LC_{50}$) For m-cresol eight concentrations from 19 mg $l^{-1}$ (no mortality) to 26 mg $l^{-1}$ (100 % mortality) were chosen for the final 96-h test to determine the 50 % lethal concentration ($LC_{50}$) Fishes transferred to tanks containing no toxicants served as control Water in the control tanks and water and toxicant in the experimental tanks were renewed daily to remove the debris, taking care to give minimum disturbance to the fish The fishes were not fed during the entire exposure period Fishes were checked for mortality at every 24 hours interval The $LC_{50}$ levels and 95% confidence limits were calculated using Probit analysis (Finney, 1971) The lethal toxicity experiments were repeated wherever necessary **(Figure 1)**.

**Figure 1: Collection and maintenance of Labeo rohita (Rohu) fish for phenol toxicity study**

**(iii) Experimental design for sub-lethal toxicity studies**

For conducting the biochemical study, fishes were taken in two separate tanks which contained desired concentrations of toxin, 1/10$^{th}$ of $LC_{50}$ value of phenol and m-cresol Six replicates were kept for each experiment The experimental fishes were dosed for 21 days Daily the contents in the tanks were replaced with the same concentrations of toxicant so as to avoid any possible degradation of constituents of toxicant During the experimental period of 21 days the fishes were fed on the same diet so as to avoid the effects of starvation on normal physiological processes Any other factors likely to influence the toxicity were nullified by maintaining suitable controls in tanks that contained no toxicant **(Figure 2)**.

**Figure 2:** *Labeo rohita* taken out of the tank after experimental period of dosing for biochemical studies

**Preparation of tissue samples for the study**

After the experimental period (21days) the fishes were killed by pithing (by damaging the brain and severing the spinal cord between the head and trunk region using a sharp needle) and the tissues such as liver, gills, kidney and muscle were removed from its body, wiped thoroughly, using blotting paper to remove blood and other body fluids Then they were washed in ice cold 0.33 M sucrose and again blotted dry and the desired amounts of the tissue were weighed and used **(Figure 3)**.

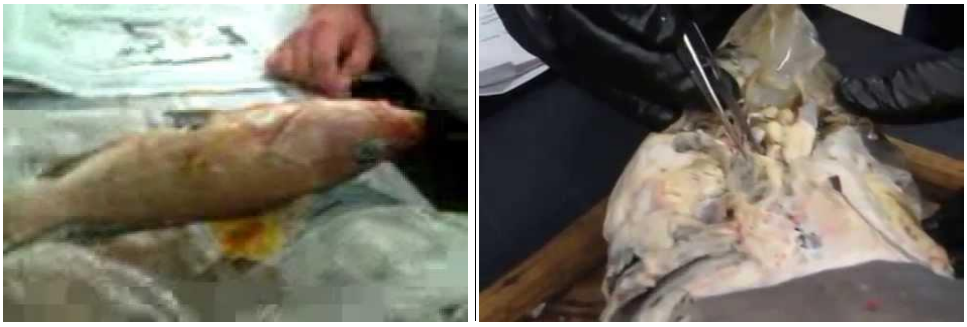

**Figure 3:** Preparation of tissue samples of Labeo rohita (Rohu) for studying of biochemical parameters

**Preparation of serum samples**

Blood was drawn from the common cardinal vein using 1 ml syringe The blood collected was then kept at room temperature for 30 minutes to separate the serum The serum thus obtained was then subjected to centrifugation at 3000 rpm for 3 minutes The serum separated was then stored at -20°C until assayed.

**Parameters Investigated**

**Estimation of serum cortisol**

The level of serum cortisol was estimated by electro-chemiluminescence immunoassay (ECLIA) To 20µl of serum sample, cortisol-specific biotinylated and a ruthenium complex labelled cortisol derivative were added It was incubated at 37°C for 9 minutes Streptavidin coated microparticles were added and were incubated at

37°C for 9 minutes This forms complex which gets bound to the solid phase via interaction of biotin and streptavidin The reaction mixture was then aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode Unbound substances were then removed with ProCell Voltage was applied to the electrode which induced chemiluminescent emission It was measured by a photomultiplier Results thus obtained were determined via a calibration curve which was instrument specific generated by a 2-point calibration and a master curve provided via the reagent barcode The results obtained were then expressed as µg/dl.

**Estimation of Total carbohydrate**

Total carbohydrate content was estimated by the method as prescribed[12] The 05% homogenate of liver and 10% homogenate of gills, kidney and muscle tissues were prepared in 10% TCA and centrifuged at 1000 × g for 15 minutes To 02 ml supernatant, 5 ml of anthrone reagent was added and boiled for 15 minutes The tubes were cooled and the absorbance was read at 620 nm in spectrophotometer against a reagent blank The standards were also treated similarly The values were expressed as mg of glucose / g wet wt of tissue.

**Assay of Glucose 6-phosphatase (EC 3.1.3.9)**

Glucose 6-phosphatase was assayed according to the method prescribed [13] The 10 % homogenate of liver tissue was prepared in 033 M sucrose solution and centrifuged at 11,000×g for 30 minutes in a refrigerated centrifuge The supernatant obtained was again centrifuged for 60 minutes at 10,500×g and the supernatant was discarded The pellet was suspended in ice-cold 033 M sucrose solution and homogenized in a glass-Teflon homogenizer The homogenate obtained was used as the enzyme source The incubation mixture in a total volume of 1ml contained 03 ml of buffer, 05 ml of substrate and 02 ml of enzyme preparation The incubation was carried out at 37°C for 60 minutes Arrested the reaction by the addition of 1 ml of 10% TCA and centrifuged The phosphorus content of the supernatant was estimated by the method as prescribed.[13] The enzyme activity was expressed as µg of inorganic phosphorus liberated / min / mg protein.

**Estimation of Blood Glucose**

Blood Glucose was estimated by the method as prescribed [14] To 02 ml of blood, 08 ml of 10 % TCA was added The contents were mixed well The tubes were centrifuged at 1000×g for 5 minutes 05 ml of supernatant was taken To this 20 ml of ortho- toluidine reagent was added The tubes were then heated in a boiling water bath for 15 minutes The standards were also treated in the same manner along with the reagent blank The values were expressed as mg glucose / dl.

**Assay of Lactate Dehydrogenase (LDH) (EC 1.1.1.2.7)**

Lactate Dehydrogenase was assayed according to the method as prescribed [15] To 10 ml of the buffered substrate, added 02 ml of sample and incubated at 37° C for 15 minutes After adding 02 ml of $NAD^+$ solution, continued the incubation for another 30 minutes and then added 10 ml of DNPH reagent Incubated the mixture for a period of 15 minutes at 37°C Then added 70 ml of 04 N NaOH solutions and measured the colour developed at 520 nm in a spectrophotometer Treated the standards also in the same manner along with blank The enzyme activity was expressed as µ moles of pyruvate liberated / h / mg protein.

**Estimation of Pyruvate**

Pyruvate was estimated by the method as prescribed [16] About 5% homogenate of liver and 10% homogenate of gills, kidney and muscle tissues were prepared in 10% TCA and centrifuged at 1000× g for 15 minutes To 20 ml of supernatant, 05 ml of 01% 2, 4-DNPH reagent was added and the tubes were kept at room temperature for 5 minutes and 30 ml of 25 N NaOH solution was added After 10 minutes the absorbance was read at 540 nm in a spectrophotometer against a reagent blank The blank consisted of 20 ml of 10% TCA, 05 ml of 01% 2, 4-DNPH and 30 ml of 25 N NaOH solutions Treated the standards also in the same manner The values were expressed as µ moles of pyruvate / g wet wt of tissue.

**Assay of Alanine aminotransferase (ALT) (EC 2.6.1.2)**

Alanine aminotransferase was assayed by the method as prescribed [17] About 10% homogenate of gills, liver, heart, kidney and muscle were prepared in 033 M sucrose solution and centrifuged at 1000× g for 15 minutes The supernatant obtained was used as the enzyme source Pipetted out 1ml buffered substrate into 'test' and 'control' Added 02 ml of the enzyme source into the 'test' and incubated the tubes at 37 ° C for 60 minutes After incubation, 02 ml enzyme was added to the control 1ml of 2, 4 - DNPH reagent was added and kept at room temperature for 20 minutes The reaction was stopped by the addition of 10 ml of 04 N NaOH, vortexed and kept at room temperature for 5 minutes The absorbance was measured at 540 nm in a spectrophotometer against a reagent blank The ALT activities were expressed as µ moles of pyruvate liberated / h / mg protein.

**Assay of Aspartate aminotransferase (AST) (EC 2.6.1.1)**

Aspartate aminotransferase was assayed by the method as prescribed[18] About 10% homogenate of gills, liver, heart, kidney and muscle were prepared in 0.33 M sucrose solution and centrifuged at 1000× g for 15 minutes The supernatant obtained was used as the enzyme source Pipetted out 1ml buffered substrate into 'test' and 'control' Added 0.2 ml of the enzyme source into the 'test' and incubated the tubes at 37 °C for 60 minutes After incubation, 02 ml enzyme was added to the control 1ml of 2, 4 - DNPH reagent was added and kept at room temperature for 20 minutes The reaction was stopped by the addition of 10 ml of 04 N NaOH, vortexed and kept at room temperature for 5 minutes The absorbance was measured at 540 nm in a spectrophotometer against a reagent blank The AST activities were expressed as µ moles of pyruvate liberated / h / mg protein.

**Estimation of Protein**

Protein was estimated by the method as prescribed.[19-21] Pipetted out 0.2 ml of tissue homogenate to the test tube and added 1 ml of 10 % TCA. The tubes were centrifuged at 5000 × g for 10 minutes. The supernatant was discarded and the precipitate was dissolved in 1 ml of 01 N NaOH Added 5 ml of alkaline copper reagent and kept for 10 minutes at room temperature After 10 minutes added 05 ml Folin- Ciocalteu Phenol Reagent and kept in dark for 30 minutes The absorbance was read at 620 nm against a reagent blank. A set of graded volumes of protein standard were also run simultaneously The values are expressed as mg of protein/ g wet wt of tissue.

## RESULTS

It was observed that, the malfunctioning of different organs in *Labeo rohita* occurs due to the alterations in different parameters and enzymes The results thus show the abnormal and lethargic effect of phenolic compounds as pollutants/xenobiotics on *Labeo rohita* The results of each of the biomarker assessed are as follows:

### Lethal Toxicity Study

The calculated $LC_{50}$ value for phenol and m-cresol exposure in *Labeo rohita* over periods of up to 96 hours was found to be 3212 mg $l^{-1}$ and 2957 mg $l^{-1}$ respectively The results are presented in **Table 1**

**Table 1:** $LC_{50}$ value for phenol and m-cresol in *Labeo rohita* (up to 96 h)

| **Phenolic Compound (s)** | **Acute Toxicity Range (mg/l)** | |
|---|---|---|
| Phenol | 3212 | 2440 |
| m-cresol | 2957 | 2464 |

### Serum Cortisol

Among phenol treated group showed the least cortisol level. The results are shown in **Table 2**

**Table 2:** Effect of different phenolic compounds on cortisol in *Labeo rohita*

| | **Control (**µg / dl) | **Phenol (**µg / dl) | **m-cresol (**µg / dl) |
|---|---|---|---|
| **Cortisol level** | 2764 | 1734 | 1934 |

*Values in the same row with different upper case letters vary significantly (P<005) between treatment groups.

### Total Carbohydrates

A statistically significant decrease in total carbohydrate (P<005) was observed in liver and muscle of both the treated groups compared to control Among the tissues kidney showed a statistically significant elevated carbohydrate level (P<005) in both the treated groups compared to control No significant variation was observed in gills of both the treated groups compared to control The result of effect in carbohydrate concentration is shown in **Table 3.**

**Table 3:** Effect of different phenolic compounds on total carbohydrate concentration in different body tissues of *Labeo rohita* Values in the same column with different upper case letters vary significantly (P<005) between tissues and values in the same row with different lower case letters vary significantly (P<005) between treatment groups (Two-way ANOVA)

| Tissue | Total Carbohydrate (mg/g wet weight of tissue) | | |
|---|---|---|---|
| | Groups treated with phenolic compounds | | |
| | Control | Phenol | m-cresol |
| Gills | 447 ± 049 | 225 ± 001 | 449 ± 002 |
| Liver | 4566 ± 223 | 216 ± 178 | 223 ± 176 |
| Kidney | 243 ± 070 | 276± 081 | 278 ± 064 |
| Muscles | 1523 ± 239 | 1056 ± 191 | 1267 ± 096 |

*Each value represents the mean ± SD of three separate experiments

**Glucose 6 phosphatase**

There was a significant decrease (P<005) in glucose-6-phosphatase activity in both the treated groups compared to control The results are shown in **Table 4.**

**Table 4:** Effect of different phenolic compounds on glucose-6-phosphatase activity (mean ± SD) in *Labeo rohita*
*Each value represents the mean ± SD of three separate experiments

| Groups | Control | Phenol | m-cresol |
|---|---|---|---|
| Glucose 6 phosphatase activity | 923± 111 | 412± 060 | 321± 077 |

**Blood Glucose**

There was a prominent decrease in blood glucose concentration after exposure to phenolic compounds. The results are shown in **Table 5.**

**Table 5:** Effect of different phenolic compounds on blood glucose (mean ± SD) in *Labeo rohita*

| Groups | Control | Phenol | m-cresol |
|---|---|---|---|
| **Blood glucose** | 7143± 111 | 3623± 060 | 2825± 077 |

*Values are expressed as mg/dl; Each value represents the mean ± SD of three separate experiments

**Serum pyruvate**

Two-factor ANOVA followed by Tukey's test showed that there was significant variation (P<005) in pyruvate level between treatments and also between tissues. The results are shown in **Table 6.** Gills, liver and kidney of both the treated groups showed a significantly increased pyruvate level (P<005) compared to control In both the treated groups muscle showed a significantly decreased pyruvate level (P<005) compared to control.

**Table 6:** Effect of different phenolic compounds on level of pyruvate (mean ± SD) in *Labeo rohita* Values in the same column with different upper case letters vary significantly (P<005) between tissues and values in the same row with different lower case letters vary significantly (P<005) between treatment groups

| Tissue | Pyruvate (micro moles /g wet weight of tissue) | | |
|---|---|---|---|
| | Groups treated with phenolic compounds | | |
| | Control | Phenol | m-cresol |
| **Gills** | 256 ± 049 | 328 ± 001 | 487 ± 002 |
| **Liver** | 648± 223 | 1067 ± 178 | 1086 ± 176 |
| **Kidney** | 534 ± 070 | 856 ± 081 | 854 ± 064 |
| **Muscles** | 638 ± 239 | 859 ± 191 | 1077 ± 096 |

Values are expressed as µmoles of pyruvate / g wet wt of tissue

Each value represents the mean ± SD of three separate experiments

**Lactate Dehydrogenase**

LDH activity in different tissues of *Labeo rohita* treated with different phenolic compounds showed significant variations (P<005) compared to control The results are shown in **Table 7** In the phenol treated group, tissues such as liver, kidney and muscle showed significantly elevated activity (P<005) compared to control Among the tissues of m-cresol treated group the gills and muscle showed a significantly elevated activity (P<005) and the liver and kidney showed a significantly decreased activity (P<005) compared to control.

**Table 7:** Effect of different phenolic compounds on LDH activity (mean ± SD) in *Labeo rohita* Values in the same column with different upper case letters vary significantly (P<005) between tissues and values in the same row with different lower case letters vary significantly (P<005) between treatment groups

| Tissue | LDH activity | | |
|---|---|---|---|
| | Groups treated with phenolic compounds | | |
| | Control | Phenol | m-cresol |
| Gills | 333 ± 049 | 623 ± 001 | 667 ± 002 |
| Liver | 387± 223 | 512 ± 178 | 736 ± 176 |
| Kidney | 354 ± 070 | 745 ± 081 | 756 ± 064 |
| Muscles | 567± 239 | 999 ± 191 | 1067 ± 096 |

*Values are expressed as µmoles of LDH liberated / h / mg protein; Each value represents the mean ± SD of three separate experiments

**ALT/SGPT**

Two-factor ANOVA followed by Tukey's test showed that there was significant elevation in ALT activity (P<005), in both the treated groups compared to control The results are shown in **Table 8.** Liver and kidney of phenol treated group showed significantly elevated activity (P<005) compared to m-cresol treated group Gills and muscle of m-cresol treated group showed significantly elevated activity compared to phenol treated group

**Table 8:** Effect of different phenolic compounds on ALT/SGPT activity (mean ± SD) in *Labeo rohita* Values in the same column with different upper case letters vary significantly (P<005) between tissues and values in the same row with different lower case letters vary significantly (P<005) between treatment groups

| Tissue | ALT/SGPT activity | | |
|---|---|---|---|
| | Groups treated with phenolic compounds | | |
| | Control | Phenol | m-cresol |
| Gills | 116 ± 049 | 156 ± 001 | 178 ± 002 |
| Liver | 323± 223 | 923 ± 178 | 967 ± 176 |
| Kidney | 156 ± 070 | 778 ± 081 | 732± 064 |
| Muscles | 212 ± 239 | 445 ± 191 | 545 ± 096 |

*Values are expressed as µmoles of ALT liberated / h / mg protein; Each value represents the mean ± SD of three separate experiments

**AST/SGOT**

AST activity was found to be significantly elevated (P<005) in tissues such as liver, kidney and muscle of both the treated groups compared to control In the phenol treated group liver and kidney showed significantly elevated AST activity (P<005) compared to m-cresol treated group Gills of both the treated groups did not show any significant variation compared to control The results are shown in **Table 9**.

**Table 9:** Effect of different phenolic compounds on AST/SGOT activity (mean ± SD) in *Labeo rohita* Values in the same column with different upper case letters vary significantly (P<005) between tissues and values in the same row with different lower case letters vary significantly (P<005) between treatment groups

| **Tissue** | **AST/SGOT activity** | | |
|---|---|---|---|
| | **Groups treated with phenolic compounds** | | |
| | **Control** | **Phenol** | **m-cresol** |
| **Gills** | 026± 049 | 145± 001 | 163± 002 |
| **Liver** | 068± 223 | 829± 178 | 826± 176 |
| **Kidney** | 234± 087 | 645± 081 | 756± 064 |
| **Muscles** | 343 ± 239 | 369± 191 | 387± 068 |

*Values are expressed as µmoles of AST liberated / h / mg protein; Each value represents the mean ± SD of three separate experiments

**Total Serum Protein**

Statistically significant decreased protein level (P<005) was observed in liver and muscle of both the treated groups compared to control The results are shown in **Table 10** Gills and kidney of both the treated groups showed a significantly elevated protein level (P<005) compared to control

**Table 10:** Effect of different phenolic compounds on total proteins (mean ± SD) in *Labeo rohita* Values in the same column with different upper case letters vary significantly (P<005) between tissues and values in the same row with different lower case letters vary significantly (P<005) between treatment groups

| **Tissue** | **Total proteins** | | |
|---|---|---|---|
| | **Groups treated with phenolic compounds** | | |
| | **Control** | **Phenol** | **m-cresol** |
| **Gills** | 3123 ± 049 | 4334 ± 001 | 4678± 002 |

| | | | |
|---|---|---|---|
| **Liver** | 7556 ± 223 | 5423± 178 | 5867± 176 |
| **Kidney** | 3558 ± 087 | 2856 ± 081 | 3786± 064 |
| **Muscles** | 7423± 239 | 3212± 191 | 3766± 068 |

*Values are expressed as mg protein/g wet weight of tissue; Each value represents the mean ± SD of three separate experiments

## DISCUSSION

In the present study, decreased cortisol level was observed in both phenol and m-cresol treated fishes compared to control There are several studies which provided evidence that the capacity to raise plasma cortisol is impaired in fish exposed to organic pollutants and metals [22-25] As cortisol is involved in the regulation of physiological functions that helps the animal to cope with stress, inhibition of the pituitary-interrenal axis will impair the ability of the animal to cope with stressors Prolonged exposure to pollutants may lead to hyperactivity, and as a result in the exhaustion of the pituitary-interrenal axis The lack of cortisol response suggests that, similar to other xenobiotics phenol and m-cresol can act as an endocrine disruptor and as such impair steroidogenesis It is not known how phenolics can affect cortisol production However there are some possible explanations First, it might be possible that one of the primary steps in the steroid hormone synthesis pathway was compromised Total carbohydrate content was found to be decreased in liver and muscle of both the treated groups compared to control Chemical stress causes rapid depletion of stored carbohydrates primarily in liver and other tissues [28] Inhibition of glucose-6-phosphatase activity was found in the liver of both the treated groups compared to control Inhibition of glucose-6-phosphatase activity may be a reflection of damage to the microsomal membrane as the enzyme is localized exclusively in the membranes of the endoplasmic reticulum On exposure to phenolic compounds gills, liver and kidney showed an elevated pyruvate level compared to control This might be due to the higher glycolysis rate, which is the only energy-producing pathway for the animal when it is under stress conditions The end product of the glycolytic pathway is pyruvate Pyruvate occupies an important junction between various metabolic pathways It may be decarboxylated to acetyl CoA which can enter the TCA cycle or it may be utilized for fatty acid synthesis Pyruvate may be carboxylated to oxaloacetate which can be used for gluconeogenesis Muscle of both the treated groups showed a decreased pyruvate level compared to control.

Lactate dehydrogenase is an enzyme recognized as a potential marker for assessing the toxicity of a chemical LDH is an anaerobic enzyme involved in the conversion of pyruvate to lactate in glycolysis The LDH in the liver and kidney of fishes treated with phenol showed an elevated activity compared to control The studies reported a similar increase in LDH activity in juvenile Australian Bass and *Macquaria novemaculeata* in response to two different crude oil spills. [26-29] The increase in LDH activity also suggests a significant increase in the conversion of pyruvate to lactic acid, thereby leading to the accumulation of lactic acid Compared to control a significant decrease in LDH activity in liver and kidney of m-cresol treated fishes and in gills of fishes treated with phenol was observed This may be due to increased tissue damage Similar results were obtained when *Labeo rohita* were exposed to sub-lethal concentrations of organophosphorus insecticide Several reports revealed decreased LDH activity in tissues under various toxic conditions [30-32] LDH is an important glycolytic enzyme in biological systems and is inducible by oxygen stress Therefore, the activity of several regulatory enzymes may be altered in order to meet the required

energy demands under toxic stress including the activity of lactate dehydrogenase (LDH), which sustains the continued process of glycolysis under anaerobic conditions ALT is an enzyme frequently used in the diagnosis of damage caused by pollutants in various tissues such as liver, muscle, and gills. [33-37] This enzyme is known to play a key role in mobilizing L-amino acids for gluconeogenesis and function as links between carbohydrate and protein metabolism under altered physiological, pathological and induced environmental conditions Elevation in the levels of AST and ALT in different tissues of *C batrachus* can be considered as a response to the stress induced by phenolic compounds to generate keto acids like α-ketoglutarate and oxaloacetate for contributing to gluconeogenesis and/or energy production necessary to meet the excess energy demand Gills and kidneys of both the treated group showed increased protein content compared to control Liver and muscle of both the treated groups showed decreased protein content compared to control The reduction in protein content indicates that under stress conditions the tissue protein may undergo proteolysis, which may have resulted in the production of free amino acids which can be used in the tricarboxylic acid cycle for energy production This would lead to an increased free amino acid pool[36] which can be used for ATP production by transamination reactions or by gluconeogenic pathway.

## CONCLUSION

The findings of the present histological investigations demonstrate a direct correlation between exposure to phenolic compounds and histopathological disorders observed in several tissues. All the histopathological observations indicated that exposure to sub-lethal concentrations of phenolic compounds caused destructive effect in the gills, liver and kidney tissues of *Labeo rohita*. The metabolites get distributed throughout the organism by the bloodstream, causing even greater damage. The observed abnormal behaviour and altered histopathology of vital organs demonstrate the severe adverse effects to exposure of phenolic compounds in *L. rohita*.

The current study reinforces the application of histopathology as a powerful tool for monitoring anthropogenic contamination within aquatic environments. Whilst links between such pathologies and contaminants are not definitive, such surveillance provides a useful insight into individual, population and overall ecosystem quality. When these pathological endpoints are assessed in conjunction with other parameters such as parasite community structure, sediment and water chemistry, enzyme responses, bile metabolite levels and molecular damage indices, a clearer picture of the complex interactions between anthropogenic and natural environmental modifiers will emerge.

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# Rhizobacteria: A Promising Tool for Drought Tolerance in Crop Plants

**PRIYA VERMA, RAGHVENDRA SAXENA*, RAJESH SINGH TOMAR**

Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior,( M.P)

*Corresponding author email: rsaxena@gwa.amity.edu

## ABSTRACT

Abiotic stress mainly involved extreme environmental conditions like drought, salinity, cold, heat, pH etc. Such extreme conditions are likely to have direct inhibitory impact on plant growth and productivity. In the recent years plant-growth promoting rhizobacteria (PGPR) mediated enhancement in abiotic stress tolerance is gaining wide attention worldwide. Plant-growth promoting rhizobacteria (PGPR) are usually associated with roots and are capable enough to extend sufficient and effective tolerance to plants from deleterious effect of drought stress and other abiotic stresses, probably by modulating various physiological and biochemical responses against drought stress. When plant experiences the harsh environmental stresses, PGPR usually facilitates improvement in tolerance by adapting different strategies. Some of the way by which PGPR improves stress tolerances in plants by activating phytohormones like ethylene, auxin, abssiccic acid (ABA), gibberllic acid. Enzymes like 1- aminocyclopropane-1-carboxylate (ACC)-deaminase activity and production of bacterial product like exopolysacchride (EPS), formation of biofilm and secretion of volatile organic compounds(VOCs) . Further, looking forward the benefits of rhizosphere, which can provide comparatively low cost alternative strategies to improve plant growth, production and facilitate protection against harsh environmental conditions make them a viable tool.Therefore present review focus on basic approaches adopted by rhizobacteria to alleviate abiotic stress tolerance especially against drought in different crop plants.

**KEYWORDS**

Rhizobacteria, PGPR, Abiotic stress, Drought stress, Phytohormones, ACC-deaminase etc.

## INTRODUCTION

Abiotic stresses such as salinity, drought, cold, flood and pH etc are among some of the major environmental factors causing serious negative impact on crop growth and productivity worldwide. Their frequent recurrence poses strict inhibitory effect on plant growth, development and productivity. Such factors contributed approximately up to 45% of the world's agricultural lands, which are subjected to continuous or frequent drought and 19.5% of irrigated agricultural lands are considered saline .[1] However it is not the single factor, rather combination of different abiotic stresses are frequently renders by crop plants simultaneously .[2] Plants have evolved various adaptation mechanisms to deal such harsh conditions for survival. it has been widely studied that apart from various plant adaptation strategies, microorganisms play a vital role towards abiotic stress tolerance in agricultural crop plants especially for drought and salinity stress. [3]

In the current scenario, microbial role in plant adaptation towards drought stress is gaining more attention.[4] This strategy is not only easier but also cost effective. The mutual association of plant with soil microorganisms provides benefit to plants against water deficit conditions that form rhizosphere and the endosphere. The Plant Growth Promoting Bacteria (PGPB) and mycorrhizal fungi have a immense potential to alter and regulate the physiological and biochemical response to water deficit, thereby leading to enhanced plant survival under harsh and adverse environmental conditions.[5,6]

Rhizophere bacteria are known to produces biofilms and secretion of exopolysaccharates which renders plants more comfortable in extreme conditions. Further PGPR also provides beneficial effect to plants by adopting certain strategies like induction of osmoprotectors and heat shock proteins.[3] Exopolysaccharides (EPS) produced by microbes constitute the active component of soil organic matter.[7] It shares most part of extracellular matrix of bacterial weight, which ranged between 40–95% .[8] EPS exhibit various vital roles in protection like surface attachment, formation of biofilm microbial aggregation, enhancement of plant microbial interaction, and bioremediation etc.[9] Exopolysaccharides (EPS) producing bacteria i.e., *Azospirillum* confers better tolerance towards water stress.[10] by improving the soil structure, texure and soil aggregation.[11,12]

One of the strategy to address stress conditions may be to enhance root growth, to maximize more water uptake from the deep within the soil profiles. Thus, maintaining leaf water relation, a basic feed-forward response to soil drying [13] Similarly the other strategy could be the lowering of the stress mediated production of ethylene therefore ACC-deaminase containing PGPR produced higher crop yields and promote stress enhancement especially under drought conditions.[14,15]

There are certain PGPR which emitting volatile organic compounds (VOCs). volatile organic compounds are presently considered as an important parameter in plant-microbial interactions. .[16,17,18] It is still unclear that volatile organic compounds (VOCs) produced by PGPR contains any known plant growth hormones or siderophores.[18,19] However VOC-mediated regulation of plant endogenous auxin homeostasis and of iron uptake by roots has been documented.[20]There are number of PGPR strains verities are identified which play an significant role in promoting crop growth and yield belongs to *Bacillus, Azospirillium, Azotobacter, Beijerinckia, Acinetobacter, Alcaligenes, Erwinia, Flavobacterium, Enterobacter, Burkholderia, Arthrobacter, Rhizobium and Serratia*.[21,22,23,24] These rhizosphere bacteria (PGPRs) enhance plant growth and increase plant biomass.[25] There are number of studies available which showed the beneficial effects of PGPRs in many agricultural crops, for example Tomatoes , Bell peppers, Cucumbers[26,] Barley[27] Wheat[28], Tobacco, *Brassica juncea*.[29] Phytohormones play pivotal role in plant growth and there are some such examples of PGPR which are able to secrete phytohormones, though the roles of gibberellins producing PGPR have been faintly understood. There are various strain which are capable for enhancing drought tolerance in different crops i.e. *Achromobacter piechaudii* in Tomato (*L. esculentum*) and pepper, (*Capsicum annuum*).[30] *Azospirillum* in Wheat (*T. aestivum*).[31,32] *A. brasilense* in Maize (*Z. mays*)[33] and *A. brasilense* in Common bean (*P. vulgaris* ).[34]

## RHIZOSPHERE BACTERIA AND ADAPTATION IN DROUGHT STRESS

Due to irregular and unpredictable rain fall pattern, drought and water scarcity become a major limiting factor to crop productivity worldwide. Plants usually adopt fast strategy to cope drought tress by minimizing water use like, stomatal closure, accumulation of ABA, compatible solutes, enzymes and enhanced expression of aquaporins and pyrophosphatases. These events usually maintain the cell turgor through osmotic adjustments.[35] Under prolonged water deficit conditions plants frequently accommodate their physiological

status in such, that can withstand a long-term adjustments in order to relive stress conditions. Which includes increased level of ethylene concentrations inhibitory effect on plant growth, increased root to shoot ratio to increase water adsorption? Induction of antioxidant enzymes in response to accumulation of reactive oxygen species (ROS) which demag plant functionality and cell integrity under stress.[5,6]

Apart from continuous progress and advancement of several engineering technology, there is a constant need to identify low cost alternative strategies to address the rapidly increasing food demands in near future.[36] The use of free-living plant growth-promoting bacteria (PGPB) can provide a broad-spectrum solution to enhance plant growth. At the same time it also provides a relatively simple and low-cost alternative strategy towards enhancement abiotic stress tolerance. [23,37,38] The ability of PGPR to enhance and promote plant growth their role in enhancement of drought stress tolerance was initially reported by Timmusk S, Wagner EG,1999. There are large number of varieties of promising microorganisms, gram-positive rhizosphere bacteria which shows potential in commercial use under drought stress because of their easy handling and endospore formation and efficient colonization ability under environmental stress conditions.[30]

Rhizobacteria developed certain adaptive features, which improves plant growth under biotic and abiotic stresses because they have coevolved with plant roots in varied extreme environmental conditions.[39] The studies indicated that emission of stressed related volatiles compounds could be a promising candidate for assessing drought stress in crop plants.Further these volatiles could provides an efficient tool for fast screening of potential rhizospheric bacterial strains isolated of from harsh environments.[39]

Study on three indigenous bacterial strains identified as *Pseudomonas putida,* and *Bacillus megaterium* have shown plant growth promoting ability under water deficit conditions. However two strains namely *P. putida* and *B. megaterium* has shown more tolerance and involved in osmotic cellular adaptation.[40] Further these bacterial strains *P. putida* and *B. megaterium* were able to produce more IAA and proline, when subjected to osmotic stress, which indicated the bacterial resistance to drought. The increase in water content, shoot and root biomass validate their efficacy in promoting plant growth and shoot water content to augment plant drought tolerance. *B. megaterium* had performed best under drought when used alone or in association with the autochthonous arbuscular mycorrhizal fungi like, *Glomus coronatum, Glomus claroideum or Glomus constrictum*. It is because *B. megaterium* capable to colonized the rhizosphere and endorhizosphere zone.[40]

Plant-growth-promoting rhizobacteria (PGPR) enhance plant productivity and immunity, there are studies which show that PGPR also involved in eliciting 'induced systemic tolerance' to abiotic stresses like drought and salinity. PGPR also reduces the need of fertilizers by increasing nutrient uptake from soils.[23]

Study on biosynthesis of bacterial IAA from its basic precursor tryptophan,The indolepyruvate decarboxylase which is the main enzyme of the indolepyruvate pathway is well studied in E. cloacae. The mutation studies in ipdC gene, which encoding this enzyme leads to inhibition of IAA biosynthesis and thereby reduction in plant growth promotion ability.[41]

The phytohormone abscisic acid (ABA) is known to control plant stress responses under water deficit conditions. However, studies indicated that increased osmo-protection of plants treated with GB03 VOCs is not associated to ABA, or at least involved into ABA production, because osmotic stress caused ABA to increase to similar levels in plants with and without exposure to GB03 VOCs. [42] There is no direct role of ABA in PGPR-induced plant drought tolerance is supported by observations that PGPR-treated Arabidopsis and cucumber plants accumulated less ABA than control plants.[43,44]

The indirect role of ABA in such PGPR-triggered abiotic stress tolerance cannot be completely ruled out, because, of the complex cross-talk among ABA, NO, SA, and hydrogen peroxide signaling pathways in plants.[45,46,47] There are studies indicated the elevation of antioxidant responses mediated by PGPR, causes enhanced drought tolerance. PGPR induced drought tolerance was found in wheat when inoculated with

*Bacillus safensisstrain* W10 and *Ochrobactrum pseudogregnonense* strain IP8.[48] Similar studies on potato treated with PGPR showed augmentation in abiotic stress tolerance including drought, salinity, and heavy-metal toxicity. Further, increase level of proline accumulation and ROS-scavenging enzymes activity were observed in PGPR-treated potato plants.[49] Some bacterially produces VOCs such as acetic acid can induce the formation of biofilms, which consist of exopolysaccharides (EPS) as major component.[50] Therefore it is possible that such PGPR VOCs may indirectly enhance plant drought tolerance by inducing exopolysaccharide production. There is a mutual beneficial effects including enhancement of plant abiotic stress tolerance by VOCs produced by PGPR have resulted from the co-evolution of PGPR with their plant symbionts.[51]

## MECHANISMS UNDERLYING DROUGHT TOLERANCE IN CROPS BY RHIZOBACTERIA

There are various mechanisms commonly adopted by plants when exposed to environmental stresses, like drought stress, salinity, nutrient deprivation, or heavy metal toxicity. These adaptation commonly includes morphological changes in root. It is a process in which plant hormone contribute a important role.[52] Indole acetic acid (IAA), which is produced in the plant shoot and transported down to the root tips.[53] When IAA is present in low concentrations in root it promotes to enhance cell elongation, resulting into increased root growth. Further, auxin is involved in promotion of the initiation and development of lateral roots. However, the high concentrations of auxin in the root tips exhibit an inhibitory effect on root growth. This inhibition could be either directly or indirectly associated with synthesis of ethylene associated with auxin. [54] As various studies indicated the considerable relationship between IAA and the ethylene precursor, 1-aminocyclopropane-1- carboxylic acid (ACC).[55] Furthermore, bacteria colonizing the root zone may also promote such developments in root growth.[56,57] As indicated in most studies, there is sufficient number of cases where, root-associated PGPR bacteria extends enhancing effects on plant growth, which are likely to produce IAA. The different plant species, which are inoculated with such PGPR bacteria displayed positive impact on root growth enhancement and/or enhanced formation of lateral roots and root there by promote more tolerance in plants against drought stress.

There are various strategies and mechanisms studied for enhancement of plant drought stress tolerance mediated by rhizobacteria but the exact mechanisms of enhancement by rhizosphere bacteria remain mostly speculative. However, rhizobacteria mediated stress tolerance effects may involved following possible explanations which include: production of hormones like abscisic acid, gibberellic acid, cytokinins, and auxin. Enzymes production like, 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce ethylene level in the root of growing plants. Promoting induced systemic resistance by bacterially-derived compounds. And formation of bacterial biofilm i.e. extracellular matrix.[37,58,59] The extracellular matrix formed by the which form major part of extracellular matrix contains wide variety of macromolecules, which are beneficial for plant development and growth. Biofilms contains numbers of sugars and oligo- and polysaccharides that can play very essential roles in bacteria-plant interactions, improvement of water retention capacity and thus improve water availability in root medium. Some of the polysaccharides has the capacity to retain water by several-fold of their mass.[41] It also found that even small polysaccharide alginate content in the biofilm can facilitates maintenance of hydrated microenvironment, by increasing the water retention capacity.[60]

However some molecular studies on genomic analysis of sequences for alginate, ACC deaminase , and auxin production revealed that such compound could be responsible for the bacterial drought tolerance enhancement.[60,61]

It has been further studied that certain microbes containing an enzyme ACC-deaminase which hydrolyses ACC, Since ACC is precursor of ethylene into ammonia and α-ketobutyrate, hence reduce the ethylene level, therefore no longer available to impart inhibitory effect on plant growth.[62] Therefore, it is most possible that auxin (IAA) and ACC-deaminase work together to increase root growth and elongation.[63] However this mechanism of PGPR is of great agronomic importance under abiotic stress environments, which are suppose to induce increased production of ethylene.[64] observed the biosynthesis of auxins, siderophore and ACC deaminase by certain PGPR, which play a major role on plant growth. Auxin is mainly involved in improvement of root growth, where as ACC deaminase is potentially involved in decreasing the growth-inhibiting levels of ethylene. Further, in "Encyclopedia of Soils in the Environment" have explained "Inhibition of plant ethylene synthesis" as one of the important mechanism for plant growth promotion, this is because of the increased root length induced by many PGPR bacteria.[65] Volatile organic compounds (VOCs) produced by PGPR can be used to determine whether VOCs induces antioxidative processes in plants when subjected to water deficit stress. Some PGPR strains, like *Pseudomonas aeruginosa* strain Pa2, produce exopolysaccharides therefore enhances drought tolerance in plants by improving the maintenance soil moisture content.[66]

## CROPS WITH ENHANCED DROUGHT STRESS TOLERANCE BY RHIZOBACTERIA

The microbial symbiotic have potential to confer stress tolerance to a large number of different variety of plant hosts, further both monocots and dicot crop species can be benefitted by beneficial effects of PGPM. [38,67,68]

The effect of *Achromobacter piechaudii*, mediated stress tolerance on pepper and tomato, indicated the enhancement for drought and salt resistance.[30]

The studies on olive trees, tomato, grapevine, and pepper plants, using microbes isolated from the roots of one host species grown under limited water farming conditions indicated that such microbes were capable of increasing the growth of a different host species under the drought stress. It was further concluded that transfer of stress-resistance attributes from one crop species to another using microbial inoculums could be a promising tool for enhancing drought stress tolerance which has the potential to minimize years of plant breeding effort.[69] Studies on wheat indicated a positive response of ACC-deaminase containing PGPR have shown enhanced (4-14%) crop yield under field condition. [70]

The PGPR isolated from arid and semiarid regions were used as the bioinoculant on maize were more effective when used in combination with their respective Exopolysaccharides (EPS) as compared to alone to enhance drought tolerance. It was further observed that EPS have potential to improve soil moisture contents, plant biomass, root and shoot length and leaf area. Therefore consortia of inocula and their respective EPS showed greater potential to drought tolerance compared to PGPR inocula used alone.[66]

Rhizobacteria and endophytes confers a large array of plant growth promoting properties, as indicated by studies conducted on pepper plants using the rhizosphere and endosphere of pepper plants grown in traditional desert farm in Egypt. Further it is convinced that PGPR have potential to support plant growth under water stress conditions.[6]

It is widely known that drought stress induces the elevation of ethylene production in plants, which inhibits the plant growth. However, studies on wheat (*Triticum aestivum* L.) inoculated with different rhizobacteria collected from wheat rhizosphere cultivated in the a semi-arid region. Screening of rhizobacteria for ACC-deaminase activity and their potential to confer drought tolerance in wheat crop indicated the better drought tolerance in wheat. it was evident from lowering the endogenous ethylene levels in the rhizosphere.[71] The increase in root-shoot mass, root-shoot length and lateral root number in PGPR inoculated wheat plants corroborated enhanced drought stress tolerance in crop by highly developed root system .

The studies on cucumber plants using rhizobacteria (PGPR) strains viz. *Burkholdera cepacia* SE4, *Promicromonospora sp.* SE188 and *Acinetobacter calcoaceticus* SE370 on plant growth indicated a significant effect on plant growth by increasing chlorophyll contents and biomass under salinity and drought stress, further there was reduced electrolytic leakage and increased water potential.[44] Further, the cucumber plants inoculated with PGPR has shown tolerance for oxidative stress by reducing the activities of various antioxidant enzyme like catalase, peroxidase, polyphenol oxidase, and total polyphenol. Therefore the PGPR could be used to mitigate stress tolerance and increased crop productivity.[44]

## CONCLUSION

Extreme environmental conditions, especially drought stress effect severely by inhibiting the plant growth and productivity, however there several strategies which plants perused against drought stress, rhizobacteria can provide better and cost effective alternative towards drought tolerance in crop plants. Since, rhizobacteria can mediate diverse beneficial interactions with plants, therefore various rhizobacteria (PGPR) were studied for their ability to mitigate stress tolerance. Further the various studies conducted on rhizobacteria. It is summed up from this review that rhizobacteria usually adopt very diverse and multiple approaches to ameliorate drought tolerance in plants like production of phytohormones i.e, auxin, cytokinin, gibbrellin ABA etc, active enzymes like 1- aminocyclopropane-1-carboxylate (ACC)-deaminase, sederophores, bacterial product like Exopolysacchride, biofilm, volatile organic compounds , activation of antioxidant and increased accumulation of osmolytes like proline, betain etc. Further there are several crop plants like potato, tomato, maize, papper, cucumber, wheat etc. can be mitigated for abiotic stress tolerance as various studies indicated however there is still need to understand the exact mechanism of and its intricacy in rhizobacteria mediated abiotic stress tolerance.

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# Epitope-based vaccine target screening against Human Immunodeficiency Virus

**SHUCHI KAUSHIK, RAJESH SINGH TOMAR***

Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior, India.

* Corresponding author email: rstomar@amity.edu

## ABSTRACT

Human Immunodeficiency Virus (HIV) is a major worldwide threat to public health. However, vaccine development for this pathogen lags behind as immunity associated with protection is currently largely ineffective. In this study, an immunoinformatics-driven screening strategy of vaccine targets was performed to thoroughly screen the vital and effective dominant immunogens against HIV. We have focused on gp41 as a target for vaccine development to prevent HIV infection as gp41 helps to initiate the process of membrane fusion between the virus and the host cell. The proposed data of target protein for vaccine can be very helpful for generating antigenic candidate by wet lab researchers. In fact *in-silico* approach for vaccine target prediction is definitely reducing manpower, time and cost in relation to search an effective candidate for the development of preventive measures against the drastic diseases caused by blocking its adherence and invading efficiency.

## KEYWORDS

Envelope proteins, Immunoinformatics, B-cell epitope, MHC, Antigen

## INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a dreadful disease caused by Human immunodeficiency virus (HIV) which infected about 42 million people across the globe. Approximately 20 million have died from AIDS and 5 million new infections occur annually. The immune system gets weakened in this condition and makes individual susceptible to opportunistic infections and tumors. Transmission of HIV is through many routes but mainly through direct contact of a mucous membrane or the bloodstream with a bodily fluid containing HIV.[1] In India there are 21.17 lakhs people living with HIV as revealed by the NACO 2015 Technical report on Estimation of HIV.[2]

Antiretroviral drugs the only available treatment for AIDS and HIV can make the disease process slow and reduce both the sufferings and deaths caused by HIV infection, but these drugs are not only costly but also out of reach of the poor people in developing countries. The best way to fight against any disease is to prevent its cause and spread and same is the case with controlling AIDS/HIV pandemic.[3]

There is a dire need for either finding or developing a safe and potential vaccine so as to control the incessant increase of patients and a high death rate of HIV infection. The pathogen gains entry into the host cell by adhering to the membrane and colonizing host tissue surfaces. Theoretically, nearly all viral proteins are potential immunogens and vaccine targets yet the majority of recent progresses focused on the viral envelope glycoproteins mainly gp20 & gp41.

In our present study, an immunoinformatics-based selection of vaccine targets was performed to carefully screen the imperative and efficient immunogens against HIV. We have investigated the ability of gp41 as a target for vaccine development to prevent HIV infection as gp41 helps to initiate the process of membrane fusion between the virus and the host cell.

## MATERIALS AND METHODS

In an effort to analyze preventive measure against HIV, we performed *in silico* prophecy of membrane binding protein as suitable vaccine candidate. In this context, following protein sequences of Human Immunodeficiency Virus envelope glycoprotein gp41 were retrieved from the National Centre for Biotechnology Information protein database and saved in FASTA format: >gi|30794653, >gi|110339310, >gi|310877610, >gi|90993006, >gi|90992998, >gi|90992996, >gi|310877608, >gi|90993002, >gi|90993004, >gi|90993000, >gi|90993053. Later on multiple sequence alignment of protein sequences was done to find out the conserved sequence using BioEdit. The conserved sequence was analyzed for B-cell epitope and T-cell epitope properties by EpiJen. The prediction of promiscuous MHC class-I and MHC class-II binding peptides was done by using ProPred I.[4] Then in the last part of the study, structural analysis was done by PSIPRED software.

## RESULTS AND DISCUSSION

More than 95% HIV infected individuals live in the areas where the prevalent antiretroviral therapies for combating HIV infections are available in limited amount and that too at high cost. Thus, a vaccine could be the best and only real long-term solution to the AIDS pandemic. In order to check the extensive spread of HIV worldwide, many strategies have been employed to search for a suitable vaccine. Since there has never been a recorded case of natural immunity to HIV and also no suitable animal model available for assessing the effectiveness of any of the proposed vaccines, the search for a HIV vaccine has proven to be a challenging problem.[5]

Peptides which are heterogenous in nature and are easily accessible to the immune cells are considered to be effective immunogens and are capable to elicit suitable immune response by activating either humoral or cell mediated immunity.[6] Due to advancements in research related to better understanding of the virology, pathogenesis and immunological properties of HIV, vaccine designs that integrate subunit proteins or epitope-based peptides have emerged as feasible candidates for developing effective therapeutic and preventative treatments for HIV.

It is evident that by blocking the membrane binding efficiency of HIV envelope glycoprotein gp41, invasion of the pathogen can be prevented and therefore in the present study, gp41 was considered to be used as a potential candidate for vaccine development. In fact *in silico* approach of vaccine target prediction definitely reduces the work, fasten the research process and cost effective in terms of searching for a pilot antigenic molecule.[7] In order to find out the conserved regions which may act as a suitable vaccine target,

multiple sequence alignment was performed which reveal following conserved sequence among the test sequences:

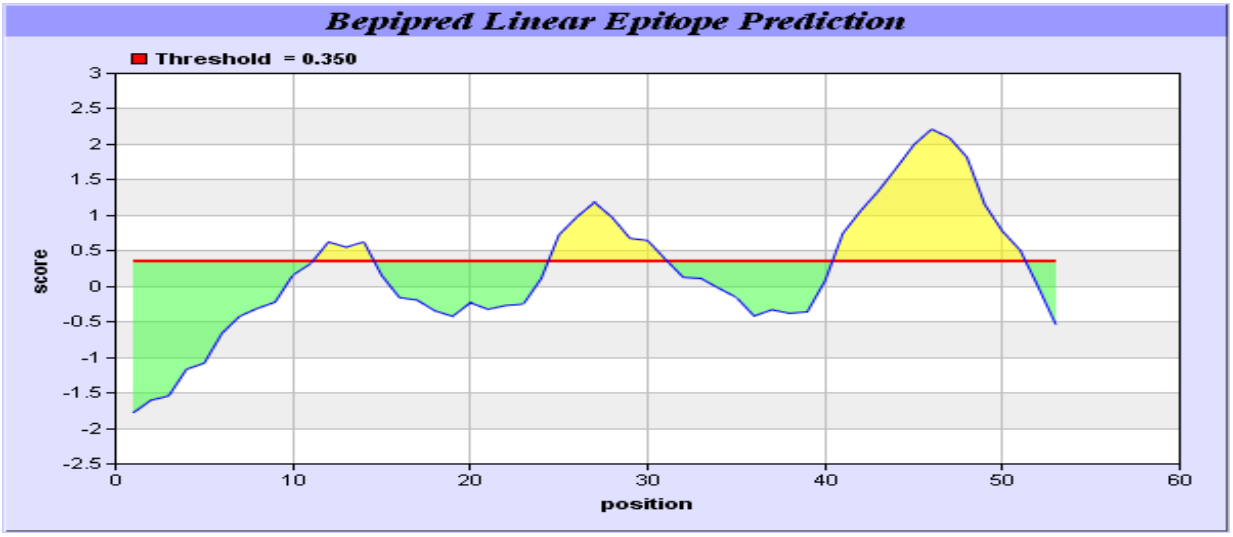
33**LWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVL**85

There are several problems associated with using peptides vaccines against HIV such as the diversity of HIV, the human leukocyte diversity antigens (HLA) and the ability to stimulate anamnestic immune response.[8] In order to combat these challenges, researchers suggest that therapeutic immunogens should contain multiple epitopes so as to be a potential target for a diversity of virus strains and HLA. Epitope-based vaccines have a wide-range of applications from curative to prophylactics. This makes the selection of most favourable epitopes an important and appealing optimization research area which should render the use of these multi-epitopes economic and practically possible.[9]

For any predicted epitope, it is important that it should induce humoral and cell mediated immune response. Such type of multiepitope vaccine development is latest investigational method for predicting vaccine targets against HPV and Influenza virus.[10-11] During the analysis of the conserved sequence, we envisaged one such type of multi-epitope peptide which could interact with B cells as well as showed good binding affinity to MHC I & II molecules.[12-13]

**Sequence:**

LWVTVYYGVP VWKEATTTLF CASDAKAYDT EVHNVWATHA CVPTDPNPQE VVL




**Average:**0.235 **Minimum:**-1.78 **Maximum:**2.214 **Threshold:** 0.350

**Figure 1. BepiPred Linear Epitope Prediction for B-cells**

The next part of the study included analysis for its binding affinity with T-cell receptors using the EpiJen server. Among 51 MHC-II alleles analyzed, the conserved region of the protein showed binding affinity with one or more than one agretope found in following alleles: DRB1_0101, DRB1_0102, DRB1_0306, DRB1_0307, DRB1_0308, DRB1_0311, DRB1_0404, DRB1_0405, DRB1_0408, DRB1_0421, DRB1_0801, DRB1_0817, DRB1_1107, DRB1_1321, DRB1_1501, DRB1_1506. As per the results of

ProPred I, the predicted epitope was found to be good binder with following MHC-I alleles: HLA-A1, A2, A*0201, A*1101, A24, A3, A*3101, A*3302, A-68.1, B*2702, B*2705, B*3501, B*3701, B*3801, B*3901, B40, B*4403, B*5101, B*5102, B*5103, B*5201, B*5301, B*5401, B*51, B*5801, B60, B61, B7, B8, Cw*0401, Cw*0702, MHC-Dd, MHC-Kd, MHC-Kk and MHC-Ld. Highest peptide score and binding was found with allele HLA-B*5401. Structure prediction of this sequence by PSIPRED revealed that there are one helix, seven coils and five strands present in this sequence. The findings of the study can be very useful for making a good antigenic candidate by researchers.

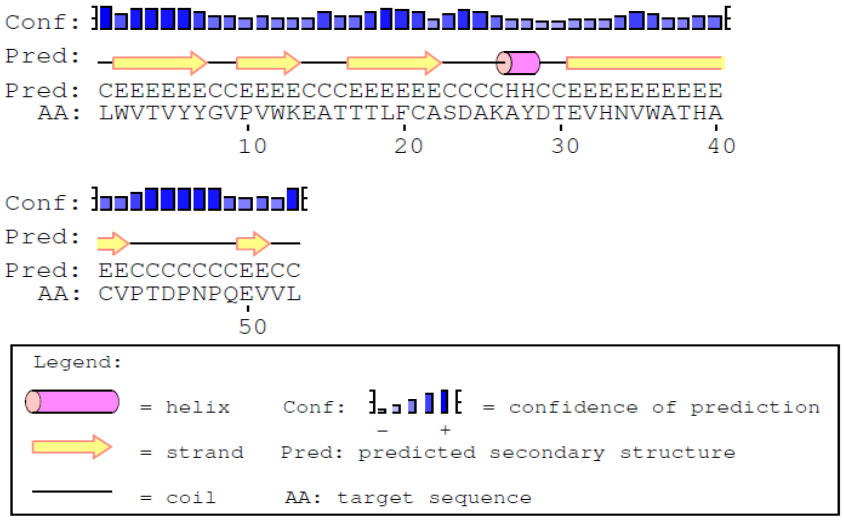



**Figure 2. PSIPRED predicted secondary structure of antigenic epitope peptide Vaccine target for HIV**

## CONCLUSION

New vaccine development is an expensive process and demands a great amount of time. However, *in-silico* approach for vaccine target prediction can definitely reduce manpower, time and cost. In relation to search for a potential cost-effective candidate for the development of preventive measures against the drastic diseases, blocking adherence and invading efficiency of pathogen may provide a better alternative to treatment. The present study was an effort to find out such a suitable vaccine target for HIV yet further investigations using more experimental paradigms are necessary for further confirmation.

## CONFLICT OF INTEREST

Conflict of interest declared none.

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# Growth inhibition of enterotoxigenic *Escherichia coli* by citrate capped copper nanoparticles

**MOHIT AGARWAL, ANURAG JYOTI AND RAJESH SINGH TOMAR***

Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior

*Corresponding author email: rstomar@amity.edu

## ABSTRACT

Nanoparticles have unique properties compared to their bulk counterparts due to their small size (less than 100 nm) and high surface-to-volume ratio. These allow a better interaction with cells and biological molecules. Because of this reason, nanotechnology has attracted a great deal of attention from the scientific community. Metal oxide nanomaterials like CuO, Ag and ZnO have been used industrially for several purposes. A common feature that nanoparticles exhibit is their antibacterial behavior against pathogenic bacteria. The present study aims on the synthesis and antimicrobial activity of CuO-NPs against Enterotoxigenic *Escherichia coli* (ETEC) and found that nanoparticles particularly copper oxide nanoparticles (CuO-NPs) can be a better alternative for diarrheagenic *E. coli*.

Synthesis of CuO-NPs was achieved by Chemical route and capped by citrate to prevent agglomeration and were well characterized. We used these nanoparticles to evaluate their antibacterial activity against ETEC. The growth inhibition study of CuO-NPs was performed followed by time dependent study. Further, to elucidate its effect on DNA, the DNA damage study was also performed. The synthesized CuO-NPs showed growth inhibitory effect activity against ETEC. Further, the time dependent study showed the similar bactericidal activity of CuO-NPs after 16 hours. The CuO-NPs were effective enough to show DNA damage. In view of these, the CuO-NPs display, in fact, enhanced antibacterial properties and their synthesis procedures are quite cost effective. We envision that this study offers novel insights into antimicrobial actions of CuO-NPs which can be used as a novel class of topical antimicrobial agent for ETEC.

## KEYWORDS

CuO-NPs and Antibacterial activity.

## INTRODUCTION

Making drinking water safe is on priority in India. Serious health problems focus in areas where the quality of drinking water is poor and deteriorating gradually. According to Bureau of Indian Standards (BIS) Peoples living in India would be provided good quality of drinking water, but still due to the extent of contamination the quality of drinking water is not under control (Bureau of Indian Standards, IS-10500-2012).[1] The presence of Enterotoxigenic *Escherichia coli,* a pathogenic strain of *Escherichia coli* in potable water is the major issue in developing countries like India which leads to diarrhea and cause for mortality and morbidity in young children. Globally, Over 6.5 million children died under age of five.[2] Basically, *Escherichia coli* reside in the intestines of human and other animals too. Most types of *E. coli* causes no harm but some of them are pathogenic and can cause disease. Enterotoxigenic *Escherichia coli* (ETEC) is the name given to a group of *E. coli* that produce special toxins which stimulate the lining of the intestines causing them to secrete excessive fluid, thus producing abdominal cramping and leads to watery diarrhea. ETEC produces two toxins, a heat-labile toxin (LT) and a heat-stable toxin (ST). It is transmitted by food or water.[3] A broad range of infectious strains now exhibit antibiotic resistance. Some of the common examples are Methecillin-resistant *Staphylococus aureus* (MRSA), Vancomycin-resistant *Enterococi* (VREs), Urinary tract infection (UTIs) and intestinal /colon infection by Enterotoxigenic *Escherichia coli* (ETEC).[4] Various different growing pathogenic strains now reveal multiple antibiotic resistance mechanism (MAR).[5] Antimicrobial agents play an essential role in the control of these infectious diseases and spread of these pathogens. The World Organization for Animal Health (OIE), Food and Agriculture Organization (FAO), and World Health Organization (WHO) all have stated on the serious intimidation posed by antimicrobial-resistant pathogenic organisms to human and animal health.[6] The over and over use of antimicrobial agents has generated the selective pressure to encourage the gradually increasing rates for antimicrobial resistance.[7] Hence there is no single antimicrobial agent available for human and animal use that has not demonstrated resistance against microorganisms. This problem had forced clinicians to rely on in vitro antimicrobial susceptibility testing for diagnoses.[8] There has been much recent interest in using silver, zinc and copper nanoparticles in new technologies with respect to their improved properties as compared to their bulk such as increased surface area and high reactivity of nano size materials.[9] Larger the surface area ensures an increased range of interaction with bio-organic material present on the viable cell surface. These synthesized nanoparticles further being incorporated into consumer products.[10, 11] The antimicrobial activities of these synthesized inorganic metal oxide nanoparticles for example CuO, ZnO, $TiO_2$, $SiO_2$ etc and their selective toxicity to bacterial cell suggests their potential application in various fields such as diagnostic, therapeutic and nano medicine based antimicrobial agent.[12] Copper and zinc slowly release their cation in small amount which are toxic to bacteria.[13] This leads to necessity for the development of potential new alternative materials in order to combat this problem. The development of antibiotics is indeed expensive (approx. $ 1 billion is required to have a new drug in the market), time consuming, risky and is also nasty because of their too short life cycle (due to bacterial resistances).[14] The present study focused on the antimicrobial activity of synthesized citrate capped metallic copper nanoparticles through a chemical means. Citrate capping is well known to control nucleation and for the stability of nanoparticles. Citrate capping has been used for the synthesis of metals such as Au, Co, Ag, Pd and Cu.[15] The antimicrobial activity of the synthesized copper nanoparticles in the present study was tested against ETEC strain isolated from potable water, including reference strain. However, it is interesting to note that our findings in this study indicate antimicrobial activity against ETEC. The ultimate purpose of this study was to analyze the effect of citrate capped copper nanoparticles by chemical means.

## MATERIALS & METHODS

**1. Sample collection:** Water samples (2L) were collected for the isolation of Enterotoxigenic *Escherichia coli* from six different sites of Gwalior city[16]. All samples were collected and brought to laboratory for further processing on the same day. Standard and modified protocols were followed to collect, concentrate and clean-up the sample.

**2. Isolation and identification of *Enterotoxigenic Escherichia coli*:** Samples collected from each site were concentrated by using centrifugation and filtered through a membrane filter (cellulose nitrate filter of 0.22 µm pore size; Millipore, USA). Membrane filter was removed aseptically and placed on culture media containing EMB (Hi-media). Further, select randomly about 10 colonies (presumptive *Escherichia coli*) with greenish metallic sheen colored colonies growing on EMB agar plates. These colonies were placed in a 25-mL Erlenmeyer flask containing 10 mL Nutrient broth, and incubated at 37 ± 1°C for 3–4 hr at 200 rpm on a rotatory shaker. A loopful of culture from Nutrient broth tubes was then spreaded on EMB Plates and incubated overnight at 37 ± 1°C. These isolates were further screened using indole, methyl red, vogous proskauer and simmon citrate tests. The isolates confirmed as *Escherichia coli* were maintained at –70°C supplemented with 15% (vol/vol) glycerol.

**3. Genomic DNA isolation and detection of virulent signature gene:** DNA was extracted by boiling prep method. Briefly, isolates of *E. coli* were incubated at $37^0$C overnight. An aliquot (1.5 ml) of the overnight culture was transferred to 2 ml tubes and then centrifuged at 7000 rpm for 3 min, remove the supernatant and resuspended the pellet by vortexing in 200 µl of sterile double distilled water. The suspension was boiled for 30 min, centrifuged (7000 rpm, 2 min), and 250 µl of the supernatant was collected and precipitation was done by using sodium acetate (0.3 M, pH 5.2) followed by ethanol. The precipitated DNA was washed thrice by 70% ethanol and resuspended in 100 µl TE (pH 8.0). Repeat the procedure for all the isolates. Quality of isolated DNA was checked on agarose gel electrophoresis. Further, we had selected 10 random isolates from each site for the presence of signature virulent gene *LT-1* (heat labile enterotoxin) using primers and thermal cyclic conditions. We used *E. coli* MTCC 723 as the positive control for *LT1* gene. The PCR reaction mixture and conditions used have been described earlier. [16] All the assays were done in duplicate. Amplicons were analyzed on 1.8% agarose gel, visualized and recorded.

**4. Antibiotics susceptibility test:** All the obtained *E. coli* isolates showed resistance to at least one of the tested antimicrobials. These selected Drug resistant strains showed high resistance against Ampicillin and Erythromycin. However, intermediate to Chloramphenicol, Co-trimoxazol were observed among some of the isolates. Resistance to amoxicillin, nalidixic acid and tetracycline in EHEC isolates has also been observed. [17] Some of the strains were found to be sensitive against ciprofloxacin. The lesser number of antibiotics having bactericidal effect as compared to more antibiotics showing ineffectiveness against ETEC isolates is crucial and alarming. Positive isolates were carried out for antimicrobial susceptible test as per CLSI guidelines.

**5. Chemicals and Reagent:** Copper sulfate ($CuSO_4.5H_2O$), Sodium Borohydrite ($NaBH_4$), tri sodium citrate ($Na_3C_6H_5O_7$**)** was purchased from Hi-media (Bangalore, India). Acetone and Ethanol were purchased from Thermo Fisher Scientific. HPLC grade water (18Ω) (Fisher Scientific) was used in this study.

**6. Synthesis of Copper nanoparticles:** For the reduction of copper ions, 0.003M Copper sulphate ($CuSO_4.5H_2O$) was placed into a erlenmeyer flask in 90 ml of HPLC grade (18Ω) de-ionized water and 0.002M sodium Borohydrite ($NaBH_4$) in 10 ml of de-ionized water. Place the flask on to the magnetic stirrer

and add the reducing agent into it drop by drop and stir it, resulting in the change in colour from light blue to yellowish, then Add 0.001 M of tri sodium citrate. There is a sudden change in colour from yellow to brown and then to dark red colour indicating the synthesis of copper nanoparticles (18). The prepared mixture was heated to 65 °C for about 2-3 hrs under continuous stirring. After incubation for 3 h, the solution was centrifuged with 12,000 rpm for 8 min, and their pellets were re-dispersed in sterile distilled water. Repeat the centrifugation and re-dispersion for 3-4 times so as to ensure the complete separation of nanoparticles. In order to remove impurities, it was precipitated and washed with absolute ethanol and acetone several times. Finally, the powder was dried at about 80 °C overnight. After drying, purified nanoparticles were resuspended in de-ionized water. The produced powder is stable for more than a month. Characterization of synthesized particles was done primary by U.V-Visible spectroscopy and T.E.M.

**7. Characterization of Synthesized Nanoparticles**:

a) Centrifugation: Centrifugation was performed in 15 ml tubes high speed centrifuge (REMI). Acetone and isopropanol were added to aqueous sample in a ratio of 1:2 to encourage precipitation and for the removal of residual impurities. After allowing the CuO-Nps to precipitate they were centrifuged again for 10 min at 12000 rpm.

b) UV-Visible Spectroscopy: UV-Visible spectra was taken in an optical quality quartz cuvette with a 1 cm path length, require 2.5 ml of solution to fill past the light path of the instrument. Spectra were seen at room temperature, while copper sulphate solution was used as a blank. Solution was diluted immediately before the analysis. Spectrum was seen from range 200 to 600 nm.

c) Transmission Electron Microscopy: CuO-Nps samples were concentrated via centrifugation prior to use for it. Transmission electron micrographs were obtained.

**8. Antimicrobial Assays.** The antimicrobial activity of CuO-NPs was tested against ETEC (Environmental isolates) and reference strain of ETEC (MTCC 723). The assays were set by preparing the CuO-NPs at different concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL, which were suspended in Nutrient Broth. Each concentration of NPs was added to $10^8$ CFU/mL of bacterial suspension and incubated at 37 °C for 18 h and 150 rpm. During the incubation the growth of bacteria was monitored by measuring the optical density (OD) at 600 nm at regular interval of two hours. Further the bacterial growth was determined by on plating the treated culture and control upon EMB agar.

**9. DNA Damage of ETEC in presence of Copper nanoparticles:** The treated culture was taken for the study of DNA damage**.** DNA form ETEC was extracted by boil prep method followed by precipitation using sodium acetate (0.3M, pH 5.2) and absolute ethanol. DNA from treated and control samples were run on agarose gel electrophoresis for the further analysis.

## RESULTS AND DISCUSSION

**1. Isolation and identification of ETEC:** On EMB Agar plates the colonies were observed to be green metallic sheen colored which preliminary confirms the presence of *Escherichia coli*, Nucleotide sequences of virulent signature *LT1* gene of ETEC were designed by using BLAST, and this confirmed the targeted strains in potable water of Gwalior city by specific computed primers using signature gene *LT1* of ETEC. Basically, ETEC produces two types of toxins, a heat-labile toxin (LT) and a heat-stable toxin (ST). These both strains

of ETEC can secrete either one or both of these toxins, while the illness caused by each toxin is similar. Infection with ETEC is the leading cause of travelers' diarrhea.

Table 1 Nucleotide sequence of candidate oligomer of *LT1* gene of ETEC

| Gene | Primer Sequence (5'- 3') | Tm($^0$C) | Amplicon |
|---|---|---|---|
| *LT1* | GGCAGGCAAAAGAGAAATGG | 54.5 | 150 bp |
| | TTGGTCTCGGTCAGATATGTG | 54.4 | |

**2. Occurrence of virulence determinants in *ETEC* isolates:** Samples collected from six different sites, three sites were positive for the ETEC. The 150 bp amplicon were observed in these samples on gel electrophoresis after PCR[18] (Figure 1). Results revealed that 50 % of ETEC strains isolated from the potable water exhibit virulent gene. Remaining isolates were negative for the presence of *LT1* gene. Our interpretation on virulence markers indicated that the drinking water is contaminated by ETEC exhibiting virulent *LT1* gene. Contaminated water and poor hygiene are the reasons for diarrheal diseases throughout the world. In present study, we found potable water samples contaminated with strains of ETEC. The presence of ETEC in potable water suggests the possibility of contamination of water supplies. Water channels flowing in the old city and other areas are often leaky and unmanaged. As a result they are often in contact with the sewage pipelines. Most of the strains were in culturable state, which shows the presence of high organic matter in potable water. Still some of the strains may have undergone in viable but not culturable (VBNC) state which could not be cultured easily.

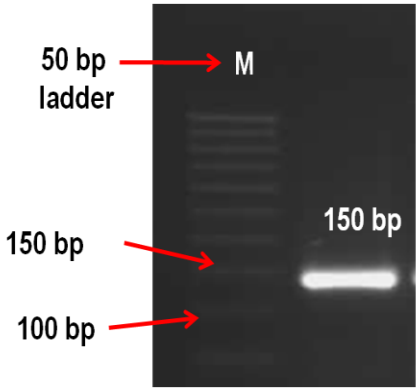


**Figure1. Agarose gel electrophoretic image of Amplicons after PCR M: Ladder (Marker) of 50); 2: Environmental samples**

**3. Determination of antimicrobials susceptibility:** ETEC isolated from contamination point were screened for susceptibility for existing antimicrobials by disc diffusion method as described by Clinical and Laboratory Standards Institute. All of the potential ETEC isolates in the present study were resistant to at least one of the antimicrobial. We found that some of isolates were resistant to more than two antimicrobials. Out of the isolates we recovered, some exhibited resistance to the β-lactam class of antimicrobials. It was found that site 3 showed high resistance pattern (45.87%) as compared to site 1 (30.22%) and site 2 (28.09%). Site 1 has high intermediate pattern (47.12%) as compared to other sites.

**4. Synthesis and Characterization of Copper nanoparticles:** Synthesis of CuO-NPs by reducing an aqueous solution of copper ions with an in-situ capping of metallic nanoparticles with citrate ions to control the particle size and also to increase its stability. The copper nanoparticles were synthesized and capped by using citrate. Citrate is a good stabilizing agent. The CuO-NPs exhibit the Surface Plasmon Resonance (SPR) related spectra in the UV-visible range at 294 nm and also show intense dark red colour (figure 2). The size and shape of the synthesized CuO-NPs were determined by transmission electron microscopy (TEM) (figure 3). TEM image of CuO-NPs synthesized by using Chemical means is predominates with spherical oval, morphologies ranging from 25 to 30 nm (approx). The nanoparticles were mono dispersed and no aggregates were found, indicating stabilization of the nanoparticles by capping agent.

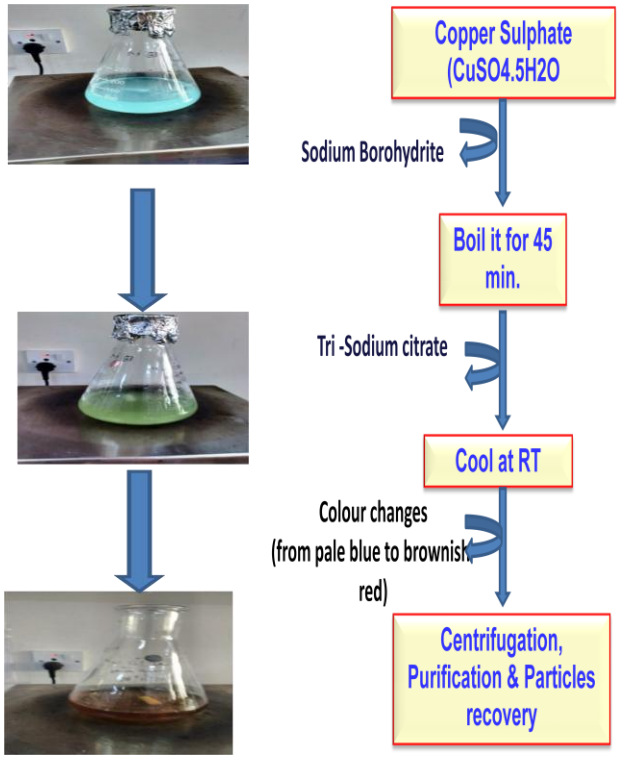


**Figure 2. Flow chart for the Synthesis of copper nanoparticles (Brownish-Red colour)**

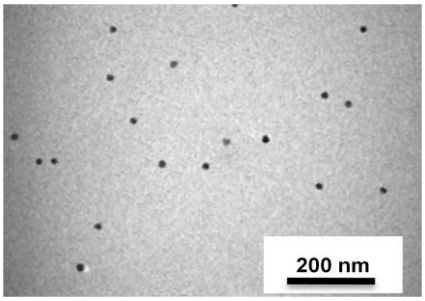


**Figure3. Transmission Electron Micrograph of Capped CuO-NPs**

**5. Antimicrobial Assay:** The antimicrobial activity of CuO-NPs against ETEC environmental strains was performed. It evident that there was an increase in the inhibitory activity as we gradually increases the concentration of nanoparticles. Growth inhibition of ETEC was examined both on EMB agar plates and also in Nutrient broth containing a range of concentration (0, as a control, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml) of CuO-NPs. ETEC at stationary phase were inoculated into media (Nutrient Broth) $10^8$ CFU/ml. The growth of bacteria was evaluated by measuring the optical density (OD) at 600 nm at different time intervals. It was found that at growth of bacteria was inhibited at concentration 0.5 mg/ml as it reaches time 2 h (Figure 3). On the plate spread with $10^8$ ETEC CFU/Plates and in broth inoculated in equivalent number of cells, bacterial growth was inhibited at 0.5mg/ml of CuO-NPs. CuO-NPs has a modest effect on cell growth, which result in the recovery of few viable cells (Figure 4). The CuO-NPs was found to exhibit the lesser antibacterial property in Nutrient broth which might be due to either prevention of interaction of copper nanoparticle via component of Nutrient broth.

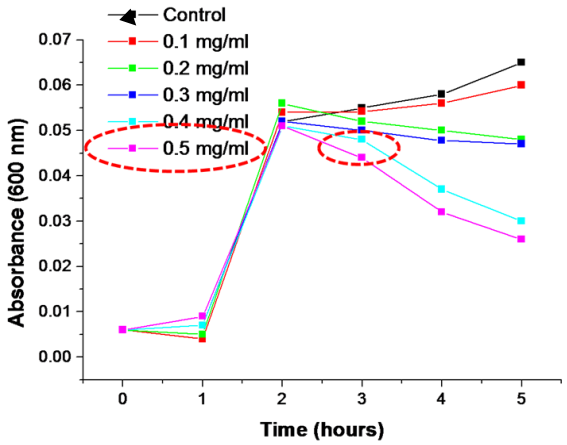


**Figure 3 Growth curve of ETEC in presence of CuO-NPs**

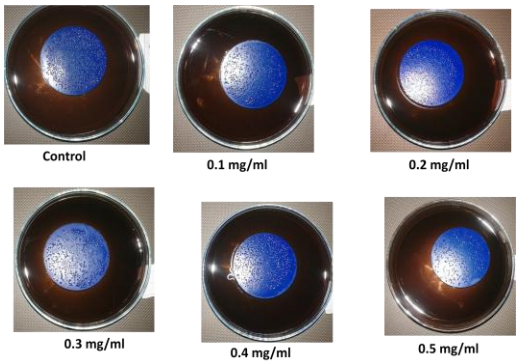


**Figure 4 Growth inhibitory effects of CuO-NPs on ETEC**

**6. DNA damage:** The culture treated with CuO-NPs was taken for the study for DNA damage**.** DNA isolated form treated ETEC culture was run on agarose gel electrophoresis. The treated culture DNA was found to be damaged as a clear smear was observed in comparison to untreated one (data not shown).

## CONCLUSION

The presence of pathogenic strains of ETEC in Gwalior city is alarming which may be the major roadblocks for the management of waterborne outbreaks. Therefore, the presence of ETEC in potable water of Gwalior city requires increased examination for risk assessment and prevention strategies for the protection of public health. CuO-NP was synthesized by chemical reduction methods. Citrate not only reduces the size but also traps on the surface of nano-particles. In view of these, the CuO-NPs show, in fact, enhanced antibacterial properties, and their synthesis procedures are quite easy and cheeper. We can visualize that this study offers novel insights into antimicrobial actions of CuO-NPs and also demonstrates CuO-NPs as a novel class of contemporary antimicrobial agent for the treatment of water borne infectious diseases.

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# Investigation of polarization and depolarization current measurements in pure and doped pvk nanocomposite

**PANKAJ KUMAR MISHRA*[#]**

[#] Dept. of Applied Physics, Amity School of Engineering and Technology, Amity University Madhya Pradesh, Gwalior-India

* Corresponding author email: pmishra@gwa.amity.edu

## ABSTRACT

TSDC and AFM studies of solution grown films of pure and nano ZnO doped PVK samples have been studied. The solution cast technique was utilized for preparation of samples. One relaxation peak was observed at 90$^{o}$C. In case, when the permanent dipoles are present, an electric field brings about their orientation and polarizes the material. The displacement of polar groups require some time and is thus dependent on frequency and temperature, which lead to different dispersion phenomena. Doping with nano ZnO found to affect the magnitudes. The activation energy decreases with the increase in poling field strength.

## KEYWORDS

PVK, TSDC, AFM, Charge Transfer Complex, ZnO

## INTRODUCTION

Polymers are complex dielectric materials. Almost all polymers are amorphous or partially crystalline macromolecular organic compounds. The increasing demand for microminiaturization of components for electronic applications has further stressed the need for growth and development of thin polymer films [1, 2]. Extensive research is underway in many laboratories on electrical properties of pure and doped polymers, copolymers, polymeric blends and polymeric compositions. The analysis of insulating materials and insulating systems has been advancing on many fronts. Extensive quantitative and qualitative basic research in the subject have been carried out resulting in a huge amount of fruitful information. A major portion of the family of insulators is taken up by polymers. In the last three decades, scientists have been showing their increasing interest in the basic electrical properties of large energy and bang gap materials. Most of the literature is available on electrical behavior, charge storage and transport or most specifically the manifestation of the "electrets state" by some organic solids, waxes, insulating dielectrics, glasses and semicrystalline and amorphous polymers[3-10].

## MATERIALS AND METHODS

Pure and nano ZnO doped PVK used in present investigation. Solution caste technique is utilized for film formation. For preparing the thermoelectrets, the solution was prepared in a glass beaker by dissolving 1 gm PVK and nano ZnO (1 mg) in chemically pure dimethyl formamide (DMF). The solution was kept for 24h to get a homogeneous and transparent solution. For measuring the TSDC, the sample was discharged by heating at the linear rate of 3°C/min. upto 120°C to achieve depolarization. The current was measured by Keithley electrometer. The thickness of the sample was of the order of 30 μm which was estimated by measuring the capacitance of the fabricated sandwiches. Atomic Force Microscope (NT-MDT, Ntegra) available in IIT Roorkee (India) was used to observe atomic and molecular scale visualization and measurement, functional mapping of surface, and molecular level manipulation.

## RESULTS AND DISCUSSION

The experimental conditions under which the thermally stimulated discharge currents and atomic force microscopy (AFM) were measured are summarized below –

| | | |
|---|---|---|
| Polarizing field strength | - | 250-700 volts |
| Polarizing temperature | - | 40- 70°C |
| Heating Rate | - | 3°C/min. |
| Electrode Material | - | Aluminium |

### THERMALLY STIMULATED DISCHARGE CURRENT (TSDC)

The TSDC thermogram for pure and ZnO doped PVK samples with fields (i.e. 250-700volts) at constant $T_p$ (i.e. 60 °C) and at $T_p$'s, (i.e. 40-70°C) with fixed poling fields (550 and 700 volts) are shown in figures 1– 6 respectively.

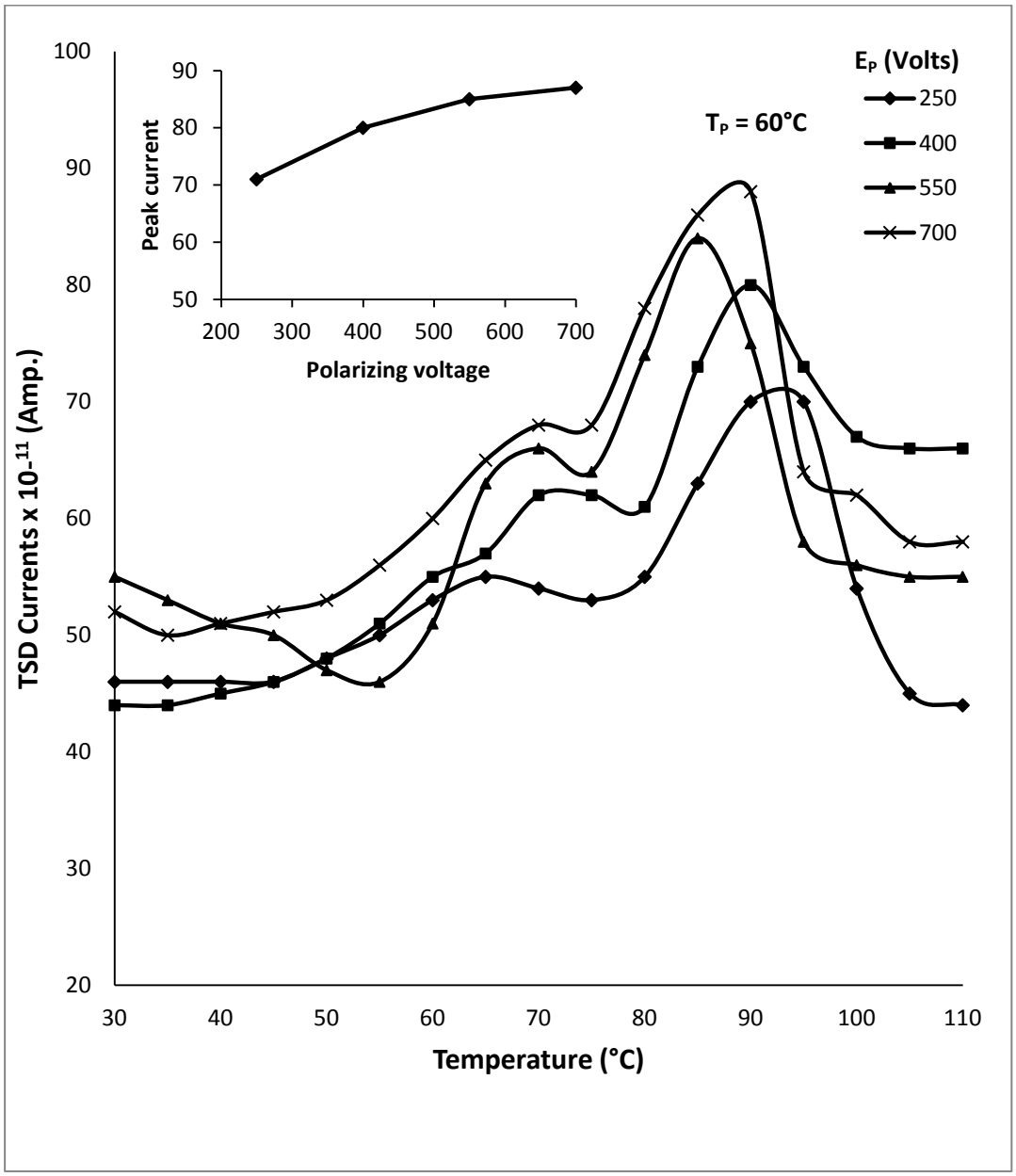



**Figure 1:** **TSDC thermograms of pure PVK samples poled at 60°C with different polarizing fields(i.e. 250, 400, 550 and 700 volts).**

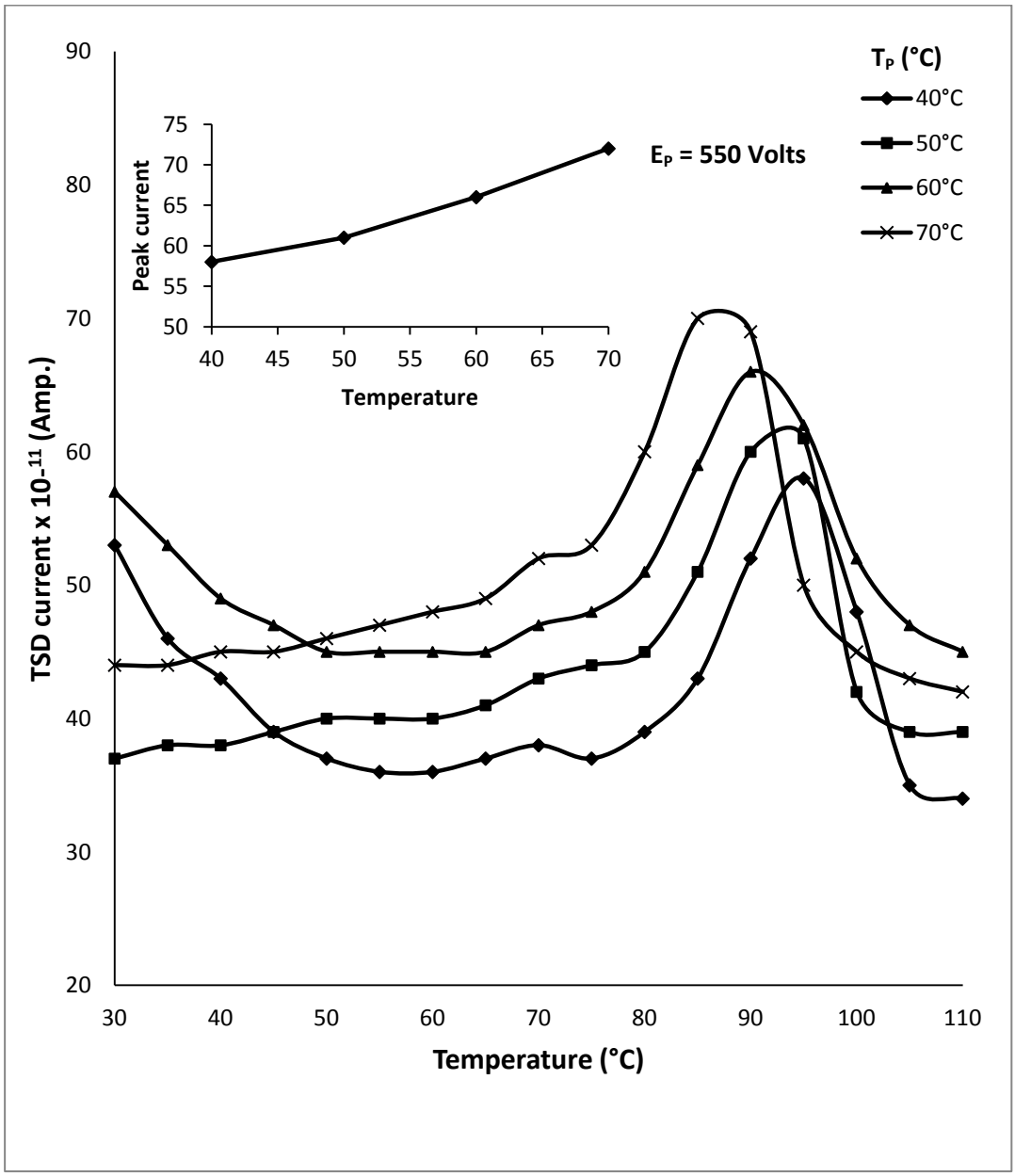



**Figure 2:** **TSDC thermograms of pure PVK samples poled with 550 volts at different polarizing temperatures (i.e. 40, 50, 60 and 70°C).**

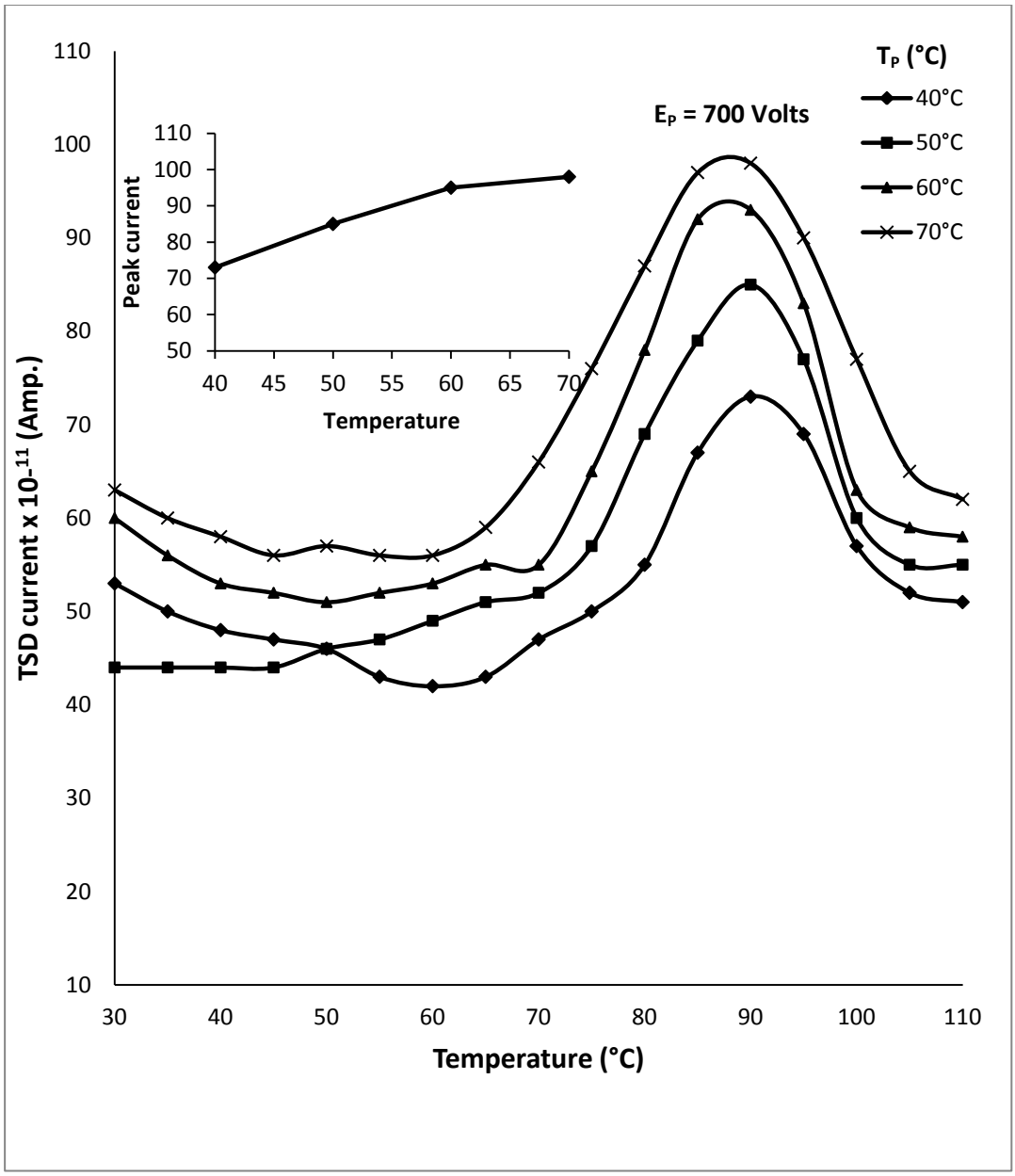



**Figure 3:** **TSDC thermograms of pure PVK samples poled with 700 volts at different polarizing temperatures (i.e. 40, 50, 60 and 70°C).**

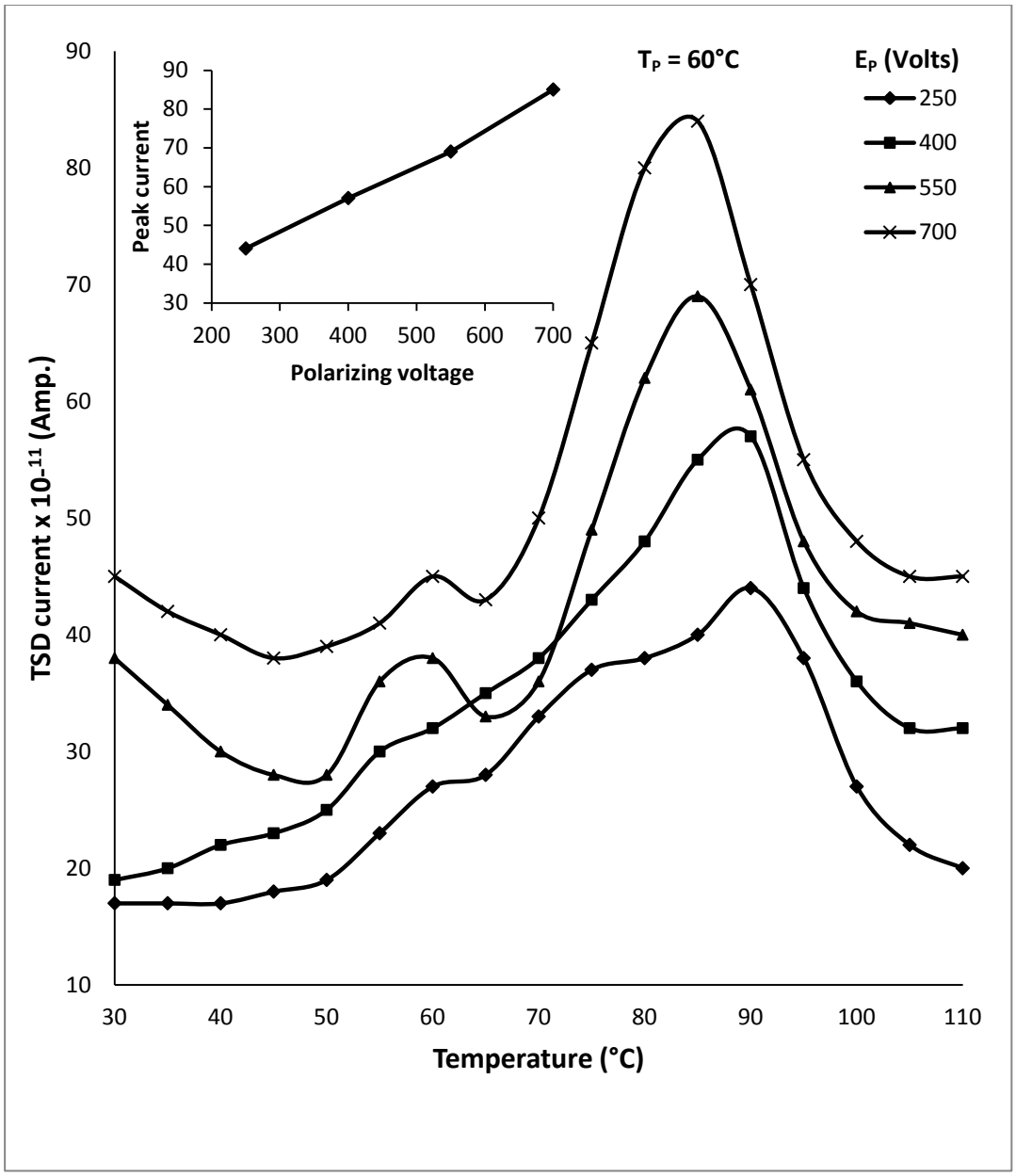



**Figure 4:** **TSDC thermograms of ZnO doped PVK samples poled at 60°C with different polarizing fields (i.e. 250, 400, 550 and 700 volts).**

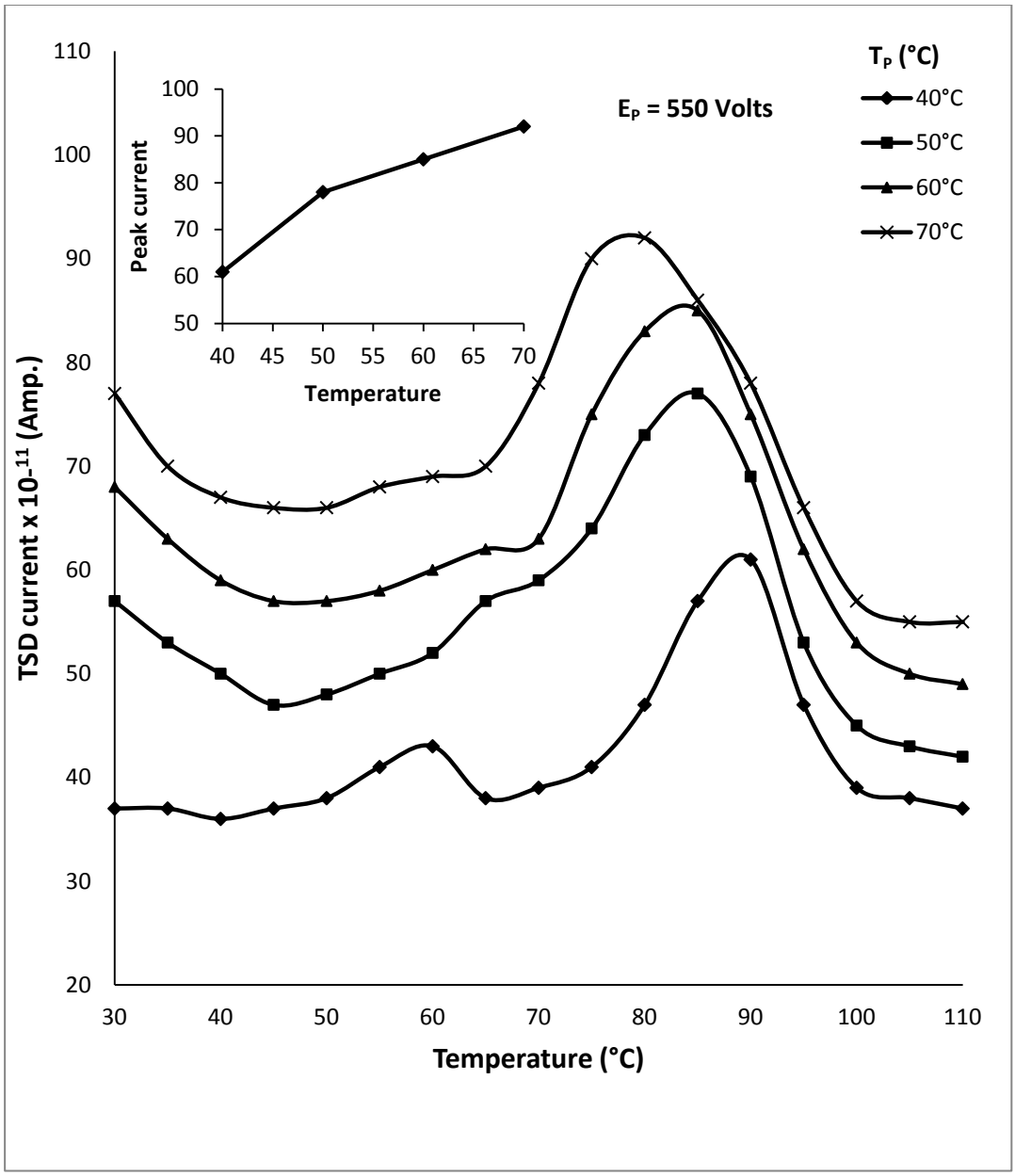



**Figure 5:** **TSDC thermograms of ZnO doped PVK samples poled with 550 volts at different polarizing temperatures (i.e. 40, 50, 60 and 70°C).**

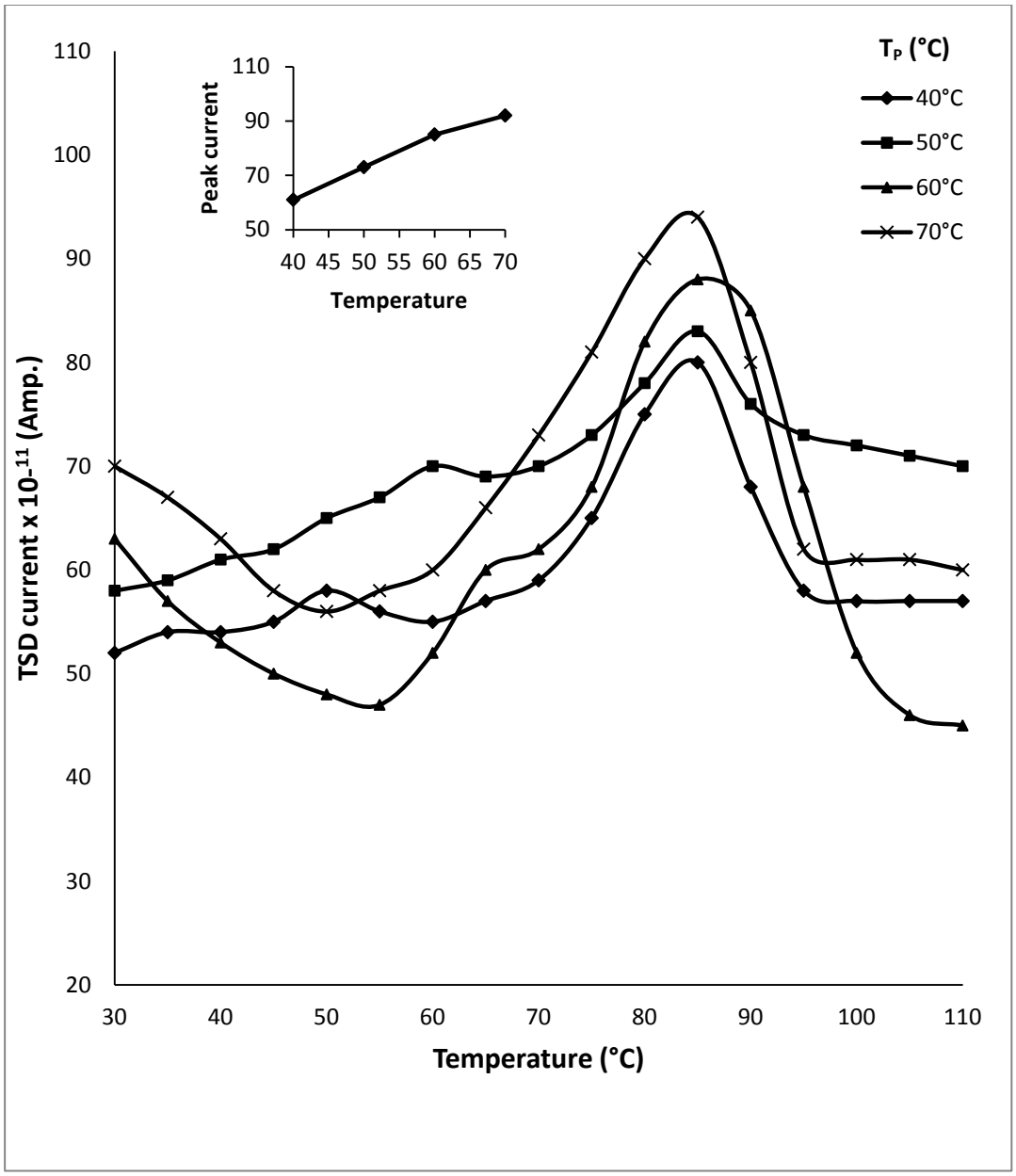
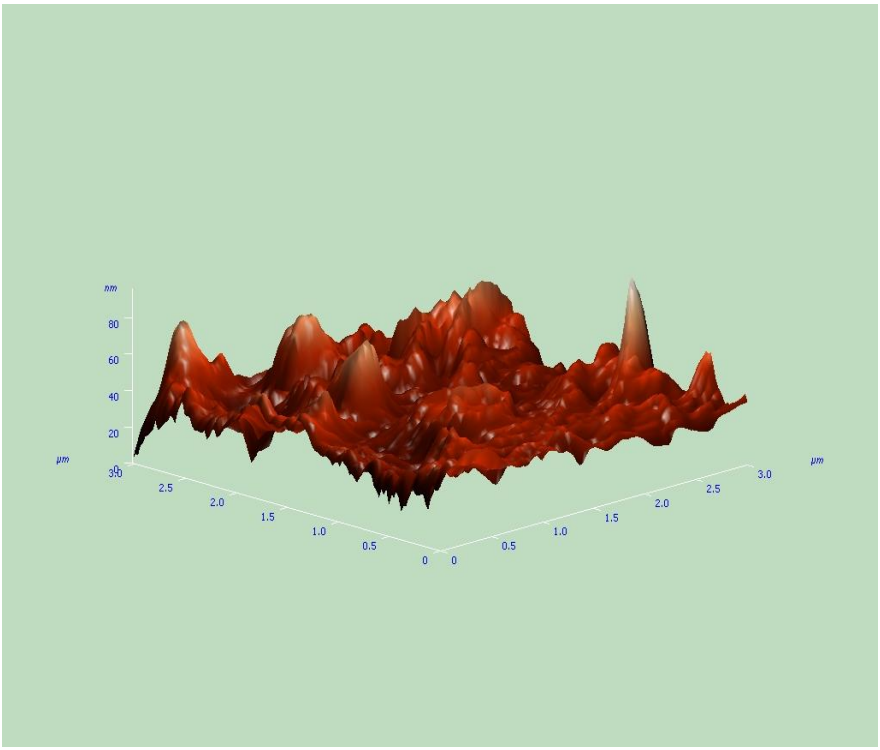


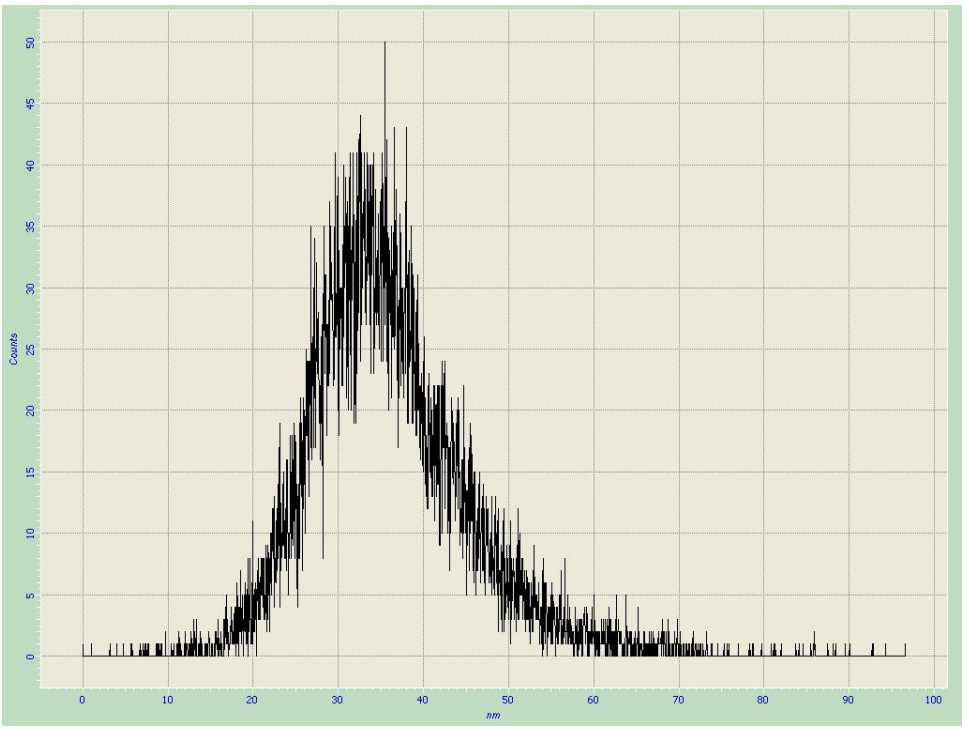

**Figure 6:** **TSDC thermograms of ZnO doped PVK samples poled with 700 volts at different polarizing temperatures (i.e. 40, 50, 60 and 70°C).**

## ATOMIC FORCE MICROSCOPY (AFM)

The roughness of the thin film was investigated using atomic force microscopy (AFM). Atomic force microscopy was performed to examine the surface morphology and to measure roughness values of pure and ZnO doped PVK samples.

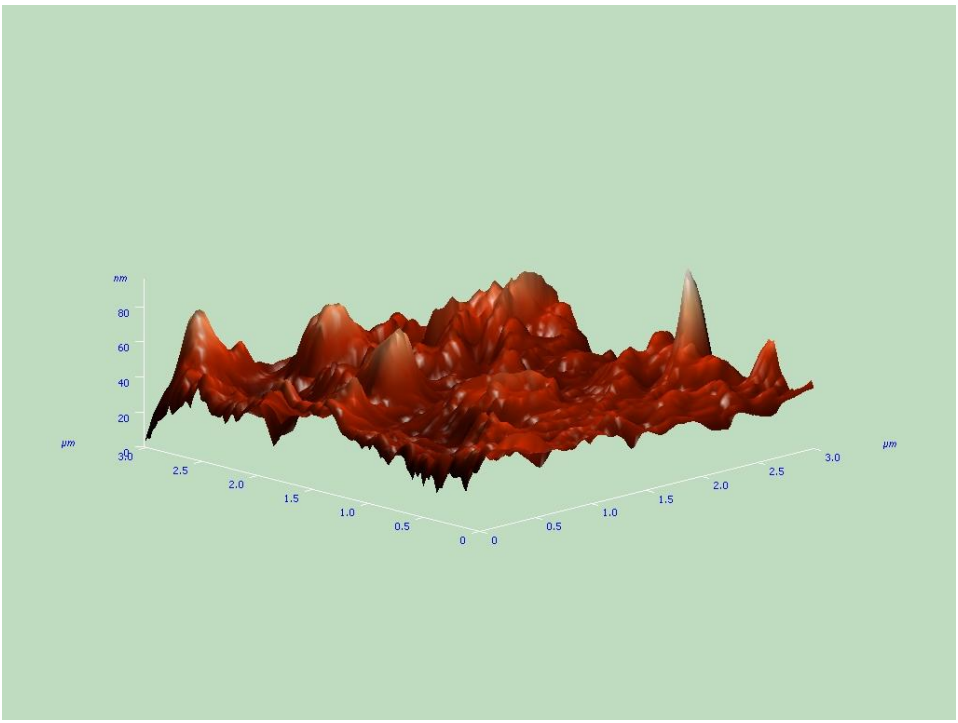



**(a)**

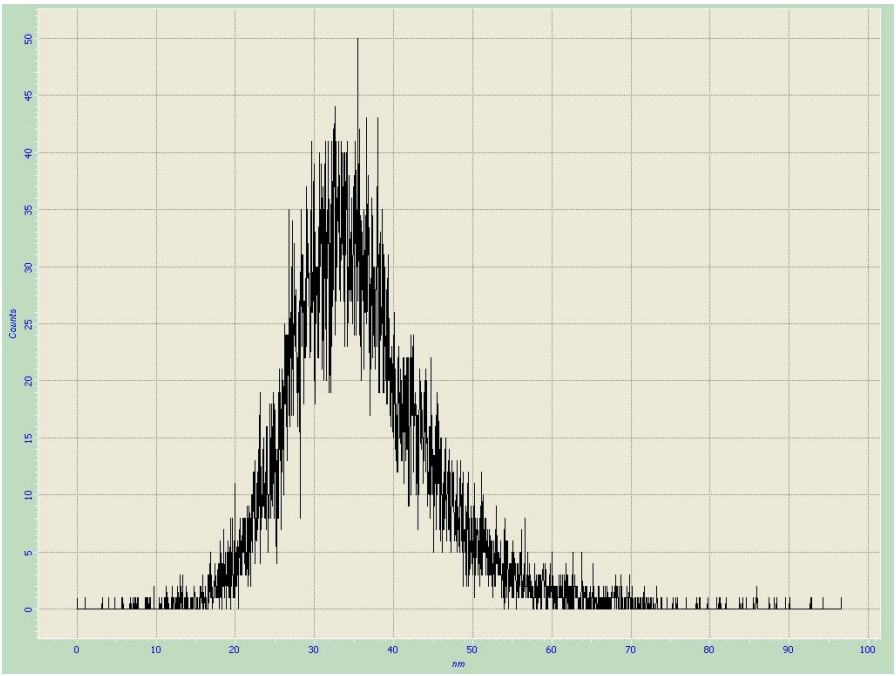



**(b)**

**Figure 7 :(a) and (b) Three dimensional topographic scan and hologram of PVK sample**




(a)




(b)

**Figure 8 : (a) and (b) Three dimensional topographic scan and hologram of nano ZnO doped PVK sample**

Figures 7 (a, b) and Figure 8 (a, b) show three dimensional topographic scan and hologram of the samples respectively. Results reveal that in case of pure PVK the roughness is 18 nm and particle size is15 nm and after doping the nano ZnO in PVK film shows a remarkable change. It reduces the roughness and particle size[11-15].

In TSDC, the appearance of peak in the high temperature region imply that the injection of ions may be significant in this polymer. It is also possible that PVK contains a high number of impurity molecules prior to field treatment and these molecules are dissociated into various ionic species by a combination of the high internal and external fields. The charge trapping in a polymer takes place at the molecular main chain, the side chain and at the interface of crystalline and amorphous regions of the polymer. The high field applied during elected formation may also produce some additional trapping sites[16-18].

The charge released from these traps occurs because of the thermal excitation and motion of the molecular chain that causes the lowering of trap depth. The released charge can recombine, retrapped in trapping sites, or may get discharged at the electrodes. The chances of retrapping of the released charge are high in a polymer having a large number of trapping sites and it is expected that the discharge will give complex TSC spectra with broad peaks indicating a distribution of activation energies. The behavior of peak current versus poling field (inset of Figures 1-6) in the higher field region indicates the space charge phenomena. The peak observed in the thermograms is not due to single relaxation but seems to be complex and may arise due to the release of the frozen dipoles by their cooperative motion with adjoining segments of the main polymer chain. The super linear increase of peak current and release charge versus poling field in the higher field region suggests that the peak is contributed both by electronic and ionic processes, arising in the bulk and injected from the electrodes[19-23]. A broad TSD peak along with a smaller peak was observed both in pure and doped PVK films at the entire range of temperature studied. The low temperature relaxation occurs due to orientation of the side groups and or local twisting of the main chain. It loses its definition under certain conditions of polarization. Shift in peak position with poling temperature shows that the charges are trapped at various trap depths.

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# Chemical composition analysis and free radical scavenging activities of microwave extracted essential oil from *Allium sativum* rhizomes

**POOJA SHARMA, RAJESH SINGH TOMAR AND RAGHVENDRA KUMAR MISHRA***

Amity Institute of Biotechnology, Amity University, Gwalior, Madhya Pradesh, India

*Corresponding author email: rkmishra@gwa.amity.edu

## ABSTRACT

Free radicals, reactive oxygen and reactive nitrogen species have been considered as mediators of inflammation, neurodegeneration, tissue injury and cell death. Also, they play a major role in food deterioration. Current research is directed towards finding naturally occurring antioxidants of plant origin. In the present study, the chemical composition analysis of *Allium sativum* rhizome essential oil (ASEO) was conducted by gas chromatography-mass spectroscopy (GC-MS). Moreover, radical scavenging activity, total antioxidant capacity and phenolic content of ASEO were investigated using different *in vitro* assays. The GC-MS analysis of essential oil obtained from microwave-assisted hydrodistillation of *A. sativum* rhizome resulted in the determination of 42 different components, representing 99.16% of total oil. The ASEO was characterized with the presence of terpenes, terpenoids, aliphatic and aromatic hydrocarbons, sulphur containing compounds and phenolics along with some other essential phytochemicals. The ASEO at the highest tested concentration (250 µg/ml) showed antioxidant capacity as the inhibition of DPPH, nitric oxide, superoxide, hydroxyl radicals and ferrous ion chelation by 82.03%, 75.66%, 79.86%, 81.11% and 71.86%, respectively. Moreover, the ASEO displayed concentration-dependent reducing power ability and remarkable inhibitory effect on ferric ion-induced lipid peroxidation in rat brain extract. The total antioxidant capacity of ASEO was found to be 94.48 ± 1.12 mg of ascorbic acid/g of the sample. In addition, the ASEO yielded 7.27 ± 0.11 mg gallic acid / g dry weight sample. The present study confirms that the ASEO had potent antioxidant, anti-lipidperoxidation and radical scavenging potential; therefore, it might be used as a natural antioxidant against food deterioration.

## KEYWORDS

*Allium sativum*, Essential oil, Microwave extraction, Reactive oxygen species, Free radicals, Antioxidants

## INTRODUCTION

The human body has a number of natural enzymatic and non-enzymatic antioxidant defenses mechanisms which neutralize the harmful effects of free radicals and other oxidants. Considerable report indicates that foods containing antioxidants may be of main importance in disease prophylaxis and treatment. Hence, a growing consent among researchers arises that a combination of antioxidants, rather than single moiety, may be more effective over the long term because of synergism among the individual constituents.[1] Free radicals are highly reactive unstable moiety containing one or more unpaired electrons and are mainly derived from oxygen (reactive oxygen species; ROS) and nitrogen (reactive nitrogen species; RNS). Free radicals are continuously generated in the human body milieu by various endogenous metabolic, physiological and pathological processes including redox reactions, bioenergetics electron transfer, as well as the result of inflammatory and neurotransmitter responses.[2,3] Moreover, they are produced by the exposure to radiation, pollution, smoking, consumption of alcohol and drugs.[4] Though, free radicals have important functions in several biological processes such as protection from microbes, different enzymatic cascades, intracellular signalling and nuclear transcriptional factors, but their excess can be hazardous to human health. Oxidative stress is an imbalance in the pro/antioxidant homeostasis that occurs through an excessive production of free radicals or low availability of antioxidants.[5]

Free radicals can adversely affect biomolecules (lipids, proteins, DNA) and have been implicated in ageing, cancer, cardiovascular disorders, diabetes and inflammation.[1,6] Free radical induce protein damage can result in loss of enzymatic activity whereas DNA damage can result in mutagenesis and carcinogenesis.[7]

Despite many dietary and nutritional components, nature has gifted living organisms with protective antioxidant mechanisms, e.g., antioxidant enzymes including catalase, glutathione peroxidases, reduced glutathione (GSH), superoxide dismutase (SOD) and antioxidant molecules including ascorbic acid and α-tocopherol.[8] Previous investigations suggest that higher intake of antioxidant rich diet lowers the risk of human morbidity or mortality.[9]

Lipids are highly susceptible to free radical induced damage, lipid peroxidation, which is a common consequence of cell death.[1, 10] Moreover, oxidation in processed foods, enriched with fats and oils, during storage leads to spoilage and quality deterioration.[11] Also, antioxidants are important in food preservation. Different manufacturers have strived to produce high quality food with superior texture, color, flavor and nutritional values in the shelf life period. However, many foods are subject to lipid peroxidation that leads to quality deterioration. Hence, food preservation against oxidative degradation has encouraged the wide usage of food additives. The use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ) have been limited because of their carcinogenic and other toxic properties. Thus, the interest in antioxidants from natural origin has increased significantly.[12]

The plant-based volatile essential oils and non-volatile secondary metabolites have wide applications in dietary supplements, food flavoring, preservation, folk medicine and fragrance industry.[13,14] The main constituents of essential oils are mono and sesquiterpenes, carbohydrates, phenols, alcohols, ethers, aldehydes and ketones which are responsible for their biological activity as well as for their fragrance. Previous investigations have established the *in vitro* and *in vivo* antioxidant efficacy of plant-based essential oils.[15,16] Application of plant materials as dietary supplements and preservatives is mainly due to their antioxidant,

antimicrobial and other biological as well as pharmaceutical potentials. Currently, essential oils and their components are gaining increasing attention because of their relatively safe status, wide consumer's acceptability and the possibility of their utilization for potential multi-purpose functional uses. Therefore, it will be significant to examine the probable roles of free radicals in various diseases and antioxidants on disease prevention.[9]

Garlic (*Allium sativum* L.) is a species of Liliaceae family and has long been used not only as spice and condiment, but also as a traditional medicine against various diseases.[17,18] Preclinical investigations have shown that dietary intake of garlic reduces the risk of gastrointestinal and prostate cancer.[19] It has been shown to possess a wide range of therapeutic effects, such as anti-cancer, antihypertensive, hypolipidemic, hepatoprotective and immunomodulatory due to the inhibition of metabolizing enzymes, suppression of mediators of inflammation and cell arrest.[20-25] Garlic contains approximately 65% water, 28% carbohydrate, 2.3% organosulphur compounds, 2% proteins, 1.2% free amino acid and 1.5% fiber.[26] The active compound allicin is responsible for pungent smell and for its therapeutic properties.[27,28] Evidences from several investigations suggest that the biological and medicinal effects of garlic are mainly due to its high content of organosulphur compounds, polyphenols, fibres and phytosterols.[29-31] The most important organosulphur compounds present in garlic are c-glutamyl-S-alkylcysteine and S-alkylcysteine sulphoxides (alliin). Alliin are rapidly converted by allinase enzyme to allicin that has potent antioxidant properties.[32]

Literature survey has confirmed that there is very little literature report available on the antioxidant and free radical scavenging capacities of essential oil obtained from *A. sativum*.[33] In addition, due to the complexity of the oxidation-reduction processes, it is obvious that no single testing method is capable of providing a comprehensive picture of the antioxidant profile of ASEO. Hence, in the present study, an exhaustive approach was applied for the assessment of antioxidant and free radical scavenging capacity of ASEO.

In this study, a microwave-extracted hydrodistillation method was applied to isolate essential oil from *A. sativum rhizome*. The objective of the present study was to evaluate the antioxidant potential and free radical scavenging capacity of ASEO using various *in vitro* models including DPPH, nitric oxide, superoxide, hydroxyl radicals and ferrous ion chelation as well as lipid peroxidation assay followed by determination of its reducing power ability, total antioxidant capacity and total phenolic content. Furthermore, the chemical composition analysis of ASEO was performed using gas chromatography-mass spectrometry (GC-MS) analysis.

## MATERIALS AND METHODS

### Chemicals and instrument

The reagents used in this study such as ascorbic acid, DPPH, sodium nitroprusside (SNP), Griess reagent, trichloroacetic acid (TCA), ferric chloride, Folin-Ciocalteau reagent; gallic acid, nitro blue tetrazolium (NBT), potassium ferricyanide, phenazine methosulphate (PMS) and α-tocopherol were purchased from Sigma-Aldrich (St. Louis, USA) and were of analytical grade. Spectrophotometric measurements were done using a 96-well micro-plate ELISA reader (Erba Lisascan II, Erba Mannheim, Germany).

**Plant material and extraction**

Fresh *A. sativum* rhizome (500 g) was purchased from authentic drug suppliers specialized in the supply of medicinal and aromatic plants. Fresh rhizomes were subjected to microwave-assisted hydrodistillation for a period of 1.5 h. The microwave extraction apparatus was purchased from a reputed company specialized in applying a range of microwave apparatus for extraction of medicinal and aromatic plants (CATA-4R, Catalyst System, Pune, India). The hydrodistillate was collected and mixed with equal amount of dichloromethane, vigorously shaken and kept in a separating funnel for a specified period of time. The process was repeated thrice. The lower organic layer of dichloromethane containing the essential oil was collected and evaporated at 25°C temperature. Finally, the oil was dried over anhydrous sodium sulphate ($Na_2SO_4$) and preserved in a sealed amber color container under dark at +4°C until tested and analyzed.

**Analysis of ASEO by gas chromatography-mass spectrometry (GC-MS)**

The detailed chemical composition analysis of *A. sativum* rhizome essential oil was performed by GC/MS-QP-2010 plus Ultra (Shimadzu company) using an Agilent DB-5 MS fused silica capillary column (30 m × 0.25 m internal diameter [i.d.]., film thickness 0.25 μm). For GC-MS detection an electron ionization system with ionization energy of 70 eV was used. Helium gas was used as the carrier gas at a constant flow rate of 1.2 ml/min. Injector and MS transfer line temperature were set at 260°C and 270°C, respectively. The initial oven temperature of 50°C is maintained for 2 min, and then increased to 210°C at a rate of 3°C/min followed by holding at 210°C for 5 min, and then increased to 210°C at a rate of 8°C/min to 280°C hold time was 10 mins. Clear and precise sample of 0.3 μl were injected through autosampler in the split mode. Split ratio was 1:100 v/v in methanol. The relative percentage of the oil constituents was expressed as percentages by peak area normalization. The percentage composition was based on flame ionization detector with peak area normalization (n = 3). Retention indices on HP-5-MS fused silica capillary column were calculated by comparing gas chromatogram with a homologous series of n-alkanes (C8-C32; Sigma-Aldrich, St. Louis, MI, USA). Identification of essential oil components was based on GC retention time of authentic reference compounds on a DB-5 capillary column while mass spectrometry tentatively identified on the basis of computer matching of mass spectra of peaks with those of standards as in Wiley 8, NIST 11, Flavor & Fragrance and Perfumery libraries for the GC-MS system.

**Determination of DPPH radical scavenging activity**

The scavenging activity of stable DPPH free radical was determined by the previously reported method with a slight modification.[34] Briefly, 75 μl of different concentrations of ASEO and reference compounds (50-250 μg/ml) were added to 25 μl of the methanolic DPPH solution (0.004%) as 3:1 ratio in a 96-well microplate. The mixture was incubated at 37°C in dark for 30 min with shaking (100 rpm). Absorbance was recorded at 517 nm using the ELISA reader (Erba Lisascan II, Erba Mannheim, Germany) against a blank sample. Ascorbic acid and α-tocopherol were also used as reference compounds (positive control) while methanol is used as negative control. All the tests were carried out in triplicate. Values are presented as means ± S.D. of three independent parallel measurements. The percent inhibition activity (% I) was calculated using the following formula:

$$I\ (\%) = 100 \times (Abs_{control} - Abs_{test}) / (Abs_{control})$$

Where, $Abs_{control}$ is the absorbance of the control reaction (containing all reagents except the test compounds) and $Abs_{test}$ represents the absorbance of a test reaction.

**Determination of nitric oxide (NO) radical scavenging activity**

In aqueous solution at physiological pH, sodium nitroprusside (SNP) spontaneously generates nitric oxide, which interacts with oxygen to generate nitrite ions ($NO_2^-$) that can be anticipated by the Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride).[35] Scavengers of free radicals result in the reduced production of nitric oxide radical. Briefly, 1 ml solution of SNP (10 mM) in phosphate buffer saline (PBS pH 7.4) was mixed with 1 ml of different concentrations of ASEO and reference compounds (50-250 µg/ml). The mixture was incubated at 25ºC for 150 min in light with shaking (100 rpm). The half quantity (1 ml) of aliquots was taken and mixed with equal quantity of the Griess reagent (1 ml). The mixture was incubated at 25ºC for 10 min in dark with shaking (100 rpm). The absorbance of pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was read at 546 nm against a blank. Ascorbic acid and α-tocopherol were used as reference compounds. All the tests were performed in triplicate. All values are presented as means ± S.D. of three independent parallel measurements. The percent inhibition activity (% I) was calculated by the formula:

$$I\ (\%) = 100 \times (Abs_{control} - Abs_{test}) / (Abs_{control})$$

Where, $Abs_{control}$ is the absorbance of the control reaction (containing all reagents except the test compounds) and $Abs_{test}$ represents the absorbance of a test reaction.

**Determination of superoxide radical ($O^{2-}$) scavenging activity**

Superoxide radical scavenging activity of ASEO was measured by the reduction of nitro blue tetrazolium (NBT) according to a previously reported method with slight modification.[36] The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce NBT to a purple color formazan. In this assay, the reaction mixture (150 µl) contained 25 µl of phosphate buffer (20 mM, pH 7.4), 25 µl of NADH (73 µM), 25 µl of NBT (50 µM), 25 µl of PMS (15 µM) and 50 µl of different concentrations of ASEO and reference compounds (50-250 µg/ml). After incubation for 5 min at 25°C temperature with gentle shaking (100 rpm), the absorbance of the reaction mixture was measured at 562 nm against an appropriate blank to determine the quantity of formazan generated. Ascorbic acid and α-tocopherol were used as reference compounds. All the tests were performed in triplicate. All values are presented as means ± S.D. of three independent parallel measurements. The percent inhibition activity (% I) was calculated by the formula:

$$I\ (\%) = 100 \times (Abs_{control} - Abs_{test}) / (Abs_{control})$$

Where, $Abs_{control}$ is the absorbance of the control reaction (containing all reagents except the test compounds) and $Abs_{test}$ represents the absorbance of a test reaction.

**Determination of hydroxyl radical ($OH^-$) scavenging activity**

Hydroxyl radical scavenging activity of ASEO was measured according to a previously reported method with slight modification.[37] The assay is based on quantification of the degradation product of 2-deoxy-2-ribose sugar by condensation with 2-thiobarbituric acid (TBA). Hydroxyl radical was generated by the Fenton reaction using ferric ion ($Fe^{3+}$)-ascorbate-EDTA-$H_2O_2$ system. The reaction mixture in a final volume of 1.3 ml contained 200 µl of 2-deoxy-2-ribose (2.8 mM), 100 µl sodium phosphate buffer (0.2 M, pH 7.4), 200 µl of ferric chloride (200 µM), 100 µl of EDTA (1 mM), 200 µl of $H_2O_2$ (1 mM), 400 µl of ascorbic acid (1 mM) and 100 µl of various concentrations of ASEO or reference compounds (50-250 µg/ml). After

incubation for 60 min at 37°C with shaking at 100 rpm, 1.5 ml of TCA (2.8% w/v aqueous solution) and 1.0 ml of thiobarbituric acid (TBA) (1.0% in 0.5M NaOH solution containing 0.02% BHA) were added in the reaction mixture, and the mixture was incubated at 100°C for 30 min (shaking at 100 rpm) to develop the pink color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. Ascorbic acid and α-tocopherol were used as reference compounds. All the tests were performed in triplicate. Values are presented as means ± S.D. of three independent parallel measurements. The percent inhibition activity (% I) was calculated by the formula:

$$I\ (\%) = 100 \times (Abs_{control} - Abs_{test}) / (Abs_{control})$$

Where, $Abs_{control}$ is the absorbance of the control reaction (containing all reagents except the test compounds) and $Abs_{test}$ represents the absorbance of a test reaction.

**Determination of ferrous ion ($Fe^{2+}$) chelating assay**

Ferrous ion chelating activity of ASEO was measured according to a previously reported method with slight modification.[38] The reaction mixture in a final volume of 200 µl contained, 150 µl of different concentrations of ASEO (50-250 µg/ml), 50 µl ferrous chloride (0.25 mM). The reaction mixture was incubated at 25°C for 5 min with gentle shaking (100 rpm). After incubation add 100 µl of ferrozine (1 mM). The mixture was again incubated at 25°C for 10 min with gentle shaking at 100 rpm. The absorbance was measured at 562 nm against an appropriate blank solution. Different concentrations of ethylene diamine tetraacetate (50-250 µg/ml) were used as reference compounds. All the tests were performed in triplicate. All values are presented as means ± S.D. of three independent parallel measurements. The percent inhibition activity (% I) was calculated by the formula:

$$I\ (\%) = 100 \times (Abs_{control} - Abs_{test}) / (Abs_{control})$$

Where, $Abs_{control}$ is the absorbance of the control reaction (containing all reagents except the test compounds) and $Abs_{test}$ represents the absorbance of a test reaction.

**Assessment of inhibition of Lipid peroxidation**

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxides formed in ferric ion ($Fe^{3+}$)/acetic acid-dependent non-enzymatic lipid peroxidation using rat brain homogenate as lipid rich media.[39] The isolated brain tissue was washed in KCl buffer (1.15% w/v). Then the tissues were homogenized using Teflon head homogenizer in ice cold KCl buffer (0.15 M). The prepared homogenate was used for study. Briefly, the reaction mixture containing, 500 µl of rat brain homogenate (10% v/v), 50 µl of ferrous sulphate (0.07 M) and 100 µl of different concentrations of ASEO or reference compounds (50-250 µg/ml). The reaction mixture was incubated at 37°C for 30 min with gentle shaking at 100 rpm. Then, 1.5 ml of acetic acid (20% v/v) was added, followed by the addition of 1.5 ml of thiobarbituric acid (0.8% w/v in 1.1% w/v sodium dodecyl sulphate) and 50 µl of trichloroacetic acid (20% w/v). The reaction mixture was vortexed and heated on a boiling water bath at 95°C for 60 mins and then cools. After cooling, 5 ml of n-butanol was added and the mixture was centrifuged at 3000 rpm for 10 mins, collect the organic layer. The absorbance of organic layer was measured at 532 nm. The hydroxyl radicals generated in the reaction initiate the lipid peroxidation, resulting in malondialdehyde (MDA) production that was measured by TBA reaction. All the tests were performed in triplicate. All values are presented as means ± S.D. of three independent parallel measurements. Ascorbic acid and α-tocopherol were used as reference compounds. The percent inhibition activity (% I) was calculated by the formula:

$$I\ (\%) = 100 \times (Abs_{control} - Abs_{test}) / (Abs_{control})$$

Where, $Abs_{control}$ is the absorbance of the control reaction (containing all reagents except the test compounds) and $Abs_{test}$ represents the absorbance of a test reaction.

***Reducing power assay***

The $Fe^{3+}$ reducing power of the ASEO was determined by the method with slight modification.[40] Aliquots (50 µl) of different concentrations of ASEO (50-250 µg/ml) and reference compounds were mixed with 50 µl phosphate buffer (0.2 M, pH 6.6) and 50 µl potassium ferricyanide (1% w/v in $H_2O$). The reaction mixture was incubated at 50°C for 20 min in dark with gentle shaking at 100 rpm. After incubation, 50 µl of TCA (10% w/v in $H_2O$) was added to terminate the reaction and the mixture was subjected to centrifugation at 3000 rpm for 10 min. For final reaction mixture, the supernatant (50 µl) was mixed with 50 µl distilled water and 10 µl $FeCl_3$ solution (0.1% w/v in $H_2O$). The reaction mixture was incubated at 25°C for 10 mins and the absorbance was measured at 700 nm against an appropriate blank solution. A higher absorbance of the reaction mixture indicated greater reducing power ability. All the tests were performed in triplicate. Values are presented as means ± S.D. of three independent parallel measurements. Ascorbic acid and α-tocopherol were used as reference compounds.

**Determination of total antioxidant capacity (TAC) by phosphomolybdenum assay**

The total antioxidant capacity of ASEO was evaluated by the method with slight modification.[41] The reaction mixture in a final volume of 1.1 ml contained 100 µl of ASEO (250 µg/ml) and 1 ml of reagent solution (0.6 M sulphuric acid, 30 mM sodium phosphate and 4 mM ammonium molybdate). The mixture was incubated at 90°C for 90 min with gentle shaking at 100 rpm. After incubation the reactant mixture had cooled to room temperature and the absorbance of mixture was measured at 695 nm against blank. A typical blank sample contained 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample preparation and it was incubated under same conditions as the rest of the samples. The anti-oxidant activity was expressed as equivalents of ascorbic acid (mg of ascorbic acid/g of sample). All the tests were performed in triplicate. Values are presented as means ± S.D. of three independent parallel measurements.

**Determination of total phenolic content**

Total phenolic content was determined using Folin-Ciocalteau reaction according to the method as described previously with slight modification.[42] An aliquot of 50 µl of ASEO (100 µg/ml) was mixed with 50 µl of 5% Folin-Ciocalteau reagent and the reaction mixture was incubated 25°C for 5 min in dark with gentle shaking at 100 rpm, followed by addition of 100 µl of $Na_2CO_3$ solution (20% w/v in $H_2O$). After incubation at 25°C for 20 min, the absorbance was measured at 730 nm against the appropriate blank solution. The total phenolic content was evaluated from a standard calibration curve of gallic acid using the concentration range of 10-50 µg/ml. All tests were run in triplicate. Value is presented as means ± S.D. of three independent parallel measurements.

## STATISTICAL ANALYSIS

All data are expressed as the mean ± SD by measuring three independent parallel replicates. Analysis of variance using one-way ANOVA followed by Duncan's test was performed to test the significance of differences between means obtained among the treatments at the 5% level of significance using "Statistical Analysis Software" (SAS Institute Inc., Cary, NC, USA).

## RESULTS AND DISCUSSION

The antioxidant activity of putative antioxidants have been attributed to various mechanisms including prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging efficacy.[43] Numerous antioxidant methods and modifications have been proposed to evaluate the antioxidant activity of natural products and to explain that how the antioxidants work in the biological system. Of these, DPPH, NO, superoxide, hydroxide radical quenching assays and reducing power, lipid peroxidation, as well as determination of total phenolic content is the most versatile methods for the evaluation of antioxidant activities of various test compounds.[44]

In this study, a microwave-assisted extraction method, which selectively extracts target compounds from various raw materials, was used for the extraction of ASEO. The main advantages of microwave extraction over the conventional extraction techniques is its increased extraction efficiency, less solvent consumption ability, environmental friendly, no provision for handling potentially hazardous fumes, shorter operational time, higher recovery rate, better reproducibility and lesser sample manipulation for extraction process.[45]

### Chemical composition of ASEO

GC-MS analysis of the ASEO led to the identification of 42 different components, representing 99.16% of the total oil. The compounds were identified on the basis of their elution order on a DB-5 MS fused silica capillary column and by matching the fragmentation patterns with those of the mass spectral data (Wiley 8, NIST 11, Flavor & Fragrance and Perfumery libraries). The identified compounds are listed in Table 1. The oil contained a complex mixture consisting of terpenes and their oxygenated derivatives, aliphatic and aromatic hydrocarbons, sulphur containing compounds, phenolics and polyhydric alcohols along with some other oxygenated essential phytochemicals. The major components detected in the oil were di-2-propenyl trisulphide (16.44%), diallyl trisulphide (11.81%), diallyl disulphide (8.90%), di-2-propenyl trisulphide (7.30%), methyl-2-propenyl trisulphide (6.88%), dimethyl disulphide (6.12%), methyl allyl trisulphide (5.26%), diallyl tetrasulphide (5.14%), methyl allyl disulphide (4.32%), diallyl sulphide (4.19%) and eugenol (2.21%). The ASEO was also found to contain 3,3-thio-bis-1-propene (1.93%), methyl-2-propenyl disulphide (1.71%), 2-vinyl[4H]-1,3-dithiane (1.24%), 1,3-dithiane (1.13%), 3,4-dihydro-3-vinyl-1,2-dithiane (1.13%), γ-cadinene (1.10%) and 3-vinyl[4H]-1,3-dithiane (1.01%) as the trace components. Fig. 1 illustrates some important components of *A. sativum* rhizome essential oil.

In recent years, several researchers have reported that hydrocarbons and oxygenated phyto-constituents of plant-based essential oils have enormous antioxidant and radical scavenging potential.[9] In general, the antioxidant compounds of essential oils are terpenes, which are phenolic in nature, and it would seem rational that their antioxidant mode of action might be related to that of other compounds. These findings were also confirmed in this study, as the ASEO was found to contain a higher proportion of terpenes, terpenoids, phenolics and importantly the presence of sulphur compounds. The sulphur derivatives like alliin and allicin are considered potent antioxidants in foods and cosmetics due to their ability to scavenge free radicals, thus, activating the status of antioxidant enzymes in the body.[46] The antioxidant activity of stable lipid soluble allyl sulfides like diallyl sulphide (DAS), diallyl disulphide (DADS), diallyl trisulphide (DATS) and diallyl tetrasulphide, which is also present in ASEO, reported previously.[47] Hence, in this study we

attempted to explore a new therapeutic agent of plant origin as ASEO, and confirmed its biological efficacy in various antioxidant models.

**DPPH radical scavenging assay**

DPPH radical scavenging assay is the most popular method for the determination of antioxidant capacity of compounds due to its simplicity, rapidity, sensitivity and better reproducibility.[48] Radical scavengers may protect cells and tissues from free radicals, thereby preventing diseases such as atherosclerosis and cancer.[49] Unlike other free radicals such as hydroxide and superoxide, DPPH has the advantage of being unaffected by certain reactions such as metal ion chelation and enzyme inhibition.[50] A freshly prepared solution of DPPH produces purple color with maximum absorption at 517 nm. The antioxidant molecule quenches DPPH molecule by donating a hydrogen atom or electron and converts it to a colorless hydrazine analogue, resulting in a decreased absorbance.

Fig. 2 illustrates a significant ($p < 0.05$) decrease in the concentration of DPPH radicals due to the scavenging ability of both ASEO and standard compounds, ascorbic acid and α-tocopherol in a concentration-dependent manner. In this assay, the ASEO, ascorbic acid and α-tocopherol at the concentration of 250 µg/ml showed maximum scavenging of DPPH radical with a percentage inhibition of 82.02% ($IC_{50}$ 98.38 µg/ml), 82.69 % and 85.02 %, respectively. These results indicated that ASEO, ascorbic acid and α-tocopherol have a noticeable effect on scavenging free radical; however, the standard compounds had little higher DPPH scavenging activity than ASEO. Consequently, the more rapidly the absorbance decreases, the more potent antioxidant capacity of ASEO observed in terms of hydrogen atom or electron donating ability. Previously the antioxidant capacity of various plant-based essential oils using a DPPH model has been confirmed.[51,52] However, our newly characterized ASEO was also verified for the presence of high DPPH scavenging capacity.

**Nitric oxide radical scavenging assay**

It is well known that nitric oxide radical has an important role in various inflammatory processes.[53] Sustained levels of the production of nitric oxide radical are directly toxic to tissues, and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis.[53] The toxicity of nitric oxide increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion ($ONOO^-$). The nitric oxide generated from SNP reacts with oxygen to form nitrite. The plant-based bioactive constituents such as essential oils inhibit nitrite formation by directly competing with oxygen in the reaction with nitric oxide.[54] Hence, it is estimated in this study that ASEO inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. In this assay, ASEO, ascorbic acid and α-tocopherol at the concentration of 250 µg/ml showed maximum scavenging of NO radical with a percentage inhibition of 75.66% ($IC_{50}$ 152.74 µg/ml), 84.62% and 80.61%, respectively. As shown in the Fig. 3, both ASEO and positive controls showed significant NO radical scavenging activity ($p < 0.05$) in a concentration-dependent manner. The NO radical scavenging activities of various plant-based essential oils have been reported previously. [52]

**Superoxide radical scavenging assay**

In the superoxide radical scavenging assay, measured by the PMS-NADH superoxide generating system, the ASEO was demonstrated a concentration-dependent inhibition of the superoxide radical (Fig. 4). At the concentration of 250 µg/ml, the ASEO, ascorbic acid and α-tocopherol evoked a maximum superoxide radical scavenging capacity by 79.86% ($IC_{50}$ 175.71 µg/ml), 87.00% and 84.45%, respectively. The values are statistically significant to the superoxide radical scavenging capacities of ascorbic acid and α-tocopherol. Based on the findings of this assay, it appears that ASEO scavenged superoxide radicals by combining with superoxide radical ions to form stable radicals, thus terminating the radical chain reaction.[55] In view to support our findings, some of the essential oils were reported to scavenge the superoxide radical effectively. [52]

**Hydroxyl radical scavenging assay**

Hydroxyl radical is a major active oxygen species causing lipid peroxidation and cellular damage.[4] This radical has the capacity to join nucleotides in DNA and it can cause strand breakage that contributes to carcinogenesis, mutagenesis and cytotoxicity.[56] The hydroxyl radical scavenging capacity of essential oil is directly related to its antioxidant activity. In this assay, ASEO, ascorbic acid and α-tocopherol at the concentration of 250 µg/ml showed maximum scavenging of OH radical with a percentage inhibition of 81.11% ($IC_{50}$ 96.71 µg/ml), 83.79% and 80.02%, respectively. As shown in the Fig. 5, both ASEO and positive controls showed statistically significant OH radical scavenging activity ($p < 0.05$) in a concentration-dependent manner. In view to support our findings, some of the essential oils were reported to scavenge the superoxide radical effectively.[52]

**Ferrous ion ($Fe^{2+}$) chelating assay**

Chelation is an important mechanism of antioxidant activity. In the body, iron is required for oxygen transport, respiration and as co-factor in various enzymatic reactions. Alternatively, because of its high reactivity, iron catalyzes oxidative degradation of lipids, proteins and other cellular biomolecules. $Fe^{2+}$ ion catalyzes Fenton-type reactions or participates in formation of OH radicals that can cause oxidative damage.[57] Therefore, $Fe^{2+}$ ion -chelating activity is considered as an important parameter in oxidative stress involving $Fe^{2+}$ ion. Among the various metal ions, $Fe^{2+}$ ion is considered as most powerful pro-oxidant commonly found in food systems.[58] Ferrozine can quantitatively form red color complexes with $Fe^{2+}$ ion. However, in the presence of chelating agents, the formation of colored complex is disturbed. Hence, measurement of color reduction is an indication of metal chelating activity of chelator. In this assay, ASEO and EDTA at the concentration of 250 µg/ml showed maximum chelation of $Fe^{2+}$ ion with a percentage inhibition of 71.86% ($IC_{50}$ 189.00 µg/ml) and 83.00%, respectively. As shown in the Fig. 6, both ASEO and EDTA showed statistically significant chelation of $Fe^{2+}$ ion ($p < 0.05$) in a concentration-dependent manner. EDTA was used as positive control and its $IC_{50}$ value was 128.40 µg/ml.

**Lipid peroxidation assay**

Lipid peroxidation is a major cause of food deterioration, affecting qualitative deterioration of food and their products. Further, it has been found that oxidative modification of low-density lipoproteins (LDLs) is crucial in the development of cardiovascular disorders including atherosclerosis.[59] This oxidative modification depends on the peroxidation of polyunsaturated fatty acids in the LDLs. Such modification can be inhibited by antioxidants.[60] Previous studies proved that dietary antioxidants are important in the

prevention of cardiovascular disorders.[61] Also an enhanced lipid peroxidation is considered to be mutagenic and carcinogenic.[62] In this study, the ASEO, ascorbic acid and α-tocopherol at the used concentration (250 µg/ml) inhibited the lipid peroxidation by 75.60% ($IC_{50}$ 147.49 µg/ml), 80.59% and 84.13%, respectively (Fig. 7). Interestingly, the ASEO showed similar inhibitory effect on lipid peroxidation activity at higher concentration when compared with reference antioxidant compounds. Moreover, the ASEO and the standard reference compounds showed significantly higher lipid peroxidation inhibitory activities at higher concentrations. The results were concentration-dependent and statistically significant ($p < 0.05$).

**Reducing Power Assay**

In recent years, different studies have indicated that the electron donation capacity reflects the reducing power of pharmacologically active compounds in a relationship with antioxidant activity.[63] Antioxidants are reducing agent, and inactivation of oxidants by reductants can be described as reduction-oxidation (redox) reaction in which one reaction species is reduced at the expense of the oxidation of the other. The reduction of $Fe^{3+}$ is often used as an indicator of electron donating ability, which is an important mechanism of phenolic antioxidant action. In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of $Fe^{3+}$ to $Fe^{2+}$ by donating an electron. Amount of $Fe^{2+}$ complex can be then be monitored by measuring the formation of Perl's Prussian blue ferric ferrocyanide, ($Fe_4[Fe(CN)_6]_3$), at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 8 shows the reducing power of ASEO as a function of their concentrations. It was found that the reducing power of ASEO, ascorbic acid and α-tocopherol also increased with the increase of their concentrations. At the concentration of 250 µg/ml, reducing power of ASEO, ascorbic acid and α-tocopherol were 1.11, 1.32 and 1.25, respectively. Moreover, the reducing power of ASEO is closer to the reducing power of ascorbic acid and α-tocopherol. The results were concentration dependent and statistically significant ($p < 0.05$). Indeed, numerous essential oil containing terpene hydrocarbons and oxygenated terpenes exhibited antioxidant activity through their reductive capacity in a $Fe^{3+}$-$Fe^{2+}$ system.[52] The data presented here indicates that the marked reducing power of ASEO seem to be attributed to their antioxidant activity.

**Total antioxidant capacity assay**

The phosphomolybdenum assay used for determining the antioxidant capacity is based on the reduction of Mo (VI) - Mo (V) by the antioxidants and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The antioxidant capacity of ASEO was estimated using the standard curve of ascorbic acid and found to be 94.48 ± 1.12 mg of ascorbic acid/g of the sample.

**Total phenolic content**

The antioxidant activity of plants is mainly contributed by the active compounds of essential oil and phenolic fraction present in them. Phenolic compounds are the main agents that can donate hydrogen atom to free radicals and thus break the chain reaction of lipid peroxidation and their ability to prevent polyunsaturated fatty acids from oxidative deterioration. Numerous studies exhibited a strong relationship between total phenolic content and antioxidant activity in fruits, vegetables, and medicinal plants.[63] Recently there is increasing interests of phenolic compounds in food industry because of their inhibitory effect on lipid peroxidation and formation of off-flavors and other objectionable compounds, thereby improve the quality and nutritional value of fresh as well as processed food.[64] The content of total phenolic compounds (mg/g) in ASEO was expressed as milligram of gallic acid equivalents (GAE) which was calculated using following

regression equation; $y = 0.16x + 3.28$ ($R^2 = 0.993$). Fig. 9 illustrates calibration curve of gallic acid as the standard compound used for the determination of phenolic compounds in ASEO. The total phenolic compounds in ASEO was found to be 7.27 ± 0.11 mg/g GAE.

## CONCLUSION

The results of this study showed that ASEO contained terpenes and their oxygenated derivatives, aliphatic and aromatic hydrocarbons, sulphur compounds, phenolics, polyhydric alcohols along with some other oxygenated essential phytochemicals, exhibited noteworthy antioxidant, free radical scavenging, ferrous ion chelation and reducing power activities. Furthermore, the ASEO exerted a potent inhibitory effect on ferric ion-induced lipid peroxidation. These *in vitro* assays indicate that ASEO can be a significant source of natural antioxidant, which might be applicable for preventing the progress of various oxidative stress induced diseases. In addition, the use of ASEO as a natural antioxidant agent will be a suitable strategy for its applications in the food, agricultural and pharmaceutical industries with lesser or no side effects. However, at this moment the antioxidant activity of individual bioactive compounds in different other antioxidant enzyme systems are unclear. Therefore, further investigation is required to isolate the compounds with the highest antioxidant capacity from the ASEO.

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**Table 1. Chemical composition analysis of microwave-extracted essential oil of *Allium sativum* rhizome.**

| S. No | Compounds[a] | PC (%)[b] | RI[c] | RT[d] | MW[e] | MF[f] | IM[g] |
|---|---|---|---|---|---|---|---|
| 1 | Di-desmethylflurazepam | 0.74 | 299 | 2.65 | 387 | $C_{21}H_{23}ClFN_3O$ | EI-MS |
| 2 | 1,2-Bis-(2-Methylpentanoyl)hydrazine | 0.29 | 374 | 2.98 | 228 | $C_{12}H_{24}N_2O_2$ | EI-MS |
| 3 | Dimethyl trisulphide | 0.54 | 416 | 3.00 | 126 | $C_2H_6S_3$ | EI-MS |
| 4 | Boric acid | 0.73 | 426 | 3.35 | 61 | $H_3BO_3$ | EI-MS |
| 5 | Scyllitol | 0.52 | 442 | 3.60 | 180 | $C_6H_{12}O_6$ | EI-MS |
| 6 | n-Butyl-n-pentyl disulphide | 0.63 | 444 | 3.78 | 192 | $C_9H_{20}S_2$ | EI-MS |
| 7 | Isoquinoline ethiodide | 0.41 | 466 | 4.08 | 283 | $C_{11}H_{10}IN$ | EI-MS |
| 8 | Ethyl N-methylcarbamate | 0.71 | 482 | 4.49 | 103 | $C_4H_9NO_2$ | EI-MS |
| 9 | Limonene | 0.12 | 490 | 4.62 | 136 | $C_{10}H_{16}$ | EI-MS |
| 10 | Benzene acetic acid | 0.37 | 496 | 5.08 | 136 | $C_8H_8O_2$ | EI-MS |
| 11 | 1,1-dimethylpropyl benzoate | 0.70 | 513 | 5.23 | 194 | $C_{12}H_{18}O_2$ | EI-MS |
| 12 | Cyercene I | 0.54 | 516 | 5.39 | 234 | $C_{14}H_{18}O_3$ | EI-MS |
| 13 | n-Octadecane | 0.30 | 553 | 6.23 | 254 | $C_{18}H_{38}$ | EI-MS |
| 14 | Quinoline ethiodide | 0.16 | 566 | 6.59 | 285 | $C_{11}H_{22}IN$ | EI-MS |
| 15 | Methyl stearate | 0.92 | 589 | 8.00 | 298 | $C_{19}H_{38}O_2$ | EI-MS |
| 16 | Phenol | 0.32 | 681 | 9.44 | 94 | $C_6H_5OH$ | EI-MS |
| 17 | Sulfamide | 0.61 | 740 | 10.22 | 96 | $H_4N_2O_2S$ | EI-MS |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 18 | Diethyl phthalate | 0.97 | 763 | 10.44 | 222 | $C_{12}H_{14}O_4$ | EI-MS |
| 19 | Dimethyl disulphide | 6.12 | 767 | 11.01 | 94 | $C_2H_6S_2$ | EI-MS |

*Table 1 continued*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 20 | 3-Hexenal | 0.15 | 770 | 12.07 | 98 | $C_6H_{10}O$ | EI-MS |
| 21 | 2-Methylpentenal | 0.15 | 776 | 12.79 | 98 | $C_6H_{10}O$ | EI-MS |
| 22 | 2-Ethylpyridine | 0.76 | 818 | 12.92 | 107 | $C_7H_9N$ | EI-MS |
| 23 | 1,3-Dithiane | 1.13 | 837 | 13.61 | 120 | $C_4H_8S_2$ | EI-MS |
| 24 | Diallyl sulphide | 4.19 | 854 | 13.74 | 114 | $C_6H_{10}S$ | EI-MS |
| 25 | Methyl allyl disulphide | 4.32 | 915 | 14.27 | 120 | $C_4H_8S_2$ | EI-MS |
| 26 | di-2-Propenyl disulphide | 7.30 | 938 | 17.69 | 146 | $C_6H_{10}S_2$ | EI-MS |
| 27 | 3,3-Thio-bis-1-propene | 1.93 | 988 | 19.27 | 114 | $C_6H_{10}S$ | EI-MS |
| 28 | Diallyl disulphide | 8.90 | 1084 | 20.21 | 146 | $C_6H_{10}S_2$ | EI-MS |
| 29 | Methyl-2-propenyl trisulphide | 6.88 | 1097 | 21.30 | 152 | $C_4H_8S_3$ | EI-MS |
| 30 | Methyl allyl trisulphide | 5.26 | 1121 | 24.24 | 152 | $C_4H_8S_3$ | EI-MS |
| 31 | 3,4-Dihydro-3-vinyl-1,2-dithiane | 1.13 | 1162 | 28.14 | 144 | $C_6H_8S_2$ | EI-MS |
| 32 | 3-Vinyl[4H]-1,3-dithiane | 1.01 | 1199 | 30.64 | 144 | $C_6H_8S_2$ | EI-MS |
| 33 | 2-Vinyl[4H]-1,3-dithiane | 1.24 | 1215 | 33.31 | 144 | $C_6H_8S_2$ | EI-MS |
| 34 | di-2-Propenyl trisulphide | 16.44 | 1240 | 35.73 | 178 | $C_6H_{10}S_3$ | EI-MS |
| 35 | Methyl-2-propenyl disulphide | 1.71 | 1277 | 37.80 | 120 | $C_4H_8S_2$ | EI-MS |
| 36 | Diallyl trisulphide | 11.81 | 1285 | 39.38 | 178 | $C_6H_{10}S_3$ | EI-MS |
| 37 | 4-Bromo-octane | 0.16 | 1295 | 40.58 | 193 | $C_8H_{17}Br$ | EI-MS |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 38 | 3-Methoxyoctane | 0.10 | 1326 | 41.77 | 144 | $C_9H_{20}O$ | EI-MS |
| 39 | Eugenol | 2.21 | 1366 | 43.12 | 164 | $C_{10}H_{12}O_2$ | EI-MS |
| 40 | γ-Cadinene | 1.10 | 1477 | 43.57 | 204 | $C_{15}H_{24}$ | EI-MS |

*Table 1 continued*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 41 | Diallyl tetrasulphide | 5.14 | 1528 | 44.14 | 210 | $C_6H_{10}S_4$ | EI-MS |
| 42 | 2,4-Dimethyl-5,6-dithia-2,7-nonadienal | 0.44 | 1635 | 45.73 | 202 | $C_9H_{14}OS_2$ | EI-MS |

[a] Compounds: Listed in order of elution from a DB-5 capillary column

[b] Percentage composition: Based on flame ionization detector (FID) peak area normalization (n = 3)

[c] RI: Retention indices on HP-5-MS fused silica capillary column were calculated by comparing gas chromatogram with a homologous series of n-alkanes (C8-C32; Sigma-Aldrich, St. Louis, MI, USA)

[d] Retention time (min)

[e] Molecular weight

[f] Molecular formula

[g] Identification method: EI-MS, identification based on retention time of authentic reference compounds; MS, tentatively identified on the basis of computer matching of mass spectra of peaks with Wiley 8, NIST 11, Flavor & Fragrance and Perfumery libraries

**Figure legends**

**Fig. 1.** Structure of some important compounds of *Allium sativum* rhizome essential oil.

**Fig. 2.** DPPH radical scavenging activity of *A. sativum* rhizome essential oil and reference compounds, ascorbic acid and α-tocopherol. All values are expressed as the mean ± S.D. of three independent parallel measurements (n = 3). Different superscripts in each column indicate the significant differences in the mean ($p < 0.05$).

**Fig. 3.** Nitric oxide radical scavenging activity of *A. sativum* rhizome essential oil and reference compounds, ascorbic acid and α-tocopherol. All values are expressed as the mean ± S.D. of three independent parallel measurements (n = 3). Different superscripts in each column indicate the significant differences in the mean ($p < 0.05$).

**Fig. 4.** Superoxide radical scavenging activity of *A. sativum* rhizome essential oil and reference compounds, ascorbic acid and α-tocopherol. All values are expressed as the mean ± S.D. of three independent parallel measurements (n = 3). Different superscripts in each column indicate the significant differences in the mean ($p < 0.05$).

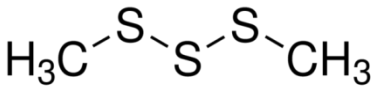
**Fig. 5.** Hydroxyl radical scavenging activity of *A. sativum* essential oil and standard compounds, ascorbic acid and α-tocopherol. All values are expressed as the mean ± S.D. of three independent parallel measurements (n = 3). Different superscripts in each column indicate the significant differences in the mean ($p < 0.05$).

**Fig. 6.** Ferrous ion ($Fe^{2+}$) chelating capacity of *A. sativum* rhizome essential oil and reference compound, EDTA. All values are expressed as the mean ± S.D. of three independent parallel measurements (n = 3). Different superscripts in each column indicate the significant differences in the mean ($p < 0.05$).

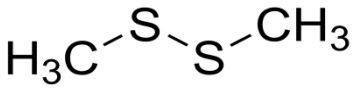
**Fig. 7.** Lipid peroxidation inhibitory effect of *A. sativum* essential oil and reference compounds, ascorbic acid and α-tocopherol. All values are expressed as the mean ± S.D. of three independent parallel measurements (n = 3). Different superscripts in each column indicate the significant differences in the mean ($p < 0.05$).

**Fig. 8.** Reducing power activity of *A. sativum* essential oil and reference compounds, ascorbic acid and α-tocopherol. All values are expressed as the mean ± S.D. of three independent parallel measurements (n = 3). Different superscripts in each column indicate the significant differences in the mean ($p < 0.05$).

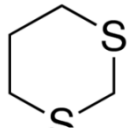
**Fig. 9.** Calibration curve of gallic acid for the determination of phenolic content of *A. sativum* rhizome essential oil. All values are expressed as the mean ± S.D. of three independent parallel measurements (n = 3).



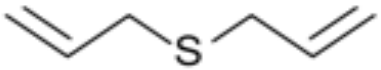
Dimethyl trisulphide



Dimethyl disulphide



1, 3-Dithiane



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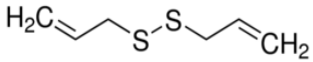
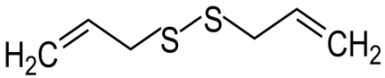
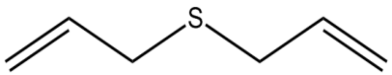


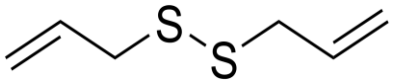
Methyl allyl disulphide



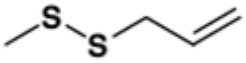
Di-2-Propenyl disulphide



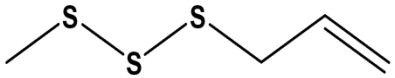
3,3-Thio-bis-1-propene



Diallyl disulphide



Methyl-2-propenyl disulphide



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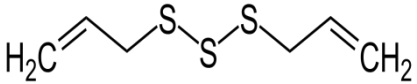
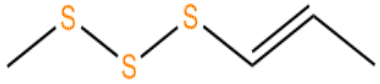
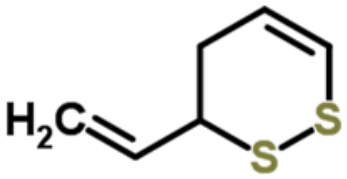


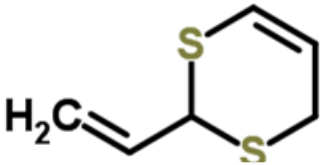
Di-2-Propenyl trisulphide



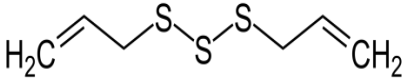
Methyl propenyl trisulphide



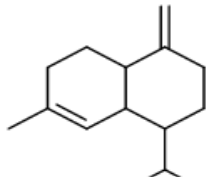
3,4-Dihydro-3-vinyl-1,2-dithiane



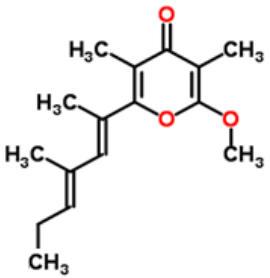
2-Vinyl [4H]1,3-dithiane

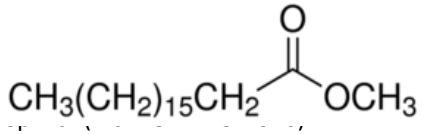


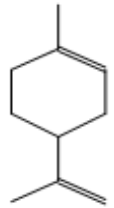
Diallyl trisulphide

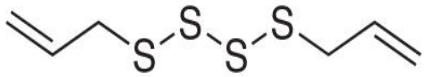


γ-Cadinene



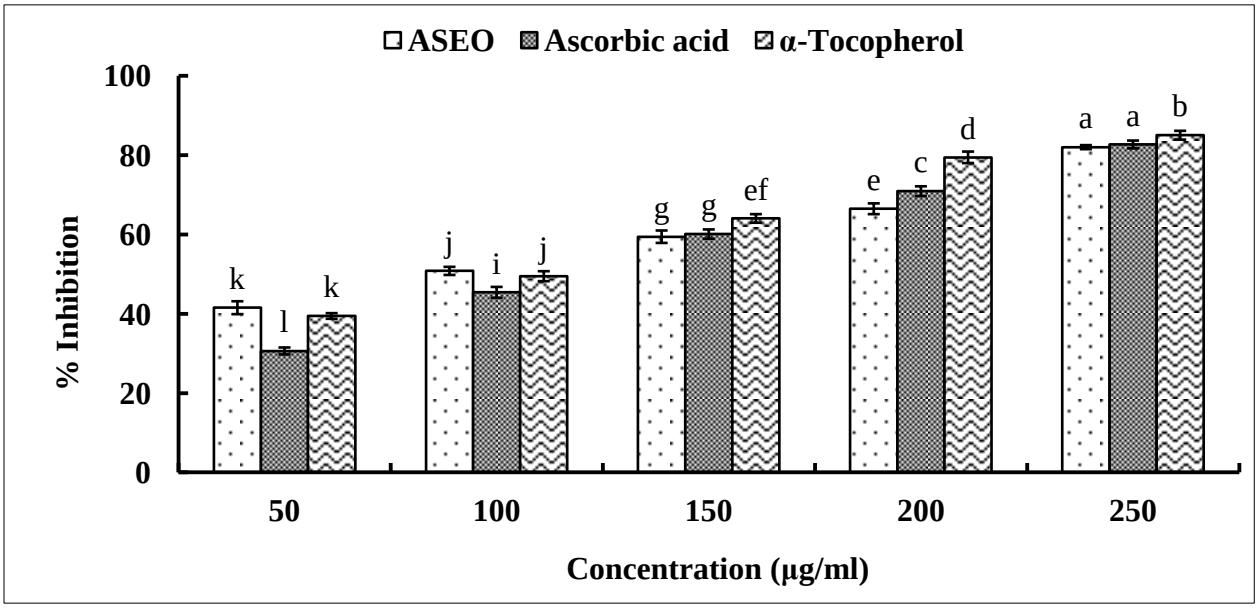






Cyercene I | Diallyl tetrasulphide | Methyl stearate | Limonene

**Figure 1**


ASEO
Ascorbic acid
α-Tocopherol
% Inhibition
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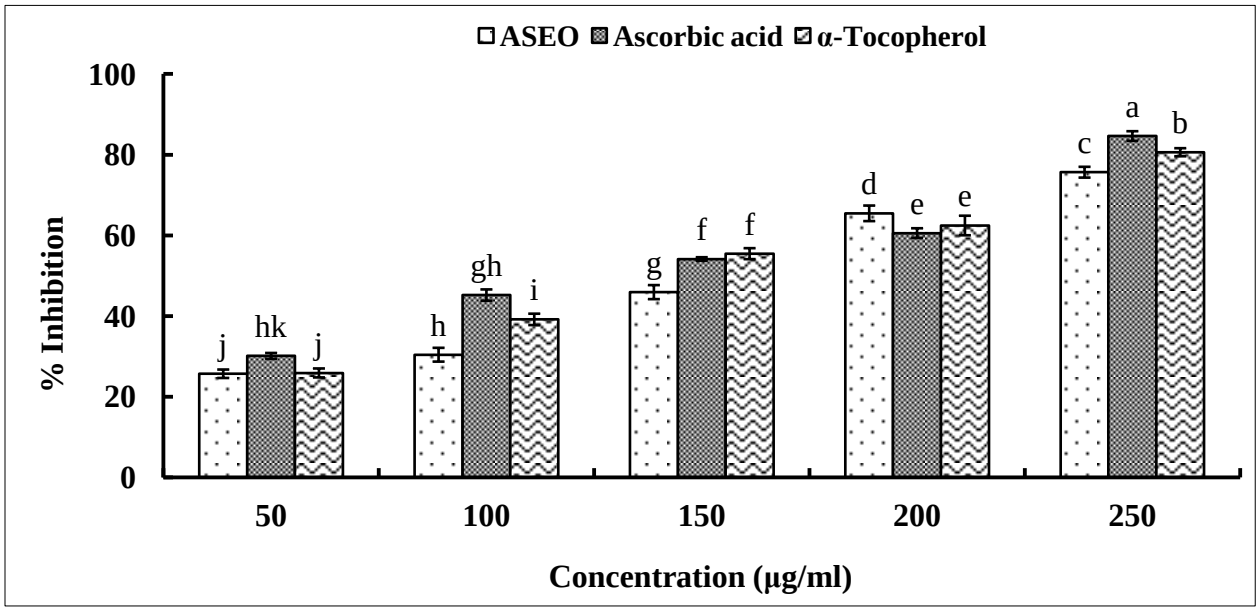
150
200
250
Concentration (µg/ml)

**Figure 2**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Ascorbic acid
α-Tocopherol
% Inhibition
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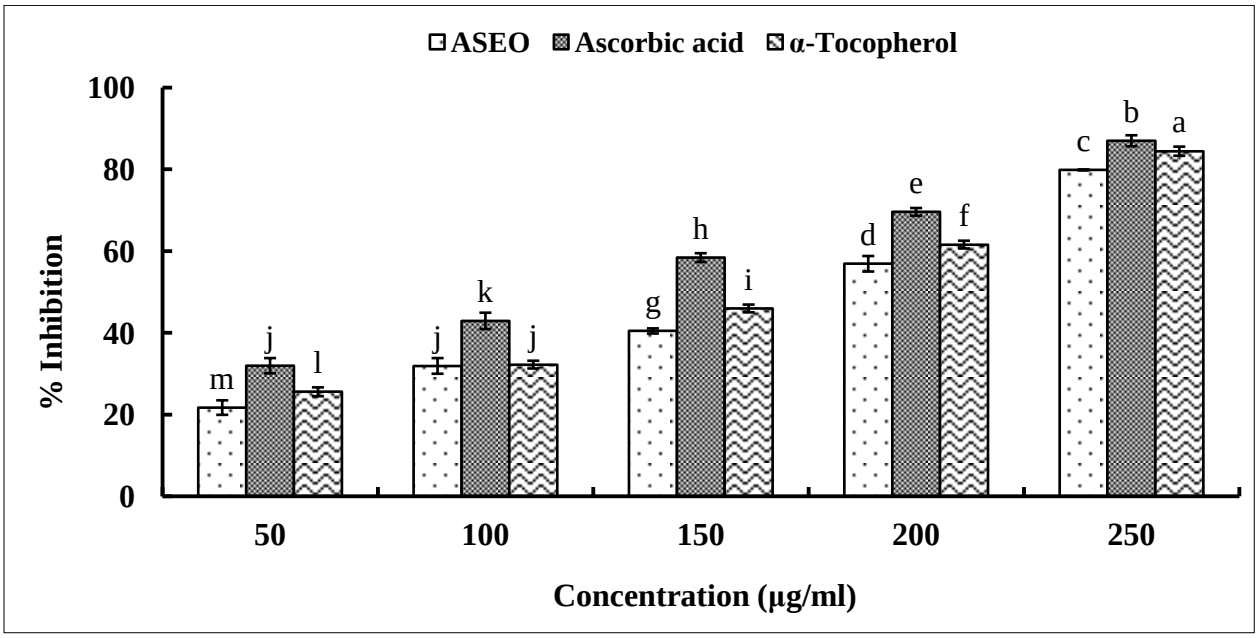
Concentration (µg/ml)

**Figure 3**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Ascorbic acid
α-Tocopherol
% Inhibition
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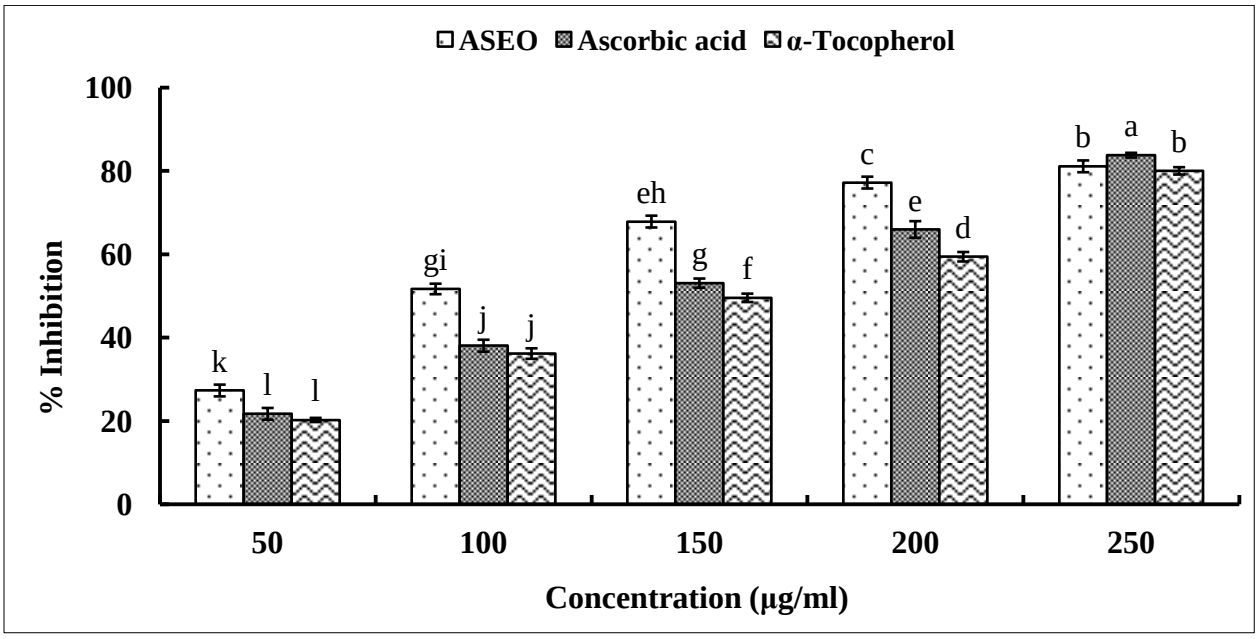
Concentration (µg/ml)

**Figure 4**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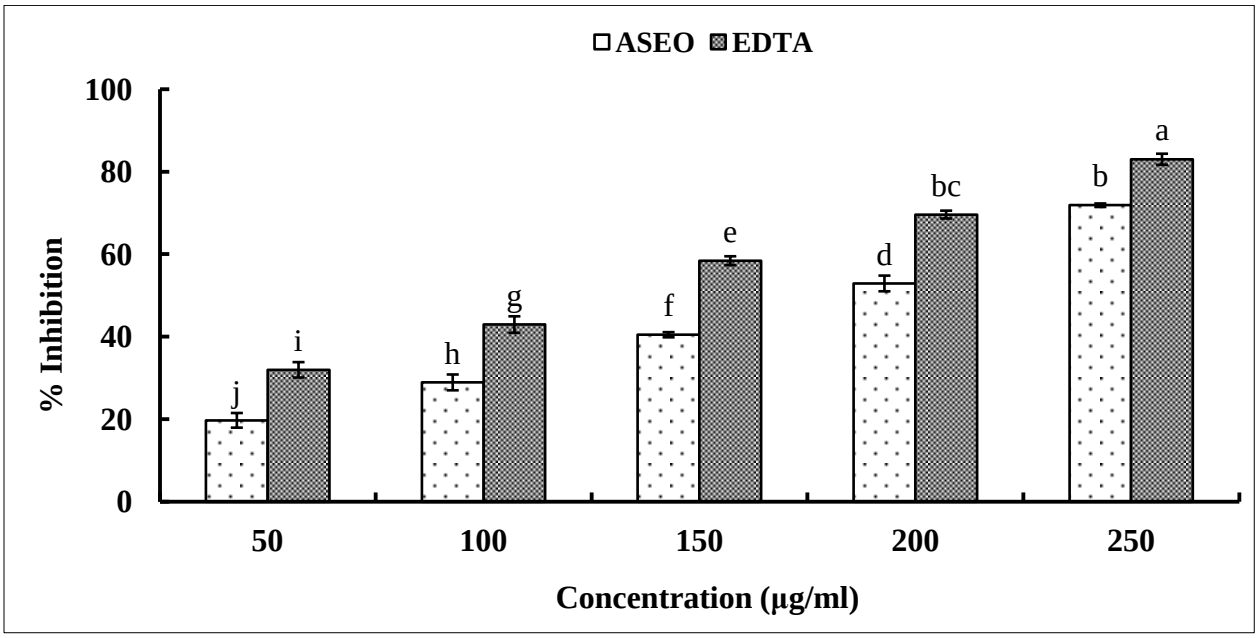
Concentration (µg/ml)

**Figure 5**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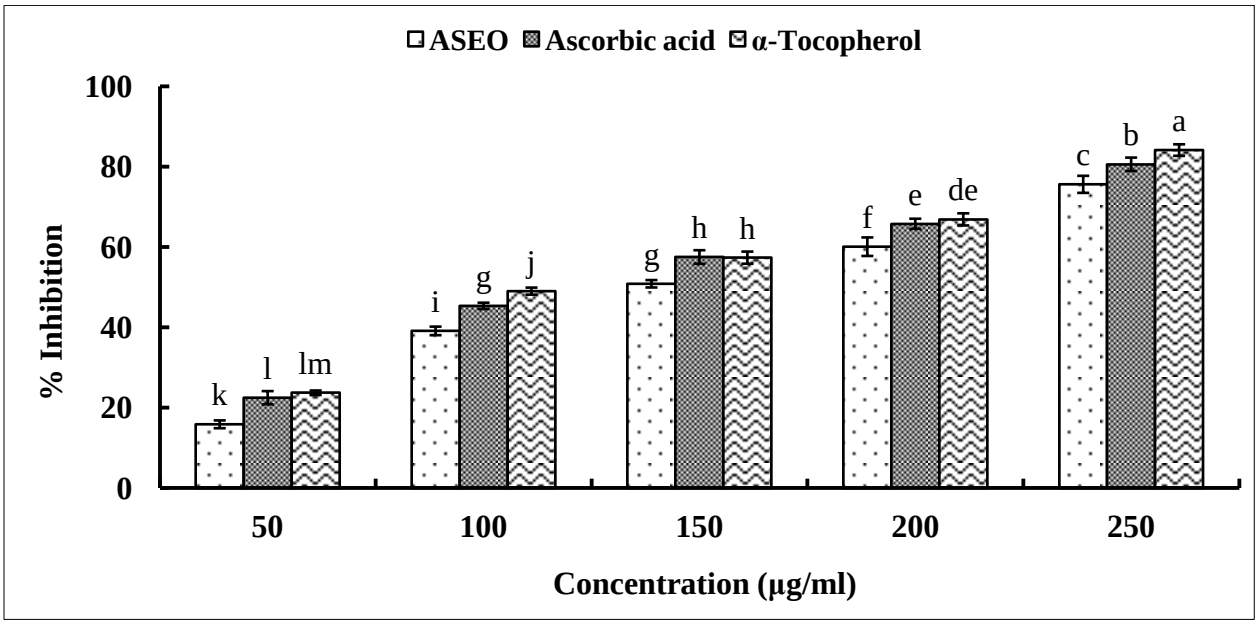
Concentration (µg/ml)

**Figure 6**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% Inhibition
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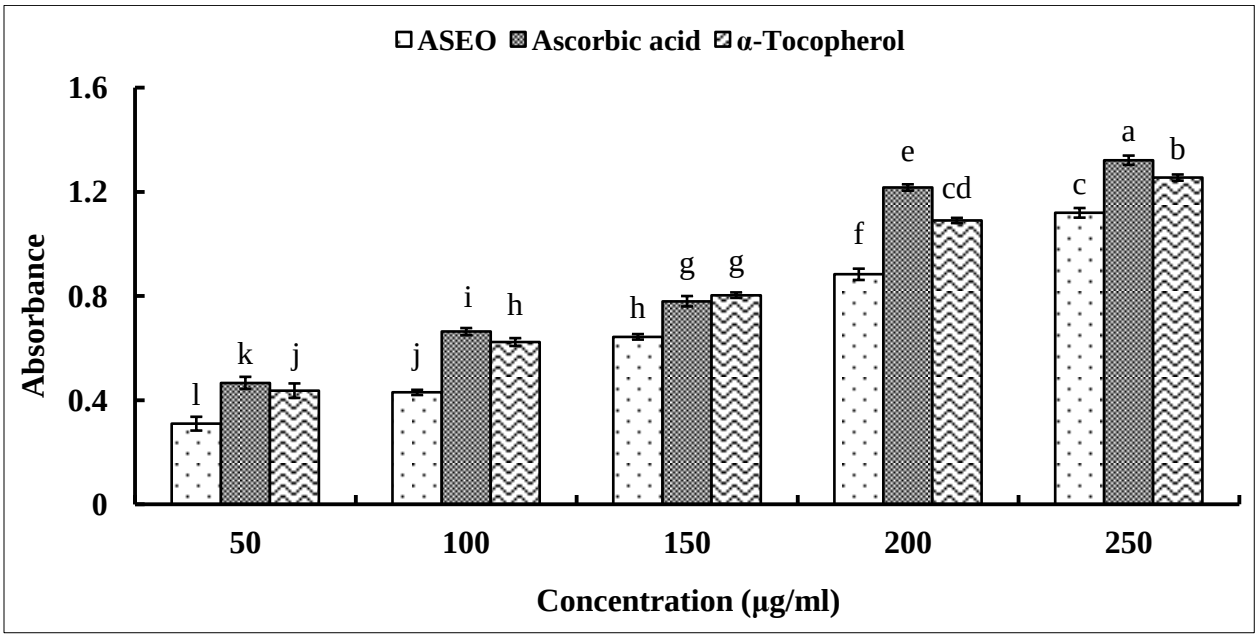
250
Concentration (µg/ml)

**Figure 7**


ASEO
Ascorbic acid
α-Tocopherol
Absorbance
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cd
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b
50
100
150
200
250
Concentration (µg/ml)

**Figure 8**

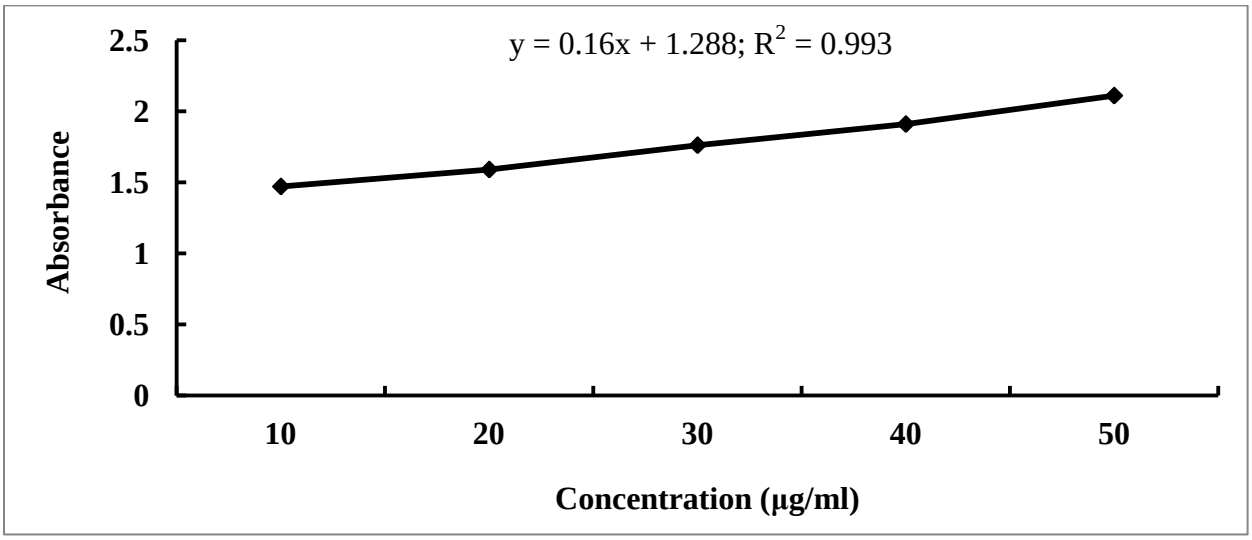
y = 0.16x + 1.288; R2 = 0.993
Absorbance
2.5
2
1.5
1
0.5
0
10
20
30
40
50
Concentration (µg/ml)

**Figure 9**

# Phytomediated synthesis of silver nanoparticles and evaluation of its antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*

**PALLAVI SINGH CHAUHAN, VIKAS SHRIVASTAVA, RAJESH SINGH TOMAR***

Amity Institute of Biotechnology, Amity University Madhya Pradesh

Corresponding author email: rstomar@amity.edu

## ABSTRACT

This study aims to provide an insight into the phytomediated synthesis of Ag nanoparticles, and its significant applications in various fields. Eco-friendly approach for fabrication of metallic nanoparticles is an important area in the field of nanotechnology. To achieve this objective it is prerequisite to use 'natural compounds' like biological components. This study provides a report on the biosynthesis of silver nanoparticles (Ag NPs) through reduction of $Ag^+$ ions by the chosen leaf extract (*Ficus Religiosa*). The synthesized silver nanoparticles were recovered and purified. The purified sample was then characterized by UV–vis spectra, FTIR, XRD, SEM and TEM. The metal salt solution after phyto treatment have shown the maximum absorbance peak at approx 410 nm and the TEM, SEM analysis showed shape of nanoparticles, with an average size of 67 nm. X-ray diffraction (XRD) analysis of the silver nanoparticles exhibited $2\theta$ values which corresponds to the silver nanocrystals, which supports the TEM results obtained. Further antimicrobial activities of synthesized nanoparticle were measured against *Bacillus subtilis*, and *Staphylococcus aureus* by agar well diffusion method. The result of the study concludes that the synthesized nanoparticle can be used as antimicrobial agent against pathogenic strains.

## KEYWORDS

Bio reduction, metal nanoparticles, characterization, plant leaf extract, antibacterial activity.

## INTRODUCTION

Nanoparticles synthesis is controlling the size of matter generally in the range of 1–100 nm.[1] It is an upcoming highlight which correlates nanotechnology with biotechnology. It has wide variety of applications in various fields like optics, electronics, catalysis, medicine, magnetics, mechanics, energy science, etc. [2-3]

The metallic nanoparticles have unique characteristics because of their larger surface area to volume ratio.[4] The fabrication of metallic nanoparticles is a wide area of "application research" in nanotechnology.

Silver NPs among various metal nanoparticles is in focus of researchers because of their varied properties such as optical polarizability and magnetic,[5] electrical conductivity,[6] catalysis,[5] antibacterial activities,[7-8] DNA sequencing,[9] and surface-enhanced Raman scattering,[10] which can be applied into various applications like antimicrobial[11-12], biosensor materials, composite fibres, cryogenic super-conducting materials, cosmetic products, and electronic components.[13]

Traditionally metal nanoparticles were synthesized by means of physical and chemical methods but these methods usually explore large area, employ use of harsh chemicals creating environmental degradation which include contamination from the chemicals and also use of hazardous products.[14] Thus the interest in green fabrication approach for metal nanoparticle synthesis is increasing,[15] so nanoparticles have been found to be relevant to numerous emerging technologies.[16]

Phyto-assisted route of metal nanoparticle synthesis received great attention because of its growing need to design environmentally-benign process for material synthesis.[17]
Herein, we followed a simple method for the fabrication of Silver NPs utilizing the medicinally important plant leaf extract of *Ficus Religiosa*[18] without any additional capping agent.

Ficus religiosa is a large and perennial tree, which are found all over the regions of India and is approximately 170m altitude in the Himalayan region.[19] Leaf extract of the chosen plant are traditionally used to treat various complications like alleviate fevers, dysentery, bruises, bleeding wounds, constipation, boils and mumps.

This study is based on the green fabrication of silver nanoparticles by an environmentally friendly procedure[20] with improved shape/size using *Ficus Religiosa* leaf extract and their antibacterial activity evaluation against various micro-organisms.

## MATERIALS AND METHODS

**Materials:**
Silver nitrate, Leaf extract (*Ficus Religiosa*), hydrogen peroxide, mannitol salt agar, starch agar, VP reagent, Simmon citrate agar, Nacl, Cell diameter (width) scale etc.
**Methods:**
**(i) Preparation of *Plant* leaf extract:**
Fresh leaves of chosen plant (*Ficus Religiosa*) were washed thoroughly and chopped into fine pieces. About 20 gm of the chopped leaves were taken into 100 ml deionised water in conical flask, mixed well, and then kept for 60 minutes in water bath usually at 80°C. The leaf extract obtained was then filtered, and the filtrate obtained was collected, finally stored at 4°C for further use. A part of the plant material was air dried for approximately 15-20 days and then is processed into powder for further use.

**ii) Synthesis of Silver Nanoparticles:**

The extract of leaves was mixed drop wise with 50 ml of 1mM Silver nitrate solution in a conical flask under aseptic condition. The flask was kept on a magnetic stirrer for shaking purpose for 4 hours, without giving

heat treatment. A change in the colour (white/foggy to brown) was observed indicating the formation of Silver nanoparticles. Recovery of the particles was then done by Centrifugation. The particles are then washed with organic solvents to remove all the impurities and unwanted particles.[3] Then the particles are allowed to dry. Then the particles are collected and stored for further characterization.

**(iii) Characterization:** In order to investigate various properties of the prepared sample, we had undergone some characterisation techniques, they are:-

**a) UV–Visible spectral analysis**

Reduction of the metal ion to metal nanoparticles is confirmed by colour change, then also characterized by UV-Vis spectroscopy.[10] Absorption of radiation by sample is measured at different wavelengths and then it is plotted so as to give the spectrum which is a plot of the wavelength v/s the absorption of light at different wavelength.[11]

**b) FTIR analysis:**

FTIR is an analysing method for measuring infrared intensity versus wavelength. It is used for analyzing possible bio molecule along with interaction of bonds between themselves. IR spectroscopy basically determines the vibration of chemical functional groups of the chosen sample. IR light first interacts with matter and then chemical bonds starts showing stretch and bend form. The chemical functional group leads to adsorb IR radiation in a specific wave number range of the structure of the rest of the molecule.

**c) X-Ray Diffraction analysis:**

XRD is used to study phase composition of a particle, crystal morphology along with orientation etc. X rays are passed through sample and the pattern resulting by this phenomena produce information of size along with shape of a single unit cell. The atoms are basically crystalline in structure and thus can diffract light at various angles.

**d) SEM analysis:**

The scanning electron microscope basically employ use of a focused beam having high energy electrons to produce a wide variety of signals at the surface of solid specimens, the signals derived from this provide information about the sample including crystalline structure and external morphology.

**e) TEM analysis:**

The size along with morphology of the Ag nanoparticles is determined by transmission electron microscopy. The samples are usually characterized by placing few drops of the nanoparticles suspension on grids, followed by allowing the solvent to evaporate slowly under the sun light before recording the TEM images.

**iv) Isolation and characterization of the microbial flora**:

The collection of the micro-flora was done from clinical samples and then the isolation of the pure Culture was done for further characterization purpose. Two dominant colonies appeared in the Culture plate, both the colonies are picked, named as Culture 1 and Culture 2 and then characterized by means of morphology estimation, staining and biochemical tests like Indole production test, Methyl red and Voges- proskauer test, catalase test, amylase activity test, catalase test and mannitol fermentation etc.

**v) Antimicrobial activity evaluation of the nanoparticle against micro-organisms:**

The antimicrobial efficacy of the biosynthesized Silver nanoparticles was evaluated against both the isolated Culture by the agar well diffusion method and then chosen amount of Silver nanoparticles were loaded in well for determining zone of inhibition. The plates were then incubated for approximately 24 hours.

**vi) Minimum inhibitor concentration (MIC) studies by tube dilution method**

This method is used to determine the levels of microbial resistance toward a particular antimicrobial agent.[21] The lowest concentration of antimicrobial agent preventing level of turbidity is considered to be the minimal / minimum inhibitory concentration of that particular agent. Thus MIC was performed to determine the concentration of biosynthesized silver nanoparticles showing growth inhibition of bacterial strains.

## RESULTS AND DISCUSSION

In this study silver nanoparticles are synthesized by leaf extract of *Ficus Religiosa*. Then the particles were subjected to characterization. The preliminary detection for AgNP formation was observed by visual color change. AgNPs were further characterized by means of UV-Vis spectroscopy, SEM, FTIR, XRD and TEM techniques.

**Visible Observation:**

Silver nanoparticle solution usually appears to be dark brown or dark reddish in colour. When the plant leaf extract is added into the Silver nitrate solution, the colour of the solution start changing from foggy white to dusty colour. This colour change observed is because of the property of quantum confinement and this property is basically a size dependent property of nanoparticles which usually affect the optical properties of the Silver nanoparticles.(Figure 1)

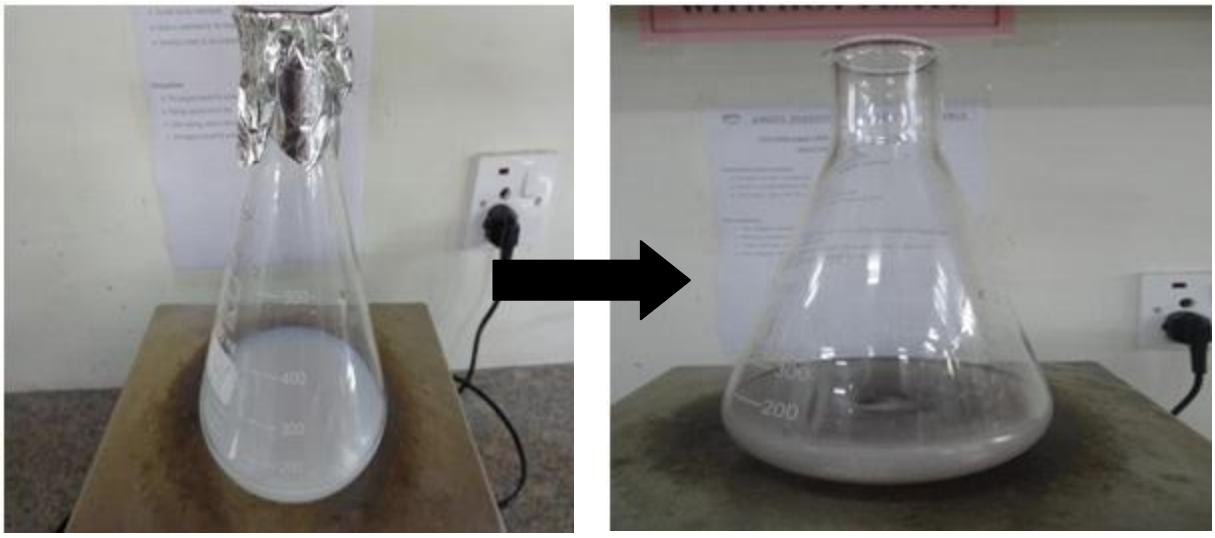

**Fig.1 showing the visible change in the colour of the suspension**

**UV–Visible spectral analysis:**

UV-visible spectroscopy is a useful tool to monitor the synthesis of the Silver nanoparticles. Reduction of Ag ions in the prepared mixtures was monitored by UV- Vis spectral analysis from 200 to 1100 nm using UV-Vis spectrophotometer. A broad absorption peak was observed at around 410 nm, which is an inference for the synthesis of the Silver nanoparticles. UV-vis spectra showed that formed Silver nanoparticles were stable in solution at room temperature.(Figure 2)

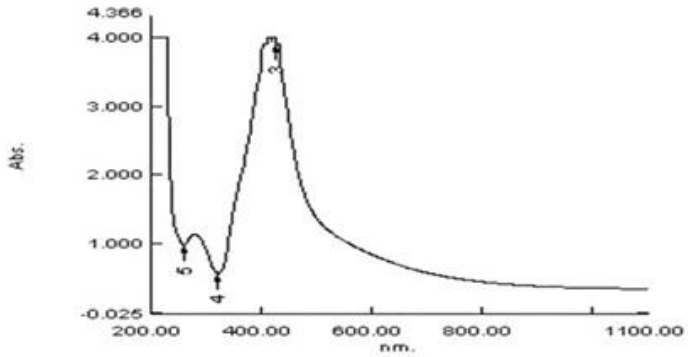


**Fig. 2 Showing UV analysis of synthesized Silver nanoparticles**

**XRD analysis:**

XRD analysis is used to determine the phase distribution, crystallinity and purity of the synthesised nanoparticles. Different Bragg reflection peaks were observed at 2θ values of 38.09, 44.16, 64.43 and 77.33 which are indexed to to the (111), (200), (220) and (311)which are reflections of FCC symmetry of silver. All

the reflections correspond to pure silver metal with face centered cubic symmetry and the intensity of peaks reflected the degree of crystallinity of the Silver NP's. Size of silver nanoparticles is calculated by means of Debye-Scherer's equation from which the average particle size is estimated to be 67.1 nm. (Figure 3)

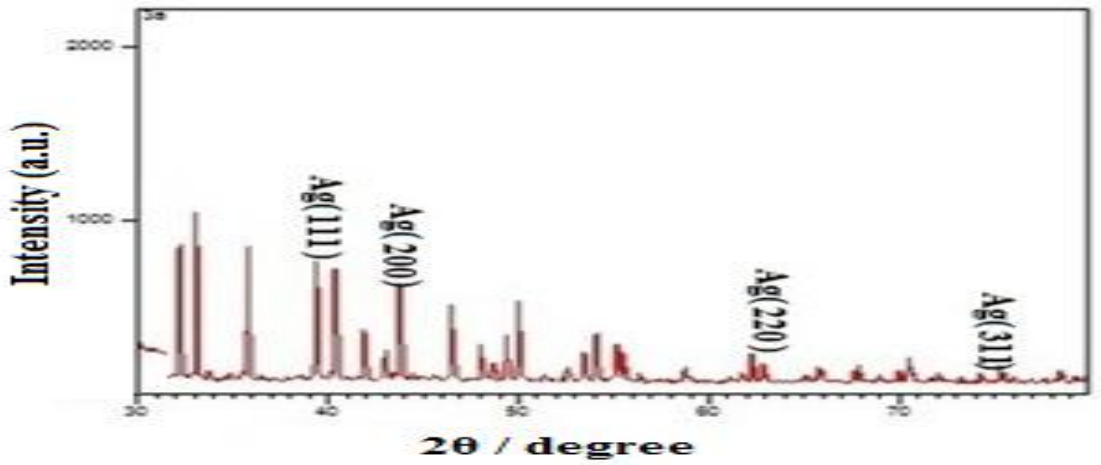


**Fig. 3 Showing XRD Analysis of biosynthesized Silver nanoparticles (a.u. = arbitrary units)**

**FTIR Analysis:**

The peak obtained at 3429.22 cm−1 refers to O-H stretch vibration of Phenol, 2734.24 cm−1 refers to O-H stretch vibration of Carboxylic acids, 1762.26 cm−1 refers to C=O stretch vibration of carboxylic acids, 1655.25 cm−1 refers to –C=C- stretch vibration of alkenes, 824.00 cm−1 and 800 cm−1 refers to C-Cl stretch vibration of alkyl halides.

Reduction of metal ions in the present study might have occur due to the availability of various water soluble phytoconstituents like flavonoids, quinones, and various other organic acids present within the plant extract. (Figure 4)

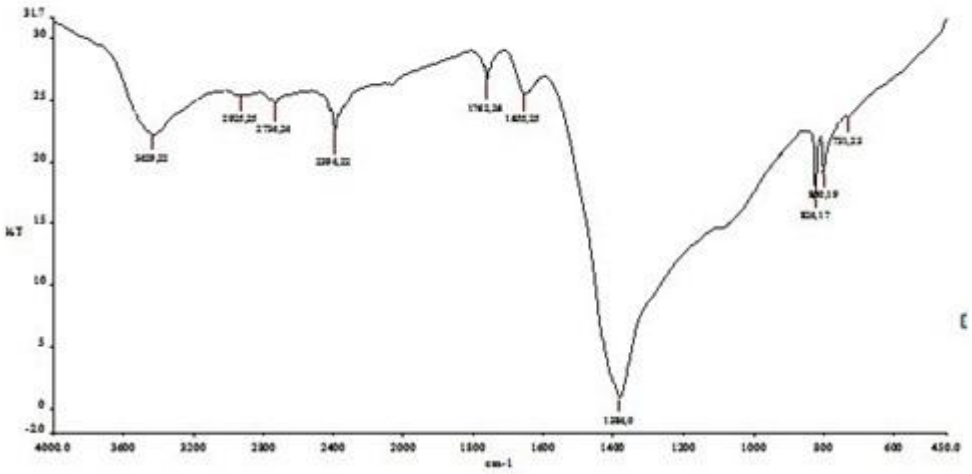

**Fig. 4 Showing FTIR Analysis of biosynthesized Silver nanoparticles**

**Scanning Electron Microscopy Analysis:**

A scanning electron microscope was employed to analyze the shape of the Silver nanoparticles that were synthesised by green method. SEM analysis shows that the plants that we have chosen have tremendous capability to synthesize Silver nanoparticles which were roughly spherical in shape and were uniformly distributed.(Figure 5)

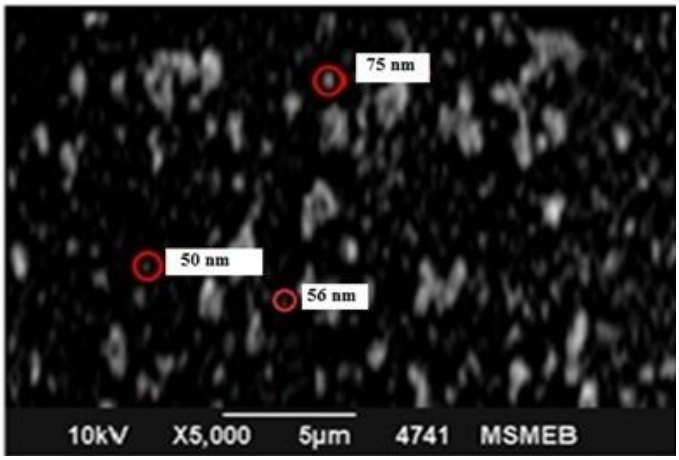


**Fig. 5 Showing SEM analysis of synthesized Silver nanoparticles**

**Transmission Electron Microscopy Analysis:**

Shape along with size of the synthesized Silver nanoparticles was characterized by transmission electron microscopy. The shape appeared to be slightly spherical and size of the NP is below 100nm. Figure clearly depicts the TEM analysis of synthesized Silver nanoparticles.(Figure 6)

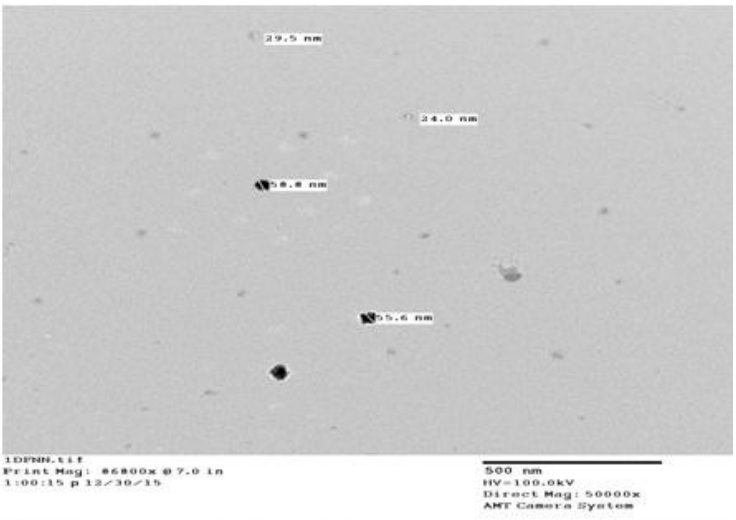


**Fig. 6 Showing TEM analysis of synthesized Silver nanoparticles**

**Identification of micro-organisms:**

The preliminary detection of the culture is done by means of gram's staining. Then the cultures were allowed for further biochemical characterization which is mentioned here as:

Culture 1 is estimated as ***Staphylococcus aureus*** which gives positive result for Catalase test and Mannitol Fermentation and negative result for the rest.

Culture 2 is estimated as ***Bacillus subtilis*** which gives positive result for Starch Hydrolysis Test, voges proskeaur, Citrate Test, 6.5% Nacl Growth and Catalase activity test and negative for the rest.(Table 1)

**Table 1 Showing the list of biochemical tests performed for detecting the presence of particular microorganisms in the water sample.**

| S.no. | Tests | Culture | |
|---|---|---|---|
| | | **Culture 1** ***Staphylococcus aureus*** | **Culture 2** ***Bacillus subtilis*** |
| 1 | Gram's Staining | Gram + *staphylococci* | Gram + *streptobacilli* |
| 2 | Indole Production Test | - | - |
| 3 | Methyl Red Test | - | - |

| | | | |
|---|---|---|---|
| 4 | Voges Proskauer | - | + |
| 5 | Catalase Activity test: | + | + |
| 6 | Glucose Fermentation | - | - |
| 7 | Sucrose Fermentation | - | - |
| 8 | Mannitol Fermentation | + | - |
| 9 | $H_2S$ Production Test | - | - |
| 10 | Starch Hydrolysis Test | - | + |
| 11 | Citrate Test | - | + |
| 12 | 6.5%Nacl Growth | - | + |
| 13 | Cell diameter ≥ 1µm(width) | - | - |

**Anti-Microbial Activity:**

The inhibitory effect of Silver NPs against tested pathogens at 0.5%. Culture 1 has shown the zone of inhibition of 18 mm and for Culture 2 it is 21 mm respectively (Figure 7 & 8). AgNO3 (metal salt solution), plant extract and solvent medium (used for nanoparticle) were kept as control. The inhibitory effect of plant extract used for the synthesizing silver nanoparticles, solvent medium, $AgNO_3$ and Silver nanoparticles against culture 1 and culture 2, are listed(Table 2) here as:

**Table 2 Antimicrobial activity test of Silver Nanoparticles**

| **S.no.** | **Culture** | **Zone of Inhibition(in mm)** | | | |
|---|---|---|---|---|---|
| | | **Plant extract** | **Solvent medium** | **Ag NP's** | **$AgNO_3$** |
| 1. | **Culture 1** | Nil | Nil | 18 | 11 |
| 2. | **Culture 2** | 10 | Nil | 21 | 13 |

**Fig. 7 Showing Antimicrobial activity of the synthesized nanoparticle against culture 1**

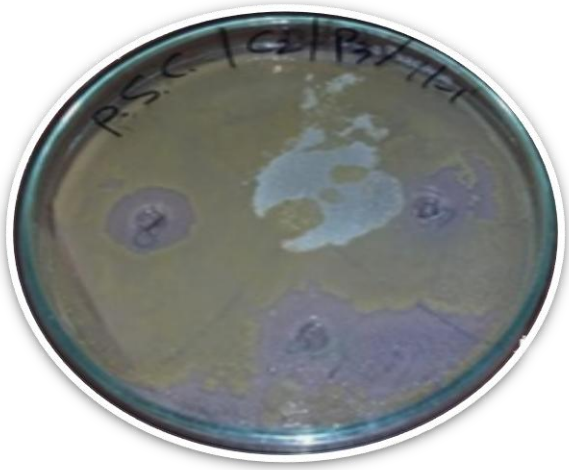

**Fig. 8 Showing Antimicrobial activity of the synthesized nanoparticle against Culture 2**

**Minimum inhibitory concentration (MIC) studies by tube dilution method:**

Different concentration of Silver nanoparticles was used to analyse its antimicrobial efficacy, out of which 400µl of 0.5% concentration was found effective as Minimum inhibitor concentration (MIC).

## CONCLUSION

Green synthesis of Silver nanoparticles by the help of green plants is a very cost effective, safe, non-toxic, eco-friendly route of synthesis which can be manufactured at a large scale. The chosen plant leaf extract showed great capability to synthesis Silver NPs at optimum temperature conditions. The UV absorption peak at 410 nm clearly indicates the synthesis of Silver NPs. The SEM and TEM studies were helpful at deciphering their morphology and size of the particle. FTIR studies confirmed the bio fabrication of the Silver NPs by the action of different phytochemicals with its different functional groups present in the extract solution. The XRD patterns confirmed the purity, phase composition and nature of the synthesised nanoparticles. The following study confirmed the synthesis of stable nanoparticles, which could be due to the presence of capping and stabilizing materials such as flavanoids and terpenoids with in the plant extract. Synthesized Silver nanoparticles have potential antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus* and thus can be used as effective antimicrobial agent in future.

## ACKNOWLEDGEMENT

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# An advance mathematical model to detect thermal data of wounded tissues of human being

**MANISHA JAIN**

Amity University Madhya Pradesh, Gwalior (M. P.)

*Corresponding author email mjain@gwa.amity.edu

## ABSTRACT

Though wound healing process area always been a center of attraction is poorly known for researchers due to its nonlinear behavior from wounding to healing processes. The mathematical modeling is helpful to derive the complex interaction mechanisms of healing process. Temperature profile during healing plays an important role which can be formulated using the mathematical models by incorporating various parameters which are meticulous for the entire process. The various internal and external factors are important and have a significant role in wound management. The present study deals with the temperature which is very important for physiological experiments and theoretical analysis of cleansing material and the time taken by the tissues to become a steady state. The Mathematical model deals with the partial differential equation having an appropriate boundary and initial conditions. A Solution of the complex model is solved using Finite Element Method and relation between tissue temperature and time taken by a wound to heal is presented in graphs for the tapered sized wound.

## KEYWORDS

Wound healing; remodeling phase; mathematical model; finite element.

## INTRODUCTION

Wound healing process is a very complex phenomenon. All deep skin dermal wounds should be well-taken care of because it may damage the organ integrity after losing tissue of any human body. In tissues wound repair, a complex interacting phase occurs after the break up in tissues of the skin i.e. Inflammation, tissue formation, angiogenesis, tissue contraction and tissue remodeling (Clark, 1989).

Tissue repair or wound repair is the body's ordinary process of regenerating dermal tissues and epidermal tissue (SST region). In normal skin, the epidermis (outermost layer) and dermis (inner or deeper layer) remains in steady-state equilibrium. These layers work a strong defending barrier against the external

environment. Once the protective barrier is broken i.e. wound appears, the normal physiologic process of wound healing starts automatically to heal. The entire wound healing process is a combination of complex biochemical events. It begins immediately after the injury happens and continues till the repair of the damaged tissues. This process may continue for days and moths. Successful wound healing requires adequate blood and nutrients to the site of damage. The overall health and nutritional status of the patient control the result of the damaged tissue (Jennings, 1992).

In all the phases, the interaction of the cells known as "The ECM (Extracellular Matrix)" has great importance. After the formation of the blood clot during the inflammation, white blood cells attack the wound site by migrating during the ECM process. In this process fibroblasts also migrate into the wounded area and start replacing the clot with collagen (Streuli C, 1999). The speed of the fibroblasts is controlled by the ECM. It is confirmed that the ECM composition has an effect on the mobility of fibroblasts that migrate more easily on fibronectin gels than on collagen gels (Wojciak-Stothard, 1997 and K.S. Midwood, 2004).

The different proteins production by fibroblasts, growth factors, cytokines that alter fibroblast behavior can be found in the ECM in the wound region. At the end fibroblasts which are the responsible for categorizing collagen fibrils into the fibrous structure in SST (skin and subcutaneous Tissue) region (A. C. Guyton, 2000).

Various mathematical models focused on the importance of Critical Size Defect (CSD) of the wound (J. A. Adam, 2000 and 2002) and the role of growth factor for a timely healing wound. Wound healing is a complicated and combination of several processes like chemotaxis (movement of cells induced by a concentration gradient), neovascularization, synthesis of extracellular matrix proteins, and scar modeling (J. C. Lawrence, 1997). Some models incorporate cell mitosis, cell proliferation, cell death, capillary density, oxygen supply, growth factor generation coupled to a cell density(J. Filion and A.P. Popel, 2004 and J. A. Sherrate, 1991), the advection- dominated models (A. Jennifer et al., 2009). Theoretical wound healing using numerical techniques and dermal wound healing-remodeling phase (Aydin Azizi, 2009) were discussed by researchers.

Temperature distribution in tissues has always been an interesting and important topic for researchers based on the revolutionary work by Pennes(1948), on the heat transfer in biological tissues, the problem of bio-heat transfer in living tissues. Thermal changes during the wound healing after plastic surgery (Manisha Jain, 2011), Effect of the convective term (Emmanuel Kengne, 2013) and temperature-dependent perfusion(Emmanuel Kengne, 2015) in biological tissue are based on the models proposed by Pannes. These problems are relevant in many diagnostic and therapeutic applications which involve changes in temperature. After performing a series of experiments Pennes derived a thermal energy conservation equation known as "Bio-Heat Transfer" (BHT) equation or the traditional BHT equation.

**Mathematical Formulation:**

Heat transfer in the biological system plays a significant role in many diagnostics and remedial treatments. Spatial distribution of thermal changes precisely monitored by the physicians during the treatment protocol is essential. Either rising (above 37oC) or lowering (below 37oC) (T.R. Gowrishankar, 2004) i.e. hyperthermia and hypothermia causes many abnormalities within the body. Most of the Heat transfer analysis on thermal-medical problems has been traditionally performed using the Pennes model. Pannes model describes thermal exchange in the human body due to passive conduction known as diffusion and perfusion of tissue by blood nicely. Mathematically it is given by

$$\rho c \frac{\partial T}{\partial T} = \nabla \bullet (k \nabla T) - c_b \omega_m (T) \rho_b [T - T_a] + Q_m + Q_r (x,t) \qquad (1)$$

Here the effect of metabolic heat production per volume $Q_m$ and blood mass flow are given by the terms and $\omega_b c_b$(T-Ta). $c_b$ and $\rho_b$ are the specific heat and the density of blood, respectively. T is the local tissue temperature, Ta, k, ρ, c, $w_b$ are: a reference arterial blood temperature, thermal conductivity, density and specific heat of the tissues, blood mass flow rate respectively. $Q_r$(x, t) is the heat deposited per volume due to spatially distributed heating. Studies indicated constant blood perfusion within each tissue in thermal distribution models but several experiments reveal that the vasculature activities in tissues are strongly temperature-dependent [25]( $w_b c_b$=a+bT). Temperature-dependent perfusions are more difficult to solve and hence numerical approaches such as finite difference method, finite element method, boundary element method, etc., are the best options for the solution. In the present work thermal propagation of human tissues is investigated during wound healing process by incorporating appropriate initial and boundary conditions.

The optimum environment for the moist environment of the wound healing is required by dressing on the wound time to time. The wound- bed temperature of patients should be measured immediately before and after dressing changes. Wound bed temperature is maintained by cleansing solution, it should be warmed at atmospheric temperature(E. Vella, 2004 and Jo Trim). Maintaining optimum wound temperature helps in increasing blood flow to the wound bed, enhancing the rate of gain of wound tensile strength and increasing oxygen tension. These are supportive conditions to get fast wound repair (C. C. MacFie et al., 2005). It also helps in preventing uncontrolled bacteria proliferation, thereby reducing the risk of infection (Lock, P. M. 1979). The outer surface of the body is exposed to the environment, so during removal of insulation, heat loss at this surface takes place due to conduction, convection, radiation and evaporation.

***Boundary Conditions:*** *Heat loss across the boundaries on x axis*

$$\left.\frac{\partial T}{\partial x}\right|_{x=P_1} = \bar{\varepsilon}, \quad -\left.\frac{\partial T}{\partial x}\right|_{x=P_2} = \bar{\theta} \qquad (2)$$

*Heat loss across the boundaries on y- axis :* Heat loss normal to the surface is expressed as

$$-K\frac{\partial T}{\partial n} = h(T - T_a) + LE \quad for \quad t > 0 \ \ at \ \ y = P_4 \qquad (3)$$

Where h, $T_a$, L, E and ∂T/∂n, are heat transfer coefficient, atmospheric temperature, the latent heat, rate of evaporation and the partial derivatives of T along the normal to the skin surface respectively.

***For inner surface:*** Human body maintains its body core temperature at 37°C therefore, the boundary condition at inner boundary is

$$T(x,y,t) = T_b \ \ for \ \ t \geq 0 \ \ \ at \ \ y = P_3 = 0 \qquad (4)$$

**Initial condition:** The outer surface of the skin has to be insulated (before dressing of wound) hence tissue temperature at time *t=0* is assumed 37°C, mathematically it is given by

$$T(x,y,0) = T_b \qquad (5)$$

**Finite Element Method (FEM)**

We use finite element method to solve (1). The tissue temperature T as a function of time t and space (x,y,z) 1D, 2D and 3D. To solve such equations numerically, it is necessary to discretize in both space and time. The Finite Element Method is the appropriate method to solve such a complex model by splitting up each spatial direction using a fixed step size. Each node of mesh T(X(x,y,z), t) at the node (xi, yj) at time $t_k$ is given by $T_{i,j,k}$ represents the tissue temperature. Solving the partial differential equation by FEM will yield a set of algebraic equations in $T_{i,j,k}$, which may be solved computationally. By using FEM any irregular size wound can be discretized into finite shape and size such as wound over bone. Physical geometry of wound is important when considering areas over any irregular shape and size of the organ like wound over the ankle. The tissue temperature of such irregular areas can be determined by discretizing the wound into triangular and rectangular shapes together (Fig.1 and 2). To solve (1), let the wound of irregular shape and finite size (thickness) 11 mm. along Y axis and length are assumed 100 mm. along X axis having triangular, rectangular elements (ne=790) nodes (nn=451).

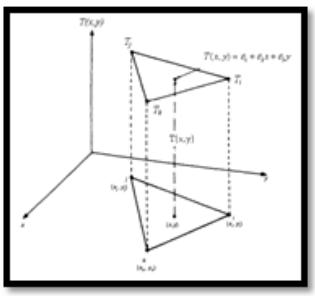

Fig. 1: A three nodes triangular element

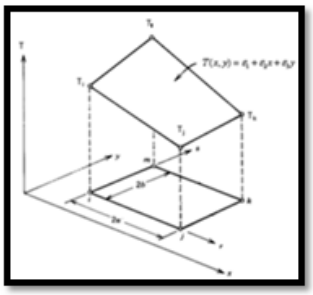

Fig. 2: A four nodes rectangular element

The variational form of eq. (1) along with the boundary and initial conditions, is given by

$$I^e = \frac{1}{2}\iint_{\Delta^e}\left[K^e\left\{\left(\frac{\partial T^e}{\partial x}\right)^2+\left(\frac{\partial T^e}{\partial y}\right)^2\right\}+M^e(T_b-T^e)^2-2S^eT^e+\rho c\ \frac{\partial (T^e)^2}{\partial t}\right]dxdy \qquad (8)$$

$$+\frac{1}{2}\int_{\Omega 1}\left[h(T^e-T_a)^2+2LET^e\right]dx\ +\int_{\Omega 2}\left[\bar{\varepsilon}^eT^e\right]dy+\int_{\Omega 3}\left[\bar{\theta}^eT^e\right]dy$$

Where $\Delta^e$ is the region contained in $e^{th}$ element and Ω1, Ω2 and Ω3 are the boundaries of the region. For triangular and rectangular elements the field variable T depends on $T_i$ , $T_j$ , $T_k$ and $T_i$ , $T_j$ , $T_k$ , $T_l$ respectively.

**The physiological parameters:**

The values of physiological parameters K, M, and S for healthy tissues are constant, almost negligible in wounded tissues (Vincent Falanga) and the increasing function of time and space (Mahanty, S. D., 1980 and S. O. Brattgard, 1978) at the time of healing. The general mathematical expression is as follows:

$$K(y,t)=\varsigma(t)\sum_{d=0}^{1}\alpha_d^e y^d,\ M(y,t)=\psi(t)\sum_{d=0}^{1}\beta_d^e y^d,\quad S(y,t)=\zeta(t)\sum_{d=0}^{1}\gamma_d^e y^d \tag{6}$$

Where; the unknown constants $\alpha_d$, $\beta_d$ and $\gamma_d$ are used to find linear expression for K,M and S respectively in the dermis.

***For wounded tissues :***

$$\varsigma(t)=\left(\nu_0+\nu_1 e^{-\nu t}\right),\ \ \psi(t)=\left(\mu_0+\mu_1 e^{-\mu t}\right),\ \ \zeta(t)=\left(\theta_0+\theta_1 e^{-\theta t}\right) \tag{7}$$

Where $\nu_o$, $\nu_1$, $\mu_o$, $\mu_1$ and $\theta_o$ and $\theta_1$ are calculated by applying suitable conditions and $\nu$, $\mu$ and $\theta$ are constants and used to change the growth rates of K, M, and S.

**(i) For sub-dermis region: (e=1(1)395)**

$$\alpha_o^e=K_1,\alpha_1^e=0,\beta_0^e=M^e,\beta_1^e=0,\gamma_0^e=S^e,\gamma_1^e=0,$$

**(ii) For dermal region: (e=396(1)632)**

$$\alpha_o^e=\left(\frac{K_1y_{329}-K_3y_{206}}{l^e}\right),\alpha_1^e=\left(\frac{K_3-K_1}{l^e}\right),\beta_0^e=\left(\frac{M_1y_{329}-M_3y_{206}}{l^e}\right),\beta_1^e=\left(\frac{M_3-M_1}{l^e}\right),$$

$$\gamma_0^e=\left(\frac{S_1y_{329}-S_3y_{206}}{l^e}\right),\gamma_1^e=\left(\frac{S_3-S_1}{l^e}\right),\ M_3=S_3=0;M_1=M,S_1=S;l^e=(y_{329}-y_{206})$$

**(iii) For epidermis region : (e=633(1)790)**

$$\alpha_o^e=K_3,\alpha_1^e=0,\beta_0^e=0,\beta_1^e=0,\gamma_0^e=0,\gamma_1^e=0,$$

***Interpolation Function :***

The shape function for each triangular-shaped wound is :

$$T(x,y)=\varepsilon_1+\varepsilon_2x+\varepsilon_3y \tag{9}$$

where $\varepsilon_1$, $\varepsilon_2$ and $\varepsilon_3$ are unknown constants for the e[th] element, nodal temperature $T_i$ , $T_j$ and $T_k$ of $e^{th}$ element can be expressed as

$$T(x,y)=N_iT_i+N_jT_j+N_kT_k=\left[N(x,y)\right]\overline{T} \tag{10}$$

where

$$N=\begin{bmatrix}N_i & N_j & N_k\end{bmatrix},\overline{T}=\begin{bmatrix}T_i & T_j & T_k\end{bmatrix}'$$

$$N_i(x,y)=\frac{1}{2\Delta}(a_i+b_ix+c_iy),\ \ N_j(x,y)=\frac{1}{2\Delta}(a_j+b_jx+c_jy),$$

and

$$N_k(x,y)=\frac{1}{2\Delta}(a_k+b_kx+c_ky),\qquad \Delta=\frac{1}{2}\begin{vmatrix}1 & x_i & y_i\\ 1 & x_j & y_j\\ 1 & x_k & y_k\end{vmatrix}$$

Where

$$a_i=x_jy_k-x_ky_j,\quad b_i=y_j-y_k,\ c_i=x_k-x_j$$

$$a_j=x_ky_i-x_iy_k,\quad b_j=y_k-y_i,\ c_j=x_i-x_k$$

$$a_k=x_iy_j-x_jy_i,\quad b_k=y_i-y_j,\ c_k=x_j-x_i$$

Rectangular shape wound having four nodes *i, j, k* and *l* in counter clock direction is written as

$$T(x,y) = \varepsilon_1 + \varepsilon_2 x + \varepsilon_3 y + \varepsilon_4 xy \tag{11}$$

Applying the four nodal conditions at nodes *i, j, k* and *l* the matrix form gives

$$\begin{bmatrix} T_i \\ T_j \\ T_k \\ T_l \end{bmatrix} = \begin{bmatrix} 1 & x_i & y_i & x_i y_i \\ 1 & x_j & y_j & x_j y_j \\ 1 & x_k & y_k & x_k y_k \\ 1 & x_l & y_l & x_l y_l \end{bmatrix} \begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \varepsilon_3 \\ \varepsilon_4 \end{bmatrix} \tag{12}$$

This gives the values of polynomial coefficient as

$$\begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \varepsilon_3 \\ \varepsilon_4 \end{bmatrix} = \begin{bmatrix} 1 & x_i & y_i & x_i y_i \\ 1 & x_j & y_j & x_j y_j \\ 1 & x_k & y_k & x_k y_k \\ 1 & x_l & y_l & x_l y_l \end{bmatrix}^{-1} \begin{bmatrix} T_i \\ T_j \\ T_k \\ T_l \end{bmatrix}$$

In terms of the nodal values the field variable is then described by

$$T(x,y) = \begin{bmatrix} 1 & x & y & xy \end{bmatrix} \begin{bmatrix} 1 & x_i & y_i & x_i y_i \\ 1 & x_j & y_j & x_j y_j \\ 1 & x_k & y_k & x_k y_k \\ 1 & x_l & y_l & x_l y_l \end{bmatrix} \begin{bmatrix} T_i \\ T_j \\ T_k \\ T_l \end{bmatrix} \tag{13}$$

The interpolation function written in local coordinates *r* and *s* as

$$T(r,s) = \varepsilon_1 + \varepsilon_2 r + \varepsilon_3 s + \varepsilon_4 rs \tag{14}$$

The values of unknowns $\varepsilon_1$, $\varepsilon_2$ , $\varepsilon_3$ and $\varepsilon_4$ for the e[th] element can be obtained by solving nodal equations of T and rearranging the terms eq.(5.13) becomes

$$T(r,s) = N_i T_i + N_j T_j + N_k T_k + N_l T_l = [N(r,s)]\overline{T} \tag{15}$$

$$N_i = 1 - \frac{r}{2a} - \frac{s}{2b} + \frac{rs}{4ab}, \quad N_j = \frac{r}{2a} - \frac{rs}{4ab}, \quad N = \begin{bmatrix} N_i & N_j & N_k & N_l \end{bmatrix},$$

$$N_k = \frac{rs}{4ab}, \quad N_l = \frac{s}{2b} - \frac{rs}{4ab} \qquad \overline{T} = \begin{bmatrix} T_i & T_j & T_k & T_l \end{bmatrix}'$$

The resultant partial differential equation after incorporating all above equations is given by

$$[P]_{n_n \times n_n}\left[\frac{d\overline{\overline{T}}}{dt}\right]_{n_n \times 1} + [Q]_{n_n \times n_n}\left[\overline{\overline{T}}\right]_{n_n \times 1} = [R]_{n_n \times 1} \tag{16}$$

Linear differential equation (16) is solved by using Crank Nicolson Method[4].

$$\frac{T_i^{n+1} - T_i^n}{\Delta t} = F_i^n(T, x, t, t_x, t_{xx});\ \frac{T_i^{n+1} - T_i^n}{\Delta t} = F_i^{n+1}(T, x, t, t_x, t_{xx})$$

$$\frac{T_i^{n+1} - T_i^n}{\Delta t} = \frac{1}{2}\left[F_i^n(T, x, t, t_x, t_{xx}) + F_i^{n+1}(T, x, t, t_x, t_{xx})\right]$$

$$T_i^n = (i\Delta x, n\Delta t)$$

**Numerical Results and Discussions**

The numerical calculations have been made for three cases of atmospheric temperatures. The values of physical and physiological parameters taken (V. Saxena, 1991) to obtain the numerical results are given in TABLE 1 and 2.

| Parameter | Value | Parameter | Value | Parameter | Value |
|---|---|---|---|---|---|
| $K_1$ | 0.060 cal/cm/min/ °C | $S_2$ | variable | $h$ | 0.009 cal/cm²/min/°C |
| $K_2$ | Variable | $S_1$ | 0.0 cal/cm³/min | $c$ | 0.830 cal/gm/°C |
| $K_3$ | 0.030 cal/cm/min/ °C | $T_b$ | 37°C | $\varepsilon$ | 0.01 |
| $M_2$ | Variable | $P$ | 1.090 gm/cm³ | $\nu$ | 0.01 |
| $M_B$ | 0.0 cal/cm³/ °C | $L$ | 579.0 cal/gm | | |

**Table 2** Values of $M_1$ and $S_1$ at different $T_a$ and E

| Parameter | $T_a$ (°C) | $M_1 = M = (m_b c_b)$ (cal/cm³/min/°C) | $S_1 = S$ (cal/cm³/min) | E (gm/cm²/min) |
|---|---|---|---|---|
| | 15 | 0.003 | 0.0357 | 0.0 |
| | 23 | 0.018 | 0.018 | 0.0, 0.00024, 0.00048 |
| Values | 33 | 0.0315 | 0.018 | 0.00024, 0.00048, 0.00072 |

**Table 1** Values of physical and physiological parameters

Here in Fig 3:(A)-(C) and Fig 4:(A)-(C) nodal temperature versus time graph has been plotted for $T_a$ = 15°C for both, normal and wounded tissues for the different rate of healing constant $\nu=\mu=\theta=0.01, 0.02, 0.03$. Fig 3(A-C) and Fig 4 (B-C) show temperature profiles for $T_a$ = 23°C and 33°C at different rates of evaporation. Graphs are plotted for the four nodes viz.: one at the core of the skin ($T_1$), one at the interface of subcutaneous - dermis ($T_5$), one at the interface of dermis - epidermis ($T_8$) and one at the surface of the skin

($T_{11}$). Fig 5 (A-C) are the graphs comparing temperature profile of the skin surface (Epidermis Layer) against time for different atmospheric temperatures ($T_a$ = 15$^o$C , $T_a$ = 23$^o$C and 33$^o$C ) at fixed rate of sweat evaporation $E$ = 0.0, 0.00024 and 0.00048 gm/cm$^2$/min respectively.

Fig (3&4) show tissue temperature at the skin core is 37$^o$C as assumed initially. With the increase in time the temperature decreases very fast for first 10 minutes and 20 minutes for normal and wounded tissues respectively.

This is due to the fact that with the release of insulation of normal as well as the wounded region more heat loss takes place from the wounded region during cleansing and dressing of wound (open wound) than that of the normal region. Hence, more and fast temperature fall is noted about 20 minutes for the wounded region and slow temperature fall about 10 minutes for the normal region. During the process, the sweat glands become active due to which sweat is evaporated by utilizing the adequate amount of latent heat of epidermis tissues through the process of evaporation. Fall in temperature is higher in wounded area than that in the normal ones. The reason behind in decrease in temperature in wounded tissues that that as soon as opening is made in the tissue, blood perfusion rate is eventually stopped in the wounded area due to vasoconstriction in the detached blood vessel due to vascular spasm of the smooth muscle in vessel wall followed by formation of platelet plug to stop bleeding. At the time of surgery the values of physiological parameters in transplanted tissues are negligible and with the increase of time, the value of these physiological parameters also increases (Vincent Falanga, Mahanty, S. D., 1980 and S. O. Brattgard, 1978).

In the healing process, biological and chemical processes take place gradually. These processes are responsible for increasing tissues temperature (Hall, J. E., 2010). Cell division takes place gradually resulting in the slow increase in temperature. This mitotic division needs normal body temperature. Therefore, very small and gradual increment for about 100 minutes is noted for transplanted tissues and tends to become steady after about 250 minutes and approaching towards normal values. The delay in resumption of steady state temperature profiles by wounded tissues can be validated on the basis of experimental findings of Gannon. Due to normal cell division in the tissues of normal region temperature becomes almost steady after 20 minutes. The steady state occurs when the physiological parameters of wounded region start attaining the values equal to that of the normal tissues. Steady state nearer to the normal values shows the proper functioning of physiological parameters thus fast healing. It is also observed from the (Fig. 3-4) that temperature of transplanted tissues tends to steady very fast for $\nu=\mu=\theta=0.02$& 0.03 but experimentally it is proved that tissue temperature of the wounded site takes about 3 hours 20 minutes to acquire normal body temperature (MacFie, C. C., 2005). Therefore, we must say that results obtained for $\nu=\mu=\theta=0.01$ are more approximate.

**Conclusion:**

In this paper, author established the mathematical relation between the parameters on the basis of the experimental phenomenon. Such mathematical models can be useful to prevent adverse effects of the wound. Thermal information is useful for biomedical scientists as well as researchers for predicting the rate of healing of healthy or unhealthy persons. This study is helpful in the treatment of various diseases, to develop protocols for medical purpose and for evaluation of the effectiveness of hyperthermic treatments.

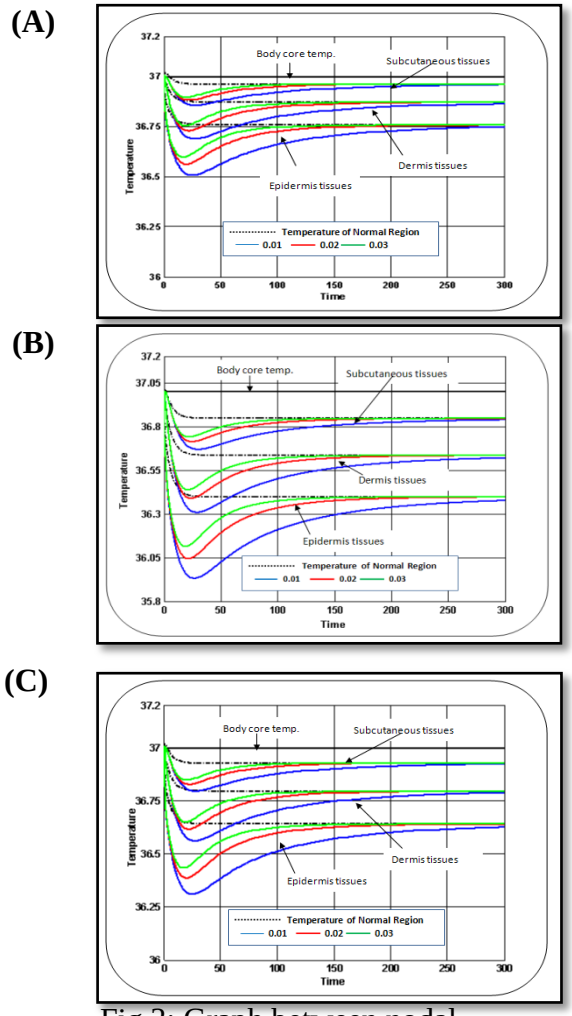


Fig 3: Graph between nodal temperature and time for $T_a$=23$^o$C, (A) E=0.0 gm/cm$^2$/min.
(B) E=0.000024 gm/cm$^2$/min.
(C) E=0.000048 gm/cm$^2$/min.

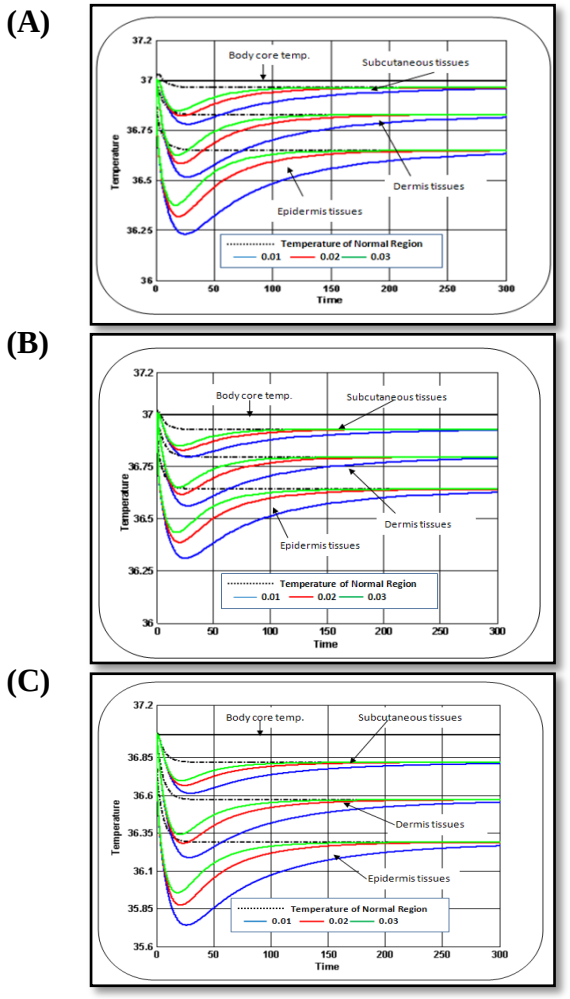


Fig 4: Graph between nodal temperature and time for (A) Ta=15$^o$C, E=0.0 gm/cm$^2$/min.
(B) Ta=33$^o$C E=0.000024 gm/cm$^2$/min.
(C) Ta=33$^o$C E=0.000048 gm/cm$^2$/min.

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# Role of P-Glycoprotein and its Inhibition in Cancer Therapy: A Review

**SHARMISTHA BANERJEE*[1], SHUCHI KAUSHIK[1], RAJESH SINGH TOMAR[1]**

[1]Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior, India

*Corresponding Author email: sbanerjee@gwa.amity.edu

## ABSTRACT

P-glycoprotein (Permeability glycoprotein or Plasma glycoprotein) commonly abbreviated as P-gp is a member of ATP-binding cassette (ABC) family of transporters that share sequence and structural homology, which bears 48 members at present. This protein was first discovered in Multi Drug Resistance (MDR)-Chinese Hamster Ovary (CHO) cells. It is estimated that among this large family of transporters, only 10 are known to provide drug resistance. They use the energy liberated by ATP hydrolysis to pump substrates out of cells. The expression of this protein is usually highest in tumors originated from tissues that usually express P-gp, i.e. epithelial cells of kidney, colon, pancreas, liver and adrenal resulting in the development of resistance to some cytotoxic agents before chemotherapy is started (intrinsic resistance). In other tumors, the expression of P-gp may be low at the time of diagnosis but increases after exposure to chemotherapeutic agents, thereby resulting in the development of MDR in those cells (acquired resistance). P-gp develops MDR by decreasing the concentration of intracellular drug which decreases the cytotoxicity of a broad spectrum of antitumor drugs including anthracyclines (e.g. DOX), vinca alkaloids (e.g. vincristine), podophyllotoxins (e.g. etoposide) and taxanes (e.g. taxol). The efflux pump is mainly inhibited in order to improve the delivery of therapeutic agents. In general, inhibition of P-gp can be achieved by three mechanisms: (i) blocking binding site of the drug either competitively, non-competitively or allosterically; (ii) interfering with ATP hydrolysis; and (iii) altering integrity of lipids in cell membrane. This review focuses on role of P-gp in MDR and several inhibitors of P-gp that would increase the efficacy of drug in cancer therapy.

## KEY WORDS

ABC; MDR; Chemotherapy; Efflux; Allosterically; Inhibitors

## INTRODUCTION

This protein was identified for the first time in multidrug resistant Chinese hamster ovary (CHO) cells by Ling and co-workers in 1974.[1]

Multi Drug Resistance in cancer cells is considered as major hindrance to the effectiveness of chemotherapy in cancer treatment. It is a process in which tumor cells *in vitro* when exposed to a cytotoxic agent evolve cross-resistance to a wide variety of structurally and functionally distinct compounds. The drug resistance that evolve in cancer cell is mainly due to increased expression of certain proteins, such as transporters of cell membranes that may increase efflux of the cytotoxic drugs from the cancer cells, which may result in lower intracellular concentrations. In addition, MDR exists intrinsically in some cancers even before administration of chemotherapeutic agents.[2] The cytotoxic drugs that are most frequently associated with MDR are hydrophobic, amphipathic natural products, such as vinca alkaloids (vinorelbine, vincristine, vinblastine), taxanes (paclitaxel, docetaxel), epipodophyllotoxins (etoposide, teniposide), anthracyclines (doxorubicin, daunorubicin, epirubicin), topotecan, mitomycin C and dactinomycin.[3]

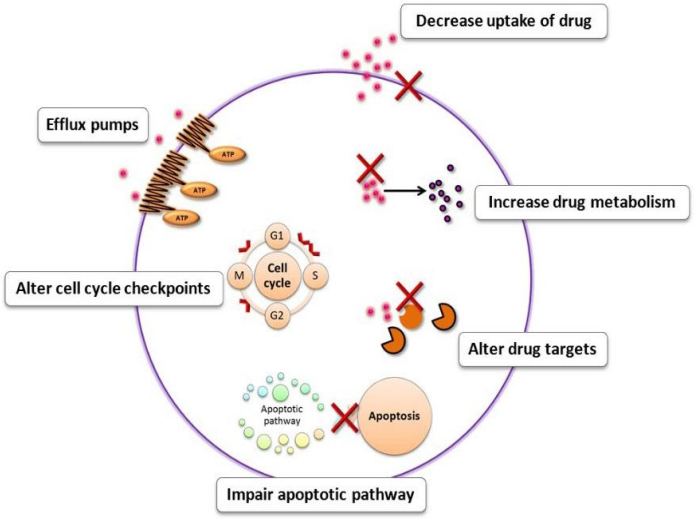



Fig.1: Mechanism of MDR development in Cell

## Substrates of P-gp

This protein has a wide range of substrates that differ not only in size and structure but also in several chemical properties. Since the basic criteria for a compound to be a substrate for P-gp efflux is its interference with lipid bilayer membrane, a large array of cationic, lipophilic and planar drugs fulfils the criteria of being P-gp substrate, inspite of their dissimilar structure. Anticancer drugs, various pharmacotherapeutic agents that affects central nervous system, cardio vascular system and antimicrobials are substrates to this efflux protein.[4,5]

## P-gp inhibition

The efflux pump is mainly inhibited in order to improve the delivery of therapeutic agents. In general, inhibition of P-gp can be achieved by three mechanisms: (i) blocking binding site of the drug either competitively, non-competitively or allosterically; (ii) interfering with ATP hydrolysis; and (iii) altering integrity of lipids in cell membrane. [6-10] The aim is to attain improved bioavailability of drug, drug uptake in the targeted organ, and more efficient cancer chemotherapy with the potential to specifically hinder the function of P-gp. Inhibitors are as structurally diverse as substrates.[10] Many inhibitors (verapamil, cyclosporin A, trans-flupenthixol, etc.) are transported with the help of P-gp itself.[11]

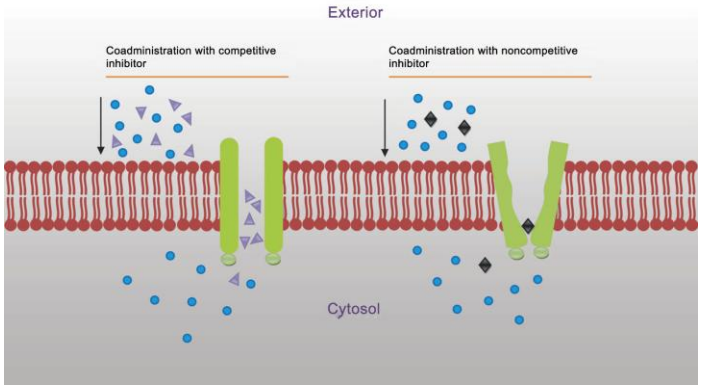
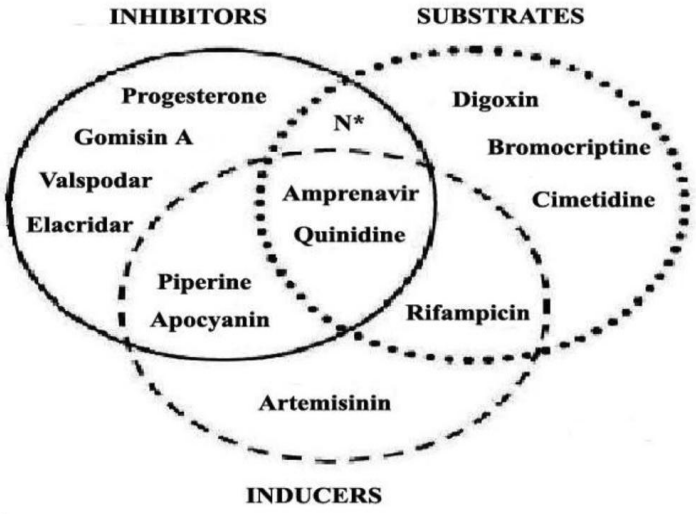



Fig. 2: Mechanism of action of P-gp12

## Types of P-Gp inhibitors

The substances which inhibit or bypass the efflux of P-gp are known as P-gp inhibitors/P-gp modulators/ chemosensitizers/reversal agents. Co-/concurrent administration of the P-gp substrate-therapeutics with these P-gp inhibitors can prevent/overcome the substrate removal and provide the expected therapeutic benefits of the P-gp substrates that could act as drugs. Though many inhibitors of P-gp are already discovered among the available drugs, their toxicity and drug interaction profiles drove the scientists to identify more rational inhibitors. Till date, the inhibitors have been discovered among various natural products, pharmaceutical inert excipients and formulations. Prodrug strategy is also being applied to prevent P-gp efflux. A few novel antitumor drugs, synthetic peptides and P-gp expression suppressors are under development to circumvent the protein action.[6]




**N* implies verapamil, diltiazem and most of the first generation inhibitors which are P-gp substrates as well as inhibitors**

## P-gp Modulators

Modulators focusing P-gp directed MDR belong to different chemical classes and are categorized as the first, second and third generation of MDR reversal compounds on the basis of their affinity towards the protein and relative toxicity towards normal cells as indicators of their side effects.[13]

Many P-gp modulators including verapamil, cyclosporin (cyclosporin A), tamoxifen, and several calmodulin antagonists were discovered in the 1980s.[14] These agents often showed discouraging results *in vivo* because of their low binding affinity which in turn demanded the higher doses of drugs, resulting in toxicity beyond the permissible limits.[15] Many scientistists are inspecting into Chinese medicine (CM) for rational MDR reversing agents.[16]

Inhibitors or modulators developed from natural sources are commonly attributed as "Fourth Generation Inhibitors". In fact, compounds from natural products are rendered as most diverse and novel chemical candidates for the discovery of new inhibitors. It is therefore not much astonishing that many scientists identify the worth of identifying novel natural product modulators, because they could be more promising in comparison to the already developed modulators. A vast array of materials that could be used as biologically active components are now isolated from plants, fungi and even marine organisms, then purified and characterized. Most importantly, natural extracts are usually low in toxicity and are well tolerated in the human body. For that reason researchers are actively identifying agents from natural sources that have a strong modulatory effect on the action or expression of P-gp.[17]

## Fourth generation P-gp inhibitors

Tetrandrine is a bisbenzylisoquinoline alkaloid extracted from the root of *Stephania tetrandra* (Fenfangji) blocks calcium channel.[18] Tetrandrine reversed MDR *in vitro* and modified concentration of drug efflux mediated by P-gp.[18,19(a),19(b),20] simultaneous administration of tetrandrine with doxorubicin or vincristine *in vitro* showed synergic action on cancer cells.[21] Tetrandrine decreased the expression of P-gp.[22]

Matrine is a quinolizidine alkaloid extracted from *Sophora alopecuroides* (Kudouzi). In resistant K562/DOX cell line, matrine (up to 50 μg/mL, non-toxic) enhanced the intracellular concentration of doxorubicin and induces apoptosis.[23] Matrine increases the cytotoxicity of vincristine in resistant K562/VCR cell line.[24] It was proposed that matrine prevented MDR by decreased expression of P-gp.[22]

Tetramethylpyrazine, a calcium channel blocker, is an active alkaloid from *Ligusticum chuangxiong* (Chuanxiong).[25] In MDR resistant HL-60/VCR cell line, tetramethylpyrazine markedly reversed MDR against many drugs namely doxorubicin, duanorubicin and vincristine.[26] It reduces drug efflux (up to 50%) in resistant MCF-7/DOX cell line that overexpresses P-gp.[27] Tetramethylpyrazine when combined with β-elemene showed stronger MDR reversal effects in resistant K562/DOX cell line.[28] Tetramethylpyrazine reduced expression of P-gp in resistant HepG2/DOX cell line.[29]

Peimine (also known as verticine) is a cevanine type isosteroidal alkaloid isolated from the bulbs of *Fritillaria thunbergii* (Zhebeimu) and other *Fritillaria* species.[30,31] In resistant K562/DOX and HL-60/DOX cell lines, peimine showed increased intracellular accumulation of daunorubicin and MDR reversal was achieved by inhibiting the expression of P-gp.[32] Berbamine is a calcium channel blocker isolated from *Mahonia fortunei* (Shidagonglao). In K562/DOX cell line, berbamine inhibited cell growth by induction of apoptosis and enhancing the concentration of rhodamine-123 and doxorubicin in the intracellular environment.[33,34] In MCF-7/DOX cell line, O-(4-ethoxyl-butyl)-berbamine, a derivative of berbamine, MDR reversal was achieved by enhancing G2/M arrest and increasing the intracellular concentration of doxorubicin.[35]

Ginsenosides are triterpenoid dammarane derivatives extracted from *Panax ginseng* (Renshen). Several ginsenosides, like Rg1, Rg3, Re, Rc and Rd prevented the efflux of drug.[36] A mixture of purified saponins containing Rb1, Rb2, Rc, Rd, Re and Rg1 reversed MDR whereas individual ginsenosides did not show any promising results.[37] Ginsenosides reversed MDR of several chemotherapeutic agents namely homoharringtonine, cytarabine, doxorubicin and etoposide in K562/VCR and in a dose-dependent manner in K562/DOX.[38] Pgp expression was decreased but expression of bcl-2 remained the same.[39]

*Panax notoginseng* (Sanqi) total saponins reversed MDR of doxorubicin in MCF-7/DOX and K562/VCR cell lines. The mechanism may be related to reduced expression of P-gp.[40-41] Quercetin is one of the most common flavonoids in natural products including Chinese medicinal herbs such as *Sophora japonica* (Huai). Quercetin inhibited the binding of heat shock factor at the MDR1 promoter, which decreases MDR1 transcription and that in turn reduces the expression of P-gp.[42] Quercetin also circumvented the overexpression of Pgp mediated by arsenite.[43] In HL-60/DOX and K562/DOX cell lines, quercetin enhanced the sensitivity of cancer cells for daunorubicin and reduces the expression of P-gp.[44(a),44(b)] MDR reversal effect of quercetin was presumably mediated by its action on membrane potential of mitochondria and the induction of apoptosis. Furthermore,

quercetin derivatives are capable of showing MDR reversal rather than quercetin itself.[45] In comparison with other active flavonoids, quercetin was less effective than kaempferol but more potent than genistein and daidzein in reversing MDR. Genistein and daidzein had no effect on Pgp expression.[46] Although quercetin may be a rational MDR reversing agent, lethal drug-drug interaction between quercetin and digoxin has been reported.[47]

Curcumin, the major ingredient in *Curcuma longa* (Jianghuang) is responsible for inhibiting the transport activity of all three important ABC transporters, i.e. Pgp, MRP1 and ABCG2.[47] Curcumin reversed MDR of doxorubicin or daunorubicin in K562/DOX cell line and reduced the expression of P-gp in a time-dependent manner.[48] Curcumin increased the anticancer sensitivity to vincristine by inhibiting Pgp in SGC7901/VCR cell line.[49] Moreover, curcumin was useful in reversing MDR by reducing the expression of bcl-2 and survivin but an increasing the expression of caspase-3 in COC1/DDP cell line.[50] Derivatives of curcumin showed MDR reversal by inhibiting Pgp efflux. A chlorine substituent at the meta-or para-position on benzamide improved MDR reversal.[51]

Bisdemethoxycurcumin modified from curcumin resulted in increased inhibition of Pgp expression.[52] Tetrahydrocurcumin, the major metabolite of curcumin, inhibited all three major ABC transporters.[53] Curcumin initiated atypical and caspase-independent cell death in MDR cells.[54] Nanoemulsion of curcumin proved to be better than conventional curcumin in increasing the efficiency of drug delivery into the cells by down-regulating Pgp expression and inducing apoptotis.[47,55]

Schizandrins, the active constituents of *Schisandra chinensis* (Wuweizi), were examined for reversal effects of MDR. Schizandrin A reversed MDR by inducing apoptosis and down-regulating Pgp and total expression of protein kinase C. The crude extract of *Schisandra chinensis* reversed the resistance against vincristine *in vivo*.[56] Deoxyschizandrin and g-schizandrin, among the nine dibenzo[a,c] cyclooctadiene lignans examined, enhanced intracellular drug concentration and induced cell cycle arrest at the G2/M phase when administered with sub-toxic doses of doxorubicin.[57] Gomisin A, on the other hand, altered Pgp-substrate interaction by binding to Pgp simultaneously with substrates.[58]

'Shengmai Injection' consisting of *Panax ginseng* and *Ophiopogon japonicus* (Maidong), down-regulated Pgp expression in peripheral blood lymphocyte membrane. When combined with oxaliplatin, 5-fluorouracil or folinic acid, the injection increased the survival rate of patients suffering from colon cancer.[59] The injection also increased the effectiveness of tamoxifen and nifedipine in combination therapy.[60]

'Shenghe Powder', consisting of Panax ginseng, *Scorophularia ningpoensis* (Xuanshen) and *Atractylodes macrocephala* (Baizhu) increased the intracellular concentration of vincristine in resistant SGC-7901/VCR cell line, possibly due to the induction of apoptosis and down-regulation of Pgp and bcl-2 expression.[61] 'Modified Sanwubai Powder', consisting of herbs such as *Croton tiglium* (Badou), *Platycodon grandiflorum* (Jiegeng) and *Fritillaria thunbergii*, induced apoptosis in SGC-7901 cell line and down-regulated the gene expressions of p53, bcl-2, rasP21CD44 and Pgp.[62]

Oil emulsion from *Brucea javanica* (Yadanzi) reversed MDR when administered with other chemotherapeutic agents like vincristine, doxorubicin, cisplatin, mitomycin C, 5-fluorouracil or etoposide, presumably due to down-regulation of Pgp expression or inhibition of TOPO II or both.[63-64]

'Sangeng Mixture Decoction', consisting of *Reynoutria japonica* (Huzhang), *Actinidia arguta* (Mihouligen) and *Geum aleppicum* (Shuiyangmeigen), reversed MDR of doxorubicin by down-regulating the expression of P-gp.[65] FFTLG, a formula containing *Actinidia arguta*, reversed MDR in K562/DOX cell line by acumumulating the intracellular concentration of doxorubicin.[66] R1, consisting of *Ligusticum chuanxiong*, *Curcuma longa* and *Millettia dielsiana* (Jixueteng), increases the anticancer activities of doxorubicin in MCF-7/DOX by down-regulating the expression of Pgp.[66-67]

**Natural and synthetic polymers with P-gp inhibitory activity**

Polymers such as polysaccharides, PEG derivatives, dendrimers, thiolated polymers and amphiphilic block copolymers were researched and reported for their P-gp modulatory activities.[68-69] Amongst natural polymers, anionic polysaccharides such as xanthan and gellan gum, dextran, fucoidan and sodium alginate have been patented and qualified as oral bioavailability enhancers and their MDR overcoming abilities.[70]

PEG derivatives and analogues offer wide array of applications[71]; and constitute large number of potential MDR modulators.[69] It was recorded that secretory transport of rhodamine 123 across isolated rat intestine was inhibited on adding different concentrations (0.1-20% w/v) of PEG and that it was not dependent on their molecular weights. Treatment with PEG led to an enhanced accumulation of paclitaxel and doxorubicin in Caco-2 cells[72]; and higher rates of intestinal transport and absorption of prednisolone and quinidine in rats.[73]

Nanoparticles and liposomes are generally PEGylated to impart stealth properties to them.[74] Few PEGylated liposomes have been evaluated for their ability to overcome MDR too.[75-76] In one study[77], pre-treatment of Caco-2 cells with surfactants composed of PEG and fatty acids/fatty alcohols led to enhanced intracellular accumulation of epirubicin.

## Antibodies and Peptides as Pgp inhibitors

Pgp dependant MDR could be reversed by hydrophobic peptides that are high-affinity Pgp substrates. These peptides, may represent a new class of compounds to be consideration as rational chemosensitizing agents.[78] Peptide analogues of TMDs (Trans Membrane Domains) are assumed to show interference with the assembly or function of the target protein. Small peptides developed to correspond to the transmembrane segments of Pgp could act as specific and potent inhibitors, suggesting that TMDs of ABC transporters can also serve as templates for the design of P-gp inhibitors.[79]

Studies suggest that immunization could be an alternative supplement to chemotherapy. A mouse monoclonal antibody directed against extracellular epitopes of Pgp was shown to inhibit the *in vitro* efflux of drug substrates.[80] Similarly, immunization of mice with external sequences of the murine gene *mdr1* evolved antibodies capable of reverting the MDR phenotype *in vitro* and *in vivo*, without developing an autoimmune response.[81]

## Reduced expression of MDR genes

Selective downregulation of resistance genes in cancer cells is an emerging approach in therapeutics. Although in cell lines MDR is often caused by the amplification of the *MDR1* gene, the overexpression of the protein has transcriptional components as well. Regulation of Pgp expression is really a complex task, and would involve different mechanisms in normal cells in comparison with the cancerous ones.[82] If mechanisms governing expression of Pgp in malignant cells were mediated through tumour-specific pathways, cancer-specific approaches to prevent overexpression of P-gp could be designed with smaller effects on constitutive expression of normal cells.[83]

## CONCLUSION

An ultimate aim in cancer therapy is to develop specialized treatment that targets growth-promoting pathways and prevent drug resistance. Anticancerous agents fail to destroy cancerous cells for numerous causes like variations in the absorption, metabolism and delivery of drug to target tissues and tumor location in parts of the body into which the drugs do not easily penetrate and also the development of multi-drug resistance phenomenon. Various generations of MDR modulators and inhibitors have represented new and improved medications, although not to the perfection. The perfect reversing agent would be the one which is efficient, devoid of unrelated pharmacological effects, shows no pharmacokinetic
interaction with other drugs and restores the treatment efficiency of the anticancer drug to that observed in MDR negative phenotype. In this context, recent studies have reported that natural compounds found in vegetables, fruits, plant derived beverages and herbal dietary supplements not only have anticancer properties, but may also affect P-gp expression. P-gp inhibitors found in natural products, especially those found in traditional medicine and dietary supplements, have the potential to be developed as MDR reversing agents which could lead to more successful chemotherapy.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# Genetic Diversity of Epiphytic Pink Pigmented Facultative Methylotrophs from Leaf Phyllosphere of Crop Plants

**GAGAN JYOT KAUR[1], MANISH KUMAR*[1], RAJESH SINGH TOMAR[1]**

[1]Amity Institute of Biotechnology, Amity University, Gwalior, M.P.

*Corresponding author email: mkumar@gwa.amity.edu

## ABSTRACT

Leaf phyllosphere of five different crop plants were selected for the isolation of epiphytic methanol utilizing pink pigmented facultative methylotrophs (PPFMs) on AMS (Ammonium mineral salt) media by leaf imprinting technique. An average of eight isolates from each plant were selected and screened on the basis of multiple PGP activities like siderophore production, IAA production and ammonia production. Finally screened epiphytic isolates from each phyllosphere were subjected to DNA extraction and 16S rRNA gene amplification. ARDRA (Amplified ribosomal DNA restriction analysis) profiling was obtained by using *Hae*III and *EcoR*I restriction enzymes. Matrix was prepared for the construction of similarity clusters using Ntsys software. Distinct major and minor clusters were obtained on the basis of presence and absence of bands appeared after restriction analysis. In major cluster I a total 21 PPFMs were grouped while rest of the PPFMs were present in 3 different sub-clusters with different similarity percentage. The obtained dendrogram provided an unambiguous similarity% among the PPFMs isolates. This study indicated a clear pattern of genetic diversity of epiphytic PPFMs in phyllosphere of crop plants.

## KEY WORDS

PPFM, ARDRA, Cluster, PGP

## INTRODUCTION

Pink-Pigmented Facultative Methylotrophs (PPFMs) are phylogenetically diverse group of bacteria utilizing reduced one-carbon compounds such as formate, formaldehyde, methanol with no carbon-carbon bond as sole source of carbon and energy. Mostly PPFMs are designated as genus Methylobacterium[1]. PPFMs come under a subpopulation of Proteobacteria of family Methylobacteriaceae. The oxidation of methanol is the peculiarity of PPFMs, one of the properties of methylotrophs based on the presence of methanol dehydrogenase gene i.e. mxaF[2]. PPFM generally residing to the plant surfaces where they utilize methanol secreted from plants[3].They are reported to secrete types of auxins[4] and cytokinins[5] that are grasped by the plants leading to plant growth promotion. In a number of studies, PPFMs have been described to enhance plant growth, ethylene reduction, siderophore production, enhanced seed germination, cytokinin production and systemic resistance[6,7,8]. PPFM bacteria are cosmopolitan in nature and present in various niches including drinking water, lake sediments, inside body, marshes, phyllosphere, rhizosphere, root nodules, dust, sewage, etc [9,1,7,8]. Methylobacterium spp. Are present on the

surface of leaf as epiphytic microbial community[10] and are predominant genera on the leaf phyllosphere[11].Most of the PPFM isolates are reported as plant colonizers and several researches have been undertaken for the genetic diversity of this pink microbial community. Molecular markers such as RFLP (Restriction fragment length polymorphism), RAPD (Restriction analysis of polymorphic DNA) and ARDRA (Amplified ribosomal DNA restriction analysis) are strong molecular fingerprinting technique. These techniques are allowing us to discriminate distinct microbial communities that are closely related or distant.

In this investigation, we have examined and explored the genetic diversity of PPFM bacteria in the leaf area of five different kharif crop plants such as rice, millet, maize, mung bean and urad bean to consider the heterogeneity of PPFMs associated with specific plant phyllosphere and across five different crop plants through PGP screening and ARDRA profiling.

## MATERIALS AND METHODS

### (i) Isolation of PPFMs

The pink pigmented methylotrophic bacteria were isolated using Ammonium Mineral Salts (AMS) medium supplemented with 0.5% (v/v) filter-sterilized methanol[11] from the leaf surface of rice, millet, maize, mung bean and urad bean cultivars raised for this investigation from the agricultural plots near Gwalior region, MP, India. There was random selection of five plants of each crop at 30 days after sowing and leaves were collected in sterile bags and PPFMs were isolated by leaf-imprinting technique[11]. Imprinted fused colonies were separated by spread plate method while PPFM isolates were purified on AMS plates by streak plate method.

### (ii) Screening of Strains for PGPR activities

### Phosphate solubilisation

To observe the phosphate solubilising ability of the isolates, Pikovskaya's medium[12] containing tri-calcium phosphate was prepared. Pure colonies of the PPFM isolates were spot inoculated to the Pikovskaya's medium plates and incubated at 30±2°C for 4-6 days. Clearing or 221oil221ate221ation zones were observed around the colonies. The clear zone of solubilisation shows the plant growth promoting activity of the pink pigmented facultative methylotrophic bacteria obtained from the leaf phyllosphere.

### Siderophore production

The method of Schwyn and Neiland[13] was applied for the detection of siderophores production by the PPFM isolates. Siderophores, the iron chelating compounds are beneficial for the plants. PPFM isolates were spotted on to CAS (Chrome azurol S) agar plates and incubated at 30±2°C for 3-4 days. Around the bacterial growth, orange halo zone was observed. The efficiency of siderophore production was observed qualitatively and designated with plus (+) sign from 1 to 4 depending on their efficiency.

**Ammonia production**

Ammonia is a volatile substance produced by many rhizobacteria that is toxic to fungi. To study the production of ammonia, bacterial isolates were grown in peptone water broth and incubated at 28°C for four days. The accumulation of ammonia was detected by addition of one ml of Nessler's reagent to each tube. A faint yellow color indicated small amount of ammonia, deep yellow to brownish color indicated maximum production of ammonia. Qualitatively the isolates were designated with plus (+) sign depending on their efficiency to produce ammonia.

**Indole Acetic Acid (IAA) production**

Ability of PPFM isolates for the production of IAA by rhizobacteria was estimated according to the method of[14.] The bacterial cultures were grown in TY broth and 1mL broth culture of each isolate was centrifuged at 10,000 rpm for 20 minutes. The supernatant was transferred to Glass tubes and pellet was used for protein estimation. To the supernatant (if alkaline) two drops of orthophosphoric acid were added followed by 4 ml of reagent (a). The tubes were incubated for 25 minutes at room temperature. The absorbance was recorded using a spectrophotometer at 530 nm. Auxin concentration values were determined by preparing standard curve using standard IAA, 0-100 µg $ml^{-1}$ and expressed as µg IAA $mg^{-1}$ protein.

**(iii) Extraction of genomic DNA**

PPFM isolates obtained from different phyllosphere under study were selected for the molecular characterization. The genomic DNA extraction method was as described by Pospiech and Neumann[15]. Inoculated broth of pure cultures were centrifuged, supernatant discarded and the pelleted cells were washed twice with TE buffer and resuspended in 0.5 ml in SET buffer (75mM NaCl, 25mM EDTA and 20 mM Tris). The cell suspension was incubated with 10 µL (10 mg/mL) of lysozyme solution and kept at 37°C for 1hr. The following reagents were then added: 10 µL of sodium dodecyl 222oil222ate (10%), and 10 µL of proteinase K (10 mg/mL). The reaction mixture was incubated at 55°C for 2 hr in water bath to lyse the cells. To the mixture, 150 µL NaCl (5M), equal volume of water saturated phenol and Chloroform: Isoamyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 10 min. The aqueous layer was transferred to fresh tube. To the aqueous layer 0.1 volumes of 3M sodium acetate and 2 volumes of chilled 95% ethanol was added and kept at 4 °C for 30 minutes. The precipitated DNA was pelleted by centrifugation at 12,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol and the pellet was dried by keeping at 37 °C for 10 min. The DNA was then dissolved in 50µL of milli Q water and stored at 4 °C for further analysis.

**(iv) 16S rRNA gene Amplification**

For the amplification of 16S rDNA primers pA (5'AGAGTTTGATCCTGGCTCAG3') and pH (5'AAGGAGGTGATCCAGCCGCA3') were used to obtain approximately 1500-bp fragment from the amplification of 16S rDNA[16]. The 100µL PCR reaction mixture contains 50-90ng DNA template, 1X Taq buffer, 0.2 mM each of deoxyribonucleoside triphosphate (dNTP) mixture, 10 pmol of each primers, 1.5 mM $MgCl_2$, and 2 U of Taq DNA polymerase (Bangalore Genei, India). The PCR reaction was performed in a thermocycler (Bio-Rad) using the following conditions: initial denaturation of 5 min at 94°C, followed by 40 cycles consisting of 40 s at 94°C (denaturation), 40 s at 50°C (annealing) and 1 min 30 s at 72°C (Primer extension) and a final extension period of 7 min at 72°C. The PCR reaction mixture and the

amplification conditions were same as described above. After amplification the PCR product was resolved by electrophoresis in 1.2% agarose gel stained with ethidium bromide and visualized on a gel documentation system (Alpha-Imager) and gel images were digitalized.

### (v) ARDRA (Amplified ribosomal DNA restriction analysis)

Molecular chronometer 16S rRNA gene amplification was performed in a thermocycler (Eppendorf Master cycler, German) with a 25 µl reaction mixture containing 50 ng of genomic DNA, 0.2 mM of each dNTP, 1 µM of each primer[17] 2.5 mM of MgCl2, and 1 U of Taq DNA polymerase (Bangalore Genei, India) and the buffer supplied with the enzyme. The PCR conditions were those described earlier[17]. Approximately 1 µg of PCR-amplified 16S rDNA fragments were restricted with endonucleases HaeIII, PstI, and Sau3AI (Fermentas, USA) separately at 37°C for overnight and resolved by electrophoresis in 3.5% metaphor agarose gels. Banding patterns were visualized by ethidium bromide staining and documented in AlphaImager TM1200 documentation and analysis system. Strong and clear bands were scored for similarity and clustering analysis using the software, NTSYS-PC2 package (Numerical taxonomy analysis program package, Exeter software, USA). Similarity among the strains was calculated by Jaccard's coefficient[18] and dendrogram was constructed using UPGMA method[19].

## RESULTS

### Methylotrophic population in phyllosphere

From the phyllosphere of five different crop plants of Kharif season, a total 114 isolates were isolated on AMS medium. Dorsal leaf surface of the crop plants were selected for the isolation of PPFMs on the selective media supplemented with methanol as a source of carbon substrate. Pure cultures of PPFMs obtained on this specific media were selected on the basis of various morphotypes. The leaf impression technique revealed that bacteria colonize primarily the leaf veins. All the isolates were from the plants grown with same agronomic management and from same location. Out of 114 phyllosphere isolates, 21 were from rice, 20 from millet, 25 from maize, 28 from mung bean, and 20 from urad bean. Isolates growing on AMS medium had the capability to produce pink pigments over the media.

### Screening based on PGP (Plant growth promoting) activities

PPFM isolates were further screened on the basis of different plant growth promoting tests such as siderophore production, ammonia production, phosphate solubilisation and IAA (Indol acetic acid) production. Out of 114 isolates growing on AMS media plates, a total 40 isolates were selected having multiple plant growth promoting activities. Isolate no. 6 (strain S1), 21 (strain S8), 30 (strain S13), 37 (strain S18), 57 (strain S27) and 67 (strain S29) were observed positive for all four PGP activities test while other isolates having also multiple plant growth promoting attributes (Table1). These screening results a number of potent pink pigmented facultative methylotrophic bacteria obtained from phyllosphere habitat.

**Table 1. Plant growth promoting activities of different PPFM isolates showing multiple attributes.**

| Strains | Isolate no. | Phosphatase | Siderophore | IAA production (µg/ml) | $NH_3$ Production |
|---|---|---|---|---|---|
| S1 | 6 | + | ++ | 48.12 | + |
| S2 | 8 | + | +++ | 83.34 | - |
| S3 | 9 | + | ++++ | 83.01 | - |
| S4 | 12 | - | + | ND | + |
| S5 | 13 | - | + | 53.41 | + |
| S6 | 16 | - | ++ | 72.56 | - |
| S7 | 18 | + | + | 47.21 | - |
| S8 | 21 | + | + | 35.56 | + |
| S9 | 23 | + | ++ | ND | + |
| S10 | 25 | + | - | 87.23 | + |
| S11 | 27 | + | - | 67.43 | + |
| S12 | 29 | + | + | ND | + |
| S13 | 30 | + | ++ | 54.98 | + |
| S14 | 31 | + | - | 87.45 | + |
| S15 | 33 | + | + | ND | + |
| S16 | 34 | - | +++ | ND | + |
| S17 | 35 | - | +++ | ND | + |
| S18 | 37 | + | ++++ | 111.45 | + |
| S19 | 42 | + | + | 88.36 | + |
| S20 | 44 | - | ++ | 76.56 | + |
| S21 | 45 | - | + | 49.03 | + |
| S22 | 49 | - | + | 48.67 | + |
| S23 | 51 | + | - | 89.32 | + |
| S24 | 53 | - | + | 86.45 | - |
| S25 | 54 | + | + | ND | - |

| | | | | | |
|---|---|---|---|---|---|
| S26 | 55 | + | + | 65.76 | - |
| S27 | 57 | + | ++++ | 48.36 | + |
| S28 | 59 | + | + | 59.29 | - |
| S29 | 67 | + | + | 48.56 | + |
| S30 | 69 | + | + | 84.49 | - |
| S31 | 75 | + | ++++ | 93.48 | - |
| S32 | 78 | + | - | 85.39 | + |
| S33 | 80 | + | + | ND | + |
| S34 | 91 | + | - | ND | + |
| S35 | 99 | + | - | ND | + |
| S36 | 101 | + | + | 86.39 | - |
| S37 | 102 | + | ++ | ND | - |
| S38 | 106 | - | + | 61.43 | + |
| S39 | 109 | - | - | 84.77 | + |
| S40 | 111 | - | + | 59.49 | - |

*****Efficiency of siderophore production **(+,++,+++** and **++++),** ND: not detected

**16S rRNA gene amplification**

Genomic DNA from all screened 40 PPFM isolates was used as template for the detection of 16S rRNA gene fragments which were amplified using PCR. All of the isolates were giving an amplification of 1.5 kb amplified gene product (fig.1). The amplified product was purified by Genei Quick PCR purification kit and run on 1.5% agarose gel electrophoresis to see the authenticity of the amplified product.

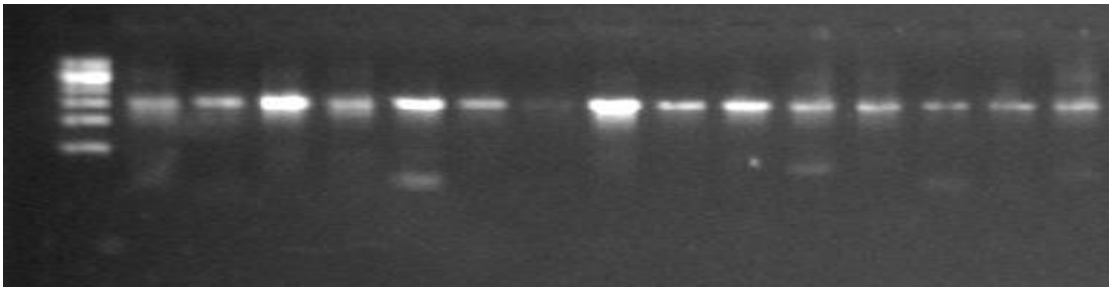

**Fig.1** 16S rRNA gene PCR product from PPFM isolates S1 to S15.

**ARDRA**

Restriction digestion of 16S rRNA gene using three endonucleases (HaeIII and EcoRI) has yielded three to six distinct restriction patterns for each enzyme (fig.2) About two to ten restricted fragments of varying

sizes were found in each of the restriction patterns. Cluster analysis of combined 16S rDNA restriction patterns based on Jaccard's similarity index grouped all the 114 isolates under four distinct groups: I, II, III, and IV. Majority of the isolates were under group I (52.5% of total isolates), and the remaining isolates shared the groups II, III, and IV as 10%, 30%, and 7.5%, respectively (fig.3). Cluster I comprised 21 isolates of rice, maize and urad bean. ARDRA cluster II comprised 4 isolates from mung bean while cluster III and IV had maize and millet isolates.

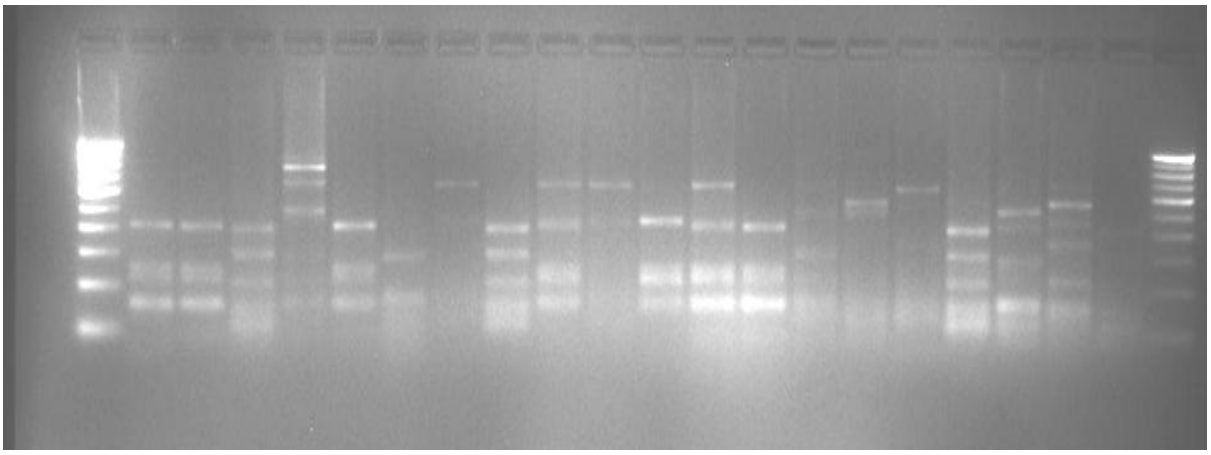

**Fig.2** ARDRA pattern of the isolates (A1 to A20) after restriction analysis of ribosomal DNA

## DISCUSSION

In present study, ribotyping method ARDRA along with plant growth promotion ability pattern were applied for studying the genetic diversity of Methylobacterium in phyllosphere of rice, millet, maize, mung bean and urad bean. At the level of physiology and taxonomy, aerobic methylotrophs are considered as a diverse subpopulation of bacterial community having multiple attributes of plant growth promotion [20,4], specifically and more especially in the phyllosphere region where they are present abundantly[21]. In the present study, various morphotypes of the PPFMs were obtained based on culture dependent approach (methanol supplemented Ammonium mineral salt media) and amplification of the 16S rRNA gene, ARDRA fingerprinting clustered and characterized a number of diversified PPFMs affiliated to genus Methylobacterium. Our results showed the presence of four distinct groups of PPFMs in crop plants distributed (Fig 3).

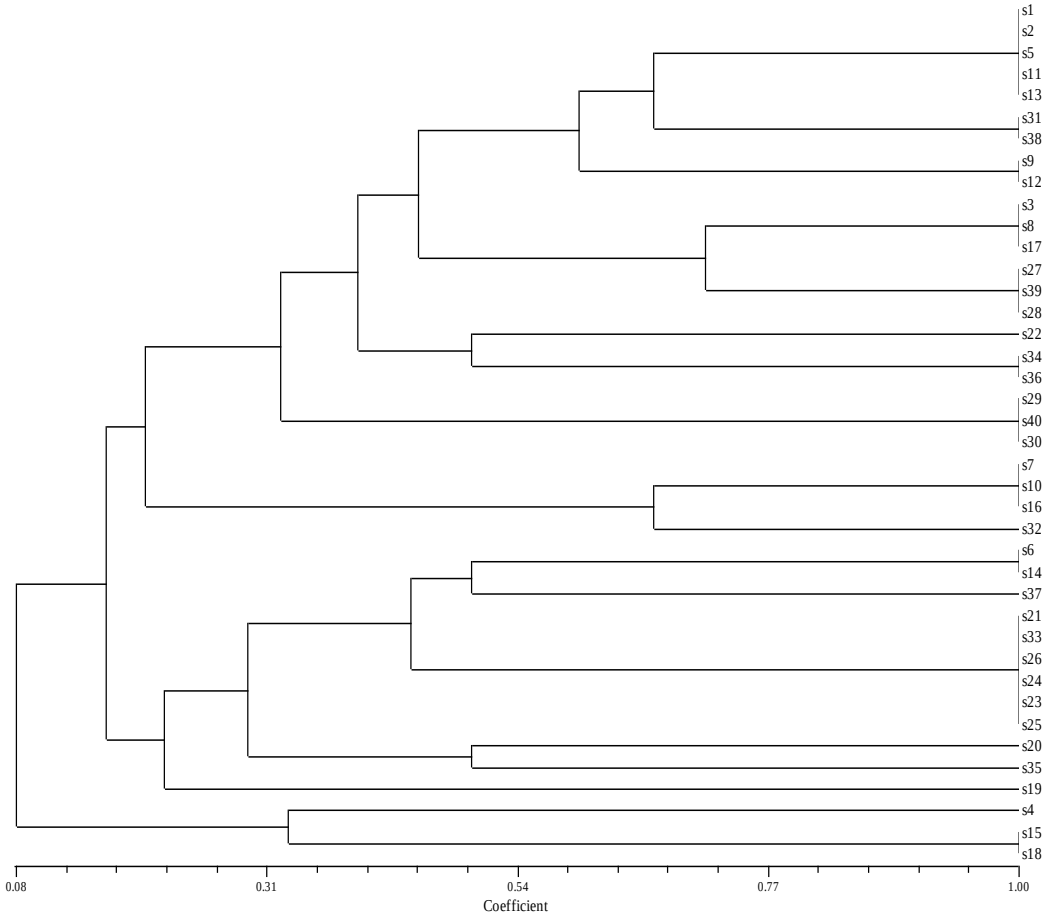


**Fig.3** Dendrogram constructed based on the banding pattern obtained after ARDRA, showing % similarity and relatedness of the PPFM isolates.

This subpopulation of methylotrophic bacteria have different potentials for plant growth promotion in many crop plants, enhancing seed germination[22,23,24,6,25,26,7] with the production of plant growth hormones such as IAA and cytokinins[4]. Our results illustrated the variety of pink pigmented facultative methylobacteria having different morphological appearance with different plant growth ability.

The classification and identification up to species and strain level, 16S rRNA gene sequence analyses will be utilized further for this diverse group of methylobacteria. Since these PPFMs have different plant growth promoting abilities, can be exploited for their application in agricultural field as bioinoculant in form of foliar spray or in solid form. Along with application in field, they can be utilized as a potent foliar spray against phytopathogens associated with the plants. The genetic diversity of cultivable pink population of methylobacteria from different crop plants is an approach to explore plant growth promoting phyllosphere inhabiting methylobacteria based on fingerprinting technique like ARDRA. In a study metabolic profiling along with genetic diversity was better illustrated for this special group of microbes[27]. Further extension of research including 16S rRNA gene sequence analysis, metagenomic approach and phylogeny based on these conserved sequences will provide an insight and exploration of these identified PPFM phytosymbionts and their evolutionary relatedness in future.

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# Growing of S*taphylococcus aureus* cells with soil components enhances virulence in mice caused by soft tissue infections.

**PRATISTHA DWIVEDI* AND RAJESH SINGH TOMAR**

Amity Institute of Biotechnology, Amity University, Madhya Pradesh.

* Corresponding author email: pdwivedi@gwa.amity.edu

## ABSTRACT

*Staphylococcus aureus* is an important human pathogen, whose occurrence is not environmental restricted. It causes skin and soft tissue infections, muscle tissue infections, gangrene, sepsis and several other diseases in the human. This organism is predominantly present in health care facilities but it has been reported to cause the infections via soil and other environment. For many more other Gram positive as well as Gram negative pathogens have abode in the soil. There they are well tolerant to the harsh environment. Also, soil is responsible for the invasion and infection of pathogen. Here in this paper, we have described the role of soil in facilitating virulence of bacteria in the host by lethality assay in mice using live cells from several strains of *S. aureus* grown in media alone and media enriched with sand (mimics wound infection by soil). Two lethal doses of total eight strains (seven environmental and one type strain) were used to test lethality in mice. Early death in animals with the injected with culture grown in sand enriched media suggested an invasive response of bacteria in presence of soil.

## KEYWORDS

Soil, lethal, pathogen, *Staphylococcus.*

## INTRODUCTION

*Staphylococcus aureus* is the human bacterial pathogen and it causes of skin and soft tissue infections (SSTIs), endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, foreign-body infections, and sepsis.[1] The organism was once can be isolated largely from hospitals, environments related to health care and those patients availing these facilities frequently. Strains were found to be rapidly disseminated among the general population in majority of the areas of the United States and it has been observed that it can infect patients with and without exposure to the health care environment. Curiously, large reservoirs of isolates now affecting patients through other environment including soil beside health care facilities.[2] Moreover, this condition is leading to implication of existing controlling methods of this organism unsuccessful, so there is urgent need to evaluate virulence factors from the environment other than health care facilities in order to control its spread and invasion.

*S. aureus* is also present in the soil, on human skin, and in the other environment. Thus, the causative agents of Staphylococcal infections are not environmentally restricted. Even operating theaters can be habitats for infecting organisms.[3]

With the advancement in the adequate medical facility, the incidence of sepsis and other infections caused by this organism has reduced. Several virulence factors are thought to be evolved in the harsh and diverse soil environment that may promote gangrenous and muscle infection in the host. Thus, extensive study on soil exposure to the muscle tissues and many other infections can improve understanding towards pathogenesis of this microorganism. In this paper we describe the effect of soil on the progression of the skin and soft tissue infection and sepsis in animal models by performing lethality assay.

# MATERIALS AND METHODS

### Bacterial Strains

*Staphylococcus aureus* strain ATCC 12600 was obtained from Becton–Dickinson India Pvt. Ltd., India, and was grown under aerobic conditions. Seven other environmental strains of *S. aureus* were isolated from different regions of India.

### Growth conditions

All the seven environmental strains and one type strain of *Staphylococcus aureus* were grown under aerobic conditions at 37°C with shaking at 250 rpm in Todd-Hewitt broth containing heart infusion (dehydrated), 3.1 g; yeast enriched peptone, 20 g; dextrose, 2 g; sodium carbonate, 2.5 g; sodium chloride, 2 g; L-cystine, 0.5 g; disodium phosphate, 0.4 g and distilled water, 1000 ml. One set of Todd-Hewitt broth media was supplemented with processed sand particles anaerobically prior to sterilization. All the strains were also grown in Todd-Hewitt broth enriched with sand particles.

### Preparation of processed sand particle

Coarse sand is collected, washed, dried and filtered by soil sieve of different mesh sizes. Fine texture of sand is collected in a broad mouthed vessel and oven sterilized (dry sterilization). This processed sand is further used for enriching Todd-Hewitt media.

### Lethality Assay

Animal experiments were approved by the Animal Ethical Committee at institutional level and all efforts were made to minimize suffering of mice. Relative virulence of different strains of *S. aureus* was determined in terms of time to death in BALB/c mice by intraperitoneal injection of washed cells grown in Todd-Hewitt media alone and Todd-Hewitt media supplemented with sand ($2 \times 10^4$ and $2 \times 10^6$ CFU) in a volume of 0.1 ml phosphate buffer saline and survival was periodically observed. One set of inoculum was supplemented with sand particles to provide loci of infection. Mice in triplicate were used for each dose of cells and mean values of time to death were calculated.

# RESULTS

Total 43 strains of *S. aureus* were isolated from different regions of India preferably from the soil. Of them, 13 strains were found to cause sepsis in animal models (data not shown). Seven environmental strain are tested here in this paper for their capability to induce sepsis and skin and soft tissue infections (SSTIs) followed by lethality in mice. Isolated strains of *S. aureus* that are screened here for lethality assay are shown in table 1.

| S. No. | Strain | Infection | colonies/ gram soil |
|---|---|---|---|
| 1. | UD02-1 | Sepsis | 20 |
| 2. | UD02-8 | SSTI | 10 |
| 3. | KO08-2 | Sepsis | 55 |
| 4. | RA13-2 | Sepsis | 23 |
| 5. | IN05-2 | Sepsis | 41 |
| 6. | IN08-2 | SSTI | 15 |
| 7. | KO08-2 | SSTI | 21 |

Mouse lethality assay by total eight strains of *S. aureus*, using culture grown in media with sand and without sand, showed a significant difference in virulence of mice which is observable by time to death. Mice that were injected with the culture grown in media enriched with sand are having very less value of time to death shown in Figure 2 compare to the mice that were given lethal dose of culture grown in media alone (Figure 1). KO08-2 and IN08-2 were the strains that showed a marked difference between two conditions. These finding demonstrated the role of soil in the skin and soft tissue infection of *S. aureus*. With our results, it is clearly shown that soil provides loci of infection and the media supplemented with sand was well mimicking wound contamination with soil.

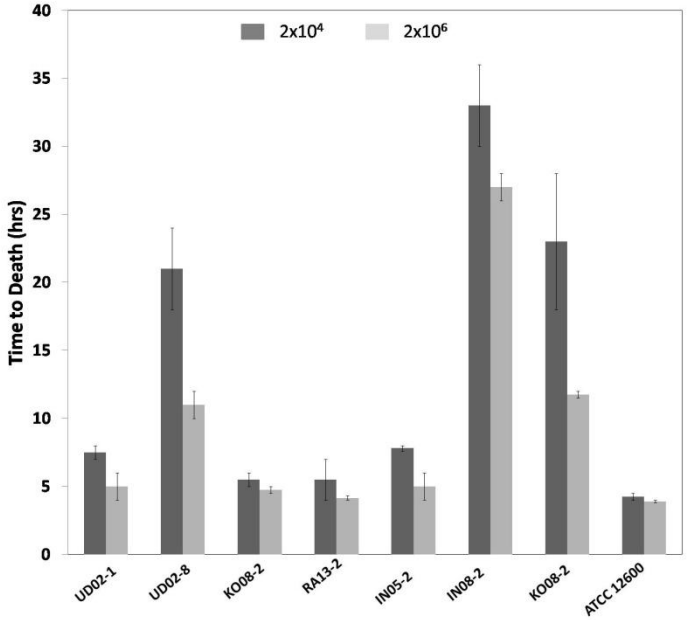

**Figure 1:** Lethality assay of 8 strains of *S. aureus* on mice using two doses ($2x10^4$ and $2x10^6$ cfu/ml) of active cultures grown in Todd-Hewitt media alone.

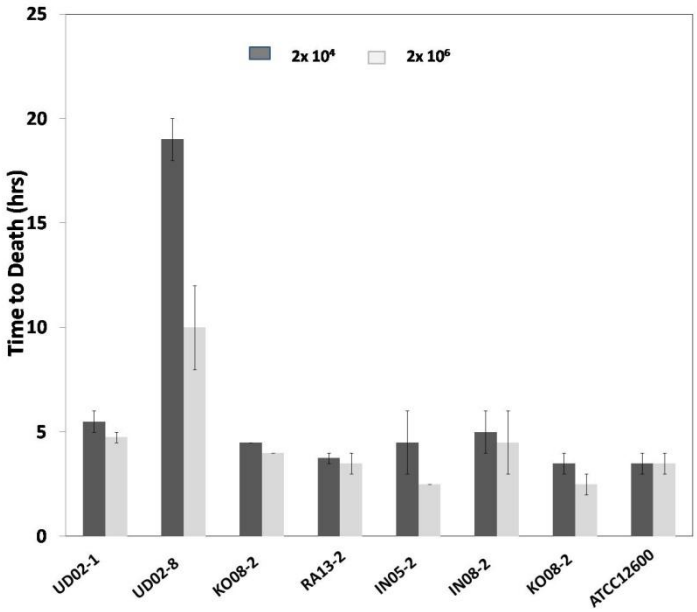


**Figure 2:** Lethality assay of 8 strains of *S. aureus* on mice using two doses ($2x10^4$ and $1.2x10^6$ cfu/ml) of active cultures grown in Todd-Hewitt media supplemented with sand.

## DISCUSSION

Significant consideration is given to food and water related infections. However, reason unknown, very little attention is given to a wide variety of soil-related infections, includes wound, respiratory tract, or gastrointestinal infections. Soil is a multilayered surface complex of mineral and organic (humus) constituents present in solid, liquid, and gaseous states. Pathogens may be indigenous or enter the soil through deliberate introduction (for purposes of biological control) or indirectly by animal deposits, manure application, or from flooding with sewage or contaminated water.[4,5]

Strategies that bacteria acquire to survive this in-soil competition may, in turn, provide the ability to infect animals. Horizontal gene transfers and the occurrence of pathogenicity islands (blocks of acquired DNA encoding for multiple virulence factors) may further allow the emergence of opportunistic pathogens from these microenvironments. Pathogenic fungi or bacteria may enter humans via direct inoculation into wounds. Soil minerals introduced simultaneously may promote infection by suppressing local host defenses.[5]

SSTIs (Skin and soft tissue infections) caused by *S. aureus* strains are the most common clinical manifestations. Furthermore, these infections are recurrent in nature and the factors associated with their recurrence are not clearly known. It is general observation that after first infection of SSTI, patients tend to

lose their immunity but mechanism behind this process is largely unknown. There is possibility of reinfection due to acquisition of virulence associated factors from environmental contamination.[6]

*Bacillus cereus* gastroenteritis is an important food-borne disease worldwide. Pathogenicity is aided by a variety of toxins. Emetic toxin (a peptide) induces nausea and vomiting a few hours after ingesting a meal contaminated with the toxin. Spores germinate within an insect or animal host or on contact with organic matter, entering the soil via the droppings of an animal host or upon the host's death. Saprophytic growth in soil, including transition from a single cell to a multicellular form, then ensues. Cells and spores may then contaminate plant material and enter food processing areas.[7,8] *Listeria monocytogenes* causes an estimated 1591 cases of gastroenteritis in normal humans per year in the United States,[9] in addition to meningitis and focal infections in the immunocompromised and serious infections in pregnant women and babies. It is ubiquitous in soil and other material such as sewage, silage, groundwater, and vegetation. Thus, some cases of listeriosis may involve infection via the soil.

Similarly, it has been hypothesized that soil may serve as a source of many other bacterial infections like *Campylobacter*[10], *E. coli* 0157[11], *Burkholderia pseudomallei*[12], *Legionella*[13], and mycobacterial infections.[14] Finally, soil-acquired primary cutaneous *Nocardia* infection may be seen after trauma in gardeners and other outdoor or agricultural workers.[15]

In our study, the use of sand particles in Todd-Hewitt media has efficiently contributed in the growth of *S. aureus* cells in numbers high enough to exert sufficient virulence for inducing lethality in mice. Comparison of the lethality assay on mice against several strains of *S. aureus* cells grown under Todd-Hewitt media alone and Todd-Hewitt media supplemented with sand was showing differential virulence in mice of almost all strains used for the study. The cultures were more lethal when grown in the media supplemented with sand warrants a detailed analysis and validation with enough biological and technical replicates with reference to the factors in the soil that participate in enhancing virulence caused by *S. aureus* infections.

## CONCLUSION

A variety of bacterial and fungal microorganisms are capable of departing a soil environment to cause serious focal or systemic infection. Specific evolved virulence factors or the ability to grow in diverse, sometimes harsh, microenvironments may promote human infection. Our data show that soil offers *Staphylococcus aureus* pathogen a new and healthy environment for thriving and producing infection. Many more other pathogen gets a suitable environment for them to cause pathogenesis so questions regarding travel and soil exposure by various routes should be further included during diagnosis and treatment of pathogens. Additionally, the mechanism and in depth study of virulence determinants present in the soil should be required for elucidation of disease progression.

## ACKNOWLEDGEMENT

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# Antimicrobial Efficacy of Leaf Extract of Some Medicinal Plants against *Staphylococcus aureus*

**SURENDRA SINGH PARIHAR[1*], RAJ NARAYANGUPTA[2], RAJESH SINGH TOMAR[1]**

[1]Amity Institute of Biotechnology, Amity University Madhya Pradesh, Gwalior, India
[2]Department of Biochemistry, All India Institute of Medical Sciences (AIIMS) Bhopal (M.P.) India

* Corresponding author email ssparihar@gwa.amity.edu

## ABSTRACT

*Ipomeafistulosa, Thivetianerrifolia & Ghaopatta* leaves were analyzed for capability of antibacterial activity by preparing their aqueous and ethanol extracts against gram-positive bacteria (*Staphylococcusaureus*). Observation of disc diffusion method revealed that the pattern of prohibition depends upon the plants part, solvent used for extraction and the organism tested.Ethanol extracts showing potent antibacterial activity as compared to aqueous extracts. Ethanolic extract of *Ghaopatta* were observe more sensitive as compared to *Ipomea fistulosa & Thivetia nerrifolia.* This study concluded that *Ghaopatta* leaf extract contain the potentially active antibacterial agents and may also use to isolate the refined molecules for treatment for scavenging the infectious agents like *S. Aureus*.

## KEYWORDS

*Ipomea fistulosa, Thivetia nerrifolia & Ghaopatta,* Antibacterial activity, Drug resistance, Sterile Disc,

## INTRODUCTION

Medicinal value of plants is important to human life. Twenty thousand medicinal plants were found in all over the world. In India 15 percent are available out of 20,000. Various climatic zones of the country have about 90% of the medicinal plants.[1]. It is Reported that 70 to 80% of the people depended in traditional medicine or herbal medicines.[2&3]Thousand species of bacteria, fungi, animals and plants were identified in india.[4]Synthetic drugs are not only expensive but it is harmful of human life for the treatment of diseases. Therefore, Now a days is required to develop new Herbal drugs to control Microbial infection and also used as therapeutic agents.[5,6&7]Naturally occur compounds are safer than synthetic compounds.[8]More world population (three quarters) are depends on plants and plant extracts for health care.[9] The disease caused by common pathogens can be cured by herbal medicines.[10] Experiments on the use of plant compounds against microbes were first recognized in the late 19th century.[11]Naturally occurring compounds play an important role for the treatment of different microbial infections.[12]

The world has growing great interest in scientific and commercialization of medicinal plants and plant-based products mainly due to their immense economic potential and widespread cultural acceptability.[13] Plant extracts have great potential as antimicrobial compounds, especially in the treatment of infectious diseases caused by resistant microorganisms.[14]The medicinal actions of plants are unique for particular

plant species consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct.[15]
The plant extracts have been used for centuries as a popular method for treating several health disorder and great significance in therapeutic treatments and helpful to cure the problem of multidrug resistant microorganism.

1. ***Ipomoea fistulosa***

   It is also known as besharam. It belongs to the family convovulaceae. It is worldwide distributed, being found in Latin America from Colombia to Paraguay, Bolivia, and Argentina. It is also found in Africa and India. It is found in large groups at the rivers and lake shores. It is a bush with a height up to 3m, few ramified, thick stems, fistulous finely striated. Its flowers are light violet colored, grouped in bunches of 2-3 units, with very light colors.

2. ***Thevetia neriifolia***

   *Thevetia neriifolia* is a small tree having glossy dark green leaf and fragrant saffron yellow to orange or peach colored flowers. It is used medicinally in Philippine Islands, Guiana, Brazil and Gold Coast. *T. neriifolia* is used to treat various inflammatory and cardiovascular diseases, beside the antiviral and antifungal properties Generally, *T. neriifolia* leaf are applied in cardiac disorder, fever, ringworms, and measles treatment.

3. ***Ghaopatta***

   It has thick green leaves which are used in healing of wound by people of tribal and most of the rural areas in Indian population.

## MATERIALS & METHODS

### Collection of Raw materials

Fresh plants of *Ipomea fistulosa, Thivetia nerrifolia, & Ghao patta*, were collected in month of April. These plants were identified and confirmed by the Botanical taxonomist, in Gwalior. Then these plants were washed thoroughly 2- 3 times with running tape water and then sterile water and kept at room temperature in departmental laboratory for 10 days for drying. After drying, all parts were separated from plants. The dried parts of plants were pulverized into powdered form with the help of pestle and mortar. The powder of all plants is preserved in the sterile boxes for further processing and in making extraction.

### Test Microorganism

The bacterial strains were further selected for the present study, collected from Microbial Type Culture Collection (MTCC), Chandigarh, India. Gram positive Bacteria, namely *Staphylococcus aureus* was screened for present investigation. Bacterial culture was grown in nutrient agar slants at 37 °C. Bacteria was revive prior to susceptibility testing by transferring them into a separate test tube containing nutrient broth and incubated overnight at 37°C.

### Chemical & reagents

The chemicals and media used for this study were from Hi Media, Qualigens and Merck and the glass wares from Borosil.

### Extract Preparation

### Aqueous Extract

Weigh the 10gm of dried plants leaf material were crushed in motor pestle and then it was dissolved in 100 ml sterile distilled water in a warring blender for 10 minutes. The macerated were first filtered through double layered muslin cloth. Then this extract was centrifuged at 4000g for 30 minutes. Supernatant was filtered through whatman no.1 filter paper and sterilized at 121°c for 30 minutes. The extract was preserved aseptically in a brown bottle at 5°C until further use.[16]

### Ethanol Extract

Weigh the shade, dried plants leaf sample (10gm) in conical flasks with cotton plug which containing 100 ml of 95% ethanol solvent. Flask was kept in shaking incubator for overnight shaking at 35 °C at 110 rpm. After overnight shaking, filtration was done with the help of what man no. 1 filter paper into another clean conical flask. After it, filtrate was subjected to centrifugation at 7000 rpm at18 °C for 15 minutes. The supernatant of the leaves were collected into a separate clean air tight bottles and were stored in a refrigerator at 4 °C for further antimicrobial testing.[16]

### Antibacterial Assay

In-vitro bacterial test was carried out by Disc Diffusion method. Tetracycline antibiotic discs were used as positive controls while corresponding solvent was used as Negative Control. The discs of tetracycline were prepared by impregnating disc with 5mg/ml concentration and then left for air drying. Then disc of the plants samples were made by impregnating the discs with extraction of each sample and left for air drying. The discs for negative control were prepared by impregnating the discs with respective solvents used to prepare extracts. After then bacterial cultures were taken by loop and were spread on Muller hinton agar plates with the help of glass spreader. Then discs of the samples, disc of positive control and discs of negative control were placed on seeded agar plates. The inoculated plates were incubated at 37°c for 24 hours and antibacterial activities were calculated and evaluated by measuring the zone of inhibition against the tested bacteria.

## RESULTS AND DISCUSSION

Sample extracts of the plants leafwere used and tested against Gram positive Bacteria (*Staphylococcus aureus)***.** These extracts were aqueous and ethanol extracts of leaf of *Ipomea fistulosa, Thivetia nerrifolia, & Ghaopatta.* Ethanol extract of *Ghaopatta* plants leaf shows good antimicrobial activity against *Staphylococcus aureus* then compare to *Ipomea fistulosa & Thivetia nerrifolia.* The zone of inhibition is shown in Figure 1, 2 & 3 and Table 1. These studies reveals that the use of ethanol solvent in the preparation of plant extracts provides more consistent antibacterial activity while aqueous extract of *Ipomea fistulosa, Thivetia nerrifolia, &Ghaopatta* showed no significant activity against the microorganism. The extract nature and mode of action of the active constituents is quite obscure at this stage. Further work may however reveal whether these components act as intracellular bacterial enzyme inhibitor or impair the cell wall synthesizing system of the cell, or any other biological reaction destruction which causes inhibition of bacterial growth.[17] These results clearly indicates that the polarity of antibacterial compounds make them more readily extracted by ethanol solvent.

**Table 1: Effect of Ethanolic and Aqueous extracts of Medicinal Plants on *Staphylococcus aureus***

| Name of Plants / Control | Zone of inhibition of Ethanol Extract (In MM) | Zone of inhibition of Aqueous Extract (In MM) |
|---|---|---|
| Tetracycline (Positive Control) | 35 | 35 |
| Negative Control | - | - |
| Ipomoea Fistulosa | 10 | - |
| ThevetiaNeriifolia | 10 | - |
| Ghaopatta | 13.5 | - |

**Fig:1. Zone of inhibition of ethanol extract of *Ipomoea fistulosa* against *Staphylococcus aureus***

**Fig:2. Zone of inhibition of ethanol extract of *Thevetia neriifolia* against *Staphylococcus aureus***

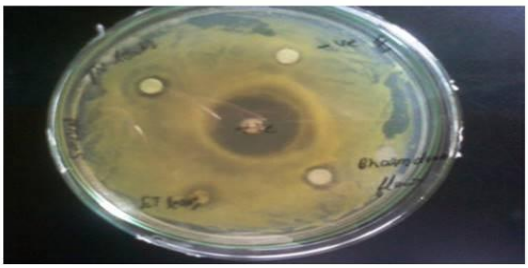
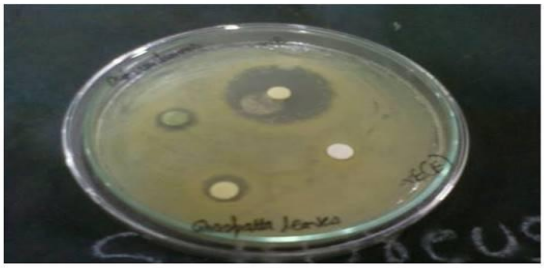


**Fig:3. Zone of inhibition of ethanol extract of *Ghaopatta* against *Staphylococcus aureus***



## CONCLUSION

The present study demonstrates the antibacterial potential of crude extracts of *Ipomea fistulosa, Thivetia nerrifolia, & Ghaopatta* leaves. The results indicate that Ethanol extracts of *Ghaopatta* have significant antibacterial potential against the bacterial strains of clinical significance and observe the traditional medicinal value of the plant. The study concludes that the plants reserved biologically active substances. The extracts obtained by the plant can potentially be used in the treatment of infectious diseases caused by microorganisms that are showing emergence of resistance to currently available antibiotics. These studies also support the folkloric usage of the studied plants and suggest with antibacterial agents in new drugs for the therapy of infectious diseases caused by microbial pathogens.

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