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QUANTITATIVE EVALUATION OF PYTOCOMPOUNDS FROM *TERMINALIA CHEBULA* BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) METHOD AND ITS ANTIBIOFILM ACTIVITY

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ABSTRACT

The efficacy of phytocompounds such as tannic acid (TA) and ellagic acid (EA) from dried fruits of *Terminalia chebula* Retz against Multiple Drug Resistant Uropathogenic *Escherichia coli* (MDRUPEC) by using High Performance Thin Layer Chromatography (HPTLC) and by Confocal Laser Scanning Microscopy (CLSM) techniques. The effectiveness of these phytoconstituents against biofilm formation has been studied in which methanol extract of *T.chebula* have shown best activity against biofilm forming organisms compared to other standard compounds. Due its paramount action, the qualitative and quantitative evaluation of its phytocompounds was made by using HPTLC analysis. Simultaneously, anti-biofilm activity of these phytocompounds have also be evaluated against MDRUPEC with CLSM technique. The present research has shown that, amongst an assortment of concentrations of the plant extracts used, 40µg/ml has exhibit best antibacterial activity against the test strains. The evaluation of phytocompounds such as Gallic Acid (GA), Tannic Acid (TA) and Ellagic Acid (EA) were quantitatively estimated 279.42 ng (nanogram), 16.13 µg (microgram) and 22.00 µg respectively by using HPTLC method. This newer approach have formed a frame work for development of new phytochemical therapeutic agents for the effective treatment of various alarming diseases of human population by the drug resistant microbes.

KEYWORDS: ***pathogenesis, drug resistance, phytocompounds, biofilm formation***

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INTRODUCTION

Medicinal plants are the rich source of developed secondary metabolites which are potential remedies for different ailments in human beings. Due to the indiscriminate use of antimicrobials, the pathogens develop resistance which leads to its treatment very difficult. Hence, plant compounds are widely accepted due to the perception that they are safe and they have a long history of use in folk medicine as immune boosters and for the prevention and treatment of several diseases. The discovery of bioactive compounds from plant origin offers an attractive approach to control infectious diseases in a safety margin and lesser costs. *T. chebula* is called as 'King of Medicine' in Tibet and is always listed at the top of the list in Ayurvedic Materia Medica due to its extra ordinary power of healing[1] and it possess numerous pharmacological activities due to the presence of tannins, chebulic acid, glycosides, sugar, triterpenoids, steroids and good quantity of phosphoric acid [2]. Uropathogenic *Escherichia coli* (UPEC), the primary causative agent of urinary tract infections (UTIs), have been presumed to be a predominantly extracellular pathogen. Biofilm formation by uropathogenic *E.coli* is a known virulence factor [3]. Fimbriae were probably one of the factors which contribute to form biofilm on the surface. Various structures such as flagella, fimbriae, outer membrane proteins, curli and extra cellular polymeric matrix are involved in biofilm formation[4].The main factor contributing to microbial resistance is the biofilm formation by the microbes. The use of traditional medicinal plants for primary health care has steadily increased worldwide in recent years. Currently, out of 80 % of pharmaceuticals derived from plants, very few are now being used as anti microbial. Plants are rich in a wide variety of secondary metabolites that have found anti microbial properties [5]. Current research relies on HPTLC, is becoming a routine analytical technique due to its advantages of low operating cost, high sample through put and need for minimum sample clean up. The major advantage is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis [6]. HPTLC chromatogram pattern comparison seems to be promising for finger printing the active compounds in plant extracts.TLC and HPTLC are methods commonly applied for the identification, the assay and the testing for purity, stability, dissolution or content uniformity of raw materials (herbal, and animal extracts, fermentation mixtures, drugs and excipients) and formulated products (pharmaceuticals, cosmetics, nutrients)[7]. These flexible and cost effective techniques present the advantage of the simultaneous processing of standards and samples with versatile detection possibilities, including great variety of post – chromatographic derivatization reagents. Due to several advantages, such as the rapidity, the fewer amounts of sample, and an extremely limited solvent waste, HPTLC has gained widespread interest as a favorable technique for the determination of pharmacologically interesting compounds in biological matrices, such as plants, leaves and flowers and herbal formulations. Recently, HPTLC has been widely employed for the quantification of secondary metabolites[8]. Based on these backgrounds, this first research has been focused on the determination of antibiofilm efficacy of phytocompounds from *T.chebula,* as an effective therapeutic potential for the mankind.

MATERIALS AND METHODS

Instrumentation

Analysis was performed by using

- Camag HPTLC (High Performance Thin Layer Chromatography) system equipped with a sample applicator Linomat V, twin trough development chamber (10x10) size, TLC Scanner III, and Win CATS integration software.

Reagents and Chemicals

Analytical grade of Toluene, Ethyl acetate, Methanol, and Formic acid, were obtained from SD fine chem. Ltd, Mumbai. The standard Tannic acid (TA) and Ellagic acid (EA) were received from Sigma Aldrich Pvt. Ltd, Chennai. The accessories of pre coated TLC aluminium sheets silica gel F_{254} (10 x10 cm, 0.2 mm thick) plates, microbiological media and antibiotic discs were procured from Hi-Media, Mumbai.

Collection of plant material

The dried fruits of *Terminalia chebula* (Haritaki) were collected from the Siddha Medical centre, situated at Erode, Tamilnadu and received the certificate of authentication from Botanical Survey of India (BSI), Tamil Nadu Agricultural University (TNAU) campus, Coimbatore, Tamil Nadu to confirm its botanical properties. From this, a voucher specimen was preserved using a standard procedure for future reference. The dried fruits were then processed to obtain fruit pulp and crushed well it to make a fine powder form by using a sterile mixer grinder. The powder was stored in a closed vessel for future analytical works.

Preparation of plant extracts [1]

Methanol extract preparation

Weighed about 500 gm of coarsely powdered plant material and soaked in methanol. From this, the extract was separated by using Soxhlet apparatus and it was again reconstituted by adding 5% Dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/ml.

Bacterial pathogen used

The antibacterial activity of methanol extract of *T.chebula* was checked against various strains of Multiple Drug Resistant (MDR) Uropathogenic *Escherichia coli* (UPEC). The resistant uropathogens of *Escherichia coli* were obtained from the Department of Microbiology, Tiruppur Kumaran College for Women, Tiruppur, who were isolated and identified on the basis their drug resistant profile. The results of this experiment were assessed by using the reference strain of *Escherichia coli* 25922 which purchased from Microbial Type Culture Collection Center (MTCC), Chandigarh, India.

Antibacterial activity of T.chebula

The methanol extracts of *T.chebula* with different concentrations were screened for their antibacterial

activity by using agar disc diffusion method [9]. The bacterial cultures were calibrated in accordance with McFarland's turbidity standard method. From this, 20 µl of the bacterial culture was spread on the sterile Mueller Hinton agar (MHA) plates. On this, the sterile discs containing different concentrations (10, 20, 30, 40 and 50µg) of methanol extracts of *T.chebula* were placed, and the zone of inhibition were scaled using a standard procedure. The antibiotics of DMSO and Gentamycin were used as reference negative and positive controls respectively. All these plates in triplicates were then incubated at 37^{0}C for 24 hours. After incubation, the zone of inhibition was measured and recorded.

***Phytochemical screening*[10]**

The incidence of various phyto constituents were analyzed from methanol extracts of *T.chebula* includes phenols by ferric chloride test, alkaloids by Mayer's test, flavanoids by alkaline reagent test, carbohydrates by Fehling's test, terpenoids by Knollar's test, saponins by foam test and tannins by Folin Ciocalteu method.

Quantitative evaluation of certain phytocompounds by HPTLC

Preparation of standard solutions

Taken 10 mg of Tannic acid and Ellagic acid in 10 ml volumetric flask, and it was then made up to 10ml with methanol (1mg/ mL^{-1}). From this dilution, 1ml was pipette out and diluted again up to 10 ml to obtain the final concentration of 100 µg /ml.

Preparation of sample (methanolic extract)

Taken 1 gm of the methanolic extract of *T.chebula*) in 10 ml volumetric flask, and dissolved in 5 ml of methanol. The mixture was mixed well and filtered by Whatmann No 1 filter paper and it was again made up to the mark with methanol.

***Development of HPTLC technique* [11]**

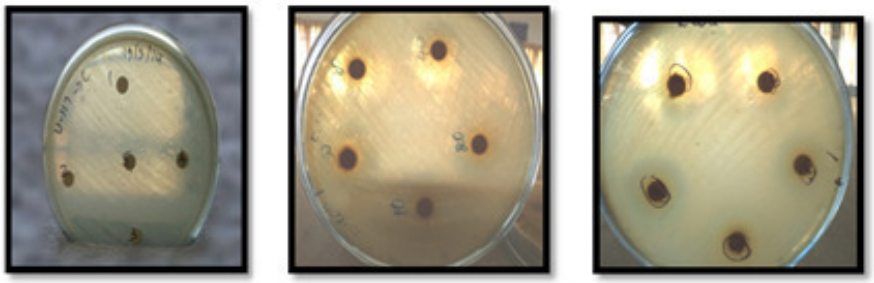
The samples of methanolic extracts of *T.chebula* and the standards of TA, GA and EA were spotted individually 10mm from the bottom on a pre-coated TLC aluminium sheets silica gel F_{254} (10x10cm, 0.2mm thickness) plates. The mobile phase used was Toluene: Ethyl acetate: Formic acid: Methanol (4.3:4.3:1.2:0.3 v/v/v/v). The sampled TLC plates were kept in a saturated Camag twin trough chamber for 15min. After development, the TLC plates were dried in hot air oven at 60^{0}C for 5 minutes and scanned by using CAMAG TLC Scanner III with absorbance at 254nm and operated by Win CATS software 4.03 versions.

Detection of Antibiofilm Activity of T.chebula

Different conventional methods such as Congo red agar method [12], Tube adherence method[13] and Tissue Culture Plate method [14] were performed for detection of antibiofilm activity of *T.chebula* against biofilm forming bacterial pathogens.

RESULTS AND DISCUSSION

In the present study, an interaction between the natural phytomedicines and Multiple Drug Resistant Uropathogenic *Escherichia coli* (MDRUPEC) strains were evaluated. Currently, developing resistance to different antibacterial agents by bacterial pathogens is recognized as a major global public health problem. Continuous use of *Terminalia chebula* in ayuverdic traditional medicine may be due to their potential in producing the diversity of secondary metabolites against wide range of diseases. Thus, antibacterial activities of *T.chebula* were observed against MDRUPEC, in which 40µg/ml of the extract showed a clear zone (figure – I). Similar results have been made by many researchers[1].

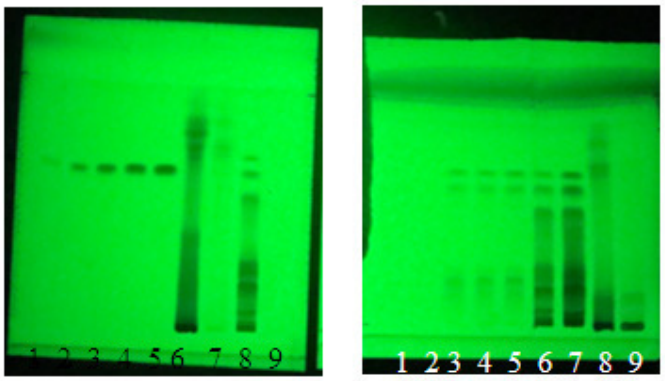


a) Control (DMSO) b) MDRUPEC strain 1 c) MDRUPEC strain 2

Figure 1

Shows Antibacterial Activity of Methanolic extract of T.chebula against MDRUPEC strains by disc diffusion method

Among the results, it was observed that the strains *E.coli* would lose their activity at the concentration of 40µg/ml of the concentrate of *T.chebula.* In this study, use of methanol to extract *T.chebula* may be due to its nature of enhancing the activity of *T.chebula* metabolites such as alkaloids, flavanoids, sterols and tannins *etc*.,[15-18], and increased a greater number of active constituents responsible for antibacterial activity. The results of HPTLC analysis exhibited that the high incidence of specific phytocompounds such as Tannic acid and Ellagic acid from *T.chebula* as portrayed in table I and figure – II.



a) Tannic acid *b) Ellagic acid*

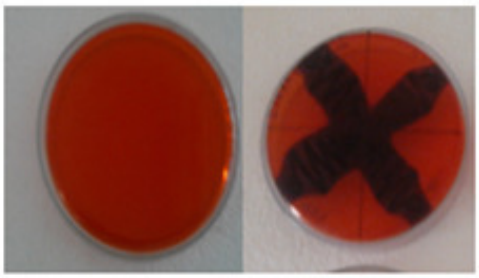
Figure 2
Incidence of phytocompounds on HPTLC at 254 nm

Figure 2 showed that, lane 1 to 5 indicated the increasing concentration of respective standard phytocompounds (1, 3, 6, 9 and12µl). The lane 6 denoted (12 µl) methanolic extract of *T.chebula* and similarly lane 7 to 9 denoted unknown compounds. Ellagic acid was appeared as 4 distinguished bands due its robustness. The results on HPTLC exhibited that the various chromatograms of the samples which required for further purification using various instrumental methods and this can be interpreted in other research paper.

Table 1
Recovery of specific phytocompounds from T.chebula by HPTLC method

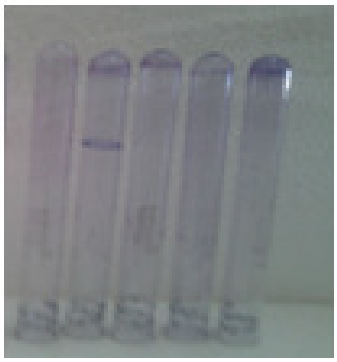
S.No	Name of the compounds	Rf values	Area	Recovery	Yield % (w/w)
1	Tannic Acid	0.78	5103.82	16.13 µg	5.3
2	Ellagic Acid	0.63	23818.41	22.00 µg	7.3

The Rf values of used standards such as Tannic Acid and Ellagic Acid were 0.78 and 0.63. The peak areas 5250.32 and17109.96 were represented the incidence of Tannic Acid (20 µg) and Ellagic Acid (20µg) respectively. The result also indicated that the amount of TA and EA in methanolic extract of *T.chebula* were 5.3 % (w/w) and 7.3 % (w/w) respectively (Table 1). In this analytical study, instrument built WINCAT software was used in order to observe the specific principles of bioactive phytocompounds. Of many compounds, tannic acid, propyl gallate and methyl gallate, were found to be effective inhibitor to the growth of many intestinal bacteria including *Escherichia coli* (ATCC 25922)[1]. It is found that tannic acid has a greater relative binding efficiency to iron than propyl gallate, methyl gallate or gallic acid[1]. The inhibitory effect of tannic acid to the growth of intestinal bacteria may be due to the strong iron binding capacity, whereas the effect of propyl gallate and methyl gallate probably occurs by a different mechanism[19]. Comparatively, tannic acid possesses a greater relative binding efficiency to iron than gallic acid. Tannic acid may act like a siderophore to chelate iron from the medium and make it unavailable to microorganisms. Microorganisms growing under aerobic conditions need iron for a variety of functions, including reduction of the ribonucleotide precursor of DNA, formation of haem, and other essential purposes. In previous research study[20] reported that the inhibitory effect of tannic acid on the growth of intestinal bacteria may be caused by its strong iron-binding capacity. Recently[21] reported the multidrug resistant uropathogen's efflux - pump inhibition by their fruit extract compound gallotannin, 1, 2, 6 – tri ortho galloyl – beta – D – glucopyranose. The present investigation on antibiofilm activity of various phytocompounds exhibited that the methanolic extract of *T.chebula* extended its greater inhibition activity against MDRUPEC. The activity of biofilm eradication were recorded as methanol extract < ellagic acid < tannic acid as depicted in fig. 3

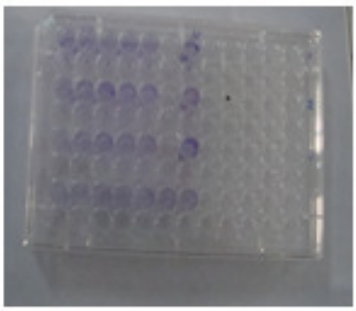


Control ***biofilm producers***

a) Growth on Congo red agar



b) tube adherence test



c) TCP method

Figure 3
Biofilm formation

The antibiofilm activity due to combination of phyto constituents in the methanolic extract of *T.chebula* are enlisted in Table 2.

Table 2
Phytochemical screening of dried fruit of methanolic extracts of T.chebula

S.No	Phytoconstitutents	Tests Used	Significance
1	Phenolics/ tannins	Ferric chloride / Folin ciocalteu method	+++
2	Alkaloids	Mayer's test	+++
3	Flavonoids	Shinoda test	+++
4	Carbohydrates	Fehling's test	++
5	Saponins	Foam test	-
6	Terpenoids	Knollar's test	++

Note: '+++' indicates copiously present: '++' indicates moderately present: '-' indicates absent.

Due to the presence of various pharmacological activities and phytochemicals of this extract and some of the identified compounds of this plant as well as their toxic effects in a bid to highlighting the importance of this untapped resource in the fight against the human diseases.

CONCLUSION

This explorative research finding has form a frame work for the development of novel therapeutics from the natural phyto medicines by using HPTLC, in a cost effective, reliable and flexible way to evaluate the ingredients of plant materials in a systematic approach. In addition the inhibition of biofilm formation by various pathogens has also been effectively carried out in a more updated analysis of structural changes by using CLSM analysis. Further studies envisioned to high light the detailed study on specific identification of individual compounds from the plant origin and its elaborate inhibitory mechanism on bacterial cells.

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CONFLICT OF INTEREST

Conflict of interest declared none

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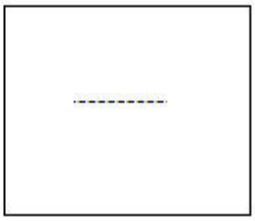

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