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PHYTOCHEMICAL SCREENING, ISOLATION, ANTIBACTERIAL AND ANTICANCER ACTIVITY STUDIESOF *CAESALPINIA PULCHERRIMA LINN* LEAVES BY HPTLC ANALYSIS

ANJU.V*[1] AND SUBIN MARY ZACHARIAH[2]

[1]Assistant Professor, Department of Pharmaceutical Chemistry, Pushpagiri College of Pharmacy, Thiruvalla, Kerala.
[2]Associate Professor, Department of Pharmaceutical Chemistry and Analysis, Amrita School of Pharmacy, Amrita Vishwa Vidyapeetham , Amrita University, India

ABSTRACT

Caesalpinia pulcherrima Linn commonly known as peacock flower belonging to family *Caesalpiniaceae* is an ornamental plant widely distributed in Asia. The plant and its parts are widely used in traditional medicine for the treatment of various disorders. The aim of the work was to study the pharmacological activities of the leaf extract and isolated fractions. Preliminary phytochemical analysis was carried out to determine the constituents present in different extracts of plant. The methanolic extract showed maximum active constituents and their quantitative determination were carried out and matches with their standards. The TLC and HPTLC analysis of the methanolic extract for determination of major phytoconstituents were carried out. Further isolation of the total flavanoid and phenolic fraction were done using suitable solvent systems. The methanolic extract and isolated fractions were tested for their antibacterial, invitro antioxidant and anticancer activities. The antibacterial activity was carried out using agar well diffusion method and the MIC was determined using broth dilution method. The invitro antioxidant assay were carried out by different methods. The invitro anticancer activity of the extract and fractions were carried out in HepG2 cell line using MTT assay .The results showed that *Caesalpinia pulcherrima* leaf constituents showed good anticancer activity.

KEYWORDS:**Cytotoxicity, antibacterial, antioxidant, phytoconstituents, secondarymetabolites**

ANJU.V

Assistant Professor, Department of Pharmaceutical Chemistry,
Pushpagiri College of Pharmacy, Thiruvalla, Kerala.

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INTRODUCTION

Phytochemistry is the branch of chemistry which deals with the chemicals derived from the plants. They are naturally occurring chemical compounds from plants that are responsible for their organoleptic properties**,** biosynthesis, metabolism, normal distribution and biological functions [1].The study of phytochemicals or secondary metabolites become important since they exhibit a number of protective functions like attack against insects [2,3]. The determinations of the major phytoconstituents in plants are important since they can serve the quality control purpose and elucidation of their therapeutic mechanism[4].The purpose of the study was to extend the use of herbal medications and to study their positives and negatives to treat health conditions. Thus the secondary plant metabolites also called phytochemicals which are earlier found to have an unknown pharmacological action are extensively used as a source of medicinal agents[5,6]. Herbal medicines are becoming one of the mainstream as improvements in analysis and quality control along with clinical research signifies the value of herbal medicines in treating and preventing disease [7].There is a positive role for botanicals in combination with conventional treatments in oncology. This may be due to the clinical data supporting a potentially positive role for botanicals in combination with the conventional treatment methods[8].Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. An interaction between traditional medicine and modern biotechnological tools represents a vast potential resource to be established towards New Drug development. The interface between cell biology, in vitro assays and structural chemistry will be the best way forward to obtain valuable leads. In recent years, focus on plant research has increased all over the world. As with all areas of phytomedicine, the value of medicinal plants lies in the potential access to extremely complex molecular structures that would be difficult to synthesize in the laboratory. Medicinal plants continue to be subject to extensive screening worldwide [9].*Caesalpinia pulcherrima* is a tropical perennial shrub or small tree in the pea family that can reach 15-20 ft in height, though typically 8-12 ft tall in cultivation. It is evergreen in the warmer tropics, and semi-deciduous to deciduous in sub-tropical regions. A fast-growing bush with low branches in an open and spreading habit, growing as far and wide as its height is 4-6 ft (1.2- 1.8m) 6-8ft (1.8-2.4m). The trunk and branches are usually armed with few scattered spines. They have large fern-like or feathery leaves on petioles up to 2 ft long. They are alternate at wide intervals, bi pinnately arranged with 4-8pairs of pinnas bearing simple and smooth 6-10 pairs of obovate leaflets. The leaflets are stalkless with 7-11 pairs. The flowers that it bears are born in racemes upto 20 cm long, in single colour of red or orange to scarlet. It also produces flat and green seed pods about 6-12 cm long abundantly, emerging along the bottom of the inflorescence that quickly darken to brown when ripe and containing shiny brown flat beans which can be used for propagation [10].Peacock flower is believed to be native to tropics and sub-tropics of America and West Indies and widely distributed in Asian countries. The leaves are used as a purgative and the different parts of this herbal plant have been used in common remedies for treatment of a number of disorders including pyrexia, menoxenia, wheezing, bronchitis and malarial infection fever, jaundice, colic, flatulence and malignant tumours. Decoction of roots were used for fevers. The infusion of bark was used for washing teeth and gums. Flower extract were found to possess anti-inflammatory activity. Leaf extracts were used for renal stones, against bronchitis, fever, skin ailments etc. Fruits have astringent and used for diarrhoea and dysentery. Seeds have activity against chest pain and breathing difficulty. Roots induce abortion in the first trimester [11, 12].Various components have been identified and isolated from the various parts of the plant. Diterpenoids, isovouacapenol C, pulcherrimin A in root. Stems contain peltogynoids, bhonducellin and 6-methoxypulcherrimin, homoisoflavonoids. Flowers showed the presence of lupeol, B-sitosterol, flavonoids, and myricetin. The leaves contain hydrocyanic acid, tannins, and benzoic acid. The bark contains terpenoid that gives antimicrobial and cytotoxic activities. Plant is used as an emmenagogue, purgative, stimulant, and abortificient, also used in bronchitis, asthma, malarial fever. 5,7-dimethoxyflavanone,5,7- dimethoxy- 3′,4′ methylenedioxyflavanone , isobonducellin , 2′-hydroxy-2,3,4′,6′ tetramethoxychalcone and bonducellin [13]. One new homoisoflavonoid, (3E)-2,3-dihydro-6,7-dimethoxy-3(3-hydroxy-4-methoxyphenyl)methylene- 4H-1-benzopyran-4-one and four naturally new analogues,(3E)-3-(1,3-benzodioxol-5-ylmethylene)- 2,3-dihydro-7-hydroxy-4H-1-benzopyran-4-one, (3E)-3-(1,3-benzodioxol-5ylmethylene)-2,3-dihydro-7- methoxy-4H-1-benzopyran-4-one,(3E)-2,3-dihydro-7- hydroxy-3-(3-hydroxy-4 -methoxyphenyl) methylene- 4H-1-benzopyran-4-oneand(3E)-2,3- dihydro-3-(3,4dimethoxyphenyl)methylene-7- methoxy-4H-1-benzopyran-4-one,along with four knownhomoisoflavonoids, bonducellin, sappanone A, 2'-methoxybonducellin and 7-Omethylbonducellinwere isolated from the aerial parts of *Caesalpinia pulcherrima*. Study of the stems isolated a cassane-type diterpene ester, pulcherralpin. Medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry.Plants have been a common source of medicaments, either in the form of traditional preparation or as pure active principles. The plant or plant parts found to be ornamental are also good medicines and can be used to cure several diseases and disorders. The plant extract have been used in folkloric medicinal practices for the treatment of various ailments. The constituents present in the plants can be separated by various methods. Modern techniques like HPLC, HPTLC etc are a suitable way for the separation. The separated constituent having specific activity can be isolated and purified. They can be can be further utilized for determining specific activity study [15, 16]. The aim of the work was to perform the phytochemical screening of different extracts of plant for identification of chemical constituents, standardization of plant based on WHO guidelines, quantitative determination of the constituents present in the plant extract, determining the HPTLC profile of the screened constituents, isolation of total flavonoids and phenolic compounds from methanol extract, antimicrobial activity of the methanolic extract and isolated fractions, In vitro antioxidant activity of the methanolic extract, In vitro anticancer studies on the

methanolic, hydro alcoholic extract and isolated components.

MATERIALS AND METHODS

Collection of material
The leaves of *Caesalpinia pulcherrima Linn* were collected from the Kooroppada village areas of Kottayam district and the plant was authentified by Dr V.J.Dominic H.O.D, Dept of Botany S.H. college Thevara, Kochi. The leaves were washed with water dried in shade and stored.

Extraction Procedure
The leaves were washed, cleaned and dried under shade and grinded in a blender and passed through sieve size of uniform porosity.100g of the powdered plant material were successively extracted with 500 ml of dichloromethane, methanol, hydro alcohol and water in the order of increasing polarity for 72 hrs using Soxhlet apparatus. The extracts were concentrated under reduced pressure using a rotary evaporator. The percentage yields of various extracts were noted and found to be 5.004%, 21.39%,11. 801, 10% successively.

Phytochemical Screening
A small quantity of the various extracts i.e.; dichloromethane, methanol, hydro alcoholic and aqueous extracts were taken for the qualitative chemical test.

Alkaloids
The extracts were treated with few drops of Dil HCl and filtered. The filtrate was tested with the following reagents:

Mayer's Test
The extracts were dissolved in Conc .HCl and filtered. A few drops of the solution are poured into the centre of a watch glass. Mayer's reagent is added in drops to the sides of the watch glass with the help of a glass rod. Formation of gelatinous white precipitate at the junction of two liquids shows the presence of alkaloids.

Dragendroff's Reagent
The extract was dissolved in methanol and few drops of Dragendrorff's reagent are added. Orange red precipitate shows the presence of alkaloids.

Hager's Test
To the extract add few drops of Hager's reagent (saturated solution of picric acid) gives a yellow precipitate.

Wagner's Test
To the extract add few drops of Wagner's reagent (iodine in potassium iodide) gives a reddish brown precipitate**.**

Glycosides
The extracts were hydrolysed with HCl for few hrs in a water bath and the hydrolysate was subjected to various tests.

Legal's Test
To the hydrolysate, 1 ml of pyridine and few ml of sodium nitroprusside solution were added and then it was made alkaline with NaOH solution. Appearance of pink/red colour.

Borntrager's Test
The hydrolysate was treated with chloroform and the chloroform layer was separated .To this equal quantity of dil.ammonia solution was added. Ammoniacal layer shows rose-pink colour.

Flavanoids
Extract was treated with few drops of aq. NaOH solution. A blue to violet colour was observed, yellow colour (flavones), yellow to orange (flavanones)

Extract was treated with conc. Sulphuric acid, yellowish to orange

Shinoda Test
The extract was dissolved in alcohol, to that pieces of Mg were added followed by Conc: HCl drop wise and heated. Presence of magenta colour shows the presence of
flavanoids.

Carbohydrates
Molisch Test
To 2-3 ml of extract add Molisch reagent (α-naphthol in alcohol), Shake well and add conc.sulphuric acid from the sides of the test tube. Violet ring at the junctions of two liquids were obtained.

Reducing Sugar
Fehling's Test
Mixed 1 ml of Fehling's A and Fehling's B reagent and boiled for 1 min.Add equal volume of test solution. Heated in a boiling water bath for 5-10 min. First a yellow colour is obtained followed by a red precipitate.

Benedict 's test
Mixed equal volume of Benedict's reagent and test solution in test tube. Heated in a boiling water bath for 5-10 min. Solution appears green, yellow or red depending upon the reducing sugar present.

Barfoed's Test
Mixed equal volume of barfoed's reagent and test solution. Heated for 1-2min in boiling water bath and cooled. Red precipitate was obtained.

Tannins
Small quantities of various extracts were taken separately in water and tested for the presence of tannins
a) Extract was treated with dilute FeCl3 solution. A blue-dark green or violet colour was obtained.
b) Extract was treated with 10 % lead acetate solution. A white precipitate was obtained.

Terpenoids
The extract was treated with 1 ml of dihydrophenyl hydrazine dissolved in 100 ml of 2M HCl. A yellow-orange colour indicates the presence of terpenoids.

Saponins
The extract was diluted with 20 ml of distilled water and

it was agitated on a graduated cylinder for 15 min. The formation of 1 cm foam shows the presence of saponins. [17,18]

Standardisation

The standardization was carried out to determine whether the plant or plant parts correspond tovarious parameters in the WHO guidelines. Each plant constituents vary depending upon that these evaluation parameters show the results. The major standardization techniques involve determination of their macroscopic and microscopic features, determination of ash value, Bitterness value, Swelling and foaming index, Determination of Haemolytic activity, Moisture content etc [19, 20]

Organoleptic Characters

Colour: Outer-dark green in colour
Inner-light green in colour
Odour and Taste: Characteristic
Length and Width: 15-25 mm and 10-15 mm
Shape: Ovate
Surface: Glabrous

Microscopic characteristics

The transverse section of leaves shows the presence of lamina and midrib. The lamina exhibits upper and lower epidermis with wavy walls. The epidermis is covered by a cuticle and it consists of an anamocytic stomata. The mesophyll tissue comprises of the palisade and spongy parenchyma. The palisade cells are columnar cells except in the mid rib region. The mid rib consist of arc shaped vascular bundles enclosed by pericyclic fibres. The vascular bundles consist of xylem and phloem. The xylem consists of lignified fibres covered by non-lignified fibres. The phloem consist of multilayered thick walled collenchymatous cells present above the lower epidermis in the midrib region. The non-lignified fibres consist of calcium oxalate crystals[21].

High performance thin layer chromatography (HPTLC)

High performance thin layer chromatography [HPTLC] method is an enhanced form of TLC and is the suitable method for estimation of chemical constituents present in plant materials HPTLC analysis of all the phytoconstituents like alkaloids, flavanoids, phenolic compounds, saponins and tannins were carried out. The program used was WINCATS and the device is Linomat 4. [22]

Test solution

1 mg of methanol extract was dissolved in 25 ml methanol and used for HPTLC analysis

Flavanoids

Solvent system**:** - Ethyl acetate: Water: Formic acid: Acetic acid (10:0.5:1.1:1.1)
Derivatizing Reagent **:-** Anisaldehyde-sulphuric acid

Phenolic Compounds

Solvent system**:**-Ethyl acetate: 1, 4-Dioxane: Formic acid: Water (5:3:1:1)
Derivatizing Reagent :- Fast blue

Saponins

Solvent system**:** - 1-Butanol: Water: Acetic acid (5:4:1)
Derivatizing Reagent**:-** Anisaldehyde- sulphuric acid

Alkaloids

Solvent system:- Toluene: Ethyl acetate: Diethyl amine (7:2:1)

Tannins

Solvent system**:** - Toluene: Ethyl acetate (98:2)

Procedure

The methanolic extract of the plant were spotted on a pre-coated TLC plate. Two spots each of 7 and 9 µl were spotted on the pre coated TLC plates (5×10 cm) after keeping the tank for a saturation period of 30 min. The plates were developed in the solvent system upto 90mm. After the development the plates were observed under 254 and 366 nm and after derivatization [23,24].

Quantitative determination

The quantitative determination were carried out to know the exact amount of active constituents present in the methanolic extract of leaf [25].

Quantitative determination of tannins

Procedure

Extraction of tannin:
0.5g powdered material + 75 ml H_2O+ Boil for 30 min + centrifuged at 2000 rpm-20 min. Made upto 100ml. Transferred 1 ml of sample extract + 75 ml water+ 5 ml folin-denis reagent + 10 ml Na_2CO_3 and dilute to 100ml with water+ Shaken well+ Absorbance measured at 700nm after 30 min. Prepared a blank with water instead of sample [26].

Quantitative determination of phenolic content

Procedure

Preparation of standard curve

A series of aliquots of standard gallic acid solution in a 10 ml standard flask + 400µl of Folin Ciocalteau Reagent (FCR) + volume made up with Na_2CO_3solution. After 2hr the suspension was centrifuged at 5000 rpm for 5 min+ Absorbance measured at 760nm against the reagent blank prepared in similar manner without gallic acid.

Preparation of Sample

10mg of plant extract + 10 ml of methanolic solution. The coloured complex was developed in the similar manner and absorbance was measured [27].

Quantitative determination of flavanoids

The total flavanoidal content was determined by Dowd's Method with some modifications

- 5 ml of 2% $AlCl_3$in hydro alcoholic solution + same volume of extract solution (0.4mg/ml)
- Absorption readings were taken at 415 nm after 10 min without AlCl3.
- The total flavanoid content was determined using a standard curve with quercetin standard (0-100µg/ml) [28].

Invitro antioxidant assay

Free radicals are highly reactive and unstable compounds produced in the body. Plants develop antioxidants that protect plants against damage caused

by active O2 formed due to exposure to U.V radiation

Reducing Power Assay:-
Preparation of standard ascorbic acid solution:-
50 mg of L-ascorbic acid into a 50 ml standard flask. Made up the volume to 50 ml using methanol. Concentrations of ascorbic acid solution (100-1000µg/ml) were prepared by taking 2,4,6,8 10ml from above solution and diluted to 10 ml using methanol.

Preparation of Sample solution
50mg of extract into a 50 ml standard flask. Made up the volume to 50 ml using methanol. Concentration of sample solution (100-1000µg/ml) were prepared by taking 2,4,6,8 ml from the above solution and diluted to 10 ml using methanol.

Procedure
0.5 ml of different concentration of sample and standard (100-1000µg/ml) + 2.5 ml of 0.2M phosphate buffer of PH 6.6 + 2.5 ml of 1% w/w potassium ferricyanide solution and kept in a water bath for 20 min at 50^0C. Cooled rapidly + 2.5 ml of 10% trichloro acetic acid and 0.5 ml of 0.1% of FeCl3 solution. Absorbance was taken at 700 nm after 10 min.[29]

DPPH radical scavenging assay
Different concentrations of solvent extracts and ascorbic acid (standard) namely 10, 20, 30, 40, 50 mcg/ml were prepared in methanol. DPPH (0.003% in methanol) was used as free radical. 1ml of different concentrations of solvent extracts + 3 ml of DPPH + incubated at room temperature in dark for 30 minutes. The optical density was measured at 517nm using UV-Visible Spectrophotometer. The degree of stable DPPH decolourization to DPPH (reduced form of DPPH) yellow indicated the scavenging efficiency of the extract. The scavenging activity of the extract against the stable DPPH was calculated using the following equation. DPPH was used as the positive control [30].

Scavenging activity (%) = A - B / A x 100

Where A = absorbance of DPPH
B = absorbance of DPPH and extract combination

Nitric oxide scavenging assay
0.5 ml of sample+ 0.5 ml phosphate buffer + 2 ml sodium nitroprusside solution(PH7.4).The mixture was incubated at 25^0C for 2.5hrs.0.5 ml of reaction mixture was pippeted+ 1 ml sulphanilic acid and allowed to stand for 5 min for complete diazotization+ 1 ml of 0.1 % N-naphthylethylene diamine dihydrochloride .Mixed and allowed to stand for 30 min to form pink coloured chromophore. The absorbance was then measured at 530 nm against the corresponding blank solution. The scavenging % or inhibition % or antioxidant activity was calculated.

Control
0.5 ml buffer +2 ml sodium nitroprusside were incubated .Add 1 ml sulphanilic acid +0.1% 1 ml naphthyl ethylene diamine dihydrochloride. Absorbance was taken at 530 nm .[31]

Hydrogen peroxide assay
Hydrogen peroxide when mixed with buffer solution get reduced to water and oxygen. Hydrogen peroxide solution (2 mM) was prepared with standard phosphate buffer (pH, 7.4). Extract samples (10,20,30,40,50µg/ml) in distilled water were added to hydrogen peroxide solution (0.6 ml).Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of both plant extracts and standard (L-ascorbic acid) compound was determined .[32]

Antimicrobial activity
Collection of Microorganisms
Stock cultures of 4 bacteria collected from NCIM, Mumbai were taken for performing the antimicrobial activity. The selected microorganisms are *Staphylococcus aureus, E-Coli, Pseudomonas aeuroginosa* and *Salmonella aboni* and are non-pathogenic in nature.

Preparation of Medium
The medium for the present study was Muller Hinton Agar .Suspended 9.5 gm of agar in 250 ml distilled water and the stock was prepared. It was heated to boiling for completely dissolving the medium. The medium was sterilized by autoclaving at 121^0 c for 15 min at 15 Lbs pressure. Broth or media was enriched with saline upto the consistency of starch. Sample organisms were inoculated into the saline and enriched for 1 hr and poured into a petri dish and solidified.

Antimicrobial activity screening by agar well diffusion method
The antimicrobial activity of the crude methanol extract, isolated flavanoids and isolated phenolic compounds were screened against 4 bacterial strains. The microbial activity was carried out using methanol as a control and various standard depending upon the organism i.e.(Amoxicillin for E-coli and *Staphylococcus aureus,* Ciprofloxacin for Pseudomonas and Bactrim for Salmonella). Wells were prepared by using a sterile cork borer of diameter 10mm and 100µl (To get the final concentration of 250 and 500 µg/well) of the test substance, standard antibiotic and the solvent control were added in each well separately. Inoculated the extract into the wells. The plates were placed at 40^0C for 1 hr to allow the diffusion of test solution into the medium and plates were incubated at a temperature optimal for the test organism and for a period of time sufficient for the growth of at least 10 to15 generations (usually 48 hours for fungi at 280C). The zone of inhibition and mean diameter of microbial growth around the well was measured in mm. For methanol extract only *Staphylococcus aureus* (zone of inhibition 15mm) and *Pseudomonas aeruginosa* (zone of inhibition 16 mm) showed activity. Isolated flavanoid fraction showed activity against *Staphylococcus aureus* (zone of inhibition 10 mm) and *E-coli*(zone of inhibition 8 mm).Isolated phenolic fraction showed activity against *Salmonella aboni* (zone of inhibition 7 mm)and *E-coli* (zone of inhibition 6 mm).[33,34]

Minimum inhibitory concentration
The Minimum Inhibitory Concentration (MIC) of the test

substances against *pseudomonas* and *staphylococcus* were determined by liquid broth method of serial dilution technique. For this assay, a series of assay tubes were prepared containing uniform volume of sterile nutrient broth and equal volume of known concentration of test substance was added. The test substance in the first tube was serially diluted in decreasing concentrations through the fifth tube and sixth tube as negative and seventh tube was left without test substance as positive control. The tubes with the test substance i.e. from one to seven were inoculated with 1 ml of inoculums (1x106 CFU per ml). The final concentration of test substance ranged from 100 to 10 µg/ml. Solvent control and sterility controls were maintained in the experiment. The tubes were incubated at 280^0C for 48 hr. The tubes were inspected visually to determine the growth of the organism as indicated by turbidity. In experimental terms the MIC is the concentration of the drug present in the last clear tube, i.e. in the tube having the lowest concentration in which growth is not observed [35].

Invitro cytotoxic activity

Cell lines and Culture medium

HepG2 (Human liver hepatocellular carcinoma cell line) was cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin(100 IU/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml) in an humidified atmosphere of 5% CO2 at 37□C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2culture flasks and all experiments were carried out in 96 microtitre plates

METHOD

Preparation of Test Solutions

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS (Fetal Bovine Serum) to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay

Principle

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x105 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 ml of different test concentrations of test drugs were added on to the partial monolayer in micro titre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24h interval. After 72 h, the drug solutions in the wells were discarded and 50 ml of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37o C in 5% CO2atmosphere. The supernatant was removed and 100 ml of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50%(CTC50) values is generated from the dose-response curves for each cell line. [36, 37]

$$\text{test group}\\ \%\ \text{Growth Inhibition} = 100 - \left\{ \frac{\text{Mean OD of individual test group}}{\text{Mean OD of Standard}} \right\}$$

RESULT AND DISCUSSION

Extraction

The extraction of Caesalpinia pulcherrima Linn Leaves (100 gm) was carried out successively with dichloromethane, methanol, hydro alcohol and water for 72 hrs. The extracts were concentrated in rotary vacuum evaporator and the % weight of extracts were noted

Table 1
Percentage yield of various extracts

EXTRACTS	% WEIGHT
Dichloromethane	5.004%
Methanol	21.39%
Hydro alcohol	11.801%
Water	10%

Standardisation

The standardization procedure for the plant material were carried out using WHO guidelines.
The total ash, bitterness value, swelling and foaming index, extractive value and haemolytic activity were determined.

Table 2
Standardisation Methods Observation

METHODS	OBSERVATION
Total Ash	19.75% w/w
Acid Insoluble Ash	1% w/w
Water Soluble Ash	3.7% w/w
Bitterness Value	960
Swelling Index	2.38cm
Foaming Index	100
Alcohol Soluble Extractive Value	0.21
Water Soluble Extractive Value	0.18
Total Haemolytic Activity	No haemolysis

Preliminary phytochemical screening

The preliminary phytochemical screening of all the extracts were carried out to determine the phytoconstituents present in the various extracts.The results reported the presence of flavanoids, phenolic compounds, terpenoids and tannins in the methanolic and hydroalcoholic extracts .All extracts showed the absence of alkaloids and reducing sugars.

Table 3
Phytochemical screening of various extracts of Caesalpinia pulcherrima

Tests	Dichloromethane Extract	Methanolic Extract	Hydroalcoholic Extract	Aqueous extract
ALKALOIDS				
a)Dragendorff's Test	–	+	–	–
b)Mayer's Test	–	+	+	+
c)Hager's Test	–	–	+	
d)Wagner's Test	–	+	+	–
GLYCOSIDES				
a)Legal's Test	–	+	–	+
b)Borntrager's Test	–	+	+	+
FLAVANOIDS				
a)Shinoda Test	–	+	+	–
b)NaOH	+		+	–
c)Conc H2SO4	–	+	–	–
TANNINS		+		
a)5% FeCl3	–		+	+
b)10% lead acetate	–	–	+	+
PHENOLICS				
a) Dil HNO3	–	+	+	–
b) Potassium dichromate	–	+	+	+
REDUCING SUGAR				
a)Fehling,s Test	–	+	+	+
b)Benedict's Test	–	+	–	+
c)Barfoed's Test	+	–	+	+
TERPENOIDS				
a)Salkowski Test	–	+	+	–
SAPONIN	+	+	+	+

Quantitative determination

The quantitative determination of the phytoconstituents like flavanoids, tannins and phenolic compounds were found out to determine their total content in the plant extract. This may further lead the work for isolation parameters

Figure 1
Caesalpinia pulcherrima Linn

Quantitative Estimation of Tannin

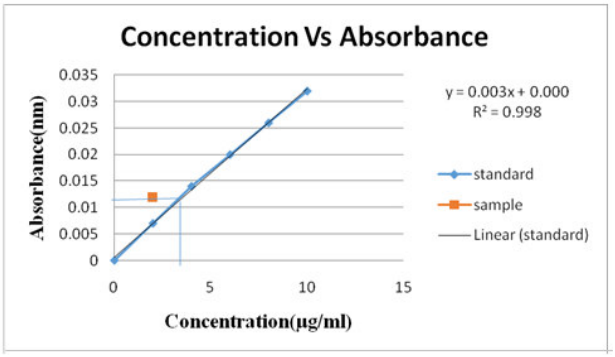

Figure 2
Quantitative Estimation of Tannin

Quantitative Estimation of Phenolic compounds

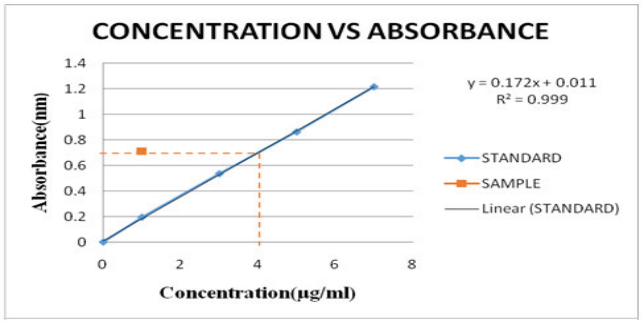

Figure 3
Quantitative Estimation of Phenolic compounds

The total phenolic compounds present in the sample were expressed as their gallic acid equivalence value.The concentration of phenolics present was 4 µg/ml equivalence of gallic acid.The R^2 value was found to be 0.999.

Quantitative Estimation of Flavanoids

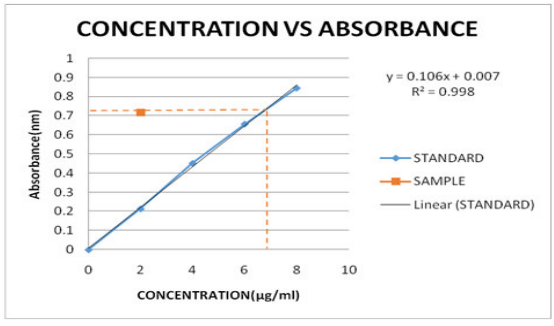

Figure 4
Quantitative Estimation of Flavanoids

The total flavanoids present in the methanolic extract of *Caesalpinia pulcherrima* was found to be 7 µg/ml equivalence of quercetin.

Phytochemical screening using HPTLC technique

The phytochemical screening of methanolic extract for the separation of chemical constituents was carried out .This was a preliminary screening for detection of various phyto constituents present in the plant and was done in 5 * 10 plates with each spot about 9 µl .

HPTLC Chromatogram

Caesalpinia pulcherrima – Flavanoids

Mobile phase: Ethyl acetate: Water: Formic acid: Acetic acid (10:0.5:1.1:1.1)
Detection/visualization: At 366nm, 254nm & after derivatization
Derivatization: Anisaldehyde –Sulphuric acid
The antimicrobial activity of the extract and isolated fraction were shown below and the Zone of inhibition of each was determined and they are compared with the values of standard antibiotic.

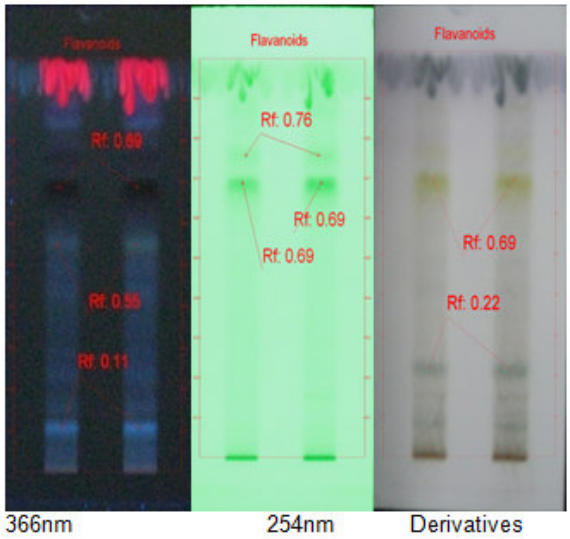

Figure 5
HPTLC Chromatogram for flavanoids at 366 nm, 254 nm and after derivatization

The HPTLC chromatogram for flavanoids were developed using solvent system (Ethyl acetate: Water: Formic acid: Acetic acid in the ratio (10:0.5:1.1:1.1).The flavanoids got prominent spots at an Rf value of 0.11, 0.22 and 0.69 at 254,366 and after derivatization.

Caesalpinia pulcherrima – Phenolic compounds

Mobile phase: Ethyl acetate: 1,4-Dioxane:Formic acid: Water (5:3:1:1)
Detection/visualization: At 366nm, 254nm & after derivatization
Derivatization: Fast blue

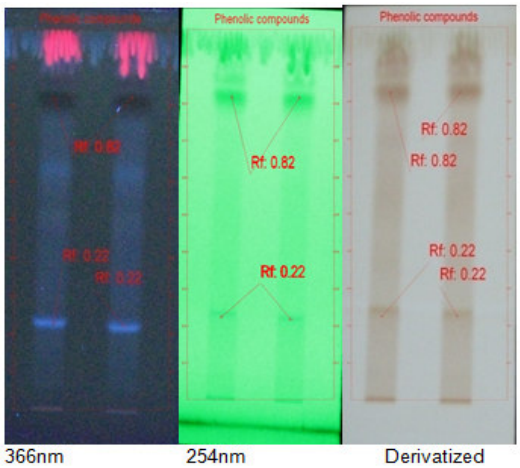

Figure 6
HPTLC Chromatogram for phenolics at 366 nm, 254 nm and after derivatization

The HPTLC profile of phenolic compounds were developed using solvent system (Ethyl acetate: 1, 4-Dioxane: Formic acid: Water) in the ratio (5:3:1:1) and the results were analyzed at 366nm, 254 nm and after derivatization. The corresponding Rf value of the spots were at 0.22 and 0.82.

Caesalpinia pulcherrima– Saponins
Mobile phase: 1-Butanol; Water; Acetic acid (5:4:1)
Detection/visualization: At 366nm, 254nm & after derivatization
Derivatization: Anisaldehyde –Sulphuric acid

Caesalpinia pulcherrima– Alkaloids
Mobile phase: Toluene: Ethyl acetate: Diethyl amine (7:2:1)
Detection/visualization: At 366nm, 254nm & after derivatization.

Caesalpinia pulcherrima – Tannins
Mobile phase: Toluene: Ethyl acetate (98:2)
Detection/visualization: At 366nm, 254nm & after derivatization
Derivatization: Fast blue
So further isolation of the total flavanoid and phenolic fraction were carried out. HPTLC plates of 20 * 10 size were used for the detection and the isolation were carried out by spotting 40 µl of the extract for 16 times in the plates.

Isolation of total flavanoids from the methanolic extract of caesalpinia pulcherrima

Caesalpinia pulcherrima – Flavanoids
Mobile phase: Ethyl acetate: Water: Formic acid: Acetic acid
Detection/visualization: At 366nm, 254nm

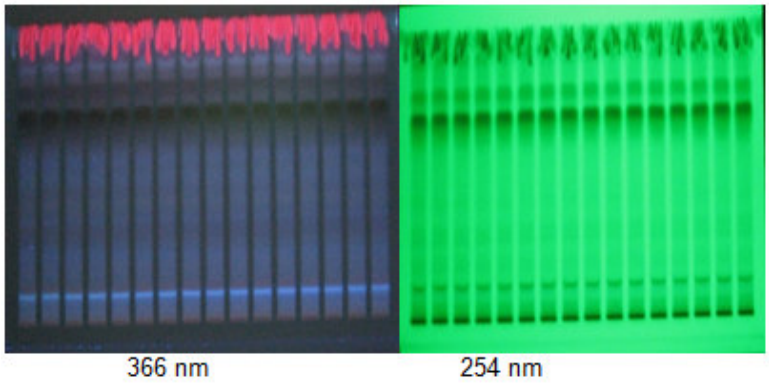

Figure 7
HPTLC Chromatogram for flavanoids isolation at 366 nm and 254 nm

The plant extract were spotted on 20* 10 plates and the spots obtained can be viewed under 254 and 366 nm. The total flavanoids obtained were scrapped off and isolated fraction were sonicated and centrifuged at 1000 rpm to separate the silica from the components. The obtained flavanoid fraction were dried and used for activity studies

Isolated phenol fraction from the methanolic extract of Caesalpinia pulcherrrima

Caesalpinia pulcherrima – Phenolic compounds
Mobile phase : chloroform: ethyl acetate: formic acid (7.5:6:0.5).
Detection/visualization: At 366nm, 254nm

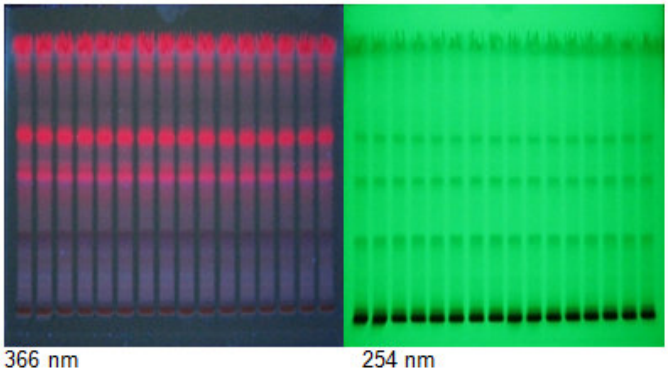

Figure 8
HPTLC Chromatogram for phenolic compound isolation at 366 and 254 nm

The similar procedures for the flavanoids were conducted to obtain the total phenolic compounds. The spots were scrapped, sonicated and centrifuged followed by separation and drying. The phenolic compounds showed the presence of gallic acid at an Rf value of 0.26 during the isolation step.

Isolated flavanoid fraction spotted
The HPTLC chromatogram after the isolation of total flavanoids were determined by spotting the solution obtained after isolation of the methanolic extract in the solvent system for flavonoids (Ethyl acetate: Water: Formic acid: Acetic acid in the ratio (10:0.5:1.1:1.1). The fraction showed prominent spots with Rf value 0.11 and

0.22 at 366 nm

Figure 9
HPTLC Chromatogram of Isolated flavanoid fraction

Isolated phenol fraction spotted

The HPTLC chromatogram was developed for the total phenolic fraction isolated from the methanol extract. The solvent system used was the same as that for isolation ie.(chloroform: ethyl acetate: formic acid in the ratio(7.5:6:0.5).They showed one active spot at an Rf value of 0.92 at 254 nm . Based on the content in the scratched fraction the spots were developed

Figure 10
HPTLC Chromatogram of Isolated phenol fraction

Methanolic extract when treated with solvent system for phenolics showed the presence of Gallic acid. The solvent system used was chloroform: ethyl acetate: formic acid in the ratio (7.5:6:0.5). The spot at Rf value of 0.26 for methanol extract and Gallic acid proved the presence of Gallic acid in extract.

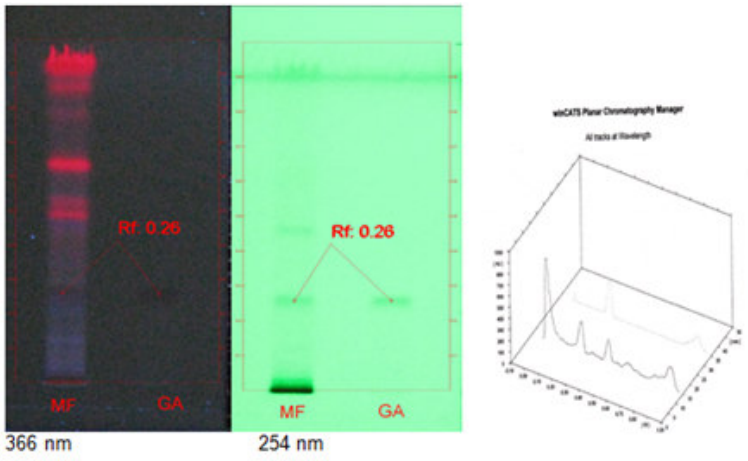

Figure 11
Methanol extract and Gallic acid comparison at 366 nm and 254 nm

Antimicrobial activity

Table 4
Zone of inhibition of various extracts

MICRO ORGANISM	ZONE OF INHIBITION(mm)			
	Methanol extract (0.1 mg/ml)	Isolated flavanoid fraction(0.1mg/ml)	Isolated phenol fraction(0.1mg/ml)	Standards used(0.1 mg/ml)
S. aureus	16 mm	10 mm	-	Amoxicillin (33 mm)
E-coli	-	8 mm	7 mm	Amoxicillin (20 mm)
P.aeruginosa	15 mm	-	-	Ciprofloxacin (40 mm)
S.aboni	-	-	8 mm	Bacitrin (25 mm)

The antimicrobial activity of the methanolic extract of *Caesalpinia pulcherrima* were carried out and the corresponding zone of inhibition were noted. The standards for each organism also vary and the corresponding zone of inhibition was shown in the table above. Both the extract and sample were taken at a concentration of 0.1 mg/ml .

Staphylococcus aureus

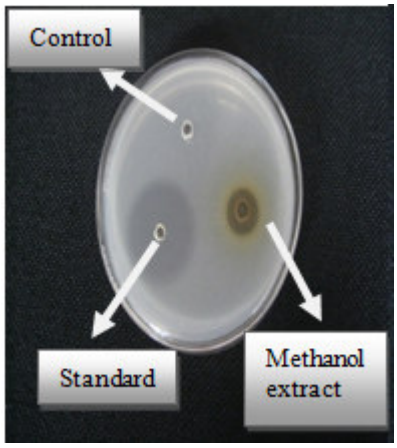

Figure 12
ZOI of S. aureus

Pseudomonas aeruginosa

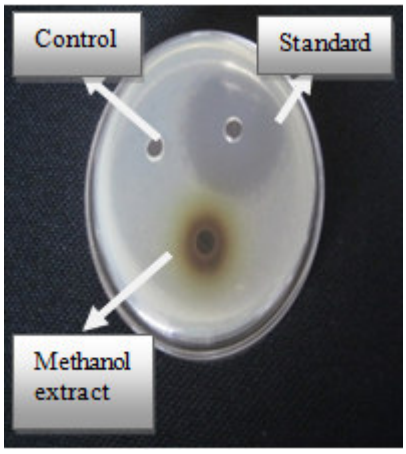

Figure 13
ZOI of P. aeruginosa

Methanol extract methanol extract
The methanolic extract showed activity against *S.aureus* and *P.aeruginosa* with a zone of inhibition of 16mm and 15 mm respectively. The methanol extract showed no activity against *E-coli* and *S. aboni* .So the extract was effective for the treatment of fever.

ISOLATED FLAVANOID FRACTION

Staphylococcus aureus Escherichia coli

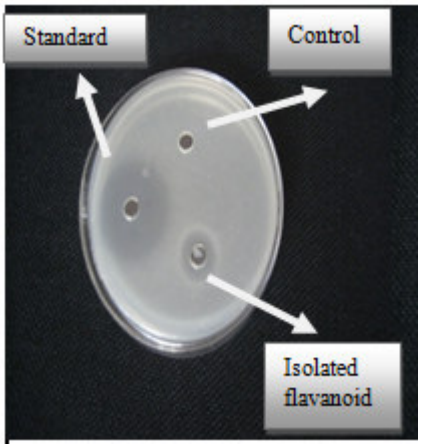

Figure 14
ZOI of S.aureus isolated flavanoid fraction

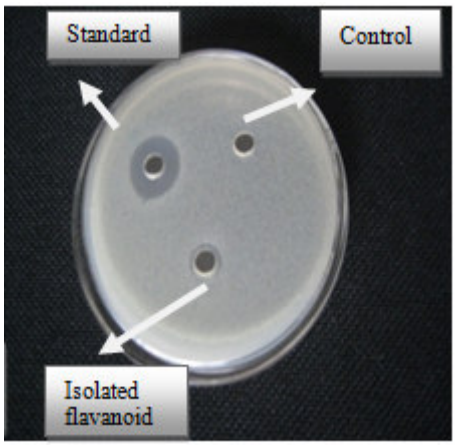

Figure 15
ZOI of E-coli isolated flavanoid fraction

ISOLATED PHENOLIC FRACTION

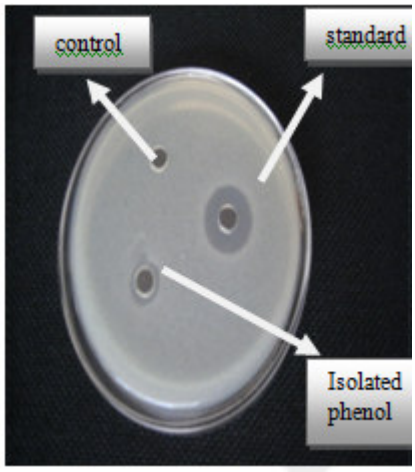

Figure 16
ZOI of E-Coli isolated

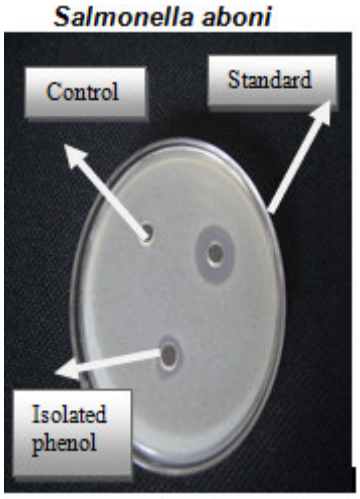

Figure 17
ZOI of S.aboni

The isolated flavanoidal fraction showed activity against *S.aureus* and *E-coli* with a zone of inhibition of 10 mm and 8 mm respectively. The *P.aeruginosa* and *S. aboni* was not inhibited by the isolated flavanoid fraction. The isolated phenolic fraction showed activity against *E coli* and *S.aboni* with a zone of inhibition 7 mm and 8 mm respectively. The fraction was inactive against *S.aureus* and *P.aeruginosa.*

Minimum inhibitory concentration

Serial dilution of 500, 100,75, 50,25,10 µg/ml solutions were taken for the study by considering100 µg/ml as the mother liquor. The analysis was carried out in nutrient broth by utilizing *pseudomonas* and *S.aureus* as the culture. A positive control (without organism) and a negative control (with organism) is also placed to identify the turbidity present in the tubes

Invitro antioxidant activity

Table 5
Reducing power assay

CONCENTRATION (µg/ml)	ABSORBANCE	
	STANDARD	SAMPLE
0	0	0
10	10	0.725
20	20	0.701
30	30	0.632
40	40	0.497
50	50	0.259
Control		0.862

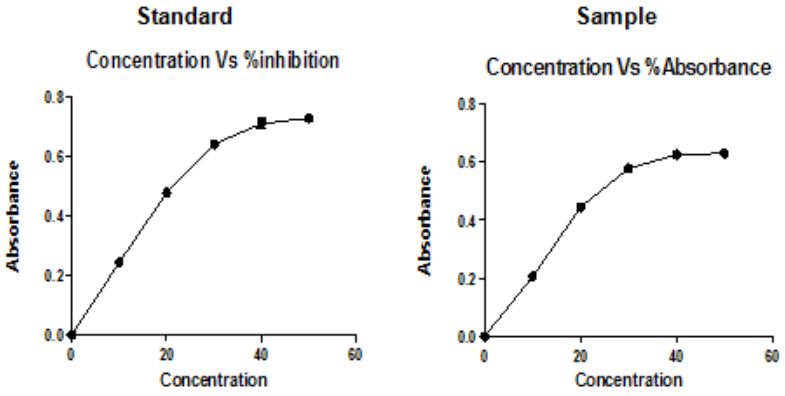

Figure 18
Concentration Vs % Absorbance graph of standard and Sample by Reducing power Assay

Table 6
DPPH Assay

CONCENTRATION	ABSORBANCE		% INHIBITION	
	STANDARD	SAMPLE	STANDARD (Mean±S.D)	SAMPLE (Mean±S.D)
0	0	0	0	0
10	0.392	0.45	32.17±0.122	22.14±0.77
20	0.207	0.257	64.18±0.36	55.53±0.27
30	0.103	0.179	82.17±0.15	71.26±0.22
40	0.039	0.092	93.25±0.20	85.23±0.16
50	0.019	0.044	96.95±0.21	92.38±0.27
control	0.578			

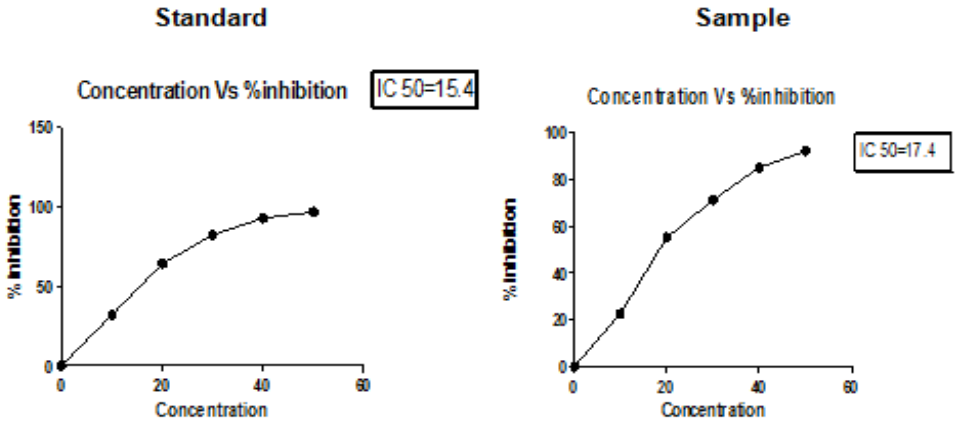

Figure 19
Concentration Vs % inhibition graph of standard and sample by DPPH ass

Table 7
Nitric oxide scavenging assay

CONCENTRATION	ABSORBANCE		% INHIBITION	
	STANDARD	SAMPLE	STANDARD (Mean±S.D)	SAMPLE (Mean±S.D)
0	0	0	0	0
10	0.516	0.555	20.37±0.07	14.35±0.34
20	0.429	0.478	33.79±0.76	26.23±0.85
30	0.346	0.394	46.6±0.06	39.19±0.14
40	0.237	0.267	63.42±0.23	58.79±0.38
50	0.153	0.175	76.38±0.34	72.99±0.59
control	0.648			

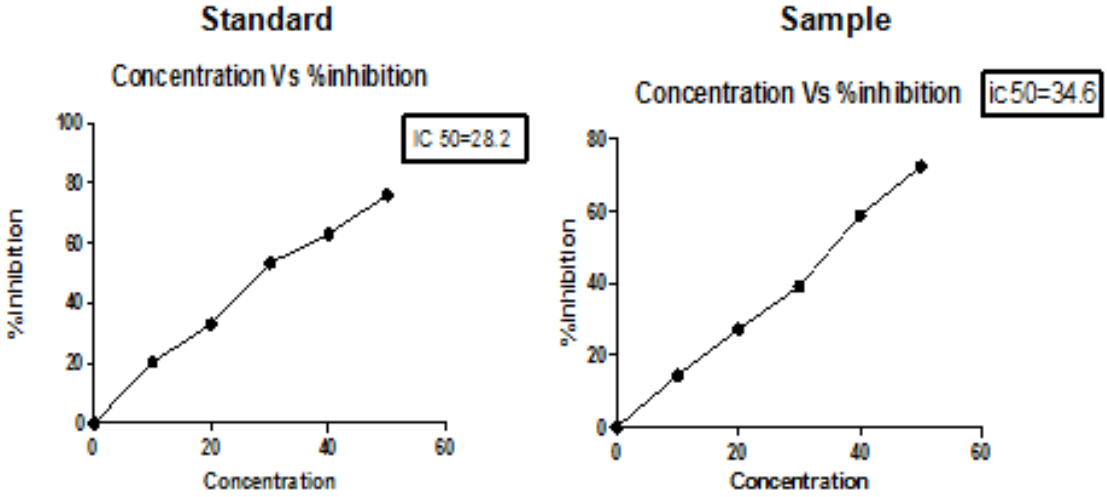

Figure 20
Concentration vs % inhibition graph of standard and sample by Nitric oxide scavenging Assay

Table 8
Hydrogen peroxide assay

CONCENTRATION	ABSORBANCE		% INHIBITION	
	STANDARD	SAMPLE	STANDARD (Mean±S.D)	SAMPLE (Mean±S.D)
0	0	0	0	0
10	0.482	0.521	28.9±0.518	23.15±0.422
20	0.316	0.39	53.39±1.463	42.47±0.18
30	0.227	0.265	66.51±0.72	60.91±0.757
40	0.147	0.189	78.31±0.473	72.12±0.37
50	0.053	0.099	92.18±0.507	85.39±0.248
CONTROL	0.678			

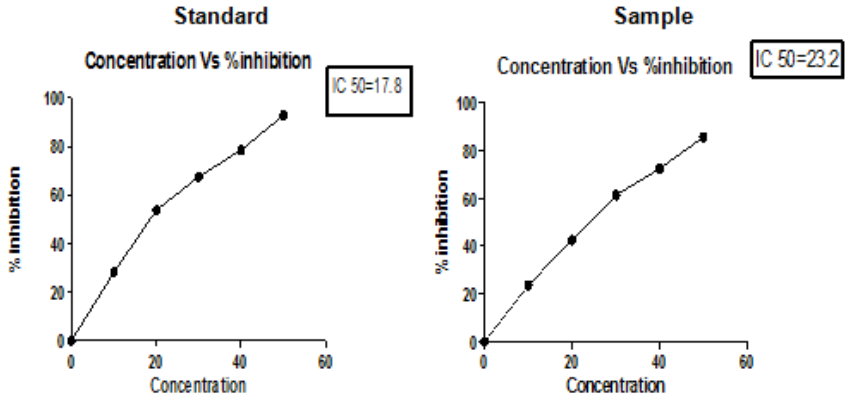

Figure 21
Concentration Vs % inhibition graph of standard and sample by Hydrogen peroxide assay

Invitro anticancer activity

The anticancer activity of the methanol extract and hydro alcoholic extract were carried out in Hep G2 (hepatocarcinoma cell line) and compared with the normal cell BRL3A (Rat liver cell line).The analysis was carried out at different concentrations of sample ranging from 1000 to62.5180 µg/ml. The % of cytotoxicity was analysed and a dose response curve was drawn based on the results. The corresponding CTC 50 value was determined from the curve

.

Table 9
Invitro cell inhibition of extracts and isolated fractions

Sl. No	Name of drug	Test Concn. (µg/ml)	% Cytotoxicity	CTC50 (µg/ml)
1	**C.pulcherrima Methanolic Extract (HepG2)**	1000	71	180.00
		500	68.2	
		250	52.1	
		125	35.14	
		62.5	24	
2	**C.pulcherrima Hydroalcoholic Extract (HepG2)**	1000	76.27	210.00
		500	75.75	
		250	48.71	
		125	30.05	
		62.5	4.84	
3	***C.pulcherrima Flavonoid fraction***	1000	76.09	700.00
		500	34.83	
		250	13.10	
		125	8.85	
		62.5	8.51	
4	***C.pulcherrima phenolic fraction***	1000	65.86	550.00
		500	46.78	
		250	11.15	
		125	9.08	
		62.5	8.16	

The isolated total flavanoids and phenolic compounds were also undergone anticancer MTT assay. The isolated phenol shows a CTC $_{50}$value of 550 µg/ml and the isolated flavanoid fraction shows a CTC $_{50}$ value of 700 µg/ml. The lower value shows best result. Thus the phenol fraction is capable of inhibiting 50 % of hepatocarcinoma cells

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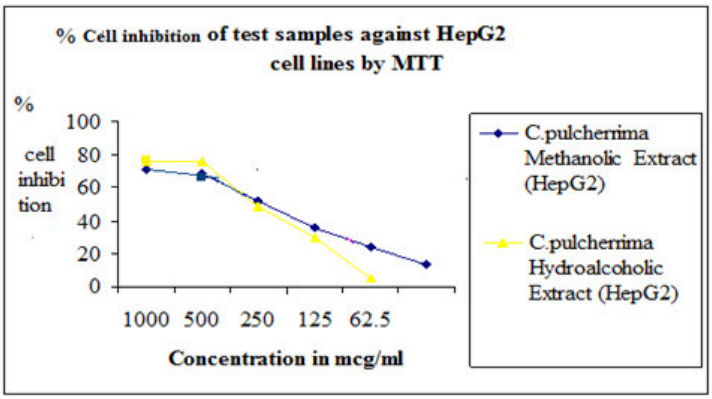

Figure 22
% cell inhibition of test samples against Hep G2 cell line by MTT assay

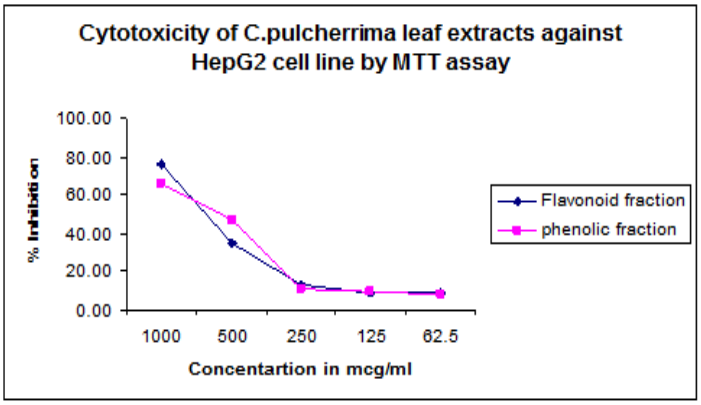

Figure 23
% cell inhibition of isolated flavanoid and phenolic fraction against Hep G2 cell line

CONCLUSION

The *Caesalpinia pulcherrima* Linn leaves belonging to family Caesalpiniaceae has been examined to determine its phytochemical and invitro pharmacological activities. Authenification and standardization and phytochemical screening of the plant were carried out initially. Quantification of the major constituents like flavanoids and phenolics and total isolation based on the analysis were performed. Determined the antimicrobial, invitro antioxidant and anticancer activities of the extracts and isolated fractions. The plant extracts and fractions are active against microorganisms act as good antioxidant and anticancer agents from the analysis. The study can be further extended for determining the major single constituent that is responsible for these activities for developing as a herbal product for the sake of humanity.

CONFLICT OF INTEREST

Conflict of interest declared none.

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India

Mr. Anubrata Paul M.Sc. Biotech (Research)

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