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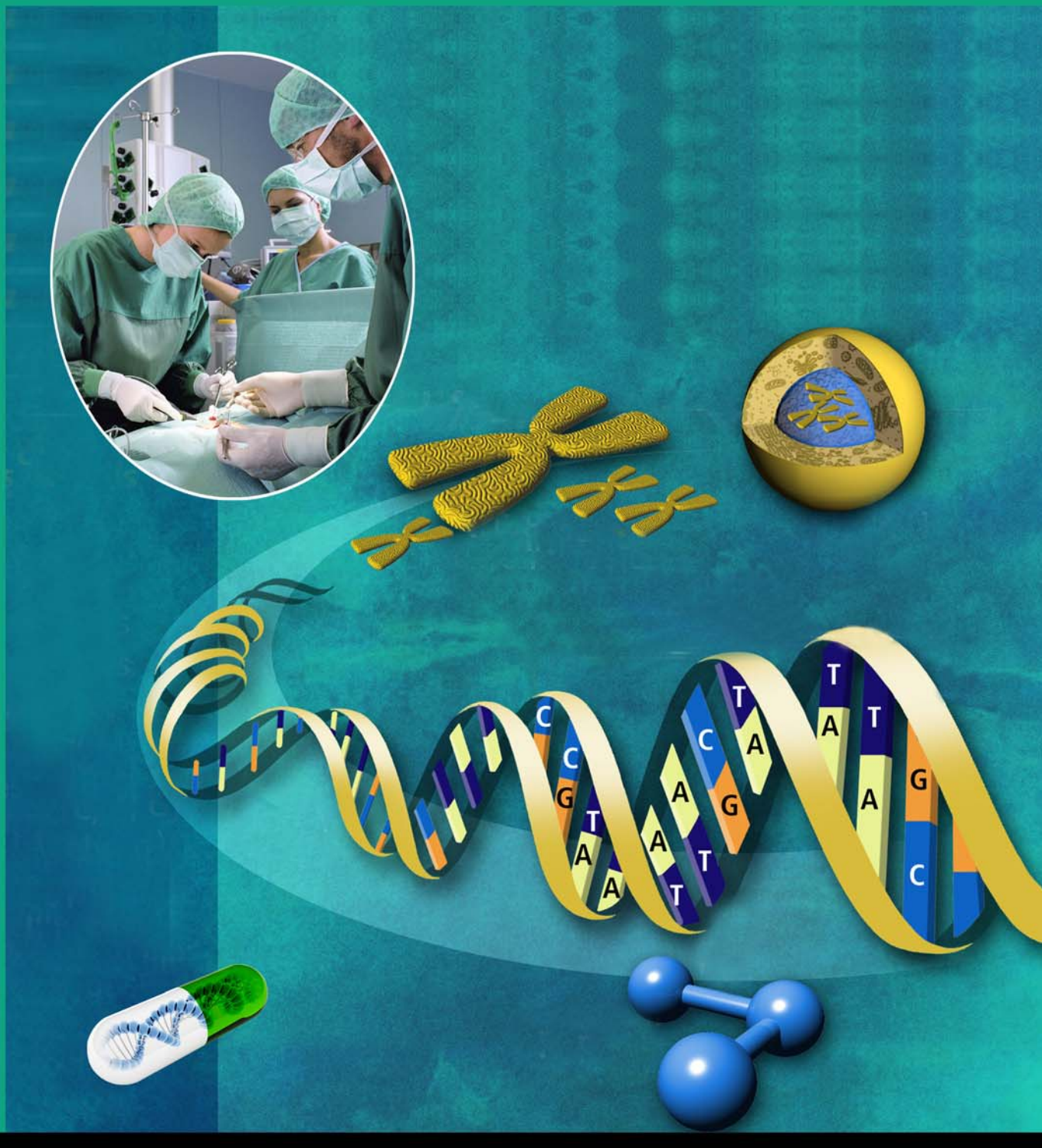

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Research Paper

PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL PROPERTY OF *CARMONA RETUSA* (VAHL.) MASAM

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Three different extracts have been prepared with petroleum ether, methanol and chloroform separately from *Carmona retusa*. Methanol extract have yielded alkaloids, flavonoids, saponins, phenols, tannins, cardiac glycosides, terpenoids, cardenolides except anthraquinones and phlobatannins. Petroleum ether extract have yielded flavonoids, phenols, cardiac glycosides and terpenoids. Chloroform extract have yielded saponins, cardiac glycosides, terpenoids and cardenolides. Antibacterial activity of petroleum ether, chloroform and methanol extracts obtained from the leaf of *Carmona retusa* were tested against four bacteria using the agar diffusion method. The experimental data indicated that all extracts exhibit moderate to appreciable antibacterial activities against *Bacillus subtilis*, *Klebsiella pneumoniae, Shigella flexneri* and *Pseudomonas aeruginosa* .These results may suggest that *Carmona retusa* could be used as an antibacterial agent.

Keywords: *Carmona retusa*, phytochemicals, antibacterial activity, agar diffusion method

INTRODUCTION

Carmona retusa is an evergreen shrub to small tree. Leaves in clusters of 3-5, blade obovate or oblanceolate, 1.5-4 cm x 0.8-2.5 cm, base decurrent onto petiole, coarsely 3-5 toothed towards apex, apex acute to obtuse or rounded, when young both surfaces with stiff white hairs, upper surface becoming scabrid, petiole 1-5 mm long. Flowers 3-12 flowered scorpioid cymes, unbranched or branched once, sepals 4-5, lanceolate, 3-4 mm long; corolla white, rotate, 8-10 mm in diameter, lobes 4-5, 3-4 mm long. Fruit globose, 4-5 mm in diameter, ripening brownish orange, pericap thin, pyrene white, bony (Lorence *et al*., 1995).

Carmona retusa leaf decoction is being used to treat cough and stomach ache root as antidote (Shrisha *et al*., 2011). Many plants have proved to successfully aid in various ailments leading to mass screening for their therapeutic components. Today, the search for natural compounds rich in antimicrobial, antioxidant and anti-inflammatory

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properties is escalating due to their medicinal importance in controlling many related chronic diseases (Govindappa *et al.,* 2011).

Present investigation was aimed to identify phytochemicals in leaves and stem of *Carmona retusa* using different solvents and our extracts have been tested against four important bacteria.

MATERIAL AND METHODS

Collection of Plant Materials

Fresh leaves and stem of *Carmona retusa* were collected from Hemagangothri Campus, Hassan district of Karnataka, India. The collected plant was authenticated with herbarium, Government Ayurvedic College, Mysore, India and Department of Studies Botany, Mysore University, Mysore.

The collected plant parts were separated as leaf and stem and were cleaned with deionized water and dried under shade for two weeks at room temperature. Dried leaves and stem were grounded and filtered using 0.3mm mesh. The plant powder was stored in air tight container and maintained at 4°C until use separately.

Preparation of Sample

Solvent systems used for the extractions were petroleum ether, methanol and chloroform. Flask extraction procedure was adapted for extraction. 25 grams of the powdered leaf sample was soaked in the conical flask containing solvent, wrapped with aluminum foil and placed in shaker for 48 hours at 120-130 rpm (Aiyegoro and Okoh, 2010).

After 48 hours, the extracts were filtered using Whatman filter paper No 1. Concentrated all the solvent extracts in an air circulating oven at 54°C until total dryness. Dried extracts were stored at 4°C for further analysis (Assam *et al.,* 2010).

Qualitative Phytochemical Screening

Phytochemical analysis of each extract has been carriedout according to standard protocols (Anyasor *et al.,* 2010).

Screening for Alkaloid

0.5 g of the extract was stirred in 5 ml of 1% HCl on a steam bath and filtered while hot. Distilled water was added to the residue and 1 ml of the filtrate was treated with a few drops of Wagner's reagent. A reddish brown precipitate indicates the presence of alkaloids.

Screening for Flavonoids

2 ml of dilute sodium hydroxide was added to 2 ml of the extract. The appearance of a yellow colour indicates the presence of flavonoids.

Screening for Saponins

1 ml of distilled water was added to 1 ml of the extract and shaken vigorously. A stable persistent froth indicated the presence of saponins.

Screening for Phenols

Equal volumes (1 ml) of extract and Iron (III) chloride were mixed. A deep bluish green solution gave an indication of the presence of phenols.

Screening for Tannins

A portion of the extract was dissolved in water, after which the solution was clarified by filtration. 10% ferric chloride solution was then added to the resulting filtrate. The appearance of a bluish black colour indicates the presence of tannins.

Screening for Anthraquinones

0.5 g of the extract was shaken with 10 ml of benzene and filtered. 10% of ammonia solution was added to filtrate and the mixture was shaken. The formation of a pink, red or violet colour on the ammoniacal phase indicates the presence

of anthraquinones.

Screening for Cardiac Glycosides

0.5 g of the extract was dissolved in 2 ml glacial acetic acid containing 1 drop of ferric chloride solution. This was under layered with 2 ml of concentrated sulphuric acid. A brown ring formation at the inter phase indicates the presence of deoxy sugar characteristics of cardiac glycosides.

Screening for Phlobatannins

A few drops of 1% HCl was added to 1 ml of extract and boiled. A red precipitation indicates the presence of phlobatannins.

Screening for Terpenoids

0.5 ml of acetic anhydride was mixed with 1 ml of sample extract and a few drops of concentrated H_2SO_4. A bluish green precipitate indicates the presence of terpenes.

Screening for Cardenolides

2 ml of benzene was added to 1 ml of the sample extract. The formation of a turbid brown colour is an indication of the presence of cardenolides.

Quantitative Phytochemical Analysis

Phenol Content Determination

The concentration of total phenolic compounds was determined using Folin-Ciocalteau reagent adapted by McDonald *et al.* (2001). Dried samples and standards were prepared in distilled water. Test solutions (samples and standards) of 0.1 ml with slight modifications were added to 4.0 ml of 1M Na_2CO_3. Five ml of Folin-Ciocalteau reagent (1:10, v/v) were added and the solutions allowed to stand at room temperature for 10 min. Absorbance were measured at 720 nm. The blank consisted of all reagents and solvents without test compounds or standard. The standard was tannic acid prepared in concentrations of 50 to 200 mg/L. This is commonly used as a reference compound. The phenolic concentrations were determined by comparison with the standard calibration curve. Total phenol values were expressed as Tannic acid equivalents (mg g^{-1} dry mass) (Olayinka *et al.*, 2010).

Alkaloid Determination Using Harborne (1973) Method

5 g of the samples were weighed into 250ml beaker and 200ml of 20% acetic acid was added and covered to stand for 4h.This was filtered and the extract was concentrated using a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitate was complete. The whole solution was allowed to settle and the precipitate was collected by filtration and weighed (Harborne, 1973; Obadoni and Ochuko, 2001).

Determination of Flavonoids

10g of the plant samples were extracted repeatedly with 100ml of 80% aqueous methanol at room temperature. The extract was filtered through Whatman filter paper No. 42 (125mm). The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed (Boham and Kocipai, 1994).

Determination of Saponins

20 g of each sample was dispersed in 200ml of 20% ethanol. The suspension was heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200ml of 20 % ethanol. The combined extracts were reduced to 40ml over water bath at about 90°C. The concentrate was transferred into a 250ml separatory funnel and 20ml of diethyl ether was

added and shaken vigorously. The aqueous layer was recovered and the purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight. The saponin content was calculated as percentage of the initial weight of sample taken (Obadoni and Ochuko, 2001).

Proteins Determination

1g plant material was extracted using 10 ml water added with few drops of triton X- 100. Supernatant was extracted in acetone and the pellet obtained was dissolved in 0.1 M NaOH. Aliquots were prepared and final volume was made to 1 ml by distilled water. 5 ml of copper reagent was added to tubes, mixed well and incubated for 10 minutes. 1 ml of Folin's reagent was mixed. Tubes were incubated for 30 min at room temperature and absorbance was taken at 700 nm. Standard curve was prepared using 50 mg % BSA (Lowry *et al*., 1951).

Antibacterial Activity

Preparation of Microorganisms

The test organisms used in this study were *Bacillus subtilis* (MTCC441), *Klebsiella pneumoniae (*MTCC*39), Shigella flexneri* (MTCC1457) and *Pseudomonas aeruginosa* (MTCC1034). The strains were maintained on nutrient agar slants at 4°C. A loop ful of each bacterial strain was added to a 10 mL sterile nutrient broth in a test tube. The tubes were then incubated in an incubator for 24 h to activate the strain.

Agar Well Diffusion Method

The strains that had been incubated for 24 h were used for the assay. A sterile cotton swab was dipped into the bacterial suspension and then evenly streaked over the entire surface of a sterile Mueller Hinton agar plate to obtain a uniform inoculum. Wells were made on the seeded plates using a 1000 μL sterile microtip (8 mm) and the plates and allowed to dry for 5 min. The plant extracts (20 μL) were dispensed into each well using a sterile micropipette. Dimethyl sulfoxide was used as a negative control and ampicillin (10 μL) was used as a positive control. The plates were incubated overnight at 37°C and the antibacterial activity was determined by measuring the diameter of zone of inhibition (mm) (Ganjewala *et al.,* 2009) with slight modification.

RESULTS AND DISCUSSION

Screening of the plant material revealed the presence of the alkaloids, flavonoids, saponins, phenols, tannins, cardiac glycosides, terpenoids and cardenolides were summarized in Table 1. Methanol extract yielded almost all above mentioned phytochemicals. Chloroform extracts yielded saponins, cardiac glycosides, Terpenoids and cardenolides whereas petroleum ether extract yielded flavonoids, cardiac glycosides and terpenoids only.

Since our plant extract have revealed the presence of alkaloids, we can say alkaloids also have shown the antibacterial activity. Okwu and Igara (2009) evaluated antibacterial activity of alkaloid of *Datura metel* Linn leaves against *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabis, Solmonella typhi, Bacillus subtilis* and *Klebsiella pneumonia* but could not inhibit *Escherichia coli*. Flavonoids are ubiquitous in photosynthesing cells and are commonly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. For centuries, preparations containing these

Table 1: Qualitative Phytochemical Analysis of Leaf Extract of *Carmona retusa*

Phytochemical	Petroleum ether Extract	Methanol Extract	Chloroform Extract
Alkaloid	–	++	–
Flavonoids	+++	+++	–
Saponins	–	+++	++
Phenols	+	+	–
Tannins	–	+++	–
Anthraquinones	–	–	–
Cardiac glycosides	++	++	++
Phlobatannins	–	–	–
Terpenoids	+	+	+
Cardenolides	–	+	+

Note: + =Presence ++ = Moderate +++ = More presence – = Absence; * Repeated the each experiment thrice.

compounds as the principal physiologically active constituents have been used to treat human diseases. Increasingly, this class of natural products is becoming the subject of anti-infective research, and many groups have isolated and identified the structures of flavonoids possessing antifungal, antiviral and antibacterial activity (Cushnie *et al.,* 2005).

The n-butanol purified saponin extract of sorghum bicolor were screened for anti-bacterial activity against three pathogenic microbes; *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. The extract inhibited the growth of the *S. aureus*. It was concluded that the saponins have inhibitory effect on gram-positive organism but not on gram negative organism and the fungi (Soetan *et al.,* 2006).

Saponin extract from Sider (*Ziziphus spina_christi*) were evaluated for his antibacterial activity against gram negative bacteria like *E.coli*, *Proteus mirabilis* and gram positive like *Staphylococcus aureus* and *Streptococcus pneumoniae*. The inhibitory effect in *in vitro* was defined to appear inhibition zone around the paper disc. The study revealed that 30% (w/ v) concentration most active against bacteria (Kredy 2010).

Compounds of pharmacological interest (tannins) were isolated from the plant species, *Solanum trilobatum* Linn and assayed against the bacteria, *Staphylococcus aureus, Streptococcus pyrogens, Salmonella typhi, Pseudomonas aeruginosa, Proteus vulgaris* and *Escherichia coli* using agar diffusion method. Tannins exhibited antibacterial activities against all the tested microorganisms (Doss *et al.,* 2009).

Studies on the antibacterial activity of ethanol, methanol and aqueous extracts of dry flower and

ethanol, methanol and acetone extracts of fresh flower of *Cassia auriculata* was conducted using agar disc diffusion method. The microorganisms used include *Staphylococcus aureus, Enterococcus faecalis, Bacillus subtilis, Salmonella typhi, Salmonella paratyphi A, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Vibrio cholerae* and *Shigella dysentrae.* The maximum activity was observed against all organisms except *Pseudomonas aeruginosa* and *Klebsiella pneumonia (*Maneemegalai *et al.,* 2010).

Quantitative estimations of bioactive constituents are summarized in Table 2. Saponins contain in large quantity followed by flavonoids, alkaloids and phenols.

Antibacterial Activity

The antibacterial activity of the petroleum ether, chloroform and methanol extracts of the stem of the plant is presented in Table 3. The antibacterial activity of the methanol extract of the stem of the *C. retusa* is much higher than chloroform and petroleum ether extract against *Pseudomonas aeruginosa* (MTCC1034).The chloroform extract

Table 2: Quantitative Phytochemical Analysis of Stem Extract of *Carmona retusa*

Name of the phytochemical	Quantity/100g of plant material (i.e., g%)
Alkaloid	0.038
Flavonoids	0.064
Saponins	0.1
Phenols	0.033
Proteins	0.12

Note: *Repeated the each experiment thrice.

Table 3: Antibacterial Activity of Various Extracts of *Carmona retusa* (Zone of Inhibition (mm))

Organism	Extracts/Positive and Negative Control				
	PE	CE	ME	AMP	DMSO
Pseudomonas aeruginosa (MTCC1034)	18 + 0.067^{a}	25 + 0.65^{b}	28 + 0.65^{a}	26 + 0.65^{ab}	–
Klebsiella pneumoniae (MTCC39)	14 + 0.057^{bc}	20 + 0.057^{a}	18 + 0.067^{a}	13 + 0.057^{c}	–
Shigella flexneri (MTCC1457)	13 + 0.057^{c}	20 + 0.057^{a}	17 + 0.067^{a}	13 + 0.057^{c}	–
Bacillus subtilis (MTCC441)	15 + 0.8^{a}	20 + 0.057^{a}	19 + 0.057^{a}	15 + 0.057^{b}	–

Note: **PE-** Petroleum Extract**; CE-** Chloroform Extract**; ME** – Methanol Extract; **AMP** – Ampicillin**; DMSO** – Dimethylsulfoxide; Ampicillin concentration- 5mg/10ml); * Repeated the each experiment thrice.

of *C.retusa* have shown maximum inhibition against *Klebsiella pneumoniae* (MTCC39), *Shigella flexneri (*MTCC1457*)* and *Bacillus subtilis* (MTCC441) followed by methanol and petroleum ether extract.

CONCLUSION

The antibacterial activity of the plant can be due to the presence of various phytochemicals, which are known to be synthesized by plants in response to microbial infection. All extracts of *Carmona retusa* have shown antibacterial activity against four bacteria and hence it can be used as a source of antibacterial agent in future. This is the first report of *C. retusa* extracts of antibacterial activity in India.

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