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Original Research Article

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Studies on phytochemical and antibacterial activity of methanol, ethanol and acetone extracts of gewia orientalis leaves

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Abstract

Three solvent extracts (methanol, ethanol and acetone) of *Grewia orientalis* leaves were screened for potential antibacterial activity against *Escherichia coli*, *Bacillus substilis*, *Staphylococcus aureus, Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Bacillus thuringienis*. The agar disk diffusion method using filter paper disks was used to study the antibacterial activity of *Grewia orientalis* leaf extracts against 6 microbial strains. All the three extracts did not exert any inhibitory action on *Bacillus thuringienis*. The highest antibacterial potentials were observed for the acetone extract, followed by methanol and ethanol extracts. The Minimum Inhibitory Concentration (MIC) of the plant extracts were ranging from 50 to 250 μg/ml. The preliminary phytochemical screening of extracts was carried out for major phytochemical derivatives in *Grewia orientalis*.

Keywords: phytochemical and antibacterial activity of methanol

Introduction

For a long period of time, medicinal plants find application in pharmaceutical, cosmetics, agricultural and food industry [1]. Human beings depend on medicinal plants for prevention and treatment for most of the diseases. Approximately 80,000 species of plants have been utilized in the treatment of various dreadful diseases in different systems of medical practice in Indian medicine [2]. The use of the medicinal plants for curing diseases has been documented in history of all civilizations. The therapeutic principles contained in the medicinal plants become responsible for curative action. With the knowledge of scientific procedures, the researchers were able to understand about therapeutic principles present in the plants. The phytochemical analysis by several researchers all over the world also reveal that the major therapeutic principles of plants are alkaloids, steroidal sapogenins, flavonoids, tannins, unsaturated sterols, triterpenoids, essential oils etc., [3-6]. As per World Health Organization medicinal plants form the best source to get a variety of drugs [7,8].

About 80% of the individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants [9]. Over 50% of all modern clinical drugs are of plant origin and plant products play a vital role in drug development programs in the pharmaceutical industry [10,11]. Therefore, such plants should be investigated to understand their properties, safety and efficiency.

Plant preparations exhibit comparatively lower incidence of adverse reactions to modern conventional pharmaceuticals, at reduced cost [12]. Considering both the consuming public and national health care institutions and thus motivating the use of plant medicines instead of synthetic drugs. Plants extracts with possible antibacterial, antiviral and antifungal activity should be tested against an appropriate bacterial, viral and fungal model, respectively to confirm the activity. The effects of plant extracts on bacteria, virus and fungi have been studied by a very large number of researchers in different parts of the world [13-28]. It has been suggested that methanolic [13-16], acetone [17-20], aqueous [21-24] and ethanolic [25-28] extracts from plants are used as potential sources of antiviral [13,17,21,25], antibacterial [14,18,22,26], antifungal [15,19,23,27] and antitumoral [16,20,24,28] agents.

In the present investigation *Grewia orientalis* was screened for phytochemical properties and potential antibacterial activity. *Grewia orientalis* belongs to the family Tiliaceae. These are climbing shrubs upto 2 m tall; branchlets densely tomentose. Leaves ovate or lanceolate, 6-10 x 4-6 cm, glabrous, base subcordate, obtuse, margin crenulate, apex acuminate or acute. Flowers white, small, in 1-3 axillary, leaf-opposed cymes. Sepals 5, lanceolate. Petals 5, lanceolate. Stamens numerous. Ovary globose, densely stiff-woolly, 4-locular; ovule 1 per locule, axile; stigma 4-lobed. Drupes globose, obscurely 4-lobed, wrinkled, velvety. *Grewia orientalis* leaves were used by tribals of southern Rajasthan to induce sterility in women and are given orally to animals as fodder for relief from impaction [29, 30]. The obvious indications from the literature survey show insufficient scientific studies which confirm the antibacterial and phytochemical properties of *Grewia orientalis.* The present study was conducted to determine the antibacterial activities and phytochemical properties of *Grewia orientalis* leaves, extracted using methanol, ethanol and acetone, against the selected microorganisms.

Materials and Methods

Collection and identification of samples

The plant materials were collected from the hilly region (Gunadala konda) near the city of Vijayawada, Andhra Pradesh, India during July-August 2010. The taxonomic identification of the selected plant species was confirmed by Sri. Ch. Srinivasa Reddy, Department of Botany, Andhra Loyola College, Vijayawada, Andhra Pradesh. Plant materials were stored at department of Biotechnology, J.K.C College, Guntur, Andhra Pradesh until further studies.

Preparation of solvent extracts

The leaves were washed thoroughly 2-3 times with running water and once with sterile distilled water and were then air dried under shade for 7 days, since certain compounds can be denatured in sun light, and then powdered by using electric grinder. 20 gm of powdered material was filled in the thimble and extracted successively with solvent (methanol, ethanol and acetone) in Soxhlet extractor for 48 hours. The methanol, ethanol and acetone extracts were concentrated under reduced pressure and preserved at 5 C in airtight bottle for further studies.

Preliminary phytochemical studies

Preliminary phytochemical screening was performed to identify phytochemicals present in the methanol, ethanol and acetone extracts of *Grewia orientalis* leaves used in this study. Several sophisticated techniques like thin layer chromatography, ultra violet spectroscopy, infrared spectroscopy, nuclear magnetic resonance and HPLC have been used for identification of various groups of phytochemical compounds in plant extracts. In the present investigation, the phytochemical compounds were detected by simple color tests. 10 mg of methanol, ethanol and acetone extracts were dissolved in 20 ml of its corresponding solvents. The preliminary phytochemical tests as described by S. Shanmugam et al. [31] were conducted upon these specified extracts.

Test for flavonoids

A small piece of filter paper is dipped to about 1 ml of each extract and is exposed to ammonia vapour. The formation of yellow color spot on the filter paper indicates the presence of flavonoids.

Test for alkaloids

To 1 ml of each extract, 1 ml of Hager's reagent (saturated solution of picric acid) was added and mixed. The appearance of crystalline yellow precipitate indicates the presence of alkaloids.

Test for tannins

To 2 ml of each extract, 1 ml of 10% lead acetate was added. The presence of tannins was indicated by the formation of white precipitate.

Test for saponins

To 1 ml of each extract taken in a test tube, 9 ml of distilled water was added and shaken vigorously for 15 minutes and allowed to stand for 10 minutes. The formation of stable foam indicates the presence of saponins.

Test for steroids

To 0.5 ml of each extract, 2 ml of acetic anhydride and 2 ml of concentrated sulphuric acid was added along the sides of the test tube. The appearance of green color indicates the presence of steroids.

Test for glycosides

To 1 ml of each extract, few drops of glacial acetic acid and ferric chloride, and 3-4 drops of concentrated sulphuric acid were added. The presence of glycosides was indicated by the appearance of blue-green color.

Test for terpenoids

To 5 ml of each extract, 2 ml of chloroform and 2 ml of concentrated sulphuric acid were added. Formation of yellow color ring at the interface of two liquids that turns reddish brown color after 2 minutes indicates the presence of terpenoids.

Test for triterpenoids

To 1 ml of each extract, saturated solution of antimony trichloride in chloroform containing 20% acetic anhydride was added. Formation of red color on heating indicates the presence of triterpenoids.

Test for anthroquinones

To 1 ml of each extract, 1 ml of 10% ferric chloride and 0.5 ml of concentrated HCl were added. It is boiled in water bath for few minutes and filtered. Then the filtrate is treated with 1 ml of diethyl ether and concentrate ammonia. Appearance of red or pink color indicates the presence of anthroquinones.

Microorganisms

The following strains of bacteria were used as antibacterial test organisms: Escherichia coli (NCIM No.2563), Bacillus substilis (NCIM No.2709), Staphylococcus aureus (NCIM No.2079), Pseudomonas aeruginosa (NCIM No.5031), Klebsiella pneumoniae (NCIM No.2957) and Bacillus thuringienis (NCIM No.2130). All cultures were collected from the Department of Microbiology, J.K.C College, Guntur, Andhra Pradesh, India. The bacterial strains were maintained on nutrient agar at 4 C and sub-cultured once in a month in our laboratory.

Preparation of Inoculum

The gram positive (*Bacillus subtilis, Staphylococcus aureus and Bacillus thuringienis*) and gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) were pre-cultured in nutrient broth overnight in a rotary shaker at 37 C. This was further centrifuged at 10,000 rpm for 5 min and pellet was suspended in double distilled water. Then the cell density was

standardized spectrophotometrically (A_{610} nm) to obtain a final concentration of approximately 10^6 cells/ml.

Antibacterial testing

Antibacterial activity of the crude methanol, ethanol and acetone extracts of selected plant leaves was determined by disk diffusion method [32]. The bacterial cultures [Escherichia coli, Bacillus substilis, Staphylococcus aureus, Pseudomonas aeruginosa Klebsiella pneumoniae, and Bacillus thuringienis] were grown in nutrient broth liquid medium at 37 C. After 24 h of growth, 10 µl of each bacterial culture, at a concentration of 10^6 cells/ml, was inoculated on the surface of nutrient agar plates by spread plate method. Subsequently, filter paper disks (5 mm in diameter) saturated with plant extracts were placed on surface of each inoculated plate. Aseptic conditions were maintained throughout the experimental process. The plates were incubated at 37 C for 24 hours. Antibacterial activity was expressed as the zone of inhibition (mm) produced by the plant extracts. Dimethylsulphoxide (DMSO) was used to dissolve the extracts when necessary. The controls were the solvents used for each extract. The control activity was deducted from the test. Ampicillin (25 µg/ml), a product of HIMEDIA, was used as positive control. The determination of antibacterial activity of each extract was independently done three times from which the mean and standard deviation (S.D.) were calculated.

Minimum Inhibitory Concentration

A Minimum Inhibitory Concentration [2] (MIC) is the lowest concentration of an antibacterial that inhibits the growth of an organism after 24 hours. The extracts that showed antibacterial activity were tested to determine the Minimal Inhibitory Concentration (MIC) for each bacterial sample. A stock solution of methanol, ethanol and acetone plant extract was prepared in methanol, ethanol and acetone, respectively and was serially diluted with their respective solvents to obtain concentrations from 50 g/ml to 1000 g/ml. Filter paper disks (5 mm in diameter) impregnated with different concentrations of methanol, ethanol and acetone plant extracts were placed on surface of nutrient agar plates inoculated with the test organisms. The plates were incubated at 37 C for 24 hours. The diameter of circular inhibition zones were measured in millimeters. Ampicillin (25 g/ml) was used as positive control and solvents (methanol, ethanol and acetone) were used as negative control. Each assay was performed three times from which the mean and standard deviation (S.D.) were calculated. The minimum concentration of the extracts that showed inhibition zone was taken as the minimum inhibitory concentration.

Results and Discussion

Phytochemical constituents such as alkaloids, flavonoids, tannins, steroids, saponins, terpenoids etc, are secondary metabolites of plants that serve a defense mechanism against prediction by many microorganisms, insects and other herbivores [33]. Preliminary phytochemical screening showed that the ethanol extract contain most of the phytochemicals like flavonoids, alkaloids, tannins, steroids, glycosides, terpenoids and triterpenoids. Terpenoids and triterpenoids were present only in ethanol extract. Phytochemical analysis of methanol and acetone extracts showed the presence of flavonoids, alkaloids, tannins, saponins, steroids and glycosides. Anthraquinones are absent in all the three extracts. The phytochemical constituents of the plant extracts investigated are summarized in Table 1.

Table 1. Phytochemical constituents of extracts of *Grewia orientalis* leaves

Phytochemical	Methanol	Ethanol	Acetone
Flavonoids	+	+	+
Alkaloids	+	+	+
Tannins	+	+	+
Saponins	+	-	+
Steroids	+	+	+
Glycosides	+	+	+
Terpenoids	-	+	-
Triterpenoids	-	+	-
Anthroquinones	-	-	-

+ = Present
- = Absent

The phytochemical compounds are known to act by different mechanism and exert antimicrobial action. The mechanism of alkaloids remains unclear. Sawer et al. [34] demonstrated that the alkaloid, cryptolepine, causes cell lysis and morphological changes of *S. aureus*, but the antimicrobial effects of the alkaloid may be through another mechanism, since the compound is known to be a topoisomerase II inhibitor and inhibits the primarily DNA synthesis [35]. As a response to microbial infection, plants are known to synthesize flavonoids. Various studies have revealed that flavonoids exhibit anti-inflammatory, vascular activities, antioxidant, antimicrobial and others medicinal properties [36-39]. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls [40]. Tannins exert its antimicrobial activity through iron deprivation or binding to proline rich proteins and interfere with the protein synthesis [41,42]. The leakage of proteins and certain enzymes from the cell is the result of the antimicrobial property of saponin [43]. Steroids specifically associate with membrane lipid and exert its action by causing leakages from liposomes [44]. Steroids, terpenoids and triterpenoids have been reported to have antibacterial properties [45-50]. Many plants contain non-toxic glycosides that can get hydrolyzed to release phenolics that are toxic to microbial pathogens [51]. Therefore, the antibacterial activity of methanol, ethanol and acetone extracts of *Grewia orientalis* leaves may be due to the presence of detected phytochemical compounds.

The methanol, ethanol and acetone extracts of *Grewia orientalis* were tested against the pathogenic microbes viz., *E.coli* (virulent strains of *E.coli* can cause gastroenteritis, urinary tract infections, neonatal meningitis), *Klebsiella pneumonia* (a cause for pneumonia), *Staphylococcus aureus* (which may cause septicemia, endocarditis and toxic shock syndrome), *Pseudomonas aeruginosa* (causes infection of urinary tract, respiratory system, gastrointestinal, soft tissue, bone joints and also causes dermatitis, bacteremia), *Bacillus substilis* (generally it is not a human pathogen, but contaminate food and causes food poisoning). The results obtained in the present investigation showed that the tested methanol, ethanol and acetone leaf extracts of *Grewia orientalis* possess potential antibacterial activity against the *E.coli, Bacillus substilis*, *Staphylococcus aureus, Pseudomonas aeruginosa and Klebsiella pneumoniae.* Neither of the extracts (methanol or ethanol or acetone) was able to inhibit *Bacillus thuringienis.* The acetone extract showed considerably more antibacterial activity than the methanol and ethanol extracts.

Ethanol leaf extract of *Grewia orientalis* showed varied in the zone of inhibition from 13.1-15.4 mm against the tested bacteria (Table 2). Lowest activity was observed in *Pseudomonas aeruginosa* (13.1 mm zone of inhibition) and highest activity in *Bacillus substilis* (15.4 mm zone of inhibition). Methanol leaf extract of *Grewia orientalis* was found to inhibit the bacterial growth with zone sizes in the range of 13.6-16.1 mm (Table 2). The methanol extract was found to have strong antibacterial activity against *Staphylococcus aureus* with inhibition zone of 16.1 mm and weak activity against *Pseudomonas aeruginosa* with inhibition zone of 13.6 mm. The acetone extract of *Grewia orientalis* showed zones of inhibition ranging from 15.1 to 16.8 mm against the test bacterial species (Table 2). The results showed that acetone extract was significantly active against *Pseudomonas aeruginosa*. It showed less inhibition zone in *Klebsiella pneumoniae* and no inhibition zone was seen in *Bacillus thuringienis*.

Table 2. Antibacterial activities of extracts of *Grewia orientalis* leaves against the selected organisms

Organism	Zone of Inhibition** (mm) at a concentration of 1 mg/ml			
	Methanol	Ethanol	Acetone	Ampicillin*
Escherichia coli	15.1 ± 0.381	14.5 ± 0.248	16.2 ± 0.478	10.3 ± 0.359
Bacillus substilis	14.7 ± 0.348	15.4 ± 0.436	16.1 ± 0.331	17.6 ± 0.423
Staphylococcus aureus	16.1 ± 0.460	15.3 ± 0.624	16.8 ± 0.624	19.7 ± 0.675
Pseudomonas aeruginosa	13.6 ± 0.214	13.1 ± 0.514	14.7 ± 0.416	15.9 ± 0.269
Klebsiella pneumoniae	13.8 ± 0.764	14.0 ± 0.560	15.1 ± 0.512	24.6 ± 0.623
Bacillus thuringienis	ND	ND	ND	ND

* = 25 μg/ml

** = Values are mean inhibition zone (mm) ± S.D of three replicates

ND = not detected

The acetone extract shows more antibacterial activity when compared with methanol and ethanol extracts. This indicates that acetone leaf extract of the *Grewia orientalis* was the most successful solvent in extracting phytochemical compounds responsible for the antibacterial property than methanol and ethanol solvents. All the three extracts of *Grewia orientalis* were found to have strong antibacterial activity against gram positive strains *Bacillus substilis* and *Staphylococcus aureus.* The inhibition zones for the standard antibiotic ampicillin against all bacterial strains ranging from 10.3 to 24.6 mm (Table 2).

The MIC of the three extracts ranged from 50 to 250 μg/ml. Crude extracts of *Grewia orientalis* leaves in all three solvents; methanol, ethanol and acetone exhibited the lowest MIC value (50 μg/ml) against *Pseudomonas aeruginosa* and *S. aureus* with methanol extract, *Bacillus substilis, S. aureus* and *Klebsiella pneumoniae* with ethanol extract and *Bacillus substilis* and *S. aureus* with acetone extract. Low MIC indicated high antibacterial efficacy of plant extracts. The results of MIC were summarized in Table 3.

Table 3. Minimum Inhibitory Concentration of extracts of *Grewia orientalis* leaves

Organism	Minimum Inhibitory Concentration* (μg/ml)		
	Methanol	Ethanol	Acetone
Escherichia coli	250	100	100
Bacillus substilis	250	50	50
Staphylococcus aureus	50	50	50
Pseudomonas aeruginosa	50	100	100
Klebsiella pneumoniae	100	50	100

* = Values are mean Minimum Inhibitory Concentration (μg/ml) ± S.D of three replicates

Conclusions

This study for the very first time reported the antibacterial activity of *Grewia orientalis.* Methanol, ethanol and acetone extracts of *Grewia orientalis* leaves shows varying degrees of antibacterial activity on the microorganisms tested. The results of this study showed that the acetone extract exhibited a stronger antibacterial activity, followed by methanol and ethanol extracts. The minimum inhibitory concentration of the extracts ranged from 50 to 100 µg/ml (acetone and ethanol extracts) and 50 to 250 µg/ml (methanol extract). *Grewia orientalis* could be a source of new antibiotic compounds. Further studies have to be done in the isolation of the secondary metabolites from the extracts which have been studied so far to test their specific antibacterial activity.

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