Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(2): 63-66
Received: 21-01-2016
Accepted: 22-02-2016

Jinku Bora
Department of Food Technology, Faculty of Interdisciplinary Science and Technology, Jamia Hamdard, New Delhi-110062, India.

Sourav Chakraborty
Department of Food Engineering and Technology, School of Engineering, Tezpur University, Tezpur-784028, India.

Charu L Mahanta
Department of Food Engineering and Technology, School of Engineering, Tezpur University, Tezpur-784028, India.

Correspondence
Jinku Bora
Department of Food Technology, Faculty of Interdisciplinary Sciences and Technology, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi-110062, Delhi, India.

Antimicrobial activity of selected medicinal plants against the pathogenic bacteria isolated from soil

Jinku Bora, Sourav Chakraborty, Charu L Mahanta

Abstract
Microbes are omnipresent in nature and soil is the favourable nourishing flora and fauna for the growth of the microbes. In the present study, soil samples were collected from hospital nearby areas and pathogenic bacteria were isolated and identified. The three isolates were cultured and two of them were identified to be as *Micrococcus spp.* and other one as *Pseudomonas spp.* Three Indian medicinal plants: *Ocimum sanctum*, *Azadirachta indica* and *Glycyrrhiza glabra* were selected to examine the antimicrobial activity against the *Micrococcus species* and *Pseudomonas species* using Agar well diffusion method. Methanolic extraction of the plant leaves and roots were done and phytochemical screening of these plants was performed for constituents such as flavonoids, saponins, terpenoids, glycosides, tannins, anthraquinones, alkaloids, etc. Among all the plants *Azadirachta indiaca* showed maximum antimicrobial activity (Minimum Inhibitory Concentration ranged from 0.05-0.1 mg/ml) against the isolated microbes. However, the isolated microbes were susceptible to the methanolic extracts of the above mentioned plants. In future, these plants can be further subjected to isolation of the therapeutic antimicrobials for health purposes and food applications.

Keywords: medicinal plants**,** phytochemicals**,** antimicrobial activity**,** therapeutic antimicrobials

1. Introduction
Soil microbiota represents one of the primitive evolutionary origins of antibiotic resistance and is a reservoir of resistance genes available for exchange with clinical pathogens [1]. A gram of soil may contain up to 5000 or more different species of bacteria [2]. Multidrug resistant strains of pathogenic bacteria such as *Escherichia coli* and *Klebsiella pneuminiae* are prominent in hospital areas and are increasing being isolated from community acquired infections [3]. *Pseudomonas aeruginosa* can be found in nature from sources as diverse as water, soil and plants. *P. aeruginosa* is more widely known as an opportunistic pathogen for humans and animals than as soil bacteria and can produce severe infections in immune-compromised hosts [4]. So, soil from the hospital nearby areas was thought to be the suitable source for the isolation of pathogenic bacteria.

Plants are rich in wide variety of secondary metabolites known as phytochemicals such as tannins, flavonoids, steroids, terpenoids, napthoquinone, inulin, alkaloids, water soluble phenols and phlobtannins. These phytochemicals have wide applications from the ancient times in the treatment of infectious diseases and have fewer side effects and reduced toxicity. Previous studies were carried out on the antimicrobial activity of methanolic extracts of *Acacia nilotica*, *Sida cordifolia*, *Tinospora cordifolia*, *Withania somnifer* and *Ziziphus mauritiana* and they showed significant antimicrobial activity [5]. A study reported that *Cinnamomum zeylanicum* bark can be used to design reliable and safer herbal drug and could be used in the pharmaceutical preparations for the treatment of infectious and malignant diseases [6]. So, the agar diffusion study and phytochemical screening of the extracts of *Glycyrrhiza glabra* roots, *Ocimum sanctum* leaves and *Azadirachta indica* leaves can help in the understanding of the antimicrobial activity of the extracts.

In the present study, biochemical, confirmatory, antibiotic sensitivity and some additional tests was done to identify the isolated bacteria from soil. Based on the above considerations, agar well diffusion study, phytochemical tests were carried out to examine the antimicrobial activity of the medicinal plants extract against the isolated bacterial species. The aim of the research is to search for the effective medicinal plants with significant antimicrobial activity against pathogens.

2. Material and Methods

2.1 Sample preparation

Soil sample were collected from the hospital areas of Delhi, India. It was stored at room temperature until use. 0.5g of the collected soil sample was dissolved in 5mL of normal saline (NS) in a test tube. Shake the test tube for about 15 minutes. Serial dilution was done until a dilution of 10^{-6} was obtained. Pour plate, spread plate and streak plate method of pure culture techniques were performed in nutrient agar medium with incubation time and temperature for 48 hrs and 37 ˚C respectively to have well isolated colonies.

Three medicinal plant samples namely *Glycyrrhiza glabra* roots, *Ocimum sanctum* leaves and *Azadirachta indica* leaves were purchased from a local market of Delhi, India. The plant samples were washed, dried in hot air oven maintained at 40-50 ˚C for 24-48 hrs. 100 g of the dried sample was extracted with methanol in Soxhlet apparatus. The dried plant extract was then dissolved in DMSO (Dimethyl sulfoxide) and stored at -80 ˚C for further use.

2.2 Biochemical and Confirmatory tests of isolates

Gram staining technique was applied to differentiate the isolated bacterial species. Biochemical tests such as Catalase test, Indole test, Methyl Red test, Voges Proskauer test and Citrate test were carried out for the identification of the isolates [7]. Confirmatory test by observing the growth of the isolates on MacConkey agar medium, Cetrimide agar, Mannitol salt agar, Nutrient broth (1% starch + 1% glycine), 5% NaCl agar and 10% NaCl agar were also performed to aid the identification process [8]. The growth of the isolates on nutrient agar medium at different temperatures (5 ˚C, room temperature and 42 ˚C) was also observed.

2.3 Antibiotic sensitivity of the isolates

The isolated bacterial cultures were inoculated in Nutrient broth and kept overnight in incubator at room temperature for 3-5 days. Nutrient agar was prepared and autoclaved for 15-20 min at 15 psi. The agar was poured in sterile petriplates and kept for cooling. Antibiotic sensitivity test was done to check the sensitivity of the isolates against 12 antibiotics namely Ampicillin (2mg/ml), Tetracycline (2mg/ml), Chloramphenicol (2mg/ml), Nalidixic acid (2mg/ml), Ciprofloxacin (2mg/ml), Penicillin (2mg/ml), Erythromycin (2mg/ml), Rifamycin (2mg/ml), Dapson (2mg/ml), Gentamycin (2mg/ml), Amoxycillin (2mg/ml) and Isoniazid acid (2mg/ml).

2.4 Phytochemical screening of plant extracts

The dried plant extract was tested for the presence of phytochemicals. Phytochemicals screening tests were done for the presence of flavonoids, saponins, tannins, steroids, terpenoids, napthoquinone, inulin, alkaloids, water soluble phenols and phlobtannins [9]. Additional tests such as Biuret test, Iodine test, Ninhydrin test, Molish test and Benedict's test were also checked for the presence of proteins, starch, amino acids, carbohydrates and reducing sugar respectively.

2.5 Determination of Minimum Inhibitory Concentration

Sensitivity of the bacterial isolates was also checked against the three medicinal plant extracts. To perform the tests, 50 µl of the bacterial isolates cultured in Nutrient broth were spread in the agar containing petri plates with the help of an L-shaped glass spreader. Wells of size 9mm were made in the agar plates and 100 µL of the antibiotic or plant extract were poured in the wells. The plant extract or antibiotic was allowed to diffuse in the well for some time and then the petriplates were kept overnight in the incubator at room temperature. Diameters of zone of inhibition (if formed) were measured in mm to determine the sensitivity of the isolates against the antibiotics and plant extracts. Data from the zone of inhibition of the plant extracts were used to determine the minimum inhibitory concentration (MIC).

3. Results and Discussion

3.1 Identification and antibiotic sensitivity of isolates

The soil samples were collected from 10 different places and after culturing three isolates were selected for further studies. We have done the biochemical tests and confirmatory tests against all the isolates, and checked in the probabilistic identification database (www.microbeid.com) for the identification of the bacterial isolates. The results of the biochemical tests and the confirmatory tests (Table 1), when compared with the online database predicted that Orange isolates and Yellow isolates belong to the *Micrococcus spp.* and the green isolate belong to *Pseudomonas spp.* The three isolates from the soil sample were found to be multiple drugs resistant (Orange isolate, Yellow isolate and Green isolate), so only their biochemical tests were done. The Orange colony and Yellow isolate was found to be resistant against Ampicillin, Amoxicillin and Isoniazid acid. The Green isolate was found to be resistant against Penicillin, Chloroamphenicol, Dapson, Ampicillin, Amoxicillin and Isoniazid acid. The resistance to antibiotics may be due to the presence of resistance genes in the bacteria. Moreover, expression of the resistant genes in bacteria is controlled by the environment and may acquire resistant to antibiotics [10].

Table 1: Biochemical tests and confirmatory tests result of the isolated bacterial cultures.

Bacterial isolates	Biochemical tests and Confirmatory tests	
	Positive results	**Negative results**
Orange isolate	Glucose, Lactose, Sucrose, Citrate, Catalase, Gelatine, Dextrose, Maltose, Mannitol salt agar, 5% NaCl, 10% NaCl, 5 ˚C growth, room temperature growth, 42 ˚C growth and King's B media.	Indole, Methyl red, Voges Proskauer, Urease, Starch, Cetrimide and MacConkey's agar.
Yellow isolate	Glucose, Lactose, Sucrose, Catalase, Gelatine, Dextrose, Maltose, Mannitol salt agar, 5% NaCl, 10% NaCl, room temperature growth, 4 2 ˚C growth and King's B media.	Indole, Methyl red, Voges Proskauer, Citrate, Urease, Starch, 5 ˚C growth, Cetrimide and MacConkey's agar.
Green isolate	Glucose, Lactose, Sucrose, Citrate, Gelatine, Dextrose, Maltose, 5% NaCl, 42 ˚C growth, room temperature growth, Cetrimide, King's B media and MacConkey's agar.	Indole, Methyl red, Voges Proskauer, Urease, Catalase, Starch, Mannitol salt agar, 10% NaCl and 5 ˚C growth.

3.2 Phytochemical tests

In a research study, 55 plant methanolic extracts were investigated for antimicrobial activity against 13 phyto-pathogens of *Gossypium* using agar ditch diffusion method. In vitro antimicrobial assays reported that 6 plant extracts exhibited antimicrobial activity and among them the highest activity was observed from the root extracts of *Rubia cordifolia* and *Glycyrrhiza glabra*. Qualitative phytochemical tests and column chromatography of the two extracts

demonstrated the presence of anthraquinones and flavonoids as the active constituents [11]. In present study, the antibacterial activity of various concentrations of the methanolic *O. sanctum* leaves, *A. indica* leaves and *G. glabra* root extracts were evaluated by the agar cup diffusion method against all the three isolated pathogenic bacteria. Phytochemical tests of the plant extracts indicated the presence of the inulin, water soluble phenols and alkaloids in all the three plant extracts, terpenoids in *O. sanctum* and *G. glabra* extracts, and tannins, flavonoids and steroids only in *G. glabra* extracts (Table 2). All the methanolic plant extracts showed good antimicrobial activity against the three isolated pathogenic bacteria. The presence of the phytochemicals in the plant extracts could be responsible for the observed antimicrobial activity against the pathogenic bacteria.

Table 2: Phytochemical and some additional tests of the three plant extracts, (+) indicate the presence of the compound in the extracts and (-) indicate the absence of the compound in the extracts.

Phytochemical tests and additional tests	*Azadirachta indica*	*Ocimum sanctum*	*Glycyrrhiza glabra*
Flavonoids, Tannins, Steroids	–	–	+
Saponins, Napthoquinone and Phlobtannins	–	–	–
Terpenoids	–	+	+
Inulin, Alkaloids, Water soluble phenols	+	+	+
Protein, Starch	–	–	–
Amino acids	+	+	+
Carbohydrates	+	+	–
Reducing sugar	+	–	+

3.3 Minimum Inhibitory Concentration

Acacia nilotica, *Syzygium aromaticum* and *Cinnamum zeylanicum* ethanolic extracts had strong antimicrobial activity against the multidrug resistant strains of *E. coli*, *K. pneumoniae* and *C. albicans*. The most potent antimicrobial plant was *A. nilotica* with MIC ranged 9.75 – 313 µg/ml [12]. Another study reported that the hexane, chloroform and ethanolic extracts of *Mallotus philippensis* had concentration dependent antimicrobial activity against *Aspergillus flavus* and *C. albicans* with the zone of inhibition ranged from 16-22 mm at various concentrations [13]. In the current research, the zone of inhibition ranged from 9-22 mm, 9-20 mm and 9-20 mm for the methanolic extracts of *A. indica*, *O. sanctum* and *G. glabra* respectively in various concentrations (0.05-80 mg/ml), during the agar well diffusion study against the three isolated pathogenic bacteria (Figure 1). *A. indica* and *O. sanctum* methanolic extracts showed the maximum zone of inhibition for the orange isolates, whereas *G. glabra* methanolic extracts showed maximum zone of inhibition for the green isolates. *G. glabra* showed quiet similar antimicrobial activity against the orange and yellow isolates. The most potent antimicrobial plant in the study was *A. indica* (MIC range 0.05 – 0.1 mg/ml), whereas other two plant extracts studied in this report were found to exhibit higher MIC values than *A. indica*.

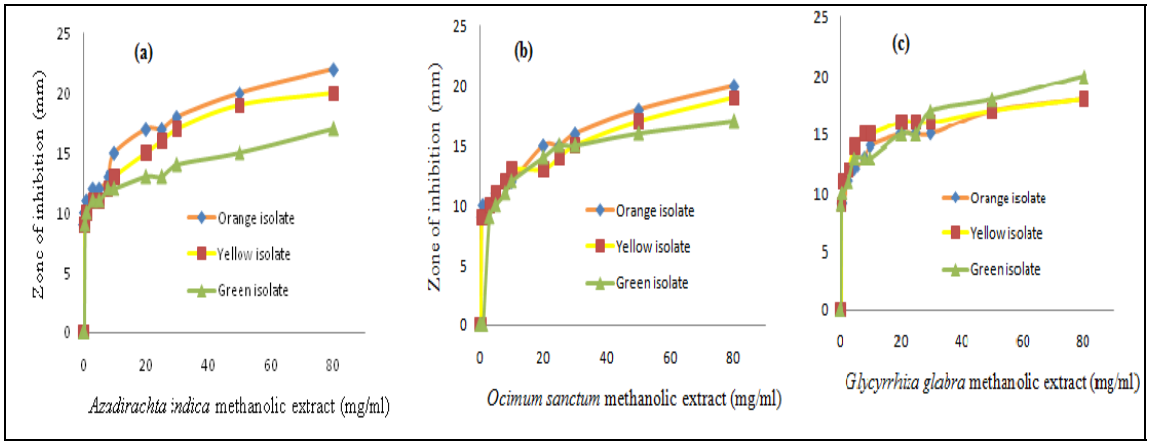

Fig 1: Plots of zone of inhibition (mm) against the plant extract dilutions (mg/ml): (a) Sensitivity of the isolates for *Azadirachta indica* extract; (b) Sensitivity of the isolates for *Ocimum sanctum* extract; and (c) Sensitivity of the isolates for *Glycyrrhiza glabra* extract.

The study can lead to the better understanding of the role played by the phytochemicals in inhibiting the growth of the pathogenic bacteria. The results of the present work clearly indicate that antimicrobial activity vary with the species of plants used and is a concentration dependent activity. However, further research is necessary to explore the antimicrobial activity of the plant extracts against the pathogenic bacteria.

4. Conclusions

This study concludes that *A. indica*, *O. sanctum* and *G. glabra* can be used against the multidrug resistant or pathogenic bacteria. So, there is a clear need for exploration of new antimicrobial agents with novel mode of action from plant sources and to study the potentiality for applications in food systems. Considering the impact of the antibiotics on pathogens and normal flora, search for potential antimicrobial agents from plant sources is a good alternative aspect. Pharmacological evaluations and understanding the mechanism of these biologically active compounds in the inhibition of the pathogens is a promising area of research. Future research is also necessary to use the phytochemicals as food additives or as functional ingredients in food.

5. Acknowledgements
The authors thank Mr. Kapil Kumar and Mr. Anup Kumar for their help in the field work.

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