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Antibacterial evaluation and phytochemical analysis of *Medicago sativa* L. against some microbial pathogens

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Abstract

Successive solvent extract *viz*., petroleum ether, chloroform, benzene, methanol, ethanol and water extracts of *Medicago sativa* was evaluated for antibacterial activity, against seven important bacterial strains by agar-well diffusion method. Methanol extract showed significant activity against all the tested bacteria followed by chloroform and ethanol extract. Benzene and Petroleum ether extracts did not show any significant activity. The antibacterial activity is more significant in solvent extracts compared to aqueous extract in all the plants indicating that the active principle responsible for antibacterial activity is more soluble in organic solvents. The results suggest that *Medicago sativa* is scientifically validate the use of this plant in the traditional medicine for isolation and characterization of the active principle for further exploitation in medical microbiology.

Keywords: *Medicago sativa*, Antibacterial activity, Methanol, Well diffusion, Phytochemical analysis

Introduction

Infectious diseases account for about half of the death in tropical countries. Antibiotic resistance has become a global concern. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multi-drug resistant pathogens (Parekh & Chanda, 2007). Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Medicinal plants remain the most common source of antimicrobial agents. This usage as traditional health remedies is the most popular for 80% of world population in Asia, Latin America and Africa and is reported to have minimal side effects (Safary *et al*., 2009). There are about 45,000 plant species in India with concentrated hotspot in the region of Eastern Himalayas, Western Ghats and Andaman and Nicobar islands. Approximately 20% of the plants found in the world have been submitted to pharmacological or biological tests (Shariff *et al*., 2006). The systemic screening of antimicrobial plant extracts represents a continuous effort to find new compounds with the potential to act against multidrug resistant pathogenic bacteria and fungi.

Description (*Medicago sativa* L.)

Perennial herb; stems erect or sometimes decumbent, 0.3–1 m long, 5–25 or more per crown, much-branched, 4-angled, glabrous or the upper part hairy; rhizome stout, penetrating the soil as much as 7-9 m; stipules united 1/3 to 1/2 length, free portion triangular-lanceolate, tapering, basally entire or with 1-2 teeth, glabrous or sparingly appressed-hairy; leaves pinnately trifoliolate; leaflets obovate-oblong, ovate or linear, tapering to base, crenate above middle mostly retuse and mucronate, 10–45 mm long, 3–10 mm broad, glabrous or appressed hairy, paler green beneath; racemes oval or rounded, 1–2.5 cm long, 1–2 cm broad, axillary, 5–40-flowered; peduncle slender, firm, always exceeding the subtending leaf, glabrous or appressed-hairy; calyx tubular, with linear-subulate teeth longer then tube; corolla yellow or blue to purple or violet, 6–15 mm long; bracteoles whitish, linear-subulate, mostly equaling the pedicel; pod slightly pubescent or glabrous, 3–9 mm in diameter, with 2–3 spirals, prominently reticulate-veined; seeds 6 or 8 per pod, yellow, castaneous or brown, ovoid, irregularly cordate or reniform.

Pharmacological Properties

Medicago sativa (Leguminosae), which is one of the most reputed medicinal plant traditionally used to improve the memory, to cure kidney pain, cough, sore muscles, as rejuvenator, antidiabetic, antioxidant, anti-inflammatory, antimicrobial and in CNS disorders. Moreover, MS has a long tradition of use as ayurvedic and homoeopathic medicine in CNS disorders. Phytochemical reports on *M. sativa* indicate that the plant contains flavonoids, alkaloids, phytoestrogens, coumarins, digestive enzymes, triterpenes, saponins and phytosterols. Several clinical and animal studies indicate that the ingestion of MS reduces cholesterol absorption and atherosclerotic plaque formation in the arteries. MS is beneficial in cardiovascular complaints, convalescence and debility, diabetes and also when used as a tonic after blood loss and during anemia. The plant has been shown to have anti-tumor activity against certain types of leukemia cells in mice and selective toxicity in dog cancer cells grown in vitro (Kundan Singh Bora & Anupam Sharma, 2011).

Materials and Methods

Fresh plant materials were collected from the villages of Coimbatore District, Tamilnadu, India. The collected plant was authenticated by Botanical Survey of India (Souhern Circle), Coimbatore and the voucher specimen of the plant has been preserved at RVS Microbiology Laboratory. The collected plants were washed with running tap water, air dried, homogenized to a fine powder and stored in air-tight bottles at 4^0C.

Solvent extraction

About 100 g of dried plant material was extracted with 200 mL of petroleum ether kept on a rotary shaker for 24 h. Thereafter, it was filtered and centrifuged at 5000 g for 15 min. The supernatant was collected and the solvent was evaporated to make the final volume as $1/5^{th}$ of the original volume (Sasikumar *et al*., 2005). In the

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Table 1. Antibacterial activity of different crude extracts of Medicago sativa on some important microbial pathogens

Microorganims	Zone of Inhibition in mm (mean ± SD)						
	P	E	C	B	M	Water	Chlo
S.aureus	-	11.6 ± 0.1	12.1 ± 0.1	-	18.1 ± 0.05	10.1 ± 0.1	23
S.pyogens	-	10.2 ± 0.1	10.9 ± 0.05	-	14.9 ± 0.15	9.0 ± 0.1	21
E.coli	-	-	10.1 ± 0.1	-	11.9 ±0.15	-	21
P.mirabilis	-	9.3 ± 0.01	8.0 ±0.1	-	8.8 ± 0.12	-	23
P.aeruginosa	-	8.9 ± 0.05	-	-	13.8 ±0.15	8.1 ± 0.05	14
S.typhi	-	-	6.9 ± 0.05	-	9.5 ± 0.11	-	20
K.pneumoniae	-	9.13 ± 0.11	10.0 ± 0.11	-	8.3 ± 0.05	8.19 ± 0.11	15

P- Petroleum ether, C-Chloroform, M-Methanol, E-Ethanol, B-Benzene, Cho - Chloramphenicol; Values are mean inhibition zone (mm) ± SD of replicates (-) no activity; Concentration of the extracts was 100mg/ml

same conditions was obtained benzene, chloroform, methanol and ethanol extracts. It was stored at 4°C in airtight bottles for further studies.

Aqueous extraction

About 100 g of dried plant material was extracted in distilled water for 6 h at slow heat for every 2 h, it was filtered through, 8 layers of muslin cloth and centrifuged at 5000 g for 15 min. The supernatant was collected. This procedure was repeated twice and after 6 h the supernatant was concentrated to make the final volume 1/5th of the original volume (Sasikumar *et al.*, 2005).

Phytochemical components

This was carried out according to the methods described by Trease and Evans (1997). Qualification phytochemicals analysis of the crude extracts of the selected plant was used for the identification of phytochemicals like tannins, alkaloids, steroids, phenols terpenoid and flavonoid etc.

Bacterial strains

Microorganisms were obtained from the Microbial Type Culture Collection Centre (MTCC), Chandigarh, India. Amongst the seven microorganisms investigated, two Gram-positive bacteria are *Staphylococcus aureus & Streptococcus pyogens* MTCC 1928, while five Gram-negative bacteria were *Proteus mirabilis* MTCC 425, *Escherichia coli* MTCC 2961, *Pseudomonas aeruginosa* MTCC 4676, *Klebsiella pneumoniae* MTCC 432 and *Salmonella typhi* MTCC 733. All the microorganisms were maintained at 4^{0}C on nutrient agar slants.

Antibacterial activity

The antimicrobial assay was performed by agar well diffusion method for solvent extract. The molten Mueller Hinton agar was inoculated with 100 µl of the inoculum (1 x 10^8 cfu/ml) and poured into the Petri plate (Hi-media). For agar disc diffusion method, the disc (0.7 cm) (Hi-Media) was saturated with 100 µl of the test compound, allowed to dry and was introduced on the upper layer of the seeded agar plate. For agar well diffusion method, a well was prepared in each plates with the help of a cork-borer (0.85 cm). 100 µl of the test compound was introduced into the well. The plates were incubated overnight at 37^{0}C. Antimicrobial activity was determined by measuring the diameter of zone of inhibition around the colonies. For each bacterial strain, controls were maintained where pure solvents were used instead of the extract. The experiment was done thrice and the mean values are presented.

Results

Different organic solvents and water extracts were tested at 50µl concentrations against seven important microbial pathogens (Table 1). Among the five solvents (Petroleum ether, benzene, chloroform, methanol and ethanol) the tested against seven microbial pathogens, methanol, chloroform and ethanol extracts recorded significant antibacterial activity against all the tested pathogens. Antibacterial activity was not observed in petroleum ether and benzene extracts against all the pathogens. Among methanol, chloroform and ethanol extracts, methanol extracts recorded significant antibacterial activity followed by chloroform and ethanol. *Staphylococcus aureus* was to be found highly susceptible to methanol extract, where as *Klebsiella pneumoniae* was less susceptible to methanol extract but was found highly susceptible to chloroform extract. Methanol extract exhibited similar antibacterial activity against, *Streptococcus pyogens*, *E.coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Proteus mirabilis*. Antibacterial activity of water extract varied greatly among the tested pathogenic bacteria. Highest antibacterial activity was observed against *Staphylococcus aureus* followed by *Streptococcus pyogens*, even though antibacterial activity was observed against other pathogens, it was not found significant.

Phytochemical analysis revealed that cardiac glycosides are generally present in all the extracts. Steroids were found only in Petroleum ether extract and flavonoids in ethanol extract. Tannins were found only in benzene extract. Other secondary metabolites such as alkaloids, saponins and oil and gums were absent in all the extracts (Table 2).

Table 2. Preliminary phytochemical analysis of Medicago sativa

Phytochemicals	P	E	C	B	M	Water
Alkaloids	-	-	-	-	-	-
Tannins	-	-	-	+	-	-
Cardiac glycosides	+	+	+	+	+	+
Steroids	+	-	-	-	-	-
Saponins	-	-	-	-	-	-
Flavonoids	-	+	-	-	-	-
Oil and Gums	-	-	-	-	-	-

P- Petroleum ether, C-Chloroform, M-Methanol, E-Ethanol, B-Benzene; (+) - Present, (-) - absent

Discussion

All plant parts synthesize some chemicals by themselves, to perform their physiological activities. In our present study, the investigated plants have exhibited different kinds of secondary metabolites. The medicinal value of these secondary metabolites is due

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to the presence of chemical substances that produce a definite physiological action on the human body. The most important of these substances include, alkaloids, glucosides, steroids, flavonoids, fatty oils, resins, mucilages, tannins, gums, phosphorus and calcium for cell growth, replacement, and body building (Kubmarawa *et al*., 2008).

Gram-positive bacterial strains were more susceptible to the extracts when compared to gram negative bacteria. This may be attributed to the fact that these two groups differ in their structure of the cell wall components. The ability of tannin compounds to cause the bacterial colonies to disintegrate, probably results from their interference with the bacterial cell wall (Venkataswamy *et al*., 2010).

Saponins are glycosides occurring widely in plants. They are abundant in many foods consumed by animals and man. Saponin is used as mild detergents and in intracellular histochemistry staining to allow antibody access to intracellular proteins. In medicine, it is used in hypercholesterolemia, hyperglycemia (Rupasinghe *et al*., 2003), antioxidant, anti-cancer, anti-inflammatory (Manach *et al.,* 1996) and central nervous system activities (Argal & Pathak, 2006). Plant steroids are known to be important for their cardiotonic activities, possession of insecticidal, anti-inflammatory (Akindele & Adeyemi, 2007), analgesic properties (Malairajan *et al.,* 2006), central nervous system activities (Argal & Pathak, 2006) and antimicrobial properties. They are also used in nutrition, herbal medicine and cosmetics.

Tannins were reported to exhibit antidiabetic (Cherian & Augusti, 1995), anti-inflammatory (Latha *et al.,* 1998), antibacterial and antitumor activities. It has also been reported that certain tannins were able to inhibit HIV replication selectively besides use as diuretics. Plant tannins have been widely recognized for their pharmacological properties and are known to make trees and shrubs a different meal for many caterpillars (Haslem, 1989). Glycosides were reported to exhibit anti-diabetic characteristics (Cherian & Augusti, 1995). Cardiac glycosides on the other hand are known to hamper the Na^+ / K^+ pump. This results in an increase in the level of sodium ions in the myocytes which then enhance the level of calcium ions. This consequently increases Ca^{2+} ions available for contraction of the heart muscle, which improves cardiac output and reduces distention of heart and thus are used in the treatment of congestive heart failure and cardiac arrhythmia.

The results of the present study support the traditional use of the *Medicago sativa* as an ethnomedicine. It also suggests that the tannins isolated from the *Medicago sativa* possess remarkable antimicrobial activity against microbial pathogens.

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