

DETERMINATION OF ANTIBACTERIAL PROPERTIES OF *MEDICAGO SATIVA* L. (ALFALFA) EXTRACTS

Miss Anila Ghani, Asma Rehman

Associate professor Government Frontier College for Women's Peshawar,
Student of BS botany frontier college Peshawar

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Abstract: Alfalfa, also called lucerne and called *Medicago sativa* in binomial nomenclature, is a perennial flowering plant in the legume family Fabaceae. Antibacterial activity of different solvent extracts of medicinal plant *Medicago Sativa* Leaves, Root and Stem against clinical pathogen of human origin was found out. The antimicrobial activity of different solvents crude extract of *Medicago Sativa* plant root, stem and leaves was tested by disc diffusion and turbidity assay method against four bacterial pathogens *E. coli*, *S. typhi*, *K. pneumoniae* and *S. aureus*. After the result it showed that the antibacterial activity of the *medicago sativa* extract were increased with the increasing concentration of the crude extracts and polarity of the solvents used for extraction.

Key Words: Anti-microbial activity of *medicago sativa*, *E. coli*, *S. typhi*, *K. pneumoniae* and *S. aureus*, disc diffusion, polarity of *medicago sativa*.

Introduction:

Plants have been considered valuable for their therapeutic and comforting qualities in the whole world. People depend generally on the medicinal plants. The Food and Agriculture Organization estimated in 2002 that over 50,000 medicinal plants are used across the world. The Royal Botanic Gardens, Kew more conservatively estimated in 2016 that 17,810 plant species have a medicinal use, out of some 30,000 plants for which a use of any kind is documented. According to W.H.O 20% of the individuals living in India are utilizing allopathic medicines while the remaining 80% depend on medicinal herbs as essential pill. The reason behind this dependence is that people believe in medicinal plants as a safe medication way. The engineered anticancer cures are past the span of normal

man on account of heavy expense element. The only alternative safe way for cancer therapy is herbal medicines which can play a major role in cancer prevention. Common man can easily afford herbal medicine and medicinal herbs are easily available. (Sakarkar & Deshmukh, 2011) *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, in axillary, 5–40-flowered; peduncle slender, firm, always exceeding the subtending leaf, glabrous or appressed hairy; calyx tubular, with linear-subulate teeth longer than 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 (Doss et al., 2011).

Phytoconstituents are the natural bioactive compounds found in plants. These phytoconstituents work with nutrients and fibers to form an integrated part of defense system against various diseases

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and stress conditions. Phytochemicals are basically divided into two groups, i.e. primary and secondary constituents according to their functions in plant metabolism. Primary constituents comprise common sugars, amino acid, proteins and chlorophyll while secondary constituents consists of alkaloids, terpenoids, saponins, phenolic compounds, flavonoids, tannins and so on. Phytochemicals are naturally occurring biochemicals in plants that help to give plants their characteristic color, flavour, smell and texture. Apart from that, phytochemicals could prevent diseases (including cancer and cardiovascular diseases) and inhibit pathogenic microorganisms (Gomathi et al., 2013).

MATERIAL AND METHODS Plan of work:

The work was planned as:

The work takes 8 month of time period in completion which was performed in a very convenient way step by step.

Step I the collection of the plants

Step II: Crude Extract Prepared

Step III: Determined of Extractive values

Step IV: Determined of different Phytochemicals

Step V: Determined of Antibacterial Activity

Procedure:

Samples Collection:

The plants were collected from district Peshawar and identify were processed for different extractions and antibacterial activity in the Microbiology Laboratory of Abasyn University, Peshawar .

Preparation of Crude Extract:

Vegetative Parts of medicago sativa along with root, were first chopped and then each part powder on the grinding machine. 150gm of powder on root stem and leaves of the plant have been taken and soaked in separate conical flasks (500ml) in distilled methanol (98 %) for overnight. On the next day the diluted extract were decant to another flask and filtered. The diluted extract was kept for evaporation of the solvent on the vacuum rotary evaporator at a temperature between 40-50°C and under reduced pressure. Again Methanol was added to the plant extract. This process was repeated three times for root, stem and leaves separately and the extract was dried completely.

Determination of Extractive values:

The extract obtained was then clarified and concentrated on a rotary evaporator under control pressure and temperature between 30-45°C. The semisolid extract was taken and weighed in a china dish and kept in a water bath at about 45 °C dried. After complete drying these extracts was again weighed and the percentage yield of the extract was calculated using the equation. (Banso and Adeyemo, 2006).

$$\text{Extractive value yield \%} = \frac{\text{Weight of Extract}}{\text{Weight of Ground Plant Material}} \times 100$$

Determination of different organic solvent extraction:

On the bases of miscibility and different electronic configuration, different organic solvent, like hexane, chloroform, ethyl acetate and isobutanol used that phytochemicals were separated from each other. Each compound was evaporated and completely dried on a vacuum rotary evaporator under a control pressure and temperature between 40-50°C. Dried extracts was diluted in 200ml distill water and transfer to separating funnel. About 150ml of Hexane were mixed to the separating funnel and was kept in shaker. Some of the substances were dissolved in the hexane. After 10-15min aqueous portion (water) and hexane were separated because hexane is insoluble in water. Due to high density aqueous portion were beneath the hexane. Three times was washed the aqueous portion with hexane. All the hexane soluble substances were removed from the extract and dried with vacuum rotary evaporator at a temperature 40°C under reduced

pressure. The same process was applied for chloroform, ethyl acetate and isobutanol.

Phytochemical screening;

Phytochemical screening of the prepared extracts was conducted with various qualitative tests to identify the presence of various chemical constituents. Performed the tests of the following chemicals and reagents were used. These were identified by characteristic color changes using standard procedures. (Ghani2003)

Chemical Test:

Tests for Alkaloids: Dragendroff's test. **Mayer's test.**

Tests for Glycosides: Modified

Borntrager's Test. Tests for Flavanoids:

Shinoda test. **Test of Triterpenoids:**

Liebermann -Burchard's test. **Detection of phytosterols:**

Salkowski's Test. **Detection of Saponins: Foam test.**

Detection of phenols: ferric chloride test. Detection of amino acid:

Ninhydrin test.

Antibacterial Activity:

The extract of different parts of the plant has been tested for its sensitivity against the selected resistant bacteria. The species of Bacteria have been obtained from microbiology laboratory Abasyn University, Peshawar. These microbes were grown and further processed for susceptibilities against these extract according to the standard microbiological procedure.

Bacterial strains

Bacterial strains used in this study were the isolated pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhi*. All the strains were confirmed by cultural and biochemical characteristics and maintained in slants for further use.

Medicago SativaL. leaves Phytochemicals extraction:

Leaves dry powder weight 248gm

Round Bottom Flask empty weight 358.5gm

Antibacterial sensitivity testing using Disc diffusion method:

This method depends on the diffusion of antibiotic from vertical cylinder or cavity through the solidified agar layer of petridish or plate to an extent such that growth of added microorganism in a circular area is presented. Inoculum of each of the bacterial strains (single colony) was suspended in 5 ml of broth (nutrient broth) and incubated at 37°C for 18 hr. The antibacterial activity was tested by the disc diffusion assay. 0.1 ml of inoculum (105 CFU/ml) was spread on sterile Mueller Hinton plates and sterile paper discs were placed on the inoculated surface. After solidifying media, holes were made by 5mm cork borer. Each hole was filled with 50ul of plant extract. The discs were impregnated with 50ul of each of the extract at two different concentrations (.2/2mg/ml & 2/5mg/ml), kept at room temperature for absorption of extract in the medium and then incubated at 37°C in the incubator for 24 hr. The antibacterial activity was evaluated by measuring the diameter of inhibition zone as per the procedure described by Kim *et al.* Ciprofloxacin was used simultaneously as control. (Doss et al.2012)

Minimum Inhibitory Concentration (MIC)

For determination of MIC, 1 ml of broth medium was taken into 10 test tubes for each Bacteria. Different concentrations of plant extracts ranging from 0.125 to 8 mg/ml concentration were incorporated into the broth and the tubes were then inoculated with 0.1 ml of inoculum of respective

Results

bacteria (105 CFU/ml) and kept at 37°C for 24 hr. The test tube containing the lowest concentration of extract which showed reduction in turbidity, when compared with control was regarded as MIC of that extract.

The strongest antibacterial activity with MIC values of 0.05mg/ml-0.50mg/ml, moderate activity values between 0.6mg/ml-1.50mg/ml and weak activity above 1.50 mg/ml. (Doss et al.2012)

R B F +Methanolic extract weight 379.8gm

Crude methanolic extract weight 21.3gm

Extractive value yield %age = Weight of Extract/ Weight of Ground Plant Material×100 (1)

Hexane fraction		Ethyl acetate fraction		Isobutanol fraction		Water fraction	
RBF Empty wt	189.9gm	Vail empty wt	13.21gm	RBF empty wt	106gm	RBF empty wt	189.9 gm
RBF+ Extract	194.5gm	Vail+extract	14.021gm	RBF+ extract	109.5gm	RBF+ extract	196gm
Hexane extraction value	4.6gm	Extractiv e value	0.841gm	Extractive value	0.841gm	Extractive value	6.1gm
Extective value yield%	1.9%	Extective value yield%	.339%	Extective value yield%	.339%	Extective value yield%	2.5%

Table2 : List of Medicago sativa different extracts used to evaluate antimicrobial activity.

No	Name	Colour of used extract
1	Ethyl acetate	Light green
2	isobutanol	Dark green
3	Hexane	Green yellow
4	Hot aqueous extract	Green
5	Cold aqueous extract	Green

Medicago sativaL. root Phytochemicals extraction

Root dry powder weight 246gm

China dish empty weight 777.1gm

China dish + extract weight 788.8gm

Crude methanolic extract weight 11.7gm

Table3

Hexane fraction		Ethyl acetate fraction		Isobutanol fraction		Water fraction	
RBF Empty wt	106gm	RBF empty wt	106gm	RBF empty wt	106gm	RBF empty wt	105.8 gm
RBF+ Extract	107.437g m	RBF+ extract	106.8gm	RBF+ extract	108.9gm	RBF+ extract	110.5 gm
Hexane extraction value	1.437gm	Extractiv e value	0.8gm	Extractive value	2.9gm	Extractive value	4.7gm
Extective value yield%	.581%	Extective value yield%	.325%	Extective value yield%	1.17%	Extective value yield%	1.9%

Medicago sativa L. Stem Phytochemicals extraction

Root dry powder weight 153gm

China dish empty weight 358.5gm

China dish + extract weight 378.4gm

Crude methanolic extract weight 19.9gm

Table4

Ethyl acetate fraction		Isobutanol fraction		Water fraction		
106gm	RBF empty wt	113gm	RBF empty wt	106gm	RBF empty wt	113 gm
110.7gm	RBF+ extract	113.5gm	RBF+ extract	108.5gm	RBF+ extract	122.5 gm
4.7gm	Extractive value	0.5gm	Extractive value	2.4gm	Extractive value	9.5gm
3.07%	Extective value yield%	.33%	Extective value yield%	1.57%	Extective value yield%	6.2%



Fig:1

Antibacterial activity of Medicago Sativa leaf against selected pathogens

Table 8

	Extracts	Conc, (mg/ml)	Zone of inhibition(mm)			
			E coli	S.typhi	K.pneumoniae	S aureous
			S17mm	S 22mm	S11mm	S22mm
Medicago Sativa leaves	Hexane	.2/2	14mm	10mm	12mm	Nil
		.2/5	nil	9mm	nil	Nil
	Ethyl acetate	.2/2	15mm	12mm	20mm	13mm
		.2/5	7mm	11mm	nil	10mm
	Isobutanol	.2/2	16mm	10mm	17mm	13mm
		.2/5	nil	12mm	nil	Nil
	Water	.2/2	12mm	10mm	14mm	15mm
		.2/5	nil	7mm	nil	Nil

Table 9
Antibacterial activity of Medicago Sativa stem against selected pathogens

	Extracts	Conc, (mg/ml)	Zone of inhibition(mm)			
			E coli	S.typhi	K.pneumoniae	S aureous
			S17mm	S22mm	S11mm	
Medicago Sativa stem	Hexane	.2/2	13mm	Nil	13mm	20mm
		.2/5	Nil	Nil	Nil	10
	Ethyl acetate	.2/2	15mm	12mm	11mm	14mm
		.2/5	Nil	9mm	11mm	20mm
	Isobutanol	.2/2	Nil	10mm	11mm	15mm
		.2/5	Nil	Nil	Nil	nil
	Water	.2/2	15mm	Nil	Nil	15mm
		.2/5	Nil	Nil	Nil	16mm

Table 10

Antibacterial activity of Medicago Sativa root against selected pathogens

	Extracts	Conc, (mg/ml)	Zone of inhibition(mm)			
			E coli	S.typhi	K.pneumoniae	S aureous
			S17mm	S22mm	S11mm	S22mm
Medicago Sativa root	Hexane	.2/2	12mm	11mm	13mm	19mm
		.2/5	nil	nil	nil	12mm
	Ethyl acetate	.2/2	nil	nil	11mm	26mm
		.2/5	nil	nil	17mm	17mm
	Isobutanol	.2/2	19mm	10mm	11mm	Nil
		.2/5	11mm	nil	nil	16mm
	Water	.2/2	14mm	nil	nil	8mm
		.2/5	nil	nil	nil	19mm

Antibacterial activity of .2mg/2ml Medicago Sativa leaf concentration against selected pathogens

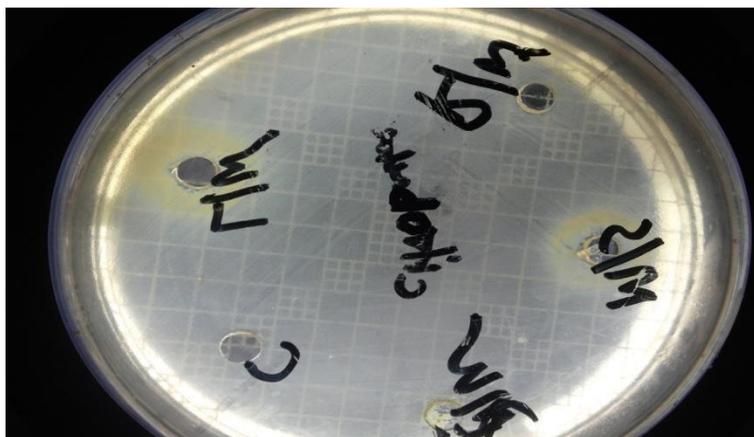


Fig16

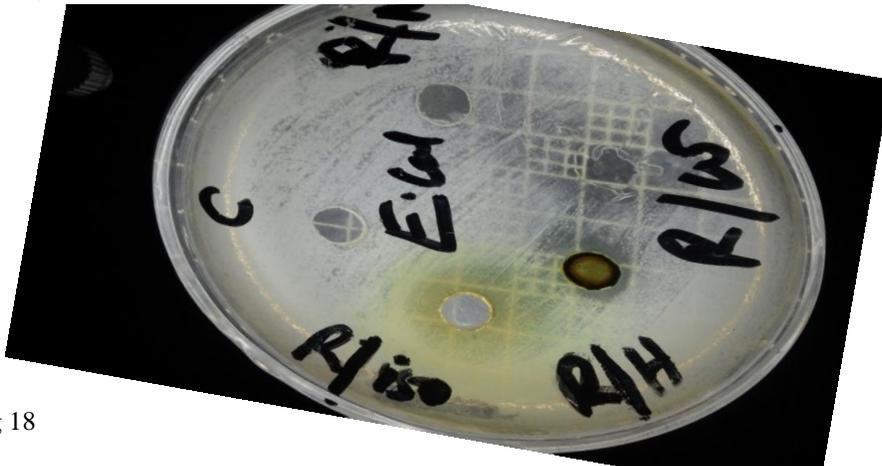


Fig 17ig 18



Fig 19



E.Coli ;stem extract applied

,w=water C= stander E=ethyl acetate ,I=isobutanol, H=exane

In the Disc Diffusion assay the zone of inhibition around the disc impregnated with plant extract over the bacterial culture plates quantitatively determined the antibacterial activity. The result showed that the antibacterial activity of the plant extract were increased with the increasing concentration of the crude extracts and polarity of the solvents used for extraction.

The plates were incubated overnight at 37°C. Antimicrobial activity was determined by measuring the diameter of zone of inhibition around the colonies (Blum, U, 1985). For each bacterial strain, controls were maintained where pure solvents were used instead of the extract.

Medicago Sativa leaves water extract showed more prominent result against *Klebsella pneumoniae* *S aureus*, better result in *E coli*, *S typhi*.

The inhibition activity was determined by measuring the diameter of the clear zone. The diameter of the clear zone indicated the inhibition activity. The iso butanol extract is the most effective against *E.Coli* and *Klebsella pneumonia* followed by Ethyl acetate extract showed significant antibacterial activity against *Klebsella pneumonia*, *S aureus* and *Ecoli*. The hexane extract showed strong inhibition against *K pneumonia* and aqueous extract did not show any significant antibacterial activity.

Antibacterial activity of .2/5mg/ml concentration of *Medicago Sativa* leaf/stem/root against selected pathogens



Fig 21



Fig25

Extract applied on S.Typh

LW=leaf water extract , LIS=Leaf isobutanol extract ,LH=Leaf hexane extract, LE,Leaf ethylacetate extract

RW=Root water extract , RIS=Root isobutanol extract ,RH=Root hexane extract, RE=Root ethylacetate extract

SW=Stem water extract ,SIS=Stem isobutanol extract ,SH=Stem hexane extract, SE=Stem ethylacetate extract



The use of herbs in medicine is common all over world. About one third of adults in the world use of alternative therapies including plants which may be used distinctly or united in a mixtures. In the contrary to chemical Company). drugs, plant sainted to be harmless, due to its natural defilement, pollution, toxicity, substitution. This useess truth maintain the study of medicinal plants and plant compounds utilized in medicine. extract is the only extract showed high antibacterial effect source. However, problems may generate due to wrong identification and require of standardization . However the revolution of man-made antibiotics, the diseases

due to bacteria is silent of main be anxious in medicine, due to the amount of antibiotic anti strains which enhancing the interest to use natural products due to their advanced biodegradability and accessibility. In this view, the study was achieved to discover the antimicrobial activity of Medicago sativa aqueous and solvent extracts on some medically important human pathogen (S. aureus, E. coli, Salmonella typhimurium, and K. pneumoniae) by agar well diffusion method.

All plant parts in order to perform their physiological activities form some chemicals by themselves, in the present study, M. sativa produce different kinds of secondary

metabolites which play an important role medicinally. A plant studies involve extraction of the active component in the plants using organic solvents. But plants as used in traditionally, using organic solvent extraction for the antibacterial properties should not be done often; therefore, in the present study the commonly used plants extracts are made with distilled water , different organic solvent extracts and tested for its antimicrobial effect against *S. aureus*, *E. coli* and *K. pneumoniae* and *S typhimurium*. All plants used in the herbal medicine mostly have best antimicrobial activities as determined earlier.

According to Venkataswamy et al. the Grampositive bacterial strains were more susceptible to the activity of the aqueous *M.sativa* leaf ,stem and root extracts when compared to gram negative bacteria. This may be explained due to the fact that these two groups have different cell wall structure. The capability of tannin compounds which detrmined to be present in *M. sativa* break ups the bacterial colonies, by its disruption with the bacterial cell wall. Considering the above results, our result was found the extreme antibacterial activity in *Medicago sativa* against Gram positive *S.aureus* *K. pneumoniae* , as estimate to Gram negative *E.coli*.as shown in Table (8) and Figure (16) The antimicrobial activity of saponins isolated from *M. Sativa* against selected medically important Gram-positive and Gram-negative bacteria has been examined. Increasing antibiotic activity was observed going from the isobutanol extracts. Activity was found particularly high against Grampositive bacteria (9) declared that the superior antibacterial activity of *M.sativa* extract to *E.coli* and where the antibacterial effects on *E.coli* and *S. aureus* were normal. However this search of antimicrobial activity was carried out on six different extracts of *M. sativa* as shown in (Table 10. The screening step in the startning study for antimicrobial activity was done using the Agar well Diffusion Method. The diameter of the clear zone showed the inhibition activity. The The iso butanol extract is the most effective against *E.Coli* and *Klebsella pneumonia* followed by Ethyl acetate extract showed significant antibacterial activity against *Klebsella pneumonia* ,*S aureus* and *Ecoli*. The hexane extract showed strong inhibition against *K pneumonia* and aqueous extract did not show any significant antibacterial activity.The isobutanol extract is the mainly successful against *S. aureus* followed by other extract observed major antibacterial activity against *Salmonella typhimurium*, *K. pneumoniae*, *S.aureus* and *E. coli*. as shown in Table (8) and Figure (17) The results of our study showed that ethanol extract 5. In-vitro of *Medicago sativa* possess very potential antibacterial effect on *K pneumonia* as shown in Table (9) and Figure (18).,(20,21,22)Also All phytochemical tests results indicated that *Medicago sativa*

contain flavonoids, tannins, alkaloids, saponin and glycosides compounds as shown in Table (9). Antimycobacterial properties of the plant could be due to the abundant flavonoids, saponins and tannins that were found in it Table (3). Phytochemical constituents such as tannins, saponins, flavonoids, alkaloids and several other aromatic compounds are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms, insects and other herbivores Therefore, in order to get valuable antimicrobial activity at low concentrations without any consequences on the health researches should center of attention on the optimization of purification and uses.

CONCLUSIONS

From all the above, it is concluded that the *Medicago sativa* extract exhibited significant antimicrobial activity against microbial pathogens and can be initiated as an choice to chemical antimicrobial drugs, but it entails broader search. Also the data propose that the methanolic extract of *M. sativa* could be a rich source of antimicrobial agents. Those outcomes have been promote to perform further tests to authenticate the precise use of the studied extracts to treat the bacterial and caused by bacteria found to be receptive to our extracts

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