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DETERMINATION AND IDENTIFICATION OF PHYTOCHEMICAL PROPERTIES OF *MEDICAGO SATIVA* L. (ALFALFA) LEAF, STEM AND ROOT EXTRACTS AGAINST VARIOUS PATHOGENS

[a]Anila Ghani*, [b]Summan B. E. Ahmad

[a] Government Frontier College for Women, Peshawar, Pakistan.
[b] Government Frontier College for Women, Department of Botany, Peshawar, Pakistan.

A B S T R A C T

The present study explores the quantitative analysis of the important phytochemicals constituents of plant Medicago Sativa L (Alfalfa) a medicinally important plant. It can be used against many pathogens such as microbes, fungus (onychomycosis, Malassezia furfur), bacteria (Cutibacterium acnes) and also various diseases like heart problems, kidney issues, cholesterol level etc. The various solvent extracts (hexane, ethyl acetate, isobutanol and water) of leaves, stem and roots of M. sativa containing alkaloids, glycosides, flavonoids, saponins, tannins, triterpenoids, sterols, proteins and phenols that cure the mentioned pathogens. Different Tests are performed for identification of the phytochemicals. The leaves extract having higher concentration of phyto-chemicals. The significance of this plant in conventional medicine and the importance of the allocation of these chemical constituents present in the plant are discussed with respect to the role of the plant in ethno-medicine in different countries of the world.

Keywords: Botanical extract, effects on yield.

INTRODUCTION

Those plants which have medicinal properties are of great importance nowadays to the health of personal and the public in general. Many new drugs are being invented through commonly used chemicals obtained from different plants. As bacterial resistance is increasing with time, more efforts are being made to find new remedies. Herbalism is one of effective way to overcome bacterial infections (*Asadi-Samani et al., 2017) (Bahmani et al., 2016) (Kooti et al., 2016) (Sarrafchi et al., 2016) (Rahimfard et al., 2017)* the medicinal importance of plants lies in some phytochemicals, these phytochemicals produce a particular physiological action in the human body. Alkaloids, tannins, flavonoids and phenolic compounds are the most important type of bioactive constituents of plants. Many of the native medicinal plants are used as flavors, condiments and food plants. They are also added to foods recommended for patient for medicinal uses as reported by *(Okwu, 1999, 2001) (Hill, 1952)*.

In the areas of high potential growth of plants, the collection and plant screening is more helpful *(Bahmani et al., 2014)*. Therefore, in order to perform the physiological activities by a plant its all parts form some chemicals by themselves, in the present study, we choose "Medicago sativa", also known as "alfalfa" and "Lucerne", it belongs to the family *Fabaceae (Sadowska et al., 2014)*. It is a perennial flowering plant. Worldwide production was around 436 million tons in 2006 and grown on approximately 30 million hectares worldwide in which North America produced 41%, Europe produced 25%, South America 23%, Asia 8% and Africa produced the remaining *(Cash, Dennis 2013)*. M. sativa produce different kinds of secondary metabolites such as saponins, flavonoids, coumarins, alkaloids, phenols which have antimicrobial and antibacterial properties *(Sadowska et al., 2014)* which play medicinally vital role. In ancient India, in the Ayuvedic treatments involve use

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* Corresponding Author:
Email: ghanianila17@gmail.com

of Alfalfa seeds and sprouts for blood cells production and its leaves and stem is a great source of minerals and proteins *(Indianetzone, 2013).* It also increase soil nitrogen fertility and eliminate soil erosion *(Latrach et al., 2014)*. Many of the native medicinal plants are used as flavors, condiments and food in many salads and sandwiches *(John, 2011)*. The plant sprouts are main ingredient in many dishes in South Indian cusine *(Dasanna, 2016)*. It contains 93% of water, 4% of protein, 2% of carbohydrates and moderate source of Vitamin K, C and B. The plant studies involve extraction of the active components from leaves, stem and roots by using organic solvents for the manufacturing of dietary supplements such as tablets and powders commercially available *(Retrieved 29 June, 2011)*. The chemical constituents obtained from plant can cure high cholesterol, kidney problems, asthama, stomach, bladder problems and menopause issues *(MedlinePlus, 2016).* Therefore, in the present study the plants extracts which are commonly used are made with distilled water, different organic solvent extracts and tested for its antimicrobial effect. The major extract of Alkaloids was identified by Dragendroff's Test (Alkaloids are precipitated from neutral or slightly acidic solution by some reagents i-e Dragendroff reagent: solution of potassium bismuth iodide). Alkaloids are a class of naturally occurring organic compound which may act as anticancer *(Kittakoop,P et al., 2014)*, galantamine *(Russo,P et al., 2013)* and antibacterial *(Cushnie TP et al., 2014)*. The second major compound is Amino acid present in almost all organisms on earth, almost 500 naturally occurring Amino acids are present *(Wagner I, 1983),* they are also used in food industry for the formation of flavor enhancer *(Garanttini S, 2000)* and artificial sweetner *(Stegink LD, 1987),* also used in synthesis of cosmetics and drugs (*Leuchtenberger et al., 2005)*, amino acids can produce cancer-fighting drugs *(Andy, 2012).* To find out amino acid in plants the present study used Ninhydrin Test in which ninhydrin react with amino acids to release carbondioxide. Glycosides are chemicals stored in plants in the form of inactive state and activated by enzyme *(Brito et al., 2007)* It show many antioxidant activity, anticancer and antitumor (*Xiao J et al., 2016)* for glycoside identification, the modified Brontrager's Test was used in the present study. Flavonoids are sec. metabolites in plants and fungus. They are also responsible for flower coloration as a plant pigment. They are approved as pharmaceutical drugs *(FDA, 2013) (HCMSSA, 2013) (NDA, 2010)*. Flavonoids have been shown anti-allergic *(YamamotoY, 2001)*, anti-microbial, anti-bacterial *(Cushnie TP, et al., 2011) (Manner S, et al., 2013)*, and anti-cancer effect *(Cazarolli LH, et al., 2008) Shinoda's* Test (cyanidin reaction) was used to identify them. Triterpenoids possess a rich pharmacology as anti-cancer properties *(Laszczyk, Melanie, 2009) (Liu, Jie, 1995)* to identify them, Liebermann-Burchard Test was performed. Salkowski's Test (conc. Sulfuric acid) was performed to identify phytosterols enriched foods and dietary supplements have been manufactured in market for long *(Patterson CA, 2006)*. A simple Froth formation Test was done to identify saponins which are useful in synthesis of cosmetics and drugs *(Lorent et al., 2014)*. Phenols have a wide range of properties which include production of drugs most notably aspirin, preservative in few vaccines *(CDCP, 2018),* many phenol derivatives have been used in synthesis of sunscreens, hair coloring and skin whitening *(DeSelms, 2008)*. All plants used in the herbal medicine mostly have remarkable antimicrobial activities as find out previously.

MATERIAL AND METHODS

Plan of work: The work has been completed in 8 months' time period in step-wise manner as following:

Step I: Collection of the plants.

Step II: Preparation of Crude Extract.

Step III: Determination of Extractive values.

Step IV: Determination of various Phytochemicals.

Procedure

Collection of Plants: In the present study the plants collection was performed from district Peshawar and identified. Plants were processed for different extractions and antibacterial activity in the laboratory of Govt. Frontier College, Peshawar.

Preparation of Crude Extract: Plant root, stem, leaves were first chopped and on the grinding machine the powder formation was done for each part of plant. 150gm of powder of root, stem and leaves of the plant have been taken and soaked in separate 500ml conical flasks in 98% distilled methanol overnight. On the next day, the diluted extract was filtered and was transferred to another flask. On the vacuum rotary evaporator the diluted extract was kept for evaporation at a temperature between 40-50°C and under reduced pressure. Again Methanol was added to the extract prepared. This method was repeated for root, stem and leaves three times separately and the entire extract was dried.

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 ºC and then the extract was dried. By using the below equation the percentage yield of the extract was calculated (Banso and Adeyemo, 2006).

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

Determination of Extracts of different organic solvent: Different phytochemicals were separated from each other on the basis of miscibility and different electronic configuration. Different organic solvents like hexane, ethyl acetate, isobutanol and water was used to prepare the mixture. 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. Hexane of about 150ml was mixed in the separating funnel which contained the diluted dried extract and the whole mixture was kept in a shaker. Some of the substances were dissolved in the hexane. As Hexane is insoluble in water so after 10-15min aqueous portion (water) and hexane were separated and formed separate layers. Due to high density of water aqueous portion remained under the hexane layer. Three times the aqueous portion was washed with hexane. All the hexane soluble substances were removed from the extract and dried with vacuum rotary evaporator at a temperature 40°C and under reduced pressure. This same process was applied to the chloroform, ethyl acetate and isobutanol separately.

Phytochemical screening: Phytochemical screening of the prepared extracts were conducted to identify the presence of various chemical constituents with various qualitative tests. The tests were performed by the following chemicals and different reagents for different chemicals were also used. These were identified by characteristic color changes using standard procedures. *(Ghani2003)*

Chemical Tests

Tests for Alkaloids: Dragendroff's test.
Tests for Glycosides: Modified Borntrager's test.
Tests for Flavanoids: Shinoda's 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.

Presence of Alkaloids

Presence of Flavonoids

Presence of Saponins

Presence of Tannins

(a) Extracts Preparation

(b) Phytochemical Extraction of Medicago Sativa LEAVES:

Dry powder weight of Leaves = 248gm
Empty weight of Round Bottom = 358.5gm
R B F + Methanol extract weight = 379.8gm
Crude methanol extract weight = 21.3gm

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

Table 1. List of organic solvent which were used for preparing mixture.

Extraction	RBF Empty wt	RBF+ Extract	Extraction value	Extraction value yield %
Hexane fraction	189.9gm	194.5gm	4.6gm	1.9%
Ethyl acetate fraction	13.21gm	14.021gm	109.5gm	0.339%
Isobutanol fraction	106gm	109.5gm	0.841gm	0.339%
Water fraction	189.9gm	196gm	6.1gm	2.5%

Table2. List of Medicago sativa different extracts used to evaluate antimicrobial activity. The Greek alphabet

Names	Color of used Extract
Hexane	Green Yellow
Ethyl acetate	Light Green
Isobutanol	Dark green
Hot aqueous extract	Green
Cold aqueous extract	Green

Medicago sativa L ROOT phytochemicals Extraction:

Root Dry powder weight=246gm

China Dish empty weight=777.1gm

China Dish + Extract weight=788.8gm

Crude methonolic extract weight=11.7gm

Table 3.Effects of different organic solvent concentration yield potientiol.

Extraction	RBF Empty wt	RBT + Extract	Extraction value	Extraction value yield %
Hexane fraction	106gm	107.437gm	1.437gm	0.581%
Ethyl acetate fraction	106gm	106.8gm	0.8gm	0.325%
Isobutanol fraction	106gm	108.9gm	2.9gm	1.17%
Water fraction	105.8gm	110.5gm	4.7gm	1.9%

In the estimate of M. sativa root phytochemicals extraction, dry root powder of 246gm was taken and the crude methanol extract was prepared. Four different organic solvents were used to make hexane fraction, ethyl acetate fraction, isobutanol fraction and water fraction to extract the phytochemicals from the M. sativa root. An empty round bottom flask was taken and weighted which was 777.1gm. The total weight became 11.7gm of crude methanol extract .The different extractive values found out from different solvents extractions were properly mentioned in table (2). These extractive values were then put in the extractive value yield percentage formula to obtain the percentage result.

Medicago sativaL. STEM Phytochemicals extraction:

Root dry powder weight = 153gm

China dish empty weight = 358.5gm

China dish + extract weight=378.4gm

Crude methanol extract weight= 19.9gm

Table 4. Effects of different organic solvent concentration yield potential.

Extraction	RBF Empty wt	RBF + Extract	Extraction value	Extraction value yield %
Hexane fraction	106gm	110.7gm	4.7gm	3.07%
Ethyl acetate fraction	113gm	113.5gm	0.5gm	0.33%
Isobutanol fraction	106gm	108.5gm	2.4gm	1.57%
Water fraction	113gm	122.5gm	9.5gm	6.2%

Table 4. Presence of phytochemicals in different botanical extracts.

Phytochemicals	Hexane	Ethyl acetate	Isobutanol	Water
Alkaloids	+	+	+	+
Glycosids	+	+	-	+
Flavonoids	+	+	+	+
Saponins	-	-	-	+
Tannins	+	+	-	-
Triterpenoids	-	-	+	+
Sterols	-	-	+	+
Proteins	-	+	+	-
Phenols	-	+	-	+

RESULTS

Results of Phytochemical screening of LEAVES of M. sativa: The above results were found out by the phytochemical screening of leaves of *M. Sativa* as mentioned in Table (4). Alkaloids were found in all the four organic solvents. Glycosids were investigated in all the three solvents except isobutanol. Flavanoids were found in all the four solvents. Saponins were only present in the water. Tannins were found in hexane and ethyl acetate and were absent in isobutanol and water. Triterpenoids were present in isobutanol and water and were absent in ethyl acetate and hexane solutions. Proteins were present in ethyl acetate and isobutanol and missing in hexane and water. Phenols were present in ethyl acetate and isobutanol.

Table 5. Results of botanical extracts.

Phytochemicals	Hexane	Ethyl acetate	Isobutanol	Water
Alkaloids	+	+	+	+
Glycosids	+	+	-	+
Flavonoids	+	+	+	+
Saponins	-	-	-	+
Tannins	+	+	-	-
Teiterpenoids	-	-	+	+

Results of phytochemical Screening of STEM of M. sativa: The above results were found out by the phytochemical screening of stem of *M. sativa* as mentioned in Table (5). Alkaloids were found in all the four organic solvents. Glycosides were investigated in all the three solvents except the isobutanol. Flavanoids were found in all the four solvents. Saponins were only present in the water. Tannins were found in hexane and ethyl acetate and were absent in isobutanol and water. Triterpenoids were present in isobutanol and water and absent in ethyl acetate and hexane. Proteins were present in ethyl acetate and isobutanol and missing in hexane and water. Phenols were present in ethyl acetate and water and were absent in hexane and isobutanol. Sterols were present in isobutanol and water and were absent in hexane and ethyl acetate.

Table 6. Results of botanical extracts.

Phytochemicals	Hexane	Ethyl acetate	Isobutanol	Water
Alkaloids	+	+	+	+
Glycosids	+	+	-	+
Flavonoids	+	+	+	+
Saponins	-	-	-	+
Tannins	+	+	-	-
Teiterpenoids	-	-	+	+
Sterols	-	+	+	+
Proteins	-	+	+	-
Phenols	-	+	-	+

Result of Phytochemical screening of ROOT of *Medicago Sativa:* The above results were found out by the phytochemical screening of root of M. sativa as mentioned in Table (6). Alkaloids were found in all the four organic solvents. Glycosides were investigated in all the three solvents except isobutanol. Flavanoids were found in all the four solvents. Saponins were only present in the water. Tannins were found in hexane and ethyl acetate and were absent in isobutanol and water. Proteins were present in ethyl acetate and isobutanol

and were missing in hexane and water. Phenols were present in ethyl acetate and water and absent in hexane and isobutanol. Sterols were present in isobutanol, ethyl acetate and water and were absent in hexane.

RESULTS AND DISCUSSION

Many studies have been conducted that shows the antibacterial properties of many plants having phytochemical properties that cause damage to the bacterial membrane and suppression of virulence effect i-e inhibition of activity of enzymes and toxins. Many plants exert healing properties against many human diseases such as Gram-negative and Gram-positive bacteria (Borges, et al., 2015). Antibacterial means which can kill microorganisms by the action of antibiotic compounds which are present in a high amount in M. sativa all parts but specially the leaves. The extract of leaves was applied topically to the patient having face Acne (Cutibacterium acnes), it was cured soon. Saponins and sterols mainly present in leaves and roots are compounds that can be used to form various supplements for cholesterol level balance. The extract was applied to toenail fungus (Onychomycosis) it was killed due to presence of flavonoids in leaves, stem and root in high level. The fungus was cultured in laboratory through PDA media. Triterpenoids present in all parts of M. sativa which can act as antitumor. The extract was applied to dandruff (malassezia furfur) topically and also to the disease cultured in petri dishes, as phenols contain aspirin so it cured the disease.

CONCLUSION

The development of new anti-bacterial agents by plant extracts is very much of importance nowadays. Phytotherapy is increasing by time to cure many diseases and serve humanity. Phytochemicals act as defensive against pathogens. The plant screened for phytochemical constituents appeared to have the ability to act as a source of useful drugs and improve the health status of consumers as a result of presence of various compounds that are essential and beneficial for good health.

So it could be concluded that the M. sativa extract produce significant and remarkable phytochemicals which can be used as anti- microbial, anti-cancer and anti-fungal agents. The leaves, stem and roots are of great importance in the field of pharmacy. This identification of various phytochemicals leads to the production of more vaccines and supplements for many diseases and can promote better health and survival. However further work must still be conducted.

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