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Antibacterial activity of leaves extracts of *Trifolium alexandrinum* Linn. against pathogenic bacteria causing tropical diseases

Abdul Viqar Khan^{1*}, Qamar Uddin Ahmed², Indu Shukla³, Athar Ali Khan¹¹Department of Botany, Faculty of Life Sciences, Aligarh Muslim University, Aligarh–202002, State of Uttar Pradesh, India²Department of Pharmaceutical Chemistry, Faculty of Pharmacy, International Islamic University Malaysia, 25200–Kuantan, Pahang Darul Makmur, Malaysia³Department of Microbiology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh–202002, State of Uttar Pradesh, India

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ABSTRACT

Objective: To investigate antibacterial potential of *Trifolium alexandrinum* (*T. alexandrinum*) Linn. against seven gram positive and eleven gram negative hospital isolated human pathogenic bacterial strains responsible for many tropical diseases. **Methods:** Non-polar and polar extracts of the leaves of *T. alexandrinum* i.e., hexane, dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH) and aqueous (AQ) extracts at five different concentrations (1, 2, 5, 10 and 15 mg/mL) were prepared to evaluate their antibacterial value. NCCL standards were strictly followed to perform antimicrobial disc susceptibility test using disc diffusion method. **Results:** Polar extracts demonstrated significant antibacterial activity against tested pathogens. EtOAc and MeOH extracts showed maximum antibacterial activity with higher inhibition zone and were found effective against seventeen of the tested pathogens. While AQ plant extract inhibited the growth of sixteen of the test strains. EtOAc and MeOH plant extracts inhibited the growth of all seven gram positive and ten of the gram negative bacterial strains. **Conclusions:** The present study strongly confirms the effectiveness of crude leaves extracts against tested human pathogenic bacterial strains causing several tropical diseases. Since Egyptian clover is used as a fodder plant, it could be helpful in controlling various infectious diseases associated with cattle as well.

1. Introduction

Man has been using plants to cure different diseases associated with pathogenic bacteria since antiquity. According to a study conducted by the World Health Organization (WHO) based on publications on pharmacopoeias and medicinal plants in 91 countries, the number of medicinal plants is nearly 21 000. Nearly 6–7 thousand species of medicinal plants out of about 17–18 thousand flowering plants are known to be in use in folk and officially recognized systems of medicine in India, i.e., Ayurveda, Sidha, Unani and Homeopathy. Such a big figure of medicinal plants is the highest percentage of flowering plants in any particular country of the world for the existing flora of that country. The wide range of medicinal plants existing in India owing to a vast range of agroclimatic

variability is accountable for increased demand of Indian medicinal plants in the international market in recent years. WHO has projected that the global herbal market for medicinal plants has been estimated to be worth around US \$120 billion which is growing at 7%–10% every year and it is likely to increase to more than US \$5 trillion by 2050[1,2]. Plants grown in this region are not systematically tested for their biological activities in general and antimicrobial activity in particular. Exceptional ways to available antibiotics for disease management have been increasingly felt due to the increase in the resistance of bacterial isolates. This has urgently demanded the requirement of second and third line drug and plants are considered potent candidates to overcome such inevitable problems associated with the complications of antimicrobial resistant bacterial[3–12].

Plants from the genus *Trifolium* have been used in traditional medicine by many cultures. In Turkish folk medicine, for example, some *Trifolium* species are used for their expectorant, analgesic, antiseptic properties and are also used to treat rheumatic aches. Some species are also grown as pasture crops for animals in the Mediterranean.

*Corresponding author: Dr. Abdul Viqar Khan, Department of Botany, Faculty of Life Sciences, Aligarh Muslim University, Aligarh–202002, State of Uttar Pradesh, India.

E-mail: viqarvicky@gmail.com

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The plant, *Trifolium alexandrinum* (*T. alexandrinum*) Linn. (Family: Fabaceae) (common name: Egyptian clover, berseem clover) is a fodder plant and is distributed all over Asia, Europe and USA. Berseem clover is a winter annual legume with oblong leaflets and hollow stems. It grows upright and produces yellowish–white flowers. Berseem may grow as tall as 18 to 30 inches and contains from 18 to 28 percent crude protein, which is equal to or better than crimson clover and alfalfa^[13].

Earlier phytochemical investigations of *T. alexandrinum* have revealed the presence of terpenoid glycosides, amino acids and their derivatives, proteins, flavonoids and their glycosides, isoflavonoids and fatty acids in different parts of the same plant^[13,14]. Daily intake of (water, hexane and ethanolic) extracts of the flower head of *T. alexandrinum* in drinking water for 4 weeks immediately after diabetes induction in male rats has been reported to cause significant decrease in glucose and glycated hemoglobin levels and increase in insulin level. It also greatly improved the levels of serum lipid parameters and significantly decreased lipid peroxidation in addition to increase the hepatic glutathione (GSH) content significantly^[15].

Detection of bioactive compounds *i.e.*, terpenoids, steroids, flavonoids, amino acids and proteins in appreciable amount in the leaves extracts of *T. alexandrinum* in our laboratory and antimicrobial effect of phenolic extract of another *Trifolium* species *i.e.*, red clover (*Trifolium pratense*) on the ruminal hyper ammonia–producing bacterium, *Clostridium sticklandii*^[16] prompted us to evaluate the possible antibacterial properties of *T. alexandrinum* *in vitro*. Hence, polar and non–polar extracts of the leaves were explored against eighteen human pathogenic bacterial strains, responsible for many of the tropical diseases. Bacteria included in this study were isolated from the patients admitted at the Jawaharlal Nehru Medical College, AMU, Aligarh, India.

2. Materials and methods

2.1. Plant material

Fresh leaves (10 kg) of *T. alexandrinum* were collected during winter from different localities of Aligarh district, U.P., India. Voucher specimen numbers (AV24, AV206) of the plant were deposited in the Herbarium of Department of Botany, Faculty of Life Sciences, Aligarh Muslim University, Aligarh–202002, U.P., India.

2.2. Preparation of polar and non–polar extracts

Shade dried plant material (2.7 kg) was pulverized in an electric grinder. Powder so obtained was stored in a dessicator. 500 g plant powder was macerated with 95% methyl alcohol (MeOH) in a round bottom flask at room temperature for about 24 h. Mother liquor (crude MeOH extract) was filtered out and residual plant material was again

macerated with 95% MeOH for another 24 h. The process was repeated four times to ascertain the maximum yield of MeOH extract. The extract was evaporated to dryness at 35 °C under reduced pressure using rotary evaporator and finally freeze dried at –50 °C to obtain final yield (64.5 g) of crude methanol extract which was kept in the chiller at –18 °C till further use^[17].

Freeze dried methanol extract (64.5 g) was refluxed with hexane over hot water bath for 5 h. After filtration, the residual methanol extract was again refluxed with hexane for another 5 h and filtered. This process was repeated three times. Hexane was evaporated under reduced pressure to obtain hexane soluble extract. Hexane insoluble fraction of methanol extract obtained in above process was refluxed with dichloromethane (DCM) for 5 h. Thereafter, it was filtered and refluxed again with DCM for 5 h and filtered. The process was repeated three times. DCM was evaporated under reduced pressure to obtain DCM soluble extract. DCM insoluble fraction was refluxed with ethyl acetate (EtOAc) for 5 h. Thereafter, it was filtered and refluxed again with EtOAc for 5 h and filtered. The process was repeated three times. EtOAc was evaporated under reduced pressure to obtain EtOAc extract. EtOAc insoluble fraction was refluxed with MeOH (95%) for 5 h and filtered. The process was repeatedly refluxed for three times with methanol. The methanol soluble fraction was evaporated under reduced pressure to obtain MeOH extract, while methanol insoluble residue was discarded^[12]. All resultant extracts were freeze dried at –50 °C with yields of hexane extract (14.5 g), DCM extract (13.0 g), EtOAc extract (8.5 g) and MeOH extract (25.0 g).

2.3. Preparation of aqueous extract

Shade dried plant material (500 g) was ground to a fine powder then it was poured with double distilled water, and left for 72 h at room temperature. The flask was then refluxed over hot water bath for 5 h and the mother liquor was filtered. The distilled water was again added, refluxed and filtered. This process was repeated for four times. The filtrate, thus obtained, was evaporated to complete dryness under reduced pressure and finally freeze dried at –50 °C to give final yield (37.0 g) of aqueous (AQ) extract which was stored in labeled sterilized screw capped bottle at –18 °C^[17].

2.4. Phytochemical analysis

2.4.1. Test for carbohydrates (Molish test)

Five mL aqueous solution of each extract was mixed with few drops of Molish reagent (alpha naphthol) and concentrated H₂SO₄ was added from side wall of test tube. Formation of purple coloured ring at junction indicated the presence of carbohydrates^[17].

2.4.2. Test for lipids (Sudan IV test)

Hexane soluble portion of each extract was added in a test tube and few drops of Sudan IV were also added. Lipids stained red when Sudan IV was added and formed a separate

ring which indicated positive test^[17].

2.4.3. Tests for proteins and amino acids (ninhydrin test)

Aqueous solution of each extract was heated with ninhydrin reagent. Characteristic deep blue or pale yellow colour due to formation of complex between two ninhydrin molecule and nitrogen of free amino acid was an indication of positive test^[17].

2.4.4. Tests for steroids and triterpenoids (Liebermann–Bruchard test)

Extracts were evaporated to dryness and extracted with CHCl_3 and few drops of acetic anhydride followed by concentrated H_2SO_4 from side wall of test tube to the CHCl_3 extract were added. Formation of violet to blue coloured ring at the junction of two liquid was an indication of positive test^[17].

2.4.5. Tests for flavonoids

Five mL of dilute ammonia solution was added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2SO_4 . A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing^[17].

2.5. Microorganisms

Leaves extracts were tested for possible antibacterial activity using disc diffusion assay against eighteen human pathogenic bacteria. The bacteria were obtained from the bacterial stock, Department of Microbiology, Jawaharlal Nehru Medical College, Aligarh, India. The bacterial cultures were maintained at 4 °C on nutrient agar.

2.6. Antibacterial activity (bacterial susceptibility tests)

National Committee for Clinical Laboratory Standards (NCCLS) were strictly followed to perform antimicrobial disc susceptibility testing using disc diffusion method to test

plant extracts against test strains^[13]. Standardized inoculums ($1-2 \times 10^7$ CFU/mL 0.5 McFarland standard) were introduced on the surface of the plates containing Mueller Hinton agar (MHA), which was spread evenly with a glass spreader. The paper discs (6 mm in diameter; Whatman No. 1 filter paper) containing 1, 2, 5, 10 and 15 mg/mL plant extracts were dried and placed aseptically on the agar surface with the help of a sterile forceps. Finally paper discs were pressed slightly with forceps to make complete contact with the surface of the medium. Plates were allowed to stand at room temperature for 30 min and then incubated aerobically at 37 °C and examined for the zone of inhibition after 24 h. The zone of inhibition was measured in diameter. The experiments were repeated thrice. Chloramphenicol (10 µg/disc) was used as a standard drug.

3. Results

0.5 kg pulverized leaves of *T. alexandrinum* yielded 37.0 g (7.4%) freeze-dried AQ extract. 0.5 kg pulverized leaves of *T. alexandrinum* yielded 64.5 g (12.9%) freeze-dried methanol extract. Methanol extract upon successive extraction with non-polar and polar organic solvents at room temperature subsequently yielded hexane (14.5 g, 2.9%), DCM (13.0 g, 2.6%), EtOAc (8.5 g, 1.7%) and MeOH (25.0 g, 5.0%) soluble freeze-dried fractions.

Qualitative analysis of polar and non-polar extracts of the leaves of *T. alexandrinum* L. revealed the presence of different class of phytochemicals in different proportion (Table 1). Hexane extract displayed positive results for the presence of lipids, steroids and terpenoids and negative for carbohydrates, proteins, amino acids and flavonoids. DCM extract showed positive results for the presence of lipids, steroids, terpenoids and flavonoids only. EtOAc extract revealed negative tests for proteins only while MeOH and AQ extracts showed absence of lipids, steroids and terpenoids. Phytochemical analysis of EtOAc, MeOH and aqueous extracts explicitly revealed greater abundance for carbohydrates, proteins, amino acids and flavonoids while

Table 1

Qualitative analysis of the phytochemicals of polar and non-polar extracts of the leaves of *T. alexandrinum* L.

Leaves extracts	Carbohydrates	Lipids	Proteins	Amino acids	Steroids	Terpenoids	Flavonoids
Hexane	–	++++	–	–	+	++	–
Dichloromethane	–	++++	–	–	++	++	+
Ethyl acetate	+	+	–	+	++	++	++++
Methanol	++	–	+	++	–	–	++++
Water	++++	–	++++	++++	–	–	+

–: negative; +: small amount; ++: average; +++: high.

hexane and DCM extracts revealed appreciable amount for the presence of lipids in the leaves of *T. alexandrinum*.

In the present study, the investigation of antibacterial activity of non-polar and polar extracts of the leaves of *T. alexandrinum* against seven gram-positive and eleven gram-negative bacteria was studied using disc diffusion method. The data pertaining to the antimicrobial potential

of non-polar (hexane & DCM) and polar (EtOAc, MeOH & AQ) extracts of the leaves of *T. alexandrinum* were presented in Table 2. The results revealed variability in the inhibitory concentrations of each extract against given bacteria. All extracts presented antimicrobial activity to at least six of the tested microorganisms. None of the extracts of the leaves of *T. alexandrinum* was active against

Table 2
Antibacterial activity of non-polar and polar extracts of *T. alexandrinum* L. leaves.

Extracts (mg/mL/disc)	Zone of inhibition (mm)																		
	Gram positive bacteria									Gram negative bacteria									
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	8	9	10	11	
Hexane	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	2	–	–	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	5	–	–	11	–	7	–	–	–	–	–	8	–	–	9	9	7	–	–
	10	–	–	13	–	9	–	–	–	–	–	11	–	–	13	10	9	–	–
	15	–	–	15	–	14	–	–	–	–	–	14	–	–	15	12	11	–	–
Dichloromethane	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	2	8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	5	11	–	–	–	7	–	7	–	7	–	–	–	–	9	–	–	–	–
	10	13	7	–	–	9	–	9	–	9	–	–	–	–	12	–	–	–	–
	15	15	10	–	–	13	–	13	–	11	–	–	–	–	13	–	–	–	–
Ethyl acetate	1	7	7	8	8	7	8	7	–	8	7	8	8	–	–	–	9	9	–
	2	10	9	11	13	9	11	8	7	9	9	10	10	–	9	9	11	10	8
	5	17	15	17	18	13	16	11	11	12	12	11	13	–	14	13	14	16	10
	10	20	19	20	20	18	20	17	16	18	18	14	14	–	17	17	17	18	15
	15	24	22	22	24	22	24	23	20	23	22	16	16	–	21	20	23	20	18
Methanol	1	8	–	9	–	–	–	–	–	–	–	7	7	–	–	–	–	–	–
	2	9	7	9	9	7	9	8	–	9	7	9	10	–	7	–	8	–	–
	5	13	9	14	10	12	13	11	7	10	11	14	15	–	11	9	13	9	7
	10	16	12	19	14	15	17	14	12	15	15	16	16	–	13	12	15	12	11
	15	19	18	21	18	17	20	16	15	18	17	19	18	–	16	15	18	15	12
Aqueous	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	2	9	–	–	9	–	–	–	–	–	–	–	–	–	7	–	7	–	–
	5	11	8	9	13	7	8	9	7	8	9	9	9	–	11	–	11	–	–
	10	15	10	12	17	11	12	13	8	12	13	11	12	–	13	8	13	11	–
	15	19	14	15	20	15	17	17	12	16	17	16	15	–	15	12	16	15	–
Chloramphenicol 10 µg/disc	18	18	16	–	–	–	16	18	16	–	16	18	–	16	17	19	18	20	

Gram positive bacteria:

1: *Staphylococcus aureus*; 2: *Staphylococcus aureus* ATCC 25953; 3: *Staphylococcus albus*; 4: *Streptococcus haemolyticus* Group-A; 5: *Streptococcus haemolyticus* Group-B; 6: *Streptococcus faecalis*; 7: *Bacillus subtilis*.

Gram negative bacteria:

1: *Escherichia coli*; 2: *Edwardsiella tarda*; 3: *Klebsiella pneumoniae*; 4: *Proteus mirabilis*; 5: *Proteus vulgaris*; 6: *Pseudomonas aeruginosa*; 7: *Salmonella typhi*; 8: *Shigella boydii*; 9: *Shigella dysenteriae*; 10: *Shigella flexneri*; 11: *Plesiomonas shigelloides*.

–: no inhibition.

Pseudomonas aeruginosa. Moderate biological activity was demonstrated by non-polar extracts as hexane extract was found to be effective against two of the gram positive (*Staphylococcus albus*, *Streptococcus haemolyticus*) and four gram negative (*Proteus mirabilis*, *Salmonella typhi*, *Shigella boydii*, *Shigella dysenteriae*) pathogens only. Dichloromethane extract inhibited the growth of four gram positive (*Staphylococcus aureus*, *Staphylococcus aureus* ATCC 25953, *Streptococcus haemolyticus*, *Bacillus subtilis*) and only two of the gram negative (*Edwardsiella tarda*, *Shigella boydii*) pathogens. While AQ extract showed no inhibitory activity against *Pseudomonas aeruginosa* and *Plesiomonas shigelloides*.

In contrast, polar extracts *i.e.*, EtOAc and MeOH extracts presented the highest activity, *i.e.*, they were able to inhibit 17 (94.44%) types of microorganisms of interest. Moreover, they also had the highest activity rate against bacteria. On the other hand, AQ extract showed inhibitory activity against 16 (88.88%) types of microorganisms and non-polar extracts (hexane and DCM) revealed antimicrobial activity against 6 (33.33%) microorganisms of interest only. The antibacterial activity was more prominent on the gram-positive bacteria

than the gram-negative bacteria. Gram-positive bacteria were the most susceptible to growth inhibition by EtOAc and MeOH extracts. The results showed that all the extracts had variable degree of antibacterial activity and that the inhibition of bacterial growth was dose dependent as inhibitory action of the extracts was found to increase with the increase of concentration against all bacterial strains as evidenced by the higher zone of inhibitions at higher concentration of each extract.

All extracts displayed similar antibacterial activity compared with positive control (chloramphenicol, 10 µg/disc) though at higher concentration. Non-polar (hexane and DCM) and AQ extracts up to 1 mg/mL did not exert any antibacterial activity on all bacterial strains. The highest antibacterial activity was observed at 15 mg/mL by all extracts against most of the bacterial strains. EtOAc extract exhibited the highest antibacterial activity *i.e.*, higher inhibition zones against most of the bacterial strains in comparison with other extracts of the leaves of *T. alexandrinum* (Table 2).

4. Discussion

The discovery of penicillin by Alexander Fleming in 1928 is a milestone in the history of medicine. As more antimicrobial compounds were discovered, it was predicted that infectious diseases would be eliminated through the use of these antimicrobials. Unfortunately, the development of bacterial resistance to these antimicrobials quickly diminished this optimism^[7]. Secondly, there is also reported reduction in the discovery of new antimicrobial agents globally. Ethnomedicinal reports have brought to light that our rich floristic heritage is one of the reliable sources which can be traced pharmacologically for their possible antibacterial potential. It is known that plants bio-constituents have been a good source of antibacterial agents. Still many of the plant species remained unexplored or under explored^[19]. Plants are important sources of potentially useful substances for the development of new chemotherapeutic agents. Various phytochemical compounds which are naturally present in plants as secondary metabolites have been implicated in the conferment of antibacterial activities^[20,21]. The presence of some of such secondary metabolites in a significant amount in the investigated part of *T. alexandrinum* may have conferred the strong antibacterial activity on the leaves extracts. In this regard, higher concentration of these phytochemicals may have been responsible for a higher degree of inhibition on the bacterial strains.

Previously, in many studies of similar kinds, ethanol and methanol are used as extractants, however, it could not demonstrate the greatest sensitivity in yielding antimicrobial compounds^[22]. For this reason in the present study five extractants based on their different degree of polarity and solubility, *i.e.*, hexane, dichloromethane, ethyl acetate, methanol and water were used to obtain maximum active compounds in the extracts. It is worth mentioning to note that a correlation was observed between the extract solubility and antibacterial activity of different fractions. This suggests that in sequential extraction, maximum antibacterial compounds were solubilized according to their degree of solubility in polar solvents as EtOAc and MeOH extracts displayed the highest antibacterial activity followed by AQ, DCM and hexane extracts. These results further confirm that significant antibacterial compounds are polar in nature as evidenced by the higher degree of antibacterial activity of EtOAc and MeOH extracts of the leaves of *T. alexandrinum* *in vitro*.

All the extracts highly affected the activity of gram positive bacteria in comparison to gram-negative bacteria. The greater susceptibility of gram-positive bacteria towards various plant extracts than that of gram-negative bacteria was also reported earlier^[23–25]. The activity of *T. alexandrinum* leaves extracts against both gram-positive and gram-negative bacteria might indicate the presence of broad spectrum antimicrobial compounds. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity^[26–47]. These results suggest that the bioactive compounds present in polar extracts have good potential for development of

novel antibacterial herbal products.

Results obtained from our study explicitly confirm antibacterial potential of the leaves of *T. alexandrinum*. Polar extracts *i.e.*, EtOAc and MeOH extracts demonstrated the most pronounced antibacterial activities against bacterial strains taken into consideration. Hence, EtOAc and MeOH extracts of the leaves of *T. alexandrinum* deserve further investigations to develop new antibiotics that may help in combating several bacterial diseases in tropical countries. Since this plant is used as a fodder plant, it could be helpful in controlling various infectious diseases associated with cattle as well. This is the first ever report on the antibacterial potential of the leaves of *T. alexandrinum*.

Conflict of interest statement

We declare that we have no conflict of interest.

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