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ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF CRUDE METHANOLIC AND ETHANOLIC EXTRACTS OF *MEDICAGO POLYMORPHA*

Amin Ullah[2], Muhammad Imran Khan*[1], Yaqoob ur rehman[2], Muhammad Waqas[2], Muhammad Ajmal Khan[2], Sher Ali [3], Gohar Zaman[1] and Hazrat Ali[1]

[1]Department of Biotechnology, University Of Science and Technology, Bannu District, Khyber-Pakhtunkhwa, Pakistan.
[2]Center of Biotechnology & Microbiology, University Of Peshawar, Khyber-Pakhtunkhwa, Pakistan.
[3]Department of Zoology, Quaid-i-Azam University, Islamabad 45320, Pakistan.

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Abstract: Microorganisms such as bacteria and fungi are not only the major cause of different severe diseases of animals and humans but also the leading cause of reduction in crops yield. Every year crops yield decreases due to fungal and bacterial attacks. Modern chemicals and synthetic pharmaceutical drugs have been failed in achieving their goals because of side effects, microbes resistivity and high prices. Plants have been proved good and reach sources of antimicrobial agents. Present research is a good attempt towards medicinal plants as perfect and safe sources of antifungal and antibacterial natural phytoconstuents. Methanolic and Ethanolic extracts of *Medicago Polymorpha* was tested against different fungal and bacteria species. The methanolic extract of *Medicago polymorpha* showed relatively low activity against *Aspergillus niger about 30%* And relatively high activities against, *Aspergillus flavus* (55%) and *Aspergillus fumigates* (45%) (Table I). Ethanolic extract of *Medicago polymorpha* showed antifungal activities against the three fungal strains in the range *Aspergillus flavus* (40%), *Aspergillus fumigates* (39%) and *Aspergillus niger* (36%) (Table II). Antibiotic Terbinafine and DMSO were used as positive and negative controls respectively. The positive control terbinafine inhibits growth of all fungus strains to an average of 98%. All fungus strains showed full growth on DMSO (Tables I and II). In antibacterial assay the methanolic extract of *Medicago Polymorpha* showed comparatively high activity against *Pseudomonas aeruginosa* (22mm) and *Micrococcus luteus* (21.5mm) and low activity against *Staphylococcus aureus* (16mm) and *Escherichia coli* (18.5mm) (Table III). The ethanolic extract showed highest antibacterial activities against *Escherichia coli* (20mm) and *Micrococcus luteus* (20mm) and comparatively some less activities against *Staphylococcus aureus* (15mm) and *Pseudomonas aeruginosa* (19mm) (Table IV). The results are in comparison with the standards cefixime-USP and roxithromycin (positive controls) (Table III& IV).

Key words: Terbinafine, antibacterial, *Aspergillus flavus*, roxithromycin, Medicinal plants

INTRODUCTION

Medicinal plants have played very basic role in keeping the standard of human health. Different parts of medicinal plants (fruits, leaves and roots etc.) have been playing very basic role in improving human health. Since long they have paid their services for human health and served humans in every field of life like cosmetics, beverages, dyes and medicines. Medicinal plants have some biologically active compounds such as flavonoids, saponins, steroids, vitamin C, cardiac glycosides, carotenoids and phenolic compounds which increase the immune system of the body. According to Bruneton, (1995) the anti-inflammatory and antioxidant properties are due to their phenolic and flavonoids compounds. In drug discovery there are two main approaches i.e. Indiscriminate/random screening and Ethno medical knowledge which are mostly used for the selection of plants species for medicinal purposes and the ethno medical strategy because of traditional therapeutical uses is of great importance (Pieters and Vlietnick, 2005).

World health organization (WHO) reported that throughout the world different species of medicinal plants are the best source of getting effective herbal drugs. Among the population 80% peoples use traditional medicines even in the developed countries, basically because of the potent Compounds derived from these medicinal plants. Therefore we should also screen different parts of the plants to obtain safe and good anti-microbial agents and to use it in future against the multi drugs resistant strain of different microbes (Fungi and Bacteria etc.). Plant's extracts (phytochemicals) are used for the treatment of different chronic diseases. Medicinal plants provide 30% drugs world widely (Grabley and Thiercke, 1999). These compounds (phytochemicals) are synthesized during secondary metabolism in medicinal plants, and thus they are used as anti-infectious agents. Medicinal plants have been used for the treatment of various diseases because of antimicrobial properties, due to this reason plants are the good and rich natural source of different types of medicines.

From ancient times medicinal plants which are a good source of those compounds which plays basic role in the maintenance and improvement of human health. As commercial Antibiotics are used for different infections but it has also been noted that some microbes develop multiple resistivity against used antibiotics and also have negative effects on the host such as they produce hyper sensitivity and Allergic

***Corresponding Author:**
Muhammad Imran Khan,
Department of Biotechnology,
University Of Science and Technology,
Bannu District,
Khyber-Pakhtunkhwa, Pakistan.

reactions. So, for the treatment of these diseases alternative anti-microbial drugs from natural plant's parts (barks, fruits, stems, roots, leaves and flowers etc.) can be isolated and used.

Thus these isolated compounds will be used to inhibit different pathogens and have no or less toxicity to the host cells, so can help us in developing new potent anti-microbial drugs (Sieradzki *et al.*, 1999). According to Monthana and Lindequista, (2005) and Bajpai *et al.*, (2005), some nutrient and non-nutrient molecules of the aromatic and medicinal plants shows anti-microbial properties, thus they can protect us from different types of specific pathogens.

Beside the prevention of pathogens, food preservation is also nowadays became very serious problem, which also requires the protection against microbes (Marino *et al.*, 2001), Therefore more safety to our food is necessary by adding anti-microbial additives such as oil etc., isolated from different plants (Mistscher *et al.*, 1987). Bagamboula *et al.*, (2003) reported that Medicinal plants have anti-microbial activities therefore it can be used against food spoilage and also for pathogens such as bacteria and fungi etc.

MATERIALS AND METHODS

Medicago Polymorpha was collected from district Bannu, Khyber Pakhtunkhwa, Pakistan. In March 2014, Identification was confirmed by taxonomist, Professor Abdur-Rehman, Government Post Graduate College, Bannu. The plant material was washed by distilled water. After being dried fine powder of the plant materials was prepared and 200 mg was dissolved in methanol. The solution was filtered through whatman filter paper and the filtrate was dried through rotary evaporator. After drying stock solution was prepared in the ratio 1:1 of extract and methanol. This stock was then store at 4C° for further using in bioassays.

Antifungal assay

To check the antifungal activity of the methanolic crude extract of *Medicago Polymorpha* the standard protocol of Duraipandiyan and Lgnacimuthu, (2009) was followed with little modifications.

Samples preparation

The methanolic crude extract of *Medicago Polymorpha*) was prepared in the DMSO as a stock solution of 1mg/ml (5mg/5ml) then DMSO as a diluting solvent was used and further 1ml solution of 200µg/ml was prepared from this stock solution by using the formula $M_1V_1=M_2V_2$. In the same way the stock solution of 1mg/ml of the terbinofine (positive control) was prepared in the DMSO and further dilution in the same way was made i.e. 1ml solution of the required concentration that is 200µg/ml was prepared in the DMSO and then 1ml DMSO was taken from the bottle which was used as a negative control.

Media for antifungal assay

SDA (Sabouraud Dextrose Agar) of MARCK was used to grow fungus for inoculums preparation. This SDA was composed of agar 15g/L, peptone complex 10g/L and glucose 40g/L.

Assay procedure

6.5gm of the SDA was dissolved in 100ml distilled water in the conical flask to prepare the media for fungus growth then autoclave it for 20 minutes at 121 C°, now 4ml of this prepared media was poured in the autoclaved test tubes up to 10cm in the slanting position for fungus growth and all this was performed in the Laminar flow then from the required concentration i.e. 200µg/ml, the volume of 67µl of the extract solution was taken by micropipette and putted in all the test tubes which were labeled for this purpose for four different fungal strains in duplicates. In the same way 67µl of the terbinafine (positive control) solution of the required concentration that is 200µg/ml were putted in the eight test tubes for the fungus strains in duplicate, in the same manner 67µl of the DMSO i.e. negative control was put in the other eight labeled test tubes for the growth of fungal strains. All the test tubes in the laminar flow were placed in slanting position for the solidification of the media at room temperature in the test tubes, then approximately 8 spores from the 1 week old culture of the each fungus strain were placed in the test tubes of the extract (COME), positive control (terbinofine) and negative control (DMSO) very carefully. Then all the test tubes were packed air tightly and then placed it at 28C° in the incubator for 1 week.

Antibacterial assay

This antibacterial activity/ potency of the methanolic crude extract of *Cardia obaliqua* (COME) was carried out by following the protocol of Bagamboula *et al.*, (2003) with slight modifications.

Media for bacteria

In this antibacterial experiment two types of media i.e. nutrient agar medium and nutrient broth medium were used. The Nutrient agar medium (MERCK) was composed of peptone (5g/L), meat extract (3g/L) and agar-agar (12g/L) used for Bacterial growth in the petri plates while the Nutrient broth medium was composed of peptone (5g/L) and meat extract (3g/L) used for Bacterial strain inoculums preparation. Nutrient broth medium was prepared by dissolving 0.8g/ 100ml of distilled water while the nutrient agar medium was prepared by dissolving 2g/

100 ml of distilled water of pH 7.0 and then all these were autoclaved.

Preparation of Samples

Methanolic crude extract of *Cardia obaliqua* (COME) solution of 1mg/ml (1000µg/ml) was prepared in DMSO (dimethyl sulfoxide). Similarly 1mg/ml (1000µg/ml) solutions were prepared of the standard Antibiotics drug i.e. Ciprofloxacin (Cipro), Ampicillin or Norfloxacin (Ampicil) in DMSO used as positive control. DMSO was also used as a negative control.

Preparation of inoculums/ microorganisms

Four strains of Bacteria i.e. *Shigella, Salmonilla*, *Escherichia coli* (ATCC15224), and *Pseudomonas aeruginosa* were used in this study. 24 hours long-standing culture in nutrient broth (MERCK) of the selected bacterial strain was mixed with physiological saline (0.9% NaCl w/v.) and its turbidity was corrected by the addition of sterile physiological saline up to a level that the standard turbidity of McFarland 0.5 $BaSO_4$ (ten raise to the power of six colony forming unit (CFU) per ml density) was obtained. These inoculums were used for seeding the nutrient agar.

Assay Procedure (Agar diffusion method)

For this Antibacterial assay 2 gram of nutrient agar was dissolved in 100 ml of distilled water of pH 7.0 in the conical flask, then this prepared media was then autoclaved for 20 min at 121° C under 15 PSI and after autoclaving it was allowed to cool up to 45°C. After this in laminar flow the suitable amount of this autoclaved cooled agar medium was poured/ put into the autoclaved petri plates of 10 cm and placed inside the laminar flow for solidification. Then a loop/ sterile cotton swab full of 104-106 suspension of hours old broth of each bacterium streaked/ seeded on the surface of agar media in the petri plates very carefully in a scientific method. Hereafter six wells were dug by means of sterile cork bore of size 8mm in the solidified agar media of petri plates at equal distance. Then 100µl (one hundred micro litter) from 1mg/ml (1000µg/ml) of the sample extract (COME) solution were put by micro pipette into the respective wells. Similarly in same way 100 micro L from 1mg/ml solution of the Ciprofloxacin (Cipro), Ampicillin or Norfloxacin (Ampicil) i.e. both positive control were also put into the respective well and then 100µl of the DMSO (the negative control) was also put in the particular wells. Now at the end all the petri plates were packed tightly in the laminar flow and then incubated at 37C° for 24 hours, after the passage of 24 hours in an incubator the zone of inhibition of all the extracts (COME) and controls were measured in millimeter (mm) and compared with the control.

RESULTS

As synthetic drugs have produced antibiotic resistant strains and also have side effects therefore researchers and investigators have shifted their efforts towards natural sources to investigate potent antimicrobial agents with no or less resistivity and side effects. Our results showed that *Medicago polymorpha* had significant antibacterial activity against all pathogenic strains. Our results are supported by the findings of Ndhlala *et al.,* (2009).

Fungi destroy food material and cause diseases by producing mycotoxins (Janardhana *et al.*, 1998). Our findings proved the antifungal activity of *Medicago polymorpha* crude extracts against various fungal strains. Many researchers proved that the inhibitory effect of plant materials against microbes are due to the presence of phenolic compounds (Baydar *et al.,* 2004; Rodriguez et *al.,* 2007; Kabuki *et al.,* 2000; Aboaba and Efuwape, 2001; Mohanta *et al.,* 2007).

Table I: % Inhibition of fungus growth by DMSO, Terbinafine and Methanolic crude extract of *Medicago polymorpha*. Data is the mean value of three different experiments performed in duplicates.

Substances / fungus	*A. fumigatus*	*A. flavus*	*A. niger*
DMSO (67µl)	0	0	0
Terbinafine 67µl (200µg/ml)	98.5	99	94.5
Extract 67µ l (200µg/ml)	45	55	30

Table II: % inhibition of fungus growth by DMSO, Terbinafine and Ethanolic crude extract of *Medicago polymorpha*. Data is the mean value of three different experiments performed in duplicates.

Substances / fungus	*A. fumigatus*	*A. flavus*	*A. niger*
DMSO (67µl)	2	0	0
Terbinafine 67µl (200µg/ml)	100	98	92.5
Extract 67µl (200µg/ml)	39	40	36

Table III: % growth inhibition of various bacterial strains by positive controls cefixime-USP and rox and roxithromycin different concentration of Methanolic crude extract of *Medicago polymorpha*. Data represents the mean value of three different experiments performed in duplicates.

Concentration (mg/ml)	*M. luteus* (mm)	*E. coli* (mm)	*P. aeruginosa* (mm)	*S. aureus* (mm)
1 mg/ml cefix	22	31	25	27
1 mg/ml rox	26	22	23	24
1 mg/ml extract	14	11.5	16	12
3mg/ml extract	18	17	19.5	17
5 mg/ml extract	21.5	20.5	22	18.5

Table IV: % growth inhibition of various bacterial strains by positive controls cefixime-USP and roxithromycin and different concentration of Ethanolic crude extract of *Medicago polymorpha*. Data represents the mean value of three different experiments performed in duplicates.

Concentration (mg/ml)	*M. luteus* (mm)	*E. coli* (mm)	*P. aeruginosa* (mm)	*S. aureus* (mm)
1 mg/ml cefix	27.5	28	23	**22.5**
1 mg/ml rox	29	26	20	**18.5**
1 mg/ml Extract	16	18	14	**10**
3 mg/ml Extract	17	18.5	19	**15**
5 mg/ml Extract	20	20	19	**15**

CONCLUSION

The plant *Medicago polymorpha* has medicinal value. Crude ethanolic and methanolic extracts of *M. polymorpha* possesses antifungal and antibacterial properties. Isolation and determination of the phytochemicals responsible for the concerned properties of the plant M. polymorpha are being suggested.

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