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Full Length Research Paper

Antibacterial and antifungal potential of leaves and twigs of *Viscum album* L.

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The leaves and twigs of *Viscum album* L. (Family: Loranthaceae) were extracted successively with various organic solvents and water. These crude extracts were assessed for antimicrobial activities against three gram positive bacteria that is *Staphylococcus aureus, Bacillus subtilis, Enterococcus faecium,* five gram negative bacteria that is *Escherichia coli*, *Bordetella bronchisiptica, Salmonella typhi, Pseudomonas aeruginosa, Pseudomonas syringae,* one yeast that is *Saccharomyces cerevisiae* and one filamentous fungus *Aspergillus flavus* by using disc diffusion method. The ethyl acetate, chloroform, ethanol, and methanol crude extracts of selected plant parts had significant antimicrobial activities on both gram positive and gram negative bacteria. The ethyl acetate and methanol crude extracts of leaves and twigs of *V. album* exhibited prominent activities against gram positive and gram negative bacteria used in comparison to other extracts which had moderate activity against all the tested bacteria. The antimicrobial activities of the crude extracts of the selected plant parts were more active against gram negative bacteria than gram positive bacteria. The standard reference antibiotics, ciprofloxacin (100 um/ml) and nystatin (1500 u/ml) were used as positive control.

Key words: Antibacterial, antifungal, disc diffusion, *Viscum album,* ciprofloxacin, nystatin.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. Various medicinal plants have been used for years in daily life to treat disease all over the world. They have been used as a source of medicine. In fact, plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. Higher plants, as sources of medicinal compounds, have continued to play a dominant role in the maintenance of human health since ancient times (Farombi, 2003). Over 50% of all modern clinical drugs are of natural product origin (Stuffness and Douros, 1982) and natural products play an important role in drug development programs in the pharmaceutical industry (Baker et al., 1995). There has been a revival of interest in herbal medicines. This is due to increased awareness of the limited ability of synthetic pharmaceutical products to control major diseases and the need to discover new molecular structures as lead compounds from the plant kingdom. Plants with possible antimicrobial activity should be tested against an appropriate microbial model to confirm the activity and to ascertain the parameters associated with it. The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world (Reddy et al., 2001; Urul, 2002; Ateb and Urul, 2003). Much work has been done on ethnomedicinal plants in India (Maheshwari et al., 1986; Rai, 1989; Negi, 1993). Interest in a large number of traditional natural products has increased (Taylor et al., 1996).

The extracts and powder of the leaves of *Viscum album* were official in the French pharmacopoeia stabilizers. In subcontinent, the leaves, stems and barriers constitute a drug used for hypertension, arteriosclerosis, tumors and arthrosis. Also used for hysteria, epilepsy and ear diseases. The drug is reputed as a cardio tonic, antiseptic, antispasmodic, diuretic, purgative and emetic with narcotic action. It is employed as a decoction or tincture in intermittent fevers.

Also considered useful in splenic and hepatic enlargements, in lumbago and pills. Different extracts from plants used in allopathic medicine are restricted to mainly a few medicinal plant species. Most of them

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require a detailed study particularly with regard to the antimicrobial properties, so that in future they can effectively replace the chemically synthesized antibiotics which have a large number of side effects. Keeping in view the above information the aim of the present study is to determine the antibacterial and antifungal activity of leaves and twigs of *V. album*.

MATERIALS AND METHODS

The plant material was collected from Rialy, Muzaffarabad Azad Jammu and Kashmir. The whole plant was withdrawn and dried carefully under shade and then homogenized to fine powder and stored in airtight bottles.

Aqueous extracts

50 g of ground plant parts material was extracted successively with distilled water in soxhlet extraction apparatus (Thomas, 1977). All these extracts were collected separately and each extract was dried in vacuum rotary evaporator under reduced pressure and low temperature that is 70°C. The last traces of the water were evaporated at water bath, which was used as a source of heat (Rawlins and Tindall, 1977).

Organic solvent extraction

The 25 g portions of each dried powdered plant part material was soaked separately in 250 ml petroleum ether, acetone, ethyl acetate, chloroform, ethanol and methanol. The extraction was carried out by maceration for 7 days in each solvent at room temperature (25±2°C). The solvents extracted material was filtered in separate flaks (Rawlins and Tindall, 1977). All extracts were then dried in a vacuum rotary evaporator, weighed and stored at 4°C until further analysis.

Preparation of dilution

The dried aqueous, methanol, ethanol, petroleum ether, acetone, ethyl acetate, and chloroform extracts were then dissolved in their respective solvents in a proportion of 100 mg/ml. The concentration of reference antibiotics that is Ciprofloxacin was 100 µg/ml and nystatin 1500 µ/ml.

Microorganisms

In the present study gram positive bacteria that is *Staphylococcus aureus, Bacillus subtilis, Enterococcus faecium,* gram negative bacteria that is *Escherichia coli*, *Bordetella bronchisiptica, Salmonella typhi, Pseudomonas aeruginosa, Pseudomonas syringae,* yeast that is *Saccharomyces cerevisiae* and filamentous fungus *Aspergillus flavus,* were used to evaluate the antimicrobial potential of different extracts of the selected plant parts.

Antimicrobial assay

A 24 h old culture of each bacterium and 72 h old culture of fungus was used as an inoculum for the test. The slants were prepared in test tube. The nutrient agar medium was used for bacterial growth and similarly for fungus the sabouraud's dextrose agar medium was used. *In vitro* antimicrobial screening was performed by disc diffusion method as described by Vander and Vlientnck (1991). The sterilized nutrient agar medium when temperature reached between 40 and 45ºC was poured in the petri dishes containing bacterial suspension. The sabouraud's dextrose agar medium was poured in the petri dishes containing fungal suspension. Two series of experiments were conducted. In first crude extracts were tested for their antimicrobial activity against already mentioned bacteria, yeast and fungus. In the second series of experiment, antibiotic discs were prepared from the dilution of commercially available standard reference antibiotics, that is, ciprofloxacin and nystatin were placed on the top of the medium in the centre of petri dishes by following the disc diffusion method (Vander and Vlientnck, 1991).

The purpose of this experimental set was to compare the antimicrobial activity of the standard reference antibiotics with that of the solvent extracts of leaves and twigs of *V. album*. The plates containing bacterial culture were incubated at 37°C for 24 h. On the other hand, the plates with fungal suspension were incubated at 25°C for 72 h. After the incubation time, all the plates were examined for the presence of inhibition as a property of antimicrobial activity.

Statistical analysis

All values were expressed as means ± standard error means. The data for each microorganism were analyzed by using one way analysis of variance (ANOVA) technique and means were compared by using LSD at 5% (0.05) probability level (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Plant extracts have been studied against bacteria for years, but in a more intensified way in the last three decades. During this period, a lot of antimicrobial screening evaluations have been published based on the traditional use of Chinese, African and Asian plant drugs (Nishibe et al., 1982; Vlientinck et al., 1995; Perez and Anesini, 1994; Macfoy and Cline, 1990; Maikere et al., 1989; Forestiere et al., 1988). Reinsch carried out the first investigations into the chemical content of mistletoe in 1860 (Franz, 1985). Recent years, although technology and medicine have developed extensively, due to the decrease in natural richness made it obligatory to use natural products for many goals. For these reasons, like in other countries, in Pakistan, *V. album* L. is used for the treatment of various diseases.

In this study, the antimicrobial influence of acetone, petroleum ether, ethyl acetate, chloroform, ethanol, methanol and water crude extracts of *V. album* leaves and twigs were determined. This plant is known to have healing properties and is used for the treatment of various diseases in people. The results of the antimicrobial screening of different solvents crude extracts of leaves and twigs of *V. album* against 8 bacteria, 1 yeast and 1 filamentous fungus were presented in Tables 1 and 2. The statistical analyses of results have been tabulated in Tables 3 and 4.

It is evident from the results that the ethyl acetate extract of leaves of *V. album* showed prominent activity

Table 1. Antimicrobial activity of leaves of *viscum album* l. Concentration of crude extracts 100 mg/ml, ciprofloxacin 100 µg/ml and nystatin 1500 µ/ml**.**

Mean diameter of zones of inhibition (mm) ± standard error of mean (S. E. M.)										
S/N	Strains	AC	PE	EA	CH	ET	MT	WT	CF	NS
1	*S. aureus*	---	---	24.33±0.17	---	15.9±0.35	15.16±0.08	9.66±0.13	32.13±0.13	---
2	*B. subtilis*	---	---	19.66±0.33	---	15.83±0.16	15.83±0.17	10.66±0.33	31.93±0.06	---
3	*E. faecium*	---	---	---	---	15.9±0.1	19.83±0.17	9.83±0.17	30.03±0.03	---
4	*E. coli*	---	---	24.96±0.23	19.76±0.23	16.66±0.33	16.93±0.06	9.16±0.17	31.83±0.17	---
5	*B. bronchisiptica*	---	---	24.83±0.17	---	16.9±0.2	16.13±0.06	10.00±0.00	31.5±0.1	---
6	*P. aeruginosa*	---	---	19.66±0.33	10.00±0.00	13.83±0.16	19.83±0.17	9.9±0.1	29.83±0.17	---
7	*P. syringae*	---	---	19.66±0.33	15.16±0.16	13.66±0.33	20.93±0.06	9.83±0.17	30.83±0.17	---
8	*S. typhae*	---	---	---	---	15.83±0.17	15.26±0.13	9.00±0.00	29.93±0.06	---
9	*S. cerevisiae*	---	---	---	---	---	---	---	---	23.00
10	*A. flavus*	---	---	---	---	---	---	---	---	15.00

Key: AC. Acetone, PE. Petroleum ether, EA. Ethylacetate, CH. Chloroform, ET. Ethanol, MT. Methanol, WT. Water, CP. Ciprofloxacin, NS. Nystatin.

Table 2. Antimicrobial activity of twigs of *Viscum album* L. concentration of crude extracts 100 mg/ml.

Mean diameter of zones of inhibition (mm) ± standard error of mean (S. E. M.)								
S/N.	Micro organisms	Acetone	Petroleum ether	Ethyl acetate	Chloroform	Ethanol	Methanol	Water
1	*S. aureus*	---	---	15.83±0.17	10.00±0.00	11.67±0.33	19.83±0.16	---
2	*B. subtilis*	---	---	15.06±0.06	9.66±0.33	11.83±0.17	19.9±0.1	---
3	*E. faecium*	---	---	20.06±0.06	9.66±0.33	12.00±0.00	15.03±0.3	---
4	*E. coli*	---	---	24.83±0.17	20.06±0.06	14.83±0.17	24.83±0.17	---
5	*B. bronchisiptia*	---	---	15.06±0.03	10.06±0.06	10.66±0.33	14.83±0.17	---
6	*P. aeruginosa*	---	---	19.66±0.33	15.1±01	12.76±0.23	17.03±0.03	---
7	*P. syringae*	---	---	22.66±0.33	9.66±0.33	11.66±0.33	15.83±0.17	---
8	*S. typhae*	---	---	15.06±0.06	9.66±0.33	9.06±0.06	10.06±0.06	---
9	*S. cerevisiae*	---	---	---	---	---	---	---
10	*A. flavus*	---	---	---	---	---	---	---

Table 3. Antimicrobial activity of leaves of *Viscum album* L. least Significant difference (LSD).

Concentration of extracts 100 mg/ml, ciprofloxacin 100 µg/ml and nystatin 1500 µ/ml								
S/N	Strains	Ethyl acetate	Chloroform	Ethanol	Methanol	Water	Ciprofloxacin	Nystatin
1	*S. aureus*	24.33^{b}	0.00^{e}	15.9^{c}	15.16^{c}	9.66^{d}	32.13^{a}	---
2	*B. subtilis*	19.66^{b}	0.0023^{e}	15.83^{c}	15.83^{c}	10.66^{d}	31.93^{a}	---
3	*E. faecium*	0.023^{e}	0.023^{e}	15.9^{c}	19.83^{b}	9.83_{d}	30.03^{a}	---
4	*E. coli*	24.96^{b}	19.76^{c}	16.66^{d}	16.93^{d}	9.16_{e}	31.83^{a}	---
5	*B. bronchisiptica*	24.83^{b}	0.023^{f}	16.9^{c}	16.13^{d}	10.00^{e}	31.5^{a}	---
6	*P. aeruginosa*	19.66^{b}	10.00^{d}	13.83^{c}	19.83^{b}	9.9^{d}	29.83^{a}	---
7	*P. syringae*	19.66^{c}	15.16^{d}	13.66^{e}	20.93^{b}	9.8^{f}	30.83^{a}	---
8	*S. typhae*	0.023^{e}	0.023^{e}	15.83^{b}	15.26^{c}	9.00^{d}	29.93^{a}	---
9	*S. cerevisiae*	---	---	---	---	---	---	23.00
10	*A. flavus*	---	---	---	---	---	---	15.00

against *S. aureus, B. subtilis, E. coli, B. bronchisiptica, P. aeruginosa,* and *P. syringae,* that is 24.33±0.7, 19.66±0.33, 24.96±0.23, 24.83±0.17, 19.66±0.33 and 19.66±0.33 mm, respectively, while ethyl acetate extract of leaves showed no activity against *E. faecium, Salmonella typhae, S. cerevisiae* and *A. flavus* (Table 1).

Table 4. Antimicrobial activity of twigs of *Viscum album* L. Least significant differences (LSD).

Concentration of extracts 100 mg/ml and Ciprofloxacin 100 µg/ml						
S/N	**Strains**	**Ethyl acetate**	**Chloroform**	**Ethanol**	**Methanol**	**Ciprofloxacin**
1	*S. aureus*	15.83^{c}	10.00^{e}	11.66^{d}	19.83^{b}	32.13^{a}
2	*B. subtilis*	15.06^{c}	9.66^{e}	11.83^{d}	19.9^{b}	31.93^{a}
3	*E. faecium*	20.06^{b}	9.66^{e}	12.00^{d}	15.03^{c}	30.03^{a}
4	*E. coli*	24.83^{b}	20.06^{c}	14.83^{d}	24.83^{b}	31.83^{a}
5	*B. bronchisiptica*	15.06^{b}	10.06^{d}	10.66^{c}	14.83^{b}	31.5^{a}
6	*P. aeruginosa*	19.66^{b}	15.1^{d}	12.76^{e}	17.03^{c}	29.83^{a}
7	*P. syringae*	22.66^{b}	9.66^{e}	11.66^{d}	15.83^{c}	30.83^{a}
8	*S. typhae*	15.06^{b}	9.66^{c}	9.06^{d}	10.06^{c}	29.93^{a}
9	*S. erevisiae*	---	---	---	---	
10	*A. flavus*	---	---	---	---	

The ethylacetate extract of twigs of selected plant also represented appreciable activity against *S. aureus, B. subtilis, E. faecium, E. coli, B. bronchisiptica, P. aeruginosa, P. syringae,* and *S. typhae.* Which were 15.83±0.17, 15.06±0.06, 20.06±0.6, 24.83±0.17, 15.06±0.03, 19.66±0.33, 22.66±0.33 and 15.06±0.06 mm, respectively but *S. cerevisiae* and *A. flavus* showed no sensitivity (Table 2). This confirms previous report by Eloff et al. (2005) in which they suggested that ethyl acetate was the best extractant with an average minimum inhibitory concentration.

The chloroform extract of leaves showed activity against *E. coli* (19.76±0.23 mm), *P. aeruginosa* (10.00±0.00 mm), *P. syringae* (15.16±0.16 mm) *S. aureus, B. subtilis, B. bronchisiptica, S. typhae, S. cerevisiae* and *A. flavus* were not affected by the extract (Table 1). The chloroform extract of twigs of *V. album* exhibited moderate activity against all the microorganisms used except *S. cerevisiae* and *A. flavus.* The mean diameter of zones of inhibition of the extract against *S. aureus, B. subtilis, E. faecium, E. coli, B. bronchisiptica, P. aeruginosa, P. syringae,* and *S. typhae* were 10.00±0.00, 9.66±0.33, 9.66±0.33, 20.06±0.06, 10.06±0.06, 15.1±0.1, 9.66±0.33 and 9.66±0.33 mm, respectively (Table 2). These results were also comparable with the previous studies of Farjana et al. (2003) in which they explained that the chloroform extract showed comparatively more *in vitro* antimicrobial activity against some bacteria.

Pokhrel et al. (2002) described that the alcoholic extract of *Bauhinia variegate* was found to have antimicrobial activity against *B. subtilis, P. aeruginosa, S. typhae, Shiegella dysenteriae, S. aureus,* and *Vibrio cholerae.* The largest zone of inhibition was found to be exhibited against *B. subtilis*. The extract was found to be more effective against Gram positive than Gram negative bacteria. In present study it was observed that the ethanolic extracts of leaves and twigs of *V. album* exhibited more or less similar zones of inhibition against all the bacteria tested. The *S. cerevisiae* and *A. flavus* were resistant to the extracts (Tables 1 and 2).

Kianbaknt and Jahaniani (2003) described that antimicrobial activity of methanolic extracts of different parts of *Tribulus terrestris* was evaluated against four bacteria by broth dilution assay and agar diffusion assay. The methanolic extracts of all parts of the plant showed considerable activity against all bacteria but in this study methanolic extracts of leaves and twigs also exhibited considerable activity against all the bacteria used. The largest zones of inhibition produced by the leaves extract against *E. faecium, P. aeruginosa and P. syringae* were 19.83±0.17, 19.83±0.17 and 20.93±0.06 mm, respectively. The largest zones of inhibition produced by methanolic extract of twigs against *S. aureus* and *E. coli* were 19.83±0.16 and 24.83±0.17 mm, respectively. *S. cerevisiae* and *A. flavus* were not influenced by the extracts of leaves and twigs (Tables 1 and 2)

The water extract of leaves represented less activity against *S. aureus, B. subtilis, E. faecium, E. coli, B. bronchisiptica, P. aeruginosa, P. syringae,* and *S. typhae* that is, 9.66±0.13, 10.66±0.33, 9.83±0.17, 9.16±0.17, 10.00±0.00, 9.9±0.1, 9.83±0.17 and 9.00±0.00 mm, respectively, but *S. cerevisiae* and *A. flavus* were resistant to the extract. The water extract of twigs had no activity against all the microorganisms tested (Tables 1 and 2).

The antibiotic Ciprofloxacin showed high activity against all the microorganisms used except *S. cerevisiae* and *A. flavus, which* were resistant to the Ciprofloxacin. The mean diameter of zones of Inhibition against *S. aureus, B. subtilis, E. faecium, E. coli, B. bronchisiptica, P. aeruginosa, P. syringae, S. typhae* were mentioned in Table 1. The nystatin exhibited activity against yeast and fungus tested while all the bacteria were resistant to nystatin (Table 1). Avato et al. (1997) reported that extracts from *Bellis perennis* have a high antimicrobial activity against bacteria than fungus. The results of Zavala et al. (1997) were similar to ours. They showed

that extracts from some plants have high activity against bacteria than yeast and fungus. Present results were comparable with the previous reports but some extracts were more effective against gram negative bacteria than gram positive bacteria while yeast and fungus tested was resistant to the extracts tested.

Conclusion

On the basis of present investigations, it is concluded that there exists a great potential in the search of new and more potent antimicrobial substances from the natural sources. The potential for developing antimicrobials from plants appears rewarding as it will lead to the development of phytomedicines to act against microbes. Plant based antimicrobials have enormous therapeutical potential as they can serve the purpose without any side effect that are often associated with synthetic antimicrobials. The tested drugs (organic solvents extracts of leaves and twigs of *V. album*) showed excellent antimicrobial activity against tested bacteria. So it can be concluded that leaves and twigs of the selected plant can be regarded as good natural antibiotics with considerable degree of antimicrobial activity.

As a consequence of this study, we will try to isolate pure compound, which is present in fractions showing large inhibitory activity to bacteria as well as any pharmacological or toxicological properties that such compound might have.

REFERENCES

Ateb DA, Erdo UOT (2003). Antimicrobial activities of various medicinal and commercial plant extracts. Turk. J. Biol., 27: 157-162.

Avato PP, Vitali M, Tava A (1997). Antimicrobial activity of polyacetylenes from *Bellis perennis* and synthetic derivatives. Planta Med., 63: 503-507.

Baker JT, Borris RP, Carte B (1995). Natural product drug discovery and development: New perspective on international collaboration. J. Nat. Prod., 58: 1325-1357.

Eloff JNE, Famakin JO, Kathrene DRP (2005). *Cembretum woodii* leaf extracts have high activity against Gram-negative and Gram-possitive bacteria. Afr. J. Biokethnol., 4(10): 1161-1166.

ErdoUrul OT (2002). Antibacterial activities of some plant extracts used in folk medicine. Pharm. Biol., 40: 269-273.

Farjana N, Saud ZA, Habib-ur-Rehman M, Ekram-ul-haque M (2003). *In vitro* antimicrobial activity of the compound isolated from chloroform extract of *Moringa oleifera* Lam. Pak. J. Biol. Sci., 6(22): 1888-1890.

Farombi EO (2003). African indigenous plants with chemotherapeutic potentials and biotechnological approach to the production of bioactive prophylactic agents. Afr. J. Biotech., 2: 662-671.

Forestiere AM, Pizzimenti FC, Monforte TM and Bisignano G (1988). Antibacterial activity of some African medicinal plants. Pharmacol. Res. Commun., 20(5): 33-36.

Maheshwari JK, Singh KK, Saha S (1986). Ethnobotany of tribals of Mirzapur District, Uttar pradesh, Economic Botany Information Service, NBRI, Lucknow.

Macfoy CA, Anesini EI (1990). *In vitro* antimicrobial activity of there plants used in traditional medicine in *Sierra Loeone*. J. Ethnopharmacol., 28: 323-327.

Maikere FR, Puyvelde LV, Mutwewingabo A, Habiyaremye FX (1989). Study of Rwandese medicinal plants used in the treatment of diarrhea. J. Ethnophamacol., 26: 101-109.

Negi KS, Tiwari JK, Gaur RD (1993). Notes on ethnobotany of five districts of Garhwal Himalaya, Uttar pradesh, India Ethnobot., 5: 73-81.

Nishibe S, Okabe K, Tsukamoto H, Sakushima A, Hisada S, Baba H, Akisada T (1982). Studies on the Chinese crude drug *Forsythia fructus.* The structure and antimicrobial activity of suspensaside isolated from *Forsythia* suspense*.* Chem. Pharm. Bull., 30: 4548-4553.

Perez C, Anesini C (1994). *In vitro* antibacterial activity of Argentine folk medicinal plants against *Salmonella typhae*. J. Ethnopharmacol., 44: 41-46.

Pokhrel NR, Adhikari RP, Baral MP (2002). *In vitro* evaluation of the antimicrobial activity of *Bauhinia variegate* locally known as koiralo. J. Microbiol. Biotechnol., 18(1): 69-71.

Rai MK (1989). Ethnomedicinal studies of Chhindwara district (M.P.). I. Plants used in stomach disorders. Indian Med., 1: 1-5.

Rawlins EA, Tindall B (1977). Bently's Text Book of pharmaceutics 8th edition, London, pp. 174-198.

Reddy PS, Jamil K, Madhusudhan P (2001). Antibacterial activity of isolates from *Piper longum* and *Taxus baccata*. Pharm. Biol., 39: 236-238.

Stuffness M, J Douros (1982). Current status of the NCI plant and animal product program. J. Nat. Prod., 45: 1-14.

Taylor RSL, Manandhar NP, Hudson JB (1996). Antiviral activities of Nepalese medicinal plants. J. Ethnopharmacol., 52: 157-163.

Thomas EH (1977). A hand book of pharmaceutical and clinical measurements and analysis. Preston Publishing Company, pp. 79-80.

Vander BDA, Vlietnck AJ (1991). Screening methods for higher plants Assay for Bioactivity K. Hostiettman (Ed). Academic press, London, pp. 43-69.

Vlietinck AJ, Hoof LV, Tott J (1995). Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. J. Ethnopaharmacol., 46: 31-47.