ORIGINAL ARTICLE

Evaluation of antimicrobial efficacy of flavonoids and alkaloids of *Andrographis paniculata* nees

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Context: The persistent increase in the number of antibiotic resistant strains of microorganisms has led to the development of more potent but more expensive antibiotics. Synthetic drugs are mostly associated with side effects and are generally costly, hence are not affordable to economically poor class of the society when long term treatment is required, thus interest has been developed in the use of herbal medicines which have been reported to have either very little or no side effects. **Aims:** Present work was carried out to assess the antimicrobial activity of *A. paniculata* against some multidrug resistant pathogenic bacteria. **Materials and Methods:** Different parts (leaf, stem, root) of *A. paniculata* were collected and air dried and soxhlet extracted by using standard methods for flavonoid and alkaloid extraction. These extracts were then tested for antimicrobial activity using disc diffusion method. Minimum inhibitory concentration, Minimum bactericidal concentration and Total activity were also calculated. **Statistical Analysis:** Mean value and Standard Deviation were calculated for the test bacteria and fungi. Data were analysed by one-way analysis of variance and *P* values were considered significant at $P < 0.05$. **Results:** *C. albicans* was found to be the most susceptible organism followed by *P. mirabilis, S. aureus, E. coli, P. aeruginosa* and *A. flavus*. *A. niger* and *T. mentegrophyte* were observed to be resistant as none of the tested extracts showed activity against them. Free flavonoid extract of root showed best activity against *C. albicans* (IZ 14 mm, MIC 0.156), whereas leaf free flavonoid extract showed maximum 21 mm inhibition zone against *P. mirabilis*.The range of MIC and MBC was found to be 1.25-0.039 and 2.5-0.078 respectively. **Conclusion:** Results of the present study reveal that extracts of *A. paniculata* are showing great antimicrobial potential against tested microorganisms, and may be exploited for future antimicrobial drugs

Key words: Flavonoids, minimum inhibitory concentration, minimum bactericidal concentration and total activity

INTRODUCTION

Plants remain the most common source of antimicrobial agents. Their usage as traditional health remedies is the most popular choice for 80% of the population in Asia, Latin America and Africa and is reported to have minimal side effects.[1,2] In recent years, pharmaceutical companies have spent a lot of time and money in developing natural products extracted from plants, to produce more cost effective remedies that are affordable to the population. The rising incidence of multidrug resistance among pathogenic microbes, has further necessitated the need to search for newer antibiotic sources. Higher plants produce hundreds and thousands of diverse chemical compounds with different biological activities.[3] The antimicrobial compounds produced by plants are active against plants and human pathogenic microorganisms.[4] The substances that can kill them and have no or least toxicity to host cells are considered potential compounds for developing new antimicrobial drugs.

Access this article online	
Quick Response Code:	Website: www.greenpharmacy.info
	DOI: 10.4103/0973-8258.111617

Andrographis paniculata (Acanthaceae) is an annual herb. It is found in Sri Lanka and throughout the plains of India specially Maharashtra, Karnataka, Uttar Pradesh, Tamilnadu, Orissa. Various medicinal properties like choleretic, antidiarrhoeal, immunostimulant and anti-inflammatory have been attributed to this plant in the traditional system of Indian medicine.[5-9] Further reported activities are hepatoprotective, antimalarial, antihypertensive, antipyretic, antithrombotic and antidote for snake bites. The present investigation was undertaken to find out the antibacterial potentiality of the flavonoid and alkaloid extracts of the different parts of *A. paniculata* against some Gram positive and Gram negative bacteria and some fungi. Flavonoids are becoming the subject of medical research. They have been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity,[10,11] antiallergic activity, antioxidant activity and cytotoxic antitumor activity. The antibacterial activity of flavonoids is being increasingly documented. Flavonoid rich plant extracts from species of Hypericum,[12] Capsella[13] and Chromolaena[14] have been reported to possess antibacterial activity.Review of the current literature reveals that no work has been carried out for extraction and screening of specific compound from selected plant. Hence, in the present work an extraction and screening for antibacterial activity of the flavonoids and alkaloids of *A. paniculata* has been undertaken.

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Received: 24-01-2013; **Accepted:** 20-03-2013

MATERIALS AND METHODS

Different parts of *A. paniculata* (leaf, stem and root) were collected in the month of February to April from the western parts of India (Jaipur, Rajasthan). Plants were identified by senior taxonomist at department of Botany, university of Rajasthan and (voucher specimen no: RUBL20873) was submitted to the herbarium, Botany department, university of Rajasthan.

Preparation of Extracts

Flavonoid extraction

Selected plant parts were separately washed with sterilized water; shade dried, and finely powdered using a blender. Each sample was subjected to extraction, following the method of Subramanian and Nagarjan, 1969.[15] One hundred grams of each finely powdered sample was soxhlet extracted with 80% hot methanol (500 ml) on a water bath for 24 h and filtered. Filtrate was re-extracted successively with petroleum ether (fraction I), ethyl ether (fraction II), and ethyl acetate (fraction III) using separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances, where as ethyl ether and ethyl acetate fractions were analyzed for free and bound flavonoids respectively. The ethyl acetate fraction of each of the samples was hydrolyzed by refluxing with 7% H_2SO_4 for 2 h (for removal of bounded sugars) and the filtrate was extracted with ethyl acetate in separating funnel. Ethyl acetate extract obtained was washed with distilled water to neutrality. Ethyl ether (free flavonoids) and ethyl acetate fractions (bound flavonoids) were dried in vaccuo and weighed [Table 1]. The extracts were stored at 4°c and were re-suspended in their respective solvents to get 10 mg/ml for antimicrobial assay.

Extraction of alkaloids

Alkaloids were extracted from different parts of the selected plants by well established methods[16] after preliminary detection of alkaloids. Finely powered sample (100 g) of plant parts were extracted with 10% acetic acid in ethanol for 4 h. Extracts were concentrated and were made alkaline by NH_4OH. The precipitate thus obtained was collected by centrifugation, washed with 1% NH_4OH, filtered, dried in vaccuo and weighed. Extracts thus obtained were stored at 4°C in air tight glass vials for further use.

Selected test microorganisms

Eight pathogens were screened in total which include four bacteria, viz., *Escherichia coli* [Microbial type culture collection and gene bank (MTCC) no. 46], *Staphylococcus aureus* (MTCC no 87), *Proteus mirabilis* (MTCC no. 425), *Pseudomonas aeruginosa* (MTCC no. 1934) and four fungal strains, viz., *Aspergillus flavus* (MTCC no. 277), *Aspergillus niger* (MTCC no. 282), *Candida albicans* (MTCC no. 183), and *Trycophyton mentagrophyte* (MTCC no. 7687). The pathogens were procured from IMTECH (Chandigarh, Punjab, India). Bacterial strains were grown and maintained on Muller-Hinton Agar medium, while fungi were maintained on Sabouraud Dextrose Agar medium.

Antimicrobial assay

Disc diffusion assay[17] was performed for screening. Muller-Hinton agar and Sabouraud Dextrose agar base plates were seeded with the bacterial and fungal inoculums respectively (inoculum size 1×10^8 CFU (colony forming unit)/ml for bacteria and 1×10^7 cell/ml for fungi.) Sterile filter paper discs of Whatmann no. 1 (6 mm in diameter) were impregnated with 100 µl of each of the extract of concentration (10 mg/ml) to give a final concentration of 1 mg/disc. Discs were left to dry in vaccuo so as to remove residual solvent, which might interfere with the determination. Discs with extract were then placed on the corresponding seeded agar plates. Each extract was tested in triplicate along with streptomycin (1 mg/disc) and

Table 1: MIC and MBC of active extracts of *A. paniculata* against different pathogens

Microorganisms plant part	MIC and MBC	Leaf			Stem			Root		
		E1	E2	A1	E1	E2	A2	E1	E2	A3
E. coli	MIC	0.625	0.312	–	0.625	–	0.625	0.078	0.039	–
	MBC	1.25	0.625	–	0.312	–	0.625	0.156	0.039	–
S. aureus	MIC	–	0.312	–	0.312	0.625	–	0.312	0.078	0.312
	MBC	–	0.625	–	0.312	1.25	–	0.625	0.156	0.625
P. mirabilis	MIC	0.039	0.156	0.625	–	0.312	0.625	0.156	0.312	–
	MBC	0.078	0.312	1.25	–	0.625	1.25	0.312	0.625	–
P. aeruginosa	MIC	–	–	–	–	0.312	1.25	0.625	–	0.625
	MBC	–	–	–	–	0.312	2.5	1.25	–	0.625
A. flavus	MIC	–	–	–	0.312	0.156	–	–	0.312	–
	MBC	–	–	–	0.625	0.312	–	–	0.312	–
C. albicans	MIC	–	0.312	0.625	–	0.156	0.312	0.156	0.312	0.625
	MBC	–	0.625	1.25	–	0.312	0.625	0.156	0.625	1.25

E1 – Free flavonoids; E2 – Bound flavonoids A1, A2, A3 – Alkaloid of respective parts; MIC – Minimum inhibitory concentration; MBC/MFC – Minimum Bactericidal/Fungicidal concentration

terbinafine (1 mg/disc) as standard for bacteria and fungi respectively. The plates were kept at 4°C for diffusion of extract, thereafter were incubated at 37°C for bacteria (24 h) and 27°C for fungi (48 h). Activity index for each extracts was calculated [Table 2] by the standard formula viz

Activity index = IZ produced by extract/IZ produced by standard

Where, IZ = inhibition zone.

Determination of minimum inhibitory concentration and minimum bactericidal/fungicidal concentration

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against test pathogens. Broth micro dilution method[18] was followed for determination of MIC values. Plant extracts were resuspended in acetone (which has no activity against test microorganisms) to make 10 mg/ml final concentration. Two fold serially diluted extracts were added to broth media of 96-wells of micro titer plates. Thereafter 100 µl inoculum (for bacteria 1×10^8 CFU/ml and 1×10^7 cell/ml for fungi) was added to each well. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. Micro titer plates were then incubated at 37°C for 24 h for bacteria and 28°C for 48 h for fungi. Each extract was assayed in duplicate and each time two sets of micro plates were prepared, one was kept for incubation while another was kept at 4°C for comparing the turbidity in the wells of micro plate. The MIC values were taken as the lowest concentration of the extracts in the well of the micro titer plate that showed no turbidity after incubation. The turbidity of the wells in the micro titer plate was interpreted as visible growth of microorganisms. The minimum bactericidal/fungicidal concentration (MBC/MFC) was determined by sub culturing 50 µl from each well showing no apparent growth. [Table 3]. Least concentration of extract showing no visible growth on sub culturing was taken as MBC/MFC.

Total activity determination

Total activity is the volume up to which test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g[19] [Table 1].

Statistical Analysis

Mean value and standard deviation were calculated for each test bacteria and fungus. Data were analysed by one-way ANOVA and *P* values were considered significant at $P < 0.05$.

Table 2: Antimicrobial activity of extracts of *A. paniculata* against some pathogenic bacteria

Microorganisms plant part	Extract	*E. coli*		*S. aureus*		*P. mirabilis*		*P. aeruginosa*	
		IZ	AI	IZ	AI	IZ	AI	IZ	AI
Leaf	E1	9.5	0.730±0.10	–	–	21	0.807±0.021	–	–
	E2	13.5	1.038±0.023	12	0.571±0.015	12.5	0.595±0.020	–	–
	A1	–	–	–	–	10	0.384±0.003	–	–
Stem	E1	11	0.846±0.007	13	0.619±0.010	–	–	–	–
	E2	–	–	12.5	0.595±0.051	13	0.50±0.010	13	0.65±0.010
	A2	12.5	0.961±0.017	–	–	9.5	0.365±0.070	8	0.4±0.017
Root	E1	15.5	1.192±0.070	12.5	0.595±0.03	14.5	0.557±0.020	9.5	0.475±0.001
	E2	16.5	1.269±0.010	15.5	0.738±0.010	14.5	0.557±0.017	–	–
	A3	–	–	12	0.571±0.017	–	–	10	0.5±0.023

All values are mean ± SD; n-3; IZ – Inhibition zone in mm (mean value: Including 6mm diameter of disc). AI – Activity index (IZ developed by extract/IZ developed by standard), ±=SEM. (-) – No activity E1 – Free flavonoids; E2 – Bound flavonoids IZ of standard drug streptomycin against *E. coli* (13 mm); *S. aureus* (21 mm); *P. mirabilis* (26 mm); *P. aeruginosa* (20 mm)

Table 3: Antimicrobial activity of some extracts of *A. panicualta* against some pathogenic fungi

Microorganisms plant part	Extract	*A. flavus*		*C. albicans*		*A. niger*		*T. mentegrophyte*	
		IZ	AI	IZ	AI	IZ	AI	IZ	AI
Leaf	E1	–	–	–	–	–	–	–	–
	E2	–	–	13.5	0.964±0.05	–	–	–	–
	A1	–	–	11	0785±0.021	–	–	–	–
Stem	E1	11.5	0.638±0.126	–	–	–	–	–	–
	E2	13.5	0.75±0.010	13.5	0.965±0.010	–	–	–	–
	A2	–	–	8.5	0.607±0.030	–	–	–	–
Root	E1	–	–	14	1.0±0.010	–	–	–	–
	E2	11.5	0.638±0.023	12.5	0.892±0.023	–	–	–	–
	A3	–	–	11	0.785±0.012	–	–	–	–

All values are mean ± SD; n-3;IZ – Inhibition zone in mm (mean value: Including 6 mm diameter of disc). AI – Activity index (IZ developed by extract/IZ developed by standard), ±=SEM. (-) – No activity E1 – Free flavonoids; E2 – Bound flavonoids IZ of standard drug candid v6 against *C. albicans* (14 mm); *A. flavus* (18 mm)

RESULTS

Antimicrobial potency of flavonoids (free and bound) and alkaloids were assessed by inhibition zone, activity index [Tables 2 and 3], Minimum inhibitory concentration and minimum bactericidal/fungicidal concentration [Tables 1 and 4]. Quantity of extracts per gram of plant material was also calculated [Table 4]. In the present investigation a total 9 extracts were tested, among which each of the extracts were active against one of the tested pathogens. *A. niger* and *T. mentagrophyte* were observed to be resistant as none of the tested extracts showed activity against them. The most susceptible organism in the investigation was *C. albicans* against which most of the plant extracts showed inhibition zone which were persistent as compared with the standard, and best activity was observed by free flavonoids of root (IZ = 14 mm, AI = 1.0 ± 0.010 MIC = 0.156 mg/ml). The leaf free flavonoid extract showed best activity against *P. mirabilis* (IZ = 21 mm, AI = 0.807 ± 0.021, MIC = 0.039), whereas bound flavonoid of leaf was most active against *E. coli* (IZ-13.5 mm) and *C. albicans* (IZ-13.5 mm). Stem free flavonoid was found most active against *S. aureus* (IZ = 13 mm, AI = 0.619 ± 0.010) while stem bound flavonoid showed satisfactory activity against *A. flavus* (IZ = 13.5 mm.AI = 0.75 ± 0.010) and *C. albicans* (IZ = 13.5 mm, AI = 0.965 ± 0.010).*E. coli, S. aureus, P. mirabilis, P. aeruginosa* and *C. albicans*, were found susceptible to free flavonoids of roots, whereas roots bound flavonoid indicated sufficient bio-activity against *E. coli* (IZ = 16.5 mm, AI = 1.269 ± 0.010). Among all the alkaloid extracts, stem alkaloids were observed as most bioactive substance, as it has shown activity against four, out of eight pathogens. MIC and MBC values [Table 1] were evaluated for plant extracts which had shown activity, in diffusion assay. The range of MIC and MBC/MFC of extracts recorded was 1.25-0.039 mg/ml and 2.5-0.078 mg/ml, respectively. In the present investigation lowest MIC value 0.039 mg/ml was recorded against *P. mirabilis and E. coli*, whereas, against *S. aureus* lowest MIC observed was 0.078 mg/ml, indicating significant antimicrobial potential of test extracts. MIC and MBC/MFC values were found to be same for five extracts indicating their bactericidal/fungicidal nature.

Quantity of extract obtained per gram from plant parts and total activity (TA) was calculated and recorded [Table 4]. Total activity indicates the volume at which extract can be diluted without losing ability to kill microorganism. Most of the extracts showed high values of TA against *E. coli, P. mirabilis* and *C. albicans*.

DISCUSSION

There is a continuous and urgent need to discover new antimicrobial compounds as there is an alarming increase in the incidence of new and re-emerging infectious diseases. Medicinal plants may be a viable alternative source to costly antibiotics (against which microbes are developing resistance rapidly), as most of the medicinal plants are safe with little or no side effects, cost effective and have ability to affect a wide range of antibiotic resistant microorganisms.

Present study is an effort towards this direction. *A paniculata* had previously been studied for antibacterial and antifungal activities, but still the literature available is meager. Water extract of whole aerial part of *A. paniculata* had already been screened against different pathogenic bacteria.[15] Chloroform, acetone, ethanol and water extract of *A. paniculata* leaf were also tested against *S. aureus, P. aeruginosa, B. subtilis, A. niger and P. chrysogenum*.[20] Aqueous, andrographolide and arabinogalactan protein from *A. paniculata* were evaluated for antimicrobial activity.[21]

Screening of plant under investigation (*A. paniculata*) so far has not been worked out for flavonoids and alkaloids. Mostly the crude extracts have been screened of the whole aerial part, without MIC, MBC/MFC and TA determination. Such studies could only indicate their antimicrobial potential but are not helpful in establishing them as an antibiotic, hence cannot replace the existing antibiotics. In the present investigation IZ, AI, MIC, MBC/MFC and TA

Table 4: Total activity of extracts of *A. paniculata*

Plant part	Extract	Quantity of extract mg/g dwt.	Total activity (ml/g)					
			E. coli	*S. aureus*	*P. mirabilis*	*P. aeruginosa*	*C. albicans*	*A. flavus*
Leaf	E1	27	43.2	–	692.30	–	–	–
	E2	17.5	56.08	56.08	112.17	–	56.08	–
	A1	1.01	–	–	1.616	–	1.61	–
Stem	E1	10.67	17.07	54.71	–	–	–	34.19
	E2	6.67	–	10.672	21.37	21.37	42.75	42.75
	A2	18.5	29.6	–	14.8	14.8	59.29	–
Root	E1	18.67	119.67	59.83	119.67	29.87	119.67	–
	E2	2	12.82	25.64	6.41	–	6.41	6.41
	A3	10.2	–	32.69	–	16.32	16.32	–

E1 – Free flavonoids; E2 – Bound flavonoids; A1, A2, A3 – Alkaloid of respective parts. Total activity = $\frac{\text{Extract per gram dried plant part}}{\text{MIC of extract}}$

have been evaluated for each extract. For most of the extracts MIC values recorded was very low, indicating strong bio efficacy of the plant. Extracts with higher MBC/MFC values than MIC values against microorganisms tested, indicate their bacteriostatic/fungistatic effects. Bound flavonoids of root were found bactericidal against *E. coli*. Extracts under study showed activity against both gram positive as well as gram negative bacteria. Although it is considered that Gram negative bacteria are less sensitive to plant extracts, possibly as a result of their extra lipo-polysaccharide and protein cell wall that provides permeability barrier to the antibacterial agent.[22] Susceptibility difference between Gram positive and Gram negative bacteria may be due to cell wall structural differences between these classes of bacteria. Activity of the tested extracts against both the classes of bacteria suggests its wide range of effectiveness against pathogens. In the light of the fact that microorganisms are becoming resistant against the drugs in use, the present investigation is of great significance, as far as the future drugs are concerned and uses of selected plant by the pharmaceutical industries for preparing plant based antimicrobials drugs.The findings of the present investigation offer a scientific evidenceto support the ethno medicinal use of *Andrographis paniculata* as an alternative medicine.

ACKNOWLEDGMENT

The authors are thankful to the Head, Department of Botany, University of Rajasthan. Jaipur, India, for providing all facilities for the present investigation. Special thanks to CSIR, New Delhi for financial assistance.

REFERENCES

1. Bibitha B, Jisha VK, Salitha CV, Mohan S, Valsa AK. Antibacterial activity of different plant extracts. Short Communication. Indian J Microbiol 2002;42:361-3.
2. Zeggwah M, Michel N and Eddouks M. Antihypertensive effect of *Lepidium sativum* in spontaneously hypertensive rats. J Ethnopharmacol 2005;102:193-7.
3. Newman DJ, Cragg GM, Snader KM. The influence of natural products upon drug discovery. Nat Prod Res 2000;17:215-34.
4. Yoganarasimhan N. Medicinal plants of India Vol. 1. Bangalore: Interline Publishing Pvt Ltd; 1996.
5. The Wealth of India. X-Z and cumulative indexes. Vol. 11. New Delhi: National Institute of Science Communication, CSIR; 1998. A dictionary of Indian Raw Material and Industrial Products. 1998. p. 123-4.
6. Nadkarni KM. Indian Materia Medica revised and enlarged by Nadkarni AK. 2nd ed. Vol. 1. Mumbai: Popular Prakashan Pvt; 2000. p. 1319.
7. Mukherjee PK. Quality control of herbal drugs. 1st ed. New Delhi: Business Horizons Pharmaceutical Publishers; 2002. p. 701.
8. Kokate CK, Purohit A, Gokhale SB. Pharmacognosy, 8th ed. Pune: Nirali Prakashan; 2002. p. 106-12.
9. Agarwal VS. Drugs plants of India. Vol. 1. Ludhiana: Kalyani Publishers; 1997. p. 182-3.
10. Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol 1983;32:1141-8.
11. Harborne JB, Baxter H. The handbook of natural flavonoids, Vols. 1 and 2. Chichester, UK: John Wiley and Sons; 1999.
12. Dall'Agnol R, Ferraz A, Bernardi AP, Albring D, Nör C, Sarmento L, *et al*. Antimicrobial activity of some Hypericum species. Phytomedicine 2003;10:511-6.
13. El-Abyad MS, Morsi NM, Zaki DA, Shaaban MT. Preliminary screening of some Eryptian weeds for antimicrobial activity. Microbios 1990;62:47-57.
14. Subramanian SS, Nagarjan S. Flavonoids of the seeds of *Crotolaria retusa* and *Crotolaria striata*. Curr Sci 1969;38:65.
15. Harborne JC. Phytochemical methods: A guide to modern techniques of plant analysis, 2nd ed. London, New York: Chapman and Hall Ltd; 1984.
16. Andrews JM. BSAC standardized disc susceptibility testing method. J Antimicrob Chemother 2001;4:43-57.
17. Basri DF, Fan SH. The potential of aqueous and acetone extracts of gall of *Quercus infectoria* as antibacterial agents. Indian J Pharmacol 2005;37:26-9.
18. Eloff JN. Quantifying the bioactivity of the plant extracts during screening and bioassay-guided fractionation. Phytomedicine 2004;11:370-1.
19. Zaidan MRS, Noor Rain A, Badrul AR, Adlin A, Norazah A and Zakia I. *In vitro* screening of five local medicinal plants for antibacterial activity using disc diffusion method. Trop Biomed 2005;22:165-70.
20. Hosamani PA, Lakshman HC, Sandeepkumar K and Rashmi C Hosamani. Antimicrobial activity of leaf extract of *A. paniculata* wall. Sci Res Report 2011;1:92-5.
21. Singha PK, Roy S, Dev S. Antimicrobial activity of *A. paniculata*. Fitoterapia 2003; 74:692-4.
22. Akinyemi KO, Oladapo O, Okwara CE, lbe CC, Fasure KA. Screening of crude extracts of six medicinal plants used in South-west Nigerian unorthodox medicine for antimethicillin resistant *Staphylococcus aureus* activity. BMC Complement Altern Med 2005;5:6.

How to cite this article: Gautam K, Kumar P, Jindal A. Evaluation of antimicrobial efficacy of flavonoids and alkaloids of *Andrographis paniculata* nees. Int J Green Pharm 2013;7:57-61.

Source of Support: CSIR, New Delhi, **Conflict of Interest:** None declared.