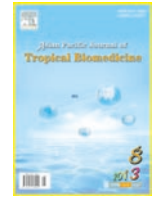




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Evaluation of anti-bacterial and anti-oxidant potential of andrographolide and echiodinin isolated from callus culture of *Andrographis paniculata* Nees

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PEER REVIEW

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Comments

This is an important novel research on active principles of *A. paniculata* *in vitro* callus cultures. The present study succeeded in providing supporting scientific evidence for its ethnopharmacological uses. Furthermore, isolation of andrographolide and echiodinin from callus cultures gave new dimensions to the study which may be helpful in mass production and exploitation of metabolites for future studies.

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ABSTRACT

Objective: To evaluate the anti-bacterial and anti-oxidant activity of andrographolide (AND) and echiodinin (ECH) of *Andrographis paniculata*.

Methods: In this study, an attempt has been made to demonstrate the anti-microbial and anti-oxidant activity of isolated AND and ECH by broth micro-dilution method and 2,2-diphenyl-2-picryl-hydrazyl (DPPH) assay, respectively. Structure elucidation was determined by electro-spray ionization-MSD, NMR (¹H and ¹³C) and IR spectra.

Results: AND was effective against most of the strains tested including *Mycobacterium smegmatis*, showing broad spectrum of growth inhibition activity with Minimum inhibitory concentration values against *Staphylococcus aureus* (100 µg/mL), *Streptococcus thermophilus* (350 µg/mL), *Bacillus subtilis* (100 µg/mL), *Escherichia coli* (50 µg/mL), *Mycobacterium smegmatis* (200 µg/mL), *Klebsiella pneumoniae* (100 µg/mL), and *Pseudomonas aeruginosa* (200 µg/mL). ECH showed specific anti-bacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* at a concentration higher than 225 µg/mL. Both AND and ECH were not effective against the two yeast strains, *Candida albicans* and *Saccharomyces cerevisiae* tested in this study.

Conclusion: This preliminary study showed promising anti-bacterial activity and moderate free radical scavenging activity of AND and ECH, and it may provide the scientific rationale for its popular folklore medicines.

KEYWORDS

Andrographolides, Disk diffusion method, Folklore medicine, King of bitters, Minimum inhibitory concentration, Phenolic compounds

1. Introduction

Anti-microbial compounds are of key interest nowadays in research areas due to the outbreak of newer infectious diseases, the increase in resistance of microorganisms towards

the existing microbial compounds and harmful side effects of the compounds. For this reason, there is a need for the development of compounds with less side effects and more targeted activity on the microorganisms. Research is going on to identify compounds with anti-microbial activity mainly

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from plant origin as they have lesser side effects^[1]. Most of the researchers concentrate on screening and minimum inhibitory concentration (MIC) determination of extracts of plants rather than identifying compounds with activity, which makes most of the researches unuseful for drug discovery. In this attempt, we isolated active constituents of the plant and screened for anti-microbial activity which can be used further in research to develop anti-microbial compounds with the isolated compounds or their synthetic analogues.

Free radicals produced in the body due to oxidative processes are responsible for many diseases including cancer and cardiovascular diseases^[2]. Although the human body has mechanisms to neutralize the free radicals produced, they are not sufficient enough due to the diets most people follow. It is recommended to have diets rich in anti-oxidants to increase the body's resistance to free radicals. Most of the previous researches were carried out on extracts of plants. In this research, along with anti-microbial activity of the isolated compounds, we also screened for anti-oxidant activity.

The genus *Andrographis* (Acanthaceae), comprising of about 40 species, is essentially distributed in southern India and in tropical Asia. *Andrographis paniculata* Nees (*A. paniculata*) is commonly known as Creat or King of bitters in English due to its bitter taste, Kalmegh in India, Hemptedu bumi in Malaysia, Fah Tah Lai in Thailand, Chuan-Xin-Lin in China and Senshinren in Japan. It is a popular ethno-medicinal herb used for treating infection, inflammation, cold, fever, snake bite, diarrhoea, dysentery, jaundice, kidney diseases, and anti-oxidant agent^[3]. Previous phytochemical studies on *A. paniculata* Nees has resulted in isolation of flavonoids and andrographolides (AND)^[4], and various parts of this plant have been reported to possess multiple therapeutic properties including anti-cancer^[5], anti-diabetes^[6], anti-inflammatory^[7], anti-malarial, antipyretic, anti-typhoid, antiviral, hepatoprotective^[8], anti-human immunodeficiency virus and immunostimulatory activity^[9].

The crude aqueous extract of *A. paniculata* has long been used for treating dysentery and diarrhoea with overall effectiveness of 91.3%^[10]. Prajjal *et al.* have investigated the anti-microbial activity of aqueous extract of *A. paniculata* and showed antibacterial activity against few microbes at higher concentration (10 mg/disc)^[11]. Youhong *et al.* have also studied the anti-microbial activity of aqueous fractions isolated from this species and found that neither the aqueous extract nor andrographolide were bacteriostatic or bactericidal against the tested microorganism^[12]. Sule *et al.* showed the effectiveness of *A. paniculata* extracts in controlling bacteria that cause skin diseases^[13]. According to Arash Rafat *et al.*, the anti-oxidant activity of *A. paniculata* is related to the total phenolic content of the extract, and there is a correlation between the content of phenolic compounds and antioxidant activity^[14]. The ethanol extract has more phenolic content and shows stronger activity than aqueous extract, which is also supported by the research of Wasman *et al.*^[15]. Considering the importance of this species in traditional therapy against bacterial infections, wound healing and diarrhoea, the present study was therefore undertaken to

evaluate the anti-bacterial and total anti-oxidant activity of the secondary metabolites – AND and echiodinin (ECH) isolated from organogenic callus culture of *A. paniculata* Nees. Plant cell and organ cultures are promising technologies to obtain plant-specific valuable metabolites and can be exploited successfully to meet the increasing demands of enhancing the yield of bioactive compounds and to expedite the pace of conservation and propagation of an important ethno-medicinal plants^[16]. Leaf explants of *A. paniculata* were used for callus culture on MS medium containing different concentrations of auxins and the active constituents of the plant were isolated and identified. The isolated compounds were used to screen antimicrobial activity by modified agar-disc diffusion method and MIC determination by micro-dilution technique against four Gram positive bacteria, three Gram negative bacteria and two yeast strains. The antioxidant activity of the isolated compounds was determined by using DPPH method.

2. Materials and methods

2.1. *In vitro* callus induction

Mature leaves collected from field grown plants were used as explants; they were surface sterilized in 70% (v/v) ethanol for 30 sec followed by repeated rinsing in sterile distilled water. Then the leaf explants were treated with 0.1% HgCl₂ (w/v) for 2 min, followed by washing in sterile distilled water. The cut ends of the explants were further trimmed with sharp edge sterile surgical blades. Then the explants were blotted on sterile filter paper discs before planting them on agar gelled MS medium taken in 150 mL conical flasks. Morphogenic callus induction on MS medium supplemented with various concentrations of auxins (NAA, 2,4-D and IAA) were studied. The optimum and bulk quantity of callus induction was observed in presence of 1.0 mg/L IAA and was used for characterization of bioactive compounds.

2.2. Extraction and isolation of active constituents

The dried powdered callus (750 g) was extracted with acetone and methanol in a 500 mL soxhlet apparatus for 24 h. The acetone and methanol extracts were column chromatographed on silica gel (100–200 mesh size) using hexane-ethyl acetate (3:7, v:v) and ethyl acetate-methanol (9:1, 8:2, v:v) step gradient, respectively. Fractions (50 mL each) were collected and subsequently monitored by preparative thin layer chromatography (TLC, silica glass plates of 20 cm × 20 cm) using hexane-ethyl acetate and ethyl-acetate methanol step gradient, respectively. This resulted in the isolation of two compounds previously reported from *A. paniculata* as echiodinin (yield 20 mg, melting point 264–265 °C; molecular formula, C₁₆H₁₂O₅, molecular weight, [M+H]⁺ 285) and andrographolide (yield 70 mg, melting point 232–234 °C; molecular formula, C₂₀H₃₀O₅, molecular weight, [M+H]⁺ 351). The IR, ¹H NMR, ¹³C NMR and mass spectra

of the compound were matched with previously reported in the literature[4,17].

2.3. Tested microorganisms

The following microbial strains were employed in the screening investigation: *Staphylococcus aureus* (*S. aureus*) MTCC 96, *Streptococcus thermophilus* (*S. thermophilus*) MTCC 1938, *Bacillus subtilis* (*B. subtilis*) MTCC 736, *Mycobacterium smegmatis* (*M. smegmatis*) MTCC 6, *Escherichia coli* (*E. coli*) MTCC 739, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 8162, *Klebsiella pneumonia* (DRL, Tezpur) (*K. pneumonia*), *Candida albicans* (DRL, Tezpur) (*C. albicans*), and *Saccharomyces cerevisiae* (*S. cerevisiae*) MTCC 3980.

2.4. Anti-microbial assay

The *in vitro* anti-bacterial and anti-fungal assays were carried out by adopting the modified agar-disc diffusion method[18]. Mueller-Hinton Agar (MHA, Sigma) was inoculated with (overnight, 12 h) bacterial cell suspension (200 μ L in 20 mL medium, 5×10^5 CFU/mL). Sterile filter paper discs of 6 mm diameter were impregnated with 20 μ L purified compounds – AND and ECH (concentration 1 mg/disc). After complete evaporation, the discs were placed on the surface of the inoculated agar plate. Gentamicine (30 μ g/disc) and myconazole (1 mg/disc) were used as positive controls, and negative controls were done using paper discs loaded with 20 μ L of the solvent (dimethyl sulfoxide, DMSO). The plates were incubated at 37 °C for 18 h. Similarly, Sabouraud dextrose agar was inoculated with yeast overnight cell suspension and incubated at 28 °C for 48 h. At the end of the incubation period, the anti-microbial activities were evaluated by measuring the zone of inhibition. An inhibition zone of 14 mm or greater (including diameter of the disc, 6 mm) was considered as high anti-microbial activity. All determinations were carried out in triplicate and the results were averaged.

2.5. Determination of MIC using the micro-dilution technique on the different strains of bacteria and yeast

The *in vitro* antibacterial and antifungal assays were carried out by adopting the micro-dilution techniques according to the National Committee for Clinical Laboratory Standards procedures for aerobic testing. Briefly, each of the microbes tested were sub-cultured twice on Mueller-Hinton agar, and the colonies (5–7) were then transferred aseptically into individual tubes containing sterile nutrient broth (10 mL). The tubes were incubated for a period of 8–12 h at 37 °C to attain growth at log phase. Subsequently, these inoculates were diluted with sterile distilled water to obtain a density corresponding approximately to 0.5 McFarland standard turbidity scale (1×10^6 CFU/mL). Stock solutions of test compounds – AND and ECH were prepared in 100% DMSO at a concentration of 1 mg/mL. A known volume (100 μ L) of each solution was placed in the first well of a 96-well microplate and 2-fold serially diluted with sterile distilled water

until the least concentration is achieved. A known volume of inoculum (100 μ L) was then added to each well. The plates were then incubated at 37 °C for 24 h. After incubation, 40 μ L of MTT (0.2 mg/mL) was added to each well and incubated for further 10–15 min. Bacterial growth is denoted by a blue coloration of the wells. The well of the lowest concentration in which no blue coloration observed was taken as the MIC.

2.6. Evaluation of anti-oxidant (antiradical) activity

The free radical scavenging activity was measured by using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method of Nihal Turkmen et al.[19], with minor modifications. Each purified compounds – AND and ECH were precisely diluted in methanol and methanol was used as a control. The reaction mixture contained 500 μ L of test compounds and 125 μ L of DPPH in methanol. Different concentrations of test compounds (10, 20, 40, 80 and 160 μ g/mL) were prepared in methanol while the concentration of DPPH was 0.1 mmol/L in the reaction mixture. These reaction mixtures were taken in microfuge tubes, vortexed and incubated at 37 °C for 30 min. The absorbance was measured at 517 nm using a spectrophotometer with methanol as blank. Ascorbic acid was used as positive control.

3. Results

In this study, we investigated the anti-bacterial and anti-oxidant activity of AND and ECH isolated and purified from *in vitro* callus cultures of *A. paniculata*. The profile of andrographolide content in organogenic callus and leaf extracts from field grown plant of *A. paniculata* (Figure 1) showed variable content of AND, 0.027 5% and 0.014 5%, respectively, as analyzed by high performance liquid chromatography (HPLC) (Figures 2 and 3), which indicates the establishment of secondary metabolite production by callus culture is better than extracting from field grown plants with many practical problems related to quality and quantity of AND. Anti-bacterial activities of AND and ECH are presented in Table 1. Among the two compounds, the AND showed a broad spectrum of anti-bacterial activity against Gram (+) and Gram (–) bacteria, while ECH was only effective against *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa* among the bacteria tested. Both AND and ECH were inactive against *C. albicans* and *S. cerevisiae* at given concentrations. *B. subtilis*, *S. aureus*, *E. coli*, *K. pneumonia*, *P. aeruginosa*, and *M. smegmatis* were the most sensitive microorganisms (zone of inhibition above 14 mm in this study) to the concentration of purified AND used in this assay. The MIC value of each microbe was determined and the lowest concentration at which no visible microbial growth observed was defined as MIC. As shown in Table 2, the AND showed MIC value as low as 50 μ g/mL against enteric bacteria *E. coli* and *S. aureus* at 100 μ g/mL, while the ECH revealed specific growth

Table 1

Anti-bacterial properties of andrographolide and echiodinin against various microorganisms using agar–disc diffusion method.

Test organisms	Zone of inhibition (mm)			
	Andrographolide (1 mg/disc)	Echiodinin (1 mg/disc)	Gentamicine (30 µg/ disc)	Myconazole (1 mg/disc)
<i>Staphylococcus aureus</i>	17.0±0.10	14.0±0.25	22.3±1.08	–
<i>Streptococcus thermophilus</i>	9.8±0.22	x	17.6±1.73	–
<i>Bacillus subtilis</i>	16.2±0.25	13.0±0.12	21.7±1.51	–
<i>Escheria coli</i>	18.5±0.40	12.0±0.25	23.4±0.76	–
<i>Mycobacterium smegmatis</i>	16.0±0.32	x	17.0±0.55	–
<i>Klebsiella pneumonia</i>	16.5±0.12	x	21.6±1.08	–
<i>Pseudomonas aeruginosa</i>	15.0±0.00	12.0±0.25	20.3±1.52	–
<i>Candida albicans</i>	x	x	–	16.7±3.5
<i>Saccharomyces cerevisiae</i>	x	x	–	17.0±1.5

“x” indicates no activity. The values for zone of growth inhibition are presented as mean±SD.

Table 2

Minimal inhibitory concentrations (MIC) of andrographolide and echiodinin using micro–dilution technique (µg/mL).

Test organisms	Andrographolide	Echiodinin
<i>Staphylococcus aureus</i>	100	225
<i>Streptococcus thermophilus</i>	350	x
<i>Bacillus subtilis</i>	100	325
<i>Escheria coli</i>	50	350
<i>Mycobacterium smegmatis</i>	200	x
<i>Klebsiella pneumonia</i>	100	x
<i>Pseudomonas aeruginosa</i>	200	300
<i>Candida albicans</i>	x	x
<i>Saccharomyces cerevisiae</i>	x	x

“x” indicates no activity.

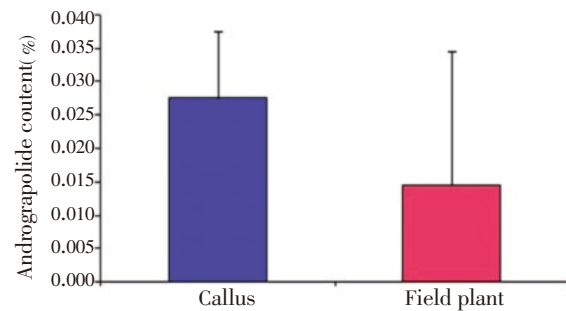
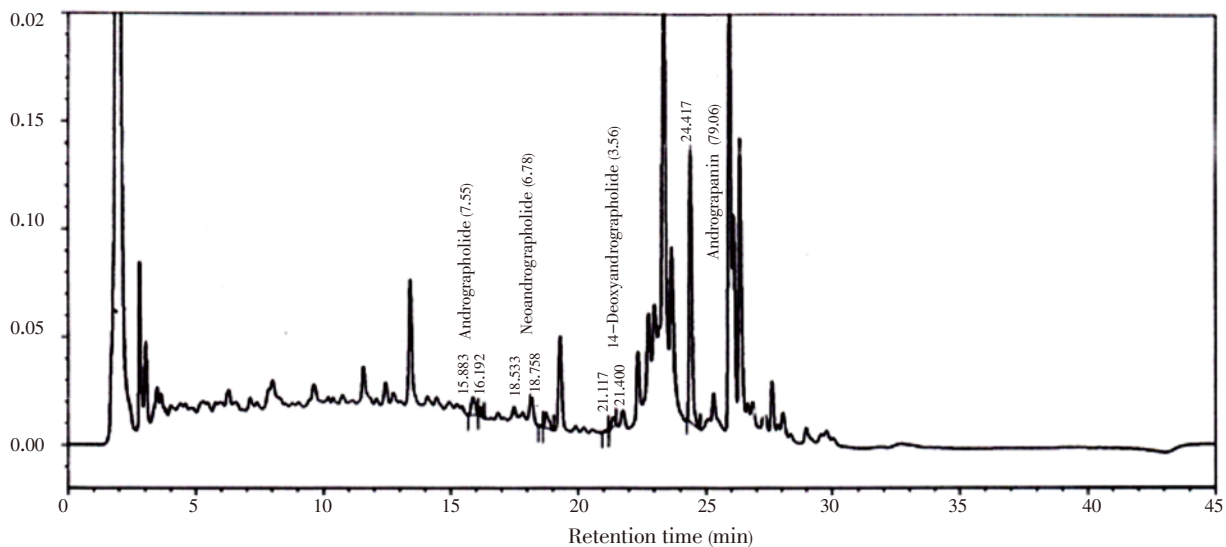


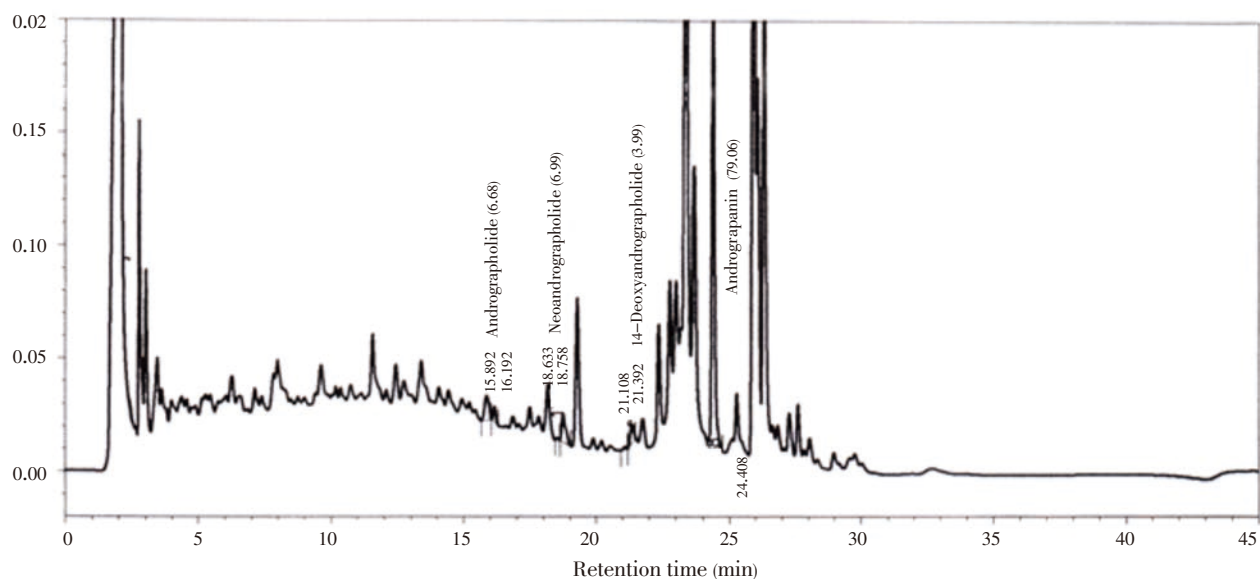
Figure 1. Analysis of andrographolide content in organogenic callus and leaf extracts from field grown plant of *A. paniculata* by HPLC.



Detector A-1
(223 nm)

PK#	Retention time (min)	Name	Area	Area percent (%)
1	15.883	Andrographolide	90 905	7.55
2	16.192	Andrographolide	58 619	2.38
3	18.533	Neoandrographolide	2 884	0.24
4	18.758	Neoandrographolide	81 572	6.78
5	21.117	14-Deoxyandrographolide	5 199	0.43
6	21.400	14-Deoxyandrographolide	42 796	3.56
7	24.417	Andrograpanin	951 612	79.06
Totals			1 203 587	100.00

Figure 2. Analysis of andrographolide content from leaf extracts from field grown plant of *A. paniculata* Nees by HPLC.



Detector A-1
(223 nm)

PK#	Retention time (min)	Name	Area	Area percent(%)
1	15.883	Andrographolide	125 184	6.68
2	16.192		46 891	2.50
3	18.533		5 663	0.30
4	18.758	Neoandrographolide	130 852	6.99
5	21.108		9 103	0.49
6	21.392	14-Deoxyandrographolide	74 716	3.99
7	24.408	Andrograpanin	1 480 213	79.04
Totals			1 872 622	100.00

Figure 3. Analysis of andrographolide content in organogenic callus of *A. paniculata* Nees by analytical HPLC.

inhibition activity against *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa* at a concentration higher than 225 $\mu\text{g/mL}$. Further, the isolated compounds from *A. paniculata* – AND and ECH were subjected to free radical scavenging activity by DPPH assay. The results showed the free superoxide anion scavenging activity ranging from 10% to 22% for AND, from 12% to 30% for ECH at a given concentrations of 10–160 $\mu\text{g/mL}$ (Table 3).

Table 3

Free radical scavenging activities of andrographolide (AND) and echinodin (ECH) determined by DPPH assay (%).

Compound	Radical scavenging activity at different concentrations ($\mu\text{g/mL}$)				
	10	20	40	80	160
AND	10 \pm 0.34	11 \pm 0.00	15 \pm 0.22	20 \pm 0.12	22 \pm 0.22
ECH	12 \pm 0.33	17 \pm 0.00	23 \pm 0.33	25 \pm 0.02	30 \pm 0.04
Ascorbic acid	ND	61 \pm 0.33	ND	ND	ND

ND: not determined.

4. Discussion

Plant secondary metabolites such as flavonoids, terpenoids, glycosides, and steroids have gained considerable attention in recent years due to their diverse pharmacological properties including antibacterial

and antioxidant activities. In the present study, we investigated the anti-bacterial and anti-oxidant activities of AND and ECH isolated and purified from *in vitro* callus cultures of *A. paniculata*. In the present study, the AND showed a broad spectrum of anti-bacterial activity against Gram (+) and Gram (-) bacteria including avirulent *M. smegmatis*, indicating the need of further research of the compound on virulent *M. tuberculosis*. A previous study on crude methanol and chloroform extract of *A. paniculata* has shown significant anti-microbial activity only against *B. subtilis* and *C. albicans* at a concentration of 10 mg/disc^[11]. Youhong *et al.* reported that neither the aqueous extract nor AND of *A. paniculata* were active against both Gram (+) and Gram (-) bacteria except that the ethanol fractions showed direct anti-bacterial activity against only two human pathogens like *Legionella pneumophila* and *Bordetella pertussis* at 5 mg/disc^[12]. Sule *et al.* reported that dichloromethane, methanol and aqueous extracts have shown activity on all microbes tested at 1 mg/disc^[13]. On contrary to the previous observation, AND showed a broad spectrum of growth inhibition activity, while ECH showed specific anti-bacterial activity against only *S. aureus*, *E. coli*, *B. subtilis* and *P. aeruginosa* at a concentration higher than 225 $\mu\text{g/mL}$. The differences in cell wall structure between Gram (+) and Gram (-) bacteria and polarity nature of compounds might be the

probable reasons for their differential susceptibility to the bioactive compounds tested in this study. However, either of the isolated compounds did not show any anti-fungal activity against *C. albicans* and *S. cerevisiae* carried out in the present study, which does not support the antifungal activity of aqueous extracts and the fractions that were positive for arabinogalactan proteins and andrographolides reported previously^[11]. This finding may be related with inability of the isolated compounds to penetrate the hydrophobic and hydrophilic lipid bilayers of cell wall architecture of yeast. Inhibition of non-pathogenic strain of *M. smegmatis* growth in this study is highly predictive of activity against human pathogenic *M. tuberculosis*^[20].

Previous studies on crude extract of *A. paniculata* showed potent free radical activity with scavenging rate ranging from 48.0% to 66.8% at concentrations of 1–50 µg/mL^[21]. In this study, the isolated compounds from *A. paniculata* – AND and ECH showed a moderate increase in the free superoxide anion scavenging activity ranging from 10% to 22% for AND and from 12% to 30% for ECH at a given concentrations of 10–160 µg/mL. These results suggest that the purified compounds possess potent free radical scavenging activity. As expected, the ECH showed relatively higher total antioxidant activity in a given tested concentration as compared to AND, which could be explained partially with respect to the number and position of free hydroxyl group attached to phenol rings^[22]. The free hydroxyl group of ECH is more acidic in nature and can readily donate the phenolic hydrogens or electron to the acceptor molecules. This observation on total anti-oxidant activity of isolated compounds correlates well with a recent report^[21]. Thus the observed weak and moderate free radical scavenging activity could be due to the absence of other potent anti-oxidant compounds present in the crude extracts of *A. paniculata*.

In conclusions, this study may provide pharmacological evidence for folklore medicinal uses of *A. paniculata* Nees against the treatment of microbial related ailments such as respiratory tract infection, acute diarrhoea, wound healing, skin inflammations and bacterial infections. The anti-bacterial and anti-oxidant efficacy of AND and ECH isolated from callus culture of *A. paniculata* Nees, an ethno-medicinal species of India, are reported for the first time, to our knowledge. Further studies are under progress in the laboratory to understand precise pharmacological properties of other isolated bioactive compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

From antiquity, medicinal herbs represent a rich source of antioxidants and antimicrobial agents. Unfortunately, in recent years, antimicrobial resistance, age related diseases and life style have become major public health concern issues. Therefore, there is a surge for novel antimicrobial and antioxidant agents from plant sources owing to its low cost and less side effects. Hence, the present research is a constructive step to fulfill the current need of the society.

Research frontiers

The isolation of secondary metabolites and their evaluation for biological potential are the starting point for pharmaceutical development. At the same time, the introduction of tissue culture technique for obtaining specific metabolite opens new avenue for further research and large scale production if required. Thus, the present study has demonstrated the amalgamation of above statements and reported the biological activities of andrographolide and echiodinin obtained from *in vitro* callus cultures of *A. paniculata* Nees for the first time.

Related reports

The isolated compounds exhibited only antibacterial activity and no antifungal activity in contrary to previously reported studies of Youhong *et al.* (2006) and Prajjal *et al.* (2003). The activity level of each compound may be attributed to the extent of compound interaction with the cell wall of tested organisms. However, the moderate antioxidant activity of compounds is in accordance with previous findings of Lin *et al.* (2009) on andrographolide. The better antioxidant potential of echiodinin may correspond to the greater free radical scavenging tendency of phenolic OH which is lacking in andrographolide structure.

Innovations and breakthroughs

The data concerning biological activities of pure compounds from *A. paniculata* callus culture are scarce. The present study provides adequate evidence for the traditional use of *A. paniculata* by evaluating the antimicrobial and antioxidant potential of andrographolide

and echiodinin. The reported observations related to antimicrobial activity of isolated compounds and antioxidant activity of echiodinin are novel to the best of our knowledge.

Applications

The isolation and identification of potential bioactive compounds are a vital step towards future pharmaceutical development. The isolated compounds can be further explored for other pathogenic bacterial and fungal stains. In order to obtain efficient activity of the compounds, their structural analogues can also be studied.

Peer review

This is an important novel research on active principles of *A. paniculata* *in vitro* callus cultures. The present study succeeded in providing supporting scientific evidence for its ethnopharmacological uses. Furthermore, isolation of andrographolide and echiodinin from callus cultures gave new dimensions to the study which may be helpful in mass production and exploitation of metabolites for future studies.

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