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Cytotoxic and Antimicrobial Activity of *Anaphalis lawii* (Hook.f.) Gamble and *Helichrysum buddleioides* DC

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Abstract

The present study was carried out to determine cytotoxic and antimicrobial activity of methanol extract of two Asteraceae members namely *Anaphalis lawii* (Hook.f.) Gamble and *Helichrysum buddleioides* DC. The whole plant materials were extracted using methanol by maceration process. The extracts were subjected to preliminary phytochemical analysis. Flavonoids, saponins and steroids were detected in both plants. Cytotoxic activity was evaluated using brine shrimp lethality bioassay. Extracts caused dose dependent mortality of brine shrimp larvae. Among extracts, marked lethal effect was displayed by *H. buddleioides* (LC_{50} value 33.34 µg/ml) when compared to *A. lawii* (LC_{50} value 85.54 µg/ml). Antibacterial activity of extracts was determined against Gram positive and Gram negative bacteria by agar well diffusion assay and MIC determination. Among extracts, marked antibacterial activity was observed in case of *H. buddleioides*. Extract of *H. buddleioides* inhibited the growth of test bacteria at low concentration when compared to *A. lawii*. Antifungal activity was evaluated against phytopathogenic fungi by poisoned food technique. Poisoning of medium with extracts resulted in considerable reduction in the mycelial growth of test fungi. Here also, extract of *H. buddleioides* exhibited marked inhibitory activity when compared to *A. lawii*. The plants used in this study appear to contain antimicrobial and cytotoxic principles which are to be isolated and subjected for bioactivity determinations.

Keywords: *Anaphalis lawii; Helichrysum buddleioides; Phytochemical; Antimicrobial; Cytotoxic; Brine Shrimp Lethality*

Introduction

Plants are an integral part of daily life. Humans depend on plants for food, shelter, medicine and other needs. Plants are important component of ethnomedicine. The term ethnomedicine refers to the study of traditional medical practices and the practice of ethnomedicine is a complex and multi-disciplinary system. It is well known that a vast majority of world's population (70 to 80%) meets the primary healthcare needs through traditional medicine. Herbal medicine has been widely practiced in several parts of the world, in particular countries such as China and India. The medicinal systems such as Ayurveda, Sidda and Unani use plants to treat several ailments. Plants and their formulations have been traditionally used in the treatment of cough, arthritis, cancer, anemia, diabetes, skin disease, inflammation, dental diseases etc. Plants are useful either in their crude or advanced state, offering a source of drugs in their pure state [1-7]. The medicinal or healing properties of plants lies in some chemicals that are present in plants. These chemicals are called phytochemicals and are produced by secondary metabolism. Many of these phytochemicals such as alkaloids, tannins, steroids, terpenoids and glycosides have multifold beneficial effects. Plants with such phytochemicals can be the important sources of many drugs. Some of the well-known drugs that have been developed from plants Vinblastine and Vincristine from *Catharanthus roseus* (anticancer), Quinine from Cinchona

species (antimalarial) and Scopolamine from *Datura metel* (sedative). Many of plant compounds have shown to exhibit several biological and pharmacological activities, which could serve as the starting point in the development of modern medicines. Phytochemicals have least or no side effects when compared to other drugs [1,3,8,9].

In the present study, we selected two Asteraceae plants namely *Anaphalis lawii* (Hook.f.) Gamble and *Helichrysum buddleioides* DC. *A. lawii* is an erect, densely woolly herb, up to 30 cm tall. It is distributed in Western Ghats, Coorg, Bababudan hills of Karnataka, Brahmagiris, hills of Coimbatore, N. Nilgiris, Anamalais, Pulneys and hills of Tinnevelly, at 5000 - 7000ft. Leaves narrow, oblanceolate, 2 cm long, sessile. Heads in corymbs, terminal, woolly, 5 mm across, heterogamous, not rayed, white-pinkish. Involucral bracts multiseriate, outer one sessile, larger. Female florets outer, filiform. Bisexual florets tubular, 5-lobed. Pappus of white hairs, connate at base. Achenes terete. Flowering and fruiting occurs between May and January [10,11]. The whole plant of *A. lawii* is air-dried, powdered, and consumed with food as Kayakalpa by the Malasars of the Velliangiri hills in the Western Ghats of Nilgiri Biosphere Reserve, India [12]. Biological activities namely antimicrobial, antioxidant and wound healing activities of *A. lawii* have been reported [13-17]. *H. buddleioides* is an undershrub, up to 1 meter tall. It is distributed in Western Ghats, Mysore, Bababooduns, Anamalais and Ceylon. Leaves broad, oblong-oblanceolate, thin, apex obtuse-acute, nerves not impressed. Heads forming dense, terminal corymbs. Involucral bracts yellow, 4-many-seriate, woolly tomentum. Ray florets female, filiform, 2.5mm long, 4-lobed. Disc florets bisexual, corolla 2 mm long, 5-lobed. Achenes 0.3 mm long, puberulous, pappus of white hairs. Flowering and fruiting occurs between July and March [11,18]. The Badaga population in the Nilgiri district of Tamilnadu uses the plant juice of *H. buddleioides* to treat cut and wounds for rapid healing [19]. More recently, Kekuda., *et al*. [17] reported antifungal activity of extract of *H. buddleioides* against *Bipolaris sorokiniana*. In the present study, we determined cytotoxic and antimicrobial potential of extract of *A. lawii* and *H. buddleoides*.

Materials and Methods

Collection and identification of plant materials

The plants were collected at Bababudan Giri, Chikmagaluru during November 2015. Authentication of plants was done by referring standard flora and with the assistance by taxonomists [10,11,18].

Extraction and phytochemical analysis

The whole plant materials (leaves, inflorescence, roots and stems) were washed under running water, dried under shade and powdered. Extraction was carried out by simple maceration process in which a known quantity of plant material (20g) was transferred to a conical flask containing 200 ml of methanol. The flasks were sealed and left for 48 hours. The flasks were stirred occasionally. The contents of each flask were filtered through Whatman No. 1 filter paper. The filtrates thus obtained were evaporated to dryness at 50°C [20]. The extracts were subjected standard phytochemical tests in order to detect the presence of various phytochemicals [13,21].

Cytotoxic activity of extracts

Brine shrimp lethality assay was performed to screen the cytotoxic nature of extract of both plants. In brief, the eggs of brine shrimp *Artemia salina* were hatched in a container filled with air-bubbled artificial sea water. After 36 - 48 hours, the shrimps were collected by pipette and used for bioassay. The shrimps were exposed to different concentrations (0 – 500 µg/ml) of positive control i.e., potassium dichromate and extracts for 24 hours and surviving shrimps were counted after 24 hours. The mortality of shrimps (%) was determined. The LC_{50} value was calculated using Origin 6 software [22].

Antibacterial activity of extracts

The extracts were prepared in Dimethyl sufloxide (DMSO). The potential of extracts to inhibit Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative bacteria (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) was evaluated by Agar well

diffusion method. In brief, 24 hours old Mueller-Hinton (M-H) broth cultures of test bacteria were swab inoculated aseptically on sterile Mueller-Hinton agar plates followed by punching wells of 8mm diameter in the inoculated plates using sterile cork borer. 100 µl of plant extracts (10 mg/ml of DMSO), standard antibiotic (Chloramphenicol, 1 mg/ml of sterile distilled water) and DMSO were transferred into respective wells and the plates were incubated at 37°C for 24 hours in upright position. Using a ruler, zones of inhibition were measured [20].

Determination of minimum inhibitory concentration (MIC)

The MIC of extracts was determined by broth tube dilution method [23]. A series of extract dilutions (two fold dilution ranging from 10 mg/ml to 0.0 mg/ml) prepared in M-H broth was used against test bacteria. The tubes containing different concentrations of extracts were inoculated with the 100 µl of M-H broth cultures of test bacteria and the tubes were incubated for 24 hours at 37°C. The MIC was determined by visual observation for growth of test bacteria. The dilution tube in which any visible growth of test bacteria was absent was considered to be the MIC of extract.

Antifungal activity of extracts

The antifungal effect of extract of *A. lawii* and *H. buddleioides* was determined by Poisoned food technique against two phytopathogenic fungi namely *Colletotrichum capsici* and *Fusarium oxysporum* f. sp. *zingiberi*. Test fungi were inoculated aseptically into control (without extract) and poisoned (0.5 mg extract/ml of medium) potato dextrose agar (PDA) plates followed by incubating the plates at 28 ± 2°C for 72 hours. The diameter of colonies in mutual perpendicular directions was measured using a ruler. Antifungal activity, in terms of inhibition of mycelial growth of test fungi, was determined using the formula:

Inhibition of mycelial growth (%) = (C – T / C) x 100, where C and T denotes the diameter of colonies in control and poisoned plates respectively [20].

Results and Discussion

Phytochemicals detected in extracts

Plants produce diverse types of metabolites. Some are produced during secondary metabolism and are referred to as secondary metabolites. Important among these are alkaloids, terpenoids, steroids and polyphenolic compounds. These chemicals (phytochemicals) are distributed in various parts of the plants. The exact role of these metabolites is not fully understood, however, most of these metabolites are of significance for plants in terms of preventing herbivores, pathogens and insects, attraction of pollinators, coping with abiotic stress etc. Besides, these phytochemicals are known to exhibit a range of bioactivities such as antimicrobial, anticancer, antioxidant, antiherbivore and insect repellent activity. These plant secondary metabolites have a great potential for medicine, industry, agriculture and food sciences. It is of profound importance to detect the phytochemicals in plants so as to relate their presence with bioactivity observed and to know the possible therapeutic role played by them [1,8,24-28]. In the present study, the extract yield was more in case of *H. buddleioides* (4.61%) when compared to *A. lawii* (3.38%). Extract of *A. lawii* was greenish brown while extract of *H. buddleioides* was dark greenish brown in color. The result of preliminary phytochemical analysis of extracts is presented in Table 1. Flavonoids, saponins and steroids were detected in both extracts. Alkaloids were detected in extract of *A. lawii* only while terpenoids and glycosides were found in extract of *H. buddleioides* only. Tannins were not detected in extract of both plants.

Phytoconstituents	*A. lawii*	*H. buddleioides*
Alkaloids	+	-
Flavonoids	+	+
Saponins	+	+
Steroids	+	+
Terpenoids	-	+
Glycosides	-	+
Tannins	-	-

Table 1: *Phytoconstituents detected in extracts.*
('+' detected; '-' not detected)

Brine shrimp lethality of extracts

Brine shrimp bioassay utilizing a simple zoologic organism *A. salina* is a simple and rapid in vivo lethality assay. The assay can be used for screening, discovering and monitoring various bioactivities such as cytotoxic, phototoxic, pesticidal, trypanocidal, enzyme inhibition, and ion regulation activities of various kinds of compounds including plant extracts. The assay can be extrapolated for cell-line toxicity and antitumor activity. The assay utilizes only 24 hours and is inexpensive and needs no special equipment and aseptic conditions to perform. This assay has been extensively employed to determine cytotoxic activity of plant extracts [3,22,29,30,31]. The result of lethal effect of extracts and potassium dichromate against the larvae of brine shrimp is shown in Table 2. Extracts caused dose dependent mortality of brine shrimps. Among extracts, marked lethal effect was shown by *H. buddleioides* (LC_{50} value 33.34 µg/ml) when compared to *A. lawii* (IC_{50} value 85.54 µg/ml). However, the lethal nature of extracts was lower than that of potassium dichromate which caused mortality of larvae with LC_{50} value 28.36 µg/ml.

Concentration (µg/ml)	Mortality of shrimps (%)		
	A. lawii	*H. buddleioides*	Potassium dichromate
0.0	0.0	0.0	0.0
50.0	35.14	44.14	62.14
100.0	48.66	52.33	78.33
250.0	58.68	64.14	96.06
500.0	65.34	71.33	100.0

Table 2: *Brine shrimp lethality of extracts and Potassium dichromate.*

Antibacterial activity of extracts

Infectious diseases caused by pathogenic bacteria are treated using antibiotics. The discovery and subsequent use of antibiotics has revolutionized the field of chemotherapy in terms of drastic reduction in morbidity and mortality. The successful therapy of diseases is facing a serious challenge due to antibiotic resistant strains of bacteria that are of serious concern in hospital as well as community settings. Besides, other drawbacks such as high cost and side effects are associated with the use of antibiotics. This triggered huge interest in scientific community to search alternatives for disease therapy. Plants, plant based formulations and plant metabolites have been investigated for antibacterial activity and several literatures have shown the potential of plants to inhibit various bacteria including antibiotic resistant bacteria [1,6,13,32-35]. Table 3 shows the inhibitory potential of extract of *A. lawii* and *H. buddleioides* against Gram positive and Gram negative test bacteria. Both extracts were effective against the all test bacteria. Among extracts, marked antibacterial activity was observed in case of *H. buddleioides* as it produced wider zones of inhibition. Among test bacteria, *K. pneumoniae* was shown to exhibit high susceptibility to both extracts. Inhibition of both Gram positive bacteria was more or less similar. Overall, Gram negative bacteria exhibited marked sensitivity to extracts than Gram positive bacteria. Standard antibiotic displayed high inhibitory efficacy when compared to extracts. Antibiotic was more effective against Gram positive bacteria. DMSO did not cause inhibition of test bacteria. In an earlier study, flower extract of *A. lawii* displayed marked inhibitory activity against test bacteria when compared to leaf extract [13]. The study of Dileep., *et al*. [15] showed the potential of leaf extract of *A. lawii* to inhibit clinical isolates of *Streptococcus mutans* dose dependently.

Test bacteria	Zone of inhibition in cm			
	A. lawii	*H. buddleioides*	Standard	DMSO
B. subtilis	1.5	1.8	2.5	0.0
S. aureus	1.5	1.8	2.6	0.0
P. aeruginosa	1.5	2.0	2.2	0.0
K. pneumoniae	2.0	2.2	2.3	0.0

Table 3: *Antibacterial activity of extracts.*

The MIC of extracts against test bacteria is shown in Table 4. It was observed that extract of *H. buddleioides* inhibited the growth of test bacteria at low concentration when compared to extract of *A. lawii*. The MIC value was least against *K. pneumoniae* (0.156 mg/ml) while other test bacteria were inhibited at MIC value of 0.312 mg/ml. The MIC of *A. lawii* ranged between 0.312 and 1.250 mg/ml. The low and high MIC of *A. lawii* was against *K. pneumoniae* (0.312 mg/ml) and *S. aureus* and *P. aeruginosa* (1.250 mg/ml) respectively.

Test bacteria	MIC (mg/ml)	
	A. lawii	***H. buddleioides***
B. subtilis	0.625	0.312
S. aureus	1.250	0.312
P. aeruginosa	1.250	0.312
K. pneumoniae	0.312	0.156

Table 4: *MIC of extracts of A. lawii and H. buddleioides.*

Antifungal activity of extracts

Plants are vulnerable to a variety of infectious agents among which fungi are considered to be the most important disease causing agents. Fungi cause a number of diseases in agricultural and horticultural crops leading to decreased crop productivity and economic loss to farmers. Apart from the crop losses, some also elaborate mycotoxins, which pose serious health hazards and impact on the commercial trade of agricultural produce. Fungal diseases of plants are commonly controlled by the use of synthetic fungicides. The use of chemical agents results in environmental pollution and side effects on non-target organisms. Moreover, the frequent usage of these agents favored the development of resistance in the phytopathogenic fungi. Natural products seem to be the best alternatives for synthetic chemicals as they are cheaper, effective against pathogens and do not cause adverse effects on health. Plants and their metabolites are activity against a wide range of pathogenic fungi including phytopathogenic fungi [36-39]. Poisoned food technique is one of the most widely used protocols for evaluating antifungal activity of various kinds of samples including plant extracts. Reduction in the colony size in poisoned plates is an indication of antifungal activity of samples. In the present study, we evaluated the antifungal potential of extracts by poisoning the PDA medium with extracts and the result is shown in Table 5. When compared to control plates, poisoned plates showed considerable reduction in the mycelial growth of test fungi. Among fungi, *C. capsici* was inhibited to high extent when compared to *F. oxysporum*. Both fungi were inhibited to > 50% by extract of *H. buddleioides* while *A. lawii* inhibited only *C. capsici* to > 50%. The extract of *H. buddleioides* exhibited marked inhibitory activity when compared to *A. lawii*. In a similar study, extract of *A. lawii* and *H. buddleioides* displayed inhibitory activity against Bipolaris sorokiniana and the effect of *H. buddleioides* was marked when compared to *A. lawii* [17]. In a study, Kekuda., *et al*. [13] observed antifungal effect of leaf and flower extract of *A. lawii* against *Cryptococcus neoformans*. In another study, leaf extract of *A. lawii* displayed high inhibition of *C. capsici* when compared to flower extract [14].

Test fungi	Colony diameter in cm (% inhibition)		
	Control	***A. lawii***	***H. buddleioides***
C. capsici	3.8	1.8 (52.63)	1.6 (57.89)
F. oxysporum	4.0	2.3 (42.50)	1.9 (52.50)

Table 5: *Antifungal activity of extracts.*

Conclusion

The extract of *A. lawii* and *H. buddleioides* displayed antimicrobial and cytotoxic potential. The observed bioactivities could be attrib-

uted to the presence of phytochemicals that have been detected in extracts. The results of the present study is an addition to the scientific literature, however, detailed investigations on pharmacological activities and active ingredients of plants could provide leads to development of pharmaceuticals of plant origin.

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