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Original Research

Antiradical and Antimicrobial Activity of *Atylosia lineata* Wt. and Arn.

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Abstract

The present study was conducted to determine antimicrobial and radical scavenging activity of *Atylosia lineata* Wt. & Arn. (Fabaceae). The shade dried and powdered leaves were extracted using methanol. Antibacterial and antifungal activity of extract was determined by agar well diffusion and poisoned food technique respectively. Antiradical efficacy was determined by DPPH and ABTS radical scavenging assays. Total phenolic content of leaf extract was estimated by Folin-Ciocalteau Reagent method. The extract was effective against all test bacteria with marked inhibitory effect against *Pseudomonas aeruginosa*. Gram negative bacteria were susceptible to higher extent than Gram positive bacteria. The extract inhibited the growth of all test fungi to >50%. Among fungi, *Colletotrichum capsici* was inhibited to higher extent. The extract scavenged DPPH and ABTS radicals in dose dependent manner. At extract concentration 100µg/ml and higher, >90% scavenging of both radicals was observed. The total phenolic content of leaf extract was found to be 81.36mg GAE/g of extract. The plant appears promising to treat infectious diseases and oxidative damage.

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INTRODUCTION

The treatment of diseases began long ago with the use of plants. Plants form an important component of traditional medicine worldwide and are considered to be an important source of valuable medicines. It is estimated that >80% of world's population depends on traditional medicine to meet primary health care needs. Worldwide, the methods of folk healing commonly utilize plants and plant based formulations as part of tradition. The practice of traditional medicine based on plants is most common in countries like China, India, Japan, Pakistan, Sri Lanka and Thailand. Besides, many plants are widely used in various systems of traditional medicine like Ayurveda, Unani, Homeopathy and Sidda. These plants are the sources of lead compounds for the development of several drugs (Kumar and Janagam, 2011; Sahoo and Manchikanti, 2013; Naik *et al*., 2015). *Atylosia lineata* Wt. & Arn. is an erect undershrub frequently found in the wet deciduous forests of Western Ghats. Branches are terete. Leaflets are elliptic or oblanceolate, subacute, membranous and pubescent beneath. Flowers are in pairs on minute peduncles. Corolla is yellow colored, pods are grey, silky-pubescent, 2-3 seeded (Saldanha and Nicolson, 1976). The present study was carried out to investigate antimicrobial and antiradical activity of leaf extract of *A. lineata*.

MATERIALS AND METHODS

Collection and Identification of Plant Material

The plant material was collected near Guddekeri, Shivamogga district, Karnataka during January 2015. The plant was authenticated by Prof. D. Rudrappa, Department of Botany, S.R.N.M.N College of Applied Sciences, Shivamogga.

Extraction

The leaves were separated, washed well to remove extraneous matter, dried under shade and powdered in a blender. The leaf powder (25g) was transferred into a conical flask containing methanol and left for two days with occasional stirring. The content of flask was filtered through 4-fold muslin cloth followed by Whatman filter paper No. 1 (Naik *et al*., 2015). The filtrate was evaporated to dryness and used for bioactivity determinations.

Antibacterial Activity

Agar well diffusion assay was performed to determine inhibitory activity of leaf extract against two Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). 24 hours old Nutrient broth cultures of test bacteria were swabbed uniformly on sterile Nutrient agar plates. Wells of 8mm diameter were punched in the inoculated plates using sterile cork borer. Using sterile pipettes, leaf extract (20mg/ml of DMSO), standard antibiotic (Chloramphenicol, 1mg/ml of sterile distilled water) and DMSO (25%, in sterile distilled water) were transferred into respectively labeled wells. The plates were left for 30 minutes and were then incubated at 37^{o}C for 24 hours in upright position. The zones of

inhibition formed around wells were measured using a ruler (Naik *et al*., 2015).

Antifungal Activity

Poisoned food technique was employed to determine the efficacy of leaf extract to inhibit the mycelial growth of *Colletotrichum capsici* (from anthracnose of chilli), *Fusarium oxysporum* f.sp. *zingiberi* (from rhizome rot of ginger) and *Alternaria alternata* and *Aspergillus flavus* (isolates from moldy grains of sorghum). The control (without extract) and poisoned (0.5mg extract/ml of medium) potato dextrose agar plates were inoculated aseptically with the test fungi by point inoculation method. The plates were incubated in upright position for 3 days at room temperature. The mycelial growth (diameter) in mutual perpendicular directions was measured using a ruler. The inhibition of mycelial growth (%) was calculated using the formula:

Mycelial growth inhibition (%) = (C – T / C) x 100

where C and T refers to diameter of fungal colony on control and poisoned plates respectively (Naik *et al*., 2015).

Antiradical Activity

Two *in vitro* assays *viz*., DPPH (1,1-diphenyl-2-picryl-hydrazyl) and ABTS (2,2-azinobis 3-ethylbenzothiazoline 6-sulfonate) assay were carried out to investigate radical scavenging efficacy of leaf extract.

DPPH Free Radical Scavenging Assay

1ml of different concentrations (12.5-200µg/ml) of leaf extract in methanol was mixed with 3ml of DPPH (0.004% in methanol) in separate tubes. The tubes were incubated in dark for 30 minutes. The absorbance was measured in a spectrophotometer at 517nm. Methanol replacing extract served as control. Ascorbic acid (HiMedia, Mumbai) was used as reference standard. Scavenging activity (%) of leaf extract was calculated using the formula:

Scavenging of DPPH radicals (%) = (C – T / C) x 100

where C and T refers to absorbance of DPPH control and absorbance of DPPH in presence of extract/ascorbic acid. The IC_{50} value was calculated. IC_{50} denotes the concentration of extract required to scavenge 50% of DPPH radicals (Rakesh *et al*., 2013).

ABTS Radical Scavenging Assay

ABTS radical was generated by mixing 7mM ABTS stock solution with 2.45mM potassium persulfate and leaving the mixture in the dark for 16 hours at room temperature. The resulting solution was diluted using distilled water to an absorbance of 0.7 at 730nm. In clean and labeled test tubes, 1ml of different concentrations of leaf extract (12.5-200µg/ml of methanol) was mixed with 4ml of ABTS radical solution and the tubes were incubated for 30 minutes at room temperature. The absorbance was measured in a spectrophotometer at 730nm. Ascorbic acid was used as reference standard. The radical scavenging activity of leaf extract was calculated using the formula:

Scavenging activity (%) = (C - T / C) x 100,

where C is the absorbance of the ABTS solution without extract/ ascorbic acid and T is the absorbance of ABTS solution in the presence of extract/ascorbic acid. The IC_{50} value was calculated. IC_{50} denotes the concentration of extract required to scavenge 50% of ABTS free radicals (Rakesh *et al*., 2013).

Total Phenolic Content of Extract

The content of total Phenolics in the leaf extract was estimated by Folin-Ciocalteau reagent (FCR) method. A dilute concentration of leaf extract (0.5 ml) was mixed with 0.5 ml of FC reagent (1:1) and 2 ml of sodium carbonate (2%). The tubes were left for 30 minutes at room temperature. The absorbance was measured at 765nm in a spectrophotometer. A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000 µg/ml). The total phenolic content of leaf extract was expressed as mg Gallic Acid Equivalents (GAE) from the graph (Rakesh *et al*., 2013).

RESULTS AND DISCUSSION

Antibacterial Activity of Leaf Extract

Antibiotic resistance is a global problem and is challenging the healthcare sector in a large part. The spread of multidrug-resistant bacteria in both hospital and community settings is a heavy burden to health services. Despite great advances in antibiotic therapy, infectious microbes in particular drug resistant strains cause marked mortality and morbidity of patients. Overuse and abuse of antibiotic resulted in emergence of several drug resistant microbial strains. Besides, the use of synthetic drugs suffers from other drawbacks such as high cost and harmful side effects. Hence, search for new sources having antibacterial activity is of much interest. Since time immemorial, plants are traditionally used to treat various diseases caused by pathogenic microbes. Plants produce a wide array of bioactive compounds having therapeutic properties. These plant metabolites can either inhibit the growth of bacteria or kill them, with no toxicity or minimum toxicity to host. In recent years, antimicrobial activities of various medicinal plants are reported from different parts of the world (Adwan *et al*., 2010; Zwetlana *et al*., 2014; Jr. Valle *et al*., 2015). In the present study, the leaf extract of *A. lineata* was found to inhibit all test bacteria to varied extent as revealed by the presence of zones of inhibition around wells. Gram negative bacteria showed higher susceptibility to extract when compared to Gram positive bacteria. Among test bacteria, *P. aeruginosa* was shown to be highly susceptible followed by *E. coli* and others. *S. aureus* and *B. subtilis* were inhibited to similar extent. However, standard antibiotic caused marked inhibition of Gram positive bacteria than Gram negative bacteria. DMSO did not inhibit the growth of any test bacteria (Table 1).

Table 1: Antibacterial activity of extract of *A. lineate*

Test Bacteria	Zone of inhibition in cm		
	Extract	Standard	DMSO
P. aeroginosa	3.7	2.6	0.0
E. coli	3.0	2.2	0.0
S. aureus	2.8	3.2	0.0
B. subtilis	2.8	3.0	0.0

Antifungal Activity of Leaf Extract

Plants have been used for various purposes such as food, fodder and medicine. However, plants are prone for infection caused by various pathogens such as bacteria, viruses, fungi, and nematode parasites. Among these, fungi cause more number of diseases in plants. The pre- and post-harvest crop diseases result in economic losses in the range 5 to 50%, or even higher. Synthetic fungicides are widely used to control plant diseases. However, the use of these chemicals suffers from drawbacks such as emergence of resistant strains,

residual effect and toxicity to non-target organisms. Hence, much interest is focused on developing antifungal agents from natural sources which have little or no side effects. Extracts of plants and their metabolites are known to inhibit a range of phytopathogenic fungi (Park *et al*., 2008; Dellavalle *et al*., 2011; Panea *et al*., 2013; Kekuda *et al*., 2014; Vivek *et al*., 2014). In the present study, we evaluated inhibitory effect of leaf extract of *A. lineata* against fungi by poisoned food technique. This technique is one of the widely used methods to determine antifungal activity of various types of samples. The result of antifungal activity of leaf extract is shown in Table 2. Poisoning of the medium with extract resulted in considerable suppression of mycelial growth of test fungi. Extract suppressed the mycelial growth of all test fungi to >50%. *C. capsici* was susceptible to high extent to extract (84.37% inhibition). Least inhibitory activity was observed against *F. oxysporum* (54.16% inhibition).

Table 2: Colony diameter (in cm) of test fungi on control and poisoned plates

Test Fungi	Colony diameter (% inhibition of fungi)	
	Control	Extract
C. capsici	3.2	0.5 (84.37)
A. alternata	2.8	1.0 (64.28)
F. oxysporum	4.8	2.2 (54.16)
A. flavus	3.4	1.5 (55.88)

Antiradical Activity of Leaf Extract

A free radical can be defined as any atom or molecule which has an unpaired electron in an outer shell. These radicals are produced during normal metabolism and on exposure to environmental pollutants, radiation and drugs. These reactive oxygen species (superoxide radical, hydroxyl radical, peroxyl radical, alkoxy radical and nonradical species hydrogen peroxide) are known to react with biomolecules namely nucleic acids, lipids, proteins and carbohydrates causing damage, mutations, protein inactivation and cell death. Oxidative damage induced by reactive oxygen species occurs when the balance between free radical generation and antioxidant defense is disrupted. This oxidative stress is implicated in several diseases/ disorders such as aging, cancer, neurodegenerative diseases, cardiovascular diseases and others. Strong restrictions have been placed on the use of synthetic antioxidants because of their carcinogenic potential. Plants are considered as an important and richer source of antioxidants. Polyphenolic compounds including flavonoids of plant kingdom are shown to be excellent antioxidants (Gulcin *et al*., 2011; Pavithra *et al*., 2013; Urquiza-Martínez and Navarro, 2016).

Assay involving scavenging of DPPH radicals is one of the most widely used *in vitro* radical scavenging assays. DPPH is a stable, nitrogen centred free radical with an absorption band at 515-520nm. In the presence of an antioxidant, it loses the absorption as it is reduced (purple color changes to yellow). The DPPH assay is simple and the results are easily reproducible. The assay is frequently used to determine radical scavenging nature of plant extracts and purified plant metabolites (Akinmoladun *et al*., 2010; Wan *et al*., 2011; Rakesh *et al*., 2013; Shalaby and Shanab, 2013; Kambar *et al*., 2014). In the present study, the leaf extract of *A. lineata* was found to exhibit concentration dependent scavenging of DPPH radicals with an IC_{50} value of 48.17µg/ml. At extract concentration 100µg/ml and higher, a scavenging of >90% was observed (Figure 1). Ascorbic acid scavenged DPPH radicals more efficiently (IC_{50} value of 3.27µg/ml) than the leaf extract. Although leaf extract displayed lesser radical scavenging potential than that of ascorbic acid, it is evident from the study that the leaf extract has the hydrogen donating property and hence the extract could serve as free radical scavengers, acting possibly as primary antioxidants (Chung *et al*., 2006).

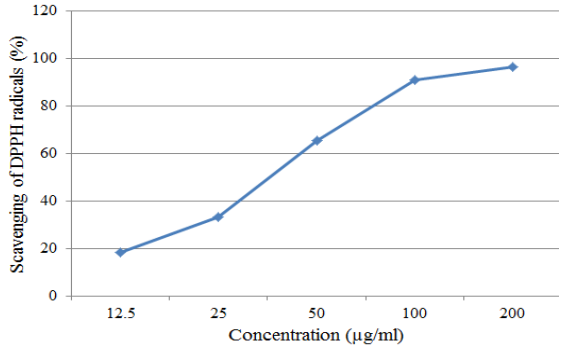

Figure 1: Scavenging of DPPH radicals by extract of *A. lineate*

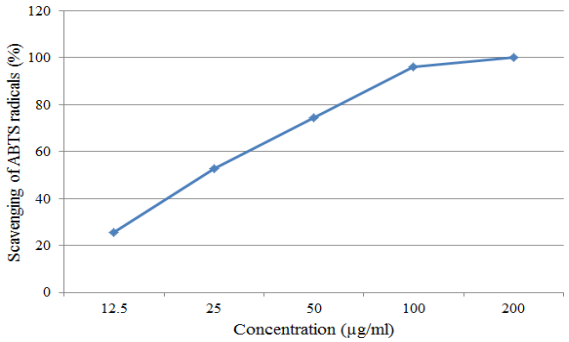

Figure 2: Scavenging of ABTS radicals by extract of *A. lineata*

The scavenging of ABTS radicals is another commonly used radical scavenging assay. Here, the ABTS radical is generated by reacting the ABTS salt with a strong oxidizing agent which may be potassium permanganate or potassium persulfate. An electron donating compound (antioxidant species) can reduce the blue-green ABTS radical solution to colorless neutral form by suppression of its characteristic long wavelength absorption spectrum (Shalaby and Shanab, 2013). This assay is widely used to determine radical scavenging activity of several kinds of samples including plant extracts (Wan *et al*., 2011; Rakesh *et al*., 2013; Shalaby and Shanab, 2013). In the present study, we evaluated the potential of leaf extract of *A. lineata* to scavenge ABTS radicals. The leaf extract scavenged ABTS radicals more efficiently in a dose dependent manner with an IC_{50} value of 33.16µg/ml. The scavenging of ABTS radicals was more marked than scavenging of DPPH radicals. At extract concentration 100µg/ml and higher, a scavenging of >90% was observed (Figure 2). However, ascorbic acid scavenged ABTS radicals to more extent (IC_{50} value of 2.17µg/ml) than the leaf extract. Although leaf extract displayed lesser radical scavenging potential than that of ascorbic acid, it is evident that the leaf extract has the electron donating property and hence the extract could serve as free radical scavengers.

Phenolic Content of Leaf Extract

Polyphenolic compounds (including flavonoids) of plant kingdom play an important role in stabilizing lipid

oxidation and are known to be associated with antioxidant activity. The phenolic compounds contribute directly to antioxidant function and may exhibit inhibitory effects on mutagenesis and carcinogenesis in humans. The antioxidative activities of phenolic compounds could be due to the different mechanisms such as reactive oxygen species scavenging, inhibition of the generation of free radicals and chain-breaking activity and metal chelation (Chang *et al*., 2007). FCR method is the oldest and most widely employed method to estimate the total phenolic content of many kinds of samples including plant extracts. Under basic conditions, the phenolic compounds reacts with FCR and form blue colored complex having maximum absorption near 750nm. The FCR method is simple, convenient and reproducible (Chung *et al*., 2006; Gulcin *et al*., 2011; Rakesh *et al*., 2013; Pavithra *et al*., 2013). In the present study, the total phenolic content of leaf extract of *A. lineata* was estimated by FCR method and expressed as mg GAE/g of extract. The phenolic content was found to be 81.36mg GAE/g of extract.

CONCLUSIONS

The findings of the present study showed the potential of extract of *A. lineata* leaves for developing novel antimicrobial and antiradical agents which can be used for treating infections and oxidative damage. In suitable form, the plant can be used to control phytopathogenic fungi. Further studies on isolation of active principles from the leaves and their toxicological and pharmacological studies are to be carried out.

Conflict of Interest

Conflict of interest none declared.

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