ISSN: 2520-7695 (Print) and XXXX-XXXX (Online)
Medical and Health Sciences Research Journal
Med. Health Sci. Res. J., Jan-Apr 2017, 1(1): 76-82
Journal Homepage: http://www.mhsrjournal.com/
http://www.wollegajournals.com/

Original Research

Radical Scavenging, Cytotoxic and Antimicrobial Activity *Flacourtia indica* (Burm. f.) Merr.

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Abstract

In the present study, we determined antiradical, cytotoxic and antimicrobial potential of leaf extract of *Flacourtia indica* (Burm. f.) Merr. (Salicaceae). Antiradical activity of extract was evaluated by DPPH and ABTS assay. Cytotoxic activity was screened by brine shrimp lethality assay. Antibacterial and antifungal activity of extract was assessed by Agar well diffusion and Poisoned food technique respectively. Leaf extract was found to scavenge DPPH and ABTS radicals dose dependently with an IC_{50} value of 20.41 and 1.20µg/ml respectively. The extract was shown to exhibit concentration dependent mortality of brine shrimp larvae with an IC_{50} value of 283.27µg/ml. Extract exhibited inhibitory activity against all test bacteria with highest and least activity against *B. subtilis* and *E. coli* respectively. The extract has shown varied antifungal activity against 6 seed-borne mycoflora. *Curvularia* sp. and *Aspergillus fumigatus* exhibited highest and least susceptibility to leaf extract. From the results of this study, it is found that the plant is a good resource that can be used for developing antiradical, cytotoxic and antimicrobial agents.

Article Information

Article History:
Received : 29-01-2017
Revised : 19-04-2017
Accepted : 23-04-2017

Keywords:
Flacourtia indica
Brine shrimp,
Agar well diffusion
Poisoned food technique
DPPH

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INTRODUCTION

Plants are known to play an important role in human wealth since time immemorial. Human beings depend on plants for fulfilling basic needs such as food, shelter and medicine. People especially from developing and under-developing countries rely on several plants for their primary healthcare needs. It is estimated that about 80% of world population depends on traditional medicine that utilizes the use of plants in certain kinds of formulations. Traditional healers all over the world use several plants singly or in combination to treat various diseases or disorders. Higher plants have provided lot of lead compounds for developing several pharmaceutical drugs. Several life-saving drugs such as vincristine, vinblastine and quinine are from plant origin. The healing power of plants is due to the presence of various phytoconstituents such as alkaloids, flavonoids, phenolic compounds and terpenes. Most of these metabolites protect the plants from infectious agents, herbivores and insects. Plant based medicines have been shown to cure several dreadful diseases including cancer. Plants have been an integral part of medicinal systems such as Ayurveda, Unani and Sidda (Cowan, 1999; Karou *et al*., 2005; Nair *et al*., 2005; Kavya *et al*., 2010; Poornima *et al*., 2012; Yoon *et al*., 2013; Martins *et al*., 2015; Savithramma *et al*., 2017).

The genus *Flacourtia* (belonging to the family Salicaceae) includes trees or shrubs, often armed with thorns. The leaves may be alternate, spiral, toothed or crenate. Flowers are small, dioecious and found in small branched racemes or clusters. Petals are absent. Fruit is represented by a few seeded berry (Bhat, 2014). One of the important species of the genus is *Flacourtia indica* (Burm. f.) Merr. It is a shrub or a small tree with stout thorns. Leaves are ovate or obovate (2-10x1-5cm), entire to crenate-serrate and glabrous beneath. Sepals are ovate and ciliate. Fruit is a globose berry and is dark purple in color when ripe. Fruits are edible. Roots and leaves are medicinal (Bhat, 2014). Various parts such as root, bark and fruit of the plant are used traditionally in the treatment of several ailments (Varkey and Thomas, 2011; Ancy *et al*., 2013; Biswas and Battu, 2016; Savithramma *et al*., 2017). The plant is reported to exhibit various bioactivities such as hepatoprotective (Varkey and Thomas, 2011; Biswas and Battu, 2016), antioxidant (Tyagi *et al*., 2010), diuretic (Ancy *et al*., 2013), anti-asthmatic (Tyagi *et al*., 2011) and antibacterial (Eramma and Devaraja, 2013) activity. The present study was undertaken to evaluate antiradical, cytotoxic and antimicrobial potential of leaf extract of *F. indica*.

MATERIALS AND METHODS

Collection of Plant Material

The plant was collected at outskirts of Sagara, Shivamogga district, Karnataka in the month of February 2017. The plant was identified on the basis of its typical characters by referring standard flora (Bhat, 2014).

Extraction

The leaves were separated, washed well to remove adhering matter, dried under shade and powdered. Extraction was done by maceration process using methanol. A known quantity of powdered leaf material (10g) was transferred to a clean container and 100ml of methanol was added. The container was closed, left for 48 hours and stirred occasionally. The content of flask was filtered through Whatman No. 1 filter paper and the filtrate was subjected to evaporation at 40^{o}C. The extract was stored in refrigerator (Kekuda *et al*., 2016).

Antiradical Activity of Leaf Extract

We conducted two in vitro assays namely DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2-azinobis 3-ethylbenzothiazoline 6-sulfonate) free radical assay to determine antiradical nature of leaf extract of *F. indica*.

DPPH Radical Scavenging Activity

DPPH radical solution and different concentrations (12.5 to 200µg/ml) of leaf extract and ascorbic acid (reference standard) were prepared in methanol. 1ml of various concentrations of leaf extract and ascorbic acid was added to 3ml of DPPH radical solution in clean and dry test tubes. The tubes were kept in dark for 30 minutes and the absorbance of each tube was measured at 517nm spectrophotometrically. A combination of 1ml of methanol and 3ml of DPPH served as control. Radical scavenging activity of each concentration of extract/standard was calculated using the formula:

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

where C and T denotes absorbance of DPPH control and absorbance of DPPH in presence of extract/standard. IC_{50} (Inhibitory concentration) value was calculated for both extract and ascorbic acid by regression analysis. This value indicates the concentration of extract/standard required to scavenge 50% of radicals (Kekuda *et al*., 2016).

ABTS Radical Scavenging Activity

The generation of ABTS radicals was done by mixing and incubating ABTS stock solution (7mM) and potassium persulfate (2.45mM) in dark for 16 hours at room temperature. Different concentrations (12.5 to 200µg/ml) of leaf extract and ascorbic acid (reference standard) were prepared in methanol. In brief, 1ml of various concentrations of leaf extract and ascorbic acid was mixed to 3ml of ABTS radical solution in clean and dry test tubes. The tubes were kept in dark for 30 minutes and the absorbance of each tube was measured at 730nm spectrophotometrically. A combination of 1ml of methanol and 3ml of ABTS radical solution served as control. Radical scavenging activity of each concentration of extract/standard was calculated using the formula:

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

Where C and T denotes absorbance of ABTS control and absorbance of ABTS in presence of extract/standard. IC_{50} (Inhibitory concentration) value was calculated for both extract and ascorbic acid by regression analysis. This value indicates the concentration of extract/standard required to scavenge 50% of radicals (Kekuda *et al*., 2016).

Brine Shrimp Lethality of Leaf Extract

Cytotoxic nature of leaf extract was evaluated by Brine shrimp lethality bioassay using the larvae of brine shrimp *Artemia salina*. The eggs of *A. salina* were hatched in artificial sea water. Various concentrations (0 to 1000µg/ml) of extract and potassium dichromate (standard, 0 to 50µg/ml) were prepared in artificial sea water (prepared by mixing commercial salt and water). 25 larvae (nauplii) were transferred into vials containing different concentrations of leaf extract and potassium dichromate and the vials were incubated for 24 hours at 25^{o}C. After 24 hours, the number of surviving shrimps was counted by using a magnifying lens (3X). Lethal nature of extract/standard in terms of mortality of larvae (%) was calculated by using the formula:

Mortiality of larvae = (number of dead larvae / total number of larvae) x 100.

LC_{50} values were determined by linear regression using Origin 6.0 software (McLaughlin, 1991).

Antibacterial Activity of Leaf Extract

Two Gram positive bacteria namely *Bacillus subtilis* NCIM 2063 and *Staphylococcus aureus* NCIM 5345 and two Gram negative bacteria namely *Pseudomonas aeruginosa* NCIM 2200 and *Escherichia coli* NCIM 2065 were screened for their susceptibility to leaf extract of *F. indica*. Inocula of test bacteria were prepared in Nutrient broth medium. The pure cultures of test bacteria were inoculated into sterile Nutrient broth tubes and the tubes were incubated at 37^{o}C for 24 hours. The broth cultures thus obtained were used to assess their susceptibility to leaf extract by agar well diffusion method. The broth cultures of test bacteria were swab inoculated on sterile Nutrient agar plates using sterile cotton swabs. Using a sterile cork borer (sterilized by dipping in alcohol followed by flaming), wells of 8mm diameter were punched in the inoculated plates. The wells were labeled and filled with leaf extract (25mg/ml of dimethyl sulfoxide [DMSO]), standard antibiotic (Chloramphenicol, 1mg/ml of sterile distilled water) and DMSO. The plates were incubated for 24 hours in upright position at 37^{o}C. Zones of inhibition were measured using a ruler (Raghavendra *et al*., 2016).

Antifungal Activity of Leaf Extract

A total of 6 seed-borne fungi namely *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *Curvularia* sp., *Alternaria* sp. and *Fusarium* sp. recovered previously from moldy grains of sorghum were tested for their susceptibility to leaf extract of *F. indica* by Poisoned food technique. In brief, the control (without extract) and poisoned (1mg leaf extract/ml of medium) Potato dextrose agar plates were inoculated aseptically with the test fungi. The plates were incubated at room temperature for 96 hours in upright position. Colony diameter of test fungi on both control and poisoned plates was measured in mutual perpendicular directions using a ruler. Antifungal activity of leaf extract, in terms of inhibition of mycelial growth of test fungi, was calculated using the formula:

Inhibition of mycelial growth (%) = (C – T / C) x 100

Where C and T represents the colony diameter of fungi on control and poisoned plates respectively (Raghavendra *et al*., 2016).

RESULTS AND DISCUSSION

In the present study, we extracted the powdered leaf material of *F. indica* by maceration process using methanol. Maceration is a simple process of extracting plant materials being used commonly by several researchers (Al-Dhabi *et al*., 2012; Okhale *et al*., 2014; Swapna *et al*., 2015; Banu and Cathrine, 2015; Raghavendra *et al*., 2016). We have selected methanol as extraction solvent. It is shown that methanol is the best solvent to extract a number of secondary metabolites such as polyphenols, flavonoids, terpenoids and saponins (Cowan, 1999; Tiwari *et al*., 2011). Maceration process resulted in obtaining a dark green colored leaf extract and the yield of extract was 14.86%.

Reactive oxygen species (ROS) are natural byproducts of metabolic pathways which involve oxygen. Superoxide, hydroxyl, peroxyl, and alkoxy radicals are the most common ROS. In normal non-stressed conditions, production and scavenging of these ROS is in equilibrium. However, under certain conditions such as pollution, radiation exposure and drought there is an increased production of ROS which leads to oxidative stress and damage to biomolecules like proteins, lipids and DNA. Oxidative stress is closely associated with conditions such as cancer, neurodegenerative diseases and cardiovascular diseases. In pathophysiological conditions, there is an extra need for antioxidants from exogenous sources such as diet. Search for antioxidants from natural sources such as plants has been increased due to negative effects associated with the use of synthetic antioxidants. It is shown that frequent consumption of plants is linked with reduced incidence of several diseases which are associated with free radical production. Plant compounds such as phenolics, flavonoids, terpenes and vitamins are shown to be excellent antioxidants (Hsu, 2006; Hazra *et al*., 2008; Shukla *et al*., 2009; Kusirisin *et al*., 2009; Maruthappan and Shree, 2010; Thaipong *et al*., 2012; Kekuda *et al*., 2016).

DPPH free radical scavenging assay is one of the most popular in vitro radical scavenging assays because the assay is simple, rapid, requires very less sample and the results are reproducible. DPPH is a stable radical having absorption maximum at 517nm in alcoholic solution. In radical form it is purple in color and its color bleaches and changes to yellow (DPPHH, non-radical) in the presence of a compound which has the hydrogen donating property. The extent of bleaching of color to yellow depends on hydrogen donating property of antioxidant (Molyneux, 2004; Chung *et al*., 2006; Thaipong *et al*., 2006; Park *et al*., 2008; Krishna and Nair, 2010; Akinmoladun *et al*., 2010; Rakesh *et al*., 2013; Kekuda *et al*., 2016). In the present study, we monitored bleaching of color of DPPH radical solution in presence of different concentrations of leaf extract and ascorbic acid at 517nm. The result of radical scavenging potential of leaf extract against DPPH radicals is shown in Figure 1. A dose dependent scavenging of DPPH radicals was shown by extract with an IC_{50} value of 20.42µg/ml. A scavenging potential of >50% was observed at concentration 25µg/ml and higher. At concentration 100µg/ml and higher, >90% scavenging of radicals was observed. Ascorbic acid scavenged radicals more effectively than leaf extract with an IC_{50} value of 10.62µg/ml. In an earlier study, Ndhlala *et al*. (2008) observed marked scavenging of DPPH radicals by peel of *F. indica*. The study of Tyagi *et al*. (2010) showed dose dependent scavenging of DPPH radicals by aqueous and methanolic extract from leaves of *F. indica* with IC_{50} value of 26µg/ml and 18µg/ml respectively. In the present study, the leaf extract of *F. indica* exhibited lower scavenging potential when compared to ascorbic acid, it is evident that the leaf of *F. indica* possess hydrogen donating property and hence the leaf extract can serve as free radical scavenger, acting possibly as primary antioxidant.

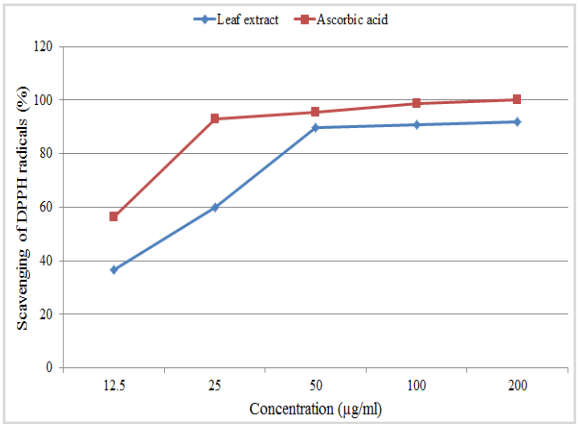

Figure 1: Scavenging of DPPH radicals by *F. indica* and ascorbic acid

Like DPPH assay, the assay involving scavenging of ABTS radicals is another popular in vitro radical scavenging assay which is commonly used to evaluate antiradical activity of plant extracts. However, this method differs from DPPH assay in that it needs the generation of radicals. The ABTS radical is generated by treating the ABTS salt with a strong oxidizing agent such as potassium permanganate or potassium persulfate. This method has been used widely to determine radical scavenging nature of plants. In this assay, an electron donating compound (antioxidant species) reduces the blue-green colored ABTS radical solution to colorless neutral form which is shown by the suppression of its characteristic long wavelength absorption spectrum (Thaipong *et al*., 2006; Wangcharoen and Morasuk, 2007; Park *et al*., 2008; Osman *et al*., 2009; Krishna and Nair, 2010; Wan *et al*., 2011; Rakesh *et al*., 2013; Shalaby and Shanab, 2013). The result of potential of leaf extract of *F. indica* to scavenge ABTS radicals is shown in Figure 2.

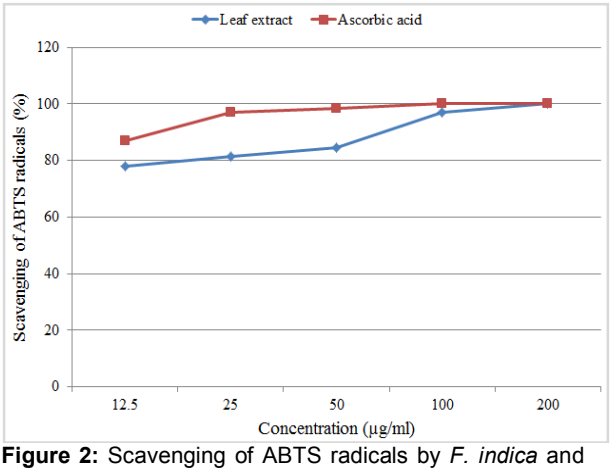

Figure 2: Scavenging of ABTS radicals by *F. indica* and ascorbic acid

Leaf extract scavenged radicals dose dependently with an IC_{50} value of 1.20µg/ml. A scavenging activity of >75% was shown by the lowest concentration of leaf extract tested. At 200µg/ml, extract exhibited 100% scavenging activity. When compared to leaf extract, ascorbic acid displayed slightly higher scavenging activity with an IC_{50} value of 1.14µg/ml. It is evident that the leaf extract possess the electron donating property and thereby the extract can serve as a free radical scavenger.

Cytotoxicity determination by brine shrimp lethality bioassay (proposed by Michael and coworkers) is a rapid, simple, easily mastered and inexpensive method and the assay requires small amounts of sample. The assay has a good correlation with cytotoxic activity in some human solid tumors. Since its introduction, the test has been successively used for frontline screening which can be followed by more specific and more sophisticated bioassays (McLaughlin *et al*., 1998; Apu *et al*., 2010). The result of cytotoxic potential of leaf extract of *F. indica* in terms of lethal effect against larvae of *A. salina* is shown in Figure 3. The extract exhibited concentration dependent mortality of larvae of *A. salina*. At concentration 10 and 50µg/ml, extract failed to cause mortality of larvae. A mortality of 50% and higher was observed at extract concentration of 250µg/ml and higher. At extract concentration 1000µg/ml, 80% mortality of brine shrimp larvae was observed. The LC_{50} value for extract was found to be 283.27µg/ml. the cytotoxic potential of leaf extract was much lesser when compared to standard (potassium dichromate) which exhibited potent cytotoxic effect with an LC_{50} value of 36.88µg/ml. In an earlier study of Ravikumar *et al*. (2010), the hydromethanolic extract of leaves of *F. indica* did not show any killing effect on the larvae of *A. salina*. Although leaf extract displayed lesser cytotoxic activity when compared to standard compound, it is evident that the leaf extract possess cytotoxic principles which can be used to develop cytotoxic agents.

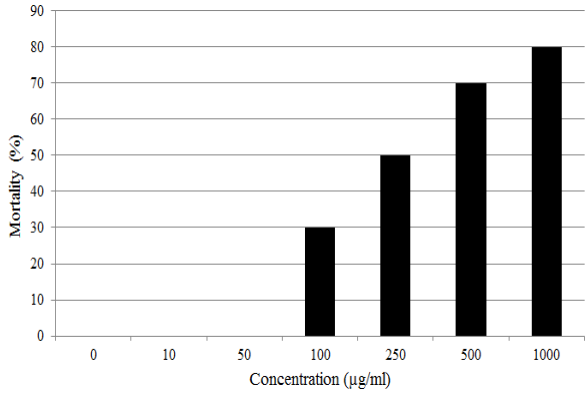

Figure 3: Mortality of larvae at different concentrations of extract of *F. indica*

Discovery of antibiotics is considered as an important milestone in the field of chemotherapy. However, soon after discovery and subsequent use, there has been upsurge of development of resistance in pathogenic bacteria against many of the antibiotics such penicillin, methicillin and vancomycin. Besides, the use of antibiotics is also associated with certain side effects. Antibiotic resistance is a global problem in hospital and community settings and is of more concern in developing and under-developing countries where these resistant pathogens are responsible for huge number of deaths. This triggered immense research on searching alternates for therapy. One of the best alternates is the use of plant based medicines. Higher plants have been used as medicine since time immemorial. It is estimated that around 80% of world population rely on traditional medicine which is chiefly based on the utilization of plants in suitable formulations. The formulations containing plants or plant metabolites have shown to act against a wide range of pathogenic bacteria including antibiotic resistant strains (Karou *et al*., 2005; Adwan *et al*., 2010; Murgananthan and Pabbithi, 2012; Martins *et al*., 2015; Wikaningtyas and Sukandar, 2016). In the present study, we evaluated antibacterial potential of leaf extract of *F. indica* by Agar well diffusion method. The presence of inhibition zone around the well was considered positive for antibacterial activity of leaf extract. The leaf extract was effective against all test bacteria. Among bacteria, Gram positive bacteria have shown high susceptibility when compared to Gram negative bacteria. *B. subtilis* was inhibited to high extent while *E. coli* showed least susceptibility to leaf extract. Inhibitory activity displayed by reference antibiotic was higher when compared to leaf extract. DMSO was ineffective against all bacteria. In an earlier study, Eramma and Devaraja (2013) showed antibacterial potential of methanol extract of root of *F. indica*.

Table 1: Antibacterial activity of *F. indica*

Test bacteria	Zone of inhibition in cm		
	Extract	Antibiotic	DMSO
S. aureus	1.3	3.2	0.0
B. subtilis	1.5	3.4	0.0
P. aeruginosa	1.2	2.8	0.0
E. coli	1.1	2.4	0.0

The development of human civilization is linked closely to cultivation of various food crops however, the plant diseases caused mainly by fungi have been a major concern to mankind as fungal infections of plants results in huge economic losses in severe cases. Management of fungal infections of plants is usually done with the use of synthetic fungicides. Indiscriminate use of these chemical agents resulted in adverse effects on non-target organisms including humans, residual effect on environment and the development of resistance in various fungal pathogens. Besides, high cost of these fungicides is also not affordable for many farmers. Higher plants have been shown to be promising alternates for synthetic fungicides and many literatures have highlighted the potential antifungal activity of several plants against a range of phytopathogenic fungi including seed mycoflora (McCartney *et al*., 2003; Hasan *et al*., 2005; Deising *et al*., 2008; Koch *et al*., 2013; Yoon *et al*., 2013; Junaid *et al*., 2014; Kekuda *et al*., 2016). In the present study, we evaluated the potential of leaf extract of *F. indica* to inhibit seed mycoflora by poisoned food technique and the result is shown in Table 2 and Figure 4. The colonies of test fungi were smaller in plates poisoned with extract indicating the antifungal potential of leaf extract. Extract displayed marked inhibition of all test fungi but to a varied extent. Highest and least inhibitory activity was observed against *Curvularia* sp. and *A. fumigatus* respectively. *Curvularia* sp., *Alternaria* sp., *Fusarium* sp. and *A. niger* were inhibited to >50% by leaf extract. Among *Aspergillus* species, *A. niger* and *A. fumigatus* were susceptible to highest and least extent respectively to leaf extract.

Table 2: **Mycelial growth of test fungi in control and poisoned plates**

Test fungi	Colony diameter in cm	
	Control	Extract
A. niger	5.7	2.7
A. flavus	4.3	2.6
A. fumigatus	3.9	2.6
Curvularia sp.	4.5	1.4
Alternaria sp.	5.2	2.0
Fusarium sp.	4.3	2.1

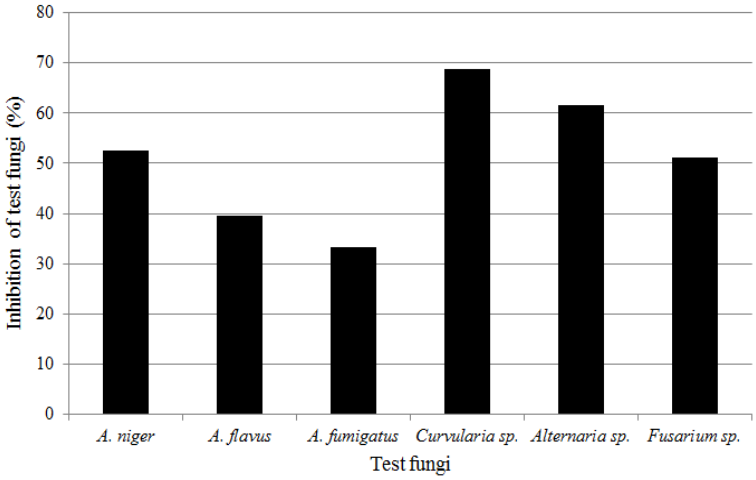

Figure 4: Inhibition of test fungi (%) by extract of *F. indica*

CONCLUSIONS

Plants are widely used all over the world to treat varieties of ailments. In the present study, the leaf extract of *F. indica* was found to exhibit antiradical, cytotoxic and antimicrobial activities. In suitable form, after safety assessment, the plant can be used in the treatment of oxidative damage, cancer and infectious microbes. It is evident form the study that the leaf possesses bioactive principles which needs to be isolated, characterized and subjected for bioactivity determinations.

Conflict of Interest

None declared.

Acknowledgements

Authors thank Head of the Department of Microbiology and Principal of S.R.N.M.N College of Applied Sciences for providing facilities to conduct work. Authors thank N.E.S for moral support. Authors also thank Ms. Shilpa M and Ms. Tejaswini Petkar for assisting in work.

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