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Evaluation of antimicrobial and cytotoxic properties of *Leucas aspera* and *Spilanthes paniculata*

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Abstract

Plants are rich source of antibacterial agents, which could be exploited in human disease management. The main aim of this study was to find out the antibacterial and cytotoxic activity of the n-hexane, ethyl acetate and ethanol extract of *Leucas aspera* and the ethanol fraction of chloroform, methanol and ethanol extract of *Spilanthes paniculata were studied*. The fraction and extracts of *S. paniculata* showed potential antimicrobial activities against 14 strains of microorganisms whereas *L. aspera* did not show any antimicrobial response. For antimicrobial test, Disc diffusion technique was used against five Gram positive and eight Gram negative bacteria and one fungi. The zone of inhibition of microorganisms was measured in mm. *In vitro* cytotoxicity test was also studied with both of the plants by Brine Shrimp Lethality Bioassay and results illustrated significant ($p<0.05$) cytotoxicity against *A. salina,* that were expressed as LC_{50}.

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Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many of which based on their use in traditional medicine. It has been noted that the original source of many important pharmaceuticals currently in use have been plants used by indigenous people (Balick *et al*., 1996). It has been reported that about 64% of the total global population remains dependent on traditional medicine and medicinal plants for provision of their health-care needs (Cotton, 1996). In order to promote the use of medicinal plants as potential sources of antimicrobial compounds, it is pertinent to thoroughly investigate their composition and activity and thus validate their use (Nair *et al*., 2006). Some phytochemicals produced by plants have antimicrobial activity allowing these plants to be studied and used for the development of new antimicrobial drugs (Nascimento *et al*., 2000). Secondary plant metabolites are largely unexplored in 'conventional' animal production systems. In the past, plant metabolites were generally considered as sources of antinutritional factors. Recent bans and restrictions on the use of animal antibiotic growth promoters stimulated interest in bioactive secondary metabolites of plant source as alternative performance enhancers (Greathead, 2003).

Leucas aspera (Family - Labiatae) is an annual herb found throughout South Asia as a weed in cultivated fields, wastelands and roadsides. The juice of the leaves is used as local application for psoriasis, chronic skin eruptions and chronic rheumatism (Kirtikar *et al*., 1991). The flowers are given with honey to treat cough and cold in children. The leaves are applied to the bites of serpents, poisonous insects and scorpion sting. *L. aspera* leaves are also used as insecticides and mosquito repellent in rural area (Reddy *et al*., 1993). The plant extract with honey is a good remedy for stomach pain and indigestion.

Spilanthes paniculata (Family- Asteraceae) is an important medicinal plant with rich source of therapeutic constituents. The genus *Spilanthes* (Asteraceae) comprises 30 species and 9 additional intraspecific taxa that are mainly distributed in the tropical and subtropical regions around the world (Jansen, 1985). In particular, this species is famous as a folklore remedy for toothache and for throat and gum infections, earning it the English nickname, the "toothache plant". *S. paniculata* all showed larvicidal activity against *Anopheles* mosquitoes suggesting a possible role for *Spilanthes* in not just the treatment but also prevention of malaria (Pandey *et al*., 2007). *Spilanthes* contains a number of biologically active compounds (Prachayasittikul *et al*., 2009), of which the most studied have been the alkylamides, which this plant possesses in abundance (Nakatani *et al*., 1992). Isolated alkylamides from *Spilanthes* have demonstrated activity against mosquito larvae. Although there are no published reports of antiplasmodial activity of isolated *Spilanthes* alkylamides, alkylamides from other plants have shown such activity (Sittie *et al*., 1998). Roots of *S. paniculata* and foliage of both species had a C:N of < 25 and released more than 90% of N, P and K within 150 d. They can be considered to be good resources for the improvement of soil fertility. Thus, these residues, particularly those of *S. paniculata*, can play a significant role in soil nutrient enrichment in poorly managed shifting cultivation systems (Majumder *et al*., 2008).

The aim of the present work was to evaluate the cytotoxic and antimicrobial assays to support the pharmacological effects and phytochemical investigation of these plants as well. Although numerous studies have shown the medicinal values of these plants, there still remains ample scope for further in depth research. So far, for the first time an attempt was taken to investigate the antimicrobial and cytotoxic effect of *Spilanthes paniculata*. Accordingly, we disclose herein the antimicrobial and cytotoxic

effects of the whole parts of *Leucas aspera* and *Spilanthes paniculata* to further establish the scientific basis of the traditional uses of these plants.

Materials and methods

Collection of the plant material

Leucas aspera and *Spilanthes paniculata* were collected from Kushtia and the Dhaka University, Dhaka, Bangladesh, respectively. For this present investigation the plants were identified by the Bangladesh National Herbarium, Dhaka, with the accession no DACB-35032 and DACB-35019.

Extraction of the plant material

The whole plants were taken and selected for the study. They were sun dried for five days and heated through oven to be fully dried at below 40°c for 24 hours. Then the fully dried plants were grinded into coarse powder with the help of a mechanical grinder. The whole powders were extracted by cold extraction with three solvents (ethanol, methanol and chloroform) and (ethanol, n-hexane, ethyl acetate extract) in case of *Spilanthes paniculata* and *Leucas aspera* respectively and kept for a period of 3 days accompanying occasional shaking and stirring. The whole mixture were then underwent a coarse filtration by a piece of clean, white cotton material. Then these were filtered through whatman filter paper. The filtrates (Ethanol, methanol and chloroform extract) and (ethanol, n-hexane, ethyl acetate extract) obtained were evaporated by Rotary evaporator (Bibby RE-200, Sterilin Ltd., UK) at 5 to 6 rpm and at 68°c temperature. It rendered a gummy concentrate of chocolate black colour. The gummy concentrate was designated as crude extract. Then the crude extract was dried by freeze drier and preserved at 4°C (Haque *et al*., 2008).

Phytochemical analysis

Qualitative phytochemical tests for the identification of alkaloids, flavonoids, steroids, glycosides, saponins, tannin and terpenoids were carried out for all the extracts by the method described by Harborne (1998) and Sazada *et al*. (2009). The freshly prepared extracts of *Spilanthes paniculata* and *Leucas aspera* were qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extracts was performed using the following reagents and chemicals: Alkaloids with Wagner reagent, flavonoids with the use of conc HCl, tannins with 5% ferric chloride, and saponins with ability to produce suds. Gum was tested by using Molish reagents and concentrated sulfuric acid, steroids with sulfuric acid, reducing sugar with the use ά-napthol and sulfuric acid and terpenoids with chloroform and conc. HCl.

Antibacterial assay

The antimicrobial activity for different extracts was determined by the disc diffusion method (Bauer *et al*., 1966). Both gram positive, gram-negative bacterial strains and fungi were used for the test. The bacterial strains used for the investigation are listed in Table 1. Solutions of known concentration (mg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs (Kanamycin 30µg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control. These plates were then kept at low temperature (4°C) for 24 h to allow maximum diffusion. There was a gradual change in concentration in the media surrounding discs. The plates were then incubated at 37°C for 24 h to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment

was carried out three times and the mean of the reading is required.

Cytotoxicity screening

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds by the method Meyer (Meyer *et al*., 1982). Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The eggs of the brine shrimp were collected and hatched in artificial seawater (3.8% NaCl solution) for 48 hr to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method. The test samples (extract) were prepared by dissolving them in distilled water (20mg/ml). 5, 10, 20, 40, 80, and 160µl of solutions for each test sample were taken in 6 vials and 4ml of sea water was added to each vial containing 30-35 brine shrimp napulii. So the concentrations of the test sample in the vials were 25, 50, 100, 200, 400, and 800µg/ml respectively. A vial containing 50µl DMSO diluted to 5ml was used as a control. Standard Colcichine was used as positive control. Then matured shrimps were applied to each of all experimental vials and control vial. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial were counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

Statistical analysis of cytotoxicity screening data

Data from the experiments were analyzed using the Statistical Package for Social Science(SPSS) software for windows version 17 (SPSS Inc., Chicago, Illinois, USA). All the triplicate data were expressed as Mean ± SD as appropriate. Statistical analysis of the results was performed by using the ANOVA (analysis of variance) followed by Bonferroni post hoc and Dunnett test. The limit of significance was set at $p<0.05$. The LC_{50} values were calculated from linear regression analysis.

Results

Phytochemical screening

Preliminary phytochemical screening of the extract of *Leucas aspera* and *Spilanthes paniculata* revealed the presence of various bioactive components of which alkaloids, cardiac glycosides, flavonoids, terpenoids and tannins were the most prominent and the result of phytochemical test has been summarized in the Table 2. Our results indicate the presence of phytochemicals in the order of, ethanol>methanol>chloroform extract of *Spilanthes paniculata* and ethanol extract >ethyl acetate>n-hexane of *Leucas aspera*. The data showed the higher yield of phytochemicals in ethanol extract in both of the plants.

Brine shrimp lethality assay

Following the procedure of Mayer, the lethality of the extracts of *Leucas aspera* and *Spilanthes paniculata* to brine shrimp was determined on *A. salina* after 24 hours of exposure of the samples and the positive control, colchicine. The results of the different extracts of *L. aspera* and *Spilanthes paniculata* (% mortality at different concentrations and LC_{50} values) were shown in Table 3 and Fig. 1-7. The percent mortality increased with an increase in concentration. In case of *L. aspera,* the ethyl acetate, n-hexane and colchicine showed almost 100% mortality to brine shrimp at 800 µg/mL and on the other hand all of the extracts of *Spilanthes paniculata* showed 100.0% mortality to brine shrimp at 800 µg/mL. The LC_{50} obtained from the best-fit line slope were found to be 114.70 µg/ml, 43.97 µg/ml and 30.32 µg/ml for ethanol, ethyl acetate and n-hexane extract of *L. aspera* and 104.95 µg/ml, 94.62 µg/ml and 111.33 µg/ml for ethanol, methanol and chloroform extract of *Spilanthes paniculata* respectively.

Table 1. Antimicrobial activity of *Leucas aspera* and *Spilanthes paniculata*.

		Leucas aspera			*Spilanthes paniculata*		
Test organisms	Kanamycin (30µg/disc)	n-hexane Extract (500µg/disc)	Ethanol Extract (500µg/disc)	EF of EA Extract (500µg/disc)	EF of CE extract (500µg/disc)	Methanol Extract (500µg/disc)	Ethanol Extract (500µg/disc)
Gram Positive Bacterial strain							
Bacillus cereus	29±0.0	--	--	--	11±1.2	15±1.1	15±0.8
Bacillus megaterium	30±0.5	--	--	--	9±1.1	12±0.7	12±0.7
Bacillus subtilis	30±0.2	--	--	7 ± 0.7	10±0.9	15±1.2	13±0.5
Sarcina lutea	28±0.0	--	--	7 ±1.0	8±1.5	11±1.4	15±0.6
Staphylococcus aureus	27±0.0	--	--	7±0.5	8±1.2	11±0.7	11±1.6
Gram Negative Bacterial strain							
Salmonela paratyphi	30±0.8	--	--	7±1.4	11±1.2	14±0.7	14±0.5
Salmonela typhi	30±0.6	--	--	7±0.8	10±1.6	11±1.2	14±1.2
Vibrio parahemolyticus	30±0.9	--	--	7±0.6	8±0.6	12±0.5	15±1.2
Vibrio mimicus	30±0.6	--	--	7±0.7	11±0.6	14±0.6	17±1.0
Escherichia coli	28±0.0	--	--	7±1.2	10±1.1	17±0.6	17±0.5
Shigella dysenteriae	30±0.0	--	--	7±1.2	12±0.6	11±0.7	12±1.2
Psedomonas aureus	30±0.9	--	--	7±1.2	11±1.1	15±0.5	13± 0.7
Shigella boydii	32±1.8	--	--	7±1.2	1±1.6	15±0.5	14±1.0
Fungi							
Aspergilus niger	30±0.0	--	--	7±1.5	10±1.5	12±1.3	14±0.6

Table 2. Qualitative analysis of the phytochemicals of *L. aspera* and *S. paniculata* extracts.

Chemical Constituents	*L. aspera*			*S. paniculata*		
	n-hexane	Ethylacetate	Ethanol	EF of CE	Methanol	Ethanol
Alkaloids	++	++	+++	+ + +	+	+ +
Glycosides	+	+++	+++	+ + +	+ + +	+ ++
Flavonoids	-	+	++	+	+	+ + +
Saponins	-	+	++	-	+ +	+ + +
Tannins	+	++	+++	-	+ + +	+
Terpenoids	+++	+++	+++	+ + +	+ + +	+ + +

Symbol (+) indicates presence and (-) indicates absence of phytochemicals. EF = Ethanol fraction, CE = Chloroform extract.

Table 3. Results of Brine Shrimp Lethality Assay for *Leucas aspera* and *Spilanthes paniculata*.

	Extracts	% mortality at different concentration						
		25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml	LC50 µg/ml
L. aspera	Ethanol	36.66±0.38*	38.99±0.67*	46.84±0.27*	52.29±0.09*	61.30±0.65*	78.49±0.64*	114.70
	EF of EA	41.62±1.44	50.22±0.29*	60.66±0.58*	81.02±1.60*	91.58±1.42*	97.90±0.85*	43.97
	n-hexane	43.36±1.05*	59.20±1.07*	68.77±0.55*	79.17±1.27*	89.49±1.63*	92.87±0.13*	30.32
	Colcichine	40	56.95	75	100	100	100	30.81
S. paniculata	EF of CE	15.83±0.27*	29.18±0.63*	47.89±0.70*	62.56±0.47*	79.16±0.22*	100±0.00	104.95
	Methanol	19.61±0.46*	32.02±0.79*	52.14±1.01*	67.75±0.42*	86.16±0.22*	100±0.00	94.62
	Ethanol	16.09±0.93*	32.02±0.53*	48.11±0.83*	65.68±0.44*	81.20±0.34*	100±0.00	111.33

Data were expressed as mean±SD and analyzed by one way ANOVA, Post hoc and Dunnett test. All of the results were compared with the standard (colchicine); $P<0.05$.

Discussion

Isolation of pure, pharmacologically active constituents from plants remains a long and tedious process. For this reason, it is necessary to have methods available which eliminate unnecessary separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or useful constituents with potential activities. This procedure enables recognition of known metabolites in extracts or at the earliest stages of separation and is thus economically very important.

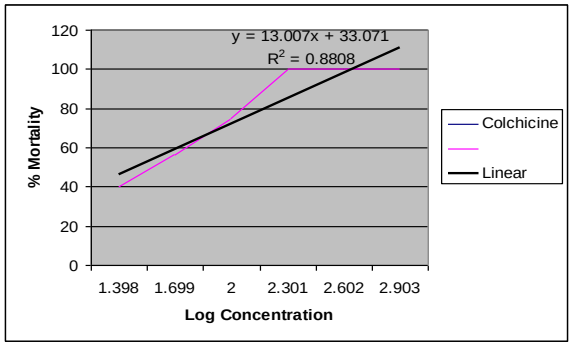

Fig. 1. Determination of LC_{50} of cholchicine against brine shrimp napulii.

Flavonoids, (a large group of naturally occurring plant phenolic compounds including flavones, flavonols, isoflavones, flavonones and chalcones), also known as nature's tender drugs, possess numerous biological/ pharmacological activities. Recent reports of antiviral, anti-fungal, antioxidant, anti-inflammatory, antiallergenic, antithrombic, anticarcinogenic, hepatoprotective, and cytotoxic activities of flavonoids have generated interest in studies of flavonoid-containing plants. Of these biological activities, the anti-inflammatory capacity of flavonoids has long been utilized in Chinese medicine and the cosmetic industry as a form of crude plant extracts (Aguinaldo *et al.*, 2005; Moon *et al.*, 2006; Veitch 2007; Jiang *et al.*, 2008; Kim *et al.*, 2004; Wu *et al.*, 2008).

Triterpenoids have a range of unique and potentially usable biological effects and reference to the use of plants with high saponin / triterpenoid content can be found in the first written herbarium. Triterpenoids are studied for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory and tonic

effects. They are used in the prevention and treatment of hepatitis, parasitic and protozoal infections and for their cytostatic effects. The disadvantage of using triterpenoids is the toxicity associated with their hemolytic and cytostatic properties. Hand in hand with ongoing extraction and isolation of natural products therefore, is the development of synthetic derivatives with lower toxic and higher therapeutic potential (Dzubak *et al*., 2006).

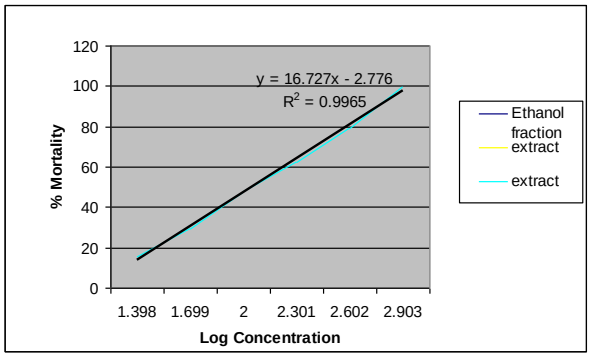

Fig. 2. Determination of LC_{50} of ethanol fraction of Chloroform extract against brine shrimp napulii.

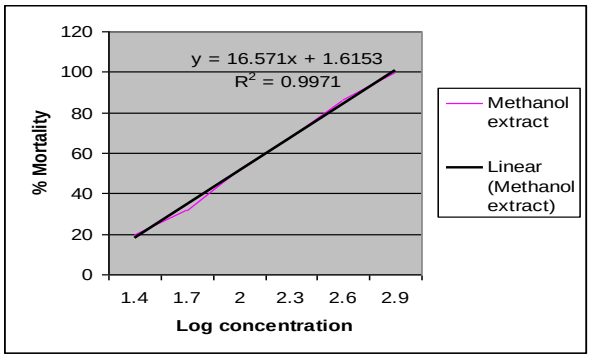

Fig. 3. Determination of LC_{50} of methanol extract against brine shrimp napulii.

The antimicrobial potential of plants was compared according to their zone of inhibition against the several pathogenic organisms. None of the *Leucas aspera* extracts showed antimicrobial effects with the dose of 500µg/ml which is consistent with other investigators (Mohana *et al*., 2008). On the other hand, the *S. paniculata* plant extract, from various extraction processes, showed their potential activity against both bacteria and fungi. None of the crude extracts of *S. paniculata* demonstrated significant inhibition of growth of the test microorganisms. This was probably due to the development of partial or complete resistance of the microorganisms against the test samples, which might be the indiscriminate use of antibacterial agents (Chowdhury *et al*., 2008). However, the results of this study showed that the extracts used can inhibit the growth of gram-positive and gram negative bacteria as well as fungi. Based on the statistical analysis, this inhibition is moderately significant than the standard broad spectrum antibiotic (30µM of Kanamycin). From the result, regarding to the antimicrobial activity, it can be concluded that the ethanol fraction of chloroform, methanol and ethanol extract of whole plant of *Spilanthes paniculata* possesses prospective broad spectrum anti-microbial potency against the given test organisms and the order of the potency should be methanol > ethanol > chloroform.

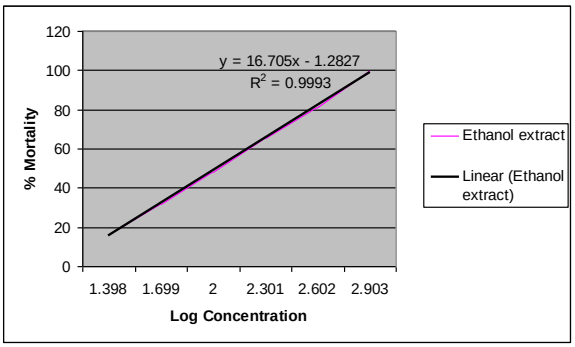

Fig. 4. Determination of LC_{50} of ethanol extract against brine shrimp napulii.

The brine shrimp lethality assay (BSLA) has been used routinely in the primary screening of the extracts as well as isolated compounds to assess the toxicity towards brine shrimp, which could also provide an indication of possible cytotoxic properties of the test materials. Brine shrimp nauplii have been previously utilized in various bioassay systems. Among these applications have been the analyses of pesticidal residues, mycotoxins, stream pollutants,

anesthetics, dinoflagelate toxins, morphine-like compounds, carcinogenicity of phorbol esters and toxicants in marine environment. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay (McLaughlin *et al*., 1991; Meyer *et al*., 1982; Sam, 1993).

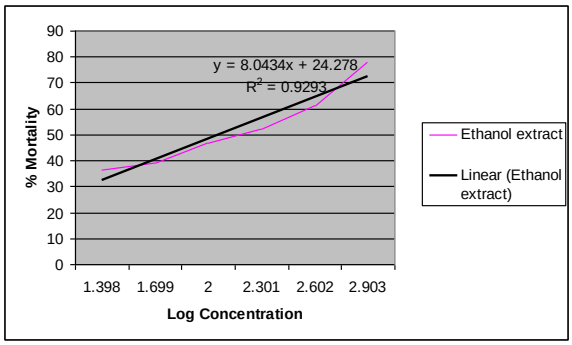

Fig. 5. Determination of LC_{50} of ethanol extract of *L aspera* against brine shrimp napulii.

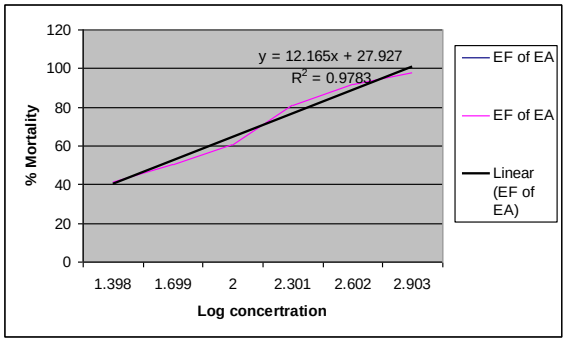

Fig. 6. Determination of LC_{50} of ethanol fraction of Ethyl acetate extract of *L. aspera* against brine shrimp napulii.

The variation in BSLA results (Table 4) may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins, flavonoids or triterpenoids) present in the extracts. The cytotoxic effect of the *Leucas aspera* was consistent with some other investigators though they investigated this activity by using the root of this plant (Rahman *et al*., 2007). Moreover, this significant lethality of the crude plant extracts (LC_{50} values less than 100 ppm or µg/mL) to brine shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds which warrants further investigation. BSLA results may be used to guide the researchers on which plant extracts/fractions to prioritize for further fractionation and isolation of these bioactive compounds. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds later.

The present study indicates that the extracts of *Leucas aspera* (ethanol, ethyl acetate and n-hexane) and *Spilanthes paniculata* (ethanol, methanol and chloroform) have got profound cytotoxic and antimicrobial (except *L. aspera*) effect and may have potential use in medicine. From the previous studies and our current investigation it may be concluded that the flavonoids and tannins are responsible for aforementioned activity. This novel finding will aid us to conduct bioactivity guided isolation and characterization of leading compounds in due course.

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