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ORIGINAL RESEARCH

Ex-vivo cytotoxic, antibacterial and DPPH free radical scavenging assay with ethanolic leaf extract of *Glycosmis pentaphylla* to justify its traditional use

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Abstract— Aim: *Glycosmis pentaphylla* belongs to the family Rutaceae. It is a shrub and locally common in the treatment of hepatic impairment. We have designed this study to provide a scientific basis with the traditional use of leaf of *G. pentaphylla* in the treatment of hepatitis. Methods: The well-established DPPH free radical scavenging activity was tested for antioxidant property evaluation. On the other hand, disk diffusion and brine shrimp method was respectivelyused to determine antibacterial and cytotoxic activity. Results & Discussion: In the evaluation of antioxidant property IC_{50} found 204.91± 2.223µg/ml, in cytotoxicity testing, it is found that the plant part shows 30.49 ± 1.976 µg/ml of LC_{50}. The ethanolic extract of *G. pentaphylla* leaves also have efficiency in bacterial growth inhibition; this extract is effective against for both gram, negative and positive. The zone of inhibition at 500 µg/ml dose in *E. coli* and *C. albican* culture was 18 mm and 15 mm, respectively. In thin layer chromatography analysis, we found presence of couple of non-polar and polar component, presence of three non-chromatophoric component are also evident. Conclusion: Appropriate isolation and identification of mechanism is suggested in further study.

Keywords— Antimicrobial; Antioxidant; Cytotoxic; G. pentaphylla; TLC.

Introduction

From the ancient era, it is human's nature to find cure in the herb source. This practice is still popular among people on all continents, and most of them have their own enriched prehistory. There is evidence that plants are still widely used in ethnomedicine around the world. There is around 250,000 to 500,000 species of plants on Earth (Borris, 1996). Only a small fraction of them most likely 1-10% of them are used as food by both humans and other animals. Therefore, there is huge possibility to use plants in medical practice and remedy purposes (Moerman, 1996).

An antibacterial agent that either kills microorganism orsuppresses its growth is often termed as antibiotic. The termantibiotic covers a broad range of agents like antimicrobials, including antifungal and other compounds (Dorland, 2010). Waksmanin first used antibiotic in 1942; heusedit to describe any substance that intersect the replicationor kills microorganisms (Waksman, 1947). With theapplication of modern science, most of today's antibioticsare either structural modification or use of optical isomerism of the 1st generation antibiotics that used to be natural com-

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pounds, for example, Penicillin, Cephalosporin, Sulfonamide, Quinolone, and so forth (von Nussbaum et al., 2006). Plant chemicals that are supposed to be responsiblefor antibacterial effects, likely to have phenolic ring, alkaloid, tannins. For example, common herbs thyme and tarragon possess effective antibacterial, antifungal, and antiviral activities, containing caffeicacid in phytochemical list (Brantner et al., 1996; Duke, 1985; Mason and Bruce P, 1987; Thomson, 1978). The mechanisms areyet not clear but might be due to phenolictoxicity to microorganisms that inhibit enzymesby the oxidation, possibly through reaction with sulfhydrylgroups or through other nonspecific interaction with theproteins (Ya C., 1988).

The liver is a highly sensitive organ, which plays a major role in maintenance and performance of thehomeostasis in our body. It is the major organ where processes like metabolism and detoxification takes place. Therefore, there isa chanceof injury because of chronic exposure to drugs, environmental toxicants and otherxenobiotics (Amacher, 2002). Liver disorders are one of the serious health issue, at present time. Ethanol is a lipid-soluble non-electrolyte and is readily absorbed from the skin and gastrointestinaltract.It quickly diffuses to the circulatory system, dispersed evenly through out the body (McDonough, 2003). Ethanolis metabolized in the liver and person who consume regularly and get addicted to alcohol (drinks 4 to 5 per day) are at risk of chronic liver diseases (Zakhari and Li, 2007). Moreover, both acute and chronicin take of ethanol produces cytokines in large amounts, particularly TNF-α by hepatic κ-cells, which plays a major role in causing liver injury (Thurman, 1998; Tsukamoto et al., 2001; Zhou et al., 2003). These things results into accumulation of hepatic lipids also the lipid peroxides and lead to auto-oxidation of hepatic cells either by acting asa pro-oxidant or by decreasing the antioxidant levels, thereby resulting in a remarkable hepatotoxicity. Lipid peroxidation by ethanol induces hepatic oxidative stress, which identified as a reason to play a pathogenic role in Alcoholic Liver Disease (ALD) (Bunout, 1999). There is evidence that almost 5% of oxygen, from total oxygen consumed, converts into oxygen derived free radicles (Halliwell, 1988; Yu, 1994). Meanwhile, those free radicals are known as reactive oxygen species or ROS (e.g., O_2^-, H_2O_2, OH^-), that are formed in body as a byproduct of different metabolism process and from exogenous sources. ROS molecules produces a stressed condition in human body that causes each cell to face about 10000 hits per second (Lata, 2003). If the generation of ROS exceeds the antioxidative defense of body, cells become saturated. Then the free radicals targets macromolecule (like lipid, protein, carbohydrate) of human body and different disease condition appears (Byung et al., 1992; Campbell and Abdulla, 1995; Cotran, 1999). Free radicals are responsible for pathogenic condition of degenerative disease like Alzheimer's, they also involved in consequence of diabetes, cardiovascular disease, nephrotoxicity, neurotoxicity and so far (Marx, 1987). Many plants contains molecules like vitamin C and E, flavonoids, carotenoids, phenolic content etc. that have ability to prevent oxidation and remove excess free radicals from body (Pratt, 1992).

Glycosmis pentaphylla is an evergreen shrub or small tree that reaches up to 5 m. The branches are hairless, unarmed, young parts, finely rusty and puberulent. Leaves are alternate, pinnate with an unpaired terminal leaflet. The plant is locally known as Motali. The whole plant has medicinal value and used locally as an anti-pyretic and anti-diarrheal agent. Particularly, its leaves extract are important in the treatment and recovery from Hepatitis. This folkloric use of this plant makes us interested to carry out thepresent evaluation with this plant.

Material and Methods

Collection and identification of plant

The plant part was collected from Madhupur of Tangail forest region of Bangladesh, in between April-May 2013. Taxonomist of National Herbarium Bangladesh, Dhaka, identified the plant and an accession number was submitted (35483).

Extraction

Extract was prepared from leaf part of the collected plant by usingorganic solvent (Ghani, 2005). The fresh leaves of *Glycosmis pentaphylla* were pieced; washed and air-dried at room temperature (24 ± 2^0C) for about 10 days. Dried leaves was milled into coarsepowder. Coarse powder, weighing about 200 grams, takenin a bottle and dissolved in ethanol. Then the mixture kept for 2 days with uninterrupted shaking. The extract was collected using Buckner funnel, where theethanolic mix of the powder was poured under vacuumsuction. The filtrate contained the crude drug extract ofethanol. The ethanol was evaporated and a concentrated crude drug extract of *Glycosmis pentaphylla*

leaves was obtained, which wasweighed to be 29 grams and was preserved into alpine tubefor further use at 4^{0}C. The percent yield was 14.5%.

Antioxidant assay

DPPH scavenging assay: The DPPH scavenging activity of *G. pentaphylla* was measured according to the method of Liu and Zhao (2006) (Liu and Zhao, 2006). The reaction mixture contained 2 ml of 95% ethanol, 0.1 M DPPH and 2 ml of the ethanolic leaf extract of *G. pentaphylla* (50, 75, 100, 200, 300 μg/ml). The solution was incubated at 25ºC for 15 min, and the absorbance of *G. pentaphylla* was determined at 517 nm. The antioxidant activity of *G. pentaphylla* extract was evaluated according to the following formula:

Scavenging rate (%) = [1-A]/ A_0 X 100

Where A is absorbance of *G. pentaphylla* extract and A_0 is the absorbance of negative control (DPPH solution). Ascorbic acid used in this method as positive control, to compare the effectiveness.

Cytotoxic assay

In vitro Brine shrimp lethality bioassay (Rahman and Rashid, 2008) technique applied,using nauplii of *Artemia salina*, for the determinationof general toxic property of *G. pentaphylla*. In this method *Vincristinsulphate* was used as a positive control, for the comparison. Eggs kept in a smalltank containing 3.8% NaCl solutionfor hatching, a light source was attached to that tank, we hatched eggs for 2 days and then it is ready for experiment. Four milligrams of the extract wasdissolved in DMSO to geta concentration of varying concentrations 100, 50, 25, 12.50 and 6.25 μg/ml. 10 brine shrimp nauplii were then placed in eachvial and allowed to stand for 24 hour. The vials were observed using a magnifying glassand the number of survivors in each vial werecounted and noted. From these data, the percentage of mortality of the nauplii was calculated for eachconcentration and the 50% lethal concentration (LC_{50}) values were determined.

Antimicrobial property investigation

Antimicrobial Activity: Stock solution was prepared by dissolving 10 mg of the ethanolic crude drug extract in ethanol. The disk for drug dissolving was prepared using sterilized filter paper. Papers were punched uniformly to exactly 6mm in diameter. Sample solutions of desired concentrations (100, 200, 400 and 500 μg/disk) were applied with the help of the micropipette in an aseptic condition. These disks were left for a few minutes in aseptic condition for complete evaporation of the solvent. In this study, commercially prepared Kanamycin disk, K-30 disks containing 30 μg/disk, was used as a standard for comparison purpose. The in vitro disk diffusion assay (Perez C, *et al*., 1990), of antibacterial screening was used to determine the susceptibility of the pathogenic microorganisms to the test compound applied.

Preparation of fresh culture of the pathogenic organisms: The nutrient agar medium was prepared and dispersed in a number of test tubes to prepare slants (5 ml in each test tube). This was done to prepare (Axenic) cultures from the supplied cultures (Madigan M and Martinko J, 2005). The test tubes were sterilized at 121^{0}C temperature and a pressure of 15lbs/sq inch for 15 minutes. After sterilization, they were kept in an inclined position, for solidification, and then was incubated at 37.5^{0}C. The test organisms were transferred to the agar slants from the supplied cultures with the help of an inoculating loop in aseptic condition. The culture was kept at 4^{0}C or less for bacterial growth for 12 hours. Then incubated at 37^{0}C for 24 hours to assure the growth of test organisms. These fresh (Axenic) cultures were then used for the sensitivity test.

The test plates were prepared for the disc diffusion test of the test samples. Bacterial suspensions were transferred to the sterile petri dishes in an aseptic area. The petri dishes were rotated several times, first clockwise and then anticlockwise to assure homogenous distribution of the test organisms. The media was poured into petri dishes in such a way, in order to give a uniform depth of approximately 4mm.

Finally, the medium was cooled to room temperature in laminar airflow unit and it was kept in refrigerator at (4^{0}C) and the sample impregnated discs and standard disc were seeded, in the sub-solidified medium. The medium was congealed to room temperature in laminar airflow unit, then refrigerate at (4^{0}C) for 24 hours in order to provide sufficient time to diffuse the antibiotics into the medium. Hence, the zones of inhibition of different samples were compared (Brown and Kothari, 1975).

TLC analysis of the fraction

Extracts were checked by thin layer chromatography (TLC) on analytical plates over silical gel. The solvent systems used was H-EA= 2:1 where, H= hexane, EA = ethyl acetate. In this case, the spots were visualized by exposure of the plates to UV lamp. Different bands were observed and corresponding Rf values are de-

termined. Rf value of each spot was calculated, R_f = (*Distance traveled by solute/Distance traveled by solvent*)

Statistical Analysis

The statistical analysis was performed using Graph-Pad Prism-6 software. Values are represented in tabular sheet as mean ± SD and ANOVA was performed for anti-microbial assay. The significant limit for that particular case was set $p<0.05$.

RESULTS

Antioxidant assay

DPPH is a relatively stable free radical and the assay determines the ability of ethanolic extract of *G. pentaphylla*to reduce DPPH free radicals to the corresponding hydrazine by converting the unpaired electrons to paired ones. Antioxidant can act by converting the unpaired electron to paired one. The dose dependent inhibition of DPPH radicals (**Fig. 1**) indicates that selected extract causes reduction of DPPH radical in a stoichiometric manner (Murray, 1999; Sanchez-Moreno, 2002; Vani et al., 1997); with the inhibitory concentration (IC_{50}) 204.91 ± 2.223 µg/ml; where the comparable standard have 56.182 ± 2.016 µg/ml of IC_{50}value (**Table 1**). From this point of view, it is clear that the extract have moderate antioxidative capacity, through which it can yet reduce the exacerbation free radicals.

Antimicrobial assay

The antimicrobial activity of the ethanolic extract ofleaves of *G. pentaphylla* was measured by disc diffusion method. Different concentrations of 100 µg/disk, 200 µg/disk, 400 µg/disk, and 500 µg/disk were measured and compared with the zone of inhibitions, which was produced by the standard. The zones of inhibition were seen against selective bacteria at a particular concentration (**Table 2**). The studied ethanolic extract of leaves of plant *G. pentaphylla* showed higher activity against *E. coli*. At higher concentrations of 400 µg/disc and 500 µg/disc, the extract also showed goodinhibitions against other studied microorganism. However, the extract showed negligible or no activity against *S. dysenteriae*, which is a gram-negative bacteria.

Cytotoxic assay

In cytotoxic test activity, percent of mortality increased gradually with the increase in concentration of the test samples. LC_{50} values obtained from the best-fitline slope (**Fig. 3**) were 30.49± 1.976 µg/ml and 24.879 ± 2.413 µg/ml for *G. pentaphylla* and vincristine sulphate, respectively.

The brine shrimp lethality bioassay is very useful toassess the bioactivity of the plant extracts, which inmost cases correlates reasonably well with cytotoxicand anti-tumor properties (McLaughlin et al., 1993). LC_{50} values of *G. pentaphylla* revealed its considerable cytotoxic potency. Sufficient amount of phenolics and flavonoidsmay be present and it might be responsible for its promising cytotoxic activity (Moreira et al., 2007; Okwori, 2007) and the possible mechanism of cytotoxicityagainst brine shrimp nauplii due to poisonous effecton cell mitosis.

TLC assay for compound detection

Observation of the TLC plates under UV lamp results following (**Table 3**). Four non-polar compounds were present with R_f values of 0.12, 0.15 and 0.23 and 0.33. Two compounds are in between polar and non-polar with R_f value of 0.44 and 0.53. Two polar compounds were present with R_f values of 0.63 and 0.70. A fluorescent compound with an R_f value of 0.81 could also be detected. Three non-chromatophoric compounds with R_f values of 0.04 (nonpolar) and 0.23 (nonpolar) and 0.64 (partially polar or polar). Thus, many compounds were present and isolation of pure compound is necessary.

Table 1. Absorbance recorded at different concentration of ethanolic extract of *G. pentaphylla* and ascorbic acid.

Group	Concentration (µg/ml)	Absorbance	IC_{50}
Ascorbic acid	50	0.445 ± 0.008	56.182 ± 2.016 µg/ml
	75	0.374± 0.012	
	100	0.298± 0.005	
	200	0.265± 0.009	
	300	0.235± 0.005	
G. pentaphylla	50	0.688±0.011	204.91± 2.223µg/ml
	75	0.623± 0.006	
	100	0.578± 0.010	
	200	0.542± 0.008	
	300	0.522± 0.006	

Absorbance represented here as mean ± SD, the sample size was 3.

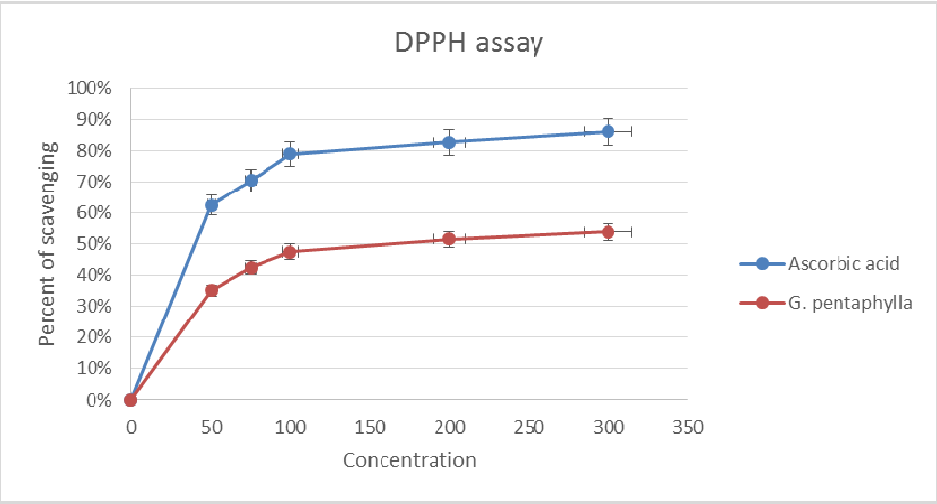

Figure 1. Antioxidant property evaluation of ethanolic extract of *G. pentaphylla*; from the graphical representation it is clear that our plant extract shows dose dependent reduction of free radicals.

Discussion

The extractive preliminary phytochemical analysis that was performed earlier, results the presence of alkaloid, flavonoid, steroid, saponin etc. (Ansari P, *et al*., 2015) Flavonoids have the hepatoprotective reputation as anti-oxidant phytoagent (Fauré et al., 1990). Tannins (Hong et al., 1995) and polyphenols (Toda et al., 1991) are also reported as they have significant antioxidant properties. Accordingly, these compounds have shown to have antioxidant activity (Dong, 2003; Leung, 2000). Total phenolic constitutes are one of the major groups responsible for primary antioxidant or free radical termination, detected in the herbal preparation. Flavonoids are the most widespreadgroup of natural compounds and probably the most important natural phenolics. The medicinal effects of plants are often attributed to the antioxidant activity of phytochemical constituents mainly phenolics, flavonoids and flavonols (Miliauskas et al., 2004). It is claimed that the phenolic compounds are powerful chain breaking antioxidants (Shahidi et al., 1992). Herbal preparation revealed well effects in DPPH scavenging in this present study.

Table 2. Tabulation of zone of inhibition from agar media bacterial culture.

Group	Concentration (µg/disk)	Microbial culture with zone of inhibition (mm)				
		E. coli	*S. aureus*	*S. dysenteriae*	*S. typhi*	*C. albican*
G. pentaphylla	100	10.33 ± 1.528	7.67 ± 0.577	0	7.33 ± 1.155	10± 2.000
	200	14± 2.646	11 ± 1.732	0	10±1.414	10.50 ± 1.291
	400	16.33 ± 1.528	12.33 ± 1.528	2± 1.000	13± 1.000	15.33 ± 1.528
	500	17.67 ± 3.786	15.67 ± 1.155	3.5± 1.291	16.25 ± 1.258	15.50 ± 0.577
Kanamycin	30	30.33 ± 3.512	33.33 ± 3.055	24.67 ± 2.082	26± 4.000	29± 3.606

Data represented here as mean ± SD, the significant limit was found p<0.001 when compared to control, sample size was 4 and on-way ANOVA was performed to estimate the significance limit.

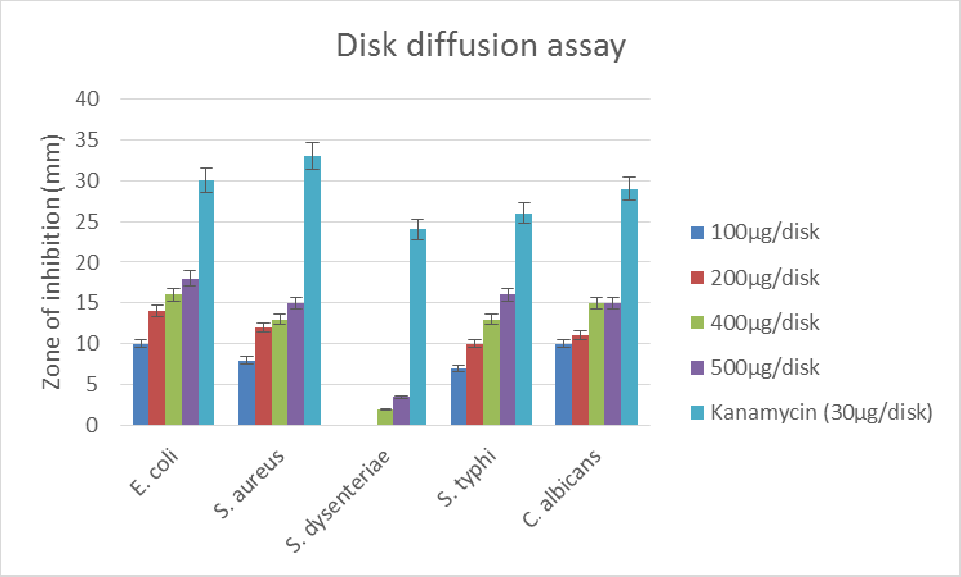

Figure 2. Schematic presentation of bacterial growth inhibition; crude extract revealed its potent effectiveness at 500 µg/disk concentration; there was two gram-positive and three gram-negative microbes used, studied extract found similar effective for both class of organism.

The crude extracts of plants are pharmacologically potent may bedue to presence of various components in the whole extract, that are claimed to possess antioxidant activity by several investigator (Hamburger and Hostettmann, 1991).

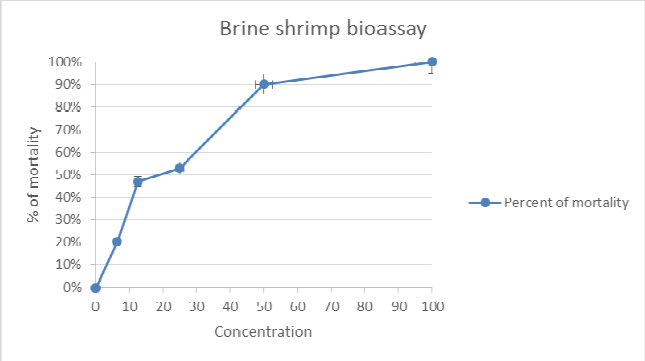

Figure 3. Graphical representation of brine shrimp lethality bioassay; with the increase of extract concentration percentage of mortality increases, the test was performed three times and the data presented in the graph, is the mean.

In the present study, we evaluated the antibacterial activity of the ethanolic crude extracts of *G. pentaphylla*. The study of antimicrobial activity was carried out against *E. coli, S. aureus, S. dysenteriae, S. typhi* and *C. albican*. The results are showed in **Table 2**. In this study, crude extract of *G. pentaphylla* leaves have more potent antimicrobial activity against gram positive thangram-negative bacteria.The antibacterial activity-demonstrated by ethanolic extract of *G. pentaphylla* may be due to presence of flavonoids. Many crude extracted from plants by several research groups have a history of use in folk medicine, as antibacterial agent. Most of the time it is reported that the flavonoid rich plant extracts possess better activity. Flavonoid enriched species of *Hypericum* (Dall'Agnol et al., 2003), *Capsella* and *Chromolaena* (El-Abyad et al., 1989) have been reported to have antibacterial activity. Many other phytochemical preparations with high flavonoid content have also been reported to exhibit antibacterial activity (Al-Saleh et al., 1997; Aladesanmi et al., 1986; Mahmoud et al., 1989; Quarenghi, 2000; Rauha et al., 2000; Singh and Nath, 1999; Tarle and Dvorzak, 1990; Tereschuk et al., 1997; Torrenegra et al., 1989), and so forth. From phytochemical analysis, reported earlier (Ansari et al., 2015), it is clear that the antimicrobial activity possessed by our plant extract may be due to presence of flavonoid content.

Based on the present study, the brine shrimp lethality of the crude extract was found to be concentration-dependent. The observed lethality plant extracts against brine shrimp indicates the presence of potent cytotoxic and probably antitumor components of the plant. According to Meyer *et al*. (1982) (Meyer et al., 1982), crude plant extract is toxic if the LC_{50} value isbelow 1000 µg/ml, but the plant extract is non-toxic if LC_{50} is higher than 1000 µg/ml. The LC_{50} value we obtained from this study was 30.49 ± 1.976 µg/ml, which means it is more potent according to Meyer *et al.* and probably containing active anti-tumor constituents.

Table 3. Observed R_f values under UV lamp.

Color of spot	R_f
Light violet	0.12
Light violet	0.15
Deep violet	0.23
Yellow	0.33
Yellow brown	0.44
Light yellow	0.53
Yellow	0.63
Yellow brown	0.70
Flurscence	0.81

CONCLUSION

This work has demonstrated that the ethanolic extracts of *G. pentaphylla* leaves possesses different pharmacological property. This plant extracts contains several active constituents. The antioxidant, cytotoxic and antimicrobial potentiality is the result or evidence of their presence. However, this plant has been used in traditional medicine for many years, our present study reportalso support the traditional use of the plant in infectious and inflammatory disorders. Further study need to be carried onto under stand the exact mechanisms of suchactions and to isolate the active principles responsible for the observed activity.

Competing interests

The authors declare that they have no competing interests.

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