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Biological activities of *Sarcanthus pauciflorus*

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ABSTRACT

Sarcanthus pauciflorus is a pendulous epiphytic orchid belonging to the family Orchidaceae. The present study was conducted to determine antimicrobial, antioxidant, anthelmintic and insecticidal efficacy of methanol extract of *S. pauciflorus*. Antimicrobial activity of methanol extract was determined against four bacteria and two fungi by Agar well diffusion method. Antioxidant activity was performed by DPPH free radical scavenging and Ferric reducing assay. Anthelmintic activity was studied on the basis of time taken for paralysis and death of adult Indian earthworms by the extract. Insecticidal activity, in terms of larvicidal effect, was evaluated using II instar larvae of *Aedes aegypti*. Total phenolic content of extract was estimated by Folin-Ciocalteau reagent assay. Phytoconstituents *viz*., tannins, saponins and glycosides were detected in methanol extract. Content of total phenolics was found to be 258.65 mg GAE/g of extract. All test bacteria and fungi were susceptible to extract of orchid. *Bacillus subtilis* and *Cryptococcus neoformans* were susceptible to high extent among bacteria and fungi respectively. Gram positive bacteria have shown greater susceptibility than Gram negative bacteria to extract. The extract exhibited marked dose dependent scavenging of DPPH free radicals. An increase in absorbance at 700nm revealed reducing power of the extract. The extract caused paralysis and death of adult Indian earthworms in a dose dependent manner. The lethal effect of extract on II instar larvae of *Aedes aegypti* was found to be dose dependent. The results of the present study shows that the methanol extract of *S. pauciflorus* is found to possess antimicrobial, antioxidant, anthelmintic and insecticidal activities which might be attributed to the presence of secondary metabolites. Further experimentations concerned with isolation of the bioactive components present in the orchid and determination of their biological activities are under progress.

INTRODUCTION

Orchidaceae (Orchid family) is the second largest family of flowering plants with approximately 20,000 species belonging to >850 genera. This diversity increases towards the tropic; where the epiphytic species predominate that almost constitute 73% of the family. In India, there are nearly 1600 species which constitute about 9% of the total flora. Historically, utilization of orchids by humans probably started with their use for medicinal purposes. Traditional systems of medicine like Ayurveda, Siddha, Yunani, Homeopathy, and Traditional Chinese Medicine. In India, orchids have been used in indigenous medicinal systems since Vedic period. Ashtawarga, a group of 8 drugs in ayurvedic system, which are used for preparation of tonics such as Chyavanprash, includes 4 orchid species. They were also used for treating rheumatism, malaria, tuberculosis, cuts, wounds, burn injuries, asthma, bronchitis and several other ailments. Being rich in alkaloids, flavonoids, glycosides, carbohydrates and other phytochemicals, orchids have high therapeutic value and are being extensively used in local systems of medicine to treat a variety of diseases or disorders (Gutiérrez, 2010; Kant et al., 2012; Basker et al., 2012).

The Western Ghats of peninsular India are repositories of orchids at various altitudes. Of about 275 taxa from the Western Ghats, at a rough estimate, about 100 are endemics and many may be endangered. Sringeri of Chikmagalur and Agumbe of Shimoga Districts of Karnataka in peninsular India are very rich in Orchid flora (Geetha, 2000). Sarcanthus pauciflorus belongs to the family Orchidaceae and is a pendulous epiphytic orchid. Stem slender, pendulous, leafy, 10-12 inches long; leaves linear, straight or falcately curved, obtusely acuminate, narrowed at the base, 2.5-6 inches long, 2-3 in wide; sheaths ribbed; racemes shorter than the leaves; flowers yellow with red margins, lip white or yellowish, the sides lobes often purple, sepals elliptic, obtuse, 16 inches long,

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petals smaller, speathulate, side lobes of lip small, acute, midlobe incurved, spurconical, subacute, dilated above; flowering May to August (Gamble, 1993; Geetha, 2000). In the present study, we have determined antimicrobial, antioxidant, anthelmintic and insecticidal activity of methanol extract of *S. pauciflorus*.

MATERIALS AND METHODS

Collection, extraction and phytochemical analysis of orchid

The orchid *S. pauciflorus* was collected from outskirts of Shivamogga and authenticated in the department for future reference. For extraction, about 50g of the dried and powdered orchid material was taken and added to 100ml of methanol. The mixture was shaken for 30 minutes and left at room temperature overnight. The extract was filtered through Whatman No 1 and the filtrate was concentrated under reduced pressure to pasty mass (Yilmaz *et al.*, 2004). The methanol extract was subjected to phytochemical tests to screen the presence of various secondary metabolites namely alkaloids, saponins, flavonoids, glycosides, tannins, steroids and terpenoids (Kekuda *et al.*, 2012a).

Total phenolic content of extract

The total phenolic content of extract was determined by Folin-Ciocalteau reagent (FCR) method employed by Junaid *et al.*, (2013) with minor modifications. A dilute concentration of extract (0.5 ml) was mixed with 0.5 ml of FC reagent (1:1) and 2 ml of sodium carbonate (7%). The reaction mixtures were allowed to stand for 30 minutes and the optical density was measured colorimetrically at 765nm. A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000 µg/ml) and the TPC of extracts was expressed as µg Gallic acid equivalents (GAE) from the graph.

Antibacterial activity of extract

Antibacterial activity of extract was tested against *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* by Agar well diffusion method (Kekuda *et al.*, 2012b). The test bacteria were inoculated into sterile Nutrient broth (HiMedia, Mumbai) tubes and incubated overnight at 37^{o}C. The broth cultures of test bacteria were aseptically swabbed on sterile Nutrient agar (HiMedia, Mumbai) plates using sterile cotton swabs. Using sterile cork borer, wells of 6mm diameter were punched in the inoculated plates and 0.1 ml of extract (50mg/ml of 25% DMSO), standard (Streptomycin, 1mg/ml) and DMSO (25%) were transferred into respectively labeled wells. The plates were incubated at 37^{o}C aerobically for 24 hours and the zone of inhibition formed around the wells were measured. The experiment was repeated twice and the average value was recorded.

Antifungal activity of extract

Antifungal activity of extract was tested against two fungi viz., *Candida albicans* and *Cryptococcus neoformans* by Agar well diffusion method (Kekuda *et al.*, 2012b). The test fungi were inoculated into sterile Sabouraud dextrose broth (HiMedia, Mumbai) tubes and incubated for 48 hours at 37^{o}C. The broth cultures of test fungi were aseptically swabbed on sterile Sabouraud dextrose agar plates using sterile cotton swabs. Using a sterilized cork borer, wells of 6mm diameter were made in the inoculated plates and 0.1 ml of extract (50mg/ml of 25% DMSO), standard (Fluconazole, 1mg/ml) and DMSO (25%) were added into respectively labeled wells. The plates were incubated aerobically at 37^{o}C for 48 hours and the zone of inhibition formed around the wells was noted. The experiment was repeated two times and the average inhibition zone was recorded.

Antioxidant activity of extract

DPPH free radical scavenging assay

The radical scavenging ability of was tested on the basis of the radical scavenging effect of extract on the DPPH free radical. 1 ml of different concentrations of extract (10-400 µg/ml) was mixed with 3 ml of DPPH solution (0.004% in methanol) in separate tubes. The tubes were left at room temperature in dark for 30 minutes and the optical density was measured at 517 nm using UV-Vis spectrophotometer. The absorbance of the DPPH control was also noted. Ascorbic acid was used as reference standard. The scavenging activity of the extract/standard was calculated using the formula:

Scavenging activity (%) = [(Ao – Ae) / Ao] x 100, where Ao is absorbance of DPPH control and Ae is absorbance of DPPH and extract/standard combination **(Elmastas *et al.*, 2006)**.

Ferric reducing assay

In this assay, various concentrations of extract (10-400 µg/ml) in 1 ml of methanol were mixed in separate tubes with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The tubes were placed in water bath for 20 minutes at 50^{o}C, cooled rapidly and mixed with 2.5 mL of trichloroacetic acid (10%) and 0.5 ml of ferric chloride (0.1%). After 10 minutes, the amount of iron (II)-ferricyanide complex (Perl's Prussian blue) formed was measured at 700 nm. The increase in absorbance of the reaction mixtures indicates increased reducing power. Ascorbic acid was used as reference standard (Junaid *et al.*, 2013).

Anthelmintic activity of extract

Adult Indian earthworms (*Pheretima posthuma*) were used to assess anthelmintic effect of extract. The worms were washed using normal saline (0.85%) to remove extraneous matter. Six worms of equal size (6 cm long) were transferred into normal saline (0.9% NaCl) containing standard drug (Pierazine citrate, 1%) and different concentrations of extract (0.1, 0.5 and 1.0mg/ml). The time taken for paralysis of worms was noted when no movement was observed (except when the worms were shaken vigorously). The death time was taken when worms failed to exhibit movement on shaking vigorously or on dipping in slight hot water (50°C). Normal saline served as control (Kumar *et al.*, 2010).

Insecticidal activity of extract

The insecticidal effect in terms of larvicidal efficacy of different concentrations of extract (0.1, 0.5 and 1.0 mg/ml) was tested against II instar larvae of *Aedes aegypti* mosquito. Briefly, twenty larvae were placed in beakers containing extract. A control was kept without adding extract. The larvicidal effect of extract was determined by counting the number of dead larvae after 24 hours. Dead larvae were identified on the basis of no movement after probing with a needle in siphon or cervical region. The experiment was repeated twice and average mortality (%) was noted **(Vinayaka *et al*., 2009)**.

RESULTS AND DISCUSSION

Preliminary phytochemical analysis of methanol extract of *S. pauciflorus* showed the presence of phytoconstituents namely tannins, saponins and glycosides. Terpenoids, flavonoids, steroids and alkaloids were not detected. Content of total phenolics, as estimated by FCR method was found to be 258.65 mg GAE/g of dry extract. The inhibitory efficacy of methanol extract of *S. pauciflorus* against test bacteria and test fungi was assessed by Agar well diffusion method and the result is as shown in Table 1 and 2. Among bacteria, *B. subtilis*, was more inhibited followed by *S. aureus*, *P. aeruginosa* and *E. coli*. Overall Gram positive bacteria were more susceptible to extract and standard than Gram negative bacteria. Inhibition caused by standard was higher than that of extract. In case of fungi, highest susceptibility to extract and standard was showed by *C. neoformans*. DMSO did not exhibit any inhibition of test bacteria.

Table. 1: Antibacterial activity of extract of *S. pauciflorus.*

Test bacteria	Zone of inhibition in cm		
	Methanol extract	Streptomycin	DMSO (25%)
S. aureus	1.8	3.5	0.0
B. subtilis	2.4	3.5	0.0
E. coli	1.3	2.8	0.0
P. aeruginosa	2.0	3.0	0.0

Table. 2: Antifungal activity of extract of *S. pauciflorus.*

Test fungi	Zone of inhibition in cm		
	Methanol extract	Streptomycin	DMSO (25%)
C. albicans	1.3	3.2	0.0
C. neoformans	1.5	3.8	0.0

DPPH free radical scavenging activity of different concentrations of methanol extract of *S. pauciflorus* and Ascorbic acid (standard) is presented in Figure 1. The extract and standard exhibited marked antioxidant activity by scavenging DPPH* (free radical) and converting into DPPHH. A dose dependent radical scavenging activity was observed in this study. The scavenging activity of ascorbic acid was greater than that of methanol extract.

The result of reducing power of different concentrations of methanol extract of *S. pauciflorus* and tannic acid is represented in Figure 2. The absorbance at 700nm was found to increase with the dose of extract and standard which is suggestive of reducing power. The reducing potential of extract was lesser when compared to tannic acid.

The result of anthelmintic activity of extract and standard is shown in Table3. The anthelmintic effect was observed as loss of motility and no response to external stimuli which eventually progressed to death. The extract caused paralysis and death of worms in a dose dependent manner. Strong anthelmintic activity was shown by piperazine citrate as evidenced by lesser time taken for causing paralysis and death of worms. The worms were unaffected in normal saline (control).

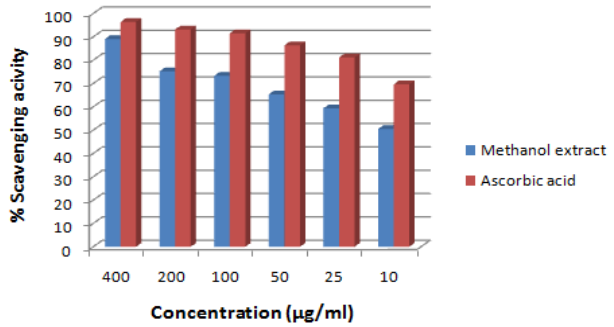

Fig. 1: DPPH free radical scavenging activity of extract of *S. pauciflorus*

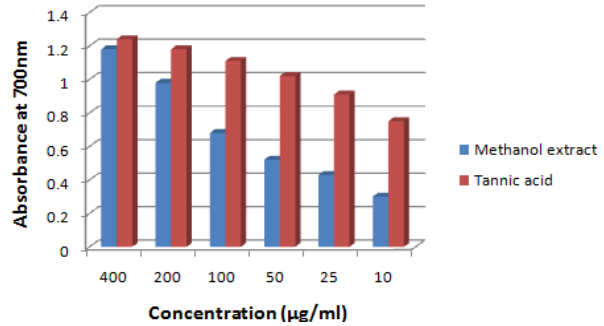

Fig. 2: Ferric reducing activity of extract of *S. pauciflorus.*

Table . 3: Anthelmintic activity of extract of *S. pauciflorus.*

Treatment	Concentration	Average time in minutes	
		Paralysis	Death
Normal saline	0.85%	-	-
DMSO	10%	-	-
Standard	1%	29	44
Methanol extract	0.1mg/ml	165	289
	0.5mg/ml	93	203
	1mg/ml	71	181

Table 4 depicts insecticidal activity, in terms of mortality (%) of II instar larvae of *A. aegypti*, by different concentrations of extract. The lethal effect of extract was found to be dose dependent and the larval mortality was 100% at concentration 0.5mg/ml and higher.

Table . 4: Insecticidal activity of extract of *S. pauciflorus.*

Treatment	Concentration	No. of dead larvae	% mortality of larvae
Control	10%	0/25	00.00
Extract	0.1 mg/ml	10/25	40.00
	0.5 mg/ml	25/25	100.00
	1.0 mg/ml	25/25	100.00

DISCUSSION

One of the most significant health-related events of modern times is the discovery and subsequent use of antibiotics.

These antibiotics have revolutionized the field of medicine and saved countless lives. However, indiscriminate use of these antibiotics resulted in the potential development of resistance in microorganisms. The problem with resistance development and other problems such as high cost and side effects resulted in an increased interest in plants and plant products as antimicrobial agents. Plants have the ability to produce a variety of metabolites. The secondary metabolites of plants have various functions like Growth regulation, inter and intra-specific interactions and defense against predators, herbivores and infection. Over 50% of all modern clinical drugs are from natural origin. Vast majority (>80%) of world's population relies on traditional medicine for their primary healthcare needs. Plants have been considered as an essential component of traditional medicine. Ayurveda and other systems of medicine have vast record of medicinal plants being used for the treatment of various types of ailments. A vast number of plants have been screened for antimicrobial activity (Cowan, 1999; Nair *et al*., 2005; Tilak *et al*., 2004; Steenkamp *et al*., 2004; Vinayaka *et al*., 2009; Duraipandiyan *et al*., 2006; Conforti *et al.*, 2008; Hemaiswarya *et al*., 2008; Davies and Davies, 2010). In the present study, we have determined antimicrobial activity of extract of *S. pauciflorus* against a panel of microorganisms that included four bacteria and two fungi. The extract has shown marked effect on the test bacteria and fungi. The inhibitory efficacy might be related to the phytochemical constituents present in extract viz., tannins, glycosides and saponins which have been shown to possess antimicrobial activity (Mandal *et al*., 2005; Akiyama *et al*., 2001; Nazemiyeh *et al.*, 2008).

Reactive oxygen species including free radicals (superoxide radical, hydroxyl radical, peroxyl radical) and no radical species (hydrogen peroxide, singlet oxygen) are produced by various means such as radiations, chemical reactions and several redox reactions. These ROS are implicated in oxidative stress which is due to imbalance in generation of ROS and antioxidant defense system of the body. These ROS contribute to protein oxidation, DNA damage and lipid peroxidation in living systems and are implicated in various pathophysiological conditions such as cancer, cardiovascular diseases, diabetes, liver cirrhosis, neurological disorders such as Parkinson's and Alzheimer's disease and others (Dasgupta and De, 2004; Choi *et al*., 2007). Cells have several antioxidant defense mechanisms that help in prevention of damaging effect produced by ROS and include antioxidant enzymes *viz*., superoxide dismutase, catalase, glutathione oxidase and small molecules such as vitamin C and vitamin E. However, in oxidative stress, there is an extra need for antioxidants from exogenous sources. Strong restrictions have been placed on the use of synthetic antioxidants such as BHT, BHA and gallates due to their doubtful safety and potential adverse effects. This led to an increasing interest in natural antioxidants. Plants have been considered as richer sources of antioxidants (Mates, 2000; Fang *et al.*, 2002; Gulcin *et al*., 2011; Da Silva and Paiva, 2012; Junaid *et al*., 2013). DPPH is one of the few, commercially available stable N-centred organic free radical and has an UV-Visible absorption maximum at 515-517nm in methanol. On accepting hydrogen from a corresponding donor, the solution of DPPH loses the characteristic deep purple color and become yellow colored Di-phenyl Picryl hydrazine. DPPH radical scavenging activity is one of the widely used assays to determine antioxidant activity of many compounds including plant extracts (Tirzitis and Bartosz, 2010; Huang *et al.,* 2005; Elmastas *et al.,* 2006; Kekuda *et al.,* 2011; Junaid *et al.,* 2013). In our study, the bleaching of the color of DPPH solution increased with increasing amount of extract in a given volume of solution. Methanol extract of *S. pauciflorus* demonstrated scavenging of free radicals. Although the scavenging abilities of extract was less when compared to ascorbic acid, it was evident that the extract showed hydrogen donating ability and therefore the extract could serve as free radical scavengers, acting possibly as primary antioxidants (Chung *et al.,* 2006).

A number of assays are designed to measure overall antioxidant activity, or reducing potential, as an indication of a host's total capacity to withstand the adverse effect of free radical stress. Reducing power reflects the electron donating capacity of bioactive compounds, is associated with antioxidant activity. The reducing capacity of a compound can be measured by the direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. Addition of free Fe^{3+} to the reduced product leads to the formation of the intense Perl's Prussian blue complex, $Fe_4[Fe(CN)_6]_3$, which has a strong absorbance at 700nm. An increase in absorbance of the reaction mixture would indicate an increase in the reducing capacity due to an increase in the formation of the complex. The ferric ion reducing antioxidant power assay takes the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of its potential antioxidant activity (Gulcin *et al.,* 2011; Meir *et al*., 1995; Chung *et al*., 2006; Kekuda *et al*., 2011). Even though the reducing ability of extract was lesser than that of reference standard, it is evident from the present study that the extract possesses reductive potential and could serve as electron donors, terminating the radical chain reactions (Chung *et al.,* 2006). In the present study, total phenol content of the orchid extract was estimated by FCR method. This method was initially intended for the analysis of proteins and later, the method was adapted to estimate total phenols in wine and plant extracts. Even though, chemical nature of FCR is undefined, the total phenolic assay by FCR method is convenient, simple and reproductive assay for studying phenolic antioxidants in plant extracts. Phenolic compounds react with FCR under basic conditions (pH~10, adjusted by Na_2CO_3). Dissociation of phenolic anion, which is capable of reducing FCR and blue color is formed (Huang *et al.,* 2005). Polyphenolic compounds are a large and diverse group of plant metabolites and are known to exhibit diverse biological activities, most of which are attributed to antioxidant activity. Phenolic compounds have strong *in vitro* and *in vivo* antioxidant activity properties and the antioxidant efficacy is associated with their ability to scavenge free radicals, break radical chain reactions and chelate metal ions. It has been reported that increased consumption of phenolic compounds is associated with a reduced risk of cardiovascular diseases and certain types of cancer. The

phenolic compounds are widespread and are distributed in fruits, vegetables and cereals (Kaisoon *et al.,* 2011). Helminthiasis, or worm infestation, is one of the most prevalent disease and one of the most serious public health problems in the world, particularly in the tropical countries. Hundreds of millions if not billions of human infections by helminthes exist worldwide and is the major cause of morbidity and is rarely fatal. They have been also found to infect livestock and crops, affecting food production with a resultant economic impact. Lack of sanitation and supply of pure water coupled with poverty and illiteracy are the factors which are responsible for this condition. Helminthiasis is common in developing countries with environmental hygiene. It contributes to the prevalence of malnutrition, anemia, eosinophilia, and pneumonia. Chemical control of helminthes coupled with improved management has been the important worm control strategy throughout the world. However, increasing problems of development of resistance in helminthes and high cost of drugs have led to the proposal of screening medicinal plants for their anthelmintic activity. In the present study, we investigated anthelmintic activity of plant extract using adult Indian earthworms due to their ready availability and anatomical and physiological resemblance to the human intestinal roundworm parasite. The standard anthelmintic piperazine citrate has been found to be more potent than plant extract. Flaccid paralysis is the major cause which results in the expulsion of the worm by peristalsis (Kumar *et al.,* 2010; Mali and Wadekar, 2008). The methanol extract of plant in the present study not only demonstrated this property but also killed the worms. Studies have shown that tannins, flavonoids and glycosides possess anthelmintic effect (Iqbal *et al.,* 2007; Silva *et al.,* 2008; Ali *et al.,* 2011). The presence of phytochemicals such as tannins and others in extract might be the reason for the observed anthelmintic effect of *S. pauciflorus*. Among arthropods, mosquitoes are able to transmit more diseases than any other group and they have been responsible for the cause of millions of people all over the world. Among all other countries India has been considered with highest incidence of mosquito borne diseases. Life threatening diseases like malaria, yellow fever, dengue fever, chikungunya ferver, filariasis, West Nile virus infection and others are the vector borne diseases in most of the tropical and subtropical countries and many other parts of the world (Ghosh *et al.,* 2012). Control over these mosquito borne diseases is essential in order to prevent their proliferation and to improve quality of environment and public health at large. Use of synthetic insecticides such as organo chlorine and organophosphate compounds are the major strategies used in mosquito control. However, the use of many of the synthetic insecticides chemicals can be equally toxic to beneficial insects as to the target species and residual nature with higher rate of biological magnification (Vinayaka *et al.,* 2009; Ghosh *et al.,* 2012). Thus the strategy is not so useful due to human, technical, operational, ecological, and economic factors. As a result of which an urge has been formed to look for alternate, eco-friendly, cost-effective, target specific agents. According to recent investigations by several authors use of natural compounds and the possible role of insecticides of botanical origin can be more effective than synthetic insecticides are based on a single active agent. Whereas plant derived insecticides comprise of a mixture of phytocompounds and there is little chance for pests to develop resistance to such botanicals. Plants have been shown to possess marked insecticidal properties and may act as suitable alternative product to fight against mosquito borne diseases (Promsiri *et al.,* 2006; Vinayaka *et al.,* 2009; Ghosh *et al.,* 2012). In this study, we have assessed larvicidal effect of methanol extract against second instar larvae of *Aedes aegypti* which is an important vector of arboviruses such as dengue fever, urban yellow fever and chikungunya. Life cycle of mosquito has four stages *viz*., egg, four larval instars, pupa and adult. The mosquito is fundamentally aquatic and it reaches the terrestrial environment only as an adult (Promsiri *et al.,* 2006; Farnesi *et al.,* 2012). The plant extract of *S. pauciflorus* has shown marked larvicidal effect. It is observed that the carbohydrates, saponins, phytosterols, phenols, flavonoids and tannins are having mosquito larvicidal activity. Prenylated xanthones, tetracyclic phenols and saponins are reported to be effective in controlling mosquito *A. aegypti* (Khanna *et al.,* 2007; Marston *et al.,* 1993). The larvicidal activity of plant extract could be possibly due to the presence of secondary metabolites such as tannins, flavonoids and glycosides.

CONCLUSION

In the present study, the methanol extract of *S. pauciflorus* is shown to exhibit antimicrobial, antioxidant, anthelmintic and insecticidal activity. The observed biological activities might be attributed to the secondary metabolites contained in the extract. Further studies are under progress to recover the active principles present in the orchid and to determine the biological activities of isolated phytochemicals.

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