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Research Article

STUDIES ON *IN VITRO* ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF *SPHAERANTHUS INDICUS* (LINN)

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ABSTRACT

The present investigation was carried out for the evaluation of antioxidant and anti bacterial activities of leaf, flower, stem, root methnolic extracts of Sphaeranthus *indicus*. Antioxidant activity was conducted using various methods viz., DPPH, Hydroxyl radical, Nitric oxide, Superoxide anion and Hydrogen peroxide. Among the extracts tested flower and leaf extracts were exhibited highest scavenging activity at 150 µg/ml concentrations. Whereas, antibacterial activity was tested with human pathogenic species viz., Staphylococcus *aureus*, Bacillus *subtilis*, Bacillus *cereus*, Klebsiella *pneumonia*, Escherichia *coli*, Salmonella *paratyphi.* All the extracts tested produced minimum zones of inhibitions against all the species tested. The highest zone was exhibited by the flower extract of 8 against Staphylococcus *aureus*. The total phenolic content and flavanoid assay was carried to understand better relation with antioxidant activities of plant extracts.

KEY WORDS

Sphaeranthus *indicus*, Human pathogenic species, Antioxidant activity, Antibacterial activity.

INTRODUCTION

Herbal industry and naturally derived products has been rapidly become immense popular over the past three decades. [1, 2].The traditional medical systems particularly, of Asian origin have been attained highest recognition value for the identification and screening of several medicinal plants which are off efficient in therapeutic properties [3]. In recent years, pharmaceutical industries are scheduled to invest lots of amount for research and development programs for the discovery of natural drugs that have been attributed to possess antimicrobial, antioxidant, anticancer properties etc. India, with wide variety of climatic and geographical conditions has been bestowed with rich biodiversity in all the levels viz., species diversity, genetic diversity and habitat diversity and as well as rich in the traditional medicine. According to World Health Organization, 21,000 species have been reported with medicinal importance around the world. Among these, the Central Drug Research Institute (CDRI), Lucknow, India alone has been screened approximately, 3,800 plant species for the identification of bioactive compounds and their biological activities [4].

One of such medicinal plant is *Sphaeranthus indicus* belongs to family *Asteraceae*. It is commonly known as *Gorakhmundi* in Hindi, and grows approximately 15-30 cm in height distributed widely throughout the wetlands in India, Sri Lanka and Australia. The extracts of various parts of this plant are practiced as folk medicine due to its wide range functions viz., treating hemicranias, mental illnesses and epileptic convulsions, vitiated conditions of leprosy, hepatopathy, pectoralgia, diabetes, fever, jaundice, gastropathy, hernia, cough, hemorrhoids,

dyspepsia helminthiasis,skin diseases and also used as a nervine tonic. It also treats piles and hepatitis [5-7]. Reportedly, the essential oils, of this plant were obtained by steam distillation, contains wide range of phytochemicals viz., a-citral, methyl-chavicol, p-methoxycinnamaldehyde, a-terpinene, β-ionone, geraniol, a-ionone, ocimene, d-cadinene and an alkaloid sphaeranthine. The solvent extraction of powdered caputula reported to contains β-sitosterol, stigmasterol, sesquiterpene lactone, hentriacontane, sesquiterpine glycoside, sphaeranthanolide, isoflavone glycosides and flavones [8-13].

With the context to the above cited literature, the present investigation was carried out to evaluate the antioxidant and antibacterial properties of leaves, flowers, stem and roots of *Sphaeranthus indicus*.

MATERIALS AND METHODS

Plant material

Leaves, Flowers, Stem and Roots of *Sphaeranthus indicus* were collected from wet lands of Kakatiya University, water filter bed, Warangal district, Andhra Pradesh, India. Plant has been authenticated by Professor V. Thirupathiah Taxonomist, Department of Biotechnology, Chaitanya Postgraduate College, Warangal. All the parts of this plant were maintained in the Departmental herbarium.

Chemicals

Dragendorff's reagent, Agar-Agar type I (Bacteriological), Ascorbic acid, tannic acid, catchien are purchased from Hi-Theme chemical laboratories, Hyderabad. Whereas, all other chemicals purchased were off research grade.

Bacterial cultures

The bacterial species which were selected for anti-bacterial assay are Staphylococcus *aureus* ATCC 96, Bacillus *subtilis* MTCC 441, B. *cereus* ATCC 9372, Klebsiella *pneumonia* MTCC 109, Escherichia *coli* ATCC 8739, Salmonella *paratyphi* ATCC 4420. All these bacterial strains were obtained from department of Microbiology, Chaitanya Post Graduate College Hanamkonda, Warangal, India.

Extraction procedure

Leaves, Flowers, Stem and Roots were made in to smaller fragments, shade dried and grinded in homogenizer in to coarse powder. The 100 g of each powdered material is extracted with methanol and concentrated under rotavapour at 40-50 °C. The extracts of leaves, flowers, stem and roots are further abbreviated as SIL-Sphaeranthus *indicus* leaf, SIF- Sphaeranthus *indicus* fruit, SIS-Sphaeranthus *indicus* stem and SIR- Sphaeranthus *indicus* root for better understanding.

Reagents preparation

Preparation of Nash Reagent: 75.0 g of ammonium acetate, 3 ml of glacial acetic acid and 2ml of acetyl acetone were mixed and distilled water was added to total volume of 1 L.

Preparation of Griess Reagent: 1% sulphanilamide, 2% Phosphoric acid and 0.1% N-1- napthylethylenediamine di hydrochloride in distilled H_2O.

Preparation of Ferrous EDTA: 0.13% ferrous ammonium sulfate and 0.26% EDTA in distilled H_2O.

Determination of total phenol content

The amount of total phenolics in methanolic extracts was determined with Folin–Ciocalteu reagent method [14]. Separately, 1ml of each methanolic extract of SIL, SIF, SIS and SIR of different concentrations (50,100 and 150 µg/ml) and standard solution of tannic acid (10, 15, 20 µg/ml) was added separately to 100 ml volumetric flask separately, that contained about 60 ml distilled water and followed by the addition of 5 ml of Folin–Ciocalteu reagent. The content was mixed thoroughly and kept constant for about 10 min. To this, add 15 ml Na_2CO_3 (20 %) and make up to 100 ml using distilled water. The mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm using a UV-visible spectrophotometer.

Total flavonoid assay

Flavonoid content was measured by aluminum chloride colorimetric assay [15]. 1 ml of each SIL, SIF, SIS and SIR methanolic extracts with different concentrations (50, 100 and 150 µg/ml) and standard solution of catechin (10, 15, 20 µg/ml) was added separately to 10 ml volumetric flask containing 4 ml of distilled water. To the above

mixture 0.3 ml of 5% $NaNO_2$ was added, followed by the addition of 0.3 ml of 10% $AlCl_3$ after 5 min. After incubation period of 6 min 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm.

Antioxidant assay

DPPH radical scavenging activity

Free radical scavenging capacity of SIL, SIF, SIS and SIR methanolic extract was determined by using DPPH as described elsewhere [16]. DPPH radical scavenging activity was done by serial dilution by taking diluted methanol (1:20) as standard. 10 ml of various diluted methanolic extracts of various concentrations (50,100 and 150 µg/ml) were added to 1 ml DPPH solution (0.004%) and incubated for 10 min at room temperature. Absorbance of test and reference standard, ascorbic acid was measured at 517 nm. The amount of DPPH scavenging was calculated by using the following formula:

% DPPH radical scavenging = [(Absorbance of control – Absorbance of test sample)/ (Absorbance of control)] × 100

Hydroxyl radical activity

Hydroxyl radical activity was measured by the method described elsewhere [17]. 1 ml of each SIL, SIF, SIS and SIR methanolic extracts of various concentrations (50,100 and 150 µg/ml) were placed in tubes and evaporated to dryness. 1 ml of ferrous- EDTA (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5ml of 0.018% EDTA, 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) and 0.5 ml of freshly prepared 0.22% ascorbic acid were added to each tube. The tubes were capped tightly and heated in a water bath at 80-90 ^{0}C for 15 min. The reaction was terminated by adding 1 ml of ice cold TCA (17.5% w/v). Latter 3 ml of Nash reagent was added to each tube and kept at room temperature for 15 min for color development. The intensity of color formed was measured at 412 nm against the reagent blank. The percentage inhibition was compared with standard and test compounds.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was determined by the method described by [18]. Briefly, 5 mM sodium nitroprusside was prepared in phosphate buffered saline and mixed with different concentrations of SIL, SIF, SIS and SIR methanolic plant extracts (50,100 and 150 µg/ml) followed by incubation at 25 ^{0}C for 30 min. A control without the extracts but with equivalent amounts of methanol was taken. After 30 min, 1.5 ml of incubated solution was pipette out and diluted with 1.5 ml of Griess reagent. The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1- napthylethylenediamine dihydrochloride was measured at 546 nm and percentage scavenging activity was measured with reference standard.

Super oxide radical scavenging activity

The super oxide radical scavenging activity was measured by the method described by [19-20]. 1ml of each SIL, SIF, SIS and SIR methanolic extracts of various concentrations (50,100 and 150 µg/ml) were mixed with 1 ml of nitro blue tetrazolium (NBT) solution (156 mM NBT in phosphate buffer of pH 7.4) and 1 ml NADH in phosphate buffer of pH 7.4. The reaction was initiated by adding 100 ml of phenazine methosulfate (PMS) solution (60 mM PMS in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 ^{0}C for 5 min and the absorbance was measured at 560 nm against blank sample and compared with standards. Decreased absorbance of reaction mixture indicates increased superoxide anion scavenging activity. The percentage of inhibition of superoxide anion generation was calculated using the following formula:

% inhibition = [(Absorbance of control – Absorbance of test sample)/ (Absorbance of control)]

× 100

Scavenging of hydrogen peroxide

Scavenging of hydrogen peroxide was measured by the method described elsewhere [21]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). 1 ml of each SIL, SIF, SIS and SIR methanolic plant extract of different concentrations (50,100 and 150 µg/ml) were added to 0.6 ml of 40 mM hydrogen peroxide solution. Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank containing phosphate buffer

without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of plant extract and standard compounds was calculated using the following formula:

% scavenged [H_2O_2] =
[(Absorbance of control – Absorbance of test sample)/ (Absorbance of Control)] × 100

Antibacterial activity:

The antibacterial activity testing of the selected cultures was carried out according to the method described by Perez.et.al (1990) [22]. Each selective medium was inoculated with the microorganism suspended in nutrient broth. Once the agar was solidified, it was punched with the wells of six millimeters diameter and was filled with 25 µL of the plant extracts and some were kept as blanks (Sterilized distilled water,). Gentamycin sulfate were used as positive control at a concentration of 10µg/ml. The dilution medium for the positive control was sterile distilled water. The plates were incubated at 35 ± 2°C for 24 hrs and the antimicrobial activity was observed and calculated.

Statistical analysis

The data from the antioxidant activity experiment are presented as mean S.E.M (n=3). Student's *t*-test was used for statistical analyses (SAS software 9.0). Values were considered statistically significant when $p<0.05$

RESULT AND DISCUSSION

Total phnolic content

All the extracts which were determined revealed to possess higher amounts of phenolic and flavanoid content. The percentage yield of phenolic and flavanoid content which were noticed was found to be 38, 59,77 and 35, 57, 75 and 26, 48, 61 and 28, 46, 58 at 50, 100, 150 µg/ml of SIL, SIF, SIS and SIR respectively. The highest yield was noticed with fruit and leaf extracts and were comparable with reference standard tannic acid 53, 67, 85 at 10, 15, 20 µg/ml respectively (**Figure 1**).

Total flavonoid assay

The percentage yield of total flavonoid content is found to be 33, 49, 66 and 30, 52, 68 and 20, 38, 54 and 18, 31, 48 at 50, 100, 150 µg/ml of SIL, SIF, SIS and SIR respectively. The highest yield was noticed with fruit and root extracts and are comparable with reference standard catechin 58, 70, 88 at 10, 15, 20 µg/ml respectively (Figure 2).

Anti-oxidant assay

DPPH radical scavenging activity

Methanol extracts of SIL, SIF, SIS and SIR were investigated for the anti-oxidant activity by using DPPH scavenging assay. The DPPH scavenging activity of all extracts is compared to the standard antioxidant, ascorbic acid 10, 15 and 25µg/ml. Among the SIL, SIF, SIS and SIR extracts the highest scavenging activity was noticed at 250 µg/ml with root extract and then followed by leaf extract (**Table 1**).

Hydroxyl radical activity

The present study reveals that the hydroxyl radical scavenging activity was significantly found with all extracts. However, highest percentage of inhibition at 250 µg/ml with root and followed by stem extract which were comparable with reference standard ascorbic acid 10, 15 and 25 µg/ml (**Table 1**).

Nitric oxide scavenging activity

Scavenging activity of nitric oxide (NO) of all extracts which were tested was found to be significant. Among all the extracts, stem and leaf extracts exhibited significant percentage of inhibition at 250µg/ml. The values are comparable with reference standard ascorbic acid 10, 15 and 25µg/ml (**Table 1**).

Super oxide radical scavenging activity

The superoxide anion radical scavenging activity of plant extract was assayed by the PMSNADH method. As shown in Table 1, the inhibition percentage of superoxide radical generation by SIL, SIF, SIS and SIR were compared to standard reference compound ascorbic acid. The highest scavenging activity was observed with fruit and stem extract at 250µg/ml which were comparable with reference standard ascorbic acid 10, 15 and 25 µg/ml (**Table 1**).

Scavenging of hydrogen peroxide

In the present study, H2O2 scavenging activity of SIL, SIF, SIS and SIR at all tested concentrations (50, 150, 250 µg/ml) exhibited notable results which were in comparison to ascorbic acid 10, 15 and 25 µg/ml as reference standard. Among all the extracts stem and root extracts are found to be high scavenging activity at 250 µg/ml which were

comparable with reference standard ascorbic acid 10, 15 and 25 µg/ml (**Table 1**).

Antibacterial activity

All the extracts which were tested were proved to be minimum significant on gram negative strains and found significant in all gram positive strains. Among the gram positive strains staphylococcus *aureus* found to be highest susceptibility towards flower extract and remaining all other extracts showed considerable activity. The zone of inhibition was noticed against staphylococcus *aureus* with flower extract is 8 at 150 µg/ml compared to the standard gentamycin 14 at µg/ml (**Table 2**).

Fig.1. Determination of total phenolic content from various parts of *Sphaeranthus indicus* at various concentrations compared with standard tannic acid.

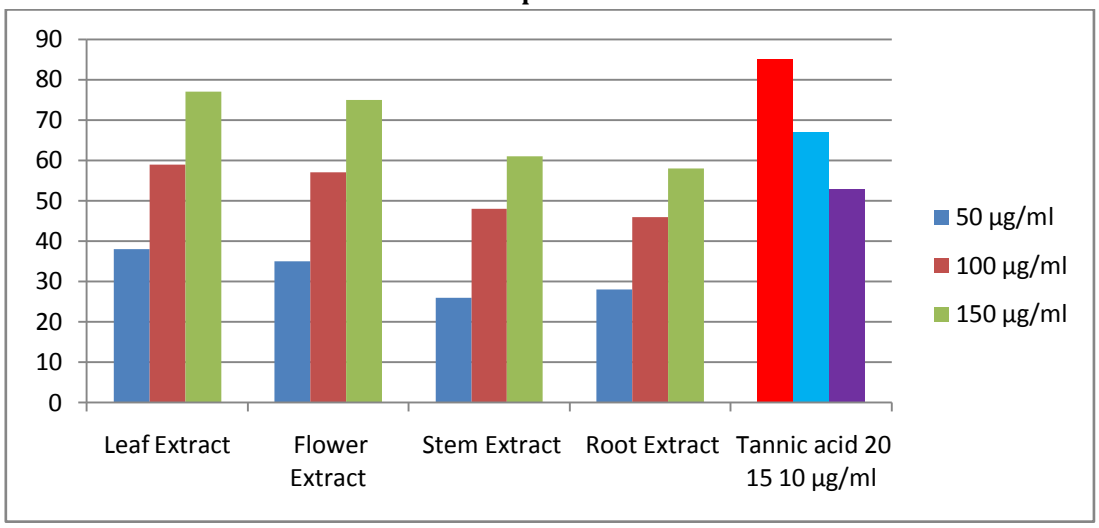

Fig.2. Determination of total flavanoid content from various parts of *Sphaeranthus indicus* at various concentrations compared with standard catchien.

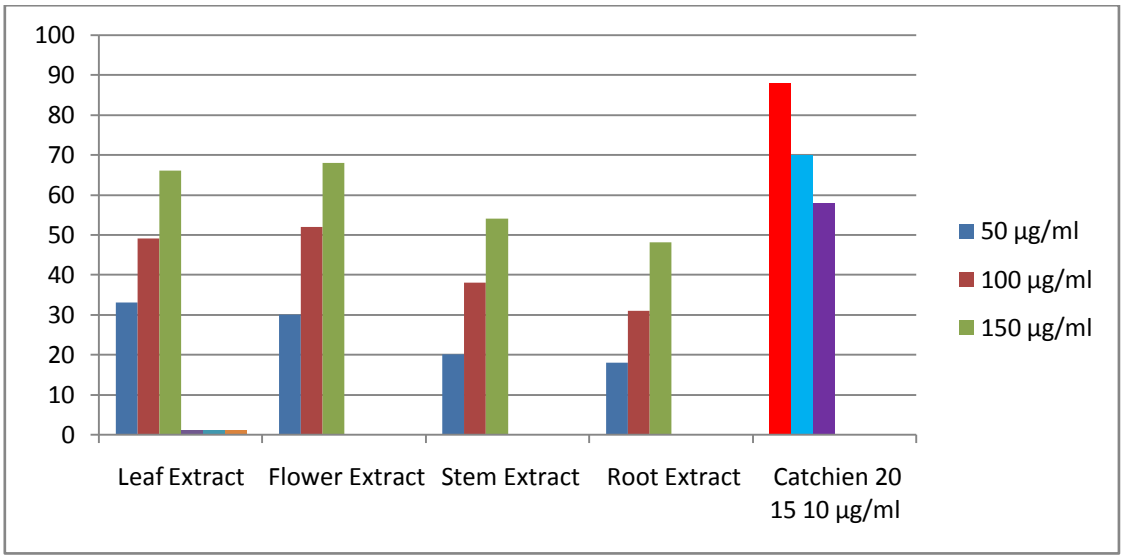

Table.1. Determination of antioxidant activity of various parts of *Sphaeranthus indicus* at various concentrations

Plant Extract	DPPH			Hydroxyl radical			Nitric oxide			Super oxide			H_2O_2		
	50	100	150	50	100	150	50	100	150	50	100	150	50	100	150
Leaf	0.2±0.8	0.2±0.11	0.3±0.17	0.2±0.11	0.3±0.20	0.3±0.38	0.2±0.17	0.3±0.4	0.3±0.27	0.2±0.11	0.2±0.19	0.2±0.28	0.3±0.20	0.4±0.20	0.5±0.2
Flower	0.2±0.18	0.2±0.14	0.3±0.24	0.5±0.29	0.2±0.15	0.3±0.17	0.1±0.17	0.2±0.9	0.3±0.5	0.4±0.23	0.2±0.14	0.3±0.17	0.3±0.2	0.3±0.17	0.4±0.2
Stem	0.1±0.16	0.3±0.5	0.3±0.11	0.2±0.11	0.4±0.26	0.3±0.17	0.2±0.12	0.1±0.08	0.2±0.18	0.2±0.1	0.2±0.14	0.2±0.17	0.5±0.3	0.6±0.3	0.2±0.14
Root	0.1±0.05	0.3±0.17	0.2±0.14	0.2±0.14	0.1±0.08	0.3±0.17	0.1±0.19	0.2±0.11	0.3±0.37	0.2±0.6	0.17±0.1	0.2±0.29	0.7±0.4	0.8±0.4	0.9±0.5

Table.2. Antibacterial activity of methanolic extracts of *Sphaeranthus indicus* compared with gentamycin sulfate. The zone of inhibition were showed in mm

Plant Extract	Leaf			Flower			Stem			Root			G*
	50*	100*	150*	50*	100*	150*	50*	100*	150*	50*	100*	150*	10*
S. *aureus*	3	4	4	4	5	8	-	-	-	2	3	3	14
B. *subtilis*	4	6	7	3	4	5	1	3	3	3	4	6	10
B. *cereus*	2	3	5	2	4	5	1	2	2	3	3	4	12
K. *pneumonia*	1	3	4	2	4	5	-	-	-	-	-	-	17
E.*coli*	3	5	6	2	3	4	2	4	5	2	3	5	12
S. *typhi*	-	-	-	-	-	-	-	-	-	-	-	-	8

G - Gentamycin Sulphate

* Concentration of plant extract and standard are µg/ml

DISCUSSION

Since, ancient times immemorial man has been become dependent on plants for his minimum nutritional requirements for comfortable living. Higher plants synthesize and accumulate extractable chemical compounds are used as raw material for various scientific and commercial utilities [23]. *Sphaeranthus indicus* possess variety of phyto chemicals with broad spectrum of medicinal activities and is scientifically exploited to use as source for universal remedies in the ayurvedic medicine [24-25]. A notable antioxidant activity was exhibited by all extracts which were tested by various radicals viz., DPPH (1, 1 - diphenyl-2- picrylhydrazyl), Hydroxyl radical, Nitric oxide, Superoxide and Hydrogen Peroxide. Among all the extracts profound scavenging activity was found with the leaves and flower extracts comparable to standard ascorbic acid.

Leaf and flower extracts reported good concentration dependent inhibition of scavenging activity by reducing the radical to the corresponding hydrazine after immediate reaction with hydrogen donors. DPPH (1,1-diphenyl-2-picrylhydrazyl) is commonly used free radical for the evaluation of scavenging activity by the plant extracts which is determined by decrease in its absorbance due to absolute change of color from purple to yellow which is directly proportional to reduction of DPPH radical in stoichometric manner [26]. However, the antioxidant activities of stem and root extracts are also comparable to standard ascorbic acid.

Depolymerisation of hyaluromic acid (polysaccharide) by the hydroxyl radical produced by the reaction of superoxide and hydrogen peroxide leads to the premature connective tissue destruction and fragmentation of DNA strand [27]. Thus it is necessary to scavenge the excess hydroxyl radical from the living systems. Among all the extracts, leaf extract revealed to possess highest scavenging activity. Whereas, the other extracts are also showed significant activity is comparable.

Hydrogen peroxide, in general known to less reactive, but however, sometimes it can generate hydroxyl radical which attains potentiality to cause cell damage. Thus, it is important to remove H_2O_2 for better protection. All the extracts exhibited good scavenging activity of hydrogen peroxide. However, leaf extract showed highest activity than other extracts.

Nitric oxide is a diffusible free radical act as an effector molecule in diverse biological systems [28]. The scavenging activity of methanolic leaf, flower, stem and root were found to be concentration dependent. Among, these extracts flower and stem exhibited greater inhibition percentage rather than others, in comparison to the known standards.

The excess production of Reactive Oxygen Species (ROS) are produced by the influence of superoxide formation involve in several metabolic and physiological processes [29-32]. The present study reports all the extracts noticed significant scavenging activity of superoxide radical at all tested concentrations of various extracts noticed significant scavenging effect ($P < .005$).

It has been reported that the infusion and soxhelt extractions of leaves of *Sphaeranthus indicus* exhibited significant antioxidant and antibacterial activities [33]. However, the present investigation results are quite different with earlier reports because studies are conducted in all parts viz., leaves, flowers, stem and roots extracted with methanol, but not restricted to only single part i.e., leaves as earlier reports. Literature survey, on antioxidant activities of this plant provides subtle information. Methanolic extracts of flower heads were reported to possess good antioxidant activity which correlated our work that showed much scavenging activity [34]. However, as mentioned above that the current work in not restricted to single part but various parts. It is also reported that the ethanol extracts of ground parts of this plant were also evaluated for antioxidant activities showed good inhibition percentage of free radicals [35]. Based on our study reports, it is confirmed that all the plant extracts noticed significant antioxidant activities and highest in the leaf and flower extract. There might be several reasons can be elucidate for the possessed activity by the plant extract. An important point can be discussed here is among all the extracts leaves and flower extracts showed highest amounts of phenolic compounds and flavanoids rather than extracts of stem and root. Phenolic compounds and flavanoids are well

known to act as effective hydrogen donors and inhibits the lipid oxidation and chelating metal ions, proving them as good antioxidants which protect us from serious disorders viz., such as strokes, heart attack and even cancer, etc [36] Thus, the leaf and flower extract established a relation with antioxidant properties.

Enormous generation of free radicals leads to oxidative stress which is potential to entangle several disorders in the living systems. The use of synthetic antioxidants is quenched to minimize hazardous effects and infer to use potential antioxidants derived naturally. In the current scenario researches are majorly focused to search and investigate novel biologically active drugs which are sustainable and don't attribute any side effects [36].

The antibacterial activity of these extracts revealed that most of extract are effective on gram positive strains compare to gram negative strains. Evidently, it has been reported that the leaves of this plant extracted with methanol, ethanol and butanol revealed that most of the gram positive strains are susceptible towards methanolic extract [33]. The present investigation results exploited that stem and root extracts showed minimum susceptibility on both gram positive and negative strains and good results were obtained with the extracts of leaves and flower. It is to state here that salmonella typhi has been found to be resistant with all the extracts tested and staphylococcus *aureus* and K. *pneumonia* were also found resistant towards stem and root extracts. It has been reported that aerial parts and flower extracts were found to be significant on various human pathogenic organisms. By pondering to previously reported articles we understand the expected reasons for high antibacterial activity associated with leaves and flower extracts are due to presence of phenolic compounds in the plant extracts in higher concentrations [37].

CONCLUSION

Based on the reports obtained in the current study it is concluded that among the methanolic extracts of leaves, flowers, stem and roots of Sphaeranthus *indicus*, leaves and flower extracts exhibited high antioxidant and antibacterial activities. Thus, it is a direct correlation to use these extracts in traditional medicine system.

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