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Research Article

PHYTOCHEMICAL, QUALITATIVE AND QUANTITATIVE DETERMINATION OF SECONDARY METABOLITES AND ANTIOXIDANT POTENTIAL OF *SPHAERANTHUS INDICUS* SEED EXTRACTS

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ABSTRACT

Plants have served human beings as a natural source for treatments and therapies from ancient times, amongst them medicinal herbs have gain attention because of its wide use and less side effects. In the recent years plant research has increased throughout the world and a huge amount of evidences have been collected to show immense potential of medicinal plants used in various traditional systems. The aim of the present study was to evaluate physicochemical, qualitative and quantitative phytochemical analysis and in vitro antioxidant activities of seed of *Sphaeranthus indicus* collected from Bhopal region of Madhya Pradesh. The physicochemical evaluations carried out in terms of moisture content and ash value. Qualitative analysis of various phytochemical constituents and quantitative analysis of total phenolics and flavonoids were determined by the well-known test protocol available in the literature. The *in vitro* antioxidant activity of petroleum ether and methanolic extract of the seeds was assessed against DPPH and superoxide radical scavenging assay method using standard protocols. Phytochemical analysis revealed the presence of carbohydrates, terpenoids, flavonoids, alkaloids, glycosides and steroids. The total phenolics content of seeds of methanolic extract was (438mg/gm), followed by flavonoids (208mg/gm). The activities of both seeds extract against DPPH and superoxide radical scavenging assay method were concentration dependent. These studies provided information for standardization and correct identification of this plant material. The diverse array of phytochemicals present in the plant thus suggests its therapeutic potentials which may be explored in drug manufacturing industry as well as in traditional medicine.

Keywords: *Sphaeranthus indicus,* Physicochemical, Qualitative, Quantitative phytochemical, Antioxidant

1. INTRODUCTION

About 75 to 80% of the world population is comprised of medicinal plants and especially in developing countries, the herbal drugs play a central role in many health care programs. The broad definition of medicinal plants has been incorporated in an ancient Indian literature which portrays that all plant parts to be potential sources of medicinal substances [1]. The lack of citations and inflexible quality control has hindered the acceptance of the alternative medicines in the developed countries by serving a lead obstacle. Hence, documentation is very essential part of research work to be carried out on traditional medicines [2]. It becomes extremely important to make an attempt towards consistency of the plant material to be used as medicine in this scenario. WHO has also recommended the evaluation of physicochemical and phytochemical parameters of medicinal plants for its efficacy, due to lack of confined synthetic drugs [3]. These evaluation parameters help in identification and authentication of the plant material. The safety and efficacy of herbal medicine depends mainly upon the exact identification and quality assurance of the starting materials. Molecular oxygen is required to maintain life, but it can be toxic through the formation of reactive oxygen species (ROS). ROS includes superoxide radical, hydroxyl radical, singlet oxygen and H_2O_2 which have been found to play an important role in the initiation and progression of various diseases such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease [4]. Oxidative stress, initiated by these free radicals, seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. But organisms have multiple mechanisms to protect cellular molecules (DNA, RNA and proteins) against ROS induced damage. These include repair enzymes (DNA glycosylases, AP endonucleases etc), antioxidant enzymes (SOD, catalase, and glutathione peroxidase) and intra as well as extracellular antioxidants (glutathione, uric acid,

ergothioneine, vitamin E, vitamin C and phenolic compounds [5]. However, this natural antioxidant mechanism can be inefficient for severe and/or continued oxidative stress. Based on this idea, there has been a strong demand of therapeutic and chemo preventive antioxidant agents with limited cytotoxicity to enhance the antioxidant capacity of the body and help attenuate the damage induced by ROS. Antioxidants are a loosely defined group of compounds characterised by their ability to be oxidised in place of other compounds present [6]. *Sphaeranthus indicus* Linn (Family- Asteraceae) is a branched herb with purple flowers that grows abundantly in rice field and distributed throughout India. It is used indigenously in the Indian system of medicine as an anthelmintic [7]. The plant has a wide range of medicinal value and has been used in hemicranias, jaundice, leprosy, diabetes, fever, pectoralgia, cough, gastropathy, hernia, hemorrhoids, helminthiasis, dyspepsia, skin diseases and nerve tonic [8, 9]. Pharmacological activities such as immunomodulatory [10], antimicrobial [11, 12], antibacterial [13, 14], anxiolytic [9], wound healing action [15] were reported on this plant. Phytoconstituents isolated from this plant are eudesmanolides [16], isoflavonoids [17], 7-hydroxy eudesmanolides [18], sterol glycoside [19], essential oil (cadiene, ocimene, citral, p-methoxycinnamaldehyde, geraniol, eugenol and geranyl acetate) [20] and eudesmanolides [21]. In India, the tribals of Madhya Pradesh used this plant for the treatment of diabetes [22]. Globally, type 1 DM affects considerable percentage of population and it leads to morbidity and mortality of the diabetic patients. Antioxidants play a major role in the prevention of diabetes and its complications by scavenging free radicals. The aim of this work was to determine the quality (types), quantity (amount) of bioactive compounds and in vitro antioxidant activity of seed of *Sphaeranthus indicus*.

2. MATERIAL AND METHODS

2.1. Plant material

Seeds of *Sphaeranthus indicus* were collected from Pinnacle Biomedical Research Institute (PBRI), Near, Bharat Scout and Guides Campus, Shanti Marg, Shyamla Hills Road, Depot Chouraha, Bhopal, Madhya Pradesh 462003, India. The identification and authentication of plant was done by Dr. Saba Naaz, Botanist, from the Department of Botany, Saifia College of Science and Bhopal. A voucher specimen number 189/Saif./Sci./Clg/Bpl. was kept in Department of Botany, Saifia College of Science, Bhopal for future reference.

2.2. Chemical reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

2.3. Organoleptic characters

Plant material (seeds) selected for the study were washed thoroughly under running tap water and then were rinsed in distilled water; they were allowed to dry for some time at room temperature. Then the plant material was shade dried without any contamination for about 3 to 4 weeks. Dried plant material was grinded using electronic grinder to obtain a powdered form and then subsequently used for organoleptic characterization. A small amount of powdered plant part was spread on a white tile and physically examined for general appearance i.e. color, taste, texture etc. Dried plant material was packed in air tight container and stored for phytochemical and biological studies [23].

2.4.Physicochemical study [24-26]

2.4.1. Moisture content

40gm of the cleaned sample was weighed and dried in an oven at 80°C for 7 hrs and the weight was measured in and after every 2 hrs intervals. The procedure was repeated until a constant weight was obtained. After each 2 hrs intervals the sample was removed from the oven and placed in the desiccators for 30 mins to cool. It was then removed and weighed again. The percentage of moisture content in the seeds was calculated by the following formula:

$$\text{Moisture} = 100(W1 - W2)/W2\%$$

Where W1= Original weight of the sample before drying, W2 = Weight of the sample after drying.

2.4.2. Total ash value

5g fine powder of seeds of *Sphaeranthus indicus* was taken in crucible and to burn to ashes at temperature increase up to 450°C and cooled at room temperature then to measured weight. The total ash was measured in percentage.

$$\text{Ash}\% = \text{Loss in weight} / W \times 100$$

2.4.3. Acid-insoluble ash

The ash + 25 ml 2N HCl boiled for five min. The remaining insoluble matter was collected, wash off with water and hot water cooled, and weighed. Acid insoluble ash was measured in percentage.

$$\text{Ash\%} = \text{Loss in weight/W} \times 100$$

2.4.4. Water-soluble ash

Take 5g ash and added 25 ml D/W boiled for 5 min insoluble material, collects less paper filter, washed in hot water is turned on 15 minutes is not higher than 450°C. Water-soluble ash from drug powder was calculated by the formula:

$$\text{Ash\%} = \text{Loss in weight/W} \times 100$$

2.5. Hot soxhlet extraction method

Dried pulverized seeds of *Sphaeranthus indicus* were placed in thimble of soxhlet apparatus. Soxhlation was performed at 60°C using petroleum ether (40-60°C) as non-polar solvent at first. Exhausted plant material (marc) was dried and then extracted with methanol. For each solvent, soxhlation was continued till no colour was observed in siphon tube. For confirmation of exhausted plant marc (i.e. completion of extraction), colorless solvent was collected from siphon tube and completion of extraction was confirmed by absence of any residual solvent, The entire extract was concentrated to dryness using rotary flash evaporator under reduced pressure and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts [27, 28].

2.6. Qualitative phytochemical analysis of plant extract

The *Sphaeranthus indicus* seeds extract obtained was subjected to the preliminary phytochemical analysis following standard methods by Khandelwal and Kokate [29, 30]. The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

2.7. Quantification of secondary metabolites

2.7.1. Total phenolic content estimation

The amount of total phenolic in extracts was determined with the Folin Ciocalteu reagent. Concentration of (20-100µg/ml) of gallic acid was prepared in methanol. Concentration of 100µg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced in to test and mixed with 2 ml of a 10 fold dilute folin Ciocalteu reagent and 4 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and it was then incubated at room temperature for 30 min with intermittent shaking and the absorbance were taken at 765 nm against using methanol as blank. Total phenolic content was calculated by the standard regression curve of gallic acid and the results were expressed as gallic acid equivalent (mg/g) [31].

2.7.2. Total flavonoid content estimation

Different concentration of rutin (20 to 100µg/ml) was prepared in methanol. Test sample of near about same polarity (100µg/ml) were prepared. An aliquot 0.5ml of diluted sample was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% $NaNO_2$ solution. After 6 min, 0.15 ml of a 10% $AlCl_3$ solution was added and allowed to stand for 5min, and then 2 ml of 4% NaOH solution was added to the mixture. The final volume was adjusted to 5ml with distilled water and allowed to stand for another 15 min. Absorbance was determined at 510 nm against water as blank. Total flavonoid content was calculated by the Standard regression curve of Rutin/ Quercetin [32].

2.8. Antioxidant activity

2.8.1. DPPH radical scavenging activity

For DPPH assay, the method of Gulçin *et al.,* 2006 [33] was adopted. A solution of 0.1mM DPPH (4mg/100ml) in methanol was prepared and 1 ml of this solution was mixed with 1 ml of different concentrations of the different extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Ascorbic acid was used as reference standard while methanol was used as control. Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of *Sphaeranthus indicus* extracts. The change in colour was measured at 517 nm wavelength using methanolic solution as a reference solution. This was related to the absorbance of the control without the plant extracts. The percentage inhibition of free radical DPPH was calculated from the following equation:

% inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%.

All the tests were carried out in triplicates. Though the activity is expressed as 50% inhibitory concentration (IC50), IC50 was calculated based on the percentage of

DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity.

2.8.2. Superoxide radical scavenging activity

The reason behind this assay was the ability to prevent the reduction of nitro blue tetrazolium (NBT) in the NBT system [34]. For calculation of superoxide dismutase activity, a method developed by Martinez et al. was used with a little modification [35]. Each 3 ml reaction mixture comprised of 50 mM sodium phosphate buffer, pH 7.8, 13 mM methionine, 2 mM riboflavin, 100 mM EDTA, NBT (75 mM) and 1 ml sample solution. The formation of blue colour formazan was accompanied by perceptive increase in absorbance after 10 min lighting from a fluorescent lamp at 560 nm. The entire reaction assembly was surrounded within a box, covered with aluminium foil. Tubes with reaction mixture were kept in the dark which served as blanks.

% inhibition = [(Absorbance of control – Absorbance of test sample)/ (Absorbance of control)] × 100

3. RESULTS AND DISCUSSIONS

The crude extracts so obtained after each of the successive Soxhlet extraction process were concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The percentage yield of extraction is very important in phytochemical extraction in order to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from the seeds of the plants using petroleum ether and methanol as solvents are depicted in the Table 1.

Table 1: Yield of crude extracts of *Sphaeranthus indicus* seeds

Solvent	% Yield
Pet. Ether	2.33
Methanol	10.16

Table 2: Physical parameters of extracts of *Sphaeranthus indicus*

Extract	Colour	Texture	Taste	Smell
Pet ether	Dark Brown	Semi solid	Bitter	Pungent
Methanol	Dark Brown	Semi solid	Bitter	Pungent

The crude extracts were prepared in two different solvents and their physical parameters were observed. Both extracts have semi solid appearance and dark brown in colour. The extracts have a bitter taste and pungent smell Table 2. The moisture content is an important parameter for checking the purity of the extract. In the present study the moisture content of *Sphaeranthus indicus* was found to be 0.64 %. Ash value was the essential tool used for the standardization of the crude extract. The total ash was important in the determination of purity of the extract which shows the presence or absence of foreign organic matter such as metallic salts or silica. Ash values were utilized to calculate the quality and purity of crude extract. It indicates the presence of various impurities like oxalate, carbonate and silicate Table 3.

Table 3: Ash value of plant material

Sample	Ash Content (% w/w)		
	Total	Water soluble	Acid insoluble
S. indicus	20.21	7.56	6.10

Table 4: Qualitative phytochemical evaluation of *Sphaeranthus indicus* seeds

Test	Pet Ether	Methanolic
Test for carbohydrates		
Molisch's	+ve	+ve
Fehling's	+ve	+ve
Benedict's	+ve	+ve
Barfoed's	+ve	+ve
Test for glycosides		
Borntrager's	+ve	+ve
Keller-killani	+ve	+ve
Test for alkaloids		
Dragendorff's	+ve	+ve
Mayer's	+ve	+ve
Hager's	+ve	+ve
Wagner's	+ve	+ve
Test for saponins		
Froth test	-ve	-ve
Test for flavonoids		
Lead acetate	-ve	+ve
Alkaline reagent	-ve	+ve
Test for triterpenoids and steroids		
Salkowski's	-ve	+ve
Libermann-burchard's	-ve	+ve
Test for tannin and phenolic compounds		
Ferric chloride	-ve	-ve
Lead acetate	-ve	-ve
Gelatin	-ve	-ve

The results of qualitative phytochemical analysis of the crude powder of seeds of *Sphaeranthus indicus* are shown

in Table 4. Methanolic extracts of sample of *Sphaeranthus indicus* showed the presence of carbohydrates, terpenoids, flavonoids, alkaloids, glycosides and steroids but in petroleum ether extracts carbohydrates, alkaloids and glycosides phytoconstituents are only present. Quantitative phytochemical assay was performed by calculating total phenolic content (TPC) and total flavonoid content (TFC). The TPC was calculated with respect to gallic acid (standard) and TFC was then calculated with respect to rutin taken as standard. The TPC and TFC in methanolic extract were found to be 438mg/gm and 208mg/gm respectively Table 5 & Fig 1, 2.

Table 5: Total phenolic and flavonoid content of extract

Test	Methanolic extract
TPC	438 mg/gm equivalent to Gallic acid
TFC	208 mg/gm equivalent to Rutin

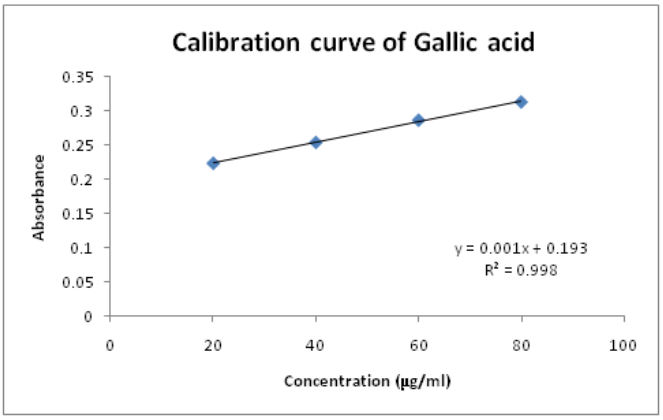

Fig. 1: Graph of estimation of Total Phenolic Content

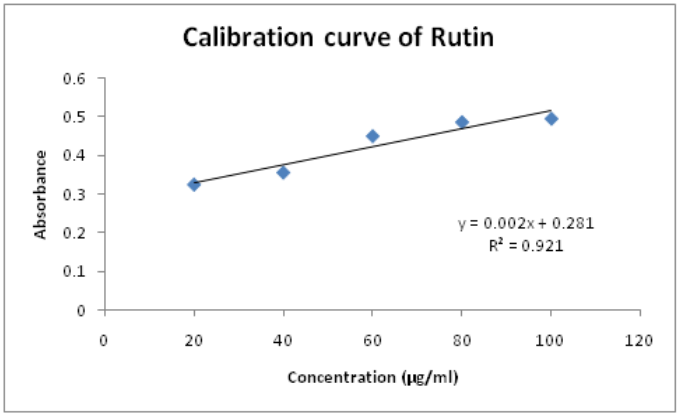

Fig. 2: Graph of estimation of Total Flavonoids Content

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. This free radical remains stable at room temperature and gets decreased in the presence of an antioxidant molecule, which give rise to colorless ethanol solution**.** The scavenging activity of extracts and standard on the DPPH radical expressed as IC50 value of methanol was 20.15, petroleum ether was 114.3 and ascorbic acid was 13.5. IC_{50} value of methanolic extract was effective and close to ascorbic acid which is a well-known antioxidant(Table 6).

Table 6: DPPH assay of ascorbic acid, petroleum ether and methanolic extract

S. No.	Conc. (µg/ml)	(% Inhibition)		
		Ascorbic acid	Pet ether Extract	Methanolic Extract
1.	**20**	54.64	31.52	50.61
2.	**40**	58.49	35.37	55.34
3.	**60**	63.74	39.75	58.66
4.	**80**	74.25	43.95	65.14
5.	**100**	82.83	46.58	71.27
IC 50 Value		**13.5**	**114.3**	**20.15**

Table 7: Superoxide radical scavenging activity of extract of *Sphaeranthus indicus*

Concentration	% Inhibition		
	Ascorbic acid (std.)	Petroleum ether extract	Methanolic extract
20 µg/ml	23.67	17.33	21.33
40 µg/ml	46.33	37.00	42.67
60µg/ml	56.00	45.67	52.33
80µg/ml	71.67	55.33	64.33
100µg/ml	84.00	66.33	75.00

Superoxide that is the one-electron reduced form of molecular oxygen, is a precursor of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen that have the potential of reacting with biological macromolecules and there by inducing tissue damages and also it has been implicated in initiating oxidation reactions associated with aging (Table 7).

4. CONCLUSION

It can be concluded that from present investigation the physicochemical and preliminary phytochemical investigation study of *Sphaeranthus indicus* seeds yielded a set of standards that can serve as an essential basis of evidence to determine the identity and to determine the quality and purity of the plant material as per its future perspectives. The phytochemical investigation gave valuable information about the different phytoconstituents present in the plant, which helps the future investigators concerning the selection of the particular extract for further investigation of isolating the active principle and also gave idea about different phytochemical have been found to possess a wide range of activities. the total phenolic and flavonoid content in methanolic seeds extract was found to be higher than all the extracts which is further proved by *in vitro* antioxidant studies. Potential antioxidant activity has good correlations with the therapeutic use in the treatment of cardiovascular disorders. Further research to isolate individual compounds, there *in vivo* antioxidant activities with different mechanism is needed.

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