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Antimicrobial activity of terpenoids from *Sphaeranthus indicus*. L

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ABSTRACT

Sphaeranthus indicus L. were tested against the uropathogenic organisms Escherichia coli, Klebsiella pneumoniae, Proteus mirabils, and Pseudomonas aeroginosa. Acetobector. It was found that out of the four extract the methanolic extract possessed higher degree of antibacterial activity against Escherichia coli. However against Klebsiella pneumonia ethanolic extract of plant showed higher activity than any other extract, whereas against Proteus mirabils, Chloroformic extract show similar inhibition zone as that with Kanamycin. It is also observed that against Acetobector and Pseudomonas methnolic and ethanolic extract shows similar activity, however it was higher than chloroform extract.

Key words: *Sphaeranthus indicus. L,* Chromatography, I.R spectrum

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INTRODUCTION

Nature has been a source of medicinal agents since times immemorial. The importance of herbs in the management of human ailments cannot be over emphasized. It is clear that the plant kingdom harbours an inexhaustible source of active ingredients invaluable in the management of many intractable diseases. Furthermore, the active components of herbal remedies have the advantage of being combined with many other substances that appear to be inactive However, these complementary components give the plant as a whole a safety and efficiency much superior to that of its isolated and pure active components.

Sphaeranthus indicus Linn is a herb commonly known as Mundi, 30 cm or 1 foot high with spreading branches, found all over the Indian plains, especially in hills, as weed in the rice-fields. All parts of the plant is used as medicine. The roots were recommended by Hakims as a stomachic and antihelminthic in doses of 40 grains daily in the form of powder. The seeds are used as stomachic and antihelminthic. Powder of the roots and seeds is given with honey, in cough. Flowers heads are highly esteemed as alternatives, depuratives, useful as blood-purifiers in skin diseases. The root-bark grounded and mixed with whey is a useful remedy in bleeding piles, and is also used as paste for local application. The oil obtained from the roots by steam distillation, and boiled in Sesamum oil, is recommended by Hakim as a valuable aphrodisiac. Its other uses are in glandular swellings in the neck and also in jaundice. Shade dried leaves in powder-form are used twice a day in chronic skin diseases as ant syphilitic and nervine tonic. This drug is also useful in urethral discharges.

Different national and international pharmaceutical companies are utilizing such plant based information in treatment of various diseases and disorders world around.**[1]** Many of the plant species have been documented pharmacologically and clinically which are endowed in phtyochemical with marked activity on human pathogenic bacteria.**[2]**

Antibiotic resistance has become a global concern; there has been an increasing incidence of multiple resistances in human pathogenic microorganism in recent years, largely due to indiscriminate use of commercial antimicrobial

drugs commonly employed in the treatment of infectious diseases. This has forced scientist to search for new antimicrobial substances from various sources like the medicinal plants common to tropical countries. **[3]** It can grow to a height of 40 cm. The stem is slender and often reddish in colour, covered with yellowish bristly hairs especially in the younger parts. The leaves are oppositely arranged, lanceolate and are usually greenish or reddish underneath measuring about 5 cm. long. In the axils appear very small dense round clusters of flowers. The small green flowers constitute the inflorescence characteristic of the euphobias. The stem and leaves produce white or milky juice when cut. **[4]**

MATERIALS AND METHODS

Collection of Plant Materials: The plants of *Sphaeranthus indicus* L. were collected from areas around District Raisen, M.P. in India. A much branched strongly scented annual with winged stem and the wings toothed leaves obviate, oblong, narrowed to the base, dentate or serrate, glandular hairy. Heads terminal and Leaf opposed, compound, bracteates, compound heads, globose avoid.

Sample preparation and extraction procedure: The fresh leaves were air dried for about one week and ground into fine powder using a mechanical grinder. 20g of the fine powder was weighed into 250ml of ethanol (95%) in a conical flask. The solution was subsequently shaken and filtered using Whatman filter paper. The filtrate was evaporated to dryness using a rotary evaporator (Model type 349/2, Corning Ltd.). A yield of 9.1% was obtained. The extract was then stored below ambient temperature. The crude extracts were dissolved in 30% dimethylsulphoxide and further diluted to obtain 250, 200,150, 100 and 50 mg /ml concentrations then were stored at 15^{o}C until required.

Application of crude extract as concentrated solution was done with a pipette placed against the column wall just above the cotton plug soaked in the solvent kept to project the top the column while the it did not touch the silica gel or the walls. When all the crude extract has been absorbed on the top of the column, the vacant space above it was filled with solvent and the column allowed to run, the supply of the solvent and combinations of solvent was replenished from a separating funnel. The various fraction thus obtained were collected in small glass vials. The different solvents used were according to the procedure given by Herborne. The fractions obtained for different plant extracts were tested for their purity by using thin layer chromatography method.

Table 1.Showing yield of crude extract in different solvent of *Sphaeranthus indicus* L

S. No.	Name of Plant	Solvent	Wt. powdered material (gm)	Vol. of Solvent (ml)	Wt. of extract obtained	% yield
1.	*Sphaeranthus indicus* Linn.	P. ether	400	500	5.2	5.2
		Acetone	400	500	6.4	6.4
		Methanol	400	500	4.12	4.12

Primary test of crude extract of *S.indicus* L

Libermann- Burchard Test: Small quantity of extract treated with few drops of acetic anhydride, boil and cool then add conc. H_2SO_4 from the side of the test tube brown ring is formed at the junction two layers. Deep red colour indicates the presence of terpenoids.

Salkowski test: Trace amount of crude is treated with few drops of conc. H_2SO_4 yellow colour at lower layer indicates presence of terpenoids.

Sulphur test: Add small amount of sulphur powder to the test solution, it sinks at the bottom purification of crude extract. The crude extracts obtained from the plants were subjected to purification process by different chromatographic techniques followed by spectroscopic methods:

a. Column Chromatography

The biologically active compounds were separated from the crude extracts by the column Chromatography.

Table2. Showing column chromatographic fractions of *Sphaeranthus indicus L*

Solvent system	Fractions	Amount of fractions	Color of fractions
n–Hexane:Chloroform(3:2)	Fr. – I		Light Blue
	Fr. – II		Dark Blue
	Fr. – III		Bluish
	Fr. – IV		Light Brown
	Fr. – V		Light Blue

b. Thin Layer Chromatography

The column purified fractions was further assessed on TLC plates for their purity. For the same, the glass plates (20 x 5 cm) were cleaned thoroughly before use. First wash with detergent and water and then with acetone to make grease free completely. Touching the surface of the cleaned plates with fingers were avoided. These dried plates were kept on a commercially obtained "moving spreader". The first step was to make the absorbent silica gel 'G' into slurry with water, usually in the proportion 20g of silica gel and 20 x 20cms of water. The slurry was thoroughly stirred and poured in the rectangular hopper which was then passed over the plates. This hopper has bottom and its trailing face has an adjustable lower edge to give an even layer of 0.25 mm thickness. After spreading the slurry uniformly, the plates were allowed to dry for 15-20 minutes and activated by heating in the chromatographic oven at 1000^{o}C for at least 3-5 minutes. These plates were ready for use. The purified samples were applied with the help of a capillary tube as a minute spot at the start line marked at 1 cm from the edge of the plate. The sot allowed to dry and kept carefully in large glass bottles containing solvent system. For alkaloids, terpenoids, saponins, flavonoids and hydrocarbons, different solvent systems were used according to the method of Herborne. The spots were located exposing the plates in iodine chamber or in UV chamber and the position of the spots and the solvent run marked and measured with a centimetre scale for determining the Rf value defined by.

$$RF = \frac{\text{Distance travelled by the substance}}{\text{Distance travelled by the solvent front}}$$

Table 3. Showing TLC on Silica Gel G acetone extractof *Sphaeranthus indicus*. L

S. No.	Fraction	Visual light	Iodine Chamber	R_f(x100) value
1.	I	Yellow	Yellow	100
2.	II	Dark Green	Dark Green	98.33
3.	III	Light Green	Light Green	95
4.	IV	Grey	Grey	91.67
5.	V	Pale Yellow	Pale Yellow	86.67
6.	VI	Yellow	Yellow	67.5
7.	VII	Yellow	Yellow	51.67
8.	VIII	Yellow	yellow	46.67
9.	IX	Yellow	Yellow	6.7

Table 4. Showing TLC on Silica Gel G P. ether extract of *Sphaeranthus indicus* L

. S. No.	Fraction	Visual light	Iodine Chamber	R_f(x100) value
1.	I	Yellow	Yellow	100
2.	II	Dark Green	Dark Green	98.33
3.	III	Light Green	Light Green	95
4.	IV	Grey	Grey	91.67
5.	V	Pale Yellow	Pale Yellow	86.67
6.	VI	Yellow	Yellow	67.5
7.	VII	Yellow	Yellow	51.67
8.	VIII	Yellow	yellow	46.67
9.	IX	Yellow	Yellow	6.7

c. INFRARED SPECTROSCOPY

An invaluable tool in organic structure determination and verification involves the class of electromagnetic (EM) radiation with frequencies between 4000 and 400 cm^{-1} (wave numbers). The category of EM radiation is termed infrared (IR) radiation, and its application to organic chemistry known as IR spectroscopy Radiation in this region can be utilized in organic structure determination by making use of the fact that it is absorbed by inter atomic bonds in organic compounds. Chemical bonds in different environments will absorb varying intensities and at varying frequencies. Thus IR spectroscopy involves collective absorption information and analyzing it in the form of a spectrum. The frequencies at which there are absorptions of IR radiation ("peaks" or "signals") can be correlated directly to bonds within the compound in question.

Because each inter atomic bond may vibrate in several different motions (stretching or bending). Individual bonds may absorbate more than one IR frequency. Stretching absorptions usually produce stronger peaks than bending; however the weaker bending absorptions can be useful in differentiating similar types of bonds (e.g. aromatic substitution). It is also important to note that symmetrical vibrations do not cause absorption of IR radiation. For example, neither of the carbon-carbon bonds in ethane or ethylene absorbs IR radiation.

Regions of the IR Spectrum

Over time organic chemists have recorded and catalogued the types and locations of IR absorptions produced by a wide variety of chemical bonds in various chemical environments. These data can be quickly referenced through tables of IR absorption ranges and compare to the spectrum under consideration. As a general rule, the most important factors determining where a chemical bond will absorb are the bond order and the types of atoms joined by the bond. Conjugation and nearby atoms sift the frequency to a lesser degree. Therefore the same or similar functional groups in different molecules will typically absorb within the same, specific frequency ranges. Consequently tables of IR absorptions are arranged by functional group – it some versions these may be further subdivided to give more precise information.

In IR absorption tables, signal intensities (height) are usually denoted by the following abbreviations: w = weak, m= Medium, s = strong, v = variable. A broad signal shape is sometimes indicated by br. occasionally absorption frequency is given as a single approximation denoted with an – rather than a range.

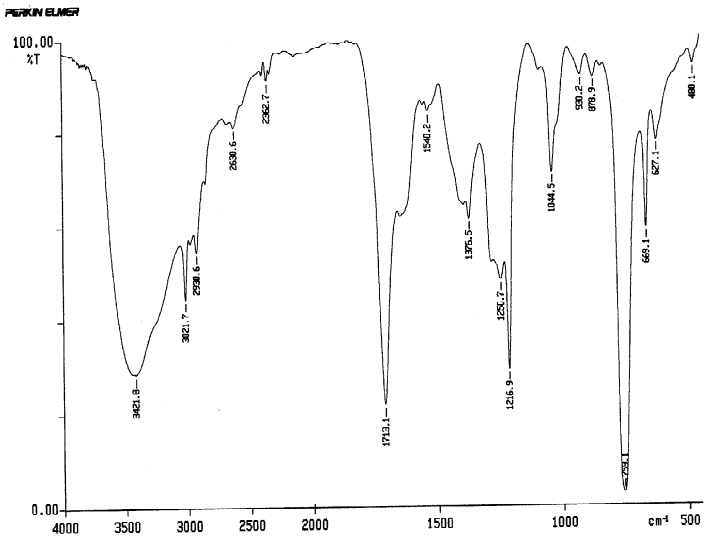
Upon first inspections, a typical infrared spectrum can be visually divided into two regions. The left half, above 2000 cm^{-1}, usually contains relatively few peaks, but some very diagnostic information can be found here. First, alkane C-H stretching absorptions just below 3000 cm^{-1} demonstrate the presence of saturated carbons, and signals just above 3000 cm^{-1} demonstrate instauration.

A very broad peak in the region between 3100 and 3750 cm^{-1} indicates the presence of exchangeable protons, typically from alcohol, amine, amide or carboxylic acid groups (see further discussion of this below). The frequencies from 2800 to 2900 cm^{-1} are normally void of other absorptions, so the presence of –C-H stretch can be easily seen here.

In contrast, the right half of the spectrum, below 2000 cm^{-1}, normally contains many peaks of varying intensities, many of which are not readily identifiable.

Table 5. Showing IR spectra of *Sphaeranthus indicus*Fr-I

Wave Number	Functional group
3752.8	Free O-H stretching
3446.0	Broad inter molecular Hydrogen bonded O-H stretch
2927.3	C-H asymmetric stretch
2337.8	C=C stretch
1643.7	C=O stretch
1397.1	C-O-H bending bond
1223.3	CH_2 wagging
1029.9	C-O stretch
770.0	Out of plane aromatic C-H bending



I.R Spectrum of Terpenoids

Evaluation of antimicrobial activity

Escheria coli, Staphylococcus aureus Salmonella typhi and Psudomonas aeruginosa were obtained from the department of microbiology Gandhi Medical College Bhopal. They were re- isolated and the pure culture were stored at 4 ^{o}C until required.

The agar diffusion method as described by Esimone was adopted for study about 15 ml of sterile molten nutrient agar in a Petri dish was seeded with 1.0 ml standardized broth culture of the bacteria and swirled gently to ensure uniform distribution of the microorganism and then allowed to solidify on a flat surface .Three holes were made in the plates using a sterile cork borer and equal volumes of the extracts were transferred into the holes using a Pasteur's pipette. Two Petri dishes containing a particular microorganism were used for each concentration of the extract. The plates were allowed to stand for one hour for prediffusion of the extract. The plates were allowed to stand for one hour for prediffusion of the extract to occur and were incubated at 37 ^{0}C for 24h.At the end of incubation, zones of inhibition that devised were measured and the average of zones of inhibition was calculated.

RESULTS

Results of the antibacterial screening of the different concentrations of the extract on the test isolates are shown. The results show that increase the zone of growth inhibition of some of the microorganisms. The extract did not inhibit the growth of S. typhi at any of the concentrations administered. The highest zone of growth inhibition of 13.5mm diameter was exhibited by 250 mg/ml concentration of the extract against *S. aureus*. Only the 200 and 250 mg/ml concentration had effects against *B. subtilis, E. coli* and *P. aeruginosa*.**[5]**The lowest Zone of growth inhibition was observed with 200mg/ml concentration of the extract against B. subtilis which gave a zone of inhibition measuring 5.6mm.The preliminary phtyochemical screening of the methanolic extract showed the presence of carbohydrate, protein, amino acid phenol, tannin, alkaloids triterpenoids, flavonoids, volatile oil and glycoside in the plant *Sphaeranthus indicus* and its part. Steroid was not present in methanol extract The antimicrobial activity of methanolic, ethanolic, chloroformic and aqueous extract of *Sphaeranthus indicus L.* were tested against the uropathogenic organisms *Escherichia coli, Klebsiella pneumoniae, Proteus mirabils,* and *Pseudomonas aeroginosa. Acetobector.* It was found that out of the four extract the methanolic extract possessed higher degree of antibacterial activity against *Escherichia coli*. However against *Klebsiella pneumonia* ethanolic extract of plant showed higher activity than any other extract, where as against *Proteus mirabils,* Chloroformic extract show similar inhibition zone asthat with Kanamycin. It is also observed that against *Acetobector* and *Pseudomonas* methnolic and ethanolic extract shows similar activity, however it was higher than chloroform extract. The minimum inhibitory concentrations of the extract on the test isolates are shown in Table 6. The lowest minimum inhibitory concentration (MIC) was produced against S. aureus with a concentration of 22.55mg/ml while the highest MIC was against B. subtils with a concentration of 74.61mg/ml. The extract had MIC of 58.09 and 57.64mg/ml.

Table6.Antibacterial screening of the different concentrations of crude ethanolic extract of *Euphorbia hirta*

Concentrations of extract (mg/ml)	Zones of inhibition (mm)				
	E.coli	*S.aureus*	*P.aeruginosa*	*B.subtils*	*S.typhi*
250	12.9^{a}	14.5^{b}	13.1^{a}	7.4^{c}	NI
200	8.8^{a}	13.9^{b}	12.3^{c}	6.6^{d}	NI
150	70^{a}	12.5^{b}	6.2^{a}	6.6	NI
100	6.8^{a}	11.6^{b}	6.1^{a}	NI	NI
50	6.8	7.8^{a}	NI	6.6	NI

Values are means of triplicate readings. NI=No inhibition ^{a,b,c}Values with different superscripts on the same row are significantly different (p=0.05).

Table 7. Minimum inhibitory concentrations of the ethanolic extract of *Euphorbia hirta* against test isolates

Plant	*E.coli*	*S.aureus*	*P.aeruginosa*	*B.subtils*	*S.typhi*
E. hirta	69.09^{a}	24.55^{b}	5864^{a}	79.61^{a}	NIL

^{a,b,c}Values with different superscripts on the same row are significantly different (p=0.05).

DISCUSSION

In the study, the results obtained indicated that the ethonolic extract of the E. hirta inhibited the growth of the test isolates except S. typhi. This, therefore, shows that the extract contains substance (s) that can inhibit the growth of some microorganisms. The observed antibacterial effects on the isolates is believed to be due to the presence of alkaloids , tannins and flavonoids which have been shown to possess antibacterial properties.

The observed antibacterial properties corroborate its use in traditional medicine. Traditionally, extracts of the plant are used in sore and wound healing, as ear drop for boils in the ear and treatment of boils. They are also used in the control of diarrhoea and dysentery.**[6]**

The large zones of inhibition exhibited by the extract against S. aureus and P.aeruginosa justified their use by traditional medical practitioners in the treatment of sores, bores and open wounds. *S. aureus* and P. aeruginosa have been implicated in cases of boils, sores and wounds**.** Also the moderate growth inhibition against *E. coli* justifies its use in the control of diarrhoea and dysentery. *E.coli* is the common cause of traveller's diarrhoea and other diarrheagenic infections in humans**.** The low MIC exhibited by the extract against *S. aureus* is of great significance in the treatment of infections caused by these microbes, especially as they frequently develop resistance to known antibiotics. Their use also will reduce the cost of obtaining health care. The relatively high zone of inhibition exhibited by the extract against *E.coli* is also of significance, since *E. coli* is a common cause of diarrhoea in developing countries.

The inability of the extract to inhibit *Salmonella typhi* may be that it possesses a mechanism for detoxifying the active principles in the extract Some bacteria are known to possess mechanisms by which they convert substances that inhibit their growth to non-toxic compounds for examples *S. aureus* produces the enzyme penicillinase which converts the antibiotic penicillin to penicillinoic acid which is no longer inhibitory to its growth. **[7]**

Statistical analysis revealed that for RBC there was no significant ($p \geq 0.05$) between the values obtained for the different concentrations of the extract injected and the control. This shows that the extract did not affect either the circulating red blood cells or the erythropoetic centres of the animals. Some extracts of plants do not have deleterious effects on RBC even up to 400 mg/kg body weight after 28 days of administration. This is also true for the WBC counts. Thus, the extract did not induce production or destruction of the WBC. The same trend was also observed for the Hb content which indicates that the extract did not affect synthesis of haemoglobin by the animal. Some plants have been suggested to interfere with the synthesis of Hb by inhibition of the uptake and utilization of iron.

CONCLUSION

From the above results it can be concluded that plant *Sphaeranthus indicus* extracts have great potential as antimicrobial compounds against micro-organisms and that they can be used in the treatment of infectious diseases caused by resistant micro-organisms. Antibiotic resistance has become a global concern. There has been an increasing incidence of multiple resistances in human pathogenic micro-organism in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This has forced scientist to search for new antimicrobial substances from various sources like the medicinal plants common to tropical countries. It can grow to a height of 40 cm. The stem is slender and often reddish in colour, covered with yellowish bristly hairs especially in the younger parts.

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