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IN VITRO EVALUATION OF PHYTOCHEMICAL, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF CALYCES OF ROSELLE (*HIBISCUS SABDARIFFA* L.)

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ABSTRACT: Diversity of plants has attracted a great deal of research interest in search of natural antioxidants and antimicrobials. Many natural substances having anti-oxidant and anti-microbial properties have been used in health foods for medicinal and preservative purposes. Fleshy calyces of *Hibiscus sabdariffa* L. of family Malvaceae including the fleshy calyces were tested positive for presence of phytochemicals like Phenols, tannin, flavonoids, alkaloids, saponin, anthraquinone, triterpenoids and steroids etc. Both Petroleum ether extract and ethanol extract had shown similar antimicrobial activities. Petroleum ether extract was very effective against bacteria like *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus cereus* and *Lactobacillus brevis*. But ethanol extract showed moderate antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus*. Antioxidants like phenolics and flavonoid content of roselle was found to be high. Petroleum ether extract also showed better antioxidant activity by scavenging DPPH free radical. This plant has the prospect of being a source of new clinically efficient antimicrobial or antioxidant compounds.

INTRODUCTION: The diversity of pathogenic bacteria is extensive and so is the variety of diseases caused by them. Despite the existence of many potent antimicrobial agents, multi-resistant pathogenic strains are continuously emerging, imposing the need for a continuous search and development of new drugs¹.

Most drugs possess many severe side effects. Many medicines of natural origin had been used since long time without any serious adverse effects.

It is therefore essential that efforts should be made to introduce new natural resources to develop cheaper drugs with lesser side effects². Despite immense technological advancement in modern medicine, many people still rely on the natural healing practices for their daily health care needs^{2,3}. World Health Organization has also encouraged studies for the treatment and prevention of different diseases on traditional medical practices⁴. Hence, many efforts have been exploited to discover new antimicrobial compounds from various kinds of sources such as soil, microorganisms, animals and plants. Plants represent still a vast untapped source of structurally novel compounds that might serve as lead for the development of novel drugs. Plants also represent a rich source of antimicrobial agent⁵. Plants generally produce many secondary metabolites which constitute an important source of

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microcides and anti-oxidants⁶. Many natural substances having anti-oxidant and anti-microbial properties have been used in health foods for medicinal and preservative purposes⁷.

Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines^{8,9}. Medicinal plants constitute to be source of both traditional and modern medicines⁴. The screening of extracts for antibacterial activity has revealed the potential of higher plants as a source of new anti-infective agents¹⁰.

Roselle (*Hibiscus sabdariffa* L.) belonging to the family Malvaceae, locally called "Lal-mesta", is an important annual crop grown successfully in tropical and sub-tropical climates. It is distributed in the Indian subcontinent, Bangladesh, Myanmar, Thailand, Senegal, Mali, Niger, Congo, France, Gambia, Nigeria, Egypt, Sudan, Namibia, Caribbean Panama, Indonesia and Malaysia⁷. It is an annual woody shrub growing to 2–2.5 m tall, cultivated for its leaves, stem, seed and calyces¹¹. The most commercially important part of the plant is the fleshy calyx (sepals) surrounding the fruit (capsules). The whole plant can be used as beverage or the dried calyces can be soaked in water to prepare a colorful cold drink, or may be boiled in water and taken as a hot drink. It also has some medicinal properties¹². Roselle is a flexible plant with a number of uses. The two botanical types of roselle are *Hibiscus sabdariffa* var. *sabdariffa*, grown for its fleshy, shiny-red calyx, and *Hibiscus sabdariffa* var. *altissima* grown for its phloem fiber¹². Calyx extract of the former is traditionally used for the treatment of several complaints like high blood pressure, liver diseases and fever. The extract also showed in vivo antihypercholesterolaemic, antinociceptive and antipyretic activities¹³.

The present study has been aimed to estimate the phytochemicals and to evaluate the antimicrobial and antioxidant activities of *Hibiscus sabdariffa* L. of family Malvaceae in vitro.

MATERIALS AND METHODS:

Plants collection and preparation

Ripe fleshy calyces of *Hibiscus sabdariffa* L. of family Malvaceae were collected in September, 2012, from village of Taldi at South 24 Parganas District, West Bengal, India. The fleshy calyces were cleaned thoroughly to make those completely free from any possible contamination. The fleshy calyces were cut into pieces and dried at 50°C for 5-6 days, then separately ground into fine powder using a mechanical grinder and sieved. The powder was kept in dark coloured glass bottles and subsequently used.

Preparation of solvent extraction

Two types of organic solvents were used for extraction. 40 g of each dry powder was mixed either in 100 ml sterile petroleum ether or ethanol (100%) for 48 hours at 24°C with stirring¹⁴. The extracts were centrifuged and filtered through Whatman No.1 filter paper and bacterial 0.45µm filter (Millipore). Then the extracts were evaporated using vacuum rotary evaporator to near dryness and stored in glass vials in dark at 4°C. Extractive values were calculated in terms of percentage considering the weight of plant material as 100%. These crude solvent extracts were diluted with either sterile double distilled water or 10% dimethyl sulphoxide (DMSO) which are to be used as negative control respectively to obtain required concentration before experiments.

Phytochemical evaluations

The extracts were tested to phytochemical evaluation using standard techniques of plant secondary metabolites according to Harborne and Turner¹⁵ and Evans¹⁶. Extracts were tested for phenolics, tannin, flavonoids, alkaloids, triterpenoids, saponin, steroids, coumarin, anthraquinone and glycosides.

Test micro-organisms

Four enteropathogenic, three food-spoiler and one probiotic bacterial strains were chosen for the antimicrobial activities of the extracts. The strains used were *Salmonella enterica* serovar typhimurium MTCC 3224, *Serratia marcescens* MTCC 4822, *Staphylococcus aureus* MTCC 7405, *Escherichia coli* MTCC 3221, *Klebsiella pneumoniae* subsp. *pneumoniae* MTCC 6644,

Proteus vulgaris MTCC 7299, *Bacillus cereus* MTCC 6909, *Lactobacillus brevis* MTCC 4460 and those strains were obtained from MTCC, IMTECH, Chandigarh, India. All bacterial cultures were maintained on tryptic soy agar (TSA, HiMedia) and subcultured regularly. The fungal strain *Aspergillus niger* was taken from laboratory collection (isolated from bread) and grown on Sabouraud dextrose agar (HiMedia). Standard inoculum was prepared by sub-culturing 4-5 freshly grown isolated colonies of each strain in Tryptic soy broth (TSB) and incubated at 35-37 °C for 24 hours. Inocula were standardized with sterile TSB to give final cell load of 10^6 - 10^7 CFU/ml.

Disc diffusion bioassay

The disc diffusion test was carried out as described by Jorgensen et al.¹⁷ A 0.5 ml standardized inoculum suspension of each bacterial strain was spread on TSA plates with a sterile bent glass rod spreader. Sterile 6-mm Whatman no.1 filter paper discs were aseptically placed on plates. Sample decoctions or extracts of standard concentrations (10 mg dry weight) were aseptically poured on the discs along with sterile double distilled water or 10% DMSO as negative and ampicillin as positive controls. Plates were allowed to stand for 30 minutes at room temperature prior to incubation at 35-37°C for 24 hours. The inhibition zone diameters were measured three times and means were represented.

Total phenolic Content (TPC)

The total phenolic contents of extracts were determined spectrophotometrically¹⁸. One ml of Folin-Ciocalteu's reagent (Merck, India), previously diluted (1:20), was added to one milliliter of sample (250 µg/ml) and mixed thoroughly. To the mixture, 4 ml of sodium carbonate (75 g/L) and 10 ml of distilled water were added and thoroughly mixed. The mixture was allowed to stand for 2 h at room temperature. Contents were then centrifuged at 2000 g for 5 min and the absorbance of the supernatant was taken at 760 nm. A standard curve was obtained using various concentrations of gallic acid (Sigma-Aldrich, Germany). Results were expressed as percentage of gallic acid equivalents (GAE) per 100 g of fresh mass. The total phenolic compounds

(TPC) was expressed as gallic acid equivalents (GAE)/g of dry weight (mgGAE/g dw) using the following equation obtained from a standard gallic acid graph $y = 3.9207x + 1.0607$ ($R^2 = 0.9932$).

Total flavonoid content

Total flavonoid content was measured by aluminum chloride colorimetric assay¹⁹. 1ml of sample or standard solution of catechin (500 µg/ml) was added to 10 ml volumetric flask containing 4 ml of distilled water. To the above mixture, 0.3 ml of 5% NaNO₂ was added. After 5 minutes, 0.3 ml of 10% AlCl₃ was added. After 5 minutes, 2 ml of 1 M NaOH was added and total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared control at 510 nm. Total flavonoid content of the sample was expressed as percentage of catechin equivalent per g dry weight (mgCE/g dw).

Ascorbic Acid Content

Ascorbic acid content in the juice was determined with DCPIP visual titration method described earlier by Ranganna²⁰. The DCPIP dye, which is blue in alkaline solution, is reduced by ascorbic acid to a colorless form. About 5 mL of extract solution was titrated with DCPIP dye to a pink color end point. The ascorbic acid content was determined by the titration of the standard ascorbic acid solution with DCPIP dye.

DPPH free radical scavenging activity

The antioxidant or free radical scavenging activity of the extracts was measured on the basis of decrease in the absorbance of methanol solution of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical²¹. DPPH is one of the few stable and commercially available organic nitrogen radicals exhibiting a dark purple color at absorbance 517 nm²². When free radicals are scavenged, DPPH will be reduced, producing a light yellow coloration reducing the absorbance. 05 ml of DPPH (25 mg /L) solution was added to 1 ml of sample solution (at different concentrations). Mixture was shaken vigorously and kept at room temperature for 30 min in dark. Then the absorbance was measured at 517 nm and compared with standards. Scavenging

activity was calculated as the percentage inhibition (I%) using the following formula:

% DPPH Anti-radical activity (I%) =

$$\frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} \times 100$$

Radical-scavenging potential was expressed as IC₅₀ value (calculated from linear regression of the graph of concentration vs. I%) representing the concentration, which scavenged 50% of the DPPH radicals.

Statistical analyses: For all tests three replicates were done and the mean values and standard deviations were determined.

RESULTS AND DISCUSSION: The fleshy calyces extract was tested to phytochemical evaluation. It was found that glycosides and coumarins are completely absent (Table 1). Phenols, flavonoids, alkaloids, saponin, anthraquinone and steroids are present. Tannin and Triterpenoids are found in ethanol extract but absent in petroleum ether extract.

TABLE 1: PHYTOCHEMICAL QUALITATIVE EVALUATION OF *HIBISCUS SABDARIFFA* L. FLESHY CALYCES EXTRACTS

Phytochemicals	Pet. Ether extract	Ethanol extract
Phenolics	++	++
Tannin	--	++
Flavonoids	++	++
Alkaloids	++	++

Table 2: ANTIBACTERIAL ACTIVITIES, INDICATED BY DIAMETER OF INHIBITION ZONE (DIZ, MM, FOR 10 MG DRY WT./ DISC, MEAN±SD) OF EXTRACTS OF *HIBISCUS SABDARIFFA* FLESHY CALYCES AGAINST THE MICRO-ORGANISMS [- MEANS <7MM DIZ I.E DIZ OF NEGATIVE CONTROL]

Extracts	Solvent types	<i>E. coli</i>	<i>S. aureus</i>	<i>S. enterica</i>	<i>S. marcescens</i>	<i>K. pneumoniae</i>	<i>P. vulgaris</i>	<i>B. cereus</i>	<i>L. brevis</i>	<i>A. niger</i>
	Pet. Ether	11±1	12±1.527	11±1	8±0.577	12±0.577	9±0.577	11±1.527	11±1	8±1.527
	Ethyl Alcohol	12±0.577	13±1	9±1	-	11±1	10±1	12±0.577	11±0.577	9±1.527

TABLE 3: PHYTOCHEMICALS ESTIMATION AND ANTIOXIDANT ACTIVITIES OF *HIBISCUS SABDARIFFA* EXTRACTS

Phytochemicals	Pet. Ether extract	Ethanol extract
Phenolics (mgGAE/g dw)	41±4	34±5
Flavonoids (mgCE/g dw)	24.45±1.8	27.56±1.52
Ascorbate (mg/g dw)	1.92±0.21	1.68±0.25
DPPH-IC ₅₀ (µg/ml)	64	58

Triterpenoids	--	++
Saponin	++	++
Steroids	++	++
Coumarin	--	--
Anthraquinone	++	++
Glycosides	--	--

The disc diffusion assay revealed that the calyces extracts had different degrees of bacterial and fungal growth inhibition, depending on the microbial strains (Table 2). Both extracts had shown similar antimicrobial activities against all tested strains. Petroleum ether extract was very effective against bacteria like *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus cereus* and *Lactobacillus brevis*. But ethanol extract showed moderate antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus*. *Serratia marcescens* strain is the least sensitive among all strains tested against both extracts. The antimicrobial activity of extracts of *Hibiscus sabdariffa* calyces might be due the presence of secondary metabolites like tannin, alkaloids and anthraquinones which are known to have antimicrobial properties²³.

Phytochemicals estimation and antioxidant activities of fleshy calyces extracts were shown in Table 3. Total Phenolic contents were expressed as mg gallic acid equivalent as this compound represents the most simple form of a phenolic compound.

Petroleum ether extract contained higher amounts of phenolics and ascorbates in comparison to ethanol extract. Phenolics are non-nutritive dietary components found to be associated with the inhibition of cancer, atherosclerosis, as well as for age-related degenerative brain disorder²⁴. The polyphenol content in *Hibiscus sabdariffa* is beneficial as a potential source of natural antioxidant. Ethanol extract contained slightly higher amounts of flavonoids. Flavonoids help in reducing the risk of heart diseases and various cancers as well as prevent menopausal symptoms²⁵. Ascorbate was in the range of 1.68-1.92 mg/g.

DPPH scavenging assay is applied extensively for the determination of free radical scavenging or antioxidant activity of any compound. DPPH assay measures the capability of the extract to donate hydrogen to the radical. In DPPH assay the lower the IC₅₀ the better it is able to scavenge the radicals, particularly peroxy-radicals which are the propagators of the autoxidation of lipid molecules and thereby break the free radical chain reaction²⁶. Petroleum ether extract showed slightly better antioxidant activity. Farombi and Fakoya found similar scavenging activity by roselle extracts against hydrogen peroxide and superoxide radicals²⁷. Mohd-Esa *et al* showed that seeds of roselle possess higher antioxidant activity than calyces and other parts²⁸.

CONCLUSION: Fleshy calyces of *Hibiscus sabdariffa* have shown potentially superior antimicrobial and antioxidant efficacy. Both extracts had shown similar antimicrobial activities against all tested strains. Petroleum ether extract also showed strongest antioxidant activity. Differential antimicrobial or antioxidant activity of extracts against different bacteria might be due to presence of different active phyto-chemicals.

Among those antimicrobial compounds, phenolic compounds, terpenoids, and steroids are very important compounds in antimicrobial or antioxidant effects. Further study is necessary to establish different active compounds from this plant and their full spectrum of efficacy. This plant has the prospect of being a source of new clinically efficient antimicrobial or antioxidant compounds and the knowledge can be extended for future

investigation into the field of pharmacology for better drug discovery.

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