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Studies on the therapeutic properties of Roselle (*Hibiscus sabdariffa)* calyx: A popular ingredient in the cuisine of North East India

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Abstract
Roselle plant (*Hibiscus sabdariffa*) of Malvaceae family finds its mention in folk medicine of North East India for its use in various ailments. In the present study, the proximate value and nutritional quality, antioxidant activity, antimicrobial properties, anti-proliferative activity and apoptosis-inducing capacity of roselle found in North East India was evaluated. Nutritional evaluation of the calyx establishes it as a good source of dietary antioxidants and ascorbic acid. Antimicrobial activity of the aqueous extract upto 25mg/ml concentration showed complete growth inhibition of both gram positive (*Staph. aureus*) and gram negative *(E. coli, K. pneumoniae)* bacteria while antioxidant activity with 100 mg/ml concentration was high (90% scavenging activity) that gradually decreases with dilution in concentration. The antiproliferative and apoptotic activities were evaluated in cervical (HeLa) cancer cell lines. It exhibited anti-proliferative activity in a concentration-dependent manner; the concentration @100mg/ml can induce upto 51.8% apoptosis. The studies indicated its potential benefits for health which can further be tested and validated for use as supplement in the food.

Keywords: *Hibiscus sabdariffa*, cancer cells, antioxidant, antimicrobial activity

Introduction
The roselle plant, with an attractive flower believed to be native to Africa, is cultivated in Sudan, India, Malaysia and Taiwan. It is also one of the signature food ingredients of indigenous delicacies such as Jingtah jajewsaw in Meghalaya, Masor tenga in Assam, Gakro ghapha in Nagaland, Silok sougri Mapan Metpa in Manipur etc. The flowers are also used as a dye by virtue of its bright red colour. In addition to their use in food, various parts of the roselle plant have been used in traditional medicine for the prevention of diseases. It has been used in herbal tea to treat hypertension, pyrexia and liver damage although the pharmaceutical components are poorly defined [1].Various workers reported the roselle calyx possesses various beneficial effects in human health as mediated through its antioxidant property [2, 3]. Supplementation of roselle calyx extract in *ex vivo* murine hematopoietic stem cells culture increased the survivability of cells [4]. It also had an inhibitory effect on biofilm formation of oral pathogenic bacteria [5].
The calyces are also rich in vitamins, especially ascorbic acid [6]. Ascorbic acid is an essential dietary component. Various workers have reported variable content suggesting the type of soil influences its ash and mineral content causing variations within the same species [7, 8, 9, 10]. The antimicrobial activity, antibacterial and cytotoxicity studies in dose-dependent manner have also been studied [11, 12]. Moreover, the antimicrobial activity on isolates from food, veterinary, and clinical samples have indicated that roselle extract contain potential antimicrobials in foods [13].Antioxidative activity of the extract was also reported in cancerous cell lines while the antioxidant potential in the form of scavenging free radical and inhibitory effect on XO activity has been evaluated [14, 15]. The effects of roselle-anthocyanins (HA) on human cancer cells (HL-60) showed apoptosis of cells in a dose and time-dependent manner [16]. It has also been reported to induce apoptosis in leukemic cells via reduction of retinoblastoma phosphorylation and Bcl2 expression [17].
In the present study, the nutritional constituents, antioxidant activity, antimicrobial properties, antiproliferative activity and apoptotic potential of locally available roselle plant, commonly used in the indigenous cuisines, is evaluated to determine its therapeutic benefits.

Materials and Method
Sample collection and preparation
The roselle calyces (Figure 1) were harvested between November-December from Bhoirymbong village in Ri-Bhoi district, located between 25°40´ to 25°21´N latitude and 90°55´15´´ to 91°16´E longitude in the state of Meghalaya, India. The calyces were dried and powdered for proximate analysis of nutrient contents and others. The extracts @100 mg/ml (w/v) concentration with water was prepared, homogenized and clarified by centrifugation at 10000 rpm for 30 min at 4°C. The aqueous extract was collected and filtered

with 0.45 µm syringe filter for further *in vitro* studies. One gram of the powder was extracted overnight in 80% methanol and filtered. The filtered extract was used for estimation of Total antioxidant capacity (TAC) and phenols, respectively.

Fig 1: Roselle plant (a) flowering stage and (b) calyces
Tables and Figures

Estimation of Proximate analysis

The proximate analysis of roselle calyces was done as per the method prescribed by AOAC for estimating moisture content, dry matter (DM), total ash (TA), crude protein (CP), ether extract (EE) and crude fibre (CF) [18]. The nitrogenous free extract (NFE) was calculated from 100 - (% moisture + % CF + % CP + % EE + % TA).

Total phenols content

Phenol content was determined with Folin-Ciocalteau reagent by the method described by Singleton *et al.* [19]. 0.5 ml of extract was initially made up to 3 ml with 80% methanol. 1 ml of DMSO and 1 ml of 10% Folin-Ciocalteau reagent was added and mixed. 3 ml of 1% Na_2CO_3 was finally added after 3 minutes, and the tubes were incubated for 2 h at room temperature. A standard curve was plotted using different concentrations of gallic acid (50-300 µg/ml). Absorbance was measured at 760 nm. The phenolic contents were calculated on the basis of the calibration curve of gallic acid (50-300 ug/ml) and expressed as gallic acid equivalents (GAE), in milligrams per gram of dry weight.

Ascorbic Acid content

Vitamin C content was determined by the method described by Sadasivam and Manickam [20]. Two gram of the powder was extracted with 4% oxalic acid overnight. The extract was filtered and used for the analysis. To 10 ml of the extract, a few drops of bromine water was added until the solution became coloured, confirming the completion of the oxidation of ascorbic acid to dehydroascorbic acid. The final volume was then made to 50 ml with 4% oxalic acid. 2 ml of sample aliquots was made up to 3 ml of distilled water. To this 1 ml of 2, 4-Dinitrophenylhydrazine reagent was added and 1-2 drops of 10% thiourea was added to remove excess bromine. The reaction mixture was mixed and incubated at 37ºC for 3 h. 5 ml of 80% sulphuric acid was then added to dissolve the osazone crystals formed. A standard curve was plotted using different concentrations of Ascorbic acid (20-120 µg/ml). Absorbance was measured at 540 nm. The total phenolic content was expressed in milligrams per gram of dry weight.

Total Antioxidant Capacity

Total antioxidant capacity was estimated with phosphomolybdenum reagent by the method described by Prieto *et al.* [21]. To 0.3 ml of sample extract, 3 ml of phosphomolybdenum reagent solution (0.6 M sulphuric acid, 28 mM potassium phosphate and 4 mM ammonium molybdate) was added. The tubes were then incubated at 95ºC for 1 h. After cooling at room temperature the absorbance was measured at 695 nm. A standard curve of ascorbic acid was plotted (20-100 µg/ml) to calculate the values. Total antioxidant capacity was expressed as Ascorbic acid equivalents (AAE), in milligrams per gram of dry weight.

Free radical scavenging activity assay

Antioxidants are compounds with free radical scavenging activity and its activity in the extract was measured using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay as per the method described by Yamasaki *et al.* [22]. 20 µl of plant extracts (five serial dilutions RD1-RD5 of 100, 50, 25, 12.5 and 6.25 mg/ml) or standard solution (BHT) is added to 200 µl of DPPH reagent in 96 well plates in triplicates. Absolute methanol is used as reagent blank. All reagents is mixed and incubated for 30 min at room temperature, protected from light. The absorbance is measured at 490 nm with an ELISA reader (Lab systems Multiskan Plus, Thermo Fisher Scientific, USA).The percentage of DPPH free radical scavenging activity is calculated as % inhibition = 100 - $[(A_s-A_b)/A_c \times 100]$ Where A_s is the absorption of extract, A_b is the absorption of the blank sample and A_c is the absorption of the control.

Antimicrobial assay

The activity of antimicrobial properties was studied using agar dilution method [23]. One ml of 0.5 McFarland microbial inoculum preparation by direct colony suspension (CLSI guidelines) of *Escherichia coli* (*E.coli*) -ATCC 25922, *Staphylococcus aureus (Staph. aureus)* -ATCC 25923 and *Klebseilla pneumoniae (K. pneumoniae)* - ATCC 700603 having approximately 10^6 bacterial/ml was added to 1 ml of serial two-fold dilution (100, 50, 25, 12.5, 6.25, 3.125 mg) of aqueous extract of roselle in tubes and incubated at 37°C for 24 h. The incubated tubes were poured into agar plates in duplicate and incubated at 37°C for 24-48 h. The minimum inhibitory concentration (MIC) endpoint is recorded as the lowest concentration of roselle extract that completely inhibits growth under suitable incubation conditions using methanol and non-treated control.

Anti-proliferative assay

Two-fold serial dilution of the aqueous extract was prepared and upto five dilutions (100, 50, 25, 12.6 and 6.25 mg/ml) were used for studying the anti-proliferative effects in HeLa cells by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay as per Mosmann with slight modification [24]. The cells @1x10^6/ml in Earle's Minimum Essential Medium (EMEM), (Sigma-Aldrich, USA) with 10% FBS were suspended in a 96 well plate @100 µl/well and 100 µl of each dilution were added in triplicates with mitogen-Concanavalin A (ConA) and Phytohaemagglutinin A (PHA) @ 10mg/ml as positive control. The cells were incubated at

37°C with 5% CO_2 for 72 h. 10 µg MTT dye was then added to each well and further incubated for 4 h. The supernatants were removed and dimethyl sulfoxide (DMSO) @150 µl was added to each well and incubated at room temperature by gentle shaking for 30 min. The supernatants were collected and the absorbance was measured at 570 nm with an ELISA reader (Lab systems Multiskan Plus, Thermo Fisher Scientific, USA).

Apoptosis assay

The apoptosis assay was done using Annexin V- FITC apoptosis detection kit (Calbiochem, Germany) in flow cytometer (BD – LSR Fortessa, USA). Two-fold five serial dilution (100, 50, 25, 12.6 and 6.25 mg/ml) of the aqueous extract was prepared for studying apoptosis in HeLa cells along with non-treated control. The cells @1x10^6/ml in Earle's Minimum Essential Medium (EMEM), (Sigma-Aldrich, USA) with 10% FBS were suspended in a 96 well plate @100 µl/well and 100 µl of each dilution were added in triplicates. The control wells were treated with 100 ml PBS (pH 7.2). The cells were incubated at 37°C with 5% CO_2 for 24 h. The cells were then processed using apoptosis detection kit following manufacturer's instructions. The percentage of apoptosis was measured in flow cytometer with setting of unstained cells and cells stained with isotype control. Samples were acquired taking 10000 cell counts.

Statistical analysis

The experiment were done in triplicates and the results were expressed as mean ± SD. Statistical difference compared between various treated and untreated groups were analyzed by one way analysis of variance (ANOVA) followed by Tukey test for significance. Statistical analysis was considered significant if $P< 0.05$.

Results and Discussion

Nutritive value

Proximate composition of dried calyces is presented in Table 1. Dry matter, crude fibre, crude protein, ash and moisture content were 40.45, 20.83, 27.32, 4.67 and 6.79%, respectively. The NFE was calculated as 64.14% which represent the soluble carbohydrate and other easily utilizable non-nitrogenous components. The analyses present a comparable proximate composition. The nutritional content of the calyx was in the range as reported in three varieties found in Sudan with the moisture content range from 6.19 -12.07%, CP between 5.5 to 9.76%, CF from 10.74 and 12.17% and carbohydrate between 60.43 and 61.76% [25]. Carbohydrate content as high as 68.7% followed by crude fibre (14.6%) and ash content (12.2%) has also been reported [26].

Table 1: Proximate composition of roselle calyces

Content	Values (%)
Moisture	09.53 ± 1.796
Dry matter (DM)	90.47 ± 1.796
Crude protein (CP)	08.31 ± 0.360
Crude fiber (CF)	11.53 ± 0.608
Ether extract (EE)	00.80 ± 0.035
Total ash (TA)	05.69 ± 0.431
Nitrogen free extract (NFE)	64.14 ± 1.294

Values are mean of triplicates ± SD

Biochemical properties

Table 2 presents the biochemical properties of the calyx analyzed. The total phenol content of methanolic extract was measured at 7.12 mg $GAEg^{-1}$, and total antioxidant capacity at 3.39 mg AAE g^{-1}. A lower value of 2.91 mg GAE g^{-1} has also been reported [27]. The presence of polyphenols in the plants in itself is indicative of the presence of antioxidative function, due to their inherent high redox potentials which make them efficient reducing agents, hydrogen donors and singlet oxygen quenchers [28]. The total antioxidant capacity measured in the methanolic extract was 3.39 ± 0.25 mg AAE g^{-1}. Clear correlations have also been established between antioxidant activity and phenolic content in plant extracts [29]. The results, therefore, reinforce the potential of Roselle as an important source of natural dietary antioxidants. Roselle calyces in the study exhibited an Ascorbic acid content of 0.801 mg g^{-1}; the dietary conversion is 80 mg/100 g edible portion. Duke and Atchley had reported an ascorbic acid content of 14 mg/100g in fresh calyces[6]. Tee *et al.* also established that roselle calyces had higher ascorbic acid content than orange [30]. The calyces in this study exhibited a dietary ascorbic acid value of 80 mg/100 g; this value is higher than the established average values of 42.7 and 43 mg/100 g for orange and papaya, respectively [31]. Roselle calyces are, thereby a nutritionally viable source of food and condiment because of its high antioxidant capacity and an appreciable amount of ascorbic acid content.

Table 2: Biochemical Properties of calyces

Parameter	Value
Total Phenol content (mg $GAEg^{-1}$)	7.12 ± 0.20
Total Antioxidant capacity (mg $AAEg^{-1}$)	3.39 ±0.25
Total Ascorbic Acid content (mg g^{-1})	0.801 ± 0.05

Values are mean of triplicates ± SD

Free radical scavenging assay

The scavenging activity ranged from 89.7% in RD1 to 57.02% in RD5 indicating the concentration-dependent inhibition (Table 3). The standard BHT showed 99.56% inhibition. Antioxidant activity of roselle also represents the free radical scavenging activity which was found to be dose-dependent. The 100 mg/ml concentration of roselle extract exhibited 89.70% scavenging activity compared to 99% standard BHT, thereby indicating high antioxidant potentials. The ethanol crude extract from the dried flowers have capacity of quenching free radical and inhibiting xanthine oxidase (XO)

activity [32]; in animal models, calyces extracts have demonstrated hypocholesterolemic and antihypertensive properties [33].

Table 3: Inhibition/scavenging activity of serial dilution RD1-RD5 of roselle extract, C – Standard BHT contral (n=3)

Dilution	Conc (mg/ml)	% inhibition/scavenging activity
RD1	100	89.70 ± 0.639
RD2	50	63.56 ± 2.132
RD3	25	60.69 ± 1.350
RD4	12.6	58.38 ± 2.286
RD5	6.25	57.01 ± 4.045
C	Std BHT	99.56 ± 0.638

Values are mean of triplicates ± SD

Antimicrobial assay

The MIC of two-fold dilution exhibited a complete growth inhibition of both gram positive (*Staph. aureus*) and gram negative (*E. coli*, *K. pneumonia*) bacteria up to 2nd dilution (25 mg/ml). Numerous colonies (> 300 colonies) were observed from the 3rd dilution onwards indicating non-inhibition of growth (Table 4). There is no growth in methanol control plates while non-treated control plates have numerous colonies present. The antimicrobial effect on both gram positive and gram negative bacteria was dose-dependent as observed by Chau *et al.* against food spoilage bacteria using ethanolic extract [12]. The antimicrobial activity was reported by other workers on isolates from food, veterinary, and clinical samples and suggested that it might be potent agents as food additives to prevent contamination from these bacteria [13].

Table 4: Antimicrobial pattern of various concentration of roselle (n=3)

Concentration (mg/ml)	*Staph. aureus*	*E. coli*	*K. pneumoniae*
100	-	-	-
50	-	-	-
25	-	-	-
12.5	+	+	+
6.25	+	+	+
MC	-	-	-
NTC	+	+	+

+ = growth present, - = no growth. MC = methanol control, NTC = non-treated control

Anti-proliferative assay

The anti-proliferative effect of the extract on the cells was visualized by microscope at high power magnification. The formation of formazan crystal on adding MTT dye was observed (Figure 2). The stimulation index (SI) of roselle-treated cells was calculated (Figure 3). A gradual decrease in SI up to third dilution (@ 25mg/ml) was first observed, which then increased with dilutions. The initial decrease and subsequent increase of SI perhaps correspond to the apoptosis of cells in the different concentration of extract compared to SI of non-treated control which showed non-inhibition of cell growth while the standard mitogen ConA show higher stimulation than PHA. The antiproliferative activities of roselle extract on cervical (HeLa) cancer cell lines showed dose dependent inhibitory effects. Similar observations was reported on its activities on different cell lines like ovarian (Caov-3), breast (MCF-7, MDA-MB-231) and cervical (HeLa) cancer cell lines found that it exhibit the strongest anti-proliferative potency towards the MCF-7 cancer cells [14].

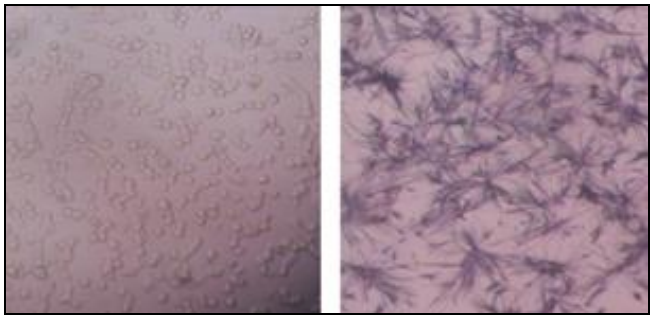

Fig 2: Photograph showing (a) inhibition of HeLa cell growth and (b) formazan crystal formation (20 X)

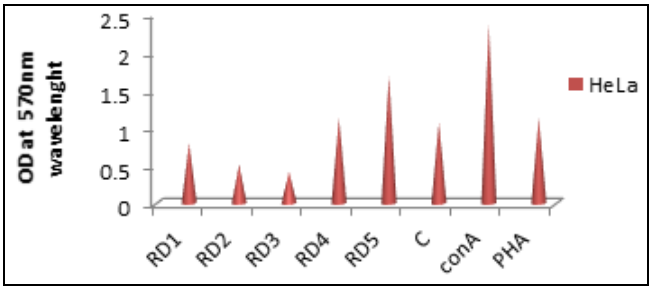

Fig 3: Stimulation index of antiproliferative assay with serial dilution (RD1-RD5) of roselle extract in HeLa cells. C - Non-treated control, Mitogen-Con A and PHA @10mg/ml. (n=3)

Apoptosis in HeLa cells

The fractions of the cells as early apoptosis, late apoptosis, necrotic and healthy cells were calculated in percentage from the total population of cells counted. The result showed the induction of apoptosis is concentration dependent (Table 5) ranges from 51.8% to 9.4 % in decreasing order of concentration in roselle-treated HeLa cells. The apoptotic activity of roselle was found to be concentration dependent in cervical (HeLa) cancer cell lines. Similar report on effects in human cancer cells (HL-60) using roselle-anthocyanins (HA) show apoptosis of cells in a dose- and time-dependent manner [16]. The 6.3% apoptosis in non-treated control cells probably represent the percentage of necrotic cell death. Therefore, the use of roselle calyces in the dietary habits definitely offers benefits in terms of its antioxidant properties, antimicrobial effects, antiproliferative and apoptotic activities against cancerous cells. The other parts of the plant, *viz.* leaves and seeds needs further investigation in this regard.

Table 5: Apoptosis of HeLa cells treated with various concentration of rosells extract (n=3)

Concentration (mg/ml)	Apoptosis (%)
100	51.8 ± 0.6364
50	25.8 ± 0.3536
25	20.5 ± 0.2121
12.5	10.6 ± 0.5657
6.25	9.4 ± 0.1414
Non-treated control	6.3 ± 0.0707

Conclusions

The oral indigenous traditional knowledge on therapeutic uses of traditional foods ingredient have been supported by research studies and documentation. Besides the regular dietary use of roselle in popular delicacies in North east region of India, the current study also reiterates roselle as a source of natural antioxidants and a good and cheaper source of Ascorbic acid, more than most fruits and vegetables. The promising preliminary results with its extracts on cancerous cells studies also provide new avenues for natural therapeutic research on cancer. Therefore, with many enriching chemical-biological knowledge from animal and human models using plant extracts, future focused studies with greater scientific robustness in terms of standardization of dose for its effectiveness, safety and tolerability will permit the formulation of safe, effective therapeutic herbal formulations which can be used as an acceptable source for curing many health issues and restoring general health.

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