Phytochemical and Biological Investigation of *Stevia rebaudiana* (Bert.) Leaves Grown in Bangladesh

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ABSTRACT: Three fractions obtained by Kupchan partitioning of 70% methanol extract of leaves of *Stevia rebaudiana* with *n*-hexane, dichloromethane and ethyl acetate designated by HSR, DSR and ESR were investigated for their antioxidant and antimicrobial activities. HSR and ESR exhibited prominent free radical scavenging activity having IC_{50} value of 11.59 and 10.38 µg/ml compared to standard, ascorbic acid (IC_{50} value 6.05 µg/ml). HSR and ESR also demonstrated significant antibacterial activity by disc diffusion method on some Gram-positive and Gram-negative bacteria. Among the fractions ESR showed the highest antioxidant and antibacterial activity. No significant antifungal activity was observed for any fraction. Preliminary phytochemical screening of all fractions showed the presence of alkaloids, saponin, flavonoids, tannin, carbohydrate and triterpenoids. Total three compounds have been isolated from ESR fraction among which two known compounds (**1-2**) were characterized as quercitrin (**1**) and physcion (**2**) through 1D NMR spectroscopic technique and other one could be a 9,10-anthraquinone derivative which was not characterized yet. The compound physcion (**2**) is first time report from the plant *Stevia rebaudiana.*

Key words: Quercitrin, Physcion, Candy leaf, Antibacterial, Free radical, DPPH, and *Stevia rebaudiana.*

INTRODUCTION

Stevia rebaudiana (Bertoni) commonly known as “sugar leaf” or ‘candy leaf ‘belongs to the family Asteraceae.[1] This perennial sweet herb is distributed in humid and wet environment and is indigenous to Brazil and Paraguay.[2] Stevia is being used as a source of natural sweetener due to its high content in sweet diterpene glycosides, such as isosteviol, stevioside, rebaudiosides (A, B, C, D, E and F), steviolbioside and dulcoside A. About 100 or more compounds have been identified in *Stevia rebaudiana*, the best known of which are the steviol glycosides, particularly stevioside, rebaudioside A and C being the most abundant which are 250-300 times more sweet than sugar[3] and less common bioactive constituents are phenolic compounds and flavonoids like quercetin, luteolin, apegenin.[4,5] Beside glycosides, the leaves of stevia also contain other phytochemicals, such as flavonoids, phenolic acids, fatty acids, proteins and vitamins. Several studies have reported that stevia extracts strived significant antimicrobial, anti-hypertensive, anti-inflammatory, hepatoprotective and immunomodulatory activities, due to its high abundance in bioactive phyto-constituents.[6-8] Now-a-days, stevia is widely cultivated in many countries of South East Asian region including Bangladesh owing to its many health benefits. The phytochemical compositions and bioactivity of any plant and plant-derived products varies greatly with the change of climate and geographical conditions such as soil, water cultivation process etc. In the present time pathogenic and spoilage microorganisms has increased due to the increase outbreaks of several type of new microorganism.[9] To our concern a very few scientific trials on antimicrobial and the antioxidant capacity of

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S. rebaudiana cultivated in Bangladesh has been evaluated. The objective of the present study was to conduct a bioactivity directed phytochemical investigation on *S. rebaudiana*. In this study, antioxidant and antimicrobial activities of different fractions derived from leaves of *S. rebaudiana* will be investigated followed by isolation of active components from the different fractions of crude extract.

MATERIALS and METHODS

Collection of plant materials. The leaves of *Stevia rebaudiana* (SR) were collected from Brac Nursery located in Joydebpur, Gazipur in late March 2018. The exsiccated plant samples were identified by Mr. Sardar Nasir Uddin, Principal Scientific Officer, Bangladesh National Herbarium, Mirpur, Dhaka where a voucher specimen has been deposited for future reference (Accession number: DACB-38588).

Instruments and materials. Column chromatography was conducted on silica gel (60-120 mesh size, E-Merck) whereas thin layer chromatography (TLC) and preparative TLC were carried out on precoated silica gel 60 F_{254} (Aluminium sheets, E-Merk, Germany). ^{1}H and ^{13}C NMR spectral data were recorded on Bruker 400 and 100 MHz, respectively. Ascorbic acid and ciprofloxacin were obtained as gifts from Square Pharmaceuticals Ltd; Bangladesh and DPPH (1, 1-diphenyl-2- picrylhydrazyl) was purchased from Sigma Chemical Co. Ltd (St. Louis, MO, USA).

Extraction. The fresh leaves were shade dried and powdered (1 kg) which were extracted with 70% methanol at room temperature for 15 days. White cotton followed by Whatman filter paper number-1 was used to get clean filtrate. The filtrate was concentrated by using a rotary evaporator to obtain greenish black mass (100g). The crude extract was then fractioned by the modified Kupchan partitioning protocol[10] to yield n-hexane, dichlormethane and ethyl acetate soluble materials of *S. rebaudiana* designated as HSR, DSR and ESR having 8%, 10% and 15% w/w yield, respectively.

Phytochemical screening. Phytochemical analysis of the different extracts was performed with the standard methods outlined by Nayek and Pereira.[11]

Isolation of compounds from ESR fraction. ESR fraction (500 mg) was used to isolate pure components by using column chromatography. At first, the ESR fraction was dissolved in methanol and mixed with small amount of silica gel (mesh size 60-120, 1 g sample) to make dry sample for column chromatography (CC). After complete mixing with spatula, methanol was initially evaporated and finally it was completely dried in a vacuum desiccator. The fine adsorbed sample was then loaded on a clean and dry glass column packed with silica gel (60-120 mesh size) and eluted with ethyl acetate: acetic acid: water (9:0.5:0.5) in order of increasing polarities. Each column fraction (10ml) collected in test tubes was monitored by TLC. Fractions with identical TLC features were combined and a total 10 (A-J) fractions obtained. The pooled fractions were then re-chromatographed by preparative thin layer chromatography (PTLC) using ethyl acetate: acetic acid: water (6:2:2) system to afford yellowish crystals of compound **1** (8 mg), white powder of compound **2** (10 mg) and white crystals of compound **3** (6 mg) with R_f values of 0.3, 0.5 and 0.7 respectively. Structure of the isolates was characterized by ^{1}H-NMR and ^{13}C-NMR spectral data.

Properties of isolated compound. Quercitrin (1): Pale yellow needle crystals, ^{1}H NMR (400 MHz, CD_3OD): δ 7.35 (1H, d, *J*=1.5 Hz, H-2'), 7.30 (1H, dd, *J*=8.0 and 1.5 Hz, H-6'), 6.9 (1H, d, J= 7.5 Hz, H-5'), 6.37 (1H, d, *J*=1.5 Hz, H-8), 6.19 (1H, d, *J*=1.5 Hz, H-6), 5.34 (1H, br. s, H-1''), 0.82 (3H, d, *J*=3.5 Hz, H-6''). ^{13}C NMR (100 MHz, CD_3OD): δ 148.4(C-2), 134.8(C-3), 177.9(C-4), 161.8(C-5), 98.7(C-6), 164.6(C-7), 93.6(C-8), 157.8(C-9), 104.4(C-10), 121.4(C-1'), 115.6(C-2'), 157.2(C-3'), 145.1(C-4'), 115.1(C-5'), 121.6(C-6'), 102.1(C-1''), 70.6(C-1''), 70.7(C-1''), 71.9(C-1''), 70.5(C-5'') and 17.4(C-6'').

Physcion (2). White powder, ^{1}H NMR (400 MHz, CD_3OD): δ 7.75 (1H, m, H-7), 6.96 (1H, d, J= 2.7 Hz, H-5), 6.66 (1H, m, H-4), 6.21 (1H, m, H-2),

5.35 (2H, s, 1-OH and 8-OH), 4.12 (3H, s, H-3), 1.98 (3H, s, H-6).

Antioxidant activity. Antioxidant activity was evaluated by using stable free radical 0.04% DPPH (1,1-diphenyl-2-picrylhydrazyl). Suitably diluted stock solutions were spotted on stained silica gel TLC plate and the plate was kept in the chambers containing solvent according to different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extract. The plate was seared at room temperature and was sprayed with 0.02% DPPH in ethanol. Due to wash out by DPPH, resolved bands were observed for 10 minutes and the color changes (yellow on purple background) were observed.[12]

Stock solution of the crude drugs that means three fraction HSR, DSR and ESR were processed in methanol from which a serial dilution was executed to reach concentration of 1, 5, 10, 50, 100, 500 µg/ml. The new concentration of samples (each 2ml) were mixed with 2 ml of a 0.004% ethanol solution of DPPH and conceded to keep for 30 minutes for reaction to be found. The absorbance was examined at 517nm and from these values the respective percentage of inhibitions were figured. IC_{50} value was calculated from the graph, which was arranged by plotting % of inhibitions against log concentration. The experiment was done 3 times and average absorption was noted for each concentration.[13] Ascorbic acid was used as standard drug.

Antibacterial activity. Disc diffusion method [14] was used for the estimation of antibacterial activity of HSR, DSR and ESR materials against a number of Gram (-)ve *Bacillus cereus*, *B. megaterium*, *B. subtilis*, *Staphylococcus aureus*, *Sarcina lutea* and the Gram (-)ve *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *S. typhi*, *Shigella boydii*, *Sh. dysenteriae*, *Vibrio mimicus*, *V. parahemolyticus*, and three unicellular fungi *Candida albicans*, *Aspergillus niger*, *Sacharomyces cerevacae*. Test samples were prepared using sterile blank discs (6 mm) which were saturated with the test samples dissolved in methanol at concentration of 500µg/disc by a micropipette and used for the assay. After incubation as per the published method, the diameter of inhibitory zones formed around each discs were computed using digital slide calipers.[15] Ciprofloxacin (5µg/disc) and fluconazole (5µg/disc) were used as positive controls for antibacterial and antifungal activities, respectively.

RESULTS

Phytochemical screening. Phytochemical screening of the different fractions of the plant extract by the standard methods revealed the presence of several phytoconstituents such as alkaloids, flavonoids, saponins, tanins and terpneoids as shown in Table 1.

Table 1. Result of phytochemical screening of extracts.

Test sample	Alkaloids	Saponins	Flavonoids	Tannins	Triterpenoids
HSR	+	+	+	+	+
DSR	+	-	+	-	+
ESR	+	+	+	+	+

+ Presence, - Absence

Evaluation of antioxidant activity. Antioxidant activity of the tested fractions was evaluated according to the method specified above and the results are summarized in Table-2. Figure 1 depicts the percent inhibition of DPPH radicals upon increasing concentration of the compounds. The IC_{50} value of HSR, DSR, ESR and ascorbic acid were found to be 11.59, 44.7, 10.38 and 6.05 µg/ml, respectively. Among the extract, ESR showed the most prominent antioxidant property which is comparable to ascorbic acid. HSR and DSR also exhibited moderate antioxidant property.

Evaluation of antimicrobial activity. The antibacterial activity of HSR, DSR and ESR were evaluated at 500µg/ disc on the growth of pathogenic

bacteria and fungi by the disc diffusion method and the results are summarized in Table 3. The crude fractions designated as HSR, DSR and ESR exhibited antibacterial activity with an average zone of inhibitions 10.3-15.3 mm, 6.2 -8.5 mm and 10.2- 16.8 mm, respectively whereas standard ciprofloxacin (5µg/ml) showed 16-23 mm. The results indicated that at the dose of 500µg/ disc ESR and HSR accorded mild to moderate activity against both Gram (+)ve and Gram (-)ve strains than DSR fraction. However, ESR, HSR and DSR did not show any antifungal activity.

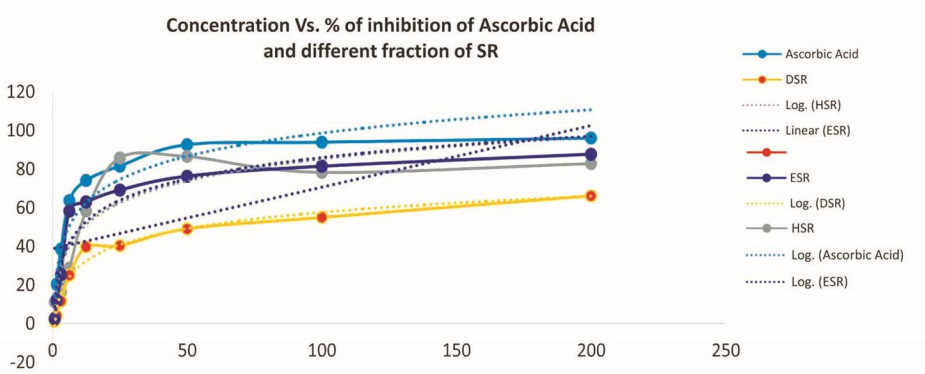

Figure 1. Concentration against % of inhibition of HSR, DSR, ESR extract and ascorbic acid

Table 2. IC_{50} values of HSR, DSR, ESR extract and ascorbic Acid.

Test sample	Regression line	R^2	IC_{50} µg/ml
Ascorbic acid	y = 17.353 In(x) + 18.713	0. 9142	6.05
HSR	y = 16.1336 In(x) + 9.905	0.8551	11.59
DSR	y = 12.092In(x) + 1.8883	0.9785	44.7
ESR	y = 15.92In(x) + 12.615	0.9197	10.38

*Ascorbic acid as standard

Table 3. Antimicrobial activity of the fractions against Gram positive and Gram negative bacteria.

Pathogens	Zone of Inhibition (mm)			
	HSR (500 µg/ml)	DSR (500 µg/ml)	ESR (500 µg/ml)	Ciprofloxacin (250 µg/ml)
Bacillus cereus	10.3 ± 0.21	-	14.2 ± 0.43	18 ± 0.11
B. megaterium	15.1 ± 0.33	-	15.1 ± 0.22	20 ± 0.32
B. subtilis	13.7 ± 0.22	7.1 ± 0.45	13.4 ± 0.33	19 ± 0.23
Staphylococcus aureus	11.1 ± 0.66	-	12.6 ± 0.44	17 ± 0.26
Sarcina lutea	14.2 ± 0.11	8.5 ± 0.29	12.4 ± 0.22	22 ± 0.32
E. coli	13.1 ± 0.23	-	10.5 ± 0.42	16 ± 0.52
P. aeruginosa	10.9 ± 0.43	6.2 ± 0.33	11.3 ± 0.34	17 ± 0.34
S. paratyphi	12.2 ± 0.21	7.4 ± 0.23	16.1 ± 0.21	23 ± 0.44
S. typhi	11.1 ± 0.31	-	10.2 ± 0.22	16 ± 0.44
Sh. boydii	13.3 ± 0.21	6.3 ± 0.23	16.8 ± 0.20	22 ± 0.74
Sh. dysenteriae	15.3 ± 0.66	6.5 ± 0.66	13.7 ± 0.13	18 ± 0.43
V. mimicus	14.7 ± 0.31	-	15.1 ± 0.22	21 ± 0.53
V. parahemolyticus	11.6 ± 0.11	-	13.7 ± 0.12	18 ± 0.23

*values mentioned here Mean ± standard deviation (n = 3)

Isolation of pure compounds from ESR extract. As the ESR fraction exhibited most prominent antioxidant and antibacterial properties, the fraction was subjected to isolation of active constituents. A total of three compounds (Figure 2) were isolated from ESR fraction of SR by column chromatography and PTLC. The high field NMR analysis was used for elucidation of structure of the isolated compounds and compared with published NMR data. The ^{1}H- NMR spectrum (400 MHz, CD_3OD) of compound **1** revealed a pair of meta-coupled aromatic doublet at δ 6.37 ppm and 6.19 ppm corresponding to H-6 and H-8 protons, respectively and an ortho-coupled double doublets at δ 7.30 ppm (J = 8.0 and 1.5 Hz) for H-6′ protons. Another meta-coupling was observed between the protons of H-2′ and H-6′ at 7.35 and 7.30 ppm, respectively. The compound **1** was composed of quercetin, a flavonoid which linked to rhamnose, in the position of C_3 via C-O-C bonding. The ^{13}C NMR spectrum of compound **1** displayed carbonyl group at 177.9 ppm and other signals between 93.6- 164.6 ppm for aromatic carbons. The ^{1}H NMR and ^{13}C NMR spectral data of compound **1** was compared to reference value of quercitrin and found to be identical.[16,17] Thus, compound **1** was identified as a flavonoid glycoside, quercitrin.

Compound **2** is a natural pigment, gave intense benzoid absorption band on the UV region ranges between 240-260 nm.[18] Compound **2** displayed two pairs of meta-coupling proton at the position of H-7 & H-5 and H-2 & H-4, respectively. Additionally, it showed a singlet for methoxy group at C-3 and a singlet at 5.35 ppm for hydroxyl group corresponding to H-1 and H-8 which is resemblance to physcion and confirmed by comparison with previous report.[19] On the other hand, the unknown compound gave two peaks only among which a singlet at 4.98 ppm and another singlet at 1.91 with integrating 3H. All these features indicated that unknown compound may also be a 9,10-anthraquinone derivative which is still under investigation. Thus, the two isolated compounds were characterized as quercitrin (**1**) and physcion (**2**). This is the first report of isolation of compound **2** from this plant.

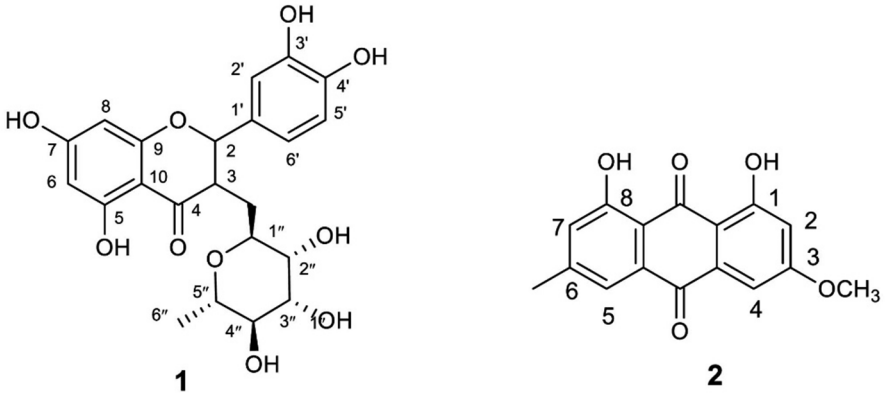

Figure 2. Structures of quercitrin (1) and physcion (2) isolated from *S. rebaudiana*

DISCUSSION

In this study, the plant extracts were fractionated into three major fractions namely HSR, DSR and ESR fractions. These fractions contained major phytoconstitutents which might be responsible for their activities. All the fractions exhibited antioxidant property. Antioxidant activity of the extracts depends on the ability to scavenge DPPH radical. DPPH stable free radical method is a most common method to determine the antioxidant activity of plant extracts.[20,21] Many disorders like neurodegenerative diseases, cancer and AIDS are associated with over activity of free radicals and it is accepted that antioxidants due to their scavenging properties are

useful for the management of those disease. Among the extracts, HSR and ESR provided promising antioxidant activity compared to standard ascorbic acid. The capability of *S. rebaudiana* extracts to scavenge DPPH could be accredited to the content of phenol, flavonoids as well as alkaloid as accentuated in several reports.[22, 23] Redox properties is mainly responsible for this activity which could be due to adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides.[24] *S. rebaudiana* extract could play a vital role as a human diet to improve antioxidant intake. [25] As it was found that ESR fraction showed most prominent antioxidant effect, it could be rationalized that the flavonoids and anthraquinone derivatives (compound **1-2**) isolated from the fraction might be responsible for antioxidant activity because antioxidant is the common biological effects of both edible and non-edible plants containing flavonoids and phenolic compounds.[26] Moreover, a study about the relationship of anthraquinone derivatives and their antioxidant activities concluded that hydroxyl groups either at meta or para position are responsible for the antioxidant activity.[27] It has also been reported that not only the hydroxyl group, but also the carbonyl group of anthraquinone skeleton is responsible for the antioxidant potential.[27]

The drug from plant sources act as a prototype to produce less toxic and more effective medicines against the growth of microorganism. [28] It is worthwhile to note that there are very few research works that have been done for antimicrobial activity of *Stevia rebaudiana*. In this study, we isolated three components quercitrin, physcion and an unidentified 9,10-anthraquinone derivatives from ESR fraction and this fraction showed prominent antioxidant and antibacterial activities than other fractions. It has been shown that the increase in concentration of quercitrin in culture media increase the linear value of inhibition zone. Quercitrin, a flavonoid, isolated from *Dendrophthoe pentandra* (L.) leaves is known to exhibit prominent antioxidant and antibacterial activities.[16] Many reports have already explained the antibacterial and antifungal activities of anthraquinone isolated from natural sources. Two anthraquinones identified as emodin and physcion isolated from *Vintilago madraspatana* revealed antibacterial activity against three species of *Bacillus*.[29] Therefore, It is well accepted that chemical compounds present in *S. rebaudiana* such as anthraquonone, alkaloids, flavonoids, tanins and triterpenoids play an important role in its antibacterial activity *in vitro*.[30] As the crude drugs are complex in nature, all components might be synergistically or cumulatively responsible for their antibacterial activity.

CONCLUSION

In conclusion, three fractions (HSR, DSR and ESR) from 70% methanolic extract of *S. rebaudiana* were evaluated for antioxidant and antimicrobial properties. HSR and ESR fractions of the plants have demonstrated significant antioxidant and moderate antibacterial activities. Phytochemical investigation of ESR fraction provided three compounds (**1, 2** and unidentified one). Among the three compounds, physcion is isolated first time from the plant *S. rebaudiana*. The plant material can be further studied extensively to isolate novel natural compounds by considering the potential bioactivity and scope which may persuade new drug development.

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