Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2021; 10(4): 303-306
Received: 19-05-2021
Accepted: 23-06-2021

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Antioxidant and cytotoxic study of extract of vegetative and flowering stages leaves *Stevia rebaudiana*

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Abstract
This study was performed to determine the anti-oxidant and in-vitro cytotoxic activity of *Stevia rebaudiana* plant extract collected during vegetative and flowering stages of plant against the commercial sweetener and Stevioside and Rebaudioside A standards. The anti-oxidant activity was determined by α, α-diphenyl-β- Picryl hydrazyl (DPPH) and Ferric reducing activity potential (FRAP) assay and cytotoxic activity by MTT assay. Flowering phase extract of Stevia showed greater antioxidant activity in FRAP assay. The *in-vitro* study was performed on 3 cell lines 3T3 cell line (murine), RAW cell line (murine) and MDA MB231 human cell line where the flowering stage extract showed 55% inhibition in cell growth.

Keywords: anti-oxidant, *in-vitro* cytotoxic, *Stevia rebaudiana*, vegetative, flowering, DPPH, FRAP, MTT, 3T3, RAW, MDA MB231 cell line

1. Introduction
Increasing prevalence of diabetes in the world population has necessitated the need for developing natural plant-based sweeteners that can confer both safety and efficacy in terms of diabetes management. A group of compounds called Steviol glycosides (SG's) isolated from *Stevia rebaudiana* has garnered attraction as a natural sweetener.
Stevia rebaudiana, is a perennial shrub of Asteraceae family (Rizzo B. *et al*., 2013) [1]. It is found in parts of Brazil, Paraguay and Argentina. Stevia gained popular interest due to its sweetening ability and calorie free nature (Rizzo B. *et al*., 2013) [1]. Among all the known SGs, Rebaudioside- A (Reb-A) is better suited for commercial use due to its pleasant taste. It is believed that Rebaudioside is up to 400 times sweeter than sucrose. *Stevia rebaudiana* is a natural alternative to artificial sweetener and found to contain over 100 phytochemicals including well characterised stevioside and rebaudioside A. (Ghosh S. *et al*., 2008) [4]. It is also rich source of terpenes and flavonoids and has high antioxidant properties. Researches have also shown antimicrobial and antitumoral properties of the Stevia plant extract. It has found its applications in beverages, energizers as well as medicinal uses such as low uric acid treatment, vasodilator, cardio-tonic, anaesthetic and anti-inflammatory (Jayaraman *et al*., 2008) [2].
This plant is a short day (SD) plant and starts flowering as soon as it gets SD conditions. As the flowers begin to develop, the leaves start to lose their quality and irreversibly become bitter. Harvesting time thus becomes the critical factor influencing the quality of Stevia (Tavarini S. *et al*., 2013) [3]. So, it is important to find alternative value addition product from this to make it sustainable for farmers.
This study was taken up to compare the antioxidant activity of commercial stevia sweeteners and the leaf extract during the vegetative and the reproductive phase of plant. The comparative cytotoxicity of the leaf extracts, Stevioside and RebA was also done. This will allow us the development of the other value addition application product of the Stevia after extraction of sweet taste imparting compounds from the leaves. Besides, it will benefit farmers in case of harvest loss due to flowering.

2. Materials and methods
A. Antioxidant studies
Collection and preparation of plant material
Leaves of *Stevia rebaudiana* were collected from the field plot in Mumbai during different phases of plant i.e., vegetative and reproductive. The leaves were washed with distilled water, and then dried under shade. The dried leaves were crushed and extracted in methanol over a rotary shaker at 100 RPM. To ensure maximum extraction, the process was repeated twice.

The solvent was then evaporated under reduced pressure using a rotary evaporator to obtain extracts. The extract obtained was further assayed for the antioxidant and antimicrobial tests. Commercially available Steviol glycosides sweeteners were used for comparison with the crude extract by dissolving in methanol at known concentration.

DPPH assay

The DPPH free radical scavenging activity of methanolic extracts of Stevia leaves and SGs were determined. The assay is based on the measurement of the scavenging capacity of antioxidants towards α, α-diphenyl-β- Picryl hydrazyl (DPPH). Known concentration dilution of plant extracts were prepared. 500µl of extract was mixed with 500µl of DPPH and incubated for 45 minutes in dark. The absorbance was measured at 517 nm. The percent antioxidant or radical scavenging activity was calculated using the following formula:

$$\%\text{Antioxidant activity} = [(Ac - As)/Ac] \times 100$$

Where Ac and As are the absorbance of control and sample, respectively. The control contained 500µL methanol in place of the plant sample.

Ferric reducing activity potential (FRAP) assay

FRAP assay performed to check the antioxidant potential of plant. Different concentrations of the methanolic extract were prepared by adding 0.2 M sodium phosphate buffer (pH 6.6). To 500 µl of the extract, 500 µl of 1% potassium ferricyanide [K3Fe (CN)6] solution was added. The reaction mixture was vortexed and incubated at 50°C for 20 min. At the end of the incubation, 500 µl of 10% Trichloro Acetic Acid (TCA) was added to the mixture and centrifuged at 3,000 rpm for 10 min. The supernatant 500µl was mixed with 500µl of deionised water and 100µl of 0.1% ferric chloride (FeCl3). The coloured solution was read at 700 nm against the blank with reference to standard using UV Spectrophotometer. Ascorbic acid was used as a reference standard, the reducing power of the samples were reported as amount of ascorbic acid in the test samples.

B. Cytotoxicity studies

Extract preparation

Methanolic extract of Stevia leaves was used for the cytotoxicity assays. 4 samples were analysed on 3 cell lines.

Samples

1. Stevia leaf: Vegetative phase
2. Stevia leaf: Flowering phase
3. Stevioside
4. RebA

Different dilutions 10 mg/mL, 5 mg/mL, 2.5 mg/m, 1.25 mg/mL, 0.625mg/ml and 0.312 mg/ml were prepared in media.

Cell lines

1. 3T3
2. RAW
3. MDA MB231

3T3, RAW and MDA-MB-231 cells were cultured in DMEM containing 1% Pen-strep and 10% fetal bovine serum. Culture were trypsinized and seeded in microtiter plate at 104 cells/mL/well concentration.

MTT assay

The assay to determine cytotoxicity potential of Stevia extract was conducted by MTT assay. 3T3, RAW and MDA MB231 were seeded separately at 104 cells/mL/well in sterile 96-well microtiter plates. Cells were incubated overnight at 37 C0 and 5% CO2 at 98% humidity till they reached log phase of their growth curve.

The 10 mg/mL extracts were serially diluted to concentrations 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.625mg/ml and 0.312 mg/ml. 10 µl of extract was added to 90 µl of media in each well. Three replicate wells were used per concentration. The treated cells were then incubated for next 24 h at 37 C0 and 5% CO2. After incubation, 5 µl of 5 mg/mL MTT was added to each well. The cells were again incubated at 37C0 and 5% CO2 for 4 h. media was then aspirated from each well after which 100 µl DMSO was added to each well. Absorbance was read by using the ELISA plate reader at 570 nm. The concentration required to kill 50% of the cell population or IC50 was computed by using linear regression of the graph of absorbance against Concentration.

3. Results and discussion DPPH and FRAP

This study was undertaken to see what form of Stevia is better for human consumption: whole leaves or commercial Stevia sweeteners? What value addition can be done once Steviol glycosides are isolated?

When screened for their antioxidant potentials by DPPH and FRAP assay, it was observed that leaves from both vegetative and flowering phase of the plant were rich in antioxidants and gave good antioxidant activity. The commercial sweetener had negligible antioxidant potential. Results reveal that it is better to consume leaves in decoctions to gain the advantage of its antioxidant nature or the compound responsible for antioxidant activity can be isolated and used for value addition in nutraceuticals.

Flowering stage leaves showed higher Ferric reducing property than the Vegetative stage leaves. The reported decline in Steviol glycoside profile during flowering stage leaves the plant of no use once flowering is initiated. The plants are pruned for next harvest. The leaves can be used be used for the isolation of antioxidants.

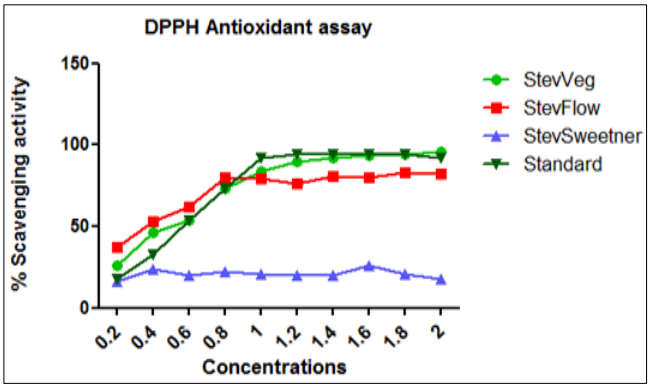

Fig 1: Antioxidant activity by DPPH method of different Stevia extracts and commercial sweetener

(Stemflow- Stevia Flowering phase extract; SteVeg- Stevia vegetative phase extract; StevSweetner-Commercially available Stevia sweetener.)

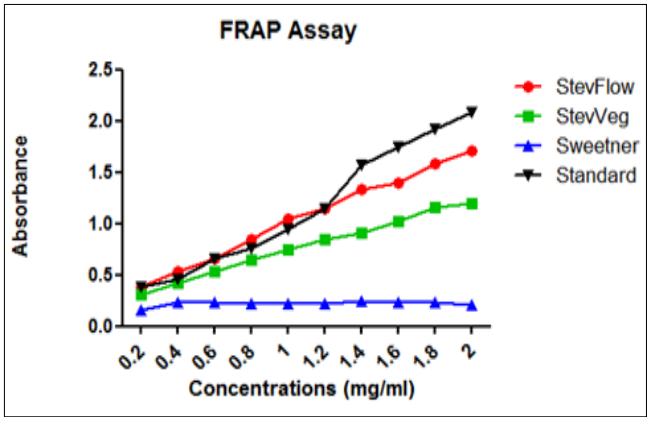

Fig 2: Antioxidant activity by FRAP method of different Stevia extracts and commercial sweetener

(Stemflow- Stevia Flowering phase extract; SteVeg- Stevia vegetative phase extract; StevSweetner- Commercially available Stevia sweetener.)

***In Vitro* cytotoxicity studies**

Due to the side effects of the chemically synthesized chemotherapeutic agent as anti-cancer drug, pharmaceutical companies have been searching natural products obtained from plants that can be potent sources presenting promising results as potential anti-cancer agents. One of the most common cancer Breast cancer has widely been studied. Stevia extract has been widely studied for its potential use as pharmaceutics against many ailments. Many cancerous lines have been screened with Stevia extract.

However due to lack of any report on Stevia in MDA MB 231, this study aimed to investigate the anti-proliferative, cytotoxic effects of *Stevia rebaudiana* leaf extracts during vegetative and flowering phase on metastatic MDA-MB-231 breast cancer cell lines.

Cell viability assay on 3 cell line was carried out, with a range of stevia extracts (Leaf extract from Vegetative phase, Leaf extract from Flowering phase, Stevioside and Rebaudioside A at concentrations (1 – 0.03125 mg/ml), over a 24-hour period.

- 3T3 Cell line (murine)
- RAW Cell line (murine)
- MDA MB231 Cell line (Human)

On 3T3 and RAW cell line no cytotoxic effect of Stevia extracts, Stevioside and RebA was observed. In RAW cell the viability increased up to 300 % in the presence of Stevia extract. In experiments with MDA MB231, leaf extract from reproductive phase i.e., flowering phase (Sflo) showed 55% inhibition in cell growth at 1000 µg/mL. Whereas all the other extracts did not show much variation in their activity and no IC 50 was obtained as cell viability did not fall below 70% (see fig.3). These results demand further investigation in the chemotherapeutic potential of Stevia against this cell line. Stevia leaves extract from different geographical region and different growth period needs to be screening and studied in detail.

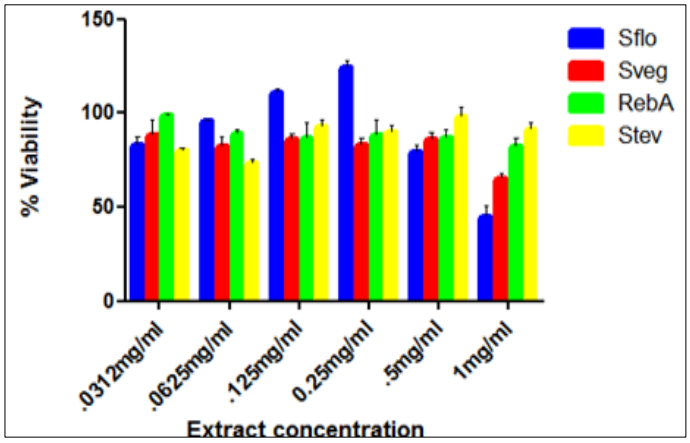

Fig 3: Cytotoxicity data of different Stevia extracts against MDA MB 231

Sflo- Stevia Flowering phase extract
Sveg- Stevia vegetative phase extract
RebA- Rebaudioside A
Stev- Stevioside

4. Conclusion

This study compared the antioxidant activity of *Stevia rebaudiana* leaves collected during the vegetative and reproductive phases with the commercially available stevia products. This plant can be used as a good antioxidant agent in various applications. The antioxidant activity of the crude extract is much higher than the antioxidant activity found in the commercial sweetener products. Results revealed that it is better to consume leaves in decoctions to gain the advantage of its antioxidant nature or the compound responsible for antioxidant activity can be isolated and used for value addition in different preparations. Cytotoxicity studies revealed that the flowering stage extract of stevia showed greater inhibition than the other extracts (see fig 3). Thus, it can be used as a potential chemotherapeutic agent against the MDA MB 231 cell line, though further research needs to be done for the same.

5. Acknowledgement

We would like to thank the Department of Science (DST) for funding the project. We sincerely express our gratitude towards the Department of Biology and Scientific Research Centre (SRC) for supporting us for completion of the project.

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