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Analysis Of The Anti-Oxidant And Cell Cytotoxicity Properties Of Stevia Rebaudiana Extract By Using In- Vitro Model

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Abstract

Leaves of *Stevia rebaudiana* are rich source of sweet glycosides of steviol. The major glycoside, stevioside, diterpenoid glycoside--is used in India and various countries as a food sweetener for diabetic patients. Its medical use is also reported in various ailments like heart, liver, pancrease etc. Besides this it is also being used against obesity, stomach burn and to lower uric acid levels in blood. In present study the leaf extract with various extract were tested for immunomodulatry properties. We performed MTT assay to check the cell viability and cytotoxicity in peripheral blood mononuclear cell (PBMC) and also calculated the Trolox equivalent values to assess the antioxidant properties of Stevia *rebaudiana.* . Percent cell viability values of cells were found to be more than 50 percentages with increase in concentration. At higher dose all these extract showed significant inhibitory effects on proliferation. The aqueous extracts shown the maximum value of Trolox equivalent with 1:16 dilution. It means with the increase in the concentration of extract there is substantial increase in antioxidant property or we can assume antioxidant is dose dependent. But in same time cell viability gets reduced with increase in concentration.

Keywords: stevia rebaudian, antioxidant, MTT assay, Soxhlet extractor

Introduction

Stevia is a natural sweetener and sugar substitute derived from the leaves of the plant species Stevia rebaudiana, native to Brazil and Paraguay (**Arumugam *et al***). Stevia belongs to *Asteraceae* family and is related to ragweed and daisy. It is commonly called as candy leave in its native place – Texas, New Mexico, and Arizona. For hundred of years, Brazilian and Paraguans have been using Stevia rebaudiana as sugar in their food. Stevia has natural sweetening and medicinal properties used for diabetes, helps in weight control, help to reduce blood pressure, stevia does not cause allergy and reduces the risk of pancreatic cancer (**Ajami**

et al). The sweet taste of stevia is due to the presence of glycosides - steviol. The glycosides present is basically a diterpene glycoside which is neither broken or absorbed by our digestive tract. Due to this the glucose level in blood remains unaffected even on high intake of stevia. Apart from being much more sweeter than regular sugar, it is non-toxic or mutagenic when compared to synthetic sugar. For diabetic patients the uptake of 2mg/kg/bw is prescribed of stevia **Prakash *et al.*, 2017; Abo Elnaga *et al.*, 2016; Sharma *et al.*, 2016**). It is approved by WHO (World Health Organisation) and FAO (Food Agriculture Organisation). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compound (**Siddique *et al.* 2014**) The documented properties of Stevia are anti-bacterial, anti- fungal, anti-inflammatory, anti-microbial, anti-viral, anti-yeast, cardio-tonic, diuretic, hypoglycaemic, hypotensive and as a vasodilator (**Goyal *et al.* 2010**) It also has a healing effect on blemishes, wounds, cuts and scratches. Not much work is available in the knowledge database about *Stevia redaudiana*, as much of the work is focused on organic solvent based extraction. So, here in our work we investigated the phytochemical composition of stevia.

Stevia is widely grown for its leaves, from which extracts can be manufactured as sweetener products known generically as stevia and sold under various trade names (**Cardello *et al.* 1999**). Immunomodulation is process by which the immune function of an organism can be altered as it interferes with its basic mechanism. This may lead to either suppression or stimulation of immune system. Immunomodulators are the agents of this phenomenon. They regulate the immune system in a way that it optimizes the immune response. Stevioside was found effective in increasing phagocytic activity, haemagglutination antibody titre and delayed type hypersensitivity. In parallel, stevioside substantially increase proliferation in the LPS and Con A stimulated B and T cells, respectively (**Sehar *et al* 2008**). The present study deals with the assessment of immunomodulatory properties of local stevia plant leaves extract by using human lymphocyte.

2. Material and Method:

2.1.1. Collection and preparation dried powder of plant material

Different parts of the Stevia *rebaudiana* was collected from SFRI (State Forest Research Institute), Jabalpur, Madhya Pradesh India. Drying of the stem and leaves was done in the shade for a week until a stable weight is achieved. Using mechanical grinder dried part was converted to fine powder. Before storing in an air tight container the powder was sieved using a 100 µm mesh for constant particle size.

2.1.2. Extraction of chemical constituents fom plant material

Different organic solvents were tried for the extraction of plant.

1. Aqueous Extract-

10gm of sample was weighed and added to 250 ml of cold distilled water and incubated for 48 hours. The solution was stirred in regular intervals over magnetic stirrer. After incubation the solution is filtered through muslin cloth and residue is again subjected to above process for complete extraction. The entire filtrate was passed through filter paper and concentrated in water bath at 40°C. The extract was stored at 4°C. the residue was dried overnight in hot air oven (40°C) and stored in dark for further process.

2. Methanol Extract-

The obtained dried residue was subjected to methanolic extraction. A filter paper is rolled (thimble) and residue is placed inside it. Thimble was then placed in Soxhlet extractor arm. Reservoir was filled with methanol and Soxhlet was operated at constant temperature of 35°C. The Soxhlet was run for 10 cycles and then solution was filtered using Whatman filter paper no. 1. The residue was dried in hot air oven at 40°C.

3. Ethyl acetate extract-

The residue is further treated with ethyl acetate. Residue was placed in thimble and kept in Soxhlet filled with ethyl acetate and heated at constant temperature of 30°C. Six cycles were processed. Solution was filtered and residue was dried overnight.

4. Petroleum ether extract-

Further the residue was placed in thimble and kept in Soxhlet at 25°C having petroleum ether. Six cycles were run. Solution was filtered and residue was dried.

2.1.3. Phytochemical screening test:-

1. Qualitative tests for primary metabolites:-

(a) Test for carbohydrates-

(i). Molisch's test- To determine the presence of starch in solution, 1 ml of plant extract was mixed with 0.4 ml of Molisch reagent and then 1 ml of concentrated H_2SO_4. Molisch reagent was prepared by mixing 15g of α-naphthol in 100 ml of ethanol. A purple ring formed at the junction of two liquids.

(ii). Benedict's test- For identifying reducing sugar, 1 ml of extract was treated with 1 ml of benedict's reagent and heated for 5 minutes. An orange precipitate was seen.

(iii). Fehling's test- Plant extract was also subjected to Fehling's test to identify the presence of glucose. 2 ml of extract was mixed with 2 ml of Fehling's solution and heated for few minutes. Formation of reddish brown precipitate confirmed the presence of glucose.

(b) Test for protein –

For testing presence of protein, extract was subjected xanthoproteic and biuret test. Both the tests showed positive results.

(c). Test for lipids –

Glycerol one of the base molecule for lipid synthesis was assessed by mixing plant extract with 1% $CuSO_4$ and few drops of NaOH. A clear blue solution was obtained confirmed the presence of glycerol. Sudan test was also conducted that gave positive result.

2. Qualitative test for secondary metabolites –

(a). Test for Alkaloids - Alkaloids are nitrogen containing compounds found in plants. To assess alkaloids the plant extract (about 1 ml) was mixed with 1.5% HCl (about 2 ml) and filtered and divided into 3 aliquots.

(i). Mayer's test- Freshly prepared mayer's regent was used. Equimolar amount of (1:1)Mayer's reagent and extract was mixed. A white precipitate was formed indicating the presence of alkaloids

(iii). Wagner's test- With prepared Wagner's reagent extract was mixed which formed brown ppt, indicating the presence of alkaloids

(iv). Dragendroff's test- 1 ml of extract was mixed with 2 ml of dragendroff's reagent which produced orange white precipitate which is a positive test.

(b). Test for saponins - To test for saponins, 1 ml of extract is mixed with small quantity of water and then sodium bicarbonate is added. Foam ws formed indicating the presence of saponins

(c). Test for Flavonoids - For assessing flavonoids, 0.5 ml of extract is ixed with 1 ml of conc. H2SO4 and 5 ml of NH3. The disappearance of yellow color shows positive result.

(d). Resins – Resins which are volatile terpenes are produced by plants. To check its presence 0.5 ml of extract was mixed with 2 ml of acetic acid and few drops of H2SO4. Initially a purple color ws obtained which turned to violet indicating the presence of resin.

(e). Tannins –

Tannins a polyphenolic compound present in plants is assessed by mixing of dried extract in water and filtered. The filterate is used as

(i). Gelatin solution test- 1% of gelatin is mixed with extract which shows formation of white precipitate, indicating a positive result.

(ii). Lead acetate test- A white precipitate is obtained on mixing filterate with CH3COOPb, showing the presence of tannins.

(iii). Ferric chloride test- Blue green color is obtained on mixing 5% ferric chloride with filterate. It is a positive test.

(f). Test for Sterols – Salkowski test is used for testing the presence of sterols. Sterols are lipids. For testing a solution of chloroform and conc. H2SO4 (1:1) is mixed with 0.5 ml of extract. The mixture is shaken vigorously to develop a red color which indicates the presence of lipids.

(g). Cardiac glycosides – Keller killiani test is conducted for testing the presence of cardiac glycosides the extract was evaporated to get the residue. The residue is mixed with little amount of water. A drop of ferric chloride is mixed with glacial acetic acid. This mixture is mixed with extract in water and H2SO4 is added. Formation of brown ring at the junction indicates the presence of deoxy sugar characteristics of cardiac glycosides.

(h). Triterpenes – Few grams of extract was mixed with 5ml of chloroform and heated at 40°C for 30 minutes. Few drops of conc. H2SO4 is added, which gives red color. It is confirmatory test for the presence of triterpenes.

2.1.5 Lymphocyte isolation and Cell Counting

Make a 1:1 dilution of whole blood in physiological saline. Aseptically transfer 2 ml of HiSep™ (LSM1077; Himedia) to a 15 ml clean centrifuge tube and overlay with 6 ml of diluted blood. The ratio of LS001 to diluted blood should be 1:3 (do not mix). The quality of the separation is dependent upon a sharp interphase between lymphocytes and the solution. Centrifuge at 1000x g, at room temperature (25°C) without brake for 30 mins. After centrifugation band of mononuclear lymphocytes formed above the HiSep solution. Discard by aspirating most of the plasma and platelet containing supernatant above the interface band (granulocytes and erythrocytes will be in the red pellet). Using pipette carefully aspirate the mononuclear cells and transfer it to a clean centrifuge tube. Counting of live and dead lymphocyte was done by using dye tryphan blue with haemocytometer.

2.1.7 Cytotoxic activity assays

Multiple procedures are available for determination of cell proliferation and cytotoxicity. Simple and cheap methods for estimating cell viability (or death) are Trypan Blue exclusion. However, these methods are not sensitive enough and cannot be used for high throughput screening. The cytotoxic effects of Stevia extract on human blood lymphocyte were determined by using two methods, the direct counting of living and dead cells using haemocytometer with inverted microscope ant by MTT assay (**Talib and Mahasneh 2010.**

Cytotoxicity was assayed by MTT method. It is based on the fact th MTT salt is reduced by mitochondrial enzymes dehydrogenases into a colored formazan product which is insoluble, which is an evidence of prove for cell viability. Formazan can be measured spectrophotometrically (**Mosmann, 1983**).

MTT powder dissolves slowly in the buffer with final concentration of the resulting solution is 5mg/ml. Lymphocyte cells were seeded in a cell culture flask in an amount appropriate for the assay and incubate at 37°C in a 5% CO_2 environment. Allow the cells to grow for up to 24 hours or till confluence is reached. Before going for the test pre-assay optimization procedure needs to be performed once for each cell line to determine optimum plating density and incubation time. Harvest the cells. Adjust the cell density to 1×10^6 cells/ml. Serially dilute the cell suspension from 1×10^6 to 1×10^3 cells/ml using appropriate culture medium (DMEM; Himedia). Seed 100µl of each dilution in 96-well microtiter plate in duplicate. Add medium control in duplicate. Incubate the cells in dark at 37C for 4 hrs. Add 10µl of MTT to each well including controls. Wrap the plate with aluminium foil to avoid exposure to light. Return the plate to the incubator for 2 to 4 hours. Observe the cells at periodic intervals under an inverted microscope for presence of needle-shaped crystals. Slow growing cell lines require longer time to develop formazan crystals. After incubation period, add 100µl of solubilization solution to each well. Stir gently on a gyratory shaker to enhance dissolution of the crystals. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm with a reference wavelength than 650nm. Determine the average values from duplicate readings at 570nm and subtract from this value the average value for blank and average value at the reference wavelength. Plot absorbance against cell density. Number of cells to be used in the cell proliferation assay should lie within linear portion of the plots

2.3 ANTIOXIDANT ASSAY -

The trolox equivalent antioxidant capacity (TEAC) assay was first developed as a simple and convenient method for total antioxidant capacity (TAC) determination. This test is based on ability of antioxidants in the sample to reduce copper (II)-chromogen oxidant complex. Absorbance of the reduced copper(I)-chromogen complex is indicative of antioxidant capacity of the test sample (EZAssayTM Antioxidant Activity Estimation Kit (CUPRAC). Prepare chromogenic substrate in an amber-coloured bottle by using given combination in kit. To prepare the the cell lysate collect 2×10^7 cells/ml in Phosphate Buffer Saline. Sonicate 3 times with 5 second intervals at 40V over ice. Use whole homogenate for the assay. For preparation of standard curve serially dilute 2500µM standard solution in 1:1 ratio to obtain 1250µM, 625µM, 312.5µM, 156.25µM, 78.13µM, 39.06µM solution. Label the 96 well plate with different concentrations of standard, control, sample and blank in duplicate. Add 100µl of deionized water to each well. Add 10µl sample to sample well, 10µl standard to standard well and 10µl deionized water to blank well. Add 100µl chromogenic to each well and incubate for 10 minutes in dark at room temperature. Measure the absorbance at 450nm using microplate reader.

3. Results

3.1 COLLECTION OF PLANT MATERIAL

Plant material (Stevia *rebaudiana*) was collected from SFRI (State Forest Research Institute), Jabalpur.

3.2DRYING THE PLANT MATERIAL –
Plant material was shade dry and took 5 days to dry till a constant weight was achieved.

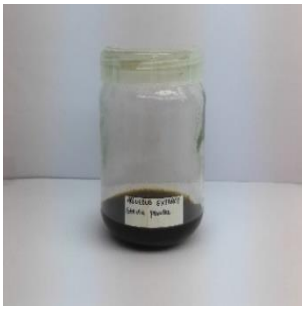
3.3 GRINDING AND SIEVING OF PLANT MATERIAL –
The plant material was grinded using pestle-mortar and sieved through 100 μm mesh sieve.

A. Plant of Stevia

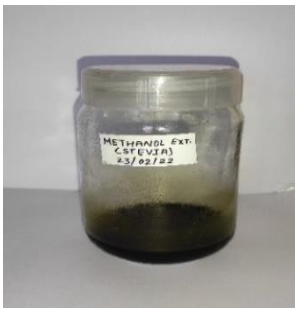
B..Drying of Stevia

C. Sample after

D. Aqueous



E. Aqueous extract



F. Methanol extract

G. Ethyl acetate

H. Petroleum ether

Fig1. Plant Material and their various extract

3.4 EXTRACTION OF CHEMICAL CONSTITUENTS IN PLANT MATERIAL –
Extraction of plant material was done through Soxhlet Extractor using 4 different solvents. Aqueous Extract – Cold percolation method was used to extract the aqueous extract followed by the filtrates which was kept in refrigerator for further use. Methanol Extract – Methanol extraction was done by Soxhlet extractor. Ten cycles was performed to get the desired concentration of the solvent. Ethyl acetate Extract – Ethyl acetate extraction was done similarly as of Methanol. Six complete cycles was performed of the solvent. Petroleum ether Extract – Five cycles of extraction was performed same as of above.

3.5 Phytochemical screening of plant Stevia rebaudiana

S.No	Primary metabolite	Aqueous extract		Methanol extract	Ethyl acetate extract	Petroleum ether extract
1.	**Carbohydrate**					
	A. Molisch's test	-		-	+	+
	B. Benedict test	-		-	-	-
	C.Fehling's test	+		-	-	-
2.	**Protein**					
	Xanthoproteic	-		-	-	-
	B. Biuret	-		-	-	-
3.	**Lipid**					
	A. Solubility					
	B. Glycerol	-		-	+	+
	C.sudan	-		-	+	+

	Secondary metabolite					
4.	**Alkaloids**					
	A. Mayer's test	-		+	-	-
	B. Dragendroff's test	-		-	+	-
	C.Wagner's test	-		-	-	-
5.	**Saponins**					
	A. Foam test	+		+	-	-
6.	**Flavionoids**	-		+	-	-
7.	**Resins**	-		-	-	-
8.	**Tannins**					
	A. Gelatin test	-		+	-	-
	B. Lead Acetate	+		-	-	-
	C.Ferric chloride	-		-	-	-
9.	**Sterols**					
	Salkowski	+		+	--	-
10.	**Cardiac glucosides**					
	Keller-Kiliani	-		-	-	-
11.	**Triterpenes**	-		-	-	-

Table 1. Table showing the result of phytochemical screening of primary and secondary metabolites of plant sample (Stevia rebaudiana). (+) positive sign shows the presence of metabolites in Stevia *rebaudiana* (-) negative sign shows the absence of metabolites in *Stevia rebaudiana*.

3.6 Cell Cytotoxicity Direct Count Method –

In direct method we have calculated percentage viability by using given formula.

Percentage viability= Total number of viable cells/ Total number of cells seeded x 100.

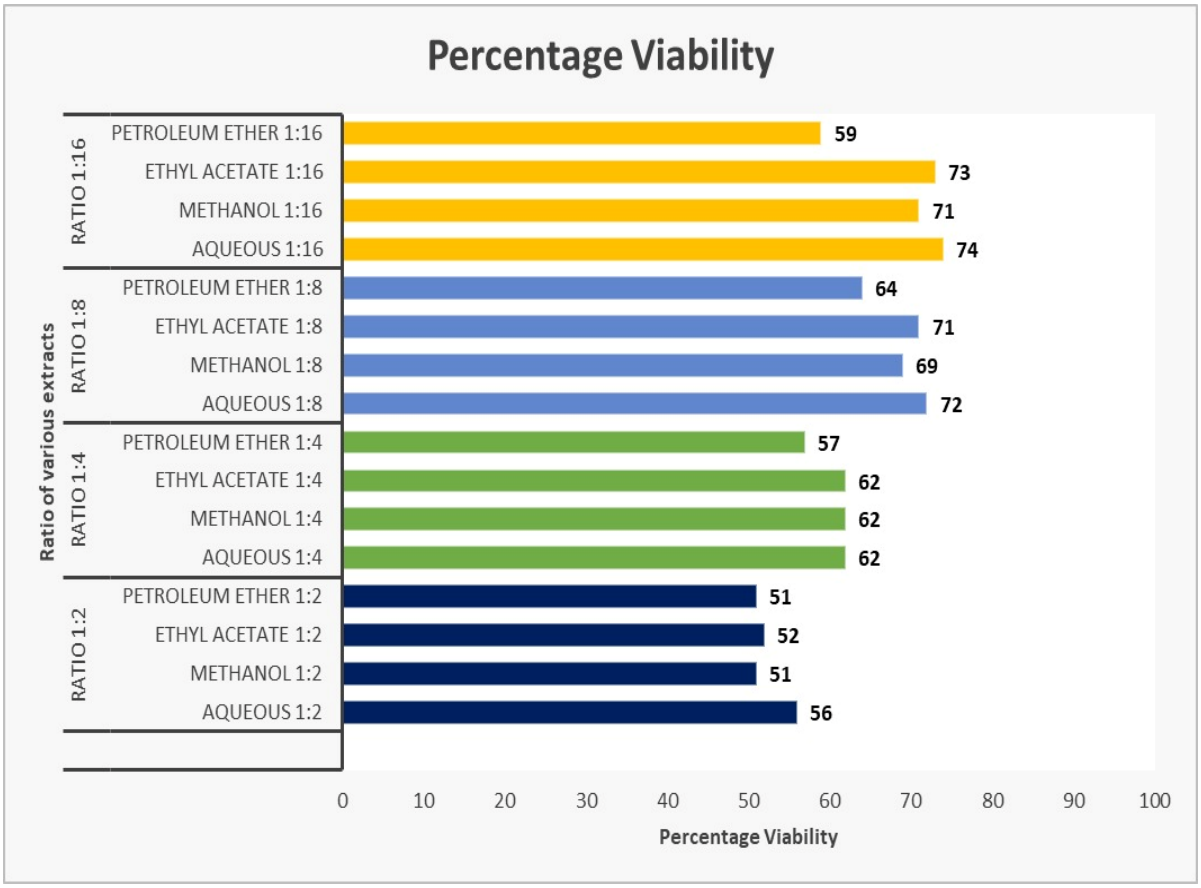
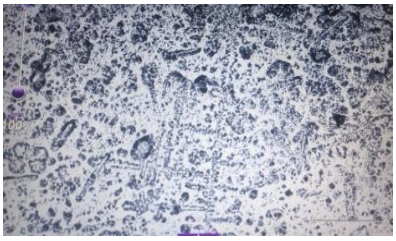
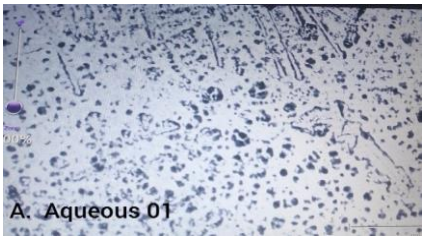


Fig 2.: graph plotted between percentage viability and ratio of various extract.

3.7 Cell Cytotoxicity MTT ASSAY –
MTT Assay has been done to check the cell viability of cells by different plant extracts of Stevia rebaudiana. Different plant extracts include aqueous extract, methanol extract, ethyl acetate extract and petroleum ether extract. And observe the cells at periodic intervals under an inverted microscope for presence of needle-shaped crystals called as formazan crystals.
After observing the formazan crystals, absorbance was read on an ELISA reader at 630nm wavelength and plotted absorbance against cell density. The percentage cell viability was calculated using the formula:
%cell viability = Absorbance of treated cells−background absorbance (b)/ Absorbance of untreated (c)−background absorbance (b) × 100 (Where b= blank and c = control)
MTT assays are presented in the table 2 shows percent viability values of the lymphocyte cells incubated for 72hrs with Aqueous, methanol, ethyl acetate and petroleum ether extracts. Percent cell viability values of cells were found to be more than 50 percentages with increase in concentration. At higher dose all these extract showed significant inhibitory effects on proliferation.

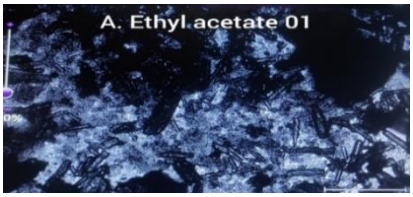


A . Control

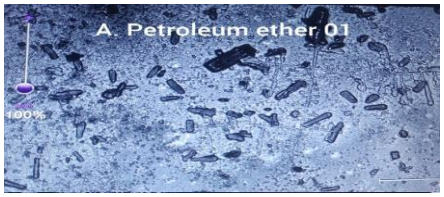


B. Aqueous extract

C. Methanol extract



D. Ethyl acetate extract



E. Petroleum ether extract

Fig 3. Results of MTT assay showing the crystal formation observed under the inverted microscope of various extract

3.8 ANTIOXIDANT ASSAY –
The average values determined from duplicate readings at 450nm. From the value obtained in step 1 subtract the average value of blank. The value obtained is corrected absorbance.
Corrected absorbance = Absorbance (450nm) (Test / standard) - Absorbance(450nm) (Blank)
Plot the corrected absorbance of Trolox standards (Yaxis) against standard Trolox concentrations (X-axis) to obtain the standard curve. Determine slope of the standard curve (y = mx + c). Calculate the Antioxidant concentration of each sample as Trolox Equivalents using the equation of the standard curve.
Troloxs Equivalent (uM) = (corrected absorbance) -(y- intercept)/ Slope. The values of trolox equivalent for different concentration of extracts are shown in table 2 and graph 1.

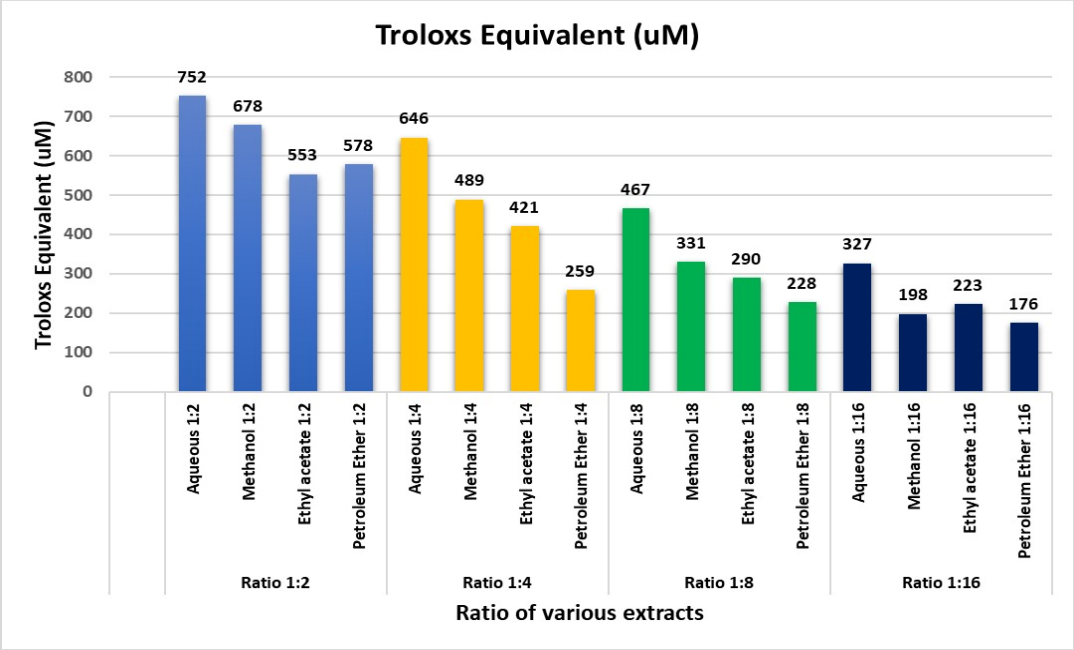


Fig 4. Graph plotted between Trolox Equivalent to ratio of various extract.

S.No	Extract	Percentage Viability	Troloxs Equivalent (uM)
1	Aqueous 1:2	**56**	**752**
	Aqueous 1:4	**62**	**646**
	Aqueous 1:8	**72**	**467**
	Aqueous 1:16	**74**	**327**
2	Methanol 1:2	**51**	**678**
	Methanol 1:4	**62**	**489**
	Methanol 1:8	**69**	**331**
	Methanol 1:16	**71**	**198**
3	Ethyl acetate 1:2	**52**	**553**
	Ethyl acetate 1:4	**62**	**421**
	Ethyl acetate 1:8	**71**	**290**
	Ethyl acetate 1:16	**73**	**223**
4	Petroleum Ether 1:2	**51**	**578**
	Petroleum Ether 1:4	**57**	**259**
	Petroleum Ether 1:8	**64**	**228**
	Petroleum Ether 1:16	**59**	**176**

Table 2. Shows Percentage cell viability* and Trolox Equivalent values of the lymphocyte cells incubated for 72hrs with various extract.

*Percentage cell viability = Absorbance of treated cells−background absorbance (b)/ Absorbance of untreated (c)−background absorbance (b) × 100 (Where b= blank and c = control).

Troloxs Equivalent (uM) = (corrected absorbance) -(y- intercept)/ Slope

4. DISCUSSION-

The proximate analysis showed that Stevia *rebaudiana* leaves are good source of protein and carbohydrates**.** The presence of secondary plant products in the leaf that are biologically important e.g. tannins, alkaloids, cardiac glycosides, sterols and triterpenes, reducing compounds and anthraquinones contribute to its medicinal value as well as exhibiting physiological activity. However, to our knowledge we have been reported that small amount of saponins and flavonoids also found in Stevia *rebaudiana.* The polyphenolic compounds found in plants also demonstrate several pharmacological properties such as antioxidant and anti-carcinogenic activities. Stevioside isolated from Stevia rebaudiana leaves affirmed the anticancer activity

and sensitization effect on breast cancer cell line MDA-MB-231 and SKBR. But we have been reported that the cytotoxic effects were related to their secondary metabolites content We tried to find out the percentage viability with different solvent extract in which Stevia *rebaudiana* showed the maximum cell viability with an aqueous extract in PBMC.

Antioxidants are compounds that have gained importance in recent years due to their ability to neutralize free radicals. The antioxidant compounds present in edible plants have recently been promoted as food additives since they display little or no toxic side effects. Our results emphasize that the Stevia *rebaudiana* leaves contains several flavanol glycosides. Their antioxidant activity has been measured and seems to be phenomenal. In the present study we find out maximum trolox equivalent value with aqueous extract with increase in concentration. Similar results were also reported by **Ruiz et al**. The presence of antioxidant properties in aqueous extract of the Stevia *rebaudiana* may be related to the various health beneficial properties assigned to this plant. Stevia leaves could be considered as a possible new antioxidant ingredient for the nutritional or functional food market. To understand the mechanism of death at high dose of extract we use flow cytometer to study the cell cycle stages and also perform the DNA laddering studies.

5. CONCLUSION

In this study it is interpret that Stevia *rebaudiana* is a good source of some primary and secondary metabolites which showed various health benefits, the Soxhlet apparatus is a good source, beneficial and advantageous for the phytochemical screening. Cell cytotoxicity effects were related to their secondary metabolites content. In which Stevia *rebaudiana* showed the cell viability in an aqueous extract, which had the ability to proliferate the immune cells. In Trolox equivalent antioxidant activity assay, stevia *rebaudiana* showed the antioxidant activity in all extract but the best result was found in aqueous extract as compared to others.

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