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Research Article

BIOLOGICAL ACTIVITIES OF NOVEL *IN VITRO* RAISED *STEVIA* PLANT

NEETA RAJ SHARMA[1], VINEET MESHRAM[2], MAHITI GUPTA[1]*

[1]Department of Biotechnology, Lovely Professional University, Phagwara, Punjab, India. [2]Department of Biochemistry, DAV University, Jalandhar, Punjab, India. Email: mahitigupta@gmail.com

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ABSTRACT

Objective: This communication explores a lead fraction from methanolic extract of novel *Stevia* species raised under *in vitro* conditions for its various biological activities.

Methods: The dried *Stevia* leaves were crushed in methanol to get the polar extract. This methanol extract was tested for pancreatic lipase and alpha-amylase inhibitory activity using quantitative plate assays. Antibacterial property of the extract was also evaluated against *Staphylococcus epidermidis*, *Escherichia coli*, *Bacillus subtilis, Staphylococcus aureus,* and *Pseudomonas aeruginosa.* Further, the antioxidant potential was evaluated using 1,1-diphenyl-2-picrylhydrazyl.

Results: The methanolic extract inhibited pancreatic lipase with IC_{50} of 5.74 µg/ml in a similar manner to a well-known anti-obesity drug in the market orlistat. The methanolic extract also showed a better pancreatic α-amylase inhibitory activity (IC_{50}=88 µg/ml) than acarbose. Further, the lead fraction exhibited 88.48% antioxidant activity. It also exhibited broad spectrum antimicrobial activity against the spectrum of Gram-positive and Gram-negative bacteria tested under laboratory conditions with a minimal inhibitory concentration ranging from 1.95 to 31.25 µg/ml.

Conclusion: Thus, this study signifies the vast potential of the lead fraction from a novel *Stevia* species for further development into a herbal formulation for prevention of various infectious and non-infectious diseases.

Keywords: Antioxidants, Enzyme inhibitor, Minimal inhibitory concentration, *Stevia*.

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INTRODUCTION

Obesity is a daunting health problem which occurs due to an imbalance between calorie uptake and utilization. Today obesity is becoming the major cause of preventable deaths, both in developed and developing nations [1]. It has been reported by World Health Organization (WHO) that every third individual around the globe is obese. It was previously known as disorder but recently WHO has declared it as a disease [2]. Pancreatic lipase is the major contributing enzyme that starts lipid metabolism by acting on triacylglycerides and resulting in the formation of monomers that are absorbed and accumulated in the body resulting to obesity. As action of pancreatic lipase is inhibited, most of the triacylglycerides are not converted to their simpler forms and hence are excreted out of the body [3]. Further, the most common disease related to obesity is Type 2 diabetes. Obesity and diabetes are two interlinked global health problems. Degradation of starch into sugars by α-amylase leads to increased postprandial hyperglycemia. Hence, inhibiting the action of α-amylase is the best way to control diabetes [4]. The current armamentarium of drugs available for the treatment of obesity suffers from various unwanted effects including fecal urgency, oily stools, abdominal cramps, flatulence, and oozing of oil from the anus leading to oily spotting [1]. Thus, there is an utmost requirement for exploring alternate avenues for development of new drugs for the treatment of these diseases with less or no side effects. Presently, there has been a lot of emphases given on the development of novel drugs from natural products due to their lesser antigenicity [5].

Stevia, commonly known as honey leaf or sweet leaf plant is a small perennial herb of South American origin belonging to the Asteraceae family [6]. Recent scientific trails have shown that leaves of *Stevia* possess broad spectrum antimicrobial, antiviral, and antifungal properties [7,8]. Further, the leaves of the plant also possess antihypertensive [9], antihyperglycemic [10], antitumor [7], and immunomodulatory effects [11]. Recently, *Stevia* grown under laboratory condition exhibited the presence of steviosides such as glycosides, steviosides, and rebaudioside. The ethanolic extract of these plants exhibited various medicinal properties including antioxidant and antimicrobial property [12]. *Stevia* plant contains a well-known non calorie natural sweetener stevioside [13]. Non calorie sweeteners are potential agents for obesity management [14] as they reduce the calorie intake of sugars. However, there exist very scanty reports on exploration of *Stevia* for anti-obesity properties. This is the first report of *Stevia* extract inhibiting pancreatic lipase with a potential similar to orlistat. Here in this study, we report the potential of a lead fraction obtained from a methanolic extract of *in vitro* raised *Stevia* plant for anti-obesity and antidiabetes, antioxidant, and antimicrobial activity leading to safer solution of obesity and diabetes management by a natural herbal agent with possible fewer side effects.

METHODS

Procurement of plant sample and preparation of lead fraction

In vitro raised novel *Stevia* strain was procured from GVS Biotech Pvt. Ltd., Punjab, India. The leaves of the *Stevia* plant were air dried at 37°C and crushed into a fine powder using pestle mortar. Further, 2 g of the powdered leaves was extracted using methanol (Merck GR, USA) over a rotatory shaker for 16-18 hrs at 130 rpm, 28±1°C. The solvent was filtered using Whatman filter Paper 1. The solvent was completely evaporated, and the leftover extract was reconstituted in dimethyl sulfoxide (DMSO) (Merck GR, USA). The crude extract was tested for its various biological activities.

Dose response activity of methanolic extract of *Stevia* against pancreatic lipase

In vitro pancreatic lipase inhibitory activity was evaluated using a slight modified protocol described by Gupta *et al.* [1]. Varying concentrations

of *Stevia* formulation ranging from 20 to 120 µg/ml were pre-incubated with 20 µl of porcine pancreatic lipase (40 U) at 37°C for 1 hr. Then, 100 µl of *p*-nitrophenyl laurate (PNPL) (2 mM) was added to start the reaction. The volume was made up to 250 µl using Tris buffer (pH 7.4). The plate was then subsequently incubated at 37°C for 3 hrs. The reading of the 96-well plate was measured at 410 nm using a 96-well plate reader. Orlistat was used as a positive control whereas negative control only comprised enzyme and PNPL.

***In vitro* pancreatic amylase inhibition assay**

Inhibition of pancreatic amylase by *Stevia* formulation was done according to modified protocol [15]. All the concentrations were reduced to 300 µl, and the test was performed in 96-well plate. In this assay, acarbose served as positive control.

***In vitro* assay for anti-oxidant activity**

The free radical scavenging property of methanolic extract of *Stevia* was determined by recording the change in the optical density of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (DPPH, Sigma-Aldrich, USA). Briefly, to 1 ml of DPPH (100 uM), 50 µl of extract (1 mg/ml) was added and incubated at 37°C for half an hour. Further, the absorbance was recorded at wavelength 517 nm using a ultraviolet-visible (UV-visible) spectrophotometer (Hitachi U-2900, Japan). Both positive (gallic acid) and negative (DMSO) controls were used in assay [16].

Free radical scavenging activity was calculated as:

$$\% \text{ Free radical scavenging} = \left[\frac{\text{OD control} - \text{OD test}}{\text{OD control}}\right] \times 100$$

Total phenolic content of methanol extract

Total phenol content of the mixture was estimated using Folin–Ciocalteu reagent based assay using gallic acid as standard. The methanolic extract (1 mg/ml) was mixed with 500 µl (HiMedia, Mumbai, India) of Folin–Ciocalteu reagent followed by addition of 1.5 ml of 2% sodium carbonate (HiMedia, Mumbai, India). The final volume was made to 5 ml using double distilled water. This mixture was then subsequently incubated at 37°C for half an hour. Using a UV-visible spectrophotometer, the absorbance was measured at 765 nm. The total phenolic content was estimated from the regression equation: y=0.026x–0.008 with R^2=0.99 [16].

***In vitro* broth dilution assay for minimal inhibitory concentration (MIC) estimation**

In vitro broth dilution assay to estimate MIC of the extract was performed as per described by Gomber and Saxena [17]. Briefly describing, 125 µl of Mueller-Hinton broth was dispensed into each well. Subsequently, 50 µl of 0.5 McFarland adjusted 18 hrs old test organisms were added into wells, and the plate was kept at 37°C for 2.5 hrs. Further, 25 µl of the test extract (i.e., two-fold serial dilutions of concentrations between 62.5 and 0.48 µg/ml) was dispensed into each well, and the plate was incubated at 37°C for 24 hrs. Further, 20 µl of 0.02% of 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) was added, and the plate was again incubated at 37°C for 60 minutes. The change in color in the wells due to difference in antimicrobial activities from pink (live) to yellow (dead) was observed and MIC was calculated. All the tests were performed in triplicates.

RESULTS

Dose response activity of methanolic extract of *Stevia* against pancreatic lipase

The quantitative plate assay ascertained the reduced pancreatic lipase activity by the methanolic extract of novel *Stevia* sp. and the only lipase inhibitor orlistat. *Stevia* extract inhibited pancreatic lipase with an IC_{50} of 2.7 µg/ml which was quite comparable to orlistat (IC_{50}=5.7 µg/ml). The inhibition pattern of *Stevia* extract was also similar to orlistat (Fig. 1).

In vitro* pancreatic α-amylase inhibitory activity of methanolic extract of *Stevia

This study showed the greater potential of *Stevia* species for inhibition of pancreatic amylase. The IC_{50} value of the methanolic extract was found to be 88 µg/ml as compared to that acarbose (IC_{50}=91.1 µg/ml) (Fig. 2).

In vitro* assay for antioxidant activity of methanolic extract of *Stevia

The methanolic extract of *Stevia* in the DPPH assay exhibited 87.48±2.08% antioxidant activity which was better than as compared to standard gallic acid which showed 84.16±1.49% antioxidant activity. There was a noticeable change in color from purple to yellow following scavenging reaction. Further, the total phenolic content of the lead fraction amounts to 21.42 µg/ml.

***In vitro* broth dilution assay for MIC estimation**

The methanolic extract of *in vitro* raised *Stevia* exhibited better MIC value (1.95-3.91 µg/ml) for *Staphylococcus epidermidis*, *Escherichia coli,* and *Bacillus subtilis* whereas it showed higher MIC values (15.625-31.25 µg/ml) for *Staphylococcus aureus* and *Pseudomonas aeruginosa* when compared to streptomycin under *in vitro* conditions (Table 1).

DISCUSSION

Stevia is a safe, calorie-free, and natural sweetener with no side effects and it is also considered as a potential antidiabetic supplement also

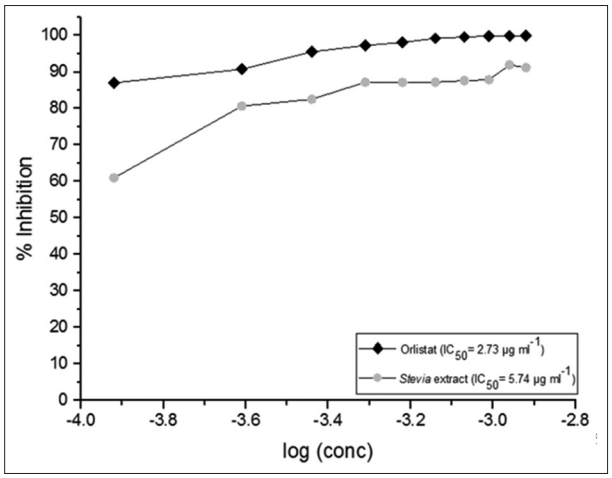

Fig. 1: Dose-response curves for inhibition of pancreatic lipase by *Stevia* formulation. Orlistat served as positive control

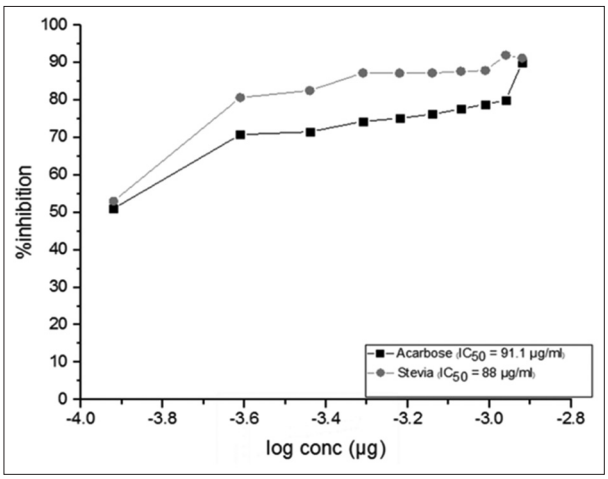

Fig. 2: Dose-response curve for inhibition of α-amylase by *Stevia* extract. Acarbose served as positive control

Table 1: MIC of methanolic *Stevia* extract and streptomycin against Gram-positive and Gram-negative bacteria

Test organism	MIC in µg/ml	
	***Stevia* extract**	**Streptomycin**
Staphylococcus aureus	15.62*	7.18
Staphylococcus epidermidis	3.91*	7.81
Escherichia coli	1.95	3.91
Bacillus subtilis	1.95	3.91
Pseudomonas aeruginosa	31.25*	15.625

*p<0.05 (Tukey's *post hoc* analysis). MIC: Minimal inhibitory concentration

possessing antioxidant and antimicrobial properties [18,19]. There are over 240 species of *Stevia,* and they have been largely focussed on their sweetening properties and in the development antidiabetic agent [20]. Apart from this, several studies have been carried which determined *in vitro* antimicrobial, anticancer, antioxidant, and anticancer properties of the *Stevia* plant [6]. However, very few or almost no efforts have been made to explore the lipase inhibitory (anti-obesity) activity and correlate it with the antidiabetic property of the plant. There exists only one report regarding pancreatic lipase inhibitory activity of *Stevia rebaudiana* [21]. Since obesity and diabetes are interrelated; this would be a novel avenue for management of two of the most threatening disease of the modern world. In this context, we chose to evaluate both the anti-obesity and antidiabetic property of the *in vitro* raised *Stevia* plant. The study was focussed on inhibition of two enzymes, *viz.*, pancreatic lipase and α-amylase for anti-obesity and antidiabetic property, respectively [1,4]. The lipase inhibitory property of the methanolic extract of *in vitro* raised *Stevia* plant (IC_{50}=5.74 µg/ml) in this study was found to be much better than that observed in *Stevia* (IC_{50}=530 µg/ml) [21]. Pancreatic lipase inhibitory potential of the lead fraction was better than hesperidin and carnosic acid isolated from citrus fruits and *Salvia officinalis,* respectively [22,23]. It is believed that further purification of the methanolic fraction will increase the inhibition response, which would further able to demonstrate exact IC_{50} of the purified compound [1]. Similar pattern was observed in several microbial lipase inhibitors such as vibralactone and Percy Quin whose phospholipids inhibitory efficiency increased on purification [24,25].

Alpha-amylase is a key enzyme which is accountable for degradation of starch into monosaccharides. Alpha-amylase hydrolyses complex sugar molecules into simpler sugars that are absorbed by the villi of small intestine, hence passing into the hepatic portal vein. These sugars are responsible for increase in postprandial glucose levels. The inhibitors of alpha-amylase have been given another name as starch blockers because they prevent absorption of dietary starch in the body ultimately lowering postprandial glucose levels [26]. The lead fraction in this study also exhibited comparable α-amylase inhibitory activity. In this study, the α-amylase inhibitory activity (IC_{50}=88 µg/ml) was found to be far better than aqueous extracts of *S. rebaudiana* reported by Ruiz-Ruiz *et al*. [15] and Patil *et al*. [27]. The extract might exhibit amylase inhibitory activity by binding at substrate binding site/active site of alpha-amylase, an enzyme responsible for the breakdown of α-1,4 glycosidic bonds in starch and other polysaccharides that increase the sugar level in body and subsequently leading to postprandial hyperglycemia. Therefore, this report supports the theory that moieties from medicinal plants having a potential to inhibit α-amylase can be used as a pharmacophore for managing postprandial hyperglycemia with minimal side effects [15].

Calculation of MIC using MTT is a widely known precise technique to estimate the response of a microorganism to a specific antibiotic [28]. This study reports the potential of methanolic fraction of *Stevia* plant to inhibit *S. epidermidis*, *E. coli,* and *B. subtilis* with a lower MIC value (1.95-3.91 µg/ml) whereas it displayed higher MIC value (15.62-31.25 µg/ml) for *S. aureus* and *P. aeruginosa* when compared to streptomycin. The antibacterial property reported in this study is far better than the earlier studies reporting MIC of *S. rebaudiana* against *B. subtilis* and *E. coli* to be 500 mg/ml [29]. Earlier studies have also reported that the aqueous and ethanolic extract of *Stevia* possesses broad spectrum antimicrobial activity against microorganisms such as *S. aureus, S. epidermidis, B. subtilis,* and *P. aeruginosa*. *Stevia* has been known to possess antibacterial, antifungal, and antiviral properties [30]. Further, the lead fraction also exhibited strong antioxidant activity via free radical scavenging activity. The antioxidant activity of this methanolic extract (88.48%) was better than that of antioxidant activity reported from earlier *Stevia* extract (71.75% and 86.4%) [12,31]. Following scavenging reaction, there was a noticeable change in color from purple to yellow. The change in color was due to the reaction of the extract with the antioxidant molecule which ultimately resulted in the scavenging of the radical by hydrogen donation [12]. *Stevia* has been reported to have antioxidant and antidiabetic properties in diabetic mice [32]. Thus, the *Stevia* plant offers to be a promising source of bioactive compounds with efficient medicinal properties. To date, there are no reports on determination of lipase inhibitory and α-amylase inhibitory activity from an extract of *in vitro* grown *Stevia* plant. This study is the pioneering work where the lipase and α-amylase inhibitory activities of the methanolic extract of *Stevia* plant along with potential antioxidant and antimicrobial activities were explored.

CONCLUSION

In vitro raised novel strain of *Stevia* plant contains a potential pharmacophore which can be used as an herbal formulation for a safer management of obesity and related diseases. Market today needs a natural drug for the two most prevailing and interrelating diseases. Purification, structural and biochemical characterization of the bioactive compound in the lead extract will lead to a natural drug for obesity and diabetes management.

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