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Determination of Stevioside-Rebaudioside-a & Gymnemic-Gymnemasins-a in *Stevia rebaudiana* & *Gymnema sylvestre* Leaves via TLC and Preparative HPLC

Susmit Kosta[1]☼, Dangi CBS[1], Manpreet Kaur[1], Malviya SN[1]

Department of Biotechnology, RKDF University, Gandhi Nagar, Bhopal (M.P.), India

☼**Correspondence to:** Department of Biotechnology, RKDF University, Gandhi Nagar, Bhopal (M.P.), India; Email: susbiotech@gmail.com

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ABSTRACT

Stevia rebaudiana and *Gymnema sylvestre* leaves known as sweet leaf and it contain with non-caloric sweeteners (Stevioside and Gymnemic acid) whose consumption could exert beneficial effects on human health. In India, the applicable of stevioside-gymnemic acid was very limited. The sweetness of steviol-glycosides was about 300 times more than sugar produce from sugar cane. Nowadays, sugarcane has critical value due to its high consumptions in community. The objective of this study is to determine stevioside-rebaudioside-A & gymnemic-gymnemasins-A in both leaves indentify and qualitatively by using soxhlet extraction method analyzed via preparative HPLC and TLC. A series of standard solution for both stevioside-rebaudioside-A & gymnemic-gymnemasins-A were analyzed. Samples were sequentially extracted using methanol as the solvent and the column had been used in this study was separated by means of a reversed phase HPLC system (Agilent technologies 1220 Infinity LC) comprised of quaternary pump, a column oven, sample freeze and UV detector. A ZORBAX Eclipse XDB-C_{18} column (4.6x150 mm, 5 µm) was employed for stevioside and Wakosil II 5C 18Φ4.6mm x 250mm for gymnemic acid, at 30°C. Separation was made in isocratic mode, using acetonitrile: water (80:20 v/v) at a flow rate of 1ml/min with 20 µl injection volume; detector and column temperature were set at 30°C. The detection wave length was 210 nm. Also, it is a good practice if the sample was extracted using solid phase extraction method compare to the conventional practice using sohxlet extraction method.

Key word: SRB, GS, Stevioside-rebaudioside-A and Gymnemic-gymnemasins-A

1. INTRODUCTION

Stevia (*Stevia Rebaudiana Bertoni*) SRB and Gurmar *(Gymnema sylvestre)* GS leaves use in human diabetics came almost a century back when it was demonstrated that the leaves of GS reduce urine glucose in diabetics (Pandey et

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al., 2013). In an animal study, Paliwal et al., (2009) have investigated that GS leaf powder had positive and encouraging effects over blood glucose levels. No adverse effect was observed on the health status of the subjects and thus, it can thus be concluded that SRB and GS powder is effective in lowering the fasting as well as postprandial blood glucose levels (Paliwal et al., 2009). Moreover, Saneja et al., (2010) have investigated the antihyperglycemic action of a crude saponin fraction and five triterpene glycosides derived from the methanol extracts of SRB and GS (Saneja et al., 2010).

Stevia (*Stevia Rebaudiana* Bertoni) (SRB) is an herbaceous native South American perennial plant containing two major compounds, stevioside and rebaudioside. It is reported that steviosides has insulinotropic effects in the beta-cell (in vitro), increase the insulin secretion and thereby decreasing blood glucose level. Oviedo *et al* found an antihyperglycemic effect in rats when supplementing the diet with dried SRB leaves (Oviedo et al., 1979).

Gurmar *(Gymnema sylvestre)* (GS) is also one of the important medicinal plants of India widely used in the treatment of diabetes mellitus (Veerashree et al., 2012). GS is popularly known as gurmar or madhunashini for its remarkable property of temporally paralyzing the sensory perception of sweet. The active principle of the drug is complex mixture of gymnemic acids found in the leaves (Shiyovich et al., 2010). Presently there is a huge market demand for this plant in national and international market.

The research of determining the two active compounds stevioside and rebaudioside of SRB leaves and gymnemic acid and gymnemasins of GS leaves, one of the best ways is by extracting among the several types of extraction method such as soxhlet extraction, hydro distillation, ultrasonic, and others. The soxhlet extraction is the most conventional of all methods and consists of a simple distillation process repeated a number of times. Soxhlet extraction method is a straight forward method. The sample phase is always in contact with fresh solvent, thereby enhancing the displacement of target compound from the matrix and the compound are not discomposed due to the moderate extraction condition.

In this study, the experimental procedure for this research in SRB and GS leaves focus on one main instrument method which is preparative HPLC for study the qualitative analysis of the stevioside-rebaudioside-A compounds and gymnemic-gymnemasins-A from both leaves. The preparative HPLC Agilent technologies 1220 Infinity LC System with the sample freeze and UV detector is used in this research. While for the most important component role play in the HPLC, is the HPLC column itself. So for this research, using the analytical column (A ZORBAX Eclipse XDB-C_{18} column (4.6x150 mm, 5 µm) was employed for stevioside and Wakosil II 5C 18Φ4.6mm x 250mm for gymnemic acid). This column has own unique itself which is it gives maximizing column efficiency. This column packing incorporate the use of well-characterized, state of the art, proprietary procedures for bonding and endcapping, that make it show very little retention loss and exhibit a column lifetime equivalent to that sterically hindered C18 silica bonded phase. The objective of this preliminary study is to determine the alternative method of analysis for extraction of the stevioside-rebaudioside-A and gymnemic-gymnemasins-A compounds using sohxlet extraction method via preparative HPLC qualitatively.

2. MATERIALS AND METHODS

Dried SRB and GS leaves material which come from a zero calorie plant known for its sweetness was used about 40-50 grams in this study. All leaves were collected from Agriculture University, Jabalpur. All the plants were identified taxonomically by department of Botany, Agriculture University, Jabalpur. The chemical reagent, Methanol (QRec, HPLC Grade, 99.9%, M.wt 32.04 g/mol,) was used on 1.2 liter as the main solvent in this research.

2.1. Thin layer chromatography (TLC) analysis

TLC analysis of SRB and GS leaves powder was performed to identify the stevioside-rebaudioside-A & gymnemic-gymnemasins-A compounds that are present in our extract, with the reference one that is in close proximity with the reference Rf value. The 500 mg of SRB and GS leaves powder was dissolved in 10ml of 50% (v/v) ethanol, and then 2ml of KOH was added and heated on a boiling water bath under relux for an hour and then cooled (Zhang et al., 1998). To this 1.8ml of 12N HCl was added and heated on water bath. After cooling the pH was adjusted to 7.5-8.5 with 11% KOH. This solution was dissolved with 50% (v/v) ethanol and filtered. 10µl was applied on TLC plate (Silica gel 60 F254) and TLC plate kept it in Solvent system of Chloroform: Methanol: Acetic acid (5:1:1). Solvent front was run upto 8 cms and TLC plate was detected by spraying Vanillin Sulphuric Acid reagent (Wagner et al., 1996).

2.2. Standard solution preparation for HPLC analysis

The both dried powder was divided into five samples with different quantities, from 1.0 g to 10.0g as shown on the table of data list samples. The first sample of dried SRB and GS leaves was put into extraction thimble and place sample into soxhlet extractor. The soxhlet apparatus using 200 mL of methanol was assembled. The thimbles are inserted and heat the reflux within period three hours or got two cycles solutions. Heating mantle was set at 70 ^{0}C.

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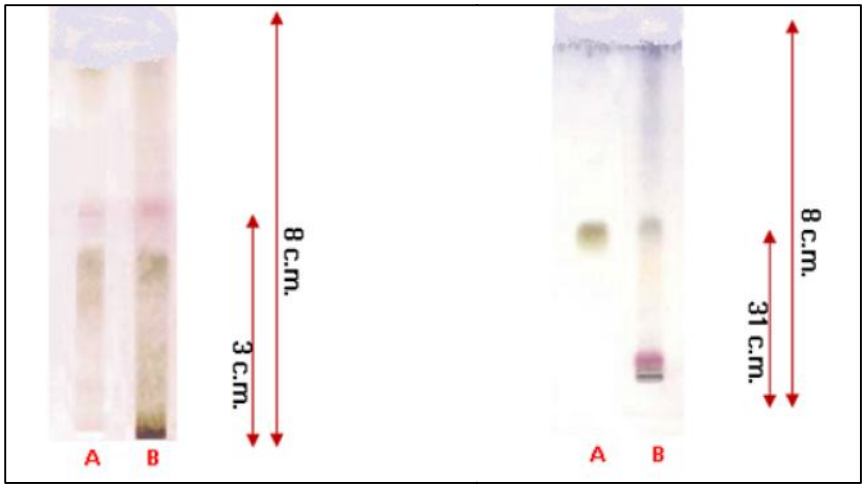

Figure 1

A. TLC of (A) Stevioside (B) Glycoside

The completed extraction, when got two cycles extraction solutions, and removed the flask. The other four samples were repeated same steps until got two cycle's extraction. The equipments are soxhlet extractor apparatus and High Performance Liquid Chromatography (Preparative HPLC Agilent technologies 1220 Infinity LC System). The chemical reagent, Methanol (QRec, HPLC Grade, 99.9%, M.wt 32.04 g/mol,) was used on 1.2 liter as the main solvent in this research. The 2.5 mg of each two standards solution were prepared by diluting with 80% methanol in water into volumetric flask respectively in prior to sonicate them for 15 minutes.

2.3. Preparative High performance liquid chromatography (HPLC) analysis

HPLC analysis of SRB and GS crude extract was carried out to determine the stevioside-rebaudioside A and gymnemic-gymnemasins A compounds content found in the leaves of SRB and GS in comparison with a reference standard. Acetonitrile-water (80:20 v/v) was used as the mobile phase. stevioside-rebaudioside A and gymnemic-gymnemasins A compounds was separated by means of a reversed phase HPLC system (Agilent technologies 1220 Infinity LC) comprised of quaternary pump, a column oven, sample freeze and UV detector. A ZORBAX Eclipse XDB-C18 column (4.6x150 mm, 5 µm) was employed for Stevioside and Wakosil II 5C 18Φ4.6mm x 250mm for gymnemic acid, at 30°C. Separation was made in isocratic mode, using acetonitrile: water (80:20 v/v) at a flow rate of 1ml/min with 20 µl injection volume; detector and column temperature were set at 30°C. The detection wave length was 210 nm (Wagner et al., 1996).

3. RESULTS AND DISCUSSION

3.1. Relatively identification of stevioside-rebaudioside-A in SRB crude extract

The crude extract of SRB was administrated to TLC plate covered with Silica gel 60 F254 to evaluate the presence of stevioside-rebaudioside-A in the crude extract Chloroform: Methanol: Acetic acid (5:1:1) system was used as mobile phase. Vanillin Sulphuric Acid reagent was used to determine stevoside. Calculation of SRB by Rf value A- stevioside-rebaudioside-A that is 0.36 c.m. and (Reference Sample) B- SRB 0.35 c.m. there was minuscule difference in value of the stevioside-rebaudioside A that might be because of some residual impurity. Among eight sweet diterpene glycosides, the most exhaustively studied is stevioside-rebaudioside-A (Ahmed et al., 2007).

The therapeutic value of stevioside-rebaudioside-A consists in the possibility to replace sugar and the ability of this compound to stimulate insulin secretion in the pancreas in the treatment of diabetes and other disturbances of carbohydrate metabolism (Silva et al., 2005). Recently, it was reported that the extract of the above-ground part of SRB possesses antiviral properties and produces a positive therapeutic effect in the treatment of neuralgia, anemia, lumbago, rheumatism, eczema, dermatitis, etc. (Geuns et al., 2003). In this context, it is especially important to develop a simple, reliable, and universal method for the determination of stevioside-rebaudioside-A in raw plant material. Many techniques were proposed for determining stevioside-rebaudioside-A, including capillary electrophoresis, gas and liquid chromatography, thin-layer chromatography in combination with optical densitometry and IR spectrophotometry (Kurihara et al., 1969). However, these techniques are nonspecific and did not find wide application. In this study we developed a simple and reliable quantitative chromatographic method for the determination of stevioside-rebaudioside-A in raw plant material, including the sample preparation stage. We have also developed a TLC technique for the qualitative analysis. The TLC separated stevioside-rebaudioside-A compounds showed the inhibitory activity of the individual compounds in SRB to be test verified (Figure 1A).

3.2. Relatively identification of and gymnemic-gymnemasins-A in GS crude extract

500 mg of GS extract was dissolved in 50ml of methanol. 10µl was applied on TLC plates. By spraying modified Vanillin Sulphuric Acid reagent (Figure 1B). Modified Vanillin Sulphuric Acid reagent was prepared by dissolving 1gm of vanillin in 90ml of ethanol, to this 5ml of acetic anhydride and 5ml of sulphuric acid was added.

Calculation of GS by Rf value gymnemic-gymnemasins-A that is 0.38 c.m. and (Reference Sample) B- GS 0.38 c.m. there was minuscule difference in value of the gymnemic-gymnemasins-A that might be because of some residual

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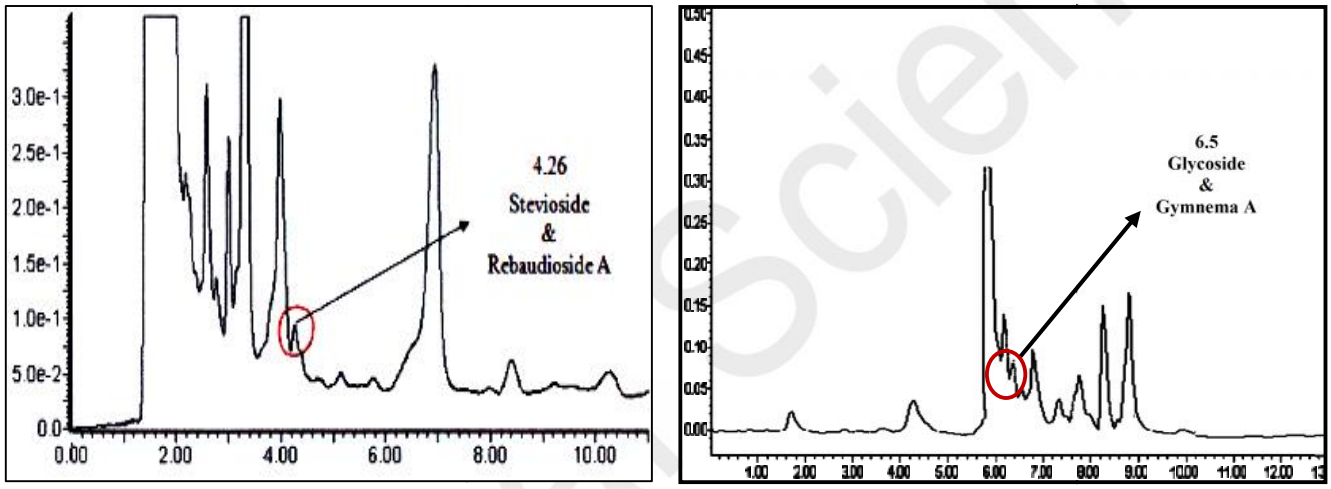
Table 1

Differences mass against area percentage of SRB and GS analyst

No.	SRB and GS Mass Sample (g)	Area Percentage (%)		Retention Times (Mins.)	
		SRB	GS	SRB	GS
1	1.0	40.2445	39.4154	26.457	28.454
2	2.5	35.4257	25.4544	26.424	28.145
3	3.0	30.2453	24.5446	26.145	28.457
4	5.0	20.2554	18.2341	27.012	27.124
5	10.0	18.2454	16.1247	27.014	27.052



A B

Figure 2

A. Chromatogram of stevioside and rebaudioside-A in SRB sample
B. Chromatogram of Glycoside and Gymnema-A in GS sample

impurity. A more attractive method for the determination of gymnemic-gymnemasins A is offered by TLC because this method, being simple and reliable, possesses specificity with respect to some gymnemic-gymnemasins-A.

GS leaves contain triterpene saponins belonging to oleanane and dammarene classes. Oleanane saponins are gymnemic acids and gymnemasaponins, while dammarene saponins are gymnemasides. Beside this, other plant constituents are flavones, anthraquinones, hantri-acontane, pentatriacontane, chlorophyl, phytin, resins, *d*-quercitol, tartaric acid, formic acid, butyric acid, lupeol, amyrin related glycosides and stigmasterol. The plant extract also test positive for alkaloids. Leaves of the species yield acidic glycosides, anthraquinones and their derivatives. Among these bioactive compounds of gymnemic-gymnemasins-A have anti-diabetic, anti-saccharine and anti-inflammatory activities (Kanetkar et al., 2007). The present study is the first ever attempt for the isolation, purification and characterization of gymnemic-gymnemasins-A from the aqueous plant extract. The identification and separation of the components present in extract of GS was carried out by TLC. The TLC of resinous extract, gymnemic-gymnemasins-A isolated after acid hydrolysis and sugar portion was performed using different solvent systems. The conversion of the blue color of the litmus to red was a positive test for the acidic nature of gymnemic-gymnemasins A. The addition of a small pinch of sodium bicarbonate in aqueous solution of all four samples evolved carbon dioxide which confirmed that the gymnemic acid was an acid (Vanek et al., 2001, Cacciola et al., 2010) also reported these compounds as the other plant constituents in addition to gymnemic-gymnemasins-A.

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3.3. Determination of SRB and GS

In this study, dried SRB and GS leaves powders are extracted linear in the range mass amount of (1.0 – 10.0 g) by through soxhlet extraction method for first run analysis, prior to further run analysis via preparative HPLC. Thus, mass of sample 1.0 g is selective for the further analysis with stevioside- rebaudioside-A and glycosides- gymnemasins-A, standard solutions of both leaves. This is due to the optimum mass of 1.0 g (37.3939 %) compare to other four samples at the UV wavelength detection signals, 210 nm as show in Table 1.

The sample solutions of dried SRB and GS leaves powders are extracted linear in the range mass amount by through soxhlet extraction method for first run analysis, prior to further run analysis via preparative HPLC. Thus, mass of sample 1.0 g is selective for the further analysis with steviosides and glycosides which are rebaudioside-A and gymnemasins-A, standard solutions.

This is due to the signal UV wavelength is in the range 205-215 nm and the retention time for the stevioside compound in the sample is 4.26 minutes close to the retention time of stevioside standard solution, 4.28 minutes. While referring the Vanek et al., (2001) research about the determination of stevioside in SRB plant material and fruit teas, which used same type Waters HPLC instrument method and C_{18} column, was determined the stevioside compound at the retention time, 15 minutes with UV signal at 205 nm.

Rebaudioside-A and gymnemasins-A compound (Figure 2), also was identified in the SRB and GS leaves sample at the same retention time of stevioside compound, 4.26 minutes or glycoside side compound, 6.5 minutes. Although the retention time of both compounds in their standard solutions respectively, were different in retention time about 5 to 7 minutes. This can be said that, after through a few references journal, they reported that the percentages extracted between them are similar in the extracted SRB and GS sample.

This statement above, was approved by taken one of a few previous researches, that the 63rd JECFA reported, where the commercially available extracts of SRB and GS leaves are stevioside , rebaudioside A and Glycoside, gymnemasins A, in various amounts ranging from about 10-70% stevioside and 20-70% rebaudioside A. On the other hand, in the journal of Cacciola et al., (2010) and Pol et al., (2007) also reported that the stevioside compound most abundant steviol glycoside (4-13% w/w) in plant leaves followed by rebaudioside A (2-4% w/w) and other seven minor active compounds of steviol and glycoside.

The chromatogram for the separation stevioside and rebaudioside-A in the SRB sample were showed that, their peaks are broadening, give poor result of resolution. The soxhlet extraction method applies in this research produces large quantities of stevioside and rebaudioside-A. The mass of dried SRB leaves sample extracted is 1 gram. Due to the Pol et al., (2007), Woelwer-Rieck et al., (2010) and Gardana et al., (2010) researches currently, used the amount of mass dried SRB leaves less than 1 gram in the range 0.25 – 0.8 grams.

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