Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2014; 6(2); 204-209

ISSN: 0975-4873

Research Article

Comparative HPTLC Fingerprinting and Antioxidant Activity of the Leaves of *Stevia rebaudiana* Bertoni from Different Geographical Sources

Mandal B[1], *MadanS[2], Gullaiya S[2]

[1]*Institute of Biomedical Education and Research, Mangalayatan University, Beswan, Aligarh (U.P.) India*
[2]*Amity Institute of Pharmacy, Amity University, Noida (U.P.) India*

Available online: 1st June 2014

ABSTRACT

The world today faces a large number of growing maladies that make the normal sustenance of an individual very arduous and challenging. Health concerns related to oxidation in the body is a major concern among these ailments which damages cell membranes and other structures including cellular proteins, lipids and DNA in the human body through free radicals that are produced during this process. Control and cure of these health conditions require a source that can overcome these health concerns and that has a minimal potential to cause adverse effects. *Stevia rebaudiana* Bertoni, the nature's sweetener, is one of the effective sources to combat the damage related to oxidative reactions in the human body. The present study involves comparative evaluation of antioxidant activities of the dried leaves of five varieties of *Stevia rebaudiana* procured from five different geographical locations of India viz., Delhi, Surat, Kangra, Bangalore and Indore using DPPH radical scavenging assay. Total phenolic and total flavonoid content were also determined using Folin-Ciocalteu reagent method and aluminum chloride colorimetric method respectively. A comparative HPTLC fingerprinting of the methanolic extracts of all the varieties was also carried out using CAMAG system consisting of Linomat 5 spotting device and Scanner 3 and the content of rebaudioside A was also compared among the different varieties with respect to standard rebaudioside A. The content of rebaudioside A was found to be the highest in the variety procured from Delhi i.e., 1.63% w/w as compared to other varieties. The variety from Kangra showed the most potent antioxidant activity with IC_{50} of 54 µg/ml among all the varieties.

Keywords: Antioxidant, DPPH radical scavenging, *Stevia rebaudiana*, total phenolic, total flavonoid.

INTRODUCTION

Oxidation reactions occur when life essential oxygen combusts within the human body and produces by-products referred to as oxygen free radicals which steal electrons from other molecules, like proteins, lipids, DNA and causing damage. In case of DNA, the problem intensifies and genetic cell mutations may occur which may become a common cause of cancer. Uninhibited over time, free radical damage builds in the body, thus causing aging. An overload of free radicals has been linked to certain diseases, including heart disease, liver disease and some cancers.

Stevia rebaudiana (*S. rebaudiana*)**,** a non-caloric substitute to conventional sucrose, distinctly possesses good antioxidant activities [1, 2, 3] and thus has the ability to boost the immune system and prevent free radical mediated diseases. It contains micronutrients like selenium, zinc, manganese which play an important role as antioxidants[4]. It is a small perennial shrub growing upto 1m tall, leaves 2-3cm long[5] which is native to regions of Paraguay and Brazil. It is popular as the "sweet herb of Paraguay"[6].

The current research involves comparative HPTLC fingerprinting and antioxidant activities of five varieties of *S. rebaudiana* procured from five different geographical locations of India viz., Delhi, Surat, Kangra, Bangalore and Indore to find out which variety contains the highest content of rebaudioside A, the bioactive glycoside, as well as to conclude which variety possesses the most potent antioxidant activity amongst all the varieties.

EXPERIMENTAL

Collection of plant material: Dried leaves of *S. rebaudiana* were procured from different suppliers of India: Saico Healthcare Pvt. Ltd. (Delhi), Keshal Nursery (Surat), Deepak Trading Co.(Bangalore), Shri Krishna Herbal (Indore) and locally field grown leaves from Chachiyan Village (Kangra) between the months of September to November, 2010. The identity of the leaves was verified by Dr. H. B. Singh, Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi and a voucher specimen for the leaves was deposited at the Herbarium of National Institute of Science Communication and Information Resources, New Delhi respectively.

Preparation of standard rebaudioside A solution: 10 mg of rebaudioside A was dissolved in 50 ml methanol and finally adjusted to a concentration of 0.4 mg/ml.

Preparation of sample extracts: 1 gm of coarsely powdered

*Author for correspondence: E-mail:smadan3@amity.edu

Table.1. R_f value of different varieties of *S. rebaudiana*

Sample Name	No. of spots and R_f of the spots
S. rebaudiana methanolic extract	
Delhi	8 spots- 0.09, 0.14, 0.18, 0.26, 0.37, 0.40, 0.49
Surat	8 spots- 0.09, 0.14, 0.17, 0.25, 0.29, 0.38, 0.48, 0.76
Bangalore	9 spots- 0.09, 0.14, 0.18, 0.27, 0.39, 0.43, 0.50, 0.66, 0.75
Kangra	8 spots- 0.10, 0.15, 0.18, 0.27, 0.42, 0.50, 0.66, 0.76
Indore	7 spots- 0.09, 0.14, 0.18, 0.28, 0.39, 0.75, 0.78

Table.2.Quantitative analysis of rebaudioside A in methanolic extract of different varieties of *S. rebaudiana*

S. rebaudiana	Content of rebaudioside A (% w/w)
Delhi	1.63
Surat	0.73
Bangalore	0.81
Kangra	0.95
Indore	0.64

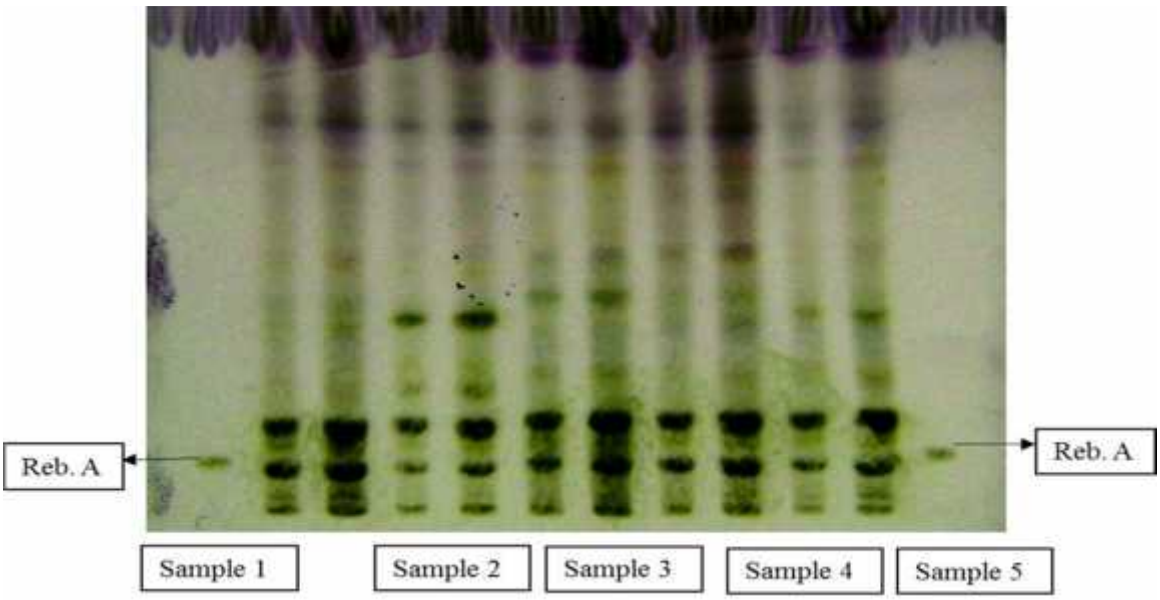

Fig. 1: HPTLC fingerprint of rebaudioside A in the five varieties of S. rebaudiana (Reb. A=Rebaudioside A)

Table.3. Results of the total phenolic content of different varieties of *S. rebaudiana*

S. rebaudiana	Total phenol (mg GAE/L)	Average (mg GAE/L)
	3.80	3.93
Delhi	4.06	
	3.53	3.64
Surat	3.76	
	5.38	5.66
Bangalore	5.94	
	5.90	5.87
Kangra	5.84	
	3.62	3.72
Indore	3.82	

air dried leaves from each sample were accurately weighed and allowed to leave for cold maceration in 25 ml of methanol for 24 hours. Sonication of the various samples in methanol was performed after 24 hours in a PCi Sonicator (Mumbai) for about 30 minutes along with heating at a temperature of about 40°C.The different samples were filtered while hot and fresh methanol was

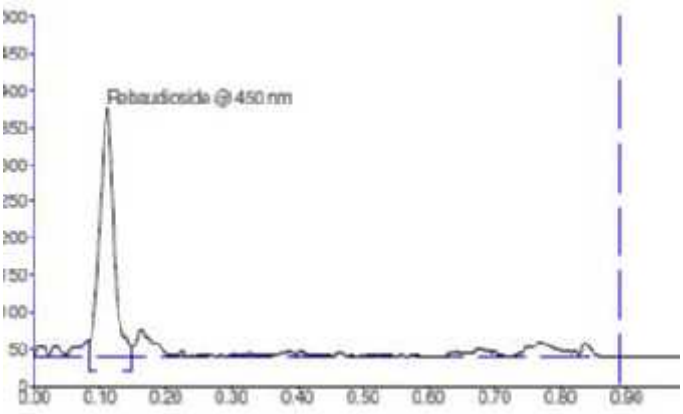

Fig. 2: HPTLC chromatogram of standard rebaudioside A

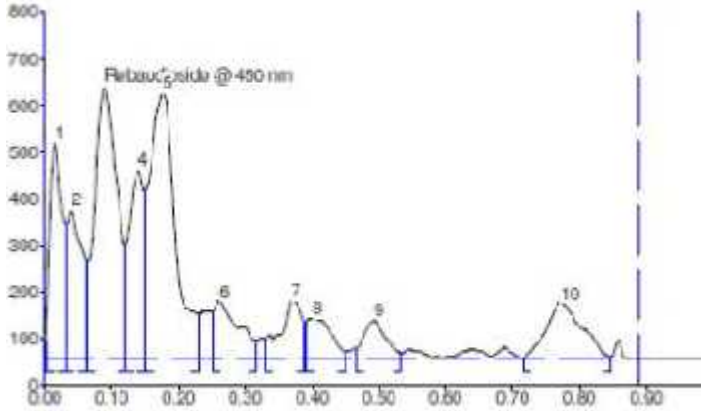

Fig. 3: HPTLC Chromatographic profile of S. rebaudiana (Delhi)

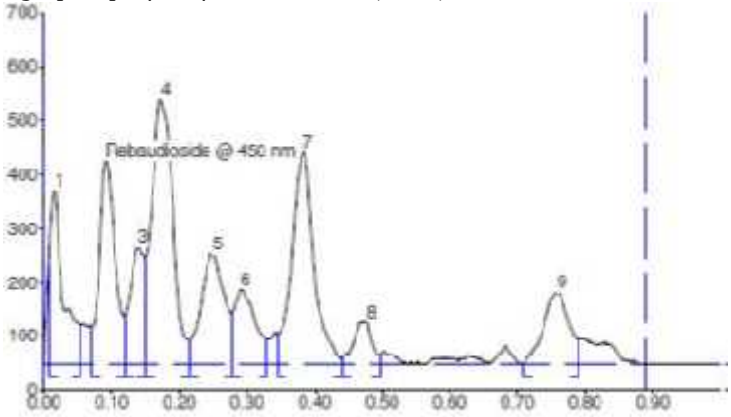

Fig. 4: HPTLC Chromatographic profile of S. rebaudiana (Surat)

again passed through the marc. The different sample extracts thus filtered were concentrated on a water bath and volume was finally adjusted to 5 ml to get concentration of 200 mg/ml of extract.

Development of HPTLC fingerprints of different samples of *S. rebaudiana:* Fingerprinting was carried out using CAMAG system consisting of Linomat 5 spotting device and Scanner 3. The solvent system used was ethyl acetate: ethanol: water (78:16:6 v/v/v). Precoated aluminium sheet (10×10 cm, Merck, Darmstadt, Germany) with silica gel 60 F_{254} of thickness 0.2 mm were used on different samples which were applied in the form of band with the help of Linomat 5 applicator attached to HPTLC system which was programmed through win CATS, the software which were installed with the apparatus. 1µl of each of the sample was applied in the form of band of 3mm and chromatogram was developed in CAMAG twin trough TLC chamber using the solvent system ethyl acetate : ethanol : water (78:16:6 v/v/v). The developed chromatogram was then scanned using CAMAG TLC Scanner 3 at 254 nm and 366 nm using slit dimension 4×0.30 mm. The plate was then sprayed with anisaldehyde-sulphuric acid reagent and after heating at 110°C for 5 minutes, scanning was done at 450 nm using the same slit dimension 4×0.30 mm.

Quantitative estimation of rebaudioside A: The percentage content of rebaudioside A in different samples of *S. rebaudiana* was calculated with respect to rebaudioside reference standard.

Determination of antioxidant activity by DPPH Radical Scavenging Assay: The antioxidant activity of the different

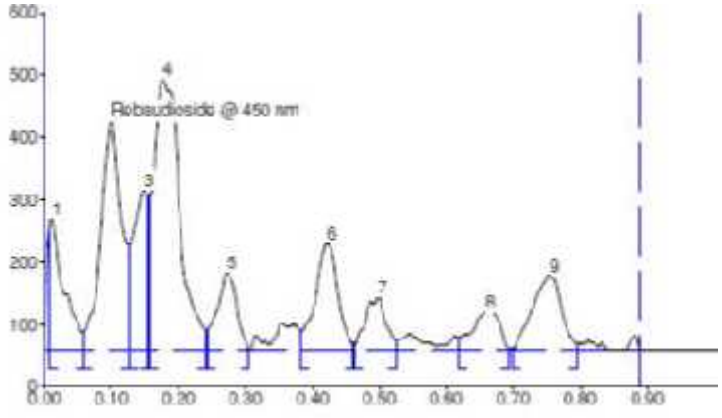

Fig. 5: HPTLC Chromatographic profile of S. rebaudiana (Kangra)

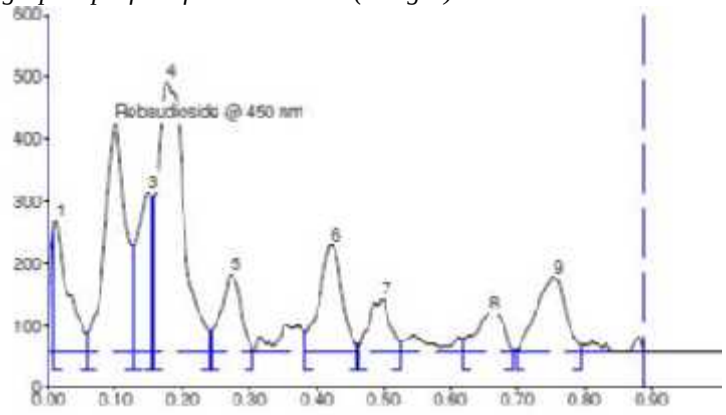

Fig. 6: HPTLC Chromatographic profile of S. rebaudiana (Bangalore)

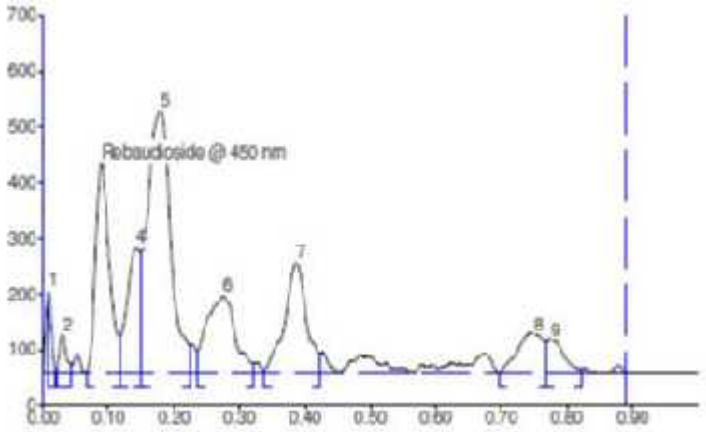

Fig. 7: HPTLC Chromatographic profile of S. rebaudiana (Indore)

methanolic extracts was evaluated using the method of Schmeda-Hirschmann *et al*[7]. 10 mg of the sample was weighed and dissolved in 1 ml of methanol using a vortex mixer (Touch Type) followed by adding to it 19 ml of methanol and finally mixing through sonication to prepare a sample of concentration 0.5 mg/ml. DPPH solution was prepared by dissolving 4 mg of DPPH in 100 ml of methanol. Various dilutions of the sample were prepared with methanol resulting in concentrations 5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, 100 µg/ml and 150 µg/ml. To each dilution was added 2 ml of the prepared DPPH solution in methanol. A control was also prepared simultaneously consisting of 2 ml methanol and 2ml DPPH solution. The prepared dilutions were then left for colour development in the dark for 20 minutes. Finally, the absorbance was measured at 517 nm against a reagent blank. Same procedure was repeated for all the five varieties of S. *rebaudiana*. The standard curve was obtained using ascorbic acid. A plot of concentration vs. the percentage inhibition of DPPH radical gave the IC_{50} value which is the concentration of sample required to inhibit 50% of DPPH radical.

Determination of total phenolic content by Folin-Ciocalteu Reagent: Total phenol estimation was carried out with Folin-Ciocalteu reagent (FCR) method[8]. 0.5 mg of the sample was weighed and dissolved in 1 ml of 50% methanol using a vortex mixer (Touch Type) followed by adding to it 4 ml of 50% methanol and finally mixing

Table.4. Results of the total flavonoid content of different varieties of *S. rebaudiana*

S. rebaudiana	Total flavonoid (mg QE/L)	Average (mg QE/L)
Delhi	54.16 54.27	54.21
Surat	43.45 42.35	42.90
Bangalore	52.83 52.91	52.87
Kangra	61.72 62.72	62.22
Indore	41.21 38.51	39.86

through sonication to prepare a sample of concentration 1 mg/ml. 0.5 ml of this solution was pipette out in a test tube to which was added 3.5 ml of distilled water followed by addition of 0.25 ml of Folin-Ciocalteu reagent (FCR). This was left for incubation for 1-8 minutes at room temperature. Lastly, to this was added 0.75 ml of 20% sodium carbonate solution and the final sample solution in the test tube was left to incubate for 2 hours. The sample was prepared in duplicate. Finally, the absorbance was measured at 765 nm against a reagent blank. Same procedure was repeated for all the five varieties of *S. rebaudiana.* The standard curve was obtained using gallic acid monohydrate .The total phenol content was expressed as gallic acid equivalent to % w/w of the extracts[9].

Determination of total flavonoid content by aluminium chloride colorimetric method: The total flavonol content of the extracts was determined by aluminium chloride colorimetric method[10]. 10 mg of the sample was weighed and dissolved in 1 ml of 80% ethanol using a vortex mixer (Touch Type) followed by adding to it 9 ml of 80% ethanol and finally mixing through sonication to prepare a sample of concentration 1 mg/ml. 0.5 ml of this solution was pipetted out in a test tube to which was added 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate aqueous solution and 2.8 ml of distilled water. A yellow color indicated the presence of flavonoids. The final sample solution in the test tube was left to incubate for 30 minutes at room temperature. The sample was prepared in duplicate. Finally, the absorbance was measured at 415 nm against a reagent blank. Same procedure was repeated for all the five varieties of *S. rebaudiana*. The standard curve was obtained using quercetin using solution in the range of 1-10 µg/ml. The results were expressed as mg quercetin/g dry weight by comparison with quercetin standard curve, which was made under the same conditions.

RESULTS AND DISCUSSION

The current study aimed at finding out the variety of *S. rebaudiana* with the highest content of rebaudioside A among the five varieties procured from five different geographical locations of India viz., Delhi, Surat, Kangra, Bangalore and Indore. It also focused on finding out the best variety with highest antioxidant potential which can fight against various oxidative stresses in the human body and hence included a comparative evaluation of antioxidant activity of these five varieties

HPTLC finger print analysis of methanolic extract of five varieties of *S .rebaudiana*: HPTLC fingerprint analysis was carried out on methanolic extracts of all the five varieties of *S .rebaudiana*. The HPTLC chromatograms of the methanolic extract are shown in (Figure 1) using standard rebaudioside A as standard where the R_f value of rebaudioside A was found to be 0.09. The HPTLC chromatogram of standard rebaudioside A and the HPTLC chromatogram profile of *S .rebaudiana* of all the five varieties are shown in (Figure 2-7). Quantitative estimation of methanolic extract showed that the variety from Delhi had the highest percentage content of rebaudioside A i.e., 1.63 % w/w as compared to other varieties shown in (Table 2). Thus, it may serve as a source for formulation of efficacious *Stevia* products. The climatic conditions like temperature, rainfall, humidity as well as soil conditions, altitude etc. that vary from region to region as well as the time of collection may be responsible for such a variation in the glycosidal content of rebaudioside A.

Total Phenolic content and Total Flavonol content determination: Total phenolic content and the total flavonoid content were determined by Folin-Ciocalteu reagent method and through aluminium chloride colorimetric assay. The variety from Kangra showed the highest phenolic and flavonoid content of 5.87 mg GAE/L and 62.22 mg QE/L respectively as shown in Table 3 and Table 4.

Antioxidant activity of all five varieties of *S. rebaudiana* by DPPH method: DPPH free radical scavenging assay was used to evaluate the antioxidant activity of the different varieties and the variety from Kangra showed the maximum potency of activity with an IC_{50} of 54 µg/ml and hence, this can serve as a source for curbing various health problems related to oxidative damage as presented in Table 5. As these compounds contribute to the antioxidant efficacy of natural products hence this observation can be directly correlated to the leaves from Kangra showing the most potent antioxidant activity.

CONCLUSION

Hence, the current research enables to find out which variety is the best with the highest content of the bioactive glycoside, rebaudioside A, responsible for various pharmacological activities of *S. rebaudiana*. The HPTLC fingerprinting and quantitative analysis assisted in finding out that the variety from Delhi was the best with highest content of rebaudioside A, and thus, this variety can be

Table.5. Results of *in vitro* antioxidant activity by DPPH free radical scavenging assay of different varieties of *S. rebaudiana*

S. rebaudiana	IC_{50} (µg/ml)
Delhi	68
Surat	63
Bangalore	56
Kangra	54
Indore	60

used in manufacturing of efficacious *Stevia* based products. Further, it also helps to find out the variety having the most potent antioxidant activity by a comparative evaluation of their antioxidant activities which was estimated with the help of DPPH free radical scavenging assay according to which the variety from Kangra was found to possess the highest antioxidant activity which can be used to curb various health problems related to oxidative stress. Furthermore, the total phenolic content (Folin Ciocalteu reagent method) and the total flavonoid content (aluminium chloride colorimetric assay) which was found to be the maximum in the variety from Kangra also gave an idea of correlating the antioxidant activity to the flavonoid and phenolic content.

REFERENCES

1. Vignais PV, Duee ED, Vignais PM and Huet J. Effects of atractyligenin and its structural analogues on oxidative phosphorylation and on the translocation of adenine nucleotides in mitochondria. *Biochem. Biophys. Acta* 1966; 118: 465- 483.
2. Ghanta S, Banerjee A, Poddar A and Chattopadhyay S. Oxidative DNA Damage Preventive Activity and Antioxidant Potential of *Stevia rebaudiana* (Bertoni) Bertoni, a Natural Sweetener. *J. Agric. Food Chem* 2007; 55 (26):10962-10967.
3. Tadhani MB, Patel VH and Subhash R. *In vitro* antioxidant activities of *Stevia rebaudiana* leaves and callus. *Journal of Food Composition and Analysis* 2007; 20(3-4): 323-329.
4. Talwar GP, Srivastava LM and Mudgil KD. Textbook of Biochemistry and Human Biology, 2nd Edn., Prentice. Hall of India Private Ltd. 1989.
5. Mishra PK, Singh R, Kumar U and Prakash V. *Stevia rebaudiana*- A Magical Sweetener. *Global Journal of Biotechnology & Biochemistry* 2010; 5(1): 62-74.
6. Sharma M, Thakral NK and Thakral S. Chemistry and *in vivo* profile of ent-kaurene glycosides of *Stevia rebaudiana* Bertoni-An overview, *Natural Product Radiance* 2009; 8(2):181-189.
7. Schmeda-Hirschmann G, Razmilic I, Gutierrez MI and Loyola JI. Proximate composition and biological activity of food plants gathered by Chilean Amerindians, *Econ. Bot*.1999; 53:177-187.
8. Arbianti R, Utami TS, Kurmana A and Sinaga A. Comparison of antioxidant activity and total phenolic content of *Dillenia indica* Leaves extracts obtained using various techniques, 14th Regional Symposium on Chemical Engineering 2007.
9. Sadasivam S and Manickam A. *Biochemical Methods for Agricultural Sciences*, Wiley Eastern Ltd., New Delhi.1992:187.
10. Woisky R and Salatino A. Analysis of Propils: some parameters and procedures for chemical quality control. *J. Agric. Res* 1998; 37: 99-105.