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U-HPLC (ULTRA-HIGH-PRESSURE LIQUID CHROMATOGRAPHY) SEPARATION OF INDOLE ALKALOID STRYCHNINE

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ABSTRACT

Ultra-high-pressure liquid chromatography (UHPLC) is an updated version of HPLC platform. It is a modern tool, useful for quick analysis, in short time and its column equilibration, is apt for method development. *Strychnos* genus (Loganiaceae) is cosmopolitan in distribution and many species related to it commonly used in several native medical practices. The majority of the members of *Strychnos* genus share similar chemical composition and contain common indole alkaloids. Purification and separation of Indole alkaloids from few species of *Strychnos* is already reported. In one study we also reported purification and quantification of Indole alkaloids by isocratic RP (Reverse Phase)-HPLC and further structural elucidation by IR (Infra-red),^{1}H-NMR(Nuclear magnetic resonance),^{13}C-NMR and LC –MS (Liquid Chromatography and Mass Spectroscopy) spectral analysis. However for the first time, we report the separation of the Indole alkaloid strychnine by U-HPLC. A simple and highly efficient RP-HPLC method was developed for the detection of alkaloid strychnine and also useful for qualitative and quantitative analysis. After optimization of the separation conditions, the indole alkaloid strychnine will be separated within 6 min and quantified with high sensitivity.

KEYWORDS: Alkaliods, HPLC, TLC, Natural products, *Strychnos*.

INTRODUCTION

The *Strychnos* genus belongs to the family *loganiaceae*, cosmopolitan in distribution, majorly indigenous to tropics covering from Africa to Asia. They are worthwhile targets for various phytochemical investigations (Deng et al. 2016; Rao and Prasad, 2008; Rao et al 2009; Rao and Prasad 2013). In traditional ethno medicine, they are used widely for the treatment of various diseases and disorders (Rao and Prasad, 2008). The *Strychnos* genus has been explored for centuries and reported with variety of indole alkaloids, which are known to possess anti-cancer, anti-bacterial, anti-fungal, anti-viral and anti-inflammatory properties (Philippe et al. 2003; Deng et al. 2006; Rao et al 2009; Rao and Prasad, 2013). Particularly the alkaloids from *Strychnos* (African and Asian) species has been well studied, characterized and structurally elucidated (Philippe et al. 2003; Rao and Prasad, 2009; Rao and Prasad, 2013). However, the complete chemical composition of many species is yet to be explored. The strychnine and brucine are the major alkaloids predominately found in *Strychnos* species along with others which include pseudostrychnine, N-methyl-sec.-pseudo-β-coubrine, 14-hydroxyicajine, strychnine-N-oxide, brucine-N-oxide icajine and novacine (Philippe et al. 2003; Frederich et al.,2003;2004; Deng et al. 2006; Rao et al 2009; Rao and Prasad, 2013). Few alkaloids in large doses are reported to be toxic to humans, whereas in small doses used as medicine such as antidote for snake poison. Strychnine and Brucine are used as alcohol denaturants and nervous system stimulants. Strychnine is used as valuable tool in experimental physiological and neuro-anatomical research (Biala et al., 1996: Bandopadhyay and De B, 1997; Rao and Prasad, 2013). Strychnine toxicity is demonstrated, through its interaction with sensitive glycine receptor in the lower brain stem and the spinal cord, thereby disrupting normal nerve signaling and leading to over excitation of the motor system and intense muscular convulsions (De B and Bisset NG, 1991; Philippe et al. 2003; Duverneuial et al., 2004; Deng et al. 2006; Rao et al 2009; Rao and Prasad, 2013).

Studying secondary metabolites in plants has become an active field, since they are potential sources for novel drugs (Biala et al., 1996: Bandopadhyay and De B, 1997; Rao and Prasad, 2008; 2013). The secondary metabolites from plants were separated, using various chromatographic techniques, with appropriate procedures, which includes extraction, purification and quantification (Philippe et al. 2003; Deng et al. 2006; Rao et al 2009; Rao and Prasad, 2013). Plant germplasm comprising of vegetative parts were shade dried, lyophilized, further extracted with suitable organic solvent using soxhlet extractor to dissolve secondary metabolites. After extraction, required metabolites were separated, purified and

quantified by various chromatographic techniques. HPLC is one among them, which is an important chromatographic tool to assess these metabolites either by qualitative or quantitative methods. In qualitative analysis, it produces a "fingerprint" under optimal conditions, which can be very useful for studying quality control of various metabolites, where as in quantitative mode one can predict its metabolite concentration. Compared to HPLC, TLC (Thin Layer Chromatography) is very simple and powerful technique used for this purpose, there are situations in which it can produce doubtful results. Earlier studies revealed that TLC and colorimetry has been widely used for the separation and quantification of the indole alkaloids from *Strychnos* genus, but the obtained results raised doubts, because it possess variety of alkaloids and TLC is not capable to separate efficiently when compared to HPLC (Zang et al, 2003). Moreover HPLC is also used as tool in chemo-systematics in differentiating the species based on the composition of secondary metabolites.

Most studies reveal that HPLC has been widely used in the analysis of Indole alkaloids from *Strychnos* genus (Biala et al., 1996: Bandopadhyay and De B, 1997; Rao and Prasad, 2008; 2013). In one study we also reported purification and quantification of Indole alkaloids by isocratic RP (Reverse Phase)-HPLC and further structural elucidation by IR (Infra-red), ^{1}H-NMR(Nuclear magnetic resonance), ^{13}C-NMR and LC –MS (Liquid Chromatography and Mass Spectroscopy) spectral analysis (Rao and Prasad, 2008). However till date there is no studies on *Strychnos* alkaloids separation by U-HPLC (Ultra-high-pressure liquid chromatography). UHPLC is an updated version of HPLC platform. It is a modern tool, useful for quick analysis, in short time and its column equilibration, is apt for novel method development. Hence for the first time, we report the separation of the indole alkaloid strychnine by U-HPLC. A simple and highly efficient RP-HPLC method was developed for the detection of alkaloids like strychnine, which is also useful for qualitative and quantitative analysis.

MATERIALS AND METHODS

Chemicals

Strychnine purchased from Fluka, HPLC grade Methanol, Chloroform, from Qualigens.0.45 and 0.2 filters from sortorius, Disposable 2ml Syringes form local company.

Apparatus

The U-HPLC analysis performed by Thermo fisher scientific Model. The configuration of our UHPLC is a four pump gradient with WPS and column thermostat, detectors like UV and

ECD 3000 two channel (6011RS, 6020RS). The Thermo fisher scientific dionex ultimate 3000 UV Absorbance detector fitted with a Acclaim TM 120 C18 5µm 120 Å (4.6x 250 nm) RP analytical column (product no 059149) packed with 5-µ particles was used. An automatic injection loop was used. Software was used to analyze the data.

Standard solution and mobile phase

Approximately 5mg of Indole alkaloid strychnine was precisely weighed and dissolved in 5ml of chloroform and stored at 4^0C. The mobile phase was Methanol: water (20:80). The flow rate was 0.75 ml/min. The eluate was monitored at 254 nm.

Calibration and Peaks identification.

Stock solution is used for making serial dilutions. Calibration was done ranging from 5 µg to 1mg. Regression equations for strychnine is obtained from peak area [y] and concentration [x]. Identification of peak was established with the UV spectra, following peak purity determination was based on the absorbance at 254 nm along with the retention time of the alkaloid.

Statistical analysis

Sigma plot was employed to establish means, standard deviation [S.D] of the alkaloid. The coefficient of variance or relative standard deviation (RSD) was calculated by expressing the standard deviation as a percentage of the relative mean.

RESULTS

The alkaloid strychnine has been limited by lack of accuracy in terms of purity and quantification by the existing methods. Hence the present study is carried in an aim to develop a fast and rapid Chromatographic technique. Fig-1 explains the detailed structure of Strychnine with Molecular formula ($C_{21}H_{22}N_2O_2$) and as well as its Molecular wt (335). Initially authentic alkaloid, strychnine was studied individually, to record its UV spectrum. Optimal chromatographic conditions were established after testing different mobile phases with a reverse-phase C18 column, which resulted in good separation of the peak. In most cases the mobile phase was the key step to achieve the best separation. Apart from this, the analysis time is another major factor in analytical work, with short run time allows less solvent consumption and also usage of equipment will be minimal. In the present study, for the first time we report the separation of strychnine by UHPLC Method. We also report the

separation of strychnine by isocratic elution with short run time and less solvent consumption. Further Qualitative and Quantitative analysis was performed.

Qualitative analysis

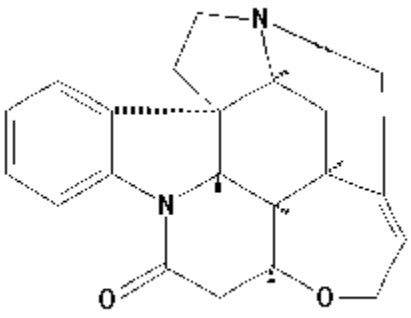
Initially qualitative analysis was performed. To achieve this, the U-HPLC fingerprinting method was optimized and further developed for quick analysis of the Indole alkaloid strychnine. The mobile phase was combination of methanol: water (20:80), with a flow rate of 0.75ml/min and the eluate was monitored at 254 nm. The standardized conditions allowed good separation of the peak and found to be pH independent. The strychnine peak, which could be identified in the chromatogram at Rt =5.3(Fig-2).

Quantitative analysis

In quantitative analysis the authentic indole alkaloid strychnine was analyzed by increasing concentrations ranging from 5 µg to 1mg. Concentrations of strychnine was calculated from peak area [y] and concentration [x] with UV detection at 254nm. Calibration curves showed linearity in the concentration range. Fig-2 describes the separation of Strychnine. The RT values of separation of Strychnine, remained constant (RT, 5.3) even with increasing concentration (5, 10 and 20µg) in a dose dependent manner, which were identified in the chromatogram (Fig2-A, B and C). The samples were injected and analyzed in triplicates and the resulting curves had a very good linear correlation coefficient.

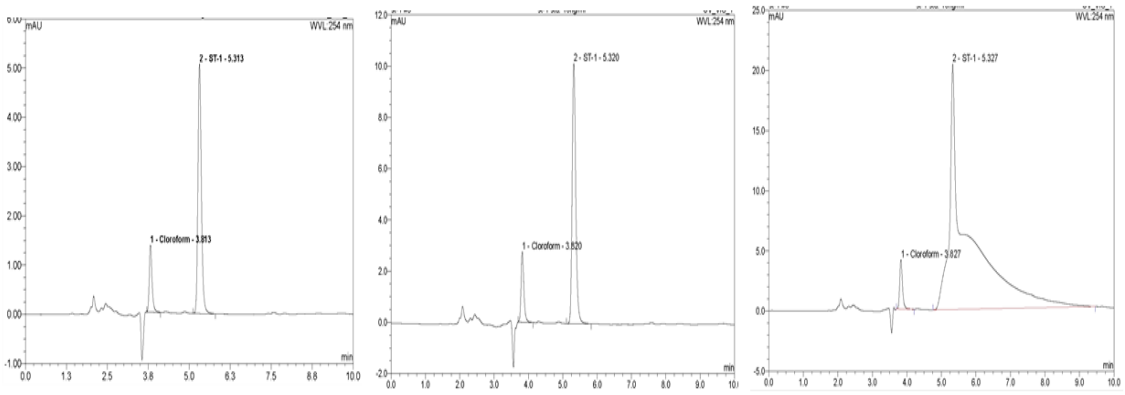
Strychnine: showed regression equation,

y = 2448.01x + 878906.6, Correlation coefficient 0.99



Strychnine $C_{21}H_{22}N_2O_2$

Fig-1. The structure of Strychnine along with Molecular formula ($C_{21}H_{22}N_2O_2$) and Molecular wt (335).



2Fig-A Fig-B Fig- C

Fig-2. The UHPLC separation of Indole Alkaloid strychnine.

A. Strychnine separation identified in the chromatogram with RT 5.3 B & C. Separation of Strychnine, with constant RT(5.3) even in the increasing concentration (5,10 and 20µg) in a dose dependent manner identified in the chromatogram.

DISCUSSION

The TLC and HPLC chromatographic techniques are widely used in evaluating the natural or synthetic compounds that fight with various diseases and disorders. Recent advances in the modern molecular biological tools like DNA sequencing, genetic engineering, gene targeting and transgenic methodologies has been shown the new way to better analyze and understand the infections, diseases and disorders and to develop new age drugs (Suresh et al., 2014; Suresh et al., 2016a; 2016b; Singh et al., 2015). To combat various diseases like cancer (Suresh et al., 2014; chetan et al., 2013) and disorders like diabetes (Reddy et al., 2013), several efficient drug development technologies has been developed, such as in silico drug designing and synthesis of novel molecules (Rao et al, 2012; Rao and Prasad, 2013; Avinash et al, 2015; Hymavathi et al, 2017). Apart from this, in traditional medicine, many herbs have been suggested for the treatment of various diseases and disorders (Satyanand et al, 2013a; Satyanand et al, 2013b; Satyanand et al, 2013c; Rao and Prasad 2008). Currently, use of the medicinal plant/herbal extracts/formulations is rapidly progressing, which is presumed to have minimal side effects. The active composition in these formulations have been responsible for this effect. The active composition may encompass a variety of natural products which include polysaccharides, pigments, steroids, terpenoids, flavonoids and alkaloids (Rao et al, 2009; Satyanand et al, 2013).

Strychnos is one such genus known to produce a variety of indole alkaloids, which has anti-cancer, anti-viral, anti-fungal and anti-bacterial properties. The chemical constituents of

various African and Asian *Strychnos* species has been studied extensively (Philippe et al. 2003; Frederich et al., 2003; 2004; Deng et al. 2006; Rao et al 2009; Rao and Prasad, 2013). Majorly indole alkaloids from these plants is isolated and well structurally characterized (Rao and Prasad, 2008). For assessing these indole alkaloids various chromatographic techniques were employed. Among these chromatographic techniques TLC (thin layer chromatography) and HPLC is widely used for the effective analysis of these chemical constituents. TLC is very simple, powerful technique used for studying these alkaloids, but there are few situations which give rise to some doubtful results. Few studies also revealed that TLC and colorimetry has been employed to isolate and quantify *Strychnos* alkaloids from its various *Strychnos* spps plant extracts, but the results obtained is always occupied with ambiguity, since these plant extracts contain various types of alkaloids and TLC alone is not capable to separate these alkaloids as efficiently when compared to HPLC (Zang et al., 2003). Thus HPLC is always ahead of TLC. The separation of alkaloids is a complex process. To estimate the alkaloid content in the *Strychnos* one has to follow appropriate procedures for extraction, purification and characterization. To separate alkaloids, one has to eliminate lipids, proteins, pigments and other plant secondary metabolites, from these extracts. Once after completion of this process HPLC is used for initial qualitative assessment and further quantitative assessment. As mentioned above Strychnine and brucine are the major alkaloids which have been reported from *Strychnos* genus. These molecules serve as the best templates, for the generation of new entities based on the structure activity drug designing. Out of two molecules, strychnine is to be found in few strychnos species ie., *S.nux-vomica, S.lucida R. Br., S.ignatti Berg., in Asia, S.icaja Bail* in Africa and *S.panamensis Seem* in Central America. (Bandopadhyay and De 1997; Philippe et al. 2003; Rao and Prasad, 2008). TLC and HPLC is widely used for the analysis of these molecules. Most studies also reveal that HPLC has been widely used in the analysis of Indole alkaloids from *Strychnos* genus (Biala et al., 1996: Bandopadhyay and De B, 1997; Rao and Prasad, 2008; 2013). In one study we also reported purification and quantification of Indole alkaloids by isocratic RP (Reverse Phase)-HPLC and further structural elucidation by IR (Infra-red),^{1}H-NMR(Nuclear magnetic resonance),^{13}C-NMR and LC –MS (Liquid Chromatography and Mass Spectroscopy) spectral analysis (Rao and Prasad,2008). However till date there is no studies on *Strychnos* alkaloids separation by U-HPLC (Ultra-high-pressure liquid chromatography). UHPLC is an updated version of HPLC platform. It is a modern tool, useful for quick analysis, in short time, and its column equilibration, is apt for novel method development. Hence for the first time, we report the separation of the indole alkaloid strychnine by U-HPLC. In the present study the

authentic alkaloid strychnine were studied first and UV-spectrum have been recorded. Optimal chromatographic conditions were achieved after experimenting with different mobile phases using a reverse- phase C18 column. The mobile phase methanol: water (20:80) combination with a flow rate of 0.75 ml/min was found to be significant and the eluate was monitored at 254 nm. The standardized conditions allowed good separation of the peak. The strychnine peak, which could be identified in the chromatogram at Rt =5.3(Fig-2). Finally the isocratic elusions led to good separation of the peak, however the separation of indole alkaloid strychnine, found to be pH independent. The obtained results in the present study is well correlated with our earlier studies (Bandopadhyay and De 1997; Philippe et al.2003; Rao and Prasad, 2008). Few studies revealed that Accuracy of the pH was essential, but whereas it is quite opposite in our study (Rao and Prasad, 2008). Moreover most studies reveal that the selection of mobile phase was crucial to achieve the best separation. Along with this, analysis time is another key factor in analytical work, with short run time allows minimal consumption of solvent, as well as cost effective and less usage of equipment (Rao and Prasad, 2008). In the current study, for the first time we report the separation of strychnine by isocratic elution with short run time and less solvent consumption in UHPLC Method. Further Qualitative and Quantitative analysis was performed.

In qualitative analysis, the U-HPLC fingerprinting method was optimized for quick analysis of the Indole alkaloid strychnine, which could be identified in the chromatogram at Rt =5.3(Fig-2). Whereas in quantitative analysis strychnine was analyzed by increasing concentrations ranging from 5 µg to 1mg, by calculating peak area [y] and concentration [x] with UV detection at 254nm. The results of calibration curves exhibited linearity in the concentration range. Moreover the RT values of Strychnine, remained constant (RT, 5.3) even with increasing concentration (5, 10 and 20µg) in a dose dependent manner, observed in the chromatogram (Fig2-A, B and C). The experimental samples were analyzed in triplicates and the resulting curves had a very good linear correlation coefficient. (Strychnine: showed regression equation y = 2448.01x + 878906.6, Correlation coefficient 0.99). The results obtained in the present study are comparable with other studies (Bandopadhyay and De B, 1997; Rao and Prasad, 2008).

CONCLUSIONS

The present findings demonstrate that the latest UPHLC method proposed in this study was simple, useful and repeatable for identification and determination of indole alkaloids like

strychnine. Moreover, for the first time we report the separation of strychnine by isocratic elution using UHPLC, with short run time and less solvent consumption.

REFERENCES

1. Deng XK, Yin W, Li WD, Yin FZ, Lu XY, Zang XC, Hua ZC, Cai BC. The anti-tumor effects of alkaloids from the seeds of Strychnos nuxvomica on HepG2 cells and its possible mechanism. Journal of Ethnopharmacology. 2006; 106: 179-186.
2. Rao PS, Prasad MNV. Extraction, Purification and Characterization of Indole Alkaloids from Strychnos wallichiana L. – an Endangered Medicinal Plant from India. Medici Aroma Plant Sci Biotech, 2008; 2: 63-67.
3. Rao PS, Ramanadham M, Prasad MNV. Anti-proliferative and cytotoxic effects of Strychnos nux-vomica root extract on human multiple myeloma cell line - RPMI 8226. Food Chem Toxic, 2009; 47: 283–288.
4. Rao PS, Prasad MNV. The Strychnos nux-vomica root extract induces apoptosis in the human multiple myeloma cell line-U266B1. Cell Biochem Biophy, 2013; 66: 443–450.
5. Philippe G, Angenot L, Tits M, Frederich M. About the toxicity of some Strychnos species and their alkaloids. Toxicon. 2004; 44: 405-416.
6. Frederich M, Choi YH, Angenot L, Harnischfeger G, Lefeber AWM, Verpoorte R Metabolomic analysis of Strychnos nux-vomica, Strychnos icaja and Strychnos ignatii extracts by 1H nuclear magnetic resonance spectrometry and multivariate analysis techniques. Phytochemistry. 2004; 65: 1993-2001.
7. Frederich M, Choi YH, Verpoorte R. Quantitative analysis of strychnine and brucine in Strychnos nux-vomica using 1H-NMR. Planta Medica. 2003; 69: 1169-1171.
8. Bandopadhyay J, De B. Seasonal variation of strychnine and brucine in vegetative parts of Strychnos nux-vomica. International Journal of Pharmacognosy. 1997; 35: 349-353.
9. Biala RG, Tits M, Wauters J-N, Angenot L. A new HPLC method for the assay of alkaloids in Strychnos nux-vomica and Strychnos ignatii. Fitoterapia, 1996; 67: 163-165.
10. De B, Bisset NG. Separation of Strychnos nux-vomica alkaloids by highperformance liquid chromatography. Journal of Chromatography A, 1991; 20: 318-320.
11. Duverneuial C, Grandmasion G, de La L, de Mazancourt P, Alvarez. Liquid chromatogaraphy? Photodiode array detection for determination of strychnine in blood: A fatal case report. Journal of Forensic Science International. 2004; 141: 17-21.
12. Suresh G, Gopi Krishna S, Nayudu N, Sravanthi M, Rao PS, Kumar VKM, Sowdamani G, Rudramadevi K, Nagesh N, Manjula B, Kondaiah K, Varadacharyulu N. Contribution

of cyclin D1 (CCND1) and E-cadherin (CDH1) alterations to colorectal cancer susceptibility: a case–control study. Tumor Biology, 2014; doi 10.1007/s13277-014-2505-9.

13. Suresh G, Sravanthi M, Bulle S, Dasi D, Prathap BN, Bramhachari PV, Nagesh N, Shivaji S, Bhanoori M, Rao RT, Rao PS, Varadacharyulu N. Manganese-superoxide dismutase (Mn-SOD) overexpression is a common event in colorectal cancers with mitochondrial microsatellite instability. Tumor Biol. 2016a feb-4, DOI 10.1007/s13277-016-4918-0.
14. Suresh G, Bulle S, Sravanthi M, Krishna MT, Nagesh N, Manjula B, Varadacharyulu N, Rao PS*; Association of Mitochondrial Displacement Loop Polymorphisms with Risk of Colorectal Cancer in South Indian Population. Mitochondrial DNA, 2016, http://dx.doi.org/10.3109/24701394.2016b.1160076.
15. Singh S, Kotakonda A, Kapardar R, Kankipati H, Rao PS, Mambatta PS, Vetaikorumagan SR, Gundlapally S, Nagappa R, Shivaji S. Response of bacterioplankton to iron fertilization of the Southern Ocean, Antarctica. Front. Microbiol. 2015; 6: 863. doi:10.3389/fmicb.2015.00863
16. Chetan R, Veeresalingam B, Kumar KM, V, Teja PD, Rao PS* A study on the clinical manifestations and the incidence of benign and malignant tumors in a solitary thyroid nodule. Int J Res Medi Sci, 2013 Nov; 1(4): 429-434.
17. Reddy S A, Dasu K, Venkat Krishnan, Reddy MR, Jithendra K, Rao PS. Prevalence of asymptomatic bacteriuria and its antibiotic sensitivity in type-2 diabetic women along the sea coast. Int J Res Medi Sci, 2013; 1(4): 487-495.
18. Rao PS, Muvva C, Geethanjali K, Babu BS, Kalashikam R. Molecular docking and virtual screening for novel protein tyrosine phosphatase 1B (PTP1B) inhibitors. Bioinformation, 2012; 8(17): 834-837.
19. Avinash A, Swarupa SS, Siva K, Sirisha D, Riyaz S, kumar ND, Sreenivasulu M, Rao PS. Design and Evaluation of Famotidine Floating Tablets. Int J Innov Pharmace Res., 2015; 6(1): 440-445.
20. Hymavathi, R, Suresh, G, Sumanth, K.M, Swapna, V.K, Sravanthi, M, Rao, P.S, Manjula, B, Varadacharyulu, N. Therapeutic effect of green tea extract on alcohol induced hepatic mitochondrial DNA damage in albino wistar rats. J Adv Res., 2017; 8(3): 289-295.
21. Satyanand V, Reddy CB, Rama Mohan P, Kumar MR, Narayanaswamy DL, Seelam A, Ramalingam K, Rao PS. Effects of Garlic extract (Allium sativum) in combination with

Amlodipine in mild to moderate essential hypertensive patients: An Open randomized parallel group study. J Pharmace Res Dev., 2013a; 2(4): 181-188.

22. Satyanand V, Venkata Krishnan, Ramalingam K, Rao PS , Priyadarshini S. Blockade of voltage dependent calcium channels lowers the high blood pressure through ginger Int J Analy, Pharmace Biomed Sci, 2013b; 2(1): 64-66.
23. Satyanand V, Venkat Krishnan, Madhavi D, Revathi, Indira S, Shaik AB, Rao PS. The effect of peppermint juice for indigestion among old age people- A preliminary study. J Pharmace Res Dev, 2013c; 2(7): 238-243.
24. Zhang J, Wang S, Chen X, Hu Z, Ma X Capillary electrophoresis with field-enhanced stacking for rapid and sensitive determination of strychnine and brucine. Analytical and Bioanalytical Chemistry. 2003; 376: 210-213.