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Formulation of health drinks using natural sweetener, its HPTLC method development and validation

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Ashwagandha, Tulsi, Mulethi, Awala, Shatavari, Gokharu, Arjun, Giloy, Safed musli, Kalimirchi, Haldi, Jaiphal **was used as an active ingredients and aqueous extract of *Stevia rebaudiana* as natural sweetener with nutraceutical in health dinks. The product was developed by treating concentrates of each crude drug with purified water. TLC profile, HPTLC method development and validation were carried out using Gallic acid as a standard. A new simple, sensitive, selective, precise and robust HPTLC method for analysis of Gallic acid in health drink was developed. Precoated silica health drink aluminium plate 60F-254 (20 × 10 cm) with 200 µm thickness was used as stationary phase while toluene: ethyl acetate: formic acid: ethanol (6: 4: 0.3: 0.4) system was developed as a mobile phase. Spectrum analysis showed the same Rf values and spectrum pattern of standard and sample. The method was validated by using accuracy, precision, linearity, robustness, ruggedness and recovery as applicable parameters. The developed method was quite good and most sensitive for the present products. The unpleasant and bitter taste of the product was masked by different concentrations of aqueous extract of *Stevia.* Sweetness potency was determined by taste evaluation method. 1% *Stevia* extract is sufficient to produce most palatable and acceptable sweet preparation.**

Key words: Formulation, TLC profile, HPTLC method development, validation, gallic acid, health drinks, *Stevia rebaudiana.*

INTRODUCTION

The herbal drug preparation in it's entirely is regarded as the active substance and the constituents are either of known therapeutic activity or are chemically defined substance generally accepted to contribute substantially to the therapeutic activity of the drug. Photochemical screening involves botanical identification, extraction with suitable solvents, purification and characterization of the active constituents of pharmaceutical importance (Wickramasimaghe M). Quality control for the officially and safely of herbal product is essential .the quality control of photochemical may be defined as the status of a drug which is determined either by identity, purity, constant and other chemical physical biological properties or by manufacturing process .compound with synthetic drug. The critical and approach for herbal drug are much more complex. Phytopharmaceutical are always mixtures of many constituents and are therefore vary variable and difficult to characterize. The active principles in Phytopharmaceutical are not always known.

The quality criteria for herbal drugs are based on a clear scientific definition of the raw material. Depending on the type of preparation, sensory properties, physical constants, moisture, ash content, solvent residues and adulterations have to be checked to prove identity and purity. Microbiological contamination, foreign materials, heavy metals, pesticide residues, all toxins and radio activity also need to be tested. To prove the constant composition of herbal preparations, appropriate analytical methods have to be applied and different concepts have to be used in order to establish relevant criteria for uniformity (Farmsorth NR, 1982).

Health drinks contain *Ashwagandha, Tulsi, Mulethi, Awala, Shatavari, Gokhru, Arjuna, Giloy, Safed musli,Ka-limirchi, Haldi, Jaiphal* have been reported as nervine tonic, immunomodulatory agents, antioxidants, tonics for heart and liver, blood purifier.

Withania somnifera (*Ashwagandha*) (Withania Somnifera, 2004) is a tonic, abortifacient, astringent, deobstruent, nervine, aphrodisiac and sedative. It has been used in diseases such as rheumatism, leprosy and arthritis. It

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is used to treat general debility, arthritis, depression, chronic fatigue, insomnia, anxiety, depressed immunity, infertility and memory loss. It increases the iron content. *Myristica fragrans* (Varro E, 1981) (*Jaiphal*) is aromatic, carminative, digestive, anti-inflammatory, diuretic, lactagogue, aphrodisiac, hypnotic, hallucinogenic, antispasmodic and stimulant agent. *Piper nigrum* (Trease and Evan`s, 1997) (*Kalimirchi*) stimulates appetite, encourages peristalsis, tones the colon muscles and is a general digestive tonic. Sometimes it is used in gonorrhoea. On account of its stimulant action it aids digestion and is especially useful in a tonic dyspepsia and turbid condition of the stomach. *Tinospora cardiofolia* (Singh SS, 2003) (*Giloy*) is antiperiodic, antipyretic, alterative, diuretic, anti-inflammatory. It is a constituent of several compound preparations. It clears out brain toxin that hinders mental activity. *Curcuma longa* (Chattopadhyay I, 2004) (Haldi) is also used as an anti-inflammatory agent, and remedy for gastrointestinal discomfort associated with irritable bowel syndrome, and other digestive disorders. It is currently being investigated for possible benefits in Alzheimer's disease, cancer and liver disorders. *Terminalia arjuna* (Kokate CK, 2008) (*Arjuna*) is mainly used in heart disease, contusions, and fractures. It is prescribed for all sorts of conditions of cardiac failure and dropsy. The tonic made from bark is believed to have a stimulant effect on the heart. It helps strengthen the body's natural rejuvenate processes, hastening the replacement of dead or weak cells with fresh, vital ones. *Chlorophytum borivilianum* (Kokate CK, 2003) (*Safed musli*) is a rare divine-graced herb to offer all the effects required for achievement of health par excellence or for attaining the ultimate positive health. It treats male sexual inadequacies like oligospermia, lack of libido, impotency, etc, general debility. *Asparagus racemosus* (Velavan S, 2007) (*Shatavari*) has been used in Ayurveda for various conditions. Its main use has been as a galactogogue to increase milk secretion during lactation. It is also used as a general tonic, and as an aphrodisiac. It is useful in nervous disorders, dyspepsia, and tumors, scalding of urine, throat infections, tuberculosis, cough bronchitis and general debility. *Tribulus terrestris* (Trease and Evan`s, 1997) (*Gokhru*) is used in the treatment of urinary disorders and impotence, kidney diseases and gravel, diseases of the genito-urinary system, calculus affections, gout etc. It is also useful for diseases of the heart, and many other conditions. It is considered as a miracle herb in India and used as a physical rejuvenation tonic. It is being studied as a potential herbal remedy against AIDS. *Glycyrrhiza glabra* (http://openmed.nic.in/3195/01/glycyrrhiza-final.pdf) (*Liquorice*) is a popular remedy for coughs, some complications of tuberculosis, and chest complaints in general, such as bronchitis. It is also highly regarded as a soothing ingredient for sore throat and laryngitis. It is also used to strengthen and balance the female reproductive system. *Ocimum Sanctum* (Kokate CK, 2008) (*Tulsi*) is widely used as a cough medicine as well as used to expel worms. Basil is the essential ingredient used to ease headaches. It is used in malaria, bronchitis and gastric disorders. It also lowers blood sugar levels and its powder is used for mouth ulcers. *Emblica officinalis* (Kokate CK, 2008) (*Awala*) fruit is the richest known source of vitamin 'C' and used as a diuretic, appetizer, laxative, hair dye, and shampoo etc. It cures insomnia and is healthy for hair .It is also used as a Cardio protective, useful in hemorrhage, menorrahagia, leucorrhoea, and discharge of blood from uterus. *Stevia rebaudiana* (Savita SM, 2004) is a natural sweetener with nutraceutical. *S. rebaudiana* contains stevioside and rubaudioside as diterpene glycosides. These are 300 times sweeter than sugar. It also contains sequiterpene lactones that are responsible for bitter after taste. *S. rebaudiana* acts as antidiabetic, antihypertensive, anti-hyperlipidemic, anti-yeast, antibacterial, anticarcinogenic and antifungal. It is considered as GRAS by USFDA.

Health drinks are the liquid preparation that contains vitamins, amino acids, minerals and other dietary supplements. Heath drinks are useful (Naram KU, 2000) for body maintenance, to prevent and treat disease. As per the guidelines given in different references, different samples of health drink were prepared. Five different samples were developed using 0.25, 0.5, 0.75, 1 and 1.25% aqueous extract of *S. rebaudiana.* Sweetness potency of different samples was determined by taste evaluation method. Stevioside is a marker compound that could not absorb in UV region. *Stevia* contains 10 diterpene glycolsides and gallic acid. TLC is possible for evaluation of health drink using stevioside as a standard. Therefore it was our intention to develop HPTLC method using gallic acid as a standard.

Thin layer chromatography (TLC), (Scinto S, 1988) also known as planar chromatography or flat bed chromatography is like all other chromatographic techniques, a multi-stage distribution process.

HPTLC is a powerful analytical technique (Agarawal H, 2004) due to its merits of reliability, simplicity, reproducibility and speed. A new, simple, sensitive, selective, precise and robust high-performance thin-layer chromatographic (HPTLC) method for analysis of Gallic acid was developed and validated for the determination of Gallic acid in health drinks. Gallic acid is mainly present in *Embllica officinalis* but also present in every crude materials used in the formulation. The aim of this work was to develop an accurate, specific, repeatable and robust method for the determination of Gallic acid.

The proposed method was validated in compliance with ICH guidelines (Q2A, ICH, 2005).

EXPERIMENTAL

Materials

Ashwagandha, Tulsi, Mulethi, Awala, Shatavari, Gokharu, Arjun, Giloy, Safed musli, Kalimirchi, Haldi, Jaiphal were procured from authentic sources (Satara Arkshala Satara) and also authenticated by botanist, Prof. B. D. Patil, Botany department, SGM College, Karad, (M.S.). Standard Gallic acid was purchased from Loba Chemie

Pvt., Ltd. Mumbai, India. All chemicals and reagents used were of analytical grade and were purchased from Loba Chemie Pvt. Ltd., Mumbai.

Formulation development of Health Drink:

The developed formula for health drink was as follows:

Ashwagandha concentrate	100 mg
Brahmi concentrate	100 mg
Tulsi concentrate	100 mg
Mulethi concentrate	100 mg
Awala concentrate	200 mg
Shatavari concentrate	100 mg
Gokharu concentrate	100 mg
Arjuna concentrates	100 mg
Giloy concentrate	100 mg
Safed musli concentratev	100 mg
Kalimirchi concentrate	50 mg
Haldi concentrate	50 mg
Jaiphal concentrate	50 mg
Stevia concentrate	05 mg

This formula was developed for 25 ml of health drink.

Aqueous extract of each crude drug was obtained separately by cold maceration method (WHO Quality control). The health drink was prepared by aqueous extraction method.

Each concentrate of different crude drugs was mixed together and was treated with purified water for 7 days with occasional shaking at room temperature and homogeneous product was developed. Such five preparations of 500 ml capacity were obtained each containing 0.25, 0.5, 0.75, 1 and 1.25% *Stevia* extract respectively. All the five samples of heath drinks were observed for its general appearance, color, odour and taste A slightly viscous yellow colored health drinks having suitable sweet taste were prepared. The sweetness potency (Rangari, 2005) of final product was determined by taste evaluation method. The sensory profile from a panel of 10 experts was noted by considering sucrose as a standard.

TLC profile (Wagnar H, 2004)

TLC plates were developed by using mobile phase as toluene: ethyl acetate: formic acid: ethanol (6: 4: 0.3: 0.4). Rf value was calculated by the ratio of the distance traveled by the spot to the distance traveled by the solvent.

For TLC, silica G was used as a coating material. The TLC plates were prepared by applying silica G on glass plate by pouring method that is, a measured amount of slurry of silica health drink was poured on glass plate which is kept on a level surface. The plate was then tipped back and forth to spread the slurry uniformly over the surface. Dried the plate at room temperature for 30 min then activate the plate at 110°C for thirty minutes in an oven.

The sample of health drinks and standard Gallic acid were applied by using capillary tubes then again dry the plate at room temperature. The plate was then placed in a development chamber. Thebottom of the chamber was covered up to nearly one mm by the solvent system after solvent has traveled one half to two thirds the length of the plate. Plates were removed and dried at room temperature. The positions of the spots were determined. The Rf value of the spots were determined

HPTLC (Wagnar H, 1996) Method development

Instrumentation and chromatographic conditions: The samples were spotted in the form of bands of width 6 mm with a Camag microlitre syringe on precoated silica health drink aluminium plate $60F_{254}$ (20 cm×10 cm) with 200 µm thickness (E. Merck, Germany) using a Camag Linomat V (Switzerland) sample applicator. A constant application rate of 150 nL s^{-1} was employed and space between two bands was 10 mm. The slit dimension was kept at 5 × 0.45 mm and 20 mms^{-1} scanning speed was employed. The mobile phase consisted of Toluene: Ethyl Acetate: Formic Acid: Methanol (3:3:0.8:0.2 v/v). Linear ascending development was carried out in a twin trough glass chamber saturated with mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25 ± 2°C) at relative humidity of 55 ± 5%. The length of chromatogram run was 80 mm. Subsequent to the development; TLC plates were dried in a current of air with help of an air-dryer. Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 254 nm. The source of radiation utilized was a deuterium lamp.

Calibration curve of Gallic acid

A stock solution of Gallic acid (100 µgmL−1) was prepared in methanol. Different volumes of stock solution 1, 2, 4, 6, 8, 10, 12µL were spotted on the TLC plate to obtain concentrations of 100, 200, 400, 600, 800, 1000, 1200 ng spot−1 of Gallic acid, respectively. The data of peak areas plotted against the corresponding concentrations were treated by least-square regression analysis.

Method validation (Ansari MJ, 2005)

Precision

Repeatability of the sample application and measurement of peak area were carried out using six replicates of the same spot (600 ng spot−1 of Gallic acid) and was expressed in terms of percent relative standard deviation (%R.S.D.) and standard error (S.E.). The intra- and inter-day variation for the determination of Gallic acid was carried at three different concentration levels of 400, 600 and 800 ng spot−1.

Robustness of the method

By introducing small changes in the mobile phase composition, mobile phase volume, duration of mobile phase saturation and activation of prewashed TLC plates with methanol, the effects on the results were examined. Robustness of the method was done in triplicate at a concentration level of 600 ng spot−1 and the %R.S.D and S.E. of peak areas was calculated.

Limit of detection and limit of quantification

LOD was considered as 3:1 and LOQ as 10:1. LOD and LOQ were experimentally verified by diluting the known concentrations of Gallic acid until the average responses were approximately 3 or 10 Times the standard deviation of the responses for six replicate determinations.

Recovery studies

The pre-analyzed samples were spiked with extra 50, 100 and 150% of the standard Gallic acid and the mixtures were reanalyzed by the proposed method. The experiment was conducted six times. This was done to check for the recovery of the Gallic acid at different levels in the formulations.

Ruggedness

A solution of concentration 1000 ng spot−1 was prepared and analyzed on day 0 and after 6, 12, 24, 48 and 72 h. Data were treated

for %R.S.D. to assess ruggedness of the method.

Specificity

The specificity of the method was ascertained by analyzing the Standard drug and Health Drink. The spot for Gallic acid in the sample was confirmed by comparing the *R*f values of the spot with that of the standard. The peak purity of the Gallic acid was assessed by comparing the spectra at three different levels, viz. peak start (S), peak apex (M) and peak end (E) positions of the spot.

Detection of related impurities

The related unknown impurities were determined by spotting higher concentrations of the Gallic acid. Gallic acid solution was prepared at a concentration of 2000 μgml^{-1} in methanol, and this solution was termed as sample solution. One milliliter of the sample solution was diluted to 40mL with methanol and this solution was termed as standard solution (50 μgml^{-1}). Two microlitres of both the standard (100 ng $spot^{-1}$) and the sample solution (4000 ng $spot^{-1}$) were applied on HPTLC plate and the chromatograms were run as described.

Analysis of gallic acid in health drink

An accurately weighed quantity of Health Drink equivalent to about 100 ng of Gallic acid, that is, 8.5 g of Health Drink was extracted with 25 ml methanol by sonication for 30 min. This extract was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was filtered and the filtrate was dried to constant weight at room temperature. The residue was redissolved in 5 ml of methanol. Six micro liters of the filtered solution was applied on the TLC plate followed by development and scanning as described in Section 2.2. The analysis was repeated in triplicate.

RESULT AND DISCUSSION

Development of the optimum mobile phase

The TLC procedure was optimized with a view to quantify the Health Drink. Initially toluene: ethyl acetate: formic acid: methanol in varying ratios was tried. The mobile phase toluene: ethyl acetate: formic acid: methanol (3:3: 0.8:0.2 v/v) gave good resolution with *R*f = 0.59 for Gallic acid but typical peak shape was missing. Finally, the mobile phase consisting of (3:3:0.8:0.2 v/v) gave a sharp and well-defined peak at *R*f = 0.59.Well-defined spots were obtained when the chamber was saturated with mobile phase for 30 min at room temperature.

Calibration curves

The developed HPTLC method for estimation of Gallic acid showed a good correlation coefficient (r^2 = 0.9991 ± 0.0002) in concentration range of 100 - 1200 ng $spot^{-1}$ with respect to the peak area. Fig. 3 displays three-dimensional image of the calibration samples at 254 nm. The mean value (±S.D.) of slope and intercept were 4.1312 ± 0.0491 and 208.2135 ± 4.5092, respectively. No significant difference was observed in the slopes of standard curves (ANOVA, P > 0.05).

Method validation

The %R.S.D. for repeatability of sample application (600 ng $spot^{-1}$) and measurement of peak areas were found to be 0.09 and 0.15% respectively. The measurement of the peak area at three different concentration levels showed low values of S.E. and % R.S.D. (<1%) for inter- and intra-day variation, which suggested an excellent precision of the method (Table 1). The low values of S.D., %R.S.D. and S.E. obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method (Table 2). Detection limit and quantification limit with signal-to-noise ratio of 3:1 and 10:1 were found to be 2.27 and 7.58 ng respectively, which indicates the adequate sensitivity of the method.

The proposed method when used for extraction and subsequent estimation of Gallic acid from the formulation afforded recovery of 98.91 - 101.34% as listed in (Table 3). Low %R.S.D. value of 0.0551 between the peak area values proved the ruggedness of the method indicating that Gallic acid is stable during the extraction procedure as well as during analysis. The peak purity of Gallic acid was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot as shown in Graph 1 and 2. Good correlation (r = 0.9992) was obtained between the standard and the sample overlain spectra of Gallic acid.

Analysis of the prepared formulation health drink

A single spot at *R*f = 0.59 was observed in the chromatogram of the Gallic acid extracted from health drink (Graph 2). There was no interference from the excipients and the other active components present in the herbal health drink formulation. The % recovery of the Gallic acid from the health drink formulation was found to be 98% and was well within the limits (label claim ±5%).

By considering Rf values of standard Gallic acid and spots observed of samples, fingerprint analysis, presence of this active chemical marker compound is detected. Chromatogram of sample solution showed other peaks than those of standards which might be due to the presence of other minor of major phytoconstituents present in it

Stevia extract in various concentration potentiate sweetness index of health drink. 0.5% aqueous extract of *Stevia* is sufficient to produce acceptable and palatable product. Stevioside have no any chromospheres group that can absorb in UV region and hence gallic acid is the only one chiefly available marker compound which was used as a standard for HPTLC work.

Conclusion

The sweet formula for health drink was prepared which

Table 1. Intra and Inter-day precision of HPTLC method (n = 6).

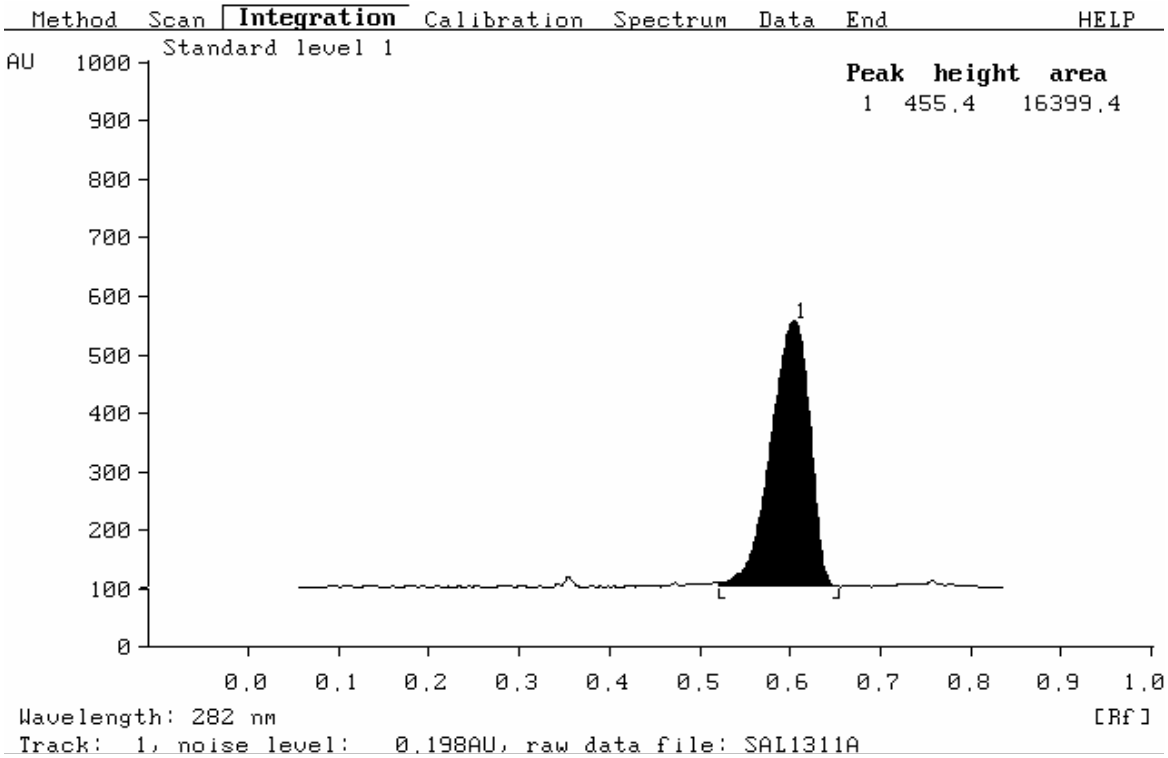
Amount (ng spot^{-1})	Intraday precision				Inter-day Precision			
	Mean area	S.D.	% RSD	S.E.	Mean area	S.D.	% RSD	S.E.
400	5826.10	1.55	0.025	0.584	5898.58	1.51	0.082	0.62
600	6720.68	1.51	0.054	0.52	6742.35	1.98	0.075	0.82
300	7450.55	1.41	0.048	0.59	7421.81	2.91	0.055	0.75

Table 2. Robustness of the HPTLC Method (n = 3,600 ng/spot).

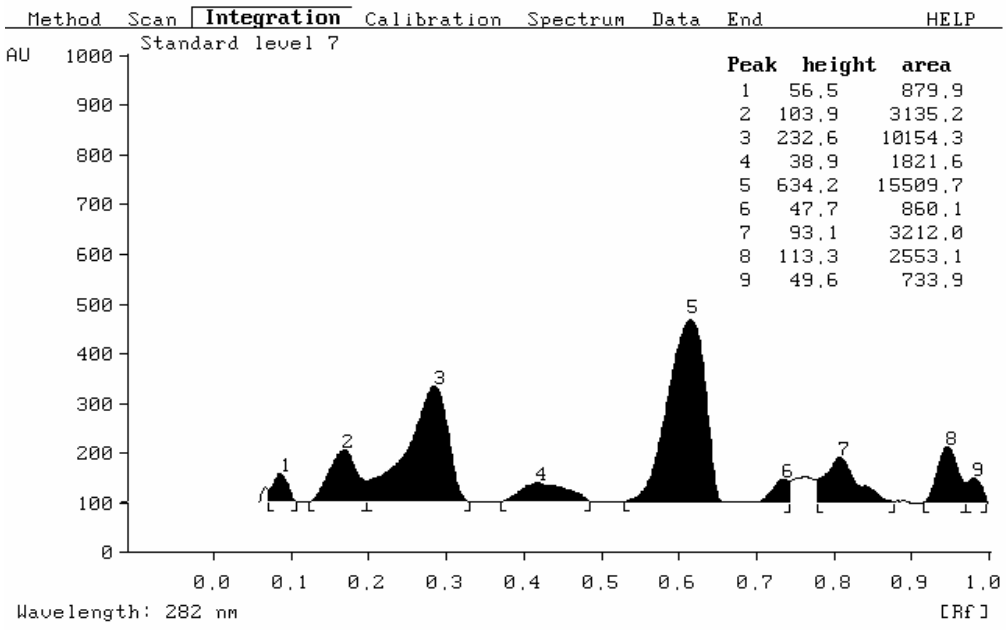
Parameters	S.D. of peak area	% RSD	S.E.
Mobile phase composition	1.62	0.3859	0.1575
Mobile phase Volume (18,20,22 ml)	1.79	0.2857	0.1167
Duration of Saturation (20,30,40 min)	1.42	0.2541	0.1180
Activation of prewashed TLC plates (2.5 and 7 min)	1.25	0.1874	0.0850

Table 3. Recovery Studies (n = 6).

Excess drug added to analyte (%)	Theoretical content (ng)	Amount Found (ng)	Recovery (%)	% RSD	S.E.
0	400	395.54	98.85	0.254	1.85
50	600	601.91	100.15	0.161	1.71
100	800	805.71	100.35	0.995	1.35
150	1000	981.90	98.19	0.105	1.81



Graph 1. Chromatogram of standard Gallic acid.



Graph 2. Chromatogram of Gallic acid and other constituents present in health drink.

renders the product of appropriate sweetness potency. The developed sweet health drink constitutes major and minor chemical moieties including stevioside as a natural target molecule. The present work gives a scientific data about the formulation and development of herbal medicines using natural sweetener and a qualitative analysis or presence of antioxidant by HPTLC method. Gallic acid is a common chemical constituent of crude drugs in the formulation, used as a chemical marker compound in present study. TLC profile ensures (Soni Sapna, 2008) best resolution of a standard Gallic acid and sample. The developed HPTLC technique is a precise, specific, accurate and robust for the determi-nation of Gallic acid. Statistical analysis proves that the method is reproducible and selective for the analysis of Gallic acid. Since the proposed mobile phase effectively resolves Gallic acid, the method can be used for qualitative as well as quantitative analysis of Gallic acid in Health Drink .Further the proposed method can be extended to study the degradation of Gallic acid under different stress conditions, as per the recommendations of ICH guidelines.

As *Stevia* acts as antidiabetic, antihypertensive, antihyperlipidemic, antioxidant, antimicrobial and nutraceutical, its use as a natural sweetener or versatile excipients in herbal preparations can provide a new vista to pharmaceutical industry so as to produce the product most elegant, sweet, acceptable and palatable by diabetic consumers.

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