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DETERMINATIONOF CATECHIN AS BIOMARKER IN ENTOCID POLYHERBAL DIGESTIVE SYRUP BY HPLC/DAD

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ABSTRACT

Entocid syrup is a balanced composition of vigilantly selected and formulated with effective herbs of proven efficacy in intestinal ailments covering the segment of hyper-acidity symptoms. Entocid is scientifically formulated in such a way that it not only neutralizes the acid secretion but at the same time it strengthens the stomach thus acts promptly and relieves the symptoms of GI upsets and in the long-run prevents the recurrence of such symptoms. The ingredients of Entocid syrup are rich in alkaloids, proteins, vitamins, glycosides, bioflavonoid and trace elements, formulated using ten potent herbal drugs namely *Amomum sabulatum*, *Berberis aristata*, *Cinnamomum tamala*, *Coriandrum sativum*, *Cuminum cyminum*, *Foeniculum vulgare*, *Vitis vinifera*, *Mesua ferrea*, *Glycyrrhiza glabra* and *Mentha piperita*. The flavonoid compounds exhibit preventive effects from colon carcinogenesis, lipid lowering effects, anti-oxidant, anti-inflammatory, anti-depressant and anti-atherogenic properties. To develop the assay method, high-performance liquid chromatography (HPLC) with a diode-array detector (DAD) was used. Using HPLC/DAD, catechin was eluted with gradient program. The UV characteristic of catechin, the detection wavelength was monitored at 265 nm. Method validation including limit of quantitation, the accuracy, inter and intra day and limitation was performed by using recovery tests. The future development of flavonoids-based drugs is limitation and assumes to provide significant effects on digestion related diseases.

Keywords: Polyherbal, Entocid, Catechin, HPLC-DAD, Digestive syrup.

INTRODUCTION

Herbal medicines are gaining more and more attention all over the world, due to their long historical clinical practice and less side effects[1]. Major categories of Traditional/Complementary and alternative medicines (TCAM) invogue in the developing and developed countries are categorized as: whole body systems(Ayurveda, homeopathy, Unani, and Traditional Chinese Medicine); mind–body medicine(meditation, prayer, mental healing); biologically based therapies (use of natural substances,such as herbs, foods, vitamins, dietary supplements, herbal products); manipulative andbody-based practices (massage); and energy medicine (Reiki)[2]. In Pakistan traditional medicines have been a strong part of our cultural heritage and playing asignificant role in providing health care to a large part of the population. However, there hasbeen lack of concerted efforts for proper utilization of traditional medicines in the health care system. Primarily three categories i.e. Tibb-e-Unani, Ayurveda and Homoeopathy arein vogue whereas Chinese Traditional System, Reiki, Acupuncture and aromatherapy hasbeen introduced in certain parts of the country in the last few years[3].Herbal dosage form that are being manufactured customary should meet the quality assessment and quality control as a prerequisite for clinical efficacy and that secondary metabolites present in these herbal ingredients are considered as the compounds responsible for their bioactivity and efficacy.

The bactericidal property of catechin [(2R,3S)-3',4',5,7-tetrahydroxyflavan-3-ol]plays several roles in the digestive tract. In the small intestine, catechins inhibit α-amylase activity, and a certain amount is absorbed into the portal vein. Although catechins are bactericidal, they do not affect lactic acid bacteria. Catechins in the diet for several weeks decreases putrefactive products and increases organic acids by lowering pH enhances preventive effects from colon carcinogenesis[4] .Catechin is known to possess preventive effect in cardio vascular diseases due to its involvement in oxidative process in atherogenesis[5]. Being antioxidant, catechin is able to modulate cellular signaling pathways that lead to elevation of vascular reactivity, platelet aggregation, and reduction of inflammation [6].

Desai et al determined Quercetin glucoside in Azadirachta indica and reported the optimization and validation parameters [7]. Whereas Lu Zhao and associate worked on HPLC-DAD detector methods for the simultaneous determination of five compounds in Sea Buckthorn with 280 nm as detection wavelength and methanol-0.4% phosphoric acid solution (gradient elution) as mobile phase under chromatographic conditions; the five compounds were well separated [8].

Alam et al [9] carried out study on quantification of glycyrrhizin biomarker in Glycyrrhiza glabra rhizome and baby herbal formulations by validated RP_ HPLC methods. A thin-layer chromatographic method has been used for quantification of glycyrrhizin in Glycyrrhiza glabra rhizome. The method was validated, as per ICH guidelines for precision, accuracy, and robustness.

In present paper, polyherbal formulation designated as Entocid syrup prepared from ten potent herbal drugs namely *Amomum sabulatum*, *Berberis aristata*, *Cinnamomum tamala*, *Coriandrum sativum*, *Cuminum cyminum*, *Foeniculum vulgare*, *Vitis vinifera*, *Mesua ferrea*, *Glycyrrhiza glabra* and *Mentha piperita*. This formulation was subjected for the standardization and there in biomarker was quantitative evaluated. The different herbal component utilized in Entocid syrup cited in the literature is delineated here with in Table 1.

MATERIALS AND METHODS

Reagents.

All the chemicals used were of AR grade purchased from Merck. Reference standard compound catechin hydrate was purchased from Sigma-Aldrich. Authenticated herbal samples of Entocid formulations were purchased from Insaf Kirana store, Jodia Bazar, Karachi. The identity was of different herbal drugs was established by Prof. Dr. Iqbal Azhar, Department of Pharmacognosy and specimens were deposited in Herbion Sample Collection as *Amomum sabulatum*, *Berberis aristata*, *Cinnamomum tamala*, *Coriandrum sativum*, *Cuminum cyminum*, *Foeniculum vulgare*, *Vitis vinifera*, *Mesua ferrea*, *Glycyrrhiza glabra* and *Mentha piperita*.

Preparation of Entocid Syrup

Cleaned and grinded herbs were extracted in an extractor filled with deionized water. After extraction, filter the aqueous extract through muslin cloth. The filtrate is transferred to evaporator. Concentrate the filtrate through thin film rotary evaporator thus yielding thick syrupy reside extract.

Manufacturing tank was filled with deionized water and it was heated to boil for 10-15 minutes. When boiling starts sucrose was added slowly with constant stirringand heating for 30 minutes so that it become homogenized syrup (solution 1).

Desired flavor was added in solution 1 and mixed the contents vigorously. In a separate tank, dissolve methyl paraben, propyl paraben, propylene glycol, citric acid anhydrous and glycerin in deionized water. Heat with constant stirring so that it is mixed properly, filter the solution and mix it with solution 1. This should be continued stirring for another 10 minutes (Solution 2).Take deionized water in jacketed kettle and add both solutions into it (Solution 1 + Solution 2) continue heating and stir the mixture till mixedwell so as to obtain in the syrup form. Now start the chilled water circulation to allow the syrup cool down to room temperature.Filter and transfer the obtained syrup to storage tank.

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Table 1 Active ingredients of Entocid syrup

S. No.	Plant Name	Common Name	Medicinal Uses
1.	Ammomum subulatum	Ilaichikalan	Treatment of indigestion, vomiting, biliousness, abdominal pains and rectal diseases
2.	*Berberis aristata*	Zarishk	Anti-inflammatory, anti-tumor and anti-diabetic properties
3.	*Cinnamomumtamala*	Tezpat	Antioxidant, anti-diabetic
4.	*Coriandrum sativum*	DhaniyaKhushk	Treatment of indigestion, vomiting, diarrhea and colon ulcers
5.	*Cuminum cyminum*	Zeera	Treatment of fever, loss of appetite, diarrhea, vomiting, abdominal distension, edema and puerperal disorders
6.	*Foeniculum vulgare*	Saunf	Antioxidant, anti-inflammatory, antimicrobial, bronchodilator, estrogenic, diuretic, lithontripic, galactogogue, emmenagogue, antithrombotic, hypotensive, gastro protective, hepatoprotective, memory enhancing, and antimutagenic properties
7.	*Vitis vinifera*	Kishmish	Treatment ofconstipation, thirst, cholera, smallpox, nausea, skin and eye infections as well as kidney and liver diseases
8.	*Mesua ferrea*	Narmushk	Anti-microbial, anti-inflammatory
9.	*Glycyrrhiza glabra*	Mulethi	Anti-ulcer, laxative, anti-diabetic, anti-inflammatory, immunomodulatory, antitumor and expectorant properties
10.	*Mentha piperita*	Podina	Treatment of nausea, vomiting, abdominal pain, indigestion, irritable bowel, and bloating

HPLC analysis

Sample Preparation.

Weigh about 12.0 gm of Entocid syrup in a 50 ml volumetric flask. Add 40 ml of 80% aqueous methanol in 50 ml volumetric flask containing syrup, further add 80% aqueous methanol to make up the volume up to mark. Stir the mixture for 15 mins, then sonicate the mixture for 5 minutes. Filter the mixture with filter paper (0.45 µm), thereafter further filter the solution with 0.22 µm HPLC filter paper as require and proceed for HPLC analysis.

Reference standard solution preparation.

Quantitative standard 3.0 mg of catechin hydrate reference standard in a vial and dissolve it in 10 ml 80% aqueous methanol. Filter the obtained solution with HPLC filter (Whattman) and proceed for HPLC analysis.

Analysis.

Analysis of catechin was conducted using an Agilent 1200 series HPLC equipped with a diode array detector (DAD). The dissolved sample was separated on Luna® 5 µm C18 100 Å, LC Column 250 x 4.6 mm, Ea . The mobile phase was composed of two solvents: solvent A was degassed water with 0.1% phosphoric acid, and solvent B was degassed with pure methanol with 0.1% phosphoric acid. An elution gradient was performed as follows: initially 65.0%of solvent A, followed to 25% A over 10 min, 65% A at 25 min. The flow rate was 0.7 mL/min, and the column temperature was 20 °C.The DAD acquisitions were integrated at 265 nm ((+)-catechin hydrate). The identification of flavonoid catechin was achieved by comparing retention times of sample with reference standard compound.

RESULTS AND DISCUSSION

Phytochemical analysis.

Phytochemical analysis conducted on the 80% aqueous methanol extract of the formulation is shown in Table 2. The presence of catechin contributed to the activity of the formulation. Pure isolated peaks of reference standard compound catechin, catechin present in Entocid syrup batch No. 016 as well as placebo of Entocid syrup, respectively (Fig. 1; Fig. 2; & Fig. 3).

Table 2 Catechin in Entocid syrup.

S. No.	Batch No.	Compound	mg/ 10 ml syrup
1.	016	Catechin	0.05 mg

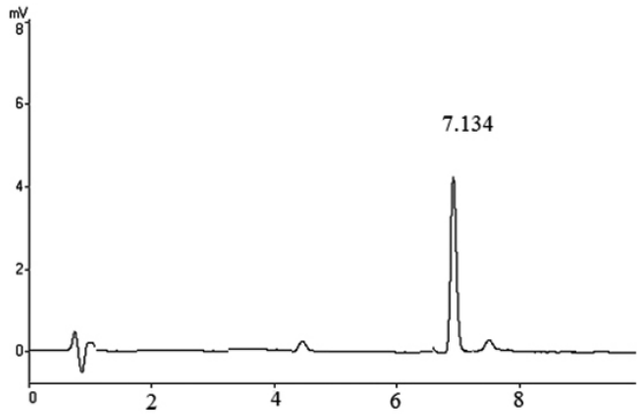

Fig. 1 Isolated peak of reference standard compound Catechin.

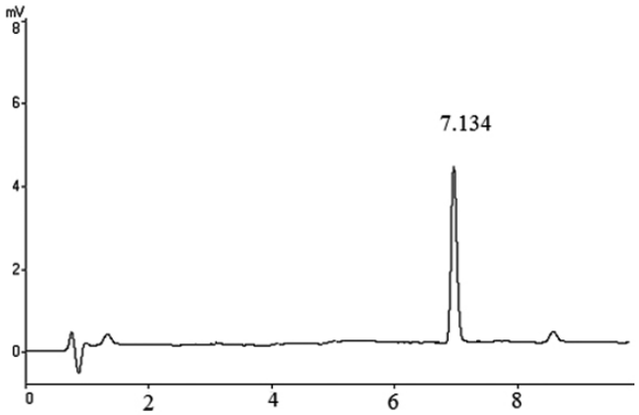

Fig. 2 Isolated peak of compound Catechin in Entocid syrup batch no. 016.

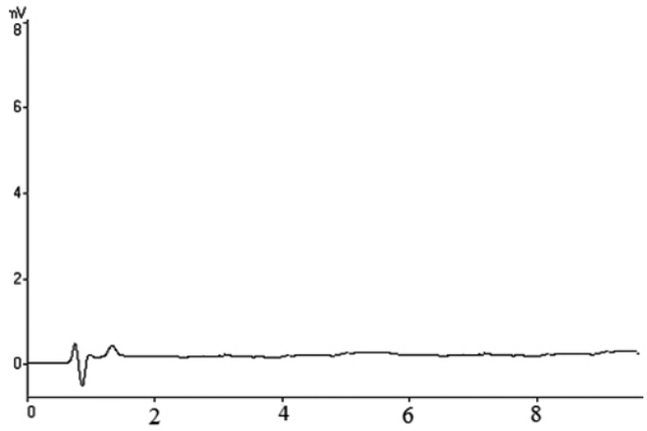

Fig.3 Placebo of Entocid syrup.

Validation of developed method

Limit of detection and limit of quantization. LOD and LOQ of the drug were derived by calculating the signal-to-noise ratio (S/N, 3.3 forLOD and 10 for LOQ) using the following equation designated by ICH guidelines. The residual standard deviation of regression line or standard deviation of Y intercept of regression lines was used to calculate LOD and LOQ.

LOD = 3.3 × (D/S)
LOQ = 10 × (D/S)

Where; D=Standard deviation of y intercept on regression lines and S =Slop of calibration curve.

Accuracy.

To check the accuracy of the method, recovery studies were carried out 80%, 100% and 120% of the test concentration as per ICH guidelines. The recovery study was performed three times at each level.

Interday and Intraday precision.

The intraday precisions were determined by estimating the corresponding response 3 times on the same day for catechin; whereas the interday precision were determined by estimating the corresponding response on 3 different days over a period of 1 week. The results were reported in terms of relative standard deviation (RSD).

Linearity.

Linearity range for catechin was 2-10 µg/ml at wavelength i.e. 265 nm. The coefficients of correlation for catechin at 265 nm were 0.999. Drug showed good regression value at its respective wavelength and the results of recovery study revealed that any small change in the drug concentration in the solution could be accurately determined by the developed method. Percentage estimation of catechin in herbal formulation was found to be 97.02±0.13 with standard deviation <2. The validity and reliability of developed method was assessed by recovery studies. Sample recovery for the compound was in good agreement, which suggested no interference of other extracted content in estimations. Precision was determined by studying the interday and intraday precision. In both intra and inter day precision study for the herbal drug % RSD were not more than 2.0%, indicates good repeatability and intermediate precision Table 3 & Table 4).

The retention time of quercetin-3-O-β-d-glucoside was 11.213 min. for Azadirachta indica, whereas in case of Quercetin it was 7.134 min in Entocid polyherbal formulation. The method was found to be linear in the range of 4.0-60 µg mL-1 (LOD 0.0535 µg/ml, LOQ 0.1621µg/ml). Limit of detection and limit of quantitation of the proposed method were found to be 1.33 and 4.0 µg mL-1, respectively. The mean recoveries were found to be within 93.53-103.75% (97.32). The method can be used as quality control tool for routine analysis of herbal extracts and formulations containing Azadirachta indica[7].

The work on Glycyrrhiza was conducted and results on the developed plate was scanned and quantified densitometrically at 256 nm However Entocid polyherbal formulation also contains Glycyrrhiza and it was Linearity range for catechin was 2-10 µg/ml at wavelength i.e. 265 nm. Glycyrrhizin peaks from Glycyrrhiza glabra rhizome formulations were identified by comparing their single spot at Rf = 0.63 ± 0.01. Linear regression analysis revealed a good linear relationship between peak area and amount of glycyrrhizin in the range of 2000-7000 ng/band. Also Quercetin also gave better performance in linear regression.

Table 3 Validation parameter for Catechin estimation.

Parameter	Catechin
λ_{max}	265.0 nm
Linearity equation	y = 0.06x + 0.004
Slope	0.06
R^2	0.999
LOD	0.0535 µg/ml
LOQ	0.1621µg/ml
Intraday precision (%RSD)	0.877±0.0034
Interday precision (%RSD)	0.993±0.0054

Table 4 Recovery study of Catechin.

Concentration Variation	Concentration Prepared (µg/ml)	% Recovery (w/w)
	Catechin	Catechin
80%	8	98.13
100%	10	96.10
120%	12	98.99
	Mean Recovery	97.32
	±S.D.	1.47

Standardization based on a single chemical markers serves to promote quality control and batch-to-batch consistency as has been observed in case of Quercetin.

Even validation of an Isocratic LC method for determination of Quercetin in was done in Nano formulation. Quercetin was analyzed on a Grace smart RP C18 column (250.0 × 4.6mm, 5µ) at ambient temperature under isocratic elution conditions using phosphate buffer pH 3.5: acetonitrile (65:35% v/v) as mobile phase at a flow rate of 1.0 mL/min. The column effluent was monitored at 254 nm. The developed method was validated according to ICH guidelines Q2(R1). The proposed method is found to be precise and accurate with a linearity range of 0.25 to 20.0µg/mL for quercetin. The method was found to be accurate with mean recovery of quercetin 99.5±0.52%. The presence of excipients did not show any interference on the determination of quercetin compounds indicating method specificity [10].

The isolated catechin is a known flavonoid which is present in many plant species. It is known to have anti-inflammatory, anti-mutagenic, anti-cancer activity. It is very effective in prevention and treatment of stress related disorders as it preserves antioxidant levels by inhibition of lipid peroxidation[11]. Good recovery values showed that HPLC method is free from interferences. This method was successfully used for the determination of catechin from polyherbal formulation Entocid. The developed HPLC method is simple, rapid, accurate, precise, and economic and validated as per ICH guidelines. This method can be successfully used for estimation of catechin in herbal formulation.

Conflict of Interest: The authors declare no conflict of interest.

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