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A novel HPTLC method for quantitative estimation of biomarkers in polyherbal formulation

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ABSTRACT

Objective: To explore the quantitative estimation of biomarkers gallic acid and berberine in polyherbal formulation Entoban syrup.
Methods: High performance thin layer chromatography was performed to evaluate the presence of gallic acid and berberine employing toluene: ethyl acetate: formic acid: methanol 12:9:4:0.5 (v/v/v/v) and ethanol: water: formic acid 90:9:1 (v/v/v), as a mobile phase respectively.
Results: The R_f values (0.58) for gallic acid and (0.76) for berberine in both sample and reference standard were found comparable under UV light at 273 nm and 366 nm respectively. The high performance thin layer chromatography method developed for quantization was simple, accurate and specific.
Conclusions: The present standardization provides specific and accurate tool to develop qualifications for identity, transparency and reproducibility of biomarkers in Entoban syrup.

1. Introduction

During the past decades, public interest in herbal medicine has increased exponentially [1–3]. According to the World Health Organization, mass population (65%–80%) in developing countries depends essentially on plants for primary health care needs owing to poverty and lack of access to modern medicine [4]. The resurgence of herbal medicines has increased the international trade enormously. Herbal medical database indicates that herbal medicine markets in Asia and Japan had reach $2.3 and 2.1 billion, respectively [5]. Pharmaceutical companies have established renewed concern in exploring plants as a major source for new lead structures and for the development of standardized phytotherapeutic agents with promising safety, efficacy and quality [6,7].

Revival of significance and the emergent market of herbal medicinal products necessitate strong commitment by stakeholders to safeguard the end users. Variability in constituents of plant material, coupled with the variety of extraction techniques and processing steps used by different manufacturers, results in distinct inconsistency in the quality of herbal products. Furthermore, various hazardous side effects, hypersensitivity reactions, effects from adulterants, and interactions with herbal drugs have been confirmed, drawing the consideration of many regulatory agencies for the standardization of plant based drugs [8]. The World Health Organization has developed specific guiding principles to support the associated countries to instigate nationalized policies on plant based drugs and to study their prospective safety, efficacy and quality, as a prerequisite for global harmonization [7,9,10].

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Standardization is a system ensuring predefined set of the quantity, quality and therapeutic effect of the constituents in each dose [11]. It is an imperative stride in establishing a quality assurance plan for production and manufacturing thereby, curtailing batch to batch variation and reassuring acceptability, safety, quality and efficacy of the polyherbal formulations [12–15]. The validation of plant based drugs and recognition of adulterants from authentic curative herbs are important for both pharmaceutical industries and community health [16]. Establishment of suitable analytical methods which can consistently determine quantitative evaluation of marker/bioactive compounds and other key components, is a challenging task for scientists.

Technological advancements which take place in the processes of isolation, purification and structural elucidation of natural compounds have made it probable to generate appropriate strategies for the analysis of quality and standardization of plant based medicines [7]. An appliance of highly oriented hyphenated techniques provides a definite tool in herbal investigations. A variety of sophisticated methods such as spectrophotometric, chromatographic, polarography, electrophoresis, and the use of molecular biomarkers in fingerprints are presently employed in standardization of plant based medicines. Thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) fingerprint profiles are used for ensuring the identity, transparency and potency of herbal formulations [17]. TLC is the common fingerprint method that is commonly used for evaluation of stability and consistency of polyherbal preparations from different manufactures [18]. HPTLC fingerprint is mostly used for evaluating the compounds with low or moderate polarities [19]. Combined chromatographic fingerprinting with metabolomics facilitates to control the intrinsic quality of herbal drugs [20]. The innovation of analytical techniques provides a specific and rapid tool in the herbal research, permitting to set quality specifications of plant based medicines.

The present study was directed to polyherbal formulation Entoban syrup which integrates an outstanding blend of herbs that have been used for decades to eradicate microorganisms and worms from gastrointestinal tract. It is the combination of *Holarrhena antidysenterica* (*H. antidysenterica*), *Berberis aristata* (*B. aristata*), *Symplocos racemosa*, *Querecus infectoria* and *Helicteres isora*. Gallic acid is a common phytoconstituent present in Entoban syrup which has been reported to possess antimicrobial and antioxidant activity. Therefore it was thought that quantification of gallic acid can be helpful in routine quality control of formulation [21]. Berberine is specific marker from *B. aristata* used to treat many health concerns, including intestinal problems, bacterial infections, and inflammation. Therefore the present study was directed towards the quantitative estimation of biomarkers gallic acid and berberine in polyherbal formulation Entoban syrup to ensure the quality of product using HPTLC.

2. Materials and methods

2.1. Chemicals

Chloroform, formic acid, ethyl acetate, toluene were purchased from Merck, Pakistan. Methanol and ethanol of analytical reagent grade (Merck, Darmstadt, Germany) were used. Gallic acid and berberine reference standard were purchased from Sigma–Aldrich GmbH, Germany. All other solvents and chemicals were of the highest analytical grade.

2.2. Apparatus

Linomat V Automatic Sample Spotter (CAMAG, Muttenz, Switzerland), 100 μL syringe (Hamilton, Bonaduz, Switzerland), glass twin trough chamber (20 cm × 10 cm × 4 cm) (CAMAG), TLC Scanner 3 linked to Win Cats software (CAMAG), 0.2 mm thickness pre-coated with silica gel 60 F254 (Merck) were used in this study. The experiment was carried out under the conditions with temperature of (25 ± 2) °C and relative humidity of 40%.

2.3. Quantitative estimation of gallic acid and berberine

2.3.1. Standard preparation of gallic acid

The standard solution was prepared containing known concentration of 0.4 mg/mL by dissolving 4 mg standard of gallic acid monohydrate in 10 mL of methanol.

2.3.2. Sample preparation of gallic acid

A total of 12.0 g of syrup was weighed accurately in 100 mL conical flask; 30 mL of water was added and mixed thoroughly. The solution was transferred carefully in 250 mL separating funnel and 50 mL of ethyl acetate was added in the funnel and was shaked carefully for 3 min. After complete separation of layers, upper ethyl acetate layer was filtered through the paper filter with anhydrous sodium sulphate (about 10 g) in 500 mL round bottom flask. Extraction was repeated four times more and ethyl acetate fraction was collected into the same round-bottomed flask. The organic fraction was evaporated under vacuum. The dry residue was dissolved in 5 mL of methanol and transferred quantitatively into a 10 mL volumetric flask. The solution's volume was brought up to the mark with methanol.

2.3.3. Standard preparation of berberine

The standard solution was prepared containing known concentration (0.1 mg/mL) by dissolving 1 mg standard of berberine hydrochloride in 10 mL of methanol.

2.3.4. Sample preparation of berberine

About 40.0 g of syrup was weighed accurately in 100 mL conical flask; 30 mL of water was added and mixed carefully. The resulting solution was transferred in 250 mL separating funnel. The solution was extracted by adding 50 mL of chloroform in the separating funnel and shaked carefully for 3 min. The layers were allowed to separate, after full division, lower chloroformic layer was filtered through the paper filter with anhydrous sodium sulphate (about 10 g) in 250 mL conical flask.

The top water layer was further extracted with 50 mL of chloroform. The extraction was repeated using 50 mL portions of chloroform (5 times in total). The extract was evaporated to dryness under vacuum. The dry residue was dissolved in 5 mL of methanol and transferred quantitatively into a 10 mL volumetric flask. The solution's volume was brought up to the mark. The obtained solution was filtered through Whatman filter paper No. 44 and filtrate was used as a sample.

2.3.5. Procedure

Analysis was performed on 20 cm × 10 cm HPTLC silica gel G60 F254 plates with fluorescent indicator. Before starting the analysis, HPTLC plates were cleaned by predevelopment with methanol by ascending method. HPTLC plate was immersed in a CAMAG glass chamber (20 cm × 10 cm), containing 30 mL methanol (HPLC grade) as solvent system. The chamber was covered with glass lid and left till development of the plate to the top with methanol. After complete development, the plate was removed from TLC glass chamber and dried in an oven at 105 °C for 5 min. Three spots of 10 µL were applied (in the form of band) of standard preparation along with three spots of 10 µL of sample preparation as the bands on the same plate by means of a CAMAG Linomat 5 (automated spray-on applicator equipped with a 100 µL syringe and operated with the settings band length 6 mm, distance between band 15 mm, distance from the plate side edge 15 mm, and distance from the bottom of the plate 15 mm).

2.3.6. TLC development and scanning for gallic acid

The plate was developed by immersing sample HPTLC plate in a CAMAG glass chamber (20 cm × 10 cm) containing the solvent system toluene: ethyl acetate: formic acid: methanol 12:9:4:0.5 (v/v/v/v). After complete development, the plate was allowed to dry by keeping in fume cupboard for 10 min and then kept in hot air oven for 5 min at 105 °C. The plate was scanned in the densitometer by linear scanning at 273 nm for gallic acid by using a TLC Scanner III CAMAG with a D2 source, and integrated the area of the spots corresponding to gallic acid standard.

2.3.7. TLC development and scanning for berberine

The plate was developed by immersing sample HPTLC plate in a CAMAG glass chamber (20 cm × 10 cm) containing the solvent system ethanol: water: formic acid 90:9:1 (v/v/v). After complete development, the plate was allowed to dry by keeping in fume cupboard for 10 min and then kept in hot air oven for 5 min at 105 °C. The plate was scanned in the densitometer by linear scanning at 366 nm for berberine by using a TLC Scanner III CAMAG with a mercury source, and integrated the area of the spots corresponding to berberine hydrochloride standard.

Amount of gallic acid and berberine in Entoban syrup was calculated by following formula:

$$\frac{A_{SMP}\times W_{STD}\times f\times \text{Dilution of Smp}\times \text{Application vol. of sample}\times P\times D\times 10}{A_{STD}\times \text{Dilution of Std}\times W_{SMP}\times \text{Application of vol. of standard}\times 100}$$

where A_{SMP} is average area of sample; A_{STD} is average area of standard; W_{STD} is weight of standard, mg; W_{SMP} is weight of sample, g; Dilution of Smp is dilution of sample, mL; Dilution of Std is dilution of standard, mL; P is percent purity of standard; f is conversion factor; D is density of syrup, mg/mL.

3. Results

In the current study quantitative estimation of specific biologically active gallic acid and berberine components were conducted in the polyherbal formulation using HPTLC. Gallic acid is a common phytoconstituent; therefore in the quantitative estimation of gallic acid, it is well represented in chromatogram (Figure 1). For optimization of method, different mobile phase compositions were employed to achieve good separation. Among the various solvent systems tried the solvent system containing toluene: ethyl acetate: formic acid: methanol in the volume ratio of 12:9:4:0.5 resulted in good separation of the gallic acid. TLC plate was observed under UV light for the presence of gallic acid, which was detected by prominent dark brown spots. The spots developed were dense, compact and typical peaks of gallic acid were obtained. The R_f value (0.58) for gallic acid in both sample (Figure 1) and reference standard (Figure 2) was found comparable under UV light at 273 nm. Peaks were symmetrical in nature and no tailing was observed when plates were scanned at 273 nm.

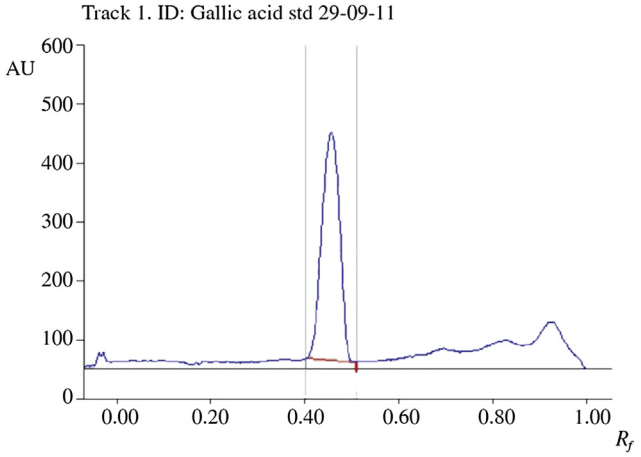

Figure 1. Peak response of gallic acid in Entoban syrup.

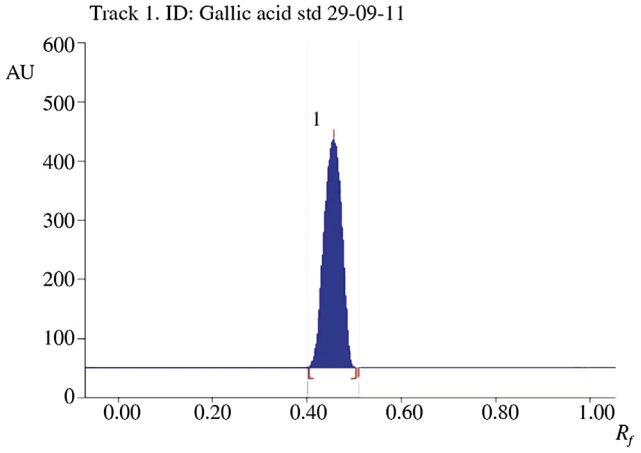

Figure 2. Peak response of gallic acid standard.

Different solvent systems were used for the detection of berberine of which the solvent system containing ethanol: water: formic acid 90:9:1 (v/v/v) resulted in good resolution of berberine in the presence of other compounds in formulation. TLC plate was observed under UV light for the presence of berberine, detected by prominent violet color spot. The R_f value (0.76) for berberine in both sample (Figure 3) and reference standard (Figure 4) was found comparable under UV light at 366 nm. An accurate, simple and specific HPTLC method for quantitative estimation of biomarkers present in Entoban syrup has been developed. The method employed in current study resulted in good peak shape of berberine and gallic acid. There was no interference from the excipients present in the

formulation. Standardization of specific biologically active gallic acid and berberine components were identified in the polyherbal formulation thereby establishing the standard of those particular compounds for validation.

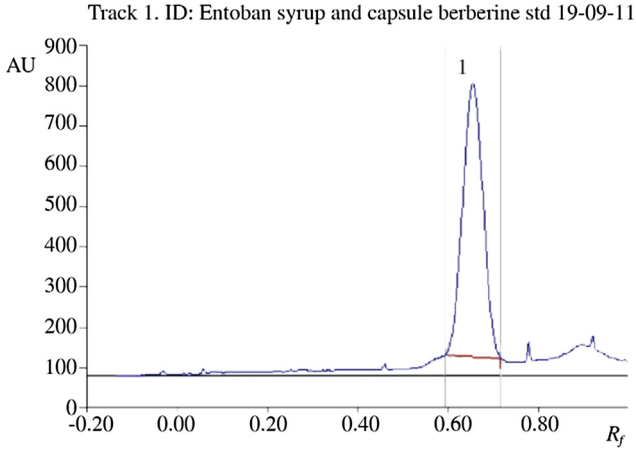

Figure 3. Peak response of berberine in Entoban syrup.

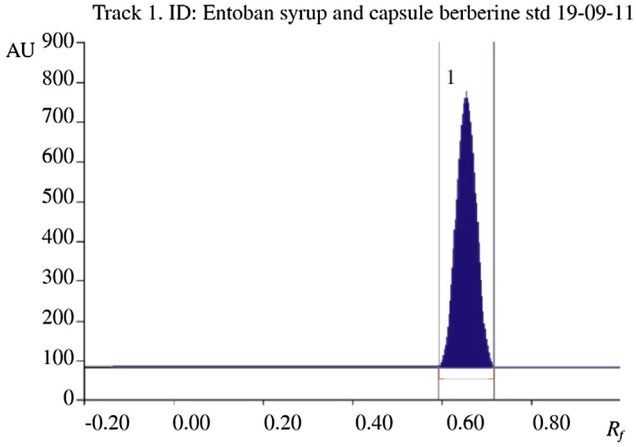

Figure 4. Peak response of berberine standard.

4. Discussion

Acute gastroenteritis (AGE) is one of the most prevalent ailments in children, and the second leading reason of morbidity and mortality round the globe. All children are probable to practice AGE in the earlier 3 years of life. AGE is epitomized by diarrhea, coupled with nausea, vomiting, fever, and abdominal pain [22]. Most cases of mild diarrhea are of viral etiology, while severe diarrhea, especially associated with fever, tends to be of bacterial etiology [23]. Though there are splendid advancements in modern medicine, traditional medicine has always been accomplished for treating gastrointestinal infections. The traditional medicine sector has become an imperative resource in health care, particularly in rural and tribal areas of the country [24]. Herbal remedies are extremely successful in curing chronic diarrhea and acute diarrheal diseases.

Herbal medicines are usually obtainable as a mixture of more than one plant constituent and its therapeutic activity depends on its phytochemical constituents [25]. Accurate identification and quality reassurance is an essential prerequisite to make sure reproducible quality of herbal medicines [26]. Standardization is an imperative aspect for evaluating the quality and safety of polyherbal formulation as these formulations are combination of more than one herb to accomplish the desired therapeutic effect. Phytochemical assessment signifies the quality measurement, including preliminary phytochemical screening, chemo-profiling, and marker compound analysis employing innovative analytical techniques. HPTLC has been emerged as a significant tool for the qualitative, semiquantitative, and quantitative phytochemical analysis of the naturally occurring drugs [27].

The present study was targeted to Entoban syrup, a polyherbal formulation which integrates an outstanding blend of herbs that have been used for decades to eradicate microorganisms and worms from gastrointestinal tract. It is the combination of *H. antidysenterica*, *B. aristata*, *Symplocos racemosa*, *Querecus infectoria* and *Helicteres isora*. Research has shown that different parts of *H. antidysenterica* executed various medicinal properties [28]. It is reported that bark of the plant showed antidiarrheal and astringent activity. The bark extract has been active against enteropathogens like enteroinvasive *Escherichia coli*, *Shigella flexneri*, *Salmonella typhimurium*, *Salmonella enteritidis* and *Vibrio cholera* [29]. In addition, the plant has been reported to possess astringent, antiamoebic activity, appetizing and antihelminthic properties. The seeds of *H. antidysenterica* are used in the treatment of dysentery, diarrhea and fever [28]. *B. aristata* is useful as anti-pyretic, anti-oxidant, anti-microbial, anti-hepatotoxic, anti-hyperglycaemic, anti-bacterial, anti-cancer, and anti-lipidemic agent [30,31].

In the current study, quantitative estimation of specific biologically active gallic acid and berberine components were conducted in the polyherbal formulation using HPTLC. Gallic acid is a common phytoconstituent, therefore in the quantitative estimation of gallic acid, it is well represented in chromatogram (Figure 1). The R_f value (0.58) of gallic acid in both sample and reference standard was found comparable under UV light at 273 nm. The gallic acid inhibits different forms of microbiological organisms so it is useful in AGE. It is already reported in the literature that *B. aristata* contain biomarker berberine, a quaternary alkaloid which has antibacterial, antiamoebic, antifungal, antihelminthic, leishmanicidal and tuberculostatic properties [32]. HPTLC was performed to confirm the quantitative presence of berberine (R_f value 0.76) employing ethanol: water: formic acid 90:9:1 (v/v/v) as a solvent system at a wavelength of 366 nm. Sample preparation and development of appropriate mobile phase are two imperative stages in analytical procedures, which becomes more considerable for plant based medicines owing to their complexity of the chemical compounds and their affinity towards different solvent systems [33]. Therefore in present study the development of mobile phases for biomarkers were optimized by using the appropriate mixture of solvents.

Standardization promises constant composition of all herbals including analytical operations for identification, markers and assay of active principles. TLC and HPTLC are routinely used as valuable tools for qualitative determination of small amounts of impurities [14]. Different researchers have proposed that HPTLC method enables high-quality resolution and can be used for quantization of biomarkers. HPTLC method was found to be simple, reliable, and convenient for routine analysis [34–36]. The present work confirms such findings. The method can be used conveniently for the estimation of gallic acid and berberine in other herbal preparations and may be utilized for standardization purpose. Its main advantages include its simplicity, accuracy and selectivity [37–39].

An accurate, simple and specific HPTLC method for quantitative estimation of biomarkers present in Entoban syrup has been developed. The method employed in current study resulted in good peak shape of berberine and gallic acid. The present standardization provides a specific and rapid tool in the herbal research, permitting to set quality specifications for identity, transparency and reproducibility of biomarkers in Entoban syrup.

Conflict of interest statement

We declare that we have no conflict of interest.

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