Isolation and HPLC Quantification of Berberine Alkaloid from *Alpinia galanga* and *Alpinia calcarata*

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ABSTRACT

Purpose: To isolate and quantify berberine alkaloid for the first time from the rhizomes of *Alpinia galanga* and *Alpinia calcarata.*

Methods: Isolated from the chloroform fraction of aqueous extract and quantified by high-performance liquid chromatography (HPLC). The purity and the structure of the identified compound (berberine) were characterised by NMR and LC-MS.

Result and conclusion: A new, simple, sensitive and selective HPLC method was developed for the quantification of berberine. The developed method was validated according to the International Conference on Harmonization guidelines. The HPLC analysis showed that *Alpinia galanga* contained an amount of 1340 and *Alpinia calcarata* had 1355 mg/Kg of berberine. The isolated berberine and its rich fractions of *Alpinia galanga* and *Alpinia calcarata* were found to scavenge the DPPH free radical with IC_{50} values of 22.5, 49.5 and 50.4 µg/ ml respectively, though its activity is less than that of a standard polyphenolic compound.

Keywords: Berberine, Alkaloid, HPLC, *Alpinia galanga, Alpinia calcarata*

Introduction

Alpinia is the largest genus of the family Zingiberaceae and the members of this genus possess many bioactive compounds with complex chemical profiles (Ghosh and Rangan, 2013). *Alpinia galanga* and *Alpinia calcarata* are two important aromatic plants of this genus. They are widely used in cooking, especially in Indonesian and Thai cuisines and also in various Ayurveda formulations for the treatment of rheumatoid arthritis and inflammatory conditions. Phytochemical constituents such as acetoxychavicol acetate, hydroxychavicol acetate, 1'-acetoxyeugenol acetate, *trans-p*-acetoxycinnamyl alcohol were identified from *A. galangal.* Pinocembrin, galangin-3-methyl ether, 5,7-dihydroxyflavanol (galangin) and zerumin were isolated from *A. calcarata*. Flavonoids, tannins and terpenes are reported as the key bioactive constituents responsible for the therapeutic efficiency. However the complete chemical constituents responsible for all biological activities of these plants are still unknown.

Berberine, a quaternary protoberberine isoquinoline alkaloid, is present in the root and the stem bark of some important medicinal plants such as *Berberis aristata* and *Berberis vulgaris* (Zuo *et al.*, 2006). Berberine has multiple therapeutic actions. It shows significant antimicrobial activity towards a variety of organisms including bacteria, fungi, protozoans and viruses (Hayashi *et al.*, 2007; Birdsall and Kelly, 1997). It has also been reported to have many biological effects including antimalarial (Tran *et al.*, 2003), antihypertensive (Ko *et al.*, 2000), antihyperglycemic (Pan *et al.*, 2003), antitumor (Kettmann *et al.*, 2004) and antiinflammatory (Kupeli *et al.*, 2002) activities. It has been reported that berberine helps in reducing cholesterol and lipid accumulations in both the plasma and in the liver (Doggrell, 2005; Battu *et al.*, 2010). Berberine is found to inhibit the single-strand cleavage of DNA (Choi *et al.*, 2001). It exhibits a strong superoxide anion radical quenching ability and a protective action against $ONOO^-$, $NO^\cdot$, and $O_2^{\cdot -}$ radicals induced oxidative damage (Yokozawa *et al.*, 2013; Rackova *et al.*, 2007).

Usually the extraction of alkaloids was done by continuous extraction method using organic solvents in Soxhlet apparatus (Gonzales *et al.*, 2014). The above method required lot of time and energy. Other techniques for berberine isolation were based on the extraction by alcohol in the neutral medium or with addition of acetic acid, further removal of side substances and the precipitation of berberine as berberine chloride and hydrogen sulphate or iodide. In order to isolate berberine, researchers also used microwave radiation or liquid extraction under pressure, an ultra filtration technique, chromatographic separation using macro porous or ion-exchange resins (Nechepurenko *et al.*, 2010). As these separations of alkaloids is costly, and requires more sophisticated instruments and can be applied for particular plants, there is need for simpler and unique method for the extraction and isolation of alkaloid from a particular plant.

To the best of information available in the literature, the berberine alkaloid in the *Alpinia* species was not identified and quantified by any method. The present study deals with the isolation and quantification of berberine alkaloid from the *Alpinia galanga* and *Alpinia calcarata* and evaluation of the antioxidant potential.

Materials and methods

Plant material

The medicinal plants were collected at Kottayam district, Kerala, India and authenticated by approved taxonomist. The voucher specimen of *Alpinia galanga* (MKU/NPC/004) and *Alpinia calcarata* (MKU/NPC/005) were deposited in our lab for future reference.

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, xanthine, xanthineoxidase, deoxyribose, thiobarbituric acid (TBA), and bovine serum albumin (BSA), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aluminum chloride was obtained from Merck (Darmstadt, Germany). All other chemicals used were of standard analytical grade. For the quantification of berberine HPLC grade solvents were used.

Isolation and quantification of berberine

The rhizomes of *Alpinia calcarata* and *Alpinia galanga* were powdered and then extracted separately with water. This crude aqueous extract was acidified with 1N HCl and then treated with 1N NaOH. The precipitate was extracted with diethyl ether and that fraction was concentrated. The aqueous layer was further extracted with chloroform. The extraction was repeated until the last fraction did not give any precipitate with Dragendorff's reagent. The collected chloroform fraction concentrated at reduced pressure to yield a yellow coloured solution and purified by column chromatography using silica gel (100-200 mesh) with pet. ether, pet. ether: $CHCl_3$, $CHCl_3$, $CHCl_3$: MeOH (in ratios of 98:2, 96:4, 95:5, 90:10, 80:20 and 70:30) as successive eluents. The $CHCl_3$: MeOH (90:10) fraction yielded a single peak in TLC, which was identified as berberine by UV-Visible spectroscopy, FT-IR and ^{1}H NMR and LCMS.

HPLC quantification of berberine

HPLC was used for the analysis of berberine. A C18 reverse phase column (Phenomenex (Torrance, USA) ODS-2.5 lm, 50 mm x 4.6 mm) was used for the separation. Five microliters of extracts (5 mg/ml in methanol) and standards (1 mg/ml) were loaded and injected manually and eluted through the column with an isocratic mobile phase system consisting of 1% formic acid in water (A) and acetonitrile (B) in the ratio of 99:1, with flow rate of 0.3 mL/min. Detector was set at 350 nm, the λ- max of berberine.

Validation of methods

Validation of the HPLC method is important in the quantification compounds. Typical analytical characteristics evaluated in an LC validation include measurement of precision, accuracy, specificity, limit of detection, limit of quantification and linearity range. By comparing the retention time and UV spectra of the peaks with those of reference berberine, the specificity of the method was determined. Peak purity was assessed by comparing the spectra acquired. The linearity assessed by determining the detector responses to a series of solutions of reference standard of different concentration. Five analyses per concentration were conducted, and calibration plots were constructed. Limits of detection and quantification of the methods were calculated using signal to noise ratio method. The precision of the method was validated in terms of repeatability and intermediate precision, expressed as % relative standard deviation (RSD). The concentrations of samples were selected so that berberine contents were in the experimental ranges.

Determination of DPPH radical scavenging activity

The antioxidant activity of extracts was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH method (Liu, 2010). The reaction mixture contains 2.8 mL of methanolic DPPH and 0.2 ml of extract at various concentrations. The contents were mixed well immediately and incubated for 30 min at room temperature (25–29 $^{\circ}$C). The degree of reduction in absorbance was recorded at 517 nm. The concentration of an antioxidant needed to trap 50% DPPH is designated as IC_{50} to express the antioxidant capacity. A low value of IC_{50} indicates higher activity.

Statistical analysis

The experimental results were expressed as mean ± SD (standard deviation) of triplicate measurements. The data were subjected to one-way analysis of variance (ANOVA) and the significance of differences between means was calculated by Duncan's multiple range test using SPSS for Windows, standard version 7.5.1, with the significance accepted at $p < 0.05$.

Results and discussion

Identification of berberine by HPTLC

The alkaloid fractions of *A. calcarata and A. galanga* were analyzed by HPTLC at 350 and 265 nms along with standard berberine in a solvent system of toluene: ethyl acetate: formic acid: methanol in the ratio 3:3:0.1:1. From the HPTLC profiles (**Fig. 1**) it is clear that both the plants contain berberine alkaloid.

Isolation and characterization of berberine

In the present study, isolation, identification and quantification of berberine alkaloid was reported for the first time in *A. galanga* and *A. calcarata*. Berberine was isolated from the chloroform fraction by column chromatography. The isolated berberine was characterised by proton NMR spectroscopy (**Fig. 2**) and also by LC-MS (**Fig. 3**) analysis which is in agreement with previous findings (Shigwan *et al.*, 2013; Battu *et al.*, 2010).

HPLC quantification of berberine

HPLC is currently the most widely used method of quantitative analysis in the pharmaceutical industry and research laboratories because of its reliability, simplicity, reproducibility, and speed (Kupiec, 2004). The amount of berberine in the rhizomes of *A. galanga* and *A. calcarata* is estimated by HPLC method. The peak corresponding to berberine is identified by comparing its retention time with standard compound and UV spectra. HPLC profile of standard berberine (**Fig. 4**) gives a retention time at 6.1 min under the experimental conditions. It is also confirmed by spiking studies. The amount of berberine in the extracts is calculated by use of a calibration plot. The alkaloid fraction of *A. calcarata* (**Fig. 5**) and *A. galanga* (**Fig. 6**) given a prominent peak at 6.1 min indicates the berberine alkaloid. The HPLC analysis showed that *A. galanga* contains an amount of 1340 ± 4 mg/ Kg and *A. calcarata* has 1355±5 mg/ Kg of berberine. Even though some previous reports are available on the HPLC quantification of berberine (Shigwan *et al.*, 2013), the present method is simple, accurate, reproducible and enables highly reliable quantification even at low concentrations.

Method Validation

The method was validated for linearity, limits of detection and quantification, precision, accuracy and robustness, in accordance with ICH guidelines (2005). The linearity of detector responses was evaluated for 0.03µg to 1 µg/ml. Response was found to be linear over that range. The correlation coefficient (r^2) was found to be 0.9985. Limits of Detection (LOD) and quantification (LOQ) of the methods were calculated using signal to noise ratio method (according to the standards of international Conference Harmonization (ICH). The signal to noise ratio for LOD was set at 3:1 and for LOQ it was 10:1. The limit of detection (LOD) was determined by successively decreasing the concentration of berberine as long as a signal-to-noise ratio of 3:1 appeared .The LOD and LOQ of the method were found to be 0.01 µg and 0.03µg. The repeatability and intermediate precision of the method are found to be 1.45 and 1.52% respectively. The accuracy of the method was evaluated by % recovery at low, medium, and high concentrations of berberine (**Table 1)**. The robustness of the method was validated by introducing small changes in some of the chromatographic conditions, the composition and amount of the mobile phase (±10%), and temperature (±2°C). Variations in HPLC analysis are ≤1.8 (*RSD* [%]).

Evaluation of free radical scavenging activity of alkaloid fractions

Berberine is reported to exhibit a wide range of pharmacological activities. To find out the antioxidant potential of the alkaloid fractions of *A. galanga* and *A. calcarata,* DPPH radical scavenging activity was studied and compared with a known polyphenolic compound. Alkaloid fraction of *A. galanga* and *A. calcarata* showed a DPPH radical scavenging activity with IC_{50} values of 49.5 and 50.4 µg/ ml. Berberine gives an IC_{50} value of 22.5 µg/ ml (Table 4.2). The studies revealed that the free radical scavenging capacity of berberine alkaloid was comparatively less than the phenolic compound gallic acid (1.41 µg/ ml). The above result is rationalized as follows: The hydroxyl groups of the phenolic compounds were known to easily interact with the free radicals and neutralise them immediately whereas berberine does not have a phenolic OH group in its structure and hence it showed mild radical scavenging action compared to polyphenols. This result was supported by the previous findings, in which hydroxylated alkaloid jatrorrhizine showed three times higher antiradical reactivity than its non-hydroxylated analogue (Rackova *et al.*, 2007).

Conclusions

Berberine alkaloid is identified and quantified for the first time in *A. galanga* and *A. calcarata*. The developed HPLC quantification method is precise, specific, accurate and robust. The method could be used to quantify berberine even at a low concentration with high accuracy. The HPLC analysis showed that *A. galanga* and *A. calcarata* contains significant amount of berberine. Free radical scavenging activity studies revealed that of berberine rich fractions of *A. galanga* and *A. calcarata* had some antioxidant activity.

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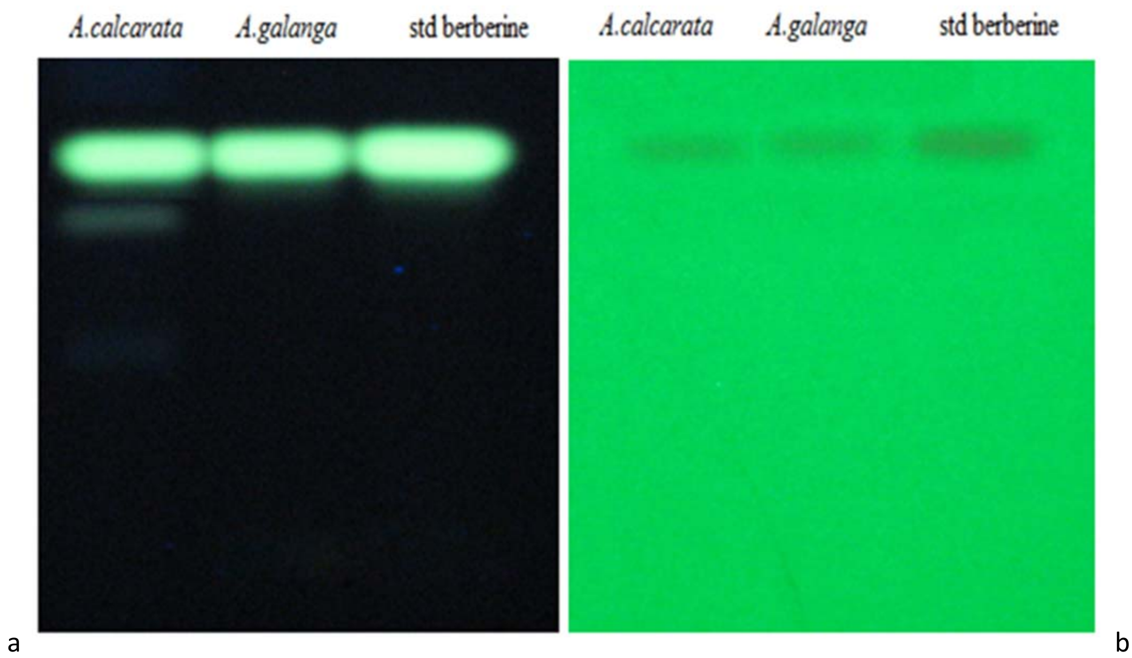

Fig. 1 HPTLC profiles of *A. calcarata*, *A. galanga* and standard berberine

a. at 350 nm b. 265 nm

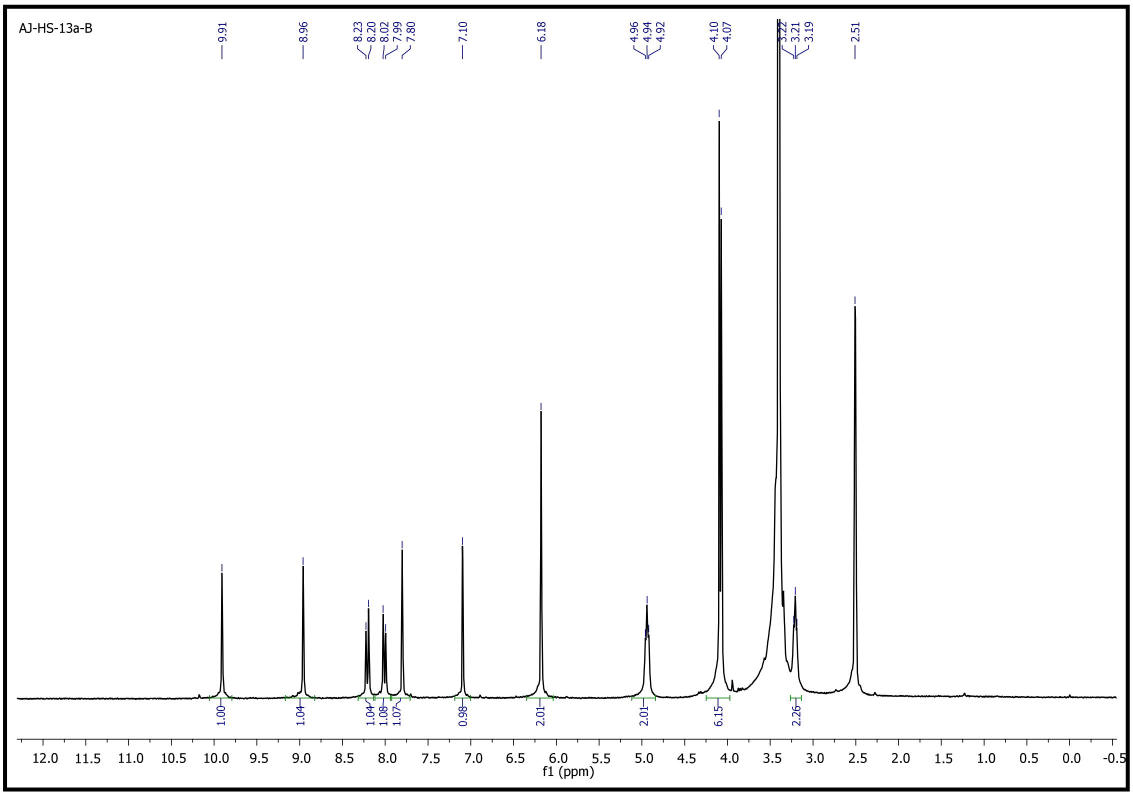

Fig.2 Proton NMR spectrum of berberine

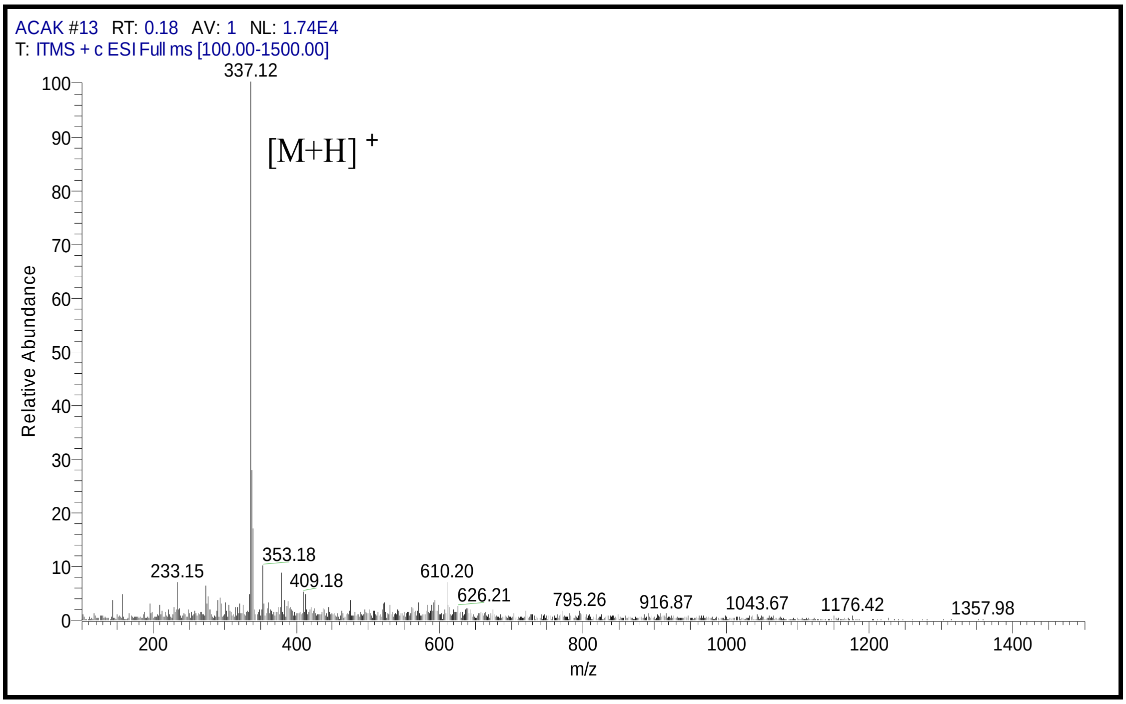

Fig. 3 Mass spectrum of berberine

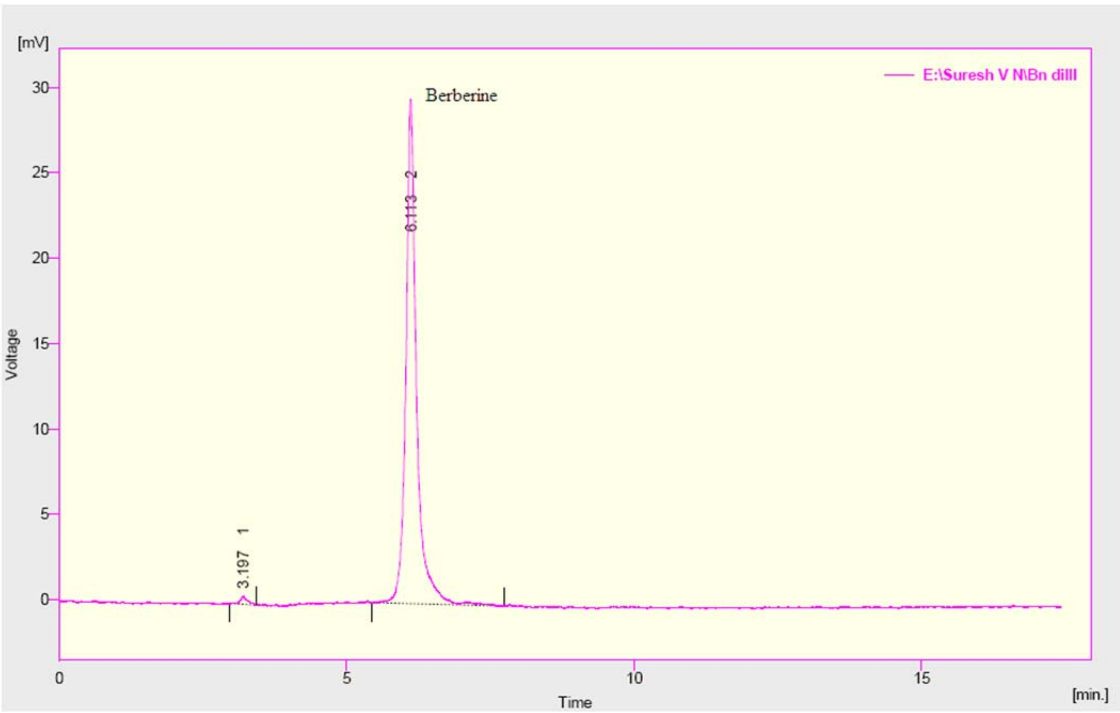

Fig. 4 HPLC profile of standard berberine

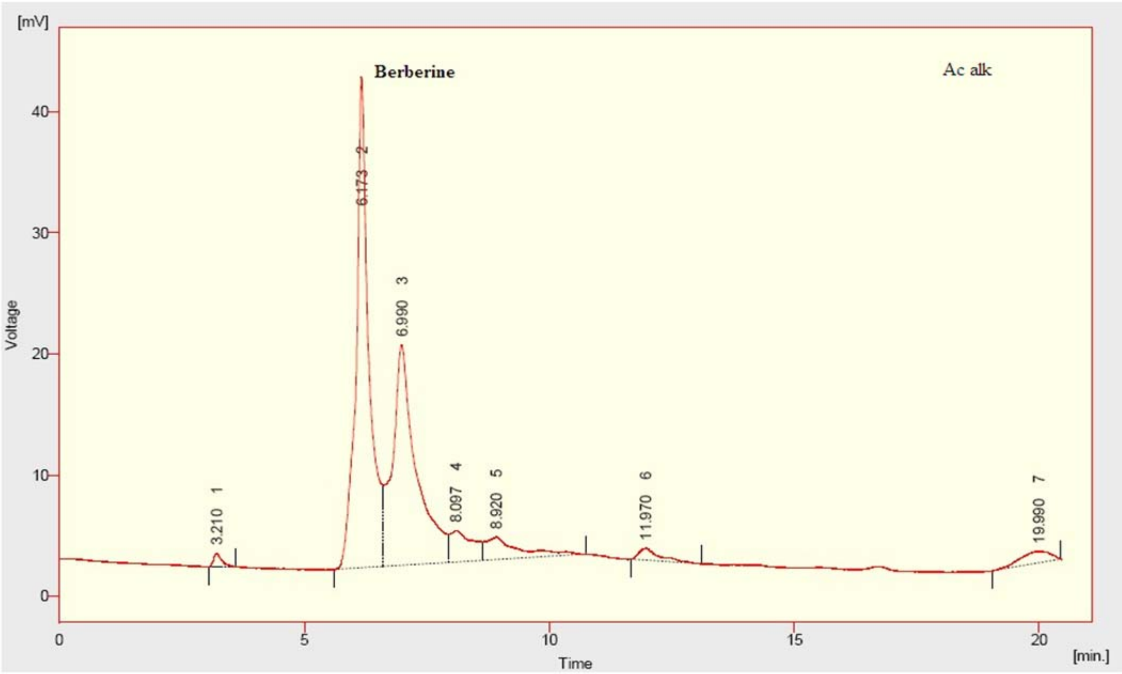

Fig. 5 HPLC profile of alkaloid fraction of *A. calcarata*

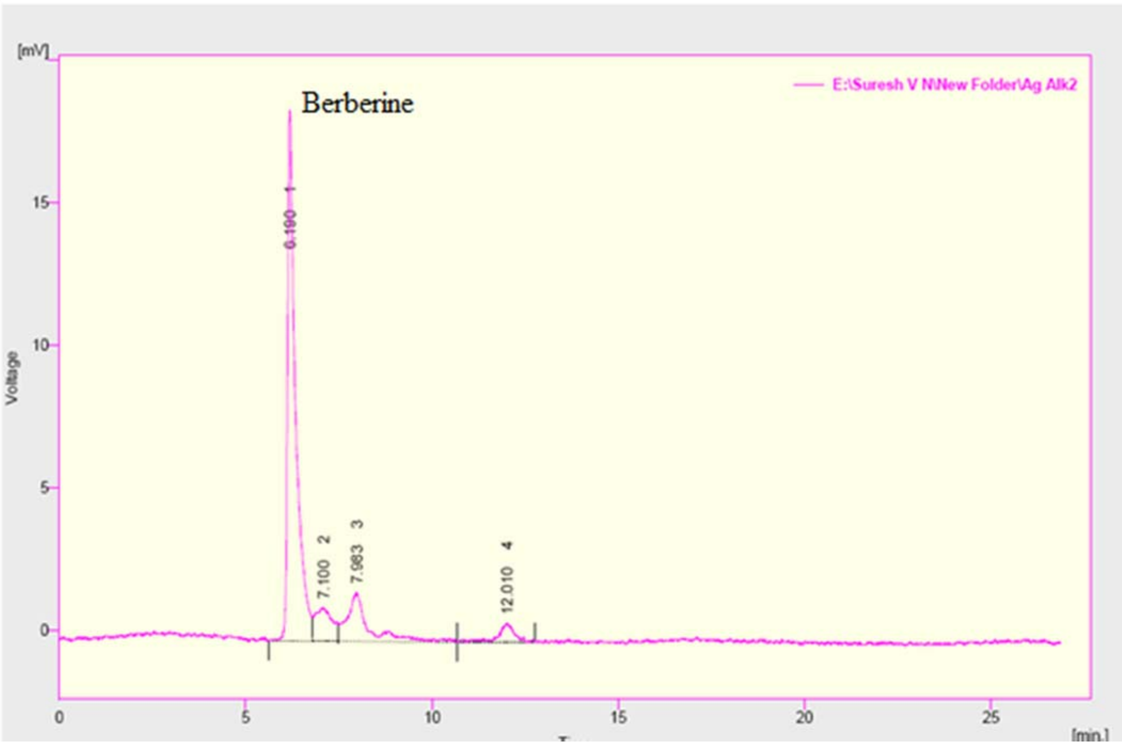

Fig. 6 HPLC profile of alkaloid fraction of *A. galanga*

Table 1. Accuracy of the HPLC quantification method

D:\ijpsr\documents\IJPSRVOL8NO06\01-IJPSR17-08-06-011-Suresh. Vnampoothiri\Excess of berberine added (%)	% Recovery
50	98.5
100	100.5
150	101.9