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A HPTLC Method for Standardization of *Curculigo orchioides* Rhizomes and its Marketed Formulation Using Gallic Acid as Standard

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In the present work, a rapid, reliable and validated HPTLC method, which allows the quantitative determination of gallic acid in rhizomes of *Curculigo orchioides* and its marketed formulation has been developed. Silica gel $60F_{254}$ plate were used as stationary phase and toluene:ethyl acetate:glacial acetic acid (12.5:7.5:0.5 v/v) as mobile phase. The wavelength selected for analysis was 260 nm. The amount of gallic acid in the rhizome was found to be 2.54% (w/w) and in its formulation it was found to be 5.13% (w/w). The developed HPTLC method can be used as a routine method for the estimation of gallic acid in raw material and marketed formulations containing *C. orchioides*.

Curculigo orchioides Gaertn, family *Amaryllidaceae*, is an herbaceous, tuberous, geophilous, perennial widely distributed all over India. The rhizomes are sweet, cooling, diuretic, aphrodisiac, virilgenic, antipyretic and tonic that can be used against haemorrhoids, leucorrhoea, pruritis, skin diseases, bronchitis, jaundice and diarrhoea[1]. It is used in Chinese traditional medicine as an analeptic agent for the treatment of decline[2]. In India it is a common ingredient in many ayurvedic formulations (such as *Muclikahadiradi Karayam, Vidaryadi Ghartam, Vidaryadileham, Marmagalika,* Liv-52) for treatment of leucorrhoea, bleeding and tonic for aphrodisiac activity. The alcoholic extract is reported to have adaptogenic, anti-inflammatory, anticonvulsive, sedative, androgenic immunopromotion and antiozidant activities[3,4]. The tubers of the plant contain phenolic compounds, steroids, aliphatic compounds, nitrogen constituents[5] triterpenoidal saponins such as curculigenin A and curculigosaponins A, B, C, E, F, G, H, I and J.[6,7]

Though *C. orchioides* rhizomes is used in various ayurvedic formulations[8] there is only one HPLC method reported for the determination of curculigoside[9] which is time consuming and tedious. In the last one decade HPTLC emerged as an important tool for qualitative, semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations which includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers[10-12].

Since the rhizomes is reported to contain mainly polyphenolics, gallic acid and phenolic glycosides, i.e. an orcinol glycoside and corchioside[13-17], we have estimated its total phenolics content colorimetrically and developed a simple, reproducible, sensitive and rapid method of HPTLC analysis for the estimation of gallic acid in *C. orchioides* and in one of its marketed monoherbal formulation.

MATERIALS AND METHODS

Rhizomes of *Curculigo orchioides* were collected from Thattekkad, Ernakulam district of Kerala and authenticated at the Post Graduate and Research Department of Botanical Survey of India, Coimbatore. A Camag-HPTLC system comprising of Linomat V automatic sample applicator and Camag-TLC scanner 3 with CAT'S version 4.0 software were used for sample application and quantitative evaluation respectively. Gallic acid was purchased from Loba

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Chemicals Pvt. Ltd, Mumbai. The herbal formulation containing *C.orchioides* rhizomes as major ingredient (70%) was purchased from Ayurvedic Pharmacy, Coimbatore. All other chemicals used were of analytical grade obtained from Glaxo Laboratories Ltd, Mumbai.

For the estimation of total phenolics a double beam Shimadzu 160A UV/Vis spectrophotometer with two matched quartz cells of 1 cm light path was employed. 1,1-diphenyl-2-picryl hydrazyl (DPPH) and ascorbic acid obtained as gift samples from Cadi'a Pharmaceuticals Ltd, Ahmedabad, were used.

Extraction:

The collected material was thoroughly washed under running water, chopped, air dried at 35-40^0 for a week and pulverized in electric grinder. The powder obtained was successfully extracted in alcohol by using Soxhlet extractor. The extract was dried and the percentage yield was found to be 2.9% (w/w) which was stored in refrigerator for further use.

Preparation of ethanolic extract of *Curculigo orchioides* rhizomes:

C. orchioides rhizome powder (250 mg) was extracted with ethanol in Soxhlet apparatus for 72 h. The extract was dried and the percentage yield was found out to be 21.9% w/w, which was stored in refrigerator for further use.

Phytochemical evaluation of the ethanolic extract of *C. orchioides* rhizome:

A stock solution was prepared by dissolving 500 mg of ethanolic extract in 20 ml of ethanol and it was subjected to preliminary phytochemical testing for the detection of major chemical group[18,19].

Estimation of total phenolics:

The total phenolic content of the ethanolic extract was estimated colorimetrically according to the method described by Singleton and Rossi.[20,21]. A stock solution (1 mg/ml) of the extract was prepared in ethanol. From the stock solution suitable quantity of the extract was taken into a 25 ml volumetric flask and 10 ml of water and 1.5 ml of Folin Ciocalteau reagent were added to it. The mixture was kept for 5 min, and then 4ml of 20% sodium carbonate solution was added and made up to 25 ml with double distilled water.

The mixture was kept for 30 min and absorbance was recorded at 765 nm. Percentage of total phenolics was calculated from calibration curve of gallic acid plotted by using the above procedure and total phenolics were expressed as percentage gallic acid.

Development of TLC fingerprint profile of rhizome of *C. orchioides*:

TLC fingerprint profile of alcoholic extract was established by using HPTLC. Suitable diluted stock solution of alcoholic extract was spotted on a precoated silica gel G 60F^{254} TLC plate (E. Merck) using Camag Linomat V Automatic Sample Spotter and the plate was developed in the solvent system of toluene: ethyl acetate: glacial acetic acid (12.5:7.5:0.5 v/v)

The plate was dried at room temperature and scanned by using Camag TLC Scanner 3 at UV 260 nm and R*f* values, spectra λmax and peak area of the resolved bands were recorded. Relative percentage area of each band was calculated from peak areas. The TLC plate was developed by spraying with 5% ferric chloride solution for the detection of phenolic compounds.

Estimation of Gallic Acid in the rhizome of *C. orchioides* and its formulation using HPTLC method:

Standard solution of pure gallic acid (10 mg/100 ml) was prepared in 95% ethanol in a volumetric flask. From this stock solution standard solution of 10-70μg/ml were prepared by transferring aliquots (1.0 to 7.0 ml) of stock solution to 10ml volumetric flask and adjusting the volume to 10 ml with ethanol. Ten microlitres of each of standard solution were applied on precoated silica gel G 60F_{254} TLC plate (E. Merck) using Camag Linomat V automatic sample applicator.

The plate was developed in the solvent system of toluene: ethyl acetate: glacial acetic acid (12.5:7.5:0.5) in a twin trough chamber to a distance of 7.7 cm. Plate was dried in air for 15 min and scanned at 260 nm. Data of peak area of each band was recorded. Standard curve for gallic acid in the range of 10-70 μg/ml was generated by plotting the peak area against concentration of gallic acid.

Sample preparation and procedure:

About 250 g of rhizome powder was made to pass through No: 60 mesh sieve and 100 g from it was accurately weighed and exhaustively extracted with 95% ethanol. The extract was concentrated and transferred to 100 ml volumetric flask and the volume was made up to 100 ml

with ethanol. Five microlitres from the above sample solution was spotted in triplicate along with 10 ml of standard solution (60 µg/ml) on precoated silica gel G $60F_{254}$ TLC plate. The plate was developed and scanned as mentioned above. Peak areas were recorded and the amount of gallic acid present in rhizome (raw material) was estimated using standard addition method.

Accurate quantity of herbal formulation containing weight equivalent to 10 mg of *C. orchioides* extract as per label claim was taken, transferred to a conical flask, extracted with 95% ethanol (3x25 ml) and filtered. The filtrates were pooled and concentrated to 25 ml. 5µl from the above sample solution was spotted in triplicate along with standard solution (60 µg/ml) on precoated silica gel G $60F_{254}$ TLC plate. The plate was developed and scanned as mentioned above. Peak area was recorded and the amount of gallic acid present in the formulation was estimated using the calibration curve for gallic acid.

Validation of method:

The method developed was validated as per ICH guidelines for specificity, instrumental precision, repeatability and accuracy. The method is found to be specific for gallic acid since it resolved the peak of gallic acid in *C. orchioides* rhizomes (R_f value 0.19) and its formulation (R_f value 0.19) effectively as shown in fig. 1 and fig. 2, respectively. The chromatogram of standard gallic acid (R_f value 0.19) is shown in fig. 3. The specificity was also confirmed by overlaying the spectra of standard with the spectra of sample recorded on TLC scanner in UV range. Precision of the instrument was checked by repeated scanning of the same spot of gallic acid (300 ng/5 µl) six times and the coefficient of variation (%CV) was calculated. Repeatability of the method was tested by applying 400 ng/5µl spot of standard solution of gallic acid on a TLC plate (n=6) and calculating % CV. Accuracy of the method was evaluated by carrying a recovery study. A varying known concentration of the standard gallic acid, i.e. 1.0, 2.0 and 3.0 mg, was added to about 1 g of finely powdered test samples separately in which the content of gallic acid were estimated previously by the proposed method. The samples were extracted and analyzed separately as per the procedure maintained above. Each set of additions was repeated 7 times and the recovery of added standard was calculated.

RESULTS AND DISCUSSION

C. orchioides Gaertn of Amaryllidaceae family is an herbaceous tuberous geophilous perennial with rootstock bearing several fleshy lateral roots (rhizomes). It is widely distributed in India. The rhizome of this plant possess medicinal properties and are sweet, cooling, diuretic, aphrodisiac, viriligenic and tonic which can be used against hemorrhoids, leucorrhoea, pruritis, skin diseases, asthma, bronchitis and jaundice, etc.

The preliminary phytochemical testing showed the presence of high amount of phenolics along with flavonoids and saponin glycosides. The total phenolics content was estimated to be 5.78 % w/w colorimetrically. The TLC studies, for the detection of phenolic compounds, showed 6 distinct bluish coloured bands when sprayed with 5% ferric chloride solution having R_f values 0.20, 0.31, 0.37, 0.54, 0.72 and 0.88, respectively.

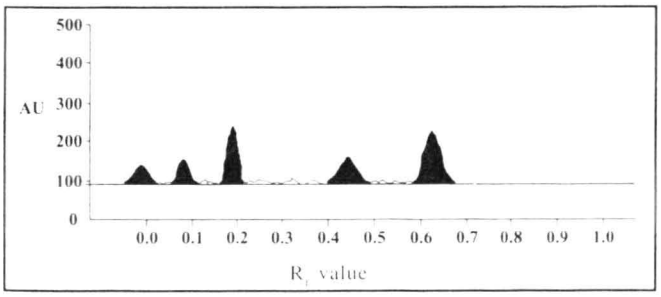

Fig. 1: The HPTLC chromatogram of *C. orchioides* rhizome at 260 nm.

The chromatogram of *C. orchioides* rhizome extract shows 4 numbers of peaks when scanned at 260 nm using solvent system toluene: ethyl acetate: glacial acetic acid (12.5:7.5:0.5 v/v). Peak no. 3 is of gallic acid with R_f value 0.19.

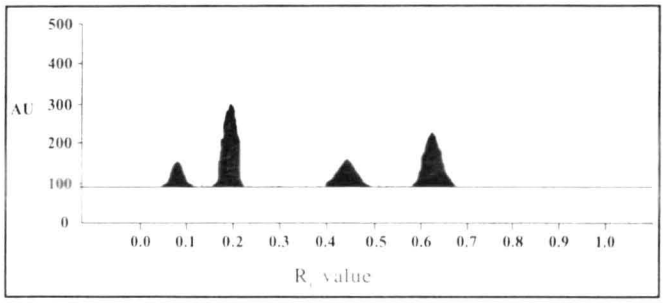

Fig. 2: The HPTLC chromatogram of formulation at 260 nm

The chromatogram of formulation shows 5 numbers of peaks when scanned at 260 nm using solvent system toluene: ethyl acetate: glacial acetic acid (12.5:7.5:0.5 v/v). Peak no. 2 is of gallic acid with R_f value 0.19.

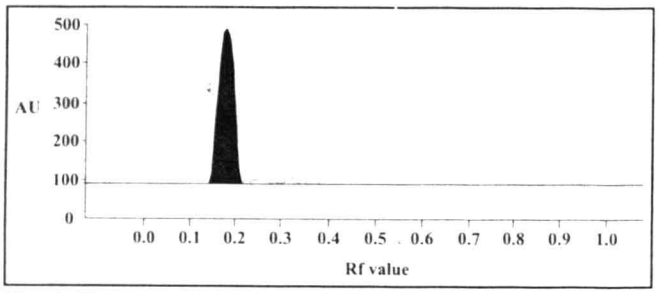

Fig. 3: The HPTLC chromatogram of standard Gallic acid at 260 nm

The chromatogram of standard gallic acid shows a prominent peak when scanned at 260 nm using solvent system toluene:ethyl acetate:glacial acetic acid (12.5:7.5:0.5 v/v) with R_f value 0.19

The HPTLC method described utilizes a precoated silica gel G $60F_{254}$ on aluminum foil and a mobile phase comprising of toluene: ethyl acetate: glacial acetic acid (12.5:7.5:0.5 v/v) which gave good separation of gallic acid (R_f = 0.19). The detector response to gallic acid was found to be linear in the range of 150 to 750 ng/5 μl with a correlation coefficient of 0.996 (± 1.7). The instrumental precision was checked and % CV was found to be 0.046. Repeatability of the method was carried out and the % CV for peak areas was found to be 0.61. The limit of detection and limit of quantitation for gallic acid was found to be 75 ng/5μl and 150 ng/5μl respectively The recovery for this component in the samples is between the prescribed limit of 98 to 100% which shows that the method is free from interference from other excipients present in the formulation. Low value of standard deviation and coefficient of variation are indicative of the high precision of the method. The content of gallic acid was found to be 3.541% w/w in *C. orchioides* rhizome and 4.13% w/w in its formulation.

Hence this proposed HPTLC method was found to the highly accurate, precise, quick and reliable for routine quality control monitoring of gallic acid in the raw material, processed powder and in herbal preparations containing C. orchioides.

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