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Phytochemical Screening and Quantification of Ellagic Acid in *Cliestanthus collinus* using RP-HPLC-DAD Method and its Insecticidal Activity against Stored Grain Pests

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This study reports the preliminary phytochemical analysis followed by the quantitative determination of ellagic acid in different parts of *Cliestanthus collinus* using reverse phase liquid chromatography (HPLC) in association with a diode array detector (DAD). The developed RP-HPLC method achieved separation of ellagic acid within 10 min of run time through gradient elution using methanol and water with 1% acetic acid as mobile phase on a C-18 reverse phase column at room temperature and 365 nm of detection wavelength. The method showed excellent linearity across a range of 0.01 µg mL^{-1} to 10 µg mL^{-1} with LOD and LOQ values of 0.027 µg mL^{-1} and 0.091 µg mL^{-1}, respectively, demonstrating lower precision characterized by RSD% of 0.73% and higher accuracy as recovery of 99.5%. Additionally, the insecticidal activity of ellagic acid obtained by isolation from the leaves, bark and fruits of *C. collinus* was examined against two stored grain pest, *Tribolium castaneum* and *Corcyra cephalonica* larvae. The results indicated that ellagic acid showed significant pesticidal activity against *T. castaneun* with a mortality rate of 98% than against *C. cephalonica* larvae with a mortality rate of 76% suggesting its use as a natural biopesticide. To our best of knowledge, this is the first report pertaining to phytochemical screening of the whole part of *C. collinus* and quantitation of ellagic acid in different parts of *C. collinus* suggesting this plant as a new natural source of ellagic acid along with pesticidal activity of ellagic acid against stored grain pest.

Keywords: Ellagic acid, RP-HPLC-DAD, *Cliestanthus collinus*, Quantification, Insecticidal activity.

INTRODUCTION

Cliestanthus collinus (Euphorbiaceae family), known as Karra in Chhattisgarh state, India, is a common plant in southern and central India, Africa and Malaysia. Plant is known by various names depending on the region, such as Garari (Hindi), Oduvan (Tamil), Kadise (Kannada), Odutu (Malayalam), Vadisaku (Telugu) and Karlajuri (Bengali) [1]. Approximately, all parts of this plant are found to possess poisonous constituents and are generally used as lethal, homicidal and abortifacient agents by people from rural areas of these regions [2]. Various case studies reported that aqueous leaf extract or decoction of the extract of this plant is responsible for slow death within 3-5 days after ingestion [3,4]. The reason behind these toxic effects is the presence of numerous bioactive compounds in different parts of plants, such as cliestanthin A, cliestanthin B, luteoline, diadizine, coumaric acid, ellagic acid, gallic acid, quercetin, arylnaphthalide (collinusin, diphylline, cliestanthin, taiwanin), *etc*. [1,5,6]. Apart from its poisonous effects, *C. collinus* is reported to have various pharmacological and therapeutic properties. For instance, a leaf extract of *C. collinus* was found to be competent against HIV-1 [5]. The crude leaf extract of the plant shows adult emergence inhibition activity against *Aedes aegypti*, *Anopheles stephensi* and *Culex quiquefasciatus* [2]. Additionally, leaf extract of plant shows larvicidal activity against *C. quinquefasciatus*, cytotoxic effect, antibacterial, antifungal, antiseptic, insecticidal and anticancer activity against human epidermoid carcinoma [7-9]. In many rural regions, the leaves of *C. collinus* are used as an insecticide for crop protection purposes. Cliestanthin A and cliestanthin B isolated from the leaves of *C. collinus* show diuretic effects in Wistar rats [10]. Although *C. collinus* contains various bioactive compounds in different parts, such as leaves, bark, fruits and roots, only the leaf extract is extensively studied and only a few compounds, such as cliestanthin A and cliestanthin B, are studied analytically and biologically. Also, evidence about the compound

responsible for the poisonous effect of *C. collinus* is lacking. Moreover, the phenolic compound content in different parts of plants has not been studied yet.

Ellagic acid, chemically recognized as 2,3,7,8-tetrahydroxy[1]benzopyranol[5,4,3-*cde*]benzopyrane-5,10-dione, is a polyphenolic compound reported to be present in *C. collinus* leaf extract [5]. Ellagic acid is reported as a highly thermodynamically stable phytoconstituent, having four rings demonstrating its lipophilic properties as well as two lactone and four phenolic groups representing its hydrophilic properties [11]. Due to these functional groups, ellagic acid shows a variety of biological activities. Studies show that this phytocompound is present in various medicinal and flowering plants such as pomegranate peels, nuts, berries (strawberries, respberries, *etc.*), mango pulp, *Junglans nigra*, *Castanea sativa*, *Quecus robus*, *Quercus alba*, *Epilobium angustifolium*, *Oroxylem indium* Vent, triphala churna, *Eugenia punicifolia*, *Eugenia uniflora*, *Decalepis hamiltonil*, *etc*. [11-19]. Nowadays, ellagic acid is gaining tremendous attention because of its potential to show various pharmacological and therapeutic properties. For instance, ellagic acid can act as a promising antimalarial agent against *Plasmodium falciparum* by inducing alkalinization or altering the pH of food vacuole through the blocking of proton pump, which regulates acidifying of these organelles [20]. Additionally, ellagic acid shows potential antioxidant, antimutagenic, anti-inflammatory, antiviral activity, cytoprotective effect against Ehrlich Ascites tumor cells and many other advantages to human health [13,19]. Also, ellagic acid shows apoptosis inducing activity in human osteogenic sarcoma (HOS) cells [21]. Reports also demonstrated that ellagic acid shows promising molecular interactions with pyruvate dehydrogenase kinase 3 (PDK3) and acts as an efficient anticancer agent [22]. This plant-based compound is also reported to show protective effects against several natural and synthetic toxicants such as aflatoxin, acrylamide, lipopolysaccharides, rotenone, *etc*. [23]. Moreover, ellagic acid can be used as an efficient biopesticide against *Spodoptera litura* even at lower concentrations without posing any significant impact on the evolution of its parasitoid *Bracon hebetor* [24]. A recent study demonstrated that ellagic acid can serve as a prominent agent for defending against perioperative neurocognitive disorders in elderly patients by limiting neuroinflammation and oxidative stress *via* the activation of IGF-1 pathways [25]. Although numerous biological activities of ellagic acid have been extensively studied but pesticidal activity regarding agricultural and residential applications has not been reported yet.

High performance liquid chromatography (HPLC) is widely used in the pharmaceutical industry for analytical and phytochemical chemistry for the identification, quantitation and decontamination of individual drugs or compounds from complex matrices [14]. Nowadays, this technique is gaining tremendous attention among various techniques used for fingerprinting studies for quality control of plant samples. Many reports suggest the use of HPLC for identification, characterization and determination of varieties of phytochemicals such as alkaloids, phenolic compounds, flavonoids, steroids, *etc.* in plant extract [14]. As well, there are many reports offering the quantification of ellagic acid from various natural sources using HPLC. It is documented that all the reported studies offer a longer run period and more retention time, due to which a high amount of mobile phase solvent is required for quantification. Thus, this study aimed to develop an easy, fast and precise RP-HPLC-DAD method for the estimation of ellagic acid in different parts of *C. collinus*. The main goal of this study was to achieve the separation of ellagic acid within 10 min of run time using a C-18 reverse phase column with gradient elution. Various analytical criterion such as linearity, LOD, LOQ, accuracy, precision, % recovery and % RSD were also studied for the validation of developed method. Also, the phytochemical screening of different parts of *C. collinus* such as leaves, bark and fruits, was done to determine the presence of secondary metabolites in the plant. Additionally, this study also reports the insecticidal activity of ellagic acid against stored grain pests such as *Tribolium castaneum* and *Corcyra cephalonica* larvae.

EXPERIMENTAL

For qualitative estimation (preliminary phytochemical screening), methanol, acetone, hydrochloric acid and sulphuric acid of analytical grade were purchased from Merck Specialties (Pvt. Ltd., India). Potassium iodide, sodium hydroxide, ferric chloride, mercuric chloride and lead acetate were purchased from Hi Media (India). Ellagic acid (purity > 98%) was obtained from Hi Media (India). For quantitative determination of ellagic acid, HPLC-grade water, methanol and acetic acid were obtained from J.T. Baker (Avantor Performance Materials, India Ltd.).

Plant sample collection: Different parts of *Cliestanthus collinus* such as bark, leaves and fruits, were collected in polyethylene bags from the Bastar division of Chhattisgarh state, India. Plant samples were thoroughly rinsed with running water and dried in an open room at room temperature. Dried samples were converted into fine particles using a mortar and pestle and kept in a glass bottle for further use.

Ultrasonication assisted maceration extraction: The ultrasonication assisted maceration extraction of different parts of *C. collinus* was carried out in accordance with the method suggested by Patle *et al.* [26] with a slight modifications. Ultrasonication-assisted maceration extraction was carried out in an ultrasonic bath (PCI Analytics Pvt. Ltd., India) using 44 kHz ultrasonic frequency with various transducers. Approximately 100 g of finely powdered plant samples were soaked in 200 mL of different solvents such as methanol, acetone and chloroform for 20 min and extraction was carried out in triplicate at 15 min of extraction time at 28 ± 2 ºC. The extract was filtered using Whatmann filter paper No. 41 and the filtrate was dried at 40-45 ºC using a hot air oven. The natural crude extract was then stored at 4 ºC for further application.

Recovery percentage of extract: To determine the recovery percentage of yield obtained from ultrasonication-assisted maceration extraction, the methanol, acetone and chloroform extracts of the leaves, bark and fruits of *C. collinus* were filtered with Whatmann paper No. 41 and the filtrate was poured into a pre-measured beaker. After that, the extraction solvent was evaporated upto dryness at 40-45 ºC in a hot air oven. Now, the beaker with dried extract was again weighed and the % yield

of crude extract was determined by using the following formula [26]:

$$\text{Yield (\%)} = \frac{\text{Weight of dried crude extract (g)}}{\text{Weight of dried plant sample taken (g)}} \times 100$$

Preliminary phytochemical screening: The primary phytochemical screening of methanol, acetone and chloroform extracts of bark, leaves and fruits of *C. collinus* was performed using chromophoric reagents. For this, firstly, the untreated crude extract was dissolved in 15 mL of diluted DMSO solvent and filtered with Whatmann filter paper No. 41. The filtrate was poured into a 5 mL glass vial for profiling of phytochemicals such as alkaloids, phenolic compounds, flavonoids, terpenoids, saponins, carbohydrates, *etc.* in the plant samples. All chemical reagents, such as 1% HCl, Mayer's reagent ($HgCl_2$ + KI), 2% $FeCl_3$, 1% NaOH and Molish reagent (α-naphthol + ethanol), were prepared before performing the phytochemical screening. The experimental processes for qualitative assessment of secondary metabolites are shown in Table-1.

HPLC analysis of ellagic acid: A stock standard solution of pure ellagic acid (100 µg mL^{-1}) was prepared in HPLC grade methanol. This stock standard solution was diluted into the required concentration ranges of 0.01, 0.5, 1, 2, 4, 6, 8 and 10 µg mL^{-1}. These solutions were kept in Amber-coloured reagent bottle at 4-5 ºC to avoid sunlight exposure. 100 µg mL^{-1} sample (leaves, bark and fruits of *C. collinus*) solutions were prepared by dissolving crude extracts in HPLC grade methanol. These solutions were used as working solutions. All solutions were filtered by a 0.22 µm filter syringe before the introduction of the HPLC instrument. Quantification of ellagic acid in different samples was performed using ultra-high-performance liquid chromatography (Ultimate 3000, Thermo-Fisher Scientific, USA) coupled with a diode array detector (DAD). A reverse phase C-18 column (100 × 4.6 mm × 5 µm; Hypersil GOLD™, Thermo Fisher Scientific, USA) was used for the quantitation. Separation and identification of ellagic acid were carried out using gradient elution by taking solvents A (methanol) and B (1% acetic acid) in water at room temperature. The flow rate was retained at 1 mL min^{-1} during the analysis and the wavelength was 365 nm. The gradient program was set as: 0-2 min, 100% B; 2.1-4 min, 60% B, 4.1-6 min, 40% B; 6.1-8 min, 10% B; 8.1-9 min, 50% B; 9.1-10 min, 100% B. The total run time was 10 min. Various analytical parameters, including linearity, accuracy, precision, LOD and LOQ, were also assessed for method validation.

HPLC method validation: The optimized RP-HPLC-DAD method for quantitative analysis of ellagic acid in different parts of *C. collinus* was validated in the manner of International Conference on Harmonization (ICH) and various analytical parameters were also evaluated.

Linearity was estimated by examining 8 distinct concentrations of pure ellagic acid in triplicate, with concentrations ranging from 0.01, 0.5, 1, 2, 4, 6, 8, 10 µg mL^{-1}. All solutions were pre-filtered using a PTFE 0.22 µm syringe filter before injecting the solution into the instrument. The calibration curve was obtained by plotting peak areas obtained *versus* their corresponding concentrations. The statistical analysis was carried out by the least squares method [27].

The accuracy or percentage recovery of the developed method was assessed by spiking a known concentration of standard ellagic acid solution into a real sample (leaves of *C. collinus*). The recovery % was determined by using the following formula [27]:

$$\text{Recovery (\%)} = \frac{\text{Conc. after spiked} - \text{Conc. before spiked}}{\text{Conc. after spiked}} \times 100$$

The precision was determined by 5 replicate assessment to ensure the repeatability of the developed method. For this, 5 repetitions of 1 µg/mL of standard ellagic acid solution were introduced into HPLC instrument and result was evaluated in terms of percentage RSD [27].

The limit of quantification (LOQ) and limit of detection (LOD) of the proposed method were determined using the values of the slope and intercept of the regression equation as LOQ = 10 × SD/S and LOD = 3 × SD/S where SD represents standard deviation of the intercept of the Y-axis and S represents the slope of the calibration curve [27].

The specificity or selectivity of the developed method was evaluated by introducing foreign chemical substances that might be present in the real sample. Various solvents, substances were injected into the HPLC instrument to find interference in the targeted compound's peak. If there were no peak at the reten-

TABLE-1
EXPERIMENTAL METHODS FOR PRELIMINARY PHYTOCHEMICAL SCREENING BY APPLYING CHROMOPHORIC REAGENTS [26]

Name of secondary metabolites (phytochemicals)	Procedure for preliminary phytochemical screening	Observations
Alkaloid	0.5 mL extract solution + 4-5 drops of 1% HCl solution + 0.5 mL of mayer's reagent	Pale yellow solution or cream coloured precipitation or light brown solution
Phenolic compound	0.5 mL extract solution + 0.5 mL of 2% $FeCl_3$ solution	Blue-green or black coloured solution
Flavonoid	(1) 0.5 mL of extract solution + few drops of 1% NaOH solution	Intense yellow coloured solution
	(2) Add few drops of 1% HCl solution	Yellow colour disappeared
Terpenoid	0.5 mL of extract solution + 0.5 mL of $CHCl_3$ + 0.5 mL of conc. H_2SO_4	Brown, reddish or violet coloured solution
Tannins	0.5 mL of extract solution + 0.5 mL distilled water + 0.5mL of 1% lead acetate	Yellow or white coloured solution
Saponins	0.5 mL of extract solution + 1mL distilled water + shaken	Stable foam formation
Carbohydrate	0.5 mL of extract solution + 3-4 drops of molish reagent + 0.5 mL of conc. H_2SO_4	Red or violet coloured solution

tion time of the targeted compound (ellagic acid), this would ensure the specificity of the developed method [27].

FTIR and UV-vis analysis of ellagic acid and isolated compound: For this, ellagic acid was isolated by an HPLC instrument with the help of an outlet pipe at the precise retention time of ellagic acid and its functional group was identified and characterized using FTIR (Bruker Alpha Eco-ATR, Bruker India Scientific Pvt. Ltd.) equipped with zinc selenide crystal cell in the wavenumber ranges of 4000-400 cm^{-1}. Pure ellagic acid (20 µg mL^{-1}) and isolated ellagic acid were prepared using methanol as solvent and the FTIR spectra of both compounds were compared to identify the isolated compound. The characterization of isolated elagic acid was also performed using the UV-Vis spectrophotometer (Evolution 300 series; Thermo Fisher Scientific, USA) in the wavelength range of 200-800 nm. The absorption spectra of the isolated compound were compared with the spectra of standard ellagic acid to identify the isolated compound.

Insecticidal activity assessment: For the evaluation of the insecticidal activity of isolated ellagic acid obtained from different parts of *C. collinus*, two stored grain pests, such as larvae of *Corcyra cephalonica* and *Tribolium castaneum* were cultured in 5 jars covered with muslin cloth for proper aeration at 25 ± 2 ºC with a relative humidity of 60-70% using a porridge diet purchased from a local shop. These insects were cultured, male and female, in each jar for 5-7 days to permit spawning. For the insecticidal activity study, different amounts (50-250 ppm) of isolated ellagic acid dissolved with methanol as a solvent were sprayed on Whatmann filter paper No. 1 and placed on petridish. After evaporation of solvent, the porridge diet and about 50 larvae of *C. cephalonica* and *T. castaneum* were transferred to the petriplate and covered with muslin cloth for determination of mortality rate at different intervals of time at 1, 6, 12, 24 and 48 h of exposure. This experiment was done in five replicates and the mortality rate was calculated according to the formula suggested by Abbot [28] as below:

$$\text{Mortality } (\%) = \frac{\text{Number of dead insects}}{\text{Total numbers of insects taken}} \times 100$$

RESULTS AND DISCUSSION

Effect of organic solvents on the yield of crude extract: The choice of organic solvents to get a better crude extract yield is a salient factor affecting the separation of a target compound from complex samples matrices. For this, various organic solvents differing from polar to non-polar (methanol > acetone > chloroform) were selected as extracting solvents and it was found that the polar solvent (methanol) provided an outstanding yield compared to the semi-polar or non-polar solvents (acetone and chloroform). The function of polar solvents is to intensify the cell wall penetrability of chemical compounds, resulting in better interaction between solute and solvent and in that way, increasing the percentage yield of extraction [29]. The % yield of the untreated crude extract with various solvents such as methanol, acetone and chloroform with triplicate analysis is shown in Table-2. The better crude extract yield was obtained from leaves (12.84 ± 0.31%), followed by bark (8.17 ± 0.10%) and fruits (6.04 ± 0.12) using methanol as the extracting solvent. Higher extraction with methanol solvent is attributed to the polarity of solvent responsible for extracting majority of the polar substances from green samples. Therefore, methanol was used as an extracting solvent for the abstraction of phytochemicals from different plant samples.

TABLE-2
EFFECT OF VARIOUS ORGANIC SOLVENTS ON % YIELD OF CRUDE EXTRACTS OF *Cliestanthus collinus*

Cliestanthus collinus	Percentage yield of untreated crude extract in various organic solvents (%)		
	Methanol	Acetone	Chloroform
Leaves	12.84 ± 0.31	8.33 ± 0.14	4.87 ± 0.19
Bark	8.17 ± 0.10	5.12 ± 0.25	2.15 ± 0.22
Fruits	6.04 ± 0.12	3.11 ± 0.21	0.85 ± 0.34

Preliminary phytochemical screening from plant samples by applying chemical reagents: The crude extracts of leaves, bark and fruits of *C. collinus* with different solvents were employed to preliminary phytochemical screening to recognize the varieties of secondary metabolites such as alkaloids, phenolic compounds, tannins, flavonoids, saponins, terpenoids and carbohydrates in the selected plant samples (Table-3). Methanol, acetone and chloroform crude extracts of leaves, bark and fruits were first dissolved in dilutions of DMSO solvent and filtered using Whatmann filter paper No. 41. The filtrate was poured separately in a 5 mL glass vial. After that, this extract was treated with appropriate chemical reagents according to the procedure described in Table-1.

The methanol extract exhibited the presence of alkaloids, phenolic compounds, flavonoids, terpenoids, carbohydrates and tannins, in all parts of the plant taken for study, whereas saponin was found in leaves and fruits but not in bark. Next,

TABLE-3
PRELIMINARY PHYTOCHEMICAL SCREENING OF METHANOL, ACETONE AND CHLOROFORM EXTRACTS OF LEAVES, BARK AND FRUITS OF *Cliestanthus collinus*

Phytochemicals	Methanol extract			Acetone extract			Chloroform extract		
	Leaves	Bark	Fruits	Leaves	Bark	Fruits	Leaves	Bark	Fruits
Alkaloid	+	+	+	+	+	–	+	+	–
Phenolic compound	+	+	+	+	+	+	+	+	+
Flavonoid	+	+	+	+	+	+	+	+	–
Terpenoid	+	+	+	+	+	+	+	+	–
Tannins	+	+	+	+	+	–	+	+	–
Saponins	+	–	+	+	–	–	+	–	–
Carbohydrate	+	+	+	+	+	+	+	+	+

the acetone extract exhibited the appearance of phenolic compounds, flavonoids, carbohydrates and terpenoids in all parts of the plant taken, whereas alkaloids and tannins were found in leaves and bark; saponins were found in leaves only. Furthermore, the chloroform extract exhibited the presence of carbohydrates and phenolic compounds in all the parts of plant; flavonoids, alkaloids, terpenoids and tannins in leaves and bark; saponins in leaves only. The preliminary phytochemical screening led us to believe that the polarity of solvents has an effect on the extraction of secondary metabolites. It was indicated that the most phytochemicals were extracted by using methanol as an extracting solvent. This is because of the polarity of methanol, which dissolved the maximum number of polar molecules of plant samples, whereas acetone and chloroform provided fewer phytochemicals due to their less polar nature compared to methanol. Suman & Elangomathavan [9] studied the phytochemical profile of the leaf extract of *C. collinus* with different solvents such as aqueous, ethanol, ethyl acetate and methanol. Findings indicated that methanol extract provided the most secondary metabolites being studied, followed by ethanol, aqueous and ethyl acetate, suggesting that polar solvents are compatible for extracting maximum polar molecules [9].

Optimization of HPLC conditions for determination of ellagic acid: The optimization of HPLC conditions for determining ellagic acid in an extracted plant sample is crucial for accurate analysis. Various parameters for optimization such as mobile phase composition, detection wavelength, elution system and flow rate measurement, assure the separation and identification of ellagic acid amongst complex plant matrices. This optimization offers the enhancement of sensitivity, reproducibility and specificity across the plant samples selected for study [18]. The polar nature of ellagic acid offers hydrophilic interaction with the stationary phase. Thereby, RP-HPLC associated with a diode array detector and a Hypersil Gold™ C-18 column was opted for analysis because it is a silica-based column and useful for the segregation of polar compounds across complex plant samples [30]. The choice of elution program is vital for better separation of targeted compounds, as it may affect the detection of the respective compounds in sample matrices. For this, the isocratic and gradient elution programs were optimized. The results indicated that isocratic elution provided various co-eluted compounds with ellagic acid, whereas the gradient program provided a sharp, symmetrical and single peak of ellagic acid at 3.9 min. of retention time. Also, the mobile phase, including methanol and water with 1% acetic acid at different pHs (2.5, 3.5 and 4), was optimized for better resolution of the chromatographic peak. Additionally, various flow rates such as 0.5, 0.8 and 1 mL min^{-1} and detection wavelengths of 254 nm, 280 nm and 365 nm were methodically optimized.

Effect of flow rate on separation of ellagic acid: Flow rate plays a crucial role in optimum separation and detection of targeted compounds. For this, different flow rates, such as 0.5, 0.8 and 1 $mLmin^{-1}$, were selected for optimization. Numerous coeluted compounds were observed at 0.5 and 0.8 mL min^{-1} of flow rate, whereas a uniform shape with a higher resolution signal was obtained at 1 mL min^{-1} of flow rate. Therefore, a 1 mL min^{-1} flow rate was chosen for further investigation.

Effect of detection wavelength on separation of ellagic acid: Each analyte shows a prominent chromatographic signal at a significant wavelength. In such a way, the detection wavelength has a vital effect on the separation of targeted analyte. In this context, several wavelengths such as 254, 280 and 365 nm were selected for study. A small peak was observed at a wavelength of 254 nm and a co-elution with a lower intensity peak of ellagic acid was obtained at 280 nm whereas a symmetrical high-intensity signal was observed at a wavelength of 365 nm. Thus, a wavelength of 365 nm was selected for the detection of ellagic acid.

Effect of pH of water as mobile phase for separation of ellagic acid: pH is a vital parameter for the separation of marked compounds to enhance height, shape, area and resolution of the signal. A previous study reported that ellagic acid has been well separated in an acidic medium [31]. Thereby, different pH ranges of water, such as 2.5, 3.5 and 4, were selected at a fixed 6 μg mL^{-1} concentration of ellagic acid at 365 nm of detection wavelength with 1 mL min^{-1} of flow rate. At 2.5 pH, a peak with negative absorbance of ellagic acid was found and a peak with a shoulder at 3.5 min of retention time was observed at pH 4, whereas a symmetrical shape with a higher resolution peak was obtained at a pH of 3.5. Therefore, pH 3.5 was selected for the detection of ellagic acid.

Finally, ellagic acid was well separated within 10 min of run time using a gradient elution of mobile phase methanol and water with 1% acetic acid at 3.5 pH. Start with 0-2 min, 100% B; 2.1-4 min, 60% B; 4.1-6 min, 40% B; 6.1-8 min, 10% B; 8.1-9.0 min, 50% B; 9.1-10.0 min, 100% B with a flow rate of 1 mL/min. The retention time of ellagic acid was observed at 3.9 min at 365 nm of wavelength, as depicted in Fig. 1.

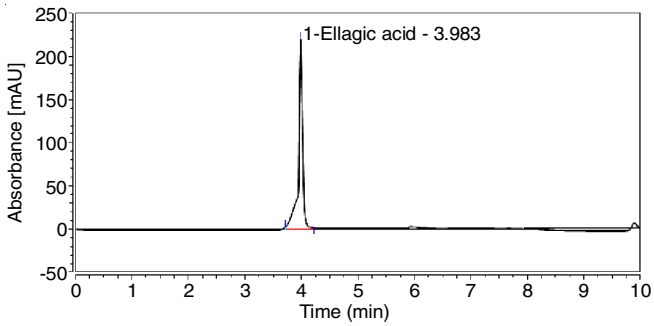

Fig. 1. Chromatographic separation of ellagic acid using gradient mobile phase of methanol and water with 1% acetic acid, with a retention time of 3.9 min and detection wavelength of 365 nm at 1 mL min^{-1} of flow rate

Analytical evaluation of validation parameters of developed method: The developed method was validated for quantification of ellagic acid in *C. collinus* by applying a standard calibration curve. For this, an eight-pointed calibration curve was drawn with a concentration ranges varying from 0.01 μg mL^{-1} to 10 μg mL^{-1} of standard ellagic acid. The correlation coefficient $R^2 = 0.9991$ with a linear regression equation ($y = 11.955x + 2.0275$) was found, indicating that the developed method is linear and specific for the separation of ellagic acid as shown in Fig. 2. The limits of quantification and detection of the developed method were found to be 0.027 μg mL^{-1} and

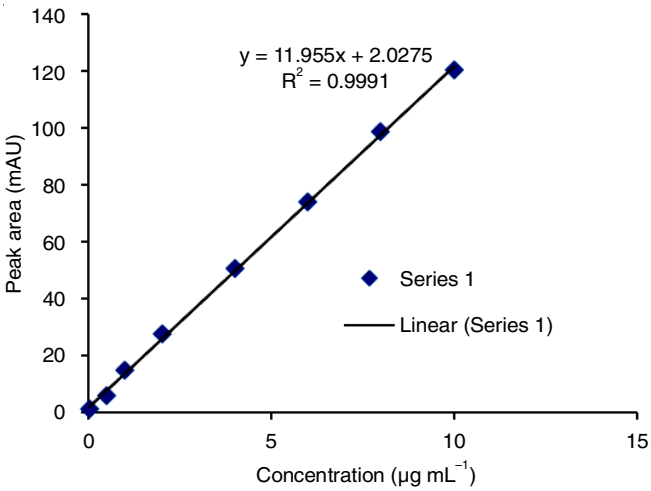

Fig. 2. Standard calibration curve of ellagic acid at optimized RP-HPLC-DAD condition

0.091 µg mL^{-1}, respectively. The value of precision was analyzed in terms of RSD% by 5 replicates measurement of 1 µg mL^{-1} of standard ellagic acid and found to be 0.73 %. Furthermore, accuracy was determined in terms of recovery% and 99.5% indicated that the developed method is accurate for the quantitative analysis of ellagic acid.

Application of developed RP-HPLC method for the determination of ellagic acid in *C. collinus*: Ellagic acid was quantified in different parts of *C. collinus* such as bark, leaves and fruits, by using the developed RP-HPLC method. For this, 100 µg mL^{-1} of crude plant extract was injected into the HPLC instrument after equilibrating the reverse phase column with water for 25 min, followed by methanol and water (70:30) for another 30 min. Fig. 3 shows the chromatograms of the leaves, bark and fruits of *C. collinus*. The concentration of ellagic acid in selected plant samples was calculated using a standard calibration curve (Table-4). The highest concentration of ellagic acid was measured in leaves (8.04 g/100 g), followed by bark (5.95 g/100 g) and fruits (2.55 g/100 g). Although studies have revealed that *C. collinus* consists of various polyphenolic compounds, including ellagic acid, there is a lack of evidence regarding the quantity of ellagic acid in *C. collinus*. As far as our knowledge, this is the first report that provides information about the concentration of ellagic acid present in different parts of *C. collinus*.

Comparison of developed RP-HPLC-DAD method with other reported method: Various analytical parameters, such as linearity ranges, LOD, LOQ, RSD% and recovery%, of present developed method were compared with the previously reported method shown in Table-5. The developed RP-HPLC-DAD method is found to be sensitive and selective for the detection of ellagic acid in different parts of *C. collinus*. The proposed method is significant in terms of linearity range, LOD, LOQ, recovery% and RSD%. The HPLC method developed for quantifying ellagic acid in pomegranate fruit peels and six different plant samples was comparable with present method in terms of linearity ranges and recovery percentage, while RSD%, LOD and LOQ values were less significant to the present method [11,32]. Similarly, the HPLC-PDA, HPLC-UV and HPLC-DAD methods reported in *Terminelia bellarica*, *Eugenia uniflora* and *Eugenia punicifolia*, respectively, were found to be less significant in terms of linearity ranges, LOD, LOQ and precision (RSD%), while the recovery percentage was found to be higher than in the present study [17,18,33]. The RP-HPLC reported for quantification of ellagic acid in triphala churna was also found to be less significant in terms of linear range, LOD, LOQ and RSD%, while recovery% was found to be higher than in the present study [16]. Moreover, the MSPD-HPLC method developed for quantifying ellagic acid in pomegranate peels was found to be less significant regarding linearity, LOD, recovery% and RSD%; however, the retention time was comparable with the present method [34]. Among all the discussed methods were less significant in terms of total run time and retention time of ellagic acid than the present developed method.

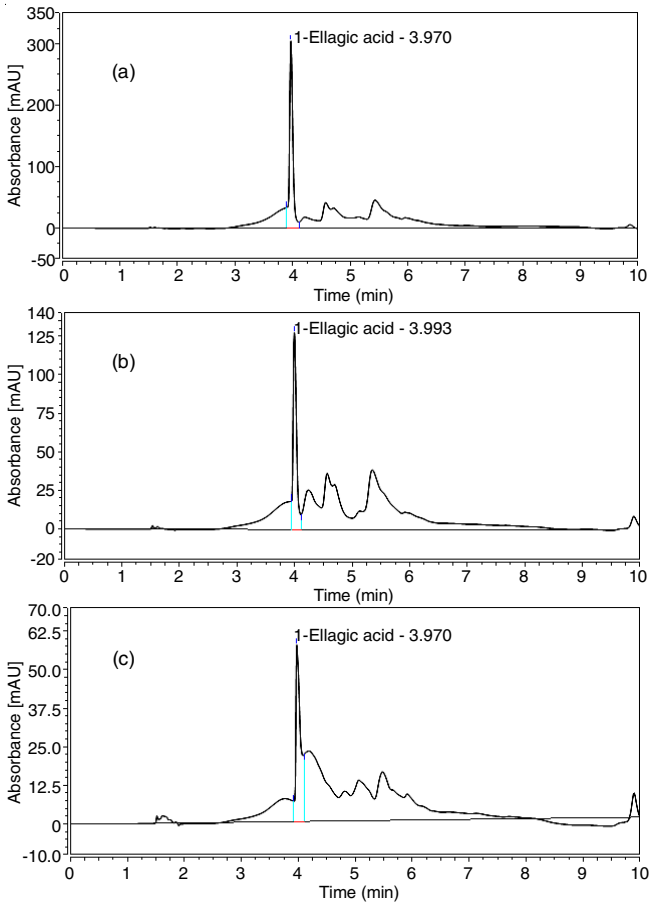

Fig. 3. The chromatograms of (a) leaves, (b) bark and (c) fruit samples of *Cliestanthus collinus*

TABLE-4
QUANTIFICATION OF ELLAGIC ACID IN LEAVES, BARK AND FRUITS OF *Cliestanthus collinus*

Plant samples (*Clistanthus collinus*)	Concentration of ellagic acid (g/100 g), (n = 3)
Leaves	8.04 ± 0.03
Bark	5.95 ± 0.12
Fruits	2.55 ± 0.01

Comparison of ellagic acid content using HPLC method with other plant samples: The ellagic acid content in various

TABLE-5
COMPARISON OF DEVELOPED RP-HPLC-DAD METHOD FOR DETERMINATION OF ELLAGIC ACID WITH OTHER REPORTED METHOD

Method	Run time (min)	Retention time (min)	Linearity ranges (μg mL^{-1})	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	Precision (RSD%)	Accuracy (recovery, %)	Plant samples	Ref.
HPLC-PDA	35	21.5	250-750	-	-	1.46	99.79	*Terminalia bellarica*	[33]
HPLC-UV	20	12.22	14.5-33.8	0.66	2.22	3.51	105.13-110.6	*Eugenia uniflora* L.	[18]
HPLC-DAD	20	13.6	32-48	1.9	5.8	4.11	81.28-105.22	*Eugenia punicifolia*	[17]
RP-HPLC	30	20.44	10-100	0.42	1.9	2.12	101.3	Triphala churna	[16]
HPLC	20	5.1	5-50	-	-	1.34	99.3-100.4 (Intra-day) 98.6-100.3 (Inter-day)	*Punica ganatum turning, Punica ganatum red, Euphorbia antisyphyllitica, Flourensia cernua, Tumera diffusa, Jatropha dioca*	[11]
MSPD-HPLC	30	4	5-800 (mg L^{-1})	1.3	-	5.9	96.7	Shirin shahvar, Siah, Abdandan, Bidaneh, Yousof khani, Malas saveh	[34]
HPLC	45	7.7	3-50	1	2.50	1.38	98.4	Pomegranate fruit peel	[32]
RP-HPLC-DAD	10	3.9	0.01-10	0.027	0.091	0.73	99.5	Leaves, bark and fruits of *Cliestanthus collinus*	Present work

plant samples reported previously using the HPLC method was compared with the plant samples of *C. collinus*. Results show that the leaves of *C. collinus* contain a higher amount of ellagic acid than other reported samples. Various plant samples, such as pomegranate peels, *Punica ganatum* turnings, *Euphorbia antisyphyllitica*, *Flourensia cernua*, *Punica ganatum* red, *Tumera diffusa*, *Jatropha dioca*, triphala churna, *Eugenia punicifolia*, *Eugenia uniflora*, *Terminelia bellarica*, *etc.* were analyzed for the quantification of ellagic acid and found to be comparatively lower than the present study. A good amount of ellagic acid was found in different parts of *C. collinus* collected from the Bastar division of Chhattisgarh state of India, as demonstrated in Table-6. Thus, the selected plant, *C. collinus*, can be used as a new source for ellagic acid.

FTIR and UV-Vis spectrometric studies: In this study, we isolated a compound at a retention time of ellagic acid precisely using an HPLC outlet pipe and the isolated compound was characterized using a FTIR spectrophotometer ranging from 4000-400 cm^{-1}. The IR spectra of the isolate compound were compared with the IR spectra of standard ellagic acid and the absorption peaks related to stretching and bending vibrations of various functional groups are shown in Fig. 4. The FTIR bands of different functional groups are generally positioned from 4000-1500 cm^{-1} whereas strong and sharp absorption bands in the range of 1500-500 cm^{-1} represent the fingerprint region [26]. The FTIR spectra of ellagic acid exhibited significant signals at 3544, 3248, 1665, 1613, 1516, 1450, 1310, 1205, 1007 and 810 cm^{-1}. The IR spectra of standard ellagic acid showed a prominent, sharp signal at 3544 cm^{-1}, which indicates the existence of a hydroxyl (-OH) group. The spectrum showed a broad band signal at 3248 cm^{-1} represents the hydrogen stretching in the aromatic ring. A second prominent signal was observed at 1665 cm^{-1} due to the twisting force (torsion effect) of the lactone carbonyl group (C=O). IR signals at 1613 cm^{-1} and 1516 cm^{-1} are because of the stretching of C=C bond indicating the presence of aromatic ring. Spectra showed a mixed signal at 1310 cm^{-1} and 1007 cm^{-1}, which are generated by asymmetric and symmetric stretching of the C-O bond. Additionally, the FTIR peak at 1205 cm^{-1} indicated the stretch of the phenolic C-O bond and the signal at 810 cm^{-1} attributed to the existence of a substituted aromatic ring [35]. The FTIR spectrum of the isolated compound was compared with these values. Findings indicated that both spectra were comparable and resemblant to each other. Thereby, the isolated compound was identified as ellagic acid, as shown in Fig. 4b.

TABLE-6
COMPARISON OF ELLAGIC ACID CONTENT WITH OTHER REPORTED PLANT SAMPLES

Plant samples name	Concentration of ellagic acid	Method employed	Ref.
Terminalia bellarica	0.468 mg/50 g	HPLC-PDA	[33]
Eugenia uniflora L.	0.026 mg/g	HPLC-UV	[18]
Eugenia punicifolia	0.065 mg/g	HPLC-DAD	[17]
Triphala churna	21.7 mg/g	RP-HPLC	[16]
Punica ganatum turning,	33.79 mg/g	HPLC	[11]
Punica ganatum red,	12.80 mg/g		
E. antisyphyllitica,	2.18 mg/g		
Flourensia cernua,	1.59 mg/g		
Tumera diffusa,	0.87 mg/g		
Jatropha dioca	0.81 mg/g		
Shirin shahvar,	1.3 mg/g	MSPD-HPLC	[34]
Siah,	2.5 mg/g		
Abdandan,	6.1 mg/g		
Bidaneh,	3.4 mg/g		
Yousof khani,	7.2 mg/g		
Malas saveh	18.1 mg/g		
Pomegranate fruit peel	7.6 mg/g	HPLC	[32]
Cliestanthus collinus			
Leaves	8.04 g/100g	RP-HPLC-DAD	Present work
Bark	5.95 g/100g		
Fruits	2.55 g/100g		

The isolated compound was further characterized using a UV-Vis spectrophotometer, a vital analytical technique for determining the numbers of chromophoric compounds and aromatic rings in plant samples *via* measuring the electronic transitions of lone pair electrons and π and σ bond electrons. In this study, we compared the UV-Vis absorption spectra of standard ellagic acid and compounds isolated from HPLC. Both compounds exhibited an absorption band at 365 nm of wavelength, which indicates the isolated compound is ellagic acid (Fig. 5).

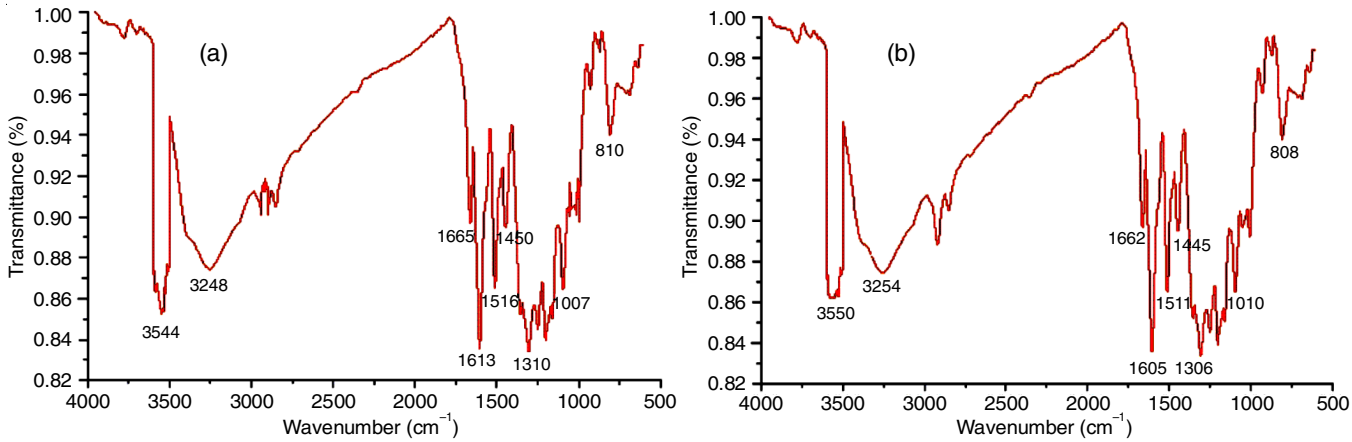

Fig. 4. FTIR spectra of (a) standard ellagic acid and (b) isolated ellagic acid

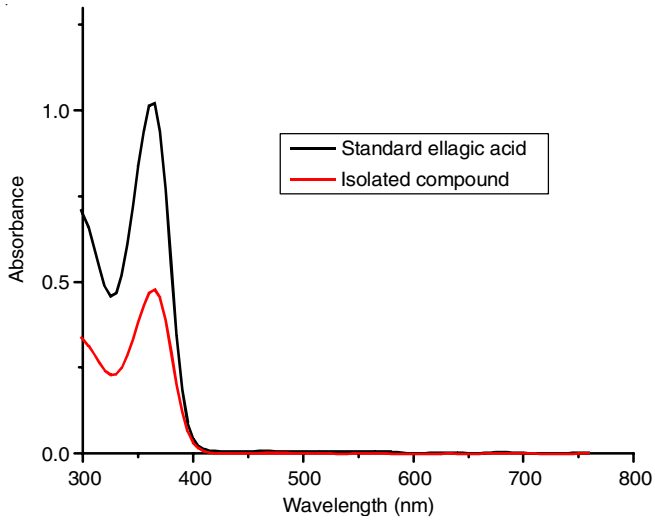

Fig. 5. UV-vis spectra of standard ellagic acid and isolated compound

Application of ellagic acid in insecticidal activity: *T. castaneum* and *C. cephalonica* larvae are the most common and destructive stored grain pests, largely affecting the quality and quantity of grains, cereals, dry nuts, dry fruits, *etc.* and necessary to control them [7]. Ellagic acid shows larvicidal activity against *Spodoptera litura* larvae by impairing their growth and further development [24]. Additionally, the repellent activities of ellagic acid make it attractive and useful for inhibiting insect overspreading in both domestic and agricultural settings. The insecticidal activity of ellagic acid against *T. castaneum* and *C. cephalonica* as evidenced by the provided data, shows its efficiency as an effective insecticide against *T. castaneum*. The data revealed increasing mortality rates with increasing concentrations of ellagic acid and longer exposure times. For *T. castaneum,* the mortality rate increases from 1% at 1 h to a higher rate of 98% at 48 h with a 250 ppm concentration, whereas *C. cephalonica* larvae shows a higher mortality rate of 76% at 48 h of exposure with the same concentration. The results demonstrated the ability of ellagic acid as a potential insecticide against *T. castaneum* within a short period of time (Fig. 6). These results suggest that ellagic acid can be used as a natural and potential insecticide for resisting stored grain pests and also for agricultural practices.

Conclusion

In present study, preliminary phytochemical screening was performed in different solvents such as methanol, acetone and chloroform to find secondary metabolites in the leaves, bark and fruits of *Cliestanthus collinus.* It was found that the methanol

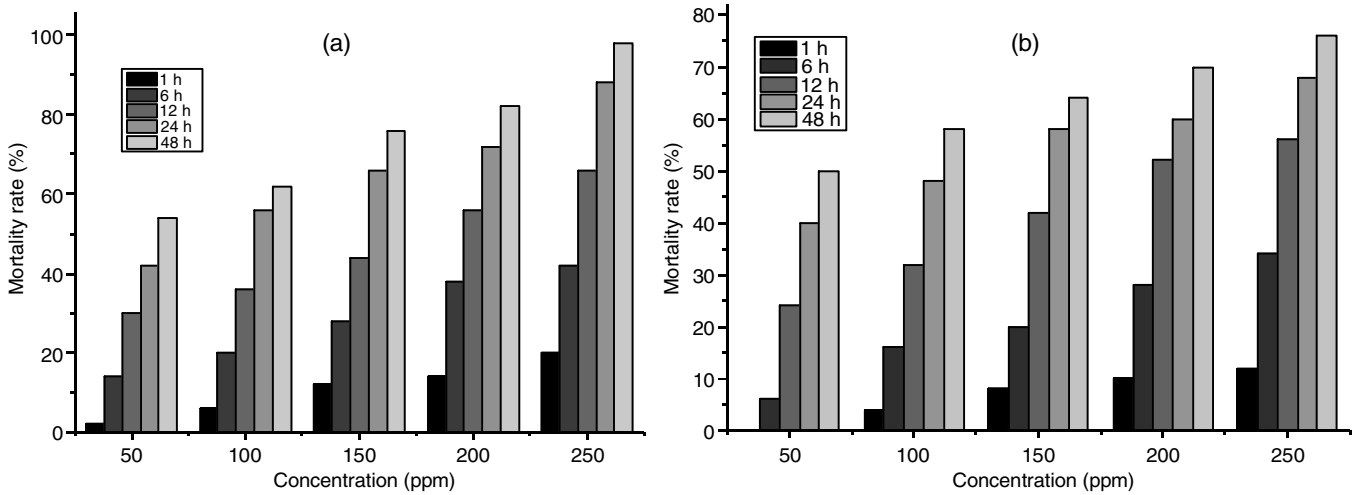

Fig. 6. The mortality rate of ellagic acid against (a) *Tribolium castaneum* and (b) *Corcyra cephalonica* larvae

extract was rich with secondary metabolites along with a higher extraction yield. A fast and precise RP-HPLC-DAD method developed was found to be significant for the detection and quantitative analysis of ellagic acid in the leaves, bark and seeds of *C. collinus*. The developed method showed better linearity, LOD and LOQ compared to the previously reported samples, with a good RSD and recovery rate. Therefore, the present developed method can be used for the quantification and identification of other new sources of ellagic acid. Moreover, ellagic acid was found to be a potential insecticide against *Tribolium castaneum* and *Corcyra cephalonica*. Thereby, the present study suggests that ellagic acid can be used as an efficient biopesticide for the pest studied.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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