Research paper

Assessment of Phytochemical Screening, TLC, and HPTLC fingerprinting Analysis of Fresh Leaves and Stems of *Leea asiatica* (L.) Ridsdale from Valmiki Tiger Reserve, West-Champaran District, Bihar

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ABSTRACT

Background: To determine the secondary metabolites, TLC and HPTLC fingerprinting analysis of the fresh leaves and stems of *Leea asiatica* (L.) Ridsdale.

Aim: The aim of this work was designed for extraction, preliminary phytochemical screening, and isolation of medicinally active flavonoid molecule from *Leea asiatica* (L.) Ridsdale.

Methodology: *Leea asiatica* (L.) Ridsdale was screened out for their secondary metabolites in the six selected solvents using standard methods. TLC and CAMAG HPTLC system was used for separation, identification, and determining the purity of flavonoids.

Result: Qualitative phytochemical screening of *Leea asiatica* revealed that the plant extract contained various types of secondary metabolites such as alkaloids, flavonoids, cardiac glycosides, tannins, terpenoids, and phenolic compounds. TLC fingerprinting analysis was carried out for different mobile phases with different ratios and it helps to precisely acknowledge the solvent system; chloroform : ethyl acetate : methanol (6 : 3 : 1) and toluene : ethyl acetate : chloroform : formic acid (6 : 6 : 4 : 1) capable of showing best resolution in separating quercetin and kaempferol flavonoids under investigation. The results from the HPTLC fingerprinting analysis of the ethanol extract of this plant revealed the presence of quercetin and kaempferol flavonoids. The ethanol extracts indicated the presence of higher flavonoid content as compared to other solvent extracts.

Conclusion: This research was to elucidate the secondary metabolite such as flavonoids in the ethanol extract of *Leea asiatica*, using TLC and HPTLC fingerprinting analysis. The ethanol

extract has elucidated peaks that were corresponding with standard peaks on undertaking chromatographic studies.

Keywords: *Flavonoid, HPTLC, Leea asiatica (L.) Ridsdale, Mobile phase, Secondary metabolites, TLC.*

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1. Introduction

Ethnomedicinal plants are a good source of biologically active compounds known as phytochemicals [1]. Our sources are *Chemical Abstracts*, *Biological Abstracts*, and current issues of 70 journals which we have found by experience to contain the majority of new information concerning natural products [2]. Every year, phytochemistry reports literally thousands of newer organic molecules or compounds. Research on these natural products represents a new horizon for the discovery and development of new therapeutic agents [3]. The most accepted definition of secondary metabolites considers them to be naturally produced substances that do not play an explicit role in the internal economy of the organism that produces it and stands in direct contrast to primary metabolites, which maintain fundamental cellular life processes. These secondary metabolites are argued to play an important role in the survival of the species that produce them via critical interactions with their environment. The most active secondary metabolites are alkaloids, flavonoids, cardiac glycosides, tannins, terpenoids, phenolic compounds, and several other aromatic compounds in the plants [4].

The term chromatography (color-writing) was derived from the Greek word chroma means color, and graphein means to write. The first reported use of TLC method for separation of extract from plants in 1938 by two Russian scientists, N.A. Izmailov and M.S. Schreiber. In 1949, this technique was first described in analytical chemistry by J. E. Meinhard and N. F. Hall. The applications of TLC method for the analysis of plant extracts began in the 1960s, with the work of Stahl and Randerath [5]. Thin layer (planar) chromatography is a very economical and physical technique of separation of the components. It was employed for routine use because the consumption of solvent is very low, and it is possible to analyze numerous samples on the same plate. In plant analysis, TLC is very extensive, with the most important contributions being those of Stahl and Randerath, Harbone, and Wagner, Fried and Sherma, who is our main reference in this research. Most TLC studies are listed in the different pharmacopoeias of the world, as reported by Wagner.

HPTLC chemical analysis is an inexpensive method used for the separation and identification of major active phytoconstituents of medicinal plants. HPTLC provides much better separation and resolution, and the results are much more reliable and reproducible than TLC [6,7]. It also exposed a better separation of individual secondary metabolites. India is a mega biodiversity countries in the world, and endowed with higher medicinal plant species richness. A large number of

ethnomedicinal plants are still not analysed/ examined phytochemically with particular reference to secondary metabolites of medicinal importance [7].

Leea asiatica (L.) Ridsdale, a member of the family Vitaceae is a shrub to small tree, distributed in the forests of Valmiki Tiger Reserve, West-Champaran District, Bihar, India on the side of river Gandak. *Leea* species (*Leea indica, Leea macrophylla, Leea asiatica, Leea aequata, Leea rubra and Leea guineensis*) are an important ethnomedicinal plant species in the Valmiki Tiger Reserve used both by traditional medicine practitioners and tribal communities residing in West Champaran district with enormous potentials and treatment of various ailments [8]. West Champaran district has 3 sub-division, 18 blocks and 1483 villages [9]. The tender leaves and stems of this species are prescribed by the tribal community other than tharu in this landscape are Oraon, Munda, Hohra, and Bhuiya, for various medical ailments, mainly for the treatment of inflammation, cough and cold, snake-bite, blood coagulation, bone fracture, osteoarthritis, diabetes. It is being prescribed/ used by the people living in villages of West-Champaran District, Bihar viz. Bettiah, Bagaha, and Narkatiaganj for the treatment of jaundice, hyperglycemia, coagulation and hepatic disorder. The leaves are used as cattle-fodder. Roots are pasted and applied on mouth sores to cure [10,11]. Previous reports exhibited more pronounced antioxidatnt, antibacterial, anthelmintic, hepatoprotective, cytotoxic, and nephroprotective effects of *Leea asiatica* (L.) Ridsdale [12]. A detailed literature survey on the phytochemical constituents of this plant is reported to possess steroids, tannins, flavonoids, glycosides, phenolic compounds, diterpenes, triterpenes, saponins, alkaloids and so forth [13]. Literature survey carried on the bioactive phytoconstituent of *Leea asiatica* (L.) Ridsdale is reported to contain eight known flavonoids: (+)-catechin, (-)-epicatechin, (-)-epiafzelechin, juglanin, mearnsetin 3-*O*-rhamnopyranoside, myricitrin, afzelin, quercitrin [12].The following known flavonoid compounds; gallic acid, quercetin, kaempferol, quercitrin, and mearnsitrin is reported from other species of genus *Leea* (*Leea indica*, *L. guineense*) [14]. However, there is no report is available on phytochemical constituent profiles for *Leea asiatica* (L.) Ridsdale. To the best of our knowledge, *Leea asiatica* (L.) Ridsdale is having a traditional medicine (TM), we intend to investigate the assortment of phytochemical profiles for flavonoids using the HPTLC work that exerted the observed biological effects, safety information, and medicinal use of this plant [7,12]. Ethnobotanical uses and the presence of phytoconstituents are correlated with the therapeutic activity of other medicinal plants of Vitaceae [15].

2. Material and methods

2.1 Instruments, chemicals and solvents:

CAMAG Linomat 5, CAMAG TLC Scanner 3, Muttenz, Switzerland, Arbro Pharmaceuticals Pvt. Ltd, New Delhi, were used for study. Rotary vacuum evaporator (Royal Scientific, India), UV cabinet (DESAGA, Germany), Silica gel 60 F_{254} (Merck, India), and other common glasswares are used for the present study.

All the chemicals and reagents used were of analytical grade (SDFCL, SIGMA, SRL, RFCL, India). Reference standards rutin, kaempferol and quercetin were purchased from Yucca Enterprises, Mumbai, India.

2.2 Collection of the plant material:

The species *Leea asiatica* was collected from forests of Valmiki Tiger Reserve, West-Champaran District, Bihar, India. The plant specimen was authenticated taxonomically by Dr. Narendra Kumar, Scientist, CSIR- Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow – 226015, India (Specimen No. CIMAP/Bot-Pharm./2018/10).

2.3 Preparation and extraction of the plant material:

The fresh leaves and stems of *Leea asiatica* were thoroughly washed with distilled water and air-dried at room temperature for about one week. The dried leaves and stems were crudely powdered with a mechanical grinder and subjected to cold extraction. 500 g dried *Leea asiatica* samples were extracted with different solvents in their increasing order of polarities such as petroleum ether, chloroform, ethyl acetate, methanol, ethanol, and water. The resultant filtrate was concentrated in powdered form by evaporation of the solvents using a rotary vacuum evaporator (Flask Capacity: 800 ml) which was stored in a refrigerator till use (at 4°C) used for phytochemical analysis as per the standard methods of Harborne [16].

2.4 Qualitative Phytochemical Screening

Petroleum ether, chloroform, ethyl acetate, methanol, ethanol, and aqueous extracts were subjected to qualitative phytochemicals analysis for the presence of various secondary phytoconstituents using standard methods of Sofowora, Trease and Evans, Kokate, Kokate and Purohit, Edeoga, Harbone, Wallis [17,18,19,20,21,22,23].

Detection of alkaloids:

a) Dragendroff's/ Kraut's test: A little quantity of solvent-free extract was mixed with few ml dil. HCl and then filtered. In a few ml filtrates added 1-2 ml Dragendorff's reagents. The formation of a red precipitate shows the presence of alkaloids.

b) Wagner's test: A little quantity of solvent-free extract was mixed with few ml dil. HCl and then filtered. In a few ml filtrates added 1-2 ml of Wagner's reagent along the sides of test tube. The formation of a brown/ reddish precipitate shows the presence of alkaloids.

c) Mayer's/ Bertrand's/ Valser's test: A little quantity of solvent-free extract was mixed with few ml dil. HCl and then filtered. In a few ml filtrates added 1-2 ml of Mayer's reagent. The formation of a yellow colored precipitate shows the presence of alkaloids.

d) Hager's test: A little quantity of solvent-free extract was mixed with few ml dil. HCl and then filtered. In a few ml filtrates added 1-2 ml of Hager's reagent along the sides of test tube. The formation of a creamy white precipitate shows the presence of alkaloids.

Detection of flavonoids:

a) Alkaline reagent test: 1 ml extract was treated with 2 ml of 2% NaOH solution and a few drops dil. HCl. The formation of intense yellow color, which becomes colorless on the addition of dilute acid, shows the presence of flavonoids.

b) NH_4OH test: 3 ml extract was treated with 5 ml of 10% NH_4OH solution, which produced a yellow fluorescence shows a positive test.

c) Mg turning test: The plant extract was dissolved in 5 ml alcohol and the alcoholic extract was treated with fragments of magnesium ribbon and added few drops of conc. HCl, which produced a crimson red color shows the presence of flavonoids.

d) Pew's test: A few ml aqueous extract solution was treated with 0.1gm metallic zinc and 8 ml conc. H_2SO_4, which produced a red color shows the presence of flavonoids.

e) Lead acetate test: 1 ml plant extract was treated with a few drops of 10% lead acetate solution, which produced a yellow precipitate shows the presence of flavonoids.

Detection of proteins and amino acids:

a) Biuret's test: 2 ml filtrate was treated with 1 drop of 2% $CuSO_4$ solution, 1 ml of 95% ethanol, and KOH pellets, which produced a pink-colored solution (in the ethanolic layer) shows the presence of proteins.

b) Millon's test: 2 ml filtrate was treated with a few drops of Millon's reagent, the appearance of a white precipitate which changed to a brick red on heating shows the presence of proteins.

c) Ninhydrin test: 2 ml filtrate was treated with 2 drops of Ninhydrin solution (10 mg ninhydrin + 200 ml acetone) which was boiled on a water bath for 10 min. The change in color of the solution to purple or blue shows the presence of amino acids.

Detection of carbohydrates:

a) Molisch's test: A few mg of the extract was shaken with 2-3 ml of water. To this 2-3 drops of alcoholic alpha naphthol solution (Molisch's reagent) were added and about 1ml of concentrated sulphuric acid was added from the side of the tube to form two layers. The development of a violet ring at the junction of two liquids indicates the presence of carbohydrates.

b) Benedict's test: An equal volume (2 ml each) of Benedict's solution and extracts were mixed in a test tube and heated in a boiling water bath for 10 min. the changes in color to yellow, green, and red show the presence of reducing sugars.

c) Fehling's test: Fehling's A and Fehling's B solutions, each 1 ml were mixed and boiled, for 1 ml and 2 ml of extracts were heated on water bath for 10 min, the appearance of a yellow and then brick-red precipitate shows the presence of reducing sugars.

Detection of cardiac glycosides:

a) Legal's test: Extracts were treated with sodium nitroprusside in pyridine and sodium hydroxide. The formation of pink to red color indicates the presence of cardiac glycosides.

b) Keller-Killiani test: To 5 ml of the extract few drops of ferric chloride solution was added and mixed, then sulphuric acid containing ferric chloride solution was added, it forms two layers showed reddish brown while the upper layer turns bluish-green indicating the presence of cardiac glycosides.

c) Baljet's Test: To 5 ml of the extract few drops of sodium picrate was added to observe yellow to orange color.

Detection of saponin glycosides:

a) Foam test: 0.5 gm of the extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of phenolic compounds:

a) Ferric Chloride test: Dissolved the extract in water, added a neutral solution of ferric

chloride slowly dropwise, and observed the color change. A green coloration indicated the presence of phenol.

Detection of tannin:s

a) Ferric Chloride test: 0.5 ml of the extract were added 1 ml water and 1-2 drops of $FeCl_3$ solution. Green black color was showed the presence of catechol tannins.

b) Gelatin test: Dissolved the extract in water, added 1% gelatin solution and 10% NaCl solution. A white precipitate showed the presence of phenol.

Detection of terpenoids:

a) Salkowski's test: Dissolved 0.5 ml of the extract in 2 ml of chloroform and evaporated on a water bath. 3 ml of conc. H_2SO_4 was added and boiled on a water bath. A grey-coloured solution showed the presence of terpenoids.

b) Libermann Burchard's test: Dissolved 2 ml of the extract in 2 ml chloroform and 2 ml of acetic anhydride. Then 2-3 drops of conc. H_2SO_4 was added and a red-violet color was observed for terpenoids.

Detection of triterpinoides:

a) Salkowski test: An equal quantity of chloroform was treated with plant extract and filtered. The filtrate was shaken well with a few drops of conc. H_2SO_4 and allowed to stand. A golden yellow layer was observed at the bottom.

b) Lieberman Burchard's test: An equal quantity of chloroform was treated with plant extract and filtered. The filtrate was shaken well with a few drops of acetic acid and 1 ml conc. H_2SO_4 gives deep red at the junction of two layers.

c) Tschugajen test: An equal quantity of chloroform was treated with plant extract and filtered. The filtrate was shaken well with an excess of acetyl chloride and a pinch of zinc chloride and warming on a water bath gives eosin red color.

Detection of diterpenes:

a) Copper acetate test: The plant extract was dissolved in distilled water and treated with 3-4 drops of $Cu(CH_3COO)_2$ solution. The formation of an emerald green color showed the presence of diterpenes.

Detection of phytosterols:

a) Libermann Burchard's test: 50 gm extract was dissolved in 2 ml acetic anhydride. Then 1-2 drops of conc. H_2SO_4 was added slowly (along the side of the test tube) and a green bluish color was observed for steroids.

b) Salkowski's test: An equal quantity of chloroform is treated with plant extract and filtered. The filtrate was shaken well with a few drops of conc. H_2SO_4 and allowed to stand for few minutes. A red color was observed in the lower layer.

Detection of fixed oils and fats:

a) Spot test/ Stain test: A little quantity of plant extract was pressed in between two filter papers and kept aside. The oil stain on the paper showed the presence of oils and fats.

Detection of gum and mucilage:

a) Alcohol test: Dissolve 100 mg extract in 10 ml distilled water and 25 ml absolute alcohol with constant stirring. The formation of a white or cloudy precipitate showed the presence of gum and mucilage.

2.5 TLC method for the identification of flavonoids (TLC Fingerprinting Analysis):

Thin-layer chromatography (TLC) is very common technique for separation, identification, and determining the purity of chemical compounds. The principle of separation in TLC is based on the adsorbent.

TLC fingerprinting studies were performed using the methodology of Stahl, Harborne, and Wagner [16,24,25].

Methodology:

The studies were performed on size 5 x 20 cm and thickness 250 µm precoated silica gel 60 F_{254} plates. The prepared ethanol extracts (2 mg/ml in their respective solvents), and the standard quercetin and kaempferol solution (1mg/ml in methanol), respectively were applied to the TLC plates by using a capillary tube and the plates were allowed to air dry for 15-20 minutes after that the plates were kept inside Hot-air oven at 100-105°C for one hour, for activation of plates. The activated plates were immersed in a development chamber and TLC studies were carried out for different mobile phases with different ratios, covered with a proper lid, and then it was allowed to develop. After drying, then the TLC plates were kept inside the UV cabinet at short wavelength, 254 nm, and long-wavelength, 366 nm, for the visualization of the separated bands. Then, the R_f value of each different spot that was observed was calculated.

2.6 HPTLC method for the estimation of flavonoids (HPTLC Fingerprinting Analysis):

HPTLC study was carried out using standard methods of Harborne and Wagner [7,25,26].

Methodology:

Solubility of ethanolic extract of aerial parts

It is more soluble in water, methanol, and pyridine, less soluble in ethyl acetate, butanol, ethanol, and acetone, not soluble in toluene, chloroform, benzene, petroleum ether, and hexane.

Sample Preparation

10 mg ethanol extract of *Leea asiatica* was taken and dissolved in 1 ml of HPTLC grade methanol and centrifuged this solution for five minutes at 3000 rpm.

Development Chamber

HPTLC of the ethanolic extract was performed by using several solvent systems, but the solvent chloroform : Ethyl acetate : Methanol (6 : 3 : 1) and toluene : ethyl acetate : chloroform : formic acid (6 : 6 : 4 : 1) provided a good satisfactory resolution for the separation of phytoconstituent flavonoid quercetin and kaempferol, respectively.

Instrumentation

CAMAG Linomat 5 "Linomat5_131223" S/N 131223 (1.00.12), CAMAG TLC Scanner 3 "Scanner3_131211" S/N 131211 (1.14.26), Muttenz, Switzerland, Arbro Pharmaceuticals Pvt. Ltd, New Delhi, consisting of sample application - CAMAG Linomat 5, chamber type - Twin Trough Chamber 20x10cm, detection - CAMAG TLC Scanner 3, Data filtering - Savitsky-Golay 7, Lamp D2 with wavelength 254 nm and HPTLC photo documentation chamber (CAMAG REPROSTAR 3) were used for the study.

Sample Application

Applied volume (5 and 10 µl) of standard solution and test solution was applied as band with a 100 µL sample syringe on precoated silica gel 60 F_{254} HPTLC plate (5 × 10 cm) with 250 µm thickness (E. Merck, Mumbai, India) using a Linomat 5 sample applicator at the application position Y 10 mm. After the sample application, prederivatization was performed by exposing the plate to the oven, at 60°C for 5 minutes.

Development of chromatogram/ Development - Glass tank

After the application of the sample, the chromatogram was developed in a Twin trough chamber 20 x 10 cm saturated by using solvents chloroform : ethyl acetate : methanol (6 : 3 : 1) and toluene : ethyl acetate : chloroform : formic acid (6 : 6 : 4 : 1) for the separation of flavonoids phytoconstituent, respectively for 15 min. The length of solvent front position was 80 mm. HPTLC plate was air-dried with the help of an air dryer. The slit dimensions of 6.00 × 0.30 mm, Micro and scanning speed was 20 mm/sec, data resolution 100 μm/step, optical filter second order, and data filtering Savitsky-Golay 7.

Detection of Spots

The detection of chromatogram was performed at 254 nm under a D2 lamp with a CAMAG TLC Scanner 3. After observation, the central points of spots that appeared on the chromatogram were marked with a needle. The Rf values and fingerprint data were recorded by Win CATS Planar Chromatography Manager software.

Image information/ Photo-documentation

The image of the plate (fingerprint profile) was captured at 254 nm using an illumination instrument CAMAG Reprostar 3.

3. Results and Discussion

3.1 Qualitative Phytochemical Screening:

Qualitative phytochemical screening of various extracts of this plant was conducted and the result revealed the presence of alkaloids, flavonoids, carbohydrates, cardiac glycosides, saponin glycosides, phenolic compounds, tannins, terpenoids and triterpenes. The results were represented in table no. 1.

Table 1: Qualitative phytochemical screening of various extracts of fresh leaves and stems of *Leea asiatica* (L.) Ridsdale

S.No.	Constituents	Test	Pet. ether	Chloroform	Ethyl acetate	Methanol	Ethanol	Aqueous
1.	Alkaloids	Dragendroff's test	+	+	+	+	+	+
		Wagner's test	+	-	+	+	+	+
		Mayer's test	+	-	-	+	+	+
		Hager's test	+	-	-	+	+	+
2.	Flavonoids	Alkaline reagent test	+	+	+	+	+	+
		NH_4OH test	+	+	+	+	+	+
		Mg turning test	+	+	+	+	+	+
		Pew's test	+	+	+	+	+	+
		Lead acetate Test	+	+	+	+	+	+
3.	Proteins and amino acids	Biuret's test	-	-	-	-	-	-
		Millon's test	-	-	-	-	-	-
		Ninhydrin test	-	-	+	+	+	+
4.	Carbohydrates	Molisch's test	-	-	+	+	+	-

		Benedict's test	-	-	+	+	+	-
		Fehling's test	-	-	+	+	+	-
5.	Cardiac glycosides	Legal's test	+	+	+	+	+	+
		Keller-Killiani test	+	+	+	+	+	+
		Baljet's test	+	+	+	+	+	+
6	Saponin glycosides	Foam test	-	-	+	+	+	+
7	Phenolic compounds	FerricChloride test	+	+	+	+	+	+
8	Tannins	Ferric Chloride test	+	+	+	+	+	+
		Gelatin test	+	+	+	+	+	+
9	Terpenoids	Salkowski's test	-	-	+	+	+	+
		Libermann-Burchard's test	+	+	-	+	+	+
10	Triterpinoides	Salkowski's test	+	+	+	+	+	+
		Libermann-Burchard's test	+	+	+	+	+	+
		Tschugajen test	+	+	+	+	+	+
11	Diterpenes	Copper acetate test	-	-	-	-	-	-
12	Steroids	Salkowski's test	-	+	-	-	-	-
		Libermann-Burchard's test	-	+	-	-	-	-
13	Fixed oils and fats	Spot test/ Stain test	-	-	-	-	-	+
14	Gum & mucilage	Alcohol test	-	-	-	-	-	+

'+' indicates phytochemicals present, '-' indicates phytochemicals absent

3.2 Chromatographic Analysis:

Thin Layer Chromatography (TLC)

Various mobile phases with varying ratios were tried for the separation of standard and ethanol extract of *L. asiatica.* In which, the most suitable mobile phase for the separation of secondary metabolite quercetin, kaempferol and rutin was found to be chloroform : ethyl acetate : methanol (6 : 3 : 1), toluene : ethyl acetate : chloroform : formic acid (6 : 6 : 4 : 1) and ethyl acetate: glacial acetic acid : formic acid : water (10 : 1.1 : 1.1 : 2.5), respectively. The results were listed in table no. 2, 3 and 4.

Table 2: Various mobile phases and R_f values of standard Quercetin and the ethanol extract of *Leea asiatica* (L.) Ridsdale

S. No.	Mobile Phase (Ratio in v/v)	Standard Quercetin	Ethanol Extract
1	Toluene : ethyl acetate : formic acid (6 : 4 : 0.3)	0.84	0.80

2	Chloroform : acetic acid : formic acid (7 : 1 : 15)	0.60	0.63
3	Chloroform : ethyl acetate : methanol (6 : 3 : 1)	0.85	0.84
4	Ethyl acetate : chloroform : formic acid (5 : 3 : 1)	0.85	0.82
5	Ethyl acetate : n-butanol : formic acid (2.5 : 1.5 : 0.5)	0.85	0.87
6	Toluene : ethanol : formic acid (5 : 4 : 1)	0.83	0.81
7	Toluene : acetic acid (5 : 1)	0.78	0.80
8	Toluene : pyridine : formic acid (5 : 4 : 1)	0.68	0.66
9	n-Hexane : ethyl acetate : acetic acid (5 : 4 : 3)	0.73	0.70
10	Carbon tetrachloride : acetone : formic acid (5 : 4 : 1)	0.55	0.51

Table 3: Various mobile phases and R_f values of standard Kaempferol and the ethanol extract of *Leea asiatica* (L.) Ridsdale

S. No.	Mobile Phase (Ratio in v/v)	Standard Kaempferol	Ethanol Extract
1	n-Butanol : acetic acid : water (2 : 2 : 4)	0.89	0.92
2	Chloroform : acetic acid : formic acid (7 : 1 : 15)	0.84	0.80
3	Toluene : ethanol : formic acid (5 : 4 : 1)	0.90	0.92
4	Toluene : ethyl acetate : formic acid (6 : 4 : 0.3)	0.93	0.90
5	Toluene : ethyl acetate : chloroform : formic acid (6 : 6 : 4 : 1)	0.92	0.93
6	Ethyl acetate : n-butanol : formic acid (2.5 : 1.5 : 0.5)	0.73	0.71
7	Ethyl acetate : acetic acid : formic acid : water (10 : 1.1 : 1.1 : 2.7)	0.76	0.80
8	n-Hexane : ethyl acetate : methanol : water (5 : 6 : 5: : 4)	0.76	0.78
9	n-Hexane : ethyl acetate : formic acid (5 : 4 : 1)	0.71	0.69
10	Cyclohexane : ethyl acetate : formic acid (5 : 4 : 1)	0.46	0.44

Table 4: Various mobile phases and R_f values of standard Rutin and the ethanol extract of *Leea asiatica* (L.) Ridsdale

S. No.	Mobile Phase (Ratio in v/v)	Standard Rutin	Ethanol Extract
1	n-butanol : acetic acid : water (2 : 2 : 4)	0.68	0.62
2	Toluene : ethyl acetate : formic acid (5 : 4 : 1)	0.56	0.59
3	Methanol: glacial acetic acid : formic acid : water (3 : 0.9 : 0.9 : 0.5)	0.60	0.63
4	Ethyl acetate : acetic acid : formic acid : water (10 : 1.1 : 1.1 : 1.5)	0.44	0.41
5	Ethyl acetate : n-butanol : ethanol : water (4 : 1: 0.25 : 5)	0.46	0.48
6	Ethyl acetate : n-butanol : ethanol : water (4 : 1 : 0.1 : 5)	0.37	0.34
7	Ethyl acetate : methanol : water (10 : 1 : 10)	0.63	0.61
8	Ethyl acetate : n-butanol : water (1 : 4 : 5)	0.61	0.58
9	Ethyl acetate : n-butanol : water (4 : 1 : 5)	0.45	0.43
10	Ethyl acetate: glacial acetic acid : formic acid : water (10 : 1.1 : 1.1 : 2.5)	0.69	0.68

After TLC, three spots resolved that were named as S_1, S_2 and S_3. The R_f values of the separated compounds was 0.68, 0.84 and 0.93 respectively. TLC profile of ethanolic extract of fresh leaves and stems of *Leea asiatica* (L.) Ridsdale were shown in Figure 1.

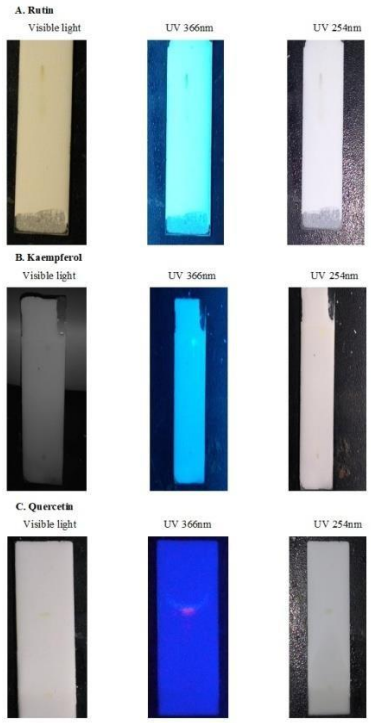

Figure 1: TLC profile of ethanolic extract of fresh leaves and stems of *Leea asiatica* (L.) Ridsdale

High Performance Thin Layer Chromatography (HPTLC)

The applied volume of standard solution and test solution was listed in table no. 5 and 6. Different proportions of selected mobile phases were tried for the separation of secondary metabolite (quercetin and kaempferol), and a ratio of chloroform : ethyl acetate : methanol (6 : 3 : 1), toluene : ethyl acetate : chloroform : formic acid (6 : 6 : 4 : 1) gave good separation and resolution. The peaks resolving at R_f 0.44 and 0.26 in ethanol crude extract sample were superimposed with respective standards of kaempferol and quercetin. Kaempferol and quercetin on derivatization with boric acid (10%), oxalic acid (3%) reagent gave blue and light green fluorescence (Figure 2, 3, 6 and 7). Standard kaempferol (R_f = 0.47) and quercetin (R_f = 0.28) showed single peak in HPTLC chromatogram and HPTLC chromatogram of *Leea asiatica* (L.) Ridsdale (Figure 4, 5, 8 and 9). The amount of kaempferol and quercetin in ethanol crude extract was calculated on the basis of peak area. The amount of kaempferol and quercetin was found to be 0.84% w/w and 0.75% w/w in in ethanol extract, respectively.

Qualitative phytochemical screening of the various extracts of *Leea asiatica* (L.) Ridsdale showed the presence of various secondary phytoconstituents. The ethanolic extract was found to have a high content of alkaloids, flavonoids, saponins, and saponin glycosides. This shows that high polar

secondary phytoconstituents were extracted with ethanol when compared to other solvents such as ethyl acetate, chloroform, and hexane. Flavonoids were found to be extractable in all the solvents such as petroleum ether, chloroform, ethyl acetate, methanol, ethanol, and water. Chromatographic methods (TLC and HPTLC) for the detection of flavonoids like quercetin and kaempferol from *L. asiatica* have not been reported in the current literature. In TLC, the standard R_f values of kaempferol were greater than quercetin and rutin. The R_f value (0.93) of S_3 compound was related to kaempferol and S_1 was related to rutin. R_f value (0.84) of S_2 compound was related to the standard R_f values of quercetin. After continuous trials by using different mobile phases for the separation of ethanol crude extract (ECE) sample of *Leea asiatica* (L.) Ridsdale by HPTLC, the desired resolution of quercetin and kaempferol with reproducible peaks were succeeded using chloroform : ethyl acetate : methanol (6 : 3 : 1) and toluene : ethyl acetate : chloroform : formic acid (6 : 6 : 4 : 1) as the mobile phase using applied volume (5 and 10 µl) of standard solution and the test solution. The amount of kaempferol and quercetin in ethanol crude extract was calculated based on peak area. Recently, HPTLC is used in developing countries as a globally accepted practical solution for small molecule characterization in quantity and quality assessment.

Table 5: Sample application - CAMAG Linomat 5

S. No.	Appl. position	Appl. volume	Vial	Sample ID (Code)	Active
1	15.0 mm	5.0 µl	1	Keampferol (STD)	Yes
2	38.3 mm	10.0 µl	1	Keampferol (STD)	Yes
3	61.6 mm	5.0 µl	2	Ethanol Crude Extract (ECE)	Yes
4	84.9 mm	10.0 µl	2	Ethanol Crude Extract (ECE)	Yes

Table 6: Sample application - CAMAG Linomat 5

S. No.	Appl. position	Appl. volume	Vial	Sample ID (Code)	Active
1	15.0 mm	5.0 µl	1	Querctin (STD)	Yes
2	38.3 mm	10.0 µl	1	Querctin (STD)	Yes
3	61.6 mm	5.0 µl	2	Ethanol Crude Extract (ECE)	Yes
4	84.9 mm	10.0 µl	2	Ethanol Crude Extract (ECE)	Yes

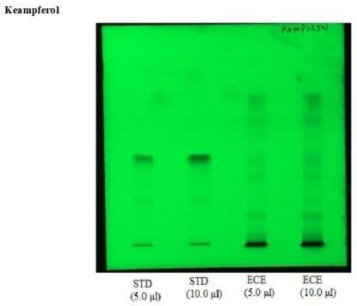

Figure 2: Chromatogram before derivatization and fractions (codes are explained in Table IV) Visualization was under UV light 254 nm

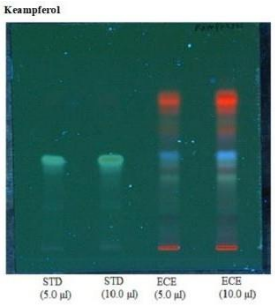

Figure 3 :Chromatogram after derivatization and fractions (codes are explained in Table VI) Visualization was under UV light 254 nm

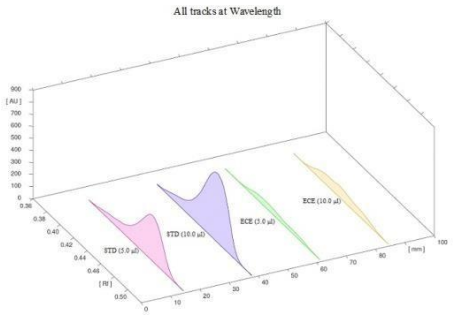

Figure 4: 3D diagram (Integration) of HPTLC densitogram for ethanol crude extract (ECE) of fresh leaves and stems of *Leea asiatica* (L.) Ridsdale with respective standard kaempferol (STD).

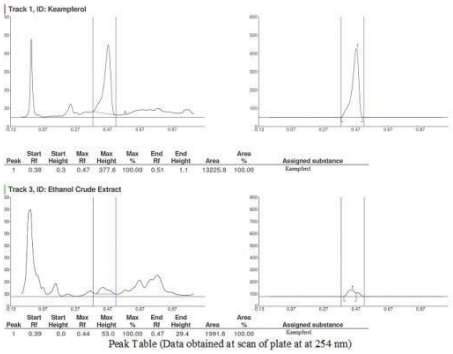

Figure 5: Chromatogram and R_f values of peaks in HPTLC fingerprint of kaempferol (STD) and ethanolic ethanol crude extract (ECE) of fresh leaves and stems of *Leea asiatica* (L.) Ridsdale

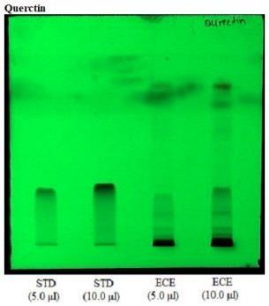

Figure 6: Chromatogram before derivatization and fractions (codes are explained in Table VII) Visualization was under UV light 254 nm

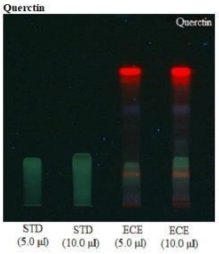

Figure 7: Chromatogram after derivatization and and fractions (codes are explained in Table V) Visualization was under UV light 254 nm

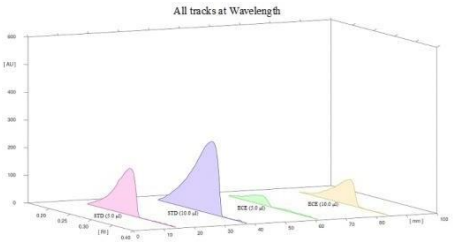

Figure 8: 3D diagram (Integration) of HPTLC densitogram for ethanol crude extract (ECE) of fresh leaves and stems of *Leea asiatica* (L.) Ridsdale with respective standard querctin (STD).

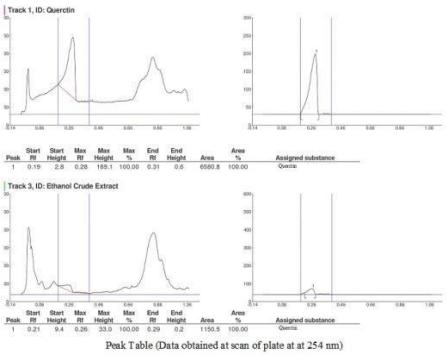

Figure 9: Chromatogram and R_f values of peaks in HPTLC fingerprint of querctin (STD) and ethanolic ethanol crude extract (ECE) of fresh leaves and stems of *Leea asiatica* (L.) Ridsdale

4. CONCLUSION

Studies of qualitative phytochemical screening is a crucial step in determining the class of phytochemical presents in the extracts which may lead to their quantitative determination, identifying the source of therapeutically active phytochemicals and are usually applied in judging the quality of the drug, to make the best and judicious use of available natural wealth. The fresh leaves and stems of *Leea asiatica* contain three identified compound as rutin, quercetin, kaempferol. The results reveal the presence of high quercetin and kaempferol content was extracted successfully from the fresh leaves and stems of *Leea asiatica*. Moreover, we can conclude that the content of quercetin and kaempferol may vary among the different cultivars and different plant parts and is also influenced by the geographical locations. Ethanol is a more polar solvent , it reveal the presence of more flavonoid content when compared to chloroform and ethyl acetate. Also additional work should be embarked upon with a view to isolate, characterize and elucidate the chemical structure of the bioactive compounds which were responsible for potent pharmacological activity.

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Conflict of Interest

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

Ethics approval and consent to participate

Not applicable

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