Research Article

Qualitative and quantitative phytochemical analysis of *Heterophragma quadriloculare* (Roxb.) K. Schum. leaves

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ABSTRACT

Objectives: *Heterophragma quadriloculare* (Roxb.) K. Schum. (HQ) is belonging to Bignoneace family. This plant has been traditionally utilized as anti-diabetic, antifungal, antiseptic and in skin disease like toe sores and chilblain. Since, scientific documentation is not well available it was thought of interest to develop scientific database for HQ leaves. With this respect we have attempted qualitative and quantitative evaluation of various phytochemicals from HQ leaves.

Methods: Successive extracts of HQ leaves have been analyzed by qualitative chemical tests and TLC. HPTLC fingerprints have also been developed for all extracts. Lupeol and ursolic acid have been identified and simultaneously estimated in petroleum ether extract of HQ leaves. Amount of secondary metabolites i.e. alkaloids, tannins, phenolics and flavonoids, have been estimated using reported methods.

Results: Carbohydrates, proteins, aminoacids, fats, alkaloids, steroidal compounds, flavonoids, terpenoids, tannins and phenolics were found in HQ leaves. By HPTLC method, 0.50 % and 0.93 % w/w of lupeol and ursolic acid have been observed in HQ leaves respectively. Percentage yield of alkaloid rich fraction was found to be in the range of 0.03-0.78 % w/w at different pH. Alkaloid rich fraction prepared at pH 10 was found to be best as per % yield and TLC profile. HQ leaves have been found to contain 3.14 %, 4.03 % and 4.09 % w/w of tannins, phenolics and flavonoids respectively.

Conclusions: This report can create the way for further identification and isolation of phytoconstituents especially alkaloids and terpenoids. Chromatographic fingerprint can also serve the purpose of drug standardization. The work can be further extended for drug discovery and drug development by isolation and characterization of phytoconstituents.

Keywords: *Heterophragma quadriloculare* (Roxb.) K. Schum., Phytochemical profile, Quantitative phytochemical analysis, HPTLC fingerprinting, Alkaloid, Secondary metabolite

1. INTRODUCTION

Heterophragma is a genus of family Bignoneace. *Heterophragma quadriloculare* (Roxb.) K. Schum. (HQ) is representative of genus and known as Warras [1]. In India it is found in different regions of Madhya Pradesh, Gujarat, Maharashtra, Andhra Pradesh, Karnataka and Tamil Nadu [2-4]. This plant is utilised as anti-diabetic, antifungal, antiseptic and in skin disease like toe

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sores and in chilblain [5, 6]. Utility of HQ plant material has been claimed traditionally by many authors but scientific documentation is not yet available. So it was thought of interest to develop scientific database for HQ leaves. Data on pharmacognostic and phytochemical characterization provide means to authenticate raw material as well as their usage. Pharmacognostic work has already been published [5] but detailed phytochemical profiling is yet to be carried out. Hence in present work we have attempted qualitative and quantitative evaluation of phytochemicals to step ahead in the direction of drug development and drug discovery. In present work alkaloids have been focused which were remained unexplored till now.

2. MATERIALS AND METHODS

2.1. Chromatographic condition for HPTLC

Whenever required separation was performed on TLC plate precoated with silica gel 60 F_{254} (E. Merk, Darmstsdt, Germany). Before sample application TLC plates were per-washed with methanol and dried in oven at 50 ˚C for 10 min. Samples were spotted on TLC plate 15 mm from the bottom edge using CAMAG Linomat V semi-automatic spotter. The TLC plate was developed in twin trough chamber. The TLC plate was scanned and analysed by CAMAG TLC Scanner IV and WinCATS software respectively.

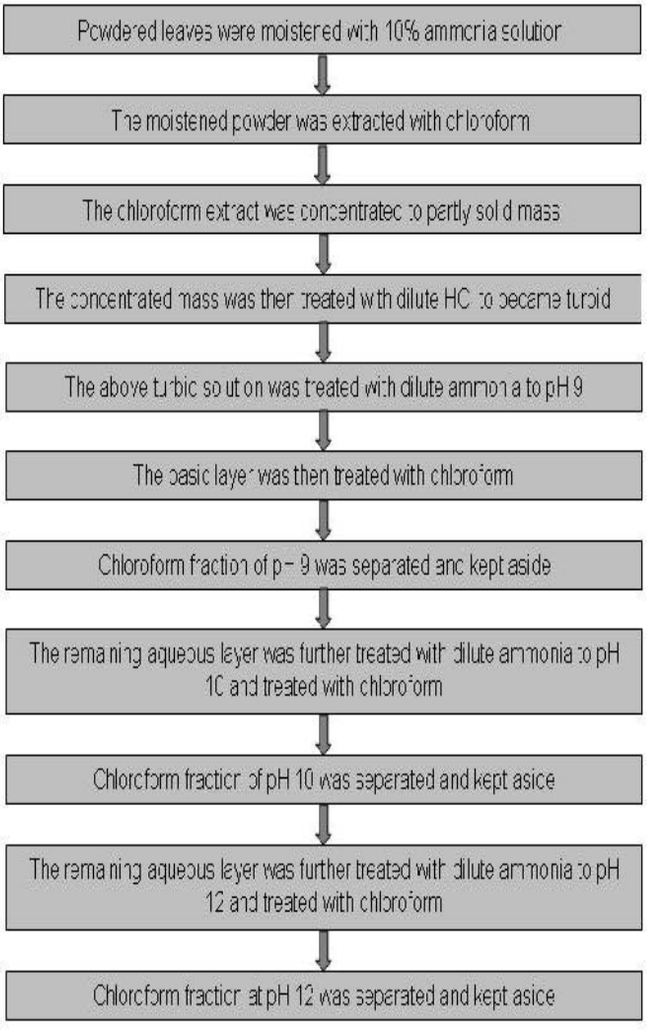

Figure 1: Schematic presentation of preparation of alkaloid fractions by basification method (Method-1)

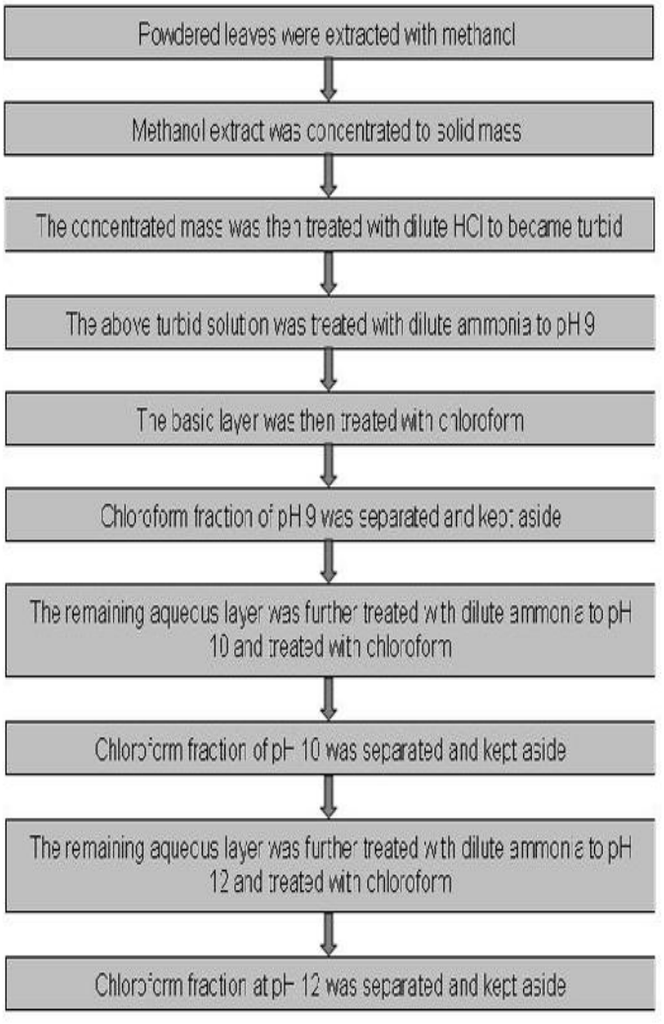

Figure 2: Schematic presentation of preparation of alkaloid fractions by acidification method (Method-2)

2.2. Preliminary phytochemical screening of HQ leaves

2.2.1. Preparation of extract from leaves by successive solvent extraction

Dry powdered leaves of HQ (Voucher number: Pharmacy/HDT/HQ/09-10/01/BS) weighing 40 gms was extracted using soxhlet apparatus with solvents of increasing polarity starting from petroleum ether followed by toluene, chloroform, ethyl acetate, acetone, methanol and water. Each time before extracting with new solvent, the powdered leaf material was air dried. All extracts were concentrated by distilling the solvent followed by drying on water bath. Consistency, colour, appearance of the extracts and their percentage yield were recorded [7, 8].

2.2.2. Preliminary phytochemical evaluation of extracts by chemical test

All successive extracts of leaves were subjected to various qualitative chemical tests using reported methods to determine the presence and/or absence of metabolites viz., alkaloids, glycosides, carbohydrates, proteins and amino acids, phytosterols, tannins and phenolics, terpenoids etc [7, 8].

2.2.3. Preliminary phytochemical evaluation of extracts by TLC

All successive extracts were subjected to thin layer chromatographic studies using reported methods to verify presence of various phytoconstituents like alkaloids, glycosides, carbohydrates, proteins and amino acids, phytosterols, phenolics and terpenoids [9, 10].

2.2.4. HPTLC fingerprinting of successive extracts of HQ

All successive extracts and methanol extract were dissolved in appropriate solvents and filtered. Clear solutions having concentration of 2 mg/ml were used for HPTLC fingerprinting. Precoated HPTLC plate (10 x 10 cm) of silica gel 60 F_{254} was used. The plate was developed with toluene: ethyl acetate (9:1, v/v) as mobile system. Air dried developed plate was scanned at 254 nm and 365 nm. For visualization of spots, plate was treated with anisaldehyde sulphuric acid reagent (AS), heated at 110 °C for 5 min and scanned in visible mode at 540 nm [9, 11].

2.3. Qualitative and quantitative evaluation of secondary metabolites

2.3.1. Identification and simultaneous estimation of lupeol and ursolic acid

Lupeol and ursolic acid were simultaneously identified in petroleum ether extract by co-TLC using aluminium backed precoated TLC plate of silica gel 60 F_{254}. A mixture of petroleum ether: ethyl acetate: toluene (7:2:1, v/v/v) was used as mobile phase and plate was treated with anisaldehyde sulphuric acid reagent for visualisation of band of phytoconstituents.

Lupeol and ursolic acid have also been simultaneously estimated in petroleum ether extract by HPTLC method. Separation was performed on aluminium backed plates precoated silica gel 60 F_{254}. The TLC plate was developed in twin trough chamber saturated with petroleum ether: ethyl acetate: toluene (7:2:1, v/v/v). The TLC plate was dried, sprayed with anisaldehyde sulphuric acid reagent and analyzed at 580 nm. Amount of lupeol and ursolic acid was calculated from peak area in terms of % w/w of dry leaves [12].

2.3.2. Qualitative and quantitative evaluation of alkaloids

Alkaloid fractions were prepared using pH dependant method. Procedures adopted are given in Figure 1 and Figure 2 [13].

Alkaloids are compound having nitrogen atom in heterocyclic ring and/or out of heterocyclic ring. Hence it was our first step to confirm the presence of nitrogen in separated fraction. Lassaigne test was performed for detection of nitrogen in each fraction [14]. Further, each fraction was tested with dragendorff's reagent, mayer's reagent, hagger's reagent and wagner's reagent for presence of alkaloids [7, 8].

TLC profile of each alkaloid fraction was developed on precoated silica gel 60 F_{254} TLC plates using chloroform: methanol (8.5:0.5, v/v) as mobile phase and dragendorff's reagent as a visualizing agent [9].

Table 1: Preliminary phytochemical profile for leaves of HQ

Successive extract	Colour	Consistency	% Yield (w/w)
Petroleum Ether	Greenish black	Sticky mass	1.76
Toluene	Green	Sticky mass	0.38
Chloroform	Green	Sticky mass	0.2
Ethyl Acetate	Blackish green	Sticky mass	0.48
Acetone	Green	Sticky mass	0.47
Methanol	Brownish black	Sticky mass	6.94
Water	Brownish black	Dry powder	11.88

2.3.3. Estimation of total tannins

Total tannins were estimated in defatted powder of dried leaves by titrimetry method using indigo carmine as an indicator and $KMnO_4$ as titrant. Amount of total tannins in powdered leaves was calculated using formula given below [15].

% Total Tannins = [(Sample reading – Blank Reading) x Normality of $KMnO_4$ solution x 0.004157 x Dilution factor]/ [Weight of drug sample taken x 0.1]

2.3.4. Estimation of total phenolics

The phenolic content in methanolic extract of HQ leaves was determined using Folin Ciocalteu reagent as per the method reported by A. Blainski *et. al.* [16]. Percentage of total phenolics was calculated from calibration curve of gallic acid.

2.3.5. Estimation of total flavonoid

Total flavonoid content was determined by aluminium chloride colorimetric method and 2, 4- dinitrophenyl hydrazine colorimetric method as described by Chami Arumughan [17]. In both methods, flavonoid content was measured in HQ leaves using methanolic extracts of leaves.

3. RESULT AND DISCUSSION

3.1. Preliminary phytochemical screening of HQ leaves

3.1.1. Preparation of extract from leaves by successive solvent extraction

Powdered leaves of HQ were subjected to successive solvent extraction (except water extract, which was prepared by decoction). The different extracts obtained are recorded in Table 1 with their % yield, colour, and consistency.

3.1.2. Preliminary phytochemical evaluation of extracts by chemical test

Results of qualitative chemical test for identification of various phytoconstituents like alkaloids, glycosides, carbohydrates, proteins and amino acids, phytosterols, tannins and phenolics, terpenoids etc., in extracts obtained from successive solvent extraction process are shown in Table 2.

3.1.3. Preliminary phytochemical evaluation of extracts by TLC

TLC profile for various phytochemicals as per reported text is explained in Table 3. Colour of respective spot is also mentioned with their Rf value. Qualitative test of all extract shows presence of carbohydrate, protein and amino acid. These results strengthen the writings showing that some part of the plant *Heterophragma quadriloculare* beeing used as food in Maharashtra region [18]. Qualitative test were negative for alkaloid, anthracene glycoside, steroidal glycoside and saponin but TLC showed that alkaloid, anthracene glycoside, steroidal glycoside and saponin are present in some extract. This would be due to low amount of such phytoconstituents. Further petroleum ether extract of HQ leaves was studied for identification and estimation of phytochemicals.

3.1.4. HPTLC fingerprinting of successive extracts

HPTLC fingerprinting of successive extract and methanol extracts showed many peak at Rf ranging from 0.03 to 0.96.

Table 2: Qualitative chemical test of different extracts of HQ leaves

Chemical constituent	P.E. ext	Toluene ext	$CHCl_3$ ext	E.A. ext	Acetone ext	MeOH ext	Water ext
Carbohydrate	-	-	-	-	-	+	+
Protein	-	-	-	-	+	+	+
Amino Acid	-	-	-	-	+	+	+
Fat & Oil	+	+	-	-	-	-	-
Steroids	+	+	-	-	-	-	-
Alkaloids	-	-	-	-	-	-	-
Steroidal Gly.	-	-	-	-	+	+	-
Anthracene Gly.	-	-	-	-	-	-	-
Saponins	-	-	-	-	-	-	-
Flavonoids	-	-	-	-	-	+	-
Terpenes	+	+	-	-	-	-	-
Tannins & Phenolics	-	-	-	-	-	+	+

P.E. = Petroleum ether, E.A. = Ethyl acetate, MeOH = Methanol, ext = extract, Gly = glycoside, + = Present, - = Absent.

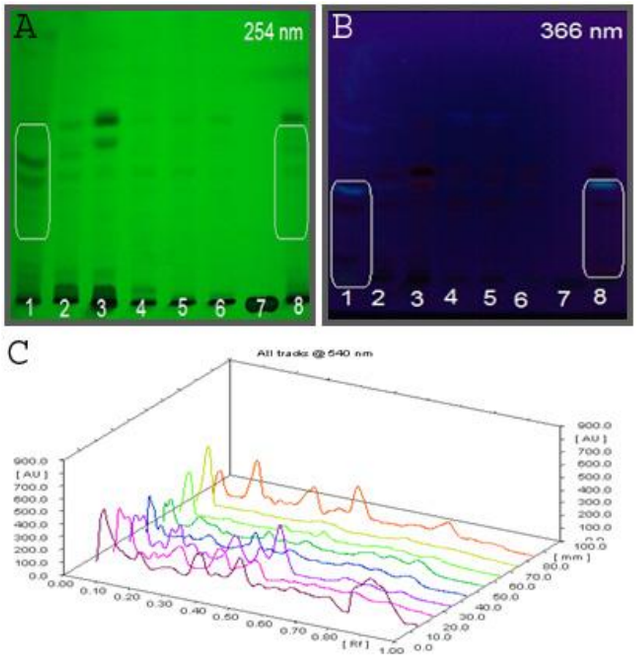

Figure 3: HPTLC fingerprinting of all successive extracts and methanol extract, A: Plate before derivatization at 254 nm; **B:** Plate before derivatization at 366 nm; **C:** 3D Chromatogram after derivatization, Track: 1= Petroleum ether extract, 2= Toluene extract, 3= Chloroform extract, 4= Ethyl acetate extract, 5= Acetone extract, 6= Methanol extract, 7= Aqueous extract, 8= Methanol extract.

Results of fingerprinting shows almost same range of compounds present in petroleum ether extract and methanol extract. But quantity of non-polar compounds present in methanol extracts were found to be very less as compare to petroleum ether extract so petroleum ether extract was selected for further investigation (Figure 3).

3.2. Qualitative and quantitative evaluation of secondary metabolites

3.2.1. Identification and simultaneous estimation of lupeol and ursolic acid

The presence of lupeol (Rf: 0.57) and ursolic acid (Rf: 0.32) has been confirmed in this work (Figure 4). Simultaneous estimation of both of them has also been carried out by HPTLC method. Dry powdered leaves of HQ was found to contain 0.50 % and 0.93 % of lupeol and ursolic acid respectively (Figure 4).

3.2.2. Qualitative and quantitative evaluation of alkaloids

Percentage yield of alkaloid fraction separated at different pH by method-1 and method-2 are summarized in Table 4.

Lassaigne test for element analysis showed green colour which confirms presence of nitrogen in each fraction. Presence of nitrogen in fraction confirms presence of alkaloids or amines. Further, each fraction gave positive result with dragendorff's reagent, mayer's reagent, hager's reagent and wagner's reagent for alkaloids. TLC profile of each alkaloid fraction with Rf value and colour of spot is shown in Figure 5.

Based on percentage yield and TLC profile alkaloid fraction derived by acidification method (method-2) and extracted at pH-10 was found to be best amongst prepared alkaloidal fractions. There

is a scope of further investigation for alkaloidal content.

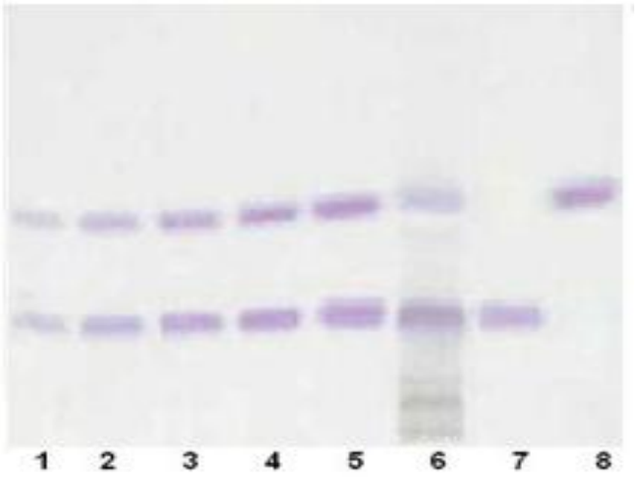

Figure 4: Photograph of HPTLC plate showing identification and estimation of lupeol and ursolic acid, Track 1-5: Calibration curve; 6: Petroleum ether extract of HQ leaves; 7: Ursolic acid; 8: Lupeol

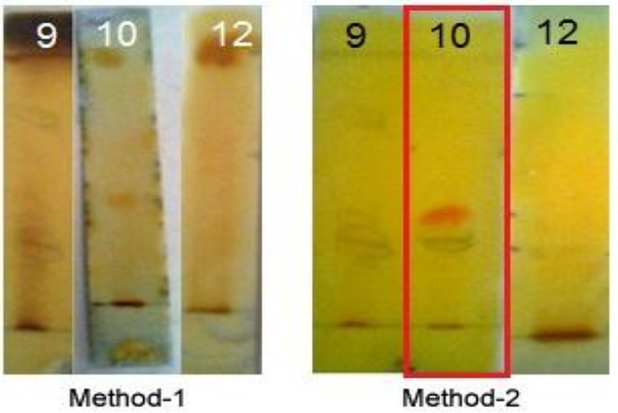

Figure 5: TLC profile of each alkaloid fraction prepared from HQ leaves

3.2.3. Estimation of total tannins

Leaves of HQ were found to contain 3.14 % w/w of total tannins. This result was obtained by average of triplicate estimation.

3.2.4. Estimation of total phenolics

Phenolic compound like gallic acid reacts with folin-ciocalteu reagent to form blue coloured complex. Estimation of phenolics involves the measurement of the absorbance of this coloured complex at 765 nm. The total phenolic content was expressed in terms of gallic acid equivalent. Phenolic content determined in the leaves by above method was found to be 4.03 % w/w of dry powder.

3.2.5. Estimation of total flavonoids

Flavonoid content estimated by aluminium chloride method and 2, 4 DNPH method was found to be 2.12 % w/w and 1.97 % w/w of dry powder respectively. Flavones, flavonols and isoflavones can form complex mainly with aluminium chloride while flavonones strongly reacts only with 2, 4 - dinitrophenyl hydrazine (2, 4 DNPH) hence results obtained by two methods were added up to evaluate the total flavonoid content. The flavonoid content determined in the sample by aluminium chloride method was found to be higher than the content determined by 2, 4 - dinitrophenyl hydrazine method.

4. CONCLUSION

With the help of detailed chromatographic study, large number of phytochemicals has been found in different extracts of HQ leaves. Amongst all phytoconstituents found in HPTLC profile, lupeol and ursolic acid have been identified and estimated in HQ leaves. Quantity of secondary metabolites like alkaloids, tannins, phenolic content, flavonoid content have also been measured in HQ leaves. This report can create the way for further identification and isolation of phytoconstituents. Chromatographic fingerprint can also serve the purpose of drug standardization hence chromatographic fingerprint has been developed for various extracts of HQ leaves.

The work can be further extended for drug discovery and drug development by isolation and characterization of phytoconstituents.

Table 3: TLC profile of successive solvent extracts of HQ leaves

Type of compounds	Detection reagent	Solvent system[a]	Extracts[b]						
			1	2	3	4	5	6	7
Carbohydrate	AS reagent	A	-	-	-	-	-	0.85, 0.75 (Brown)	0.74, 0.87, 0.35, 0.25, 0.19 (Brown)
Alkaloids	Dragendorff's reagent	B	-	-	0.32 (Brown) 0.48 (Orange)	-	-	-	-
Flavonoids	NP-PEG	C	-	-	-	-	-	0.11 (Orange) 0.37 (Orange) 0.46 (Yellow) 0.60 (Orange) 0.72 (Orange) 0.80 (Yellow) 0.90 (Green)	-
Steroidal glycosides	$SbCl_3$	A	-	-	-	0.37 (Brown) 0.47 (Blue) 0.56 (Blue)	0.56 (Blue)	0.27 (Brown) 0.37 (Blue) 0.47 (Blue) 0.56 (Brownish Blue)	-
Anthracene glycosides	Alcoholic KOH + UV-365	A	-	0.71 (Violet)	0.58 (Blue) 0.71 (Violet)	0.57 (Blue) 0.71 (Violet)	0.58 (Blue) 0.71 (Violet)	0.09 (Yellow) 0.34 (Yellow) 0.56 (Yellow)	0.12 (Yellow)
Saponins	Alcoholic KOH	D	-	0.65 (Green)	0.63 (Greenish Violet)	0.65 (Yellowish Green)	0.61 (Yellow)	0.76 (Yellow) 0.89 (Violet)	-
Terpenoids	VS reagent	E	0.05 (Violet) 0.07 (Green) 0.15 (Green) 0.23 (Brown) 0.33 (Blue) 0.38 (Blue)	0.05 (Violet) 0.07 (Green) 0.16 (Green) 0.23 (Brown)	0.05 (Violet) 0.07 (Green) 0.15 (Green) 0.23 (Brown)	0.05 (Violet) 0.07 (Green) 0.15 (Green)	0.05 (Violet) 0.07 (Green)	0.05 (Violet) 0.15 (Green)	
Phenolics	Ferric chloride	F	-	-	-	0.80 (Blue)	0.75 (Blue)	0.78 (Blue)	0.79 (Blue)

[a]Solvent systems: A = Ethyl Acetate: Methanol: Water (10:1.3:1,v/v/v), B = Toluene: Ethyl acetate: Diethylamine (6:2.5:1.5,v/v/v), C = Toluene: Ethyl Formate: Ethyl Acetate: Formic Acid (0.4:4:5:1,v/v/v/v), D = Chloroform: Glacial Acetic Acid: Methanol: Water (64:32:12:8,v/v/v/v), E = Hexane: Ethyl Acetate (9:1,v/v), F = Ethyl Acetate: Methanol: Glacial Acetic Acid (4:4:1,v/v/v); [b]Extracts: 1 = Petroleum ether, 2 = Toluene, 3 = Chloroform, 4 = Ethyle acetate, 5 = Acetone, 6 = Methanol, 7 = Water.

5. ACKNOWLEDGEMENT

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Table 4: Percentage yield of alkaloid fraction in terms of % w/w of dry powdered leaves

Method of preparation / pH at which extracted	By method-1 (% w/w)	By method-2 (% w/w)
pH-9	0.04	0.03
pH-10	0.12	0.78
pH-12	0.27	0.32

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