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SIMULTANEOUS DETERMINATION OF PHYTOCHEMICALS IN *RHYNCHOSIA CAPITATA* BY RP-HPLC AND GC/MS; ITS ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY

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ABSTRACT

The search for novel drugs from higher plants led to the analysis of a novel plant source *Rhynchosia capitata* for its phytoconstituents. GC/MS study confirm the presence of oleic acid, octadecanoic acid, phytol, hexadecanoic acid, E-11-hexadecenoic acid, Z,Z,Z-4,6,9-nonadecatriene, tetradecanoic acid, 5-azulenemethanol and1-butanol. A highly sensitive reverse phase high performance liquid chromatographic analysis is carried out to detect the presence of five plant phenolics namely gallic acid, caffeic acid, ferulic acid, rutin and quercetin. Antioxidant activity is tested via DPPH assay and antimicrobial activity for the human pathogenic strains *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* is also carried out. A comparison of the antioxidant and antimicrobial activity of the ethanol extract revealed antioxidant behavior to be dominant.

KEYWORDS: ***Rhynchosia capitata*, GC/MS, RP-HPLC, antioxidant, antimicrobial**

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INTRODUCTION

Secondary metabolites derived from plants are the richest bio resource of drugs used in traditional systems of medicine [1]. They usually exist as complex mixtures in plants and separation of individual compounds involves several multifarious steps[2]. Analytical methods like High-performance liquid chromatography (HPLC), Capillary electrophoresis (CE), Gas chromatography/Mass spectrometry (GC/MS), High pressure thin layer chromatography (HPTLC), UV detection, Liquid chromatography (LC) with diode array detector (DAD) etc., are applied for the identification of polyphenolics[3-5]. Use of standard screening method for polyphenolics identification from new source of plants is vital to assess its medicinal value. In the present study, an annual twinning prostrate plant *Rhynchosia capitata (R.capitata)* is analyzed for the first time by GC/MS for the presence of phytoconstituents followed by RP-HPLC analysis for the occurrence of five medicinally significant phenolics namely gallic acid, caffeic acid, rutin, ferulic acid and quercetin. The weed *R.capitata* is indigenous to Pakistan, India and Sri Lanka[6-7] and traditionally, the roots are known to be used as a cure to stomach ailments. Recently analgesic activity of the methanolic extract of the aerial parts has been reported[8]. A preliminary screening for phytochemicals revealed the presence of flavonoids, tannins, steroids, saponins, and terpenoids[9]. Flavonoid C-glycosides such as vitexin, isovitexin, orientin, isoorientin and vicenin 2 have been reported earlier in certain *Rhynchosia* species[10]. The limited knowledge on the chemical composition has increased the interest on the plant under study and the possibility of the weed to act as a potential drug has also been investigated. Antioxidants from plants play an important role in preventing oxidative damage related diseases such as cancer, diabetes, aging and other degenerative diseases in humans. Thus there is a need of antioxidants of natural origin that can protect damage of cells by eliminating radicals from it[11,12]. Similarly, with the increasing occurrence of bacterial resistance against available antibiotics, it has now become essential to look for newer antibiotic source. Most of the antibiotics available today come from natural origin, especially from various microbial or plant sources[13-15]. Based on the nature of chemical composition present in a plant, it may act as a potential antioxidant or an antimicrobial. In this study, comparison of these two activities of ethanolic extract of *R.capitata* has been carried out to assess its predominant behavior.

MATERIALS AND METHODS

Collection of Plant Material

Leaves of *R.capitata* were collected from Sathyamangalam, Erode district in Tamil Nadu, India during December 2012 and allowed to dry under the shade. The identification and voucher specimen number (no. 2 Gen Spec no. MH / 174354) of the plant was sorted out and deposited at the Botanical survey of India, Coimbatore. The plant was identified as *Rhynchosia capitata* (= *Glycine capitata*) Heyne ex roth.

Preparation of Extracts

Extract 1 : 20 g of the powdered leaves of *R.capitata* were soaked in 100ml of 95% ethanol for 12 h and filtered through Whatmann filter paper No. 41 along with 2 g sodium sulfate to remove the sediments and traces of water in the filtrate. The filtrate was then concentrated and the extract contained both polar and non-polar phytocomponents of the plant material used. 2 µl of this solution was used for GC/ MS analysis [16].

Extract 2: Leaves of *R.capitata* (2 g) suspended in 50 ml of 95% ethanol was extracted at 80 KHz using an ultrasonic device for 30 min (twice) at 45 °C. The resulting extract was collected, filtered and dried at 50 °C under reduced pressure. The dried crude extract was dissolved in the 100 ml mobile phase, filtered through 0.45 mm membrane filter

(Millipore) and the extract was injected into HPLC.

Preparation of Standards for HPLC

Standard stock solutions of gallic acid, ferulic acid, caffeic acid, rutin and quercetin flavonoids were prepared in methanol at concentrations of 2, 4, 6, 8 and 10 µg/ml and filtered through HPLC filter 0.45 mm membrane filter (Millipore).

Extract 3**:** 25 g of leaves were extracted with 15 ml 95% ethanol for 6 h. The resulting extract was suspended over water and partitioned using petroleum ether to remove waxes and impurities. The resulting extract was subjected to screen antioxidant activity and antimicrobial activity.

GC/MS analysis

The extract 1 was analyzed on a GC/MS CLARUS 500 Perkin Elmer system comprising gas chromatograph interfaced to a mass spectrometer (GC/MS) instrument employing the following conditions: column -Elite-5MS fused silica capillary column (30 x 0.25 mm x 0.25 µm df, composed of 5% diphenyl / 100 % Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99. 999%) was used as carrier gas at a constant flow of 1 ml/min and an injection volume of 0.5 µl was employed (split ratio of 10:1); injector temperature 250 °C ; ion-source temperature 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C/min, to 200 °C, then 5 °C /min to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da and a total mass running time of 36 min [16]. The mass spectra of the separated components were compared with those stored in the NIST database (NIST 05).

RP-HPLC analysis of flavonoids

The extract 2 was analyzed for flavonoids using a RP-HPLC method [17], Shimadzu Corp., Kyoto, consisting of a LC-10ATVp pump, SCL 10A system controller and a variable Shimadzu SPD- 10ATVp UV VIS detector and a loop injector with a loop size of 20 µl was used. The peak area was calculated with CLASSVP software. Reverse phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (250×4.6 mm i.d., particle size 5 µm, Luna 5 µ C-18; phenomenex, Torrance, CA, USA) at 25 °C. The gradient elution of solvent A (water-acetic acid; 25:1 v/v) and solvent B (methanol) had a significant effect on the resolution of compounds. Detection wavelength was 280 nm. Gallic acid, caffeic acid, ferulic acid, rutin and quercetin were used as internal and external standards. Phenolic acids present in each sample were identified by comparing chromatographic peaks with the retention time (R_t) of individual standards. The amount of each phenolic acid is expressed as µg/g.

Screening for antioxidant activity using DPPH assay

Extract 3 was analyzed for radical scavenging activity by the method followed by Blois [18]. Different volumes of extract (40 µg, 80 µg, 120 µg, 160 µg and 200 µg) were adjusted to 100 µl methanol. DPPH$^{\cdot}$ was added to 5 ml of 0.1 mM methanolic solution and allowed to stand for 20 min at 27 °C in dark and the absorbance was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radicals by the samples

DPPH radical scavenging = (control OD - sample OD / control activity OD) x 100

All the values are expressed in terms of IC_{50}.

Screening for antimicrobial activity

Antimicrobial screening was carried out using well-diffusion method [19]. Four pathogenic strains *Staphylococcus aureus* (*S.aureus*; MTCC3381), *Bacillus cereus* (*B.cereus*; MTCC430), *Escherichia coli* (*E.coli*; MTCC739), *Pseudomonas aeruginosa* (*P.aeruginosa*; MTCC424) were used. The extract 3 was dissolved in ethanol to a final concentration of 100 mg/ml. Each bacterial strain was suspended in nutrient broth and incubated for 8 h at 37 °C. Nutrient Agar (NA) plates were seeded with 8 h broth culture of different bacteria. In each of these plates, wells were cut out using sterile cork borer. Using sterilized

dropping pipettes, different concentrations (50, 100, 150 and 200 μg/ml) of plant extract was carefully added into the wells and allowed to diffuse at room temperature for 2 h. The plates were then incubated at 37 °C for 18–24 h. Gentamycin (10 μg/disc) was used as positive control and the solvent as negative control. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone.

RESULTS AND DISCUSSION

GC/MS analysis

GC/MS chromatogram of extract 1 of *R.capitata* (Fig 1) exhibited nine peaks indicating the presence of nine compounds. The chemical compounds identified in extract 1 of the leaves of *R. capitata* are presented in Table 1 and revealed the presence of oleic acid, octadecanoic acid, phytol, hexadecanoic acid, C-11-hexadecenoic acid, Z,Z,Z-4,6,9-nonadecatriene, tetradecanoic acid, 5-azulenemethanol and1-butanol.

Figure 1
GC/MS chromatogram of Rhynchosia capitata leaves

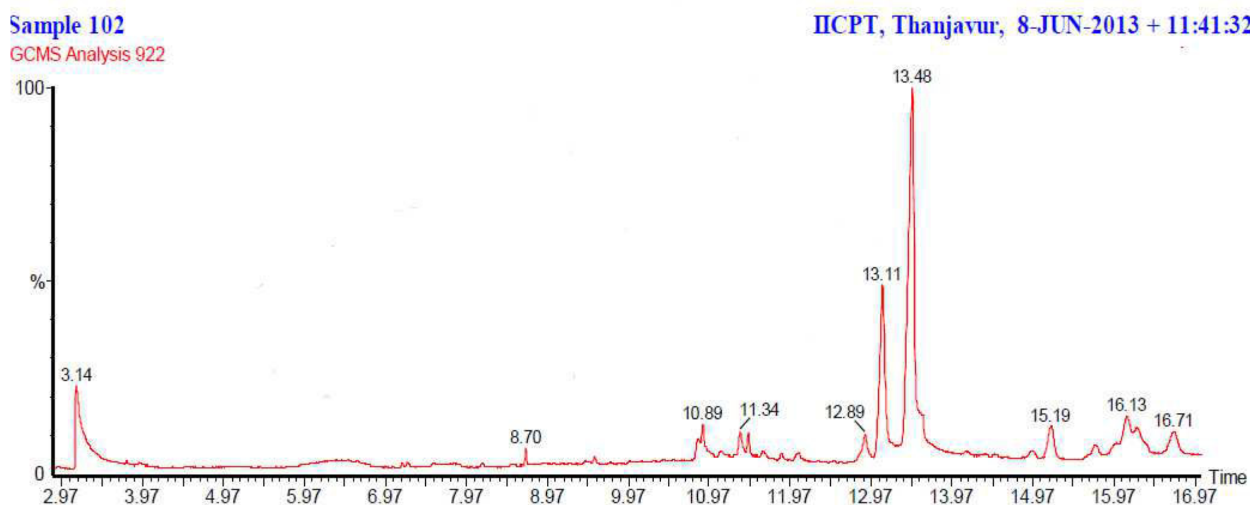


Table 1
Phytoconstituents analyzed by GC/MS for extract of Rhynchosia capitata

S.No	R_t	Name of the compound	Molecular formula	Molecular weight	Peak area(%)
1	3.14	1-Butanol, 3-methyl-, formate	$C_6H_{12}O_2$	116	15.33
2	8.70	5-Azulenemethanol	$C_{15}H_{26}O$	222	0.55
3	10.89	Tetradecanoic acid, ethyl ester	$C_{16}H_{32}O_2$	256	2.59
4	12.89	Z,Z,Z-4,6,9-Nonadecatriene	$C_{19}H_{34}$	262	2.41
5	13.11	E-11-Hexadecenoic acid, ethyl ester	$C_{18}H_{34}O_2$	282	16.12
6	13.48	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	41.89
7	15.19	Phytol	$C_{20}H_{40}O$	296	3.48
8	16.13	Oleic acid	$C_{18}H_{34}O_2$	282	8.89
9	16.71	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312	8.74

RP-HPLC analysis

RP-HPLC method is one of the most fast and reliable method for identification of plant phenolics. The leaves of *R.capitata* are found to possess C-glycosyl flavonoids [10]. Presence of phenolics such as gallic acid, ferulic acid, caffeic acid, rutin and quercetin has been investigated. The chromatographic separations at retention time (R_t) for gallic acid (R_t - 5.917), caffeic acid (R_t -9.217), rutin (R_t -10.258), quercetin (R_t -12.342) and ferulic acid (R_t - 24.108) of extract 2 is shown in Fig 2. Individual

flavonoid content for all the flavonoids were calculated from the calibration curve plotted and given interms of its R_t along with the standards as indicated in Table 2.

Figure 2
HPLC Chromatogram of Rhynchosia capitata leaves

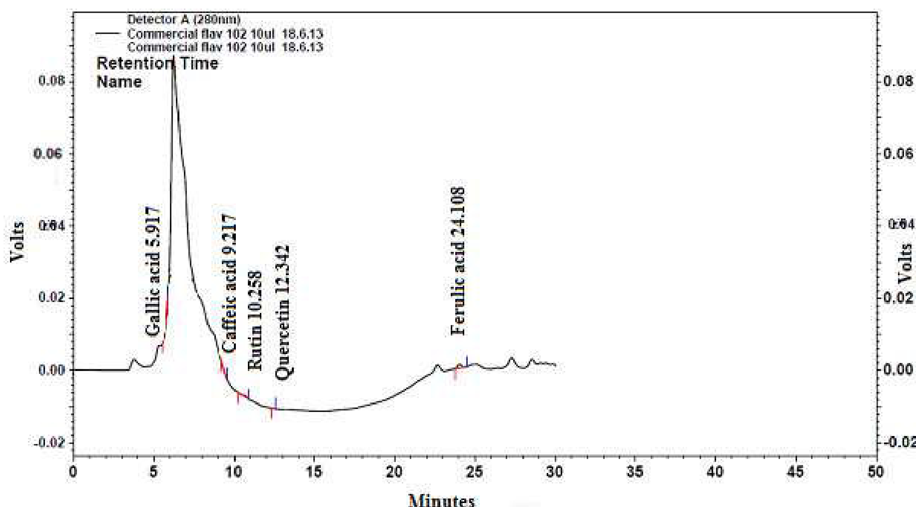


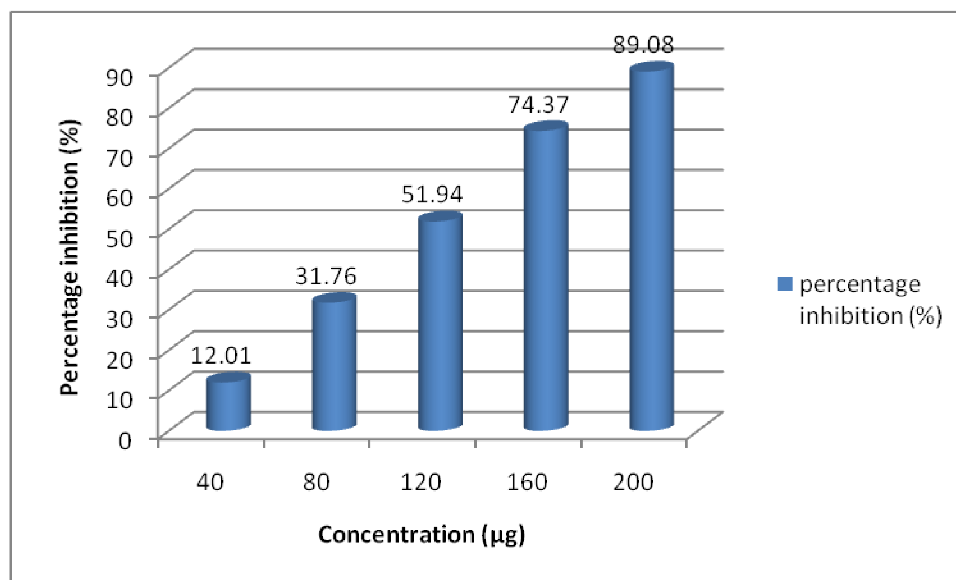
Table 2
HPLC validation data of Rhynchosia capitata leaves

Detector A (280 nm)					
Retention time(R_t) standard	**Retention time(R_t) sample**	**Area**	**Height**	**Concentration (mg/g)**	**Name of flavonoid**
5.750	5.917	29323	163	5.0	Gallic acid
9.450	9.217	3499	0	2.0	Caffeic acid
10.517	10.258	4222	0	1.0	Rutin
12.400	12.342	73	0	Below detection limit	Quercetin
24.175	24.108	16547	987	1.5	Ferulic acid

Antioxidant screening by DPPH assay

DPPH is a commercial oxidizing radical which can be reduced by antioxidants. In this assay, due to the abstraction of hydrogen atom from antioxidant compound the violet colour of DPPH was reduced to pale yellow color. Higher is the concentration of antioxidants in the extract; higher is the reduction of DPPH radical. The amount of antioxidant present in the sample necessary to decrease the initial DPPH concentration by 50% was expressed as IC_{50}. The lower the IC_{50} value, the higher is the antioxidant activity. Extract 3 obtained from *R.capitata* leaves exhibited a good radical scavenging property (44.91$\pm$0.21). The percentage inhibition of the extract 3 at different concentrations is indicated in Fig 3.

Figure 3
Percentage inhibition of extract of leaves of Rhynchosia capitata



Antimicrobial screening
The antimicrobial activity of the extract 3 of *R.capitata* leaves was analyzed *in vitro* by well diffusion method against four microorganisms and the results are tabulated (Table 3). The antimicrobial activity exhibited by the antibiotic drug gentamycin is taken as standard. In this study, extract 3 of *R.capitata* showed significant activity against *E.coli* and *S.aureus*. Among the pathogens screened, the plant extract 3 was most effective against *S.aureus* where the antimicrobial effect increased with increase in concentration of the extract. But for the pathogenic strain *E.coli* there was no significant change with increase in concentration of the plant extract of *R.capitata*. On the other hand, no zone of inhibition was observed on *B.cereus* and *P.aeruginosa* which lead to the conclusion that *R.capitata* leaves possess moderate antimicrobial behavior.

Table 3
Antimicrobial activity of extract of Rhynchosia capitata

Sample	Concentration (µg)	Zone of inhibition (mm)			
		S.aureus	*B.cereus*	*E.coli*	*P.aeruginosa*
Extract 3	50	10.5 ± 0.7	-	11.0 ± 0.0	-
	100	11.5 ± 0.7	-	11.0 ± 0.0	-
	150	15.5 ± 2.1	-	11.0 ± 0.0	-
	200	15.5 ± 2.1	-	11.0 ± 0.0	-
Gentamycin	10	23.9 ± 0.4	21.0 ± 0.5	24.3 ± 1.0	20.9 ± 0.4

CONCLUSION

GC/MS results signified the presence of nine phytochemical constituents such as oleic acid, octadecanoic acid, phytol, hexadecanoic acid, E-11-hexadecenoic acid, Z,Z,Z-4,6,9-nonadecatriene, tetradecanoic acid, 5-azulenemethanol and1-butanol. HPLC analysis provided a good platform for identification and quantification of five flavonoids compounds present in *R.capitata* leaves for the first time. From the results, it is evident that the leaf extract contained high concentration of gallic acid (5.0 mg/g of dry weight) followed by caffeic

acid, ferulic acid, rutin and quercetin. As these flavonoids have been of interest for health benefits, the present analytical study proves to be a potential application to identify and quantify the phenolic compounds in plant extracts. Phenolic compounds are known to exhibit radical scavenging and antimicrobial activity. Antioxidant behavior analysis by DPPH assay proved the leaves to be a good radical scavenger whereas the same extract was found to exhibit moderate antimicrobial activity against human pathogenic strains *E.coli* and *S.aureus*. Hence the extract of *R.capitata* leaves acts as a better antioxidant and a moderate antibiotic.

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