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Estimation of 2-hydroxy-4-methoxybenzaldehyde, lupeol and other unreported compounds in an elite ecotype of *Hemidesmus indicus* (L.) R. BR.

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Abstract

The root of *Hemidesmus indicus* (L) R. Br. is a very rich source of secondary metabolites which are used in the pharmaceutical industry. It is also used as flavoring agent in soft drinks. Qualitative and quantitative analysis of the roots of an elite ecotype were carried out for estimation of secondary metabolites and HPLC method was used for determination of important compounds like 2-hydroxy-4-methoxybenzaldehyde, lupeol and several other unreported important derivatives. The quantity of 2-hydroxy-4-methoxybenzaldehyde and lupeol in the plant material were detected as 0. 2638 mg/g, and 0.1994 mg/g, respectively. The related phenolic compounds of 2-hydroxy-4-methoxybenzaldehyde were gentisic acid and vanillic acid and those of lupeol (triterpenes) were α-amyrin and β-amyrin. The reported results would help the pharmaceutical industry and create awareness for identification and propagation of the elite ecotype of *H. indicus*, since the compounds can be widely used in traditional as well as modern medicinal systems.

Key words : *Hemidesmus indicus* (L) R. Br., secondary metabolites, 2-hydroxy-4-methoxyben zaldehyde, lupeol, related compounds, HPLC

1. Introduction

Hemidesmus indicus (L) R. Br. (Family : Asclepiadaceae), commonly known as Indian sarasaparilla, is a diffusely twining under shrub, having numerous slender wiry laticiferous branches with purplish brown bark. This plant is found throughout India, growing under mesophytic to semi dry conditions in the plains and up to an altitude of 600 m. It is quite common in open scrub jungles, hedges, uncultivated soil, *etc*. It is also found in Sri Lanka, Pakistan, Iran, Bangladesh and Moluccas (Anonymous, 2005; Nayar *et al*., 2006).

H. indicus is widely used in aromatic and medicinal properties. Due to its multiple uses, the plant has been indiscriminately collected from its natural habitat. The entire plant is uprooted for its single long tap root which has led to scarcity of raw materials for the herbal drug industry, especially in northern plains of India (Misra *et al*., 2003). *H. indicus* is commonly used in ayurvedic medicinal system (Alam *et al*., 1996; George *et al*., 2008). Apart from the roots, the aerial parts are also medicinally important. *H. indicus* has antimicrobial, anti-inflammatory, antioxidant, antidysenteric, antipyretic, hepatoprotective, antileprotic action (Sahoo, 1995). Rootstocks of this plant produce a strong fragrance, because of its typical aroma. These roots are being used as flavoring agent in sweet drinks (Sarasan *et al*., 1994; Sreekumar and Seeni, 1999). The phenolic compound 2-hydroxy-4-methoxybenzaldehyde is responsible for the fragrance of roots (Sreekumar *et al*., 1998). This aromatic aldehyde is structurally quite similar to that of vanillin. Apart from *H. indicus*, the occurrence of 2-hydroxy-4-methoxybenzaldehyde has also been detected in *Decalipus hamiltonii* (Nagarajan and Rao, 2003). Recently 2-hydroxy-4-methoxybenzaldehyde has been identified as a potent tyrosinase inhibitor from several African medicinal plants (Kubo and Ikuyo, 1999) and this is being used as an ingredient in cosmetics and other medicinal products, primarily in relation to hyper pigmentation. This compound and its derivatives are also known to possess antimicrobial (Phade *et al*., 1994) and insecticidal (George *et al*., 1999) properties. Methanolic extracts of *H. indicus* roots showed remarkable anticancer potential against MCF 7 breast cancer cell lines (Papiya *et al*., 2010).

The present study describes the analysis of roots of an elite ecotype of *H. indicus* (L.) R. Br. and separation of the bioactive secondary metabolites like phenols, *viz*., 2-hydroxy-4-methoxybenzaldehyde, its derivatives gentisic acid and vanillic acid and triterpenoids, *viz*., lupeol and its derivatives α-amyrin and β-amyrin, using RP-HPLC, and their identification, through their 1H and 13C nuclear magnetic resonance (NMR) spectra, as well as their chemical and physical properties.

2. Materials and Methods

The present study consists of qualitative and quantitative analysis of root extracts of twelve ecotypes of *H. indicus* (L) R. Br., identification of an elite ecotype with high contents of phenols and triterpenoids and estimation of the specific medicinal compounds

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by high performance liquid chromatography (HPLC). The solvents used for extraction were distilled before use. Silica gel (60-120 mesh size) was obtained from BDH (Mumbai, India). Silica Gel-G (particle size 10-40 µm) and HPLC solvents were procured from Merck (Mumbai, India).

2.1 Phytochemical analysis of root extract

Qualitative and quantitative analysis was carried out for determination of phenols and triterpenoids from roots of 12 ecotypes of *H. indicus*, collected from six ecological zones and named as OU1, OU2, HCU1, HCU2, WGL1 WGL2, MKR1, MKR2, PP1, PP2, and APMPB1, APMPB2. The *H. indicus* roots were cleaned, separated from the root and dried in a hot air oven for 24 h at 55°C, powdered and stored in air-tight containers at 10°C till further use. The root extract was prepared by grinding 0.5 gm of powdered roots in 10 ml of distilled water and made upto 100 ml.

2.2 Qualitative phytochemical analysis

The qualitative phytochemical analysis of the root extract of *H. indicus* was carried out by the following tests:

2.2.1 ***Test for identification of phenols***

Five ml of root extract (5 ml) was warmed slightly and 2 ml of ferric chloride was added for formation of green or blue color.

2.2.2 ***Test for identification of triterpenoids*** **(Salkowski test)**

Five ml of root extract (5 ml) was mixed with 2 ml of chloroform and concentrated sulphuric acid to separate a layer. A reddish brown color at the interface is the presence of triterpenoids.

2.3 Quantitative estimation of phenols and triterpenoids

Quantitative analysis of the root extract was carried out for total phenols and triterpenoids.

2.3.1 ***Determination of total phenols***

Total phenolic content of the extract was determined by Folin-Ciocalteau reagent method with modifications (Singleton *et al*., 1999). The root extract (1.0 ml) was mixed with Folin-Ciocalteau reagent and allowed to stand for 15 min and 5 ml of saturated Na_2CO_3 was then added. Absorbance was measured at A 765 nm on Shimadzu UV-visible spectrophotometer after incubating it for 30 min at room temperature. Gallic acid was used as reference standard and results expressed as gallic acid equivalents in milligrams per gram dry weight of sample (mg / g).

2.3.2 ***Determination of triterpenoids***

The root powder (50 gm) was soaked in alcohol for 24 h, filtered through muslin cloth and extracted with petroleum ether. This extract contained total triterpenoids which were expressed as mg/g of extract (Nagarajan and Rao, 2007).

2.4 Estimation of specific medicinal compounds in the root extract of *H. indicus* by high performance liquid chromatography (HPLC)

The elite ecotype of *H. indicus* was identified with the highest quantity of secondary metabolites and its root extract was analyzed by HPLC (Nagarajan and Rao, 2007) for 2-hydroxy-4-methoxybenzaldehyde, a phenolic compound, its derivatives and lupeol, a triterpene and its derivatives. The reference compounds were used as external standards to set up and calculate appropriate calibration curves. The experiments were performed using Shimadzu HPLC (LC-2010 model) (dual wave length) and LC solutions software.

2.4.1 ***Preparation of standards***

Standards of 2-hydroxy-4-methoxybenzaldehyde, gentisic acid, vanillic acid, lupeol, α-amyrin and β-amyrin were procured from Alta Vista Phytochemicals, Hyderabad, India and stock solutions prepared by dissolving 0.5 mg in methanol and made up to 50 ml.

2.4.2 ***Preparation of sample from root extract***

Fifty grams of root powder was dissolved in 200 ml of methanol (solvent) in 1:4 ratio, filtered through Whatman No.1 filter paper and incubated at 50-60ºC for 2 h. until dry to a powder. One mg of dried extract dissolved in methanol and made up to 250 ml.

2.4.3 HPLC ***operating conditions for 2-hydroxy-4-methoxy-benzaldehyde and its derivatives***

Column	: Symmetry shield RP-18 (250 × 4.6 mm) 5 µm
Eluent	: A (mobile phase A)-1 mM trifloro acetic acid in water
Eluent B (mobile phase B)	: Methanol
Flow rate	: 1 ml / minute
Diluent	: MeOH
λ max	: 280 nm
Amount of injection	: 20 µl
Weight of standard	: 0.5 mg / ml
Column temperature	: 25º C to 30ºC (Room temperature)

2.4.4 HPLC ***operating conditions for lupeol and its derivatives***

Column	: Intertsil C-8 (250×4.6 mm) 5 µm
Eluent (mobile phase)	: Acetonitrile×0.001% trifloro acetic acid in water
Flow rate	: 0.8 ml / minute
Diluent	: Methanol
λ max	: 210 nm
Amount of injection	: 20 µl
Weight of standard	: 0.5 mg /ml (5 mg/in 10 ml MeOH)
Weight of sample	: 1 mg /ml (10 mg /in 10 ml MeOH)
Column temperature	: 25ºC to 30ºC (Room temperature)

Identification of compounds was done by comparing the peak of the specific compound in the chromatogram with those of the reference standard peaks. The quantity of 2-hydroxy-4-methoxybenzaldehyde, lupeol and others was determined from the respective standard curves. For quantification of 2-hydroxyl-4-methoxybenzaldehyde, lupeol and others, the retention time and peak area were calculated from chromatogram. The percentage of compounds present in crude extract of *H. indicus* roots was calculated using the following formula and expressed as a percentage:

$$\frac{\text{Peak area of sample}}{\text{Peak area of standard}} \times \frac{\text{concentration of standard}}{\text{concentration of sample}} \times \text{Purity of standard}$$

1H and 13C NMR of the compounds were recorded at 400 MHz and 100 MHz, respectively, on a Bruker AMX 400 FT instrument (IICT, Hyderabad).

3. Results and Discussion

3.1 Phytochemical analysis of the root extracts

Qualitative analysis of the roots of twelve ecotypes of *H. indicus* revealed that all the samples contain phenols and triterpenoids (Table 1). These results were similar to Subramanian and Nair (1968) and Rekha and Parvathi (2012). Later, quantitative phytochemical analysis of roots was carried out in the twelve ecotypes of *H. indicus* for the estimation of phenols and triterpenoids. Among all the ecotypes tested, the ecotype OU-1 has been identified as an elite since it has shown the highest percentage of phenols (24.68 ± 0.2) and triterpenoids (18.34 ± 0.2) (Table 2). Hence, OU-1 was selected for the HPLC analysis for specific medicinal compounds, *viz*., 2-Hydroxyl-4-methoxybenzaldehyde, a phenolic compound, its derivatives and lupeol - a triterpene and its derivatives. Many reports suggested that plants with more phenolic content show good antioxidant activity i.e. there is a direct correlation between total phenolic content and antioxidant activity (Brighente *et al*., 2007; Subramanian *et al*., 2012). They also possess biological properties such as antiageing, anticarcinogenic, anti-inflammatory, cardiovascular protection and cell proliferation activity (Salazar *et al*., 2008; Han *et al*., 2007; Ravishankaran *et al*., 2002).

Table 1: Qualitative phytochemical analysis of root extracts of 12 ecotypes of *H. indicus*

Name of the ecotype	Collected place and District	Test for phenols in root extract	Test for triterpenoids in root extract
PP – 1	Parvathapuram, Ranga Reddy	Positive	Positive
PP – 2	Parvathapuram, Ranga Reddy	Positive	Positive
OU – 1	Osmania University Campus, Hyderabad	Positive	Positive
OU – 2	Osmania University Campus, Hyderabad	Positive	Positive
HCU – 1	University of Hyderabad Campus, Hyderabad	Positive	Positive
HCU – 2	University of Hyderabad Campus, Hyderabad	Positive	Positive
WGL – 1	Mahboobabad, Warangal	Positive	Positive
WGL – 2	Mahboobabad, Warangal	Positive	Positive
MK – 1	Mothkur, Nalgonda	Positive	Positive
MK – 2	Mothkur, Nalgonda	Positive	Positive
APMPB – 1	A.P. Medicinal Plants Board, Hyderabad	Positive	Positive
APMPB – 2	A.P. Medicinal plants Board, Hyderabad	Positive	Positive

Table 2: Quantitative phytochemical analysis of 12 ecotypes of *H. indicus*

Name of the ecotype	Phenols Mean ± S.E.	Triterpenoids Mean ± S.E.
PP – 1	19.73 ± 0.5	17.98 ± 0.2
PP – 2	20.09 ± 0.5	17.87 ± 0.3
OU – 1	**24.68 ± 0.2**	**18.34 ± 0.2**
OU – 2	24.31 ± 0.2	17.99 ± 0.2
HCU – 1	19.22 ± 0.3	18.01 ± 0.2
HCU – 2	20.12 ± 0.2	17.86 ± 0.3
WGL – 1	23.90 ± 0.4	15.46 ± 0.1
WGL – 2	22.78 ± 0.3	16.03 ± 0.2
MK – 1	22.82 ± 0.2	14.99 ± 0.3
MK – 2	23.02 ± 0.3	14.39 ± 0.3
APMPB – 1	24.09 ± 0.2	15.88 ± 0.1
APMPB – 2	23.94 ± 0.2	14.35 ± 0.2

* Phenols are expressed as gallic acid equivalent (GAE) and in mg/100 gm.

3.2 Method development (Separation and identification of 2-hydroxy-4-methoxybenzaldehyde, lupeol and their derivatives in the root extract by HPLC)

The roots of the elite ecotype OU-1 of *H. indicus* were analyzed for identification and separation of specific medicinal compounds, *viz*., 2-hydroxy-4-methoxybenzaldehyde (a phenolic compound) and its derivatives, lupeol (a triterpene) and its derivatives through HPLC. The peak of each of the compounds in chromatogram was detected with the help of its reference standard peak. For quantification of 2-hydroxy-4-methoxybenzaldehyde and its derivatives and also for lupeol and its derivatives, the retention time and peak area was calculated from the chromatogram.

3.2.1 *Quantification of 2-hydroxy-4-methoxybenzaldehyde and derivatives*

For quantification of 2-hydroxy-4-methoxybenzaldehyde and its derivatives from root organs of *H. indicus*, different stationary phases were tested. Among all the tested phases, symmetry shield RP C-18 (250×46 mm) was found to be most suitable for quantification of 2-hydroxy-4-methoxybenzaldehyde with two mobile phases (mobile phase A-1 mM trifloroacetic acid, B-methanol). Throughout the run, the flow rate was maintained at 1ml/1min. The column effluent was monitored at 280 nm with 25°C to 30°C temperature. The retention time for standard

2-hydroxy-4-methoxybenzaldehyde was recorded with three runs in HPLC and was observed as 10.231 min. at 280 nm (Figure 1). The HPLC results for quantification of 2-hydroxyl-4-methoxybenzaldehyde from root extract of *H. indicus* included the retention time of 10.627 min. at 280 nm (Figure 2, Table 3). To detect the compound exactly from chromatogram peak, both the samples (standard and root extract) (50% + 50%) were run twice in HPLC. Then, by spiking the highest peak in the chromatogram, the compound was identified as 2-hydroxy-4-methoxybenzaldehyde. The concentration of 2-Hydroxy-4-methoxybenzaldehyde in root extract of *H. indicus* was detected as 0. 2638 mg /g. Several other peaks were recorded from the roots sample of which 8.392 min. and 11.180 min. could be identified as those of gentisic acid and vanillic acid with the help of the reference standards and also quantified (1.65 mg/g and 0.164 mg/g, respectively). These results were similar to earlier reports in *Decalipes hamiltonii* and *Melissa officinalis* (Nagarajan *et al*., 2001; Karasova *et al*., 2005; Manickam and Periyasamy, 2014).

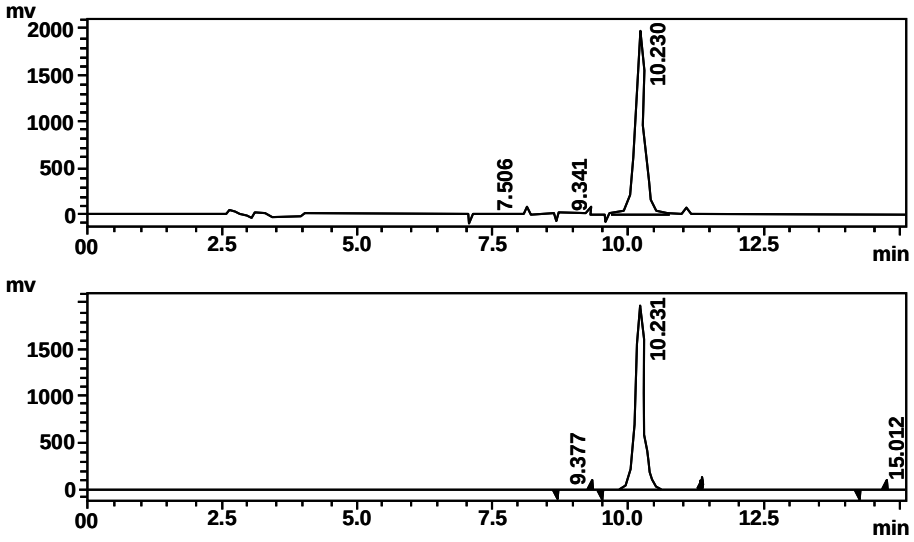


Figure 1: HPLC graph showing the standard 2-hydroxy-4-methoxybenzaldehyde

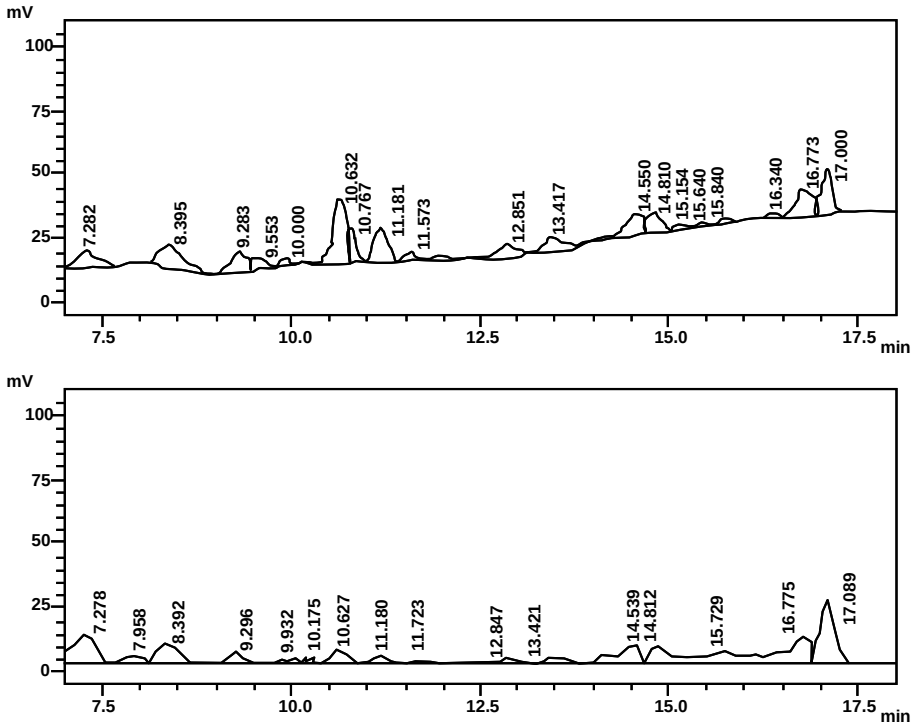


Figure 2: HPLC graph showing the analysis of root extract for 2-hydroxy-4-methoxybenzaldehyde

Table 3: The values of HPLC in relation to the peaks for 2-hydroxy-4-methoxybenzaldehyde and its derivatives

Peak #	Ret.Time	Area	Height	Height %	Area %
1	2.076	15472	1483	0.373	0.230
2	2.610	154609	33151	8.350	2.298
3	4.592	54637	5174	1.303	0.812
4	5.919	93752	4494	1.132	1.394
5	6.609	4451716	216063	54.421	66.170
6	7.278	236750	12140	3.058	3.519
7	7.958	55301	3288	0.828	0.922
8	8.392	186258	8951	2.254	2.769
9	9.296	79324	5876	1.480	1.179
10	9.932	20916	1533	0.386	0.311
11	10.175	1968	1471	0.371	0.293
12	10.627	12264	6802	1.713	1.824
13	11.180	44077	3601	0.907	0.655
14	11.723	27329	1266	0.319	0.406
15	12.847	21807	1362	0.343	0.324
16	13.421	21778	1641	0.413	0.324
17	14.539	74456	6105	1.538	1.107
18	14.812	52080	4827	1.216	0.774
19	15.729	14201	1443	0.364	0.211
20	16.775	117513	8205	2.067	1.720
21	17.089	261956	22309	5.619	3.894
22	19.393	10115	690	0.174	0.150
23	20.626	3016	357	0.090	0.045
24	22.767	22181	949	0.239	0.330
25	23.550	58591	1972	0.497	0.871
26	25.631	6283	195	0.049	0.093
27	27.136	205803	20428	5.145	3.059
28	27.759	15671	1411	0.355	0.233
29	28.164	8565	281	0.071	0.127
30	29.623	66903	4923	1.240	0.994
31	30.430	100169	7353	1.852	1.489
32	31.211	43323	2938	0.740	0.644
33	32.237	9746	918	0.231	0.145
34	34.633	12690	1347	0.339	0.189
35	36.046	687	678	0.171	0.102
36	39.193	7881	508	0.128	0.117
37	40.895	25380	887	0.223	0.377
Total		6727698		100.00	100.000

3.2.2 *Quantification of lupeol and its derivatives*

For quantification of lupeol from root organs of *H. indicus,* the inertsil C-8 (250×4.6 mm) column was found to be most suitable with single mobile phase (Acetonitrile + 0.01% trifloro acetic acid). Throughout the run, the flow rate was maintained at 0.8 ml/ min. The column effluent was maintained at 210 nm with 25ºC to 30ºC temperature. The retention time for standard lupeol was recorded with three runs in HPLC and was observed as 14.429 min. at 210 nm (Figure 3). The HPLC results for quantification of lupeol from root extract of *H. indicus* included the retention time at 14.574 min. at 210 nm (Figure 4, Table 4). To detect the compound exactly from chromatogram peak, both the samples (standard and root extract) (50% + 50%) were run twice in HPLC. Then by spiking the highest peak in the chromatogram, the compound was identified as lupeol. The concentration of lupeol in root extract of *H. indicus* was observed as 0.1994 mg /g. Several other peaks were recorded from the roots sample of which 19.318 min. and 18.240 min. could be identified as those of α-amyrin and β-amyrin, respectively with the help of the reference standards and also quantified (0.095 mg/g and 0.047 mg/g, respectively).

The derivatives of both 2-hydroxy-4-methoxybenzaldehyde and lupeol were further identified as phenols (gentisic acid and vanillic acid) and triterpenoids (α-amyrin and β-amyrin), respectively by 13C and proton NMR spectrum (Figure 5). The phenols (gentisic acid and vanillic acid) were also reported to be present in *Melissa officinalis* (Karasova *et al*., 2005). The triterpenoids (α-amyrin and β-amyrin) were reported to be present in *Decalipes hamiltonii* (Nagarajan and Rao, 2007) and *Cassia obtusifolia* and *Ficus cordata* (Vasquez *et al*., 2012). The triterpenoid compound, lupeol, reportedly has antioxidant activity, anti-inflammatory and anti-cancer activity (Rekha and Parvathi, 2012). However, in the report (Nagarajan and Rao, 2007), all the HPLC conditions for lupeol and its derivatives were different from the present conditions. These include the fact that both the columns were operated at 40°C. The detector wavelength was fixed at 205 nm for analysis, and sample peaks were simultaneously monitored by a PDA detector (200-400 nm), the mobile phase was methanol and water (94:6) and the flow rate was maintained at 1 ml/min at a pressure range of 50-350 bars. The chromatographic method described here is significant because the compounds isolated presently is a first report in *H. indicus*. These compounds were reported to possess biological activity and are potentially beneficial for respective applications, as indicated in the recent reports (Nagarajan and Rao, 2007). Further, the conditions employed in the present study for the HPLC separation of phenols and triterpenoids (as such or with minor modifications) may be useful in separating other phenols and triterpenoids from different sources. Because of the growing importance of the phenols and triterpenoids in view of their biological activities, the separation of individual compounds as described here is of the utmost importance to obtain pure components to carry out structure-activity relationship studies. Also, these bioactive compounds were isolated in reasonable quantities in their pure form from the plant, which is an edible material. The plant must, therefore, be taken up for intensive conservation and several bioassays be conducted on all the identified compounds in further studies and applications.

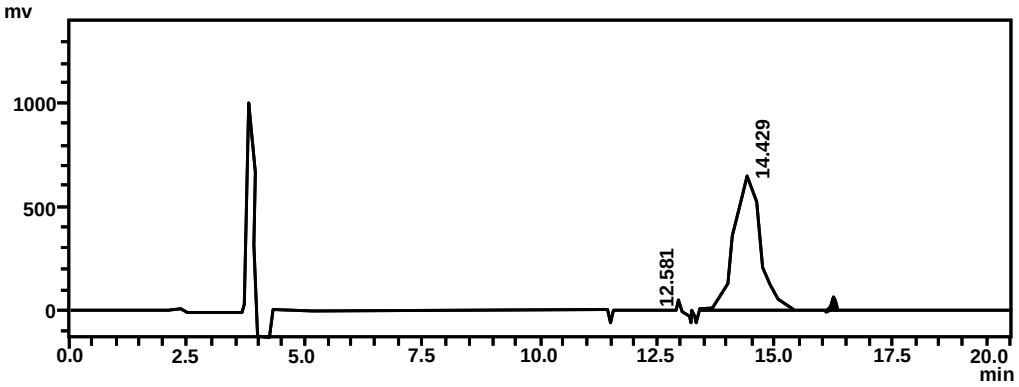


Figure 3: HPLC graph showing the standard lupeol

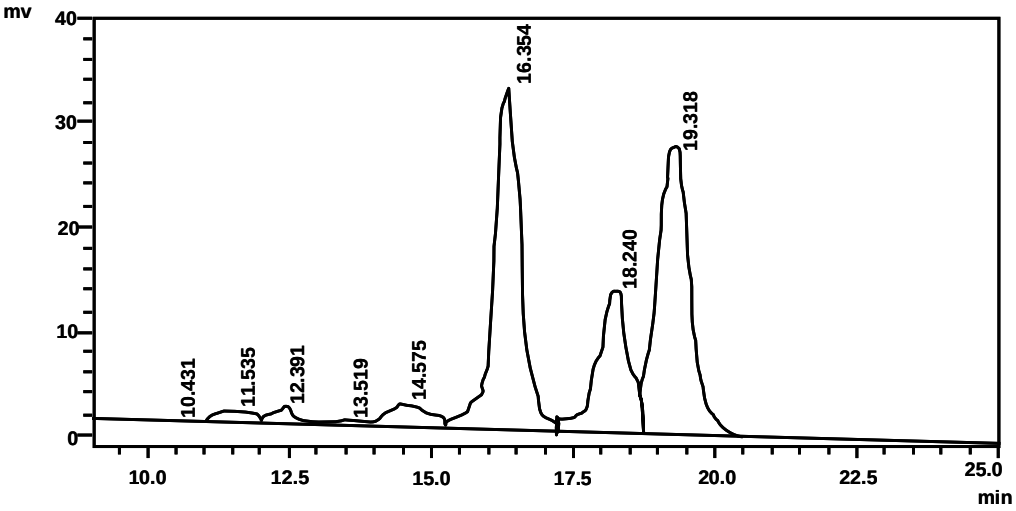
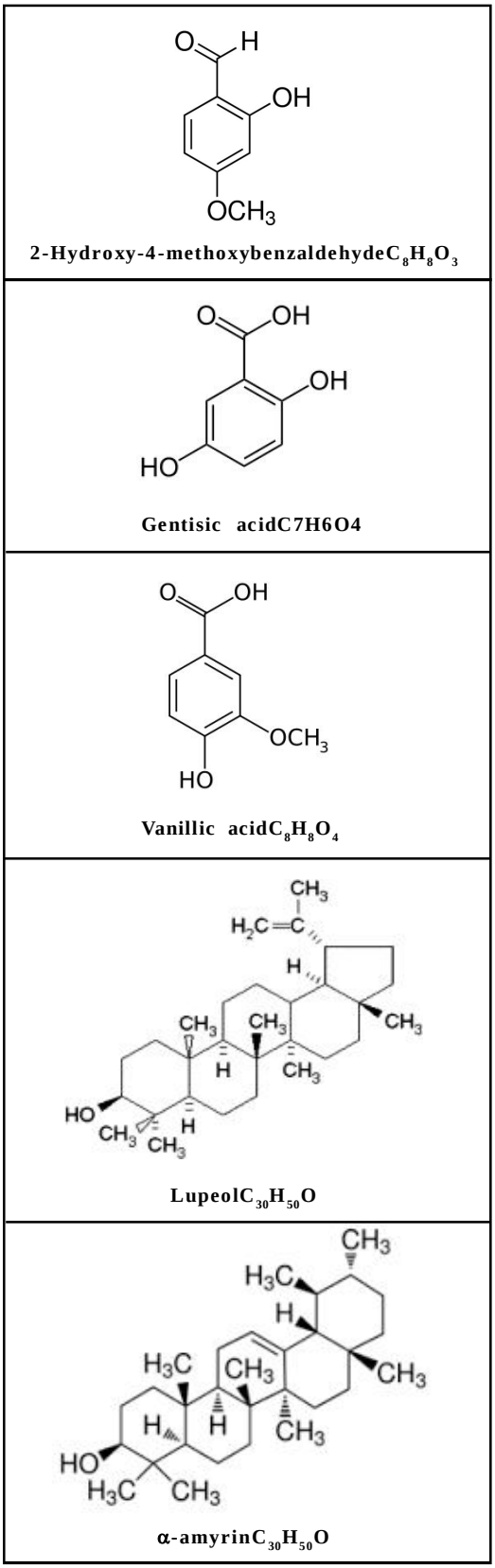


Figure 4: HPLC graph showing the analysis of root extract for lupeol and the values of HPLC in relation to the peaks for lupeol

Table 4: The values of HPLC in relation to the peaks for lupeol and its derivatives

Peak #	Ret. Time	Area	Height	Height %	Area %
1	2.405	906999	20886	0.500	2.035
2	3.187	39989501	4005105	95.954	89.732
3	4.323	318372	41865	1.003	0.714
4	4.542	213380	14531	0.348	0.479
5	5.318	34177	3474	0.083	0.077
6	5.845	58259	5807	0.139	0.131
7	7.087	38731	2421	0.058	0.087
8	10.431	3872	220	0.005	0.009
9	11.535	54074	1220	0.029	0.121
10	12.391	53057	1783	0.043	0.119
11	13.519	6590	325	0.008	0.015
12	14.575	99194	2143	0.051	0.223
13	16.354	1114414	32669	0.783	2.501
14	18.240	563675	13906	0.333	1.265
15	19.318	1111062	27619	0.662	2.493
Total		44565358		100.00	100.000

Figure 5 : Chemical structures of the compounds isolated from H. indicus



4. Conclusion

Roots of 12 ecotypes of *H. indicus* were quantitatively analyzed and an elite ecotype, OU-1 with highest contents of phenols and triterpenoids was identified, in which two specific medicinal compounds and their derivatives were identified and quantified by HPLC. The importance of the chromatographic method described is significant because the compounds isolated were reported to possess biological activity. This is a first report of the concentration of pharmaceutically important 2-hydroxy-4-mehtoxybenzaldehyde, lupeol and their derivatives from root extract of *H. indicus* and their potential applications for preparation of pharmaceuticals to treat various ailments has been highlighted.

Acknowledgement

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Conflict of interest

We declare that we have no conflict of interest.

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