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Identification of betulinic acid in ethanol extract of *Vitellaria paradoxa* leaves using spectroscopy and high-performance liquid chromatography

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Abstract
Vitellaria paradoxa tree contains antioxidant and anti-inflammatory agents. Betulinic acid (BA) is an important phytochemical. This study analysed the presence of BA in ethanol leaf extract of *V. paradoxa* by ultraviolet (UV) infrared (IR) and High-performance liquid chromatography (HPLC) techniques. The UV spectrum of the extract shows five prominent frequencies at 402.20nm, 274.00nm, 479.00nm, 345.00nm and 257.20nm, while BA standard shows two frequencies. The IR analysis of the extract showed six prominent values, 3322.9 cm^{-1}, 2929.7 cm^{-1}, 2838.4 cm^{-1}, 1654.9 cm^{-1}, 1449.9 cm^{-1} and 1015.7 cm^{-1}, comparable to the standard BA. The HPLC profile of the extract revealed seven peaks, whose retention times were 1.582 min, 2.046 min, 2.580 min, 3.124 min, 4.536 min, 5.085 min and 5.420 min, some of which are comparable to the standard BA. In conclusion, this study indicates the presence and possibility of extraction of BA from the leaves of *V. paradoxa*.

Keywords: *Vitellaria paradoxa*, betulinic acid, identification, spectroscopy, HPLC analysis

1. Introduction
Vitellaria paradoxa, commonly known as Shea butter tree, is a member of the sapotacae family of trees. It is a wild tree indigenous to the Savanna parkland of Africa, with numerous socio-economic values. [1, 3] The physicochemical profile of Shea butter has shown that it has low Saponification, Iodine, Peroxide and Acid values relative to most other vegetable oils. [4, 5] These relatively low values have been associated with the resistance of Shea butter to oxidative rancidity, hence its applications in making soaps and lubricating oils. [6] The kernel of Shea is highly rich in fat (butter), while the pulp is rich in vitamin C, making it important in the food, cosmetic and pharmaceutical industries. [7] The predominant fatty acids in Shea butter include oleic, stearic, palmitic, linoleic and arachidic acids. Oleic and linoleic acids are essential fatty acids that play important roles in reducing plasma level of low-density lipoprotein (LDL), thereby reducing the risk of coronary heart diseases (CHDs. [8] Furthermore, antioxidants, such as vitamin C, catechin and alpha-tochopherol, as well as anti-inflammatory substances have been reported to be present in Shea butter. [7, 9]
Betulinic acid is a naturally occurring triterpene reported to exhibit a variety of biological and medicinal properties such as antiretroviral,[10] anti-microbial, [11] anti-malarial, [12] anti-inflammatory, [13] antioxidant, anti-atherogenic and anti-proliferative properties. [14, 15] This triterpene and betulin, the alcohol form, are widely distributed throughout the plant kingdom, and the birch tree (*Betula* spp., Betulaceae) is an important source in which they are present in considerable amounts. [16] These two compounds have been isolated from the barks of *Doliocarpus* schottianus [17] and white birch (*Betula alba*) tree [18] using HPLC. The immense biological importance of betulinic acid has thus occasioned serious efforts towards its isolation from many botanical sources through several techniques, including Liquid chromatography-mass spectrometry (LC-MS), [19] a combination of Thin-layer chromatography (TLC) and HPLC [20] and High-performance thin-layer chromatography (HPTLC). [21, 22] In the course of literature search, we noticed a paucity of information on the presence of betulinic acid and other triterpenoids in *Vitellaria paradoxa*. This study was thus designed to isolate, identify and partially characterize betulinic acid in ethanol leaf extract of *Vitellaria paradoxa* using spectroscopic and chromatographic techniques.

2. Materials and Methods
2.1 Collection and identification of plant material
The leaves of *Vitellaria paradoxa* were collected from the Botanical garden of the College of Education (Technical) Lafiagi, Kwara state, Nigeria, on 15th of March, 2015. The plant was authenticated at the Department of Botany, Faculty of Science, University of Ilorin, Kwara

State, Nigeria, and deposited in the Herbarium Unit with a Voucher number UILH/001/952.

2.2 Drying and Extraction of plant material
The collected leaves were air-dried, and then pulverized using grinding machine and stored in a well-ventilated environment until required for use. The dry powder (500 g) was extracted with 95% ethanol (2.5 L ethanol) using soxhlet extraction method to give ethanol extract (31.21 g, 6.24%).

2.3 Chromatographic Materials and Reagents
Silica gel (50-200 mesh) for column chromatography and precoated Aluminum silica gel plate for Thin-layer chromatography (TLC) were purchased from Merck company (Germany), while phosphomolybdic acid (PMA) was purchased from Sigma Company (USA). All other chemicals and reagents used were of quality analytical grades.

2.4 Column and Thin-layer chromatography
The extract was then subjected to Column chromatography using silica gel (50-200 mesh) and elution was performed with 75% and 100% methanol sequentially. Twenty five fractions (10 ml each) were collected and monitored by Thin-layer chromatography (TLC) on a precoated Aluminum silica gel plate (Merck, Germany). The plate was developed with a mixture of ethyl acetate and n-Hexane (4:1) and stained with phosphomolybdic acid (PMA) solution (10 g PMA in 100 ml ethanol). Developed plate was subjected to heating in an oven at 105 ^{0}C for 5 minutes [23] and the samples were pooled based on comparable Rf values. The fractions were concentrated in an oven below 40 ^{0}C to give a brown semi-solid substance.

2.5 Ultraviolet (UV) and Infrared (IR) spectroscopic analyses
The UV -1800 series machine was used to analyse the brown semi-solid extract and Betulinic acid standard at a wavelength of 340nm to obtain visible spectra. The IR spectra were recorded using Agilent machine (USA), expressing the wavelength in reciprocal centimetre (cm^{-1}).

2.6 High-Performance Liquid Chromatography (HPLC) analysis
An isocratic HPLC (Mumbai machine) profiling was carried out for both the ethanol leaf extract of *V. paradoxa* and BA standard at a flow rate of 0.5 mL/min. Exactly 25 mg each of the two substances was dissolved in the mobile phase containing a mixture of acetonitrile and methanol (80:20, v/v), and the injection volume was 20µL. The C_{18} (4.5 x 250 mm, 5µm) column was maintained at the room temperature and the eluent was detected at 210nm with a run time of 30 minutes. The peaks (UV spectra) of the *V. paradoxa* leaf extract were compared with those of the BA standard.

3. Results
3.1 Ultraviolet (UV) and infrared (IR) spectroscopic analyses
The UV spectrum of *V. paradoxa* ethanol leaf extract shows five prominent frequencies at 402.20nm, 274.00nm, 4.79.00nm, 345.00nm and 257.20nm (Figure 1), while the standard BA shows two prominent peaks at 278.00nm and 260.00nm wavelengths (Figure 2). The IR profile of *V. paradoxa* depicted six prominent wavenumbers as 3322.9 cm^{-1}, 2929.7 cm^{-1}, 2838.4 cm^{-1}, 1654.9 cm^{-1}, 1449.9 cm^{-1} and 1407.1 cm^{-1}, as shown in figure 3. The result in figure 4 reveals the IR spectrum of BA standard with six prominent wavenumbers as 3322.9 cm^{-1}, 2976.3 cm^{-1}, 2946.5 cm^{-1}, 2836.5 cm^{-1}, 1654.9 cm^{-1} and 1412.7 cm^{-1}. Both the UV and IR spectral data of the ethanol leaf extract of *V. paradoxa* were observed to be comparable to the corresponding spectra of the Betulinic acid standard used in the present study.

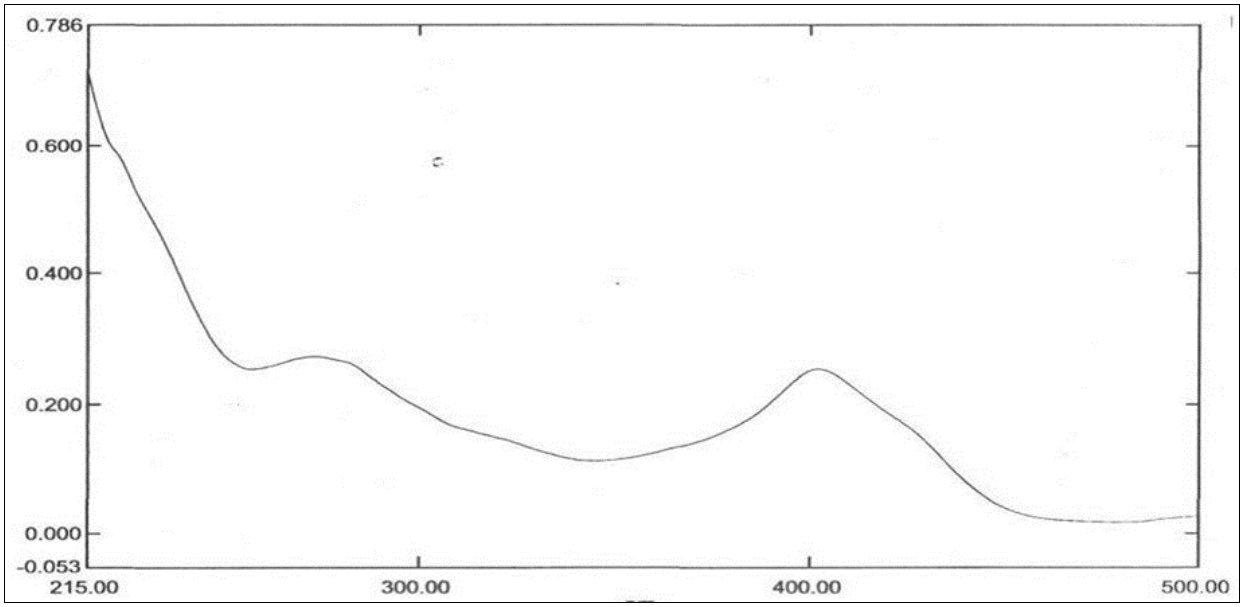

Fig 1: Ultraviolet (UV) spectrum of ethanol leaf extract of *Vitellaria paradoxa* (Shea butter)

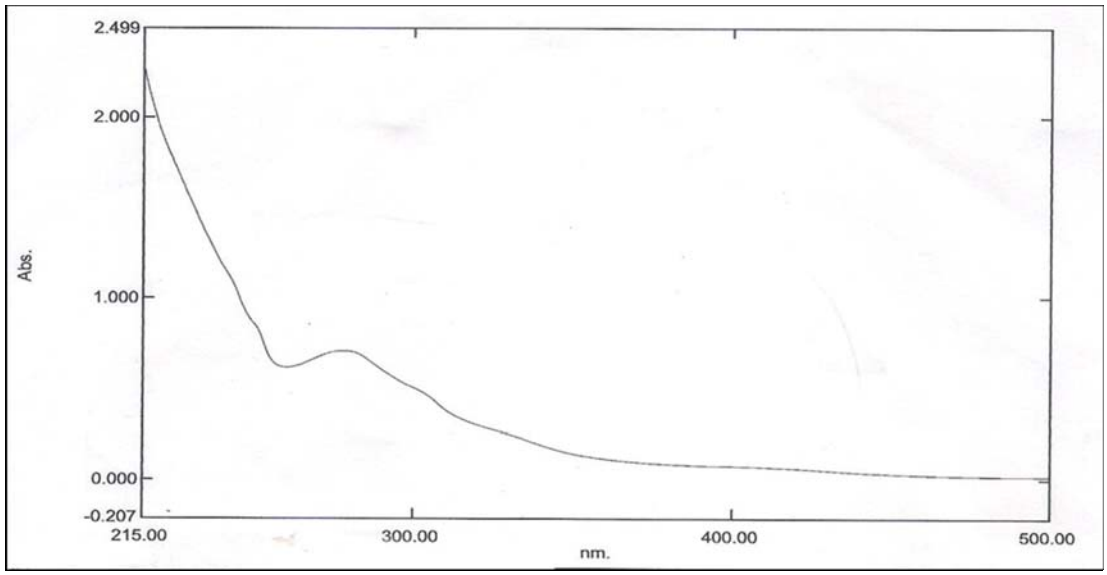

Fig 2: Ultraviolet (UV) spectrum of standard Betulinic acid

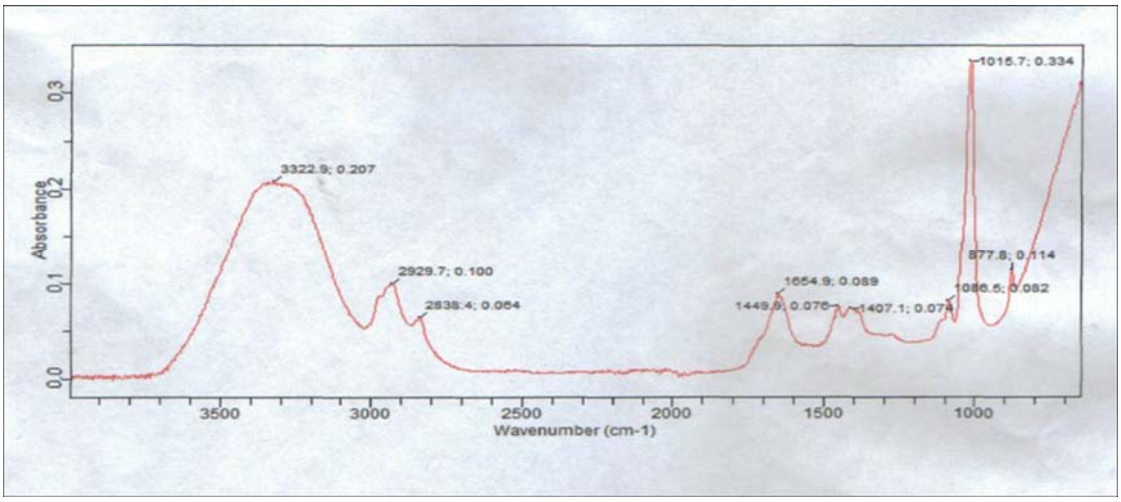

Fig. 3: Infrared (IR) spectrum of ethanol leaf extract of *Vitellaria paradoxa* (Shea butter).

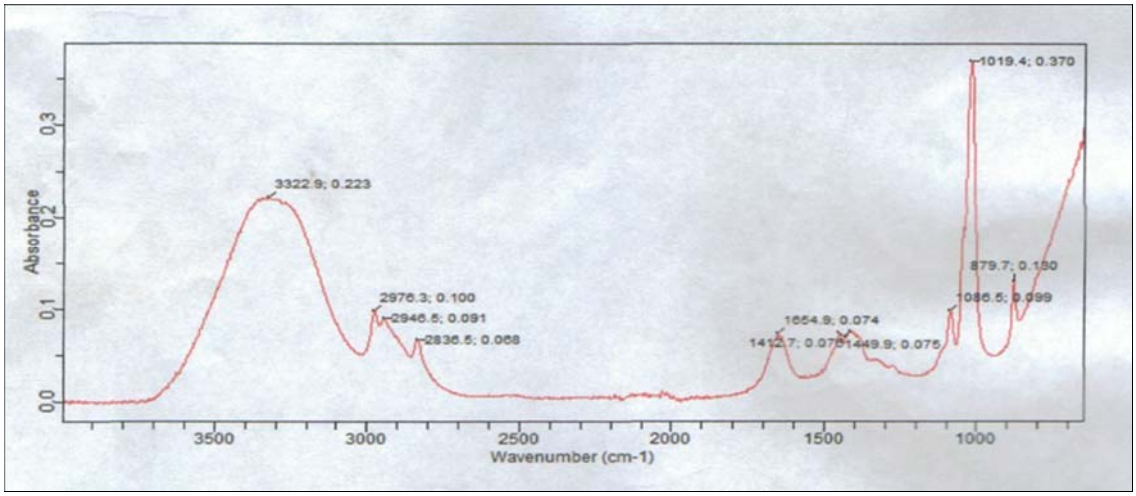

Fig. 4: Infrared (IR) spectrum of standard betulinic acid

3.2 HPLC spectral data

The elucidation of the *V. paradoxa* ethanol leaf extract using HPLC profile revealed the retention time (RT) of seven peaks as 1.582 min, 2.046 min, 2.580 min, 3.124 min, 4.536 min, 5.085 min and 5.420 min (Figure 5, Table 1), while the data from the standard Betulinic acid were found to be 0.068 min, 1.231 min, 2.089 min, 3.120 min, 4.081 min, 4.343 min and 5.702 min (Figure 6, Table 1).

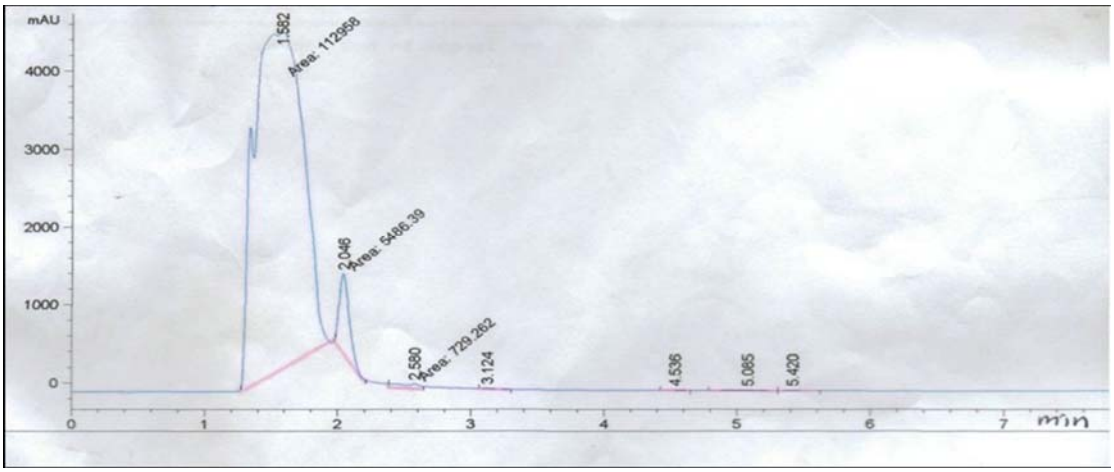

Fig 5: HPLC chromatogram of ethanol leaf extract of *Vitellaria paradoxa*

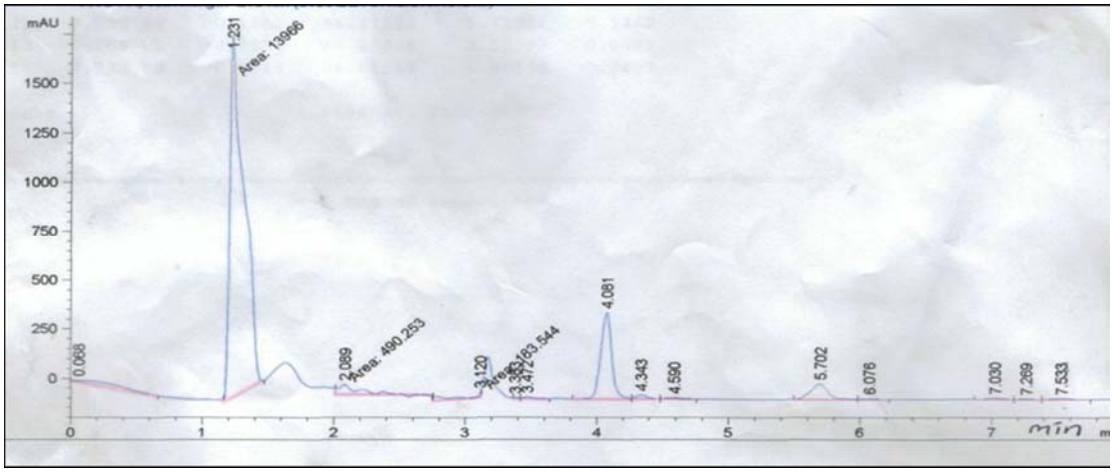

Fig 6: HPLC chromatogram of standard Betulinic acid

Table 1: HPLC profiles of ethanol leaf extract of *Vitellaria paradoxa* and Betulinic acid standard

Peaks	*Vitellaria paradoxa*			Betulinic acid (BA) standard		
	Retention time (min)	Area [mAU*sec]	Height [mAU]	Retention time (min)	Area [mAU*sec]	Height [mAU]
1.	1.582[a]	112958.000	4337.758	0.068	437.390	9.110
2.	2.046[b]	5486.392	1010.979	1.231[a]	13966.000	1854.662
3.	2.580	729.262	59.902	2.089[b]	490.253	49.343
4.	3.124[c]	38.576	7.857	3.120[c]	183.544	25.491
5.	4.536[d]	20.553	3.604	4.081	2575.525	438.326
6.	5.085	78.818	4.013	4.343[d]	104.959	18.881
7.	5.420[e]	56.946	7.828	5.702[e]	711.074	79..055

a,b,c,d,e- Comparable retention times (RT) between ethanol leaf extract of *V. paradoxa* and Betulinic acid standard, mAU- milliabsorbance Units.

4. Discussion

Vitellaria paradoxa (Shea butter) tree is a wild plant whose original home could be traced to the Savanna parkland of Africa, and has been reported to be socio-economically important. [1, 3] The widely reported biological potentials of betulinic acid, [14, 15, 24, 25] and triterpenes in general, have triggered several efforts towards isolation and characterization of these important compounds from botanical sources. In the present study, ethanol extract of *Vitellaria paradoxa*, obtained through soxhlet extraction and column chromatography, was analysed using UV, IR and HPLC techniques, and the data obtained were compared with the corresponding profiles of standard betulinic acid.

The UV profile of ethanol leaf extract of *V. paradoxa* reveals two absorption bands (274 nm and 257 nm) (Figure 1), which are comparable to the two bands (278 nm and 260 nm) obtained from the standard BA profile (Figure 2). Related studies on triterpenes isolated from botanical sources have shown absorption bands of 211 nm [26] and 257 nm, [27] while the UV absorption at 211 nm indicates the presence of carbonyl functional group, [26] the absorption at 257 nm indicates the presence of conjugated double bond, [27] which are conspicuous parts in the chemical structures of betulinic acid and related triterpenes. The IR data of ethanol leaf extract of *V. paradoxa* (Figure 3) compare well with those of the standard BA (Figure 4). However, comparing the IR result of the ethanol leaf extract of *V. paradoxa* with previous studies by Halilu *et al.* [23] and Bulus *et al*. [26] and the peaks 3322.9 cm-^{1}, 2929.7 cm-^{1} and 2838.4 cm-^{1} (Figure 3) indicate the presence of aliphatic hydroxyl (OH) and carboxylic acid (COOH) groups, methyl (CH_3) bending and methyl (CH_3) stretching, respectively. Furthermore, the work of Hossain and

Ismail [27] has supported the present study, showing the IR values 1654.9 cm-^{1}, 1449.9 cm^{-1} and 1407.1 cm^{-1} (Figure 3) to indicate the presence of unsymmetric ethylenic double bond (C=C), aromatic rings and aromatic methyl (CH_3) group, respectively, in the extract. Some other previous studies supporting the present profile on IR include those of Prince *et al*, [28] Soek *et al.* [29] and Ayotollahi *et al.* [30] This finding thus suggests the presence of betulinic acid and other triterpenes in the leaves of *V. paradoxa* (Shea butter) tree.

According to the reports of researchers, such as Olivera *et al*, [17] Zhao *et al,* [18] Kumar *et al.* [31] and Pai *et al,* [32] a single separation technique would not be adequate for the isolation of betulinic acid in HPLC profiling, rather a battery of techniques would be highly necessary. In the present study, we carried out the HPLC analysis of betulinic acid content of *V. paradoxa* leaves, following ethanol soxhlet extraction and column chromatography. The HPLC chromatograms of both ethanol leaf extract of *V. paradoxa* and standard betulinic acid have been presented as figures 5 and 6, while table 1 depicts the retention time (RT), peak area and peak height. The HPLC profile of the ethanol leaf extract of *V. paradoxa* reveals the RT of five peaks (1.582 min, 2.046 min, 3.124 min, 4.536 min, and 5.420 min) (Figure 5, Table 1), which are comparable to the data (1.231 min, 2.089 min, 3.120 min, 4.343 min and 5.702 min) exhibited by standard BA, as presented in figure 6 and table 1. The contents of betulinic acid have been determined in various plant extract using HPLC method. [33, 35] Furthermore, Taralkar and Chattopadhyay [36] have reported that the retention times (RT) of betulinic acid and ursolic acid (another triterpenoid compound) isolated from methanol leaf extract of *Vitex negundo* Linn were found to be 10.92 min and 12.36 min, respectively. These retention times are noticed to differ from our data in the present study, which could have resulted from differences in sources and prevailing experimental conditions.

5. Conclusion

This study observed comparable data between ethanol leaf extract of *V. paradoxa* and standard betulinic acid, suggestive of the presence and probability of obtaining this important triterpenoid compound from the plant. From the best of our knowledge, this study is the first to report the presence of betulinic acid in the plant used for the investigation.

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