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ISOLATION AND CHARACTERISATION OF COROSOLIC ACID FROM THE LEAF OF *F. ANDERSONII*

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ABSTRACT

The plant, *Fadogia andersonii* Robyn belongs to the family Rubiaceae. It has been employed traditionally as a therapeutic agent in the management of ailments including amoebic dysentery, inflammation, arthritis, malaria, fractures, typhoid, colicky pain, fever, wounds and boils and as an aphrodisiac. The leaf of the plant was extracted with soxhlet apparatus using methanol. The methanol extract was partitioned into ethyl acetate fraction (EFFAL), n-butanol fraction (BFFAL) and aqueous fraction (AFFAL). The qualitative and quantitative phytochemical analysis revealed the presence of saponin, cardiac glycoside, flavonoids, tannins, alkaloids, and steroids/triterpene. The highest concentrations of phenols (249.63 ± 2.88), flavonoids (117.33 ± 5.70), saponins (864.86 ± 5.07) and tannins (220.90 ± 13.38) were observed in n-butanol fraction while the highest concentration of alkaloids (288.00 ± 5.00) was found in ethyl acetate fraction. The least of all the phytochemicals were seen in the aqueous fraction except for saponin with the least concentration in methanol. Column chromatography of the ethyl acetate fraction (EFFAL) over silica gel LH 60-120 led to the isolation of corosolic acid. The structure of the isolated compound was established based on Nuclear Magnetic Resonance (NMR) and Infrared spectroscopy (IR).

Key words: *Fadogia andersonii,* soxhlet apparatus, phytochemical, column chromatography, corosolic acid

INTRODUCTION

Human beings have depended on nature for their simple requirements as being the sources of medicines, shelter, foodstuff, fragrances, clothing, flavours, fertilizers and means of transportation throughout the ages [1].

Natural products (NPs) signify large and diverse secondary metabolites with a comprehensive choice of biological activities that have been established with their numerous practices, particularly in human, veterinary and also in agriculture [2-4]. Natural Products are derived from microorganisms, marine animals, and also from plant sources. For the large proportions of the world's population plants continue to show a dominant role in the healthcare system and this is mainly true in developing countries, where herbal medicine has a continuous history of long use.

The development and recognition of medicinal and financial aid for these plants are rising in industrialized and developing nations [1]. They are also known to contain various substances that are used to perform important biological functions and defend against attack from predators such as insects, fungi and herbivorous mammals or living organisms [5,6].About 250 000 higher plant species, and more than 80 000 species are reported to have at least some medicinal value [7].

Fadogia andersonii Robyn belongs to the family Rubiaceae. It is an upright undershrub that is 1-2 feet high, with stems that are about three-angled and branchlets that are glabrous. The leaves are paired or in groups of three and are oblanceolate. Fruits are yellow, with greenish-yellow flowers. [8]. The plant is called by different Hausa speakers as *Bita Katsira*, *Dan Goyo* or *Gagai*, depending on the uses. The plant is a popular African medicinal plant, which has long been used in Africa in the treatment of diseases including amoebic dysentery, inflammation, arthritis, malaria, fractures, typhoid, colicky pain and an emetic [9].

EXPERIMENTAL

Materials: Thermoelectron UV machine, Ohaus digital weighing balance (Champ 11 CH15R, Ohaus Corporation, Pinebrook NJ, USA), Metler balance (Model P162 supplied by Gallenhamp), Syringes and needles, Mortar and pestle, sample bottles, beakers, separating funnel, conical flask and Bruka AVANCE III NMR spectrometer (500 MHz). The solvents used were of analytical grade and they included: methanol, ethanol, hexane, chloroform, ethyl acetate and n-butanol. Reagents used were freshly prepared and include those for phytochemical screening such as Molisch's reagent, Meyer's reagent, and Borntrager's reagent. Chromatographic materials used are pre-coated TLC plates (Alminium), Silica gel (60-120 mesh) and Sephadex LH 20.

Collection, identification and preparation of *Fadogia andersonii*

The aerial part of *Fadogia andersonii*plant was collected at Makwai village in Zaria Local Government Area of Kaduna state, Nigeria. It was identified and authenticated in the Herbarium unit, Department of Botany, Faculty of Life Science, Ahmadu Bello University, Zaria, with voucher specimen number 588. The leaves of the plant collected were washed and then air dried under shade at room temperature, grounded to powder and stored in an airtight container for further use.

Extraction and partitioning of the leaf of *Fadogia andersonii*

The powdered leaf (400g) of *F. andersonii* was first defatted using the soxhlet apparatus with 2 L of petroleum ether. The above procedure was repeated using methanol for extraction on the same marc. The methanol extract was concentrated, evaporated to dryness ina water bath and stored properly in a desiccator for further use. The extract was partitioned with ethyl acetate, n-butanol and to water to give the ethyl acetate n-butanol and aqueous fractions respectively.

Qualitative phytochemical screening of the leaf of *Fadogia andersonii*

Standard procedures following the methodology of Sofowara [10], Kokate, [11], and Evans [12] for the preliminary phytochemical screening were carried out on methanol extract, ethyl acetate fraction, n-butanol fraction and aqueous fraction of *F. andersonii* leaf.

1) Test for Carbohydrates

(a) *Molisch test:* Methanol extract (0.1 g) was dissolved in distilled water in a test tube and filtered. About 4 drops of molisch reagent were added followed by one millilitre of conc. H_2SO_4 by the side of the test tube, appearance of the purple coloured ring at the inter-phase as a result of interaction between molisch reagent and 5-hydroxymethylfufural was considered positive. The procedure above was repeated for ethyl acetate, n-butanol and aqueous fractions [12].

(b) *Fehling test:* To methanol extract (0.1 g) in a test tube, 5 mL of distilled water was added. The test tube was then placed in a water bath to heat, and an equal volume of Fehling solutions A and B was added drop by drop into the test tube. The appearance of brick-red precipitate was considered positive for the presence of reducing compounds. The procedure above was repeated for ethyl acetate, butanol and aqueous fractions [12].

2) Test for Saponins

a) Frothing test: Exactly 0.5 g of extract and fractions of *F. andersonii* leaf was dissolved in 10 ml of water and shaken vigorously for 30 seconds and allowed to stand for one (1) hour, the occurrence of frothing column or honeycomb-like of at least 1 cm in height and persisting for at least 30 minutes indicates the presence of saponins [10].

b) Haemolysis test: Exactly 2 ml of sodium chloride (1.8% solution in distilled water) was added to two test tubes A and B. 2 ml of distilled water was added to test tube A and 2 ml of petroleum ether extract was added to test tube B. five drops of blood was added to each tube and the tubes were inverted gently to mix the contents. Haemolysis in tube B containing the petroleum ether extract but not in tube A (i.e., control), indicated the presence of saponins in the extract [11].The procedure was repeated for methanol extract, ethyl acetate fraction, n-butanol and aqueous fraction.

3) Test for Anthraquinones

a) Bontrager test: To 0.5 g of each methanol extract and fractions (ethyl acetate, n-butanol and aqueous), 10 ml of chloroform was added and shaken. This was filtered and 5 ml of 10% ammonia solution was added to the filtrate. The presence of pink or cherished red colour in the lower layer indicates the presence of anthracenes [12].

b) Modified Bontrager's test: Exactly 0.5 g of each methanol extract and fractions (ethyl acetate, n-butanol and aqueous), were boiled with 10 ml of aqueous sulphuric acid and filtered hot. The filtrate after cooling to room temperature was shaken with 5 ml chloroform, the chloroform layer was separated and to half of its volume, 10% ammonium hydroxide was added. A pink, red or violet coloration in the ammonia phase (lower phase) is an indication of the presence of combined anthracene or anthraquinone derivatives in each extract and fractions [12].

4) Test for Cardiac Glycosides

***a) Keller-Killiani test*:** Exactly 0.5 g of each methanol extract and fractions (ethyl acetate, n-butanol and aqueous), was dissolved in glacial acetic acid containing ferric chloride and 1 drop of sulphuric acid was added to the solution. The appearance of reddish-brown coloration at the interphase indicates the presence of deoxy sugar [10].

b) Keddie's test: Exactly 0.5 g of all extracts will be treated with 1 ml of 2% solution of 3, 5-dinitrobenzoic acid in 95% alcohol. The solution was made alkaline by the addition of 5% NaOH. The presence of purple-blue colour indicates the presence of cardenolides [12].

5) Test for Flavonoids

a) Shinoda test: Exactly 0.5 g of each methanol extract and fractions (ethyl acetate, n-butanol and aqueous), was dissolved in the extracting solvent, 2 ml of 50% methanol. Pieces of magnesium fillings and 3 drops of hydrochloric acid will be added and a pink, rose or red colouration indicated the presence of flavonoids [10,12].

b) Sodium hydroxide test: Exactly 0.5 g of each methanol extract and fractions (ethyl acetate, n-butanol and aqueous), was dissolved in water and filtered. About 2 ml of 10% aqueous sodium hydroxide solution was added. The solution was observed for the presence of yellow colour. A change in colour from yellow to colourless on addition of dilute hydrochloric acid was used as an indication of the presence of flavonoids [12].

6) Test for Tannins

a) Ferric chloride test: Exactly 0.5 g of methanol extracts and fractions (ethyl acetate, n-butanol and aqueous), were dissolved in 5 ml of water each and filtered. Two drops of ferric chloride solution were added to the filtrate. The appearance of blue-black or green or blue-green (condensed/cathehic tannins) precipitate indicates the presence of tannins [12].

b) Lead sub-acetate test: To 0.5 g of each methanol extract and fractions (ethyl acetate, n-butanol and aqueous), 2 ml of ethanol was added followed by two drops of lead sub-acetate solution; the appearance of a whitish-yellow precipitate indicates the presence of tannins [12].

Quantitative phytochemical analysis of the leaf of *Fandogia andersonii* using spectrophotometric method

The methanol extract, ethyl acetate fraction, n-butanol fraction and aqueous fractions of *F. andersonii* leaf were quantified using standard procedure spectrophotometric methods.

1) Determination of total phenolic content (TPC)

Estimation of total phenol content in extract and fractions of *F. andersonii* leaf was measured spectrophotometrically by Folin–Ciocalteu colorimetric method, using gallic acid as the standard

and expressing results as Gallic acid equivalent (GAE) per gram of sample. Exactly 0.01 g of each extract, fractions and standard were diluted in 100 ml of methanol. Different concentrations (0.01-0.1 mg/ml) of gallic acid were prepared in methanol. Aliquots of 0.5 ml of the test samples and each sample of the standard solution were taken, mixed with 2 ml of Folin–Ciocalteu reagent (1:10 in deionized water) and 4 ml of a saturated solution of sodium carbonate (7.5% w/v). The tubes were covered with silver foils and incubated at room temperature for 30 min with intermittent shaking. The absorbance was taken at 765 nm using methanol as a blank. All the samples were analyzed in three replications. The total phenol was determined with the help of a standard curve prepared from a pure phenolic standard (gallic acid) [13,14].

2) Determination of total flavonoids content (TFC)

The TFC of extract and fractions of *F. andersonii* leaf was determined by aluminium chloride colorimetric assay [15]. About 0.01 g of each extract, fraction and standard were diluted in 100 ml of methanol. Briefly, 0.5 ml aliquots of each sample, standard solution (0.01-1.0 mg/ml) of quercetin. 2 ml of distilled water; and subsequently, 0.15 ml of sodium nitrite (5% $NaNO_2$, w/v) solution was added to both portions. After 6 min, 0.15 ml of (10% $AlCl_3$, w/v) solution was added. The solutions were allowed to stand for 6 min and after that 2 ml of sodium hydroxide (4% NaOH, w/v) solution was added to the mixture. The final volume was adjusted to 5 ml with the immediate addition of distilled water, mixed thoroughly and allowed to stand for another 15 min. The absorbance of each mixture was determined at 510 nm against blank. TFC was determined as mg quercetin equivalent per gram of sample with the help of the calibration curve of quercetin. All determinations were performed in triplicate.

3) Determination of total saponins content (TSC)

Total saponins content was determined according to the method described by [16]. Exactly 0.01 g of each extract, fractions and standard was diluted in 100 ml of methanol. 0.5 ml was taken, and vanillin reagent (0.25 ml; 8%) was added followed by sulphuric acid (2.5 ml; 72% v/v). The reaction mixtures were mixed well and incubated at 60°C in a water bath for 10 min. After incubation, the reaction mixtures were cooled on ice and absorbance at 544 nm (UV visible spectrophotometer) was read against a blank. The standard calibration curve was obtained from suitable aliquots of diosgenin (0.5 mg/ml in 50% aqueous methanol). The total saponins content

was expressed as mg diosgenin equivalents (DE)/g of the sample. All determinations were performed in triplicate.

4) Determination of total tannin content (TTC)

The total tannins will be determined by Folin - Ciocalteu method. Exactly 0.01 g of each extract, fractions and standard were diluted in 100 ml of methanol. About 0.1 ml of the sample extract was added to a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of Folin–Ciocalteu phenol reagent, 1 ml of 35% Na_2CO_3 solution and diluted to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard solutions of gallic acid (20, 40, 60, 80 and 100 μg/ml) was prepared in the same manner as described earlier. Absorbance for test and standard solutions was measured against the blank at 725 nm with a UV/visible spectrophotometer. The total tannin content was expressed in terms of mg of GAE/g of sample. All determinations were performed in triplicate. [17-19].

5) Determination of total alkaloid content (TAC)

Alkaloid content will be quantified by the spectrophotometric method. This method is based on the reaction between alkaloid and bromocresol green (BCG). The 0.01 g extract, fractions and standard were dissolved in 10 ml of 2 N HCl and then filtered. The pH of the phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. About 0.5 ml of this solution were transferred to a separating funnel, and then 5 ml of BCG solution and 5 ml of phosphate buffer were added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extract was collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank. TAC was determined as mg atropine equivalent/ per gram of sample with the help of the calibration curve of atropine. All determinations were conducted in triplicate [20,21]

Column chromatography of ethyl acetate fraction of the leaf of *F. andersonii*

The ethyl acetate fraction (10 g) was subjected to column chromatography. The glass column (30 × 3 cm) was packed with (100 g) of silica gel-60 (60 - 120 μm in size) using the wet slurry method. Silica gel was mixed with hexane twice its volume poured into a column andtapped gently with a glass rod to even the compaction of the particles. Ten grams (10 g) of ethyl acetate fraction was pre-absorbed on silica (dry method), loaded into the column, and stabilized overnight before elution began. Gradient elution was used, starting with hexane (100%) as the

initial eluting solvent. The solvent system's polarity was increased by gradually increasing the proportion of the more polar solvent (ethyl acetate). A total of 103 collections of 50 mL each were made. The different fractions collected were pooled together based on similarity monitored on TLC plate (Silica gel-60 F_{254} aluminium pre-coated plates) to give 5 major fractions coded EF1-EF5.Repeated silica gel filtration chromatography of column fraction (EF4)49-55 led to the isolation of HB. HB gave a single spot-on TLC with two different solvent systems (EA:CH:M: and EA:CH:M (Figure 1), indicating the purity of theisolated compound.

RESULTS AND DISCUSSION

Presented in Table 1 is the result of preliminary phytochemical screening of methanol extract, ethylacetate fraction, n-butanol fraction and aqueous fraction of the leaf of *Fadogia andersonii*

Table1: Preliminary phytochemical screening of extract and fractions of the leaf of *Fadogia andersonii*

Test	MEFAL	EFFAL	BFFAL	AFFAL
Carbohydrates				
Molisch	+	+	+	+
Fehling	+	+	+	+
Saponins				
Frothing	+	+	+	+
Haemolysis	+	+	+	+
Anthraquinones				
Bontrager	-	-	-	-
Modified Bontrager	-	-	-	-
Cardiac glycosides				
Keller- kiliani	+	+	+	+
Kedde’s	+	+	+	+
Flavonoids				
Shinoda	+	+	+	+
Sodium Hydroxide	+	+	+	+
Tannins				
Ferric chloride	+	+	+	+
Lead sub acetate	+	+	+	+
Alkaloids				
Dragendorff	+	+	+	+
Mayer	+	+	+	+
Wagner	+	+	+	+
Steroids/Triterpenes				
Salkowski	+	+	+	+
Lieberman-Burchard	+	+	+	+

MEFAL: Methanol extract of *Fadogia andersonii* leaf; EFFAL: Ethyl acetate fraction of *Fadogia andersonii* leaf; BFFAL: n-butanol fraction of *Fadogia andersonii* leaf; AFFAL: Aqueous fraction of *Fadogia andersonii* leaf. − Absent, + present

Presented in Table 2 is the result of quantitative phytochemical analysis (mg/g) on methanol extract, ethylacetate fraction, n-butanol fraction and aqueous fraction of the leaf of *Fadogia andersonii*

Table 2: Quantitative phytochemical analysis (mg/g) of *F.andersonii* leaf

Test Component	Phenol (mg GAE/g)	Flavonoid (mg QE/g)	Saponin (mg DE/g)	Tannin (mg GAE/g)	Alkaloid (mg AE/g)
MEFAL	33.47 ± 1.8	55.33 ± 9.26	345.24 ± 7.62	28.59 ± 6.41	36.50 ± 6.50
EFFAL	97.78 ± 13.14	60.67 ± 3.53	612.15 ± 7.15	159.46 ± 3.19	288.00 ± 5.00*
BFFAL	249.63 ± 2.88*	117.33 ± 5.70*	864.86 ± 5.07*	220.90 ± 13.38*	56.67 ± 3.18
AFFAL	24.68 ± 2.60	103.33 ± 5.46	678.81 ± 2.38	13.08 ± 3.49	15.67 ± 1.33

Values represented as mean ± SEM, (n=3); MEFAL-methanol extract of *F. andersonii* leaf; EFFAL-ethyl acetate fraction of *F. andersonii* leaf; BFFAL- n-butanol fraction of *F. andersonii* leaf; AFFAL-aqueous fraction of *F. andersonii* leaf. *- High value content

Physicochemical properties of isolated compound HB

Colour: white amorphous powder; Rf Values; 0.33 (hexane: ethyl acetate 7:4)

Solubility: soluble in chloroform and ethyl acetate; Melting point: 243-245 °C

Spraying reagents: Liberman- Burchard: pink coloured spot observed

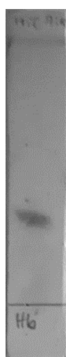

Figure 1: TLC of the isolated compound

The result of the phytochemical screening on *F. andersonii*leaf as shown in Table 1 revealed the presence of carbohydrates, saponin, cardiac glycosides, flavonoids, tannins, alkaloids, steroids/triterpenes except for anthraquinones in methanol extract and fractions (ethyl acetate, n-butanol and aqueous). This finding is consistent findings of Ameen *et al.* [9]., Mulholland *et al.,* [22], Suleiman *et al.,* [23] and Bruce *et al.,* [24]. on the leaf of *F. tetraquetra, F. cinenkowskii*, *F. erythrophlea* and stem of *F. agrestis.*

The result obtained from the quantitative phytochemical analysis of methanol extract and fractions (ethyl acetate, n-butanol and aqueous) of *F. andersonii* leaf as presented in Table 2

showed the estimation of the amount of phytochemical present from highest to least extent.The highest concentration of phenols(249.63 ± 2.88), flavonoids(117.33 ± 5.70), saponins(864.86 ± 5.07) and tannins (220.90 ± 13.38) were observed in n-butanol fraction while the highest concentration of alkaloids (288.00 ± 5.00) was found in ethyl acetate fraction. The least phytochemicals were seen in the aqueous fraction except for saponin with the least concentration in methanol as shown in Table 2. This finding demonstrates that phenols, flavonoids, tannins, saponins, and alkaloids, which may have therapeutic effects, are present in significant amounts in the leaves of *F. andersonii*. Column chromatographic separation of the ethyl acetate fraction over silica gel led to the isolation of a white amorphous powder coded HB. HB gave a single homogenous spot-on TLC indicating the purity of the compound (Figure 1). The compound tested positive for the Lieberman-Burchard test suggesting that the compound is a triterpene [25].

The IR spectrum of compound HB exhibited strong absorption bands at 3287cm^{-1}indicating an OH stretching [26]. Absorption at 2922-28.60 cm^{-1}was assigned to aliphatic C-H stretching, absorption at 1684 cm^{-1}of C=O stretching of carboxylic acid, absorption at 1617 cm^{-1} is due to C=C olefinic stretching and at 1028 cm^{-1}typical of C-O bending [27].

^{1}H NMR spectrum of compound HB revealed five (5) tertiary methyl proton at δ_H1.25, 1.20, 1.31, 1.08, 0.93 and two (2) secondary methyl resonating at δ_H0.99 and 0.94 typical of a ursane skeleton [28,29]. The two upper proton signals observed at δ_H4.31 and3.16 was due to carbinylic proton at position C-2 and C-3 of the triterpene nucleus [28]. Proton resonance at δ_H 5.27 (1H, m) was due to an olefinic proton assigned to position C-12 [29,30] and a proton doublet at δ_H 2.73 was assigned to H-18 based on chemical shift value and as well as the multiplicity pattern reported for H-18 with β-stereochemistry [31]

^{13}C NMR spectrum showed a total of thirty (30) carbon atom signals consisting of seven quaternary carbons, eight methines, eight methylenes and seven methyls. The carbon signal up field at δ_C79.14 and 77.32 was assigned to C-3 and C-2 carbon of the triterpene nucleus which was due to hydroxyl group at C-3 and C-2. The hydroxyl bearing C2 or C-3 is less deshielded by the adjacent axial than the equatorial one hence, 2β,3α [31,32] A carbon signal down field at δ_C 182.75 is attributed to the carboxylic acid at position C-28. The appearance of a signal at δ_C122.75 and 143.65 indicated the presence of a double bond in a ursane-12-ene skeleton of this triterpenoid at C-12 and C-13 [30]. The combined spectra analysis using IR, ^{1}H and ^{13}C NMR and literature data, revealed that compound HB is a pentancyclic triterpene (Figure 2) hence, named Corosolic acid

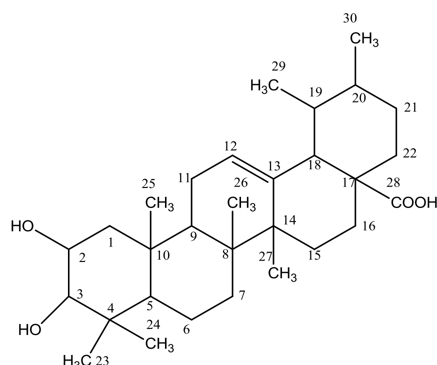

Figure 2: Structure of compound HB

The comparison of chemical shift data of corosolic acid and compound HB isolated from the leaf of *F. andersonii* obtained from the literature is presented in Table 3.

Table 3: Comparison between chemical shift data of corosolic acid and compound HB as obtained from literature

	Corosolic Acid*		Compound HB	
Position	**δ*^{1}H (ppm)**	**δ*^{13}C**	**δ ^{1}H (ppm)**	**δ ^{13}C**
1	-	48.40	-	47.72
2	4.04 (1H, ddd)	68.60	4.31 (2H, t)	77.32
3	3.36 (1H, d)	83.80	3.22 (1H, dd)	79.14
4	-	42.20	-	42.04
5	-	55.90	-	55.31
6	-	18.90	-	18.39
7	-	33.20	-	33.17
8	-	40.40	-	41.11
9	-	48.10	-	47.72
10	-	38.50	-	38.50
11	-	23.90	-	23. 67
12	5.42 (1H, br s)	122.50	5.27 (1H, brs)	122.75
13	-	144.9	-	143.65
14	-	40.10	-	39.58
15	-	28.30		28.20
16	-	23.70		23.04
17	-	46.60	-	46.96
18	2.62 (1H, d)	52.00	2.80 (1H, d)	52.70
19	-	39.50	-	39.36
20	-	42.00	-	41.72
21	-	30.00	-	30.78
22	-	34.20	-	33.90
23	1.26 (3H, s)	29.30	1.25 (3H, s)	29.01
24	1.11 (3H, s)	16.80	1.13 (3H, s)	15.43
25	1.04 (3H, s)	17.50	1.08 (3H, s)	17.09
26	0.98 (3H, s)	17.70	0.96 (3H, s)	17.21
27	1.24 (3H, s)	23.80	1.20 (3H, s)	23.50
28	-	180.20	-	182.75
29	0.94 (3H, d)	16.90	0.93 (3H, d)	15.65
30	1.02 (3H, d)	23.80	0.99 (3H, d)	23.67

CONCLUSION

The leaf revealed the presence of a phytochemical constituent of carbohydrates, saponins, cardiac glycosides flavonoids, tannins, alkaloids, steroids/triterpenes in high concentration except for the absence of anthraquinones and saponin is the most abundant in methanol extract, ethyl acetate, n-butanol, and aqueous fractions. The Ethyl acetate subjected to column chromatography whereby compound HB was isolated, revealed as pentacyclic triterpene named Corosolic acid.

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