

## Full Length Research Paper

# Isolation and characterization of terpene from leaves of *Croton macrostachyus* (Bissana)

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Received 26 February, 2016; Accepted 19 March, 2016

***Croton macrostachyus*, one of the *Croton* species found in Ethiopia, is used traditionally for treatment of a number of health ailments including diabetes, malaria, stomachache, ascariasis, abdominal pain, gonorrhoea, wounds, ringworm infestation and hemorrhoids. In the present study, phytochemical screening of ethanol extract from leaves of *C. macrostachyus* revealed the presence of terpenes, flavonoids, alkaloids and saponins. Silica gel column chromatographic separation of the ethanol extract afforded a pentacyclic triterpenoid reported for the first time from the genus. The structure of the compound was determined using combination of spectroscopic techniques via UV-Vis, IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR and DEPT-135.**

**Key words:** *Croton macrostachyus*, pentacyclic triterpenoids, anti-malaria, spectroscopic techniques, phytochemical screening.

## INTRODUCTION

Medicinal plants have been used to treat various health ailments for a long period of time in different countries. Natural products have been playing dominant role in drug discovery efforts for treatment of human and livestock diseases (Newman and Cragg, 2012). With the upsurge in the use of plants medicines, a thorough scientific investigation of these plants is imperative based on the need to validate their folklore use. *Croton* species are among the most common traditional medicinal plants used in Africa, Asia, and South America for treatment of

diabetes (Moshi et al., 2000), digestive problems (Yirga et al., 2011), malaria (Mohammed et al., 2007; Alshawsh et al., 2009; Mesfin et al., 2009), insomnia and head-ache (Bum et al., 2012), hemorrhoids and ulcers (Antonio, 2007). The genus has been reported to have a number of biological activities for instance anti-hypertensive, anti-inflammatory, antimalarial and anti-viral (Mbiantcha et al., 2013; Habtamu et al., 2012; Prozesky et al., 2001). Triterpenoids, either pentacyclic or steroidal, volatile oils containing mono and sesquiterpenoids, shikimate-derived

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compounds and phenolic compounds are among secondary metabolites reported from the genus *Croton*. Reports on the pharmacological activity of *mactostachyus* species suggested that the genus is a potential source of enormous bioactive compounds (Antonio, 2007). In Ethiopia, people use the stem and roots of the plant for diarrhea treatment and decoction of the leaf to treat malaria (Giday et al., 2007). Recent studies by Sendeku et al. (2015) and Taye et al. (2011) indicated that the methanol and ethanol extracts from leaves of *C. macrostachyus* showed antibacterial activity while the aqueous extracts were comparatively ineffective. Following the potential of the plant in traditional medicine and its efficient pharmacological activity, further analysis is required to determine the possible bioactive components.

In the ongoing study to analyze the chemical constituents of medicinal plants found in Ethiopian flora, we hereby report a comprehensive phytochemical screening, isolation and a complete characterization of one triterpenoid from the leaves of *C. macrostachyus*.

## MATERIALS AND METHODS

### Instruments

Nuclear Magnetic Resonance (NMR) analysis was recorded on a Bruker Avance 300 MHz spectrometer with deuterated  $\text{CDCl}_3$  using tetramethylsilane (TMS) as internal standard. Structural elucidations were done on the basis of NMR spectra both 1D ( $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and DEPT-135) and IR. All NMR data were obtained in  $\text{CDCl}_3$ . Chemical shifts are reported in parts per million (ppm,  $\delta$ ). Spectral splitting patterns are designated as s: singlet, d: doublet, t: triplet, q: quartet, m: complex multiplet (chemically non-equivalent H's), br s: broad signal.  $^{13}\text{C}$  NMR spectra were proton decoupled and recorded on a 100 MHz Bruker spectrometer using TMS as the internal standard. Column chromatography (CC) was performed over silica gel (Merck, 230-400 mesh). Analytical thin layer chromatography (TLC) was carried out using pre-coated 0.2 mm silica gel 60 F254 on aluminum foil and compounds on TLC were detected under UV lamp at 254 and 365 nm. UV-Vis spectrum was recorded on UNICAM UV-300 double beam spectrophotometer in the range of 200 to 1000  $\text{cm}^{-1}$  using  $\text{CHCl}_3$  as internal standard. Infrared (IR) spectrum was recorded on Perkins-Elmer Bx Infrared spectrometer using KBr disc in the range 4000 to 400  $\text{cm}^{-1}$ . Melting point was recorded by Mettler Toledo apparatus, Type FP62, and it was uncorrected. All reagents were obtained from Sigma Aldrich and used as received unless noted otherwise.

Preliminary phytochemical screening test was done based on standard procedures according to Sofowora (1982), Trease and Evans (1989) and Harborne (1973).

### Plant material

The leaves of *C. macrostachyus* (Bissana in Amharic, local language) were collected from Dawuro area, in Southern Nation and Nationalities of People Region (SNNPR), located at a distance of 512 km from the capital city, Addis Ababa, Ethiopia in July 2009. The plant material was identified by Dr. Ensermu Kelbessa, Department of Biology, Faculty of Natural Sciences, Addis Ababa University (AAU) and a voucher specimen (CM-001) was deposited

at National Herbarium of Ethiopia, Addis Ababa University. The leaves of *C. macrostachyus* samples were collected and spread to enhance drying at room temperature and then grounded into fine powder using mortar and kept in a transparent polyethylene bags until extraction.

### Preparation of crude ethanol extract

250 g powdered leaf samples of *C. macrostachyus* was soaked with petroleum ether for 3 days with occasional shaking and filtered to remove the fat contents. After filtration, the air dried marc was further soaked in 650 ml of ethanol for three days at room temperature. The ethanol extract was then filtered and concentrated using Rotary Evaporator at 40°C, air dried and weighted to yield 36.6 g (15.3%) dark green crude extract. The ethanol extract was suspended in water and partitioned with diethylether and ethyl acetate successively and combined to give a total yield of 4.7 g dark green solid. TLC analysis of the organic layer and aqueous extract showed six and eight spots, respectively, using chloroform/ethyl acetate (9:1) and *n*-hexane: chloroform (4:1) as eluent.

### Isolation and purification of compound 1

3.5 g Crude organic layer part (3.5 g) was subjected to silica gel column chromatography (silica gel, 70 g) with increasing gradient of ethyl acetate in chloroform followed by methanol in chloroform as an eluent. A total of 31 fractions, each 50 mL, were collected and analyzed by TLC. Fractions from Fr 12 to 18 (5 to 20% ethyl acetate in chloroform) showed two spots on TLC. They were combined and further purified using silica gel column chromatography to give a single clear spot (compound 1) using chloroform/ethyl acetate (1:1) as eluent.

### Isolation and purification of compound 2

Greenish ethanol extract (4.7 g) was dissolved in 15 ml chloroform, subjected to silica gel column chromatographic separation (80 g silica gel) and eluted with increasing gradient of chloroform in *n*-hexane and then ethyl acetate in chloroform successively. A total of 17 fractions (each 50 ml) were collected. The constituent profile of each fraction was monitored by TLC (40% ethyl acetate in chloroform) and visualized under UV-Vis light ( $\lambda_{\text{max}}$  254 and 366 nm). Based on their TLC profile, fractions 4 to 8 were combined and purified to give a single spot on TLC analysis (chloroform: ethyl acetate (9:1)). Fraction 9 to 14 were also combined, purified and taken to spectroscopic analysis due to their amount and level of purity. The last two fractions were dark green in color and show cloudy red spots on fluorescent UV-Vis light. Fractionation stopped after observing the dark green part at bottom. The fraction then labeled compound 2 was obtained as a pale yellow precipitate. (M.pt: 244.1°C $\pm$ 2, 44.8 mg).

### Preliminary phytochemical screening

Detections of common secondary metabolites were performed for ethanol extract of leaves of *C. macrostachyus* using the preceding analytical procedures (Kebede et al., 2015).

### Test for terpenes

250 mg of ethanol extract was mixed with 2 ml of  $\text{CHCl}_3$  and 30 ml of concentrated  $\text{H}_2\text{SO}_4$  was added carefully to form a layer

**Table 1.** Phytochemical screening tests of the ethanol extract.

Plant constituent	Observation
Alkaloids	+
Flavonoids	+
Terpenes	+
Tannins	-
Saponins	+
Anthraquinones	-

(-) Not Detected, (+) Detected

(Debjyoti, 1995). Reddish-brown coloration of the interface was inspected.

#### Test for flavonoids

250 mg of ethanol extract was dissolved in small amount of dilute NaOH and concentrated HCl (3 ml) was added (Farnsworth, 1996). A yellow solution that turns to colorless was inspected.

#### Test for tannins

Small quantity of the ethanol extract was mixed with water and heated on water bath. The mixture was filtered and small amount of solid FeCl<sub>3</sub> was added to the filtrate (Sofowora, 1982). Dark-green solution was inspected.

#### Test for alkaloids

250 mg of the crude extract was mixed with 2 ml of concentrated hydrochloric acid. The mixture was then filtered and mixed with small amount of amyl alcohol at room temperature (Ganjewala et al., 2009). The mixture was kept for observation of the color resulted from the alcoholic layer.

#### Test for saponins

250 mg of the ethanol extract was shaken with 5 ml of distilled water for 30 min and then heated to boil. Appearance of creaming mix of small bubbles (frothing) was inspected (Farnsworth, 1996).

#### Test for anthraquinones

500 mg of the ethanol extract was boiled with concentrated hydrochloric acid for few minutes in water bath and filtered. The filtrate was allowed to cool and equal volume of CHCl<sub>3</sub> was added to it. Few drops of ammonia were added to the mixture and heated in water bath. Formation of rose-pink color was inspected (Sofowora, 1982).

## RESULTS AND DISCUSSION

Preliminary phytochemical screening of the crude ethanol extract of the leaf revealed the presence of various

metabolites such as terpenoids, flavonoids, saponins and alkaloids whereas tannins and anthraquinones were not detected (Table 1).

Silica gel column chromatographic separation of the crude ethanol extract afforded one pentacyclic triterpenoid and complete characterization of the compound is presented subsequently. All the NMR chemical shift values,  $\delta$ , are presented in ppm. The IR spectrum of compound 1 indicated absorption peaks at 2926.56 and 2856.24 cm<sup>-1</sup> (sp<sup>3</sup> C-H methyl stretching and sp<sup>2</sup> C-H methylene, respectively), 1738.71 cm<sup>-1</sup> (carbonyl group of ketone moiety), 1468.91 cm<sup>-1</sup> (olefinic carbon), 1377.08 and 1365.51 cm<sup>-1</sup> (C-O of ether functionality). The UV-Vis spectrum revealed absorption peak  $\lambda_{max}$  at 380 nm attributed to n- $\pi^*$  transition.

The <sup>1</sup>H NMR spectrum showed peaks at  $\delta$  5.40 (*t*, 2H, H-7) and 5.13 (*s*, 2H, H-3) attributed to olefinic protons and a methine hydrogen next to a ketone and an ether group. An isopropyl group attached to heteroatom oxygen and a methylene attached to carbonyl group were clearly evident at  $\delta$  4.64 (*m*, 1H, H-27) and 2.43 (H-9), respectively. Furthermore, methyl groups were observed at  $\delta$  (ppm) 1.83 (H-16), 1.74 (H-30, 31), 1.32 (H-33, 34) and 1.21 (H-25, 26).

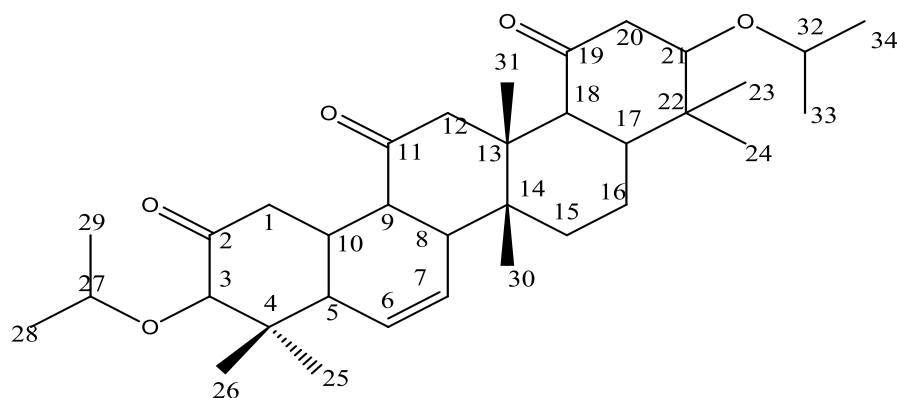
The <sup>13</sup>C-NMR spectrum showed a total of 34 well-resolved carbon signals attributed to three ketone carbonyls [ $\delta$  184.1 (C-19), 179.80 (C-11) and 175.5 (C-2)], four quaternary carbons, twelve methines, five methylenes and ten methyls, also supported by DEPT-135. Of these, four of the methines were observed to be linked to heteroatom, possibly oxygen [ $\delta$  82.3 (C-3), 78.3 (C-21), 75.6 (C-32) and 74.5 (C-27)] and two olefinic carbons  $\delta$  131.8 (C-6) and 131.6 (C-7)], also confirmed from DEPT-135 spectrum. Seven peaks observed in <sup>13</sup>C NMR spectrum [ $\delta$  184.1 (C-19), 179.8 (C-11), 175.5 (C-2), 65.7 (C-22), 65.5 (C-4), 65.2 (C-14) and 65.1 (C-13)] were not detected in DEPT-135 spectrum suggesting that these carbons belong to quaternary carbons. The down field chemical shift of methyl group (C-31) compared to methyl (C-30) is attributed to the anisotropic effect of the ketone group (C-19), in agreement with the position of the carbonyl group at C-19 (Table 2). Based on the aforementioned spectroscopic data and comparison with literature the compound was found to be a pentacyclic triterpenoid (Figure 1) reported for the first time from the genus.

Compound 2 was found as pale yellow crystalline powder with R<sub>f</sub> value of 0.58 (40% ethyl acetate in chloroform as eluent). The <sup>1</sup>H NMR spectrum of the compound showed OH protons aromatic phenyl protons. The <sup>13</sup>C NMR spectrum of the compound 2 showed a total of around sixty carbon atoms peaks of which one carbonyl carbon multiple aromatic and aliphatic carbons. The idea was supported by the IR spectrum showing broad region for OH with methylene and characteristic aromatic C=C peaks with C=O peaks. However, we are not able to elucidate the structure from

**Table 2.** Spectral data of Compound 1 <sup>1</sup>H NMR (300 MHz; CDCl<sub>3</sub>), <sup>13</sup>C NMR (100 MHz; CDCl<sub>3</sub>).

Position	<sup>1</sup> H NMR (δ ppm)	<sup>13</sup> C NMR (δ ppm)	DEPT-135
1	2.81 ( <i>d</i> )	74.1	CH <sub>2</sub> -(C=O)
2	-	175.5	C=O
3	5.13( <i>s</i> )	82.3	CH-O
4	-	71.9	C
5	2.81 ( <i>t</i> )	65.4	CH-(C=C)
6	5.40 ( <i>t</i> )	131.6	CH=CH
7	5.40 ( <i>t</i> )	131.8	CH=CH
8	2.81 ( <i>m</i> )	65.2	CH-(C=C)
9	2.43 ( <i>m</i> )	71.6	CH-(C=O)
10	2.28-2.31 ( <i>m</i> )	64.9	CH-(CH-(C=O))
11	-	179.8	C=O
12	2.81 ( <i>s</i> )	72.5	CH <sub>2</sub> -(C=O)
13	-	65.7	C
14	-	65.1	C
15	2.28-2.31 ( <i>m</i> )	65.5	CH <sub>2</sub> -(C)
16	1.83 ( <i>q</i> )	65.2	CH <sub>2</sub> -(CH)
17	2.28-2.31 ( <i>m</i> )	40.73	CH
18	2.28-2.31( <i>m</i> )	73.4	CH-(C=O)
19	-	184.1	C=O
20	2.81 ( <i>d</i> )	74.5	CH <sub>2</sub> -(C=O)
21	5.13 ( <i>s</i> )	78.3	CH-O
22	-	72.7	C
23	1.21( <i>s</i> )	30.8	CH <sub>3</sub>
24	1.21( <i>s</i> )	30.8	CH <sub>3</sub>
25	1.21 ( <i>s</i> )	30.8	CH <sub>3</sub>
26	1.2( <i>s</i> )	30.8	CH <sub>3</sub>
27	4.64 ( <i>m</i> )	74.5	CH-O
28	1.32 ( <i>m</i> )	23.8	CH <sub>3</sub>
29	1.32 ( <i>m</i> )	23.8	CH <sub>3</sub>
30	1.74 ( <i>s</i> )	18.6	CH <sub>3</sub>
31	1.74 ( <i>s</i> )	45.6	CH <sub>3</sub>
32	4.64 ( <i>m</i> )	75.6	CH-O
33	1.32 ( <i>m</i> )	23.7	CH <sub>3</sub>
34	1.32 ( <i>m</i> )	23.7	CH <sub>3</sub>

Values may be interchangeable within the same compound (CDCl<sub>3</sub>, 100 MHz).

**Figure 1.** Proposed structure of compound 1.

the spectroscopic data and further advanced spectroscopic studies are needed to elucidate the structure of compound 2.

## Conclusion

Preliminary phytochemical screening test on the ethanol extract of leaves of *C. macrostachyus* revealed the presence of alkaloids, terpenes, flavonoids and saponins. Anthraquinones and tannins were not detected in the present work. The pentacyclic triterpenoid identified in this study was reported for the first time from *C. macrostachyus*. The traditional use of the plant may be attributed to its high content of polar bioactive constituents. Apart from the phytochemical screening of the ethanol leaf extract, isolation and NMR characterization of one triterpene was successfully achieved. This work will give background record to use *C. macrostachyus* as a potential drug source. Further work on isolation of the detected bioactive molecules in concert with their biological activities including antiviral and antibacterial effects is going on.

## Conflict of Interests

The authors have not declared any conflict of interest.

## ACKNOWLEDGEMENT

The authors are grateful to Dr. Ensermu Kelbessa and staff members of the National Herbarium of Ethiopia, Addis Ababa University, for identification of the plant material. They are grateful to Dr. Alay Hagos for conducting IR spectrophotometer at Netherlands. Prof. Ermias Dagne, Department of Chemistry, Addis Ababa University is duly acknowledged for allowing us to use 300 MHz NMR spectrometer. This research is partly supported by the School of Graduate Study, Hawassa University.

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