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PHYTOCHEMICAL INVESTIGATION AND ANTIBACTERIAL ACTIVITY TEST OF THE ROOT EXTRACT OF *ALOE CALIDOPHILA*

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ABSTRACT

Aloe calidophila is one of the endemic Aloe species traditionally used for the treatment of various illnesses including stomach pain, malaria, hypertension, diarrhea and wound healing in Sidamo floristic region and southern part of Ethiopia. The aim of this study was investigate chemical constituents and subsequently characterize isolated compounds from root extract of *Aloe calidophila* using FTIR, ^{1}HNMR and ^{13}CNMR spectroscopic techniques as well as testing its antibacterial activity. The phytochemical screening tests of the dichloromethane/methanol (1:1 v/v) and methanol root extracts revealed the presence of alkaloids, anthraquinones, flavonoids, phenols, terpenoids, steroids and saponins. The chromatographic separation of dichloromethane/methanol (1:1 v/v) crude extract resulted in three compounds (ABM1, ABM2 and ABM3) using n-hexane/ethyl acetate solvent system. The chemical structures of the compounds were found to be aloesaponarin I, oleic acid and β-sitosterol, respectively, based on spectroscopic (FTIR and NMR) data and literature reports. The crude extracts and isolated compounds were evaluated for their antibacterial activities against four bacterial strains specifically (*E. coli* ATCC25922, *S. aureus* ATCC25923, *P. aeruginosa* ATCC27853 and *S. pyogenes* ATCC19615). Disc diffusion method was used for the test. The antibacterial activity tests revealed that the isolated compounds to show relatively better activities than the crude extracts. The isolated compounds showed comparative activities when compared to each other with compound AMB3 to be slightly highest activity against the bacterial strains used in the study. The present finding indicated that the roots *Aloe calidophila* could be good sources of compounds to be used as leads in the discovery of antibacterial agents provided that *in vitro* tests are carried out in several bacterial strains.

KEYWORDS

Aloe calidophila, Aloesaponarin I, Oleic acid, β-sitosterol, Antibacterial activity and Phytochemical screening.

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INTRODUCTION

The plant species that belong to the genus *Aloe* are the oldest medicinal plants known to human[1]. They are native to sub-Saharan Africa, many islands of western Indian Ocean, including Madagascar and Saudi Arabian Peninsula[2,3]. Reports revealed the use this plant species for treatment of several

human illnesses such as stomach ailments, gastrointestinal problems, skin diseases, constipation for radiation injury, for its anti-inflammatory effect, for wound healing and burns, as an antiulcer and diabetes[4-6]. There are also literature reports that state its wide use in the preparation of skin care products, cosmetics and as nutraceutical[7,8,6]. The leaves and roots *Aloe* species are well known as principal sources of many interesting phytochemicals such as anthraquinones, preanthraquinones, alkaloids, anthrones, chromones, glycosides, carbohydrates, coumarins, flavonoids, naphthalene, steroids, volatile oils etc[9,10].

In Ethiopia and Eritrea, about 46 species of Aloe have been identified so far with a high proportion of endemics adapted to harsh climates. It has been reported that the leaf latex of several Aloe species and their constituents possess wide spectrum of biological activities such as antimicrobial[11,12], antimalarial[9,13] and antiglycation[14]. The aloe species in Ethiopia are distributed in all floristic region of the country including Afar, Arsi, Bale, Gamo Gofa, Gojam, Gonder, Harerge, Kefa, Shewa, Sidamo, Tigray, Wellega and Welo floristic regions (Sebsebe and Nadal, 2010)[15]. Accordingly, the majority of them are found in Sidamo floristic region (with 14 species) followed by Harerge, Bale, Shewa, Tigray and Welo floristic regions (with 10, 8, 8, 7 and 7 species respectively). Whereas few species found in Afar and Wellega floristic regions (each possess one *Aloe* species) and one *Aloe* species (*Aloe macrocarpa*) found in many floristic regions including Arsi floristic area (Sebsebe and Nadal, 2001). The distribution of *Aloes* in Ethiopia can be correlated to vegetation types, and showed that the endemic and near-endemic species occurred predominantly in two vegetation types. These are: (a) dry montane evergreen forest (and associated montane evergreen scrub or montane grassland), and (b) Acacia-Commiphora woodland and bushland[16]. Some of the *Aloes* that occur in dry montane evergreen forest include *Aloe debrana, Aloe adigratana, Aloe percrassa, Aloe pulcherrima, Aloe elegans, Aloe camperi and Aloe yavellana.* Acacia-Commiphora wood land and bush land also host *Aloe* species such as *Aloe calidophila, Aloe ellenbeckii, Aloe gilbertii, A. Aloe friisii, Aloe retrospiciens, Aloe mcloughlinii, Aloe pirottae*, *Aloe otallensis* and *Aloe trichosantha. Aloe* species such as *Aloe bertemariae and Aloe citrine* are reported from desert and semi-desert scrubland and *Aloe ankoberensis* is from Afro-alpine vegetation of Ethiopia[17].

Aloe calidophila (Figure No.1) is one of the endemic species of Ethiopia which grows mostly in Acacia-Commiphora woodland/bushland or open wooded grassland in altitude range of 1280-1620m in Dida Cheena Plains, between Moyale and Mega, in Sidamo floristic region[17]. The different parts of *Aloe calidophila* are used for the treatment of various diseases in traditional or folk medicine in Ethiopia. For instances, its leaf and roots are traditionally used in Sidama region, South nations around Gamo Gofa, and in other central highlands of the country for the treatment of various illnesses such as stomach pain, malaria, hypertension, diarrhea, endocrine system, repel flies from infected eye, wound healing, and anthelmintic[18]. Despite its wide use in traditional medicine, there are no exhaustive studies. In other words, there are only a limited study reports on this species. For instance, a study on phytochemical and biological activities of latex from its leaves revealed the presence of phytochemicals that have been presumed to be responsible for its antileishmanial activity. Some of them are 4-dihydroisocoumaringlucoside, 7-aloin B, 8-aloin A, 9-aloinoside B, 10-aloinoside A, 11-microdontin B, 12-microdontin A[19,20]. Moreover, compounds isolated from the latex have been reported to show a better anti-promastigote activity than some standard antileishmanial drugs such as Amphotericin B[21]. To the knowledge of our team, there are no reports on phytochemical investigations and isolation of compounds from the root parts of *Aloe calidophila*. Therefore, the aim of the present study was to carry out phytochemical screening on root extract of *Aloe calidophila* and its chemical composition, and also evaluation of antibacterial

activities of the crude extracts and compounds isolated from crude extracts.

MATERIAL AND METHODS

Collection of plant material

The root of *A. calidophila* was collected from Dida Cheena, an area between the towns of Yabello and Mega in the Southern Nation, Nationalities and Peoples' Region of Ethiopia. The area is about 550 km south of Addis Ababa and 221km south of Hawassa town. The plant material was authenticated by a botanist Professor Sebsebe Demissew, Department of Biology, Addis Ababa University.

Preparation of plant material

The collected plant material (root part) was washed with tap water and chopped to small pieces and air-dried at room temperature for one month. The dried plant material was ground using a grinding machine to facilitate the extraction process.

Extraction

500g of the powdered root of *A. calidophila* was soaked in 4 L of dichloromethane/methanol (1:1 v/v) at room temperature in Erlenmeyer flask. After shaking the contents well, the flask containing the solution was placed on an orbital shaker and agitated at the speed of 120 revolutions per minute for 72 hours. Then the solution was filtered using Whatman filter paper. The filtered solution was concentrated using rotary evaporator (Heidolph, UK) at temperature of 40ºC. The marc left was further extracted with 100% methanol following similar steps used above (Scheme No.1).

Phytochemical screening tests

Phytochemical screening tests or identification of secondary metabolites were performed on the crude extract of dichloromethane/methanol (1:1 v/v) and methanol extract using standard procedures to investigate the presence of some secondary metabolites such as alkaloids, anthraquinones, flavonoids, glycosides, phenols, saponins, steroids, tannins and terpenoids using preliminary phytochemical analysis[21].

Isolation and characterization of compounds

Nine grams (9g) of dichloromethane/methanol (1:1 v/v) dried crude extract was adsorbed onto 18g of silica gel and subjected to column chromatographic isolation (180g silica gel 60-120 mesh size used as the stationary phase). The column was eluted with increasing gradient of n-hexane in ethyl acetate solvent system. The elution process was started by 100% n-hexane and progressed through various ratios of an increasing portion of ethyl acetate in n-hexane. A total of 100 fractions were collected and each fraction was checked by TLC. The spots on the TLC plates were visualized using UV light (254 and 365nm), (UV-Tcc chamber). The collected fractions were concentrated using rotary evaporator. Fractions were tested using TLC and those with the same TLC profiles were combined. The column chromatographic separation led to isolation of three compounds. The fraction number 93-95 gave compound ABM1 (65:35); fraction number 63-74 gave compound ABM2 (80:20) and the fraction number 40-45 gave compound ABM3 (85:15). Then, the structural elucidations of the compounds were carried out based on data obtained from spectroscopic data such FTIR (Perkin Elmer BX infrared spectrometer) and NMR (Bruker Avance 400 MHz) data. The NMR spectroscopic analyses were carried out at The Department of Chemistry, Addis Ababa University and the FTIR analyses were done at The Department of Chemistry, Hawassa University, Ethiopia.

RESULTS AND DISCUSSION

The yield of extracts, qualitative phytochemical tests, characterization of isolated compounds, and antibacterial activity tests are discussed in the following sections.

Masses of crude extracts

The resulting extract of *Aloe calidophila* root was 14.5g (2.9%) and 4.7g (0.97%) for dichloromethane/methanol (1:1 v/v) and methanol, respectively (Table No.1). This data showed that, the crude extract obtained from dichloromethane/methanol (1:1 v/v) was greater than methanol extracts. This indicated that most of

the components the extracts could be medium polar. This finding was consistent with literature reports[22]. The mass and TLC profile of the dichloromethane/methanol (1:1 v/v) extract were taken as criteria for selecting this extract for column chromatographic separation.

Phytochemical screening

Phytochemical screening of the dichloromethane/methanol (1:1) and methanol root extracts revealed the presence alkaloids, anthraquinones, flavonoids, phenols, terpenoids, steroids and saponins (Table No.1). According to above phytochemical observation, *Aloe calidophila* is one of the plants rich in many secondary metabolites which could be attributed to be responsible for the traditional medicinal uses and also the plant as such studies may lead to drug discovery and development.

Isolation and characterization of compounds

Three compounds (ABM1, ABM2 and ABM3) were isolated in the present study. The structures of the compounds were determined based on spectroscopic data (IR and NMR) and other physical data such as the melting point. The collected data were analyzed and compared with similar data reported in literature.

Compound ABM1 was isolated as yellow crystal (19.1mg) with a melting point of 190-193^{o}C and an R_f value of 0.4 (35% ethyl acetate in n-hexane). The FTIR spectrum (Appendix 1) of the compound showed a broad band at 3345cm^{-1} and band at 1697cm^{-1} indicated the presence of OH and stretching of unsaturated carbonyl group, respectively. Intense bands at 2925 and 2856cm^{-1} could be attributed to C-H stretching and vibration of C-H bonds of saturated groups. The absorption band around 1460 and 1579cm^{-1} could be attributed to aromatic ring C=C stretching as well as chelated –COO group[24]. The ^{1}HNMR spectrum (400 MHz, DMSO) (Appendix 2) showed the presence of two broad singlets at 2.75ppm and 3.88ppm which correspond to protons of CH_3 and OCH_3 attached to an aromatic ring[23]. There are four sets of aromatic protons of which three of them (δ7.30 (dd, 8.3, 1.3 Hz), δ7.61 (d, J=1.3 Hz), δ7.70 (t, J=7.9 Hz)) belong to ring A having ABX multiplicity pattern with ortho-para and one meta coupling systems and a singlet peak at δ7.72. Furthermore, highly deshielded signals at δ12.78 and δ11.78 could be attributed to a hydroxyl group peri to carbonyl carbon of aromatic compounds (Mudin *et al.*, 2018). These data suggest that compound is most likely anthraquinone type. The ^{13}CNMR (101 MHz, DMSO) spectrum (Appendix 3) revealed seventeen carbon signals, two of which are carbonyl carbons at δ189.65 and 182.21ppm that are typical of anthraquinone skeleton. The peaks at δ 167.64 and 52.99 reveal the presence of methyl ester group attached to an aromatic ring. The spectrum revealed eleven quaternary carbons. These are six non-oxygenated aromatic quaternary carbons a (at δ122.89, 117.13, 132.77, 124.91, 137.17 and 141.46), two oxygenated aromatic quaternary carbons at (at δ161.89 and 159.33) and three carbonyl carbons (δ 167.64, 182.21 and 189.65) (Table No.2). The DEPT-135 spectrum (Appendix 4) showed that there are four aromatic methines at δ112.45, 136.57, 118.82 and 124.52. The peaks at δ20.36 and δ52.99 could be attributed to a methyl connected to aromatic ring and methyl of ester (a methoxy group), respectively (Table No.2). Thus, based on the above spectral data and comparison with literature reported data, the structure of compound ABM1 was proposed to be similar with 1, 6-dihydroxy-8-methyl-anthracene-7-methyl ester-9, 10-dione or Aloesaponarin I (Figure No.2) (Mudin *et al*, 2018, Abdissa *et al*, 2017, Tamiru and Ebsa, 2022)[23-25]. It was reported that this compound is found in many aloe species such as *Aloe gilbertii*, *Aloe secundiflora, Aloe pulcherrima, Aloe turkanensis* and *Aloe eleganis*[23,24]. This is the first report of isolation from *A. calidophila*. The ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectra data of compound ABM1 are given below (Table No.2).

Compound ABM2

Compound ABM2 (17.3mg) was isolated as yellow crystal with a melting point of 13-14^{o}C and an R_f value of 0.58 (20% ethyl acetate in n-hexane). In the FTIR spectrum of compound ABM2 (Appendix 5), the bands at 2920cm^{-1} and 2850cm^{-1} indicated

the C-H stretch of olefinic group and alkyl groups, respectively. The band at 1707cm^{-1} indicated C=O stretch of carbonyl groups. This band could be attributed to C=O stretch of carboxylic acid whereas the intense absorption band at 1272cm^{-1} indicates C-O stretching. The absorption bands at 1467 and 1292cm^{-1} indicate characteristic bending of C-H bending of CH_2 and CH_3, respectively. Thus, the observed carbonyl group (C=O) and C-O stretching bands in the FTIR spectrum (Appendix 6) suggest that the compound ABM2 is most likely a carboxylic acid. The ^{1}H NMR (400MHz, $CDCl_3$) spectrum (Appendix 6) showed the peak at δ 0.9 could indicate the presence of terminal methyl (-CH_3) groups whereas the peaks at δ1.33 and 1.63 could suggest the presence of aliphatic methylene (-CH_2) groups. The peak at δ2.07 indicated presence of a methylene group that is directly bonded to C=C bond. Instead, the peak at δ2.37 indicates presence of methylene that is directly bonded to a carboxylic acid group whereas the peak at δ5.38 indicates presence of olefinic protons in the structure[26]. The observed spectral data and literature reported data suggest that compound AMB2 could be an aliphatic acid. The ^{13}CNMR (101 MHz, $CDCl_3$) spectrum (Appendix 7) of compound ABM2 showed peak at δ14.12 could indicate the presence of terminal methyl (-CH_3) group attached to aliphatic acids. The peaks at δ130.03 and 127.90 the presence of at least one C=C bond whereas a single peak at δ179.42 indicate C=O stretch of quaternary carbon atom of carboxylic acid. On the other hand, the peaks in the range of δ22.68 to 33.94 indicated presence of more than one methylene (-CH_2) carbons. The DEPT-135 spectrum (Appendix 8) of this compound also showed single peaks that could indicate the presence of terminal methyl (-CH_3) carbon at δ14.08, methylene carbons at δ22.71 to 33.95 and olefinic methine carbons at δ130.03 and 128.07 (Table No.4). Based on the observed spectral data and comparison of the data from literature reports, compound ABM2 was suggested to be identical with oleic acid (Figure No.3)[26-28]. The ^{13}CNMR, ^{1}HNMR and DEPT-135 spectra data of this compound from *A. calidophila* root extract are reported below (Table No.3). This is the first report of isolation of compound ABM2 from *Aloe calidophila* plant.

Compound ABM3

Compound ABM3 (15.7mg) was isolated as orange crystal with a melting point of 135-137^{o}C and an R_f value of 0.75 (15% ethyl acetate in n-hexane). The FTIR spectrum of compound ABM3 (Appendix 9) showed a broad band at 3474 cm^{-1} indicating the presence of -OH group in the compound. The absorption bands at 2933 and 2860cm^{-1} could indicate the presence of C-H stretching for asymmetrical CH_3 and symmetrical CH_2, respectively. The bands at 1739cm^{-1} and 1056cm^{-1} indicated the unsaturation that is C=C and presence of C-O stretches in the compound, respectively. The ^{1}H NMR (400MHz, $CDCl_3$) spectrum (Appendix 10) of compound ABM3 revealed the presence of methyl (CH_3), methylene (CH_2), methines (CH) protons, and one hydroxyl (-OH) group. The presence of two singlets at δ0.69 and 1.01 suggest the existence of two CH_3 attached to the quaternary carbons. The multiplet peaks at δ2.33 indicates that two methylene protons that are adjacent to the carbon attached to the hydroxyl group. A signal at δ3.55 presents a proton attached to a hydroxyl (OH) group bearing carbon. This a characteristic proton of steroids (The overlapping triplets at δ5.37 are assigned to the olefinic proton (a proton attached to a C=C double bond). The FTIR and ^{1}HNMR spectral patterns suggest that compound AMB3 is most likely a cholest-5-en-3-ol (B-sterol)[26]. The ^{13}CNMR (101 MHz, $CDCl_3$) spectrum of compound ABM3 (Appendix 11) showed the presence of twenty-nine carbons that include six methyl, eleven methylenes, nine methine and three quaternary carbon signals. The carbon peaks at δ140.75 and 121.73 could be assigned to the olefinic carbons (C=C). The peak at δ71.82 indicates a carbon atom bearing hydroxyl (-OH) group. The peaks at δ11.85 and 19.40 could be assigned to angular methyl carbons (Table No.5). The DEPT-135 spectrum (Appendix 12) compound AMB3 indicates the peaks for the CH_3, and CH while peaks that appear in the δ20-40 region

indicate the presence of eleven CH_2 groups. Three signals that do not appear in the DEPT-135 spectrum confirm the presence of three quaternary carbons in compound AMB3. Thus, based on the above FTIR, ^{1}HNMR, ^{13}CNMR and Dept-135 spectral data and comparison with previous literature reports[26,29-31], the structure of compound ABM3 was suggested to be β-sitosterol (Figure No.4). Isolation of this compound has not been reported from *Aloe calidophila* plant. The ^{13}C-NMR and ^{1}H-NMR and spectra data of this compound ABM3 (β-sitosterol) from *Aloe calidophila* root extract are reported below (Table No.4).

Antibacterial activity tests result

The antibacterial activity of the crude extracts from root of *A. calidophila* and isolated compounds (ABM1, ABM2 and ABM3) were tested at the concentration of 200 and 300µg/ml against four pathogenic bacterial strains: two Gram-positive bacteria (*S. aureus* and *S. pyogenes*) and two Gram-negative bacteria (*E. coli* and *P. auruginosa*) (Appendix 13). The result revealed that dichloromethane/methanol (1:1 v/v) and methanol crude extract showed the zone of inhibition ranging from 7 to 9mm for gram-positive bacteria and from 6.5 to 8mm for gram-negative bacteria. The dichloromethane/Methanol (1:1v/v) extract showed higher antibacterial activity against Gram-positive bacteria such as *S. aureus* (8 mm) and *S. pyogenes* (8.5mm) than Gram-negative bacteria such as *E. coli* (7.5mm) and *P. auruginosa* (7mm) at the concentration of 300 mg/ml. When dichloromethane/Methanol (1:1v/v) extract is compared with methanol extract against all selected pathogenic strain, methanol extract showed relatively higher inhibition zone at both concentrations (200 and 300mg/ml) (Table No.5). The isolated compounds showed inhibition zones ranging from 8 to 13mm for gram-positive and from 7.5 to 11.5mm for gram-negative bacteria. The isolated compounds showed significant activities specially in compound ABM3 (Aloesaponarin I) at 300µg/ml against *S. aureus*, *S. pyogen*, *E. coli* and *P. auruginosa* with inhibition zone of 12, 13, 11.5 and 10.5mm, respectively, compared to positive control (Ampicillin) (13.5, 14.5, 13 and 12.5mm, respectively). The data showed that the isolated compounds (ABM1, ABM2 and ABM3) possess relatively higher bacterial zones of inhibition as compared to the crude extracts when tested against the abovementioned bacterial strains (Table No.6). The isolated pure compound showed promising activities against *S. pyogenes* and *S. aureus* with ABM1 (Aloesaponarin I) and ABM2 (Oleic acid) showing relatively the best antibacterial activity against *S. pyogenes* when compared to the rest of one compound ABM3 (β-sitosterol) (Table No.6). Comparatively, compound ABM1 (Aloesaponarin I) has revealed better antibacterial activities against the four strains. These finding is consistent with previous literature[27]. As described in literatures, this compound occurs in the roots of many Aloe species including *A. saponaria* and also showed antimicrobial activities[24]. Compound ABM3 (Aloesaponarin I), was reported to be a promising compound to be used as a lead for development of antimalarial drugs as it showed high antimalarial activity against chloroquine-resistant strain plasmodium parasite[32]. Finally, the observed antibacterial activities from root of *A. calidophila* crude extracts and the isolated compounds could support the traditional use of the plant for the treatment of different human illnesses including bacterial infection and malaria reported in literature[18]. Thus, further tests are recommended on large number of bacterial strains to determine the potentials of crude extracts and compounds isolated from *Aloe calidophila* in order to get compounds that can be used in the discovery of new antibacterial agents.

Table No.1: Phytochemical screening test results of the dichloromethane/methanol (1:1) and methanol extracts of *Aloe calidophila* root

S.No	Secondary metabolites	Types of test	Extracts	
			Dichloromethane/methanol (1:1)	Methanol
1	Alkaloids	Mayer's reaction	+	+
2	Anthraquinones	Borntragers	+	+
3	Flavonoids	Alkaline reagent	+	+
4	Glycosides	Keller-kiliani	+	+
5	Phenols	Ferric chloride	+	+
6	Saponins	Froath Foam	+	+
7	Steroids	Liebermann-Burchardt	-	-
8	Tannins	Alcholic ferric chloride	-	-
9	Terpenoids	Liebermann-Burchardt	+	+

(+): presence of constituent; (-): absence of constituent

Table No.2: Comparison of the ^{1}HNMR (400 MHz, DMSO) and ^{13}C NMR (101 MHz, DMSO) data of the compound ABM1 (Aloesaponarin I) and reported data

Position	NMR spectral data of compound ABM1 (ppm)			Literature reported spectral data of Aloesaponarin I[24,25]	
	^{13}C-NMR data	^{1}H-NMR data	DEPT-135 data	^{13}C-NMR data	^{1}H-NMR data
1	161.89			161.5	
2	112.45	7.30 (dd)	CH (112.45)	112.0	7.31 (dd)
3	136.57	7.61 (d)	CH (136.57)	136.8	7.62 (t)
4	118.82	7.0 (t)	CH (118.82)	118.1	7.77 (dd)
5	130.07	7.72 (s)	CH(124.91)	130.82	7.80 (s)
6	159.33			159.52	
7	122.89			122.2	
8	117.13			117.5	
9	189.65			189.6	
10	182.21			182.3	
11	132.77			132.8	
12	124.91			124.5	
13	137.17			138.8	
14	141.46			142.91	
-COO	167.64			167.96	
-OCH_3	52.99	3.88 (s)	CH_3 (52.99)	52.79	4.06 (s)
-CH_3	20.36	2.75 (s)	CH_3 (20.36)	20.37	2.95 (s)
1-OH	12.78	12.78 (s)			12.91 (s)
6-OH	11.78	11.78 (s)			10.45 (s)

Table No.3: The ^{1}HNMR (400 MHz, $CDCl_3$), ^{13}CNMR (101 MHz, $CDCl_3$) and DEPT-135 spectral data of the compound ABM2 and literature reported data of oleic acid[27-29]

Position	NMR Spectra data of compound ABM2 (ppm)			Literature Spectra data[27-29]	
	^{13}C-NMR data	^{1}H-NMR data	DEPT-135 data	^{13}C-NMR data	^{1}H-NMR data
1	179.42	-	-	179.7	-
2	33.94	2.37 (t, 2H)	33.95 (CH_2)	34.0	2.36 (t, 2H)
3	24.67	1.65 (m, 2H)	24.68 (CH_2)	24.68	1.63 (m, 2H)
4	29.14	1.27 (m, 2H)	29.15 (CH_2)	29.14	1.27 (m, 2H)
5	29.08	1.27 (m, 2H)	29.08 (CH_2)	29.07	1.27 (m, 2H)
6	29.40	1.27 (m, 2H)	29.42 (CH_2)	29.4	1.27 (m, 2H)
7	29.59	1.33 (m, 2H)	29.59 (CH_2)	29.6	1.32 (m, 2H)
8	27.15	2.07 (m, 2H)	27.05 (CH_2)	27.16	2.02 (m, 2H)
9	127.90	5.38 (d, 1H)	128.07 (CH)	129.7	5.4 (d, 1H)
10	130.03	5.38 (d, H)	130.03 (CH)	130.03	5.4 (d, 1H)
11	27.21	2.07 (m, 2H)	27.21 (CH_2)	27.22	2.02 (m, 2H)
12	29.70	1.27 (m, 2H)	29.70 (CH_2)	29.70	1.27 (m, 2H)
13	29.36	1.27 (m, 2H)	29.35 (CH_2)	29.33	1.27 (m, 2H)
14	29.55	1.27 (m, 2H)	29.53 (CH_2)	29.53	1.27 (m, 2H)
15	29.24	1.27 (m, 2H)	29.24 (CH_2)	29.25	1.27 (m, 2H)
16	31.93	1.27 (m, 2H)	31.93 (CH_2)	31.94	1.27 (m, 2H)
17	22.68	1.27 (m, 2H)	22.70 (CH_2)	22.68	1.27 (m, 2H)
18	14.12	0.90 (t,3H)	14.08 (CH_3)	14.13	0.88, t

Table No.4: Comparison of the ^{1}H-NMR (400 MHz, $CDCl_3$) and ^{13}CNMR (101 MHz, CDCl3) data of the compound ABM3 (β-sitosterol) and reported data

Position	NMR Spectra data of compound ABM3 (ppm)		Literature NMR spectra data of *β-sitosterol*[27,30-32]	
	^{13}CNMR data	^{1}HNMR data	^{13}CNMR data	^{1}H-NMR data
1	37.25		37.3	
2	31.92		31.9	
3	71.82	3.55 (m)	71.8	3.52 (m)
4	42.29		42.3	
5	140.75		140.8	
6	121.73	5.37 (s)	121.7	5.36 (br s)
7	31.71		31.7	
8	32.23		32.0	
9	50.14		50.1	
10	37.00		36.6	
11	21.09		21.1	
12	39.79		39.8	
13	42.39		42.4	
14	56.12		56.7	
15	24.3		24.3	
16	28.61		28.6	

17	56.07		56.1	
18	11.85	0.69(s)	11,9	0.69 (s)
19	19.40	1.01(s)	19.4	1.01 (s)
20	36.15		36.2	
21	14.12	0.90 (dd,6.8)	18.8	0.92 (s)
22	34.84		34.0	
23	26.14		26.1	
24	45.84		45.8	
25	29.15		29.2	
26	19.82	0.84 (d,6.65)	19.8	0.814 (d, 6.5)
27	19.03	0.86 (d,6.65)	19.0	0.833 (d, 6.5)
28	23.06		23.1	
29	11.98	0.87 (t)	12,0	0.845 (t, 7.5)

Table No.5: Antibacterial inhibition zones (mm) of crude extracts and isolated compounds of Aloe calidophila

S.No	Tested samples	Sample code and Concentration	Bacteria species and zone of inhibitions (mm)			
			Gram-positive		Gram-negative	
			***S. aureus* ATCC25923**	***S. pyogen* ATCC19615**	***E. coli* ATCC25922**	***P.aeruginosa* ATCC27853**
1	Dichloromethane/Methanol Extract	A1=200ug/ml	7	7	6.5	6.5
		A2= 300ug/ml	8	8.5	7.5	7
2	Methanol Extract	B1= 200ug/ml	7.5	8	7	7
		B2= 300ug/ml	9	9	8	8
3	Compound ABM_3	C1= 200ug/ml	8	9	8	7.5
		C2= 300ug/ml	9.5	10	9	9
4	Compound ABM_2	D1= 200ug/ml	9	10	9	8
		D2= 300ug/ml	10	11	10	9.5
5	Compound ABM_1	E1= 200ug/ml	10.5	11	10	9
		E2=300ug/ml	12	13	11.5	10.5
6	Ampicillin (positive control)		13.5	14.5	13	12.5

Key: +ve= positive, *S. aureus= Staphylococcus aureus*, S. pyogenes=*Streptococcus pyogenes*, *E. coli=Escherichia coli*, *P.aeruginosa=Pseudomonas aeruginosa*

Scheme No.1: The outline of the procedures used for extraction

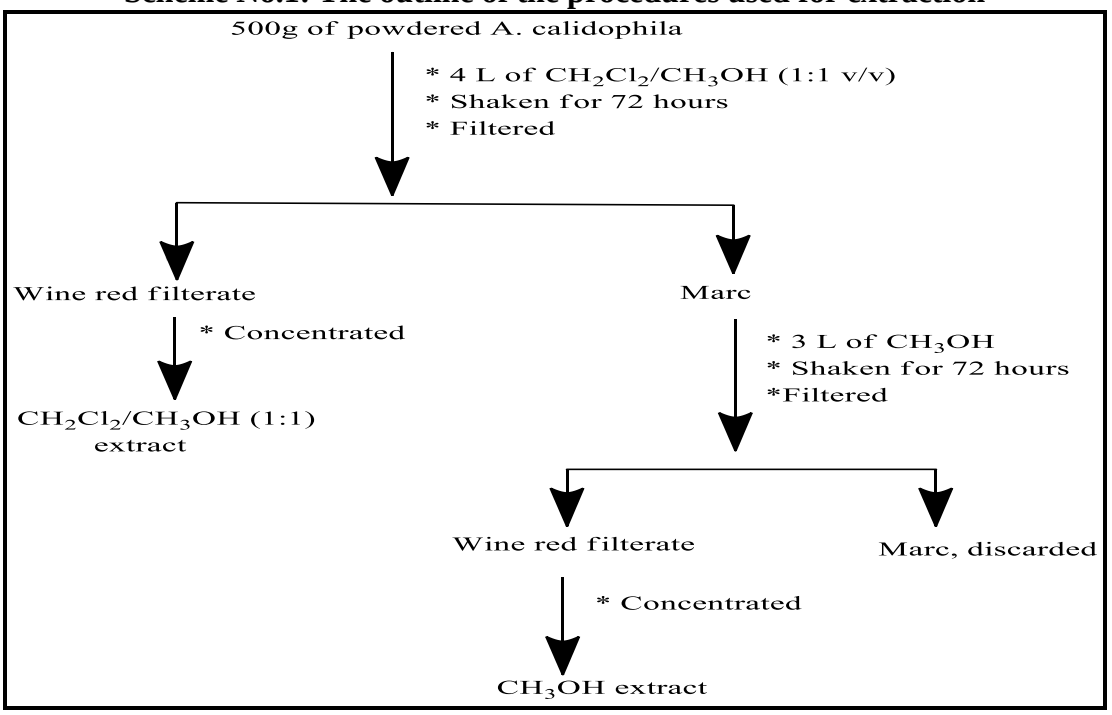

Figure No.1. The leaf (a) and root part (b and c) of *Aloe calidophila* (Photo taken by Abinet M on March **19,** (2023

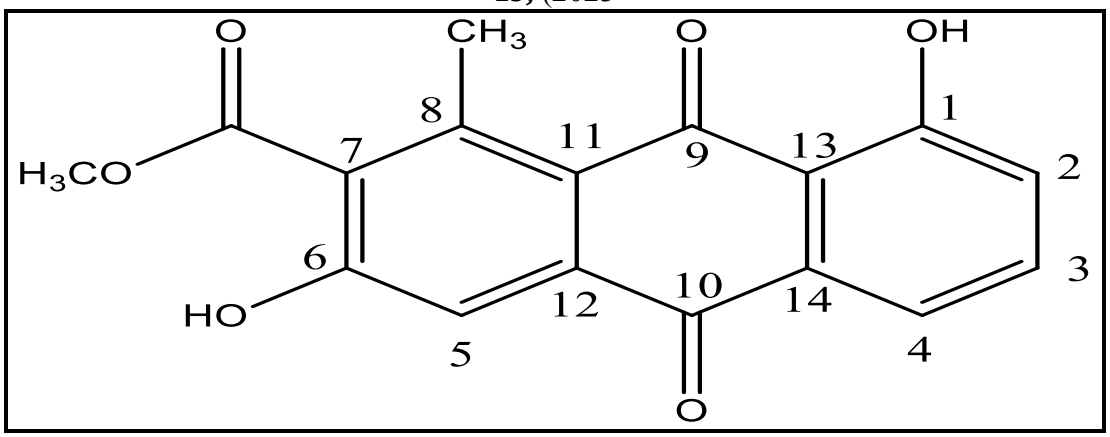

Figure No.2: The proposed structure of compound ABM1 (Aloesaponarin I)

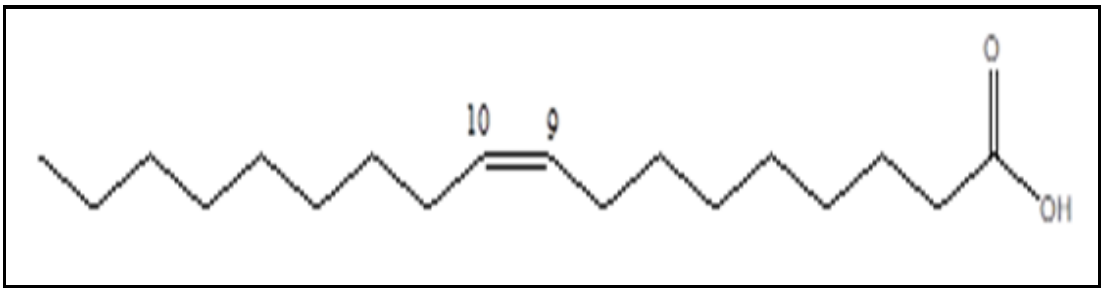

Figure No.3: The proposed structure of compound ABM2 (Oleic acid)

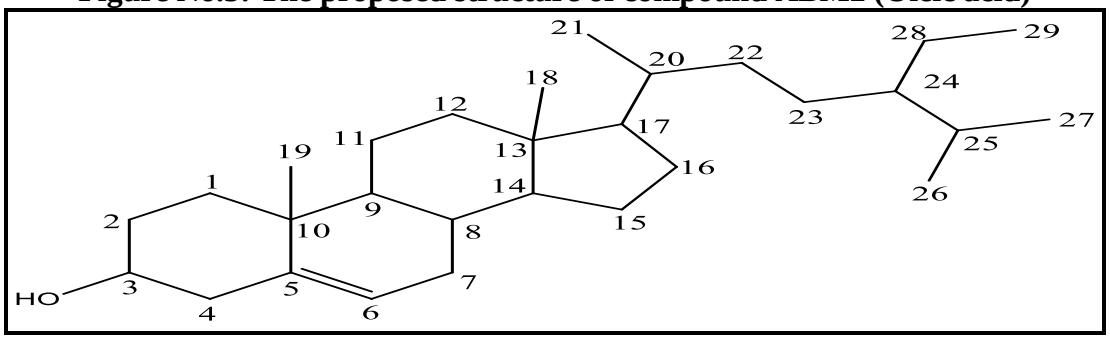

Figure No.4: The proposed structure of compound ABM3 (β-sitosterol)

CONCLUSION

The phytochemical screening tests of the dichloromethane/Methanol (1:1 v/v) and methanol crude extracts of roots of *A. calidophila* revealed the presence of alkaloids, anthraquinones, flavonoids, phenols, terpenoids, steroids and saponins. The chromatographic separation of dichloromethane/Methanol (1:1 v/v) root extract of *A. calidophila* result in isolation of three compounds namely aloesaponarin I (ABM1), oleic acid (ABM2) and β-sitosterol (ABM3). Their structures were determined based on spectral (IR and NMR) data and comparison of the data with literature reports. These compounds were isolated for the first time from *A. calidophila.* The crude extracts and isolated compounds were subjected to *in vitro* test to evaluate their antibacterial activities using disc diffusion method against four bacterial strains specifically (*E. coli* ATCC25922, *S. aureus* ATCC25923, *P. aeruginosa* ATCC27853 and *S. pyogenes* ATCC19615). The results revealed that the isolated compounds possess promising antibacterial activities with aloesaponarin I (compound AMB1) with relatively the highest activity against all the bacterial strains used in the study. Therefore, the further *in vitro* antibacterial activity tests are recommended using several bacterial strains to discover lead compounds from the roots *A. calidophila* in search of new antibacterial agents.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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