Phytochemical Analysis of Roots of *Aloe gilbertii* and *Millettia ferruginea*

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Abstract

There are about 6500 species of higher plants in Ethiopia making the country one of the most diverse floristic regions in the world. Many medicinal plants are found in southern and south western parts of the country. Nevertheless, there are limited ethnobotanical information and knowledge on the chemical constituents of these medicinal plants. As part of the ongoing project to identify the chemical constituents of medicinal plants of Southern Ethiopia, a comprehensive phytochemical analysis was conducted on the roots of *Aloe gilbertii* and *Millettia ferruginea.* Phytochemical screening tests of the crude root extracts were done accompanied by complete isolation and spectroscopic characterization of compounds isolated from the extracts. Phytochemical screening tests revealed the presence of alkaloids, anthraquinnes, and flavonoids in the roots of both plants whereas terpenoids were absent. Two flavonoids (**1** and **2**) and one known anthraquinone, 8-methoxychrysopanol (**3**), were identified from the roots of *M. ferruginea* and *A.gillbertii*, respectively. The structures of these compounds were determined using spectroscopic techniques (UV-Vis, IR, ^{1}H NMR, ^{13}C NMR, DEPT-135, COSY, gHSQC and gHMBC) and comparison with literature. The presence of flavonoid and anthraquinone derivatives, known in literature for their various pharmacological activities, in the roots may be attributed to the wide traditional use of the plants.

Key words: anthraquinone, ethnobotany, flavonoids, medicinal plants

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INTRODUCTION

There are about 6500 species of higher plants in Ethiopia making the country one of the most diverse floristic regions in the world. The greater concentrations of these medicinal plants are found in southern and south western parts of the country. Nevertheless, there are limited ethnobotanical information and knowledge on the chemical constituents of these medicinal plants. As part of the ongoing project to identify the chemical constituents of medicinal plants of Southern Ethiopia, a comprehensive phytochemical analysis was conducted on the roots of *Aloe gilbertii* and *Millettia ferruginea* Phytochemical screening tests of the crude root extracts were done accompanied by complete isolation and spectroscopic characterization of compounds isolated from the extracts. Phytochemical screening tests revealed the presence of alkaloids, anthraquinnes, and flavonoids in the roots of both plants whereas terpenoids were absent in the roots of both plants. Two flavonoids (**1** and **2**) and one known anthraquinone, 8-methoxychrysopanol (**3**), were identified from the roots of *M. ferruginea* and *A.gillbertii*, respectively. The structures of these compounds were determined using spectroscopic techniques (UV-Vis, IR, ^{1}H NMR, ^{13}C NMR, DEPT-135, COSY, gHSQC and gHMBC) and comparison with literature. The presence of flavonoid and anthraquinone derivatives, known in literature for their various pharmacological activities, in the roots may be attributed to the wide traditional use of the plants.

Throughout history, mankind has always been interested in naturally occurring compounds from prebiotic, microbial, plants and animals sources. In Ethiopia, peoples have been using plants in various forms of formulations to treat various diseases (Abebe 1986). For instance, *M. ferruginea* (Figure 1) fruits powder mixed with butter is topically applied to treat skin infection (Mesfin et al., 2009) and fruits powder mixed with honey is taken orally for amoeba and treating 'mujele' (chigger) with fruit paste mixed with butter (Teklehaymanot andand Giday, 2007). The fresh leaf and roots of *A. gilbertii* (Figure 2) is traditionally used to treat liver related disease in Ethiopia (Lulekal et al., 2008). In an

ongoing project to identify the chemical constituents of medicinal plants of Southern Ethiopia, so far we have done a comprehensive phytochemical analysis of roots of *Zanthoxylum chalybeum* (Anza et al., 2014), *Senna didymobotrya* (Alemayhu et al., 2015), *Crotalaria incana* (Anza et al., 2015), *Clerodendrum myricoides* (Esatu et al., 2015), *Lantana camara* (Taye et al., 2015), and *Vernonia auriculifera* (Albejo et al., 2016). We hereby report a comprehensive phytochemical analysis of the roots of *M. ferruginea* and *A. gilbertii*. The genus *Aloe* is known for its bioactive anthraquinones and phenolic components including C-glucosyl derivatives such as barbaloin, aloin A and B, aloenin, aloe-emodin (Fanali et al., 2010). The genus *Millettia* is known for its bioactive flavonoids components including leptobotryanone, maximaisoflavone B, robustigenin, medicarpin, maackiain, genistein, biochanin A, prunetin, chrysoeriol, kaempferol and desmoxyphyllin A (Feng et al., 2007; Sritularak et al., 2006; Rayanil et al., 2011; Pancharoen et al., 2008).

Figure 1. *Millettia ferruginea* (picture taken in Dec., 2014)

Figure 2. *Aloe gilbetii* (picture taken in Dec., 2014)

MATERIALS AND METHODS

General procedures and equipment

UV-Vis spectrum was measured with GENESY's spectrometer (200-400nm) in methanol at room temperature. Infrared (KBr pellet) spectrum was recorded on Perk-Elmer BX infrared spectrometer in the range 400-4000cm^{-1}. Nuclear Magnetic Resonance (NMR) analysis was recorded on Bruker Avance 400MHz spectrometer with tetramethylsilane (TMS) as internal standard. Structural assignments were performed on the basis of 1D NMR (^{1}H NMR, ^{13}C NMR, DEPT-135) and 2D NMR (COSY, gHMQC, gHMBC) spectra. Thin Layer Chromatography (TLC) was conducted using silica gel 60 F_{254}. Column chromatography was performed on silica gel 60 (60-100 mesh).

Plant materials

The roots of *M. ferruginea* were collected in January 2013 from Alamura hill (7° 00' N; 38° 30' E ; 1800 m a. s. l.), in Sidama Zone of SNNPR, Ethiopia whereas roots of *A. gilbertii* were collected from Jello Kebele, Shashemene Woreda, Oromia region that is found 250 km south of Addis Ababa, Ethiopia. All plant materials collected were identified and authenticated by a botanist from Department of Biology, Hawassa College of Teachers Education. Specimen of each plant species was deposited at the herbarium of Hawassa College of Teachers Education, Hawassa, Ethiopia.

Extraction and isolation

The collected plant materials were dried and grounded into fine powder with the help of mortar and pestle. The grounded roots (each 500 g) were extracted by cold percolation with CH_2Cl_2/CH_3OH (1:1) three times for 24 hrs while shaking at speed of 230 r/min and temperature controlled at 28.0 ^{O}C. The marc left was further extracted with methanol (100%) as above. The extracts were concentrated using rotary evaporator (40 ^{o}C) and gave yield of 59 g (11.8%) for *M. ferruginea*, and 49.2 g (9.84 %) for *A. gilbertii*. The crude extract obtained was screened for the presence of various classes of secondary metabolites following the standard protocols (Pradeep et al., 2014; Saleem et al., 2014). Isolation of compounds from the crude extracts was carried out using silica gel column chromatography and solvent mixture of n-hexane and ethyl acetate with gradual increase in polarity.

Phytochemical screening tests for secondary metabolites

Phytochemical screening test was carried out on the crude extract of CH_2Cl_2:CH_3OH (1:1) using standard procedures reported in literature (Pradeep et al., 2014; Saleem et al., 2014) in order to identify the type of secondary metabolites present in the crude extracts.

Alkaloid was screened by adding 1mL of 1% HCl to 3mL of the test extract, dissolved in methanol, in a test tube. The mixture was heated for 20min, cooled and filtered. Then 1mL of the filtrate was tested with 0.5mL Wagner's, Hager's and Mayer's reagents. Formation of reddish brown precipitate for Dragendorff's and Wagner's reagents, yellow precipitate for Hager's reagent and cream precipitate for Mayer's reagent indicated the presence of alkaloids (Pradeep et al., 2014; Saleem et al., 2014).

Flavonoids were determined by Mg-HCl reduction test. A piece of magnesium ribbon (powder) and 3 drops of conc. hydrochloric acid were added to 3mL of the test extract dissolved in methanol. A red coloration indicated the presence of flavonoids. Five milliliters of dilute ammonia solution was added to 5mL of the aqueous filtrate of extract followed by the addition of 1mL concentrated H_2SO_4. A yellow coloration indicated the presence of flavonoids. The yellow color disappeared on standing for 10 min (Pradeep et al., 2014; Saleem et al., 2014).

Terpenoids were isolated by Salkowski test i.e. about 5mL of the extract, dissolved in methanol, was mixed with 2mL of chloroform and 3mL of concentrated H_2SO_4 was added. A reddish brown coloration at the interface confirmed the presence of terpenes (Pradeep et al., 2014; Saleem et al., 2014).

For Tannin identification, about 0.2g of the dried powdered samples was boiled in 10mL of distilled water in a test tube and then filtered. Addition of 0.1% $FeCl_3$ solution resulted in a characteristic blue, blue-black, green or blue-green color which confirmed the presence of tannins (Pradeep et al., 2014; Saleem et al., 2014).
In order to screen saponins about 0.2g of powdered sample extract was boiled in 2mL of distilled water on a water bath and filtered. A fraction of aqueous filtrate about 1mL was mixed with 2mL of distilled water and shaken vigorously to form a stable persistent froth. The frothing was mixed with three drops of olive oil and shaken vigorously. Formation of an emulsion confirms the presence of saponins (Pradeep et al., 2014; Saleem et al., 2014).

RESULTS AND DISCUSSION

Phytochemical screening tests of the crude CH_2Cl_2/CH_3OH (1:1) extracts of the roots of the plant materials used in the experiment revealed the presence of alkaloids, anthraquinnes, and flavaonoids in the roots of both plants whereas terpenoids were absent in both plants (Table 1). Moreover, the screening test indicated the presence of tannins and saponins in the roots of *M. ferruginea* only (Table 1). The presence of flavonoid and anthraquinone derivatives, known in literature for their various pharmacological activities, in the roots may be attributed to the wide traditional use of the plants.

The crude extracts obtained from the roots of the two plant species were subjected to isolation using column chromatography. The extracts in the column were eluted using a mixture of n-hexane and ethyl acetate with gradual increase in polarity. This led to isolation of flavonoids (1, 2) and 8-methoxy chrysopanol (3) from roots of *M. ferruginea* and *A.gillbertii,* respectively. The structures of these compounds were identified by exhaustive spectroscopic analysis (UV-Vis, IR, ^{1}H NMR, ^{13}C NMR, DEPT-135, COSY, gHSQC and gHMBC) and comparing with literature spectral data.

Table 1: Phytochemical screening test results of the crude dichloromethane: methanol (1:1) extract

Plant Constituent	**Reagent used**	***A. gilbertii***	***M. ferruginea***
Alkaloids	Dragendroff's reagent	+	+
Tannins	$FeCl_3$	-	+
Anthraquinones	$HCl + CHCl_3 + NH_3$	+	+
Saponins	Warming in water bath	-	+
Terpenoides	Chloroform and conc. Sulphuric acid	-	-
Flavonoides	Dilute ammonia solution + dil. HCl	+	+

+ indicates presence, - indicates absence

Compound 1 was obtained as white crystalline solid (7mg) from the dichloromethane/methanol (1:1). The UV-Vis spectrum in H_2O showed maximum absorption band at $\lambda_{max;}$ 263nm indicating the presence of π-π^* transition in conjugated system of C=C double bond. The IR spectrum exhibited strong absorption band of conjugated carbonyl at 1641cm^{-1}, and medium absorption around 1600cm^{-1} and 1470cm^{-1} suggesting the presence of aromatic system. The ^{1}H NMR spectrum suggested the presence of two methyls at δ 1.56 (6H, *s*), two methoxy at δ 3.96 (3H, *s*) and δ 3.92 (3H, *s*), methine proton singlet at δ 7.81,H-2 (1H, s), three doublet protons at δ7.1 H-2'(1H, *d*), δ 6.83, H-5' (1H, *d*) and δ 6.76, H4" (1H, *d*) and a double doublets at δ 6.96, H-6' (1H, *dd*) in aromatic region and one doublet at δ 5.70, H-3" (1H, *d*) in sp^2 system and one methylene dioxy ($-OCH_2O-$) protons at δ 6.00, H-2"' (2H, *s*) (Table 2).

The ^{13}C NMR spectrum coupled with DEPT-135 showed twenty two carbons attributed to one carbonyl carbon at δ 175.4, six oxygenated quaternary aromatic carbons at δ 140.1, 147.6, 147.6, 149.2, 151.2 and 153.1, one olefinic quaternary carbon at δ 125.4, three quaternary aromatic carbons at δ 125.7, 113.2 and 106.2; three methine

carbons in aromatic region at δ 122.6, 110.1, 108.3,one oxygenated olefinic methine carbons at δ 150.4, two olefinic methine carbons at δ 129.4 and 115.1, one oxygenated quaternary aliphatic carbon at δ 78.2, two methoxy carbons at δ 62.3 and 61.5, one methylene dioxy (OCH_2O) at δ 101.1 and two methyl carbons at δ 28.1 (Table 2). The peaks at δ 62.25 and 61.49 suggest the existence of methoxy group attached to aromatic sp^2 system. The peak at δ 101.1 suggests the existence of oxymethylene group.

The UV-Vis (λ_{max} = 263nm) spectrum coupled with the ^{1}H NMR chemical shifts at δ 7.81, (1H, *s*, H-2) and ^{13}C NMR chemical shifts at δ 150.4 (C-2), δ 125.4 (C-3) and δ 175.4 (C-4) suggest isoflavone skeleton for this compound. Moreover, the HMQC spectrum which showed correlation between methine protons at δ 7.81, 7.1 6.96, 6.83, 6.76, 6.00 and 5.70 correlate with the carbons at δ 150.4, 110.1, 108.3, 122.6, 129.2, 101.1, and 115.1, respectively(Table 2). From the ^{1}H-^{1}H COSY spectrum evidence, the doublet protons at δ 6.76 (H-4", *d,* J= 8.0) and the doublet protons at δ 5.70 (H-3", *d,* J= 8.0) couple each other suggesting that the two protons are on the adjucent sp^2 carbon atoms. HMBC spectrum (Table 2) revealed that protons H-2 (δ 7.81, *s*) and H-2' (δ 7.1, d, J= 2.4) correlate with the same quaternary carbon at δ 125.7 (C-1') in support of isoflavone skeleton.

Additional correlations observed between the protons H-2' (δ 7.1, d, J= 2.4), H-6' (δ 6.96, *dd,* J= 2.4, 7.8), H-5' (δ 6.83, *d,* J= 7.8) and H-6'' (δ 6.00, *s*, 2H) with the same oxygenated quaternary aromatic carbon C-3' (δ 147.6) coupled with the correlation of protons H-6' (δ 6.96, *d,* , J= 2.4, 7.8), H-5' (δ 6.83, *d,* , J= 7.8) and methylene dioxy protons (H-6'', δ 6.00, *s*, 2H) suggest that the oxymethylene is connected at C-3' and C-4' and ring B have an ABX spin pattern. HMBC correlations between proton H-3" (δ 5.70, *d,* J= 8.0) and H-4" (δ 6.76, *d,* J= 8.0) with the oxygenated aliphatic quaternary carbon C-2'' (δ 78.2) and coupled with correlation of prpton H-4'' (δ 6.76, *d,* J= 8.0) with that of quaternary carbon C-8a at δ 153.0 suggest that the pyran ring is connected to ring A. Finally, from the HMBC spectrum correlation observed between H-4" (δ 6.76, *d,* J= 8.0) with that of oxygenated quaternary carbons C-8a (δ 153.1) and C-7 (δ 149.2) support unequivocal placement of the two methoxy groups at C-5 (δ 151.17) and C-6 (δ 140.06) positions of ring A. Thus, all spectral data were in good concurrence with those reported in the literature (Dagne *et al*. 1989) and this compound was previously reported from the ethanolic extract of the stem bark of the same species and the trivial name was given as 5-methoxy durmillone (1, Figure 3) (Dagne *et.al*, 1989).

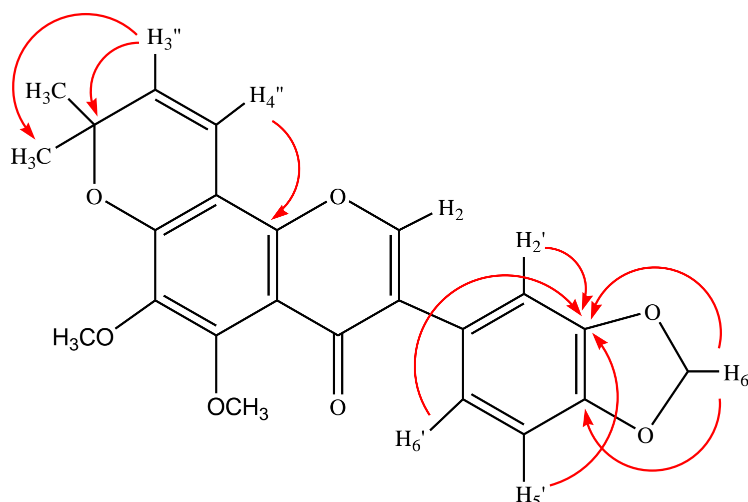

Figure 3: Important HMBC correlations of 5-methoxy durmillone (**1**)

Table 2. Complete NMR data of 5-methoxydurmillone (**1**) (chemical shift (δ) is given in ppm) (400MHz, $CDCl_3$)

Position	^{13}CNMR data of compound 1 (ppm)	^{1}HNMR data of compound 1 (ppm)	^{1}H-^{1}H correlation	HMBC		Reported ^{13}CNMR*	Reported ^{1}HMR data*
				^{2}J	^{3}J		
C-2	150.4	7.81 (H-2, s)			C-1',C-4, C-5	150.2	7.81
C-3	125.4					125.3	
C-4	175.0					175.0	
C-4a	106.7					106.5	
C_5	151.2					151.0	
C_6	140.1					140.0	
C_7	149.2					149.1	
C_8	113.2					113.1	
C_{8a}	153.0					153.0	
$C_{1'}$	125.7					125.6	
$C_{2'}$	110.1	7.10 (H-2', d, J= 2.4)			C-3"	110.0	7.08
$C_{3'}$	147.6					147.5	
$C_{4'}$	147.6					147.5	
$C_{5'}$	108.3	6.83 (H-5', d, J= 7.8)		C-3'	C-1'	108.2	6.85
$C_{6'}$	122.6	6.96 (H-6', dd, J= 2.4, 7,8)		C-1'	C-6, C-2'	122.5	6.93
$C_{2''}$	78.2					68.1	
$C_{3''}$	115.1	5.70 (H-3", d, J= 8.0)	H-7↔H-5		C-2", C-3, C-7	114.9	5.68
$C_{4''}$	129.2	6.76 (H-4", d, J= 8.0)	H-5↔H-7	C-2",	C-8, 2"-CH_3	129.0	6.76
2"-CH_3	28.1	1.56 (H-5", s)				28.0	1.55
O-CH_2-O	101.1	6.00 (H-6", s)				100.0	5.98
5-OCH_3	62.2	3.96 (H-7", s)				62.1	3.96
6-OCH_3	61.5	3.92(H-8", s)				61.3	3.90

*Dagne et al., 1989

Compound **2** have a very comparable NMR data with 5-methoxy durmillone (**1**) except the ^{13}C NMR spectrum. It showed eleven additional signals that can be attributed to two sugar units, two glucopyranosyl moiety of which two signals at δ 66.2 and δ 66.4 are due to methylene carbons of the sugar moeity. The existence of sugar moiety was also supported by signals at δ 4.98 (^{1}H NMR spectrum) and anomeric carbon peaks (^{13}C NMR spectrum) at δ 100.0 (C-1"') and δ 100.9 (C-1""). The downfield chemical shift of C-2"' (δ 87.9), 2"'' (δ 87.8) and C-6"' (66.2), C-6"" (66.3) suggested an interglycosidic linkage between the two glucopyranosyl (1""→6"'). In addition, the chemical shift values of oxygenated quaternary aromatic carbons at δ 167.4 C-7 and δ 106, C-7 and C-8, respectively, reveal the existence of -OH at C-7 and prenyl group at C-8. In agreement with this, the absence of downfield singlet which is a characteristic of the chelated OH group suggest that the position of glucose moiety to be at C-7 (δ 100.0, C-1"', δ_H 5.05). Thus, based on the above spectral data, the structure of compound **2** was proposed to be a close derivative of 5-methoxy durmillone (**1,** Figure 3).

Compound **3** is isolated as yellow powder with R_f value of 0.57 in *n*-hexane/ethylacetae (7:3) solvent system. The IR spectrum showed broad and weak absorption approximately at 3400 cm^{-1} attributed to hydroxyl group. The strong absorption band at approximately 2920 and 2850cm^{-1} revealed the presence of aliphatic C-H stretching vibration. The strong stretching vibration at 1615 cm^{-1} showed the presence of aromatic functionality in the molecule. The absorption bands at 1715 cm^{-1} and 1665 cm-^{1} indicate the absorption of the un-chelated and chelated carbonyl carbon, respectively. The ^{1}H NMR spectrums revealed a singlet signal at δ 12.90 integrated to one proton indicate the presence of chelated hydroxyl group at C-1 *peri* to carbonyl carbon C-9. A triplet peak at δ 7.64 coupled with a doublet proton at δ 7.64 indicates the presence of an ABX aromatic pattern on one ring of anthraquinone skeleton. Two protons showed a weak coupling not well resolved multiplicity (close to broad singlet) at δ7.32 and 7.28 indicates the aromatic protons on C-4 and C-2 of the other ring of the anthraquinone skeleton. A singlet peak at δ 4.08 and 2.99 ppm integrated to three protons indicates the presence of methoxy proton with *peri* effect at C-8 position of ring C

of anthraquinone and a methyl directly connected to an sp^2 carbon (C-3 of ring A) of anthraquinone skeleton. Based on close comparison with literature (Hui et al., 2014), the structure of the compound was found to be a derivative of chrysopanol except substituent on C_8 (hydorxyl replaced by methoxy group). The chemical shift of the methoxy group was desheilded to 4.1 due to the *peri* effect with the carbonyl carbon C-9. Thus, based on the above data, compound **3** was found to be 1-hydroxy-8-methoxy-3-methylanthracene-9,10-dione, known as 8-methoxychrysophanol.

Table 3. Comparison of the observed ^1H NMR (400 MHz, $CDCl_3$) spectroscopic dataof 8-methoxy chrysopnaol (**3**) with the reported value of Chrysophanol

Position	^{1}HNMR data of compound 3 (ppm)	Reported ^{1}HNMR data of Chrysophanol**
1-OH	12.9 s	12.03 *s*
2-H	7.28 *brs*	7.11 *br s*
$3-CH_3$	2.90 *s*	2.47 s
4-H	7.32 *brs*	7.30, *dd*, *J*=1.2
5-H	7.78 *dd* (*J*=7.5, 1.2)	7.83 Hz
6-H	7.66 *dd*	7.66, *t*
7-H	7.78 *dd* (*J*=8.0, 1.2)	7.81 *dd*, *J*=1.1, 7.5 Hz
8-OH	-	12.13 *s*
$8-OCH_3$	4.10 *s*	

**Hui et al., 2014

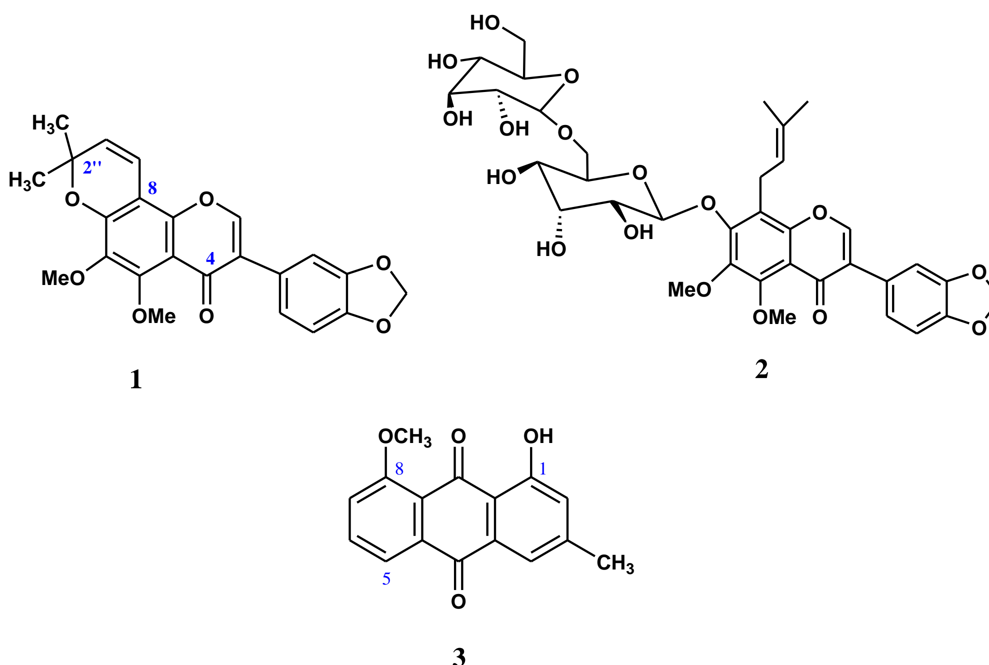

Figure 4. Structures of compounds 1-3.

CONCLUSION

In order to promote Ethiopian herbal drugs and traditional use of medicinal plants, there is an urgent need to evaluate the therapeutic potentials of the drugs as per the WHO guidelines. Bioactive extracts should be validated and standardized on the basis of phytochemical constituents. Despite the rich biodiversity of Ethiopian flora, there are limited information about the type of secondary metabolites present in most of these plants and their biological activity. This paper is a summary of the phytochemical analysis works we have carried out on two plants; *A. gilbertii* and *M ferruginea*. From the two plants, three secondary metabolites (**1**-**3**) are fully characterized. The study is one of the few attempts to phytochemically analyze the polar extracts of these

plants and further work is recommended on polar extracts of the various parts of the plants so as to identify more novel and bioactive secondary metabolites in support of their traditional use.

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