ISOLATION AND CHARACTERISATION OF ALPHA AND BETA AMYRINS FROM PROPOLIS OBTAINED FROM BENUE STATE, NIGERIA

S. S. Ipav[1*] J. O. Igoli[2], T. A. Tor-Anyiin[2] and J. V. Anyam[2]

1. Department of Chemical Sciences, University of Mkar, Mkar, Benue State.
2. Department of Chemistry, University of Agriculture, Makurdi, Benue State.

* Corresponding author: e-mail: selumunipav@gmail.com Tel.: +234 8036487889

ABSTRACT

Chemical composition of propolis has been reported to be phyto-geographical in nature. The aim of this study was to isolate and characterize chemical compounds from hexane and ethyl acetate extract of propolis obtained from Gboko, Benue State, Nigeria. To isolate compounds, extract was subjected to column chromatography by gradient elution using two solvent mixtures - hexane: ethyl acetate and ethyl acetate: methanol. The structure of the isolated compound was established using ^{1}H-NMR and further verification of data on the compound by comparison with literature reports. α-amyrin, β-amyrin, α-amyrin acetate and β-amyrin acetate, known pharmacologically active pentacyclic triterpenoids, were isolated from the hexane and ethyl acetate extract of this propolis.

Keywords: propolis, column chromatography, ethyl acetate, ^{1}H-NMR, α-amyrin, β-amyrin, α-amyrin acetate and β-amyrin acetate.

INTRODUCTION

The use of natural products to solve human challenges has gained wide acceptance. Pharmacological effects can be both good and bad. The side effects recorded from use of drugs has necessitated further research into natural products sourced variously such as from plants, animals, microbes, algae and marine sponges. Propolis, a plant natural product has been reported to exhibit several biological effects such as antioxidant, antibacterial, anti-inflammatory, anticancer [1] and anti-venom [2] among others. Propolis, a plant exudate resin is collected by honeybees to be used as glue and as draught-extruder for beehives [3]. Chemical constituents of propolis have been reported to be dependent of geographical location [4]. Report by [5] shows that chemical constituents of propolis from other regions include fatty and phenolic acids and esters, substituted phenolic esters, bioflavonoids (flavones, flavanones, flavanols and others), terpenes, steroids, aromatic aldehydes and alcohols, and derivatives of sesquiterpenes, naphthalene and stilbenes. The main types of flavonoids that have been reported include rutin (an antihypertensive agent), quercetin (a potent antidiabetic material), galangin and caffeic acid phenethyl ester [5].

In this paper, we report the isolation, structure elucidation of four pentacyclic triterpenes; α-amyrin, β-amyrin α-amyrin acetate, and β-amyrin acetate from propolis obtained from Gboko, Benue State.

MATERIALS AND METHODS

Experimental Procedures

Column chromatography using silica gel 60 (0.040–0.063 mm) (200–425 mesh ASTM) was carried out. Pre-coated aluminum sheets coated with silica gel F250 (Merck, Germany) were used to carry out Thin-layer chromatographic experiments. Nuclear magnetic resonance (NMR) analyses were carried out on a Bruker AVIII (500 MHz) spectrophotometer using $CDCl_3$ as the solvent and TMS as the internal standard. JEOL MStation JMS-700 mass spectrometer was used to acquire mass spectral data.

Preparation of Propolis Extract

Propolis sample was obtained from Beekeepers in Gboko. The vegetation surrounding the hives was

made largely of *Maranthes polyandra, Detarium microcarpum* and *Burkea africana*. The homogenized propolis sample was placed in the thimble of the Soxhlet extractor and refluxed for 24 hours successively with hexane and ethyl acetate at a maximum temperature of 40 °C. The liquid extract obtained was evaporated to dryness using a rotary evaporator at 40 ^{o}C. The dry extract was dissolved in silica gel for column chromatography.

Isolation of Compounds

Dried extract of the sample was extracted with hexane and ethyl acetate. The extracts were combined (based on similarity on TLC) and subjected to column chromatography using silica gel in a glass column. The column was packed wet in a hexane: ethyl acetate (90:10) mixture and eluted with ethyl acetate in hexane gradient starting with 10 % ethyl acetate in hexane and increasing the amount of ethyl acetate by 10% until 100% ethyl acetate yielding 10-ml vials. Further elution with ethanol in ethyl acetate starting with ethyl acetate: ethanol (90:10) mixture. The fractions were examined by TLC, and similar ones were combined and allowed to dry in a fume cupboard. 1H NMR spectroscopy and mass spectrometry were used to analyze the compounds.

One hundred and seventeen 10 mL fractions (numbered SGP-1 to SGP-117) were collected. Concentrated fractions were subjected to Thin Layer Chromatography (TLC) and similar fractions were pooled together. A total of fifteen fractions numbered SGPH-1 to SGPH-15 were collected on the basis of TLC similarity. Fractions SGPH-3 (**IG-JA-SGP 33**) (**A**),SGPH-9 (**IG-JA-SGP 26**) (**B**), SGPH-7 (**IG-JA-SGP 107**)(**C**) SGPH-6 (**IG-JA-SGP 27**) (**D**) were stored at room temperature for spectroscopic analysis. Each fraction was characterized using proton nuclear magnetic resonance (1H-NMR) spectroscopy and mass spectrometry.

RESULTS AND DISCUSSION

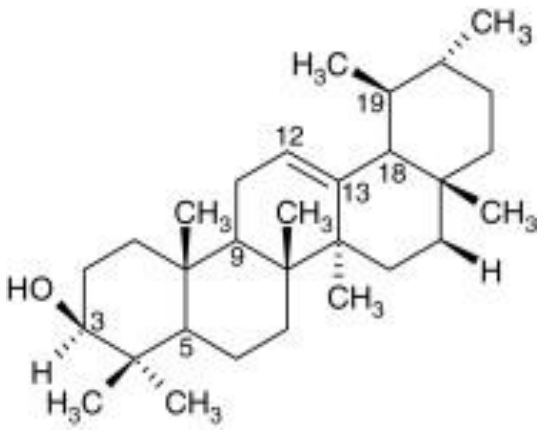
Compound A

The mass spectrum of IG-JA-SGP 33 (**A**) showed a molecular ion peak at m/z 426, which corresponded to a molecular formula $C_{30}H_{50}O$. The formula showed six double bond equivalents, five of which were adjusted in a pentacyclic carbon framework and the remainder in a C=C bond. The mass spectrum also showed fragments at 219 and 203 generated due to a retro-Diels Alder fragmentation indicative of the presence of double bond at position 12 in ring C [6].

1H NMR diagnostic peaks occurred at 5.15 (IH, H-12), 4.52 (1H, m, H-3). The H NMR analysis also showed the presence of 8 methyl groups associated to H-23 - H-30 assigned as follows 0.85 (H, d, H-30), 0.96 (3H, s, H-26), 0.88 (3H, s, H-23), 1.00 (3H, s, H-25), 0.83 (3H, d, H-29), 1.05 (3H, s, H27), 0.79 (3H, s, H-28), 0.82 (3H, s, H-24). These NMR features complied with established features of triterpenoid amyrins. The occurrence of a doublet at (H-18) indicated the presence of only one proton attached to H-19 which then coupled with the proton at H-18 to give the doublet at H-18. This implies that the other position on C-19 is occupied by CH_3- presenting a vicinal dimethyl arrangement at H-19 and H-20. This suggests an α-amyrin structure for the isolated compound. A doublet at H-18 in triterpenoids shows structure is α-amyrin (3β - hydro xy – urs-12-en-3-ol). The presence of doublet at H-29 and H-30 is also used to confirm the presence of α – amyrin. This result agrees with literature reports [7][8][9][10].

The chemical structure of the fraction (**A**) which is α-amyrin is shown below. The results of ^{1}H NMR spectrum of fraction (**A**) in this study in comparison with data from literature are shown in Table 3

α-amyrin is a pentacyclic triterpenoid that is ursane which contains a double bond between positions 12 and 13 and in which the hydrogen at the 3-β position is substituted by a hydroxy group. It is a pentacyclic triterpenoid and a secondary alcohol.



α – amyrin

Table 1: Experimental value of ^{1}H NMR spectrum of IG-JA-SGP 33 (A) in comparison with data from Literature.

Proton Position Labelled	Experimental	[7]	[8]	[9]	[10]
1					
2					
3	4.52 (m)	3.16 (s)	3.15	4.55 (t)	4.50 (dd)
4					
5			0.67		
6					
7					
8					
9					
10					
11					
12	5.15 (d)	5.15	5.12	5.15 (s)	5.12 (t)
13					
14					
15					
16					
17					
18					
19					
20					
21					
22					
23	0.88 (s)	0.98 (s)	0.95 (s)	-	0.88 (s)
24	0.82 (s)	0.77 (s)	0.76 (s)		0.88 (s)
25	1.00 (s)	0.95 (s)	0.75 (s)	0.93 (s)	1.01 (s)
26	0.96 (s)	1.12 (s)	0.89 (s)	0.95 (s)	0.98 (s)
27	1.05 (s)	0.92 (s)	1.01 (s)	1.08 (s)	1.07 (s)
28	0.79 (s)	0.81 (s)	0.95 (s)	0.80 (s)	0.79 (s)
29	0.83 (d)	0.85 (d)	0.85 (d)	-	0.88 (s)

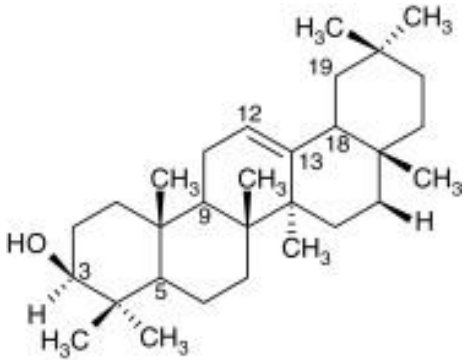
30	0.85 (d)	1.51 (d)	0.79 (d)	-	0.88 (s)

Compound B

IG-JA-SGP 107 (**B**) was obtained as white powder. The mass spectrum showed a molecular ion peak at m/z 426, which corresponded to a molecular formula $C_{30}H_{50}O$. The formula showed six double bond equivalents, five of which were adjusted in a pentacyclic carbon framework and the remainder in a CC double bond. The mass spectrum also showed fragments at 219 and 203 generated due to a retro-Diels Alder fragmentation indicative of the presence of double bond at position 12 in ring C[6]. The NMR spectrum showed the presence of eight methyl singlets, one olefenic proton at δ 5.15 (s) and an oxygenated proton at δ 3.25 (d), all suggestive of oleanane type triterpenoid (Table 4). The spectral data of IG-JA-SGP 107 (**B**) compared well with those previously reported as 3b-hydroxylolean-12-ene (β-amyrin) [11][7][8][6].

β-amyrin, also known as amyrin or (3β)-olean-12-en-3-ol, is a member of the class of compounds known as triterpenoids. Triterpenoids are terpene molecules containing six isoprene units. Thus, β-amyrin is considered to be an isoprenoid lipid molecule. Beta-amyrin is a pentacyclic triterpenoid that is oleanane substituted at the 3beta-position by a hydroxy group and containing a double bond between positions 12 and 13. It is one of the most commonly occurring triterpenoids in higher plants. It has a role as a plant metabolite and an Aspergillus metabolite.

The chemical structure of fraction **B** which is β-amyrin is shown below. The results of ^{1}H NMR spectrum of fraction **B** in this study in comparison with data from literature is shown in Table 4.



β-amyrin

Table 2: ^{1}H NMR spectrum of IG-JA-SGP 107 (B) Experimental in comparison with data from Literature

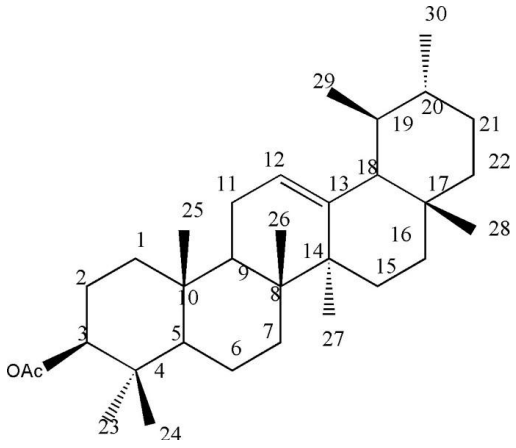
Position Labelled	Experimental	Literature			
		[11]	[7]	[8]	[6]
1					1.55, 1.49
2					1.52, 1.55
3	3.25 (d)	3.61 (dd)	3.19 (t)	3.15	3.20 (dd)
4					
5				0.67	0.71
6					1.53, 1.30
7					
8					
9					1.95
10					
11					1.84
12	5.15 (s)	5.22 (s)	5.15 (t)	5.12	5.16 (t)
13					
14					
15					
16					
17					
18					1.89

19					1.59
20					
21					1.66
22					
23	0.79 (s)	0.80 (s)	0.98 (s)	0.77 (s)	0.77 (s)
24	0.82 (s)	0.84 (s)	0.77 (s)	0.90 (s)	0.98 (s)
25	0.86 (s)	0.88 (s)	0.95 (s)	0.74 (s)	0.92 (s)
26	0.90 (s)	0.95 (s)	1.12 (s)	0.94 (s)	0.93 (s)
27	0.94 (s)	1.12 (s)	0.92 (s)	1.11 (s)	1.16 (s)
28	0.97 (s)	1.34 (s)	0.81 (s)	0.81 (s)	1.07 (s)
29	0.99 (s)	1.01 (s)	0.85 (s)	0.85 (s)	0.86 (s)
30	1.03 (s)	1.11 (s)	1.53 (s)	0.85 (s)	0.79 (s)

Compound C

IG-JA-SGP 26 (**C**) was obtained as a white powder. The mass spectrum showed a molecular ion peak at m/z 468, which corresponded to a molecular formula of $C_{32}H_{52}O_2$. The formula showed seven double bond equivalents, five of which were adjusted in a pentacyclic carbon framework and the remainder in CC and CO double bonds. The mass spectrum also showed fragments at 219 and 203 generated as a result of a retro-Diels Alder fragmentation indicative of the presence of double bond at position 12 in ring C [6]. IG-JA-SGP 26 (**C**) gave 1H-NMR signals characteristic of a triterpenoid. The 1H-NMR spectrum of IG-JA-SGP 26 (**C**) showed the presence of several signals between 0.87 and 1.05. These signals are attributable to overlapping methyl, methylene and methine protons typical of triterpenes. The triplet signal observed at δ 5.15, is typical of an olefinic proton (H-12); that at δ 4.53 corresponds to the oxymethine proton typical of hydrogen at C-3 of triterpenes. This slightly deshielded signal indicates a substitution of hydroxyl group with an acetate group at C-3. One of the methyl singlets, which appeared downfield at δ 2.07, is indicative of the presence of an acetate moiety. Deshielding of this methyl proton can be attributed to its proximity to a carbonyl functional group. Hence, its suggested attachment to the acetate moiety at position 3. These compared favorably with reports on α-amyrin acetate by other researchers including [12][13][6][10].

The chemical structure of fraction **C** which is α – amyrin acetate is shown below. The results of ^{1}H NMR spectrums of fraction IG-JA-SGP 26 (**C**) in this study in comparison with literature data are shown in Table 3.



α-amyrin acetate

Table 3: ^{1}H NMR spectrum of IG-JA-SGP 26(C) Experimental in comparison with data from Literature

Proton Position Labelled	Experimental	Literature		
		[12]	[13]	[6]
1				
2				1.61
3	4.53 (m)	4.47 (1H, dd)	4.48 (d)	4.48 (dd)

4				
5				0.81
6				1.51, 1.34
7				
8				
9				1.54
10				
11				1.89
12	5.15 (d)	5.18 (1H, dd)	5.11 (t)	5.10 (t)
13				
14				
15				
16				
17				
18	1.93 (m)	1.95 (d)		1.29
19				1.38 (m)
20				1.98
21				
22				
23	0.87 (d)	0.89 (d)	0.86 (s)	0.85 (s)
24	0.90 (m)	0.89 (d)	0.86 (s)	0.84 (s)
25	0.94 (s)	0.93 (s)	0.99 (s)	0.96 (s)
26	0.96 (s)	1.02 (s)	0.96 (s)	0.98 (s)
27	0.99 (s)	0.83 (d)	1.05 (s)	1.04 (s)
28	1.00 (s)	1.19 (s)	0.78 (s)	0.78 (s)
29	1.03 (s)	1.14 (s)	0.83 (s)	0.77 (d)
30	1.05 (s)	1.06 (s)	0.83 (s)	0.83 (d)
1’	-	-	-	-
2’	2.07 (s)	2.00 (3H, s)	2.02 (s)	2.02 (s)

Compound D

IG-JA-SGP 27 (**D**) was obtained as a white powder. The mass spectrum showed a molecular ion peak at m/z 468, which corresponded to a molecular formula of $C_{32}H_{52}O_2$. The formula showed seven double bond equivalents, five of which were adjusted in a pentacyclic carbon framework and the remainder in C=C and C=O bonds. The mass spectrum also showed fragments at 219 and 203 generated as a result of a retro-Diels Alder fragmentation indicative of the presence of double bond at position 12 in ring C [6]. IG-JA-SGP 27 (**D**) gave 1H-NMR signals characteristic of a triterpenoid. The 1H-NMR spectrum of IG-JA-SGP 27 (**D**) showed the presence of several signals between 0.74 and 1.12 as can be seen in table 6. These signals can be attributed to overlapping methyl, methylene and methine protons typical of triterpenes. The triplet signal observed at δ 5.15, is typical of an olefinic proton (H-12); that at δ 4.53 corresponds to the oxymethine proton typical of hydrogen at C-3 of triterpenes. This deshielded signal indicates a substitution of hydroxyl group with an acetate group at C-3. One of the methyl singlets, which appeared downfield at δ 2.31, is indicative of the presence of an acetate moiety. The deshielding of this methyl proton can be attributed to its proximity to a carbonyl functional group. Hence, its suggested attachment to the acetate moiety at position 3. The presence of two gem-dimethyl groups was further supported by the number of sp3-hybridized quaternary carbons of (C-29 and C-30). These results indicate that Fraction **D** is β-amyrin acetate as also reported by [14].

The chemical structure of fraction **D** which is β-amyrin acetate is shown below. The results of ^{1}H NMR spectrum of fraction **D** in this study in comparison with data from literature is shown in Table 4.

β-amyrin acetate, also known as β-amyrin acetic acid, is a member of the class of compounds known as triterpenoids. Triterpenoids are terpene molecules containing six isoprene units. β-amyrin acetate is practically insoluble (in water) and an extremely weak basic (essentially neutral) compound (based on its pKa).

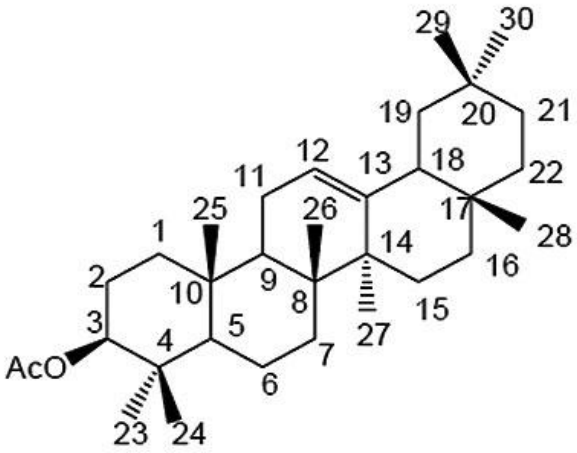


Figure 4.β-amyrin acetate

Table 4: ^{1}H NMR spectrum of IG-JA-SGP 27 (D) Experimental in comparison with data from Literature

Proton Position Labelled	Experimental	Literature [14]
1		
2		
3	4.53	
4		
5		
6		
7		
8		
9		
10		
11		
12	5.15 (t)	5.13 (1H, t)
13		
14		
15		
16		
17		
18		
19		
20		
21		
22		
23	0.82 (t)	0.83 (3H, s)
24	0.87 (m)	0.86 (3H, s)
25	0.94 (s)	0.96 (3H, s)
26	0.96 (s)	0.962 (3H, s)
27	1.12 (s)	1.13 (3H, s)
28	0.74 (s)	0.82 (3H, s)
29	0.91 (s)	0.87 (3H, s)

30	0.87 (s)	0.87 (3H, s)
1’	-	-
2’	2.31 (s)	2.3 (2H)

CONCLUSION

Known pentacyclic triterpenoids; α-amyrin, β-amyrin, α-amyrin acetate and β-amyrin acetate have been isolated and characterized from hexane and ethyl acetate extract of propolis obtained from Gboko, Benue State. The presence of these compounds may account for the pharmacologic activities of propolis such as its traditional use in the treatment of inflammation, pain and malaria fever.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no conflict of interest

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