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CYCLOARTANES AND PENTACYCLIC TRITERPENES FROM AWKA AND IJEBUODE PROPOLIS AND EVALUATION OF THEIR ANTIMICROBIAL ACTIVITY

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ABSTRACT

Chemical investigation of propolis samples from southern Nigeria led to the isolation of cycloartane-type triterpenes namely: 24-methylene cycloartanol and ambonic acid along with pentacyclic triterpenes: Lupeol and α, β-amyrins. All compounds were identified and structures elucidated using proton nuclear magnetic resonance (^{1}HNMR) spectroscopic data and comparison with literature.

Keywords: Cycloartanes, pentacyclic triterpenes, Nigerian propolis, ^{1}H-NMR.

INTRODUCTION

Propolis is a lipophilic, sticky, gummy, resinous substance collected by various species of bees, including honey bees (*Apis mellifera*) and stingless bees (*Tetragonisca angustulla lliger*). It is used to seal cracks in the hives and prevent invaders from entering, and also acts as a natural antibiotic to prevent bacterial, viral or fungal infections inside the hive (Castro, 2001; Pereira *et al.*,2003, Wagh, 2013 and Sampa *et al.*, 2015). The composition of propolis depends strongly on the plant origin and season, as well as on the bee species (Burdock, 1998; Kuropatnicki *et al.*, 2013). Propolis is typically classified into poplar propolis This Propolis type, originating from the temperate zone, contains mainly phenols (Falcao *et al.*, 2013) and tropical zone propolis, rich in other substances including prenylated derivatives of coumaric acids, diterpenes and lignans(Marucci, 1999) prenylated benzophenones(Cuesta-Rubio *et al.*, 2002) and prenylated flavonoids (Raghukumar *etal.*, 2010). This clearly demonstrated that propolis from tropical areas is variable and therefore a promising region for propolis research, potentially providing evidence for bioactive could supply components (Petrova *etal.*, 2010).

There are previous reports of the occurrence of cycloartane and pentacyclic triterpenes in African propolis (Kadar *et al.*, 2014; Tamfu *etal.*, 2020). Zhang *et al.*, (2013) examined the chemical characterization of African propolis and reported that triterpenoids were the main chemical components in more than half of the propolis samples analyzed. There is limited research on African propolis, including Nigeria, from previous studies, but its unique chemical makeup has been reported (Watson *et al.*, 2006; Sawaya *et al.*, 2007 and Petrova *et al.*, 2010).

In this research, we isolated and characterized cycloartanes and pentacyclic triterpenes from southern Nigerian propolis which afforded ambonic acid, lupeol and α, β-amyrins

MATERIALS AND METHODS

General Experimental procedure

X NMR spectra were obtained on a Bruker AVIII-400 NMR spectrophotometer operating at 400 MHz for ^{1}HNMR and spectra were processed using MestReNova. Chemical shifts are expressed in δ parts per million (ppm) and chemical shifts were referenced to the residual solvent peak at δ_H 7.26 for $CDCl_3$ while coupling constants are expressed in Hertz. Column chromatography was carried out using silica gel (40-60 µm, 60A, thermos scientific) and hexane-ethyl acetate gradients. The column used was 600 mm × 30 mm, TLC analysis was performed with Machery-Nagel precoated silica gel 60 F_{254} plates.

The antimicrobial assay was carried out using an agar well diffusion and serial dilution method on some local clinical isolates including Methicilin Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant *Enterococci* (VRE), *Helicobacter pylori, Campylobacter jejuni, Salmonella typhi, Escherichia coli, Candida albicans, Candida krusei, Candida tropicalis.*

Propolis samples were obtained from an apiary in Anambra (*6.2220 °N and 7.0821 °E*) and Ijebuode (*6. 8300 °N and 3.9165 °E)* in April 2022. Samples were confirmed by Prof. John Igoli of the Center for Natural Products Chemistry Research, Joseph Sarwuan Tarka University, Makurdi, Nigeria

Extraction and Isolation Procedure of Compound IAP 89, IAP (74-81) and IJP 28

Two propolis samples (50g) each from Awka, Anambra and Ijebuode, Ogun Nigeria were extracted with hexane, ethyl acetate and methanol in succession for 24 hours by maceration, after filtration, the ethyl acetate extracts were concentrated using rotatory evaporator at 40^{o}C to yield 9.0g and 7.3g brown extract respectively. 1g each of the ethyl acetate extract was separated by column chromatography on a silica gel packed column eluted with hexane-ethyl acetate gradients (90:100 to 100% EtOAc), several fractions were collected and were monitored by thin layer chromatography (TLC), fraction IAP 89, IAP (74-81) and IJP 28 were subjected to proton nuclear magnetic resonance to determine the structures.

RESULTS

Table 1: The Results of ^{1}H-NMR for IAP 89 and IJP 28, Experimental, in comparison with the data from literature

Position labeled	Experimental ^{1}H Chemical shiftδ (ppm), J(Hz)		Literature ^{1}H Chemical shift δ (ppm)	
	IAP 89	IJP 28	Mailafia *et al.*, 2020	Shwe *et al.*, 2019 (Literature)
1.				1.65, 0.90
2.	1.42(s)			1.52, 1.67
3.	3.16(d)	3.16(d, 2.5)	3.2(m)	3.2 (m)
4.	-			-
5.	0.67			0.67(o)
6.	1.35			1.52(o), 1.37
7.	1.39			1.39
8.	-			-
9.	1.26			1.25(o)
10.	-			-

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11.	1.20		1.40(o), 1.20
12.	1.08		1.06, 1.62(o)
13.	1.68		1.66(o)
14.	-		-
15.	1.59		1.60 (o), 1.05
16.	1.37		1.35(o), 1.45 (o)
17.	-		-
18.	1.37		1.37(o), 1.36
19.	2.38		1.45(o), 2.40(m)
20.	-		-
21.	1.19(m)		1.9(m), 1.3(o)
22.	1.18		1.18,1.37
23.	0.97(s)	0.97(s)	0.90(s)
24.	0.76(s)	0.77(s)	0.76(s)
25.	0.83(s)	0.85(s)	0.83(s)
26.	1.03(s)	1.05(s)	1.03(s)
27.	0.95(s)	0.94(d)	0.94(s)
28.	0.79(s)	0.77(s)	0.79(s)
29.	4.69, (d) 4.56 (d)	4.70, (d) 4.55 ,(d)	4.57, (d) 4.11,(d)
30.	1.56(s)	1.65(s)	1.67(s)

Table 2: The Results of ^{1}H-NMR for IJP28 Experimental, in comparison with the data from literature

Position Labeled	Experimental ^{1}H Chemical shiftδ (ppm), J(Hz)		Literature^{1}H Chemical shift δ (ppm)		
	IJP 28 (α-amyrin)	IJP 28 (β-amyrin)		IJP 28 (α-amyrin)	IJP 28 (β-amyrin)
1.	1.54	1.54	1.	1.54	1.54
2.			2.		
3.	3.20 (d, 4.8)	3.20(d, 4.8)	3.	3.20 (d, 4.8)	3.20(d, 4.8)
4.			4.		
5.			6.		
7.	1.56, 1.30	1.56, 1.30	8.	1.56, 1.30	1.56, 1.30
5.			9.		
6.			10.		
7.			11.		
8.			12.		
9.			13.		
10.	5.12(t, 3.6)	5.18(d, 3.6)	14.	5.12(t, 3.6)	5.18(d, 3.6)
11.			15.		
12.			16.		
13.			17.		
18.			19.		
20.			21.		
22.	1.89(s)	1.89(s)	23.	1.89(s)	1.89(s)
14.			24.		
15.			25.		
26.			27.		
28.			29.		
30.	0.75(s)	0.75(s)	31.	0.75(s)	0.75(s)
32.	0.99(s)	0.99(s)	33.	0.99(s)	0.99(s)
34.	0.91(s)	0.91(s)	35.	0.91(s)	0.91(s)

36.	0.95(s)	0.95(s)	37.	0.95(s)	0.95(s)
16.	1.00(s)	1.00(s)	38.	1.00(s)	1.00(s)
39.	0.82(s)	0.82(s)	40.	0.82(s)	0.82(s)
41.			42.		
43.			44.		

Table 3: The Results of ^{1}H-NMR for IAP 74 - 81 experimental, in comparison with the data from literature

Position labeled	Experimental ^{1}H Chemical shift δ (ppm), J(Hz)	Literature ^{1}H Chemical shift δ (ppm)	
	IAP 74-81	Pujirahayu *et al.*, 2019	Omar *et al.*, 2017
1.	1.85(s)	1.85	1.88
2.			
3.			
4.			
5.			
6.			
7.	1.16	1.14	1.13
8.			
9.			
10.			
11.			
12.			
13.			
14.			
15.			
16.			
17.			
18.	1.00 (s)	1.00 (s)	1.02 (s)
19.	0.79(s), 0.58 (s)	0.79(d), 0.58(d)	0.82 (d) 0.60 (d)
20.			
21.			
22.			
23.	2.04(s)	2.02	2.08 (t)
24.			
25.	3.19(q)	3.16(bq)	3.21 (q)
26.			
27.			
28.	0.91 (s)	0.91 (s)	0.91 (s)
29.	1.05 (s)	1.05	1.08 (s)
30.	1.10(s)	1.10	1.03(s)
31.	4.98(s) 4.94 (s)	4.97(s) 4.93(s)	5.0 (bs) 4.90 (s)

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Table 0: Antimicrobial Activities and Zone Of Inhibition of Isolated fractions of Awka and Ijebuode Propolis

Microorganism	**Fractions** IAP 89	IJP28	IAP 74-81	*Sparfloxacin	*Fluconazole	*Fulcin
MRS*A*	24	0	29	35	0	0
VRE	27	0	30	0	0	0
S.aureus	26	26	31	31	0	0
S. pyogenes	0	28	0	30	0	0
E. coli	25	28	31	34	0	0
K. pneumonia	23	0	30	0	0	0
P. mirabilis	27	27	30	31	0	0
C. albicans	26	28	30	0	34	0
C. krusei	0	0	0	0	32	32
A. fumigatus	0	0	0	0	0	32
F. oxysporum	28	-	31	0	25	27
F. pinicola	24	-	28	0	0	31

Key: *MRSA = Methicillin resistant staphylococcus aureus, VRE = Vancomycin resistant enterococci, S. aureus = Staphylococcus aureus, S. pyogenes = Streptococus pyogenes, E. coli = Escherichia coli, K. pneumonia = Klebsiella pneumonia, P. mirabilis = Proteus mirabilis, C. albicans*

*= Candida albicans, C. krusei = Candida krusei, A. fumigatus = Aspergillus fumigatus, F. oxysporum = Fusarium oxysporum, F. pinicola = Fomitopsis pinicola, * = standard drugs*

Table 5: Minimum inhibitory concentration (MIC), minimum bactericidal and fungicidal concentration of isolated fractions Awka and Ijebuode propolis

MIC mg/mL				**MBC/MFC mg/mL**		
Microorganism	**IAP 89**	**IJP 28**	**IAP 74-81**	**IAP 89**	**IJP 28**	**IAP 74-81**
MRSA	50	-	25	100	-	50
VRE	25	-	100	100	-	25
S. aureus	50	25	100	100	50	25
S.pyogenes	-	-	-	-	-	-
E. Coli	50	25	50	100	50	25
K.pneumonia	50	-	100	100	100	25
P.mirabilis	25	25	100	100	50	25
C. albicans	25	50	100	100	50	25
C. krusei	-	-	-	-	-	-
A. Fumigatus	-	-	-	-	-	-
F.oxysporum	25	-	100	50	25	25
F. pinicola	50	-	25	100	100	50

DISCUSSION

Characterisation of IAPe 89

Compound IAPe 89 was isolated as a white powder, the structure of IAPe 89 was elucidated by (^{1}HNMR) experiments at 400MHz.

^{1}HNMR IAPe 89($CDCl_3$): ^{1}H NMR (500 MHz, Chloroform-d) δ 4.69 (d, *J* = 2.5 Hz, 1H), 4.57 (dt, *J* = 2.6, 1.4 Hz, 1H), 3.18 (dd, *J* = 11.4, 4.9 Hz, 1H), 2.37 (td, *J* = 11.1, 5.8 Hz, 1H), 1.68 (m, 5H), 1.59 (s, 1H), 1.37 (s, 1H), 1.34 (d, = 2.1 Hz, 1H), 1.28 (s, 1H), 1.20 (s, 1H), 1.18 (d, *J* = 2.0 Hz, 1H), 1.16 (d, *J* = 2.3 Hz, 0H), 1.08 (d, *J* = 4.9 Hz, 1H), 1.03 (s, 3H), 0.97 (s, 3H), 0.95 (m, 3H), 0.83 (s, 3H), 0.79 (s, 3H), 0.76 (s, 3H), 0.70 – 0.66 (m, 1H).

The ^{1}HNMR spectrum (Appendix 1) showed a proton doublet of doublet at 3.18 ppm assigned to H-3, the methyl groups resonated at 0.97, 0.83, 0.76, 1.03, 0.70, 0.79 and 1.68ppm, respectively. The signals at 4.69 and 4.57 each gave a doublet, with each integration assigned to a proton of the methylene group at H-20. The presence of seven methyl singlets and two olefinic protons in the spectrum indicated that the compound is a pentacyclic triterpenoid (Anwer *et al.*, 2008)

Based on the ^{1}H NMR spectroscopic data and comparison with literature reports (Mailafiya *etal.,* 2020; Shwe *et al.* 2019,), IAPe 89 was identified as 20(29) lupen-3-ol (lupeol). Lupeol has previously been isolated from Cameroonian propolis (Omar, *et al.,* 2019). This is the first report of Lupeol from Awka, Anambra propolis (southeastern) Nigeria. This underscores the relationship between the two countries as they hail from the tropical areas and therefore have a similar type of vegetation

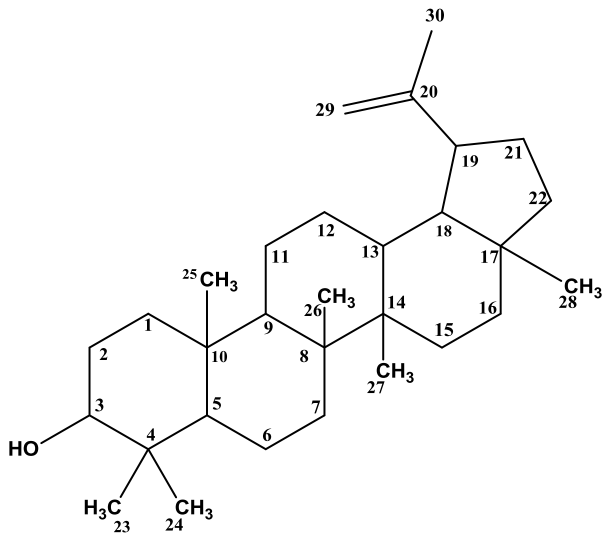

Figure 3: Structure of Lupeol from IJP 28, IAPe 89

Characterisation of IAP 74-81

Compound IAP 74-81 was isolated as a white solid, R_F (0.54) eluted at EtOAc/n-hexane (30:70) gradient.

^{1}H NMR (400 MHz, Chloroform-d) δ 4.98 (s, 1H), 4.94 (s, 1H), 3.19 (q, J = 7.0 Hz, 2H), 2.04 (s, 1H), 1.85 (s, 3H), 1.16 (s, 1H), 1.10 (s, 3H), 1.05 (s, 3H), 1.00 (d, J = 2.2 Hz, 3H), 0.91 (s, 3H), 0.79 (s, 3H), 0.58 (d, J = 4.3 Hz, 3H).

The ^{1}H NMR IAP (74-81) spectrum showed a proton quartet at 3.19 ppm assigned to H-25. The signals at 4.98(s, 1H), 4.94(s, 1H) each gave a singlet integration to a proton assigned to olefinic protons at H-31. The methyl protons showed signals at 1.85, 1.10, 0.91, 1.00, 0.79. 1.05 and 0.58, respectively. Based on the ^{1}H NMR data and comparison with literature reports (Pujirahayu *et al.*, 2019; Popova *et al.*, 2021) IAP 74-81 was identified as an ambonic acid. Ambonic acid has previously been isolated from southern Nigerian propolis from Ijebuode, Ogun State (Pujirahayu *et al.*, 2019) and from Cameroonian propolis and mango (Magnifera indica), Anarcardiacae. This could point to the plant source for the materials used

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in honey and propolis production by bees in Cameroon and throughout tropical Africa (Oleiveira *et al.,* 2022). Studies have been conducted on the biological activities of these cycloartane triterpenes, which exhibit highly preferential cytotoxicity over human pancreatic PAN-1 cancer cells (Shwe *et al.* 2019).

Triterpenes have been reported as a secondary metabolite that have extensive biological activity which are mainly produced by plants to protect themselves from various disease attack, therefore, bees look out and use them to form propolis as a protective chemical agent for nest and members of colonies, creating a fantastic bee-plant chemical interaction.

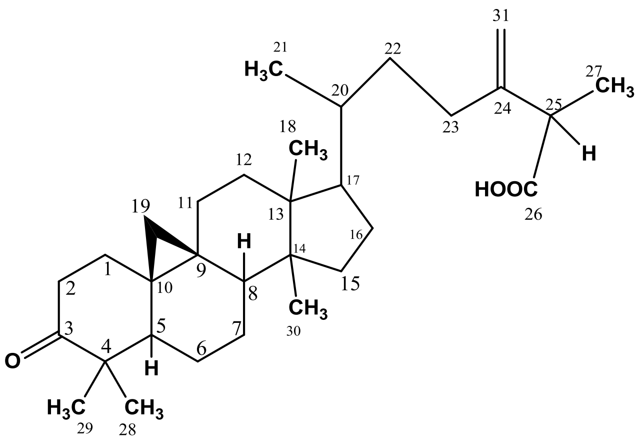

Figure 4: Structure of Ambonic acid from IAP 74-81

Characterization of IJP 28 as mixture of Lupeol, α and β -amyrins

Compounds IJP 28 was identified as a mixture. The compounds were identified and characterized by ^{1}HNMR (Appendix 3,4 and 5). ^{1}HNMR data were compared with literature and the values of chemical shifts are given in (Table 3,4 and 5).

^{1}H NMR (400 MHz, Chloroform-d) δ 5.18 (d, J = 3.8 Hz, 1H), 3.20 (d, J = 4.9 Hz, 1H), 1.56 (d, J = 3.8 Hz, 3H), 1.54 (s, 2H), 1.00 (s, 7H), 0.99 (s, 3H), 0.95 (s, 3H), 0.90 (s, 3H), 0.82 (d, J = 1.7 Hz, 3H), 0.75 (s, 3H).

^{1}H NMR (400 MHz, Chloroform-d) δ 5.12 (t, *J* = 3.6 Hz, 2H), 3.20 (d, J = 4.8 Hz, 1H), 1.89 (s, 0H), 1.54 (s, 1H), 1.30 (d, J = 2.5 Hz, 0H), 1.13 (d, J = 0.8 Hz, 1H), 0.99 (s, 3H), 0.95 (s, 3H), 0.91 (s, 3H), 0.82 (3H), 0.80 (s, 3H), 0.75 (s, 3H).

^{1}H NMR (400 MHz, Chloroform-d) δ 4.68 (d, J = 2.5 Hz, 1H), 4.56 (dt, J = 2.6, 1.4 Hz, 1H), 3.16 (d, J = 5.1 Hz, 1H), 1.56 (s, 1H), 1.42 (s, 1H), 1.35 (s, 1H), 1.13 (d, J = 0.9 Hz, 1H), 1.03 (s, 3H), 1.02 (s, 3H), 0.95 (s, 3H), 0.90 (s, 3H), 0.76 (s, 3H), 0.66 (s, 3H).

The ^{1}H NMR spectrum (CDCl3, Appendix 3) showed a triplet at 5.12 ppm, an olefinic proton assigned at H-12, a doublet at 3.20 ppm assigned to H-3, the methyl protons had signals at 1.89, 0.75, 0.99, 0.91, 0.95, 1.00 and 0.82ppm. The ^{1}H NMR spectrum (Appendix 4) showed a signal at 5.18 ppm, a doublet assigned to H-12 indicating the olefinic proton, a doublet at 3.20 ppm assigned to H-3, while signals at 1.89, 0.75, 0.99, 0.91, 0.95, 1.00, and 0.82 were assigned as methyl protons. All of these signals were consistent with the data obtained from (Okoye *et al.*, 2014; Vitor *et al.,* 2009). Based on the ^{1}H NMR chemical shift and comparison with literature data, IJP 28 was identified as a mixture of α, β-amyrins.

The ^{1}H NMR spectrum (Appendix 5) showed a proton doublet at 4.56 ppm and 4.68 ppm, respectively, assigned to the olefinic proton at H-20, also a signal at 3.16 ppm representing a doublet assigned to H-3 and methyl proton signals is seen at 0.95, 0.90, 0.85, 0.76, 1.56, 1.13 and 1.03, respectively. Based on the ^{1}H NMR spectroscopic data, IJP 28 was identified as a mixture of lupeol and, α, β-amyrins. They have been reported to possess a broad spectrum of biological and pharmacological activities, including inflammatory,hepatoprotective,antihyperglycemic, and hypolipidemic effects (Melo *et al.*, 2011; Oleveira *et al.*, 2007 and Yam-Puc *et al.*,2019). This is the first report of, α, β-amyrins in Nigerian propolis from Ijebuode, although it has also been reported in Cameroonian, Mexican and Malaysian propolis

α, β-amyrins are said to have cytotoxic, antioxidant and anti-inflammatory effects (Hernadez-Vasques *et al.*, 2012).Lupeol has previously been isolated from Cameroonian propolis (Omar *et al.*, 2017)Although this is the first time Lupeol is isolated from Awka propolis and now from Nigerian propolis from Ijebuode (Ogun). This is probably the kind of vegetation types that are similar and expected from tropical propolis in general and African propolis in particular (Bilcharska, *et al.*, 2019).

ANTIMICROBIAL ACTIVITY

The results of the antibacterial and antifungal activities were presented in tables 4 and 5, the isolated compounds inhibited the organisms except for compound IJP 28 which recorded zero inhibition in *MRSA*, *VRE* and *K. pneumonia.* The isolated fractions have considerable antibacterial activity. This is in agreement with the work of Popova *et al.*, (2013) and Bosio *et al.*, (2000). The isolated compounds also showed great anti-oxidant potential showing a good inhibition for *Candida albicans, Fomitopsis pinicoal and Fusarium oxysporum.* This is supported by the work of Tobaldini-Valerio *et al.*, (2016) in which propolis extract proved to be a good inhibitor of *Candida* virulence.

Considering that natural products can be classified as antimicrobials having MIC between 100 and 1000 mg / mL (Popova *et al.*, 2013). These isolated fractions have significant antimicrobial potentials as their MIC values are within these range and hence could use in disease control and antibiotic resistance

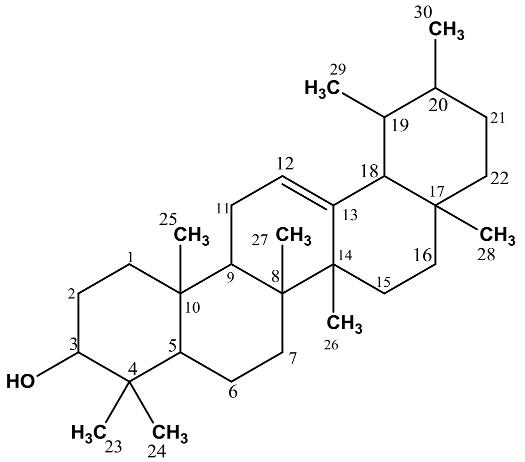

Figure 1: Structure of alpha-amyrin from IJP 28

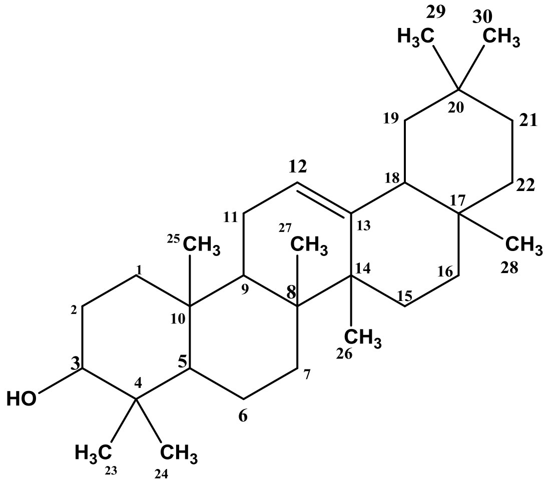

Figure 2: Structure of Beta- Amyrin from IJP 28

CONCLUSION

In summary, from this study, the mango tree *(Magnifera indica) L.* was identified as the most important tree plant source around the apiaries where the propolis was collected, hence the occurrence of these compounds identified. Mango chemical markers in these

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propolis samples examined were cycloartane triterpenes. It is very likely that the bees collect resins from mangoes that appear on the tree bark and latex on the fruit, and it also seems the bees instinctively have the ability to recognize types of compounds in resinous plants (triterpenes in this case) as protective agents against nest and colony attacks.

These cycloartanes and pentacyclic triterpenes can therefore be used in drug development to treat diseases and conditions.

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