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Chemical Composition, Total Phenolic Content, Free Radical Scavenging and Antimicrobial Activities of Resins- Juniperus Procera Resins and Relevant Propolis Produced by Apis Mellifera Jementica in Al-Baha Province, Saudi Arabia

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Abstract

The purpose of this study was to investigate the chemical compositions, total phenolic contents (TPC), free radical scavenging (FRSA), and antibacterial activities of Juniperus procera resins and the relevant propolis produced by Apis mellifer jementica in Al-Baha Province, Saudi Arabia. The results showed that both the resin and propolis samples, which were collected during April to June of 2014 and 2015, contained different compounds and included mono-, sesqui-, di-, and triterpenoids, wax esters, n-alkane, and n-alkene. The TPC levels of the resin and propolis samples were high for samples collected in 2014 relative to the samples collected in 2015. Also, the FRSA of the resin and proplois samples collected in 2014 was higher than the samples collected in 2015. All various solvents (DCM, DCM:MeOH, and MeOH) extracts of resin and propolis samples collected in 2014 showed very low inhibition against Aspergillus niger.; whereas the different solvent extracts of propolis collected in April 2015 showed significant inhibitory activity against E. coli ($P < 0.05$). In contrast, resin and propolis extracts of samples collected in 2015, showed no significant difference in their ZOI against C. albicans. Clearly, propolis extracts produced by honeybees from J. procera resins showed strong inhibitory activity against E. coli and S. aureus and comparatively weak activity against C. albicans and A. niger.

Key words: Juniperus procera resins, Propolis, Apis mellifera jemenitica

Introduction:

Different plant species and trees are found in the southwestern part of Saudi Arabia, where many of them are used as traditional medicine such as Juniperus procera Hochst. ex Endl [1]. This evergreen tree, which is locally named 'Arar', is tall (~ 8m high) [17]. Different compounds have been extracted from the bark, leaves, and essential oil of J. procera including lignan β-peltatin, deoxypodophyllotoxin, isocupressic acid, (+)-Z-communic acid, (+)-totarol and sugiol [34]. Abietane, pimarane, labdane, ferruginol diterpenes, and hinokiol were isolated from berries of J. procera [42]. Totarol and ferruginol isolated from the bark of J. procera tree by [32], whereas ferruginol, hinokiol, and 4-epi-abietinol from the aerial parts of J. procera by [26], and sugiol were extracted from the leaves of J. procera by [43].

The essential oil of J. procera acts as an antioxidant and OH-radical scavenging agent as it was evaluated by using deoxyribose degradation assay [14]. Abietanes extracted from the bark of J. procera showed antibacterial activity [44]. J. procera is used in Saudi Arabia to treat tuberculosis and jaundice [16, 43]. Most compounds that were isolated from the different parts of J. procera such as totarol demonstrated efficiency against pathogen bacteria were used with MIC 1.25-2.5µg/ml against Mycobacterium intracellulare, Mycobacterium smegmatis, Mycobacterium exenopi and Mycobacterium chelonei [12]. Ferruginol was also isolated from different parts of J. procera and exhibited strong activity against multidrug-resistant and methicillin-resistant Staphylococcus aureus [51]. Hinokiol has ability to scavenge DPPH radicals reported by [20].

Propolis is a sticky substance produced by honeybees from resin/gummy materials collected from different plants [22, 38]. It is used by honeybees to protect their hives from infectious microbes and other threats [6, 48]. Many, researchers were interested in the chemical composition of propolis and their biological activities because of its remedial properties [7, 8, 15, 46, 47]. The investigators of this research had observed that the honeybee foragers collecting organic material from J. procera resin in Feeg Village of Al-Baha province in Saudi Arabia.

Therefore, the purpose of this study was to investigate the chemical compositions, total phenol contents, antioxidant and antimicrobial activities of the *Juniperus procera* resins and the relevant propolis produced by local honeybees from the same resins.

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Materials and methods:

Apiary site:

Al-Baha province, which occupies 12.000 km^2, is located in the southwestern part of Saudi Arabia. It is situated between longitude 41˚and 42˚ E and latitude16˚ and 20˚ N (2). The vegetation of this region is diverse and covers about 190 plant species belonging to 59 families (2). These plant species include *Juniperus procera* (2), the densest plant species of the study area (Feeg Village). The apiary site is located in Wadi Feeg, between Banikabeer (Baljrashi governorate) and Al-Baha city (Fig. 1). This region has a temperate climate in summer and coldish in winter with much fog and rainfall in most months of the year (18).

Figure 1. Map showing the site of Apiary at Al-Baha Province, Saudi Arabia.

Sampling:

The resin samples from the plant source *Juniperus procera* trees and propolis from the beehives were collected during April and June of the years 2014 and 2015. During the field experiment, the researchers observed that the honeybee workers were collecting resin materials from aerial parts of *Juniperus procera* trees. These resin material were clear gluey with a nice aroma and collected by a metal tool directly from parts of trees. The honeybees *Apis mellifera jementica* used the resins to produce propolis that had a dark brown color with the same aroma of the collected resins of *Juniperus procrea*. The resin and propolis samples were collected in glass vials with Teflon caps (15 ml volume, Thermo scientific®.), labeled, dated, and stored in a refrigerator at -20 ˚C for further experiment.

Sample extraction and chemical analysis:

For chemical analysis, each sample of the resins and relevant propolis were cut into small pieces. About 0.5g of each sample of resin and relevant propolis was extracted separately in 10 ml of three different solvents including dichloromethane, a mixture of dichloromethane:methanol (DCM:MeOH 2:1, v:v) and methanol. Each mixture of the sample and solvent was placed in a shaker for 24 hours then sonicated by using an ultra-sonication bath at 25˚C for 30 minutes. Glass microfiber filters (47mm) was used to filter each extract, which was transferred to preweighed vials. The extract was then blown by nitrogen gas to dry and re-weigh it as to obtain the yield of the extraction and finally, exactly 0.5 ml of the relevant solvent was added to the vial.

The derivatization method of (3) was performed with some modification for only samples that were extracted by a mixture of DCM:MeOH and methanol. An exact volume of 20 µl of each sample was added to a 1.5 ml glass vial then it was evaporated to dryness under nitrogen gas. About 100µl of [N, O-bis (trimethylsilyl) trifluoroacetamide, BSTFA, Pierce Chemical Co.] were added to the aliquot and placed inside an

oven for three hours; then the sample was again evaporated to dryness under nitrogen gas. After dryness, 20 µl of hexane was added for each sample before the instrumental analysis.

The instrumental analysis was carried out by an Agilent 6890 gas chromatography coupled to a 5973 Mass Selective Detector (GC-MS), using a DB-5MS (Agilent) fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) and helium as a carrier gas. The GC was temperature programmed from 65°C (2 min initial time) to 310°C at 6°C min^{-1} (isothermal for 55 min final time) and the MS was operated in the electron impact mode at 70 eV ion source energy. Mass spectrometric data was acquired and processed using the GC–MS ChemStation data system.

The compounds were identified by comparison with the chromatographic retention characteristics and mass spectra of authentic standards, literature mass spectra, and the mass spectral library of the GC-MS data system. The mass spectra of unknown compounds were interpreted based on their fragmentation patterns. Compounds were quantified using the total ion current (TIC) peak area. A procedural blank was run in sequence to resin and propolis samples, presenting no significant background interferences.

Total phenolic content:

The Folin-Ciocalteu method was used to determine the total phenolic content (TPC) of the resin and corresponding propolis extracts, using a modified version of the procedure described by (50). Briefly, three dilutions were established for each resin and propolis extract by mixing 5, 10, and 15 µl of the extract with 50 µl Folin- Ciocalteu reagent in 96-well plates. The mixtures were incubated at room temperature for 5 min, adjusted to 65 µl by adding dimethyl sulfoxide (DMSO), mixed with 80 µl 7.5% sodium carbonate, and then incubated for 2 h at room temperature in the dark. Each experiment was performed in triplicate, and the absorbance of the reaction mixtures at 490–630 nm ($A_{490-630}$) was measured using a microplate reader (Model: MR-96 A. Medical Electronics CO, LTD. China®). Curve calibration of the gallic acid solution was used as standard ($A_{490-630}$ = 1562.5 × gallic acid (µg) - 16.9 (R^2 = 0.9938), and results were expressed as (mg) GAE/mg of resin and propolis extracts.

DPPH free radical-scavenge activity:

The antioxidant activities (i.e., free radical-scavenge activity (FRSA)) of the resin and corresponding propolis extracts were evaluated using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) reagent as described by (16). Briefly, for each sample, 1 mg extract was dissolved in 1 ml DMSO; 500 µl was diluted with 500 µl DMSO to obtain a concentration of 0.5 µg extract/ml; three-volume (4, 8 and 12 µl) from solutions were mixed with 180 µl DPPH reagent in 96-well plates and then the mixtures were incubated in the dark for 30 min. Each experiment was performed in triplicate, and the $A_{490-630}$ of the reaction mixtures was measured using a microplate reader and MeOH as a blank. Gallic acid was also used as a standard, and the percentage inhibition (PI) was calculated as PI = (A0 - A1/A0) × 100%, where A0 and A1 represent the absorbance of the negative control and sample, respectively.

Antimicrobial activity:

The disc diffusion method was used to evaluate the antimicrobial activities of the resins and relevant propolis samples against four microbes, including the gram-negative *Escherichia coli* ATCC 25922, gram-positive *Staphylococcus aureus* ATCC 25923, *Aspergillus niger* AUMC 8777, and *Candida albicans* ATCC 66193. All pathogen strains were obtained from the Microbiology Laboratory, Department of Botany and Microbiology, College of Science, King Saud University Riyadh. Nutrient agar was used to culture the bacterial strains at 37°C for 24 hours in an incubator. Potato dextrose agar was used to grow *C. albicans* and *A. niger* at 37°C for 48 hours. To adjust the turbidity to 0.5 McFarland standards (10^8 CFU/mL), saline solution (0.089% NaCl) was used to prepare suspensions for *C. albicans* whereas *A. niger* was directly applied by selecting spores from colonies with a sterile cotton applicator and then inoculating media in a petri dish. Sterile blank discs (6 mm in diameter) were submerged in 60 µl of each extract and placed on the surface of the plate. The diameter of the zone of inhibition (ZOI) was measured to evaluate the antimicrobial activity of the resin and propolis extracts. Each experiment was performed in triplicate. To determine the susceptibility of both gram-positive and gram-negative bacteria, ampicillin (10 µg/disc) was used as a positive control, and nystatin (100 µg/disc) was used as a standard control for fungal pathogens. To obtain the appropriate concentration, 50 mg from each dried extract was dissolved in 500 µl DMSO, and then 60 µl from the total solution was added to a blank disc.

Results:

Chemical analysis:

The results (Table 1) showed that the yields of the *J. procera* resins extracted by DCM ranged from 0.0499 to 0.438 mg/g (mean = 0.27±0.19 mg/g) for 2014, and from 0.0 to 0.39mg/g (mean = 0.25±0.2 mg/g) for 2015. The yield of resin extracted by DCM:MeOH ranged from 0.0238 to 0.363 mg/g and 2.35 to 0.390 mg/g (mean = 0.14±0.19 mg/g and 1.16±1.0 mg/g) for the years 2014 and 2015, respectively. The yield of the extracted resin by MeOH ranged from 0.201 mg/g to 0.475 mg/g with mean values of 0.36±0.14 mg/g in the year 2014 and 0.43± 0.04 mg/g for the year 2015. The yields of the DCM propolis extracts during the same periods ranged from 0.504 to 0.296 mg/g (mean = 0.36±0.12 mg/g) for the year 2014 and ranged from 2.47 to 0.960 mg/g (mean = 1.30±1.0 mg/g) for the year 2015. For the DCM:MeOH extracts, the yields ranged from 0.291 to 0.470 mg/g (mean 0.40±0.09 mg/g) and from 2.50 to 0.250 mg/g (mean = 1.07±1.0 mg/g), for the years 2014 and 2015 respectively. The yields of the methanol propolis extracts ranged from 0.249 to 0.199mg/g (mean 0.20 ±0.04 mg/g) for the year 2014 while for the year 2015 ranged from 0.66 to 0.152mg/g (0.41±0.2mg/g). (Table.1).

Table 1: The yields of the Juniperus procera resins and relevant propolis extracts (mg/g) collected during the months of April-June in 2014 and 2015 using three solvents: DCM, a mixture of DCM:MeOH, and MeOH

Type	Solvent	April	May	June	Mean(mg/g)	SD
Resin 2014	DCM	0.05	0.31	0.44	0.27	0.19
	Mixture	0.02	0.36	0.02	0.14	0.19
	MeOH	0.20	0.41	0.47	0.36	0.14
Resin 2015	DCM	0.39	0	0.36	0.25	0.2
	Mixture	2.35	0.75	0.39	1.16	1.0
	MeOH	0.40	0.41	0.47	0.43	0.04
Propolis 2014	DCM	0.28	0.50	0.30	0.36	0.12
	Mixture	0.47	0.29	0.44	0.40	0.09
	MeOH	0.25	0.20	0.16	0.20	0.04
Propolis 2015	DCM	2.47	0.96	0.47	1.30	1.0
	Mixture	2.51	0.44	0.25	1.07	1.0
	MeOH	0.66	0.43	0.15	0.41	0.2

The analytical results of the organic compound compositions of the total extracts by different solvents of the resins and propolis samples are summarized in Tables 2 and 3. The major compounds were mon-, sesquis-, diterpenoids, and wax esters and their chemical structures are shown in appendix 1. In resins, monoterpenoids were significant with average relative concentrations ranging from 12.48±9.97% in April, 7.41±12.83% in May, and 10.78±11.21% in June of 2014. In 2015 their average relative concentrations were 10.43±9.27% in April, 8.57±14.84 in May and 3.67±6.35% in June. The major compound was pinene with concentration ranging from 0% to 20.72% in 2014 and from 0% to 25.7% in 2015, where the highest concentration was detected in the DCM and DCM:MeOH extracts (Table 2). The sesquiterpenoids were minor in all extracts ranging from 0% to 0.5% where the major compounds were β-Eudoesmol and caryophyllene oxide. Diterpenoids were the highest concentrations in the extracts with average relative concentrations of 58.51±14.95% in April, 57.64±16.30% in May, and 52.46±40.92% in June of 2014. In 2015, they were 48.73+16.18 in April, 78.72+10.09% in May and 73.32+22.72% in June (Table 2). The major compounds were ferruginol (2.99-38.84%), Communic acid (1.27-48.13%), sugiol (4.05-9.8%) and totarol (0.62-23.6%) in 2014. In 2105, the major compounds were ferruginol (13.14-64.01%), Communic acid (0.0-60.7%), totarol (0.32-21.01%) and sugiol (0.40-3.39%).

Table 2. The relative concentrations (%) of compounds determined in the resin of *Juniperus procera* extracted by different solvents (dichloromethane (1), dichloromethane:methanol (2) and methanol (3)) from Al-Baha Province, Saudi Arabia in 2014 and 2015

2014	April				May				June			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
Monoterpenoids												
R. C. (%)	19.79	16.53	1.13	12.48 ±9.97	22.22	0	0	7.41± 12.83	23.52	6.43	2.4	10.78± 11.21
Major compound (%)	P(18.4), C(1.4)	P(15.3), C(1.0)	P(0.95)		P(20.7), P1(0.9), L(0.55)		P		P(19.4), L(2.27), P1(1.19)	P(5.31), C(1.03)	Pi(2.1)	
Sesquiterpenoids												
R. C. (%)	0.23	0.22	0	0.15± 0.13	0.21	0.53	ND	0.37± 0.23	0.14	0.1	0.07	0.10±0 .04
Major compound (%)	E(0.13), C1(0.1)	E(0.12), C1(0.09)			E(0.15), C1(0.06)	C (0.53)			E(0.09), C1(0.05)	C1(0.09)	C1(0.07)	
Diterpenoids												
R. C. (%)	60.88	72.13	42.51	58.51 ±14.9 5	51.2	76.17	45.54	57.64 ±16.3 0	13.36	94.99	49.04	52.46± 40.92
Major compound (%)	F(38.8), C2(11.7), S(4.05), T(3.7)	F(38.7), C2(16.2), S(9.5), T(3.8)	F(23.2), S(9.8), C2(5.3), T(3.02)		F(20.12), C2(11.43), T(6.14), S(3.99)	C2(48.13), F(22.1), S(3.14), T(2.19)	F(22.81), C2(12.33), S(7.04), T(3.36)		S(3.87), F(2.99), T(1.99), C2(1.27)	F(38.1), T(23.6), C2(16.1), S(9.5)	F(26.2), C2(16.3), S(5.92), T(0.62)	
2015												
Monoterpenoids												
R. C. (%)	16.23	16.15	0.13	10.84 ±9.27	25.7	0	0	8.57± 14.84	11	0	0	3.67±6 .35
Major compound (%)	P(16.2)	P(15.35)	P (0.9)		P (25.7)				P (11)			
Sesquiterpenoids												
R. C. (%)	0.05	0.13	0.13	0.10	0	0	0	0.00	0	0	0	0.00
Major compound (%)	C1(0.05)	E(0.08)	C1(0.13)									
Diterpenoids												
R. C. (%)	58.05	58.1	30.05	48.73 ±16.1 8	68.38	88.54	79.25	78.72 ±10.0 9	70.29	52.27	97.4	73.32± 22.72
Major compound (%)	F(38.5), C2(13.05), T(4.59), S(1.87)	F(38.1), C2(12.05), T(4.9), S(1.9)	F(13.14), C2(5.5), T(4.1), S(3.12)		F(64.01), T(3.06), S(1.22)	F(42.2), C2(17.72), S(2.96), T(1.8)	F(32.9), C2(21.21), T(21.01), S(3.39)		F(55.1), T(8.23), C2(4.8), S(1.2)	C2(28.1), F(22.1), S(1.0), T(0.32)	C2(60.7), F(34.3), T(2.0), S(0.4)	

A1 = β-Amyryl acetate, A2 = α-amytyl acetate, A3 = Amyrin, C = Camphor, C1 = Caryophyllene oxide, C2 = Communic acid, C3 = α-Cedrol, D = Dammaradienol, D1 = Dammaradienyl acetate, E = β-Eudesmol, L = Limonen, L1 = Lupeol, L2 = α-Lupeyl acetate, P = Pinene, P1 = Pincaveol, S = Sugiol, W1 = Eicosyl stearate (chemical structures are illustrated in Appendix 1)

For propolis samples, monoterpenoids were detected only in 2014 as minor compounds with average relative concentrations ranging from 0.24±0.40% in April, 0.45±0.78% in May, and 0.13±0.23% in June. The major compound was Pinene with relative concentration ranging from 0% to 1.23%, where the highest concentration was detected in the DCM extracts (Table 3). The sesquiterpenoids were traces in all extracts ranging from 0% to 0.18% in 2014 and from 0% to 2.14% in 2015 where the major compounds were α-Cedrol. Diterpenoids were major compounds in the extracts with average relative concentrations of 35.22±34.86% in April, 16.00±15.53% in May, and 20.34±17.73% in June of 2014. In 2015, they were lower in concentrations with average values of 2.07±2.94% in April, 5.90±10.22% in May, and 8.78±15.21% in June (Table 3). The major compounds were ferruginol (0.0-36.53%), communic acid (0.0-29.96%), sugiol (0.0-20.09%) and totarol (0.0-2.81%) in 2014. In 2105, the major compounds were ferruginol (0.0-17.4%), communic acid (0.0-10.93%), and totarol (0.0-2.96%). Triterpenoids were detected in the propolis samples with average relative concentrations of 0.22±0.26% in April, 1.39±2.16% in May and 7.54±6.12% in June of 2014; where the major compounds were α-lupeyl acetate (0.0-12.5%), dammaradienyl acetate (0.0-2.71%), dammaradienol (0.0-2.30%), β-amyryl acetate (0.0-0.93%), β-amyrin (0.0-0.71%), and lupeol (0.0-0.22%). In 2015, triterpenoids were relatively higher in relative concentrations with average values of 4.53±4.04% in April, 4.16±6.39% in May, and 5.31±4.79% in June. The major compounds were dammaradienol (0.0-4.8%), β-amyryl acetate (0.0-1.96%), β-Amyrin (0.0-0.61%), lupeol (0.0-8.42%), α-amyryl acetate (0.0-11.4%), and α-lupeyl acetate (0.0-5.03%). Traces of wax ester were detected only in the propolis samples collected in 2014 and the main compound was eicosyl stearate ranging from 0.0% to 1.93%.

Table 3. The relative concentrations (%) of the compounds determined in the propolis produced by honeybee *Apis mellifera jemenetica* from *Juniperus procera* extracted by different solvents (dichloromethane (1), dichloromethane:methanol (2) and methanol (3), in Al-Baha Province, Saudi Arabia in 2014 and 2015.

2014	April				May				June			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
Monoterpenoids												
R. C. (%)	0.01	0	0.7	0.24±0.40	1.35	0	0	0.45±0.78	0.4	0	0	0.13±0.23
Major compound (%)	P(0.1)		P(0.56)		P(1.23)				P(0.4)			
Sesquiterpenoids												
R. C. (%)	0	0	0.18	0.06±0.10	0.12	0	0	0.04±0.07	0.01	0	0	0.00
Major compound (%)			C3(0.18)		C3(0.08)				E(0.01)			
Diterpenoids												
R. C (%)	35.94	0	69.71	35.22±34.86	16.99	0	31.01	16.00±15.53	28.67	0	32.46	20.38±17.75
Major compound (%)	F(30.1), T(2.1), S(1.49)		F(36.53), C2(29.96), T(2.63), S(0.59)		F(12.83), T(2.81), S(0.74)		F(23.4) S(7.2), T(0.31)		S(20.09), T(1.94), F(1.73)		F(16.6), C2(13.7), T(1.2), S(0.96)	
Triterpenoids												
R. C. (%)	0.51	0.14	0	0.22±0.26	3.88	0.29	0	1.39±2.16	7.45	13.71	1.47	7.54±6.12
Major compound (%)	A1(0.51)	A1(0.14)			D(1.93), A1(0.87), D1(0.83)	A1(0.29)			D1(2.71), D(2.3), A1(0.93), L2(0.26)	L2(12.5), A(0.71), D1(0.29), A1(0.22)	L2(0.74), A(0.51), L1(0.22)	
Wax esters												
R. C. (%)	0	0	0	0.00	0.63	1.93	0	0.850.98	0.04	0	0	0.01±0.02
Major compound (%)					W1(0.63)	W1(1.93)			W1(0.04)			
2015												
Monoterpenoids												
R. C. (%)	0	0	0	0.00	0	0	0		0	0	0	0.00
Major compound (%)												
Sesquiterpenoids												
R. C. (%)	0	0	0	0.00	0	2.14	0	0.71±1.24	0	0	0	0.00
Major compound (%)						C3(2.14)						
Diterpenoids												
R. C. (%)	5.44	0	0.77	2.07±2.94	0	0	17.71	5.90±10.22	0	0	26.35	8.78±15.2
Major compound (%)	T(2.96), F(2.48)		T(0.43), F(0.34)				F(17.4), T(0.31)				F(15.42), C2(10.93)	
Triterpenoids												
R. C. (%)	7.78	5.81	0	4.53±4.04	0.96	11.52	0	4.16±6.39	6.65	9.29	0	5.31±4.79
Major compound (%)	D(4.8), A1(1.96), A3(0.61)	L1(2.2), A2(1.7), L2(1.5)			L2(0.96)	A2(11.4), L1(0.12)			L2(5.03), A2(1.62)	L1(8.42), A2(0.87)		
Wax esters												
R. C. (%)	ND	ND	ND		ND	ND	ND		ND	ND	ND	

A1 = β-Amyryl acetate, A2 = α-amytyl acetate, A3 = β-Amyrin, C = Camphor, C1 = Caryophyllene oxide, C2 = Communic acid, C3 = α-Cedrol, D = Dammaradienol, D1 = Dammaradienyl acetate, E = β-Eudesmol, L = Limonen, L1 = Lupeol, L2 = α-Lupeyl acetate, P = Pinene, P1 = Pincaveol, S = Sugiol, W1 = Eicosyl stearate(chemical structures are illustrated in Appendix 1).

Total phenolic content and free radical-scavenging activity:

Phenolic compounds are important because of their antioxidant and antimicrobial activities. Therefore, the TPC should be used to evaluate the quality of propolis samples. In the present study, the TPC of both plant resins and corresponding propolis samples were measured. Statistical analysis indicated a significant difference ($P < 0.05$) between the TPC of the June 2014 MeOH extracts of propolis and resin from the same plant sources. For example, the mean TPC of the June 2014 MeOH extract of *J. procera* propolis was 48.5 mg/g, whereas that of *J. procera* resin was 215.0 mg/g. Meanwhile, the mean TPC of the May 2014 MeOH extract of *J. procera* propolis was 126.5 mg/g, whereas that of *J. procera* resin was 42.5 mg/g. However, there was no significant difference in the TPC of the other extracts (DCM or DCM:MeOH) prepared in April, May, or June (Table 4). In 2014 (April, May, and June), the TPC of the DCM extracts of propolis and resin ranged from 88.0 to 108.0 mg/g and from 72.0 to 102.0 mg/g, respectively, whereas the TPC of the DCM:MeOH extracts of propolis and resin ranged from 88.0 to 101.0 mg/g and from 90.0 to 99.5 mg/g, respectively, and that of the MeOH extracts of resin and propolis ranged from 42.5 to 215.0 mg/g and from 48.5 to 189.0 mg/g propolis, respectively (Table 4). However, the DCM, DCM:MeOH and MeOH extracts of propolis prepared in April, May, and June 2015 possessed the highest TPCs, ranging from 66.5 ± 12.0 to 81.5 ± 27.6 mg/g and from 129.0 ± 2.8 to 109.5 ± 10.6 mg/g, respectively (Table 4).

The free radical-scavenging activity (FRSA) of all samples used in the present study was also evaluated by using a DPPH assay. Phenolic compounds are important antioxidants, owing to their FRSA (24). Antioxidant activity was defined as the ability to inhibit oxidative degradation (39). The reduction of stable DPPH radical to yellow-colored diphenyl-picrylhydrazine (DPPH-H) demonstrated the samples FRSA. In alcoholic solutions with hydrogen-donating antioxidants, DPPH is reduced to its non-radical form, DPPH-H (27). All the *J. procera* resin and corresponding propolis samples were used in the present study exhibited FRSA (Table 4). The FRSA of the resin and propolis samples of *J. procera* increased with increasing TPC, and FRSA and TPC were positively correlated. In general, there were no significant differences between the FRSA of the *J. procera* resin and propolis samples; however, for certain extracts of a few samples, the FRSA of the resin and corresponding propolis samples were significantly different. For example, for the May 2014 samples, the FRSA of the DCM resin extracts (22.7%) was higher than that of the corresponding propolis extracts (8.4%), even though the TPC of the propolis was greater (Table 4). However, for the DCM:MeOH extracts of the June 2014 samples, the FRSA of the resin extracts (57.2%) was significantly higher than that of the corresponding propolis extracts (12.1%) (Table 4). For the MeOH extracts of the April 2014 samples, the FRSA of the *J. procera* propolis extracts (53.1%) was significantly higher than of the *J. procera* resin extracts (22.3%) and for the MeOH extracts of the June 2014 samples, the FRSA of the resin extracts (57.2%) was higher than that of the corresponding propolis extracts (9.7%). (Table 4). However, there were no significant differences in the FRSA of the *J. procera* resin and propolis extracts of April, May, and June 2015 ($P < 0.05$), (Table 4).

There was a significant correlation between the TPC and FRSA of the propolis extracts of April, May, and June 2014 ($r = 0.66335$) but not between those of April, May, and June 2015 ($r = 0.34268$); the correlation between TPC and FRSA was significant for the resin extracts of both 2014 ($r = 0.65987$) and 2015 ($r = 0.46762$).

Table 4: Total phenolic content (TPC) mg/g and free radical-scavenging activity (FRSA) % of Juniperus procera propolis and resin extracts (DCM, DCM:MeOH and MeOH) prepared in April-June 2014-2015, Al-Baha Province, Saudi Arabia. Values represent means ± SD of three replicates, and different lowercase letters (a, b) indicate significant differences (P < 0.05).

Solvent	Type	Parameters	2014			2015		
			April	May	June	April	May	June
DCM	Resin	TPC	101.5±20.5a	72.0±42.4a	102.0±35.3a	40.0±2.8b	46.0±5.6a	15.5±3.5b
		FRSA	9.3±3.4 a	22.7±4.4 a	12.4±2.1 a	7.6±2.5 a	12.8±7.7 a	6.4±2.8 a
DCM:MeOH		TPC	90.0±29.7a	96.5±27.6a	99.5±12.0a	113.5±2.1a	19.5±13.4a	42.0±22.6b
		FRSA	24.5±5.8 a	16.7±3.5 a	57.2±10.1 a	12.6±2.3 a	5.6±2.2 a	12.2±2.2 a
MeOH		TPC	82.5±19.1a	42.5±7.8b	215.0±76.4a	85.5±19.1a	48.5±21.9b	133.5±7.9a
		FRSA	22.3±3.9 b	5.3±4.7 a	57.2±10.2 a	8.2±2.4 a	12.8±2.4 a	11.0±4.0 a
DCM	Propolis	TPC	88.0±15.5a	108.0±12.7a	104.0±19.8a	66.5±12.0a	20.0±9.9b	81.5±27.6a
		FRSA	7.5±2.4 a	8.4±2.1b	13.6±4.5 a	8.5±1.7 a	9.1±1.2 a	8.8±3.9 a
DCM:MeOH		TPC	88.0±7.1a	101.0±11.3a	97.0±22.6a	42.5±13.4b	27.5±6.3a	129.0±2.8a
		FRSA	16.7±3.8 a	11.7±4.1 a	12.1±6.2 b	8.5±1.1 a	8.7±1.2 a	9.5±4.3 a
MeOH		TPC	189.0±90.5a	126.5±23.3a	48.5±7.8b	133.5±29.0a	109.5±10.6a	68.0±8.5b
		FRSA	53.1±14.9 a	14.0±6.2 a	9.7±2.1 b	7.4±2.4 a	7.9±2.7a	8.2±1.5a

Antimicrobial activity:

The results showed that DCM and MeOH extracts of *J. procera* resins of June 2014 showed significant inhibitory activity against *E. coli* (P < 0.05; Table 5). Additionally, DCM and DCM:MeOH of the resin extracts of April and May 2014 showed significant inhibitory activity against *S. aureus* (P < 0.05; Table 5). Also, the DCM and MeOH extracts of resin samples collected in April 2015 showed significant inhibitory activity against *S. aureus* (P < 0.05; Table 5). Propolis produced in April, May, and June 2014 from *J. procera* significantly inhibited the growth of *E. coli*, *S. aureus*, and *C. albicans*, whereas it only had a weak effect against *A. niger*. DCM, DCM:MeOH, and MeOH propolis extracts prepared in May, April, and June 2014 showed a significant inhibitory activity against *E. coli* (P < 0.05; Table 5). Propolis extracts by DCM:MeOH or MeOH in April 2014 showed the highest inhibitory activity against *S. aureus* (P < 0.05; Table 5). Most propolis extracts as well as corresponding *J. procera* resins showed no significant difference in their ZOI against *C. albicans* (Tables 5). All extracts of resin and relative propolis prepared in April, May and June 2014 showed very low an inhibition against *Aspergillus niger*. Tables 5. On the other hand, DCM, DCM:MeOH, and MeOH extractions of propolis prepared in April 2015 showed significant inhibitory activity against *E. coli* (P < 0.05; Table 5). In contrast, propolis extracts prepared in April, May, and June 2015, as well as extracts of the corresponding resins, showed no significant difference in their ZOI against *C. albicans* (Tables 5). However, the same extracts of propolis and resins only had a weak inhibitory effect against *A. niger* (Table 5).

Table 5: Means ±SD of Zone of inhibition (mm) activity of resin and propolis (*Juniperus procera*) extracts (DCM, DCM:MeOH and MeOH) prepared in six months from April-June 2014 and 2015 were determined against *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Candida albicans* (ATCC 66193) and *Aspergillus niger* (AUMC 8777). Means with the same letter (a, b and c) are not significantly different (P < 0.05).

Pathogens	Sample	Solvent	2014			2015		
			April	May	June	April	May	June
E. coli	Resin	DCM	10.67±0.58 c	12.0±1.0 b	20.0±0.0 a	19.67±0.58 a	12.33±0.58 c	14.67±0.58 b
		DCM:MeOH	19.67±0.58 a	20.0±0.0 a	9.0±0.0 b	15.67±0.58 b	12.33±0.58 c	17.33±0.58 a
		MeOH	12.67±0.58 b	12.33±0.58 b	20.67±0.58 a	12.0±0.0 a	13.0±1.0 a	-
S. aureus	Resin	DCM	21.0±1.0 a	12.0±1.0 c	15.33±1.15 b	14.33±1.5 a	16.67±1.5a	11.33±1.15 b
		DCM:MeOH	16.67±0.58 b	19.67±0.58 a	9.33±0.58 c	14.0±1.0 b	15.67±0.58 b	18.67±1.5 a
		MeOH	-	-	16.33±0.58	13.0±1.0 b	17.33±2.3 a	15.0±0. ba
C. albi-cans	Resin	DCM	13.33±1.15 a	12.0±1.00 a	12.0±1.00 a	11.0±1.7 (a)	17.0±6.1 a	11.67±1.15 a
		DCM:MeOH	12.33±0.58 a	12.33±0.58 a	-	12.33±0.58 a	12.33±0.58 a	11.67±0.58 a
		MeOH	11.67±0.58 a	11.67±0.58 a	11.67±0.58 a	12.0±0.0 a	11.67±0.58 a	12.0±0.0 a
A. niger	Resin	DCM	-	-	-	8.33±0.58 a	-	-
		DCM:MeOH	10.0±0.0 a	10.0±0.0 a	-	-	-	-
		MeOH	-	8.67±1.15	-	12.67±1.15 a	-	-
E. coli	Propolis	DCM	10.0±1.0 c	18.67±0.58 a	13.0±1.0 b	19.3±0.58 a	12.0±1.7 b	12.67±1.5 b
		DCM: MeOH	18.33±0.58 a	12.67±1.15 b	9.33±0.58 c	17.7±2.3 a	12.67±0.58 b	11.67±0.58 b
		MeOH	20.0±0.0 b	20.0±0.0 b	22.33±0.58 a	22.3±0.58 a	15.67±0.58 b	12.0±1.0 c
S. aureus	Propolis	DCM	12.67±0.58 a	13.0±1.7 a	11.67±0.58 a	15.3±1.5 a	12.0±1.0 b	10.0±1.0 b
		DCM:MeOH	15.67±0.58 a	12.67±1.1b	-	15.0±1.0 a	14.0±1.0 a	13.67±0.58 a
		MeOH	19.33±1.15 a	15.67±1.15 b	17.0±1.0 b	17.0±1.7 a	13.33±0.58 b	13.67±1.15 b
C. albi-cans	Propolis	DCM	13.33±0.58 a	10.67±0.58 b	11.0±1.0 b	12.3±1.15 a	12.67±0.58 a	10.67±1.5 a
		DCM:MeOH	10.67±0.58 a	11.0±1.0 a	11.33±1.5 a	14.7±0.58 a	13.0±1.0 ba	11.33±1.5 b
		MeOH	12.33±0.58 a	11.67±0.58 a	10.0±1.0 b	13.3±0.58 a	14.0±1.7 a	13.67±1.15 a
A.niger	Propolis	DCM	-	-	-	13.3±0.58 a	-	11.67±0.58 b
		DCM:MeOH	-	-	-	12.3±0.58 a	-	-
		MeOH	13.0±0.0 a	13.0±0.0 a		12.7±1.15 a	-	-

Discussion:

Chemical analysis:

The results showed that no differences between yields of the different *J. procera* resin extracts and the yields of relevant propolis extracts by various solvents. In the second year (2015), the yields of extracts of both resins and relevant propolis were higher. This can be attributed to the existing secondary metabolisms, where higher plants response to environmental factors to produce more materials. Honeybees produce more propolis with fewer impurities when raw materials such as secondary metabolisms are relatively too high in the surrounding area. Also as known higher plants produce secondary metabolisms to adapt to both biotic and abiotic stress conditions (28), and also to communicate with symbiotic microorganisms as well as to attract pollinators and seed dispersers (56). The contents of secondary metabolisms generally include phenolic acids, flavonoids, terpenoids, steroids, and alkaloids (13, 24). Many literature reports mentioned that environmental factors influence the biosynthesis and accumulation of secondary metabolisms (37). The accumulation of secondary metabolisms depends on various environmental factors such as light, temperature, soil water, soil fertility, and salinity. The contents of secondary metabolisms can be changed if an individual factor is changeable while others are constant (57). The major compounds of the resin samples collected in the months from April to June 2014 were monoterpene, monoterpene derivative, monoterpene alcohol, sesquiterpene, diterpenoid, triterpenoid, fatty acids, n-alkanes, n-alkenes and biphenol. The occurrence of these compounds in both *J. procera* resins and relevant propolis is consistent with many studies in the literature (26, 4, 31, and 33). Other studies have reported the same compounds identified in resins of other species belongs to the genus *Juniperus* spp. such as *Juniperus communis* L. (21). Although this work was considered the first study proved that honeybee *A. m. jemenitica* produce propolis from resins of *J. procera*, other few studies conducted on Saudi Arabia propolis have

found different compounds beside kaempferol and *trans*-cinnamic (19), which were not detected in propolis samples of the current study. Terpenes, including monoterpene hydrocarbons and sesquiterpenes, were found in different parts of *Juniperus foetidissima* such as leaves and fruits, and the major components in that parts are limonene, α-pinene and cedrol (29); these compounds were found also in resin of *J. procera* and relevant propolis. The current study confirmed that honeybee *A. m. jemenitica* produced propolis from *J. procera* according to the chemical composition of both resins and propolis., where about 19.5% of compounds were found in both resins and propolis.

Most compounds found in the propolis samples of this study are present in the essential oils of *Cupressus sempervirens* which belong to the family (Cupressaceae) (53). Propolis samples from Yemen and Ethiopia are rich in triterpenoid (3, 41). These results are consistent with this study which showed that propolis produced by honeybee *A. m. jemenitica* from resins of *J. procera* is rich in triterpenoids and diterpenoid. This may indicate that plant species *J. procera* also dispersal in Ethiopia (12), and honeybee in that country may produce propolis from resins of this plant species. Also, the chemical groups of triterpenoids, n-alkane and n-alkene were detected in propolis samples collected from honeybee colonies in the apiary of the Bee research unit, King Saud University (5). The propolis samples from Al-Baha of Saudi Arabia contained different compounds such as sandaracopimaric acid, (+)-ferruginol, (+)-totarol cycloartenol- derivatives and triterpene acetates (27). These compounds were also found in propolis samples of the current study. Sugiol and ferruginol were isolated from *J. procera* by (45), and the results of the current study confirmed that *J. procera* was the major source of propolis in Al-Baha province of Saudi Arabia. More studies are needed to investigate different propolis samples to find out if there are other sources of propolis components and to investigate if there are new compounds in propolis samples with significant effects against complex diseases such as cancer and diabetes.

Total phenolic content and free radical-scavenging activity:

According to our knowledge, no studies have investigated TPC or FRSA of propolis from Saudi Arabia. Therefore, the present study is the first to investigate the TPC and FRSA of the corresponding plant resins and propolis from Saudi Arabia. The TPC values of propolis samples were consistent with TPC values of propolis from other countries, including Brazil, China, and Australia, for which the reported TPC values ranged from 127 to 142 mg /g (54). Meanwhile, the TPC values of the extracts of the resin samples were consistent with TPC values of resin from other *Juniperus* spp (45). The high TPC values of both *J. procera* resins and propolis samples in 2014 relative to 2015, may be attributed to the effect of adverse environmental conditions, whether abiotic or biotic, on the plants. For example, two *Juniperus* spp. responded to salt and methyl jasmonate stress differently (52). More specifically, *J. oxycedrus badia* responded to salt stress, whereas *J. phoenicea* only responded to methyl jasmonate, and both species responded to the stress by modifying their TPC levels. The TPC values of propolis may be related to the TPC of the corresponding plant sources. TPC levels of four *Juniperus* spp. were highest during the winter (October to January) and reach the lowest level during the spring (February to July) (52). In the present study, there were only a few significant differences between the TPC of the corresponding resin and propolis extracts. Pinene (IR-α-pinene, 1S-α-pinene, D-pinene, and β-pinene) are organic compounds that are considered the major components of plant resin, especially in conifers. These compounds, which may play important roles in FRSA, were identified in the DCM extracts of both *J. procera* resin and propolis. Compounds act as bronchodilators in humans and also possess anti-inflammatory, acetylcholinesterase-inhibitory activity (40). The presence or absence of such compounds in propolis confirmed the role of honeybees in the chemical composition and, thus, the biological activity of the propolis. Therefore, it would be beneficial to determine the best time to collect propolis from beehives. Communic acid is an important compound, because of its biological properties (9). In the present study, communic acid was found only in the *J. procera* resin extracts collected in May 2014 and 2015. Furthermore, the relative concentration of this compound was high (30–60%). The orientation of communic acid may differ, depending on plant sex (35). This illustrates that honeybee response-specific compounds that are present in specific orientation and structure in resins or other materials secreted by different plants. The chemical composition of MeOH extracts of corresponding resin and propolis samples differed; however, the main compounds

were ferruginol, sugiol, and totarol. The compounds pinene and communic acid may play an important role in determining FRSA, as well as other biological activities.

Antimicrobial activity:

Moreover, all resins and relevant propolis samples showed differences in their biological activity; which may be attributable to the specific compounds that were found in each resin and propolis extract. Notably, the solvents used in our study may dissolve certain specific compounds differently than that in other solvents given that compounds have variable solubility in each solvent, and the concentration of each compound in the solvent plays a major role in its biological activity. The propolis extracts produced by honeybees from *J. procera* resins showed strong inhibitory activity against *E. coli* and *S. aureus* and comparatively weak activity against *C. albicans* and *A. niger*. This inhibitory activity may be attributable to monoterpenes (pinene) and diterpenoids (ferruginol, totarol and sugiol), which were found in the DCM propolis extracts produced by honeybees in April, May, June 2014, and April 2015. This result is consistent with a study by (32), which used extracts from *J. procera* leaves and bark as an antimicrobial agent against *Mycobacterium intracellulare*, *Mycobacterium smegmatis*, *Mycobacterium xenopei* and *Mycobacterium chelonei*. In addition, another study reported that α–pinene has antibacterial and antifungal activity (49); however, the concentration of this compound was low in all except DCM extract of this study compared to that found in different propolis samples from Brazil. Sugiol was also reported to act as an antifungal agent (10). Other compounds found in propolis extracts that may contribute to its antimicrobial activity include sesquiterpenes such as α-cedrol, caryophyllene oxide (23, 25, 30, and 36). Additional compounds that exhibit antibacterial activity include triterpenoids (e.g., lupeol, amyrin, and dammaradienol) found in samples of April, May, and June 2015 (55). The propolis samples of the current study showed strong inhibitory activity against pathogenic bacteria, whereas its inhibitory effect was weak against fungal pathogens, as well as resin extracts of *J. procera*. The propolis produced in April 2014 and 2015 showed higher inhibitory activity compared to that in propolis produced in May 2014 and 2015. The varying biological activities of these propolis samples may be attributed to variable concentrations of various compounds in the propolis.

Conclusion:

The current study showed useful and significant results because: (1) propolis samples exhibited strong potency as free radical scavenging and antimicrobial activities and (2) it is the first study to prove that honeybees produce propolis from the resins of *J. procera*. Therefore, more studies are needed to investigate more propolis samples produced by honeybees from the same plant source and area in order to determine which compounds are present in significant concentration. This will help to isolate compounds that are more effective against high-risk diseases such as cancer. Despite the fact that *J. procera* is dispersal in more than one area of Saudi Arabia and is considered the major source for propolis components, many other plant sources are available and still not investigated. Therefore, more studies are needed to investigate more propolis samples and monitoring honeybee workers to find out about the plant sources for propolis production.

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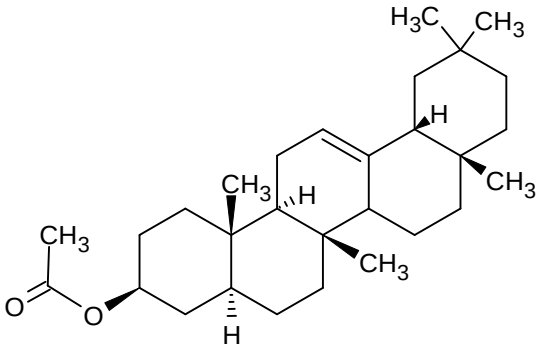
Appendix 1.



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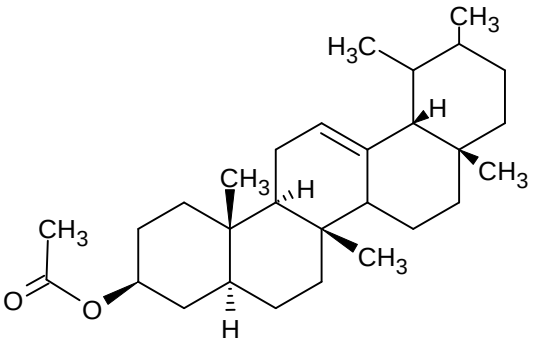

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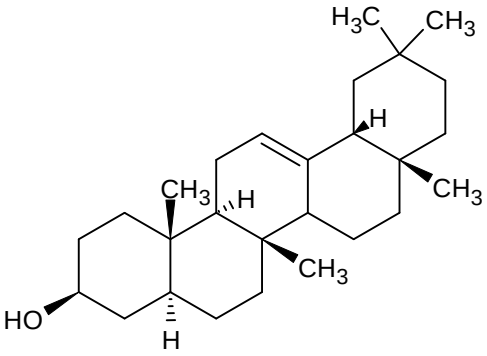

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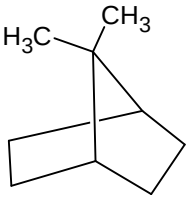

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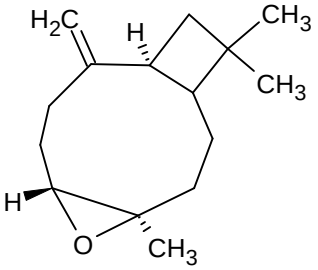

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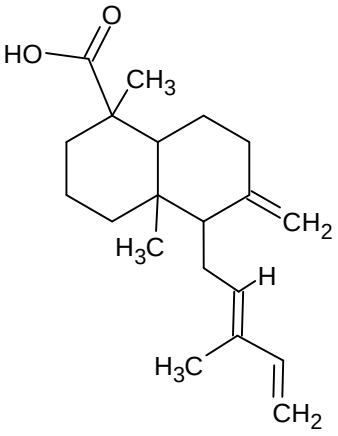

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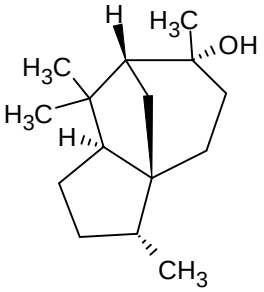

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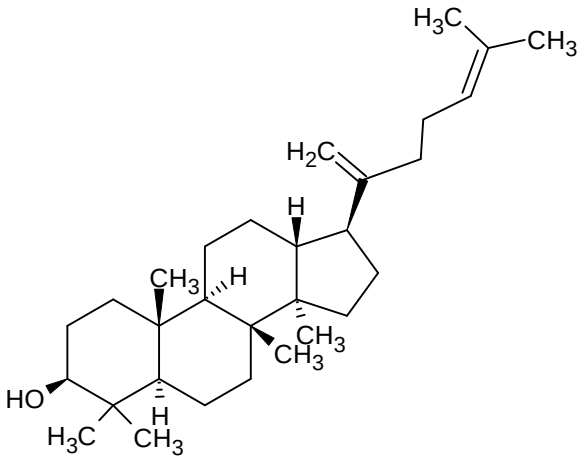

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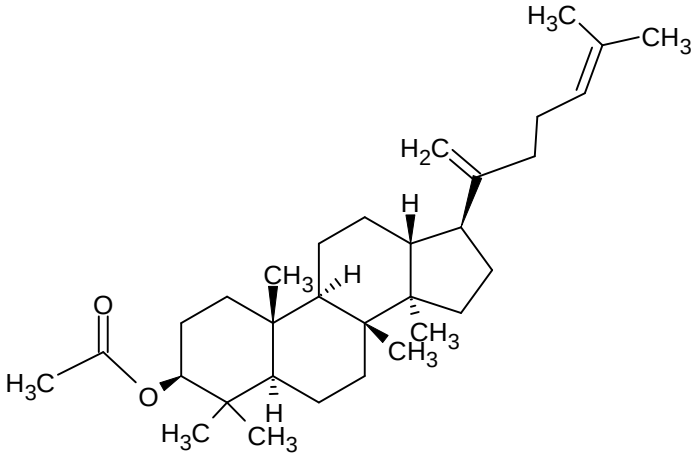

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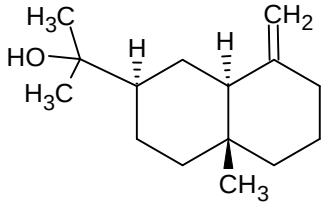

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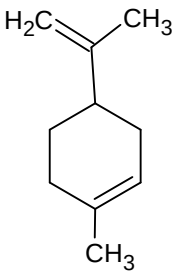

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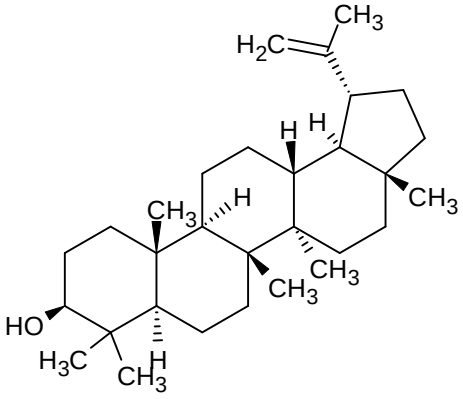

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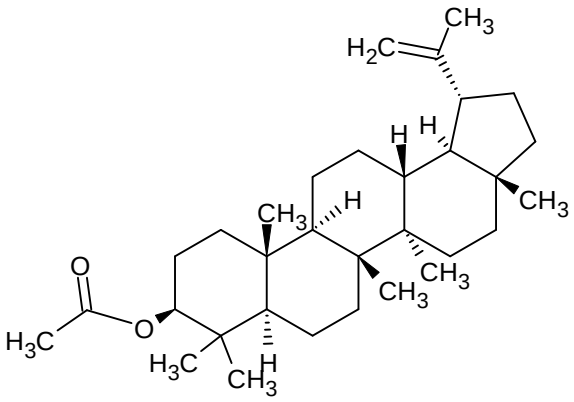

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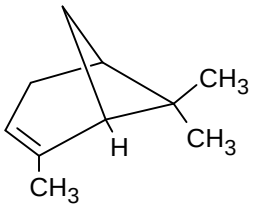

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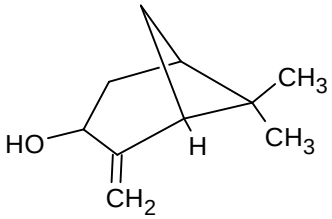

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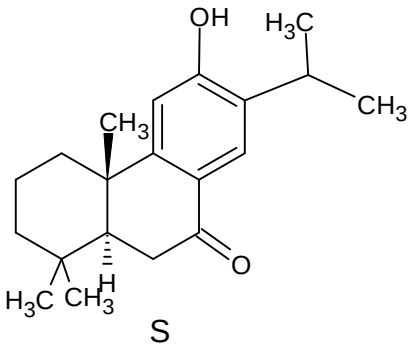

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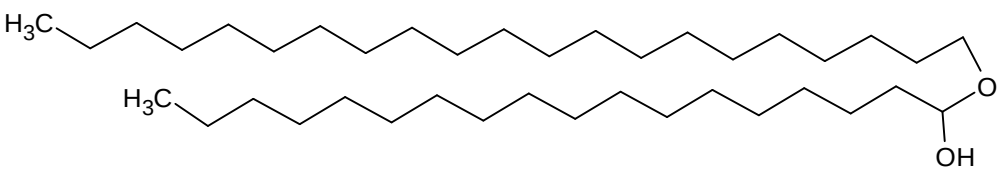

W1

التركيب الكيميائي، المحتوى الفينولي، نشاط الجذور الحرة والنشاط المضاد للميكروبات لراتينج نبات العرعر والبروبوليس المنتج بواسطة سلالة النحل اليمني في منطقة الباحة بالمملكة العربية السعودية

نوفل إبراهيم بايعقوب أحمد إبراهيم رشدي

أحمد عبدالله الغامدي

الملخص

أوضحت النتائج أن كلاً من راتينج نبات العرعر والبروبوليس يحتويان على مركبات مختلفة حيث تتضمن: .mono-,sesqui-,di-and triterpenoids,wax esters, n-alkane, and n-alkene. مستويات المحتوى الفينولي راتينج نبات العرعر كانت عالية خاصة للعينات التي تم جمعها من إبريل حتى يونيو 2014 بينما أقل في العينات التي جمعت من إبريل إلى يونيو 2015.أيضا مستويات المحتوى الفينولي في عينات البروبوليس المجموعة في الفترة من إبريل حتى يونيو 2014كانت عالية بينما أقل في العينات المجموعة في الفترة من إبريل إلى يونيو 2015. نشاط الجذور الحرة لعينات الراتينج المجموعة في الفترة من إبريل–يونيو2014 كان عالياً بينما اقل في العينات المجموعة من إبريل – يونيو 2015. في حين نشاط الجذور الحرة في عينات البروبوليس المجموعة من إبريل–يونيو2014 كان أعلى منه في عيّنات البروبوليس المجموعة في الفترة من إبريل حتى يونيو2015. كل مستخلصات الراتينج والبروبوليس لنبات العرعر المحضرة في الأشهر في إبريل، مايو ويونيو 2014 أظهرت نشاطاً تثبيطياً أقل ضد فطر العفن الأسود في حين المستخلصات المحضرة في إبريل 2015 أظهرت نشاطاً تثبيطياً معنوياً عند ($P<0.05$) ضد البكتيريا *Escherichia coli* بالمقابل مستخلصات البروبوليس المحضرة في إبريل، مايو ويونيو 2015 وكذلك مستخلصات راتينج نبات العرعر أظهرت نشاطاً تثبيطياً غير معنوي ضد الخميرة *Candida albicans* في حين مستخلصات البروبوليس أظهرت نشاطاً تثبيطياً معنوياً ضد البكتيريا *E.coli, S. aureus* بالمقابل كان نشاطها ضعيفاً ضد فطر العفن الأسود والخميرة *C.albicans*.

الكلمات المفتاحية: راتينج نبات العرعر، البروبوليس، سلالة النحل اليمني.