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Antimicrobial Activity and Chemical Analysis of Iraqi Propolis

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Abstract: In this study the antimicrobial effect of ethanolic extracts of Iraqi propolis was tested by using three different concentrations (10%, 20%, and 30%) against Staphylococcus aureus, Escherichia coli and Candida albicans compared with chlorhexidine gluconate 0.2%w/v mouth wash using the disk diffusion method. The chemical composition of 10% EEP was also investigated using high performance liquid chromatography (HPLC) and infrared spectroscopy (FTIR-Fourier Transmittance Infra -Red). The results showed that EEP10% had the most significant powerful inhibitory activity against the growth of Staph. aureus and C. albicans and the same effect against. E. coli compared with chlorhexidine gluconate 0.2% mouth wash. HPLC analysis of propolis by ethyl alcohol and acid hydrolysis releaved two active phenolic compounds (p-hydroxy benzonic acid and benzoic acid). FTIR analysis shows strong broad stretch peak at 3500 cm^{-1} region, this indicated to presence of phenolic groups.

Keywords: Propolis, antimicrobial activity, chemical analysis.

Introduction

Propolis is a resinous substance that is produced by bees. This word comes from ancient Greek, means an outer wall of a city (pro: before, polis: city) and relates to the protective properties of the substance. Bees use it to repair their structure, protect and reinforce their hives, and to cover honeycombs. It kills pathogens, prevents unwanted guests from entering the hive, protects against rain and being a very sticky substance. [1,2]. Propolis, a natural nontoxic substance collected by Apis mellifera bees from different plant sources, that is have several properties that may confer health benefits to humans. Therefore, its chemical composition and pharmacological activity are highly variable depending on its botanical origin. [3]. Propolis chemical composition is very complex. This product is mostly constituted by 50% resin (composed of flavonoids and related phenolic acids), 30% wax, 10% essential oils, 5% pollen and 5% various organic compounds. The contents and composition of these components depend on various factors: geographical origin, vegetation of the area, season, and the state of propolis (fresh or aged). [4,5].

Chemical analysis of propolis has pointed to the presence of at least 300 compounds in its composition. [6]. Among these organic compounds, we may find phenolic compounds and esters, flavonoids in all their forms (flavones, flavonoles, flavonones, dihydroflavonoles, and chalcones), beta-steroids, terpenes, aromatic aldehydes and alcohols, sesquiterpenes, and stilbene terpenes. [7,8].

In propolis most of the phenolic compounds are in the form of flavonoids whose concentration depends on various factors, including health of the plant, plant species used by the bees, season, environmental factors and so on. [9]. Multiple aromatic compounds, mainly phenolics and flavonoids, seem to be the pharmacologically active constituents in propolis [10–12], which are well-known plant compounds that have unique and multidirectional antibacterial, anti-inflammatory, antioxidant, antifungal and immunomodulative properties [13-15].

Materials and Methods

1-Preparation of propolis extract

Propolis was collected at the end of September 2013 from Shikhan city in Mosul countryside, Ninevah Province, Iraq especially between the frames and the internal walls of the hive. Propolis collected was kept in dry place and stored at 4°C until it's processing. Propolis was cut into small pieces after cooling at (-20°c) and 10gm,20gm,30gm of propolis weighted and each weight extracted with 100ml of 70% ethanol. The extraction was carried out at room temperature in dark place by hot plate magnetic stirrer with intermittent shaking for ten days.The ethyl alcohol extract was filtered through whatman filter paper No.1 and kept at refrigerator for 24 hours and refiltered again for the further filtration. Each propolis extract was evaporated by rotary vacuum evaporator. [16].

2-Antimicrobial study

A. Sources of microorganisms:

The organisms used Staph. aureus, E. coli which are obtained from Department of Biology at Collage of Science, University of Mosul and C. albicans which are isolated from inner surface of upper complete denture from old patient attending to prosthodontics department/ College of dentistry/ University of Mosul by using sabouraud's dextrose agar (Oxide), adding to it chloramphenicol antibiotic,. This isolate were identified according to gram stain and the suitable biochemical tests(germ tube test and C.H.O. fermentation test)[17].

B. Antimicrobial activity:

The disk diffusion method was used to investigate the antimicrobial activity of EEP (10%, 20%, and 30%) against Staph. aureus, E.coli and C. albicans, chlorhexidine gluconate 0.2%w/v mouth wash was used as a control treatment. In vitro antimicrobial activity was screened by inoculating activity growing brain heart broth (Oxid)(0.5MacFurland) standard of pathogenesis Staph. aureus, E.coli on Muller Hinton agar plates and C. albicans on sabourauds dextrose agar plates adding to it 50mg/L chloramphicol antibiotic, with sterile swabs which were dipped in broth culture and streaked on agar plates. The disks (6mm in diameter) of whatman filter paper No.1 were prepared by adding 1ml of each EEP (10%, 20%, and 30%) and chlorhexidine to 100 sterile disks. For each solution, disks were applied to the inoculated plates by sterile forceps, after 15 minutes the plates were incubated for 24 hours at 37° C. The plates were examined and the zone of inhibition of growth of microorganisms were noticed and measured by ruler in millimeters to evaluate the propolis antimicrobial activity. [18].

3. Chemical analysis

A. HPLC * Extraction of propolis by ethyl alcohol :

- Propolis material (10 gm) is extracted by using 70% ethanol 250ml, using mechanical starrier for 28 hours.

- After filtration and concentration of the ethanolic extract, using rotatory vacuum evaporator (RVE) to get (5-10) ml of the crude extract.

*Acid hydrolysed with 2M Hcl for (30-40) minutes. The cooled solution is extracted two times with ethyl acetate.

The combined extracts taken to dryness and the residue taken up in a small volume of ethanol for HPLC analysis. [19].

All these accomplished by HPLC apparatus Schimadzo LC Japan.

Mobile phase: acetonitrile, column: C18 (Dos-3) (4.6*250) mm.Gl-Sciences.

B. FTIR

After the samples employed for HPLC analysis, taken to drynesss and used for FTIR analysis by FTIR spectrophotometer (Bruker Tensor) made in Germany.

Statistical analysis

SPSS program version 19 was used to analyze the obtained data. ANOVA test and Duncan's multiple range tests were used for the compa- rision between the study groups. Data expressed as a mean and standard deviation values.The level of significance at $p<0.05$.

RESULTS

The results obtained in this study were represented as descriptive statistic of mean± standard deviation for the values of inhibition zone for (Staph.aureus, E-coli and Candida albicans) between EEP (10%, 20%,and 30%) as a study groups and chlorhexidine as a control.

Antimicrobial study

Table (1) Descriptive Statistics for EEP groups and chlorhexidine gluconate 0.2%w/v mouth wash.

Groups / Types of bacteria	Chlorhexidine 0.2%	Propolis 10%	Propolis 20%	Propolis 30%
Staph. aureus	8.33±1.33 AB	11.33±0.33 A	7.67±1.86 B	7.33±0.89 B
E.coli	3.0±0.58 A	2.33±0.33 A	2.0±0.0 A	2.33±0.33 A
C. albicans	8.33±0.33 B	14.67±2.03 A	10.67±1.86 AB	9.0±1.0 B

*Data describe by mean ±SE.
**Different letters mean there is significance.

The results obtained in this study show that EEP10%, 20% and 30% inhibit the growth of Staph.aureus as mean and stander error (11.33±0.33), (7.67±1.86), (7.33±0.89), respectively. While the inhibition of chlorhexidine 0.2%w/v (8.33±1.33), there was no significant differences between chlorhexidine and EEP (10%, 20%, 30%) against Staph.aureus. There was significant differences in inhibition of EEP10% compare with EEP 20% and EEP 30%. There was no significant difference in inhibition between EEP 20% and EEP 30%. Our study show there was no significant difference between EEP for all concentration and chlorhexidine against E.coli. For C. albicans, the mean and stander error for inhibition zone of EEP10% is(14.67±2.03) and this concentration had significant difference compared with EEP 30%(9.0±1.0) and with chlorhexine (8.33±0.33) while there was no significant difference with EEP 20%(10.67±1.86). This study show that only EEP10% have significant difference with chlorhexidine.The mean and stander error for inhibition zone of EEP10%(14.67±2.03) is more than that chlrorhexidine(8.33±0.33) while there was no significant differences between chlorhexidine and EEP20% and EEP30% as shown in table 1.

From the result of this study, the EEP 10% was the best among the three solutions used and statically better than chlorhexidine against staph. aureus and C. albicans and the same effect against E.coli while 20% and 30% had the inhibitory effect as chlorhexidine against all test microorganisms. The end result; EEP10% have more effect than chlorhexidine while EEP 20% and EEP30% show there is no significant difference compare with chlorhexidine.

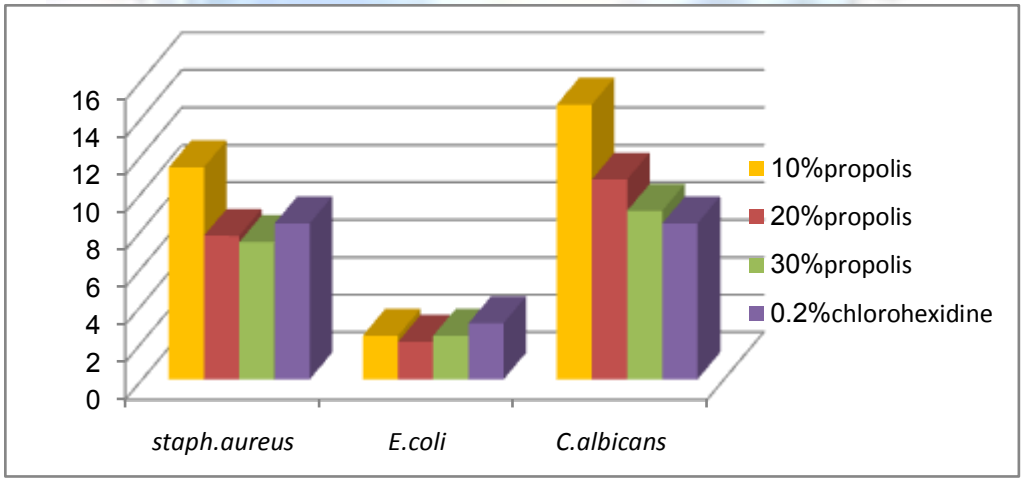


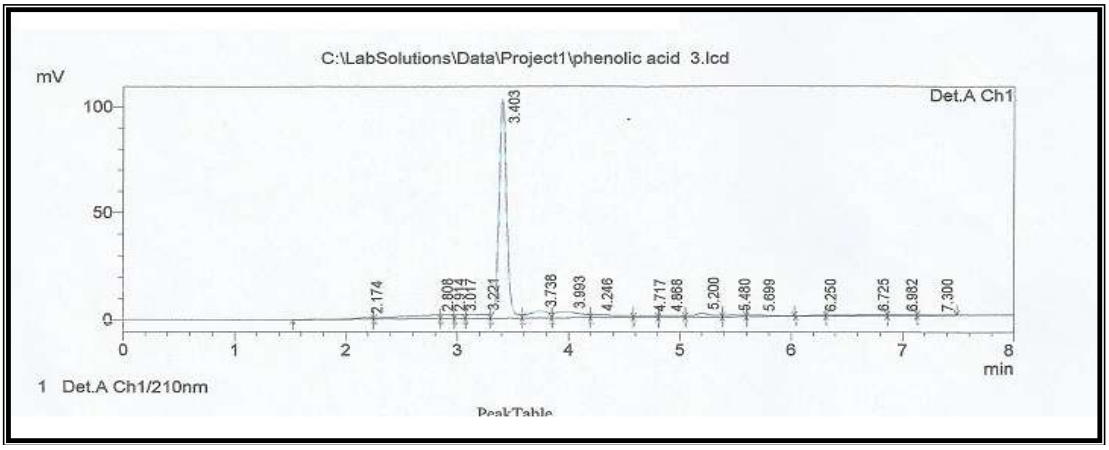
Figure1: Antimicrobial effect of EEP (10%, 20%, 30%) on Staph. aureus, E.coli and C.albicans compared with chlorhexidine 0.2%.

Chemical analysis

High Performance Liquid Chromatography (HPLC)

The EEP10% sample shows the following curve with different peaks level (3.403, 3.518) mV indicating the presence of different phenolic compounds.

*** Extraction of propolis by ethyl alcohol:** indicate the presence of p-hydroxy benzonic acid.



Retention time (min)

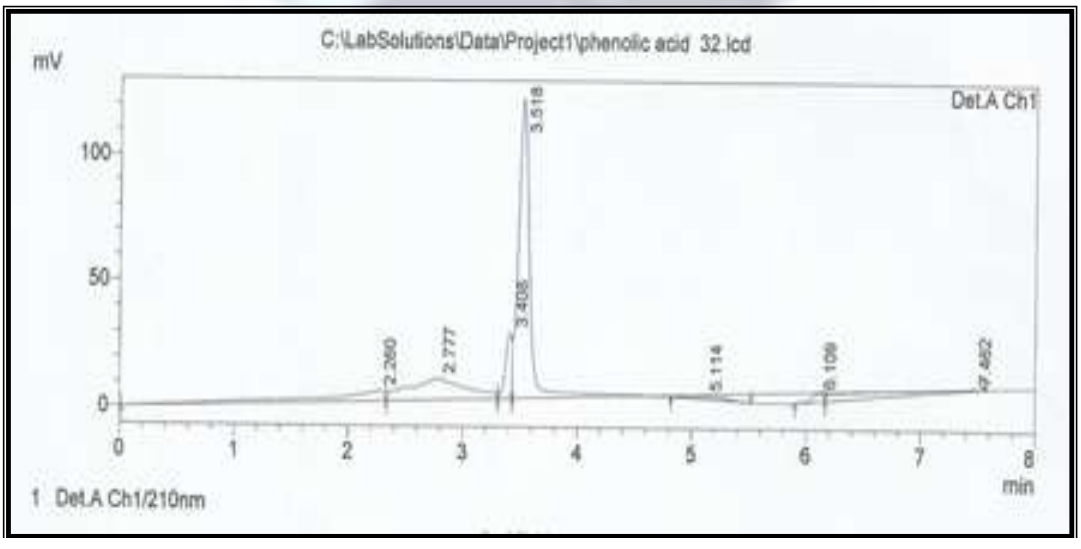
PeakTable

Detector A Ch1 210nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.174	8853	751	1.135	0.606
2	2.808	45170	1711	5.791	1.381
3	2.914	12777	1743	1.638	1.407
4	3.017	9807	1660	1.257	1.340
5	3.221	22929	1898	2.940	1.532
6	3.403	488414	103050	62.617	83.175
7	3.738	39757	3301	5.097	2.664
8	3.993	45302	2632	5.808	2.125
9	4.246	25810	1400	3.309	1.130
10	4.717	12011	891	1.540	0.719
11	4.868	11334	866	1.453	0.699
12	5.200	19276	1609	2.471	1.299
13	5.480	8213	703	1.053	0.568
14	5.699	8261	536	1.059	0.433
15	6.250	4791	444	0.614	0.358
16	6.725	11813	351	1.514	0.283
17	6.982	3732	256	0.479	0.207
18	7.300	1748	93	0.224	0.075
Total		779999	123896	100.000	100.000

Figure (2) chromatogram of EEP 10% sample as it appear in HPLC.

***Acid hydrolysis:** indicate the presence of benzoic acid**.**



Retention time (min)

PeakTable

Detector A Ch1 210nm

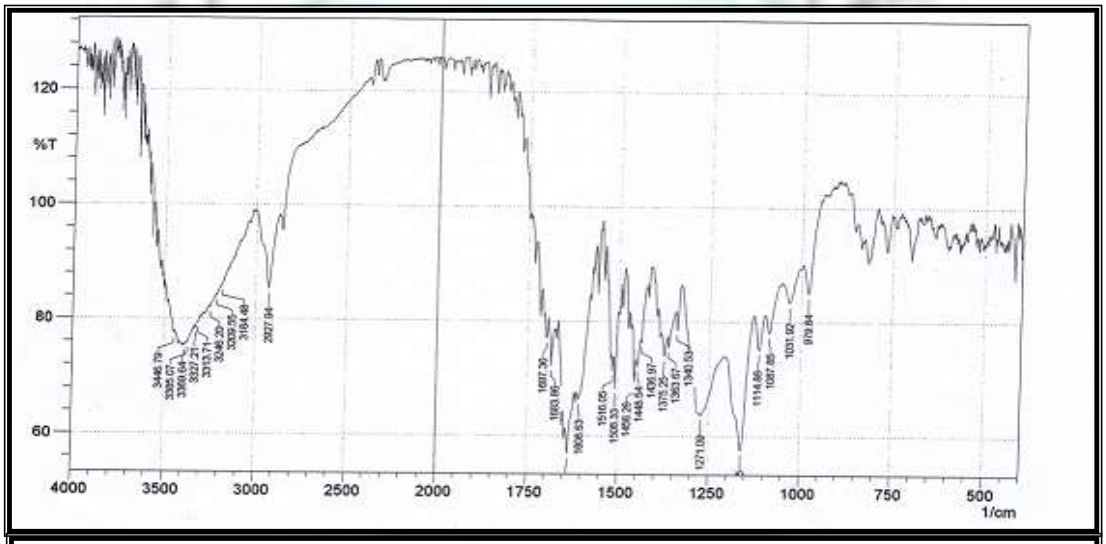
Peak#	Ret Time	Area	Height	Area %	Height %
1	2.260	176234	4068	14.982	2.525
2	2.777	296437	8368	25.201	5.194
3	3.408	105366	25843	8.958	16.042
4	3.518	428728	118311	36.448	73.441
5	5.114	24733	1008	2.103	0.626
6	6.109	22367	3088	1.902	1.917
7	7.462	122418	410	10.407	0.255
Total		1176283	161096	100.000	100.000

Figure (3) chromatogram of ethyl acetate of EEP 10% sample as it appear in HPLC.

Fourier Transmittance Infra –Red (FTIR)

The FTIR shows:

Strong broad stretch peak at 3500cm^{-1} region, this indicated to presence phenolic group. Medium sharp stretch peak at3028cm^{-1} due to aromatic(C-H).Strong sharp stretch peaks at 2927 cm^{-1}and 2856cm^{-1}due to saturated (aliphatic) (C-H). Medium sharp stretch peak at 1645cm^{-1}due to unsaturated aliphatic double bonds. Strong sharp deform at peak 1280cm-^{1} due to(C-O) from the analysis mentioned above, may be this compound has a structure similar.



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	979.84	84.994	9.039	995.27	950.91	1.641	0.811
2	1031.92	83.429	4.256	1047.35	1008.77	2.59	0.45
3	1087.85	78.076	4.81	1099.43	1060.85	3.363	0.444
4	1114.86	75.144	6.048	1128.36	1101.35	2.915	0.472
5	1161.15	57.595	20.784	1209.37	1130.29	13.303	4.596
6	1271.09	63.762	16.634	1328.95	1211.3	17.477	6.045
7	1340.53	78.463	4.61	1344.38	1330.88	1.134	0.138
8	1363.67	75.488	2.31	1365.6	1346.31	1.992	0.086
9	1375.25	74.021	3.459	1382.96	1367.53	1.849	0.133
10	1436.97	76.325	2.056	1438.9	1427.32	1.026	0.033
11	1448.54	72.151	1.704	1450.47	1440.83	1.245	0.051
12	1456.26	69.534	6.226	1462.04	1452.4	1.342	0.176
13	1508.33	68.318	8.718	1512.19	1502.55	1.227	0.213
14	1516.05	73.512	1.603	1529.55	1514.12	1.884	0.294
15	1608.63	66.393	3.245	1612.49	1581.63	4.345	0.591
16	1635.64	56.841	5.715	1641.42	1620.21	4.374	0.314
17	1683.86	72.254	7.089	1691.57	1678.07	1.6	0.243
18	1697.36	77.04	4.7	1710.86	1693.5	1.668	0.245
19	2927.94	85.337	13.061	2999.31	2877.79	4.009	3.197
20	3184.48	85.885	0.218	3186.4	3153.61	1.949	0.029
21	3209.55	84.122	0.3	3211.48	3196.05	1.106	0.009
22	3246.2	81.979	0.459	3250.05	3224.98	2.058	0.025
23	3313.71	79.291	0.195	3315.63	3302.13	1.332	0.015
24	3327.21	78.377	0.328	3329.14	3317.56	1.192	0.009
25	3369.64	75.741	0.52	3375.43	3340.71	3.975	0.061
26	3385.07	75.308	0.225	3387	3377.36	1.174	0.008
27	3446.79	77.319	1.334	3477.66	3441.01	3.559	0.065

Figure (4): EEP 10% measurement by FTIR (Infra-red) spectroscopy.

DISCUSSION

Various industries are now looking into sources of alternative, more natural and environmentally friendly antimicrobials, antibiotics, antioxidants and antibiotics crop protection agents [20]. Therefore, the goal of this study was to investigate the effect of different concentrations ethanolic extract propolis on Staph. aureus, E. coli and C. albicans. In present study, propolis showed good antibacterial and antifungal activities against gram positive microorganism and fungi, respectively. The antimicrobial effects of these extracts were comparable to the effect of chlorhexidine using disc diffusion test. [21]. In this study showed that propolis inhibited the gram positive bacteria more than gram-negative bacteria. Generally, plant extracts are usually more active against gram-positive bacteria than gram-negative bacteria. [22].Some sciences reported that ethanolic extracts of propolis showed high antibacterial activity against gram-positive cocci (Staph. aureus), but had a weak activity against gram-negative bacteria and yeast (C. albicans). [23].

From these results it appears that E.coli is more resistant than S. aureus against propolis. E.coli is considered a particularly very dangerous pathogen because of its resistance to many commonly used antibiotics. Therefore, to prevent contamination from E.coli higher concentrated propolis or more effective disinfectant/antibiotic should be applied. E.coli is a gram negative bacteria and it is notorious for its resistance to many antibiotics due to the permeability barrier afforded by it's outer membrane. [24].It may be due to differences in the composition and structure of Gram positive, Gram negative bacteria & fungal cell wall and this observation was documented by Orsi et al.2005 who had suggested that propolis shows limited action on Gram-negative bacteria. [25].

Bankova et al. 2000 is expected that antimicrobial action is always existent because of its vital importance as antimicrobial agent to the bees, independently of the region where the propolis is produced. [26]. The mechanism of antimicrobial activity is complex and could be attributed to a synergism between phenolic and other compounds, [27] this reported in 1993 by Korel et al. [28]. Katircioglu and Mercan in 2003were documented that flavonoids were the most important group of compound with propolis biological activity. [29]. Miorin et al. (2003) suggested that effectiveness of honey or propolis depends on differences in chemical composition, geographic region and bee species. [30].

The antibacterial activity and chemical composition of propolis from bees have been reported by Marcucci et al 2000, [31] and Velikova et al, [32] HPLC should be considered to evaluate the quality of herbal medicines all over the world considering multiple constituents present in the herbal medicines and its products. [33].HPLC analysis performed with the purpose of identify and quantify its mainly phenolic compounds. According to our research these compounds (benzoic acid and para-hydroxybenzoic acid). Higher amount of para-hydroxybenzoic acid is present in propolis which attributed to the antibacterial activity of propolis may be related to the presence of flavonoids. [34].

CONCLUSION

Present findings revealed that EEP 10% had higher antibacterial activity against Staph. aureus and C. albicans and same effect against E.coli when compared with chlorohexidine gluconate 0.2% w/v mouth wash. Higher concentration of propolis may be investigated to inhibit .coli. We conclude from this study that (EEP) propolis possesses antibacterial and antifungal activities, which may vary according to the geographical locations where the propolis was produced and according to bacteria species, therefore, propolis might be a useful as antibacterial and antifungal agents especially against Staph. aureus and C. albicans.

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