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Antioxidant and Antimicrobial activities of Egyptian Bee Pollen

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ABSTRACT

Bee pollen obtained from (*Trifolium alexanderinum* L.) has been collected and extracted by ethanol (Total ethanol extract TEE). Ethanol extract was further separately fractionated by solvent of increasing polarities, petroleum ether (pet. Ether), dichloromethane and (DCM) and ethyl acetate(EtoAc). The produced fractions as well as the main extract were tested for phenolic and flavonoids contents. EtoAc subfraction exhibited the highest phenolic and flavonoid contents represented by (2.3 and 0.8µg/ml).The antioxidant activities of the ethanol 70% extract and its subfractions were also measured. TEE and EtoAc subfractions showed the highest antioxidant activities (90 and 79%, respectively). Additionally, the antimicrobial activities of TEE and its subfractions were also evaluated. Which revealed that TEE, pet. ether and DCM subfractions showed the highest antimicrobial activities against three tested microorganisms but EtoAc subfraction showed the lowest antimicrobial activity.

***Keywords*:** Bee pollen, Antioxidant, total phenolic, total flavonoids, antimicrobial activity

Introduction

Antioxidant compounds have the ability to reduce oxidative damage of biomolecules, including lipoprotein and DNA from reactive oxygen species(Mărghitaş *et al.,* 2009).The DPPH radical scavenging test is one of the simplest method used to evaluate antioxidant activity in natural products (Kedare and Singh, 2011). The antioxidant activity of phenolic compounds due to their redox properities by absorbing and neutralizing free radicals (Javanmardi *et al.,* 2003). Bee pollen and other bee products are characterized by high antioxidant activity (de Arruda *et al*., 2013) .Pollen of different botanical conditions possess different antioxidant activities which is related to their content of phenolic acids and flavonoids(Almaraz-Abarca *et al*., 2004).Natural products considered as sources of new chemical diversity,the search for new antimicrobial substances exhibiting minimal side effects because of the harsh side effects of several drug (Boukraâ *et al*., 2013).Pollen collected by the honey bee (*Apis mellifera*) for feeding its larvae in the early stages of development (Muradian *et al*., 2005).When bees visiting flowers, they touch the stamens which cover all their bodies, then they use their legs to push pollen in pollen basket. The bees moisten the pollen with mouth secretionnectar and salivary substances to help pollen to adhere together at the basket hairs (Campos *et al*., 2008). The secretion of bees contains different kind of enzymes,e.g. amylase, catalase etc. Pollen contains several phytochemicals such as terpenoids, flavonoids, carotenoids and steroids (Kao *et al*., 2011).Bee pollen including other minor components like minerals and trace elements, vitamins and carotenoids, phenolic compounds, flavonoids, sterols and terpenes (Silva *et al*., 2014). In fact, bee pollen is referred as the "complete food", as it contains all the essential amino acids needed for the human bodies .The bee pollen considered a rich source of essential amino acids with branched chains, i.e., leucine, isoleucine and valine. It also contains lipids especially unsaturated fatty acids. However, the composition of bee pollen depends strongly on the plant source and geographic origin, together with other factors such as climatic conditions, soil type and beekeeper activities (Feás *et al*., 2012). Phenolic compounds especially flavonoids display a wide range of biological effects including

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antioxidant and antiviral activities, anti-inflammatory, antiallergic, antithrombotic andvasodilatory actions , also phenolic compounds has the ability to lower the risk of coronary heart diseases slowing down the aging process as well as lowering cholesterol levels (Dastan *et al*., 2017).

The present study aimed to evaluate the antioxidant activity, antimicrobial activities, total phenolic and total flavonoids of TEE and other three successive fractions.

Material and methods

Bee pollen extraction

Bee pollen of (*Trifolium alexandrinum* L.) about 4 kg were grinded and extracted with ethyl alcohol (ETOH) 70% and the extract was filtered and concentrated under reduced pressure. The dried residue about 100g was suspended in water and then fractioned with pet. ether, DCM and EtoAc. Then all solvents were evaporated till dryness under reduced pressure.
The obtained extractes were subjected to the following tests:-

1-Total phenolic contents

Total phenolic content was determined according to the Folin-Ciocalteau colorimetric method according to Singleton and Rossi, (1965). Pinocembrin was used as a standard for the calibration curve. The sample 0.5mLand 2mL of sodium carbonate 75g/L were added to 2.5mL of 10% (w/v) Folin-Ciocalteau reagent. After 30min. of reaction at room temperature, phenolic content was measured at 765nm.

2- Total flavonoids content

Total flavonoid contents were determined by the aluminum calorimetric method described by (Quettier-Deleu *et al*., 2000) using quercetin as the reference standard. Briefly, the test sample of ethanol solution (150µL) was mixed with 2% (w/v) aluminium chloride (150µL). After 15 min of incubation at room temperature, the total flavonoids were measured at 435nm by spectrophotometer.

3-DPPH radical scavenging activity

DPPH radical scavenging activity of all fractions was analyzed according to Matsushige *et al*. (1996)1ml of methanol solution for each extract (100µg/ml) was added to 1ml of methanol solution of DPPH (60µM). The prepared solutions were mixed and left for 30 min. at room temperature. The optical density was measured at 520nm using a spectrophotometer.

4-Antimicrobial activities of different fractionated extracts

The antimicrobial activity of different extract were determined according to Abdel-Aziz *et al*., (2014); Barry, (1976) by the agar cup plate method. Four different tested microorganisms namely: *Staphylococcus* aureus (*S.aureus* G+ve), *Pseudomonas aeruginosa (P.aeruginosa* G-ve), *Candida albicans* (*C. albicans* yeast) and *Aspergillus niger* (*A.niger* fungus) were used. Nutrient agar plates were heavily seeded uniformly with 0.1ml of 10^5-10^6 cells/ml in case of bacteria and yeast. A Czapek-Dox agar plate seeded by 0.1mlthe fungal inoculum was used to evaluate the antifungal activities. Then a hole (1cm diameter) was made in media by gel cutter (Cork borer) in sterile conditions Then, one drop of melted agar was poured into hole and allowed to solidify to make a base layer. After that specific amount of tested sample (0.1 ml) was poured into the hole. The plates were kept at low temperature (4°C) for 2-4 hours to allow maximum diffusion. The plates were incubated at 37°C for 24 hours for bacteria and at 30^{o}C for 48 hours in upright position to allow maximum growth of the microorganisms. The antimicrobial activity of the test agent was determined by measuring the diameter of zone inhibition expressed in millimeter (mm).

Results and Discussion

1-Total Phenolic and total flavonoid contents

The Bee pollen contains considerable amounts of phenolic compounds. Total phenolic content in the main extract of TEE was 1.6±0.07µg GAE/ml (Table 1).The total phenolic content of other fractions ranged from 2.3 ± 014 to 0.8± 0.07µg GAE/ml. Regarding to total flavonoids ,the high value were detected in EtoAc fraction (0.85±0.02 µg QE/g DW),The total flavonoid content of TEE was(0.5±0.042µg QE/g DW), while The total flavonoid content of other fractions ranged from 0.1±0.005to 0.85±0.02 µg QE/g DW .

It was found that the compounds with a flavonol skeleton always had the highest antioxidant activities (Wenjun *et al.,* 2015). In contrast, isoflavan had a slightly lower antioxidant activity than flavonol followed by chalcone, isoflavone and isoflavone glucoside had the lowest antioxidant capacities, as a result of the number and position of hydroxyl groups and the different flavonoid skeletons would largely influence the radical scavenging ability. Amić *et al.* (2003) stated that the 3-OH group would significantly increase the radical scavenging power, and among all the tested compounds, only compounds had the 3-OH group, which were found to have the highest anti-radical activities. In contrast, the compounds which had no 3-OH group, had the lowest radical scavenging power (Cai *et al*., 2006). Moreover, the presence of ortho-dihydroxy groups on the B-ring or A-ring could have also enhanced the radical scavenging potential, which is a possible explanation as to why some compounds had slightly higher antioxidant activity in comparison with others. It was studied the importance of the free hydroxyl group on position 3 of C ring and two hydroxyl groups on B ring as important sites for scavenging NO (Mac Micking *et al.,* 1997). This result was important because NO is a diffusible agent that reacts to superoxide anion generating peroxinitrite, which causes LDL (low density lipoprotein) oxidation, a key process of atherosclerosis etiologic process (Metodiewa *et al.,* 1997). There are many limiting factors affecting the concentration of phenolic compounds (Hegazi *et al.,* 2004) i.e., type of solvents, extract temperature, stirring and the origin and source of the Bee pollen. (Leja *et al.,* 2007) studied thephenolic constituents and the antioxidant capacity of bee pollen from 12 species from Poland , it was noticed that the antioxidant activity was related to phenylpropanoid content ,While the flavonoids to the total phenoliccompound contents differed as a function of floral origin from 4.78% (*L. purpureum*) to 37.3% (*C. angustifolium*).(Serra Bonveh *et al*., 2001) determined both of total phenolic ant flavonoid content in 11 bee pollen from Spain, flavonoids quercetin, miricetin and trans cinnamic acid were the major flavonoid content. Phenolic acids and flavonoids were considered as natural constituents of bee pollen, which are responsible for its biological activity (Rzepecka-Stojko *et al.,* 2015; Almeida-Muradian *et al*. 2005).

Table 1: Total phenolic and flavonoid content of Bee pollen fractions

Bee pollen fraction	Total Phenolic content mg/g GAE	Total Flavonoid content mg/g QE
Ethanol 70%	1.6±0.007	0.5±0.042
Dichloromethane	0.8±0.07	0.1±0.005
Petrolium ether	1.5± 0.010	0.2±0.007
Ethyl acetate	2.3±0.014	0.85±0.02

2-DPPH-free radical scavenging activity

DPPH radical scavenging activity is one of the most widely used method for screening the antioxidant activity of plant extract. The (fig 1) shows the antioxidant activities of some Bee pollen fractions .The TEE produced the higher radical scavenging activity when compared with other solvents i.e. EtoAc, DCM and Pet.ether. The highest DPPH scavenging activity was observed in TEE (90%), followed by EtoAc and pet.ether fractions (79%, 75%), While DCM has moderate activities (63%). For a long time, bee pollen was considered as a good source for human nourishment with high energy (Pascoal *et al*., 2014). The 70% combined mixtures of ethanolic propolis and pollen extracts (EPP70) has found to exhibit high antioxidant activity which has been measured by (DPPH) assay method (Abu Shady *et al*., 2016). In comparison between two Brazilian bee pollen it was reported

that Melipon arufiventris caused 50% inhibition of DPPH ,while T. apicalis only observed 39% of DPPH inhibition (Silva *et al.,* 2009), The difference in antioxidant activity is contributed to the difference compounds found in bee pollen extract and it could be due to specific pollen for aging activities and different diets of stingless bee itself (Nagamitsu and Inoue., 2002). EtoAc, DCM and Pet.ether extracts exhibited lower antioxidant activity than TEE, The high antioxidant activity of ethanol extract can be also attributed to its major components, i.e. gallic acid and catechin (El Gengaihi *et al.,* 2014). Our obtained data are in agreement with these findings, and the highest values of polyphenols in this fractions relate to the good DPPH radical inhibition results, this correlation between phenolics compounds and the antioxidant capacity (DPPH method) is in good agreement with the results of Mustafa *et al.* (2010). Some studies suggest that it is not always possible to correlate the total phenolics and antioxidant capacity. This can be explained by several factors, including the presence of different active compounds in the plant that can modify the antioxidant capacity, the synergistic effects of different compounds, the experimental conditions, and the mechanisms of the methods used for antioxidant reactions. Structural factors include the nature of the phenolic groups and the changes caused by glycosylation (Cho *et al*., 2003), There are also compounds that react strongly with the DPPH, and others that have a slower reaction rate (Tsimogiannis *et al.,* 2006).

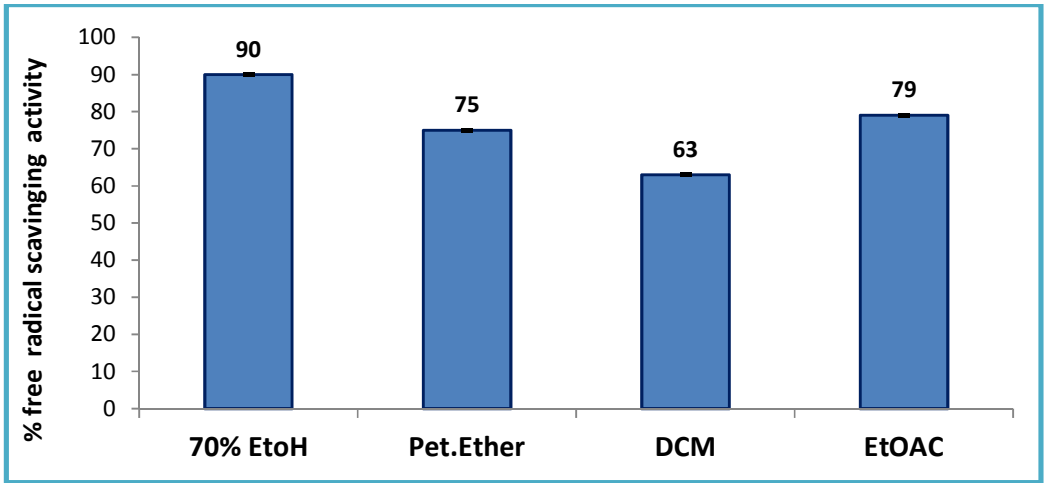

Fig. 1: DPPH-free radical scavenging activity of bee pollen ethanol extract and its subfractions**.**

3-Antimicrobial activities

The antimicrobial activities of bee pollen on four different test microorganisms namely: *S.aureus* (G+ve), *P.aeruginosa* (G-ve), *C.albicans* (yeast) and *A.niger* (fungus) by using cup plate method has been studied (Table 2 and Fig 2).EtOH 70% extract of bee pollen exhibited the highest clear zones against *S. aureus* (38 mm) ,followed by *P. Aeruginosa*(33mm). On the other hand, the pollen extracts had moderate antimicrobial efficiency against *A. niger* (17mm) and *C. albicans* (15mm), respectively .Pet.ether fraction showed the highest clear zone against *P. aeruginosa* (41 mm) followed by *S. aureus* (33mm).With respect to *C. albicans*, pet. ether fraction exhibited a clear zone represented by 18mm. However, pet.ether fraction didn't give antifungal activity against *A. niger* of DCM fraction, it has been noticed that DCM fraction had the highest activity against *P. aeruginosa* (45 mm) followed by *S. aureus* (38mm). Whereas, in case of *C. albican*s; DCM fraction showed moderate activity as compared to the other fractions (18mm).On the other hand , pet.ether fraction could not inhibit the fungus *A. niger* growth and no clear zone appeared. Moreover, the inhibitory effect of EtoAc fraction showed moderate activity against the different tested microbes: The *S. aureus* with an inhibition zone of 22mm,the *P. aeruginosa* with an inhibition zone of 19mm, the *C. albicans* with an inhibition zone of 18mm and the *A. niger* with an inhibition zone of 17mm. Generally, the pollen extracts were more effective against Gram-positive and Gram- negative bacteria**.** The best inhibitory properties of *Trifolium* bee pollen solvent extracts were found on ethanolic

extracts 70%, followed by pet. ether and DCM to *S. aureus* and *P. aeruginosa.* On the other hand, moderate inhibitory activity for EtoAc fraction was measured. All bee pollen fraction had similar antimicrobial effect to *C. albicans*, while the activity against fungi vary from moderate inhibitory properties to no activity. Morais *et al.* (2011) studied antimicrobial activity of bee pollen extract to some selected bacteria, they determined that the strongest effect of bee collected pollen extract was against *E. coli* and *S. typhi*, *Entero bacteriaceae* respectively. And the lower antimicrobial activity of bee collected pollen extract against *Bacillus cereus* and *S.aureus*,They also determined that bee collected pollen extract had antimicrobialeffect to some yeasts and fungi .It was reported that S. aureus is inhibited by extracts of Greek, Slovakia and Egyptian pollen respectively, (Khider *et al.,* 2013) and also observed antibacterial activity of pollen against *L. monocytogenes* (Graikou *et al.,* 2011). Many investigators have observed that antibacterial activity of pollen extracts could be ascribed to the high content of phenolic compounds, Examples of such phenolic compounds include p-coumaric, caffeic and ellagic acids, galangin, pinocembrinand tectochrysin and flavonoids, These are found in pollen such as glucosides, quercetin and kaempferol. All are variable depending on their floral source .(Erkmen and Ozcan, 2008) investigated the antimicrobial activity of pollen extract at concentrations from 0 % to 2.5 % (v/v) on bacteria (*B. cereus*, *B. subtilis*, *E. coli*, *S. typhimurium*, *S. aureus*, *L. monocytogenes* and Yersinia enterocolitica, Enterococcus faecalis), yeasts (Saccharomyces cerevisiae and Candida rugosa), and molds (Aspergillusniger and Rhizopusoryzae). But, these concentrations had no antimicrobial effect on the bacteria and fungi. The results showed that the antimicrobial activity depended on concentration. Teresinha Carpes *et al*. (2007) determined antibacterial activity of some pollen extracts (Parana and Alagoas state) obtained with different concentrations of ethanol by disk diffusion assay. While any ethanolic extract of Parana pollen had no effect on S. aureus and B. cereus; rate at 50, 60, 70 and 80 % of Alagoas pollen extract had an effect on S. aureus, rate at 50 and 60 % had an effect on B. cereus. Furthermore, *P. auriginosa* was inhibited by Parana pollen extracts rate at 60 and 70 %.Pollen extracts (Alagoas state, Brazil) have been obtained using different concentrations of ethyl alcohol (40-90%). All ethanolic extracts showed antimicrobial activity against *S. aureus* except 95% (Carpes *et al*., 2007).Whereas, 60% ethanolic extract inhibited *Bacillus subtilis*, *P. aeuginosa* and *Klebsiella* sp.The mode of action of flavonoids and other phenolics has been considered including cytoplasmic membrane damage, topoisomerase inhibition, NADH-cytochrome c reductase inhibition, and ATP synthase inhibition (Cushnie and Lamb, 2011). flavonoids have mechanisms of action involving cell membranes (Tsuchiya, 2015; Verstraeten *et al*., 2015). The mechanisms of action of phenolic compounds with an emphasis on structural features correlated to specific mechanisms (Gyawali and Ibrahim, 2014) were additionally studied.

Table 2: The antimicrobial activity of different solvent fraction of bee pollen against *Staphylococcus aureus* (G+ve), *Pseudomonas aeruginosa* (G-ve), *Candida albicans* (yeast) and *Aspergillusniger* (fungus)

Fraction no.	Sample Name	Clear zone (ϕmm)			
		Staphylococcus aureus	***Pseudomonas aeruginosa***	***Candida albicans***	***Aspergillus niger***
1	Ethanol 70%	35	38	15	17
2	Dichloromethane	33	41	18	0
3	Petrolium ether	38	45	18	0
4	Ethyl acetate	22	19	17	18

In case of *S. aureus* activity of the extracts represented: DCM> TEE> Pet.ether > EtoAc.
In case of *P. aeruginosa* activity of the extracts represented: DCM> Pet.ether >TEE >EtoAc.
In case of *C. albicans* activity of the extracts represented: DCM=Pet.ether >EtoAc >TEE.
In case of *A. niger* activity of the extracts represented: EtoAc>TEE> Pet.ether= DCM.

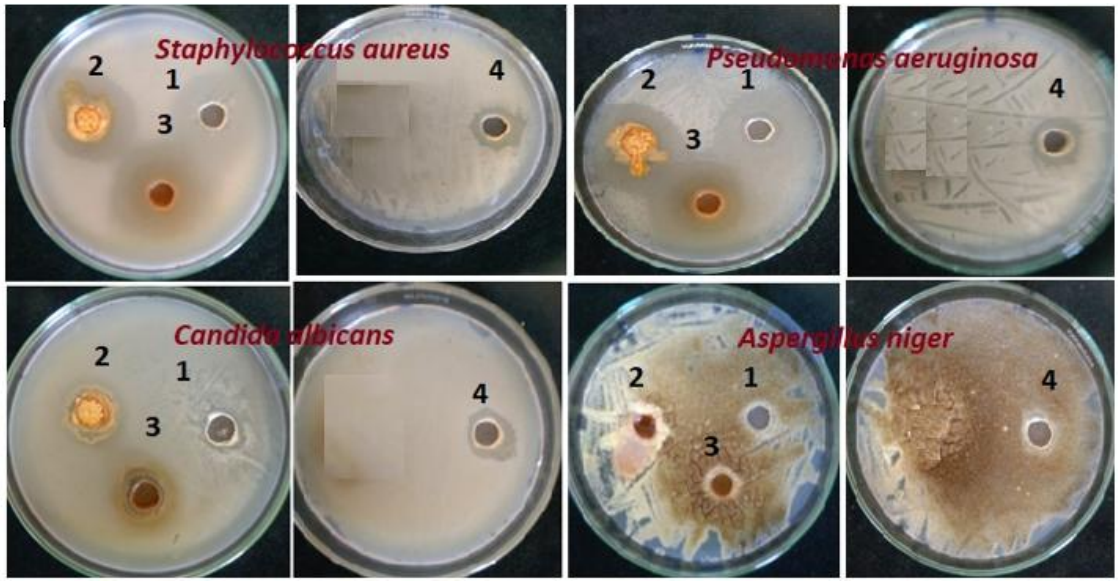

Fig. 2: The antimicrobial activity of different solvent fraction of bee pollen against *S. aureus* (G+ve), *P. aeruginosa* (G-ve), *C. albicans* (yeast) and *A. niger* (fungus)

Conclusion

These provided results suggested that bee pollen contained polyphenolic and flavonoid components which may become attractive and promising treatment for metabolic diseases and it has highly antioxidant activity. Extraction of bee pollen with different solvents leads to the production of extracts with different biological activities. Further fractionation of extracts and purification resulted in the skeleton identification of the most effective pure compound that could be responsible for the bee pollen biological activity. The antimicrobial activities of pollen extracts were evaluated and the activity was differed according to the solvent used.

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