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Synergistic antioxidant activity of honey bee products and their mixtures

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Abstract: The aim of this work was to evaluate the influence of binary combination antioxidant synergistic effects of Honeybee products (citrus honey, clover honey, sugar feeding honey, bee pollen, bee bread, bee wax, old wax comb, Egyptian propolis, Chinese propolis, royal jelly, Drone brood homogenate, Worker brood homogenate, queen brood homogenate, bee venom) and The present study compared the antioxidant activity between water and ethanol extracts of bee products and evaluated the synergistic antioxidant activity effect of binary combination of bee products (water and ethanol extracts, separately), the antioxidant activity was analysed via DPPH free radical scavenging activity assay and found that, propolis is the most powerful antioxidant of all the bee products examined, and the ethanol (80%) extraction method recorded more antioxidant activity than the water extract, but in the royal jelly, drone brood homogenate, worker brood homogenate, queen brood homogenate, bee venom the water extract were the highest. The data obtained provide that, the mixture activity of bee products understudy affected by the interaction between bioactive compounds which effect on the substances showed antioxidant activity, and some of these binary combinations recorded synergistic effects, this may be due to the antioxidant capacity may increasing by additive another antioxidant components from other products.
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1. Introduction

Antioxidants such as ascorbic acid, tocopherols, ubiquinol-10, flavonoids, polyphenols, glutathione, glutathione peroxidases, and reductase, catalases and other peroxidases protect lipids, proteins and DNA against damage by reactive oxygen species in the human body (Sies, 1997). The same antioxidant classes occur in many products, such as tea, medicinal plants, spices, fruits, vegetables, honey, beebread, *etc*. (Wettasinghe and Shahidi 2000; Riemersma *et al.*, 2001; Čeksterytė *et al*., 2006) .

Honeys and related by products have a great potential to serve as a natural food antioxidant (Silva *et al.*, 2011)Bee products such as honey, propolis, pollen, royal jelly and bee venom are among the most popular natural products used in folk medicine due to their powerful healing properties and their content in bioactive molecules (Brown *et al*., 2016). This is why many publications are currently devoted to the study of the therapeutic effects and chemical composition of these products (Fratini *et al*., 2016; Zhou *et al*., 2015).

Honey is widely used in human diseases and has been recently introduced to modern medicine as an important intervention in wound healing. It has various activities such as anti-inflammatory and antioxidant properties, and has hepatorenal protective activities (Al-Waili *et al*., 2006, 2011; Kolayli *et al*., 2016) Bee pollen has antioxidants such as phenolic acids and flavonoids which have anti-inflammatory properties. It contains polyphenols, carotenoid pigments, phytosterols, tocopherol, vitamins, enzymes and co enzymes which are attributed to its biological activities (Denisow and Denisow-Pietrzyk 2016).Drone brood homogenate (DBH), that is nearly completely unknown in Western Europe. It is, however, available in Romania and some Eastern European countries under the commercial name of Apilarnil, a bee product that is based on DBH invented in Romania during the early 1980s by Nicolae Iliesiu (Iliesiu, 1981). It is prepared through the homogenization and lyophilization of whole combs containing 7 day old male bee larvae (Sawczuk *et al*., 2019). Apilarnil is highly valued in Romania for its nutritional properties and is used in cases of disease-induced malnutrition, treatment of anorexia, and even depression (Sawczuk *et al*., 2019; Sidor and Dżugan, 2020).

Many traditionally used plants and bee products exhibit greatly improved pharmacological outcomes when used in combination than when used individually (Boukraa 2008; Xu *et al*., 2014). In fact, synergistic interactions between the constituents of natural products considerably contribute to the

enhancement of their therapeutic efficacy. Yoirish (2001) have mentioned that honey can be used in combination with a large number of medicinal plants.

Honeybee products are composed of different nutrients and have different biological activities. In recent years, consumers have been using a combination of different foodstuffs to get the maximum benefit. Therefore, there is an increasing trend in the production of foodstuffs that include a combination of different products (Nagai *et al*., 2001, Koç *et al.*, 2011, Kanbur *et al.*, 2009; Silici *et al.*, 2009).

Therefore, this study aimed to investigate the antioxidant activities of honey bee products binary combination synergistic effect using different extract solutions (water and ethanol, separately).

2. Material and methods

Pollen were collected in spring season by a standard pollen trap was mounted on the hive entrance and maintained throughout the collection period. Every day, pollen was taken from the traps, cleaned and kept in air tight plastic bags (Barreto, 2004). Beebread was obtained directly from the combs and only beebread pieces cleaned and kept in air tight plastic bags. Drone, worker and queen brood homogenate samples were collected three times during the beekeeping season. Immediately after their delivery to the laboratory, the larvae were manually removed from comb cells and deep frozen, then ground up by crushed mechanically with a grinder in ice bath (Schmidt and Buchmann 1992). Collection of Royal Jelly material was achieved in deprived colonies after 3 days of transferring the larvae. On the third day of transfer, the larvae were taken off and the royal jelly was collected from each cell, transferred to plastic vials (Chen, 1993; Zeng, 2013), Bee venom sample were collecting from experiment apiary colonies by bee venom's collection frame devices (Input Voltage: 11.5-13.5 VDC, Collector Frames: 40cm x 50 cm) and the collection time is 30 minutes. After 30 minutes, the collector frame removed from the colony then the deposited bee venom on the glass plate was scrapped using a scraping knife (Rybak *et al.*, 1995). Wax sample obtained by collecting the fresh formed wax comb pieces in the colonies.

Pollen, beebread, brood homogenate, royal jelly and bee venom, wax samples were obtained from honey bee colonies located in the experiment apiary of honey bee research department, Plant Protection Research Institute (PPRI), Agriculture Research Center(ARC), Egypt, stored in a deep freezer at -18 ^{0}C after collection and preparation until use.

The clover and citrus honey samples were collected by beekeepers between March and July of 2020 from apiaries located in different provinces of Egypt. The floral authenticity of honey samples was verified by pollen analysis (Louveaux *et al.*, 1978). Feeding honey sample was collected after feeding the colonies (with empty combs from honey) by sugar solution 50% (sucrose solution (1: 1 w/v) which continuously provided for several days in the experiment apiary, and the sample stored in dark at room temperature (25C°)

Three propolis samples were used, the first sample was Egyptian propolis which collected from honey bee colonies located in the experiment apiary through two years (2019-2020) according to (Breyer, 1995) and the second sample was Chinese propolis which imported from China and purchased commercially in Egyptian market and the third sample propolis was collected from old wax combs (3-5 years old) which collected from experiment apiary.

Preparation of sample extracts was performed using distilled water and ethanol 80% as solvents. First, five grams of the sample material was ground up mechanically with a grinder in ice bath with 100 ml extract solution (water or ethanol 80%), with continuous swirling for 3 days, then the extract was centrifuged (10 min at 4000rpm) to obtain a clear supernatant liquid at a final concentration of 5g/100ml(5%) as stock solution, but for bee venom sample the weight was 1g /20ml(5%), the a clear supernatant liquid stored in a deep freezer at -18 ^{0}C after preparation until antioxidant assay.

Water and ethanol extracts samples were diluted with water or ethanol 80% at ratio of 1:1 (2.5%) and mixed well by vortex. in addition to provide the synergistic antioxidant activity of a binary combinations of honey bee products, every two honey bee product samples (stock soluation,5%) were mixed in a 1:1 ratio for water and ethanol extracts individually.

Antioxidant activity (radical scavenging activity and antioxidant content): The radical scavenging activity of the samples for the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured as described (Martins *et al.*, 2008), with slight modifications10 µl of each sample solution was mixed with 3 mL of DPPH (Sigma-Aldrich) in methanol (40 mg/liter). Methanol was used as the blank sample. The mixtures were left for 30 min at room temperature and the absorbance were then measured at 517 nm. The radical scavenging activity was calculated as follows: % Inhibition = [(blank absorbance - sample absorbance) / blank absorbance] x 100. The mean of IC50 (concentration causing 50 % inhibition) values of each sample was determined graphically (mg/ml). The antioxidant content was determined using a standard curve of gallic acid, quercetin and ascorbic acid (vit c) (Aldrich) were treated in equivalent conditions to the samples. The average of values was obtained, expressed as mg equivalent antioxidant / g of sample.

All values were expressed as the mean ± SD. The significant differences were analyzed by using IBM SPSS Statistics 26 (to compare the antioxidant activity between water and ethanol extract for individual bee product and among the honey bee products water extract / ethanolic extract, separately). Analysis of synergistic effects was done following the method by (Qiao *et al.* 2015) with some modifications. The data from this experiment were analyzed through using CompuSyn software to determine the synergistic effect of the samples binary combination. The statistical analysis was performed and the result was presented as combination index (CI). A CI value is a mathematical and quantitative representation of the pharmacological interplay of two drugs (CI>1: antagonism; CI = 1: additive; CI<1: synergism) (Chou, 2008).

3. Results

The present study deals principally with the results of an explorative investigation into the antioxidant activity of different honey bee products extracted by two different solutions (water and ethanol, 80%) and determine the synergistic effect of the samples binary combination (table , 1,2 and 3).

The results of the DPPH radical scavenging activity (%), IC50, gallic acid, quercetin and vit C equivalents (mg/g) of different honey bee products samples extracted by water and ethanol (80%) summarised in Table 1. The antioxidant activity (IC50) varied from the highest value, which was observed in Chinese and Egyptian propolis, pollen, and old wax comb samples (66.533, 36.625, 80.012 and 55.238 mg/ml in water extract and 13.878, 19.740, 51.625 and 36.108 mg/ml in ethanolic extract, respectively), to the lowest value, which was recorded in sugar feeding honey and wax samples (1748.25, 942.78 mg/ml in water extract and 1644.7, 532.280 mg/ml in ethanolic extract, respectively). Propolis is the most powerful antioxidant of all the bee products examined. It is obvious that the ethanolic extract had more antioxidant activity than the water extracts, but in bee venom, drone, worker, and queen brood homogenates samples, the water extracts were higher than the ethanolic extracts.

Significant differences were recorded among all honey bee products in water extract or in ethanolic extract. in addition, the antioxidant activity of honey bee product ethanolic extract had a significant difference when compared with water extract. but, there was no significant differences in antioxidant activity between the water extract and ethanolic extract in the sugar feeding honey and in the wax sample.

The highest value of IC50 was found in a multifloral honey and the lowest in Persea Americana honey. Persea Americana honey had the most active radical scavenger activity (RSA: IC50= 8.0 mg/mL), (Sánchez *et al.*, 2012). results obtained from 15 honey samples. The TPC(total phenolic compounds) values ranged from 27.0 to 92.7 mg GAE (gallic acid eq.) /100g of honey, the highest values found for samples classified as polifloral honeys, when compared to samples of monofloral honeys. Variations in TPC may be due to different floral origins. All samples have radical scavenging ability with a consumption of DPPH ranging from 7.3% to 25.9%, in 30 min, compared to standard gallic acid, consuming 100% of the radical (Almeida,, *et al.*, 2016). These results are in agreement with Al-Mamary *et al.* (2002) investigated the antioxidant activity and TPC of four different Yemeni honeys (acacia and ziziphus) and three different exported honeys (USA, Sweden, and Iran) and reported the highest values for acacia (*Acacia ehrenbergina*) honey and indicated positive correlations between TPC and antioxidant activity. The values from the DPPH (0.36–3.42mg ascorbic acid eq/g honey) assays were low compared with the reported results for other unfloral honey from different regions in the world (Bertoncelj *et al.*, 2007; Silici *et al.*, 2010).

Baltrušaitytė *et al.* (2007) found that bee bread present better antioxidant activity than honey. The antioxidant capacity of bee bread was demonstrated by an IC50 of DPPH (0.05 ±0.01 mg/ml), ABTS (0.08±0.05 mg/ml), and reducing power (0.05 ±0. 04 mg/ml).

The Sidor team assessed the antioxidant activity and TPC of DBH (drone brood homogenate) at different stages of brood development (Sidor *et al.*, 2021). The lowest level of %I DPPH was shown in pupae with white eyes (6.3% in 70% ethanol extract) while its highest level was observed at the larval stage (20.5%).

The ethanolic extract bee pollen (*Trifolium alexandrinum* L.) produced the higher radical scavenging activity when compared with other solvents i.e. ethyl acetate, dichloromethane and petrolium ether. The highest DPPH scavenging activity was observed in ethanolic extract (90%), followed by ethyl acetate and petrolium ether fractions (79%, 75%), While dichloromethane has moderate activities (63%) (Abd Elsalam *et al.*, 2018).

Table 1. Antioxidant Activity of water and ethanolic extract of honey bee products (2.5%w/v)

product	Water extract					Ethanol extract					Prob.
	%	IC_{50}	Gallic acid equivalent	Quercetin equivalent	Vit C equivalent	%	IC_{50}	Gallic acid equivalent	Quercetin equivalent	Vit C equivalent	
		mg/ml	mg/g				mg/ml	mg/g			
CH	3.499±0.001	310.650	0.158	2.118	2.984	7.056±0.061	136.955	0.318	4.272	6.018	*
TH	3.903±0.176	255.630	0.176	2.363	3.329	5.307±0.001	240.135	0.239	3.213	4.527	*
FH	0.757±0.439	1748.250	0.034	0.458	0.646	0.758±0.201	1644.700	0.034	0.459	0.647	ns
P	15.560±0.001	80.012	0.702	9.420	13.271	27.623±0.001	51.625	1.246	16.723	23.560	*
B	9.469±0.181	126.930	0.427	5.732	8.076	15.621±0.543	77.900	0.705	9.457	13.323	*
W	1.539±0.620	942.780	0.069	0.932	1.313	1.807±0.799	532.280	0.082	1.094	1.542	ns
WOC	25.633±0.181	55.238	1.156	15.518	21.863	37.877±1.810	36.108	1.708	22.930	32.306	*
EPRO	35.000±0.001	36.025	1.579	21.189	29.852	58.200±0.001	19.740	2.625	35.234	49.640	*
CPRO	21.471±0.001	66.533	0.968	12.999	18.313	90.551±0.348	13.878	4.084	54.819	77.232	*
R	10.253±0.001	104.809	0.462	6.207	8.745	8.323±0.001	143.065	0.375	5.039	7.099	*
DH	8.986±0.543	131.035	0.405	5.440	7.664	5.789±0.121	298.570	0.261	3.505	4.938	*
WH	8.865±0.061	128.350	0.400	5.367	7.561	5.235±0.460	239.750	0.236	3.169	4.465	*
QH	8.624±0.302	143.655	0.389	5.221	7.356	5.728±0.001	273.985	0.258	3.468	4.886	*
V	14.822±0.107	84.290	0.668	8.973	12.642	7.288±0.001	177.855	0.329	4.412	6.216	*
*						*					

Values (%I DPPH) are means ± standard deviations, IC50: 50% inhibitory concentration, CH: citrus honey, TH: clover honey, FH: sugar feeding honey, P: bee pollen, B: bee bread, W: bee wax, WOC: old wax comb, EPRO: Egyptian propolis, CPRO: Chinese propolis, R: Royal jelly, DH: Drone brood homogenate, WH: Worker brood homogenate, QH: queen brood homogenate, V:bee venom, *: significantly different, ns: no significantly different

Propolis being the best displayer of antioxidant properties (Karadal *et al.,* 2018). (Nakajima *et al.,* 2009) demonstrate that propolis (both water extract and ethanolic extract propolis) had the strongest antioxidant effects, among the bee products tested (propolis, royal jelly, and bee pollen). Bee pollen had fairly strong antioxidant effects, especially against the H2O2 and O2·-, although its effects were only one-tenth as powerful as those of propolis.

Mohdaly *et al.* (2015) reported that propolis extract had superior scavenging activity (based on DPPH and ABTS assays) compared to pollen extract. Regarding the total antioxidant activity (TAC), the results of propolis samples were ranging from 14.20±0.47mg of ascorbic acid equivalent AAE/g in sample S10 (Bethlehem) to 80.37±1.77 mg AAE/g in sample S6 (Al-Khalil). IC50 ranging between 0.02±0.001mg/mL in sample S6 (Al-Khalil) and

1.13±0.054mg/mL in sample S8 (Ramallah) (Daraghmeh and Imtara (2020). The IC50 of antioxidant activity (DPPH methods) in Indonesian stingless bee propolis ranged from 150.20 to 207.63 ppm (Mulyati *et al*.,2019. All the samples from the seasonal study presented antioxidant activity with values higher than 80.6%, 81.5% and 78.5% in the extract concentration of 80.0 µg/mL for the Propolis A(Te Ilha do Porto apiary (Propolis A), B(Primavera apiary (Propolis B) and C (Paripueira apiary (Propolis C))samples, respectively (Do Nascimento *et al.,* 2019). De Mendonca *et al.,* 2015 showed that, Brazilian red propolis presents antioxidant activity with IC50 values between 5.0–8.0 µg/ml. Te Sonoran propolis presented good results of antioxidant activity using DPPH method in concentration range of 12.5 to 100.0 µg/ml 25. Te San Juan Propolis from Argentine presented good antioxidant activity with IC50 values between 15.0 to 42.0 µg/mL during a year seasonal cycle (Isla, *et al.,* 2009).

Research teams affirmed that the antioxidant capacity of wax extracts is higher than that of (some) honey. also royal jelly and bee venom antioxidant properties are considered (Martinello and Mutinelli, 2021).

The antioxidant activity of bee venom using classical assays revealed antioxidant properties, some data suggest that melittin alone exerts very poor antioxidant activity compared to bee venom extracts and this might be due to the influence of other venom components (Pavel *et al.,* 2014).

The propolis extracts had the highest and the honey samples had the lowest antioxidant activity among the bee products (Karadal *et al.,* 2018). Similarly, many investigators have reported propolis extracts to possess strong antioxidant effect (Nagai *et al.,* 2001) and Nakajima *et al.* (2009). Nagai *et al.* (2001) and Nakajima *et al.* (2009) have also reported that propolis extracts are the most powerful antioxidant among bee products (propolis, pollen, honey and royal jelly).

The antioxidant activity of the binary combined water or ethanolic honey bee products extracts was individually reported in table 2 for water extract and table 3 for ethanolic extract and figure 1. Furthermore, to confirm the interaction between honey bee products, the data was computed in CompuSyn software to determine the CI of the combination.

Data in table 2, 3 and fig. 1 reported that, the Egyptian propolis, Chinese propolis and pollen water extract and clover honey, pollen and Egyptian propolis ethanolic extract combination showed more synergistic effects in the majority of the binary combinations, but the royal jelly water extract and worker, drone homogenate ethanolic extract samples showed the minority synergistic combinations effect.

Evaluate the influence of supplementation of multiflower honey with bee products on the phenolic compound content and on antioxidant activity. Average total phenolic and flavonoids contents in the multiflower honeys were 36.06 ± 10.18 mg gallic acid eq./100 g and 4.48 ± 1.69 mg quercetin aq./100 g, respectively. The addition of royal jelly did not affect significantly the phenolic compound content and antioxidant activity. Supplementation of honey with other bee products, i.e. beebread, propolis, pollen, resulted in significant increase in the total phenolic and flavonoids contents, and in antiradical activity and reducing power, with the largest effect found for addition of beebread. Significant linear correlations between the total phenolic and flavonoids contents and antiradical activity and reducing power were found (Juszczak *et al.,* 2016).

Beebread prepared for use with honey and some wax particles possesses higher radical scavenging capacity than pure natural honey. The broad spectrum of different components that complement the composition of bee products could provide a synergistic effect in the latter products (Čeksterytė *et al.,* 2016).

Table 2. Synergistic Antioxidant Activity of binary combinations of honey bee product water extract (2.5%w/v)

Product	CH	FH	P	B	W	WOC	EPRO	CPRO	R	DH	WH	QH	V
CH	7.20±0.56	4.40±0.67	18.52±3.99	16.67±0.65	4.87±0.64	29.45±1.56	36.08±1.23	24.49±2.07	11.64±0.92	10.79±0.57	11.96±0.72	10.05±0.91	15.58±1.10
CI	0.9421	0.9324	0.9462	0.6650	0.9831	0.8273	1.0210	0.9440	1.1111	1.0808	0.9441	1.1354	1.1171
	sy	sy	sy	sy	sy	sy		sy			sy		
TH		4.08±1.02	17.01±3.28	12.25±1.77	4.76±0.59	26.87±2.95	38.10±5.89	23.13±0.59	12.25±0.00	11.57±0.59	10.20±1.77	10.54±1.18	16.49±3.13
CI		1.1227	1.0739	1.0032	1.1000	1.0405	0.9586	1.0303	1.0760	1.0279	1.1808	1.1095	1.0623
							sy						
FH			16.40±0.00	11.69±0.00	2.00±0.10	27.76±0.28	36.15±0.94	23.49±1.46	9.31±0.50	9.470±0.496	9.470±0.57	8.225±0.57	15.46±2.36
CI			0.9698	0.8272	1.1187	0.9185	0.9706	0.9121	1.1873	1.0040	0.9891	1.1327	0.9849
			sy	sy		sy	sy	sy			sy		sy
P				24.07±0.31	19.57±0.95	39.94±0.00	48.80±1.28	38.19±0.31	21.57±0.63	23.373±0.98	22.27±0.43	23.62±0.71	27.58±4.00
CI				0.8900	0.8048	0.8586	0.8807	0.7890	1.0607	0.8951	0.9000	0.8777	0.9362
				sy	sy	sy	sy	sy		sy	sy	sy	sy
B					11.42±0.50	30.83±2.89	42.78±1.92	23.61±4.81	15.00±2.89	16.67±0.22	15.28±2.41	14.5±1.828	22.8±0.53
CI					0.9069	1.0147	0.9192	1.2081	1.2027	0.9747	1.0781	1.1271	0.9176
					sy		sy			sy			sy
W						27.49±4.05	37.24±5.47	23.68±1.29	10.52±0.62	9.885±0.868	8.91±1.47	8.56±0.43	15.44±5.33
CI						0.9490	0.9461	0.9251	1.0881	1.0206	1.1384	1.1593	1.0250
						sy	sy	sy					
WOC							56.36±1.44	40.77±3.90	29.47±2.79	29.30±0.74	29.26±4.01	28.53±1.02	34.36±1.19
CI							0.9059	0.9811	1.1002	1.0700	1.0680	1.0952	1.0235
							sy	sy					
EPRO								54.62±0.18	44.06±0.54	47.60±2.31	44.06±1.49	47.39±2.82	49.00±0.00
CI								0.8648	0.8992	0.7981	0.8740	0.7923	0.8626
								sy	sy	sy	sy	sy	sy

CPRO		29.28±1.65	28.90±2.04	28.62±0.95	28.69±5.81	31.52±0.53
CI		0.9384	0.9151	0.9229	0.9126	0.9928
		sy	sy	sy	sy	sy
R			14.30±1.49	18.32±0.75	17.80±1.24	22.73±0.00
CI			1.2395	0.9033	0.9232	0.9559
				sy	sy	sy
DH				14.45±3.85	12.34±0.00	21.96±1.60
CI				1.1195	1.3579	0.9428
						sy
WH					14.33±0.58	22.20±1.39
CI					1.1213	0.9242
						sy
WQ						20.66±0.00
CI						1.0018

Values (%I DPPH) are means ± standard deviations, CH: citrus honey, TH: clover honey, FH: sugar feeding honey, P: bee pollen, B: bee bread, W: bee wax, WOC: old wax comb, EPRO: Egyptian propolis, CPRO: Chinese propolis, R: Royall jelly, DH: Drone brood homogenate, WH: Worker brood homogenate, QH: queen brood homogenate, V:bee venom, CI : Combination Index, SY: synergistic.

Table 3. synergistic Antioxidant Activity of binary combinations of honey bee product ethanolic extract (2.5%w/v)

Product	CH	FH	P	B	W	WOC	EPRO	CPRO	R	DH	WH	QH	V
CH	11.00±0.72	7.25±0.33	33.28±2.66	23.76±5.41	7.94±0.00	44.23±0.87	62.96±3.30	95.40±0.27	13.49±2.18	9.76±0.87	8.94±0.64	9.10±0.92	12.79±1.39
CI	1.021	1.057	0.931	0.815	1.068	0.919	0.976	1.012	1.023	1.234	1.299	1.329	1.007
			sy	sy		sy	sy						
TH		8.33±2.89	32.67±2.31	20.79±2.80	7.38±0.54	43.29±0.62	64.01±7.24	95.94±0.60	11.91±0.59	11.22±1.02	11.56±1.56	10.54±0.59	11.21±1.39
CI		0.671	0.912	0.893	0.899	0.916	0.940	0.996	1.043	0.880	0.801	0.943	1.021
		sy	sy	sy	sy	sy	sy	sy		sy	sy	sy	
FH			29.09±1.82	15.88±2.70	3.12±0.19	45.52±1.38	56.97±5.84	93.21±1.05	9.68±0.00	5.38±0.93	5.65±0.81	5.46±0.00	8.00±1.64
CI			0.950	1.014	0.783	0.797	1.033	0.985	0.900	1.210	1.035	1.132	0.976
			sy		sy	sy		sy	sy				sy
P				43.33±0.00	29.95±3.25	66.39±0.48	81.11±3.85	94.61±0.59	34.44±0.96	30.39±1.36	29.89±1.54	33.06±1.36	33.33±5.25
CI				0.825	0.938	0.826	0.964	1.269	0.920	1.014	1.022	0.908	0.934
				sy	sy	sy	sy		sy			sy	sy
B					17.72±0.92	45.56±0.48	72.22±4.81	96.56±0.315	23.28±5.30	18.63±0.50	22.38±1.10	22.76±1.96	23.79±3.87
CI					0.928	1.040	0.927	1.075	0.886	1.049	0.810	0.811	0.822
					sy		sy		sy		sy	sy	sy
W						40.35±0.00	60.98±2.16	93.51±0.40	9.25±0.50	6.67±0.72	5.975±0.865	5.63±0.55	8.55±0.79
CI						0.947	0.961	0.989	1.046	1.091	1.132	1.309	1.012
						sy	sy	sy					
WOC							74.67±0.58	93.33±0.61	41.76±0.61	38.77±0.68	38.67±4.72	39.68±1.87	40.91±0.00
CI							1.193	1.341	1.013	1.066	1.059	1.033	1.021
EPRO								97.37±0.82	63.39±3.82	62.71±0.00	59.02±5.55	61.02±2.00	62.94±3.49
CI								1.566	0.982	0.968	1.034	0.996	0.979
									sy	sy		sy	sy
CPRO									95.46±0.44	94.76±0.26	93.86±0.45	96.01±3.04	90.61±2.92

CI		1.021	1.006	1.008	0.999	1.047
					sy	
R			11.57±1.65	13.22±0.44	13.97±0.734	13.27±5.10
CI			1.121	0.913	0.887	1.063
				sy	sy	
DH				8.97±1.137	8.96±0.360	11.18±2.677
CI				1.142	1.201	1.068
WH					9.27±0.643	11.11±2.749
CI					1.092	1.541
WQ						13.33±0.001
CI						1.269

Values (%I DPPH) are means ± standard deviations, CH: citrus honey, TH: clover honey, FH: sugar feeding honey, P: bee pollen, B: bee bread, W: bee wax, WOC: old wax comb, EPRO: Egyptian propolis, CPRO: Chinese propolis, R: Royall jelly, DH: Drone brood homogenate, WH: Worker brood homogenate, QH: queen brood homogenate, V:bee venom, CI : Combination Index, SY: synergistic.

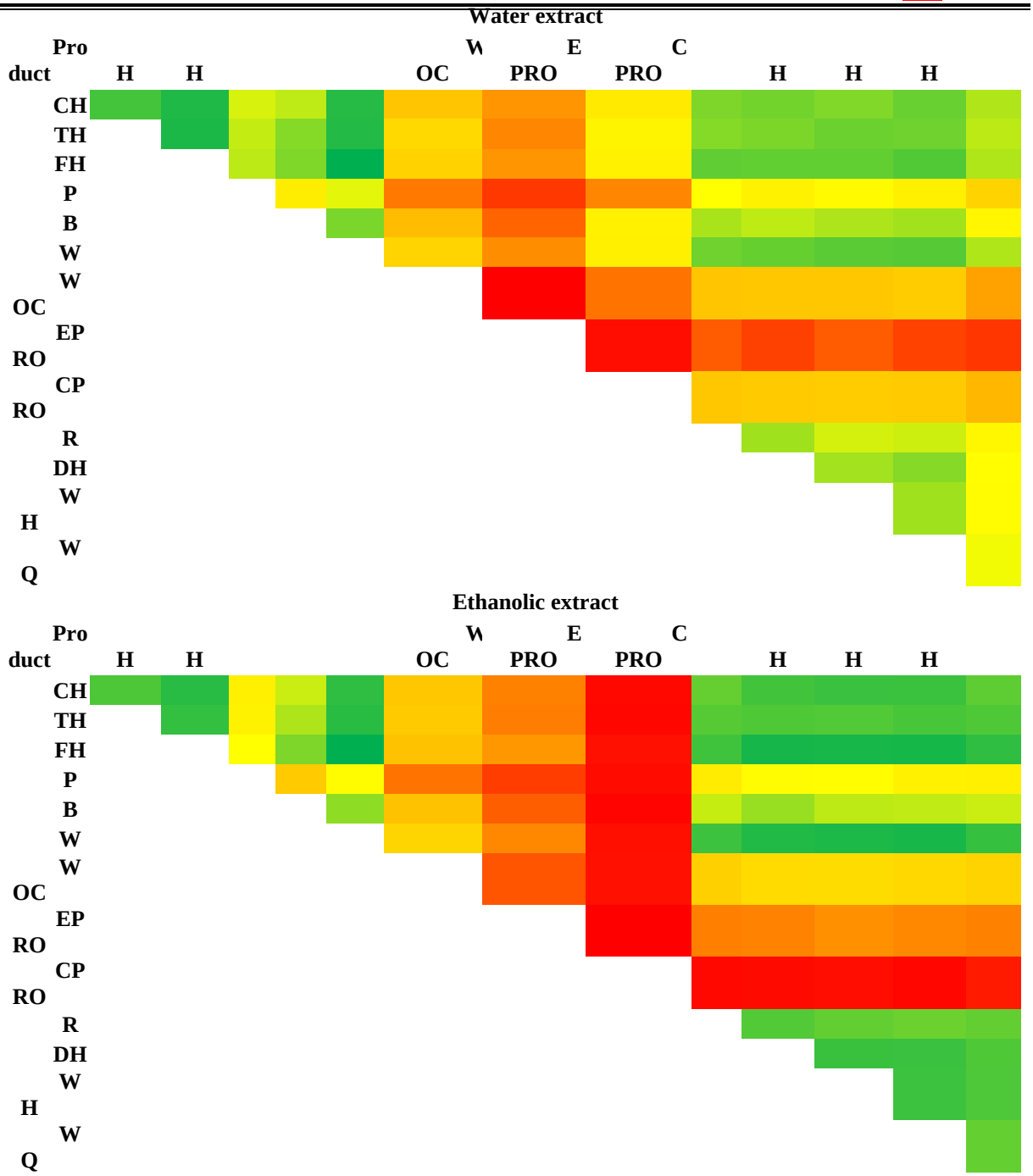

Figure 1. heat mapping of synergistic Antioxidant Activity of binary combinations of honey bee product

Values (%I DPPH), CH: citrus honey, TH: clover honey, FH: sugar feeding honey, P: bee pollen, B: bee bread, W: bee wax, WOC: old wax comb, EPRO: Egyptian propolis, CPRO: Chinese propolis, R: Royall jelly, DH: Drone brood homogenate, WH: Worker brood homogenate, QH: queen brood homogenate, V:bee venom.

Active compounds in the bee pollen extract might either enhance (synergistic) or decrease (antagonistic) the therapeutic activity of chemotherapy drugs. There was a great concern that some compounds present in the natural product might work antagonistically instead of synergistically with the therapeutic activity of drugs (HemaIswarya and Doble 2006). CI values reported in all interactions between bee pollen extract and cisplatin were less than 1. These CI values further proved the synergistic effect between the two compounds. The same data indicate that bee pollen extract works synergistically with chemotherapy drug, cisplatin and enhances the effect of cisplatin even at lower concentration (Wan Omar *et al.*, 2016).

Among the mixtures of honeybee products, the mixture of royal jelly, honey, pollen, and propolis had the greatest antioxidant activity (72.98±3.08 mg AAE/g) and the triplet mixture of royal jelly, honey, and propolis had the least antioxidant activity. The antioxidant activity of royal jelly and propolis was respectively observed as 59.02±5.98 mg AAE/g and 267.37±0.33mg AAE/g .Honey, bee pollen, and mixed samples exhibited positive correlations with TPC and antioxidant activity. Similarly, honey and mixed samples exhibited positive correlations with TPC and FRSA (Özkök and Silici, 2017).

Jin *et al.* (2018) studied the antioxidant properties of water and methanol extract from Linden bee pollen, finding that methanol extract potentiated the antioxidant effect. Borycka *et al.* (2015) compared the results of different extractions using water, ethanol, and methanol, analyzing five types of commercial bee pollen products: bee pellets, micronized bee pellets, pollen tablets, bee bread, and bee bread in honey. They concluded that the extraction method seemed to be crucial and that ethanol was the most effective solvent. TPC and AOA, as determined by FRAP and ABTS assays, was highest in the ethanol extracts taken from each investigated product, followed by methanol and water. Bee bread displayed the highest AOA and phenolic content compared to the other pollen products.

Miguel *et al.* (2010) compared water, methanol, and 70%ethanol as extraction solvents, choosing a hydroalcoholic mixture to extract phenols in propolis samples, given its good performance and lower toxicity compared to methanol. Cavalaro *et al.* (2019) also studied the effects of ethanol/water concentration, solid solvent ratio, and extraction time with regard to the TPC and antioxidant capacity of green Brazilian propolis, using ultrasound-assisted extraction. They optimized the procedure using 99% ethanol solution and a 1:35 propolis: solvent ratio (w/v), over 20 minutes.

4. Discussion

The results associated with the antioxidant activity of different bee products suggest that there are significant differences among the products under investigation. Propolis and pollen recorded the highest values of activity, but the lowest values were recorded in sugar feed honey and wax samples. That may be due to the fact that bee products are multicomponent natural substances and this component differs from honey bee products to another and therefore also contains other substances presenting antioxidant activity. That means that the difference in antioxidant activity is contributed to the different compounds in the honey bee products. The high content of phenolic compounds in propolis and pollen, which, reported by many researchers investigations, reflected the high antioxidant activity, the low antioxidant capacity in sugar feeding honey sample might be influenced by absent of the honey floral source and its content of plant secondary metabolites .

The antioxidants that naturally occur in honey contribute to antioxidant capacity. These compounds are flavonoids, phenolic acids, and some enzymes (e.g. glucose oxidase, catalase), ascorbic acid, carotenoid like substances, organic acids, Maillard reaction products, amino acids and proteins (Gheldof and Engeseth 2002). Enzymes naturally occur in honeys, including glucose oxidase, catalase and peroxidase (McKibben and Engeseth, 2002). These enzymes are known to have antioxidant properties. Different honey types have diverse phenolic content and consequently different antioxidant activity. In addition, processing, handling and storage of honey may influence its composition (Gheldof and Engeseth 2002; Turkmen *et al.*, 2005). The significant correlation was found between the antioxidant activity as determined by the FRAP assay and also as determined by the DPPH assay and the phenolic content , indicating that phenolic compounds appear to be responsible for the antioxidant activity of acacia honey (Krpan *et al.*, 2009).

Bee pollen has antioxidants such as phenolic acids and flavonoids which have anti-inflammatory properties. It contains polyphenols, carotenoid pigments, phytosterols, tocopherol, vitamins, enzymes and co enzymes which are attributed to its biological activities (Denisow and Denisow-Pietrzyk, 2016). Campos *et al.* (1997) demonstrated that the flavonoid/phenolic components must play a significant role in the free radical capacity scavenging of bee pollen based on the observation that the bee pollen which exhibit the highest activity is that with the highest level of flavonoids and phenolic acid derivatives.

An ethanolic extract of propolis contains abundant flavonoids, particularly quercetin, rutin,and kaempferol, and possesses high total antioxidant capacity (Zhang *et al.*, 2016). Phenolic compounds might be responsible for the biological activity in the three kind of ethanolic extract propolis. The phenolic

compounds content found in Egyptian ethanolic extract propolis were salicylic acid, caffeic acid, ferulic acid, quercetin ,pinocembrin, pinostrobin, genistein and daiazein higher than that in Chinese ethanolic extract propolis and old wax combs ethanolic extract propolis, in addition the phenolic compounds found in Chinese ethanolic extract propolis were phenol, para hydroxy benzoic acid, p. coumaric acid, 3,5 dimethoxy benzyl alcohol, trans – cinnamic acid, chrysin, galangin, daidzin, acacetin higher than that in Egyptian ethanolic extract propolis and old wax combs ethanolic extract propolis, on the other hand in old wax combs ethanolic extract propolis were pyrogallic acid, protocatechuic acid, catechines, higher than that in Egyptian ethanolic extract propolis and Chinese ethanolic extract propolis. It is evident that composition of phenolic constituents were different in the three kinds of ethanolic extract propolis and Egyptian ethanolic extract propolis were contained more phenolic compounds than in the Chinese ethanolic extract propolis and old wax combs ethanolic extract propolis. (Kamel *et al.,* 2013)

Some biomolecules and compounds in royal jelly were reported to have an antioxidant effect. For instance, albumin proteins in royal jelly also have ant oxidative impacts (Guo *et al.,* 2005). Protein and phenolic fractions of royal jelly have high antioxidant activity and FRSA against reactive oxygen species (Eraslan *et al.,* 2008). The antioxidant potency of royal jelly is attributed to its polyphenolic and flavonoid compounds; free amino acids, including essential ones; small peptides, such as di-peptides (Lys-Tyr, Arg-Tyr, and Tyr-Tyr) obtained from protease hydrolyzed royal jelly proteins; peptides and proteins; fatty acids (the main being 10-hydroxydecanoic acid); and vitamins (Kocot *et al.,* 2018; Giampieri *et al.,* 2018; Ramadan and Al-Ghamdi 2012).

According to Aljadi and Kamaruddin (2004), the antioxidant capacity of honey and propolis is due mainly to the phenolic com-pounds and flavonoids they contain, and there is a high degree of correlation between these substances and the antioxidant capacity of honey, although a synergic action between several compounds cannot be discounted (Johnston *et al.,* 2005; Kücük *et al.,* 2007).

It was found that antioxidant activities of the bee products varied according to their phenolic contents and could be ordered from highest to lowest as propolis, pollen, and honey (Saral *et al.,* 2016, Yildiz *et al.,* 2014). AOA (antioxidant activity) is highly correlated with phenolic compounds, but bee products are multicomponent natural substances and therefore also contain other substances presenting AOA, including minerals, amino acids, peptides, proteins, organic acids, and enzymes (Da Silva *et al.,* 2016).it was reported that flavonoid content is higher in beebread and its antioxidant capacity is remarkably stronger than that of honey (Čeksterytė *et al.,* 2006; Baltrušaitytė *et al.,* 2007).

It's possible that the variation in antioxidant activity seen in this study is attributable to the solvent used. The antioxidant activity of the ethanol (80%) extraction technique was higher than that of the water extract. This could be because the antioxidant activity of the extracted extracts is influenced by the extraction solvents, their concentration, and polarity. Because the components of bee products have different structures, and while hydrophilic ones are better soluble in polar solvents like alcohols, those with hydrophobic properties have a greater affinity for nonpolar solvents like hydrocarbons, the composition of the obtained extracts is affected by the use of different polar solvents. It was found that the different types of extraction solvent had different effects on the concentration of bioactive compounds in the extracts.

It can be seen that highest TAA(total antioxidant activity) is observed in bee pollen homogenized with ethanol, while water homogenates presented the lower TAA values. The highest TAA values are observed for ochre and brown pollen samples homogenized with ethanol. In the same way, the highest polyphenol content values were observed in ethanol bee pollen homogenates, followed by methanol homogenates and water homogenates (Sánchez *et al.,* 2012). Freire *et al.* (2012) studied about the values of total phenolic and flavonoid contents compared with other studies which used ethanol, methanol and water extraction. They found that the difference type of extraction solvent had the difference effect on concentration of bioactive compound in the extracts. it is clear that the TAC of the propolis particles increase gradually with increasing volume fractions of ethanol. The highest radical scavenging activity was found in the propolis particles extracted with pure ethanol, with the TAC measured at 317.65 mg AAE /g . The results supported the assumption that an increase in ethanol fraction in the extraction solvent should have higher capability to dissolve different types of phenolic compounds due to the change in the solvent polarity, leading to higher antioxidant activity expressed in the solution (Abdullah *et al.,* 2019). Moreover, both the chemical composition and biological properties of propolis extracts are highly dependent on the type of solvents used for the extraction (Sun *et al.,* 2015, Bittencourt *et al.,* 2015, Narimane *et al.,* 2017).

Accordingly, there could be considerable changes in AOA and phenolic compounds when comparing honey and its extract. In general, according to the studies evaluated, honey dissolved in water yields higher polyphenol values, while extraction with methanol results in higher flavonoid levels (Mouhoubi-Tafinine *et al.,* 2016, Lianda *et al.,* 2012)

But in the present study the venom, royal jelly, worker, queen, and drone homogenates, the water extract revealed more antioxidant activity than the ethanol extract. This can be explained by several factors; the high protein content in these products makes them more soluble in water, and the ethanol causes aggregation, denature, reduce solubility, and precipitate the proteins, losing its biological activity.

Ethanol affects proteins in aqueous solution. It can denature proteins (Herskovits and Mescanti 1965, Gerlsma 1968, Mousavi *et al.,* 2008, Gerlsma and Stuur 1972), often accompanied by transition in secondary structure (Dufour and Haertlé 1990,1993) and reduce their solubilities (Yoshikawa *et al*., 2012a,2012b).This study showed that ethanol at high concentrations, above 50-60 %, alters the structure or the association state of bovine serum albumin (BSA) and ribonuclease A (RNase A) pH dependently (Yoshizawa *et al.,* 2014)

bee products are multicomponent natural substances, and therefore when they are combined in a mixture, the mixture activity is affected by the interaction between bioactive components in the mixture. This has an effect on the antioxidant activity of the mixture, and some of these binary combinations had synergistic effects.

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). the mixture containing propolis had higher TPC and antioxidant activity. This was an expected outcome since propolis was reported to have the highest biologicactivity in previous studies investigating honeybee products together (Özkök and Silici 2017). This is because the overall antioxidant capacity of each sample results from the combined activity of other nonphenolic compounds, although phenols do remain the largest class of antioxidants found in nature (Sousa *et al.,* 2016).

This interaction would explain the low values detected for the activities in honey and bee pollen that contain metals compared with the control samples. If this ligation occurs, the metals complexed to the phenolic compounds in bee products will decrease any biological or chemical property of the phenolic compounds, the bee products from those species were chemically characterized and a slight antioxidant activity in honey was detected in the control samples (Mejías and Montenegro 2012), and has also been utilized in food industry (Kuzma 2010).

There are many investigations that are attributed to the antioxidant and enhancing the immune system of humans, animals, and insects. Bee nutrition comes from two main sources, nectar and pollen. Which provides bees with carbohydrates, protein, fat, minerals, vitamins, and a good source of antioxidant compounds; it might be increasing the resistance of honey bee to the pathogenic agents. So, it is a promising area to further explore in future studies.

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