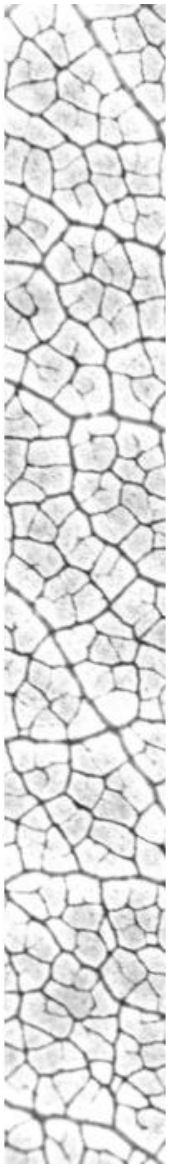

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In vitro and *in vivo* antioxidant activity of Iraqi propolis against benign prostatic hyperplasia in rats

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Abstract
The objective of the present work included evaluation of antioxidant action of Iraqi propolis extract *in vivo and in vitro.* The *in vitro* part of the study comprised studying of the scavenging activity of serial concentrations of propolis extract in two different methods DPPH scavenging assay and reducing power assay. Results showed that IC50 of the propolis extract, and the reference drug (ascorbic acid) with regard to the DPPH scavenging activity, were 1.65µg/mL, 1.74 respectively while 1.08 µg/ml and 1.11 µg/ml regarding to the reducing power assay respectively. The *in vivo* part involved studying the antioxidant and therapeutic activity of 200mg/ kg BW of propolis extract given twice daily on the exogenous testosterone induced benign prostatic hyperplasia (TIBPH) in the wister rat which conducted on 18 male rats divided randomly to the 3 groups of 6 rats for each group. After completion of treatment Prostate weight, prostate index, Malondialdehyde (MDA) content, catalase (CAT) activity, glutathione (GSH) content, and superoxide dismutase (SOD) activity markers were measured. Results showed a significant ($P<0.05$) improvement in different mentioned parameters of propolis treated rats included reduction in both prostatic weight and prostatic index, decreased in MDA content with significant ($P<0.05$) increase in GSH content, CAT and SOD activities as compared with positive control group. In conclusion, the above results proved that the propolis have a good therapeutic and antioxidant activities against (TIBPH) in rats.

Keywords: propolis, Diethyl acetate extract, BPH, antioxidant

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INTRODUCTION

Bee products are variant, one of them is Propolis which is resinous material also called a bee glue which seldom present in its pure construction. It's normally produced by bees for sealing openings or cracks and smoothing out the inner walls because of its waxy character (Bankova *et, al*. 2000). Propolis has wide health benefits such as treat cold sores diabetes, some inflammatory cases, swelling and inner mouth sores. Another medical uses of propolis are to treat genital herpes, canker sores, and burns and to improve immunity, although these uses of propolis are still controversial. It is also used as trendy alternative medicine for self-cure of wide range of diseases and involved in several cosmetics (Alencar *et, al*. 2007). Previously more than 200 substances have been identified in propolis obtained from different areas, involving flavonoids, phenolic acids, esters, sesquiterpenes, diterpenes, lignans, alcohols, aromatic aldehydes (Barbosa *et, al*. 2009) and this composition of a propolis are differ between hives, seasons and locations. Recently, propolis became more attractive substance because of its wide benefits that make it a prospective curative and preventive agent. Therefore, its products are growing, and occur at the highest of the category list including oral and wound care products (Andrea Braakhuis, 2019).

The most health benefits of propolis that had inspected comprise wound healing, cardio-protective, anti-microbial, and maintenance of perfect neural function. Antioxidant and inflammatory activity play a role in the mechanism by which propolis support health, however the range of physiological effects of the propolis is broad and diversified (Lan, et, al. 2016) (Paulis *et, al*. 2012). propolis has remained relevant in developing for various health care purposes. Honey and propolis have many helpful impacts in human care and might be used effectively as a natural drug for some human diseases (Cornara, *et, al.* 2017).

Benign prostatic hyperplasia (BPH) is enlargement of the prostate gland because of the increase in

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proliferation of glandular and stromal prostatic cells, with a prevalence of prostatic mesenchymal cells (Ahmad *et, al*. 2012). BPH threatens the patient's life, and the enlarged prostate adversely impacts the bladder or may obstruct the urethra, causing a clinical symptom in the lower part of the urinary tract (Eaton, 2003). Researchers recorded about 80% of men over than eighty years old have been suffering BPH (Dull *et, al.* 2002).

There are several factors playing an essential role in the incidence and development of BPH involving hormones, inflammatory mediators, inflammatory genes, oxidative stress (OS), and dietary factors, but there is no concurrence about which is the paramount one. Several studies conducted *In vivo* and *In vitro* suggested that the (OS) may considered one of the main pathways contribute in the BPH occurrence (Roumeguere *et, al*. 2017) (Udensi *et, al*. 2016) (Al-Hariri M. 2014). Although no evidence regarding cause and time of the occurrence of chronic prostatic inflammation, some researchers supposed that BPH might be an immune-mediated inflammatory disease and prostatitis may attribute to prostatic growth (Bostanci *et, al*. 2013). It had been found that the propolis possessing a wide pharmacological activity including anti-inflammatory, antitumor and immunomodulation (Al-Hariri M. 2014) (Vit P *et, al*. 2015) (Zabaiou *et, al.* 2017).

Several previous studies on urine and plasma have found elevation in (OS) level in patients suffering from BPH (Minciullo *et, al*. 2015). Oxidative stress is one of multiple mechanisms that initiate the chain of reactions concerned in the development and advancement of prostatic hyperplasia (Udensi *et, al*. 2014). Oxidative stress increased either due to increased generation of reactive oxygen species (ROS) or because of decrease antioxidant defense mechanisms. In last decade, studies have demonstrated that OS is correlated with the initiation and development of prostatic cancer and prostatic hyperplasia (Paschos *et, al*. 2013). So, the present study aimed to detection antioxidant action of Iraqi propolis against oxidative stress induced experimentally via high dose of testosterone in rats.

MATERIALS AND METHODS

Propolis collection and extraction:

Enough amount of propolis material was harvested in May 2019 directly from honeybee colonies located in Al-Diwaniya province. The identity of the propolis was authenticated by the honey division / department of plant protection / directorate of agriculture / ministry of agriculture in Al-Diwaniya city. The propolis samples were cleaned, free of wax and wood, made into the small pieces and ground to the powdered by a mortar and pestle then extracted by Soxhlet extraction apparatus. To extract propolis, accurately weighed samples of dried powder (approx. 30 gm) were placed in cellulose extraction thimble. The thimble was placed in the Soxhlet extraction unit and extracted for 18 hours with 500 mL of the diethyl acetate solvent. The suspension obtained was filtered with Whatman filter paper. The remaining solvent was then evaporated to dryness by rotary vacuum evaporator under low pressure to obtain sticky semisolid material. the resulting extract was kept in refrigerator at 4° C until use.

Part one: *In vitro* assays

DPPH assay:

The free radical scavenging activity of the diethyl acetate propolis extract on the stable radical 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) was evaluated according to the method reported by (Shen, *et, al*. 2020).

The prepared DPPH solution (1 ml) was mixed with propolis extract solution (3ml) in methanol to prepare different propolis concentrations of 0.38, 0.76, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µg/ml. It was vortexed well and left at room temperature for 30 minutes. To determine the sample absorbance value the UV-VIS spectrophotometer was used at wavelength 517 nm. In this experiment, Ascorbic acid is used as a positive control. The percentage of free radical scavenging activity of tested compounds was determined via following equation: % scavenging activity = [(Ac-As) / Ac] × 100 where Ac = absorbance of control and As = absorbance of tested sample. The experiment was carried out in triplicates and data were analysed. The half maximal inhibitory concentration (IC50) of propolis extract with the reference drug was then calculated.

Reducing power assay:

In order to evaluate the total reducing power activity of diethyl acetate, propolis extract was obtained using the method described by (Ferreira *et, al*. 2007). Total amount of 2.5 mL of different concentrations of the propolis extract (0.38, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µg/mL) were added to the 2.5 mL phosphate buffer solution (0.2 M, pH = 6.6) and 2.5 mL of 1% potassium ferricyanide [K3Fe (CN) 6] in separated test tubes. After that the solution warmed at 50°C in water bath, for 20 min. Then, 2.5 mL of 10% tri-chloro-acetic acid was added to the solution and agitated gently. Then, 2.5 mL of this mixture was poured into 2.5 mL of distilled water and 0.5 mL of FeCl3 (0.1% solution) and left for 10 min., The absorbance of the sample was recorded using spectrophotometer at 700 nm wavelength. In this experiment, Ascorbic acid is used as a positive control.

BPH induction:

In the present work, 18 male Wistar rats, aged 11 weeks and weight at (215-235) grams**,** were equipped at the animal house of Veterinary Medicine College in the University of Al-Qadisiyah.

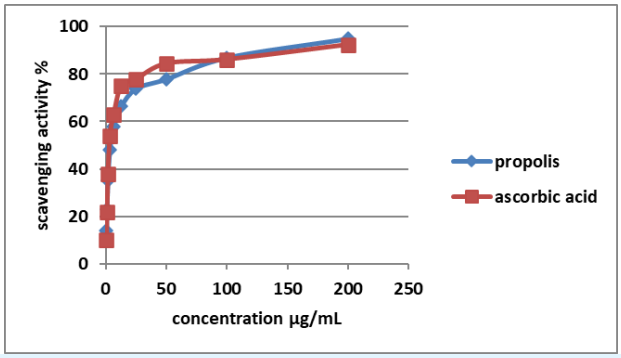

Fig. 1. DPPH radical scavenging activity percent of the different concentration of diethyl acetate propolis extract

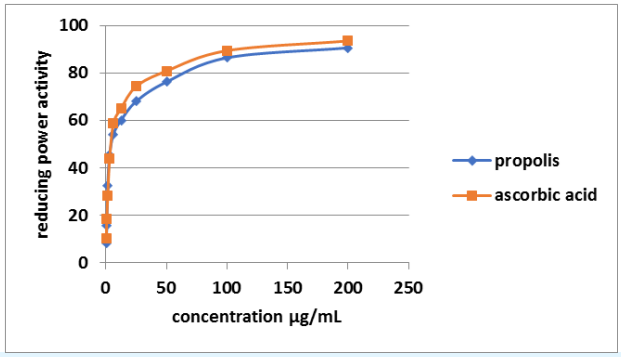

Fig. 2. Reducing power assay of the different concentration of diethyl acetate propolis extract

The rats were laid in polypropylene cages (48×34×18cm) in a controlled temperature (22°C) and light-period (12/12 hours light/dark) room, with free access to food and water. Care was taken to avoid any unnecessary stress. Prostate enlargement was conducted via inguinal S/C injection of 3 mg/kg of testosterone enanthate (TE) for 3 weeks. The rats were randomly distributed into three groups (each n = 6): the negative group, the untreated BPH group, and di-ethyl-acetate propolis extract (200 mg/kg). Animals received BPS or propolis extract via stomach tube for 21 days twice daily with TE, while the control group received BPS and 0.5 ml corn oil subcutaneously. Then, all rats were fasted for 12 hours. The animals were sacrificed under brief exposure to the chloroform vapor, after those prostates of all rats were sliced out and weighted. The whole prostates were weighted and stored at −80°C for oxidative stress markers detection.

Oxidative stress markers estimated:

The ventral portion of the isolated prostate gland was subjected to homogenization using ice-cooled phosphate-buffered saline (50 mM potassium phosphate, pH 7.5). The malondialdehyde (MDA) content that used to measure lipid peroxidation was detected and calculated according to the method described by (Mihara, *et, al*. 1978). CAT activity and reduced glutathione (GSH) content was detected and calculated according to the method described by (Rukkumani, *et, al*. 2004). Superoxide dismutase (SOD) activity was detected and calculated according to the methods described by (Kakkar *et, al*. 1984).

Statistical Analysis

Data of the experimental groups were analysed using SPSS version 25 software by one-way ANOVA test follow by LSD to compare the means of studied parameters in the different experimental groups. *p* value less than 0.05 was considered statistically significant.

RESULTS

The *in vitro* antioxidant activity of propolis extract were proved by assessment of both percentage of DPPH scavenging activity and reducing power by using series of concentrations (0.38-200µg/ml) and compared its results with reference drug (ascorbic acid)

The scavenging activity and reducing power activity of propolis extract and reference drug were increased significantly with increase the concentration of the extract or ascorbic acid **Figs. 1** and **2**. Additionally, Results showed that IC50 of the propolis extract, and the reference drug (ascorbic acid) with regard to the DPPH scavenging activity, were 1.65µg/mL, 1.74 respectively while 1.08 µg/ml and 1.11 µg/ml regarding to the reducing power assay respectively. There was no significance (P>0.05) difference in the value of LD_{50} of the propolis extract compared with ascorbic acid in DPPH and reducing power assay **Table 3**.

In regarding to the *in vivo* study, application of 3 mg/kg of exogenous testosterone enanthate (TE) subcutaneously for 3 weeks induces negative effects on prostatic tissue in rats. These include the increase in prostatic weight and prostate index along with significant (P<0.05) alteration in the oxidative stress biomarkers namely MDA, CAT, GSH, SOD compared with negative control group.

In the same time, The results showed a significant (P<0.05) improvement in different parameters in the propolis diethyl acetate extract treated group compared to the positive control group, where the prostate weight and prostate index significantly (P<0.05) decreased (**Table 1**) as well as a significant (P<0.05) improvement in different antioxidant biomarkers MDA, CAT, GSH and SOD (**Table 2**), which clearly indicate the excellent antioxidant activity of propolis.

In conclusion, the propolis has a good therapeutic and antioxidant activity on the testosterone induced BPH.

DISCUSSION

This study determined the effect of propolis diethyl acetate extract on rats with experimentally induced BPH. The results showed a positive relationship between oxidative stress and some different parameters level in rat´s prostate tissue including MDA, CAT, GSH, and SOD.

Table 1. Body weight (gm), prostate weight (gm) and prostate index in control and treated groups

Drug material	BW (gm)	PW (gm)	Prostate index(mg/gm)
Negative control	249.5±4.74A	0.413±0.009C	1.65±0.04C
Positive control (testosterone)	260.5±3.81A	0.902±0.012A	3.47±0.05A
Treatment group (Propolis diethyl acetate extract (200 mg/Kg) BW	258.1±3.11A	0.774±0.011B	2.99±0.08B
$LSD_{0.05}$	11.904	0.038	0.16

Table 2. The oxidative stress markers in prostate tissues in control and treated groups

Drug material	MDA (nmol/mg)	CAT (U/mg)	GSH (nmol/mg)	SOD (U/mg)
Negative control	0.218±0.007C	0.211±0.008B	61.6±1.61A	0.703±0.018A
Positive control (testosterone)	0.669±0.02A	0.265±0.003A	41.2±1.08C	0.538±0.016C
Propolis diethylacetate extract (200 mg/Kg) BW	0.385±0.007B	0.203±0.002B	54.4±1.83B	0.610±0.009B
$LSD_{0.05}$	0.034	0.012	4.655	0.055

Table 3. LD_{50} value of the propolis extract and ascorbic acid in DPPH and reducing power assay

substance	DPPH assay	Reducing power assay
propolis (µg/ml)	1.658±0.18	1.084±0.21
Ascorbic acid (µg/ml)	1.743±0.14	1.114±0.11

Oxidative stress may aggravate and increase number of pathological conditions including BPH. Moreover, OS may interrupt the balance between the oxidant process and the mechanism of some enzymes level resulting in generation of electrophiles and ROS leading to potential tissue and cellular damage (Minciullo *et, al.* 2014.). A number of studies referred to the link of (OS) production and development of BPH resulted from the overproduction of free radicals or reduction in the activities of free radical scavenging enzymes like GR, GST, SOD, and GST levels in the circulation, or both (Miniciullo *et, al*. 2015).

Increase the MDA level in plasma or tissue referred to increase level of peroxidase lipid and the last indicator for (OS). There is potent relationship between elevated MDA in plasma or tissue and BPH development and regression (Meagher *et, al*. 2000). Some researchers have recorded an elevation in the level of MDA in the patients suffering from BPH which point to lipid peroxidation. The concentrations and activities of the antioxidative enzymes decrease which favors the elevation of (OS) and initiation and development of BPH (Aryal *et, al.* 2007). CAT has a well-known role in detoxifying H_2O_2 which might cause increase in production of OH, the last help to prevent the intensified lipid peroxidation in the affected tissue by oxidize the protein, DNA, and lipids which may lead to mutations causing carcinogenesis (Cooke *et, al*. 2000). Different studies revealed conflicted results associated with the level of CAT in BHP; some authors pointed decreased its activity while others contradicted between increase and no difference (Aydin *et, al*. 2006) (Battisti *et, al*. 2011). GSH causing peroxides detoxification as it act as a redox buffer through reactions catalyzed via GSH peroxidase (Cooke *et, al*. 2000).

Propolis is a bee product comprises bioactive material obtained from plant-based food of the bees. Our results showed a significant curative activity for the propolis extract in a dose of 200mg / kg body weight against (TIBPH) in rats where the prostate weight and prostate index decreased significantly. This result is in agreement with the results Chiu and his colleagues (Chiu *et, al*. 2020). The mechanism by which the propolis showing its therapeutic activity on BPH may throughout scavenging activity against free radicals due to its high flavonoid and phenolic contents (Barbosa *et, al*. 2009) (Ben Ammar *et, al*. 2019) (Gul *et, al*. 2016) (Attia *et, al*. 2012). It is well known that the propolis considered a natural antioxidant (Lan, *et, al*. 2016) (Paulis *et, al.* 2012) (Gul Baykalir *et, al.* 2016) (Kurck-Gorecka *et, al*. 2012).

Moreover, it might neutralize the damages resulted from (OS) (Udensi *et, al*. 2016) or prevents generation of free radicals caused by xanthine oxidase (Gul *et, al*. 2016), along with the anti-inflammatory activity of the propolis against different inflammations including prostatitis (Lan, *et, al*. 2016) (Al-Hariri M. 2014) (Vit *et, al*. 2015) (Zabaiou *et, al*. 2017). The above mechanism by which the propolis reveals its curative effect by its antioxidant activity, is based on the results of several previous studies which suggested that one of the main causes of BPH is (OS) (Roumeguere *et, al*. 2017) (Vit *et, al*. 2015). This antioxidant activity of ethanolic extract of propolis is due to its highly contents of polyphenols especially phenolic acids and its enrichment of flavonoids (Barbosa et, al. 2009) (Andrea, 2019) (Ahmad *et, al*. 2012) (Bostanci *et, al*. 2013) (Vit *et, al*. 2015) (Zabaiou *et, al*. 2017).

The antioxidant activity of diethyl acetate propolis extract can be proved by decreasing different antioxidant markers (CAT, GSH and SOD). This result is in agreement with the result Gul Baykalir and his colleagues, Propolis may stimulate different body antioxidant enzymes primarily GSH and CAT (Gul *et, al*. 2016).

In addition, increase the weight of prostate is an important factor in BHP, which characterized by hyperplasia of both epithelial and stromal cells, resulting in prostate enlargement (Nahata *et, al*. 2012). This study

revealed a significant increase in the weight of prostate in TIBPH untreated control group when compared with negative ones. Whereas propolis treated group showed a significant decrease in the weight of prostate compared to untreated group (Agrawal *et, al*. 2012) (Mbaka *et, al*. 2017). Some researchers on animal models of BPH demonstrated a significant elevation of prostatic lipid peroxidation with a significant reduction of the prostatic levels of GSH, SOD, and catalase activities of BPH untreated rats (Cimino *et, al*. 2014). Our study showed a significant improvement of these parameters following the treatment with propolis.

CONCLUSION

The above results proved that the propolis have a good therapeutic and antioxidant activities against (TIBPH) in rats.

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