ANTIMUTAGENIC ACTIVITY OF BEE POLLEN AND PROPOLIS WATER EXTRACTS AGAINST CISPLATIN-INDUCED CHROMOSOMAL ABNORMALITIES IN BONE MARROW CELLS OF MICE

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ABSTRACT

The protective activity of Bee-collected pollen (BPE) and water-soluble derivative of propolis (WSDPE) aqueous extracts was studied on cisplatin (CDDP) induced genotoxicity in male albino mice (*Mus mascullus*). The treatment of mice with Bee-collected pollen and propolis extracts at doses 140 and 8.4 mg/kg body weight/day, respectively for 14 days synergistically with the intraperitoneal administration of cisplatin at dose of 2.8 mg/kg b.wt exhibited significant chemoprotective activity. Genotoxicity and cytotoxicity were evaluated by the bone marrow chromosomal aberration assay and mitotic index, respectively. The animals of positive control group (CDDP alone) showed a significant increase in genotoxicity. WSDPE and BPE, alone did not significantly induce chromosomal aberrations confirming their non-mutagenic effects. While, the animals in groups five and six (G5 and G6), that were injected i.p. with CDDP alone for one week and then for the next 14 days these animals were given WSDPE and BPE through oral intubation in synergistic with i.p. injection of CDDP, exhibited a significant decrease in cytogenetic damages induced by CDDP in bone marrow cells. The anti-cytotoxicity effects of WSDPE and BPE were also evident, as observed by significant increase in mitotic index, when compared to positive control group (G2). Thus, results of the present investigation revealed that WSDP and BPE have chemoprotective potentials against CDDP induced genotoxicity in bone marrow cells of male albino mice. Also, the present investigation indicated that the chemoprotective frequency of BPE was much greater than WSDPE.

Key words: Antimutagen, Albino mice, Bee pollen, Propolis.

INTRODUCTION

Cisplatin [cis-diammine-dichloro-platinum (II)] (CDDP) is a potent antineoplastic agent used for the treatment of a wide range of malignant solid tumors including testicular, ovarian, breast, lung, bladder, head and neck cancer **(Lin *et al.*, 1999; Lin *et al.*, 2001; Khynriam and Prasad, 2003 and** Pabla ***et al.*, 2008).** Nevertheless, the majority of antineoplastic drugs, besides their generic growth property, display genotoxic and cytotoxic effects which in turn contribute to growth inhibition. These toxic effects may lead to initiation of unrelated tumors **(Brozovic *et al.*, 2008).** This drug has severe toxic effects that interfere with its therapeutic efficacy, namely bone marrow toxicity, neurotoxicity, nephrotoxicity, hepatotoxicity and show the impairment of bone formation years after cessation of chemotherapy **(Wang *et al.*, 2004; Chandrasekar *et al.*, 2006 and Kim *et al.*, 2008).** Also, the oxidative stress is

one of the most important mechanisms involved in CDDP-induced toxicity **(Husain and Naseem, 2008)**.

Recently, a considerable emphasis is being laid down on the use of dietary constituents as a chemoprotective measure for the control of neoplastic and genetic diseases. Bee-collected pollen and propolis are apicultural products which are recognized as a well balanced food **(González-Güerca *et al.*, 2001).** These beehive products are composed of nutritionally valuable substances and contain considerable amounts of polyphenol substances which may have several useful pharmacological properties, such as antibiotic, anti-neoplastic, anti-inflammatory, anti-diarrhoeatic and antioxidant **(Campos *et al.*, 1997 and Aliyazicioglu *et al.*, 2005).**

Honeybee-collected pollen is a mixture of flower pollen collected by honeybees from a variety of plants and is the insect's primary food source. Pollen grains, which are flowers' male reproductive cells, contain concentrations of phytochemicals and nutrients. Bee pollen is rich in carotenoids, flavonoids and phytosterols. The exact profile varies depending on the plant sources and growing conditions; however, beta-carotene, beta-sitosterol, isorhamnetin, kaempferol, lycopene, quercetin and rutin are consistently reported **(Markham and Campos 1996 and Campos *et al.*, 1997).**

Propolis is a resinous substance collected by honeybees (*Apis mellifera*) from exudates and buds of plants and mixed with secreted beeswax. People have used propolis as a folk medicine from ancient times. Even though propolis has diverse physiologic functions such as antioxidant, anticarcinogenic, antimicrobial and anti-inflammatory effects, **(Marcucci *et al.*, 2001; Ishikawa *et al.*, 2004 and Kumazawa *et al.*, 2004).** Such effects have been associated with the presence of phenolic compounds, such as flavonoids and aromatic acids **(Heim *et al.*, 2002; Ichikawa *et al.*, 2002).**

The antioxidant activity of flavonoids present in bee collected pollen and propolis has been shown to be capable of scavenging free radicals. The radical scavenging activity of phenolic compounds is assigned to the hydrogen-donating ability of these compounds **(Surveswaran *et al.*, 2007).** Antioxidants intercept the free radical chain oxidation by donating hydrogen from the phenolic hydroxyl groups, thereby forming stable end products, which does not initiate or propagate further oxidation **(Shimizu *et al.*, 2004 and Jayaprakasha *et al.*, 2006).**

Development and utilization of more effective antioxidants of natural origin are desired. Naturally occurring polyphenols are expected to help reducing the risk of alkylating agents and various life-threatening diseases, including cancer and cardiovascular diseases, due to their antioxidant activity. The purpose of the present study was to evaluate and compare the effectiveness of the water extracts of honeybee-collected pollen (BPE) and water-soluble derivative of propolis (WSDP) from Beni-Suef, Egypt, as *in vivo* antimutagenic agents against cisplatin-induced chromosomal abnormalities in bone marrow cells of mice (*Mus musculus*).

MATERIALS AND METHODS

Chemicals

Cisplatin [cis-diammine-dichloroplatinum (II)] (CDDP) was purchased from MERCK in a form of ampoules, each contains 25 mg of CDDP in 25 ml sterile saline solution. All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Experimental animals

The experimental animals used in this work were random bred adult males of laboratory mice *Mus musculus* (20-30 gm in weight). Animals were obtained from Ophthalmology research institute. All animals were housed in plastic cages with wired covers and kept under normal laboratory conditions for the different periods of time used. The animals were not treated with antibiotics, vitamins or insecticides and were fed a standard commercial diet (ATMID Company, Egypt) and drank tap water.

Extract preparations

The honeybee collected pollen and propolis were provided by local beekeepers in Beni Suef, Egypt. These samples were harvested in September 2006. Bee collected pollen was obtained as yellow pellets which contain a mixture of pollen from the anthers of flowers of the plants growing in the surroundings of the beehives, while propolis was obtained in the form of a yellow-brown powder derived from water-soluble derivative of propolis (WSDP).

Bee pollen and propolis extracts prepared according to the methods of **Orsolic *et al.*, (2005) and Yamaguchi *et al,* (2007).** The powder of bee pollen (280 mg) was suspended in 10 ml of distilled water and mixed vigorously. This suspension was kept stand overnight in dark and centrifuged at 10000 rpm in a cooling centrifuge for 45 minutes at 10°C. The supernatant fraction was collected and filtered. The filtrate was kept in a frozen condition at -10°C until use. On the other hand, propolis extract was prepared under sterile condition by dissolving the WSDP powder in 15 ml distilled water and mixed vigorously for 10 minutes. Finally, this suspension was centrifuged at 1000 rpm for 10 minutes at room temperature. The supernatant was collected and kept under a freezing condition until used.

Doses and organization of experimental groups

The single dose (2.8 mg/kg b.wt) of CDDP used in the present study was selected with reference to the dose range that has been used in previously published studies dealing with the cytotoxicity and genotoxicity of CDDP (Nersesyan and Muradyan 2004), while doses of bee pollen (BPE) and propolis (WSDPE) water extracts used in the present study were 140 and 8.4 mg/kg b.wt, respectively **(Mani *et al.*, 2005 and Yamagushi *et al.*, 2007).**

Mice were divided into 6 groups (5 animals each). The animals of group one (G1) served as a negative control group received 0.9% of NaCl solution by intraperitoneally injection (i.p.) twice/week for three weeks. The animals of group two (G2) received i.p. injection of CDDP (2.8 mg/kg b.wt.) twice/week for three weeks. In group three (G3) 8.4 mg/kg b.wt of WSDPE was given to the animals through oral intubation once/day for 14 days consecutively. The animals of group four (G4) received oral administration of BPE (140 mg/kg b.wt) once/day for 14 days. The animals of group five and group six (G5 and G6) were injected i.p. with CDDP alone for one week, these animals were

given WSDPE (G5) and BPE (G6) through oral intubation in combination with i.p. injection of CDDP, in the following 14 days of treatment.

Preparation of the mice bone marrow cell system

Bone marrow cell preparations for the analysis of chromosomal aberrations and mitotic index were produced by the colchicine–hypotonic technique.

After completion of the treatment period, animals in each group were sacarified 24 hours post-injection of all treatments by cervical dislocation. Colchicine (4 mg/Kg b.w.) was given intraperitoneally 22 hs prior of sacrificing. The bone marrow smears of animals in each group were prepared according to **Preston *et al.*, (1987)** protocol. Slides were stained with Giemsa and 50 well spread metaphase plates/animal were analyzed for chromosomal aberrations including structural chromosomal aberrations (chromatid breakage {include break and deletion}, chromatid gap, centromeric attenuation, centric fusion and end to end association) and numerical chromosomal aberrations (polyploidy and Endomitosis) and incidence of aberrant cells for each group was also calculated. The mitotic index was obtained by counting the number of mitotic cells in 1000 cells/animal. While the percentage of suppressed aberrant cells was calculated according to **Shukla and Taneja (2002)** as follows: 100 – (% of aberrant cells in CDDP+extract treated groups (G5 or G6)/% of aberrant cells in positive control (CDDP treated) group) x 100.

Statistical analysis

Statistical analysis for the difference in the mean number of chromosomal aberrations and mitotic index between groups was carried out using student-*t*-test ($P < 0.05$ was considered significant).

RESULTS

According to the cytogenetic results presented in tables 1 and 2, seven structural and numerical chromosomal aberrations were determined in the control and the experimental groups. The results obtained in the first phase of cell cycle (24 h sampling time), revealed that cisplatin (CDDP) when given at a single dose of 2.8 mg/kg b.wt, twice/week for three weeks (G2) induced a high frequency of chromosomal aberrations in bone marrow cells of mice when compared with the control (G1) group (Tables 1 & 2). In the CDDP-treated groups the most frequent chromosomal aberration was chromatid breakage. The mitotic index was significantly decreased ($P < 0.05$) 37.75, over control, indicating bone marrow cytotoxicity (Table 2).

When the propolis extract (WSDPE) treated group (G3) was compared with the control group (G1) in terms of mean total number of structural chromosomal aberrations, the percentage of incidence of aberrant cells and the number of aberrations/cell, G3 displayed significant increase ($P < 0.05$), whereas the mean total number of numerical chromosomal aberrations was significantly decreased ($P < 0.05$). The WSDPE was not cytotoxic at this given dose (8.4 mg/kg b.wt), in which there was no significant change in mitotic index compared with group G1 (Table 2). However, in the aqueous bee pollen extract (BPE) treated group (G4) when compared with the control group (G1) in terms of mean total number of numerical chromosomal aberrations, the percentage of incidence of aberrant cells and the number of aberrations/cell displayed no significant differences ($P < 0.05$) confirming its non-mutagenicity (Tables 1 & 2). The BPE was also not found to be cytotoxic at the given dose

(140 mg/kg b.wt), where there was no significant changes in mitotic index comared to G1 (Table 2).

Moreover, in the WSDPE or BPE and CDDP treated groups (G5 and G6, respectively) there was a significant decrease in the rates of clastogenetic changes compared with the CDDP treated group (Tables 1 & 2). All types of chromosomal aberrations induced by CDDP including breaks, gaps, end to end association, centric fusion, centromeric attenuation, and other multiple damages were found to be reduced by WSDPE and BPE but still significantly higher than negative control group (G1). Also, the mitotic index was found to be increased significantly ($P< 0.05$), indicating of their anti-cytotoxicity towards CDDP (Table 2). The percentages of aberrant cells which were found to be 50.00 $\pm$ 4.147 in CDDP treated animals, were reduced to 34.80 $\pm$ 3.382 and 30.80 $\pm$ 1.743 ($P< 0.05$) by WSDPE and BPE, respectively (Table 2). Also a significant decrease in the number of aberrations per cell was observed in G5 and G6 in comparison with the CDDP treated group (G2). The calculated suppressive effect was 30.40% and 38.40%, by WSDPE and BPE, respectively (Table 2).

DISCUSSION

Propolis and bee-collected pollen are apicultural products which are composed of nutritionally valuable substances and contain considerable amounts of polyphenol substances which may act as potent antioxidants. Development and utilization of more effective antioxidants of natural origin are desired. Naturally occurring polyphenols are expected to help reducing the risk of various life-threatening diseases, including cancer diseases, due to their antioxidant activity **(Teixeira *et al.*, 2008).** Also, Phenolic compounds are known to counteract oxidative stress in the human body by helping maintaining a balance between oxidant and antioxidant substances **(Materska and Perucka, 2005 and Siddhuraju, 2006).**

Flavonoids and phenolic acids are major classes of polyphenolic compounds, whose structure-antioxidant activity relationships in aqueous or lipophilic systems have been extensively reported **(Nenadis *et al.*, 2004 and Gardjeva *et al.*, 2007).** In addition to the antioxidant activity, many phenolic compounds have been shown to exert anticarcinogenic or antimutagenic activity to a greater or lesser extent **(Tapiero *et al.*, 2002 and Awale *et al.*, 2005).** Their physiological and pharmacological activities may be derived from their antioxidant properties, which are related to their molecular structure **(Heim *et al.*, 2002).** The mechanisms of antioxidant action may include suppression of oxygen reactive species (ROS) formation, removal or inactivation of oxygen reactive species and up-regulation or protection of antioxidant defenses **(Van Acker *et al.*, 1996 and Montoro *et al.*, 2005).**

Cisplatin (CDDP) is an inorganic platinum compound with a broad spectrum antineoplastic activity against different types of human tumors **(Siddik, 2003).** CDDP has been demonstrated to have the potential for initiating genetic events in non-tumor cells in human and in animal systems. Nevertheless, both clinical and experimental studies reported a dose-limiting nephrotoxicity which restricts cisplatin's optimal usefulness in cancer chemotherapy **(Nersesyan *et al.*, 2003).** Bone marrow cytogenetic is a useful short-term technique, for elucidating the mechanism as well as to identify the substances for their clastogenic and anticlastogenic activity **(Renner, 1990 and Badary *et al.*, 1997).**

The results of the present investigation revealed that, administration of CDDP at a dose of 2.8 mg/kg b.wt, twice/week for three weeks induced cytogenotoxic effects. These results are consistent with those previously reported **(Antunes *et al.*, 2000; Khynriam and Prasad 2003; Chandrasekar *et al.*, 2006 and Brozovic *et al.*, 2008).** In the present study, the administration of Bee pollen (BPE) and propolis (WSDPE) aqueous extracts (140 and 8.4 mg/kg b.wt, respectively), by gastric intubation combination with the intraperitoneal injection of CDDP for two weeks, effectively reduced the incidence of chromosomal damages induced by CDDP in bone marrow cells and increased the frequency of the mitotic indices of bone marrow cells. These results revealed the protective efficiency of Egyptian bee pollen and propolis aqueous extract. This is consistent with those reported by **El-khawaga *et al.* (2003); Fu *et al.* (2004); Lotfy (2006); Carpes *et al.* (2007) and Teixeira *et al.* (2008).**

The anti-mutagenic actions of bee propolis extract involve enhancement of the level of glutathione S-transferase (GST), inhibiting cytochrome P-450 activity and interaction with microsome-generated proximate mutagens to generate an inactive complex **(Jeng *et al.*, 2000 and Russo *et al.*, 2006).** These effects were associated with inhibition of cell cycle progression, accelerating the detoxification of mutagens and carcinogens and induction of apoptosis **(Soni *et al.*, 1997; Varanda *et al.*, 1999 and El-khawaga *et al.*, 2003). Lotfy (2006)** indicated that, Egyptian propolis is characterized by the presence of unusual esters of caffeic acid with C12- C16 fatty alcohols, mainly saturated. Flavonoid glycones and especially flavanones are typical components of propolis **(Bankova *et al.*, 1997).** All such constituents of crude Egyptian propolis have increased its pharmaceutical demand and have rendered it an interesting subject of study.

However, the mechanism for protection of the bee pollen extract involves scavenging potentially toxic and mutagenic electrophiles and free radicals and modification of antioxidant pathways **(Ohta *et al.*, 2007).** The recent investigations indicated that, bee pollen extract contains significant amounts of polyphenolic substances, mainly flavonoids **(Di Paola-Naranjo *et al.*, 2004; Almeida-Muradian *et al.*, 2005 and Leja *et al.*, 2007).** Also several researchers found that polyphenols are antioxidants with redox properties which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers **(Okawa *et al.*, 2001 and Caldwell, 2003).** The polyphenols also have metal chelation properties **(Rice-Evans *et al.*, 1996).** These compounds chelate metals and react with free radicals, genotoxic substances and carcinogenics **(Tang *et al.*, 2005).** Epidemiologic studies have shown a correlation between an increased consumption of phenolic antioxidants and a reduced risk of cardiovascular disease and certain types of cancer **(Cook and Samman, 1996).**

According to the results obtained, bee pollen extract seems to have interesting biological properties than propolis. The protective effect of bee pollen and propolis extracts towards CDDP induced toxicity implies a good marker of its antimutagenic, activity. Further investigations are needed to elucidate the interaction of bee pollen and propolis constituents with genotoxic compounds at genetic level.

Table 1, 2

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التأثير المضاد للطفرات للمستخلصات المائية من خبز وصمغ نحل العسل ضد التغيرات الكروموسومية الناتجة عن السيسبلاتين فى خلايا نخاع عظم الفأر

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تناول هذا البحث دراسة التأثير السمى الوراثى لعقار السيسبلاتين على كروموسومات خلايا نخاع العظم للفأر (مس مسكيولس) و دراسة الكفاءة الوقائية للمستخلصات المائية لنوعين من منتجات نحل العسل منتشر الأستخدام وهما خبز و صمغ النحل وقد تم إختيار هذان النوعان لإحتوائهما على العديد من المركبات الفينولية والتى لها كفائة عالية فى مقاومة العديد من مسببات السرطان لكونها من مضادات الأكسدة. وقد تم معاملة الفئران بجرعة ١٤٠ميلجرام/كيلوجرام من مستخلص خبز النحل و ٨.٤٠ ميلجرام/كيلوجرام من مستخلص صمغ النحل بجرعات يومية عن طريق الفم لمدة ١٤ يوماً وأيضاً تم معاملة الفئران بعقار السيسبلاتين بحقنة فى التجويف البطنى بجرعة ٢,٨ ملج/كج ثلاث مرات فى الأسبوع و لمدة ثلاثة اسابيع متتالية و فى المجموعات التى يتم فيها دراسة الكفائة الوقائية لتلك المستخلصات تم معاملة الفئران بعقار السيسبلاتين لمدة أسبوع ثم السيسبلاتين والمستخلص المائى معاً لمدة اسبوعين متتاليين ويتم أخذ العينات بعد ٢٤ ساعه من أخر معاملة فى كل مجموعة. وقد أوضحت النتائج المتحصل عليها أن عقار السيسبلاتين له تأثير سمى وراثى على خلايا النخاع حيث أنه تسبب بحدوث العديد من التغيرات الكروموسومية وأيضا تأثير سمى خلوى أدى الى نقص فى معامل انقسام الخلايا مقارنة بالمجموعة الضابطة. أوضحت الدراسة أن مستخلصات خبز وصمغ النحل لها كفائة عالية على مقاومة تلك التأثيرات السمية الوراثية فى خلايا نخاع الفأر بتقليل التغيرات الكروموسومية وأرتفاع معدل معامل انقسام الخلية وكانت هذه التغيرات معنوية مقارنة بمجموعة السيسبلاتين. و أيضاً أوضحت النتائج المتحصل عليها فى تلك الدراسة أن التأثير الوقائى أو العلاجى لمستخلصات خبز النحل أقوى من مستخلصات صمغ النحل.