Letter to the Editor

Evaluation of the Effects of Cypermethrin on Female Reproductive Function by Using Rabbit Model and of the Protective Role of Chinese Propolis

AE Khatab[1], NM Hashem[2,#], LM El-Kodary[3], FM Lotfy[1], and GA Hassan[2]

The prophylactic effects of Chinese propolis against cypermethrin toxicity were evaluated by performing ovary and uterus histopathology, as well as by characterizing ovarian function, embryos, and litters. Cypermethrin induced atypia in the ovary and uterus, and decreased the ovulation sites and the number of embryos. Cypermethrin-induced oxidative stress during pregnancy, decreased the parturition rate as well as the number and weight of offspring and increased the incidence of morphological malformations in the offspring. Administration of propolis to cypermethrin-treated animals mitigated cypermethrin-induced reproductive toxicity.

Cypermethrin is a synthetic form of the naturally derived insecticide pyrethrin, and is used extensively worldwide. It degrades in soil and plants within a few days; however, its concentration stays relatively constant after a treatment indoors, and it can cause hazardous health effects. Cypermethrin can affect reproduction-related steroids, and crosses the placental barrier to interfere with fetal development[1]. Recent studies have shown that the reproductive toxicity of cypermethrin can be partially mediated by oxidative stress[2].

Propolis is a mixture of resinous plant substances that are produced by honeybees. It contains an abundance of phytochemicals (flavonoids, phenolic acids, and long-chain fatty acids) with antioxidant activities[3]. Therefore, this study aimed to identify the effects of cypermethrin on the different reproductive functions of female rabbits and their offspring, and the protective role of propolis based on its chemical constituents.

The chemical constituents of the ethanolic extract of propolis (Guang Zhou Herb & Bee Products Co., Ltd., Tianhe District, Guangzho, China) were identified by gas chromatography-mass spectrometry (GC/MS). A Thermo Scientific TRACE-1300 series GC system fitted with a fused silica DB-5 capillary column (inner diameter, 30 m × 0.32 mm; film thickness, 0.25 μm), coupled to a Triple Quadrupole Mass (TSQ 8000 Evo) was used (Thermo Fisher Scientific Inc., Austin, Texas, USA). The column temperature was set at 40 °C with an initial hold of 5 min, which was then increased to 270 °C at 2 °C/min, and maintained at 270 °C for 20 min. The splitless injection mode was used (0.5 μL of a 1:1000 methanol solution). The carrier gas was helium with a flow rate of 1.0 mL/min. Injector and detector temperatures were 250 °C and 290 °C, respectively. Mass spectra were scanned in the range of 40-700 amu, and the scan time was 5 scans/s. The constituents were identified based on a combination of retention index data and mass spectral data using the Wiley 9 library.

Forty female V-line rabbits (5 months old, weighing 2.935±0.029 kg), obtained from the Laboratory of Rabbit Physiology Research, Faculty of Agriculture, Alexandria University, Egypt, were used. The rabbits were handled in accordance with the Standard Guide for the Care and Use of Laboratory meeting the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes. Propolis (50 mg/kg body weight) and alpha-cypermethrin (50 mg/kg body weight; 1/5 lethal dose, LD_{20}) were dissolved in corn oil. Alpha-cypermethrin [α-cyano-(3-phenoxyphenyl)methyl(±) -*cis*/*trans*-3-(2,2-dichlorovinyl)2,2-dimethylcycloprop -anecarboxylate] is a racemic mixture of two isomers of cypermethrin with the molecular formula $C_{22}H_{19}C_{l2}NO_3$ and molecular weight of 416.3 g/mol (Chimac Agriphar S.A., Belgium). Female rabbits were randomly divided into four groups (*n*=10), and administered corn oil (Con group), propolis (Pro

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1. Department of Home Economics Department, Faculty of Specific Education, Alexandria University, Alexandria 21545, Egypt; 2. Department of Animal Production, Faculty of Agriculture (El-Shatby), Alexandria University, Alexandria 21545, Egypt; 3. Department of Home Economics, Faculty of Agriculture (El-Shatby), Alexandria University, Alexandria 21545, Egypt

group), cypermethrin (Cyp group), or their combination (Cyp/Pro group) by oral gavage. Each female rabbit was treated with 25 IU of equine chorionic gonadotropin (Gonaser®, Hipra, Spain) 2 d before being allowed to naturally mate with fertile male rabbits. Female rabbits of each group were divided into two subgroups (5 females each). The first subgroup received the treatments for 60 d, and allowed to mate 2 d prior to being euthanized. Reproductive organs were directly removed and weighed after the animals were euthanized. The number of ovulation points on each ovary was recorded. Excised oviducts were flushed with phosphate buffer solution containing 20% bovine serum albumin (Sigma, USA). Next, the collected embryos from each oviduct were counted and their developmental stage was recorded. Finally, the ovaries and uteri were fixed and processed for preparation of histological sections. The second subgroup received the same treatments immediately after mating until parturition for two gestation periods. The parturition rate, abortion rate, and offspring characterization data were recorded.

Blood samples of the first subgroup were collected on the day of mating to determine the plasma estradiol (E_2) concentration. In the second subgroup, blood samples were obtained on days 14 and 28 after mating. The activities of glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT) (Reactivos GPL, Barcelona, Spain) and concentrations of malondialdehyde acetate (MDA; Biodiagnostic, Giza, Egypt) and progesterone (P_4) were determined. Hormonal assessment was carried out using commercial solid-phase enzyme immuno-assay kits (DRG International Inc., Springfield, USA).

Results are expressed as mean±standard error values. Analyses of variance (ANOVA) were conducted to determine significant differences among groups, followed by Duncan's new multiple range test. Data expressed as percentages were analyzed using a Chi-square test. Statistical significant was considered at $P<0.05$. All statistical analyses were carried out using the Statistical Analysis System program (SAS Institute, 2001, Version 8. Cary, USA).

The analysis of the propolis ethanolic extract helped identify 17 chemical compounds including alkaloids such as 3,3-dimethyl-2-phenyl-2-(1-oxo-1, 2,3,4-tetrahydronaphthalen-2-yl) azirane (21.40%), N,N-dimethyl deacetyl colchinine (3.67%), 2,4-bis (4-chlorophenyl)-5,6-dihydrobenzo[h] quinazoline (3.02%); flavones such as lucenin 2 (5.17%), baicalin (3.82%), and quercetin 7,3',4'-trimethoxy ester (2.71%); and organosilicons such as cyclohexasiloxane, dodecamethyl (6.51%) and hexasiloxane,1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl (5.41%) (Table 1).

Table 1. Retention Times (RTs, minute) and Percentages of Relative Area (%) of Chemical Constituents of Propolis Ethanolic Extract Detected by Gas Chromatography-mass Spectrometry (GC/MS)

RT	Area	Compound
2.02	21.4	3,3-Dimethyl-2-phenyl-2-(1-oxo-1,2,3,4-tetrahydronaphthalen-2-yl) azirane
7.41	2.94	Cyclopentasiloxane, decamethyl-(CAS)
10.04	6.51	Cyclohexasiloxane, dodecamethyl
12.32	5.41	Hexasiloxane,1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl
27.67	3.70	Glycodeoxycholic acid
27.95	7.59	Cholestan-3-one, cyclic1,2-ethanediyl aetal, (5á)-(CAS)
28.01	3.67	N,N-Di methyl deacetyl colchinine
28.73	3.02	2,4-Bis(4-chlorophenyl)-5,6-dihydrobenzo[h]quinazoline
29.02	5.36	5-Chloro-3-(3,4-dimethoxyphenyl)-6-methyl-2H-1,4-oxazin-2-one
29.89	2.71	Quercetin7,3',4'-Trimethoxy ester (CAS)
30.11	2.47	6-Ethyl-5-(4'-trifluoro methyl phenyl) pyrimidine-2,4-diamine
30.19	3.82	Baicalin
31.93	5.17	Lucenin 2 (Luteolin 6,8-C-diglucoside)
32.04	3.85	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-Hexadecamethyloctasiloxan
34.53	5.82	3,9-Epoxypregnane-11,14,18-triol-20-one,16-cyano-3-methoxy-,11-acetate,16-cyano-3-methoxy-,11-acetate
34.80	4.23	6-Amino-5-cyano-4-(5-cyano-2,4-dimethyl-1H-pyrrol-3-yl)-2-methyl-4H-Pyran-3-carboxylic acid ethyl ester
34.89	5.77	7-Hydroxymethyl-1-bromo-4-isopropoxy-5-methoxyna phthalene

As shown in Table 2, the normal diameters of ovarian follicles lined by multiple layers of granulose cells were observed in the Con, Pro, and Cyp/Pro groups. Follicular atresia and fewer layers of granulosa cells were observed in the Cyp group. Compared to the other groups, the Cyp group also showed fewer endometrial glands and increased myometrium hypertrophy.

The highest (P<0.001) relative uterus weight was observed in the Cyp group. Treatment with cypermethrin significantly decreased (P<0.05) the number of ovulation points in the cypermethrin-treated groups, whereas the number of ovulation points was intermediate in the Cyp/Pro group. The lowest (P<0.001) concentration of E_2 was in the Cyp group. However, the combination of propolis with cypermethrin significantly improved (P<0.001) the concentration of E_2 compared to the cypermethrin-treated group. Treatment with cypermethrin significantly decreased (P<0.05) the number of embryos, whereas the number of embryos was intermediate in the Cyp/Pro group. Neither the numbers/developmental stages of the embryos collected nor the number of unfertilized ova were affected (P>0.05) by the treatment.

As shown in Table 3, cypermethrin increased the concentration of MDA (P<0.01) and decreased the activity of antioxidant enzymes (GPX, CAT, and SOD). The lowest concentration of P_4 was in the Cyp group, followed by the Cyp/Pro group and Con group. Cypermethrin decreased (P<0.05) the parturition rate, and increased (P<0.05) the abortion rate compared to those in the Con, Pro, and Cyp/Pro groups. In addition, it decreased (P<0.05) the number of offspring born and their birth weights, and increased (P<0.05) the incidence of morphological abnormalities in offspring compared with those in the Con, Pro, and Cyp/Pro groups. Propolis mitigated the cypermethrin-induced negative effects on antioxidant enzymes activities, reproductive performance, and offspring characterization.

In this study, cypermethrin caused ovarian follicle atresia and granulosa cell apoptosis, which were associated with reductions in the number of ovulation points and the plasma E_2 concentration. The number of ovulation points depends on the follicle reserves in the ovary. Furthermore, E_2 is

Table 2. Histopathology and Relative Weights of Ovaries and Uteri, Ovarian Function, and Embryo Characterization of Female Rabbits Administered Propolis (Pro), Cypermethrin (Cyp), or Their Combination (Cyp/Pro) Compared to Those in the Controls (Con)

Parameter	Experimental Groups			
	Con	Pro	Cyp	Cyp/Pro
Ovary				
Follicles atresia	no atresia	no atresia	atresia ++	atresia +
Follicles size	normal	normal	decrease ++	decrease +
Ovarian follicular cells	normal	normal	decrease ++	decrease +
Uterus				
Endometrial glands lining	normal	normal	decrease ++	Decrease +
Endometrial glands number	normal	normal	decrease ++	Decrease +
Myometrial thickness	normal	normal	increase	no increase
Endometrial glands atypia	no atypia	no atypia	increase ++	no atypia
Relative weights				
Ovary relative weight, g	0.024±0.004	0.024±0.005	0.020±0.002	0.022±0.004
Uterus relative weight, g	0.580±0.060^{b}	0.470±0.020^{c}	0.730±0.170^{a}	0.610±0.090^{b}
Ovarian functions				
No. of ovulation point/doe	12.00±1.28^{a}	13.40±2.88^{a}	9.80±0.20^{b}	10.50±2.06^{a,b}
E_2, pg/mL	119.20±1.92^{a}	118.0±3.16^{a}	57.2 ±2.86^{c}	101.40±2.07^{b}
Embryo characterization				
No. of embryos/doe	10.40±0.86^{a}	11.60±1.34^{a}	7.0±1.30^{b}	10.2±1.45^{a,b}
Stage of embryo	morula	morula	morula	morula
No. of unfertilized ova/doe	0.00±0.00	0.00±0.00	0.20±0.06	0.00±0.00

Note. $^{a\text{-}c}$Within rows, means with different superscripts significantly differ (P<0.05).

synthesized by granulosa cells, which were damaged by cypermethrin. In a previous study, women exposed to pyrethroids showed decreased ovarian follicle reserves[4]. In this study, cypermethrin caused myometrium hypertrophy, which was associated with an increase in the relative weight of the uterus. This finding could be related to the hormone mimicking effect of cypermethrin[1].

Our study showed that cypermethrin decreased the number of embryos, but did not cause deleterious effects in their developmental stage. This seems to be related to decreased ovulation sites rather than fertilization failure. Particularly, the number of unfertilized ova was not affected by cypermethrin. In addition, cypermethrin did not reduce the occurrence of pregnancy (evaluated on day 12 of pregnancy). However, cypermethrin decreased the parturition rate and increased the abortion rate (observed on day 25 of pregnancy). Collectively, the main effects of cypermethrin on pregnancy appeared during the second half of the pregnancy period. The fetal losses might be due to the direct effect of cypermethrin on fetus viability, as cypermethrin can cross the placental barrier[1], or due to the destructive effect of free radicals on luteal cell membranes, which reduces P_4 biosynthesis[5].

The fetal malformation that occurred after the administration of cypermethrin could be partially explained by the genotoxic potential of cypermethrin (teratogenic effect), which could be mediated by the damaging effect of free radicals on the DNA structure, resulting in gene mutations and thus fetal malformation[6].

When propolis was administered concomitantly with cypermethrin, it attenuated the reproductive toxicity of cypermethrin and maintained the activity of antioxidant enzymes. This is most likely due to the unique chemical constitution of propolis, where high percentages of flavones including lucenin 2, baicalin, and quercetin were detected. Baicalin is suggested to be a strong inducer of neural differentiation[7], and may attenuate the neurotoxicity of cypermethrin. Moreover, luteolin has antioxidant, anti-inflammatory, antimicrobial, anticancer, anti-allergic, and anti-platelet activities[8]. In addition, alkaloids are known to have anti-inflammatory effects by inhibiting the production of inflammatory mediators such as IL-6, IL-8, and TNF-α[9]. Additionally, organosilicon compounds are used for multi-therapeutic purposes such as the protection of hepatocytes against chemical toxicity[10].

Table 3. Concentration of Plasma Antioxidant Enzymes and Progesterone (P_4), Reproductive Performance, and Offspring Characterization of Female Rabbits Administered Propolis (Pro) or Cypermethrin (Cyp) or Their Combination (Cyp/Pro) Compared to Those in the Control (Con) During Pregnancy (mean±stander error)

Parameter*	Experimental Groups			
	Con	Pro	Cyp	Cyp/Pro
Antioxidant enzymes**				
GPX (U/mL)	15.32±0.37^{b}	17.55±0.33^{a}	8.99±0.34^{d}	10.13±0.40^{c}
SOD (μ/mL)	7.26±0.12^{b}	10.33±0.21^{a}	4.80±0.11^{d}	6.52±0.11^{c}
CAT (μmol·min^{-1}·mL^{-1})	53.90±0.60^{b}	63.77±0.53^{a}	38.77±0.54^{d}	45.20±0.55^{c}
MDA (μmol/mL)	3.24±0.15^{c}	2.81±0.12^{d}	5.33±0.13^{a}	4.53±0.12^{b}
P_4, ng/mL	23.45±0.41^{b}	25.30±0.36^{a}	15.45±0.38^{d}	17.97±0.37^{c}
Reproductive performance				
Pregnancy rate, %	80.00 (8/10)	80.00 (8/10)	50.00 (5/10)	70.00 (7/10)
Parturition rate, %	80.00^{a} (8/10)	80.00^{a} (8/10)	30.00^{b} (3/10)	70.00^{a} (7/10)
Abortion rate, %	00.00^{b} (0/10)	00.00^{b} (0/10)	20.00^{a} (2/10)	00.00^{b} (0/10)
No. of offspring borne /doe	6.10±1.10^{b}	11.63±0.95^{a}	2.60±1.20^{c}	8.62±0.96^{b}
Birth weight, g	55.0±2.76^{a}	58.38±4.75^{a}	43.67±3.42^{b}	50.62±2.89^{a,b}
No. of abnormal offspring/doe***	00.00±0.00^{b}	00.00±0.00^{b}	1.33±0.29^{a}	00.00±0.00^{b}

Note. *Parameter values were the average of two pregnancy outcomes. **GPX = glutathione peroxidase, SOD = superoxide dismutase, CAT = catalase, MDA = malondialdehyde acetate. ***Morphological abnormality in the form of white abscesses was observed on the skin of offspring. $^{a-d}$Within rows, means with different superscripts significantly differ (P<0.05).

Cypermethrin reproductive toxicity could be countered using Chinese propolis as a nutraceutical agent owing to its various biological therapeutic properties including antioxidant activity.

#Correspondence should be addressed to NM Hashem, Tel: 20-012-88719758, Fax: 20-35922780, E-mail: hashemnesreen@yahoo.com

Biographical note of the first author: AE Khatab, female, born in 1983, PhD student, majoring in maternal and child care.

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REFERENCES

1. Dewailly E, Forde M, Robertson L, et al. Evaluation of pyrethroid exposures in pregnant women from 10 Caribbean countries. Environ Int, 2014; 63, 201-16.
2. Ince S, Kucukkurt I, Demirel H, et al. Thymoquinone attenuates cypermethrin induced oxidative stress in Swiss Albino mice. Pestic Biochem Phys, 2012; 104, 229-35.
3. Hashem NM, Abd El-Hady A, Hassan O. Effect of vitamin E or propolis supplementation on semen quality, oxidative status and hemato-biochemical changes of rabbit bucks during hot season. Livestock Sci, 2013; 157, 520-6.
4. Whitworth KW, Baird DD, Steiner AZ, et al. Anti-Müllerian hormone and lifestyle, reproductive, and environmental factors among women in rural South Africa. Epidemiology, 2015; 26, 429-35.
5. Nayyar S, Jindal R. Essentiality of antioxidant vitamins for ruminants in relation to stress and reproduction. Iranian J Vet Res, 2010; 11, 1-9.
6. Assayed ME, Khalaf AA, Salem HA. Protective effects of garlic extract and vitamin C against in vivo cypermethrin-induced teratogenic effects in rat offspring. Food Chem Toxicol, 2010; 48, 3153-8.
7. Tu XK, Yang WZ, Shi SS, et al. Baicalin inhibits TLR2/4 signaling pathway in rat brain following permanent cereral ischemia. http://www.ncbi.nlm.nih.gov/pubmed/20859668?dopt=Abstract Inflammation, 2011; 34, 463-70.
8. El-Hawary SS, El-Sofany RH, Abdel-Monem AR, et al. Polyphenolics content and biological activity of Plectranthus amboinicus (Lour.) spreng growing in Egypt (Lamiaceae). Phcog J, 2012; 4, 45-54.
9. Zhang Y, Xu W, Li H, et al. Therapeutic effects of total alkaloids of Tripterygium wilfordii Hook f. on collagen-induced arthritis in rats. J Ethnopharmacology, 2013; 145, 699-705.
10. Babalola OO, Ojo OE, Oloyede FA. Hepatoprotective activity of aqueous extract of the leaves of Hyptis suaveolens (l.) Point on acetaminophen induced hepatotoxicity in rabbits. Res J Chem Sci, 2011; 7, 85-8.