IMMUNONOLOGICAL EVALUATION AND ACUTE TOXICITY STUDY WITH FERTILITY EXAMINATION FOR THE EFFECT OF AQUEOUS EXTRACT FROM DRIED FRUITS OF *Piper nigrum* L. IN MICE

Maha F. Al Tae'e , *Zaid K., Hadeel W.
Department of Biotechnology, College of Science, University of Baghdad. Baghdad- Iraq.
*The Development and Teaching Center, University of Baghdad. Baghdad- Iraq.

Abstract

The research work was conducted to investigate the effect of oral administration of water extract of black pepper at doses of (1, 5) mg/kg body weight for two weeks daily by determining the genotoxic effect (mitotic index), evaluation of immunological effect (IgG, IgM, IgA, C3, C4) and measuring fertility hormones (follicles stimulation hormone/FSH, lutenising hormone/LH) levels with histopathological examinations of female albino swiss mice ovaries in comparison with control (normal saline). A clear effect in increasing mitotic activity was reveled for both doses in comparison with control. Results also showed a significant increase in the value of the all immunological parameters at both doses in comparison with control. Also obvious raise was seen in the level of FSH and LH for both doses when compared with normal saline treated mice with no significant damage seen in female ovaries tissue, in fact there were certain clinicopathological changes in mice ovaries tissue which were represented by increasing in the numbers of primary and secondary follicles and in the numbers of corpus luteum at both doses.

تقييم مناعي ودراسة سمية حادة مع فحص الخصوبة لتأثير المستخلص المائي للثمرة الجافة لنبات الفلفل الاسود في الفئران

مها فخري الطائي، *زيد كاظم، هديل وليد
قسم التقانة الاحيائية، كلية العلوم، جامعة بغداد. بغداد-العراق.
*مركز التطوير والتعليم المستمر، جامعة بغداد. بغداد-العراق.

الخلاصة

تم اجراء هذا العمل البحثي بهدف التحري عن تاثير المستخلص المائي لنبات الفلفـل الاسـود الـذي اعطي عن طريق الفم وبجرعتين (1,5) ملغم/كغم من وزن الجسم ولمدة اسبوعان من خلال حساب التاثير السمي الوراثي (معامل الانقسام الخيطي)، تقييم التـاثير المنـاعي بقيـاس معـدل المعـاملات المناعيـة (IgG, IgM, IgA, C3, C4) وقياس معدل الهرمونات الانثوية (FSH, LH) مصحوبة بفحص التغيرات المرضية النسيجية في مبايض الاناث المعاملة بالمستخلص ومقارنتها مع السـيطرة (الـدارئ الملحـي). أظهرت النتائج وجود تأثير واضح في زيادة معدل الانقسام الخيطي ولكلا الجرعتين بالمقارنة مع السيطرة. كذلك اظهرت النتائج حدوث زيادة معنوية في قيم معاملات المناعة لكلا الجرعتين بالمقارنة مع السـيطرة.

وقد لوحظ ايضا حدوث ارتفاع ملموس في معدل هرمون FSH وLH وايضا عند كلا الجرعتين بالمقارنة مع الفئران المعاملة بالدارئ الملحي وفي الوقت نفسه لم يتم مشاهدة حدوث اي تلف فـي نسـيج مبـايض الاناث ولكن كان هنالك بعض التغيرات المرضية النسيجية والتي تمثلت بحدوث زيادة في عدد الجريبـات الاولية والثانوية والاجسام الصفراء.

Introduction

Recently there has been a renewed interest in improving health and fitness through the use of more natural products. Spices are an important part of the human diet which has been used to enhance the flavor, color and aroma of food. In addition to boosting flavor, spices are also known for their preservative and medicinal value [1].

Black pepper (*Piper nigrum, Piperaceae*) is derived from the fruit of a climbing vine native to southern India and Sri Lanka and it is one of the most widely used among spices. It is valued for its distinct biting quality attributed to the alkaloid "piperine" (a pungent alkaloid which represents the active principle of black pepper) [2]. Black pepper was shown to have certain medicinal properties (a): By increasing the bioavailability of other anti-tumourigenic spices, black pepper dramatically increases their potency and effectiveness against cancer. In addition to this important property, black pepper also counteracts cancer development directly. Its principal phytochemical, piperine, inhibits some of the pro-inflammatory cytokines that are produced by tumor cells. In so doing it interferes with the signaling mechanisms between cancer cells, thereby reducing the chances of tumor progression. Collectively, these properties make black pepper one of the most important spices for preventing cancer, (b): Black pepper contains several powerful antioxidants and is thus one of the most important spices for preventing and curtailing oxidative stress. In addition to their direct antioxidant properties, several of these compounds work indirectly by enhancing the action of other antioxidants. This makes black pepper particularly valuable in minimizing the damage caused by a diet rich in saturated fats, one of the main causes of oxidative stress, (c): Black pepper exhibits immunomodulatory properties and is capable of boosting the number and the efficacy of white cells, thereby assisting the body to mount a powerful defense against invading microbes and cancer cells, (d): Piperine increases the bioavailability of valuable phytochemicals present in other spices and can boost the activity of biochemically active compounds contained in green tea, curcumin and a variety of other spices by up to several hundred percent, depending on the molecule concerned. It does this via two principal mechanisms. First, it promotes the rapid absorption of certain chemicals from the gastrointestinal tract, protecting them from being broken down by chemicals in the intestinal lumen and by enzymes that occur in the cells lining the intestines. Secondly, once the compound has entered the blood stream, piperine provides protection against oxidative damage by liver enzymes. In this way black pepper enables us to reap optimum benefits from the medicinal phytochemicals found in other dietary spices [3, 4, and 5].

The immunoglobulins, also known as antibody-ies, are a family of proteins that exist in the plasma. The immunoglobulin family includes immunoglobulin A (IgA), immunoglo-bulin G (IgG), immunoglobulin M (IgM), immunoglobulin D (IgD) and immunoglobulin E (IgE). All of the immunoglobulins play a role in the immune system's defense mechanisms. The immune system manufactures the immunoglobulins in response to exposure to a foreign invader. After exposure to a foreign invader, such as a specific virus, bacteria, or toxin produced by an organism, a certain type of lymphocyte (a type of white blood cell) produces the immunoglobulins. Then the immunoglobulins level can be measured in the blood [6].

Complement analysis in the clinic is usually associated with the quantification of C3 and C4 and screening for complement activity together with complement activation products. These analyses have been available in routine diagnostic laboratories for decades. In recent years, however, the field of complement analysis has expanded considerably, with the introduction of novel assays to detect complement activation products, and spreading still further towards genetic analysis to reveal the basis of complement deficiencies and identify mutations and polymorphisms associated with some diseases [7].

Fertility hormones, FSH (Follicle Stimulating Hormone) is a hormone secreted by the pituitary gland in the brain. It is stimulates the follicles in the ovaries to ripen several eggs. FSH also readies the mammary glands for milk production. In men, the FSH initiates sperm production. While LH (Lutenising Hormone) is secreted by the pituitary gland to stimulate ovulation that is, the release of the egg or ovum from the follicles. LH secretion signals the remnants of the follicle to change into the corpus luteum. The corpus luteum then begins producing progesterone and estrogens [8].

The present study aims to open up a new approach in the development of natural drugs with immunomodulatory effect at the *in vivo* level with less toxicity by studying the oral acute toxic effect of black pepper on mouse bone marrow cells and fertility to make sure if it is safe or not.

Materials and Methods

All the chemicals were obtained from Sigma Chemical Co. (USA) and BDH (England).

Experimental Animals: Three groups of female albino mice, which were obtained from the Biotechnology Research Center/AL-Nahrain University, were used in this study. Their ages were ranged between 8-12 weeks and weighting 25-30 gm. They were divided into subgroups, and each group was putted in a separate plastic cage. The cages were kept in a room with 23-25 Cº temperature. The animals were fed with a suitable quantity of water and complete diet.

Administration of Experimental Animals: The animals in this experiment were treated with a cumulative dose of black pepper for 14 days. The main aim of this experiment was to evaluate the acute treatment effect of black pepper in normal mice. The mice were divided into three experimental groups. Each group consisted of 5 mice and to which the black pepper was administered orally.

Group I: Negative control, treated with 0.1ml of normal saline.

Group II: black pepper treatment, treated with 0.1 ml of 1mg /Kg

Group III: black pepper treatment, treated with 0.1 ml of 5mg /Kg.

The animals were monitored for apparent signs of toxicity for 14 days. Then they were sacrificed on the 15th day after administration and the blood was separated to measure the levels of IgG, IgM, IgA, C3 C4 and FSH, LH fertility hormones. After that the ovaries were collected and fixed in 10% buffered formaldehyde solution.

Preparation of water extract: water extraction of black pepper was prepared by boiling 100 gm in 1000 ml sterile distilled water over low flame for 15 minutes. The flask was then plugged and removed from the heat and allowed to cool. After cooling the content of the flask was filtered and dried to prepare the required concentrations [9].

Chromosomal preparation from somatic cells of the mouse bone marrow: All the chemicals were obtained from Sigma Chemical Co. (USA) and BDH (England). This experiment was done according to [10]. Each animal was injected with 0.25ml of colchicine with a concentration of 1mg/ml intraperitoneally (I.P) 2hr before sacrificing the animal, Then the animal was sacrificed by cervical dislocation and fixed on its ventral side on the anatomy plate and the abdominal side of the animal and its thigh region were swabbed with 70% ethanol. The femur bone was then taken and cleaned from the other tissues and muscles and gabbed from the middle with a forceps in a vertical position over the edge of the test tube, and by sterile syringe 5ml of PBS were injected so as to wash and drop the bone marrow in the test tube. The test tube was taken and centrifuged at speed of 2000 rpm for 10min. After that the supernatant was removed and 5ml of potassium chloride 0.075 M was added as a hypotonic solution, then the test tubes were left for 30min in the water bath at 37C º and shaked from time to time. The tubes were then centrifuged at 2000 rpm for 10min and the supernatant was removed and the fixative solution was added (as drops) on the inside wall of the test tube with the continuous shaking, the volume was fixed to 5ml and the content shaked well. The tube was kept at 4Cº for 30min to fix the cells. After that the tubes were centrifuged at 2000 rpm for 10min. The process was repeated three times and the cells were suspended in 2ml of the fixative solution. By a pasture pipette, few drops from the tube were dropped vertically on two chilled slides from a height of 3 feet at a rate of 4-5 drops to give the chance for the chromosomes to spread well. Later the slides were kept to dry at room temperature, and then stained with Giemsa stain and left for 15min and washed with distilled

water. the mitotic activity is expressed by the MI which is the number of dividing cells in 100 cell.

Assay measurements of hormones: Serum hormones (FSH, LH) concentrations were evaluated with a Bio merieux Italia S.P. a vidia campigliano, 58 50015-point A EMA (F_1) Italia miniVIDAS, following the manufacturer's recommendations.

Assay measurements of immunoglobulins and complements: serum levels of IgG, IgM, IgA, C3, and C4 were evaluated by using radial immunodiffusion plate, following the kit manufacturer's method.

Histological Examinations: This was performed by using method of [11]. At the time of death, mouse organs ovaries were taken for histopathological examination. The perfuse-fixed ovaries placed in Bouin fluid overnight, and processed for routine paraffin embedding. The ovaries were cut into 5-µm sections. Three serial sections per ovaries were mounted on slides, deparaffinized, rehydrated, and stained with hematoxyline - eosin stain. Sections of the ovaries were examined by light microscopy; primary and secondary follicles and corpus luteum diameters were assessed in each ovary using a previously calibrated micrometer eyepiece.

Statistical Evaluation: Data were analyzed by 1-way analysis of variance with ANOVA- test. Data are presented as means ± SE. The level of significance was $P < .05$. [12].

Results and Discussion

After the mice were orally given two doses 1, 5mg/kg of the water extract from the dried fruits of *P. nigrum*, neither signs of toxicity nor death of mice were observed during the 14 days of the acute toxicity experimental period, similar results were also obtained by studying much higher dose on rats in which the pepper did not cause toxicity neither at the acute or the subchronic toxicity study [13]. Significant difference in mitotic percentage between treated and untreated (control) animals were occurring as shown in Table 1. A clear effect in increasing mitotic activity was reveled for both concentrations (59.33, 66.66)% respectively in comparison with control 51.16%. The black pepper, *Piper nigrum* L. (Piperaceae) has traditionally been used as both spice and medicine. It contains small quantities of chemopreventive compounds such as B-carotene, piperine, tannic acid and capsaicin. The antimutagenic activity of black pepper could be related to the large number of theses potent chemopreventive compounds and especially piperine which were shown to be a promising antimutagenic compound [14], piperine demonstrates not only chemopreventive effect, but also genoprotective effects against B(a)-p induced mutagenesis in Swiss albino mice. Oral supplementation with piperine can reduce DNA damage and DNA protein cross-links by enhancing phase II enzymes. It can induce apoptosis in B(a) p-induced lung carcinogenesis in Swiss albino mice [15]. However, similar results were also obtained in which black pepper were given in combination with genotoxic agents and it was shown to be so effective in reducing the mutational events induced by these agents either by suppression of metabolic activation or interaction with the active groups of mutagens and this suggest to be the mechanism by which the pepper exert its antimagnetic property [16].

Table 1: Cytogenetic effects of *Piper nigrum* in comparison with control (normal saline) on mouse bone marrow cell.

Groups	Mitotic index (mean±SE)
Control	A 51.16 ± 3.76
Black pepper 1mg	B 59.33 ± 2.51
Black pepper 5mg	C 66.66 ± 2.51

Differences A, B, C are significant (P<0.05) to compression rows.

To determine the immunomodulatory effect in mice treated with the extract, immunological parameters were examined as presented in Tables 2. The concentration of immunoglobu-lins and complements (IgG, IgM, IgA, C3, C4) in the treated mice with 1 and 5mg/kg of the extract was higher than that of the control group. Results show a significant raise in the levels of them 1092, 144.8, 226.7, 243.63, 343.6 and 1130, 156.9, 231.0, 258.70, 354.8 mg/dl respectively at both doses in comparison with control 1020, 130.6, 213.62, 224.32, 320.9 mg/dl.

These results were came in agreement with [17] in which the researchers show that both black pepper extract or piperine alone have immuno-modulatory activity at doses of 10 and 1.4 mg/kg body weight of the animal respectively

on the 5th day of immunization. The pepper were seen to increase the alpha-esterase positive cells and bone marrow cellularity and this in turn lead to increase in the level of immunoglobulins and complements in additions to other types of immune system cells. Thus the black pepper had no immunotoxic effect and may be considered as immunologically safe compound.

Table 2: Immunomodulatory activity of *Piper nigrum* in comparison with control (normal saline) in mice.

Groups	Immunoglobulin mg/dl (mean±SE)				
	IgG	IgM	IgA	C3	C4
Control	A 1020 ± 84.61	A 130.6 ± 26.9	A 213.62 ± 17.51	A 224.32 ± 208	A 320.9 ± 40.2
Black pepper 1mg	B 1092 ± 93.42	B 144.8 ± 30.04	B 226.7 ± 20.3	B 243.63 ± 20.93	B 343.6 ± 38.2
Black pepper 5mg	C 1130 ± 89.45	C 156.9 ± 27.62	B 231.0 ± 21.60	CD 258.70 ± 19.48	C 354.8 ± 40.6

Differences A, B, C, D are significant (P<0.05) to compression rows.

However, with fertility aspect, black pepper shows promising results. A significant raise in the levels of fertility hormones (FSH and LH) was seen after treatment with the two doses of the extract when compared with control treated mice as shown in Table 3. The levels of FSH were 1.93, 2.04 mIu/m while LH 2.23, 2.41 mIu/m at the two doses respectively when compared with normal saline treated mice (1.46, 1.32) mIu/m.

Table 3: Fertility activity of *Piper nigrum* in comparison with control (normal saline) in mice.

Groups	FSH mIu/m (mean±SE)	LH mIu/m (mean±SE)
Control	A 1.46±0.06	A 1.32±0.63
Black pepper 1mg	B 1.93±0.04	B 2.23±0.30
Black pepper 5mg	B 2.04±0.46	C 2.41±0.36

Differences A, B, C are significant (P<0.05) to compression rows.

Moreover, these observations were further investigated by the histopathological assessment of the female ovaries tissues. The results showed that the water extract of *P. nigrum* did not produce a significant damage in these tissues, however, significant changes were seen in the number of primary and secondary follicles and corpus luteum in the pepper treated mice when compared with the control treated mice as shown in table 4. After oral administration of the two extract doses the number of the primary follicles was 5.07, 5.52 and secondary follicles 7.21, 7.43 respectively, while control was 4.36, 6.43. On the other hand, the number of corpus luteum was 5.21, 5.43 at both doses respectively, while normal saline treated mice showed 4.40.

Table 4: Effect of *Piper nigrum* on primary and secondary follicles and corpus luteum in comparison with control (normal saline) in mice.

Groups	No. of primary follicles (m±SE)	No. of secondary follicles (m±SE)	No. of corpus luteum (m±SE)
Control	A 4.36±0.82	A 6.43±1.22	A 4.40±0.86
Black pepper 1mg	B 5.07±1.32	B 7.21±2.03	B 5.21±0.93
Black pepper 5mg	C 5.52±0.93	B 7.43±1.82	B 5.43±0.82

Differences A, B, C are significant (P<0.05) to compression rows.

Black pepper was shown to have powerful antioxidant [18] and radical scavenging activity [19], and since many studies show a strong relation between antioxidants and fertility inductions [20, 21, 22] and between increased free radicals and reduced fertility [23]. These studies showed that the imbalance between antioxidant defense and free radical activity is more evident in the infertility condition, thus pepper extract shows significant effect in increasing fertility in mice.

In addition to that, black pepper is rich in vitamin E and also many researches showed a significant relation between this vitamin and fertility [24, 25], and thus this is considered to be another way or mechanism by which black pepper exert it is potent effect on fertility and reproduction.

References

1. deSouza E. L.; Stamford T. L.; Lima E. O. and Trajano V. N. **2005**. Antimicrobial effectiveness of spice: an approach for use in food conservation system. *Braz. Arch. Bio. Technol.*, **48**(4): 1516-1519.
2. Srinivasan K. **2007**. Black pepper and its pungent principle-piperine:A review of diverse physiological effects. *Criti. Rev. Food Sci. Nutrit.*, **47**(8): 735-748.

3. Web site: **2005** Black Pepper- Antioxidant, Anti - Cancer Super spice. http : / / www. ezinear -ticles.com
4. Khajuria A; Thus N. and Zutshi U. **2002.** Piperine modulates permeability characteristics of intestine by inducing alterations in membrane dynamics. *Phytomedicine*, **9**(3):224-231.
5. Platel K.; Rao A.; Saraswathi G. and Srinivasan K. **2003**. Digestive stimulation effect of three India spices mixes in experimental rats. *Nahrung*, **46**:394-398.
6. Von König C. H., Finger H. and Ormaycht D. H. **1979**. Immunoglobulins and other plasma proteins in late pregnancy (author's transl). *Immun Infect*.; **7**(3): 89-92.
7. Tom E. M.; Sakari J.; Lennart T. and Bo N. **2007**. Complement analysis in the 21st century. *Molec*. *Immun*., **44**(16): 3838-3849.
8. Web site: **2005** What are FSH and LH Pituitary Hormones. www.WikiAnswer. Com.
9. Nazia M. A. and Perween T. **2006**. Bacterial activity of black pepper, bay leaf, aniseed and coriander against oral isolates. *Pak. J. Pharm. Sci.*, **19**(3):214-218.
10. Allen, J. W.; Shuler, C. F.; Memnders, R. W. and Olatt, S. A. **1976**. A simplified technique for in vivo analysis of sister chromatid exchange using 5-bromodeoxyuridine tablets. *Cytogen. Cell Genet,* **18**:231-237.
11. Bancroft, J. D. and Stevens, A. S. **1992**. *Theory and Practice of Histological Techniques.* 2nd (ed.). Churchill Living Stone, Edinburgh, London. Pp:233-50
12. Al-Mohammed N. T.; Al-Rawi K. M.; Younis M. A. and Al-Morani W. K. **1986**. Principle of Statistics. *J. Al-Mousl University.* **2**: Pp:33-67
13. Siharat C.; Nirush L.; Umarat S.; Anongnad N.; Nadthaganya S. and Kanjana J. **2007**. Acute and subchronic toxicity study of the water extract from dried fruits of Piper nigrum L. in rats. *Songklanakarin J. Sci. Technol.*, **29**(1):109-124.
14. Selvendiran, K., Prince Vijeya Singh, J., Babakrishnan, K. and Sakthisekaran, D. **2003**. Cytoprotective effect of piperine against B (a)p induced lung cancer with reference to antioxidant systems in Swiss albino mice. *Fitoterapia*, **74**:109-115
15. Selvendiran, K., Mumtaz Banu, S. and Sakthisekaran, D. **2005**. Oral supplementation of piperine altered phase II enzymes and reduced DNA damage and DNA-protein cross links in B(a)p induced experimental lung carcinogenesis. Mol. *Cell Biochem*., **268**:141-147.
16. Hamss, R. E.; Idaomar, M.; Alonso, A. and Serrano, A. **2003**. Antimutagenic properties of bell and black pepper. *Food and Clinical Toxicology*, **41**(1):41-47.
17. Sunila, E. S. and Kuttan, G. **2004**. Immunomodulatory and antitumor activity of *Piper nigrum* Linn. and piperine. *J. Erthnopharmacology,* **90**(2):339-346.
18. Mittal, R. and Gupta, R. L. **2000**. *In vitro* antioxidants activity of piperine. *Meth. Clin. Pharmac*., **122**:163-167.
19. Gulcin, I. **2005**. The antioxidant and radical scavenging activities of black pepper (Piper nigrum) seeds. *Int. J. Food Science and Nutrition,* **56**(7):491-499.
20. Ruder, E. H; Hartman, T. J. and Goldman, M. B. **2009**. Impact of oxidative stress on female fertility. C*urrent Opinion in Obstetrics and Gynecology*., **21**(3): 219-222.
21. Vural, P.; Akgül, C.; Yildirim, A. and Canbaz, M. **2000**. Antioxidant defense in recurrent abortion. *Clin Chim Acta*.; **295**(1-2):169-77.
22. Elizabeth H. R.; Terryl J. H.; Jeffrey B. and Marlene B. G. **2008**. Oxidativ**e** str**e**ss and antioxidants **e**xposur**e** and impact on female fertility**.** *Fertility*, **15** (4): 2893–2903.
23. B**e**hrman HR, Kodaman PH, Pr**e**ston SL, and Gao S. **2001.** Oxidativ**e** str**e**ss and th**e** ovary. *J Soc Gyn**e**col Inv**e**stig.*, **8**(1): 40–42.
24. Zhou**,** X. Q. **2008**. Effect of vitamin E on reproductive function in the mice treated with 2, 3, 7, 8- tetrachlorodibenzo - p - dioxin. *Toxicology and Industrial Health*, **24**(9): 595-601.
25. Web site: **2006** Vitamins and Minerals for Fertility. http://www.thefertilityshop.com