Evaluation of the protective action of officinarum extract against bisphenol A-induced oxidative stress and DNA damage in male albino rats

Mahdi Hamzah Khashan
School of Al-Qasim intermediate, Educational directorate of Babylon, Iraq, E-mail: mehdialqasimi@gmail.com

Rasha Abdulameer Jawad
Biology Department, College of Education for pure Sciences, Karbala University, Karbala, Iraq.

Nasser Merza Hamza
Biology Department, College of Education for pure Sciences, Karbala University, Karbala, Iraq.

Abstract

Objective: Antioxidants that reduce oxidative stress, lipid peroxidation, and DNA damage, restoring the global antioxidant defense system, can be used to treat male infertility and poor semen quality associated with bisphenol A (BPA) exposure. this work, aimed to investigate the protective action of Alpinia officinarum (A. officinarum) rhizome extracts against oxidative stress and DNA damages induced by BPA.

Material and methods: Eighteen adult albino rats were used and divided into three groups; control group received 0.2mL of olive oil/rat/day, BPA group received BPA (50 mg/kg) and protected group received A. officinarum extract (400 mg/kg) then BPA (50 mg/kg) after one hour. The various doses were administrated orally for 60 days. 24 hours after the last dose, the animals were weighed and sacrificed, blood samples were collected for the assessment of oxidant/antioxidant markers, while testes and epididymis were weighed after extraction and cleaning, and epididymis sperm were used to estimate the degree of DNA damage.

Results: The weighted values of the testes and epididymis showed a significant decrease in the BPA group compared to the control group, while the decrease of those values was relatively low in the protected group, as it moved away from the BPA group and approached the control group. The data also showed a significant decline from the normal state in the indicators of oxidation/antioxidants due to the treatment with BPA , while that decline receded to a large degree in the group protected with A. officinarum extract. The results also included a significant increase in the number of sperms that suffered from DNA fragmentation and the amount of that damage in the BPA-treated group, but that damage was much less than in the protected group.

Conclusion: We conclude that A. officinarum extract is effective in reducing the harmful effects of increased oxidative stress and DNA fragmentation that result from exposure to BPA through its anti-free radical formation and scavenging activity.

Keywords: *Bisphenol A, Alpinia officinarum, Oxidative stress.*

INTRODUCTION

An endocrine disruptor is defined by the World Health Organization as "an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations".[1] bisphenol A (BPA) represents one of those environmental chemical pollutants that mimic the natural oestrogen 17-β-oestradiol (E2).[2] BPA is a crystalline chemical compound widely used as key monomer of epoxy resins and polycarbonate plastics for more than 50 years.[3] In the last decades, results of preclinical research revealed endocrine-disrupting effects of BPA on male reproductive functions, clarifying possible mechanisms by which BPA can interfere with the regulation of spermatogenesis mainly throughout the hypothalamic–pituitary–gonadal axis.[4] In rodent models, the in vivo exposure to BPA at different doses and time intervals resulted in a significant decrease in sperm counts,[5] sperm motility and normal sperm morphology,[6] increase in sperm DNA damage,[7] and poor spermatogenesis.[5,8] Independently of its hormonal disrupting effects, BPA could interfere with spermatogenesis processes even through other mechanisms. After in vivo exposure to BPA, an impaired testicular glucose homeostasis has been reported in the rat.[9] Also, an increased testicular oxidative stress has been revealed both in the rat and in the mouse.[10] BPA can also induce apoptosis in cultured Sertoli cells from rodents[11] by inducing dysfunction of mitochondria and generation of reactive oxygen species (ROS).[12] Although BPA is not an oxidizer itself, it leads to cellular changes usually manifested by lipid peroxidation (LPO) and free radicals production causing oxidative stress (OS).[13] Testicular structural damage and dysfunction are often associated with increased OS [14] In addition, increased ROS levels can induce errors during DNA replication, transcription or post-transcriptional events , resulting in sperm DNA fragmentation , chromatin condensation abnormalities, and protamine expression defects.[15]

Alpinia officinarum Hance (Lesser Galangal) belongs to the Zingiberaceae family and is one of the most popular Chinese herbal medicines.[16] Diarylheptanoids, flavonoids, and essential oils are the three major classes of bioactive compounds found in the rhizomes of A. officinarum.[17] A. officinarum is considered one of the most beneficial crude medicines in traditional Chinese medicine. A number of pharmacological studies have indicated that A. officinarum has antibacterial, antiviral, antitumor, and antioxidant properties, as well as gastrointestinal bleeding-inhibiting properties.The mechanism is likely related to its antioxidant properties (removal of oxygen free radical activity).[18] Fang et al.,[19] stated that galangin, kaempferide, and quercetin are the main constituents of this plant that possess antioxidant activity, and thus can attenuate oxidative stress and reproductive toxicity induced by BPA.

MATERIALS AND METHODS

Animals

Male adult albino rats weighing 200-220 grams, which were produced in the domestic animal laboratory in the College of Education, Karbala University for Pure Sciences, were used. These animals were kept under typical laboratory conditions with a 12-hour light/dark cycle and water available as desired. They were also fed a regular meal.

Collection of Plant and Preparation of Extract

A plant taxonomist from Karbala University, College of Education for pure Sciences

identified Alpinia officinarum rhizomes they were bought at the community market. After cleaning them with water from suspended impurities and then air drying, they were soaked in 70% ethyl alcohol for three days at ambient temperature. ALP extract and solvent were combined in a ratio of 1 to 5 to produce A. officinarum extract. After filtering using a Whitman filter, the extract was concentrated until dry using a rotatory evaporator set at 45°C. 12.5% of the dry extract was produced.[20]

Experimental Design

A total of 18 rats were equally divided into three groups and given the following treatment orally using an intragastric tube for the period of 60 days.

Group I: (control) received olive oil (0.2 ml/animal/day).

Group II: (BPA group) received BPA (50 mg/kg/day) dissolved in 0.2 ml of olive oil.

Group III: (Protected group) received A. officinarum extract (400 mg/kg/day) then BPA (50 mg/kg/day) after one hour.

Sample Collection

After 24 h of last dose, the animals were weighed and sacrificed. Heart blood samples were collected for biochemical tests. Reproductive organs (testis and epididymis) were immediately dissected out and separately weighed then used to estimate DNA damage.

Oxidant/Antioxidant biomarkers study.

Blood samples were used to estimate Oxidant and Antioxidant parameters. Serum malondialdehyde (MDA) levels were measured using colorimetric assay.[21] Activity of catalase (CAT) was measured according to method described by Hadwan and kadhum.[22] Finally, the procedure to estimate the glutathione (GSH) level followed the method described by Griffith.[23]

Estimation of DNA damage

The cauda of each epididymis was cut and minced separately in 3 ml of normal saline and filtered through nylon mesh for collection of spermatozoa. Sperm DNA damage was assessed by the single cell gel/comet assay according to method described by Almabhouh et al. [24] using a Comet Assay kit (Cell Biolabs, Inc., USA).

Statistical analysis

The results of the data analysis were presented as mean ± standard error (SE) using IBM SPSS software (version 26, Chicago, IL, USA) . The one-way ANOVA and post hoc LSD tests were used to analyze group differences. The threshold for statistical significance was fixed at $P<0.05$.

Results

Effect of BPA and hydroalcoholic extract of A. officinarum on the weights.

As in table 1, there was no significant difference in body weight gain between all experiment groups. On the other hand, the testis and epididymis weights were significantly decrease in BPA group as compared to control group, however they were significantly increase ($p< 0.05$) in protected group comparing with BPA group.

Table 1: Effect of BPA and hydroalcoholic extract of A. officinarum on the weights.

Groups	body weight gain (g)	Testis weight (mg)	Epididymis weight (mg)
Control g.	74.83±2.04[a]	1462.17± 4.98[a]	470.66± 3.05[a]
BPA g.	72.33±1.54[a]	1191.83±23.70[b]	372.33±8.87[b]

Protected g.	76.17±1.92[a]	1440.67±6.63[a]	468.33±3.09[a]

Values were expressed as Means ± SE. Values at the same column with different letters are significant at P<0.05

Effect of BPA and hydroalcoholic extract of A. officinarum on oxidant/antioxidant biomarkers.

As in table 2, malondialdehide levels were significantly increased (p< 0.05) in BPA group as compared to control group. At the same time, CAT activity and GSH levels were significantly lowered (p< 0.05) in BPA group as compared to control group. On the other hand, protected group showed significant decrease (p< 0.05) of MDA levels and significant increase (p< 0.05) of CAT activity and GSH levels comparing with BPA group.

Table 2: Effect of BPA and hydroalcoholic extract of A. officinarum on oxidant/antioxidant parameters.

Groups	MDA level (µmol/l)	CAT activity (kU/l)	GSH levels (µmol/l)
Control g.	2.41±0.05[c]	104.16±3.31[a]	72.66±2.20[a]
BPA g.	5.13±0.09[a]	64.66±3.27[c]	39.16±1.90[b]
Protected g.	3.01±0.08[b]	91.83±3.24[b]	65.50±2.65[a]

Values were expressed as Means ± SE. Values at the same column with different letters are significant at P<0.05.

Effect of BPA and hydroalcoholic extract of A. officinarum on DNA damage of spermatozoa.

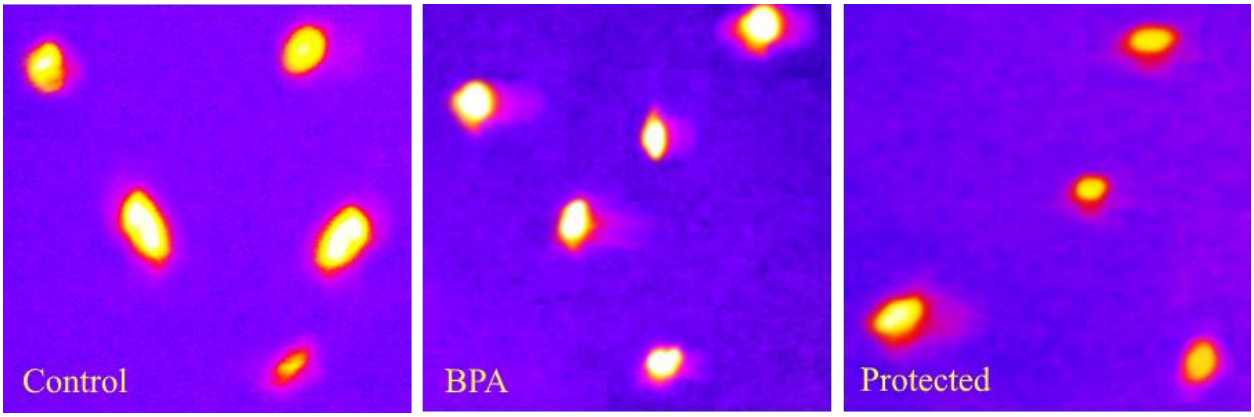
As in table 3, the number of comets/100 spermatozoa and percent of DNA in tail were significantly increased (p< 0.05) in BPA group as compared to control group. However, these changes were significantly decreased in protected group as compared to BPA group.

Table 3: Effect of BPA and hydroalcoholic extract of A. officinarum on DNA damage of spermatozoa.

Parameters	Control g.	BPA g.	Protected g.
Number of comets/100 spermatozoa	16±0.73[c]	28±1.26[a]	21±1.06[b]
% DNA in Tail	2.32±0.21[c]	8.47±0.52[a]	5.63±0.35[b]

Values were expressed as Means ± SE. Values at the same column with different letters are significant at P<0.05

Figure 1: Photomicrograph from alkaline comet assays showing types of sperm DNA damage as observed of experimental animals.



DISCUSSION

BPA is a well-known environmental toxin that has the ability to cause oestrogenic and anti-androgenic responses in both humans and animals.[25] Qiu et al.[26] showed that exposure to BPA caused higher oxidative stress and DNA damage in rats and common carp, respectively. The most widely available sources of materials that have the capacity to scavenge free radical ions are plants and their bioactive components. [27] This study examined the potential effects of an alcoholic extract of Alpinia officinarum rhizomes against BPA-induced oxidative stress and sperm DNA damage in adult male rats. No significant changes in body weight growth were seen between any of the treated groups and the control group, according to our findings. These results were in agreement with the study carried out by Ullah et al.[28] According to Wu et al. [29], BPA disrupts the endocrine system without affecting body weight. In the same context, the average weights of the testes and epididymis were significantly lower in the BPA-treated group than in the control group. This may be because that the BPA compound may be causing an endocrine problem, which lowers sex hormone levels. Also Weight loss could result from the breakdown of some significant molecules, including the proteins in the testicles.[30] These outcomes were consistent with prior research' findings that exposure to bisphenol chemicals decreased testicular and epididymis weights.[30,31] The protected group showed an improved effect of the extract in reducing weight loss, as the results reported a significant increase in the weights of testes and epididymis between that group and the BPA-treated group.

The oxidation of polyunsaturated fatty acids (PUFA) and their esters results in MDA production.[32] hence, it is frequently utilized as a biomarker of lipid oxidation caused by oxidative stress.[33] The presence of unsaturated fatty acids in the sperms' membranes makes them more vulnerable to lipid peroxidation.[34] In our study, MDA serum levels significantly increased in the BPA group, most likely as a result of oxidative damage brought on by BPA. These results agree with those of earlier research in the same field.[30] Negm and Ragheb[35] showed that A. officinarum reduced serum MDA and elevated serum SOD, indicating protection against lipid peroxidation and alleviated oxidative stress. These results can be linked to the antioxidant characteristics of A. officinarum rhizome since it contains components such polyphenols, flavonoids, and diarylheptanoid substances that scavenge reactive oxygen species.

On the other hand, CAT and other enzymatic antioxidants guard the biological system from ROS's harmful effects and lessen the oxidative damage they cause to testicular cell membranes.[36] Lipids, proteins, and DNA can quickly oxidatively deteriorate when exposed to H2O2. Reduced CAT activity reduces the testicles' capacity to expel H2O2 created as a result of BPA exposure.[37] At the same time, GSH is crucial for cellular processes like the breakdown of H2O2, lipid peroxidation, and amino acid translocation in the cell membrane.[38] When compared to control group animals, our findings clearly demonstrated that BPA exposure reduced CAT activity and GSH levels. These findings corroborated those of Kamel et al.[39], who found that exposure to BPA raises LPO levels while also altering CAT and GSH levels, both of which point to oxidative stress. On the other hand, the results of our study indicated that A. officinarum extract provides high protection against oxidative damage, as a significant increase was observed in the CAT activity and GSH levels in the protected group compared to the group treated with BPA. Many studies in this field showed the effectiveness of extracts of this plant against oxidative stress[29,35] Reid et al.,[40] stated that by encouraging the expression of antioxidative proteins, flavonoids compounds isolated from A. Officinarum contributed to the protection against oxidative stress.

By identifying broken DNA strands in diverse cell types, the comet test is a well-known and effective way to detect sperm DNA damage.[41] There are various types of DNA strand breaks that comet-assay parameters may reflect, with tail DNA% reflecting single-strand breaks and tail moment reflecting double DNA breaks, which are more mutagenic than single breaks.[42] Errors in chromatin handling and isolation,[43] hormonal deficiencies, aberrant cell apoptosis, and oxidative stress[44,45] could harm sperm DNA. Although moderate levels of ROS activity are thought to be necessary for healthy sperm function, greater levels may be harmful and may cause DNA breakage and apoptosis in spermatozoa and seminiferous tubules.[46]

Comet assay analysis in our study revealed a significant DNA fragmentation of spermatozoa in BPA group (Table 3; Fig. 1C). This may be due to the increased oxidative stress and high levels of ROS. These results can be supported by many previous in vitro and in vivo studies that used different doses and time periods.[47] These changes appeared to be significantly reduced in the group protected with Alpinia extract (Table 3; Figure 1D) which may be due to the antioxidant efficacy of this extract.

CONCLUSION

We deduce from the experimental results the effectiveness of the alcoholic extract of the rhizomes of Alpinia officinarum and through its antioxidant activity in reducing all the detrimental changes that resulted from exposure to BPA (50 mg/kg), which caused a significant decrease in the weights of the testes and epididymis, an increase in oxidative processes, a weakening of Antioxidant enzymes' effectiveness, and DNA fragmentation in a significant number of spermatozoa.

Reference

European Commission. Community Strategy for Endocrine Disrupters—A Range of Substances Suspected of Interfering with the HormoneSystems of Humans and Wildlife; Commission of the European Communities: Brussels, Belgium, 1999.

Calafat, A. M., Ye, X., Wong, L. Y., Reidy, J. A., & Needham, L. L. (2008). Exposure of the US population to bisphenol A and 4-tertiary-octylphenol: 2003–2004. Environmental health perspectives, 116(1), 39-44.

Tomza‐Marciniak, A., Stępkowska, P., Kuba, J., & Pilarczyk, B. (2018). Effect of bisphenol A on reproductive processes: A review of in vitro, in vivo and epidemiological studies. Journal of Applied Toxicology, 38(1), 51-80.

Castellini, C., Totaro, M., Parisi, A., D'Andrea, S., Lucente, L., Cordeschi, G., ... & Barbonetti, A. (2020). Bisphenol A and male fertility: Myths and realities. Frontiers in endocrinology, 353.

Grami, D., Rtibi, K., Hammami, I., Selmi, S., De Toni, L., Foresta, C., ... & Sebai, H. (2020). Protective action of eruca sativa leaves aqueous extracts against bisphenol a-caused in vivo testicular damages. Journal of medicinal food, 23(6), 600-610.

Grami, D., Rtibi, K., Hammami, I., Selmi, S., De Toni, L., Foresta, C., ... & Sebai, H. (2020). Protective action of eruca sativa leaves aqueous extracts against bisphenol a-caused in vivo testicular damages. Journal of medicinal food, 23(6), 600-610.

Dobrzyńska, M. M., & Radzikowska, J. (2013). Genotoxicity and reproductive toxicity of bisphenol A and X-ray/bisphenol A combination in male mice. Drug and chemical toxicology, 36(1), 19-26.

Kazemi, S., Bahramifar, N., Moghadamnia, A. A., & Jorsarae, S. G. A. (2016). Detection of bisphenol A and nonylphenol in rat's blood serum, tissue and impact on reproductive system. Electronic Physician, 8(8), 2772.

D'Cruz, S. C., Jubendradass, R., Jayakanthan, M., Rani, S. J. A., & Mathur, P. P. (2012). Bisphenol A impairs insulin signaling and glucose homeostasis and decreases steroidogenesis in rat testis: an in vivo and in silico study. Food and chemical toxicology, 50(3-4), 1124-1133.

Anjum, S., Rahman, S., Kaur, M., Ahmad, F., Rashid, H., Ansari, R. A., & Raisuddin, S. (2011). Melatonin ameliorates bisphenol A-induced biochemical toxicity in testicular mitochondria of mouse. Food and chemical Toxicology, 49(11), 2849-2854.

Iida, H., Maehara, K., Doiguchi, M., Mōri, T., & Yamada, F. (2003). Bisphenol A-induced apoptosis of cultured rat Sertoli cells. Reproductive Toxicology, 17(4), 457-464.

Wang, C., Qi, S., Liu, C., Yang, A., Fu, W., Quan, C., ... & Yang, K. (2017). Mitochondrial dysfunction and Ca2+ overload in injured sertoli cells exposed to bisphenol A. Environmental toxicology, 32(3), 823-831.

D'Cruz, S. C., Jubendradass, R., & Mathur, P. P. (2012). Bisphenol A induces oxidative stress and decreases levels of insulin receptor substrate 2 and glucose transporter 8 in rat testis. Reproductive Sciences, 19, 163-172.

Lamirande, E. D., & Gagnon, C. (1993). A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. International Journal of Andrology, 16(1), 21-25.

Paoli, D., Pecora, G., Pallotti, F., Faja, F., Pelloni, M., Lenzi, A., & Lombardo, F. (2019). Cytological and molecular aspects of the ageing sperm. Human Reproduction, 34(2), 218-227.

Pharmacopoeia Committee of P. R. China (2010) Pharmacopoeia of People's Republic of China. Chemical Industry Publishers, Beijing.

Nanjing University of Chinese Medicine(2006) Dictionary of Chinese Materia Medica, 2nd edn. Shanghai Science and Technology Press.

Beattie et al (2011) Ginger phytochemicals mitigate the obesogenic effects of a high-fat diet in mice: a proteomic and biomarker network analysis. MolNutr Food Res 55:203–213.

Fang, L., Zhang, H., Zhou, J., Geng, Y., & Wang, X. (2018). Rapid screening and preparative isolation of antioxidants from Alpinia Officinarum Hance using HSCCC coupled with DPPH-HPLC assay and evaluation of their antioxidant activities. Journal of Analytical Methods in Chemistry, 2018.

Kolangi, F., Shafi, H., Memariani, Z., Kamalinejad, M., Bioos, S., Jorsaraei, S. G. A., ... & Mozaffarpur, S. A. (2019). Effect of Alpinia officinarum Hance rhizome extract on spermatogram factors in men with idiopathic infertility: A prospective double‐blinded randomised clinical trial. Andrologia, 51(1), e13172..

Shakeri, F., Soukhtanloo, M., & Boskabady, M. H. (2017). The effect of hydro-ethanolic extract of Curcuma longa rhizome and curcumin on total and differential WBC and serum oxidant, antioxidant biomarkers in rat model of asthma. Iranian Journal of Basic Medical Sciences, 20(2), 155.

Hadwan, M. H., & kadhum Ali, S. (2018). New spectrophotometric assay for assessments of catalase activity in biological samples. Analytical biochemistry, 542, 29-33.

Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Analytical biochemistry, 106(1), 207-212.

Almabhouh, F. A., Osman, K., Siti Fatimah, I., Sergey, G., Gnanou, J., & Singh, H. J. (2015). Effects of leptin on sperm count and morphology in Sprague‐Dawley rats and their reversibility following a 6‐week recovery period. Andrologia, 47(7), 751-758.

Urriola-Muñoz, P., Lagos-Cabré, R., & Moreno, R. D. (2014). A mechanism of male germ cell apoptosis induced by bisphenol-A and nonylphenol involving ADAM17 and p38 MAPK activation. PLoS One, 9(12), e113793.

Qiu, W., Chen, J., Li, Y., Chen, Z., Jiang, L., Yang, M., & Wu, M. (2016). Oxidative stress and immune disturbance after long-term exposure to bisphenol A in juvenile common carp (Cyprinus carpio). Ecotoxicology and environmental safety, 130, 93-102.

Agarwal, A., Gupta, S., & Sikka, S. (2006). The role of free radicals and antioxidants in reproduction. Current opinion in obstetrics and gynecology, 18(3), 325-332.

Ullah, A., Pirzada, M., Afsar, T., Razak, S., Almajwal, A., & Jahan, S. (2019). Effect of bisphenol F, an analog of bisphenol A, on the reproductive functions of male rats. Environmental health and preventive medicine, 24, 1-11.

Wu, M., Xu, H., Shen, Y., Qiu, W., & Yang, M. (2011). Oxidative stress in zebrafish embryos induced by short‐term exposure to bisphenol A, nonylphenol, and their mixture. Environmental toxicology and chemistry, 30(10), 2335-2341.

Alboghobeish, S., Mahdavinia, M., Zeidooni, L., Samimi, A., Oroojan, A. A., Alizadeh, S., ... & Khorsandi, L. (2019). Efficiency of naringin against reproductive toxicity and testicular damages induced by

bisphenol A in rats. Iranian journal of basic medical sciences, 22(3), 315.

Munir, B., Qadir, A., & Tahir, M. (2017). Negative effects of bisphenol A on testicular functions in albino rats and their abolitions with Tribulus terristeris L. Brazilian journal of pharmaceutical Sciences, 53.

Chauhan, A., & Chauhan, V. (2006). Oxidative stress in autism. Pathophysiology, 13(3), 171-181.

Liu, H. H., Shih, T. S., Chen, I. J., & Chen, H. L. (2008). Lipid peroxidation and oxidative status compared in workers at a bottom ash recovery plant and fly ash treatment plants. Journal of Occupational Health, 50(6), 492-497.

Merker, H. J., Günther, T., Höllriegl, V., Vormann, J., & Schümann, K. (1996). Lipid peroxidation and morphology of rat testis in magnesium deficiency. Andrologia, 28(1), 43-51.

Negm, S. H., & Ragheb, E. M. (2019). Effect of (Alpinia officinarum) hance on sex hormones and certain biochemical parameters of adult male experimental rats. Journal of Food and Dairy Sciences, 10(9), 315-322.

Qu, J. H., Hong, X., Chen, J. F., Wang, Y. B., Sun, H., Xu, X. L., ... & Wang, X. R. (2008). Fenvalerate inhibits progesterone production through cAMP-dependent signal pathway. Toxicology letters, 176(1), 31-39.

Aitken, R. J., & Roman, S. D. (2008). Antioxidant systems and oxidative stress in the testes. Molecular mechanisms in spermatogenesis, 154-171.

Srividya, A. R., Dhanabal, S. P., Misra, V. K., & Suja, G. (2010). Antioxidant and antimicrobial activity of Alpinia officinarum. Indian journal of pharmaceutical sciences, 72(1), 145.

Kamel, A. H., Foaud, M. A., & Moussa, H. M. (2018). The adverse effects of bisphenol A on male albino rats. The Journal of Basic and Applied Zoology, 79(1), 1-9.

Reid, K., Wright, V., & Omoregie, S. (2016). Anticancer properties of Alpinia officinarum (lesser galangal)–A mini review. Int J Adv Res, 4, 300-6.

Agarwal, A., & Said, T. M. (2003). Role of sperm chromatin abnormalities and DNA damage in male infertility. Human reproduction update, 9(4), 331-345.

Meeker, J. D., Singh, N. P., Ryan, L., Duty, S. M., Barr, D. B., Herrick, R. F., ... & Hauser, R. (2004). Urinary levels of insecticide metabolites and DNA damage in human sperm. Human Reproduction, 19(11), 2573-2580.

Ndovi, T. T., Choi, L., Caffo, B., Parsons, T., Baker, S., Zhao, M., ... & Hendrix, C. W. (2006). Quantitative assessment of seminal vesicle and prostate drug concentrations by use of a noninvasive method. Clinical Pharmacology & Therapeutics, 80(2), 146-158..

Trivedi, P. P., Kushwaha, S., Tripathi, D. N., & Jena, G. B. (2010). Evaluation of male germ cell toxicity in rats: correlation between sperm head morphology and sperm comet assay. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 703(2), 115-121..

Zini, A., & Libman, J. (2006). Sperm DNA damage: clinical significance in the era of assisted reproduction. Cmaj, 175(5), 495-500.

Almabhouh, F. A., Osman, K., Siti Fatimah, I., Sergey, G., Gnanou, J., & Singh, H. J. (2015). Effects of leptin on sperm count

and morphology in Sprague‐Dawley rats and their reversibility following a 6‐week recovery period. Andrologia, 47(7), 751-758.

Ullah, H., Ambreen, A., Ahsan, N., & Jahan, S. (2017). Bisphenol S induces oxidative stress and DNA damage in rat spermatozoa in vitro and disrupts daily sperm production in vivo. Toxicological & Environmental Chemistry, 99(5-6), 953-965.