ASDJ
AINSHAMS DENTAL JOURNAL

AIN SHAMS DENTAL JOURNAL
Official Publication of Ain Shams Dental School
Des. 2019 - Vol. XXII

Histpathological and Immunohistochemical Evaluation of the Prophylactic Effect of Camellia Sinensis Against Sumithion -Induced Toxicity in Rat Submandibular Salivary Gland

*Nehad A. Ahmad**

.

Abstract

Objectives: The aim of the present study was to evaluat the prophylactic effect of Camellia sinensis against Sumithion -induced toxicity in rat Submandibular salivary glands.

Materials and Methods: thirty adult male Wistar rats with an average weight of 120–150 g were randomised into 3 groups, control (group I), Sumithion administration (group II), Sumithion administration 1 h after Camellia sinensis administration (groups III). The rats were then sacrificed after 28 days. The Submandibular salivary glands were examined histologically, immunohistochemically.

Results: Histopathologically the Sumithion group showed sign of acini degeneration represented by loss of normal architecture. The nuclei of the acinar cells appeared with different sizes and shape (polymorphism). While in group III The acini relatively preserved their shape. Histomorphometric analysis showed the highest mean value of Ki-67 immuno-reactivity was recorded in sumithion Group, followed by Camellia, with the least value recorded in control. ANOVA test revealed a significant difference between groups (p=0.00). Tukey's post hoc test revealed a significant difference between each 2 groups.

Conclusions: The administration of natural antioxidants has a beneficial effect on prevention of cytotoxicity induced by organophosphorous compounds.

Keywords: Sumithion, Camellia sinensis, Submandibular gland, Ki-67.

* *Lecturer of Oral biology department Faculty of Dental Medicine for Girls AL-Azhar University*

Introduction

Organic insecticide poisoning remains one of the major issues in both developing and developed communities. A great proportion of acute poisoning cases are caused by exposure to pesticides, especially organophosphate (OPs) compounds. Sumithion [O,O-dimethyl O-(4-nitro-m-tolyl) phosphorothioate] an organophosphorous insecticide, is now widely used for controlling a wide range of insects and pests. (1)

Organophosphate pesticides (OPs) are currently one of the most commonly used classes of insecticides with high potential for human exposure. OPs present a societal, health, and environmental concern as they can poison not only insects but other animals as well, including birds, amphibians, and mammals. Individuals at greatest risk are those who most frequently handle these compounds, including formulators, applicators, farmers and home gardeners. OPI are also used extensively in the home environment, and as a result, young children are at increased likelihood for exposure to these compounds. (2)

OPs are classified as anticholinesterases and thus increase the concentration of acetylcholine (ACh) within the neuromuscular junction. With high doses, this can lead to acute neurotoxicity in humans. (3)

These toxic effects probably occur through the generation of reactive oxygen species (ROS) causing damage to the various membranous components of cell. (4)

The central role of ROS in mediating the pathology in several diseases has stimulated interest in the possible role of anti-oxidant agents in preventing the development of these diseases. Human body produced it's own endogenous antioxidants to combat different free radicals. The majority of antioxidants are obtained from external resources. In particular, diet is considered as exogenous source of antioxidants as vegetables, fruits and green tea. (5). In general, herbal medicines are complex mixtures of different compounds that often act in a synergistic fashion to exert their full beneficial effect. In recent years, the health benefits effects of green tea are mainly attributed to its polyphenol content, particularly flavanols and flavonols, which represent 30% of fresh leaf dry weight. (6).

Recently, many of the aforementioned beneficial effects of Camellia sinensis (green tea) were attributed to its most abundant catechin, epigallocatechin-3-gallate (EGCG). Green tea extracts are more stable than pure epigallocatechin gallate because of the presence of other antioxidant constituents in the extract. Also epigallocatechin gallate possesses anti-cancer properties. In general, herbal medicines are complex mixtures of different compounds that often act in a synergistic fashion to exert their full beneficial effect. (7)

The current study was carried out to hypothesise that Camellia sinensis could be used as co-treatment to reduce side effects associated with use of Organophosphorous Insecticide.

Materials and methods

In this study, 30 adult male Wistar rats with an average weight of 120– 150 g were used in this study. They were randomly assigned into four groups, 10 rats each. Group I (control group) did not receive any medical treatment. Groups II, and III were given 1/30 LD50 20 mg/kg body weight of sumithion orally once per day for 28 days. Group (III) was given 60 mg green tea extract daily 1 h before sumithion administration once per day for 28 days.

At the end of 28 days, animals were sacrificed by ketamine over dose. The submandibular salivary gland were dissected. The glands were used for the light microscopic, and immunohistochemical examination.

- **Light microscopic (LM) examination**

Specimens were immediately fixed in 10% neutral formalin for 48 h, and then rinsed in distilled water. Specimens were dehydrated in ascending grades of alcohol and embedded in paraffin. From each gland sections of 5 mm thickness were cut. The sections were subjected to haematoxylin and eosin stain according to the conventional method. Histopathologic examination was performed using light microscopy.

- **Immunohistochemistry (IHC)**

A recognized indicator of cell mitotic activity is Ki-67, an increase in Ki-67 expression is indicative of increased cell mitotic activity and proliferation. Ki-67 monoclonal antibodies detect a nuclear antigen expressed exclusively at the level of cells in the proliferation phase (phases G1, S, G2 and mitoses), but not in the G0 phase. Therefore, Ki-67 antibodies allow for the immunohistochemical determination of the tissue growth fraction. In this study, immunostaining for Ki-67 was performed using polyoclonal Mouse Anti-Human Ki-67 Antigen, Clone MIB1, and Code No. M 7240 staining system. The staining procedure sections of the instructions included with detection system were followed. Positive cells expressing Ki-67 were identified by a brown precipitate in the nucleus except in mitotic cells, where the chromosomes and the cytoplasm are labeled.

- **Histomorphometric analysis**

The data were obtained using Leica Qwin 500 image analyzer computer system (England). The image analyser consisted of a coloured video camera, coloured monitor, hard disc of IBM personal computer connected to the microscope, and controlled by Leica Qwin 500 software. The Optical density (OD) of Ki 67 was measured using an objective lens of magnifi- cation 40x (a total magnification of 400). Ten fields were measured for each specimen. Areas of positive reaction were then masked by a blue binary colour. Mean values were obtained for each specimen.

- **Statistical analysis**

Values were presented as mean and standard deviation (SD) values. Data were explored for normality using Kolmogorov-Smirnov test of normality which indicated that data were normally distributed (parametric data), therefore one way analysis of variance (ANOVA) test was used for comparison. This was followed by Tukey's post hoc test for pairwise comparison. The significance level was set at $p \leq 0.05$. Statistical analysis was performed with SPSS 18.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows.

Results

1. **Histological Results**

A. Control group (Group I)

Microscopic examination of the submandibular salivary gland of Wistar albino rats were formed of typical mixed acini (serous and mucus) acini with normal architecture.

The serous acini appeared normally with definite form and arrangement of their serous cells with rounded basally located nuclei. The cells were roughly pyramidal in shape and were arranged around a central narrow lumen. The serous units contained granular basophilic cytoplasm. (Fig. 1)

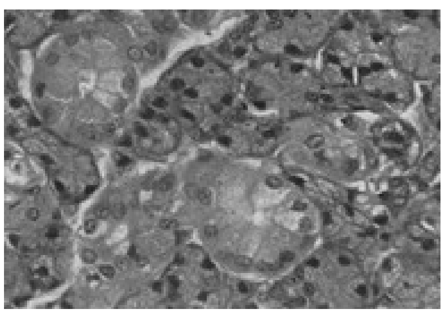
The mucous acini consist of high cuboidal or pyramidal cells that surround wide lumen. Their nuclei appeared angular, darkly stained and located mostly at the basal end of the cells. Their cytoplasm appeared empty with basophilic trabeculation due to dissolved mucigen granules (Fig. 1)

B. The sumithion group (II)

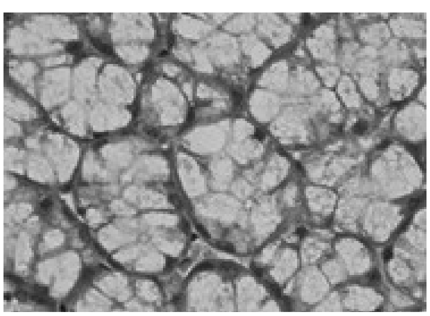
Some of acini of the submandibular salivary gland completely destroyed leaving empty spaces. while others of serous acini appeared smaller in size with loss of normal architecture. The nuclei of the serous acini revealed different sizes, shape and position. Some of mucous acini lacked acinar boundaries and the cell lining lacked their nuclei. (Fig. 2).

C. The camellia group (III)

Most of serous acini were regained their normal architecture. The acinar cells exhibited small deeply basophilic nuclei. The cytoplasm showed few vacuolization. The mucous acini revealed normal acinar form, boundaries and arrangement but still lacked nuclei in most of their cell lining. (Fig. 3).

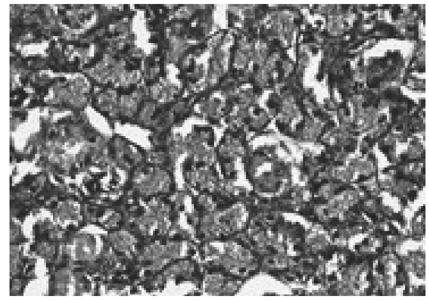


a

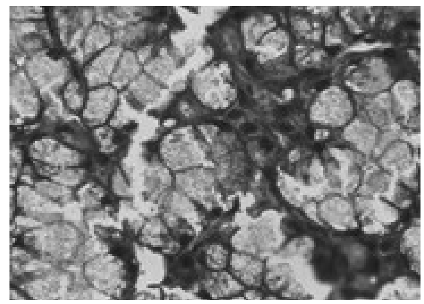


b

Fig. (1): Photomicrograph of submandibular salivary gland of control group (a: serous acini), (b: mucous acini) showing normal acinar architecture (H&E, 400X).

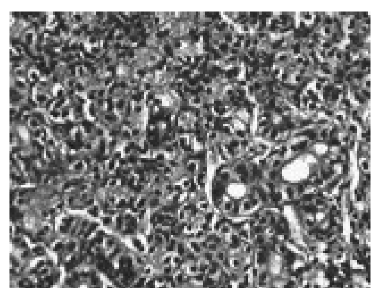


a

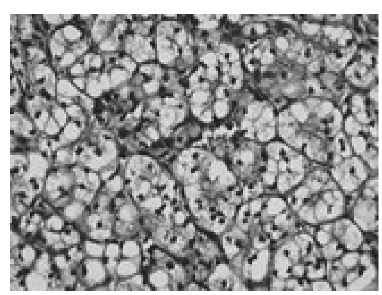


b

Fig. (2): Photomicrograph of submandibular salivary gland of group II showing some serous acini (a), and mucous acini (b) completely degenerated, lack the normal architecture of the remaining acini and complete absence of the nuclei in most of the destructive acini. (H&E, 400X).



a



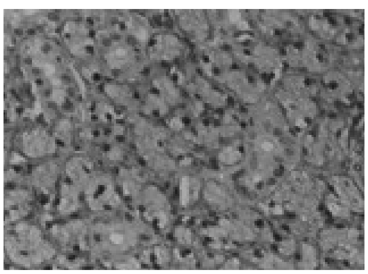
b

Fig. (3): Photomicrograph of submandibular salivary gland of group III showing normal

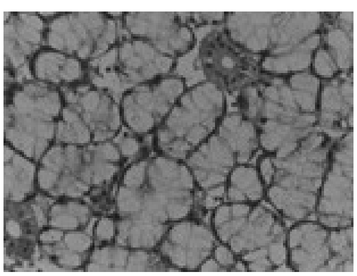
histological features of serous acini (a) and mucous acini (b). Note minor atrophy of some acini, and few cytoplasmic vacuolization, absence of nuclei in some mucous acini. (H&E, 400X).

2. **Immunohistochemical results**

We evaluated the effect of sumithion as well as the prophylactic antioxidant treatment on cell proliferation using Ki-67 IHC. A negative Ki-67 staining was observed in either nuclei or cytoplasm of acini of submandibular and sublingual salivary glands group I (Fig. 4). However, group II revealed negative nuclear and granular cytoplasmic reaction in some samples and strong positive Ki-67 staining other samples. (Fig. 5). Immuno-reactivity for Ki-67 was relatively reduced in the most acini of group III either nuclei or cytoplasm of acini of submandibular salivary glands. (Fig. 6).

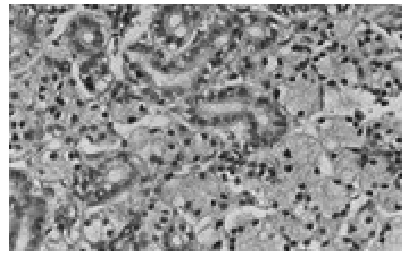


a

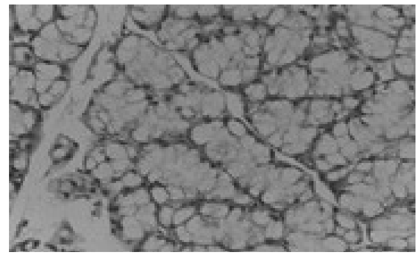


b

Fig. (4): A photomicrograph showing; Group I revealed negative nuclear and granular cytoplasmic reaction in serous and mucous acini. (IHC, 400X).

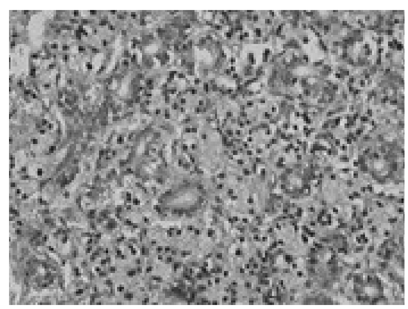


a

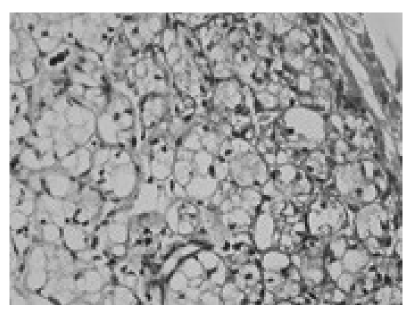


b

Fig. (5): A photomicrograph showing; Group II displayed relative negative immuno-reaction in some glands (a,b) and strong nuclear and granular cytoplasmic reaction in other acini (c,d) of Submandibular Salivary glands. (IHC, 400X).



a



b

Fig. (6): A photomicrograph showing; Group III with Immuno-reactivity for Ki-67 was relatively observed in the most acini either nuclei or cytoplasm of acini (a,b) of Submandibular Salivary glands. (IHC, 400X).

3. **Histomorphometric analyses**

Comparison between groups

The highest mean value of Ki-67 immuno-reactivity was recorded in Group 2 (Sumithion) 79.76±2.1, followed by Camellia 31.31±2.8, with the least value recorded in control (12.07±2.6). ANOVA test revealed a significant difference between groups (p=0.00). Tukey's post hoc test revealed a significant difference between each 2 groups (Table 1, Fig.7)

Table (1a) Descriptive statistics and comparison of mean values recorded in the 3 groups (ANOVA test)

	Mean	Std. Dev	Std. Error	95% Confidence Interval for Mean: Lower Bound	95% Confidence Interval for Mean: Upper Bound	Min	Max	F	P
G1 (Control)	12.07c	2.57	.81	10.23	13.91	9.25	17.34	1884.4	0.00*
G2 (Sumithion)	79.76a	2.06	.65	78.28	81.23	75.54	82.46		
G3 (Camellia)	31.71b	2.91	.92	29.63	33.79	27.44	35.93		

Significance level p≤0.05, *significant

Tukey's post hoc test: means with different superscript letter are significantly different

Table (1b) Details of Tukey's post hoc test for pairwise comparison

(I) groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval: Lower Bound	95% Confidence Interval: Upper Bound
G1 (control)	G2 (Sumithion)	-67.68910	1.13	.00	-70.50	-64.88
	G3 (Camellia)	-19.64670	1.13	.00	-22.46	-16.83
G2 (Sumithion)	G1 (control)	67.68910	1.13	.00	64.88	70.50
	G3 (Camellia)	48.04240	1.13	.00	45.23	50.86
G3 (Camellia)	G1 (control)	19.64670	1.13	.00	16.83	22.46
	G2 (Sumithion)	-48.04240	1.13	.00	-50.86	-45.23

Significance level p≤0.05, *significant

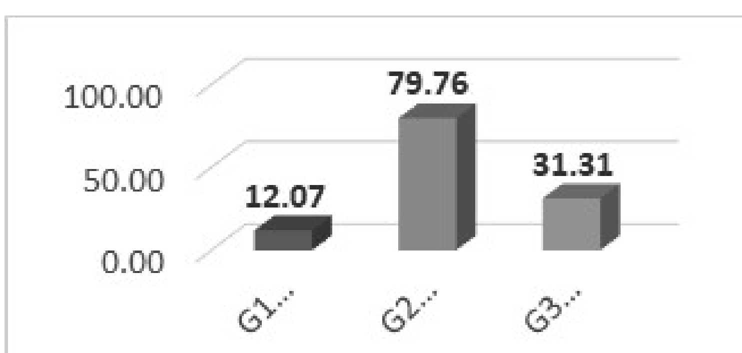


Fig. (7) Bar chart illustrating the mean recorded value in the 3 groups

Discussion

Organophosphate pesticides (OPs) are currently one of the most commonly used classes of insecticides and are applied in agricultural, residential, and community settings. (8)

Somithion belongs to the organophosphate family of insecticides (9). It is considered a cholinesterase inhibitor. Somithion is effective against a wide range of pests, i.e. penetrating, chewing and sucking insect pests (coffee leaf miners, locusts, rice stem borers, wheat bugs, flour beetles, grain beetles, grain weevils) on cereals, cotton, orchard fruits, rice, vegetables, and forests. It may also be used as a fly, mosquito, and cockroach residual contact spray for farms and public health programs (10). OPs are classified as anticholinesterases and thus increase the concentration of acetylcholine (ACh) within the neuromuscular junction. With high doses, this can lead to acute neurotoxicity in humans (11). The widespread use of pesticides is usually accompanied with serious problems and health hazards. Now it is established that many pesticides in common use can produce some toxic and adverse effects on liver, kidney, thyroid gland and other biological systems when tested on various types of experimental animals through their mode of action or by production of free radicals that damage all cell components (12,13). Several studies have demonstrated that OPs are also associated with cancer risk. (14)

In the present study, systemic administration of sumithion led to signs of degeneration of glandular epithelium cells that was demonstrated in serous and mucous acini of rat submandibular salivary gland histopathologically. In addition to high mean value of Ki-67 immuno-reactivity was recorded in Group 2 (Sumithion treated rat group) followed by (Camellia extract treated group) that was demonstrated immunohistochemically and histomorphometrically.

In the present study, Histopathologically the sumithion group showed that, some of acini of the submandibular salivary gland completely destroyed leaving empty spaces. while others of serous acini appeared smaller in size with loss of normal architecture. The nuclei of the serous acini revealed different sizes, shape and position. Some of mucous acini lacked acinar boundaries and the lining cells lacked their nuclei. On the other hand, the cytotoxic effect was reduced in the camellia group III. Our observations were in agreement with Nahed et al., who reported that fenitrothion treated rats was accompanied by similar histopathological alterations in parotid salivary gland after oral administration of fenitrothion in dose 1/30 LD50 20 mg/kg body weight orally once a day for 28 days. (1). Moreover Afshar et al., reported the same histopathological changes in liver and Kidney of wistar rats. (2). The principle mechanism by which sumithion induces acute toxicity is probably through the production of reactive oxygen species (ROS) causing oxidative damage to the various membranous components of cell. (4)

Camellia sinensis has antioxidants activity which inhibit many oxidation reaction caused by free radicals thereby preventing or delaying damage to the cells and tissues. (15)

Similar studies have described the protective effects of EGCG on the viability of lead-exposed cells and on the synaptic plasticity of Wistar rats exposed to lead. (16)

Green tea polyphenols has beneficial therapeutic effects, including anti-oxidant, anti-inflammatory, anti-cancer, anti-microbial, and immunomodulatory effects. (17)

Recent reports demonstrate that green tea may exert a positive effect on the reduction of medical chronic conditions such as cardiovascular disease, cancer, Alzheimer's disease, Parkinson's disease, and diabetes. (18)

For immunohistochemical (IHC) analysis we use marker of cell proliferation (Ki-67) which revealed that negative nuclear and granular cytoplasmic reaction in some samples and strong positive Ki-67 staining other samples of sumithion group. While in camellia group the immuno-reaction of Ki-67 was relatively reduced in the most acini either in nuclei or cytoplasm of acini of submandibular salivary glands. ANOVA test revealed a significant difference between groups (p=0.00). Tukey's post hoc test revealed a significant difference between each 2 groups.

The positive Ki-67 staining that seen in experimental groups can be explained by several studies which demonstrated that Ops compouned are associated with cancer risk through various mechanisms including increased oxidative stress, disruption of adhesion molecules, acetylcholinesterase inhibition, endocrine disruption, and induction of genomic instability. (19)

On other hand treatment with certain Ops compound resulted in increased cell proliferation and treatment with others resulting in either increased cell proliferation or cell cycle arrest and apoptosis, perhaps depending on the dosage administered. (20), hence this study was explain our finding in histological and immunohistochemical results.

The Agricultural Health Study demonstrated that exposure to Ops compound was significantly associated with risk for aggressive prostate cancer (21).

The Ki-67 antigen, protein has an important function in cell division, has been identified in the early steps of polymerase I-dependent ribosomal RNA synthesis. (22)

Ki-67, a marker for proliferation, has been extensively studied as a prognostic marker in breast cancer and predictor of response to chemotherapy, and used in recurrence indexes. (23,24)

George. et al., considered Ki-67 to be a prognostic factor in the molecular classification subgroups of breast cancer. (25)

Natural antioxidant (Camellia sinensis) is seen to be give positive results in reducing the toxic effect of sumithion on salivary gland acini resulting in improved quality of saliva.

Steven et al., investigated the effects of green tea supplementation on markers of cell proliferation, apoptosis, and angiogenesis using paired pre-surgery and surgery specimens from 28 newly diagnosed breast cancer patients. Patients in the green tea group displayed a consistent pattern of reductions in markers of cell proliferation (Ki-67) in benign and malignant cell components; this was not observed in the no-green tea group. (26)

The previous study support our finding in prophylactic group (III) in which immune-reactivity with Ki-67 was reduced.

Moreover 42 in vitro studies and 13 studies of mouse xenograft models where the therapeutic supplementation of anticancer drugs with GTP led to a synergistic effect on the inhibition of tumor development. The tumor volume was reduced by 70.3% in the combination of EGCG with anticancer drugs, while EGCG or GTE treatment alone was slightly less effective than in combination with anticancer drugs.(27)

All results of this study clearly demonstrated that oral administration of sumithion leads some histopathological changes in salivary glands in addition to cancer risk in some rats.

Conclusions

The administration of natural antioxidants has a beneficial effect on prevention of cytotoxicity induced by organophosphorous compounds.

Reference

1. Nahed S.K., Bassant A.E., Prophylactic effect of green tea and Nigella sativa extracts against fenitrothion-induced toxicity in rat parotid gland, archives of oral biology 56 (2011) 1339–1346

2. Afshar, Simin, Heidari, Reza, Farshid, Amir A., Ilkhanipour and Minoo, Oral toxicity of fenitrothion in Wistar rats: a biochemical and histopathological study. International Journal of Applied Environmental Sciences; June 1, 2008.

3. Colovic, M.B.; Krstic, D.Z.; Lazarevic-Pasti, T.D.; Bondzic, A.M.; Vasic, V.M., Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. Curr. Neuropharmacol. (2013), 11, 315–335

4. Goel A., Dani V., And Dhawan D., Protective effect of xinc on lipid peroxidation, antioxidant enzymes and hepatic histoarchitecture chlorpyriphos induced hepatotoxicity. Chemico-biological Interaction; 156 (2005): 131-140.

5. Govindarajan R., Vijayakumar M., Pushoangadan p.: Antioxidant approach to disease management and the role of 'Rasayana' herbs of ayurvede. J. Ethnopharmacol; 99(2005) 165-178.

6. Naghma K, Hasan M: Tea polyphenols for health promotion. Life Sciences. 2007, 81: 519-533. 10.1016/j.lfs.2007.06.011.

7. Chacko et al., Beneficial effects of green tea: A literature review. Chinese Medicine 2010, 5:13

8. Lerro, C.C.; Koutros, S.; Andreotti, G.; Friesen, M.C.; Alavanja, M.C.; Blair, A.; Hoppin, J.A.; Sandler, D.P.; Lubin, J.H.; Ma, X.; et al. Organophosphate insecticide use and cancer incidence among spouses of pesticide applicators in the Agricultural Health Study. Occup. Environ. Med. 72:(2015), 736–744.

9. Meister R.: Farm Chemicals Handbook, Meister publishing Co. Willoughby, OH, USA; 1994.

10. Thomson W.: Agricultural Chemicals. Book I: Insecticides. Thomson Publications, fresno, CA, USA; 2001.

11. Colovic, M.B.; Krstic, D.Z.; Lazarevic-Pasti, T.D.; Bondzic, A.M.; Vasic, V.M. Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. Curr. Neuropharmacol. 11:(2013), 315–335.

12. Poovala V., Huang H., Salahudeen A.: Role of reactive oxygen metabolism in organophosphate-Bidrin-induced renal tubular cytotoxicity. J. Am. Soc. Nephrol; 10:1746-1752, 1999.

13. Kovacic P.: Mechanism of organophosphates (nerve gases and pesticides) and antidotes: electron transfer and oxidative stress. Curr. Med. Chem; 10: 2705-2709, 2003.

14. Koutros, S.; Beane Freeman, L.E.; Lubin, J.H.; Heltshe, S.L.; Andreotti, G.; Barry, K.H.; Dellavalle, C.T.; Hoppin, J.A.; Sandler, D.P.; Lynch, C.F.; et al. Risk of total and aggressive prostate cancer and pesticide use in the Agricultural Health Study. Am. J. Epidemiol. 177(2013), 59–74.

15. Cao, T.; Zhang, X.; Yang, D.; Wang, Y.-Q.; Qiao, Z.-D.; Huang, J.-M.; Zhang, P. Antioxidant effects of epigallocatechin-3-gallate on the aTC1−6 pancreatic alpha cell line. Biochem. Biophys. Res. Commun. 2018, 495 (1), 693−699.

16. Della Latta, V.; Cecchettini, A.; Del Ry, S.; Morales, M. Bleomycin in the setting of lung fibrosis induction: From biological mechanisms to counteractions. Pharmacol. Res. 2015, 97, 122−130.

17. Shah, S.; Gani, A.; Ahmad, M.; Shah, A.; Gani, A.; Masoodi, F. In vitro antioxidant and antiproliferative activity of microwave-extracted green tea and black tea (Camellia sinensis): a comparative study. Nutrafoods 2015, 14 (4), 207−215.

18. Lujuan X.; Hua Z.; Ruili Q.; Rong T.; and Yoshinori M. Recent Advances in the Understanding of the Health Benefits and Molecular Mechanisms Associated with Green Tea Polyphenols. J. Agric. Food Chem. 2019, 67, 1029−1043.

19. Engel, L.S.; Werder, E.; Satagopan, J.; Blair, A.; Hoppin, J.A.; Koutros, S.; Lerro, C.C.; Sandler, D.P.; Alavanja, M.C.; Beane Freeman, L.E. Insecticide Use and Breast Cancer Risk among Farmers' Wives in the Agricultural Health Study. Environ. Health Perspect. 2017, 125, 097002.

20. Hu, L.; Luo, D.; Zhou, T.; Tao, Y.; Feng, J.; Mei, S. The association between non-Hodgkin lymphoma and organophosphate pesticides exposure: A meta-analysis. Environ. Pollut. 2017, 231, 319–328.

21. Koutros, S.; Beane Freeman, L.E.; Lubin, J.H.; Heltshe, S.L.; Andreotti, G.; Barry, K.H.; Dellavalle, C.T.; Hoppin, J.A.; Sandler, D.P.; Lynch, C.F.; et al. Risk of total and aggressive prostate cancer and pesticide use in the Agricultural Health Study. Am. J. Epidemiol. 2013, 177, 59–74.

22. Jouat W, Arnold N. Is the Ki-67 labelling index ready for clinical use? Ann Oncol 2011; 22:500–502.

23. Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, A'Hern R, et al. Prognostic value of Ki67 expression after short-term presurgical endocrine therapy for primary breast cancer. J Natl Cancer Inst 2007; 99:167–170.

24. Ming Chen, Shuzhong Yao, Qinghua Cao, Meng Xia, Junxiu Liu, and Mian He. The prognostic value of Ki67 in ovarian high-grade serous carcinoma: an 11-year cohort study of Chinese patients Oncotarget. 2017; 8(64): 107877–107885.

25. George P. et al., The role of Ki-67 in the proliferation and prognosis of breast cancer molecular classification subtypes. Anti-Cancer Drugs 2014, 25:950–95.

26. Steven S., DarcyV., Debra H., Chiu-ChenT., Chung S., Malcolm C. and Anna H. Biological effects of green tea capsule supplementation in pre-surgery postmenopausal breast cancer patients. Forntiers of oncology. 3: (2013)

27. Fujiki, H.; Sueoka, E.; Watanabe, T.; Suganuma, M. Synergistic enhancement of anticancer effects on numerous human cancer cell lines treated with the combination of EGCG, other green tea catechins, and anticancer compounds. J. Cancer Res. Clin. Oncol. 2015, 141 (9), 1511−1522.