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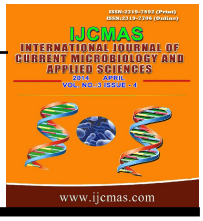

Original Research Article

Comparison study on native olive waste extract and its nano- particles effect on oxidative stress induced by aflatoxin B_1 in rat brain

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Keywords

Olive waste, oxidative stress, aflatoxin B_1, brain

A B S T R A C T

Aflatoxin B_1 is one of the most potent hepato-carcinogens known and can accumulate in the brain. In our study the protective role of the olive cake extract and its nano particle were studied against the hazard of aflatoxin B_1 in the brain of rats. Olive waste extract and its nano were investigated through an electron microscope, and the particles were in nano size. From obtained results, both olive waste extract contained nutritive compound (polyphenols and essential fatty acids). Biological evaluation was investigated using experimental rats witch designed for six groups including the control group, group 2 received AFB_1, group 3 received AFB_1 + 0.2 ml native extract, group 4 received AFB_1 + 0.5 ml native extract, group 5 received AFB_1 + 0.2 ml sonicated extract and group 6 received AFB_1 + 0.5 ml sonicated extract. Administration of AFB_1 in rats increased all histochemical parameters compared with the control group. Treatment of rats received AFB_1 with native or sonicated extracts restore all histochemical parameters towards the control values.

Introduction

The classic production of olive oil generates three phases and two wastes: olive oil (20 %), solid waste (% 30)and aqueous liquor (50 %). The solid waste olive oil cake (OOC) or "orujo") is a combination of olive pulp and stones. (Niaounakis M, Halvadakis, 2004). annually, important quantities of olive residue are produced and may be the source of ecological damages. Like agricultural residues, which are abundant, renewable, low cost raw materials (Jeanne, 2013).

Olive cake waste is very rich in polyphenol compounds and could be used as low cost edible natural antioxidants for protection against aflatoxicosis in animal

and human as well through the production of bio active compounds use as antioxidant agents to improve human health in Egypt, (Sherif et al., 2012). As well as (Nicolas et al., 2013) reported that olive mill waste water is rich in water-soluble polyphenolic compounds that show remarkable antioxidant properties. The oxidative stress defines itself as being a loss of the balance between oxidizing and antioxidants within a cell (Cardey 2007). The responsibility of these oxidative stresses belongs to amputees to the free radicals (Coulidiati, 2010).

The latter are a member of reactive species of the oxygen or of some nitrogen ,which play a very important role in diverse pathologies as the inflammatory cryptogenetic diseases of the bowel, atherosclerosis ,the cancer and the cellular ageing (Wu and Ng, 2008; Gagliardi et al., 2009; Havlik et al., 2010; Bangou et al., 2011). The previous investigations show that among the recognized biological potentialities of plants, comes first of all the antioxidizing activity in front of the arsenal of the free radicals which are produced in the body (Bangou, 2012). Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction (Sies H. et al., 1992).The compound antioxidants found in plants play an important role in the treatment and the prevention of the oxidative stress diseases (Bangou, 2012).

Aflatoxins, mainly AFB_1 and AFB_2, were found in the brain, liver, lungs, kidney and blood of children who have died from kwashiorkor, Reye syndrome or from other un identified central nervous system (CNS) diseases (Casteels and Eggermont, 1994; Hendrickse RG, Maxwell, 1989; Oyelami et al., 1995; Oyelami et al., 1999; Peraica et al., 1999). Moreover, a study investigating the effect of AFB1 intraperitoneal acute and chronic treatment on acetylcholinesterase (AChE) activity suggests that AFB1 changes acetylcholine turnover and hence cholinergic transmission in rat brain and adenohypophysis (Egbunike and Ikegwuonu, 1984). Hence, the inhibitory ability of a toxic substance therefore differs on the G1 and G4 isoforms, causing differential xenobiotic cholinergic and non-cholinergic toxicity in particular during brain developmental stages. Some studies show that simple coumarin derivatives with an in tact pyrone moiety, such as that in AFB1 inhibit acetylcholinesterase (AChE) activity by binding to the peripheral site of the enzyme (Simeon et al.,1999; Peng, 1995). This peripheral site of the enzyme has been shown to be significant in the non-synaptic functions of AChE (Johnson and Moore, 1999; Johnson and Moore, 2000).

It is well known that the essential fatty acids (EFAs) play important roles in preventing many diseases and abnormal differentiation problems. The essential fatty acids cannot be synthesized by human cells and hence have to be obtained from dietary sources. The famous diet model, which is known as the Mediterranean diet, providing oleic acid, (LAs), and alpha lenolenic acid (ALAs), antioxidant nutrition's and reduced amounts of saturated fatty acids, resulted in a 70 % reduction in coronary events and 80 % reduction in deaths (Huertas et al., 2003). The lack of EFAs causes several abnormalities and malignant transformations in the human body, such as breast cancer (Eynard, 2003), cardiovascular diseases (Kang and Leaf, 2000), as well as inflammatory and

immunological responses (Daret and Ching, 1996). According to their results, the main constituents of all hexane extracts were ALAs and LAs. It is clear that there is a significant correlation between the EFAs and antioxidant activity. The main aim of the study was to use olive waste as natural source of antioxidants for protection against oxidative stress caused by AfB1in rat brain.

Materials and Methods

Olive cake

Olive cake residues were purchased from Food Technology Research Institute, Agriculture Research Center, Ministry of Agriculture, Giza, Egypt.

Aflatoxin B1

Aflatoxin B1 was purchased from Sigma Chemical Co. (St. Louis, Mo. U.S.A.).

Kits

Histochemical analyses: Superoxide dismutase, Glutathione, Glutathione peroxidase, Malondialdehyde, adrenaline, noradrenaline kits were purchased from Biomeieux, Laboratory of Reagents and Products (France).

Experimental animals

Two months old, mature male –rats were purchased from the Animal House Colony, National Research Center, Giza, Egypt.

Extraction of polyphenols from olive cake

The polyphenols extracted from the olive waste using a mixture of water- ethanol (1:1, v/v) adjusted to pH 9 with NaOH. The ratio of olive cake and extraction mixture was 1:4 (w/v). After two successive extractions, the total ethanol extract filtered and dried using freezdrier to powder case and then dissolved in 100 ml distilled water to be suitable for analysis and administration of rats.

Electron microscope

A transmission electron microscope (type JEOL-1230 operated at 100 KV attached to a CCD camera). The sample emulsion was dropped on grids coated with carbon and left to dry in ordinary atmosphere. After that the grid was put on the holder and in TEM to investigate.

GC/MS analysis

One micro liter of each sample extract was injected into a Hewlett Packard 5890 gas chromatograph equipped with a HP-5 fused silica capillary column (50 m x 0.2 mm x 0.33 um film thickness) and connected to Hewlett Packard 5970 series mass selective detector. The carrier gas was helium, maintained at a flow rate of 1.0 ml/min. The injection part temperature was 220 ^{o}C with electron energy of 70 EV. The quadruple temperature was 208 ^{o}C. the oven programmed was as follows: 160 ^{o}C for 5 min., 3 ^{o}C/min. to 220 ^{o}C for 30 min. The mass spectrometer is turned by letting in a small amount of perfluoro-tri-butyl-amine (C12F27N) gas as a reference. The fragments of peak for m/z, 69, 219 and 502 were observed and tune results were recorded and the mass are calibrated. The mass spectrums for each of the peaks from the resulting chromatogram from analyzing samples were observed by the total ion count (TIC) mode.

Sonication method

The part of producing an olive waste

extract was subjected to the sonication process using ultrasonic equipment model (SH 80-2L DIGITAL HEATED-MTI corporation-Eumax) for 15 min.

Experimental Animals

Two-month old Sprague-Dawley male rats (100-120 g) were maintained on a standard lab. diet (protein: 160.4; fat: 36.3 and fiber 41g/kg), and housed in a room free from any source of chemical contamination, artificially illuminated and thermally controlled, at the Animal House Lab.

Experimental Design

Animals were divided into eight groups (6rats/group) and housed in filter-top polycarbonate cages and were maintained on their respective extract for 4 weeks as follows:

Group 1. Normal control animals which fed on basal diet and water without any treatment. Group 2. Fed on basal diet and AFB1 (22ug/kg b.w) dissolved in corn oil. Group 3. Fed on basal diet + AFB1 (22 ug/kg b.w) + 0.2 ml olive waste extract. Group 4. Fed basal diet + AFB1 (22 ug/kg b.w) + 0.5 ml olive waste extract. Group 5. Fed basal diet + AFB1 (22 ug/kg b.w) + 0.2 ml sonicated olive waste extract. Group 6. Fed basal diet + AFB1 (22 ug/kg b.w) + 0.5 ml sonicated olive waste extract.

The animals were observed daily for signs of toxicity and weighted as well. At the end of experimentation period, the animals were killed and brain samples were removed and kept from all animals for histochemical analysis.

Histochemical Analysis

Glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities in brain tissues were determined according to the methods of (Marklund S, Marklund, 1974). Lipid peroxidation level (malondialdehyde, MDA) was estimated as described by (Meltzer et al., 1997). Glutathione (GSH) was estimated as described by (Baker et al., 1990; Eyer et al., 1986). Adrenaline (ADR) and nor adrenaline (NAD) were determined according to the methods of (Boomsma et al., 1993; Dealsandro et al., 1990).

Total a polyphenols of olive cake extract

Total polyphenol was determined in native olive extract and its nano particles according to method described by (Price et al., 1978) as follow: sample extract for the assay was obtained by shaking 2 ml extract in 10 ml methanol at 5 min intervals for 20 min; the supernatant was obtained by centrifuging for 10 min at 1200 g. For the anthocyanidin production assay, 6 ml of 5% HCl in n-butanol (50 ml 32%) was added to 1 ml of sample extract in test tube. The test tube was covered and placed in oven at 100 ^{O}C for 50 min. Absorbance was read at 550 nm.

Statistical analysis

All data were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System (SAS Institute Inc. 1982). The significance of the differences among treatment groups was determined and all statements of significance were based on probability of $P < 0.05$.

Results and Discussion

Table 1 shows to total polyphenol of native olive waste extract and its nano particles and cleared that native olive waste extract had total polyphenol 0.70 mg/ml, while its nano particles decreased to be 0.62 mg/ml. Olive waste can provide a cheap source of phenolic compounds with strong antioxidant properties.

The olive waste extract (OWE) was subjected to the sonication process using ultrasonic waves to decrease the particle size to be in nano size to possess new properties. Fig. 1 shows the scanning electron microscopy of OWE and its particle size that was in range from 115-456 nm, when the original OWE exposed to ultrasonic waves, the particle size decreased to be in range of 4.2-9.7 nm. So, reducing the particle size of materials is an efficient and reliable tool for improving their biocompatibility.

Table 2. showed the results of GC/MS and from the obtained data cleared that OWE contains 3beta-hydroxyl-6-aza-beta-homo, Heptadecanoic acid, Tetradecanoic acid, ethyl ester, 2-chloroethyl linoleate, 9-octadecanoic acid, methyl ester, 9-hexadecanoic acid, 4,5-nonadiene,2-methyl and Ethyl oleate, whereas NOWE contains Cholestan-3-ol,4-methyl, Tetradecanoic acid, Tetradecanoic acid, ethyl ester, Linoleic acid ethyl ester, 9-octadecanoic acid methyl ester, 9-hexadecanoic acid and Ethyl oleate respectively.

Table. 3., Fig. 2, 3. shows to the effect of the olive cake extract and its nano particles on the histochemical parameters in rat brain of the experimental animals, the SOD, GSH, GPx and MDA were determined as an indicator of oxidative stress and from the results the control, and groups received OWE and its nano particles companied with AfB1 were in normal histochemical parameter, while administration of AfB1 alone induced increase histochemical parameters.

It is worthy to report that SOD was in the range from 5.06 to 6.93 except AfB1 group (G2) that was increased to 7.26, as well as GSH was in the range from 5.66 to 10.16 for control and treated groups, the highest value (10.50) in G2 and the lowest value (9.23) in G6 also observed significant among treated and control groups. In addition, MDA was in the normal range for G1 (control) but in two, groups G3, G4 received native OWE the mean value (1120 and 1090 respectively) is the low value near to control, whereas intake of AfB1 (G2) alone induced increase the same parameters to be 1156.

At the same time observed of data that elevation in hormone levels (Adrenaline and noradrenalin) that acting as a neurotransmitter in central nervous system and the brain in G4 group, the Adrenaline value is 344 and noradrenalin value is 568 but in positive control (G2), the value is 319 and 561 respectively.

On the other hand the values amounted to 319 (Adrenaline) and 551 (noradrenalin) is lower than the positive control (G2).

In fact, nanotechnology helps in overcoming the limitations of size and can change the outlook of the world regarding science (Mirkin and Taton, 2000). The total polyphenol was measured in both OWE & SOWE, in this respect (Nicolas et al., 2013) reported that olive mill waste water is rich in water-soluble polyphenolic compounds that show remarkable antioxidant properties. The material properties can differ in nano-scale, the physical, chemical, and biological in

Table.1 The polyphenol content of native and sonicated olive waste extracts

Native olive waste extract	Nano olive waste extract
0.70 mg/ml	0.62 mg/ml

The olive waste extract and nano particles were investigated using GC/MS to know their fatty acid composition and found that both olive waste extract and its nano particles are similar somewhat in the presence of the fatty acids and found are both contained Tetradecanoic acid- ethyl ester, 9-octadecanoic acid- methyl ester, 9-hexadecanoic acid and Ethyl oleate.

The olive waste extract also contains Heptadecanoic acid, 2-chloroethyl linoleate and 4,5-nonadiene,2-methyl that disappeared in the sonicated olive waste extract that contained new compounds are Cholestan-3-ol,4-methyl and Linoleic acid ethyl ester.

Table.2 Fatty acid composition of olive waste extract and its nano particles

No	OWE		NOWE	
	Compound	%	Compound	%
1	Hetradecanoic acid	14.35	Cholestan-3-ol,4-methyl	3.58
2	Tetradecanoic acid, ethyl ester	1.47	Tetradecanoic acid	15.96
3	2-chloroethyl linoleate	1.38	Tetradecanoic acid, ethyl ester	1.76
4	9-octadecanoic acid, methyl ester	5.69	Linoleic acid ethyl ester	1.38
5	9-hexadecanoic acid	29.82	9-octadecanoic acid methyl ester	5.06
6	4,5-nonadiene,2-methyl	3.35	9-hexadecanoic acid	37.05
7	Ethyl oleate	11.36	Ethyl oleate	10.94

OWE: olive waste extract, NOWE: nano olive waste extract

The olive waste extract and nano particles were used at different concentrations for protection of brain rats against hazard of AfB1 and the hisochemical parameters were carried out and found that both extracts have the protective effect against hazard of AfB1 in rat brain and treatment of rats with both tow extracts enhanced the **SOD, GSH, GPX, MDA, ADR,** and NAD and all these parameters reached the control group value, but the native olive waste extract had the protective effect better than sonicated olive waste extract.

Table.3 The effect of olive waste extracts and its Nano particles on histochemical parameters in a rat's brain

Group	SOD (μ /mg)	GSH (μ /mg)	GPx (μ /mg)	MDA (nmol/g)	ADR (ng/ml)	NAD (ng/ml)
G1	5.06 ±0.09^{c}	5.66 ±0.23^{b}	12.90 ±0.52^{a}	852 ±12.99^{c}	340 ±3.93^{a}	570 ±6.66^{a}
G2	7.26 ±0.27^{a}	10.50 ±0.38^{a}	16.53 ±0.80^{a}	1156 ±23.86^{a}	323 ±52.67^{a}	561 ±12.01^{a}
G3	7.26 ±0.22^{ab}	9.50 ±0.66^{a}	15.96 ±0.55^{a}	1120 ±3.60^{ab}	333 ±14.81^{a}	568 ±1.85^{a}
G4	6.00 ±0.32^{b}	9.26 ±0.71^{a}	15.23 ±0.28^{a}	1090 ±25.16^{b}	344 ±15.30^{a}	568 ±9.87^{a}
G5	6.50 ±0.31^{a}	10.16 ±0.67^{a}	15.66 ±0.12^{a}	1155 ±7.33^{a}	338 ±7.81^{a}	562 ±3.38^{a}
G6	6.93 ±0.03^{a}	9.23 ±0.27^{a}	16.23 ±5.88^{a}	1144 ±5.78^{a}	319 ±14.44^{a}	551 ±10.69^{a}

SOD=Superoxide-dismutase, GSH=Glutathione, GPX=Glutathione peroxidase, MDA=Malondialdehyde, ADR=Adrenaline, NAD= Noradrenaline

Fig. 1 The native olive waste extract was investigated using scanning electron microscopy to know the particle size and it was in range of 115-456 nm, when the original OWE exposed to ultrasonic waves. Reducing the particle size of materials is an efficient and reliable tool for improving their biocompatibility.

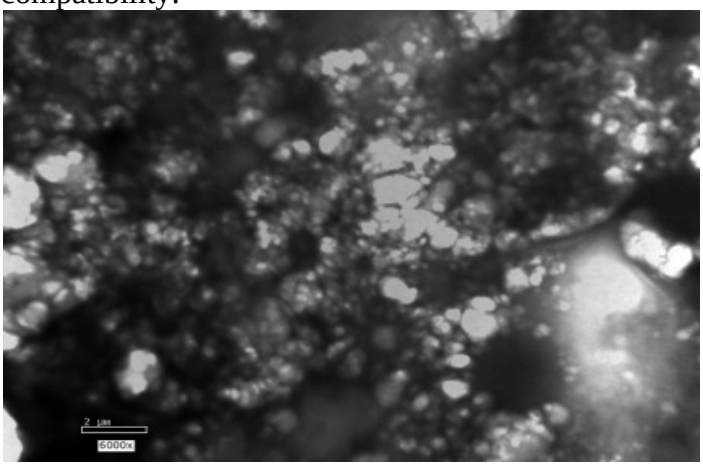


Fig.2 The native olive waste extract was subjected to ultrasonic waves to decrease its particle size to be in nano scale and was studied by using electron microscopy to know the new particle size induced and resulted from sonication process and we found that the particle size decreased to be in range, of 4.2-9.7 nm.

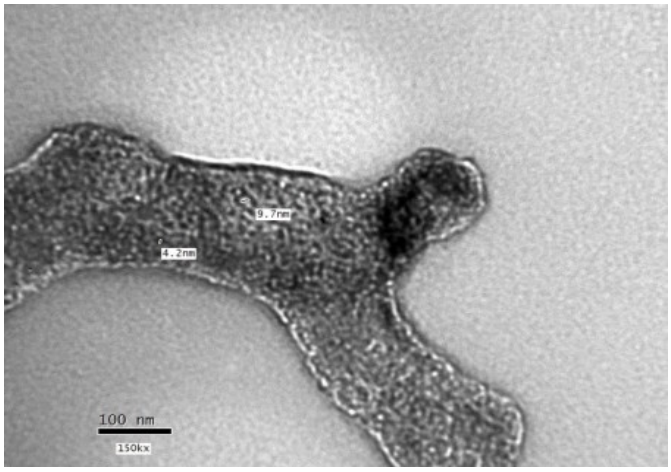


Fig.3 The olive waste extract and its nano particles were used in the treatment of rats treated with AfB1 to study their protective effect against hazard of AfB1 in rat brain and found that both extracts have the protective effect in the regard of the study and decrease of SOD, GSH, GPx towards the control group but the native extract had protective effect better than sonicated extract..

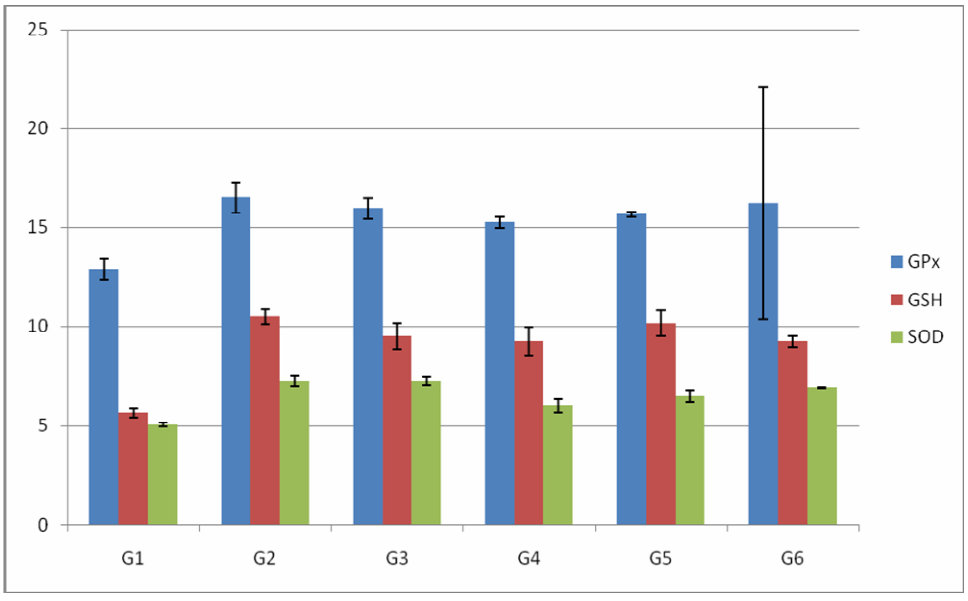
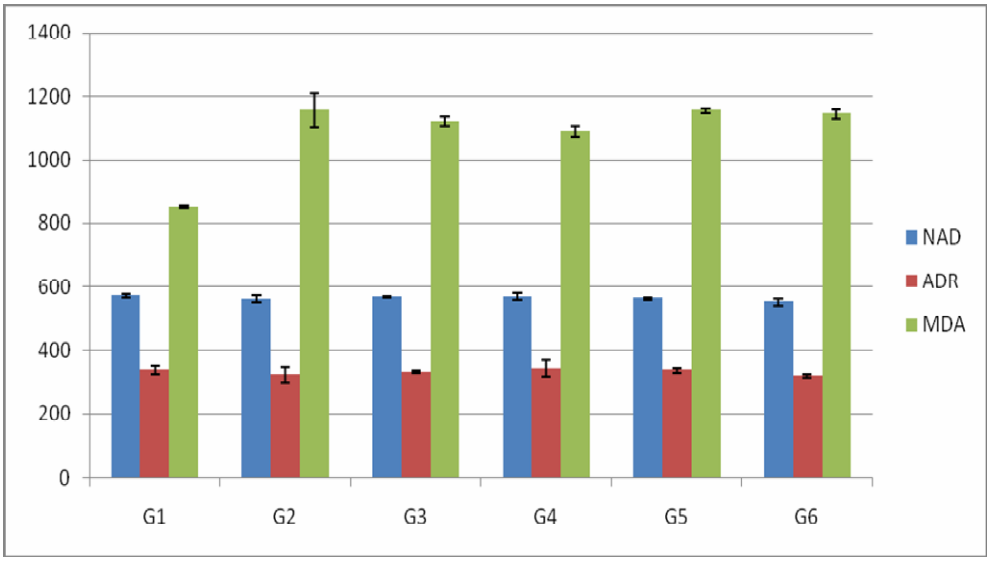


Fig.4 The olive waste extract and its nano particle that obtained by sonication process were used in the treatment of rats to study their effects on NAD, ADR, MDA of rat brain treated with AfB1 and found that the olive waste extract had the protective effect against hazard of AfB1 in brain rats and decrease the mentioned parameters towards the control group



fundamental and useful ways from the properties of individual atoms and molecules or bulk matter. Nanotechnology is directed toward understanding and creating improved materials, devices, and systems that exploit these new properties (NNISP, 2004). In fact, nanotechnology helps in overcoming the limitations of size and can change the outlook of the world regarding science (Mirkin CA, Taton , 2000).

Polyphenols were found to be protect the liver against cellular oxidative damage and maintaenance of interacellular level of antioxidant enzymes (Amat et al., 2010). Neuroprotective properties of polyphenolic antioxidant compound curcumin were reported based on its ability to inhibit homocysteine (Hcy) neurotoxicity and related Hcy-induced lipid peroxidation in animals hippocampi (Ataie et al., 2010). Studies performed in apolipoprotein E-deficient mice proved that polyphenols from wine and tea can prevent the development and/or reduce progression of atherosclerosis, probably due to their potent antioxidative activity and ability to protect LDL against oxidation (Miura et al., 2001).

But, AFB1 alters the levels of various biogenic amines (neurotransmitters) and their precursors in rat and mouse brains Acute AFB1 treatment in experimental animals has been reported to cause a decrease in regional brain acetylcholinesterase enzymes that may affect the cognitive functions as well as memory and learning of the individual while chronic exposure increases adenohypophyseal acetylcholinesterase. Mycotoxins especially aflatoxins and its metabolites and other products such as the reactive oxygen species (ROS) like the AFB-8,9-epoxides may interfere with the normal functioning of the nerve cells by forming DNA adducts, protein adducts,

oxidative stress factors, mitochondrial directed apoptosis of the nerve cells as well as inhibiting their synthesis of protein, RNA and DNA ((Johnson et al., 1997; Brown et al., 2009; Ezekiel et al., 2011; Halliwell, 2007; Verm, 2005 Thrasher JD, Crawley, 2012). Aflatoxins also cause abnormalities in mitochondrial DNA, structure and function, including defective oxidative phosphorylation in the brain cells (Vermeulen et al., 2003; Thrasher JD, Crawley, 2012; Verma, 2005).

So, observed that decline of adrenaline and nor-adrenaline in rats' administration Nano-particals, it may regard to increase of oxidative stress. The oxidative stress may be also influenced oxidative metabolism in patients with major depression (Salih *et al.,* 2012). The oxidative stress may result in damage to critical cellular macromolecules such as DNA, lipids and proteins. Cellular fatty acids are readily oxidized by ROS to produce lipid peroxyl radicals, which can subsequently propagate into MDA that may interact with cellular DNA to cause DNA-MDA, adduct that may affect energy production in the brain (Vermeulen et al., 2003; Thrasher JD, Crawley, 2012; Verma, 2005).

Aflatoxins may also deplete the myelin sheath of the nerves, an important substance that covers the nerves and hence become exposed to insults. Mycotoxins especially aflatoxins have been reported to be toxic to various aspects of brain chemistry and their function (INCHEM. 1993; Thrasher JD, Crawley, 2012).

The administration of antioxidant is very critical to those exposures to aflatoxin in food. The non- traditional source of antioxidant and rich in unsaturated fatty acids and polyphenol as OWE in native form is highly beneficial to protect neuron cells of the central nerves system and brain of injuries and inflammation. NOWE have a low effect to protect against aflatoxin as an antioxidant, but the OWE was better than NOEW as a protective agent from hazard AfB1 in brain rats.

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