ORIGINAL ARTICLES

The antioxidant effect of nutritive nanoparticles extract from Olive cake residues for protection liver and kidney injuries induced by Aflatoxin B1.

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ABSTRACT

Aflatoxin B₁ is constant contaminant of human food supply in Egypt as well as worldwide. The aim of the current study was to utilize olive cake residue and its nanoparticle from olive cake residues as low cost natural sources of antioxidants able to protect against liver and kidney injuries induced by aflatoxin B₁. Both native and sonicated olive cake extract formulated from active polyphenol compounds were used in this study and particle size of sonicated extract was decreased from 486 to 1 nm in both native and sonicated olive cake extract (N, SOCE) respectively to be in nano particle size. Native and sonicated OCE were used for the protection against liver and kidney injuries resulted from the consumption of aflatoxin B₁-contaminated diet in laboratory male rats divided for six groups including the control group, group 2 received orally AFsB₁ (22 ug/kg b.w), group 3 received AFsB₁ + 0.2 NOCE, group 4 received AFsB₁ + 0.5 NOCE, group 5 received AFsB₁ + 0.2 SOCE and group 6 received AFsB₁ + 0.5 SOCE and at the end of the experiment the blood, liver and kidney samples were collected for biochemical and histopathological examinations and cleared that administration of AFsB₁ alone induced significate decrease in body wight, increase of biochemical parameter i.e liver and kidney functions accompanined with significant negatively changes of liver and kidney tissues. Treatment of rats with both NOCE and SOCE enhanced all biochemical parameters accompanied with significant positively changes of liver and kidney tissues to reach the control group and it could be concluded that NOCE was better than SOCE as active enhancement formula able to protect against liver and kidney injuries induced by AFsB₁ in rats.

Key words: Egyptian olive cake, antioxidant, Aflatoxin, nanoparticle.

Introduction

Aflatoxin B₁ (AFB₁) is the most prevalent aflatoxin usually found in cases of aflatoxicosis, and is responsible for acute toxicity, chronic toxicity, carcinogenicity, teratogenicity, genotoxicity, hepatotoxicity and immunotoxicity. The International Agency for Research on Cancer (IARC, 1993) has classified aflatoxin B₁ as a group 1 carcinogen (that means carcinogenic to humans) since 1987, and a group 1 carcinogenic agent since 1993 due to the exposure to hepatitis B virus (Castegnaro & McGregor, 1998).

(Williams et al. 2004) estimated that 4.5 billion of the world’s population is exposed to aflatoxins because they are also everywhere. Olive biophenols are most widely studied as antioxidants and free radical scavengers (Obied et al., 2005) and Mediterranean OMW is rich in these compounds (Visioli et al., 1995).

In 2002, Egypt was ranked number eight among the top olive producers worldwide, with production of 318,000 metric tons. Over the past three years, the industry has seen improvements at each stage of production, with more sophisticated mills being installed and more trees planted. The commercial value of olive cake depends on its water and fat content. Olive cake contained high polyphenol content and unique polyphenol profile, the most abundant compound being verbascoside (Visioli et al 1999). Antioxidants are anticarcinogenic and antimutagenic properties have been ascribed to these compounds, particularly with respect to aromatic polycyclic hydrocarbons (2, 7, 10, 12, 15, 16, 18, 19, 23, and 24).

The olive oil industry generates large quantities of a deleterious by-product known as olive mill waste (OMW). Being seasonal and resistant to degradation, OMW is a major problem for the development of a sustainable olive oil industry. Raw (untreated) OMW has broad spectrum toxicity against bacteria (Capasso et al., 1995), fungi (Fodale et al., 1999), algae (Della Greca et al., 2001), plants (Capasso et al., 1992; Casa et al., 2003), insects (Capasso et al., 1994) and human cells (Capasso et al., 1995). However, fractionated OMW extracts and isolated biophenols demonstrate selective or minimal toxicity (Capasso et al., 1995). Nevertheless, European research identifies OMW as a potential source for the recovery of antioxidant (Visioli et al., 1995;
Aldini et al., (2006), anti-atherogenic (Leger et al., 2000) and anti-inflammatory biophenols (Visioli et al., 1999). The European work suggested the production of bioactive compounds such as biophenols from OMW as a viable alternative for value adding to this problematic by-product (Obied et al., 2005).

The main aim of the study was to recycling of residues from olive oil industry, which represent a disposal and potentially environmental pollution problem and used as natural sources of compounds valuable natural antioxidants for protection against aflatoxicosis.

Materials:

1- Olive cake residues were purchased from Food Technology Research Institute, Agriculture Research Center, Ministry of Agriculture, Giza, Egypt.
2- Aflatoxin B₁ were purchased from Sigma Chemical Co. (St. Louis, Mo. U.S.A.), sodium benzoate as preservative of produced drink at concentration of 0.05 % and citric acid used was produced by (El.Naser chemical company).
3- Kits:
   Serum biochemical analyses: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), uric acid, creatinine, urea, total protein, albumine and total bilirubin kits were purchased from Biomerieux, Laboratory of Reagents and Products (France).
4- Experimental animals:
   Three months old, sexually mature male –rats were purchased from the Animal House Colony, National Research Center, Giza, Egypt.

Methods:

Extraction of polyphenols from olive cake:

The extraction was performed at room temperature. In the first step, the dry olive cake 200 g extracted three times with hexane (1:4 w/v) to remove the residual oil and pigments. In the second step, the polyphenols extracted from the olive cake 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 freeze-drier to powder case and then dissolved in 100 ml distilled water to be suitable for analysis and administration of rats.

Electron microscop:

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 putted on the holder and in TEM to investigate.

Sonication method:

The part of produced olive cake extract was subjected to sonication process using ultra sonic equipment model (SH 80-2L DIGITAL HEATED-MTI corporation-Eumax) for 15 min.

Total a polyphenols of olive cake extract:

Total polyphenols were determined in NOC and SOC extracts according to method described by Swain and Hillis (1959), Burns (1971) and 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 °C for 50 min. Absorbance was read at 550 nm.
Experimental Animals:

Three-months old Sprague-Dawley male rats (100-120 g) purchased from animal house colony Giza, Egypt were maintained on standard lab diet (protein: 160.4; fat: 36.3 and fibre 41g/kg ), and housed in a room free from any source of chemical contamination, artificially illuminated and thermally controlled, at the Animal House Lab., National Research Centre, Dokki, Cairo, Egypt.

Experimental Design:

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

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

The animals were observed daily for signs of toxicity and weighted as well. At the end of experimentation period (i.e. day 28), blood samples were collected from all animals from retro-orbital venous plexus for biochemical analysis. Then all animals were killed and samples of the liver and kidney tissue of each animal were removed and hydrated in ascending grades of ethanol, cleaned in xylene and embedded in paraffin. Sections (5um) thick were cut and stained with hematoxylin and eosin (H&E) (Drury et al., 1976) for histopathological study.

Statistical analysis:

All data were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System (SAS, 1982). The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio (Waller and Duncan, 1969). All statements of significance were based on probability of P < 0.05.

Results and Discussion:

Effect of sonication process on particle size of olive cake extract:

**Fig. 1:** Scanning electron microscopy of NOCE.  
**Fig. 2:** Scanning electron microscopy of SOCE

Nanotechnology provides the ability to engineer the properties of materials by controlling their size, and this has driven research toward a multitude of potential uses for nanomaterials (Bonnemann Richards 2001) nanomaterials can be modified for better efficiency to facilitate their applications in different fields such as bioscience and medicine. The olive cake extract was exposed to ultra sonic waves to fractionation of molecules to be in nanoparticle size to have a new properties and new behavior. Fig (1) showed that the particle size of native olive cake extract and cleared that the average size was 12-486 nm. Additionally the morphological examination demonstrated that the sample is composed of several morphologies including multi-twinned nanoparticles with dark segments, while Fig (2) shows the sonicated olive cake extract had average size 1-100 nm to be in nanoparticle size and gave regular spatial arrangement with light segments. Size is a main determining factor for the distribution of (inhaled or instilled) nano in and outside of the respiratory tract (Oberdörster et al., 2004). Reducing the particle size of materials is an efficient and reliable tool for improving
their biocompatibility. In fact, nanotechnology helps in overcoming the limitations of size and can change the outlook of the world regarding science (Mirkin and Taton 2000).

Table 1: Total antioxidant of NOC and SOC:

<table>
<thead>
<tr>
<th>Total Polyphenol of NOC (mg/ml)</th>
<th>Total polyphenols of SOC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.702</td>
<td>0.620</td>
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</tbody>
</table>

Table (1) shows total polyphenol of NOC and its nano particles SOC and cleared that NOC had total polyphenols 0.702 mg/ml while its nano particles decreased to be 0.620 mg/ml. Olive waste can provide a cheap source of phenolic compounds with strong antioxidant properties.

An antioxidant is a substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents the oxidation of that substrate (Halliwell 1990). For health, antioxidants are needed to prevent the formation and oppose the actions of reactive oxygen and nitrogen species, which are generated in vivo and cause damage to DNA, lipids, proteins, and other biomolecules (Halliwell 1996). Olive biophenols are biologically active compounds and contain numerous simple and complex substances that are characterized by multifunctional moieties. The simple biophenols such as vanillic acid, gallic acid, cumaric acid, caffeic acid, tyrosol and hydroxytyrosol possess alkene, alcoholic, and carboxylic groups whereas more complex biophenols such as secoiridoids (oleuropein and ligstroside) possess glycosidic and monoterpenoid units (Bianco and Uccella 2000).

Table 2: Effect of AFB1 and treatments on total body weight of experimental animals:

<table>
<thead>
<tr>
<th>No.</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>974</td>
<td>996</td>
<td>954</td>
<td>984</td>
<td>970</td>
<td>954</td>
</tr>
<tr>
<td>2</td>
<td>1.072</td>
<td>1.046</td>
<td>1.024</td>
<td>1.074</td>
<td>996</td>
<td>1.012</td>
</tr>
<tr>
<td>3</td>
<td>1.160</td>
<td>1.122</td>
<td>1.092</td>
<td>1.144</td>
<td>1.066</td>
<td>1.082</td>
</tr>
<tr>
<td>4</td>
<td>1.266</td>
<td>1.290</td>
<td>1.254</td>
<td>1.310</td>
<td>1.264</td>
<td>1.218</td>
</tr>
<tr>
<td>5</td>
<td>1.390</td>
<td>1.374</td>
<td>1.336</td>
<td>1.424</td>
<td>1.384</td>
<td>1.310</td>
</tr>
<tr>
<td>6</td>
<td>1.468</td>
<td>1.434</td>
<td>1.442</td>
<td>1.520</td>
<td>1.444</td>
<td>1.414</td>
</tr>
<tr>
<td>7</td>
<td>1.548</td>
<td>1.502</td>
<td>1.492</td>
<td>1.602</td>
<td>1.508</td>
<td>1.494</td>
</tr>
</tbody>
</table>

Rate increase

574±  77.141
506±  73.916
538±  77.788
618±  85.522
538±  83.466
540±  73.178

Table 2 shows total body weight of rats during the experimental period to study the effect of AFs alone and/or combined with treatments i.e NOCE or SOCE on body weight and from the results cleared that control group was in normality weight increase since had 974g at initial time and increased to 1.548 at end of experimental period with rate increase 574g. Administration of AFs alone induced significant weight decrease of group AFs since had 996g at initial time to 1.502 at end of experiment with rate increase 506g by deficiency weight 68g comparing with control group. Treatment of rats with NOCE or SOCE almost restores total body weight of all rat groups to reach control group especially in case treatment with high dose NOCE. Total body weight of groups treated with NOCE increased gradually from 954 to 1.492 and 984 to 1.602 with rat increase 538 and 618 for both treatments, but total body weight of groups treated with SOCE increased gradually during experimental period from 970 to 1.508 and 954 to 1.494 with rat increase 538 and 540 for both treatments respectively.

Table 3: Effect of native and sonicated olive cake extracts on serum biochemical parameters of rats:

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Uric acid mg/dL</th>
<th>Urea IU</th>
<th>Creatinine mg/dL</th>
<th>AST U/L</th>
<th>ALT U/L</th>
<th>ALP U/L</th>
<th>Albumine U/L</th>
<th>Total protein</th>
<th>Total bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>2.29± 0.315</td>
<td>36.38± 1.088</td>
<td>0.222± 0.020</td>
<td>35.34± 3.332</td>
<td>27.93± 3.634</td>
<td>102.01± 1.894</td>
<td>3.85± 0.058</td>
<td>7.54± 0.217</td>
<td>0.49± 0.029</td>
</tr>
<tr>
<td>Group 2</td>
<td>4.16± 0.317</td>
<td>41.14± 1.510</td>
<td>0.370± 0.020</td>
<td>50.15± 4.621</td>
<td>40.53± 0.792</td>
<td>129.32± 7.956</td>
<td>2.33± 0.088</td>
<td>5.23± 0.129</td>
<td>0.78± 0.045</td>
</tr>
<tr>
<td>Group 3</td>
<td>2.64± 0.073</td>
<td>31.21± 0.684</td>
<td>0.239± 0.017</td>
<td>33.91± 1.583</td>
<td>29.68± 1.007</td>
<td>127.05± 4.611</td>
<td>3.16± 0.094</td>
<td>6.97± 0.110</td>
<td>0.48± 0.024</td>
</tr>
<tr>
<td>Group 4</td>
<td>2.40± 0.095</td>
<td>25.93± 0.635</td>
<td>0.240± 0.017</td>
<td>29.85± 2.943</td>
<td>20.95± 2.667</td>
<td>114.53± 11.35</td>
<td>3.45± 0.032</td>
<td>7.28± 0.197</td>
<td>0.47± 0.024</td>
</tr>
<tr>
<td>Group 5</td>
<td>2.70± 0.292</td>
<td>30.25± 0.515</td>
<td>0.339± 0.027</td>
<td>28.62± 5.073</td>
<td>30.84± 4.196</td>
<td>125.71± 8.632</td>
<td>3.25± 0.020</td>
<td>7.22± 0.049</td>
<td>0.61± 0.021</td>
</tr>
<tr>
<td>Group 6</td>
<td>2.27± 0.057</td>
<td>27.99± 0.599</td>
<td>0.332± 0.020</td>
<td>30.95± 3.025</td>
<td>26.19± 6.130</td>
<td>125.44± 8.153</td>
<td>3.27± 0.018</td>
<td>7.28± 0.058</td>
<td>0.62± 0.012</td>
</tr>
</tbody>
</table>
In our research, animals received basal diet without any treatments appeared in a healthy case and their weight were normal from 120 to 200g during the month of study and no clinical observations were noticed and no mortality occurred in any of the group treatments. Serum biochemical analysis (Table 3) revealed that administration of AFs resulted in a significant increase in ALT, AST, ALP, creatinine, uric acid, urea and total bilirubin while, significantly decreased of albumin and total protein.

Treatments of rat groups received AFB1 with NOCE or SOCE succeeded to restore all biochemical parameters towards the control value although some of these parameters were still differ than the control values. From the obtained results cleared that uric acid of control group was 2.29 mg/dl for control group, administration of AFB1 increases uric acid to 4.16 mg/dl. Supplementation of all groups administrated with AFB1 combined with either NOCE or SOCE succeed to restore these parameters towards control value and were in range between 2.2-2.7 mg/dl. Administration of rats with AFB1 alone increased urea from 36.38 to 41.14 but treatments of all groups received AFB1 with both NOCE or SOCE succeed also to decrease of urea toward the control group and the range was in between 25.63-31.21 mg/dl as well as creatinin increased from 0.222 to 0.370 mg/dl caused by administration of AFB1 but decreased to range of control group when treated with either NOCE or SOCE to reaches the normal control and ranges were 0.239-0.339 mg/dl. Also the liver functions are very important for efficiency liver expression, from obtained results ALT, AST were 35.34 & 27.93 for normal 50.15 & 40.53 u/l for AFs groups respectively and when the groups received AFs and treated with NOCE or SOCE decreased these parameters to range of normal control and were 33.91 & 29.85 and 30.28 & 26.19 u/l. Total protein and albumin were decreased caused by administration of AFB1 to be 5.23 & 0.78 respectively and treatments with NOCE and SOCE increased these parameters to reached the control group to be in range between 6.97-7.28 and 0.47-0.62 for total protein and albumin. The ALP was 102.01 u/l for control group and increased to 129.32 u/l for group AFs, it is worthy to report that treatments with NOCE or SOCE at low and high concentration reduced ALP toward control value, also total bilirubin was increased in group AFs to be 0.781 comparing with control which was 0.490 and the treatments decreased total bilirubin to be in range between 0.480-0.624. Generally treatments with NOCE and SOCE caused increase of these parameters to reach the control group but treatment with NOCE at different concentrations was the best treatments rather than treatments with SOCE. Shyamal et al (2010) found that rats received AFB1 and treated with plant extract as antioxidants have significant antioxidant effects, which is important for removing free radicals and reactive intermediates generated from the metabolism of environmental toxins.

Increased levels of transaminases (ALT, AST) alkaline phosphatase (ALP), createnine, uric acid, γ-GT and urea may indicate degenerative changes in the hepatic tissues and biliary system (Kaplan, 1987) and necrosis or hepatocellular injury.

AFB1 is the most abundant and toxic form of all naturally occurring aflatoxins. It is teratogenic, hepatocarcinogenic and hepatotoxic to various animal species.(Bassir & Adekunle 1970 and Brien et al 1983). AFB1 is first metabolised (Phase 1 metabolism) mainly by the Cytochrome P450 enzyme (CYP450) system found in the microsomes, producing a variety of metabolites such as AFB1 epoxide and hydroxylated metabolites (AFM1, AFP1, AFQ1 and aflatoxicol). AFB1 epoxide is a very reactive and unstable metabolite of AFB1 that will bind to cellular macromolecules like DNA, RNA, lipids and proteins, leading to lipid peroxidation and cellular injury.(Stresser et al 1994) The formation of AFB1-DNA adduct is highly correlated to the carcinogenic effect in both animal and human cancers.(Thabrew & Bababunni 1980). The major AFB1-DNA adduct formed in liver is aflatoxin B1-N7-guanine.(Essigmann et al 1977) This adduct is unstable and subjected to decomposition in the rat liver, which culminates in the destruction of hepatic cells.

**Fig. 3:** Liver of control group shows normal histological structure of hepatic lobule.

**Fig. 4:** Liver of rats received AFs alone showed fatty degeneration of hepatocytes and focal hepatic necrosis associated with leucocytic cells infiltration.
The biochemical results reported in the current study were confirmed by the histological examination for the liver and kidney tissues. The histopathological examination of liver in the control group (Figs. 3 to 8) revealed normal histological structure of hepatic lobule but the group rats administrated with AFs alone without any treatments showed fatty degeneration of hepatocytes and focal hepatic necrosis associated with leucocytic cells infiltration, in such regard, Shyamal et al 2010 reported that the liver sections of rats intoxicated with AFB1 showed disturbed lobular architecture, ballooning, fatty degenerative changes and focal necrosis.

The groups treated with NOCE at low and high dose improved liver cells and showed vacuolization of hepatocytes and sinusoidal leucocytosis and vacuolization of hepatocytes for NOCE at different concentrations, but liver of rats treated with SOCE showed fatty degeneration of hepatocytes (Fig. 7) and showed vacuolization of hepatocytes (Fig. 8) for both concentrations. Concerning the histological examination of liver tissues the control group shows normal structure of nephropathy, but the group AFs showed vacuolization and congestion of capillary tufts (Fig. 9&10), while when the groups received AFs and treated with NOCE showed no histological changes (Fig. 11 & 12) for low and high concentration. Concerning the groups treated with SOCE the kidney showed slightly congestion of capillary tufts apparent normal renal parenchyma (Fig. 13 & 14) and showed apparent normal renal parenchyma for both concentrations of SOCE. From histological examinations cleared that NOCE improved the liver and kidney tissues rather than SOCE.

**Conclusion:**

From the obtained results concluded that the 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.
Fig. 9: Kidney of control group showed normal histological structure of nephropathy.

Fig. 10: Kidney of rats received AFs alone showed vacuolization and congestion of capillary tufts.

Fig. 11: Kidney of rats received AFs+0.2 ml NOCE shows no histological changes

Fig. 12: Kidney of rats received AFs+0.5 ml NOCE shows no histological changes

Fig. 13: Kidney of rats received AFs+0.2 ml SOCE shows slightly congestion of capillary tufts apparent normal renal parenchyma

Fig. 14: Kidney of rats received AFs+0.5 ml SOCE apparent normal renal parenchyma.

Acknowledgments

We wish to express my sincere thanks, deepest gratitude and appreciation to Prof. Dr. Khayria Naguib, Prof. of Food Toxicology and Contaminants, NRC, Egypt, for continued assistance, guidance, unlimited helps to complete this work.

References


