HEPATO-CURATIVE AND REGENERATIVE POTENTIALS OF WILD OLIVE (*Olea ferruginea*) FRUIT PULP EXTRACTS AGAINST FLUORIDE-INDUCED TOXICITY IN MICE: A HISTOPATHOLOGICAL STUDY

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ABSTRACT: Ameliorative potentials of wild olive fruit pulp extract (WOFPE) were compared with vitamin E against the hepato-histopathological responses of fluoride exposure (50 ppm in drinking water) in mice. Forty adult males were divided equally into 4 groups: control (C), fluoride (F), fluoride+vitamin E (FE), and fluoride+olive (FO). The mice in the C group were provided F-free drinking water throughout the 15-day study period. The animals in the other 3 groups were given 50 ppm of F^- (from NaF) in drinking water for 10 days followed by F-free water for the next 5 days. Additionally, the FE and FO groups were given vitamin E (60 µg in 0.1 mL corn oil) or 0.1 mL WOFPE, respectively, by gavage 12-hourly on days 11–15. Liver samples were obtained from the animals on day 16 after euthanasia. In contrast to the control group, hepato-histopathological signs observed in the F group included peri-central intra-lobular focal hepatic lesions (PIFHL), misaligned hepatic cords with little or no sinusoidal spaces in-between them, and, in the hepatocytes, enlarged nuclei with vague margins and cytoplasmic vacuolations. Although aggregations of oval stem cells were seen in the FE group, PIFHL were also visible. The PIFHL were found to be completely healed in the FO group, and, in addition, large number of oval cells were also seen, both in clumped aggregations and defused between the hepatic cords. Moreover, the juvenile hepatocytes were found to be aligning to produce nascent hepatic cords and epithelial cells were also seen infesting the intra-lobular spaces among the nascent hepatic cords. Analysis of the micrometric data indicated a significant difference ($p<0.05$) in the mean cross-sectional area (MCSA) of the hepatocytes in the F (498.8 μm^2) and FO (350.8 μm^2) groups. Moreover they also differed significantly ($p<0.05$) from the C (419.5 μm^2) and FE groups (404.5 μm^2). The mean sinusoidal breadth in all four groups (C: 8.62 µm, F: 1.9 µm, FE: 5.02 µm and FO: 3.19 µm) differed significantly ($p<0.05$) from each other. These findings indicate that subchronic exposure to 50 ppm F in drinking water may cause hepatocytic damage and hepatolobular derangements while WOFPE had better curative and regenerative potentials than a standard natural antioxidant (vitamin E) against these toxicological manifestations.

Keywords: Fluoride; Histopathology; Liver; Olive; Strawberry.

INTRODUCTION

Contamination by the fluoride ion (F) has resulted in various health issues and one of its major detrimental effects is the induction of oxidative stress.[1] In this context, fluoride exposure has been found to cause hepatotoxicity through its effect on the levels of free oxygen radical scavenging enzymes (catalase and

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superoxide dismutase) in liver.[2-5] Medicinal plants are being frequently investigated for their remedial potentials against toxicologic and pathological manifestations.[6,7] It has already been established that plants rich in certain medicinal compounds such as anthocyanines, phytophenols, and flavonoids harbor excellent antioxidant and disease combating potentials.[6-12] The benefits of olives have been known for centuries and it has been traditionally used to prevent and treat different diseases that include hypertonia, arteriosclerosis, rheumatism, gout, diabetes mellitus, hypoglycemia, hypertension, atherosclerosis, vasoconstriction, and inflammation.[13-16] These health benefits of wild olives are generally attributed to their precious medicinal ingredients especially antioxidants such as phenols, triterpenes, and flavonoids.[17-18] Keeping in view these reported health benefits, wild olives were selected for the investigation of the ameliorative potential of their fruit pulp extracts against F-induced hepato-histopathology in comparison with vitamin E, a standard natural antioxidant.

MATERIALS AND METHODS

Animal care and groups: Forty, 10–12-week-old, male mice of 28–30 g weight were placed randomly (10 individuals each) into control (C), fluoride (F), fluoride+vitamin E (FE), and fluoride+olive (FO) groups. The C group mice were maintained without any treatment for 15 days. The mice in the F, FE, and FO groups were given 50 ppm F ions (from NaF) in drinking water for 10 days followed by F-free water (Khush Aab- PSQCA Licence No: CML/N-137/11, the certified mineral water product of University of Sargodha) for the next 5 days. The FE group mice received 60 μg vitamin E (diluted in 0.1 mL corn oil) in the last 5 days on a 12-hourly basis by gavage while the FO group mice received 0.1 mL wild olive fruit pulp extract (WOFPE) instead. The experimental design of this study strictly adhered to the institutional and national guidelines for laboratory animal care, maintenance and experimental use. The standard animal housing protocols included cyclic dark/light periods of 12 hr duration and 24/7 free access to food and water while the ranges of temperature and humidity were 22±2ºC and 40–44%, respectively.

Fluoride and vitamin E dilutions: Stock solution of 1000 ppm F ions was prepared by dissolving 2.25 g of laboratory grade crystalline NaF in 1000 mL of fluoride-free mineral drinking water. Soft gelatin capsules (brand name Evion) containing 200 mg Tocopherol (in 1 mL oil base solvent) from Merck Pakistan Private Limited (License No. 000043, Reg. No. 008754) were used a source of vitamin E. Dilutions were made in water and oil respectively of F and vitamin E employing the formula $C_i \times V_i = C_f \times V_f$ where C_i = initial concentration, V_i = initial volume, C_f = final concentration, and V_f = final volume.

Preparation of the wild olive fruit pulp extracts Fully ripe wild olive *(Olea ferruginea)* fruits were collected from Soon Valley (District Khushab, Pakistan). The fruits were thoroughly washed, air dried, and crushed gently in a ceramic mortar and pestle to separate the seeds from the pulp. One hundred g of pulp was finally crushed in a blender with 100 mL F-free water for 15 min. The thick juicy material obtained was centrifuged at 5,000 rpm for 10 min. The transparent darkly

colored supernatant obtained in this way was stored at –30ºC in Eppendorf® tubes (1 mL capacity) for experimental use.

Organ recovery and histological preparations: The livers from animals of all the groups were collected intact by abdominal incision after euthanasia on day 16 of the study. An appropriately sized piece (3 mm^3) from each liver was obtained from the medial lobe and immediately fixed in formol acetic alcohol (60 mL absolute ethanol + 35 mL 10% formaldehyde + 5 mL glacial acetic acid) for 24 hr, and finally placed in 90% ethanol (1–3 hours). After complete dehydration in absolute ethanol (1–3 hours), clearing was achieved in xylene (1–2 hours). Embedding was carried out in molten histological wax (at 56–58ºC) for 2–3 hours. Serial sections (5 µm) were obtained on a rotary microtome and stained with hematoxyline and eosin.

Digital photography and processing: Digital photomicrographs (400×) of the selected histological sections from all groups were obtained on a Labomid (CXR_2) trinocular microscope in an attached digital camera (Sony: DSC-W35). These snapshots were used for digital micrometry using photo-shots (400×) of the stage micrometer for calibration. Selected photomicrographs were also processed for color, contrast, cropping, labeling, etc. to highlight the histopathological changes in various groups in CoralDRAW11.

Data measurements and statistical applications: To obtain the average cross-sectional area (CSA) of the hepatocytes, 100 randomly selected cells were measured for each individual in each group of 10 mice. The 10 average CSA values thus obtained for each group (one for each member of the group) were then used to calculate the mean CSA±SEM for each group. The diameter of each randomly selected hepatocyte was digitally measured from two perpendicular lines and the values obtained were placed in the following formula to calculate the CSA.

$$\text{Cross-sectional area of the hepatocyte (CSA)} = \pi \times \frac{D_1}{2} \times \frac{D_2}{2}$$

where π = pi, D_1 = cell diameter 1, and D_2 = cell diameter 2.

Similarly, 100 measurements of sinusoidal breadth were obtained for each group of 10 mice (10 from each animal). The 10 average sinusoidal breadth values thus obtained for each group (one for each member of the group) were then used to calculate the mean sinusoidal breadth±SEM for each group. These micrometric data were subjected to statistical applications of ANOVA (one way) and Duncan's Multiple Range Test with the help of the Softonic SPSS-20 software.

RESULTS

Histological observations: Signs of healthy hepato-histological dispositions, i.e., the central vein surrounded by radiating hepatic chords containing hepatocytes with centrally placed rounded nuclei were clearly visible in the control liver slides, while the sinusoidal spaces of almost uniform breadth were easily recognizable between the adjutant hepatic cords (Figure 1A). The histopathological signs of

fluoride exposure included irregularly shaped hepatic cords containing enlarged hepatocytes showing cytoplasmic vacuolations and irregularly shaped nuclei that caused the sinusoids to shrink and peri-central intra-lobular hepatic lesions. Signs of apoptosis, including diffused nuclei, shrunken cytoplast and vague cellular margins, were observed in the hepatocytes that were present near and around the central lobular vein (Figure 1B).

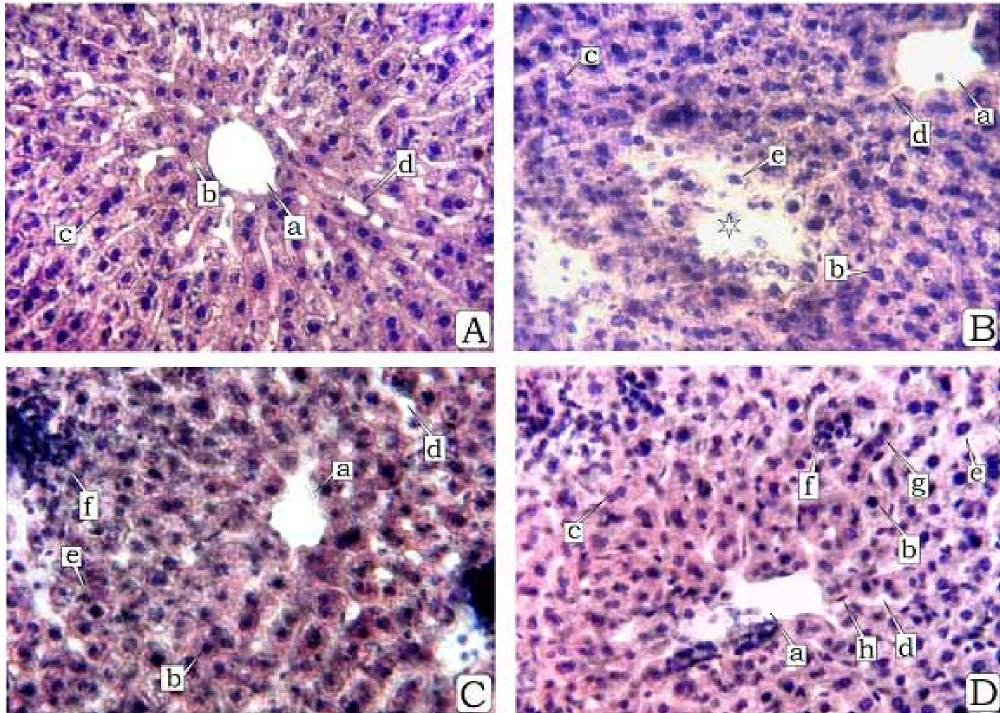

Figure 1A, B, C, and D. Selected sections of hepatic lobules from the control (C, Figure 1A), fluoride (F, Figure 1B), fluoride+vitamin E (FE, Figure 1C), and fluoride+olive (FO, Figure 1D) groups (400×). Star: peri-central intra lobular hepatic lesion; a: central lobular vein; b: mono-nuclear hepatocyte; c: binuclear hepatocyte; d: sinusoidal space; e: hepatocyte apoptosis; f: hepatic pro-generator cells; g: nascent hepatic cord; h: epithelial cells.

Although, in the FE group signs of hepatocytic proliferation were seen indicating rejuvenation of the hepatic tissues, nevertheless, in scattered places, apoptotic signs were also visible in hepatocytes and the hepatic cords were poorly aligned (Figure 1C). In the FO group, signs of recovery from the toxicological effects of fluoride exposure were clearly identifiable with normalization of hepatocytic cytoplasts and recognizably widened sinusoidal spaces. Obvious signs of hepatocytic proliferation, the presence of large number of oval cells and binuclear hepatocytes, and the emergence of nascent hepatic cords were clearly visible (Figure 1D).

Mean cross-sectional area of the hepatocytes: Analysis of the data employing ANOVA suggested highly significant variations among the groups ($p<0.001$). The *post hoc* analysis showed a significant difference ($p<0.05$) between the F and FO

groups compared to each other and as well as a significant differences ($p<0.05$) between both the F and FO groups and the C and FE groups (Table 1).

Breadth of sinusoids: The analysis of the data through ANOVA suggested a highly significant variation among the groups ($p<0.001$). The *post hoc* analysis indicated a significant ($p<0.05$) variation between any two groups which were compared (Table 1).

Table 1. Micrometric variations of the cross-sectional area of the hepatocytes and the mean sinusoidal breadth among the groups (Values are mean±SEM, n=10)

Histometric parameter	Group			
	Control	Fluoride	Fluoride + vitamin E	Fluoride + Olive
Cross-sectional area of hepatocytes (μm^2)	$419.5 \pm 16.75^{*,a}$	$498.8 \pm 23.8^{*,b}$	$404.5 \pm 25.05^{*,a}$	$350.8 \pm 22.2^{*,c}$
Sinusoidal breadth (μm)	$8.62 \pm 0.21^{*,a}$	$1.9 \pm 0.24^{*,b}$	$5.02 \pm 0.23^{*,c}$	$3.19 \pm 0.2^{*,d}$

Comparing, for the same histometric parameter, the control, fluoride, fluoride+vitamin E, and fluoride+olive groups: *$p<0.001$; comparing, for the same histometric parameter, any two groups not sharing a common lower case superscript, $^{a, b, c,}$ or ^{d}: $p<0.05$.

DISCUSSION

Evidence indicates that fluoride has the potential to cause hepatotoxicity.[19] The results of the present study indicate that particular histopathological signs occur with fluoride exposure such as peri-central intra-lobular hepatic lesions, complete obliteration of sinusoidal breadth in most of the sinusoids, and enlargement of individual hepatocytes because of a concurrent increase in cytoplasmic vacuolations together with simultaneous nuclear shape distortions and enlargements. Although post treatment with the hepatoprotective antioxidant vitamin E resulted in regenerative histological signs with the appearance of clumps of oval (hepatoblastic) cells, the hepatotoxicity signs of cytoplasmic vacuolations and shrunken sinusoids were still appreciable as indicated in the histological sections and micrometric data. In the WOFPE treated group, the rejuvenation of the hepatic architecture was quite evident with the presence of structurally normal hepatocytes and large numbers of epithelial and hepatoblastic oval cells.[20-21] The micrometric findings of cross-sectional area of hepatocytes and sinusoidal breadth also support the amelioration of the histopathological signs of fluoride exposure with WOFPE post treatment. These findings suggest the hepatoprotective and regenerative potentials of WOFPE against fluoride exposure are significantly better than those of the natural antioxidant vitamin E. The results fully support exploring the potential health benefits of wild medicinal fruits for preventing or treating the hepatoxicity of hazardous environmental chemicals.

CONCLUSION

The results indicate that wild olive fruit pulp extracts possess significant hepatoprotective and regenerative potentials against fluoride-induced hepato-histopathologies and that they can provide better protection against chemically induced histopathology of the liver than the natural antioxidant vitamin E.

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