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Attenuation of arsenic trioxide induced cardiotoxicity through flaxseed oil in experimental rats

Mathews V. Varghese[a], M. Abhilash[a], Manju Alex[a], M. V. Sauganth Paul[a], A. Prathapan[b], K. G. Raghu[b] and R. Harikumaran Nair[a]

[a]School of Biosciences, Mahatma Gandhi University, Kottayam, India; [b]Biochemistry and Molecular Mechanism Laboratory, Agroprocessing and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology (NIIST), Trivandrum, India

ABSTRACT

Objectives: Arsenic trioxide (As_2O_3) is a potent drug for acute promyelocytic leukaemia, but its clinical trials are allied with some serious adverse events mainly cardiac functional abnormalities. So the objective of our investigation is to identify the cardioprotective action of flaxseed oil (FSO), a natural compound against As_2O_3 induced cardiotoxicity.

Methods: Male wistar rats were treated with As_2O_3 (4 mg/kg) to induce cardiotoxicity. FSO (250 and 500 mg/kg) was given in combination with As_2O_3 for evaluating its cardioprotective efficacy.

Results: Treatment with As_2O_3 resulted in deposition of arsenic in heart tissue, increased cardiac marker enzymes release, lipid peroxidation (LPO), oxidative insults and pathological damages in the heart. Co-treatment with FSO (500 mg/kg) significantly reduced the arsenic accumulation, cardiac marker enzymes, LPO and cardiac structural alterations. FSO treatment significantly improved cardiac glutathione content, antioxidant enzymes and reduced the pathological damages in cardiac tissue. Gas chromatographic–mass spectrometry analysis revealed that the major fatty acid content in the FSO is alpha-linolenic acid, which has a strong milieu in cardiac health.

Conclusion: The results of the current investigation suggested that FSO is an effective agent in reducing arsenic-induced cardiac toxicity and can be used as an adjunct/dietary supplement for the cancer patients on As_2O_3 therapy.

Introduction

Arsenic has been recognized as an environmental toxicant as well as a medicinal compound throughout human history [1]. Human populations exposed to arsenic were related with cancers of skin, bladder, kidney, liver and lung [2]. Despite its reputation as a carcinogen, arsenic has been used medicinally for thousands of years [3]. The Food and Drug Administration (U.S.A.) approved arsenic trioxide (As_2O_3) for the treatment of relapsed and refractory acute promyelocytic leukaemia [4]. But the use of As_2O_3 is hampered by its cardiotoxicity characterized by action potential variations, QT interval prolongation, torsades de pointes and even sudden cardiac death [5,6]. Enhanced oxidative stress in cardiac tissue due to arsenic deposition was a major etiology of arsenic-induced cardiotoxicity [7]. Now a day, dietary supplements in the form of functional foods/nutraceuticals has been receiving lots of attention in the mitigation of cardiotoxicity induced by chemotherapeutic drugs.

Flax (*Linum usitatissimum* Linn.) belongs to the family *Linaceae* has a widespread history of conventional use both as a source of oil and fiber and is developed for commercial applications in over 30 countries of the world [8]. Flax seed oil (FSO) has a history of dietary usage in Europe and Asia for its potential health benefits, which comprise anticancer, bactericidal, anti-inflammatory, laxative and anti-atherogenic effects [9]. FSO is the richest plant source of polyunsaturated fatty acid alpha-linolenic acid (ALA) [8]. ALA (18:3 n-3, n-3 fatty acid), a precursor of eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) plays an active role in the cell membrane integrity [10]. The plant-based n-3 poly unsaturated fatty acids (PUFA) especially omega-3 fatty acids are reported to have protective effects against cardiovascular disease, cancer as well as metabolic syndromes such as obesity and diabetes [9,11].

Attenuating As_2O_3 induced toxic effects in the heart would have a tremendous impact on anti-cancer treatment. Therefore the present study was conducted *in vivo* to investigate the protective effect of FSO against the cardiac toxicity induced by As_2O_3.

Materials and methods

Chemicals and reagents

As_2O_3, sodium pyruvate, thiobarbituric acid, triton X-100, phenazine methosulphate, nitroblue tetrazolium, glutathione (GSH) and 5,5-dithio-bis 2-nitrobenzoic acid were purchased from Sigma Aldrich (Bangalore, India). L-aspartate, α-oxoglutarate, thiobarbituric acid and nicotinamide adenine dinucleotide phosphate were from Merck Specialties Pvt. Ltd. (Mumbai, India). All other chemicals used were of analytical grade and were purchased from Central Drug House (Pvt.) Ltd, Mumbai, India. FSO was obtained from local farmers of Lucknow, India and authenticated by taxonomist from Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, India.

Preparation of flax seed extract

Linum usitatissimum seeds were thoroughly washed using running tap water followed by rinsing with distilled water,

shade-dried and powdered. The soxhlet extraction procedure was carried out using n-hexane [12] and the yield of FSO obtained was 21.64 percentage (%).

Identification of fatty acid composition in FSO using GC–MS

Acid catalyzed trans-esterification reaction was followed for preparation of fatty acid methyl esters (FAME). Twenty milligram of oil sample was dissolved in 1 ml of hexane and 3 ml of sulphuric acid in methanol (2%) was added and reflux condensation was done for 3 hours at 100°C. Thin layer chromatography technique was performed to identify the reaction completion with the solvent system, namely, hexane, diethyl ether and acetic acid in the proportion of 80:20:1. The FAME was analyzed by using Shimadzu GC–MS model GC-17A equipped with mass spectrometer GC–MS QP 5050A (Shimadzu Corporation, Tokyo, Japan). Analysis was performed in triplicates with FSO. Each FAME peak was identified with its standard FAME Peak.

Animals and experimental groups

Wistar strain male albino rats weighing 200 ± 20 g were obtained from Small Animal Breeding Station of Govt. Veterinary College, Mannuthy, India. The animals were acclimatized for 7 days before commencement of the experiment. Protocol used in this study for the use of animals was approved by the Institutional Animal Ethics Committee, School of Biosciences, Mahatma Gandhi University, Kottayam, India (Approval No. B1662009/2).

To study the effect of FSO on As_2O_3 induced cardiac toxicity, thirty rats were divided into five groups with six in each group. Control rats fed with normal diet; Second group were administered with FSO 500 mg/kg body weight; Third group were administered with As_2O_3 4 mg/kg body weight; Fourth group were administered with As_2O_3 4 mg/kg body weight and FSO 250 mg/kg body weight; and the fifth group were administered with As_2O_3 4 mg/kg body weight and FSO 500 mg/kg body weight. The route of administration of As_2O_3 and FSO was through oral intubation daily for a period of 45 days was selected based on our previous study [13].

Consequently, after 24 hours of the last treatment, blood was drawn from the orbital sinus of the eye of individual rats and then animals were sacrificed. The serum samples obtained was used for biochemical assays. Heart removed immediately and a portion was homogenized in ice cold Tris–HCl buffer (0.2 M, pH 7.4). The homogenate was centrifuged at 11 200g for 20′ at 4°C and the supernatant was used for the detection of lipid peroxidation (LPO) and antioxidant status.

Detection of arsenic accumulation in heart tissue

A portion of the heart tissue was digested by thermal acid microwave digestion and diluted with double distilled water. Total arsenic concentration was analysed by standard inductively coupled plasma-optical emission spectroscopy (Optima 2000 DV ICP-OES, Perkin Elmer, Inc. U.S.A.).

Biochemical analysis

Creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), triglycerides and total cholesterol in the serum were detected by using commercially available kits (Span Diagnostic Ltd., India) and are analysed with the help of semi auto analyzer-BCA201 (Recorders & Medicare Systems (P) Ltd. Chandigarh, India).

LPO was measured by estimating using thiobarbituric acid reacting substance (TBARS) according to the method of Beuge and Aust [14]. Reduced GSH was determined as a measure of total thiols status by the method of Ellman [15]. Glutathione peroxidase (GPx) was determined by measuring the decrease in GSH content after incubating the sample in the presence of hydrogen peroxide (H_2O_2) and sodium azide [16]. Tissue catalase (CAT) was determined from the rate of decomposition of H_2O_2 [17]. Glutathione-S-transferase (GST) was determined from the rate of increase in conjugate formation between GSH and 1-Chloro-2,4-dinitrobenzene (CDNB) [18]. Superoxide dismutase (SOD) was measured by the method of Kakkar et al. [19].

Histopathology

Heart tissues were fixed in 10% neutral-buffered formalin and the processed sections were stained routinely with hematoxyline and eosin. Stained slides were examined using microscope (Motic AE 21, Wetzlar, Germany). The microphotographs were taken using Moticam-1000 (Burtons Medical Equipment Ltd, Kent, UK) at the original magnification of 100×. Fibre swelling, interstitial oedema, capillary congestion and micro-haemorrhages in myocardial tissue were scored from 0 to 5 as follows: 0 = no histopathological changes; 1+ = histopathological changes involving <25%; 2+ = histopathological changes involving 25–50%; 3+ = histopathological changes involving 50– 75%; and 4+ = histopathological changes involving 75–100%.

Statistical analysis

The data were analyzed using the statistical package program SPSS/PC+ version 18 (SPSS Inc. Chicago, IL, U.S.A.). Statistical analysis between different groups were performed with one-way ANOVA followed LSD post hoc multiple comparison test. All data were expressed as means ± standard deviation (SD). The results were considered significant at $P < 0.05$.

Results

Fatty acid composition of FSO using GC–MS revealed that FSO contains 8.93% of palmitic acid (C16:0), 5.54% of stearic acid (C18:0), 23.97% of oleic acid (C18:1), 14.62% of linoleic acid (C18:2) and 46.94% of ALA (C18:3). The highest concentration of fatty acid was found to be unsaturated fatty acid, linolenic acid (Figure 1 and Table 1).

Table 1. Fatty acid composition of FSO.

Peak	Retention Time	Area	Height	Concentration (%)
1 Palmitic acid	15.894	123 347	8624	8.928
2 Stearic acid	18.633	76 563	4977	5.542
3 Oleic acid	20.122	331 158	20 994	23.970
4 Linoleic acid	22.547	201 951	13 039	14.618
5 ALA	26.046	648 543	40 773	46.943
Total		1 381 562	88 407	100.000

Values for fatty acid distribution peaks, $n = 3$.
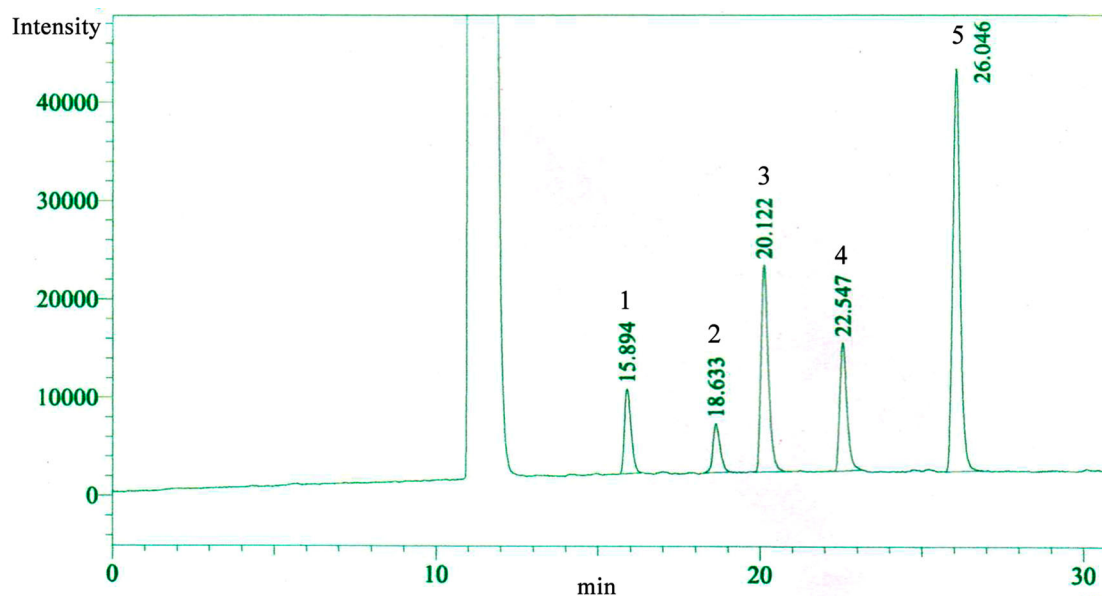
**Figure 1.** GC–MS profiling of flax seed oil: fatty acid distribution peaks, $n = 3$.

The concentration of arsenic in the rat heart tissues was determined and the data shown in Figure 2. Increased deposition of arsenic was identified in the cardiac tissue of As_2O_3 (4 mg/kg body weight) alone administered rats. Co-treatment with FSO (500 mg/kg body weight) in As_2O_3 treated rats showed decreased level of arsenic deposition than As_2O_3 alone treated rats (Figure 2). But the rats treated with lower dose of FSO (250 mg/kg body weight) did not exhibit any significant reduction in arsenic deposition when compared to As_2O_3 alone treated rats.

The levels of cardiac marker enzymes CK-MB and LDH were significantly elevated in the serum of As_2O_3 alone given rats (Table 2). As_2O_3 exposed rats supplemented with and FSO (500 mg/kg) have shown a significant decrease in the levels of these enzymes. On the other hand, rats administered with FSO alone didn't exhibit any change in the activities of CK-MB and LDH when compared to control rats. Moreover, As_2O_3 and FSO both individually and in combination treatments did not show any significant alterations in serum cholesterol and triglyceride concentrations (Figure 3).

As_2O_3 treatment induced LPO in the heart tissues of rats as indicated by elevated levels of TBARS (Table 3). Administration of FSO (500 mg/kg) significantly reduced As_2O_3 induced LPO in the heart which indicates the antiperoxidative potential of FSO. Table 3 shows the activities of antioxidant enzymes and the concentration of non-enzymatic antioxidant GSH (total thiols) in the control and treated rats. FSO (500 mg/kg) in combination with As_2O_3 for 45 days significantly elevated the GSH content in the heart tissues of rats along with increased activities of antioxidant enzymes such as CAT, SOD, GST and GPx.

Histological examination revealed cardiac structural abnormalities in As_2O_3 alone administered rats i.e. fibre swelling, interstitial oedema, fibre separations, capillary congestion and micro-haemorrhages (Figure 4(c)). There was only minor swelling and reduced capillary congestion in cardiac muscle fibres in As_2O_3 and FSO (500 mg/kg) co-treated group (Figure 4(e)). But the As_2O_3 and FSO (250 mg/kg) co-treated group did not show a significant pathological recovery (Figure 4(d)). The control groups showed normal cardiac architecture (Figure 4(a,b)). According to histopathological
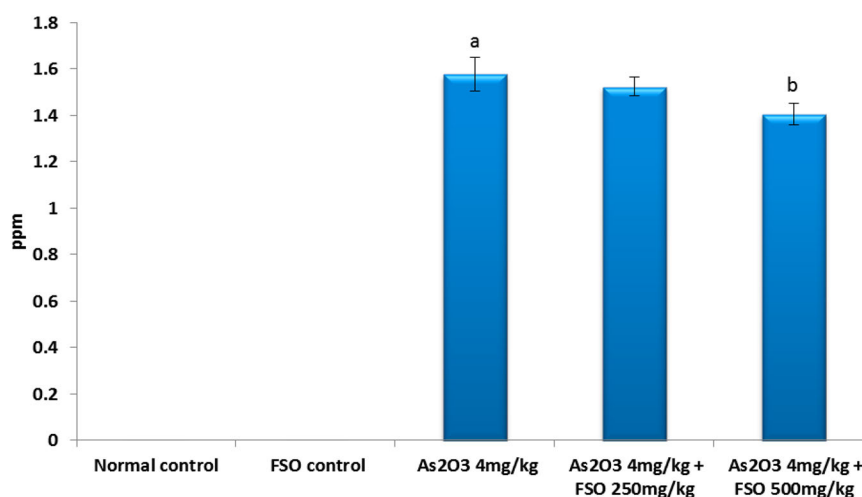
Figure 2. Effect of FSO on arsenic deposition in heart: data represented as mean ± SD, $n = 6$. [a]$P < 0.05$ vs. normal control, [b]$P < 0.05$ vs. As_2O_3 (4 mg/kg b.wt).

Table 2. Cardiac marker enzymes.

Parameters	Normal control	FSO control	As$_2$O$_3$ 4 mg/kg	As$_2$O$_3$ 4 mg/kg + FSO 250 mg/kg	As$_2$O$_3$ 4 mg/kg + FSO 500 mg/kg
CK-MB	25.83 ± 2.32	25.33 ± 2.16	55.83 ± 3.19[a]	51.67 ± 2.58	43.33 ± 2.8[b]
LDH	585.17 ± 10.93	582.17 ± 10.83	919.33 ± 12.72[a]	846.33 ± 11.02[b]	803.5 ± 12.08[b]

CK-MB (U/l), LDH (IU/l); Data represented as mean ± SD, $n = 6$.
[a]$P < 0.05$ vs. normal control.
[b]$P < 0.05$ vs. As$_2$O$_3$ (4 mg/kg b.wt).

scoring the FSO concentration 500 mg/kg was found significant in combating As$_2$O$_3$ toxicity (Table 4).

Discussion

The fatty acid composition of FSO obtained in the present study was in the following order; ALA > oleic acid > linoleic acid > palmitic acid > stearic acid. High amount of n-3 PUFAs have beneficial effect on cardiovascular health, cancer as well as metabolic syndromes [9]. The bioactive omega-3 fatty acid, ALA (46.94%) was found as the fatty acid constituent of highest concentration in FSO. ALA in flaxseed can be metabolized into DHA and EPA [10]. Meta-analysis of both dietary and biomarker studies also suggests that unsaturated fatty acid, ALA consumption is associated with a lower risk of cardiac damage [20].

In the present investigation, the administration of As$_2$O$_3$ caused a significantly increased deposition of arsenic in the heart tissue. Arsenic concentration in heart might cause direct myocardial injury, cardiac arrhythmias, and cardiomyopathy [21]. The deposition of arsenic in heart tissue may be due to the failure of its detoxification mechanism. Arsenic, by binding strongly to sulfhydryl groups, can block various enzymes and depletes GSH stores, therefore creating increased negative effects of free radical generation in higher amounts [22]. Omega-3 fatty acids in FSO showed protective effects against free radical production by regulating the fluidity of cell membrane and reducing oxidative stress [23]. In the present study, FSO treatment improved the GSH content and antioxidant enzymes. The beneficial effects of FSO may be due to its effective detoxification capacity [24] and the removal of accumulated arsenic from the cardiac tissue. Moreover, FSO administration did not induce any significant alteration in total cholesterol and triglyceride levels in the serum. This indicates that the consumption of FSO not resulted in any hyperlipidemia.

Different mechanisms have been accounted for the As$_2$O$_3$ toxicity. It can produce a wide variety of ROS including superoxide, singlet oxygen, peroxyl radical, nitric oxide, H$_2$O$_2$, dimethylarsinic peroxyls and also the dimethylarsinic radicals [22]. These free radicals can induce cellular toxicity, tissue damage and release cardiac marker enzymes into the systemic circulation. Our observation also support above fact as evidenced from increased levels of cardiac marker enzymes CK-MB and LDH in the serum of As$_2$O$_3$ alone treated rats. Omega-3 fatty acid in FSO may have a significant role in cardiac protection by improving the antioxidant status and the effective arsenic removal [7]. In the current investigation, treatment with FSO reduced As$_2$O$_3$ toxicity possibly by reducing the oxidative stress and the arsenic deposition.

Accumulation of LPO products can bring cell apoptosis, cell necrosis, as well as tissue damage. Despite the lack of specificity of the TBARS test, it continues to be described as an exact measure of malondialdehyde for LPO [14]. Lipid peroxides may cross-link with membrane proteins or phospholipids to form polymers and these polymers can induce abnormal morphology and malfunction of cells [25]. n-3 PUFA, ALA can prevent cell membrane damage from peroxidation products and oxidative stress. Membrane incorporation of PUFAs may reduce cellular susceptibility to LPO, alter membrane fluidity, enhance receptor function, elevate enzyme activity and influence the production of lipid mediators. FSO may transfer hydrogen to peroxyl free radical and can control the LPO [26,27]. In line with these reports; our observations demonstrate that FSO has an alleviating effect on arsenic-induced oxidative stress by preventing LPO and by activating antioxidant enzymes. The beneficial effects may probably by preventing the free radical conjugation with the methylene groups of fatty acids in membrane [28]. This may be the reason for reduced LPO and increased activities of antioxidant enzymes in FSO treated rats.

SOD, CAT, and GPx play key roles in the regulation of oxidant/antioxidant balance in the cell. Arsenic, by down-regulating cellular antioxidant defense system, leads to LPO and oxidative stress which results in apoptosis [22]. FSO has the
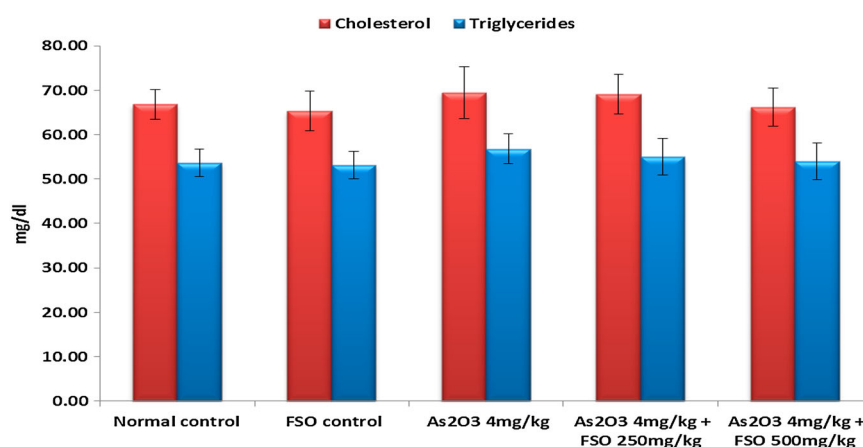
Figure 3. Effect of FSO and As$_2$O$_3$ on serum cholesterol and triglyceride: data represented as mean ± SD, $n = 6$.

Table 3. Antioxidant status of heart.

| Parameters	Normal control	FSO control	As$_2$O$_3$ 4 mg/kg	As$_2$O$_3$ 4 mg/kg + FSO 250 mg/kg	As$_2$O$_3$ 4 mg/kg + FSO 500 mg/kg
TBARS	0.73 ± 0.03	0.70 ± 0.03	0.90 ± 0.03[a]	0.87 ± 0.02	0.79 ± 0.03[b]
SOD	7.35 ± 0.43	7.37 ± 0.38	5.33 ± 0.31[a]	5.98 ± 0.2[b]	6.23 ± 0.31[b]
GPx	3.94 ± 0.23	4.1 ± 0.22	2.11 ± 0.29[a]	2.30 ± 0.25	2.99 ± 0.12[b]
CAT	20.41 ± 1.33	20.74 ± 1.16	11.35 ± 0.98[a]	14.84 ± 0.9[b]	16.03 ± 1.39[b]
GSH	6.64 ± 0.45	6.94 ± 0.46	3.75 ± 0.31[a]	4.05 ± 0.29	5.78 ± 0.22[b]
GST	3.98 ± 0.27	4.01 ± 0.28	1.57 ± 0.13[a]	1.87 ± 0.16	2.71 ± 0.21[b]

TBARS (μM/mg protein), SOD (U/mg protein), GPx (μg of GSH consumed/min/mg protein), CAT (μ moles of H$_2$O$_2$ consumed/min/mg protein), GSH (μM/g tissue), GST (μM of CDNB-GSH conjugate formed/min/mg protein); data represented as mean ±SD, $n = 6$.
[a]$P < 0.05$ vs. normal control.
[b]$P < 0.05$ vs. As$_2$O$_3$ (4 mg/kg b.wt).

potential to prevent cyclophosphamide-induced decrease in GSH and GPx in mice [24]. In hyperglycemia also, FSO supplementation significantly reduced LPO and increased the GSH content, and thereby alleviated the membrane dysfunction of human erythrocytes [23]. The use of FSO is a very cost effective approach than DHA in alleviating the cardiotoxicity of As$_2$O$_3$ [7]. In the current investigation, FSO treatment increased the activities of antioxidant enzymes and total thiol levels in cardiac tissues, suggesting the improved antioxidant defense in cardiac tissues. The potent antioxidant activity of FSO may be due to the rich amount of ALA, which can suppress ROS generation by enhancing antioxidant enzyme activity. It is also reported that enhanced antioxidant enzyme activity could reduce the arsenic-induced free radical release and thereby restore the cellular integrity [29]. In our study, structural abnormalities induced by arsenic in cardiac tissue were significantly reduced with FSO treatment. It is clear that FSO mediated cellular defense mechanism effectively reduced arsenic toxicity and there by reduced the cardiac tissue damages.
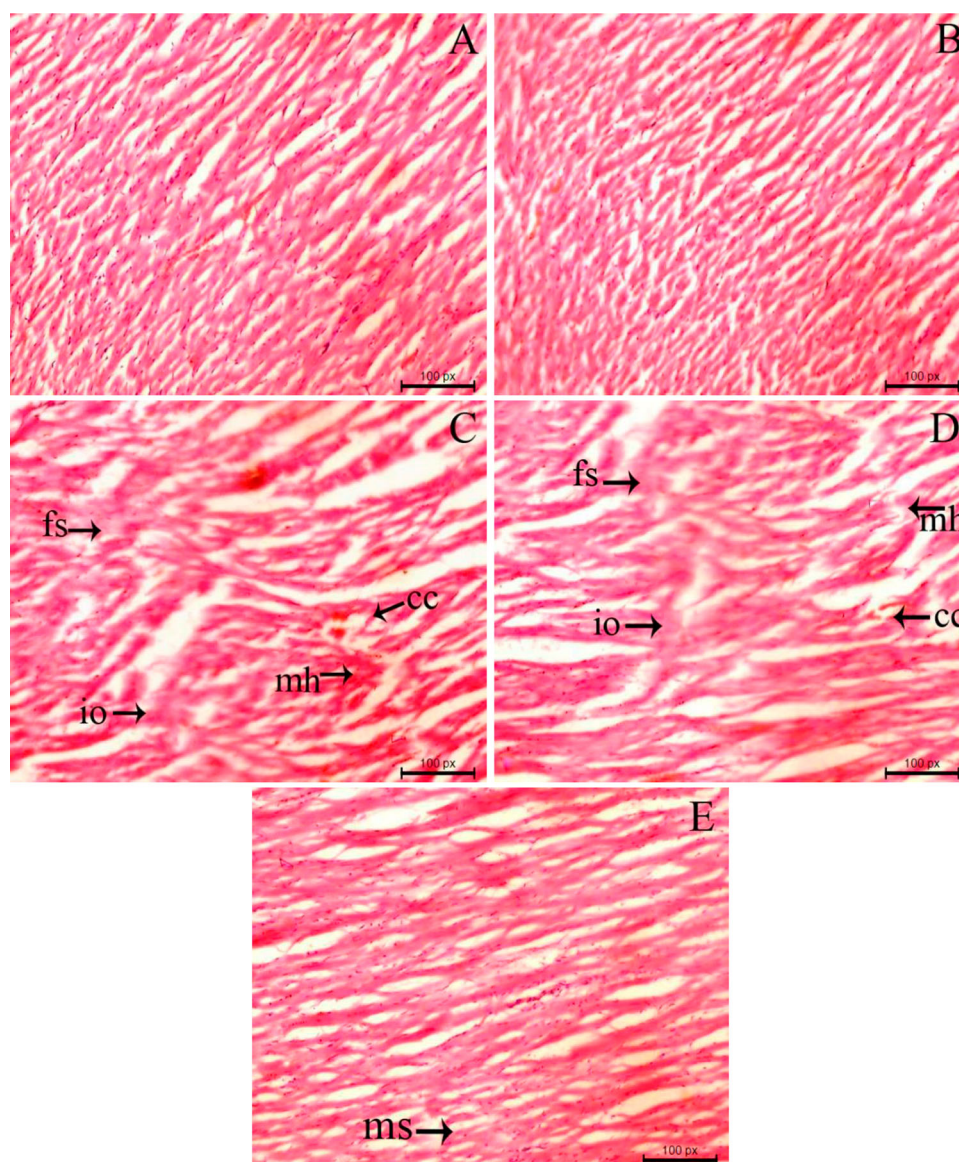
Figure 4. Histopathology of heart tissue: histopathological changes occurred in rat heart tissues after As$_2$O$_3$ administration and its amelioration with FSO treatment (Hematoxylin and Eosin, 100×). (a) – normal control; (b) – FSO (500 mg/kg b.wt); (c) – As$_2$O$_3$ (4 mg/kg b.wt); (d) – As$_2$O$_3$ (4 mg/kg b.wt) + FSO (250 mg/kg b.wt), (e) – As$_2$O$_3$ (4 mg/kg b.wt) + FSO (500 mg/kg b.wt). fs – fibre separations, io – interstitial oedema, cc – capillary congestion, mh – micro-haemorrhages, ms – mild swelling.

Table 4. Histopathological scoring.

Histopathological changes	Normal control	FSO control	As_2O_3 4 mg/kg	As_2O_3 4 mg/kg + FSO 250 mg/kg	As_2O_3 4 mg/kg + FSO 500 mg/kg
Fibre swelling	0	0	3.75 ± 0.3[a]	3.62 ± 0.24	1.92 ± 0.19[b]
Interstitial oedema	0	0	3.7 ± 0.33[a]	3.57 ± 0.35	1.1 ± 0.13[b]
Capillary congestion	0	0	3.6 ± 0.32[a]	3.43 ± 0.33	1.88 ± 0.2[b]
Micro-haemorrhages	0	0	3.58 ± 0.38[a]	3.28 ± 0.29	1.12 ± 0.13[b]

Data represented as mean $\pm$ SD, $n = 6$.
[a]$P < 0.05$ vs. normal control.
[b]$P < 0.05$ vs. As_2O_3 (4 mg/kg b.wt).

Conclusion

Cardioprotective effects of FSO may be due to the increased amount of biologically active omega-3 fatty acid, ALA (46.9%). It is evident that FSO has the ability to counteract As_2O_3 induced cardiotoxicity and can maintain the proper balance between pro-oxidant/antioxidant defense systems in the cells. Based on the findings, we suggest that, the use of FSO as a dietary supplement in As_2O_3 treatment may reduce its cardio toxic effects. In addition, more studies on detailed molecular mechanisms of arsenic removal from cardiac tissue during FSO treatment are also warranted.

Ethics approval

Institutional ethical approval obtained from the Institutional Animal Ethics Committee, School of Biosciences, Mahatma Gandhi University, Kottayam, India (Approval No. B1662009/2).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Notes on contributors

Mathews V. Varghese received his PhD in Biosciences in 2015 at the Mahatma Gandhi University, Kerala, India. He is currently a junior scientist at the Malabar Institute of Medical Science Research Foundation (MIMS RF), Kerala, India. His main research activity is devoted to study the pharmacology and toxicology of anticancer drugs.

Abhilash M received his PhD in Biosciences in 2015 at the Mahatma Gandhi University, Kerala, India. He is currently an assistant professor of Biochemistry at the St. Thomas College, Kerala, India. He is primarily interested in studying health impact of artificial sweeteners.

Manju Alex received her PhD in Biosciences in 2016 at the Mahatma Gandhi University, Kerala, India. She is currently a lecturer of Biology at Placid Vidya Vihar Senior Secondary School, Kerala, India. Her main research activity is to study about the renal calcium oxalate deposits and its relationship to oxidative stress.

Sauganth Paul M. V received his PhD in Biosciences in 2015 at the Mahatma Gandhi University, Kerala, India. He is currently an assistant professor of Biochemistry at the St. Gregorios Dental College, Kerala, India. His main research activity is devoted to study the food additives relayed free radical generation and oxidative stress.

Prathapan A received his PhD in Biochemistry in 2015 at the CSIR-National Institute for Interdisciplinary Science and Technology (NIIST), Thiruvananthapuram, Kerala, India. He is currently a postdoctoral fellow at the University of Manitoba, Winnipeg, Canada. His research involves the study of molecular mechanisms related to cardiovascular and metabolic diseases.

Raghu K. G received his PhD in Experimental Biology in 1984 at the Saurashtra University, Gujarat, India. He is currently a principal scientist at the CSIR-National Institute for Interdisciplinary Science and Technology (NIIST), Kerala, India. His laboratory is focused in studying the aspects of free radical generation and electrophysiological alterations in cardio-metabolic syndrome.

Harikumaran Nair R received his PhD in Physiology in 2000 at the Mahatma Gandhi University, Kerala, India. He is currently an assistant professor of Physiology at the Mahatma Gandhi University, Kerala, India. His laboratory is devoted to functional biology to which his group has made significant contributions. Major emphasis has been on the identification of toxicological mechanisms under chemotherapeutic stress.

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