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Artemisia maritima ameliorates Ferric Nitrilotriacetate (Fe-NTA) mediated Inflammation, Hepatic Oxidative Stress, and Tumor Promotion Response

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ABSTRACT

Ferric nitrilotriacetate (Fe-NTA) is a well known hepato and nephrotoxic agent. Escalating body of evidence suggested the role of oxidative stress in Fe-NTA mediated hepatotoxicity. Artemisia species bears an extensive medicinal value and are widely used in traditional medicine against various ailments including cancer. In the present communication, we have reported ethanolic extract of Artemisia maritima aerial parts to possess potent antioxidant activity and showed significant protection against Fe-NTA mediated inflammation, hepatic oxidative stress, hepatotoxicity and tumor promotion response. A high total phenolic content with potent reducing power and significant scavenging of free radical species (ROS and RNS) has been observed by ethanolic extract of A. maritima. The extract also significantly and dose dependently protected against oxidative damage to lipids and DNA. Single administration of Fe-NTA (9mg/ Kg body weight) significantly induced cyclooxygenase-2 (COX-2) and inducible nitric oxide (iNOS) expressions and augmented the levels of inflammatory cytokines including interleuckin-6 (IL-6) and tumor necrosis factor-α (TNF- α). Pretreatment of mice with A. maritima extract not only reduced the COX-2 and iNOS expressions, but also significantly and dose dependently ameliorated the levels of inflammatory cytokines. Intraperitoneal administration of Fe-NTA (9 mg/kg body weight, i.p.) to Swiss albino mice induced oxidative stress by depleting hepatic glutathione content and activities of antioxidant and phase-II metabolizing enzymes. Elevation in tumor promotion markers such ODC and [^{3}H]-thymidine incorporation into DNA led to a significant oxidative stress and allied damage in liver tissues and induced hyperproliferation. Fe-NTA led to a substantial hepatic injury, evident with a marked increase in levels of hepatic damage markers and histopathological changes, suggesting Fe-NTA to afflict damage to liver. Pretreatment of mice orally with A. maritima extract at a dose regimen of 100-200 mg/ kg body weight for seven days not only restored hepatic antioxidant arsenal close to normal but also significantly and dose dependently precluded against alteration in all these diagnostic parameters. The results of the present study illustrated A. maritima aerial parts to possess potent antioxidant and free radical scavenging activities and restore inflammation, oxidative damage and hyperproliferation inflicted to hepatic tissues.

Keywords: Artemisia maritima, antioxidant, ferric nitrilotriacetate, hepatoprotective, oxidative stress

1. INTRODUCTION

Living systems are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. These gents may be present in air, food, and water or they may be produced by metabolic activity within the cells and leads to the generation of reactive oxygen species (ROS). To combat oxidative insults by ROS, nature has bestowed cells with an elaborate system of antioxidant defense mechanism that protects cellular macromolecules from detrimental effects of oxidative injury. Cells are also equipped with a panel of detoxifying enzymes responsible for inactivation and subsequent elimination of carcinogens [1]. Exposure of cells and tissues to oxidative stimuli of ROS or electrophilic carcinogens, therefore, forces the cells to turn on their antioxidant-detoxification arsenal as the first line of defense. However, oxidative stress results when the balance between the productions of ROS overrides the antioxidant capability of the target cell leading to a variety of pathological conditions including cancer. Cancer is a leading cause of death in the world. Oxidative stress contributes to multistage carcinogenesis either by a direct mechanism involving damage to DNA, lipids and proteins, or indirectly by modulating cellular transduction pathways, and forcing damaged or initiated cells to undergo promotion and progression.

Nitrilotriacetate (NTA), a synthetic tricarboxylic acid, forms water soluble complexes with several metal ions including Fe^{3+} [2]. Fe-NTA, an iron complex of the chelating agent NTA, is a potent hepato and nephrotoxic agent and a potent tumor promoter mediating its effect by inducing oxidative stress [3]. Repeated intraperitoneal injections of Fe-NTA results into a significant iron deposition in hepatic parenchyma cells leading to a high incidence of hepatic carcinoma in the liver [3].

Fe-NTA mediated oxidative stress plays a vital role in inflammation and contributes to multistage carcinogenesis by distinctive mechanisms. Fe-NTA administration can induce the co-expression of cyclooxygenase type 2 (COX-2) and/or inducible nitric oxide synthase (iNOS), which synthesizes PGE_2 and NO, respectively, above and beyond inducing the expression of cytokine inflammatory response factors such as interleuckin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [4, 5] and thus inflicting the pathophysiology of different cancers.

Fe-NTA induced oxidative stress is crucial in hepatic toxicity and tumorigenesis. Elevation in the toxic lipid peroxidation (LPO) products such as thiobarbituric acid reactive substances (TBARS) and 4-hydroxy-2-nonenal (HNE) have been reported in the liver upon Fe-NTA administration [6]. Fe-NTA administration results into oxidative DNA damage leading to single and double strand breaks, DNA-protein cross-links, elevation in 8-hydroxy deoxyguanosine levels [7]. Further, Fe-NTA stimulates production of $^{\bullet}OH$, which is responsible for initiating most of these DNA mutilations [8]. Research conducted in our laboratory has made a significant contribution in unraveling the mechanism of Fe-NTA toxicity. We have revealed Fe-NTA to be a potent hepatic and renal tumor promoter attenuating glutathione and glutathione metabolizing enzymes in liver and kidney and thus bringing about oxidant buildup in these organs [9].

A great deal of clinical and experimental research conducted over last few decades has provided convincing data for the use of ethno-botanicals in the chemoprevention of cancer. *Artemisia*, the largest genera of Compositae family with more than 350 species, is widely distributed throughout world [10]. A variety of species of this genus are frequently utilized for the treatment of various disorders such as malaria, inflammation, hepatitis, cancer, and infections by fungi, bacteria, and viruses [11, 12]. This genus is receiving growing interest presumably due to the diversified biology and chemistry of the constituents and the frequent application in traditional medical practice. Although several phytochemical and biological studies on the genus Artemisia have been performed, however, only a few of them are concerned with *Artemisia maritima. A. maritima* L. (Syn: *A. brevifolia* Wall., *Artemisia fragrans* Willd.; Family: Asteraceae alt. Compositae) locally known as 'Afsanteen-ulbahr' [13], is a bitter aromatic herb or low shrub that grows abundantly in the high altitudes of the North Western Frontier including Kashmir and Himachal Pradesh [14]. The plant is known to possess antibacterial [15], anthelmintic [16, 17] and antidiabetic [18] activities. Moreover, it has a reputation in folklore regarding its use in the treatment of jaundice [19] and for intermittent as well as remittent fever [20]. Previous phytochemical studies on *A. maritima* revealed the presence of flavonoids and terpenoids, including monoterpenes, and sesquiterpene lactones with germacrane, eudesmane guaiane and elemane frameworks [21-26]. Some of the chief constituents of *A. maritima* includes artemesnin, hispidulin, luteolin, quercetin, rutin and apigenin [27]. In the present communication, attempts have been made to check the efficacy of ethanolic extract of *A. maritima* aerial parts against Ferric nitrolotriacetate (Fe-NTA) mediated inflammation, hepatic carcinogenesis, and toxicity.

2. MATERIALS AND METHODS

2.1. Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG), horseradish peroxidase, phenol red, glutathione reductase, bovine serum albumin (BSA), 1,2,dithio-bis-nitrobenzoic acid (DTNB), 1,chloro-2, 4,dinitrobenzene (CDNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), γ-glutamyl *p*-nitroanilide, flavin adenine dinucleotide (FAD), nitrilotriacetic acid (NTA), glucose-6-phosphate, Tween-20, 2,6,dichlorophenolindophenol and thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), acryl amide, bisacryl amide, nitrocellulose membrane were obtained from Sigma Chemical (St. Louis, MO, USA). Diacetylmonoxime, NaOH, urea, picric acid, trichloroacetic acid (TCA) and perchloric acid (PCA), were purchased from CDH, India. [^{14}C] ornithine (sp.act. 56 m Ci mmol) and [^{3}H] thymidine (sp.act. 82 Ci mmol) were purchased from Amersham Corporation (UK). ELIZA kits for IL-6 and TNF-α and antibodies against iNOS and COX-2 were purchased from ebiosciences, CA, USA. All other chemicals and reagents were of highest purity commercially available.

2.2. Preparation of Extract

Artemisia maritima aerial parts were collected from the hilly area of Village-Kultura-Kupwara located in temperate region of Kashmir Valley and authenticated by Dr H. B. Singh, Taxonomy section, NESCAIR, PUSA Road, New

Delhi. A specimen voucher has been deposited in our Phytochemistry laboratory. The powdered shade-dried aerial parts of *Artemisia maritima* were soxhlet extracted exhaustively with (95 %) ethanol. The extract obtained was concentrated to dryness under reduced pressure in a rotary evaporator to yield dark brownish mass (266.42 g, 12.11 %).

2.3. Phytochemical screening of extract

The ethanolic extract was subjected to preliminary phytochemical screening for the presence of different classes of compounds such as tannins, saponins, flavonoids, terpenoids, cardiac glycosides, alkaloids, anthraquinones and carbohydrates. The presence of tannins was detected by ferric chloride reagent according to Harborne (1973) [28]. The test for flavonoids was performed by the method of Kapoor *et al.* (1969) [29]. Saponins were identified by frothing test according to Sofowara (1993) [30]. The test for terpenoids was performed according to the method of Harborne (1973) [28]. 5 ml of each extract was mixed in 2 ml of chloroform, and concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the inter face was formed to show positive results for the presence of terpenoids. The identification of cardiac glycosides was performed by Legal test and Killer-Kiliani test in which 1 g of extract was added to 4 ml acetic anhydride and sulphuric acid [31]. Besides anthraquinones and carbohydreates, the test for alkaloids was performed by boiling 0.5 g aqueous extract (5 ml) with 1% HCl followed by addition of Mayer's reagent [28].

2.4. Total Phenolics Assay & Reducing Power

The total polyphenolics content in ethanolic extract of *A. maritima* aerial parts was performed according to method of Taga *et al.* (1984) [32]. The reducing power determination was tailored from Oyaizu *et al.* (1986) [33].

2.5. Scavenging of DPPH radicals

To 1 ml of the extract solution (in methanol), 0.5 ml of 0.15 mM DPPH solution (in methanol) was added. The contents were mixed vigorously and allowed to stand at 20 °C for 30 minutes, following which the absorbance was measured at 517 nm.

2.6. Scavenging of ROS and NO

Scavenging of $O^{2\bullet-}$, H_2O_2, and •OH was determined. $O^{2\bullet-}$ scavenging was determined by method of Yen and Chen (1995) based on inhibition of NBT reduction by $O^{2\bullet-}$ (generated by PMS-NADH system) [34]. •OH scavenging was determined by the method of Aruoma and Halliwell (1987) based on deoxyribose degradation by •OH [35]. NO scavenging was determined as described previously by us [49].

2.7. Lipid peroxidation (LPO) and DNA Damage Assay

LPO was performed according to Wright et al (1981) [36]. DNA damage assay was performed according to procedure adapted from Halliwell and Gutteridge (1990) [37].

2.8. Preparation of Fe-NTA solution

Fe-NTA solution was prepared according to Athar and Iqbal (1998) [38]. Briefly, ferric nitrate and NTA were dissolved in double-distilled water at a concentration of 9 mg/Kg body weight and 36 mg/Kg body weight respectively. Equal volumes of the two solutions were mixed and pH adjusted to 7.4 with sodium bicarbonate with constant stirring. Fe-NTA solution was prepared fresh immediately before its use.

2.9. Animals

Female Swiss albino mice (25-30 g) were obtained from Central Animal House of Hamdard University, New Delhi, India. Mice were housed in a temperature-controlled (25 °C) room with alternating 12-h/12-h light/dark cycles, and were allowed to acclimatize for 1 week before study. They had free access to pellet diet and water.

2.10. Treatment protocol

Mice were randomly divided into 5 groups of 6 mice each. Group I animals served as control. Group II animals received *A. maritima* extract at a dose of 200 mg/ Kg body weight p.o. by gavage for 1 week and served as control. Groups IV and V animals were given *A. maritima* extract at a dose of 100 mg/ Kg body weight and 200 mg/ Kg body weight p.o. respectively daily for 1 week. After 1 week, animals of groups III, IV and V received an i.p. injection of Fe-NTA (9.0 mg Fe/kg body weight), while mice of groups I and II were injected with equal volume of saline. All the animals were sacrificed 12 h after the treatment with Fe-NTA or saline by decapitation and liver of each animal were removed immediately. A portion of liver of each animal was homogenized in 0.1 M phosphate buffer (pH 7.4) containing 1.15 % KCl for preparing of post-mitochondrial supernatant (PMS) and cytosol as described previously [38]. The remaining portion of each liver was fixed in 10% buffered formalin for histopathological studies. Just before animals were sacrificed, blood was collected in test tubes from retro-orbital sinus for liver function tests.

2.11. Western Blotting

COX-2 and iNOS measurements were performed by western blotting according to the method of Morales *et al*, (2006) [39]. Briefly hepatic tissues were homogenized with a polytron homogenizer and lysed in ice-cold buffer containing 140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% NP-40, 20 mM Tris base, pH 7.5 to which were added protease-inhibitors in the moment of use (2 mM of PMSF and 10 mg/mL of aprotinin). The homogenized tissues were centrifuged thereafter at 40C with 14000 RPM during 25 min and supernatants were taken. The protein concentration was measured using a commercial kit. A total of 100 μg of protein per sample was separated on a 12% polyacrylamide gel and electroblotted on polyvinylidene fluoride (PVDF) transfer membranes (milipore, 0.45 μm). Incubation of the membrane was performed in 2.5% glutarylaldehyde solution for 1 hour. After rinsing with tris buffered saline (TBS), membranes were blocked in blocking buffer (5% milk and 0.5% BSA) for 1 hour followed by three washes with tris buffered saline containing 0.1% Tween 20 (TBST). The membrane was subsequently incubated overnight at 40C with respect to specific antibodies such as rabbit polyclonal anti COX-2 antibody (dilution 1:400) and a rabbit polyclonal anti-iNOS

antibody (dilution 1:500). After incubation with primary antibody, the membrane was washed with TBST thrice followed by incubation with horse-radish peroxides labeled secondary antibody at a dilution of 1: 1000 for 45 minutes at room temperature. Anti-β-actin mouse monoclonal antibody was used at a dilution of 1: 4000. The membrane was washed thrice again with TBST. Final antigen-antibody reaction was visualized by avidin-biotin complex technique using 3, 3- diaminobenzidine as a substrate. The sections were counter stained with hematoxylin followed by examination with light microscopy.

2.12. Cytokines, IL-6 and TNF-α

To measure the level of IL-6 and TNF-α in mice serum, enzyme linked immunosorbant assay (ELIZA) was performed according to the manufacturer of the kit (e-biosciences, CA). The results were expressed as ng/ml of plasma.

2.13. Ornithine Decarboxylate (ODC) Activity

ODC activity was determined by the method of O'Brien *et al.* (1975) [40].

2.14. [3H]-Thymidine Incorporation Assay

To isolate hepatic DNA and assess of incorporation of [3H]thymidine into DNA, the method of Smart *et al.* (1986) was followed [41].

2.15. Reduced glutathione (GSH) Assay

GSH was determined in hepatic PMS by the method of Jollow *et al.* (1974) [42].

2.16. Measurement of antioxidant and phase II enzyme activity

Glutathione-S-Transferase (GST) activity was measured by the method of Habig *et al.* (1974) [43]. Glutathione reductase (GR) activity was assayed according to Mohandas *et al.* (1984) [44]. Catalase (CAT) activity was performed according to Claiborne (1985) [45]. Glutathione Peroxidase (GPx) activity was measured according to method of Mohandas *et al.* (1984) [49]. Glucose-6-Phosphate Dehydrogenase (G-6-PD) activity was assayed by the method of Zaheer *et al.* (1965) [46].

2.17. Liver function tests (LFT)

AST and ALT were assayed by the method adapted from Reitman and Frankel (1957) [47] .The level of serum bilirubin and albumin levels were assayed by commercially available kits (Monozyme India Ltd.) according to manufacturer'protocols. Protein level was determined by Lowry's method [48].

2.18. Histopathological observation

For histological studies, animals were sacrificed by cervical dislocation and the liver of each animal was removed immediately and processed according to the procedure described previously by us [49]. The parameters related to microarchitecture configuration (disorganization of parenchyma structure) and hepatic injury (ballooning denegation, steatosis and necrosis) were analyzed.

3. RESULTS

3.1. Phytochemical screening

The preliminary phytochemical analysis of aerial parts of *A. maritima* extract showed the presence of tannins, flavonoids, terpenoids, anthraquinones and carbohydrates.

3.2. Total phenolics & reducing power of the extract

The extract was found to contain 398 mg/g of total polyphenolics expressed as gallic acid equivalent (GAE, mg/g of extract). Fig. 1 represents the reducing power of *A. maritima* extract as compared to gallic acid and ascorbic acid. The reducing power of the extract was found to be significant and dose dependent.

3.3. Free radical scavenging activity of Artemisia maritima extract

Artemisia maritima aerial parts scavenged several kinds of free radicals. Antioxidants reduce purple colored stable DPPH free radicals to colorless α-α-diphenyl-β-picryl hydrazine. The extent of discoloration indicates the amount of DPPH scavenged. DPPH scavenging assay is a powerful tool to elucidate antioxidant activity. *A. maritima* extract potently and dose dependently quenched DPPH radicals (IC50 was approximately 15-17 µg/ml) (Table 1).

A. maritima extract powerfully scavenged ROS including $O^{2\bullet-}$, H_2O_2 and •OH (Table 1). A dose- dependent scavenging of $O^{2\bullet-}$ radicals (IC_{50} value being approximately 93 µg/ml) was observed by the extract. *A. maritima* extract scavenged as much as 63.8% H_2O_2 at a concentration of 200 µg/ml. •OH radicals were generated by Fenton type reaction and measured by their ability to degrade deoxyribose sugar into fragments that yield a pink chromogen upon reaction with thiobarbituric acid. As much as 72.8 % •OH were quenched by *A. maritima* extract at a dose of 200 µg/ml. A dose-dependent response was observed in case of •OH scavenging as well (IC_{50} being about 100 µg/ml).

NO liberated by NO donor, SNP was also scavenged by *A. maritima* extract. Incubation of SNP solution at 25 °C for 2 h released significant amount of NO (Table 1). *A. maritima* extract potently and dose dependently scavenged NO released (Table 1). 61.2 % of NO could be quenched by the extract, at 200 µg/ml, IC_{50} being about 69-71 µg/ml. Results of the control experiments suggested no interference of the extract with the reaction between nitrite and Griess reagent even at vey high concentrations, reflecting the fact that the decrease in NO is entirely by the extract.

3.4. Effect of A. maritima extract on LPO and DNA damage

Fig 3a shows the protective effect of A. maritima on hepatic microsomal LPO induced by Fe-NTA and H_2O_2. Exposure of microsomes to Fe-NTA and H_2O_2 led to about 2.78 fold increase in LPO in hepatic microsomes. Co-incubation with *A. maritima* extract significantly and dose dependently prevented this LPO (Fig 3 a).

Exposure of calf thymus DNA to Fe-NTA and H_2O_2 caused 3.89-fold damage to DNA (Fig 3b). In the presence of *A. maritima* extract in the reaction mixture, a significant and dose-related production was observed against DNA damage.

3.5. Effect of *A. maritima* extract on Fe-NTA induced COX-2 and iNOS expressions

Western blot analysis showed a significant rise in COX-2 and iNOS expressions in the group receiving a single dose of Fe-NTA injection after 12h. Fig 2 (a, b). No significant changes in the level of COX-2 and iNOS were observed in mice pretreated with *A. maritima* followed by Fe-NTA administration as compared to control Fig 2 (a, b).

3.6. Effect of *A. maritima* extract on plasma level of cytokine after Fe-NTA injection

A significant increase in the level of IL-6 ($p<0.01$) and TNF-α ($p<0.01$) were observed after 4h of Fe-NTA treatment, however, their levels decreased significantly in groups receiving *Artemisia maritima* extract(D1 & D2). (Fig 5)

3.7. Effect of *A. maritima* extract on Fe-NTA mediated induction of hepatic ODC activity and DNA synthesis

Fe-NTA treatment caused 4.52-fold increase in ODC activity as compared to saline treated controls (Fig 4 a). Prior treatment with *A. maritima* extract restored ODC activity significantly and dose dependently (Fig 4 a).

Fe-NTA treatment led to an increase in [3H]-thymidine and hence DNA synthesis. 3.86-fold elevation was observed in [3H]-thymidine incorporation following Fe-NTA treatment (Fig 4 b). Prophylactic treatment with *A. maritima* extract prevented the increase in [3H]-thymidine incorporation significantly and dose dependently (Fig 4 b).

3.8. Effect of *A. maritima* extract on Fe-NTA induced modulation in GSH metabolism and alteration in hepatic antioxidant enzymes

Fe-NTA treatment caused depletion of hepatic GSH and suppressed activities of GST and GR (Table 2). However, prophylactic treatment of animals with *A. maritima* extract significantly and dose dependently restored GSH content and activities of both GST and GR close to saline treated control values (Table 2).

Treatment of mice with Fe-NTA caused suppression in the activities of CAT, GPx and G-6-PD by 68.4, 55.39 and 48.8 % (Table 2). Pretreatment of animals with *A. maritima* extract resulted in the recovery of reduced levels of the three enzymes. The recovery ranged from 12.50 to 32.99 % (for GPX and CAT respectively) with lower dose of *A. maritima*

extract, with higher dose level, the recovery ranged from 33.48 to 58.61 % (for G-6-PD and CAT respectively) compared to their respective saline treated controls (Table 3).

3.9. Effect of *A. maritima* extract on markers of hepatic injury

Liver function tests (LFT) were done to assess Fe-NTA induced hepatic injury. Fe-NTA modulated most of the investigated LFT parameters (Table 4). Fe-NTA elevated levels of ALT, AST, ALP and bilirubin and depleted albumin and protein level of serum. Seven days of pretreatment with aerial parts of *A. maritima* extract significantly and dose dependently protected against Fe-NTA induced modulation in LFT implying it to have a protective effect against Fe-NTA induced liver damage.

3.10. Histopathological observations

The presence of damage to liver of Fe-NTA treated mice was also evident from histopathological studies (Fig 6). Extensive fatty changes were prominent in hepatic chords with edema and mild kuffer cell hyperplasia. The hepatic sections showed mild haemorrhage in Fe-NTA treated animals. Pretreatment with *A. maritima* extract preserved hepatic architecture close to normal; prominent protection was observed against marked changes occurred by Fe-NTA (Fig 6).

4. DISCUSSION

Many phytochemicals with strong antioxidant potential have been shown to possess cancer chemopreventive attributes. A tremendous attention is therefore being paid on exploring safe and effective antioxidant compounds. Our laboratory had previously revealed the role of oxidative stress in hepatic carcinogenesis induced by Fe-NTA and their protection by several phytochemicals [49, 50]. The present study was designed to explore the antioxidant efficacy of ethanolic extract of aerial parts of *A. maritima* and to evaluate its protective effects against Fe-NTA mediated hepatotoxicity, inflammation and tumor promotion response.

Many compounds from medicinal plants have pharmacological activities and thus may serve as a potential source for novel anti-tumor agents. Species belonging to the genus Artemisia have a well-documented antitumor activity. Artemisinin, isolated from the shrub-Artemisia annua, is of special biological interest because of its outstanding antitumor activity. Eupatilin (5,7-dihydroxy-3',4', 6-trimethoxyflavone), one of the pharmacologically active ingredients derived from Artemisia plants, induces apoptosis in human gastric cancer (AGS) cells [51]. The pro-apoptotic effects of eupatilin were further verified by elevated expressions of p53 and p21. In the present study, we have shown ethanolic extract of *A. maritima* aerial parts to protect against Fe-NTA mediated inflammation, hepatic tumor promotion and its oxidative injury.

A high yield of total polyphenolics and a high reducing power was obtained in *A. maritima* extract, both suggesting the extract to possess enormous antioxidant potential. *A. maritima* extract also efficiently scavenged ROS including $O^{2\bullet-}$, H_2O_2 and •OH radicals and RNS such as NO. ROS, RNS and the products of their interaction are potently toxic and can impair the structure and function of cellular molecules. *A. maritima* extract, with its potent free radical quenching capacity, was envisaged to protect the biomolecules from oxidative damage. As expected, the extract was found to significantly inhibit Fe-NTA plus H_2O_2 induced oxidative damage to lipids and DNA. The damage to lipids and DNA is crucial to both toxicity and carcinogenesis. In fact, both LPO and DNA damage are considered vital early markers of toxicity and carcinogenesis. LPO is detrimental to cell, both at membrane and genetic level [52]; products of LPO, malonaldehyde and 4-hydroxyl-2-nonenal cross-link the membrane, induce DNA damage and/or cause mutations leading to physiological alterations [52, 53]. Thus, it seems that suppression of LPO and DNA mutilation by *A. maritima* extract plays a crucial role in antitumor activity.

Fe-NTA has been regarded as one of the inflammation related xenobiotics as it induces a complex inflammatory response in several cell types [4, 5]. Since inflammation could play a major role in the hepatic oxidative damage produced by exposition to Fe-NTA, the next purpose of our study was to assess the expression of COX-2 and iNOS, and level of inflammatory cytokines associated to inflammation. We have observed that a single administration of Fe-NTA after a week significantly induced COX-2 and iNOS expressions, augmented the serum IL-6 and TNF-α levels; however, mice pretreated with *A. maritima* extract not only reduced COX-2 and iNOS expressions but also significantly and dose dependently ameliorated the levels of inflammatory cytokines indicating its ability to inhibit oxidative stress related inflammation

Further, dysregulated proliferation is an essential characteristic of tumor promotion. ODC is the first and rate limiting enzyme in biosynthesis of polyamine, which are essential for DNA synthesis and hence for cell proliferation. Induction of ODC activity and enhancement of [3H] thymidine incorporation are therefore extensively used as biochemical markers to evaluate the hyperproliferation and tumor promtion. An augmentation in ODC activity and [^{3}H]-thymidine incorporation into DNA following Fe-NTA treatment suggests its tumor promoting potential in hepatic tissue [45]. A significant and dose dependent amelioration in ODC activity and [^{3}H]-thymidine incorporation by *A. maritima* extract suggests it to possess antihyperproliferative and antitumor efficacy. These findings are consistent with our previous observations wherein antitumor agents effectively inhibit Fe-NTA induced tumorigenesis [50].

Many botanicals show anti-tumorigenic effect through their antioxidant activity. Fe-NTA is a well-known oxidant. In the present study, Fe-NTA administration led to a significant decrease in hepatic GSH levels and activities of antioxidant enzymes: GR, GPX, CAT, and G-6-PD. *A. maritima* extract maintained the levels of all these enzymes near to normal that may be attributed to potent free radical scavenging activity of the extract. During oxidative stress, substrate (For e.g., $O^{2\bullet-}$ and H_2O_2) plays a crucial role in suppressing the activities of antioxidant enzymes. Under these

circumstances, A. maritima extract, possessing potent $O^{2\bullet-}$ and H_2O_2 scavenging activity may ameliorate the levels of these ROS, consequently restoring enzyme activities. The constituents of *A. maritima* may also induce de-novo synthesis of the antioxidant enzymes. Phase-II enzymes detoxify the metabolic products of phase I enzymes to fairly inert and extractable forms [54]. Depletion in the activities of enzymes such as GST occurs after Fe-NTA administration. Several studies have illustrated a clear correlation between chemopreventive potential of anticancer agents and induction of these enzymes (De Flora and Ramel, 1998) [54]. Enhancement in the activities of GST by *A. maritima* extract depicts its protective efficacy against Fe-NTA induced toxicity. *A. maritima* extract also recovered the elevated H2O2 levels to their normal. Thus, it appears that *A. maritima* extract abrogates Fe-NTA induced toxicity by acting at it various target points. Significant improvement in the levels of liver damage markers: AST, ALT, ALP and bilirubin following extract pretreatment suggested its protective efficacy against liver damage. Results of the histopathological investigations of the liver are also in favor of these findings, wherein a marked protection was observed by extract against Fe-NTA induced pathological changes, particularly necrosis.

In conclusion, the results of the present study inferred that aerial parts of *A. maritima* possess significant antioxidant, antiinflammatory, hepatoprotective and antihyperproliferative properties. The possible mechanisms of protective ability of the extract against Fe-NTA mediated inflammation, oxidative damage and hyperproliferation seems to be implicated through its potent antioxidant potential and free radical scavenging activities. The crucial target points at which *A. maritima* exerts its effect involves the inhibition of the induction of COX-2, iNOS, and inflammatory cytokines; along with mitigating hepatic oxidative stress. Further, by quenching the electrophilic species and inhibiting ODC induction and DNA synthesis, *A. maritima* extract may interrupt the growth promoting and mutagenic functions of polyamines and arachidonic acid metabolism.

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Table 1: Scavenging of DPPH, superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and nitric oxide (NO) by *Artemisia maritima* extract. Butylated hydroxy anisole (BHA) was taken as a standard. Results are expressed as % of control.

Group	DPPH	$O_2^{\bullet-}$	H_2O_2	$^{\bullet}OH$	NO
	(Percent of Control)				
Control	100.0 ± 1.8	100.0 ± 2.7	100.0 ± 2.8	100.0 ± 2.9	100.0 ± 3.0
***A. maritima* Ext**					
10 µg/ml	65.4 ± 3.8[c]	85.2 ± 5.7	93.9 ± 4.1	90.3 ± 4.1	86.1 ± 6.2[a]
25 µg/ml	25.7 ± 2.2[c]	78.7 ± 4.6[a]	86.3 ± 3.4[a]	81.3 ± 4.3[a]	67.3 ± 4.1[c]
50 µg/ml	0.0	71.5 ± 3.2[c]	79.5 ± 2.6[b]	70.4 ± 2.2	53.2 ± 2.4[c]
100 µg/ml	-	46.7 ± 2.6[c]	58.2 ± 5.0[c]	49.5 ± 3.6[c]	44.8 ± 5.3[c]
300 µg/ml	-	-	14.6 ± 1.5[c]	3.4 ± 0.9[c]	33.9 ± 3.7[c]
BHA					
200 µM	54.0 ± 2.4[c]				
200 µM	-	28.7 ± 2.4[c]	36.4 ± 3.2[c]	N.D.*	61.5 ± 2.5[c]

Each value is mean ± S.E. (*n*=5). [a]p<0.05, [b]p<0.01, [c]p<0.001 Vs control group.

* Not determined

Table 2: Protection afforded by *Artemisia maritima* extract against Fe-NTA induced depletion in hepatic glutathione (GSH) content and decline in the activities of glutathione-S-transferase (GST) and gluthione reductase (GR).

Treatment groups	Reduced glutathione [nmol GSH/g tissue]	Glutathione-S- transferase [nmol CDNB conjugate formed/min/mg protein]	Glutathione reductase [nmol NADPH oxidized /min/mg protein]
Saline (control)	0.497 ± 0.06	198.7 ± 7.1	293.7 ± 11.3
Fe-NTA (9mg Fe /Kg body weight)	0.232 ± 0.04[b]	91.1 ± 5.4	174.3 ± 8.2[c]
A. maritima extract (100 mg/Kg body weight) + Fe-NTA (9mg Fe /Kg body weight)	0.34 ± 0.03	127.1 ± 7.6[d]	207.4 ± 6.9[d]
A. maritima extract (200 mg/Kg body weight) + Fe-NTA (9mg Fe /Kg body weight)	0.437 ± 0.02[f]	173.5 ± 6.4[f]	257.1 ± 9.1[e]
A. maritima extract only (200 mg/Kg body weight)	0.501 ± 0.04[f]	211.3 ± 10.5[f]	295.4 ± 11.2[f]

Each value is mean ± S.E. ($n=6$). [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ compared to the corresponding saline treated controls. [d]$p<0.05$, [e]$p<0.01$, [f]$p<0.001$ compared to the corresponding Fe-NTA treated groups.

Table 3: Protection afforded by *Artemisia maritima* extract against Fe-NTA mediated decline in the activities of hepatic antioxidant enzymes: catalase (CAT), glucose-6-phosphate dehydrogenase (G-6-PD) and glutathione peroxidase (GPX) in mice. The assays were performed as detailed in materials and methods section.

Each value is mean ± S.E. ($n=6$). [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ compared to the corresponding saline treated controls.

Treatment groups	Catalase [nmol H_2O_2 consumed/min/mg tissue]	Glutathione peroxidase [nmol NADPH oxidized formed/min/mg protein]	Glucose-6-phosphate dehydrogenase [nmol NADH reduced / min/ mg protein]
Saline (control)	247.4 ± 8.2	283.1 ± 14.1	45.7 ± 4.5
Fe-NTA (9mg Fe /Kg body weight)	78.7 ± 4.3[c]	126.3 ± 9.1[c]	23.4 ± 2.4
A. maritima extract (100 mg/Kg body weight) + Fe-NTA (9mg Fe /Kg body weight)	159.7 ± 9.5[f]	161.7 ± 7.7	30.3 ± 1.6
A. maritima extract (200 mg/Kg body weight) + Fe-NTA (9mg Fe /Kg body weight)	223.1 ± 8.2[f]	227.3 ± 10.5[f]	38.7 ± 4.3
A. maritima extract only (200 mg/Kg body weight)	249.4 ± 9.3[f]	287.9 ± 5.2[f]	46.9 ± 2.4

[d]$p<0.05$, [e]$p<0.01$, [f]$p<0.001$ compared to the corresponding Fe-NTA treated groups.

Table 4: Effect of *A. maritima* extract on Fe-NTA induced modulation in the levels of serum AST (aspartate aminotransferase), ALT (alanine aminotransferase), ALP (alkaline phosphatase), bilirubin and TSP (total serum proteins).

Parameter	Gr I	Gr II	Gr III	Gr IV	Gr V
AST	100 ± 3	394 ± 9.1	280 ± 5.4 [a]	175 ± 6.5 [c]	119 ± 4.7
ALT	100 ± 3.7	321 ± 7	249 ± 7.3 [b]	139 ± 5.4 [c]	103 ± 3.1
ALP	100 ± 4.6	164 ± 8	136 ± 4.3	113 ± 4.8 [b]	97 ± 3.3
Bilirubin (mg %)	1 ± 0.1	2.5 ± 0.2	1.8 ± 0.1 [a]	1.6 ± 0.2 [b]	1.1 ± 0.1
TSP (mg protein / ml serum)	67.4 ± 3.8	51.9 ± 3.3	59.2 ± 3.4	64.7 ± 4.1	66.9 ± 3.9

Group I (Gr I) to group V (Gr V) are as described in Materials and methods section. Activities of ALT, AST and ALP are expressed as % of control. Each value is mean ± S.E. ($n=6$). [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ vs. Fe-NTA treated group.

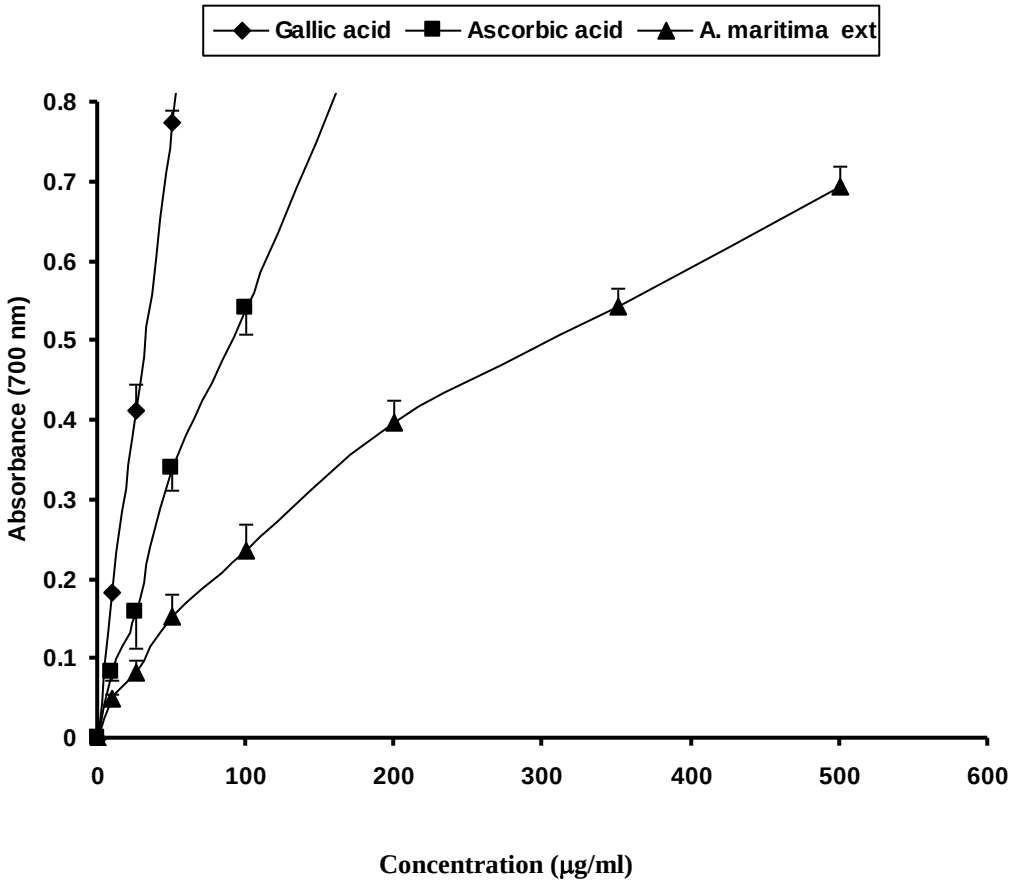

Fig 1: Reducing power of ethanolic extract of *Artemisia maritima*, ascorbic acid and gallic acid as evaluated by spectrophotometer detection of Fe^{2+} to Fe3+ transformation as described in materials and methods section. Each value is mean ± S.E. (*n=5*).

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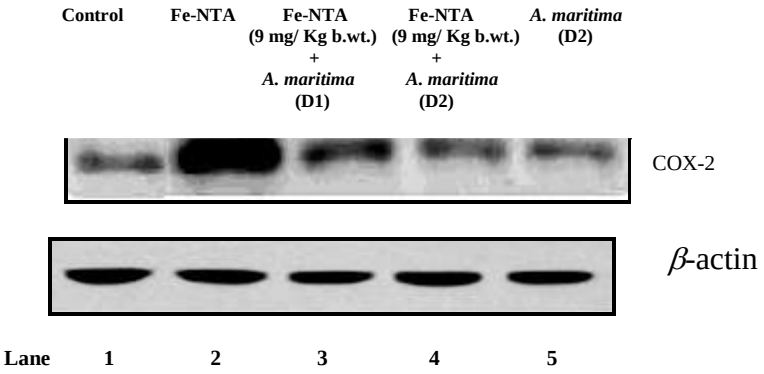


Fig 2(a). Western blot analysis of COX-2 protein level in hepatic sections of mice. Each value is mean ± S.E. (*n*=*6*). [a]p<0.05 compared to the corresponding saline treated controls. [d]p<0.05 compared to the corresponding Fe-NTA. Lane-1: Control; Lane-2: Fe-NTA (9 mg/ Kg b.wt.); Lane-3: Fe-NTA (9 mg/ Kg b.wt.); Lane-4: Fe-NTA (9 mg/ Kg b.wt.) + *A. maritima* (D1); Fe-NTA (9 mg/ Kg b.wt.) + *A. maritima* (D2); Lane-5: *A. maritima* (D2)

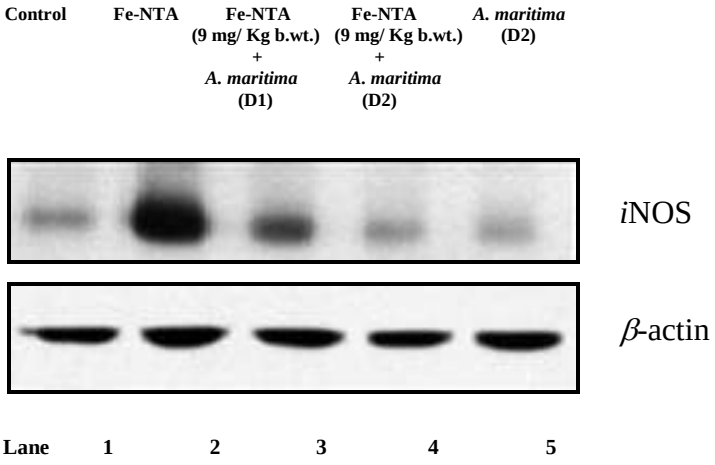


Fig 2 (b). Western blot analysis of iNOS protein level in hepatic sections of mice. Each value is mean ± S.E. (*n*=*6*). [a]p<0.05 compared to the corresponding saline treated controls. [d]p<0.05 compared to the corresponding Fe-NTA. Lane-1: Control; Lane-2: Fe-NTA (9 mg/ Kg b.wt.); Lane-3: Fe-NTA (9 mg/ Kg b.wt.); Lane-4: Fe-NTA (9 mg/ Kg b.wt.) + *A. maritima* (D1); Fe-NTA (9 mg/ Kg b.wt.) + *A. maritima* (D2); Lane-5: *A. maritima* (D2)

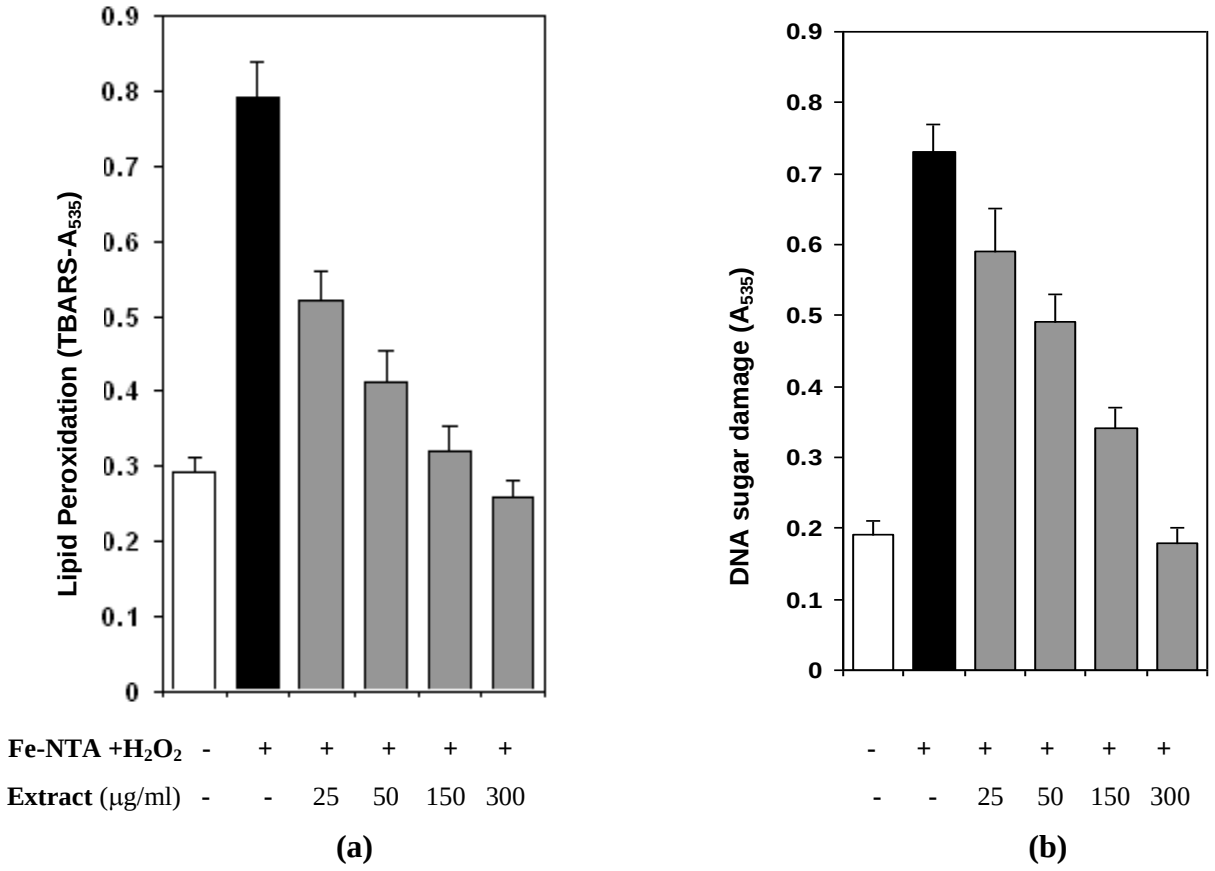
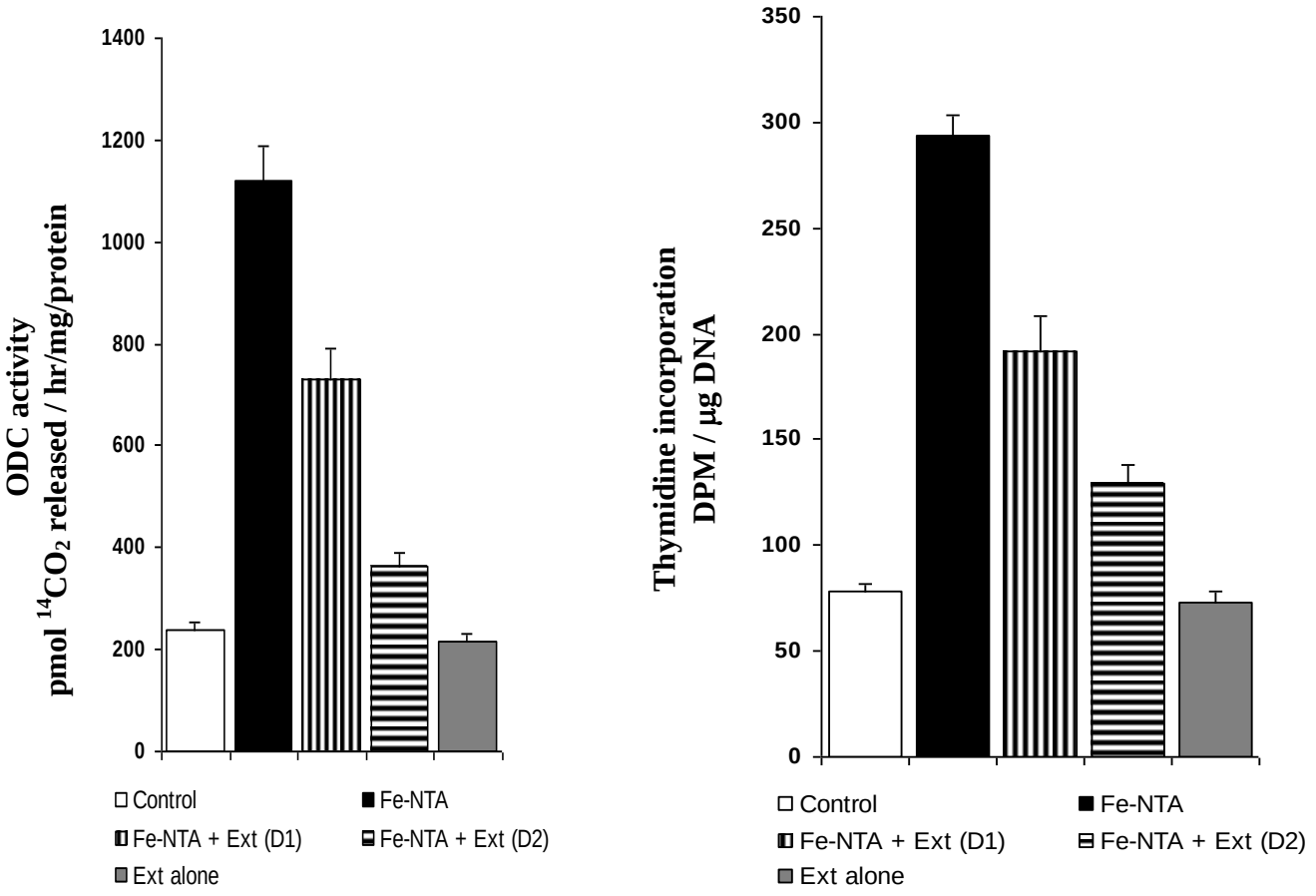


Fig 3 (a) Inhibitory effect of *Artemisia maritima* extract on lipid peroxidation induced by Fe-NTA plus hydrogen peroxide in hepatic microsomes. **(b)** Inhibitory effect of *A. maritima* extract on damage induced by Fe-NTA plus hydrogen peroxide to calf thymus DNA. Experiments were performed as detailed in materials and methods section. Each value is mean ± S.E. (*n*=*5*). $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$ Vs control group.



(a) **(b)**

Fig 4: Effect of *Artemisia maritima* extract pretreatment on Fe-NTA induced elevation in (a) hepatic ornithine decarboxylase (ODC) and (b) [^{3}H]-thymidine incorporation into hepatic DNA. Each value is mean ± S.E. (*n=6*). [a]p<0.05, [b]p<0.01, [c]p<0.001 compared to the corresponding saline treated controls. [d]p<0.05, [e]p<0.01, [f]p<0.001 compared to the corresponding Fe-NTA treated groups. Doses D1 and D2 represent 100 and 200 mg/Kg body weight respectively of *A. maritima* extract.

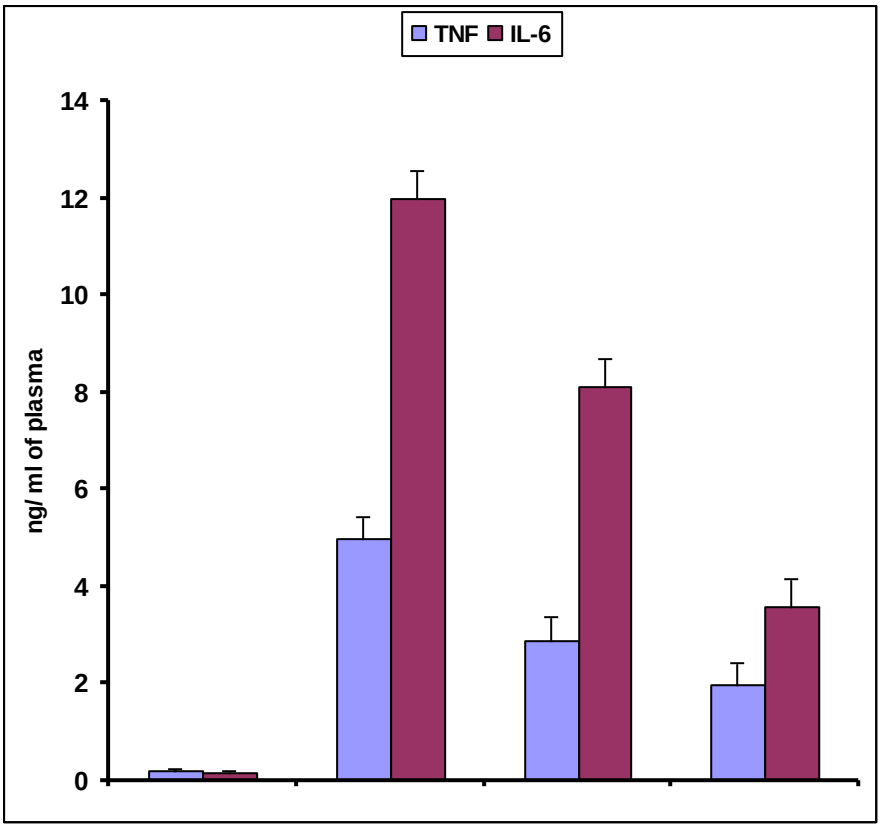


Fig 5. Plasma levels of inflammatory cytokines (TNF-α, IL-6) after 4 h of treatment. Each value represents mean ± S.E. (*n=6*). [a]p<0.05 compared to the corresponding saline treated controls. [d]p<0.05 compared to the corresponding Fe-NTA

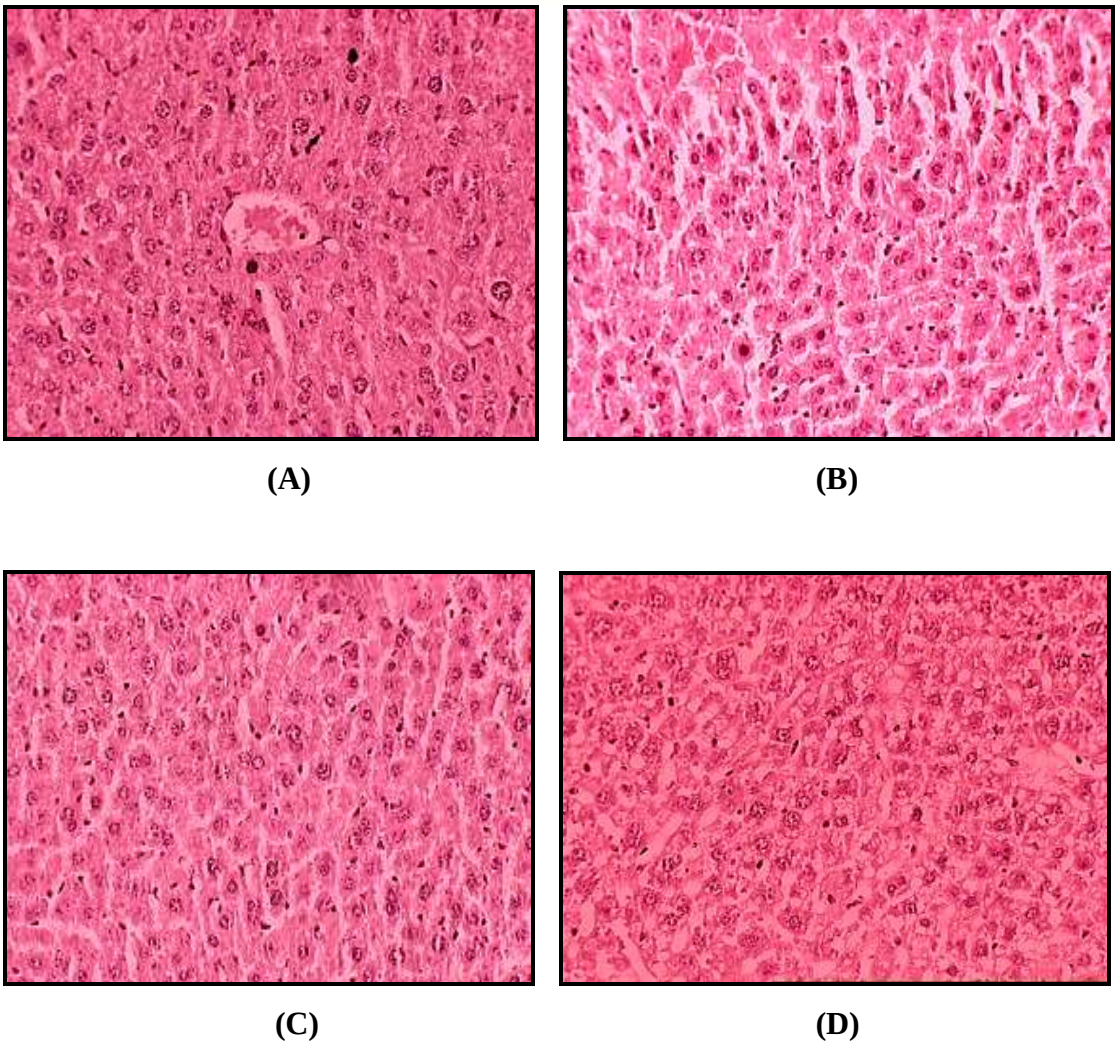


Fig 6: Photomicrographs of liver section (haematoxylin-eosin) representing suppressive effects of *A. maritima* extract on Fe-NTA induced hepatic histopathological changes. Dose regimen and experimental procedures were as described in the materials and methods section. (A) Hepatic sections of saline treated control mice (B) hepatic sections of Fe-NTA treated mice (C) hepatic sections of *A. maritima* root extract pretreated + Fe-NTA exposed mice.

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