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RESEARCH ARTICLE

Anticlastogenic and anti-apoptotic potential of *Eulophia gracilis* extract in arsenic-induced oxidative liver damage in rats

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ABSTRACT

Assessment of arsenic intoxication on human and murine tissues reveals complex toxicity resulting in variety of health damages most especially liver injury. *Eulophia gracilis* (*E. gracilis*) is an important medicinal orchid that elicits health benefit in folkloric medicine related to its antioxidant properties. However, scholarly literature lacks information on the role of *E. gracilis* in sodium arsenite-induced hepatic toxicity. Therefore, we assessed the influence of *E. gracilis* extracted in aqueous-methanol solvent on sodium arsenite-induced dysfunctional liver organ and genotoxicity in rats. Rats were intragastrically exposed to sodium arsenite (5 mg/kg) once in 7 days and concurrently with oral treatment of *E. gracilis* extract (200 mg/kg body weight) daily for 14 consecutive days. Arsenic-exposed rats displayed dysfunctional liver pathology through significant increases ($p < 0.05$) in plasma transaminases. Liver homogenate of intoxicated rats showed significantly declined ($p < 0.05$) redox activities of superoxide dismutase, catalase, arylesterase and deficit in reduced glutathione and Vitamin C alongside with severe diffuse vacuolar degeneration in liver architecture. Immunohistochemical analysis of liver showed an upregulated expression B-cell lymphoma 2 protein (BCL-2 protein) with concomitant low expression of tumor suppressor p53 protein coupled with clastogenicity depicted by increased frequency of micronucleated polychromatic erythrocytes (mPCEs) in the bone marrow of intoxicated rats. However, treatment with *E. gracilis* mitigated liver damage and improved the antioxidant enzymes and molecules in co-treated animals. Additionally, *E. gracilis* alleviated sodium arsenite-facilitated alteration in expression of BCL-2 and p53 proteins in liver, abrogated surges in the frequency of formation of mPCEs in bone marrow and reduced pathological lesions in the examined liver organ of rats co-treated with *E. gracilis*. Overall, *E. gracilis* suppressed liver dysfunction associated with sodium arsenite exposure via abrogation of oxidative stress and genotoxicity in male rats.

Keywords: sodium arsenite, *Eulophia gracilis*, hepatotoxicity, oxidative stress, genotoxicity

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INTRODUCTION

Hepatic disease is a global problem due to wide range exposure either through ingestion or inhalation of various hepatotoxicants [1]. Liver disease accounts for two million deaths annually and 4% of all deaths worldwide [2]. Deaths are largely attributable to complications of cirrhosis and hepatocellular carcinoma [3]. The cause of liver damage ranges from infection by pathogenic organisms such as hepatitis B virus to different chemical agents like inorganic arsenic, cadmium and lead [4]. Chemical compounds such as arsenic compounds were not only reported to cause liver damage but have also been classified as potential clastogen which may possibly act as carcinogen as it inhibits DNA repair mechanism and therefore increases mutation [5]. Human exposure to harmful inorganic arsenic is increasing in some parts of the world especially among developing countries through the ground water and soil due to rapid industrialization by geological and anthropogenic activities [6, 7]. Epidemiologically, arsenic intoxication due to human exposure has been linked to liver injury, cirrhosis and liver fibrosis through oxidative stress mechanism [8, 9]. This is due to the fact that liver seems to be one of

major targets for arsenic where it causes toxic and carcinogenic effects [10]. Worse still, reliable drugs for the treatment of liver are found wanting in allopathic practices though many potential drugs are still under clinical trial [11]. The absence of these reliable liver protective drugs gave chances for the utilization of herbs in the management of various liver disorders [12]. A number of natural products and plants have shown hepatoprotective property [13]. One of such natural plants is *Eulophia gracilis* (*E. gracilis*) that *is* used to treat diabetes, liver disease, hyperlipidemia and as aphrodisiac especially among Oyo and Jigawa indigenes in Nigeria. The plant has been shown to possess phytochemicals of medicinal values which may qualify it to be a chosen nutraceutical [14]. The antioxidant activities of several species of *Eulophia* have been documented and *E. gracilis* was reported to contain phytochemicals of pharmacological importance such as glycoside, alkaloid, tannins, phlobatanins and flavonoid [14, 15]. More importantly, the different successive extracts of *E. gracilis were reported* to possess selective cytotoxicity potential against laryngeal cancer cells (HEp-2), rhabdomyosarcoma (Rd), breast cancer cell (MCF-7) and cervical carcinoma (HeLa) [16]. The plant displayed the capacity to abate the onco-hematologic development and offered protective efficiency against some chemical and drug-induced liver degeneration [17-19]. Therefore, this study was designed to determine the protective effect of *E. gracilis* extract on alteration in biochemical indices, antioxidant status and clastogenicity elicited by sodium arsenite.

MATERIAL AND METHODS

Chemicals and Reagents

Glutathione (GSH), thiobarbituric acid (TBA), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5 - dithio-bis-2-nitrobenzoic acid (DTNB), hydrogen peroxide and epinephrine, were purchased from Sigma chemical company (London, UK). Alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), aspartate aminotransferase (AST) and alanine amino transferase (ALT) assay kits were products of Randox Laboratories Ltd. (Antrim, UK). Anti-Bcl-2 antibody and Anti-p53 monoclonal antibody were bought from Elabscience (Texas, USA). All other chemicals and reagents were of analytical grade and highest purity.

Animal Used and Treatments

Male rats of weight 180-220 g were got from McTemmy farm and housed in wire meshed cages at room temperature under regulated cycle of light (12-hr light: dark). The animals were fed with commercial chow and clean tap water *ad libitum*. The handling of experimental animals conformed to animal care and use in laboratory experiment [20] with ethical approval number of FNS/ERC/2023/014 from Faculty of Natural Sciences Ethical review committee of Ajayi Crowther University on 7th March, 2023.

Plant Collection and Processing

Fresh *E. gracilis* plants were harvested, identified and authenticated in the department of botany at University of Ibadan in Nigeria where it was assigned herbarium number UIH22528. The pseudobulb of the plants were removed, rinsed in water, sliced into pieces and air dried. The dried pseudobulb were then ground into powder, extracted by cold maceration in 80% methanol tagged as aqueous methanol extract of *E. gracilis* (AMEG), evaporated by rotary evaporator and then freeze-dried.

Experimental Design

A simple randomized design was employed in this research study. 24 male Wistar rats were assigned randomly into four groups with each group containing 6 rats. Group 1 is a control group and animals were administered with distilled water. Group 2 animals were administered with AMEG 200 (200 mg/kg body weight of aqueous methanolic extract of *E. gracilis* daily). The group 3 animals were given sodium arsenite (SA) alone (5 mg/kg body weight of SA once in a week). Group 4 was treated with 5 mg/kg body weight sodium arsenite once in a week and simultaneous treatment with AMEG 200. All treatments were executed orally for two weeks.

Blood Sample Collection and Plasma Separation

Blood sample was first obtained through retro-orbital plexus from rats and then sacrificed by cervical dislocation. Plasma was obtained by centrifuging blood at 3,000 rpm for ten minutes.

Preparation of Cytosolic Fractions

The liver organs were excised from rats rinsed and homogenized in 0.01 M potassium phosphate buffer (pH 7.4) and centrifuged at 12,500 g and 4 °C for 15 minutes. The supernatant obtained was used for enzymatic and non-enzymatic antioxidant assays.

Plasma Assessment for Activities of Biomarker Enzymes of Liver Function

The activities of the alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) marker enzymes were determined by using diagnostic kits from Randox. The principle described by Reitman and Frankel [21] was used for determining AST and ALT activities. ALP was evaluated following the highlighted method of Tietz *et al* [22]. Activity of GGT was assessed by method developed in [23] following manufacturing procedure on manual of assay kit.

Assessment of Non-enzymatic and Enzymatic Antioxidants

Dinitro phenyl hydrazine was used in determining vitamin C concentration in liver following the method in [24] while Ellman's reagent was used to assess hepatic glutathione as outlined in [25]. Superoxide dismutase activity in the liver was evaluated by the technique in [26] as

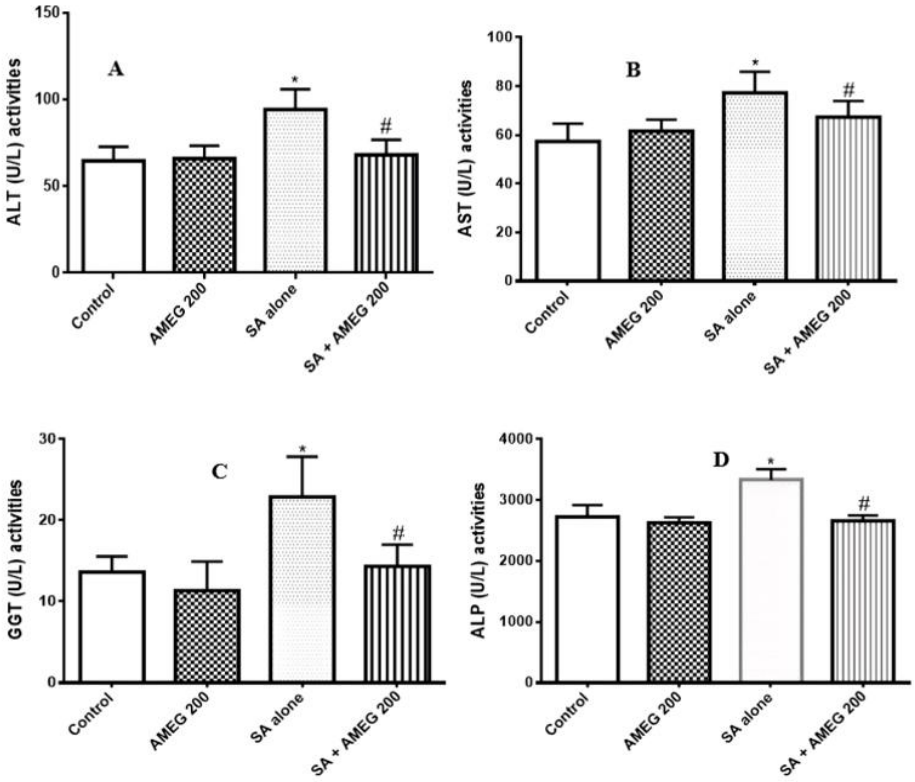

Figure 1. Influence of *E. gracilis* on sodium arsenite-induced alterations in the plasma activities of alanine amino transferase (A), aspartate amino transferase (B), gamma glutamyl transferase (C), and the alkaline phosphatase (D) in rats (data are represented as M ± SD of six animals in a group & * stand for significantly different from the control while # represents significantly different from SA group at p < 005) (Source: Authors' own elaboration)

described in [27]. The method in [28] was used for determination of hepatic catalase in which chromate acetate, a reductive product from dichromate was measured at 570 nm. Glutathione S-transferase activity in the liver homogenate was assessed by using the procedure outlined in [29].

Assessment of Biomolecular Oxidation

The extent of peroxidation of lipid biomolecule was carried out by employing the methodology in [29], where reaction between thiobarbituric acid and malondialdehyde product of lipid peroxidation (MDA) produced a pink colored chromophore at 532 nm. Hepatic level of advanced oxidized protein products (AOPPs) was evaluated as outlined in [30].

Determination of Protein

Protein content of liver homogenate was measured by the procedure in [31] where bovine serum albumin was used as standard.

Histopathology

The method described in [32] was used to process liver for histopathological examination.

Statistical Analysis

Results of the study are expressed as mean (M) ± standard deviation (SD) and then subjected to one-way analysis of variance test by using StatPac® Statistical Software. The statistical significance was based on $p < 0.05$.

RESULTS

Influence of *Eulophia Gracilis* Treatment on Sodium Arsenite- Induced Changes on Plasma Liver Function Biomarkers

Administration of *sodium arsenite* significantly increased the plasma activities of Alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT) and Alkaline Phosphatase (ALP) by 45.87%, 34.86%, 47.49% and 22.33%, respectively when compared to the control group. However, co-treatment with the *E. gracilis* significantly attenuated the SA-induced increase in plasma ALT, AST, GGT and ALP relative to exclusive SA-treated rats

Influence of *Eulophia Gracilis* on Sodium Arsenite-Induced Changes in Liver Biomarkers of Oxidative Stress in Rats

Figure 1 shows the influence of *E. gracilis* on sodium arsenite-induced alterations in the plasma activities.

The hepatic activities of enzymatic antioxidants: SOD, CAT and arylesterase (parts A, B, and C in **Figure 2**, respectively) were significantly reduced ($p < .05$) following

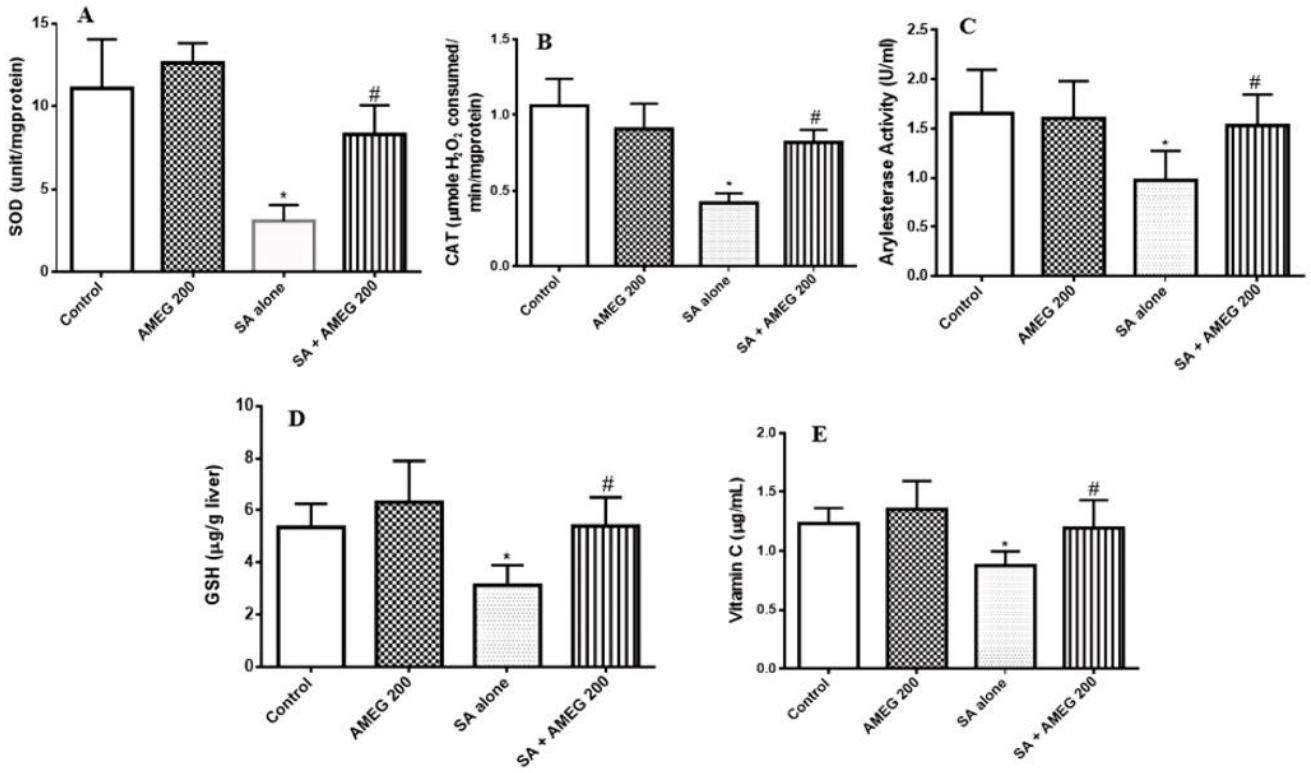

Figure 2. Ameliorative influences of *E. gracilis* on sodium arsenite-induced alterations in hepatic antioxidant status in rats: superoxide dismutase (A), catalase (B), arylesterase (C), reduced GSH (D), and vitamin C (E) (data are represented as M ± SD of six animals in a group & * stand for significantly different from the control while # represents significantly different from SA group at p < 005) (Source: Authors' own elaboration)

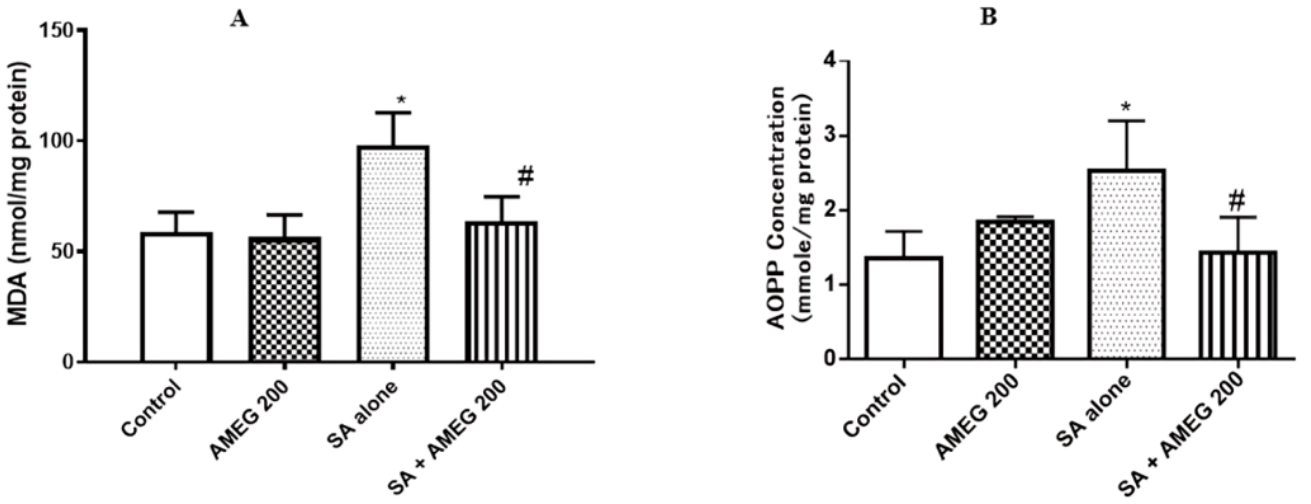

Figure 3. Ameliorative influence of *E. gracilis* on sodium arsenite-induced changes in hepatic markers of oxidative stress in rats: MDA (A) and Advanced oxidation protein product, AOPP (B) (data are represented as M ± SD of six animals in a group & * stand for significantly different from the control while # represents significantly different from SA group at p < 005) (Source: Authors' own elaboration)

sodium arsenite intoxication in rats A similar reduction was also observed in the hepatic non-enzymatic antioxidants: reduced GSH and vitamin C (parts D, C, and E in **Figure 2**, respectively). However, co-treatment with *E. gracilis* significantly ameliorated the levels of vitamin C and GSH and the activities of hepatic arylesterase, SOD, and CAT in the intoxicated rats.

Effect of *Eulophia Gracilis* on Sodium Arsenite-Induced Changes in Hepatic Concentration of MDA and Advanced Oxidation Protein Product, AOPP

MDA is one of the products of lipid peroxidation while AOPPs are the dityrosine containing protein cross linking products. Both MDA and AOPPs are products of oxidative stress. Compared to the control group, animal intoxicated

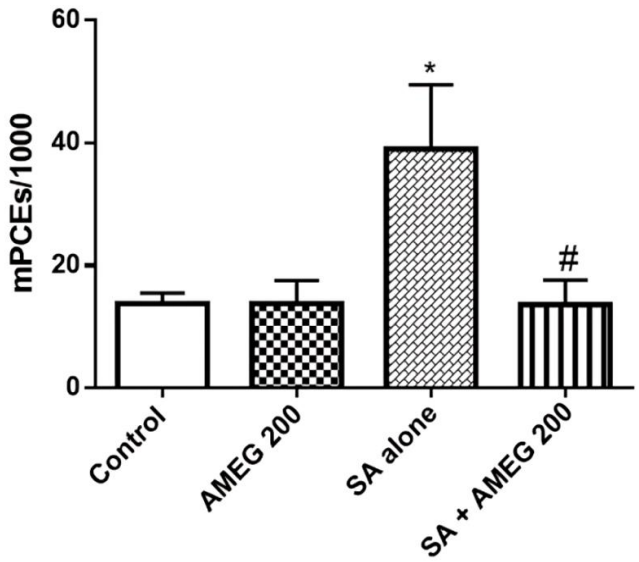

Figure 4. Effect of *E. gracilis* on sodium arsenite-induced changes in the frequency of micronucleated polychromatic erythrocytes formation in rats (data are represented as M ± SD of six animals in a group & * stand for significantly different from the control while # represents significantly different from SA group at p < 005) (Source: Authors' own elaboration)

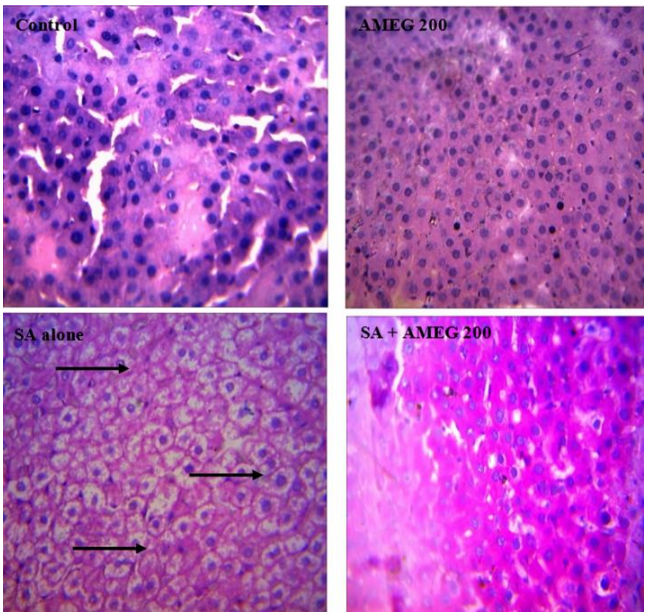

Figure 5. Histoarchitecture of rat liver showing the influence of *E. gracilis* in arsenic-induced toxicity in rats (the arrows indicate the area of vacuolar degeneration) (Source: Authors' own elaboration)

with sodium arsenite has a significant ($p < .05$) increase in the hepatic AOPPs and MDA as observed in **Figure 3**). However, co-treatment with *E. gracilis*, attenuated this increase in hepatic AOPPs and MDA levels.

Effect of *Eulophia Gracilis* on Sodium Arsenite-Induced Changes in the Frequency of Micronucleated Polychromatic Erythrocyte Formation in Rats

The depletion in antioxidant status induced by sodium arsenite intoxication was accompanied by a significant increase in the formation of micronucleated polychromatic erythrocytes in the marrow of rats as shown in **Figure 4**. This increased frequency was significantly elevated in sodium arsenite treated rats by 52.2% when compared with the control. Treatment with *E. gracilis* extract significantly attenuated this increase when compared with SA alone group

The Effect of *Eulophia Gracilis* Extract on Histoarchitecture of Liver in Sodium Arsenite-Induced Toxicity of Rats

The plates in **Figure 5** present the influence of *E. gracilis* extract on sodium arsenite-induced alteration in architecture of liver of rat. The picture in control plate shows normal histology of the liver cell where there are no visible lesions seen. Also, administration of 200 mg/kgbw of *E. gracilis* into rats does not have remarkable effect on the architectural structure of liver as there is no visible lesion observed in AMEG 200 plate of the **Figure 5**. However, intragastric exposure of rats to 5 mg/kg sodium arsenite once in seven days for fourteen days resulted in severe diffuse vacuolar degeneration of hepatocyte as shown by arrows in SA alone plate of **Figure 5**. Coadministration of 200 mg/kgbw of *E. gracilis* extract for 14 days into animals exposed to sodium arsenite intoxication nullified the histoarchitecture distortion by sodium arsenite and resulted into no visible lesions observed in SA + AMEG 200 plate.

Influence of *Eulophia Gracilis* on p53 and BCL-2 Expression in Liver Cell of Sodium Arsenite-Induced Toxicity in Rats

The p53 expression in animal intoxicated with sodium arsenite was lower with percentage high positive value of 21.87% when compared to control group which has percentage high positive value of 34.01% as presented in **Figure 6**. However, there was an improved expression of p53 protein in animal co-treated with *E. gracilis* extract.

Moreover, sodium arsenite induces increase in antiapoptotic BCL-2 protein expression with percentage high positive value of 77.08% relative to the control that has percentage positive value of 49.27% as shown in **Figure 7**. But co-administration of *E. gracilis* extract abrogated the induced increase in BCL-2 expression by sodium arsenite.

DISCUSSION

Arsenic toxicity has been associated with several defects in humans and animals majorly with progressive toxicity from liver damage to hepatocellular carcinoma [33, 34]. Sodium arsenite like other heavy metal ion interferes with the activities of thiol containing enzymes and caused genomic instability [35, 36]. The medicinal properties of plant species belonging to Eulophia genus are well documented ranging from inhibition of advanced glycation

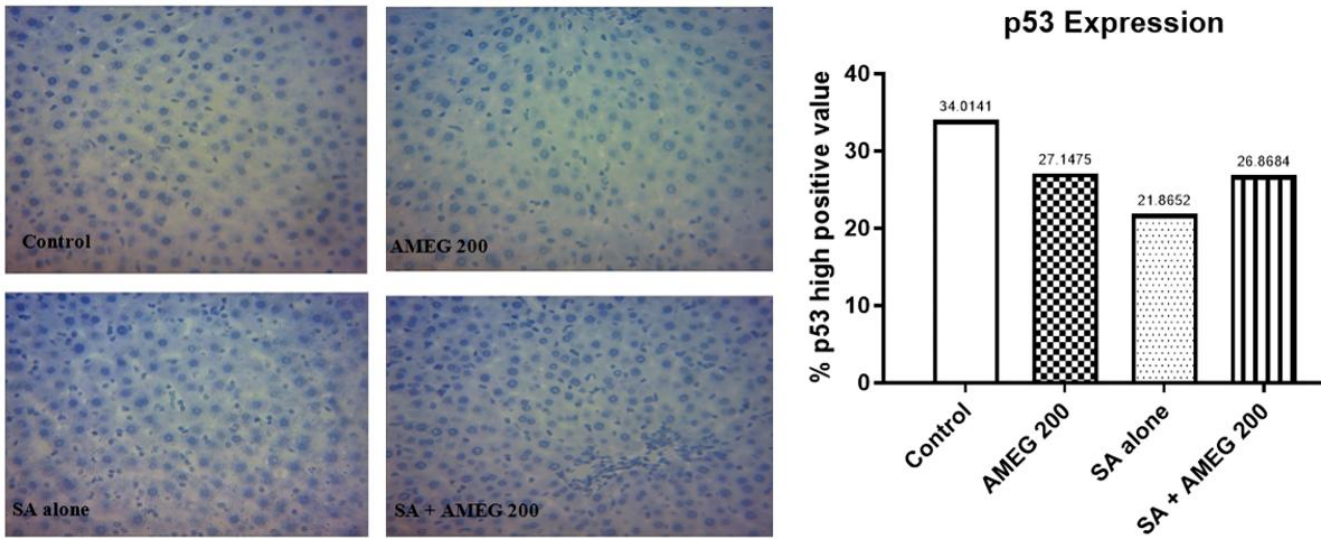

Figure 6. Influence of *E. gracilis* on p53 expression in liver cell of sodium arsenite-induced toxicity in rats with corresponding imageJ percentage high positivity in representative samples (Source: Authors' own elaboration)

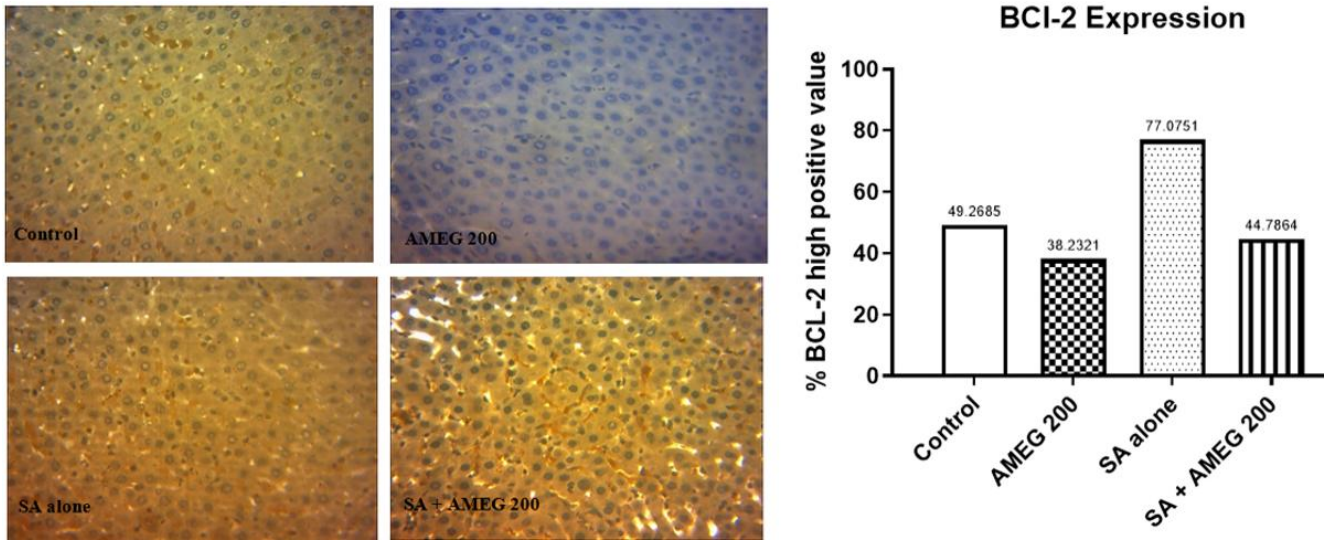

Figure 7. Influence of *E. gracilis* extract on BCl-2 expression in the liver cell of sodium arsenite-induced toxicity in rats with corresponding imageJ percentage high positivity in representative samples (Source: Authors' own elaboration)

end products, hepatoprotective, hypolipidemic and aphrodisiac effect [37, 38]. Elemental analysis of *E. gracilis* plant confirms the presence of metal ion activators like Zinc, Magnesium, copper, Iron and Manganese. The plant has been shown to contain phytochemicals like glycoside, alkaloids, tannins, phlobatanins and flavonoids which are of medicinal values [14].

In this present study, the effect of *E. gracilis* was tested on hepatotoxicity and clastogenicity induced in Wistar rats by sodium arsenite. Greater susceptibility of arsenic and its bioaccumulation in liver relative to other organs after exposure have been reported by some authors [11]. Serum ALT and AST were notably reliable markers for assessment of liver function. The surge increase in ALT and AST activities after exposure to sodium arsenite has been well documented [39]. The present work presented a significantly increase in activities of ALT and AST in sodium arsenite group relative to control animal group; this is consistent with hepatoxicity reports by other researchers on sodium arsenite where there were rise in Serum activities of AST and ALT resulting from arsenic exposure [40, 41].

In contrast, in animals treated with *E. gracilis*, the activities of ALT and AST decreased drastically. The evaluation of GGT and ALP activities is screening test with a high negative predictive value for hepatic injury [42]. Several chemical agent and drugs increase GGT activity through microsomal enzyme induction [43]. In this study, the GGT and ALP activities were elevated in SA treated Wistar rats in **Figure 1**. Significantly increased activity of GGT in SA alone animals relative to control indicates a severe damage to tissue membrane during SA toxicity because GGT is a membrane bound enzyme [44]. However, coadministration of *E. gracilis*

with SA reduced the activities of ALP and GGT significantly ($p < 0.05$) relative to animal group treated with only SA. Therefore, this could indicate the membrane stabilizing activity of *E. gracilis* and its soothing effect on liver function.

Arsenic exposure has been associated with production of reactive oxygen species and free radicals [45]. These reactive species have been associated with the pathogenesis of arsenic intoxication [46]. Oxidative stress-associated hepatic dysfunction by sodium arsenite had been earlier reported [47]. Notwithstanding, involvement of scavengers of free radicals and enzymes such as GSH peroxidase, catalase and SOD that can terminate chain reactions are parts of various mechanisms through which cells can be protected from reactive oxidants [48]. Inhibition of catalase and SOD enhanced cellular sensitivity to radical-induced damage as they prevent bioaccumulation of hydrogen peroxide and superoxide ions. SOD and catalase are marker antioxidant enzymes for hepatocellular damage when they are assessed in liver [49]. Our results showed decreased activities of SOD and catalase in SA treated group when compare with the control in part A and part B in **Figure 2**. Thus, administration of *E. gracilis* offered protection against reduced catalase and superoxide dismutase activities in co-treated animals.

The resultant influence of arsenic on SOD has been linked to GSH and vitamin C depletion as GSH and Ascorbic acid are primarily first line of defense in prevention of tissue oxidation [50]. GSH is an antioxidant molecule that directly free radicals in addition to its cofactor roles in GST and GPx that are detoxifying enzymes against oxidants. GSH also helps in regeneration of active forms of vitamins C and E [51]. In this present work, there were depletion in GSH and vitamin C levels after arsenic treatment in relation to control animals in part D and part E in **Figure 2**. This agreed with the findings of other researchers who reported GSH depletion following the exposure of rats to sodium arsenite [52]. This could be due to adjustment response mechanism to oxidative stress and this reflects the fundamental importance of GSH in the detoxification of arsenic species and enhanced excretion of methylated arsenic compounds. Also, interaction of GSH with free radicals generated by sodium arsenite metabolism may lead to its oxidation and resultant decrease in its level [53]. Our results corroborated the earlier evidences of other researchers that associated the rise in ROS in animals treated with sodium arsenite to depletion in antioxidant markers [54]. However, treatment with *E. gracilis* improved the redox status in the rat hepatocyte by statistical increase in Vitamin C and GSH levels of animal group co-treated with *E. gracilis* when compared to SA-treated animals.

Peroxidation of lipid is a biomolecular mechanism of oxidizing cellular lipids and this indicates oxidative stress consequent to overload of ROS and depleted antioxidant system [55]. Therefore, rise in the MDA concentration is considered to be a proven indicator of heightened lipid peroxidation that inferred tissue damages [56]. In this study, the significant increase in MDA concentration following sodium arsenite intoxication is an indication that sodium arsenite could enhanced oxidative stress through the induction of lipid peroxidation (part A in **Figure 3**). However, it was further shown in this study that the increased level of MDA in the rats was abated by treatment with *E. gracilis* extract.

The result obtained from micronucleus assay clearly demonstrated that SA administered induces the elevated frequency of micronucleated polychromatic erythrocytes (PCEs) in rat bone marrow as shown in **Figure 4**. Arsenic has been reported to generate radicals that could attack DNA which consequently leads to breakage of chromosome (57). It is noteworthy in this study that *E. gracilis* caused a significant reduction in mPCEs in bone marrow of co-treated animals.

AOPPs are protein products with dityrosine cross linkages that form reliable marker of evaluating the extent of protein damage by oxidants [31, 58]. Determination of AOPPs in the liver is used as a biomarker for assessing drug-induced oxidative stress mediated liver injury [59]. Oxidized proteins are produced intracellularly and in plasma when proteins are oxidized due to increased reactive oxygen generation [60, 61] and its elevated concentration has been linked to disease conditions such as nephropathies, diabetes, cancer and atherosclerosis [62]. The significant increase in generation of plasma AOPP observed in animals exposed to arsenic is an indication of sodium arsenite-induced oxidative stress with subsequent damage to proteins in the animals. However, treatment with *E. gracilis* extract alleviated the heightened level of AOPPs in co-treated animals when compared with animals that are exclusively treated with sodium arsenite. This shows that this plant extract may possess antioxidant potential and therefore prevent hepatic protein damage.

Paraoxonase (PON1) is an esterase enzyme produced in the liver but found in serum that reduces the bioaccumulation of peroxidation products due to its hydrolytic activity on organophosphate and aromatic esters [63]. It possesses antioxidant property by protecting HDL from peroxidation and prevent free radical injury to plasma membranes [64]. Previous studies reported the inhibitory influence of arsenic on paraoxonase activity [65]. Our result indicated the reduction in activity of arylesterase of PON1 following arsenic intoxication which is consistent with earlier reports from other researchers [66]. However, *E. gracilis* treatment raised the activity of arylesterase in co-treated animals.

Toxicity of sodium arsenite associated with liver injury has been confirmed in animal model by pathological aberration in liver in response to increased arsenic content in the liver. Previous studies showed that arsenic caused liver

damage with a detectable physiological change in liver [67]. The result of histopathological examination of cross section of liver organ showed a severe diffuse vacuolar degeneration of hepatocytes in SA alone treated group as compare with no observable lesion in control group. These histopathological results support the earlier results previously reported by other investigators [68]. However, administration of the extract of *E. gracilis* improve the liver architecture by mitigating histopathological lesions in livers of animal co-treated with the extract.

p53 protein is a well-recognized marker of DNA damage. Many researchers have shown that arsenite could cause DNA-protein cross link and DNA strand breakage in cells that could trigger wild type p53 protein accumulation [69, 70]. The previous finding reported that sodium arsenite downregulated the p53 expression via MAPK/ERK pathway [71]. The present results showed a reduced expression of p53 and high expression of bcl-2 in sodium arsenite-exposed animal relative to the control group in **Figure 6** and **Figure 7**, respectively. The frequent occurrence of apoptosis in liver disease is a pointer that synthesis of anti-apoptotic Bcl-2 in liver may be of therapeutic assessment. The direct link between overexpression of anti-apoptotic protein in liver and increased tumor growth and hepatocellular carcinoma development have been reported [72]. However, expression of the p53 and Bcl-2 protein were modulated in the group co-treated with *E. gracilis* extract.

In conclusion, *E. gracilis* suppressed liver dysfunction associated with sodium arsenite exposure via abrogation of oxidative stress, modulation of tumor suppressor p53 and anti-apoptotic bcl-2 proteins in liver and mitigation of genotoxicity in male rats. However, this study was performed on small sample size of six animals per group without the comparation to standard hepatoprotective drugs. We recommend further study to determine its synergistic effect and relative potential with existing hepatoprotective agent on large sample size.

Author contributions: OSO: design, conceptualization, metholodology; **OAO:** supervision; **OSO & OAO:** data curation, formal analysis, writing – original draft, writing – review & editing. Both authors have agreed with the results and conclusions.

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Ethical statement: The authors stated that the study was approved by the Research Ethics Committee, Faculty of Natural Sciences, Ajayi Crowther University, Oyo in 2017 with approval number FNS/ERC/2017/021. Written informed consents were obtained from the participants.

AI statement: The authors stated that no generative AI or AI assisted tools were used during the study.

Declaration of interest: No conflict of interest is declared by authors.

Data sharing statement: Data supporting the findings and conclusions are available upon request from the corresponding author.

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