

Ameliorative effect of gallic acid on methotrexate-induced hepatotoxicity and nephrotoxicity in rat

Ebenezer Tunde Olayinka, Ayokanmi Ore, Oluwatobi Adewumi Adeyemo, Olaniyi Solomon Ola

Biochemistry Unit, Department of Chemical Sciences, Ajayi Crowther University, Oyo, Oyo State, Nigeria

Abstract

We investigated the protective effect of gallic acid (GA) against methotrexate (MTX)-induced hepatotoxicity and nephrotoxicity. Male Wistar rats were randomized into five groups ($n = 6/\text{group}$): I, control; II, MTX-treated for seven days; III, pre-treated with GA for seven days, followed by MTX for seven days; IV, co-treated with MTX and GA for seven days and V, GA for seven days. MTX caused a significant increase ($P < 0.05$) in plasma biomarkers of nephrotoxicity (urea, creatinine) and hepatotoxicity (Bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase) when compared with control. Furthermore, MTX caused a significant decrease in the activities of hepatic enzymic antioxidants (superoxide dismutase, catalase, glutathione S-transferase) and nonenzymic antioxidants (Vitamin C and glutathione), followed by a significant increase in hepatic malondialdehyde content. However, pre-treatment and co-treatment with gallic acid ameliorated the MTX-induced biochemical changes observed. Taken together, GA protected against MTX-induced hepatotoxicity and nephrotoxicity in rats, by reducing the impact of oxidative damage to tissues.

Introduction

Methotrexate (MTX), (Figure 1A) is an antimetabolite and a 4-amino, 10-methyl analogue of folate that inhibit dihydrofolate reductase (DHFR), involved in the formation of tetrahydrofolate from dihydrofolate and consequently inhibiting DNA synthesis and cell proliferation.¹ MTX is a cytotoxic agent used in the treatment of leukaemia and various malignancies, as well as in non-neoplastic diseases such as psoriasis, rheumatoid arthritis and as anti-inflammatory and immunosuppressive agent.^{1,3} Earlier studies have revealed that MTX is converted mainly to metabolites connected to glutamate (MTX-glu) in cells and tis-

sues.⁴ The complex so formed is referred to as polyglutamated forms which is responsible for most biochemical activities of MTX.⁵

MTX is known to generate reactive oxygen species (ROS) in both normal and cancer cells resulting in oxidative damage.⁶ The anti-cancer, anti-inflammatory and immunosuppressive actions of MTX has been shown to occur via ROS generation and induction of apoptosis.⁷ MTX has been reported to induce renal and hepatic toxicity via oxidative stress and its efficacy has been limited by severe organ toxicity.^{8,9}

In the regulation of oxidative processes, cellular systems have been equipped with several antioxidant defense mechanisms, and there are several phytochemicals of plant origin with inherent antioxidant properties that are capable of boosting cellular enzymic and non-enzymic antioxidant indices thus making them excellent scavengers of free radicals.¹⁰ The chemoprotective properties of plant extracts have been extensively studied and are attributed to the presence of flavonoids, anthocyanins and phenolic compounds.¹¹ Gallic acid (GA) (3,4,5-trihydroxybenzoic acid) (Figure 1B) is a naturally occurring phenolic compound present in green tea, gall nut, grapes, red wine, hops, oak bark *etc.*¹² Several authors have reported that GA possess strong antioxidant properties and a wide array of biological and pharmacological activities such as free radical scavenging, anti-apoptotic and anti-inflammatory.¹³ Other reported biological effects include protection against doxorubicin-induced myocardial toxicity and cyclophosphamide-induced oxidative stress.¹⁴

The present study is therefore aimed at evaluating the protective effect of gallic acid pre-treatment and co-treatment on methotrexate-induced hepatotoxicity, nephrotoxicity and oxidative stress in rats. In this study, the role of GA in MTX induced hepatotoxicity and renal toxicity was evaluated by assessing plasma biomarkers of hepatic and renal function as well as selected oxidative stress markers (level of lipid peroxidation, enzymic and non enzymic antioxidants).

Materials and Methods

Drug, chemicals and reagents

Methotrexate tablets is a product of West Coast Pharmaceutical Works Ltd, Gota, Ahmedabad, India; Gallic acid, Glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5-dithio bis-2-nitrobenzoic acid (DTNB), epinephrine, and hydrogen peroxide (H_2O_2), were obtained from Sigma[®] Chemical Company, London, UK; Assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alkaline phos-

Correspondence: Ayokanmi Ore, Department of Chemical Sciences, Ajayi Crowther University, PMB 1066, Oyo, Oyo State, Nigeria.
Tel.: +2348061656814.
E-mail: oreayokanmi@gmail.com

Key words: Methotrexate; hepatotoxicity; nephrotoxicity; gallic acid; rat.

Contributions: ETO designed the study; AO, OAA and OSA were involved in the animal treatments, laboratory and statistical analysis. OAA prepared the manuscript. AO handled all correspondence regarding the manuscript. The final version was read and approved by all authors.

Conflict of interest: the authors declare no potential conflict of interest.

Received for publication: 14 June 2016.
Revision received: 13 August 2016.
Accepted for publication: 15 August 2016.

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Journal of Xenobiotics 2016; 6:6092
doi:10.4081/xeno.2016.6092

phatase (ALP), urea, Creatinine, total bilirubin were purchased from Randox[®] Laboratories Ltd. (Antrim, UK). All other reagents used were of analytical grade and of highest purity.

Animals

Male rats (Wistar strain) weighing between 160-180 g were obtained from the animal housing unit, in the Department of Chemical Sciences, Ajayi Crowther University, Oyo, Nigeria. The rats were acclimatized under laboratory conditions prior to experiment. The animals were housed in wire-meshed cages and provided with water and food *ad libitum*. They were fed with commercial rat diet (Ladokun[®] Feeds, Nigeria Ltd Ibadan, Nigeria). The study was approved by the ethical committee of the Faculty of Natural Sciences, Ajayi Crowther University, Oyo, Nigeria. Handling of the experimental animals was done in accordance with international guidelines on the care and use of experimental animals (National Research Council).¹⁵

Experimental design

Thirty rats were randomly assigned into five experimental groups (I–V) of six animals each. The animals of each group were treated as presented in Table 1. The dose for MTX (0.2 mg/kg bw) was selected based on the recommended adult dose for rheumatoid arthritis and other inflammatory diseases while the dose for GA (20 mg/kg bw) was arrived at based

on available literature.¹⁶ The respective doses were prepared based on the average weight of animals in each treatment groups and administered in one mL of distilled water. The drug doses were administered once daily by oral intubation.

Plasma and tissue preparation

Blood samples were collected from each animal, via retro orbitals plexus in heparinized sample tubes (Li heparin). Animals were sacrificed and liver was collected from each animal for preparation of the post-mitochondrial fraction (PMF).

Centrifugation of blood samples were done at 4000 rpm for 5 minutes in a bench centrifuge (Analytica, Athens, Greece). The plasma obtained were stored at -4°C for subsequent plasma assays. Liver samples were rinsed in ice-cold 1.15% KCl and homogenized in 4 volumes of 0.01 M potassium phosphate buffer (pH 7.4). The homogenates obtained were subjected to centrifugation at $12,500\times g$ for 15 min at -4°C in a refrigerated centrifuge (Eppendorf UK Ltd., Stevenage, UK) and supernatants (PMF) were collected in sample tubes and used for subsequent biochemical assays.

Biochemical analysis

Total protein

The protein concentration in the liver PMF was determined according to the biuret method of Gornall *et al.*¹⁷

Biomarkers of renal function

Plasma level of creatinine and urea were determined with RANDOX[®] diagnostic kits following the manufacturer's protocol. The method for creatinine assays was based on Jaffe¹⁸ and the method of Tietz¹⁹ was employed for plasma urea determination.

Biomarkers of hepatic function

Plasma total bilirubin (TBILI) level, and activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate

aminotransferase (AST), and gamma glutamyl transferase (γ -GT) were assayed using RANDOX[®] diagnostic kits based on the manufacturer's procedure. Assay of TBILI level was based on the method of Tietz *et al.*²⁰ Activity of ALP was determined according to Tietz *et al.*²⁰ Plasma activities of ALT and AST were measured according to the method of Reltman and Frankel.²¹ The plasma activity of γ -GT was determined following the method described by Szasz.²²

Biomarkers of oxidative stress

The level of hepatic reduced glutathione (GSH) was determined following the method of Jollow *et al.*²³ Ellman's reagent reacts with reduced glutathione, and the chromophoric product resulting from the reaction has a molar absorption at 412 nm. The level of vitamin C in the liver PMF was determined according to Jagota and Dani.²⁴ Vitamin C in samples reacts with Folin-phenol reagent resulting in blue chromophore which has maximum absorption at 760 nm. Hepatic GST activity was measured following the method of Habig *et al.*²⁵ The procedure described by Misra and Fridovich²⁶ was used for the determination of hepatic superoxide dismutase activity. Hepatic catalase activity was determined by the method described by Singha.²⁷ Hepatic level of lipid peroxidation (LPO) was determined by the method described by Vashney and Kale.²⁸ This assay involved the reaction between thiobarbituric acid and malondialdehyde (MDA) a

product of lipid peroxidation to yield a stable pink chromophore which absorbs maximally at 532 nm.

Statistical analysis

Data are presented as the mean \pm standard deviation (SD) of six replicates. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison between control and treated rats in all groups using Sigma plot[®] statistical package (Systat Software Inc., San Jose, CA, USA). P-values less than 0.05 ($P<0.05$) were considered statistically significant.

Results

Protective effects of gallic acid on MTX-induced changes in markers of hepatic and renal toxicity

As presented in Table 2, administration of MTX caused a significant ($P<0.05$) increase in plasma creatinine and urea levels (124% and 68.5% respectively) compared to control. However, pre-treatment and co-treatment of GA with MTX attenuated the observed elevated plasma urea and creatinine levels when compared with the MTX-treated group.

The plasma level of total bilirubin (TBILI) and alkaline phosphatase (ALP) activity (biomarkers of hepatobiliary damage) increased

Table 1. Experimental design.

Treatment-Groups	Treatments-Duration	
	Day 1-7	Day 8-14
I (CTRL)	-	Control; distilled water
II (MTX)	-	0.2 mg/kg bw MTX
III (MTX + GA) (pre-treated)	20 mg/kg bw GA	0.2 mg/kg bw MTX
IV (MTX + GA) (co-treated)	-	0.2 mg/kg bw MTX + 20 mg/kg bw GA
V GA	-	20 mg/kg bw GA

CTRL, control; MTX, methotrexate; GA, gallic acid.

Table 2. Ameliorative effect of gallic acid pre-treatment and co-treatment on methotrexate-induced changes in plasma biomarkers of renal and hepatic function in rat.

Group Parameter	Control	MTX	MTX + GA (pre-treated)	MTX + GA (co-treated)	GA
Creatinine (mg/dL)	0.98 \pm 0.13	2.2 \pm 0.1*	1.4 \pm 0.1 ^a	1.5 \pm 0.1 ^a	1.2 \pm 0.1
Urea (mg/dL)	35.0 \pm 1.2	59.0 \pm 2.6*	43.2 \pm 2.3 ^a	45.5 \pm 3.2 ^a	39.4 \pm 2.9
TBILI (mg/dL)	0.36 \pm 0.02	0.86 \pm 0.03*	0.47 \pm 0.02 ^a	0.49 \pm 0.02 ^a	0.43 \pm 0.02
ALP (U/L)	157.4 \pm 6.4	406.8 \pm 7.3*	268.8 \pm 1.9 ^a	327.4 \pm 1.4 ^a	287.0 \pm 7.5
ALT (U/L)	21.5 \pm 1.6	43.6 \pm 2.4*	26.9 \pm 1.6 ^a	33.0 \pm 2.2 ^a	26.8 \pm 2.4
AST (U/L)	147.2 \pm 5.3	284.1 \pm 6.3*	195.3 \pm 4.8 ^a	240 \pm 5.7 ^a	193.4 \pm 3.8
GGT (U/L)	1.5 \pm 0.2	3.9 \pm 0.2*	2.1 \pm 0.1 ^a	2.3 \pm 0.1 ^a	1.7 \pm 0.2

Data are expressed as mean \pm S.D for six rats in each group. *Significantly different from the control ($P<0.05$); ^asignificantly different from the methotrexate group ($P<0.05$); values in parenthesis represent percentage (%) increase.

significantly ($P < 0.05$) in rats by 138.8% and 158% respectively following MTX treatment (Table 2). However, the levels of TBILI and ALP activity were significantly ameliorated in the plasma of animals pre-treated or co-treated with GA when compared with the MTX group.

MTX treatment also caused a significant increase in the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transferase (γ -GT) (biomarkers of hepatocellular toxicity) in the plasma of rats by 102.8%, 93% and 106.7% respectively compared to values in control (Table 2). Pre-treatment and co-treatment of GA with MTX significantly ameliorated the elevated activities of plasma ALT, AST, and γ -GT when compared to MTX-treated group.

Protective effects of gallic acid on MTX-induced changes in markers of oxidative stress. Hepatic SOD and catalase activity (Table 3) were significantly reduced in the MTX-treated group by 34.0% and 47.8% when

compared with control ($P < 0.05$). Also, hepatic GST activity (Figure 2) was also significantly reduced by 38.3% in the MTX-treated rats when compared with the control. However, GA pre-treatment and co-treatment significantly ameliorated the MTX-induced decrease in hepatic activities of SOD, CAT, and GST relative to the MTX-treated group ($P < 0.05$).

Furthermore, hepatic ascorbic acid (AA) and GSH level (Figures 3 and 4) were significantly ($P < 0.05$) decreased following treatment with MTX by 37.9% and 38.9% when compared with the control. Conversely, pre-treatment and co-treatment with GA significantly ($P < 0.05$) protected against the MTX-induced decrease in hepatic AA and GSH levels when compared with the MTX group. In addition, the hepatic MDA level rose significantly ($P < 0.05$) in the MTX-treated rats by 58.8% when compared with the control (Figure 5). However, GA pre-treatment and co-treatment attenuated the increase in hepatic MDA relative to the MTX-

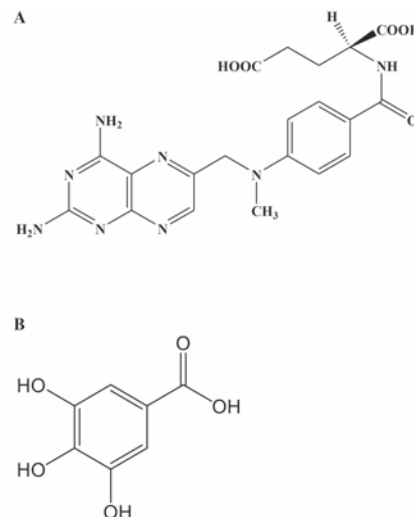


Figure 1. Chemical structure of methotrexate (A) and gallic acid (B).

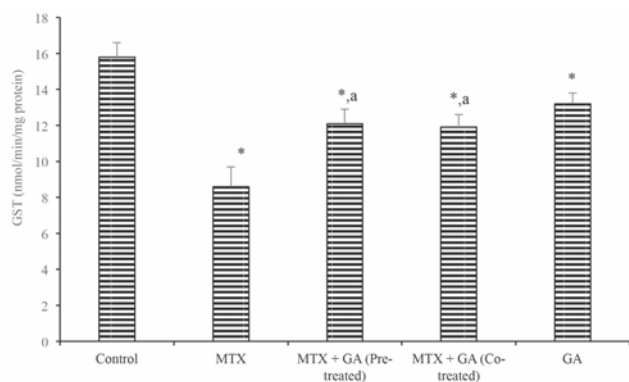


Figure 2. Ameliorative effect of gallic acid pre-treatment and co-treatment on methotrexate-induced reduction in hepatic glutathione S-transferase (GST) activity in rats. Data are expressed as mean \pm S.D for six rats in each group. *Significantly different from the control ($P < 0.05$); ^asignificantly different from the methotrexate group ($P < 0.05$).

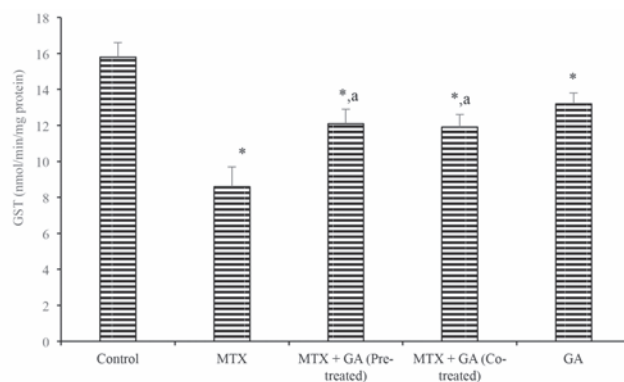


Figure 4. Ameliorative effect of gallic acid pre-treatment and co-treatment on methotrexate-induced reduction in hepatic glutathione (GSH) level in rats. Data are expressed as mean \pm S.D for six rats in each group. *Significantly different from the control ($P < 0.05$); ^asignificantly different from the methotrexate group ($P < 0.05$).

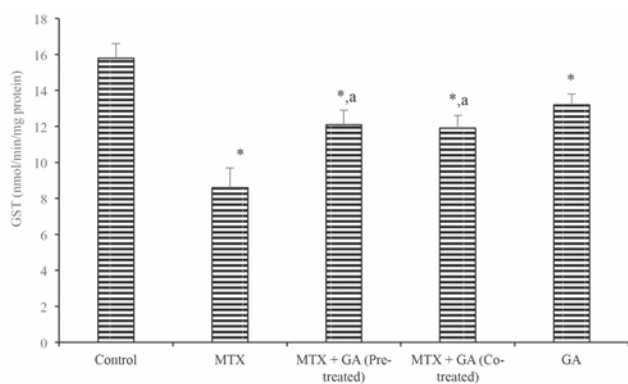


Figure 3. Ameliorative effect of gallic acid pre-treatment and co-treatment on methotrexate-induced reduction in hepatic vitamin C level in rats. Data are expressed as mean \pm S.D for six rats in each group. *Significantly different from the control ($P < 0.05$); ^asignificantly different from the methotrexate group ($P < 0.05$).

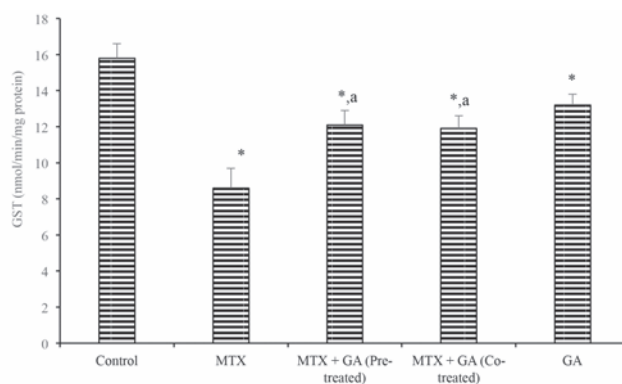


Figure 5. Ameliorative effect of gallic acid pre-treatment and co-treatment on methotrexate-induced increase in hepatic level of lipid peroxidation (MDA) in rats. Data are expressed as mean \pm S.D for six rats in each group. *Significantly different from the control ($P < 0.05$); ^asignificantly different from the methotrexate group ($P < 0.05$).

treated group.

Discussion and Conclusions

Methotrexate (MTX) is a chemotherapeutic agent indicated in conditions such as autoimmune diseases, inflammatory myopathies, leukemia etc. However it is known to be associated with side effects including hepatotoxicity and nephrotoxicity; often mediated by reactive oxygen species (ROS).²⁹⁻³¹ Recent studies have demonstrated the protective roles of phytochemicals in drug-induced organ toxicity. Gallic acid employed in the present study is a potent natural antioxidant with numerous biological activities including hepatoprotective activity.³²⁻³⁴

Biomarkers of renal function: urea and creatinine were considered in this study. Urea and creatinine are metabolic products removed by the kidney from circulation. Increase in their plasma level is an indication of loss in renal function.³⁵ The increase in plasma urea and creatinine observed in this study is in agreement with previous report on MTX.³¹ The attenuation of the level of plasma urea and creatinine by GA is an indication of improved renal function and the nephroprotective role of GA.³⁶

The liver is the target of several xenobiotics including chemotherapeutic agents such as MTX. MTX is stored in the liver cells as MTX-polyglutamates and has been linked to MTX-induced hepatotoxicity.³⁷ The plasma level of bilirubin and activities of AST, ALT, ALP and -GT are reliable indices of hepatotoxicity.³⁸ Elevation of the plasma activities of AST and ALT have been linked to structural damage to the liver.³⁹ Bilirubin is present in the liver, bile, intestine and the reticuloendothelial cells of the spleen. High plasma bilirubin, ALP and -GT levels are indicators of hepatobiliary injury.^{38,39} However, pre-treatment and co-treatment with GA ameliorated the MTX-induced cellular damages which agrees with previous finding on the hepatoprotective activity of GA.³⁷

Oxidative stress has been identified as a toxicological mechanism of most chemotherapeutic drugs. Free radicals release and ROS plays a significant role in the MTX-induced hepatotoxicity and nephrotoxicity.^{30,31} In this study, MTX caused a significant decrease in the hepatic SOD, CAT, GST, GSH and vitamin C. SOD catalyses the rapid dismutation of superoxide anions to hydrogen peroxide (H₂O₂) and molecular oxygen, while CAT converts the H₂O₂ formed to water and molecular oxygen. The low molecular weight antioxidants, vitamin C and GSH play an important role in cellular redox balance. They are the first line of defense against oxidation damages.⁴⁰ AA is

involved in the preservation of tocopherol in membranes and lipoproteins, while GSH act as a substrate for several antioxidant enzymes such as glutathione peroxidase (GPx), glutathione -S - transferase (GST).⁴¹ GSTs are a family of proteins primarily involved in the detoxification of highly reactive electrophiles including drugs by its combined action with GSH as a conjugating agent.⁴² The reduction in the levels of the antioxidant defense system occasioned by MTX may predispose the liver to oxidative injury. However, GA significantly improved the antioxidant defense systems in the liver of rats in a similar manner to previous findings.⁴³ Lipid peroxidation induced by MTX is an indication of the involvement of free radicals in MTX-mediated toxicity. The amelioration of hepatic LPO by GA in the study may be related to the free radical scavenging properties of gallic acid.^{43,44}

In summary, current findings suggest that gallic acid has the potential to protect against Methotrexate-induced hepatotoxicity and nephrotoxicity. In addition, the mechanism of protection by gallic acid may involve free - radical scavenging. Therefore, gallic acid may be employed as a co-therapy in MTX chemotherapy as a protection against chemotherapy-associated oxidative damage to tissues.

Research highlights

- Chemotherapy-associated oxidative stress is a relevant side effect of most anticancer agents.
- Methotrexate (MTX) acts as an antimetabolite as its anticancer mechanism.
- Gallic acid is a plant derived antioxidant.
- Hepatotoxicity, nephrotoxicity and oxidative stress was observed following exposure to methotrexate.
- Administration of gallic acid as a pre-treatment or co-administered with MTX ameliorated MTX induced toxicity in rats.

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