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Comparative Evaluation of Hepatoprotective Activity of Apigenin and Alpha-Tocopherol Against Methotrexate-Induced Hepatotoxicity in Rats

Vignesh .V* and Dr.N.Chidambaranathan

Department of Pharmacology, K.M College of Pharmacy, The Tamil Nadu Dr. M.G.R Medical University, Chennai, Tamil Nadu, India

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ABSTRACT

Background: Methotrexate (MTX), a folate-antagonist antimetabolite widely used in the management of malignancies, rheumatoid arthritis and psoriasis, has clinical utility that is frequently limited by dose-dependent hepatotoxicity driven largely by oxidative stress. Objective: This study was designed to comparatively evaluate the hepatoprotective potential of apigenin and alpha-tocopherol, individually and in combination, against MTX-induced hepatic injury in rats, with silymarin employed as the standard reference hepatoprotective agent. Methods: Twenty-four Wistar albino rats were allocated into six groups of four animals each: normal control; MTX control (20 mg/kg, i.p., single dose on day 7); silymarin (50 mg/kg/day, p.o.) plus MTX; apigenin (50 mg/kg/day, p.o.) plus MTX; alpha-tocopherol (100 mg/kg/day, p.o.) plus MTX; and a combination of apigenin and alpha-tocopherol plus MTX. Oral treatments were administered daily for fourteen days. Hepatic superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and malondialdehyde (MDA), together with serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), were estimated, and liver sections were examined histologically. Results: MTX administration produced a marked depletion of hepatic SOD, CAT and GSH and a significant elevation of MDA, ALT, AST and ALP relative to the normal control ($p<0.001$), accompanied by hepatocellular degeneration, cytoplasmic vacuolation and pyknotic nuclei on histopathology. Pre-treatment with apigenin, alpha-tocopherol and, most markedly, their combination significantly attenuated these oxidative and biochemical derangements and preserved hepatic architecture ($p<0.001$ versus the MTX control), with the combined regimen producing the most consistent protection among the test groups and approaching the effect of silymarin. Conclusion: Apigenin and alpha-tocopherol confer hepatoprotection against methotrexate-induced liver injury through complementary antioxidant mechanisms, and their combined use may represent a rational adjunct strategy meriting further pharmacological and clinical investigation.

Introduction

The liver occupies a central position in xenobiotic metabolism, and this metabolic burden renders it particularly susceptible to injury from a wide range of therapeutic agents. Drug-induced liver injury remains one of the most frequent causes of hepatic dysfunction encountered in clinical practice and is a recurring concern during the development and long-term use of several classes of drugs, including antineoplastic and immunomodulatory agents [1–3].

Methotrexate (MTX) is a folic-acid-antagonist antimetabolite that competitively inhibits dihydrofolate reductase, thereby blocking the conversion of dihydrofolate to tetrahydrofolate and curtailing the synthesis of purine nucleotides and thymidylate required for DNA, RNA and protein synthesis. This antiproliferative action underlies its therapeutic value in acute lymphoblastic leukaemia, non-Hodgkin lymphoma, rheumatoid arthritis and psoriasis [4–6]. Despite this broad clinical utility, prolonged or repeated MTX administration is frequently constrained by dose-limiting hepatotoxicity, which remains one of the principal adverse effects restricting its long-term use [7,8].

The mechanistic basis of MTX-induced hepatotoxicity is now reasonably well characterised and is understood to involve excessive generation of reactive oxygen species (ROS) by MTX polyglutamate metabolites, mitochondrial dysfunction, release of pro-inflammatory cytokines and hepatocyte apoptosis [9]. The resulting oxidative burden overwhelms the hepatic enzymatic antioxidant defences, superoxide dismutase (SOD) and catalase (CAT), and depletes the non-enzymatic antioxidant reduced glutathione (GSH), while simultaneously driving peroxidative degradation of membrane phospholipids, the extent of which is conventionally quantified through the end-product malondialdehyde (MDA) [10,11]. This cascade of oxidative injury culminates in loss of

* Corresponding author
✉: Vignesh .V: vignesh7101999@gmail.com

hepatocellular membrane integrity, reflected biochemically as leakage of the transaminases alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and of alkaline phosphatase (ALP) into the circulation, and structurally as hepatocellular degeneration, cytoplasmic vacuolation, inflammatory infiltration and, in more severe injury, focal necrosis on histopathological examination [3,10].

From a public-health perspective, drug-induced hepatotoxicity is among the leading causes of acute liver injury reported in clinical practice and a recurrent reason for treatment discontinuation, regulatory restriction and, in severe cases, withdrawal of otherwise effective drugs from the market. Because methotrexate continues to be used at relatively high cumulative doses in oncology and at lower but chronic doses in rheumatology and dermatology, even a modest reduction in its hepatotoxic liability through adjunct antioxidant co-therapy could meaningfully extend its safe therapeutic use. Rodent models of methotrexate-induced hepatotoxicity are widely accepted for this purpose because they reproduce, in a reproducible and controllable manner, the biochemical and histological hallmarks of the human condition, thereby providing a practical experimental platform on which candidate hepatoprotective agents can be screened before consideration for further pharmacological development [7,9,10].

Given this well-established oxidative mechanism, compounds capable of scavenging free radicals, chelating pro-oxidant transition metals, or interrupting the propagation of lipid peroxidation are of particular pharmacological interest as candidate hepatoprotective agents against MTX-induced liver injury. Apigenin, a naturally occurring flavone widely distributed in fruits, vegetables and herbs, possesses well-documented antioxidant, anti-inflammatory and free-radical-scavenging properties. Its hydroxylated flavone structure allows direct quenching of reactive oxygen species and chelation of transition metal ions that would otherwise catalyse Fenton-type reactions, while a favourable low-toxicity profile has supported its investigation across a range of experimental models of oxidative organ injury, including the liver [12–16].

Alpha-tocopherol, the principal biologically active isoform of vitamin E, is a lipid-soluble, chain-breaking antioxidant that resides within cell membranes, where it intercepts lipid peroxyl radicals and arrests the propagation phase of lipid peroxidation before it can compromise membrane integrity. Hepatic alpha-tocopherol transfer protein selectively redistributes the vitamin to peripheral tissues, underscoring the liver's role in regulating whole-body antioxidant defence, and alpha-tocopherol has previously demonstrated protective activity against oxidative hepatic injury induced by several toxicants [17–21].

Silymarin, a standardised flavonolignan complex derived from the seeds of Silybum marianum (milk thistle), is the most extensively studied and clinically established hepatoprotective agent and served in this study as the reference standard. Its protective activity has been attributed to hepatocyte membrane stabilisation, scavenging of free radicals, and stimulation of antioxidant enzyme regeneration within hepatocytes, actions that collectively support its long-standing use as a benchmark against which novel hepatoprotective candidates are evaluated [22–26].

Although the individual hepatoprotective properties of apigenin, alpha-tocopherol and silymarin have each been documented in various models of chemically induced liver injury, comparative data examining apigenin and alpha-tocopherol side by side, and evaluating whether their combination confers additive or synergistic benefit specifically against methotrexate-induced hepatotoxicity, remain limited [27–30]. Because apigenin acts predominantly as an aqueous-phase radical scavenger and metal chelator whereas alpha-tocopherol functions within the lipid membrane compartment, a rational basis exists for hypothesising that their combined administration could provide more comprehensive antioxidant coverage than either agent alone. The present study was therefore undertaken to evaluate and comparatively assess the hepatoprotective effect of apigenin and alpha-tocopherol, individually and in combination, against methotrexate-induced hepatotoxicity in Wistar albino rats, using silymarin as the standard reference hepatoprotective agent, with hepatic antioxidant status, serum biochemical markers of hepatocellular integrity, and histopathological correlation as the principal study endpoints. The aim of the present study was to evaluate and comparatively assess the hepatoprotective effect of apigenin and alpha-tocopherol against methotrexate-induced hepatotoxicity in Wistar albino rats, using silymarin as the standard reference hepatoprotective agent. The specific objectives were: (i) to induce hepatotoxicity in rats using methotrexate; (ii) to evaluate the hepatoprotective activity of apigenin; (iii) to evaluate the hepatoprotective activity of alpha-tocopherol; (iv) to evaluate the combined hepatoprotective activity of apigenin and alpha-tocopherol; and (v) to compare the effects of the test drugs, individually and in combination, with those of the standard hepatoprotective drug silymarin.

Materials and Methods

The present investigation was carried out in the Department of Pharmacology, K.M. College of Pharmacy, Uthangudi, Madurai, following approval from the

Institutional Animal Ethics Committee (IAEC approval no. TNMGRU/M.PHARM/261624500508/KMCP/08/2025-2026), in accordance with CPCSEA guidelines for the care and use of laboratory animals.

Drugs, Chemicals and Reagents

Apigenin was procured from BLD Pharmatech Pvt. Ltd., Hyderabad, India, and alpha-tocopherol (vitamin E) from SM Chemicals, Mumbai, India. Methotrexate and silymarin (the standard hepatoprotective drug) were obtained through standard commercial sources. Apigenin and silymarin, being poorly water-soluble, were suspended in 0.5% w/v sodium carboxymethyl cellulose (CMC) for oral administration; alpha-tocopherol, being practically insoluble in water, was dissolved in olive oil; and methotrexate was prepared in normal saline. Diagnostic kits for ALT, AST, ALP and total protein, together with hematoxylin and eosin stain, 10% neutral buffered formalin and analytical-grade reagents, were used as required.

Table 1: showing the solubility characteristics and vehicle used for oral/intraperitoneal administration of each drug.

Drug	Solubility	Vehicle used
Apigenin	Poorly soluble in water	0.5% w/v sodium CMC, oral
Silymarin	Poorly soluble in water	0.5% w/v sodium CMC, oral
Alpha-tocopherol	Practically insoluble in water; soluble in oils	Olive oil, oral
Methotrexate	Formulation-dependent aqueous preparation	Normal saline, i.p.

Experimental Animals

Healthy adult Wistar albino rats of either sex, weighing 180–220 g, were procured and acclimatised to the laboratory environment for seven days prior to the start of the experiment. Animals were housed under standard conditions (12 h light/12 h dark cycle, ambient temperature and humidity), maintained on a standard pellet diet with water available ad libitum, and were randomly allocated into six experimental groups of four animals each, for a total study duration of fourteen days.

Experimental Design and Treatment Protocol

Hepatotoxicity was induced by a single intraperitoneal dose of methotrexate (20 mg/kg) administered on day 7 of the experimental protocol. The treatment schedule for each group is summarised in Table 2.

Table 2. Experimental group allocation and treatment schedule (n = 4 rats/group).

Group	Treatment protocol
Group I — Normal control	Vehicle only, p.o., days 1–14
Group II — MTX (toxic) control	Vehicle, days 1–14; MTX 20 mg/kg, i.p., on day 7
Group III — Standard	Silymarin 50 mg/kg/day, p.o., days 1–14; MTX on day 7
Group IV — Apigenin	Apigenin 50 mg/kg/day, p.o., days 1–14; MTX on day 7
Group V — Alpha-tocopherol	Alpha-tocopherol 100 mg/kg/day, p.o., days 1–14; MTX on day 7
Group VI — Combination	Apigenin 50 mg/kg/day + alpha-tocopherol 100 mg/kg/day, p.o., days 1–14; MTX on day 7

All treatment drugs were freshly prepared before each administration and given orally by gavage at a fixed time daily to minimise inter-day variability. Following methotrexate challenge on day 7, the respective treatments were continued uninterrupted until completion of the fourteen-day protocol. At the end of the study, animals were fasted overnight and anaesthetised for terminal sample collection.

Estimation of Serum Biochemical Parameters

Blood samples were allowed to clot and centrifuged to separate serum, which was used for spectrophotometric estimation of ALT, AST, ALP and total protein using commercially available diagnostic kits, following the manufacturer's standard procedures.

Estimation of Hepatic Oxidative Stress Parameters

Excised liver tissue was rinsed in ice-cold normal saline, blotted dry and divided into portions for biochemical and histological analysis. A 10% w/v liver homogenate was prepared for estimation of oxidative stress parameters. Reduced glutathione (GSH) was estimated by the method of Hissin and Hilf; catalase (CAT) activity was assayed by the dichromate–acetic acid method of Sinha; superoxide dismutase (SOD) activity was determined by the nitro-blue-tetrazolium inhibition method of Kono; and malondialdehyde (MDA), the end product of lipid peroxidation, was quantified using the thiobarbituric-acid-reactive-substances (TBARS) fluorometric assay with 1,1,3,3-tetramethoxypropane as standard.

Histopathological Examination

A separate portion of liver tissue from each animal was fixed in 10% neutral buffered formalin, processed

routinely, embedded in paraffin, sectioned at 5 µm thickness and stained with haematoxylin and eosin. Stained sections were examined under a light microscope for architectural and cytological changes, and representative photomicrographs were captured for each group.

Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM) for four animals per group. Group differences were analysed by one-way analysis of variance (ANOVA) followed by an appropriate post- hoc multiple comparison test. A probability value of $p<0.001$ was taken to indicate statistical significance, with comparisons made both against the normal control and against the methotrexate (toxic) control.

Results and Discussion

Administration of methotrexate produced a consistent pattern of oxidative hepatic injury across all measured endpoints, which was variably attenuated by the test drugs, as summarised below.

Effect on Hepatic Antioxidant Status

Table 3. Effect of apigenin, alpha-tocopherol and their combination on hepatic SOD, CAT, GSH and MDA in methotrexate-induced hepatotoxicity.

Group	SOD (U/mg protein)	CAT (U/mg protein)	GSH (µg/mg protein)	MDA (nmol/mg protein)
I – Normal control	3.70 ± 0.074	2.67 ± 0.06	1.77 ± 0.08	50.25 ± 3.04
II – MTX control	1.63 ± 0.01**a	1.30 ± 0.07**a	0.71 ± 0.07**a	95.01 ± 5.01**a
III – Silymarin (std.)	3.58 ± 0.05**b	2.61 ± 0.06**b	1.73 ± 0.07**b	53.12 ± 3.02**b
IV – Apigenin	3.35 ± 0.05**b	2.42 ± 0.07**b	1.61 ± 0.08**b	59.27 ± 3.24**b
V – Alpha-tocopherol	3.12 ± 0.04**b	2.21 ± 0.08**b	1.49 ± 0.06**b	65.34 ± 3.56**b
VI – Combination	3.54 ± 0.04**b	2.57 ± 0.05**b	1.70 ± 0.07**b	54.76 ± 3.12**b

*Values are mean ± SEM (n = 4/group), analysed by one-way ANOVA followed by post-hoc multiple comparison testing. **a p<0.001 vs normal control; **b p<0.001 vs MTX (toxic) control.*

As shown in Table 3, the normal control group (Group I) displayed baseline hepatic antioxidant status. Methotrexate challenge alone (Group II) produced a highly significant fall in SOD, CAT and GSH together with a marked rise in MDA relative to Group I ($p<0.001$), consistent with overwhelming of the enzymatic and non-enzymatic antioxidant defences and accelerated lipid peroxidation of hepatocyte membranes.

Silymarin treatment (Group III) restored antioxidant status closest to normal among all treatment groups. Apigenin (Group IV) and the combination of apigenin with alpha-tocopherol (Group VI) produced the next-closest recovery, each significantly improving SOD, CAT, GSH and MDA relative to the MTX control ($p<0.001$). Alpha-tocopherol alone (Group V) also conferred significant protection but to a comparatively modest degree relative to the other treatment groups, a pattern consistent with its more restricted, membrane-localised mode of antioxidant action relative to the broader aqueous- and membrane-phase coverage achieved by apigenin and the combination regimen, respectively (Figures 1–4)

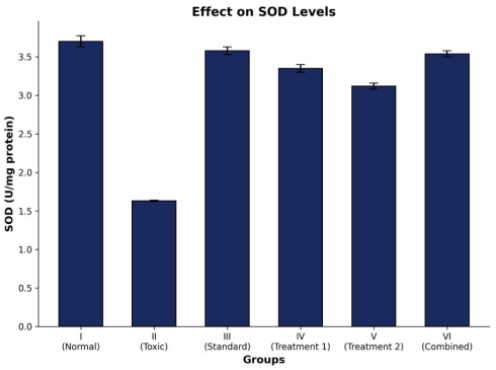

Figure 1. Hepatic SOD levels

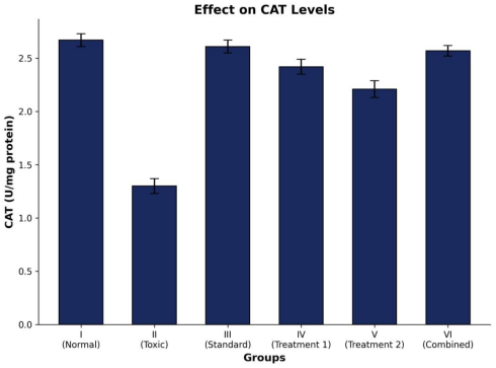

Figure 2. Hepatic CAT levels

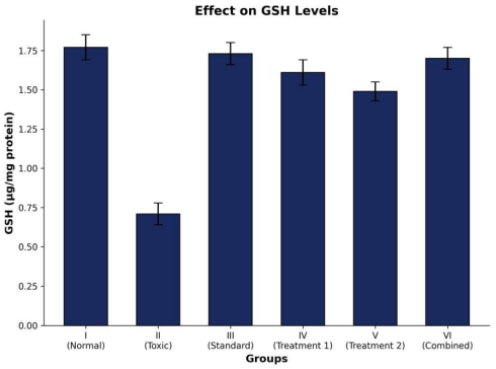

Figure 3. Hepatic GSH levels

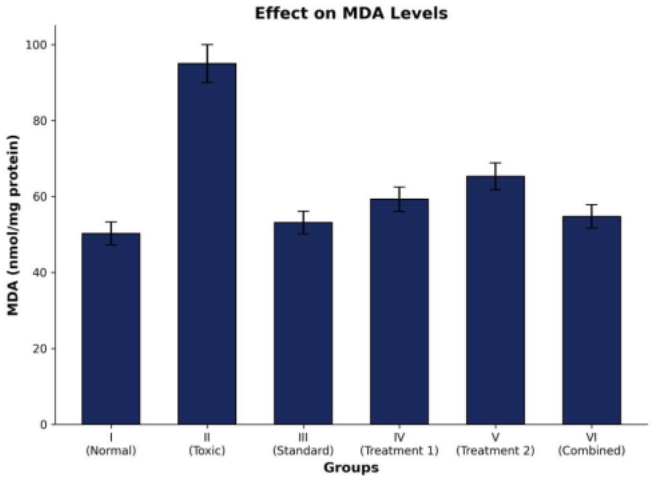

Figure 4. Hepatic MDA levels

Effect on Serum Biochemical Parameters

Table 4. Effect of apigenin, alpha-tocopherol and their combination on serum ALT, AST and ALP in methotrexate-induced hepatotoxicity.

Group	ALT (IU/mL)	AST (IU/mL)
I – Normal control	33.99 ± 1.05	30.34 ± 0.19
II – MTX control	69.28 ± 4.21**a	94.41 ± 5.06**a
III – Silymarin (std.)	50.37 ± 3.18**b	42.36 ± 1.42**b
IV – Apigenin	39.84 ± 2.31**b	33.27 ± 1.08**b
V – Alpha-tocopherol	43.76 ± 2.64**b	35.35 ± 0.26**b
VI – Combination	46.82 ± 2.87**b	39.18 ± 1.21**b

*Values are mean ± SEM (n = 4/group), analysed by one-way ANOVA followed by post-hoc multiple comparison testing. **a p<0.001 vs normal control; **b p<0.001 vs MTX (toxic) control.*

Serum ALT, AST and ALP followed the expected inverse pattern relative to the antioxidant data (Table 2). Methotrexate alone produced an approximately two-fold elevation of all three markers relative to normal control ($p<0.001$), indicating substantial hepatocellular and canalicular membrane injury consistent with the oxidative damage documented above. Notably, apigenin monotherapy (Group IV) produced the greatest reduction in serum transaminases among all treatment groups, with ALT and AST values numerically closer to the normal control than even the silymarin standard, pointing to a particularly strong direct membrane-protective action of apigenin. Alpha-tocopherol (Group V) also significantly lowered enzyme leakage, in keeping with its role in preserving membrane phospholipid integrity through inhibition of lipid peroxidation. The combination group (Group VI) recorded the lowest ALP of all treatment groups, suggesting an additional benefit of the combined regimen on biliary canalicular membrane integrity that complemented the hepatocellular protection already evident from the antioxidant findings (Figures 5–7).

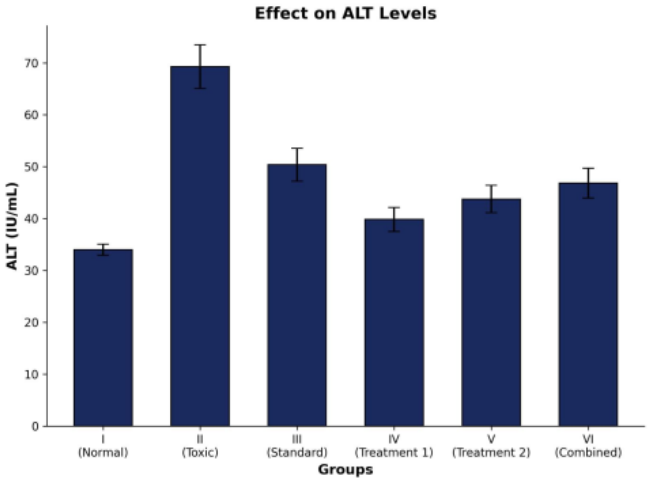

Figure 5. Serum ALT levels

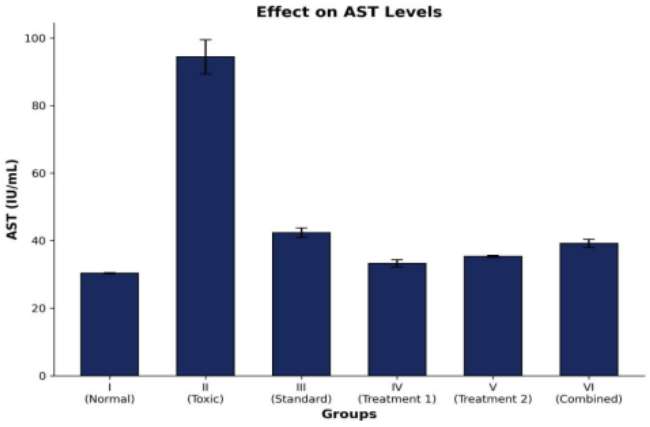

Figure 6. Serum AST levels

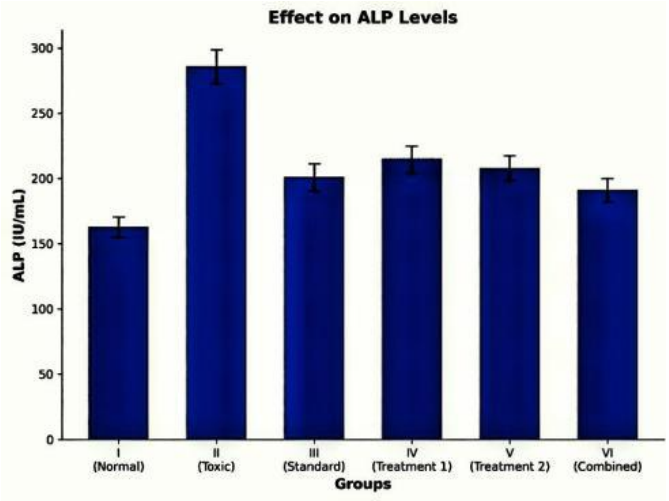

Figure 7. Serum ALP levels

Histopathological Findings

Histopathological findings closely paralleled the biochemical and antioxidant results. Liver sections from the normal control group (Figure 8) showed preserved hepatic architecture, with hepatocytes arranged in regular cords radiating from the central vein, uniform round nuclei and intact sinusoidal spaces, without evidence of vacuolation, necrosis or inflammatory infiltration.

In marked contrast, sections from the methotrexate control group (Figure 9) revealed pronounced hepatocellular degeneration with cytoplasmic vacuolation (fatty change), loss of normal cord architecture and pyknotic, condensed nuclei, changes consistent with methotrexate-induced oxidative hepatocellular injury and early necrosis.

Silymarin treatment (Figure 10) restored near-normal hepatic architecture, with well-preserved hepatocyte cords, mild sinusoidal dilation and only occasional vacuolation. Apigenin-treated sections (Figure 11) similarly showed well-preserved lobular architecture with intact hepatocyte cords, minimal vacuolation and largely normal nuclear morphology, comparable to the standard drug. Alpha-tocopherol-treated sections (Figure 12) showed moderate preservation of hepatocyte architecture with mild focal vacuolation and nuclear change, representing a partial-to-good protective effect intermediate between the toxic control and the other treatment groups. Sections from the combination group (Figure 13) showed the most favourable morphology among the test drug groups, with intact hepatocyte cords, minimal vacuolation and largely normal nuclei throughout, closely approaching normal liver histology and consistent with a protective effect superior to either agent given alone.

Methotrexate is a folate antagonist of established clinical value whose long-term use is frequently limited by dose-dependent hepatotoxicity mediated predominantly through oxidative stress and mitochondrial dysfunction [7,9]. In the present study, a single hepatotoxic challenge of methotrexate produced the expected triad of biochemical derangement: depletion of hepatic SOD, CAT and GSH, accumulation of the lipid-peroxidation product MDA, and elevation of serum ALT, AST and ALP, findings that were closely mirrored by degenerative histopathological change. This internally consistent pattern across independent biochemical and structural endpoints supports the validity of the model employed and is in keeping with previous descriptions of methotrexate-induced hepatic oxidative injury [9–11,31,32].

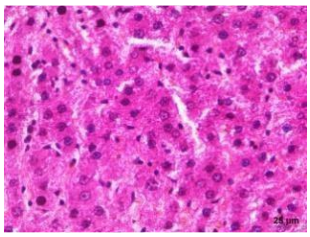

Fig. 8. Group I — Normal control (H&E)

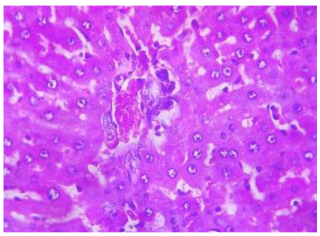

Fig. 9. Group II — MTX control (H&E)

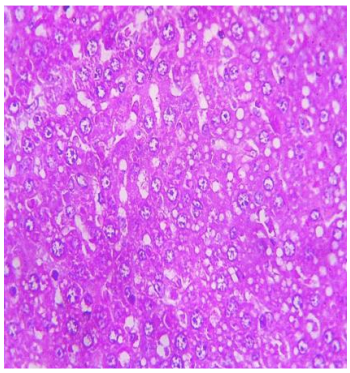

Fig. 10. Group III — Silymarin (H&E)

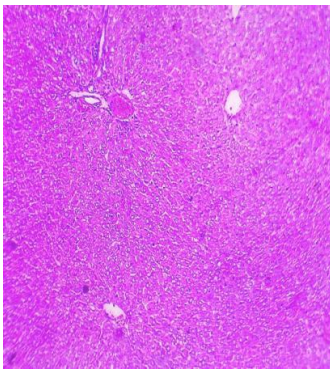

Fig. 11. Group IV — Apigenin (H&E)

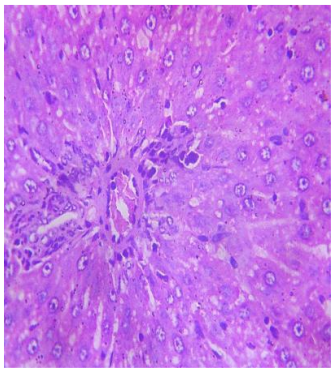

Fig. 12. Group V — Alpha-tocopherol (H&E)

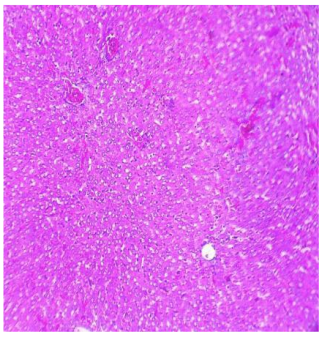

Fig. 13. Group VI — Combination (H&E)

The restoration of hepatic antioxidant enzyme activity by silymarin observed here is consistent with its recognised membrane-stabilising action and its capacity to promote regeneration of the hepatocellular antioxidant enzyme pool [22,25,26]. Apigenin monotherapy produced comparably strong protection of antioxidant status and, notably, the most pronounced reduction in serum transaminases among the treatment groups, an effect attributable to the intrinsic free-radical-scavenging and transition-metal-chelating capacity conferred by its hydroxylated flavone structure, which limits Fenton-type ROS generation at source and spares the endogenous antioxidant pool [12–15,30,33].

Alpha-tocopherol produced significant but comparatively more modest protection of the enzymatic antioxidant parameters, a pattern consistent with its principal

mechanism as a lipid-soluble, chain-breaking antioxidant that intercepts peroxyl radicals within the membrane bilayer and arrests propagation of lipid peroxidation, while exerting comparatively less direct influence on cytosolic enzymatic defences such as SOD and CAT [17–20,34]. This mechanistic distinction between the aqueous/metal-chelating action of apigenin and the membrane-restricted, chain-breaking action of alpha-tocopherol provides a plausible explanation for the superior recovery observed in the combination group relative to either agent alone: the two antioxidants appear to act in a complementary fashion across cytosolic and membrane compartments, yielding more comprehensive protection than either could achieve in isolation [35–38].

The correlation between biochemical and histopathological findings strengthens confidence in these conclusions. The severe hepatocellular degeneration, vacuolation and pyknosis observed in the methotrexate control paralleled its markedly deranged biochemical profile, while the near-normal architecture seen with silymarin, apigenin and the combination regimen paralleled their favourable biochemical recovery. The intermediate histological picture in the alpha-tocopherol group similarly mirrored its intermediate biochemical protection, reinforcing the internal consistency of the present findings [39,40].

Overall, apigenin and alpha-tocopherol each independently attenuated methotrexate-induced hepatic injury through distinct but complementary antioxidant mechanisms, and their combination produced the most consistent protection among the test groups across antioxidant, biochemical and histological endpoints, approaching that of the standard drug silymarin. These observations are in broad agreement with previous reports describing flavonoid- and tocopherol-mediated hepatoprotection in other models of chemically induced liver injury [27–30,41], and support the rationale for further evaluating apigenin and alpha-tocopherol, individually and in combination, as candidate adjunct hepatoprotective agents against methotrexate-associated liver toxicity.

From a translational standpoint, both apigenin and alpha-tocopherol are naturally occurring, orally bioavailable compounds with well-characterised safety profiles at nutritional and supra-nutritional doses, which distinguishes them favourably from several synthetic hepatoprotective candidates that have shown promise in preclinical models but faced tolerability concerns on further development. The present findings, taken together with earlier reports of apigenin's protective activity against methotrexate-induced oxidative injury and of alpha-tocopherol's established role in membrane antioxidant defence [30,34], strengthen the rationale for considering a combined apigenin–alpha-tocopherol regimen as a low-toxicity adjunct strategy that could, in principle, be co-administered alongside methotrexate in clinical settings where hepatotoxicity risk is a limiting concern, pending confirmation in more extensive pharmacological and toxicological studies.

Conclusion

The present study demonstrates that a hepatotoxic dose of methotrexate produces significant depletion of hepatic antioxidant enzymes, increased lipid peroxidation, elevation of serum liver enzymes and characteristic degenerative histopathological changes in Wistar albino rats, confirming successful induction of oxidative hepatic injury.

Pre-treatment with apigenin and alpha-tocopherol, individually and particularly in combination, significantly restored antioxidant enzyme levels, reduced lipid peroxidation, normalised serum biochemical markers and preserved hepatic architecture relative to the methotrexate control. The combination regimen afforded greater protection than either agent alone and approached the efficacy of the standard hepatoprotective drug silymarin across most parameters studied, indicating a complementary antioxidant interaction between the two agents.

These findings support the combined administration of apigenin and alpha-tocopherol as a promising hepatoprotective strategy against methotrexate-induced liver injury. Further studies incorporating a wider

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