

Antioxidant and Hepatoprotective Effects of *Betula Alnoides* Bark Extract: Insights from Methotrexate-Induced Liver Injury and FRAP Analysis

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KEYWORDS

Betula
Alnoides,
Antioxidant,
Hepatoprotective,
Methotrexate
Liver Injury
Model, FRAP,
Side Effects.

ABSTRACT:

Methotrexate (MTX), a widely used chemotherapeutic and immunosuppressive agent, is associated with significant hepatotoxicity mediated by oxidative stress and inflammation. This study explored the antioxidant and hepatoprotective potential of *Betula alnoides* bark extract (BABE) in an MTX-induced liver injury model, with a focus on its effects on biochemical and oxidative stress markers. Rats were divided into groups treated with MTX, silymarin, or varying doses of BABE (200, 400, and 600 mg/kg). Key hepatic and oxidative stress parameters, including SOD, CAT, TBARS, and GSH, were assessed, alongside lipid profile and serum protein levels. The antioxidant capacity of BABE was further evaluated using the Ferric Reducing Antioxidant Power (FRAP) assay. MTX significantly disrupted liver function, increased oxidative stress, and altered lipid and protein profiles. Co-treatment with BABE demonstrated dose-dependent hepatoprotection, restoring biochemical markers to near-normal levels, with the 600 mg/kg dose achieving results comparable to silymarin. BABE also mitigated lipid peroxidation, enhanced antioxidant enzyme activities, and restored serum protein and albumin levels. The FRAP assay confirmed BABE's strong antioxidant potential. These findings highlight the therapeutic potential of *Betula alnoides* bark extract as a natural alternative for mitigating oxidative stress and liver damage caused by MTX.

1. Introduction

Liver diseases represent a significant global health challenge, affecting millions of individuals and contributing to substantial morbidity and mortality rates. Among the numerous causes of liver dysfunction, drug-induced hepatotoxicity is a critical concern, particularly with agents like methotrexate (MTX). Methotrexate is a widely prescribed chemotherapeutic and immunosuppressive agent used in the management of malignancies, rheumatoid arthritis, and psoriasis. Despite its efficacy, MTX is associated with severe adverse effects, including hepatotoxicity. The mechanism underlying MTX-induced liver damage involves oxidative stress, mitochondrial dysfunction, and inflammation, leading to hepatic injury characterized by elevated liver enzymes, oxidative imbalance, and lipid peroxidation (Drotman and Lawhan, 1978, Wolf, 1999, Sonika and Kar, 2012, Kaplowitz and DeLeve, 2013, Bahashwan et al., 2015).

Oxidative stress, a state of imbalance between reactive oxygen species (ROS) production and antioxidant defense mechanisms, is a key driver of hepatotoxicity. Excessive ROS generation results in lipid peroxidation, protein oxidation, and DNA damage, disrupting cellular homeostasis. Antioxidants, both enzymatic (e.g., superoxide dismutase [SOD], catalase [CAT], and glutathione [GSH]) and non-enzymatic, play a crucial role in mitigating oxidative damage. The depletion of these antioxidants, as seen in MTX-induced toxicity, exacerbates hepatic injury, necessitating interventions that can restore redox balance and protect liver function (Drotman and Lawhan, 1978, Wolf, 1999, Sonika and Kar, 2012, Kaplowitz and DeLeve, 2013, Bahashwan et al., 2015, Tukov et al., 2007, Lee et al., 2008).

Natural products have emerged as promising therapeutic candidates for the management of liver diseases due to their multifaceted pharmacological activities, including antioxidant, anti-inflammatory, and hepatoprotective effects. Among them, medicinal plants are of particular interest because of their rich phytochemical profiles, which include flavonoids, phenolics, saponins, and terpenoids (Sonika and Kar, 2012, Jaswal et al., 2013, Kaplowitz and DeLeve, 2013, Bhuyan et al., 2018, Kirtikar and Basu, 1935, Chance and Maehly, 1955, Chopra et al., 1956, Drotman and Lawhan, 1978, Ravichandra et al., 2013, Talluri et al., 2016, Wang et al., 2020). These bioactive compounds have been shown to combat oxidative stress, inhibit inflammation, and promote liver regeneration. *Betula alnoides*, a deciduous tree belonging to the Betulaceae family, has been traditionally used in folk medicine for its anti-inflammatory, antioxidant, and wound-healing properties. The bark of *Betula alnoides* is rich in phenolic compounds and flavonoids, which are known for their potent free radical-scavenging activity. However, its potential for mitigating drug-induced liver injury remains underexplored (Tukov et al., 2007, Lee et al., 2008, Ravichandra et al., 2013). This study aims to investigate the antioxidant and hepatoprotective potential of *Betula alnoides* bark extract (BABE) in an MTX-induced liver injury model. The study evaluates the effect of BABE on key markers of hepatotoxicity, including liver enzymes (SGPT, SGOT, ALP, and γ -GT), oxidative stress biomarkers (SOD, CAT, TBARS, and GSH), lipid profile, and serum proteins. Additionally, the Ferric Reducing Antioxidant Power (FRAP) assay was employed to quantify the antioxidant capacity of BABE, providing mechanistic insights into its therapeutic efficacy.

A standard hepatoprotective agent, silymarin, was used as a positive control to benchmark the efficacy of BABE. Silymarin, derived from *Silybum marianum*, is a well-established antioxidant and hepatoprotective agent that restores redox balance and prevents hepatic damage. By comparing BABE with silymarin, this study provides a comprehensive evaluation of its therapeutic potential (Drotman and Lawhan, 1978, Moron et al., 1979, Zimmerman, 1999, Sonika and Kar, 2012, Kaplowitz and DeLeve, 2013). The findings of this research are expected to contribute to the growing body of evidence supporting the use of natural products in managing oxidative stress-related liver disorders. Moreover, the study highlights *Betula alnoides* as a potential natural alternative for combating drug-induced hepatotoxicity, paving the way for future research into its active constituents and clinical applications.

2. Material and Methods

Drugs and Chemicals

The study utilized high-quality reagents and chemicals sourced from reputable and verified suppliers to ensure the reliability and accuracy of the results. Methotrexate was procured from Hiltop Pharma, Kala Amb, Himachal Pradesh, India, as gift sample. Gift samples of quercetin

and catechin were generously provided by UCG Pharma, Baddi, India, ensuring the availability of pure and authentic compounds for the experiments.

Collection of the Plant Material and Identification

Betula alnoides, commonly known as the Himalayan birch or Alder birch, is a deciduous tree belonging to the Betulaceae family. It is widely distributed in the subtropical and temperate regions of Southeast Asia, including India, China, and Thailand. Traditionally, the bark of *B. alnoides* has been used in various medicinal applications due to its rich phytochemical composition, which includes flavonoids, tannins, terpenoids, and phenolic compounds. The plant material used in this study was collected from the local region of Solan, Himachal Pradesh, India. The identity of the plant was authenticated by a qualified botanist to ensure its accuracy. A herbarium specimen with the reference number MK/2022/72-284 was prepared and is being preserved in the pharmacy department for documentation and future reference. This process adhered to standard botanical collection and authentication protocols, ensuring the reliability of the plant material used in the research.

Extraction

The cold maceration method was employed to extract bioactive compounds from *Betula alnoides* bark. The collected bark was cleaned, shade-dried to preserve its phytochemical integrity, and finely powdered. A measured quantity of the powdered bark was soaked in a hydroethanolic solvent (70% ethanol and 30% distilled water) in a 1:10 ratio (w/v) at room temperature. The mixture was stirred intermittently and allowed to macerate for 72 hours to ensure thorough extraction of soluble compounds. After maceration, the mixture was filtered using Whatman filter paper No. 1 to remove debris and insoluble materials. The filtrate was then concentrated under reduced pressure using a rotary evaporator at a temperature not exceeding 40°C to prevent degradation of heat-sensitive compounds. The concentrated extract codenamed as BA-BE was further dried to obtain a semisolid residue, which was stored in an airtight container at 4°C for subsequent analysis. This method ensured the retention of key bioactive constituents.

Methotrexate (MTX) Induced Liver Damage

The methotrexate (MTX)-induced liver damage model was used to evaluate the hepatoprotective potential of *Betula alnoides* bark extract. Albino Wistar rats (weighing 180–200 g) were randomized into five groups, each consisting of six animals. The groups included a control group, an MTX-only group, and three treatment groups receiving *Betula alnoides* bark extract (BABE) at doses of 100, 200, and 400 mg/kg body weight. On the first day, a single intraperitoneal dose of MTX (20 mg/kg) was administered to induce hepatotoxicity. BABE was orally administered to the treatment groups for seven consecutive days, starting 24 hours before the MTX injection. The control group received normal saline, while the MTX-only group received no additional treatment. After the experimental period, blood samples were collected via cardiac puncture under anesthesia for biochemical analysis. Liver tissues were excised for histopathological examination and oxidative stress marker assessment, evaluating the hepatoprotective efficacy of BABE. The animal study was previously approved by IAEC as per the guidelines from The Committee for Control and Supervision of Experiments on Animals (CCSEA), India.

Experimental Design (Talluri et al., 2016) The experimental design aimed to evaluate the hepatoprotective and antioxidant effects of *Betula alnoides* bark extract (BABE) in

methotrexate (MTX)-induced liver damage in Wistar rats. The study involved 30 male albino Wistar rats (180–200 g) randomly divided into five groups, each containing six animals, as follows:

1. **Control Group:** Received normal saline (vehicle) orally for seven days.
2. **MTX Group:** Administered a single intraperitoneal dose of MTX (20 mg/kg) on day one without any treatment.
3. **BABE 100 Group:** Administered BABE (100 mg/kg) orally for seven days, starting 24 hours before MTX injection.
4. **BABE 200 Group:** Administered BABE (200 mg/kg) orally for seven days, starting 24 hours before MTX injection.
5. **BABE 400 Group:** Administered BABE (400 mg/kg) orally for seven days, starting 24 hours before MTX injection.

On the eighth day, animals were anesthetized, and blood samples were collected for biochemical assays. Liver tissues were excised, weighed, and processed for histopathological evaluation and assessment of oxidative stress markers. The experimental design ensured that the effects of BABE on MTX-induced hepatotoxicity were assessed comprehensively across various doses.

Table 1: Table Summarizing the Experimental Design for the Study.

Group	Treatment	Dose	Duration	Purpose
Control	Normal saline (vehicle)	Equivalent volume orally	7 days	Baseline for comparison
MTX	Methotrexate (MTX) only	20 mg/kg intraperitoneal	Single dose on Day 1	Induce liver damage
BABE 100	BABE + MTX	100 mg/kg orally + MTX	7 days (BABE) + MTX on Day 1	Evaluate hepatoprotection at a low dose
BABE 200	BABE + MTX	200 mg/kg orally + MTX	7 days (BABE) + MTX on Day 1	Evaluate hepatoprotection at a medium dose
BABE 400	BABE + MTX	400 mg/kg orally + MTX	7 days (BABE) + MTX on Day 1	Evaluate hepatoprotection at a high dose

Liver Profile and Lipid Profile

Blood samples were collected from all experimental animals via cardiac puncture under light anesthesia on the final day of the study. The samples were allowed to clot at room temperature and then centrifuged at 3,000 rpm for 15 minutes to separate the serum. The serum was stored at -20°C until further analysis. The liver function markers, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin, were quantified using commercially available diagnostic kits. These biochemical parameters were measured spectrophotometrically to assess hepatic injury and the hepatoprotective potential of *Betula alnoides* bark extract (BABE). Serum lipid levels were analyzed to evaluate the impact of BABE on MTX-induced alterations in lipid metabolism. The parameters included total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). These markers were determined using enzymatic

methods with specific diagnostic kits. The results from the liver and lipid profiles were statistically analyzed to compare the effects of different doses of BABE and to assess its efficacy in mitigating MTX-induced hepatotoxicity and dyslipidaemia (Latt, 1991, Finley and Tietz, 1996, Davidson and Rosenson, 2009).

Assessment of Oxidative Stress: Estimation of Catalase (CAT) and Superoxide Dismutase (SOD)

Liver tissue homogenates were prepared in ice-cold phosphate buffer (0.1 M, pH 7.4) and centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was collected and used for enzymatic assays.

Catalase (CAT) Activity

Catalase activity was estimated by measuring the decomposition rate of hydrogen peroxide (H_2O_2). The reaction mixture consisted of phosphate buffer (0.05 M, pH 7.0) and 0.1 M H_2O_2 . The reaction was initiated by adding the liver tissue homogenate. The decrease in absorbance at 240 nm was recorded spectrophotometrically for 1 minute. Results were expressed as μmol of H_2O_2 decomposed per minute per milligram of protein (Chance and Maehly, 1955, Greenwald and Claiborne, 1985).

Superoxide Dismutase (SOD) Activity

SOD activity was measured using the nitroblue tetrazolium (NBT) reduction method. The reaction mixture included phosphate buffer (0.1 M, pH 7.4), 0.1 mM EDTA, 0.1 mM NBT, and 0.1 mM riboflavin. The homogenate was added, and the reaction was initiated by exposing the mixture to a fluorescent light source. The absorbance was measured at 560 nm, and the results were expressed as the amount of enzyme required to inhibit 50% of NBT reduction per minute per milligram of protein (Misra and Fridovich, 1972, Kakkar et al., 1984).

Reduced Glutathione Activity (GSH)

Reduced glutathione (GSH) levels were determined using Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB). Liver tissue homogenates were prepared in ice-cold 0.1 M phosphate buffer (pH 7.4). An aliquot of the homogenate was mixed with 4% sulfosalicylic acid and centrifuged at 10,000 rpm for 10 minutes at 4°C. The reaction mixture consisted of supernatant, phosphate buffer (0.1 M, pH 8.0), and DTNB solution (0.01 M). The yellow color formed due to the reaction of GSH with DTNB was measured spectrophotometrically at 412 nm. The results were expressed as μmol of GSH per gram of tissue (Tietze, 1969).

Estimation of TBARS (Thiobarbituric Acid Reactive Substances)

Lipid peroxidation levels in liver tissue were quantified by measuring TBARS. Tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). An aliquot of homogenate was mixed with 15% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA) and heated in a boiling water bath for 15 minutes. The mixture was cooled, centrifuged at 10,000 rpm for 10 minutes, and the absorbance of the supernatant was measured at 532 nm. The concentration of TBARS was calculated using the molar extinction coefficient of malondialdehyde (MDA) and expressed as nmol MDA per milligram of tissue protein. These assays provided critical insights into the oxidative stress levels and the antioxidant defense mechanisms in the liver (Iqbal et al., 1996).

Ferric Reducing Ability of Plasma (FRAP) The Ferric Reducing Ability of Plasma (FRAP) assay was conducted to evaluate the antioxidant potential of *Betula alnoides* bark extract (BABE) by assessing its ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in the presence of antioxidants. The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM ferric chloride solution in a ratio of 10:1:1. The reagent was incubated at 37°C for 10 minutes to ensure stability. BABE samples were prepared in distilled water at various concentrations, with Trolox, a vitamin E analog, used as the standard antioxidant. For the assay, 200 μL of BABE or Trolox was mixed with 1.8 mL of FRAP reagent and incubated at 37°C for 4 minutes to allow the reduction of Fe^{3+} -TPTZ to Fe^{2+} -TPTZ, forming a blue-colored complex. The absorbance of the mixture was measured spectrophotometrically at 593 nm against a reagent blank. Results were calculated using a Trolox calibration curve and expressed as μmol of Fe^{2+} equivalents per gram of extract. This assay provided a quantitative measure of the ferric reducing antioxidant power of BABE, highlighting its potential to mitigate oxidative stress through free radical neutralization. (Benzie and Strain, 1996).

Statistical Analysis

The experimental data were expressed as mean $\pm$ standard deviation (SD) for all groups ($n = 6$). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to evaluate significant differences between groups. The significance level was set at $p < 0.05$. GraphPad Prism software (version 8) was used for all statistical computations and graphical representations. Correlation analyses were also performed where applicable to assess the relationship between antioxidant activities and biochemical markers. This approach ensured reliable interpretation of the data and facilitated comparisons across different experimental groups.

3. Results and Discussion

Extraction Process

The plant material, comprising barks of *Betula alnoides*, was meticulously collected from its natural habitat to ensure the quality and authenticity of the samples. The collected material was thoroughly cleaned to remove dirt and debris, followed by air drying in a shaded area to preserve its bioactive components, avoiding direct sunlight that could potentially degrade sensitive phytochemicals. Once dried, the plant material was pulverized into a coarse powder using a mechanical grinder, ensuring uniform particle size to facilitate efficient extraction. The powdered material was subjected to cold maceration extraction using a hydroethanolic solvent (70% ethanol and 30% water) to obtain a concentrated crude extract rich in bioactive compounds.

Preliminary Phytochemical Screening

The extract was subsequently subjected to preliminary phytochemical screening to identify the presence of various phytochemical classes. The analysis revealed the presence of a diverse array of secondary metabolites, including alkaloids, flavonoids, glycosides, saponins, phenols, phytosterols, fatty acids, carbohydrates, and proteins. These bioactive constituents are well-known for their pharmacological potential, contributing to the antioxidant, hepatoprotective, and therapeutic properties of *Betula alnoides*. The identification of these compounds lays a foundational understanding for exploring the plant's medicinal value and its mechanism of action in subsequent studies.

Evaluation of Hepatoprotective Activity in Methotrexate (MTX) Induced Liver Injury

Body Weight Gain The data on body weight percentage increase highlight the effects of methotrexate (MTX), silymarin, and *Betula alnoides* bark extract (BABE) on mitigating MTX-induced toxicity. The control group exhibited a normal physiological body weight increase of $42.27 \pm 0.98\%$, reflecting healthy growth under standard conditions. In contrast, the MTX-treated group showed a marked reduction in body weight gain to $30.15 \pm 0.95\%$, demonstrating the deleterious effects of MTX, including hepatotoxicity, oxidative stress, and impaired metabolism. Treatment with silymarin, a standard hepatoprotective agent, restored the body weight increase to $41.79 \pm 0.98\%$, indicating its efficacy in alleviating MTX-induced toxicity and supporting metabolic recovery. Similarly, BABE showed a dose-dependent protective effect. At 200 mg/kg, the body weight gain improved to $37.69 \pm 0.97\%$, indicating partial protection. The 400 mg/kg dose further enhanced recovery, with a weight gain of $40.79 \pm 0.99\%$, nearing control and silymarin-treated levels. The highest dose of BABE, 600 mg/kg, completely restored and slightly surpassed the control group, achieving a weight gain of $42.72 \pm 0.99\%$, suggesting optimal protection and recovery.

Liver Weight

The data on absolute liver weight demonstrate the impact of methotrexate (MTX), silymarin, and *Betula alnoides* bark extract (BABE) on liver physiology. The control group showed an absolute liver weight of 5.53 ± 0.94 , representing the normal physiological condition. MTX treatment caused a significant increase in liver weight to 10.85 ± 0.89 , indicating pronounced hepatotoxicity. This increase is likely due to MTX-induced oxidative stress, inflammation, and possible lipid accumulation, resulting in cellular swelling and liver hypertrophy. Silymarin, a well-known hepatoprotective agent, reduced the liver weight to 5.88 ± 0.89 , almost restoring it to control levels. This outcome highlights silymarin's efficacy in mitigating oxidative stress, reducing inflammation, and preserving liver architecture. Treatment with BABE showed a dose-dependent reduction in liver weight. At 200 mg/kg, the liver weight decreased to 8.27 ± 0.88 , indicating partial protection. The 400 mg/kg dose reduced liver weight further to 7.45 ± 0.97 , reflecting enhanced hepatoprotection. The highest dose of 600 mg/kg brought the liver weight to 6.23 ± 0.99 , approaching the values of the control and silymarin-treated groups. These results suggest that BABE effectively mitigates MTX-induced liver hypertrophy, likely due to its phytochemical composition rich in flavonoids and phenolics, which counteract oxidative damage and inflammation.

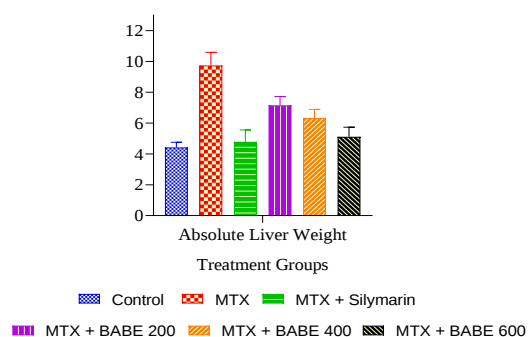


Figure 1: Gains in Body Weight.

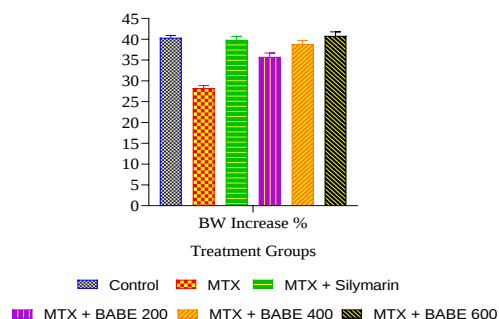


Figure 2: The Proportion of Absolute Liver Weight.

Lipid Profile

The data in Table 2 illustrate the effect of *Betula alnoides* bark extract (BABE) on the lipid profile, including total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), in methotrexate (MTX)-induced hepatotoxicity. The normal control group exhibited physiological lipid profile values with low levels of TC (2.34 ± 0.74 mg/dl), TG (3.72 ± 0.73 mg/dl), LDL (1.76 ± 0.32 mg/dl), and a higher HDL level (3.11 ± 0.73 mg/dl). In contrast, MTX administration significantly altered the lipid profile, showing a marked increase in TC (7.45 ± 0.18 mg/dl), TG (7.09 ± 0.54 mg/dl), and LDL (7.71 ± 0.38 mg/dl), accompanied by a substantial reduction in HDL levels (1.62 ± 0.48 mg/dl) ($p < 0.001$). These changes indicate severe dyslipidemia and oxidative stress induced by MTX. Co-treatment with silymarin significantly improved the lipid profile compared to the MTX group. Silymarin reduced TC, TG, and LDL levels to 2.63 ± 0.19 mg/dl, 4.12 ± 0.25 mg/dl, and 2.13 ± 0.36 mg/dl, respectively ($p < 0.001$), while partially restoring HDL to 2.94 ± 0.90 mg/dl ($p < 0.01$), reflecting its potent hepatoprotective and lipid-modulating effects. BABE treatment also demonstrated a dose-dependent improvement in the lipid profile. At 200 mg/kg, BABE partially reduced TC, TG, and LDL levels (5.30 ± 0.58 mg/dl, 6.14 ± 0.64 mg/dl, and 6.10 ± 0.35 mg/dl, respectively), although HDL levels (2.26 ± 0.77 mg/dl) did not show a statistically significant increase. At 400 mg/kg, BABE significantly reduced TC (3.35 ± 0.37 mg/dl), TG (4.02 ± 0.73 mg/dl), and LDL (4.14 ± 0.22 mg/dl) ($p < 0.001$) while moderately restoring HDL levels (2.61 ± 0.75 mg/dl, $p < 0.01$). The highest dose of BABE (600 mg/kg) exhibited the most pronounced effect, with lipid parameters approaching normal levels. TC, TG, and LDL were reduced to 2.57 ± 0.63 mg/dl, 3.74 ± 0.19 mg/dl, and 2.28 ± 0.21 mg/dl, respectively ($p < 0.001$), while HDL was restored to 2.83 ± 0.58 mg/dl ($p < 0.01$). In summary, MTX-induced dyslipidemia was significantly ameliorated by both silymarin and BABE treatment. While silymarin showed greater efficacy in restoring HDL levels, BABE, particularly at 600 mg/kg, demonstrated comparable lipid-lowering effects, suggesting its potential as a natural alternative for managing MTX-induced lipid abnormalities and oxidative stress. These results underscore the dose-dependent lipid-modulating activity of BABE, which could be attributed to its rich phytochemical composition, including flavonoids and phenolic compounds, known for their antioxidant and lipid-regulating properties.

Table 2: The Impact of BABE on the Lipid Profile

	TC (mg/dl)	TG (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
Normal Control	2.34 ± 0.74	3.72 ± 0.73	1.76 ± 0.32	3.11 ± 0.73
Methotrexate (MTX)	^a $7.45 \pm 0.18^{***}$	^a $7.09 \pm 0.54^{***}$	^a $7.71 \pm 0.38^{***}$	^a $1.62 \pm 0.48^{***}$
Silymarin + MTX	^b $2.63 \pm 0.19^{***}$	^b $4.12 \pm 0.25^{***}$	^b $2.13 \pm 0.36^{***}$	^b $2.94 \pm 0.90^{**}$
BABE 200 + MTX	^b $5.30 \pm 0.58^{***}$	^b $6.14 \pm 0.64^*$	^b $6.10 \pm 0.35^{***}$	^b 2.26 ± 0.77^{ns}
BABE 400 + MTX	^b $3.35 \pm 0.37^{***}$	^b $4.02 \pm 0.73^{***}$	^b $4.14 \pm 0.22^{***}$	^b $2.61 \pm 0.75^{**}$
BABE 600 + MTX	^b $2.57 \pm 0.63^{***}$	^b $3.74 \pm 0.19^{***}$	^b $2.28 \pm 0.21^{***}$	^b $2.83 \pm 0.58^{**}$

Results are presented as Mean $\pm$ SD (for n=6 values). ^a vs normal control group at $***p < 0.001$.
^b vs MTX treated group at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Liver Functions

The data from Table 3 demonstrate the effects of *Betula alnoides* bark extract (BABE) on biomarkers of hepatotoxicity, specifically SGPT (ALT), SGOT (AST), ALP, and γ -GT, in methotrexate (MTX)-induced hepatotoxicity. In the control group, normal levels of SGPT (58.05 ± 2.12 IU/L), SGOT (77.41 ± 1.76 IU/L), ALP (180.15 ± 2.68 IU/L), and γ -GT (104.34 ± 2.83 nM/min/mg protein) reflect healthy liver function. MTX treatment caused a significant elevation in all biomarkers, with SGPT, SGOT, ALP, and γ -GT levels increasing to 117.13 ± 3.56 IU/L, 235.63 ± 3.88 IU/L, 452.54 ± 5.64 IU/L, and 153.23 ± 2.74 nM/min/mg protein, respectively ($p < 0.001$), indicating severe hepatocellular damage and compromised liver function. Silymarin co-treatment significantly alleviated the hepatotoxic effects of MTX, with biomarker levels nearly restored to normal values. SGPT, SGOT, ALP, and γ -GT levels were reduced to 62.37 ± 1.89 IU/L, 86.87 ± 1.96 IU/L, 188.57 ± 2.80 IU/L, and 108.76 ± 3.83 nM/min/mg protein, respectively ($p < 0.001$), confirming the strong hepatoprotective effects of silymarin. Similarly, BABE treatment demonstrated a dose-dependent reduction in these biomarkers. At 200 mg/kg, partial recovery was observed, with SGPT, SGOT, ALP, and γ -GT levels decreasing to 91.78 ± 1.95 IU/L, 148.83 ± 2.86 IU/L, 303.55 ± 2.65 IU/L, and 126.76 ± 3.84 nM/min/mg protein, respectively ($p < 0.001$). The 400 mg/kg dose showed significant improvement, reducing the biomarkers to 69.67 ± 1.98 IU/L, 91.63 ± 2.37 IU/L, 207.27 ± 2.54 IU/L, and 110.54 ± 3.77 nM/min/mg protein, respectively ($p < 0.001$). At the highest dose of 600 mg/kg, BABE restored biomarker levels close to those of the control group, with SGPT, SGOT, ALP, and γ -GT values at 61.76 ± 1.95 IU/L, 83.72 ± 1.84 IU/L, 191.83 ± 2.68 IU/L, and 107.82 ± 3.78 nM/min/mg protein, respectively ($p < 0.001$). These findings highlight the dose-dependent hepatoprotective activity of BABE, with higher doses demonstrating comparable efficacy to silymarin. The significant reduction in hepatotoxic biomarkers suggests that BABE effectively mitigates MTX-induced oxidative stress and hepatocellular injury, likely due to its rich phytochemical profile, including flavonoids and phenolics. These results underscore the therapeutic potential of BABE as a natural hepatoprotective agent, warranting further studies to elucidate its mechanisms of action and bioactive constituents.

Table 3: The Effect of BABE on Biomarkers of Hepatotoxicity Induced By MTX

	SGPT (ALT) (IU/L)	SGOT (AST) (IU/L)	ALP (IU/L)	γ -GT (nM/min /mg protein)
Control	58.05±2.12	77.41±1.76	180.15±2.68	104.34±2.83
Methotrexate (MTX)	^a 117.13±3.56** *	^a 235.63±3.88* **	^a 452.54±5.64* **	^a 153.23±2.74** *
Silymarin + MTX	^b 62.37±1.89***	^b 86.87±1.96***	^b 188.57±2.80* **	^b 108.76±3.83* **
BABE 200 + MTX	^b 91.78±1.95***	^b 148.83±2.86* **	^b 303.55±2.65* **	^b 126.76±3.84* **
BABE 400 + MTX	^b 69.67±1.98***	^b 91.63±2.37** *	^b 207.27±2.54* **	^b 110.54±3.77* **
BABE 600 + MTX	^b 61.76±1.95***	^b 83.72±1.84***	^b 191.83±2.68* **	^b 107.82±3.78* **

Mean $\pm$ SD for the six numbers. At the *** $p < 0.001$ probability level, the control group's significance is shown. The MTX group's significance is indicated by b at the *** $p < 0.001$ probability level.

Total Serum Protein Level and Serum Albumin Level

The results presented in Figure 2 show the impact of *Betula alnoides* bark extract (BABE) on total serum protein and serum albumin levels in methotrexate (MTX)-induced hepatotoxicity. The control group maintained normal levels of total serum protein (approximately 8 mg/dL) and serum albumin (approximately 4 mg/dL), indicative of optimal liver function. Conversely, the positive control (MTX-treated group) displayed a significant reduction in both parameters, with total serum protein and albumin levels markedly lower than those of the control group ($p < 0.0001$). This reduction reflects impaired hepatic protein synthesis and liver dysfunction caused by MTX-induced oxidative stress and hepatocellular damage. Treatment with the standard (silymarin) significantly restored total serum protein and albumin levels to values comparable to the control ($p < 0.001$), reaffirming its established hepatoprotective efficacy. Similarly, BABE treatment exhibited a dose-dependent improvement in both parameters. At 200 mg/kg, BABE partially increased total serum protein and albumin levels, while the 400 mg/kg and 600 mg/kg doses resulted in substantial restoration of these levels to near-normal values ($p < 0.0001$). These results indicate that BABE effectively mitigates MTX-induced hepatic damage by enhancing protein synthesis and alleviating oxidative stress, with its efficacy increasing at higher doses. In summary, *Betula alnoides* bark extract demonstrated significant hepatoprotective activity, particularly at higher doses, comparable to the standard treatment. This protective effect can be attributed to its rich phytochemical composition, including flavonoids and phenolics, which are known to counteract oxidative stress and promote hepatic recovery. These findings support the potential therapeutic application of BABE in managing liver dysfunction and protein synthesis impairment caused by hepatotoxic agents like MTX.

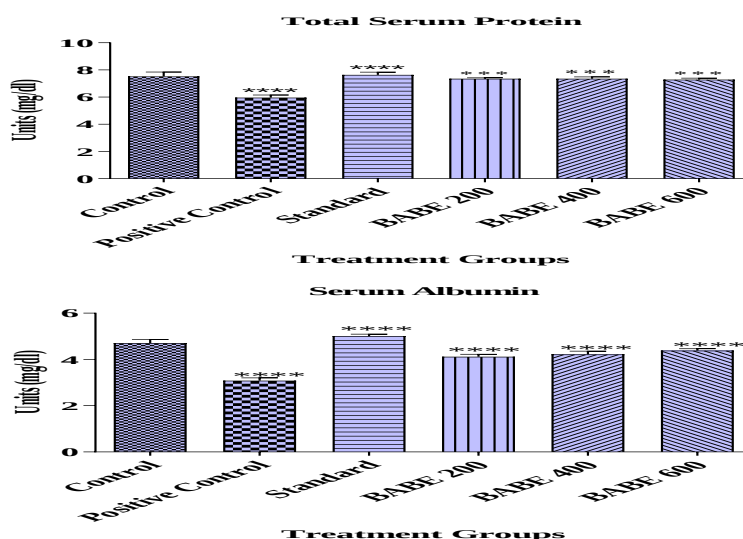


Figure 3: Effect of BABE on Total Serum Protein Level and Serum Albumin Level. Data Are Expressed As Mean $\pm$ SD (N = 6). One-Way ANOVA Tukey Post Hoc: ** $P < 0.001$, **** $P < 0.0001$

Serum Bilirubin Level and Conjugated Bilirubin Level

The results depicted in the figure illustrate the effects of *Betula alnoides* bark extract (BABE) on total serum bilirubin and conjugated bilirubin levels in methotrexate (MTX)-induced hepatotoxicity. In the control group, total serum bilirubin and conjugated bilirubin levels were maintained at normal physiological levels, approximately 0.4 mg/dL and 0.2 mg/dL, respectively, reflecting optimal liver function. The positive control group (MTX-treated) displayed a significant elevation in both total bilirubin (approximately 1.2 mg/dL) and conjugated bilirubin (approximately 0.8 mg/dL) ($p < 0.0001$), indicating severe hepatic dysfunction and bile excretion impairment caused by MTX. Treatment with the standard drug (silymarin) effectively reduced both total and conjugated bilirubin levels to near-normal values ($p < 0.0001$), reaffirming its hepatoprotective properties. Similarly, BABE treatment demonstrated a dose-dependent reduction in bilirubin levels. At 200 mg/kg, BABE moderately reduced total bilirubin ($p < 0.01$) and showed a non-significant change in conjugated bilirubin. However, higher doses of BABE (400 mg/kg and 600 mg/kg) significantly lowered both total bilirubin ($p < 0.01$, $p < 0.001$) and conjugated bilirubin ($p < 0.01$) levels, approaching those of the control group. This dose-dependent effect highlights the ability of BABE to mitigate hyperbilirubinemia and restore hepatic excretory function. In conclusion, the results underscore the hepatoprotective potential of *Betula alnoides* bark extract, particularly at higher doses, in reducing MTX-induced bilirubin elevation. The observed effects may be attributed to the antioxidant and anti-inflammatory properties of its phytochemical constituents, which alleviate oxidative stress and improve bile excretion. These findings support the potential therapeutic use of BABE in managing liver dysfunction and hyperbilirubinemia.

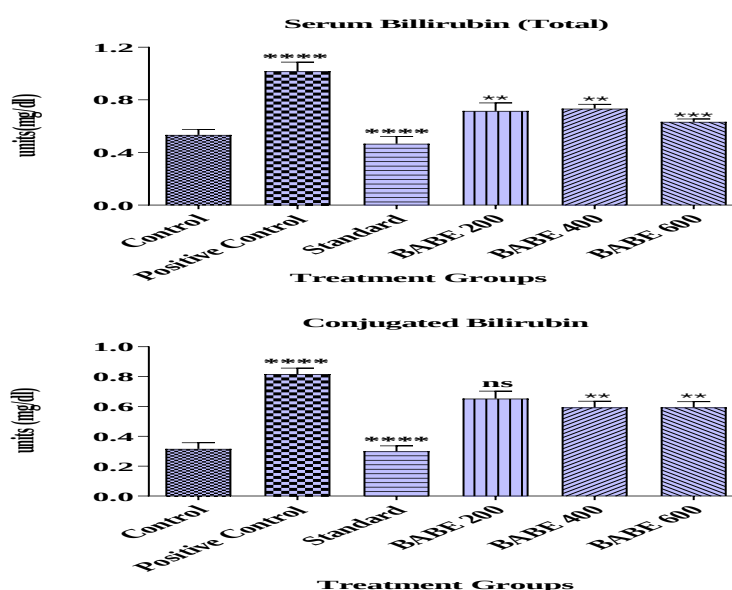


Figure 4: Effect of BABE on Serum Bilirubin Level and Conjugated Bilirubin Level. Data Are Expressed As Mean $\pm$ SD (N = 6). One-Way ANOVA Tukey Post Hoc: ** $P < 0.001$, **** $P < 0.0001$

Assessment of Oxidative Stress Markers

The data demonstrate the effect of *Betula alnoides* bark extract (BABE) on oxidative stress markers, including superoxide dismutase (SOD), catalase (CAT), thiobarbituric acid reactive substances (TBARS), and reduced glutathione (GSH), in methotrexate (MTX)-induced

hepatotoxicity. The control group maintained normal levels of SOD (18.84 ± 1.10 U/mg protein), CAT (4.07 ± 0.56 U/min), TBARS (21.56 ± 1.11 nM/min/mg protein), and GSH (2.89 ± 0.11 μ M/g tissue), reflecting a balanced oxidative state. However, MTX treatment caused a significant decline in antioxidant enzyme activities, with SOD and CAT levels reduced to 7.84 ± 0.99 U/mg protein and 2.58 ± 0.89 U/min, respectively ($p < 0.001$). Concurrently, a marked increase in TBARS (46.53 ± 1.87 nM/min/mg protein, $p < 0.001$) and depletion of GSH (0.52 ± 0.01 μ M/g tissue, $p < 0.001$) indicated severe oxidative damage and a compromised antioxidant defense system. Co-treatment with silymarin significantly mitigated the oxidative stress induced by MTX. SOD and CAT levels improved to 16.81 ± 0.99 U/mg protein and 4.57 ± 1.01 U/min ($p < 0.01$), while TBARS levels reduced to 19.76 ± 1.65 nM/min/mg protein ($p < 0.01$) and GSH levels increased to 2.79 ± 0.09 μ M/g tissue ($p < 0.001$), approaching normal levels. Similarly, BABE exhibited a dose-dependent antioxidant effect. At 200 mg/kg, moderate improvements were observed, with SOD and CAT increasing to 12.49 ± 1.02 U/mg protein and 4.25 ± 0.99 U/min, respectively, while TBARS levels decreased to 32.78 ± 1.71 nM/min/mg protein ($p < 0.01$) and GSH increased to 2.18 ± 0.02 μ M/g tissue ($p < 0.001$). At 400 mg/kg, more pronounced effects were seen, with SOD, CAT, TBARS, and GSH levels improving significantly. The highest dose of BABE (600 mg/kg) restored oxidative stress markers close to control values, with SOD at 17.75 ± 1.03 U/mg protein, CAT at 4.59 ± 0.99 U/min, TBARS at 20.83 ± 1.91 nM/min/mg protein ($p < 0.001$), and GSH at 2.98 ± 0.09 μ M/g tissue ($p < 0.001$). These results indicate that MTX-induced hepatotoxicity is strongly linked to oxidative stress, characterized by reduced antioxidant enzyme activities, increased lipid peroxidation, and GSH depletion. BABE effectively alleviated these effects in a dose-dependent manner, with higher doses demonstrating strong antioxidant activity comparable to silymarin. The phytochemical constituents of BABE, such as flavonoids and phenolics, likely contributed to its ability to scavenge free radicals, enhance endogenous antioxidant defenses, and reduce oxidative damage. These findings highlight the potential of *Betula alnoides* as a natural therapeutic agent to combat oxidative stress and its associated liver damage.

Table 4: The Impact of BABE on Biological Indicators of Oxidative Stress

	SOD (U/mg protein)	CAT (U/min)	TBARS (nM /min/mg protein)	GSH (μ M /g tissue)
Control	18.84 $\pm$ 1.10	4.07 $\pm$ 0.56	21.56 $\pm$ 1.11	2.89 $\pm$ 0.11
Methotrexate (MTX)	^a 7.84 $\pm$ 0.99***	^a 2.58 $\pm$ 0.89***	^a 46.53 $\pm$ 1.87***	^a 0.52 $\pm$ 0.01***
Silymarin + MTX	^b 16.81 $\pm$ 0.99**	^b 4.57 $\pm$ 1.01**	^b 19.76 $\pm$ 1.65**	^b 2.79 $\pm$ 0.09***
BABE 200 + MTX	^b 12.49 $\pm$ 1.02**	^b 4.25 $\pm$ 0.99**	^b 32.78 $\pm$ 1.71**	^b 2.18 $\pm$ 0.02***
BABE 400 + MTX	^b 15.48 $\pm$ 0.99**	^b 4.43 $\pm$ 0.99**	^b 24.46 $\pm$ 1.88**	^b 2.68 $\pm$ 0.09***
BABE 600 + MTX	^b 17.75 $\pm$ 1.03**	^b 4.59 $\pm$ 0.99**	^b 20.83 $\pm$ 1.91***	^b 2.98 $\pm$ 0.09***

Mean $\pm$ SD for the six numbers. At the *** $p < 0.001$ probability level, the control group's significance is shown. The MTX group's significance is indicated by b at the *** $p < 0.001$ probability level.

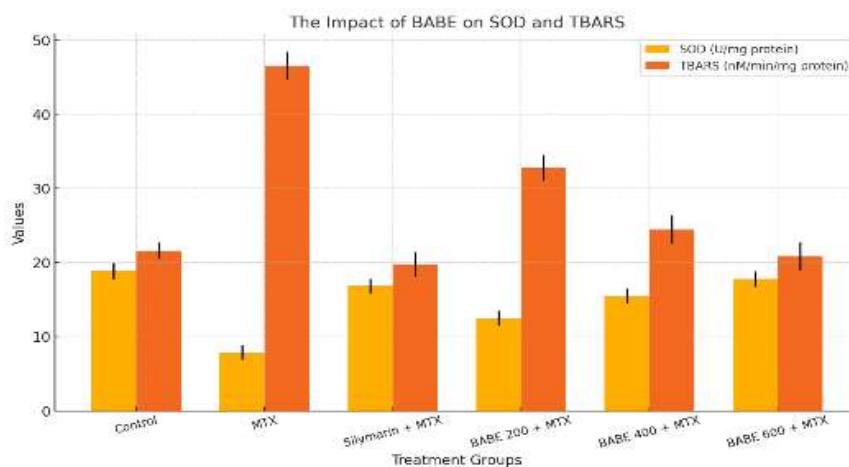


Figure 5: The Impact of BABE on Biological Indicators of Oxidative Stress (SOD and TBARS)

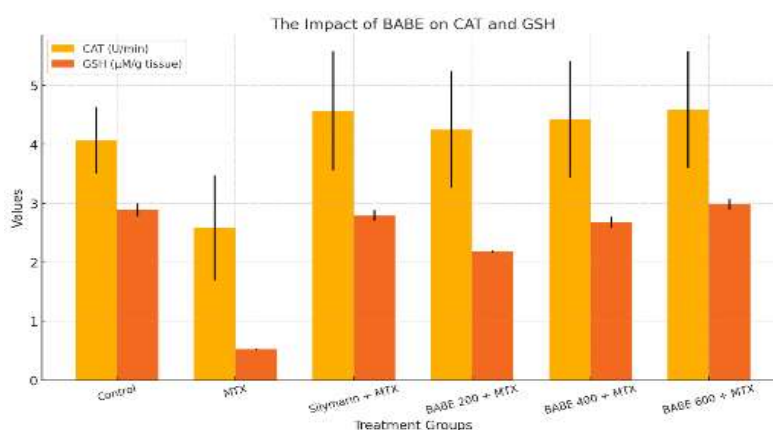


Figure 6: The Impact of BABE on Biological Indicators of Oxidative Stress (CAT and GSH)

Ferric Reducing Ability of Plasma (FRAP)

The FRAP assay results demonstrate the antioxidant potential of *Betula alnoides* bark extract (BABE) at various doses compared to standard antioxidants (Figure 7). The saline control group exhibited the lowest FRAP value, indicating minimal ferric reducing antioxidant capacity. Treatment with BABE at doses of 150 mg/kg, 300 mg/kg, and 600 mg/kg showed a dose-dependent increase in FRAP values. BABE at 150 mg/kg demonstrated a significant improvement ($p < 0.01$) in antioxidant capacity compared to the saline control, while 300 mg/kg and 600 mg/kg doses showed further enhancement, with the highest antioxidant potential observed at 600 mg/kg. The antioxidant activity of BABE at 600 mg/kg was comparable to standard antioxidants, including quercetin (50 mg/ml), vitamin C (50 mg/ml), and vitamin E (40 mg/ml), all of which exhibited significantly higher FRAP values ($p < 0.01$) than the control. These results suggest that BABE, particularly at higher doses, possesses substantial antioxidant activity, approaching the efficacy of well-established antioxidant standards. This dose-dependent increase in FRAP values highlights the potential of *Betula alnoides* bark extract as a natural antioxidant, which may contribute to its therapeutic efficacy in managing oxidative stress-related conditions.

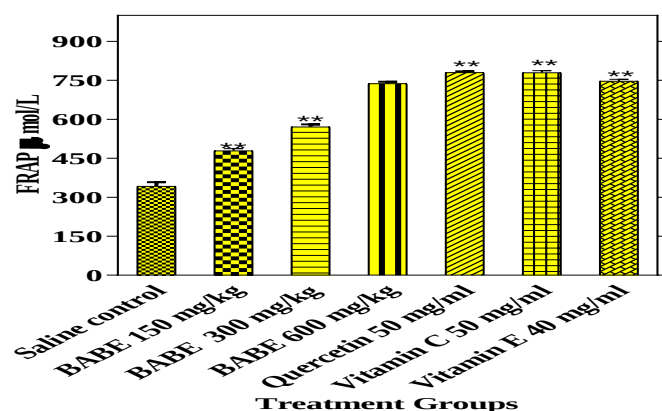


Figure 7: Ferric Reducing Ability of Plasma by BABE. Data are Expressed as Mean $\pm$ SD (N = 6). One-Way ANOVA Tukey Post Hoc: ** P < 0.01

4. Conclusions

The study underscores the hepatoprotective and antioxidant efficacy of *Betula alnoides* bark extract (BABE) in mitigating methotrexate (MTX)-induced hepatotoxicity. MTX significantly impaired liver function and caused oxidative stress, as evidenced by increased TBARS levels, depleted antioxidant enzymes (SOD and CAT), and reduced GSH levels. It also disrupted serum protein, albumin, and lipid profiles, leading to hepatic dysfunction. BABE, administered at doses of 200, 400, and 600 mg/kg, exhibited dose-dependent protective effects, with the highest dose (600 mg/kg) restoring liver function markers to near-normal levels, comparable to the standard hepatoprotective agent silymarin. The extract effectively reduced lipid peroxidation, enhanced antioxidant enzyme activities, replenished GSH levels, and normalized protein and lipid profiles. The Ferric Reducing Antioxidant Power (FRAP) assay further validated its antioxidant potential. The findings suggest that the phytochemical composition of BABE, particularly its flavonoids and phenolics, plays a pivotal role in mitigating oxidative stress and protecting liver integrity. This study highlights *Betula alnoides* as a promising natural therapeutic candidate for managing oxidative stress-induced liver damage. Further research on the isolation of active compounds and their mechanisms of action may pave the way for its clinical application in hepatoprotection.

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