

Blood Plasma as a Sampling Model during Drug-induced Thrombocytopenia: Effects of Antioxidants

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ABSTRACT

OBJECTIVE Blood plasma, a complex biological mixture, plays a part in a variety of roles including clotting, defense, and transport, and reflects the overall status of blood components. Drug-induced thrombocytopenia (DIT) is characterized by abnormally low platelet count (below ~150,000 per μ L) which can be caused by adverse effects of medications. This study aims to address a basic question, can plasma be employed as a sampling model to assess the oxidative stress (OS) changes and antioxidant status during DIT? The objective was to analyze the antioxidant status and OS in plasma during DIT, and to determine the effects of antioxidant supplementation such as CaripillTM, L-Carnitine (LC), and vanillic acid (VA) during DIT.

METHODS Male Wistar rats were used as animal models and grouped into control groups (n=5) and thrombocytopenia groups (n=5). Antioxidants were given to the thrombocytopenic and the control rats (50 mg/kg body weight) once a day for 7 days. Blood plasma from both groups was evaluated for total antioxidant capacity, antioxidant enzymes, markers of lipid peroxidation, protein oxidation, and OS.

RESULTS The antioxidants significantly increased the total antioxidant capacity (CUPRAC) and ferric-reducing antioxidant power (FRAP) of the plasma and decreased the levels of conjugate dienes. CaripillTM also reduced lipid peroxidation, significantly elevated protein sulfhydryl and nitrite/nitrate levels, LC elevated lactate dehydrogenase levels, and VA increased superoxide dismutase activity and attenuated lipid peroxidation during DIT.

CONCLUSIONS The antioxidants CaripillTM, vanillic acid, and L-carnitine were demonstrated to be beneficial during DIT and to have prospects in alternate therapeutics. This study confirms that plasma can be utilized as a sampling model to study changes during OS situations.

KEYWORDS antioxidants, caripill, L-carnitine, oxidative stress, plasma, thrombocytopenia, vanillic acid

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INTRODUCTION

Oxidative stress (OS) during thrombocytopenia is one of the most important factors that can affect platelet count. Plasma is representative of the entire OS microenvironment of the whole blood.

Proteins are the most abundant substances in plasma and play a part in a variety of roles including clotting, defense, and transport (1, 2). An imbalance of reactive species and endogenous antioxidants in the plasma causes damage to

biological molecules during disease conditions (3, 4). However, endogenous antioxidants can neutralize free radical intermediates and inhibit their oxidation to a certain extent (5). Antioxidant supplementation has gained acceptance as an alternative to complement the current therapies. Alternative therapeutics can be an effective tool for treating secondary complications and can be integrated with conventional medicine.

Drug-induced thrombocytopenia (DIT) is a condition induced by therapeutic antibiotics, cardiovascular drugs, and anti-cancer agents (6, 7). These agents can affect the platelet count by their cytotoxic effects on bone marrow megakaryocytes, thereby reducing platelet synthesis, or by accelerating the clearance of circulating platelets (8). Some of the important drugs involved in inducing thrombocytopenia are balhimycin, vancomycin, sulfonamides, trifluoperazine, carmustin, phenacetin, cisplatin, penicillin, methotrexate, lovastatin, cyclophosphamide, quinine, quinidine, methicillin, and hydroxyurea (9).

Herbal supplements have proven to be beneficial in treating thrombocytopenic conditions. For example, *Carica papaya* leaf extract contains flavonoids and polyphenols and is an active pharmaceutical ingredient of Caripill™ tablets (10). It aids the increase in platelet count in dengue infection and other thrombocytopenic conditions (11, 12). Vanillic acid (VA), a naturally occurring phenolic compound found in many edible plants and fruits possesses free radical scavenging, anticancer, antimutagenic, and antiangiogenic effects with multiple pharmacologic functions such as antimicrobial, anti-filarial, anti-inflammatory, and antioxidant activities. It can directly scavenge hydroxyl radicals and other cellular oxygen species due to the presence of the carboxyl group (13). It has proven to be beneficial in ameliorating oxidative stress by reducing lipid peroxidation products and significantly restoring enzymatic and non-enzymatic antioxidant systems (14, 15). L-carnitine (LC) is a non-protein amino acid and a free radical scavenger. The carbonyl group stabilizes free radicals on α -carbon. It prevents oxidative damage to antioxidant enzymes by chelating metal ferrous ions, thereby interfering with reactive species formation (16). The effect of LC during oxidative stress has been well demonstrated in different biological systems (17, 18).

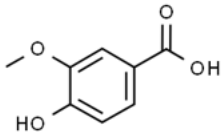
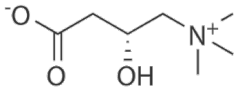
This study aims to address a basic question: can plasma be employed as a sampling model to assess the OS changes and antioxidant status? This study was designed to (i) analyze the antioxidant status in plasma under thrombocytopenic conditions, (ii) determine the status of protein oxidation and lipid peroxidation in plasma under thrombocytopenic conditions, and (iii) assess the effects of antioxidants such as Caripill™, L-carnitine and vanillic acid as supplements during thrombocytopenia. The structures and composition of the studied antioxidants are listed in Table 1.

METHODS

Chemicals

Caripill™ (*Carica papaya* leaf extract tablets, Micro Lab Limited, Bangalore, India) and hydroxyurea (Hydrea, Sarabhai Chemicals, Gujarat, India) were purchased from a local pharmacy. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), dinitrophenyl hydrazine (DNPH), epinephrine, bathocuproinedisulfonic acid disodium salt (BCS) and Griess reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA); 2,4,6-Tripyridyl-s-triazine (TPTZ), L-carnitine (LC) and vanillic acid (VA) were purchased from HiMedia Laboratories (Maharashtra, India). All other

Table 1. Antioxidants supplemented during drug-induced thrombocytopenia

Antioxidant	Composition/structure
Caripill™ (10)	Flavonoids
	Phenolic compounds
	Papain
	Chymopapain
	Cystatin
	Tocopherol
	Ascorbic acid
	Cyanogenic-glucosides
	Glucosinolates
	Cardiac glycosides
	Anthraquinones
	Carpaine
	Pseudocarpaine
Vanillic acid (13)	
L-carnitine (16)	

chemicals were reagent grade and organic solvents were spectral grade.

Instruments

All absorbance measurements were carried out using a PC-based Double Beam Spectrophotometer 2202 (Systronics, Gujarat, India) with 10 mm quartz cuvettes. All 96-well microplates were read using a LISA Plus microplate ELISA reader (Aspen Diagnostics, Delhi, India). A refrigerated centrifuge (REMI, Mumbai, India) was used for centrifugation.

Animal care

Wistar rats were used in this study due to the similarity in their physiology with that of humans and to avoid baseline variations as the rats are bred under controlled conditions. Ethics approval for animal studies was obtained from the Institutional Animal Ethics Committee, Nargund College of Pharmacy, Bangalore, India (Ethics approval number: IAEC/NCP/93/2015).

Four-month-old male Wistar rats (~200-240 g) were acclimatized for a week in animal house conditions prior to the study. The rats were housed in cages (five in each cage) at $28 \pm 1^\circ\text{C}$ with a 12/12 h light and dark cycle. They were fed lab chow and tap water ad libitum.

Experimental design

Hydroxyurea was orally administered to each rat at 400 mg/kg body weight (thrombocytopenia group) once daily for 10 days (11). The antioxidants (50 mg/kg body weight) were orally administered to the respective groups once daily for 7 days.

The animals were divided into the following groups (n=5): group I - controls; group II - control + L-carnitine (C+LC); Group III - Control + Caripill™ (C+CP); group IV - control + vanillic acid (C+VA); group V - thrombocytopenia (Thr); group VI - thrombocytopenia + L-carnitine (Thr+LC); group VII - thrombocytopenia + Caripill™ (Thr+CP); and group VIII - thrombocytopenia + vanillic acid (Thr+VA).

Sampling of blood

The Wistar rats were anesthetized with ether and then restrained in dorsal recumbency. A syringe needle was inserted into the heart, slightly below the xiphoid cartilage and to the left of the

midline. Blood was drawn into collection tubes containing citrate phosphate dextrose adenine-1 (CPDA-1) as an anti-coagulant (19).

Isolation of plasma

Whole blood was centrifuged at room temperature (RT) for 20 min at 2,000 rpm to separate the platelet-rich plasma (PRP). The PRP was then centrifuged at 4,000 rpm at 22°C for 15 min. The resulting supernatant containing platelet-poor-plasma was stored for further analysis at -20°C (20).

Superoxide dismutase (SOD)

SOD activity in the plasma samples was analyzed according to the method of Misra and Fridovich, 1972 (21). Plasma samples were added to carbonate buffer (0.05 M, pH 10.2, containing 0.1 mM EDTA). Epinephrine was added to this mixture and absorbance was measured immediately at 480 nm. SOD activity was determined as the amount of enzyme that can inhibit the oxidation of epinephrine by half, which is equal to 1 unit.

Catalase (CAT)

Catalase activity was measured according to Aebi, 1984 (22). Absolute ethanol (10 μL) was added to the plasma samples then the mixture was incubated on ice for 30 min. To this, H_2O_2 (66 mM) in phosphate buffer (0.1 M) was then added and the absorbance was measured immediately at 240 nm. Catalase activity was measured using the molar extinction coefficient $43.6 \text{ M}^{-1}\text{cm}^{-1}$.

Cupric-ion reducing antioxidant capacity (CUPRAC)

The plasma samples were treated with 0.25 mM BCS (dissolved in 10 mM phosphate buffer solutions) and the absorbance was spectrophotometrically measured at 490 nm. 0.5 mM CuSO_4 was added to the samples and the mixture was incubated at room temperature for 3 min. The reaction was terminated by adding EDTA (0.01 M). Absorbance was measured at 490 nm using uric acid in 1 M NaOH as the standard (23).

Thiobarbituric acid reactive substances (TBARS) assay

TBARS level in plasma was measured according to the method of Olas et al., 2006 (24). Plasma

samples were cooled on ice for 10 min and then 20% (v/v) cold trichloro acetic acid (TCA) in 0.6 M HCl was added. The mixture was centrifuged for 15 min, then 0.12 M thiobarbituric acid (TBA) was added to the supernatant. This was boiled for 20 min, after which absorbance was recorded at 532 nm using a microplate reader.

Conjugate dienes (CD)

Plasma was treated with diethyl ether/ethanol mixture (1:3 (v/v)) and the mixture was vortexed. It was centrifuged at 4,000 rpm. The absorbance of the supernatant was read at 235 nm (25).

Protein carbonyls (PrC)

PrC in plasma were analyzed according to Reznick & Packer, 1994 (26). Plasma samples were incubated with 10 mM dinitrophenyl hydrazine (DNPH) in the dark for 1 h at room temperature. TCA (20%) was added to the samples which were then incubated on ice for 10 min. The mixture was centrifuged at 3,000 rpm. The pellets were washed with ethanol (ethyl acetate) [1:1 (v/v)] until the color disappeared. The final precipitate was dissolved in 6 M guanidine HCl. Absorbance was measured at 370 nm. Protein carbonyl content was calculated using the absorption coefficient $22,000 \text{ M}^{-1}\text{cm}^{-1}$.

Protein sulfhydryls (SH)

Plasma samples were mixed with 0.08 mol/L sodium phosphate buffer (pH 8.0, with 0.5 mg/mL $\text{Na}_2\text{-EDTA}$ and 2% sodium dodecyl sulfate) and the mixture was vortexed. DTNB was added to the mixture which was incubated for 15 min at room temperature. Absorbance was measured at 400 nm. SH in the plasma samples was calculated using a molar absorptivity of $13,600 \text{ M}^{-1}\text{cm}^{-1}$ (27).

Ferric reducing ability of plasma (FRAP)

FRAP assay was performed using the method of Benzie and Strain, 1996 (28). Plasma samples were added to a freshly prepared FRAP reagent (300 mM acetate buffer [pH 3.6], 10 mM tripyridyltriazine, and 20 mM FeCl_3). The reaction mixture was incubated for 5 min at 37 °C and absorbance was read at 593 nm. FRAP was determined using the extinction coefficient $21,250 \text{ mM}^{-1} \text{ cm}^{-1}$.

Nitrites and nitrates

Plasma samples were mixed with Griess reagent (100 μL) and incubated for 30 minutes in the dark at room temperature. Absorbance was read at 520 nm. The amount of nitrites present in the plasma sample was determined using sodium nitrite as the standard (29).

Nitrate levels were determined following Y/egin et al., 2015. Plasma samples were treated with CuSO_4 (50 $\mu\text{g/mL}$), $(\text{NH}_2)_2\text{SO}_4$ (1 mg/mL), NaOH (8 mg/mL), and Griess reagent. This mixture was vigorously shaken and the absorbance was measured at 520 nm (30). The results are expressed as a (nitrites)/(nitrates) ratio.

Lactate dehydrogenase (LDH)

Plasma samples were treated with a mixture of reagent 1 (containing 80 mM Tris, 200 mM NaOH, and 1.6 mM pyruvate) and reagent 2 (0.2 mM nicotinamide adenine dinucleotide). This mixture was incubated at 37 °C for 5 min. The decrease in absorbance was measured at 340 nm for 3 minutes (31).

Protein estimation

The protein content in the plasma samples was estimated according to Lowry et al., 1951 (32). The diluted plasma sample (20X) was mixed with alkaline copper sulfate (50:1 mixtures of A: 2% Na_2CO_3 in 0.1 N NaOH and B: 0.5% CuSO_4 in 0.1 N sodium potassium tartrate). The mixture was incubated for 15 min at room temperature. Folin's reagent was added and the mixture was incubated for 30 min at room temperature. The optical density was measured colorimetrically at 660 nm.

Statistical analyses

Results are expressed as mean $\pm$ SEM (n=5). One-way ANOVA analysis between the groups for all the parameters was performed based on the Bonferroni post-hoc test using GraphPad Prism 6 software and $p < 0.05$ was considered statistically significant.

RESULTS

Platelet counts were 8.88 ± 0.24 lakhs/ mm^3 (control group) and 1.86 ± 0.3 lakhs/ mm^3 (Thr group). Platelet count increased by 5-fold in the Thr+CP and Thr+VA groups, and 3-fold in the Thr+LC group compared to the Thr group.

Superoxide dismutase (SOD)

SOD levels in plasma were maintained across all control groups. The Thr+VA group showed a significant increment of 143% compared to the C+VA group ($p < 0.05$). SOD increased 92% and 134% in the Thr+VA group with controls and the Thr group, respectively (Figure 1A). The control group was set as 100%.

Catalase (CAT)

Variations in CAT were similar in the control and experimental groups (Figure 1B).

Cupric ion reducing antioxidant capacity (CUPRAC)

The changes observed in CUPRAC were similar in the control and experimental groups (Figure 2A).

Ferric reducing ability of plasma (FRAP)

Changes in FRAP were statistically significant between the groups. FRAP increased significantly by 74% in C+CP and 81% in C+VA groups against controls ($p < 0.05$). FRAP increased significantly by 60–80% in the Thr+LC, Thr+CP, and Thr+VA groups compared to controls ($p < 0.05$). There were increments of 20% and 50% in the C+LC and Thr groups with respect to controls, respectively (Figure 2B).

Lipid peroxidation (LPO) products

Variations in TBARS levels were observed among the groups, but were similar (Figure 3A). Changes

between the groups in conjugated dienes were statistically significant. There was a significant decrement of approximately 30% in all experimental groups with respect to the controls ($p < 0.05$) (Figure 3B).

Protein oxidation products

Protein carbonyls (PrC) showed statistically significant changes in the different control groups. All the control groups supplemented with antioxidants (C+LC, C+CP, and C+VA) showed a significant reduction of ~40% compared to controls ($p < 0.05$). PrC also increased in the Thr+VA group by 210% with respect to the C+VA group (Figure 4A). Protein sulfhydryl (SH) was significantly higher (70%) in the Thr+CP group with respect to the controls ($p < 0.05$). There were increments of 35%, 45%, and 60% in the Thr, Thr+LC and Thr+VA groups, respectively, compared to controls. SH levels were also higher by 20%, 40%, and 65% in C+LC, C+CP and C+VA groups, respectively, compared to controls (Figure 4B).

Nitrite/nitrate ratio

The control group was set as 100%. The nitrite/nitrate levels were similar in plasma; however, there was a statistically significant increase in the Thr+CP group (140%) compared to controls. A 70% increment was observed in the Thr+CP group with respect to the Thr group, and the ratio increased by 80% in the Thr+CP compared to the C+CP group (Figure 5).

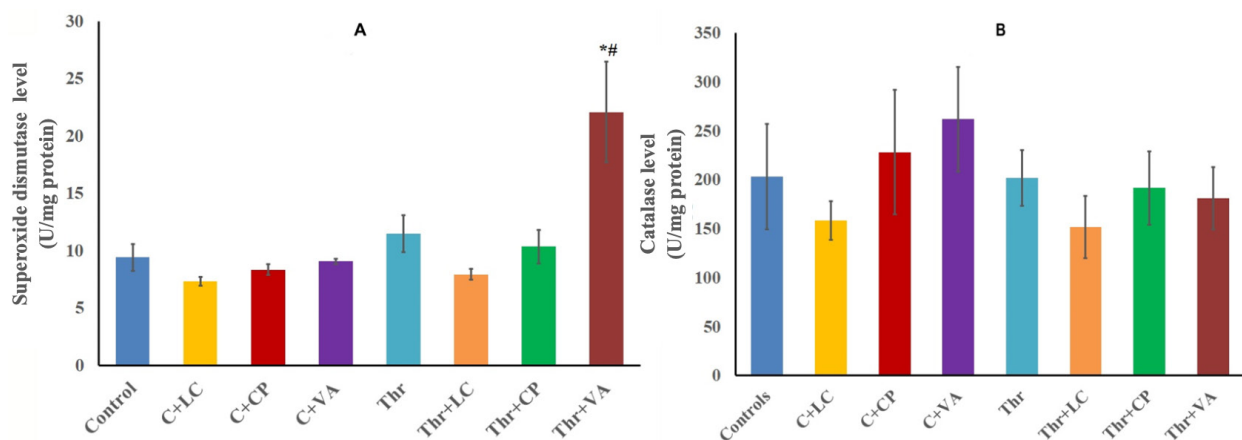


Figure 1. Superoxide dismutase (A) and catalase (B) in plasma of rats with induced thrombocytopenia. C+LC- Control + L-carnitine; C+CP- Control + Caripill™; C+VA- Control + vanillic acid; Thr- thrombocytopenia; Thr+LC- thrombocytopenia + L-carnitine; Thr+CP- thrombocytopenia + Caripill™; Thr+VA- thrombocytopenia + vanillic acid. '*' represents groups with statistically significant differences ($p < 0.05$) compared to the controls. '#' represents groups with statistically significant differences ($p < 0.05$) compared to the thrombocytopenia.

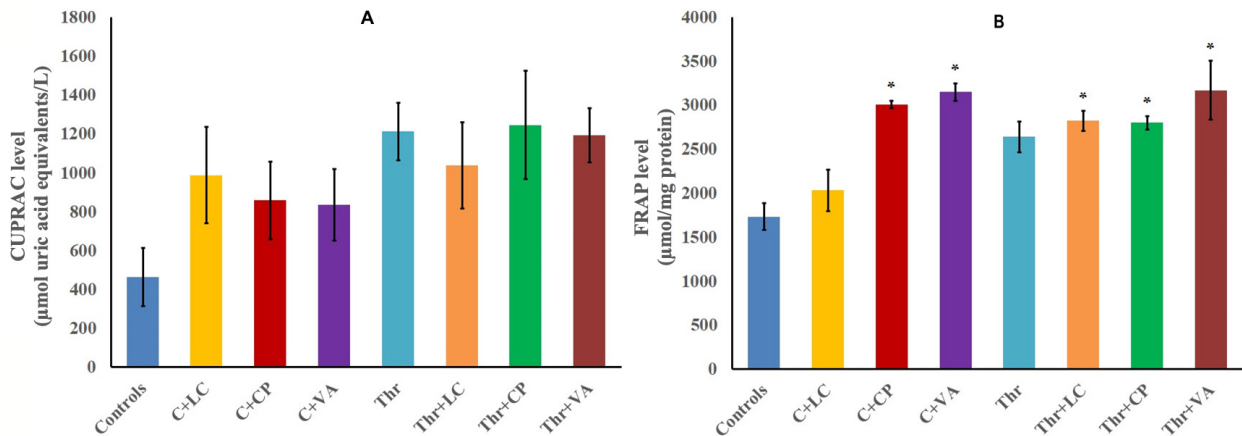


Figure 2. Cupric ion reducing antioxidant capacity (CUPRAC) (A) and ferric reducing ability of plasma (FRAP) (B) in plasma of rats with induced thrombocytopenia

C+LC- Control + L-carnitine; C+CP- Control + Caripill™; C+VA- Control + vanillic acid; Thr- thrombocytopenia;

Thr+LC- thrombocytopenia + L-carnitine; Thr+CP- thrombocytopenia + Caripill™; Thr+VA- thrombocytopenia + vanillic acid

‘*’ represents groups with statistically significant differences ($p < 0.05$) compared to the controls.

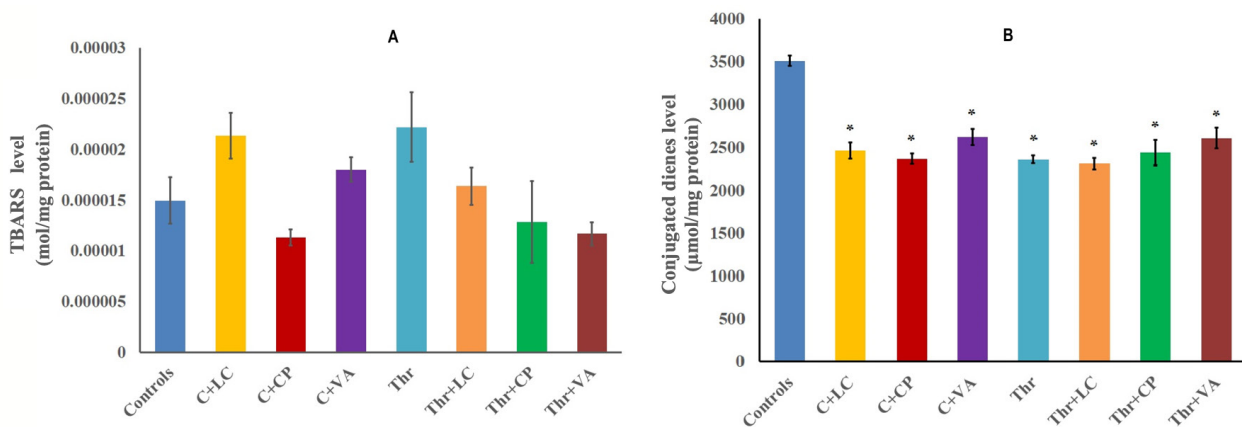


Figure 3. TBARS (A) and Conjugated Dienes (B) in plasma of rats with induced thrombocytopenia

C+LC- Control + L-carnitine; C+CP- Control + Caripill™; C+VA- Control + vanillic acid; Thr- Thrombocytopenia;

Thr+LC- Thrombocytopenia + L-carnitine; Thr+CP- Thrombocytopenia + Caripill™; Thr+VA- Thrombocytopenia + vanillic acid

‘*’ represents groups with statistically significant differences ($p < 0.05$) compared to the controls

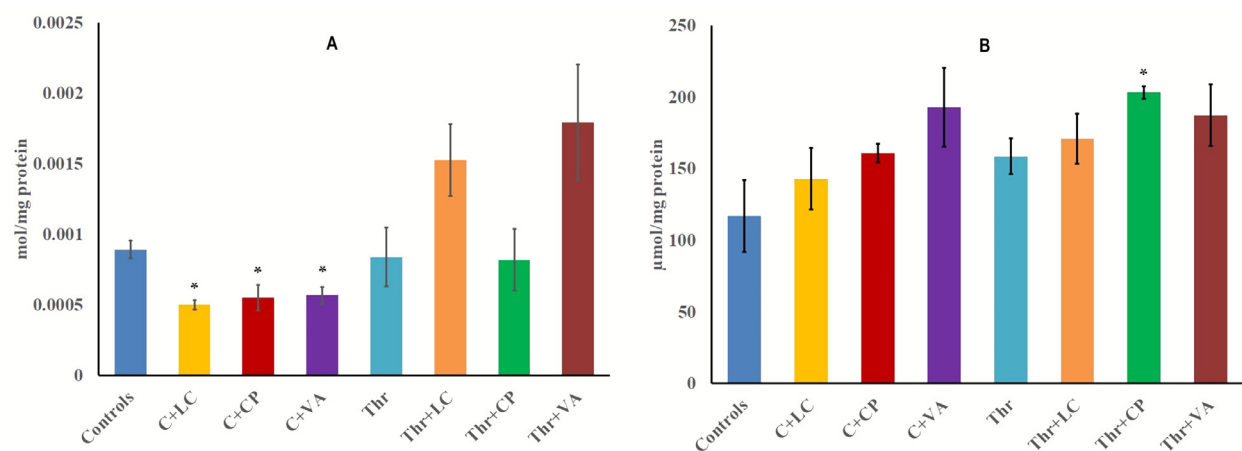


Figure 4. Protein carbonyls (PrC) (A) and protein sulfhydryl (P-SH) (B) in plasma of rats with induced thrombocytopenia

C+LC- Control + L-carnitine; C+CP- Control + Caripill™; C+VA- Control + vanillic acid; Thr- Thrombocytopenia;

Thr+LC- Thrombocytopenia + L-carnitine; Thr+CP- Thrombocytopenia + Caripill™; Thr+VA- Thrombocytopenia + vanillic acid

‘*’ represents groups with statistically significant differences ($p < 0.05$) compared to the controls.

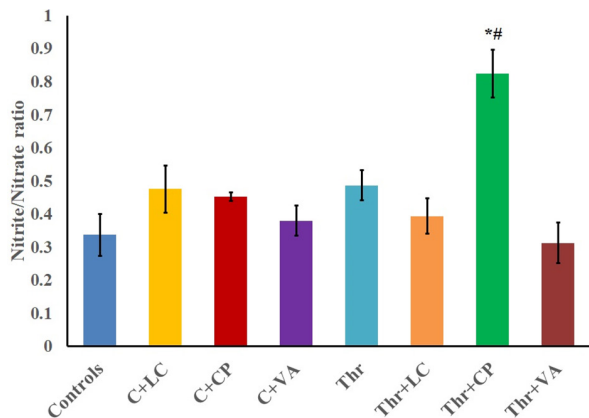


Figure 5. Nitrites/nitrates ratio in plasma of rats with induced thrombocytopenia

C+LC- Control + L-carnitine; C+CP- Control + Caripill™; C+VA- Control + vanillic acid; Thr- Thrombocytopenia; Thr+LC- Thrombocytopenia + L-carnitine; Thr+CP- Thrombocytopenia + Caripill™ ; Thr+VA- Thrombocytopenia + vanillic acid

*' represents groups with significant differences ($p < 0.05$) compared to the controls.

#' represents groups with statistically significant differences ($p < 0.05$) compared to the thrombocytopenia.

Lactate dehydrogenase (LDH)

The control group was set as 100% and the changes in LDH in the other groups were statistically significant. Groups supplemented with L-carnitine (C+LC) and Caripill™ (C+CP) antioxidants increased the LDH level significantly by 190% and 150%, respectively, compared to controls ($p < 0.05$). LDH level also significantly increased by 240% in the Thr+LC group compared to the controls ($p < 0.05$). There were increments of 140%, 200%, and 150% in the C+VA, Thr+CP and Thr+VA groups compared to controls, respectively (Figure 6).

DISCUSSION

Endogenous antioxidant machinery, comprised mainly of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), forms the first line of defense against reactive oxygen species (ROS) (33). SOD is an enzyme that acts on superoxides and converts them into less toxic H_2O_2 . SOD levels were elevated in Thr+VA, indicating that VA supplementation in thrombocytopenic rats activated SOD and thereby scavenged superoxides (34). Catalase terminates the reaction started by SOD by degrading H_2O_2 to H_2O and O_2 . H_2O_2 can also be scavenged by GPx. GPx and CAT have the same action upon H_2O_2 , but

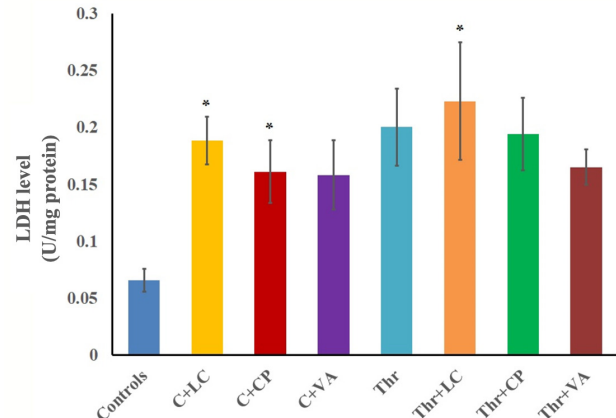


Figure 6. Lactate dehydrogenase (LDH) in plasma of rats with induced thrombocytopenia

C+LC- Control + L-carnitine; C+CP- Control + Caripill™; C+VA- Control + vanillic acid; Thr- thrombocytopenia; Thr+LC- thrombocytopenia + L-carnitine; Thr+CP- thrombocytopenia + Caripill™ ; Thr+VA- thrombocytopenia + vanillic acid

*' represents groups with statistically significant differences ($p < 0.05$) compared to the controls

GPx is more efficient during periods of low ROS levels, whereas CAT acts more effectively during high H_2O_2 concentrations. Similar variations in CAT during thrombocytopenia indicate that GPx can act on lower levels of H_2O_2 as GPx is the first line of defense against H_2O_2 (35, 36).

The antioxidant potential of the plasma was assessed based on CUPRAC and FRAP assays. CUPRAC assay provides the concentration of non-enzymatic antioxidants in the plasma sample. Similar levels indicated that endogenous non-enzymatic antioxidant defenses had maintained antioxidant potential in the plasma (37). The molar concentration of antioxidants present in plasma denoted by FRAP signifies that both the antioxidants and endogenous antioxidant systems acted to provide better protection against oxidative stress (38). Elevated FRAP in C+CP, C+VA, Thr+LC, Thr+CP and Thr+VA suggest that all three antioxidants (LC, Caripill™, and VA) show multiple mechanisms of scavenging free radicals, chelation of metal ions, and inhibition of free radicals and lipid hydroperoxides formation (39). Nonetheless, VA was the most effective antioxidant for improving the overall antioxidant capacity in plasma during thrombocytopenia. It has a carboxyl group that can transfer electrons or donate protons, and can therefore directly scavenge hydroxyl radicals and other cellular oxygen species (13). LC chelates metal ferrous ions and inhibits ROS formation. Its

carbonyl group stabilizes free radicals on α -carbon and protects cells against oxidative damage from ROS and RNS (16).

Oxidative stress in a biological system can be measured by assessing products of lipid peroxidation such as TBARS and conjugated dienes, and protein oxidation products such as sulfhydryls and carbonyls (4). Lipid peroxidation levels can be used to assess the damage caused to cell membranes such as membrane fluidity alteration, permeability, and integrity (40). Lipids upon oxidation give rise to products such as conjugate dienes and TBARS. CD is used as a marker for the identification of non-specific lipid peroxidation caused by free radicals. TBARS measure malondialdehyde (MDA) present in the plasma, one of the low molecular weight end products generated due to the decomposition of some primary and secondary lipid peroxidation products (41). Conjugated dienes levels were decreased in the experimental groups compared to the controls. Changes in TBARS levels were similar in all the experimental groups. This indicates that lipid peroxidation was negligible due to the free radical scavenging activity of antioxidants and the antioxidant capacity of the plasma. This was also corroborated in our results by CUPRAC levels. Caripill™ was the most effective in protecting the plasma from lipid peroxidation in both the C+CP and Thr+CP groups. This can be attributed to the antioxidant activity of the flavonoids and other phenolic compounds present in its active pharmaceutical ingredient, *C. papaya* leaf extract (10).

Carbonyl groups can be formed by different mechanisms, one of them being the oxidative cleavage of protein backbone, particularly at glutamyl side chains, and also oxidative deamination of lysine. The attack of hydroxyl radicals on proline, arginine, lysine and threonine side chains also leads to the formation of carbonyl groups (42). The generation of carbonyls in all control groups supplemented with antioxidants decreased, suggesting that antioxidants help protect protein from oxidation whereas similar protein carbonyl levels were maintained in the Thr groups during the disease condition, indicating that antioxidant supplementation can protect plasma against protein carbonyl formation. Protein sulfhydryl levels contribute significantly to the antioxidant status of plasma (43). Sulfhydryl (SH) becomes oxidized

to disulfide, which can be reversed by the action of ROS. ROS are mainly present in the cysteine components of proteins during low glutathione concentrations (44). SH increased in thrombocytopenic rats treated with Caripill™ compared to controls, indicating reduced oxidative stress. Caripill™ protected the SH from oxidation more effectively than both LC and VA, suggesting that CP is the most potent in maintaining SH in a reduced state.

The direct measure of reactive nitrogen species such as nitric oxide radicals is tedious due to their radical nature and short half-life. These radicals are generated in plasma by specific nitric oxide synthases (NOSs). For that reason, measurement of the stable end products of NO radicals, such as nitrite, and nitrate, in plasma is most often used for estimating NO radicals (45). One of the main factors interfering with NO is superoxide anions, which favor the generation of peroxynitrite anions. NO and its metabolites stimulate and inhibit lipid peroxidation reactions (46). An increase in the nitrite/nitrate ratio of Thr+CP showed that more RNS were generated in this group. Lactate dehydrogenase is considered one of the indicators of tissue/cell damage (47). Similar changes in the Thr+VA and C+VA groups suggest that VA supplementation is most efficient in maintaining LDH levels in thrombocytopenic conditions similar to the controls.

CONCLUSIONS

Plasma has been demonstrated to be an ideal sample as plasma is of biological origin, it is more appropriate to use sample instead of material for studying oxidative stress during DIT as it represents all the changes occurring in the blood during DIT. This study confirms that Caripill™ is effective in improving overall antioxidant status (FRAP) in plasma, reducing lipid peroxidation, and increasing the protein sulfhydryls in thrombocytopenia groups. VA upregulated SOD and attenuated lipid peroxidation more effectively in thrombocytopenia groups compared to non-thrombocytopenic controls. Nonetheless, all the antioxidants enhanced the antioxidant status of plasma in both control and thrombocytopenic conditions as seen in the CUPRAC and FRAP results. The antioxidants Caripill™, VA, and LC have been shown to be beneficial during DIT. Antioxidant

supplementation can be beneficial in thrombocytopenic conditions which opens new avenues for employing antioxidants in alternate therapeutics.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

ADDITIONAL INFORMATION

Author contribution

V.R.: conceptualization, Visualization, methodology, validation, writing–review and editing; A.B.A: writing–original draft preparation; A.H., F.A., O.O.L, P.D., S.S.R.: investigation, data curation, writing– original draft preparation; MM: supervision, investigation.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Anderson S, Lightfoot D. Principles of physiology. Cambridge: Cambridge University Press, 2002. p. 704.
2. Tortora G. Principles of anatomy and physiology. 10th Edition. New Jersey: John Wiley & Sons; 2003.
3. Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol*. 1997;82:291–5.
4. Schiller H, Reily P, Bulkley G. Antioxidant therapy. *J Crit Care Med*. 1993;21:92–102.
5. Chakraborty P, Kumar S, Dutta D, Gupta V. Role of antioxidants in common health diseases. *Res J Pharm Technol*. 2009;2:238–244.
6. Greenberg E, Kaled E. Thrombocytopenia. *Crit Care Nurs Clin North Am*, 2013;25:427–34.
7. Chong B, Choi P, Khachigian L. Drug-induced immune thrombocytopenia. *Hematol Oncol Clin North Am*. 2013;27:521–40.
8. De Silva E, Hugh K. Drug-induced thrombocytopenia: Focus on platelet apoptosis. *Chem Biol Interact*. 2018;284:1–11.
9. George J, Raskob G, Shah S, Rizvi M, Hamilton S, Osborne S. Drug-Induced Thrombocytopenia: A Systematic Review of Published Case Reports. *Ann Intern Med*. 1998;129:886–90.
10. Saeed F, Arshad M, Pasha I, Naz R, Batool R, Khan A, Nasir M, Shafique B. Nutritional and phyto-therapeutic potential of papaya (*Carica papaya* Linn.): an overview. *Int J Food Prop*. 2014;17:1637–53.
11. Gammulle A, Ratnasooriya W, Jayakody Fernando C, Chamini K, Preethi V. Thrombocytosis and anti-inflammatory properties, and toxicological evaluation of *Carica papaya* mature leaf concentrate in a murine model. *Online Int J Med Plant Res*. 2012;1:21–30.
12. Yudisthira D, Firdausi F, Alyani C, Nurkolis F, Al Rasyid H, Yusuf V, Taslim N. Potential of *Carica papaya* leaf extract as an future medicine for thrombocytopenia in dengue patients: from traditional to scientific drug discovery. *Adv Tradit Med*. 2023;1–4.
13. Chou T, Ding H, Hung W, Liang C. Antioxidative characteristics and inhibition of α -melanocyte-stimulating hormone-stimulated melanogenesis of vanillin and vanillic acid from *Origanum vulgare*. *Exp Dermatol*. 2010;19:742–50.
14. Salau V, Erukainure O, Ibeji C, Olasehinde T, Koorbally N, Islam M. Vanillin and vanillic acid modulate antioxidant defense system via amelioration of metabolic complications linked to Fe^{2+} -induced brain tissues damage. *Metab Brain Dis*. 2020;35:727–38.
15. Shabani M, Jamali Z, Bayrami D, Salimi A. Vanillic acid alleviates methamphetamine-induced mitochondrial toxicity in cardiac mitochondria via antioxidant activity and inhibition of MPT Pore opening: an in-vitro study. *BMC Pharmacol Toxicol*. 2023;24:33. PubMed PMID: 37208773
16. Gülçin I. Antioxidant and antiradical activities of L-carnitine. *Life Sci*. 2006;78:803–11.
17. Ravikumar S, Prabhu S, Vani R. Effects of L-carnitine on the erythrocytes of stored human blood. *Transfusion Medicine*. 2020;30:215–25.
18. Caballero-García A, Noriega-González D, Roche E, Drobnic F, Córdova A. Effects of L-carnitine intake on exercise-induced muscle damage and oxidative stress: a narrative scoping review. *Nutrients*. 2023;15:2587. PubMed PMID: 37299549
19. Devi S, Subramanyam M, Vani R, Jeevaratnam K. Adaptations of the antioxidant system in erythrocytes of trained adult rats: impact of intermittent hypobaric-hypoxia at two altitudes. *Comp Biochem Physiol C Toxicol Pharmacol*. 2005;140:59–67.
20. Dodge J, Mitchell C, Hanahan DJ. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch Biochem Biophys*. 1963;100:119–30.

21. Misra H, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem.* 1972;247:3170-5.
22. Aebi H. Catalase in vitro. *Meth Enzymol.* 1984;105:121-6.
23. Da Cruz G. US Patent for use of bathocuproine for the evaluation of the antioxidant power in liquids and solutions. US patent. 6613577. 2003.
24. Olas B, Nowak P, Kolodziejczyk J, Ponczek M, Wachowicz B. Protective effects of resveratrol against oxidative/nitrative modifications of plasma proteins and lipids exposed to peroxynitrite. *J Nutr Biochem.* 2006;17:96-102.
25. Olas B, Wachowicz B. Resveratrol and vitamin C as antioxidants in blood platelets. *Thromb Res.* 2002;106:143-8.
26. Reznick A, Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Meth Enzymol.* 1994;233:357-63.
27. Habeeb AFSA. Reaction of Protein Sulfhydryl Groups with Ellman's Reagent. *Meth Enzymol.* 1972;25:457-64.
28. Benzie I, Strain J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem.* 1996;239:70-6.
29. Chen L, Mehta P, Mehta J. Oxidized LDL decreases L-arginine uptake and nitric oxide synthase protein expression in human platelets. *Circulation.* 1996;93:1740-6.
30. Yegin S, Yur F, Çetin S, Güder A. Effect of lycopene on serum nitrite-nitrate levels in diabetic rats. *Indian J Pharm Sci.* 2015;77:357-60.
31. Buhl S, Jackson K. Optimal conditions and comparison of lactate dehydrogenase catalysis of the lactate-to-pyruvate and pyruvate-to-lactate reactions in human serum at 25, 30 and 37°C. *Clin Chem.* 1978;24:828-31.
32. Lowry O, Rosebrough N, Farr A, Randall R. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193:265-75.
33. Matés J, Pérez-Gómez C, De Castro I. Antioxidant enzymes and human diseases. *Clin Biochem.* 1999;32:595-603.
34. Kumar S, Prahalathan P, Raja B. Antihypertensive and antioxidant potential of vanillic acid, a phenolic compound in L-NAME-induced hypertensive rats: a dose-dependence study. *Redox Rep.* 2011;16:208-15.
35. Augustyniak A, Bartosz G, Cipak A, Duburs G, Horáková L, Luczaj W, et al. Natural and synthetic antioxidants: an updated overview. *Free Rad Res.* 2010;44:1216-62.
36. Rahman K. Studies on free radicals, antioxidants, and co-factors. *Clin Interv Aging.* 2007;2:219-36.
37. Campos C, Guzmán, R, López-Fernández E, Casado A. Evaluation of the copper(II) reduction assay using bathocuproinedisulfonic acid disodium salt for the total antioxidant capacity assessment: the CUPRAC-BCS assay. *Anal Biochem.* 2009;392:37-44.
38. Ognik K. Effect of L-carnitine on the level of biochemical and antioxidant indices of blood of Turkey hens. *Ann UMCS Zootechnica.* 2012;30:1-10.
39. Son S, Lewis B. Free radical scavenging and antioxidative activity of caffeic acid amide and ester analogues: Structure-activity relationship. *J Agri Food Chem.* 2002;50:468-72.
40. Schiller H, Reilly P, Bulkley G. Antioxidant therapy. *Crit Care Med.* 1993;21:S92-102.
41. Janero D. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic Biol Med.* 1990;9:515-40.
42. Stadtman E, Levine R. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids.* 2003;25:207-18.
43. Molicki J, Draaisma A, Verbeet N, Munneke R, Huysmans H, Hazekamp M, Berger H. Prime solutions for cardiopulmonary bypass in neonates: Antioxidant capacity of prime based on albumin or fresh frozen plasma. *J Thorac Cardiovasc Surg.* 2001;122:449-56.
44. Omparn P, Hisalaphong C, Akornchai S, Nichern S, Orales N. Comparative antioxidant activities of curcumin and its dimethoxy and hydrogenated derivatives. *Biol Pharm Bull.* 2007;30:74-8.
45. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and (15N) nitrate in biological fluids. *Anal Biochem.* 1982;126:131-8.
46. Bloodsworth A, O' Donnell VB, Freeman BA. Nitric oxide regulation of free radical- and enzyme-mediated lipid and lipoprotein oxidation. *Arterioscler Thromb Vasc Biol.* 2000;20:1707-15.
47. Klein R, Nagy O, Tóthová C, Chovanová F. Clinical and diagnostic significance of lactate dehydrogenase and its isoenzymes in animals. *Vet Med Int.* 2020;2020:5346483. PubMed PMID: 32607139