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Caesalpinia crista coat protects platelets and plasma cells from sodium nitrite induced oxidative stress and exhibits anticoagulation property

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Abstract

Current study investigates the protective effect of *Caesalpinia Crista* Coat Methanolic Extract (CCCME) on $NaNo_2$ (10mM)-induced oxidative stress on plasma and platelets along with its anticoagulation potential. As oxidative stress markers, the level of lipid peroxidization (LPO), Protein carbonyl content (PCC) endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) activities were measured. The level of LPO and PCC in sodium nitrite induced oxidative stress in platelets and plasma cells increased. While, the activity of SOD and CAT decreased. Interestingly, CCCME significantly normalized the increased level of LPO, PCC level in a dose dependent manner from the concentration 0-150µg. On the other hand, CCCME normalized the activity of SOD and CAT in sodium nitrite induced oxidative stress in platelets and plasma cells significantly and the maximum dose consumed was 200µg. The CCCME showed strong anticoagulant effect as enhanced the clotting time of both PRP and PPP from control 199s to 680s and 250s to 753s respectively. The anticlotting effect of CCCME was also further strengthened by APTT and PT test. CCCME appeared to interfere in intrinsic pathway of coagulation. CCCME could be better candidate in the regulation of stress related complications.

Keywords: CCCME, antioxidant, platelets, plasma, anticoagulant, APTT and PT

Introduction

Antioxidants are the first line of defence to counter the free radicals or reactive oxygen/nitrogen species (ROS/RNS) generated by auto-oxidation processes or enzymatic reactions in mammalian system. Numerous studies have shown that reactive oxygen species, including hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$), superoxide anion ($O_2^{-\bullet}$), peroxyl radical (ROO•), and polycyclic aromatic hydrocarbons ($PAH^{\bullet}$) are highly reactive and toxic molecules that are generated in metabolically active cells. They are very dangerous and can cause oxidative damage to lipids, proteins, including enzymes, and DNA. They have also been linked to the pathogenesis of oxidative diseases, such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure [1]. Oxidants generated in the cardiovascular system may significantly affect all components of the haemostatic system and lead to dysfunction of vascular endothelium, alterations in the coagulation process as well as impaired fibrinolytic activity of blood plasma [2]. Human plasma contains potential sites for radical formation and destruction. The oxidative stress may alter the hemostatic system by oxidative damage to platelets and plasma proteins involved in blood coagulation may lead to changes in hemostatic process [3]. It is well known that antioxidants can scavenge free radicals. However, some synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are the most widely added to food, have recently been restricted because of serious concerns about their carcinogenic potential [4]. The plant-based substances, enhancing the physiological antioxidative defence are believed to be non-toxic and also able to efficiently counteract the biological consequences of oxidative stress. Therefore, attention has turned to natural products to find natural antioxidants to replace synthetic once [5]. The role of different medicinal plants in the defence against oxidative/nitrative stress in blood platelets and human plasma is still unknown. Investigation of therapeutic properties of extracts derived from *Caesalpinia crista* (family: Fabaceae/Caesalpiniaceae) coat is a promising trend in phytopharmacological research for a variety of reasons, including the diversity of chemical components, widespread occurrence of these plants. *Caesalpinia crista* is an herb reported in ayurveda and ancient traditional system in India [6]. The seeds of *Caesalpinia crista* are common Indian folk-lore drug. lt is a ornamental tree present in all over the world. The seeds are traditionally have been used to cure Leprosy, paralysis, asthma, malaria, hydrocele, orchitis and liverpool diseases [7]. Seeds have multiple therapeutic properties (antibacterial, antiviral, antifungal, antimalarial, anti-filarial, antidiabetic, antihyperglycemic, antihypoglycemic, antilipidemic, immunomodulatory, protease inhibitory, proapoptotic, cox-2 inhibitory, anthelmintic, diuretic, anti-inflammatory, adaptogenic, antiestrogenic, antipyretic, analgesic and antispermatogenic activity) [8]. Sodium nitrite ($NaNo_2$) is an inorganic salt having both beneficial and harmful properties [9]. The diet is generally the most appropriate source of human exposure. The $NaNo_2$ is used as fertilizers, dyes, pyrotechnics and also found in drinking water. In food industry, the sodium nitrite is called as E250 and used as the preservative and colour fixative in fish and meats. Sodium nitrite is also used as a pharmacological agent for cyanide poisoning. The vasodilator effect of $NaNo_2$ could be a better therapeutic role in the treatment of pulmonary hypertension,

posthemorrhagic cerebral vasospasm and myocardial infarction [10-12]. Although the vasodialarory effect of nitrite has been widely studied, the effect of nitrite on platelet activity has little attention. The nitrite at the higher concentration 500 mM was reported as to inhibit ADP-induced platelet aggregation and increased cGMP levels in the plasma [13]. The elevation of plasma nitrite levels after the consumption of nitrrte-rich diet decreases the blood pressure and reduces the platelet aggregation induced by ADP and collagen in plasma, this has showing a effect of nitrite on platelet activity [14]. Human platelets and plasma are an ideal model system for studying the protective effects of various antioxidants against ONOO−-induced oxidative / nitrative stress. ONOO− can travel a mean distance of 3, 5.5 and 0.5 μm in mitochondria, blood plasma, and erythrocytes during half-life, respectively [15]. The ONOO− is a diffusible molecule that can penetrate the platelet membrane through a complex mechanism of transport. Oxidative stress is also known to modulate platelet function and may contribute to platelet aggregation. Hence platelet participation in thrombus formation is critical to the pathological activation of the coagulation cascade, for the treatment of atherothrombotic disease, the development of various antiplatelet therapies including antioxidant action is essential [16]. In the previous study *Caesalpinia crista* seed aqueous extract exhibited (CCSAE) strong anticoagulant effect [17]. In the present study the protective effects of the *Caesalpinia Crista* Coat Methanolic Extract (CCCME) against oxidative/nitrative protein damage to human plasma and blood platelets treated with sodium nitrite ($NaNo_2$) were examined (10mM).

Materials and methods

1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 4-dinitrophenylhydrazine (DNPH), TCA, 95% ethanol, $NaNo_2$, SDS, acetic acid, thiobarbituric acid, EDTA, calcium chloride, TEMED, Quercetin and hydrogen peroxide were purchased from Sigma Chemicals Company (St. Louis, USA).

All other chemicals used were of analytical grade.

Fresh human blood was collected from healthy donors for the Platelet-Rich Plasma (PRP) & Platelet Poor Plasma (PPP). All the experimentations were conducted in accordance with the ethical guidelines and were approved by the Institutional Human Ethical Committee (IHEC-UOM No. 47Res/2014– 15), University of Mysore, Mysore. Conducting animal experiments were permitted by the Institutional Animal Ethical Committee (UOM/IAEC/02/2016), University of Mysore. The animal handling were proceeded in accordance with the guidelines of the Committee for the Purpose of monitoring and Supervision of Experiments on Animals (CPCSEA).

Preparation of *Caesalpinia Crista* Coat Methanolic Extract (CCCME)

Caesalpinia crista seeds were purchased from local market Tumkur, thoroughly washed them and dried at room temperature for 24h. Seed coats were separated mechanically, homogenized using pestle and mortar by adding 95% methanol. The *Caesalpinia Crista* coat methanolic extract obtained was filtered using Whatman No-1 filter paper and allowed to evaporate the alcohol content. The obtained filtrate was completely dissolved in PBS and it was used for further assays

Induction of oxidative stress

The plasma was diluted with normal saline (1:1, V/V) and were treated with $NaNo_2$ (10Mm) for 2 hours at 37^0C.In order to examine the antioxidant property of *Caesalpinia Crista* Coat Methanolic Extract of (CCCME), the plasma were pre-treated with CCCME in the concentration of 50 to 200μg for 1 hr at 37 ^{0}C [18].

Evaluation of Lipid per Oxidation (LPO)

Malondialdehyde (MDA) formation was determined as a measure of lipid peroxidation, according to to the method of Ohakawa *et al* [19]. 10μl of $NaNo_2$ induced pre-treated CCCME (0-150μg) RBC lysate was taken in cleaned dry test tubes and 1.5ml of acetic acid (pH 3.5, 20% v/v), SDS (8% w/v, 0.2ml) and 1.5ml thiobarbituric acid (0.8% w/v) was added, the reaction mixture was boiled at 45-60 °C for 45min and centrifuged at 450*g* for 10min. The adducts that formed were extracted in butanol and measured TBARS (Thiobarbituric Acid-Reactive Substance) formed spectrophotometrically at 532nm. The results were expressed as nmol/g of protein.

Determination Protein Carbonyl Content (PCC)

According to the method described by Levine *et al* [20]. The 10μl of $NaNo_2$ induced pre-treated CCCME (0-150μg) RBC lysate was taken in cleaned dry test tubes and equal volume of 10mM DNPH in 2N HCl was added, incubated for 1hr shaking occasionally at room temperature. Corresponding blank was carried out by adding only 2N HCl to the sample. After incubation, precipitated the mixture with 20% Trichloroacetate (TCA) and centrifuged at 1200*g* for 15min. The precipitate was washed twice with acetone by centrifuging at 2500*g*, for 15min and finally dissolved in 1ml of Tris buffer (20mM pH 7.4 containing 0.14M NaCl, 2% SDS) and the supernatant was recorded at 360nm. and the results were expressed as nmol/mg of protein.

Super Oxide Dismutase (SOD) enzyme activity

SOD enzyme activity was determined according to the method of Kostyuk *et al* [21]. Activity was assayed in samples (2% Haematocrit) by monitoring the inhibition of quercetin autoxidation. Briefly, an aliquot of sample (0.01 mg protein) was added to the reaction mixture (1 ml) consisting of phosphate buffer (16mM, pH 7.8) containing TEMED–EDTA (8mM/0.08mM) mixture and quercetin (0.15% w/v).

The decrease in absorbance was monitored for 3min at 406nm. The amount of protein that inhibits quercetin autoxidation by 50% is defined as one unit. The results were expressed as U/mg of protein.

Catalase (CAT) enzyme activity

CAT activity was assayed by the method of Beers *et al* [22]. Briefly the method is as follows: 2.25ml of potassium phosphate buffer (50 mM, pH 7.0 or 65mM, pH 7.8) was added to 0.05 ml of 2% Haematocrit by potassium phosphate buffer (50mM, pH 7.0)) and incubated at 25ºC for 30 min. 650μl of hydrogen peroxide (to get 7.5mM final) were added to initiate the reaction. The change in absorbence was measured at 240nm for 3min. The catalase activity was expressed in IU.

One international unit (IU) of catalase is the enzyme activity, which decomposes one μmol of hydrogen peroxide per minute at 25 ºC.

Preparation of platelet-rich plasma and platelet-poor plasma

The method of Ardlie and Han *et al* [23] was employed for the preparation of human platelet-rich plasma (PRP) and platelet-poor plasma (PPP). The platelet concentration of PRP was adjusted to 3.1×108 platelets/ml with PPP. The PRP maintained at 37 °C was used within 2hr for the aggregation process.

All the above preparations were carried out using plastic wares or siliconized glass wares.

Plasma re-calcification time

The plasma re-calcification time was determined according to the method of Quick *et al* [24]. Briefly, the crude CCCME (20-80µg) was pre-incubated with 0.2 ml of citrated human plasma in the presence of 10mM Tris HCl (20µl) buffer pH 7.4 for 1min at 37 °C and clotting time was recorded in seconds.

Bleeding time

The bleeding time was described according to the method of Denis *et al* [25].

Briefly, 0-8µg of CCCME in 30µl of PBS was injected intravenously through the tail vein of a group of five mice. After 10min, mice were anaesthetized using diethyl ether and a sharp cut was made at the tail tip of a mouse in 3mm length. Immediately, the tail was vertically immersed into PBS which is pre-warmed to 37 °C. Bleeding time was recorded from the time bleeding started till it completely stopped.

APTT and PT

Briefly, 100µl of normal citrated human plasma and CCCME (0–100µg) were pre-incubated for 1min. For APTT, 100µl reagent (LIQUICELIN-E Phospholipids preparation derived from Rabbit brain with ellagic acid), which was activated for 3min at 37 °C was added. The clotting was initiated by adding 100µl of 0.02M $CaCl_2$ and the clotting time was measured. For PT, the clotting was initiated by adding 200µl of PT reagent (UNIPLASTIN–rabbit brain Thromboplastin).

The time taken for the visible clot was recorded in seconds. The APTT ratio and the international normalized ratio (INR) for PT at each point was calculated from the values of control plasma incubated with the buffer for identical period of time.

Determination of protein concentration

Protein concentration was estimated by following the protocol of Lowry *et al* using BSA as standard [26].

Results

The ability of CCCME to protect plasma and platelets from oxidative stress was measured by exposure of the plasma and platelets to oxidant $NaNO_2$, resulted in enhanced level of lipid peroxidation and protein carbonyl content. As estimated by the level of MDA (marker of lipid peroxidation), the exposure of plasma and platelets to $NaNO_2$ lead to the lipid peroxidation. CCCME in plasma and platelets treated with $NaNO_2$ reduced the $NaNO_2$-induced lipid peroxidation. CCCME decreased the MDA level in plasma and platelets in a dose dependent manner and statistically significant value were observed at the concentration of 50-100µg ($P< 0.01$) and 150µg ($P< 0.001$) of plasma (Fig 1A), where as statistically more significant values were observed at the concentration of 100-150µg of CCCME in platelets. (Fig 1B). CCCME was examined for protective effect on protein oxidation in plasma and platelets. The $NaNo_2$ treated plasma and platelets were revealed considerable elevation of the protein carbonyl content (PCC) compared to the control, while CCCME significantly ($P<0.05$) decreased the PCC at the concentration of 50-100µg and for 150µg ($P<0.01$) in the $NaNo_2$ treated plasma (Fig 2A) and protective effects of CCCME on platelets were exhibited at the dose of 100-150µg ($P<0.001$) (Fig 2B). While, $NaNo_2$ treated plasma and platelets exhibited decreased antioxidant enzymes SOD and CAT level when compared to the control. Interestingly, CCCME significantly increased the SOD ($P< 0.001$) for plasma at the concentration of 150µg (Fig 3A) and in case of platelets increased SOD ($P< 0.01$) was observed at 100-150µg (Fig 3B). The CCCME also drastically increased the CAT ($P< 0.001$) activities at the concentration of 150µg in both $NaNo_2$ treated plasma and platelets (Fig 4A&4B). When plasma re-calcification time was done using both human platelet rich and poor plasma, the addition of CCCME interestingly increased the clotting time from control 199s to 680s and 250s to 753s for PRP and PPP respectively, at the concentration of 20µg to 60µg and did not altered clot formation upto 80µg on increased dose concentration (Fig 5A). Furthermore, the *in-vivo* mouse tail bleeding assay, which showed the strong anticoagulant activity of CCCME. when the CCCMW was injected intravenously, there was a significant prolonged bleeding time recorded more than 320s ($P<0.01$) in a dose dependent manner at the concentration of 80µg by comparing with the PBS-treated control of 180s (Fig 5B). The CCCME have anticlotting effect when examined the APTT test by increasing clotting time 82s, when compare to control 40s in a dose dependent manner at the concentration of 100µg of CCCME (Table 1).

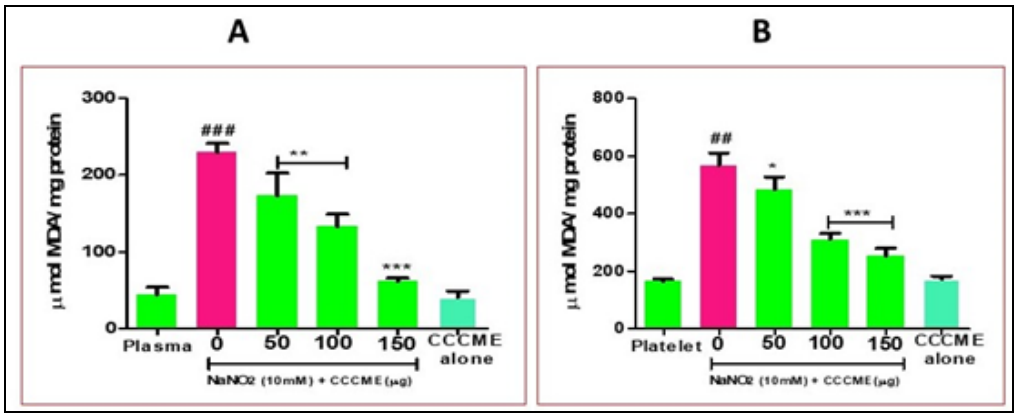

Fig 1: Effect of *Caesalpinia Crista Coat Methanolic Extract* (CCCME) on lipid peroxidation of plasma (A) and platelets (B): $NaNo_2$ (10mM) was used as an agonist in lipid peroxidation induction for about 1h of incubation period. For inhibition studies, Plasma and platelets were pre-incubated with different doses (50-150µg) of CCCME for 10min at 37 °C prior to $NaNo_2$ (10mM) treatment. Results are expressed as mean ± SEM (n = 6). # P<0.05, ## P<0.01, ### P<0.001 as compared with normal control group; * P<0.05, ** P<0.01, *** P <0.001 as compared with control group. CCCME: *Caesalpinia Crista* Coat Methanolic Extract.

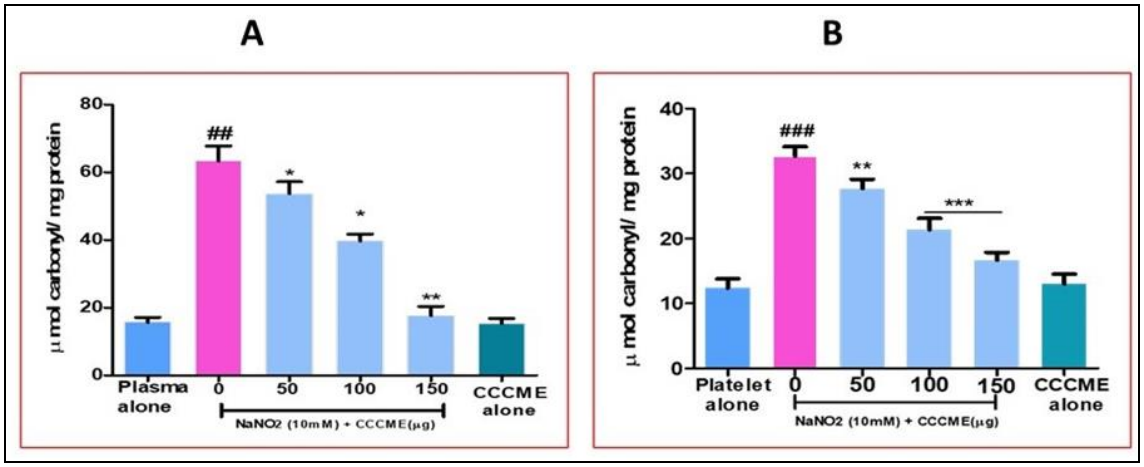

Fig 2: Effect of *Caesalpinia Crista Coat Methanolic Extract* (CCCME)) on protein carbonyl content of plasma (A) and platelets (B): The $NaNo_2$ (10mM) was used as an agonist in induction of protein carbonyl content of plasma and platelets for about 1h of incubation period. For inhibition studies, plasma was pre-incubated with different doses of CCCME for 10min at 37 °C prior to $NaNo_2$ (10mM) treatment. Results are expressed as mean ± SEM (n = 6). # P<0.05, ## P<0.01, ### P<0.001 as compared with normal control group; * P<0.05, ** P<0.01, *** P <0.001 as compared with control group. CCCME: *Caesalpinia Crista* Coat Methanolic Extract.

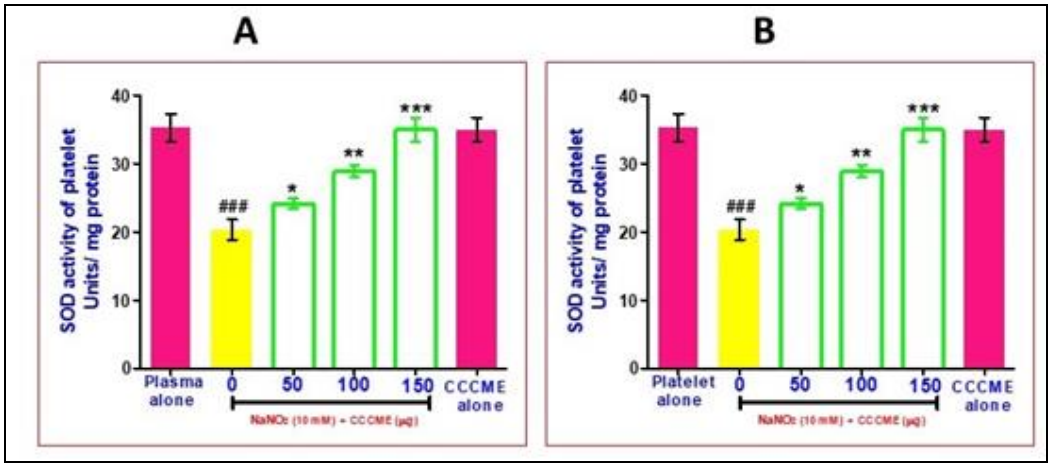

Fig 3: Effect of *Caesalpinia Crista Coat Methanolic Extract* (CCCME) on SOD of activity plasma (A) and platelets (B): To determine the SOD activity of CCCME against NaNo2 -induced oxidative damage, plasma and platelets were pre-incubated with different doses (50-150µg) of CCCME for 10min at 37 °C prior to NaNo2 (10mM) treatment. The data was expressed as average enzyme units per mg protein. Results are expressed as mean ± SEM (n = 6). # P<0.05, ## P<0.01, ### P<0.001 as compared with normal control group; * P<0.05, ** P<0.01, *** P <0.001 as compared with control group. CCCME: *Caesalpinia Crista* Coat Methanolic Extract.

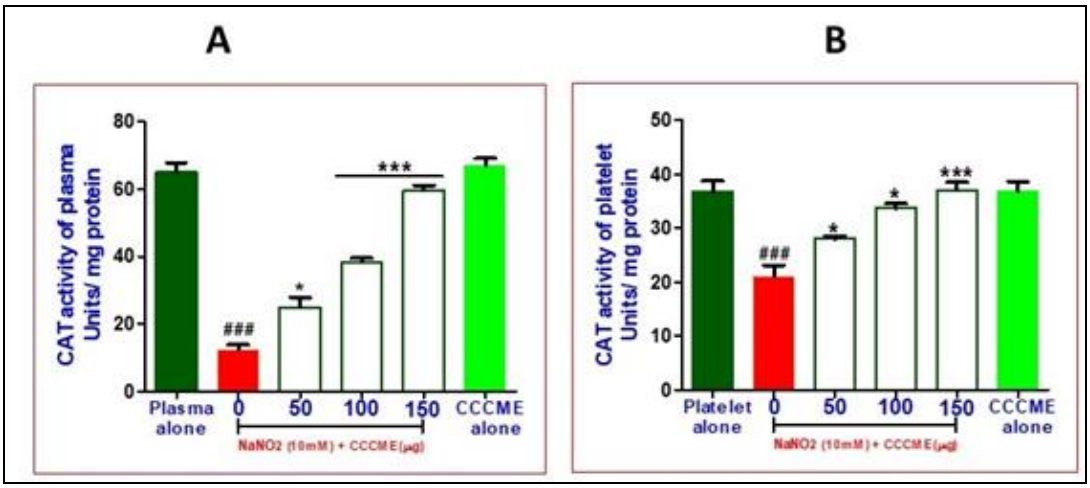

Fig 4: Effect of *Caesalpinia Crista Coat Methanolic Extract* (CCCME) on catalase activity of plasma (A) and platelets (B): To determined the catalase activity of CCCME against NaNo2 -induced oxidative damage, plasma and platelets was pre-incubated with different doses (50-150µg) of CCCME for 10 min at 37 °C prior to NaNo2 (10mM) treatment. The data was expressed as average enzyme units per mg protein. Results are expressed as mean ± SEM (n = 6). # P<0.05, ## P<0.01, ### P<0.001 as compared with normal control group; * P<0.05, ** P<0.01, *** P <0.001 as compared with control group. CCCME: *Caesalpinia Crista* Coat Methanolic Extract.

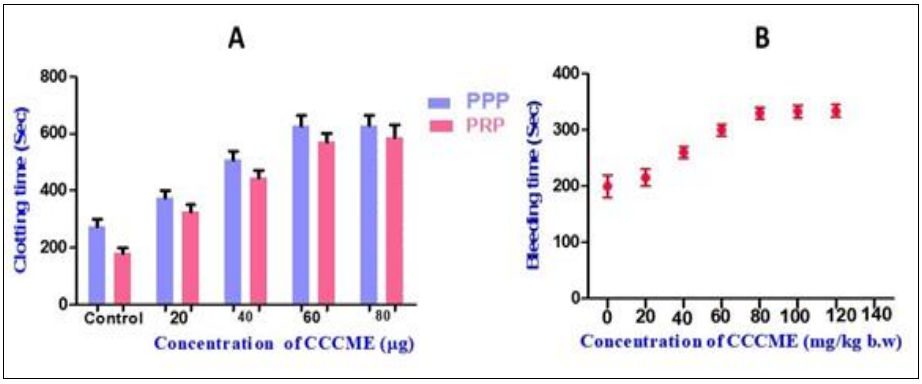

Fig 5: Plasma re-calcification time (A): CCCME (20-80µg) was pre-incubated with 0.2 ml of citrated human plasma PRP/PPP in the presence of 20 µl 10 mM Tris–HCl buffer (pH 7.4) for 1 min at 37° C. 20 µl of 0.25 M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded. Tail bleeding time (B): Tail bleeding time was measured 10min after intravenous administration of PBS or various doses of CCCME. Each point represents the mean ± SD of three independent experiments (P<0.01). CCCME: *Caesalpinia Crista* Coat Methanolic Extract.

Table 1: Anticoagulant activity of CCCME

CCCME (µg)	PT clotting time (S)	PT (INR values)	APTT clotting time (S)	APTT ratio
0	11.00 ± 0.04	0.93 ± 0.01	40.02 ± 0.03	1.41 ± 0.03
20	11.02 ± 0.02	0.96 ± 0.11	47.40 ± 0.02	1.73 ± 0.02
40	11.60 ± 0.04	1.05 ± 0.05	51.05 ± 0.02	1.88 ± 0.13
60	12.01 ± 0.03	1.13 ± 0.02	62.21 ± 0.03	2.21 ± 0.07
80	12.41 ± 0.15	1.18 ± 0.05	70.18 ± 0.09	2.26 ± 0.01
100	11.03 ± 0.06	0.97 ± 0.03	82.20 ± 0.04	2.93 ± 0.04

Discussion

Recently, considerable attention has been paid on the identification of natural plant-derived bioactive substances (e.g., antioxidants) that may be used in clinical routine. *Caesalpinia crista* coat methanol extract has been used in traditional medicine due to its valuable health benefits. Many natural compounds present in the human diet can lower the risk of developing diseases such as cancer, cardiovascular and neurodegenerative disorders [27]. Living organisms are equipped with enzymatic and chemical antioxidative mechanisms for the control of oxidants. Certain amount of oxidative damage takes place even under the normal conditions; however, increased oxidant and decreased antioxidant levels defeat the ability of the antioxidative mechanisms to prevent oxidative damage [28]. Thus, antioxidant property of CCCME was further evaluated using plasma and platelets as an *in-vitro* model of oxidative stress. The plasma and platelets were incubated with free radical generating compound $NaNo_2$ (10mM). $NaNO_2$ is the induction of methemoglobinemia, a condition in which there is a reduction in haemoglobin ability to transport oxygen. Lipid peroxidation is an autocatalytic free radical chain propagating reaction, which is known to be associated with pathological conditions of a cell. Malondialdehyde (MDA) is a 3-carbon, low molecular weight aldehyde produced by free radical-mediated chain of reactions and also MDA is the end product of lipid peroxidation, largely used as marker of lipid peroxidation, this was reported to be higher in cancer tissues than in non-diseased organ [29]. When the plasma and platelet were treated with $NaNo_2$, malondialdehyde and protein carbonyl contents were increased. The treatment with CCCME significantly reduced the plasma and platelets malondialdehyde and protein carbonyl contents. Plasma is easily obtainable from animals and humans and contains both lipid and protein components that may be susceptible to oxidation, making it appropriate to investigate the suitability of plasma oxidation variables as biomarkers of *in vivo* oxidative stress. Oxidation of plasma proteins may be discernible as an increased content of carbonyl (aldehyde or ketone) adducts on the proteins. Measurement of protein carbonyls has also been used to show accumulation of oxidative damage to proteins over the longer term, for example, in studies of aging. Measurement of protein oxidation offers several advantages over the monitoring of lipid peroxidation, including the early formation and relative stability of oxidized proteins [30]. Oxidative modification of protein has been suggested to be not only a marker for oxidative damage but also a causal factor in oxidative injury [31]. Protein carbonyls represent an irreversible form of protein modification and have been demonstrated to be relatively stable (degradation/clearance in hours/days) in contrast to lipid peroxidation products that are removed within minutes [32]. In addition, protein carbonyls are formed early during oxidative stress conditions and are not a result of one specific oxidant, thus they can be called a marker of overall protein oxidation. Several hydrazine derivatives, most commonly 2, 4-dinitrophenylhydrazine (DNPH) introduce to detectable functional groups into the oxidized protein. So, the most often used procedure to detect protein carbonyls is after their derivatization with DNPH [33]. Superoxide dismutase (SOD) protects tissues against oxygen free radicals by catalysing the removal of superoxide radical, converting it into H_2O_2 and molecular oxygen, which both damage the cell membrane and other biological structures [34]. The damage caused by increased generation of $O_2^{\bullet}$ and H_2O_2 arises from their metal dependent conversion into the strongly reactive hydroxyl radical. $O_2^{\bullet}$ is also produced by several other cell types and can be generated from accidents of chemistry during metabolism. Since SOD enzymes generate H_2O_2, this enzyme works in collaboration with catalase and glutathione peroxidases, and the H_2O_2 removes enzymes to protect from cellular damage by hydrogen peroxide [35]. Catalase is a haem-protein, in pathological conditions, which increase the rate of hydrogen peroxide production, will lead to the accumulation of hydrogen peroxide in cytosol and mitochondria. Safe disposal of hydrogen peroxide is carried out by catalase and glutathione peroxidase. The former is located only in peroxisomes, the latter functions in the cytosol and mitochondria. Elevation of hydrogen peroxide results in harmful consequences, such as depletion of ATP,

GSH, NADPH pools and induction of mitochondrial permeability, disrupting mitochondrial membrane potential that trigger the apoptotic pathway [36]. The preincubation of CCCME with plasma and platelets, enhances the activities of SOD and catalase during $NaNo_2$ induced oxidation, The $NaNo_2$ (10mM) significantly decreased the CAT and SOD activities [37]. The present study was also designed to investigate the anticoagulant effect of CCCME by assessing its effect on various coagulation parameters, that means plasma recalcification time, activated partial thromboplastin time, prothrombin time. Coagulation is defence mechanism and a complex process which leads to the blood clot formation. Coagulation is highly conserved throughout biology and in all mammals. Coagulation begins almost instantly after an injury to the blood vessel has damaged the endothelium (lining of the vessel). Medicinal plants have good anticoagulant therapeutic effect. Cardiovascular thrombotic disease results in widespread mortality and hospitalization, which can be successfully reduced through the use of anticoagulant medicines. Clotting is the body's normal response to prevent a person from bleeding to death [38]. However, blood clot formation can be dangerous if it occur within healthy blood vessels, or if not degraded after due time. Many diseases like heart attack, stroke and pulmonary embolism are associated with inappropriate blood clot formation [39]. Coagulants from plant sources that are safe, cost effective and available from indigenous plant resources. Anticoagulant from plants should definitely have better safety margin Such anticoagulant may present with little or no side effects. Our result showed the anticoagulant activity of CCCME by increasing the plasma recalcification time (*in vitro*) and tail bleeding time (*in vivo*). It revealed that anticoagulation activity of CCCME. The coagulation cascade of secondary haemostasis majorly have two pathways, the contact activation pathway formerly called as the intrinsic pathway and other pathway is the tissue factor pathway known as the extrinsic pathway. Finally lead to fibrin stands formation that is common pathway [40]. The results reported here show that while the CCCME have anticlotting property by enhancing the APTT clotting time in a dose dependent manner, the exhibited anticoagulant effect correlating with intrinsic coagulation process. This indicates that the CCCME inhibit a factor or factors in the intrinsic pathway of blood coagulation. CCCME did not show clinical significance property on PT test because the values remained in normal limits.

This indicated that CCCME mainly exhibit anticoagulant activity correlating with the intrinsic coagulation process. The anemia and hypoxia conditions caused by this deleterious events.

Conclusion

In our work, the experiments were designed to assess the anticoagulant and antioxidative activity of the CCCME. CCCME play a significant role in the protection of plasma and platelet against $NaNo_2$ induced oxidative stress by reduced lipid peroxidation and protein oxidation which was associated with an increased activity of SOD and CAT.

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Declaration of Conflict of Interest

The authors declared no potential conflict of interest with respect to the authorship and publication.

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