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Ethanol extract of *Momordica charantia* seed extract (EEMCS) exhibits poor anticoagulant but strong antiplatelet and antioxidant properties

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Abstract
The characterization and evaluation of Ethanol extract of *Momordica charantia seed* (EEMCS) on blood coagulation, platelet function and antioxidant potential was carried out. Phytochemical screening of EEMCS was positive for Polyphenols, saponins, steroids, terpenoids and glycosides. The characterization of EEMCS by TLC and GC/MS adjudged the presence of secondary metabolites. In specific the GCMS data revealed the presence of 16 compounds along with hydrocarbons and eight aldehyde, three esters, three alcohols, one ketone and one terpenic oxide groups. Interestingly, EEMCS was found to be poor anticoagulant because it did not interfere in plasma coagulation in clotting time from control 146s to 160s at 20µg concentration. Furthermore, the effect of EEMCS on platelet aggregation was analyzed using agonists such as collagen, ADP and epinephrine. Interestingly, EEMCS showed 19%, 91% and 97% platelet aggregation inhibition for said agonists in platelet rich plasma. The EEMCS was also examined for its probable antioxidant potential. Oxidative stress was induced using platelet rich plasma using sodium nitrate. To the surprise, EEMCS exhibited strong antioxidant activity that was compared with positive control vitamin C. In addition, EEMCS normalized the levels of stress markers such as, protein carbonyl content, lipid peroxidation, total thiol content, catalase and super oxide dismutase activity in platelet rich plasma. While, EEMCS devoid of RBC lysis, edema and hemorrhagic properties. Suggesting ethanol extract of *Momordica charantia* seed extract exhibits poor anticoagulant but strong antiplatelet and antioxidant properties.

Keywords: secondary metabolite, plasma coagulation, oxidative stress and platelet aggregation

Introduction
Botanicals have been extensively used for medicinal purposes from long ago. Botanicals and their derived products currently available in the market as food supplements [1, 2]. There is an cumulative evidence for the potential of plant-derived compounds to cure, diabetes, cancer, cardiovascular and infectious diseases [3]. Phytochemicals have been considered as suitable candidates in the treatment regime, could be due to safer, eco-friendly, low-cost and fast releafing properties [4]. Although, plants stores plethora of Phytochemicals attributed to therapeutic potential. Bioactive plants secondary metabolites have been extensively explored as therapeutic agents [5, 6]. In plants secondary metabolites are vital to the growth, development and predominately for defense mechanism [4]. Till date thousands of different types of secondary metabolites have been identified in plants [7]. The phytomedicines employed in phytotherapy are usually utilized as an extract (water or alcohol extracts, distillate, or essential oil), which contains dozens or even hundreds of secondary metabolites often from several structural groups [8]. Chemically, these compounds are either nitrogen-containing (alkaloids) or nitrogen-deficient (terpenoids and phenolics) [9, 10]. Several pharmaceutical agents commonly used today are basically derived from the plant compounds. For instance, well known antidiabetic drug metformin an ancient herbal medicine from *Galega officinalis* has been widely used to treat diabetes [11]. *Momordica charantia* or bitter gourd/bitter melon belongs to the family Cucurbitaceae [12]. Bitter gourd is cultivated in the regions of tropics and subtropics [13]. Bitter gourd is the not only store house for high quality Phytochemicals but also known for therapeutic potential. It found to cure cancer, diabetes, infection, inflammation, arthritis and cardiovascular diseases. The key Phytochemicals explored extensively from bitter gourd includes, cucurbitanes -type triterpenoids, charantin, karaviloside IX, momordicoside and it's a glycosides momordicosides A, B, Q, R, and T, and also polypeptide-p, vicine, and the ribosome-inactivating protein momordin [14]. The said chemicals found to exhibits potent antidiabetic property.

Despite, the said therapeutic efficacy the role secondary metabolites of *Momordica charantia* on thrombosis are least explored. Hence, the current study investigates the anticoagulant, antiplatelet and antioxidant properties of Ethanol extract of *Momordica charantia* seed (EEMCS) and results are presented.

Materials and methods
Preparation of Ethanol Extract of *Momordica Charantia* Seed (EEMCS)
Momordica charantia Seed 20g was powdered and mixed with 100ml of 80% ethanol, the seed extract was filtered through Watmann filter paper and supernatant was collected and dried in a hot air oven at 60^0C. The pellets obtained was dissolved in distilled water and stored at 4^0C till further use.

Thin-layer chromatography (TLC)
A preliminary analysis of EEMCS extract was done by TLC. A volume of 50µl of EEMCS was applied on TLC (10x5cm) silica gel plates. The plate was developed by ethyl

acetate: methanol (50:50 v/v) mobile phases. The number of compounds fraction was visualized under UV chamber.

Phytochemical screening of EEMCS

Preliminary phytochemical screening of ethanol extract of *Momordica charantia* seed was performed, according to standard protocols [15]. Presence or absence of alkaloids, flavonoids, polyphenols, saponins, tannins, steroids, terpenoids, glycosides, carbohydrate and lipids were tested based on appearance of colour or precipitation [16].

Phytochemical analysis by GC-MS

Analysis of EEMCS was performed by using a GC-MS (Model; QP 2010 series, Shimadzu, Tokyo, Japan) equipped with a VF-5ms fused silica capillary column of 30m length, 0.25 mm dia. and 0.25µm film thickness. For GC-MS detection, an electron ionization system with ionization energy was used. NIST08s.LIB and WILEY8.LIB library sources were used for matching the identified components from the EEMCS.

Collection of human blood

Blood was drawn from antecubital vein of apparently healthy, non-smoker and nonalcoholic adult volunteers provided with the written consent, as per the guidelines of Institutional Human Ethical Committee (IHEC), University of Mysore, Mysuru. The drawn blood was anticoagulated with acid citrate dextrose (ACD; 85mM sodium citrate; 71mM citric acid; 111mM dextrose) in the ratio 6:1 (blood: ACD, v/v) and centrifuged at 200g for 10min at 37°C, which results in upper platelet rich plasma(PRP) and lower packed RBCs.

Plasma recalcification time

The plasma recalcification time was determined according to the method of [17]. Briefly, the crude EEMCS (0-30µg) was preincubated with 0.2mL of citrated human plasma in the presence of 10mmol/l Tris–HCl (20mL) buffer (pH 7.4) for 1min at 37^0C. To the preincubated mixture, 20mL of 0.25 mol/L $CaCl_2$was added and the clotting time was recorded.

Antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity

The antioxidant activities of EEMCS with Ascorbic acid as positive control were evaluated by using DPPH radical scavenging assay [18]. Briefly, samples using at various concentrations (0-100µg) were taken in test tubes and the final volume was made up to 1mL using methanol. Freshly prepared 1mL of 1.2mM DPPH in methanol solution was added to the tubes containing the samples. The added mixture was incubated for 20min at room temperature in the dark. The absorbance of the resulting mixture was recorded at 517nm against blank and control using UV/Vis spectrophotometer (BioMate 3S, Thermo Scientific, USA).

Determination of oxidative stress marker

Lipid peroxidation

Lipid peroxidation was measured according to the method of [20]. Approximately 0-2mg of protein from lysate of washed platelets treated with an agonist $NaNO_2$ (500µM) and EEMCS (0-200µg/mL) was taken in dry test tubes, 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 2000rpm for 10min. The formed Adducts were extracted into1-butanol (3mL) and the TBARS (Thiobarbituric Acid-Reactive Substance) formed was read photometrically at 532nm and quantified using TMP (1, 1, 3, 3-tetramethoxypropane) as the standard.

Protein carbonyl content

DNPH assay is mainly used to measure the protein carbonyl content, according to the method described by [21], 1mg of protein from a lysate of PRP treated with an agonist $NaNO_2$ (500µM) and EEMCS (0- 200µg/mL) was taken and an equal volume of 10mM DNPH in 2N HCl was added, incubated for 1hr shaking alternatingly at room temperature. The corresponding blank was carried out by adding only 2N HCl to the sample. After incubation, the mixture was precipitated with 20% Trichloroacetate (TCA) and centrifuged at 5000rpm, for 15min. The precipitate was washed twice with acetone by centrifuging at 10000rpm, 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. The difference in absorbance is determined and expressed as µm of carbonyl groups/mg protein, using molar extinction coefficient of $22mM^{-1}cm^{-1}$.

Determination of total thiols

To determine the total thiols,the method of [22] was used, the platelets sample (0.05mg protein) was added to 0.375mL of Tris–HCl buffer (0.2 M, pH 8.2) containing di-thio-bis-nitrobenzoic acid (DTNB, 10mM) and 1.975mL of methanol. Subsequent incubation for 30 min at room temperature the tubes were centrifuged at 3,000g for 10 min. The absorbance of the supernatant was measured at 410nm and expressed as nmol of DTNB oxidized/mg protein.

Protein carbonyl content

The protein carbonyl content was measured using DNPH, according to the method described by [21]. 1mg of protein from lysate of PRP treated with an agonist *p*-TBC(50mM), and TSE (50-250 µg/mL), was taken and an equal volume of 10mM DNPH in 2N HCl was added, incubated for 1hr shaking intermittently at room temperature. Corresponding blank was carried out by adding only 2N HCl to the sample. After incubation, the mixture was precipitated with 20% Trichloroacetate (TCA) and centrifuged at 5000rpm, for 15min. The precipitate was washed twice with acetone by centrifuging at 10,000rpm, for 15min and finally dissolved in 1ml of Trisbuffer (20 mM pH 7.4 containing 0.14 M NaCl, 2% SDS) and the supernatant was recorded at 360nm. The difference in absorbance is determined and expressed as ηmols of carbonyl groups/ mg protein, using molar extinction coefficient of 22 $mM^{-1}cm^{-1}$.

Antioxidant enzymes Activities

Superoxide dismutase (SOD)

The assay of SOD was based on the reduction of nitro blue tetrazolium (NBT) to water in-soluble blue formazone. In 0.5mL homogenate 1mL of 50mM sodium carbonate 0.4ml of 24mM NBT and 0.2mL of 0.1mM EDTA were added. The reaction was initiated by adding 0.4mL of 1mM hydroxylamine hydrochloride. Zero-time absorbance was taken at 560nm followed by recording the absorbance after

5min at 25^0c. The control was simultaneously run without homogenate. Units of SOD were expressed as the amount of enzyme required to inhibit the reaction by 50%. The specific activity was expressed as units per mg protein.

Determination of catalase activity

Catalase activity was determined using a spectrophotometric method by measuring the rate of hydrolysis of H2O2 [23]. Briefly, 100µl of the EEMCS was mixed with reaction mixture and incubated with 1000µL of 8.8mM H2O2 in 6.0mM sodium phosphate buffer, pH 7.4 at 37^0C for 5min. The decrease in absorbance was monitored at 240nm and the activity was expressed as µmol H2O2 decomposed/min/ mg protein (€-43.6/mM/cm).

Preparation of platelet rich plasma (PRP) and platelet poor plasma

Ardlie *et al*. method was employed. Nine volumes of human blood from healthy donors (who were non-smokers and non-medicated at least for the previous 15days) in to one volume of acid citrate dextrose (93mM sodium citrate, 7mM citric acid and 140mM glucose pH 6.5) followed by centrifugation at 90g for 10min at room temperature. The supernatant was called platelet rich plasma (PRP). The remaining blood was centrifuged at 500g for 15min and the supernatant obtained was the platelet poor plasma (PPP). The platelet concentration of PRP was adjusted to $3.1x10^8$ platelets/ml with PPP. The PRP maintained at 37^0C was used within 2hr. All the above preparations were carried out using plastic (polypropylene tubes) wares or siliconized glassware.

Platelet aggregation

The turbid metric method [24] was followed using a Chronology dual channel whole blood/optical lumi aggregation system (Model-700).Aliquots of PRP (0.45ml) was pre-incubated with EEMCS(0-75µg) for 3min in a cylindrical glass cuvette under constant stirring. . The aggregation was initiated independently by the addition of agonists, such as Collagen, ADP and epinephrine and followed for 6min. along with aliquots of PPP (0.45mL). As platelets aggregate in response to an added agonist, light transmission decreases progressively producing an aggregation trace on the recorder. The aggregation trace was the plot of light transmission between platelet rich plasma (PRP) and platelet poor plasma (PPP) base line, which represent 0%and 100% aggregation respectively.

Indirect hemolytic activity

Indirect hemolytic activity was determined [26], using washed human erythrocytes. Briefly, packed human erythrocytes, egg yolk and PBS (1: 1: 8, v/v) were mixed; 1mL of this suspension was incubated independently with the various concentrations of EEMCS (0–250µg) for 1hr at 37^0C. The reaction was stopped by adding 9mL of ice-cold PBS and centrifuged at 1000g for 10min at 4^0C. The amount of hemoglobin released in the supernatant was measured at 540nm. Activity was expressed as percentage of hemolysis against 100% lysis of cells due to addition of water that served as a positive control, and PBS served as a negative control.

Edema-inducing activity

The procedure of Sannanaik *et al*., (26) was followed. Groups of five mice were injected separately into the right foot pads with different doses of EEMCS (0-200µg) in 20mL saline. The left foot pads received 20mL saline alone served as controls. After 1hr, the mice were anesthetized by diethyl ether inhalation. The hind limbs were removed at the ankle joint and weighed. Weight increased was calculated as the edema ratio, which equals the weight of edematous leg x100/weight of normal leg. Minimum edema dose (MED) was defined as the amount of protein required to cause an edema ratio of 120%.

Hemorrhagic activity

Hemorrhagic activity was assayed as described by Kondo [27]. A different concentration of EEMCS (0-200µg) was injected (intra dermal) independently into the groups of five mice in 30mL saline. The group receiving saline alone served as a negative control and the group receiving venom minimum hemorrhagic dose (MHD)] as a positive control. After 3hr, the mice were anesthetized by diethyl ether inhalation. A dorsal patch of the skin surface was carefully removed and observed for hemorrhage against saline-injected control mice. The diameter of the hemorrhagic spot on the inner surface of the skin was measured. MHD was defined as the amount of the protein producing 10mm of hemorrhage in diameter.

Statistical analysis

The data are presented as mean-SD. Statistical analyses were performed by Student's t test. A significant difference between the groups was considered if P value was less than 0.01.

Results

EEMCS found to contain terpenoids, steroids, phenols and saponins

The qualitative tests for EEMCS revealed the presence of terpenoids, steroids, phenols and saponins (Table.1).The TLC chromatogram of EEMCS showed five banding pattern (Figure 1). However, the GC-MS chromatogram showed 16 peaks with varied retention time, out of which four major peaks were major and rest of them were minor. Furthermore, an interpretation of mass spectrum of GC-MS was conducted using the database of NIST (National Institute of Standards and Technology) and WILEY libraries (Figure 2). According to the said experimental data the key compounds present were 1-Butanol,2-methyl, acetate (1.77%),2-Heptenal(1.71%),1-octen-3-one(2.31%), 2-Undecene, 9-methyl (1.14%), 2-Octenal(1.04%) and Nonanal (1.06%)(Figure 3).

EEMCS devoid of anticoagulant property

EEMCS did not interfere in plasma recalcification time even at the increased concentration (Figure 4**)**. It could able cause negligible amount of anticoagulation as it increased the clotting time from the control 118s to 122s even at the concentrations of 30µg and remains unaltered after this dose.

Antioxidant activity

EEMCS was subjected to free radical scavenging activity.

Interestingly, it significantly reduced DPPH radicals in a dose-dependent manner (0-30µg). There was considerable increase in the antioxidant potential of the EEMCS noticed against the positive control ascorbic acid, suggests antioxidant property of EEMCS (Figure 5**).**

EEMCS normalized NaNO2 induced Oxidative damage in platelet rich plasma

Lipid peroxidation (LP) protein carbonylation (PCC) and total thiol content (TT) are the key indicators of cells oxidative stress. Hence, an extent of lipid peroxidation and protein carbonylation and total thiol content was measured as a marker of oxidative stress. As the mark of lipid peroxidation the level of malondialdehyde (MDA) was measured. There was a remarkable increase in the level of MDA was noticed in$NaNO_2$treated mice plasma sample. While in the other hand, oxidative stress induced plasma cells pretreated with EEMCS, there was a significant decrease in the lipid peroxidation level was observed It was compared with EEMCS alone group, EEMCS alone didn't show any significant alteration in plasma cells (Figure 6). In addition, there was a remarkable elevation of protein carbonylation in the $NaNO_2$ treated plasma cells was observed that was compared with an untreated mice plasma sample. But in case of EEMCS pretreated mice group the level of PCC was significantly reached the normal level 60U. In case of EEMCS alone treated group of mice plasma sample unaltered level of protein carbonylation was noticed (Figure 7). Furthermore, effect of EEMCS on total thiols (TT) content was also measured. In case of $NaNO_2$ treated mice plasma sample group there was a significant increase in the thiol content was noticed. Whereas, in case of EEMCS pre-treated plasma cells there was a significant inhibition of thiols content was observed 80U. While in the EEMCS alone treated plasma cells there was no alteration in the level of total thiol content (Figure 8).

EEMCS normalized the antioxidant enzymes level in stress induced plasma cells

To reinforce the observed antioxidant property of EEMCS, its ability to normalize the antioxidant enzyme level was examined. Curiously, EEMCS normalized the level of catalase (Figure 9) and Super Oxide Dismutase (SOD) level in stress induced plasma cells (Figure 10). In case of $NaNO_2$ treated plasma cells there was increased level of catalase and SOD activity was identified. Whereas in case of EEMCS-treated plasma cells there was absolute normalization of catalase and SOD was observed. While, EEMCS alone did not alter the SOD and catalase activities in plasma cells.

Effect of EEMCS on platelet aggregation

Furthermore, EEMCS was examined for anti-platelet activity using agonists such as Collagen, ADP and epinephrine with platelet-rich plasma (PRP). EEMCS inhibited the collagen, ADP and epinephrine induced platelet aggregation of PRP (Figures 11, 12 and 13). The percentage of platelet aggregation inhibition potency of EEMCS was found to be 19%, 91% and 97% for the said agonists respectively. Although, EEMCS showed strong antiplatelet potential for ADP and epinephrine it showed mild antiplatelet activity for collagen (19%).

Non-toxic properties of EEMCS

In order to study the non-toxic effect of EEMCS, hemolytic and hemorrhage assays and edema inducing activities were analyzed. Interestingly, it did not hydrolyze RBC cells (Figure 14), unable to cause hemorrhagic and edematic effect in experimental animals. Whereas, positive control *Daboia russelli* venom induced hemorrhage and edema in experimental mice (Figure 15).

Discussion

Usage of plant based medicines for curing life threatening deadly disease is a prehistoric practice. The key reasons could be due to their strong therapeutic efficacy with least side effects. Although, whole plant extract has been used in the treatment regime. Several compounds/secondary metabolites/proteins/lipids/carbohydrates have been well characterized. Momordica Charantia seed stores huge amount of phytoconstituents responsible for curing cancer, diabetes and other infectious diseases [28, 29]. Despite its immense therapeutic applications the role of secondary metabolites of Momordica *Charantia* seed on thrombotic disorders was not explored. Hence, the present study deals with characterization of Ethanol extract of *Momordica Charantia* seed (EEMCS) and its role in thrombotic disorders. EEMCS was positive for Polyphenols, saponins, steroids, terpenoids and glycosides. The characterization of EEMCS by TLC, HPLC and GC/MS adjudged the presence of secondary metabolites. In specific the GCMS data reveals the presence of 16compounds along with hydrocarbons and eight aldehydes, three esters, three alcohols, one ketone and one terpenic oxide groups. *Momordica charantia* seed ethanol extract showed reproductive toxicity [30], Antileukemic Potential [31], Anti-hyperlipidemic [32], antidiabetic activity [33], antimicrobial activity [34]. Numerous investigation showed isolated bioactive components from plants different parts exhibits anti-inflammatory, antimicrobial and contraceptive properties (35,36).EEMCS did not interfere in plasma coagulation of platelet rich plasma as compared with the control, the reason could be the active compounds in ethanolic extract namely terpenoids, flavonoids and phenolics did not interact with either intrinsic/extrinsic/common pathway coagulation factors, thus poor anti-coagulation was noticed [37].United states Food and Drug Administration (UFDA) approved Natural and synthetic food additives are generally used to maintain or improve safety, the nutrient value, taste and texture of food [38]. One such food additive issodium nitrite ($NaNO_2$),it is an inorganic salt used in the manufacture of dyes, antimicrobial, flavoring, coloring and preservative agent in meat and fish product [39].In addition, it also used in pharma industries and in medicine as antidote for cyanide poisoning [40]. The hazardous effect of $NaNO_2$ derives generation of free radicals from the reaction of nitrites with amines to produce nitrosamines, and with amides to produce nitrosamides [41]. In mammals the toxic effects of nitrates and nitrites ions are well reported. They found to cause abnormality in reproductive function [42], hepatotoxicity, methemoglobenemia, growth retardation, tissue injury, dysregulation of inflammatory responses and endocrine disturbance [43, 44, 45, 46]. Overall harmful effect is due to the oxidative stress induced by the free radical generated from $NaNO_2$. Hence in this study an effort was also made on the potency of EEMCS on $NaNO_2$ induced oxidative stress. Platelet rich plasma containing large number of platelets and poor in other plasma cells such as leukocytes and growth factors [47]. Uncontrolled oxidative stress elevates the rate of platelet activation and aggregation which leads to pathological conditions such as Cardiovascular, thrombotic disorder, inflammatory disorder and complications associated with diabetes [48].In this connection oxidative

stress markers and antioxidant enzymes level were includes the protein oxidation, lipid peroxidation, total thiol status, SOD and catalase was considered [49, 50]. Surprisingly the current study revealed, improved antioxidant mechanism in all the parameters.

The relative improvement may be due to antioxidant activity of bioactive components of EEMCS. Several agonists (collagen, epinephrine, ADP, Arachidonic acid, serotonin, norepinephrine, thromboxane A2 and thrombin) activate platelets up on vascular injury [51, 52]. Activated platelets recruit additional platelets to the growing hemostatic plug by several feedbacks amplification loops by releasing platelet agonists such as ADP and thrombin stored in the α-granules under physiological condition [53]. However, Unregulated way of platelet activation also plays a key role in the pathophysiology of thrombotic disorders [54]. Thus, targeting platelet activation inhibition favors least level of unwanted clot formation in the arteries and veins. Hence, EEMCS was evaluated for platelet aggregation inhibition activity.

Although, EEMCS showed strong antiplatelet potential for ADP and epinephrine it showed mild antiplatelet activity for collagen. The mechanism of observed antiplatelet activity of EEMCS yet to be identified. Eptifibatide, derived from rattle snake venom that inhibits glycoprotein IIb/IIIa receptor on platelets is currently being used in the treatment of thrombotic disorders [55]. In addition, irreversible cyclooxygenase inhibitors (Aspirin and Triflusal), Adenosine diphosphate (ADP) receptor inhibitors (Clopidogrel, Prasugrel, Ticagrelor, Ticlopidine), Phosphodiesterase inhibitors (Cilostazol, Dipyridamole), Protease-activated receptor-1(PAR-1), antagonists (Vorapaxar)and Glycoprotein IIB/IIIA inhibitors (Abciximab, Eptifibatide and Tirofiban) and thrombolytic drugs (Alteplase, Reteplase, Tenecteplase, Anistreplase, Streptokinase, Urokinase [56, 51]. Ethanolic extracts of leaves of *Acheranthusaspera* whole plant of *Tridaxprocumbens* [52, 57], Phenanthrenes and flavonoids from leaves of *Calantheari sanensis*, latex of *Artocarpu sheterophyllus* [58, 59] found to exhibit anti platelet activity. In addition EEMCS did not cause RBC lysis, edema and hemorrhagic activities suggesting their nontoxic property.

Conclusion

In conclusion, this study for the first time attempted to identifies anticoagulant, antioxidant and antiplatelet properties of EEMCS. It exhibited strong antioxidant, anti platelet activities with poor anticoagulation. Thus, purification and characterization could help in the better understanding of the identified secondary metabolites.

Acknowledgements

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Conflicts of interest

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

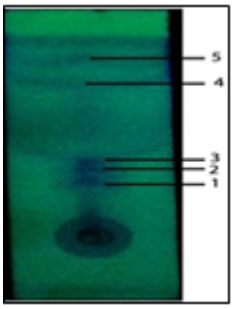

Fig 1: Represents the TLC chromatogram of EEMCS.

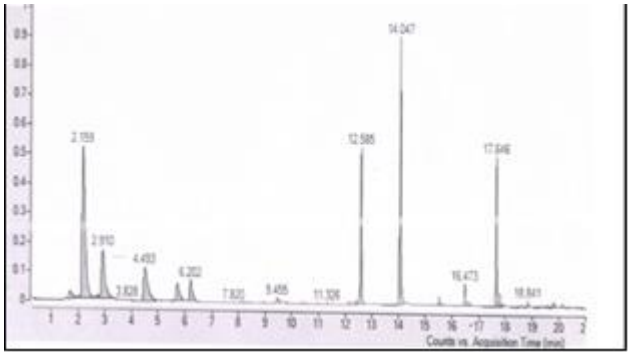

Fig 2: Represents GC/MS chromatogram of EEMCS

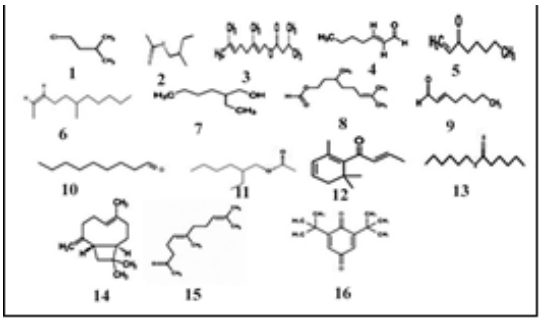

Fig 3: Represents the bioactive components identified in EEMCS using GC/MS: 1;Butane,1-chloro-3-methyl, 2-1-Butanol, 2;methyl-acetate,3;Geranylisovalerate, 4;2-Heptenal, 5; 1-octen-3-one, 6; 2-Undecene,9-methyl, 7; 1-Hexanol,2-ethyl, 8; Citronellylformate, 9; 2-Octenal, 10;Nonanal, 11; Acetic acid,2-ethylhexyl ester, 12;Damascenone, 13;Hexanoic acid, hexyl ester, 14; Caryophyllene, 15; Trans –Geranylacetone, 16; 2,6-Di-tert-butylquinone.

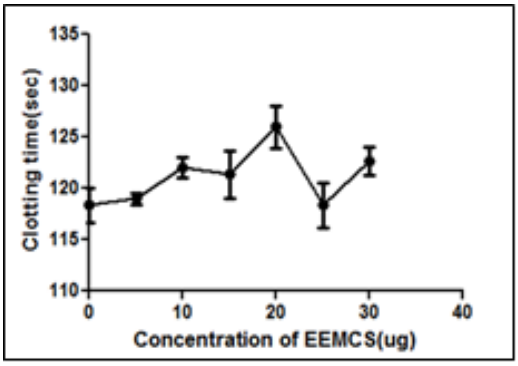

Fig 4: Plasma re-calcification time: (A) EEMCS(0-30µg) was pre-incubated with 0.2mL of citrated human plasma in the presence of 20µL 10 Mm Tris–HCl buffer pH 7.4 for 1min at 37 ^{0}C. 20µL of 0.25 M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded

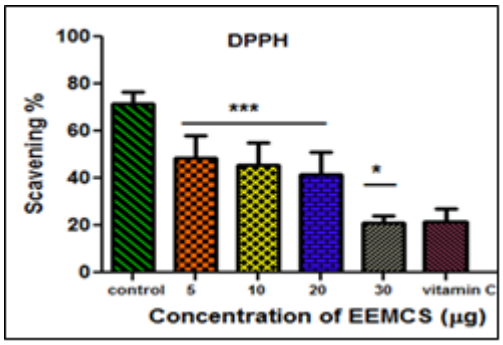

Fig 5: DPPH Radical Scavenging Activity: Dose dependent DPPH scavenging ability of EEMCS (0-30µg).

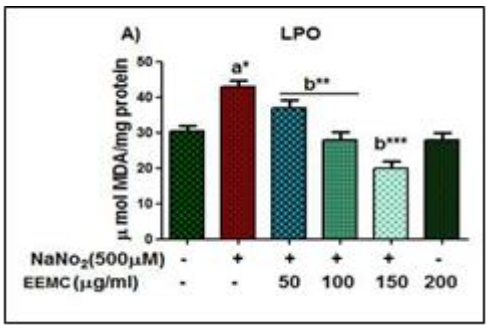

Fig 6: Effect of EEMCS on lipid peroxidation. Malondialdehyde (MDA);a* -oxidative stress inducer $NaNo_2$ treated, before,a* without treatment and sample,from b** oxidative stress inducer and EEMCS (50-200µg) with different concentrations . Each value represents the mean ± S.D. (n=3). *Significantly reduced stress in treated PRP when compared to $NaNo_2$.

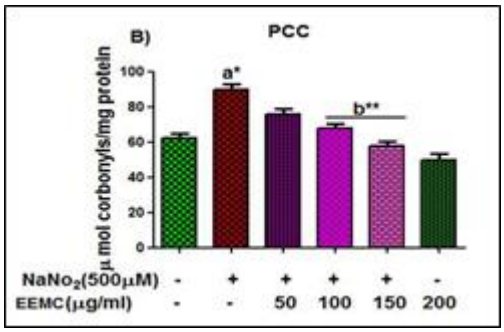

Fig 7: Effect of EEMCS on Protein Carbonyl Content (PCC): Each value represents the mean ± S.D. (n=3) and values different letters are significantly different in presence of stress inducer $NaNo_2$. And along with sample EEMCS treated ($p < 0.05$).

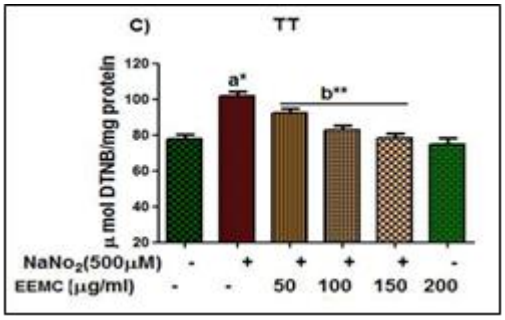

Fig 8: Effect of EEMCS on Total thiol (TT) content. Each value represents the mean ± S.D. (n=3) and values with different letters are significantly different in presence of stress inducer $NaNo_2$. And along with sample EEMCS treated ($p < 0.05$).

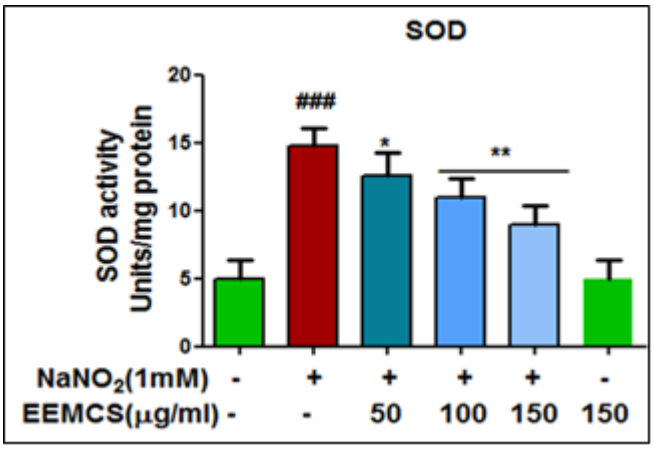

Fig 9: Superoxide radicals scavenging activity of EEMCS: superoxide dismutase (SOD) in control, $NaNO_2$ stress and EEMCS (0-150µg) treated PRP. One unit (U) of enzyme activity was expressed as 50% inhibition of NBT reduction/min/mg protein. Each value represents the mean ± S.D. (n=3).

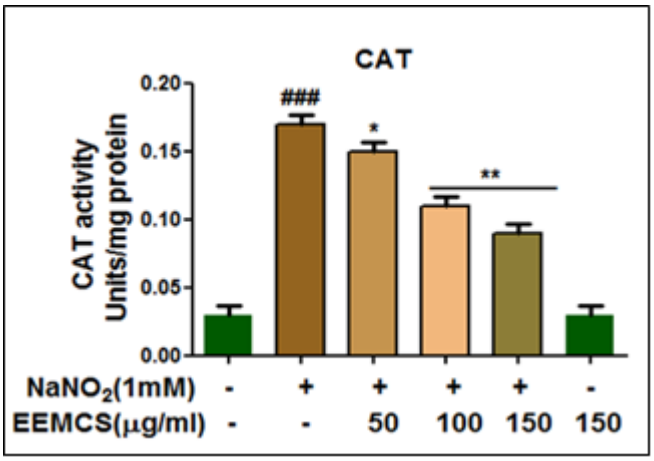

Fig 10: Peroxide radicals scavenging activity of EEMCS: Catalase (CAT) in control, $NaNO_2$ stress and EEMCS (0-150µg) treated PRP. The enzyme activity (U) was expressed as ×10-3 k/sec/mg protein. Each value represents the mean ± S.D. (n=3).

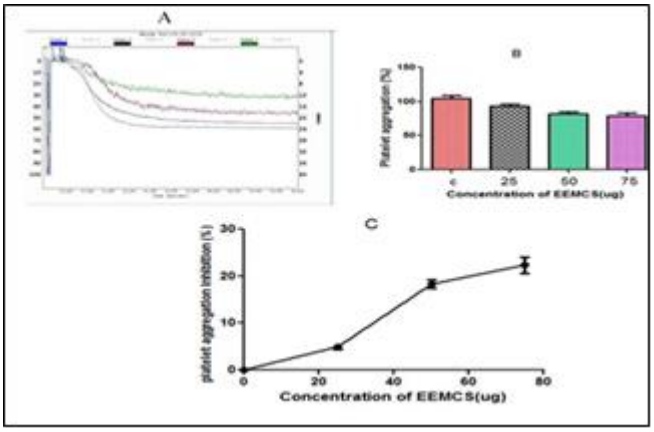

Fig 11: Platelet aggregation was initiated by adding collagen as an agonist using PRP. Platelet Aggregation was initiated by adding collagen as an agonist. A: Aggregation trace of EEMCS Trace 1 (collagen 10µM); Trace 2 (ADP 10µM +25µg EEMCS); Trace 3 (collagen 10µM +50µg of EEMCS); Trace 4 (collagen 10µM +75µg of EEMCS), The values represent ± SD of three independent experiments, B: Dose dependent platelet aggregation. C: Platelet aggregation inhibition %.

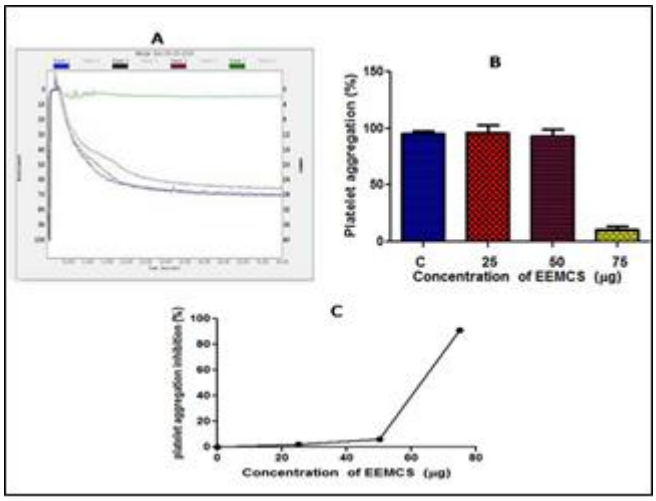

Fig 12: Platelet aggregations was initiated by adding ADP as an agonist using PRP. Platelet Aggregation was initiated by adding ADP as an agonist. A: Aggregation trace of EEMCS Trace 1 (ADP 10µM); Trace 2 (ADP 10µM +25µg EEMCS); Trace 3 (ADP 10µM +50µg of EEMCS); Trace 4 (ADP 10µM +75µg of EEMCS), The values represent ± SD of three independent experiments, B: Dose dependent platelet aggregation. C: Platelet aggregation inhibition %.

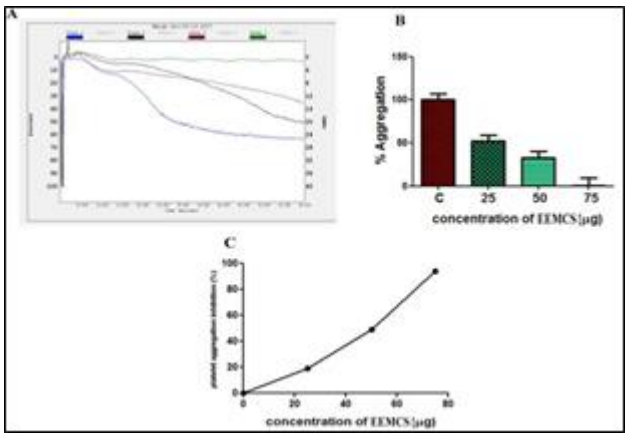

Fig 13: Platelet aggregation was initiated by adding Epinephrine as an agonist using PRP. Platelet aggregation was initiated by adding Epinephrine as an agonist. A: Aggregation trace of EEMCS Trace1 (Epinephrine 5µM); Trace 2 (Epinephrine 5µM +25µg EEMCS); Trace 3 (Epinephrine 5µM +50µg of EEMCS); Trace 4 (Epinephrine 5µM +75µg of EEMCS), The values represent ± SD of three independent experiments, B: Dose dependent platelet aggregation. C: Platelet aggregation inhibition %.

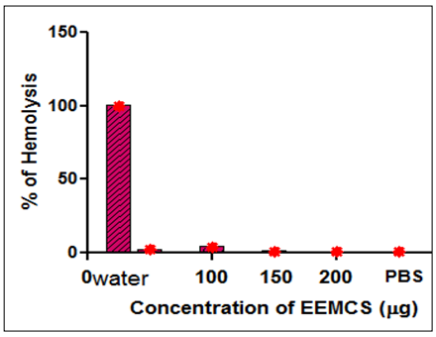

Fig 14: Indirect Hemolytic assay. Packed human erythrocytes and phosphate buffered saline (PBS) incubated with different concentration of EEMCS (0-200µg) independently for 1hr at 37°C. +ve control: Water, –ve control: PBS for % of hemolysis. The amount of hemoglobin released in the supernatant was measured at 540nm.

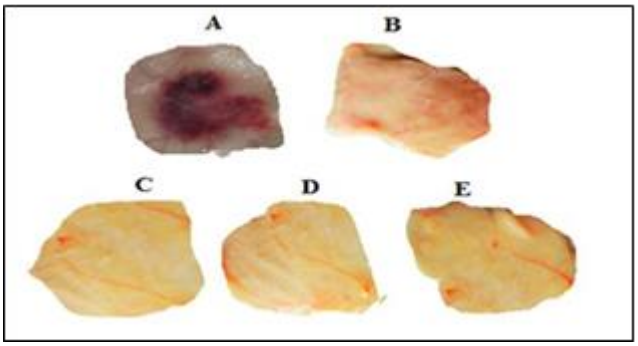

Fig 15: Dose dependent hemorrhagic activities of EEMCS. A: positive control 2 MDH venom, B: saline, C: 50µg, D: 100µg and E: 200µgof EEMCS was injected independently in to mice in a total volume of 50µL intradermally.

Table 1: Phytochemical screening of ethanol extract of *Momordica charantia* seed (EEMCS).

Sl. NO	Test	Result
1	Carbohydrates	-ve
2	Proteins	-ve
3	Lipids	-ve
4	Terpenoids	+ve
5	Alkaloids	-ve
6	Tannins	-ve
7	Steroids	+ve
8	Phenols	+ve
9	Saponins	+ve
10	Glycoside	+ve

Table 2: Bioactive components identified in EEMCS using GC/MS.

Sl.No	Retention time	Area	Area%	Compounds Names
1	4.163	61472.24	0.36	Butane,1-chloro-3-methyl
2	8.571	300830.74	1.77	1-Butanol,2-methyl-,acetate
3	11.106	10964.39	0.06	Geranylisovalerate
4	11.493	290287.17	1.71	2-Heptenal
5	12.227	392556.54	2.31	1-octen-3-one
6	12.366	194614.35	1.14	2-Undecene,9-methyl
7	14.058	149063.28	0.88	1-Hexanol,2-ethyl
8	14.832	58351.64	0.34	Citronellylformate
9	15.069	1796894.26	1.04	2-Octenal
10	16.6	179664.17	1.06	Nonanal
11	17.946	5553.65	0.03	Acetic acid,2-ethylhexyl ester
12	25.368	84290.3	0.5	Damascenone
13	25.553	11872.34	0.07	Hexanoicacid,hexyl ester
14	26.264	33297.01	0.02	Caryophyllene
15	26.778	64577.46	0.38	Trans –Geranylacetone
16	27.021	37093.89	0.22	2,6-Di-tert-butylquinone

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