Research Article

COMPARATIVE EVALUATION OF AQUEOUS AND ETHANOL EXTRACTS OF *MOMORDICA CHARANTIA* SEED ON COAGULATION CASCADE AND PLATELET FUNCTION

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ABSTRACT

The present work demonstrates on the comparative studies on Aqueous and Ethanol Extracts of *Momordica charantia* (bitter gourd) Seed on plasma re-calcification time and platelet function. Anticoagulant effect of Aqueous and Ethanol Extracts of *Momordica charantia* was screened for tests such as plasma recalcification time, platelet aggregation studies and non-toxic property was tested by edema, hemorrhage and indirect hemolytic activities. Aqueous Extract seed (AEMCS) showed strong anticoagulant effect by enhancing the plasma clotting time from control 146s to 432s, while, Ethanol Extract of *Momordica charantia* Seed (EEMCS) showed weak anticoagulant effect as it enhanced the clotting time from control 146s to 160s. Furthermore, both extracts were analyzed for agonists such as, Collagen, Adenosine diphosphate (ADP) and epinephrine induced platelet aggregation. Curiously, both the extracts were inhibited agonist induced platelet aggregation of Platelet rich plasma (PRP). The percentage of platelet aggregation inhibition for AEMCS was found to be 65%, 50% and 85% respectively for collagen, ADP and epinephrine induced platelet aggregation. However, 19%, 91% and 97% platelet aggregation inhibition for EEMCS on said agonists. In addition, AEMCS and EEMCS both devoid of RBC lysis, edema and hemorrhagic properties, suggesting its nontoxic nature. In conclusion; both aqueous and ethanol extract of *Momordica charantia* found to exhibit anticoagulant and antiplatelet activities; while, AEMCS showed strong anticoagulant potential with moderate antiplatelet efficiency. On the other hand, EEMCS exhibited strong antiplatelet activity but poor anticoagulation. Hence, it could be promising agent in the management of thrombotic disorders.

Keywords: *Momordica charantia* (bitter gourd) seed extracts, anticoagulation, platelet aggregation, Platelet rich plasma (PRP), collagen, ADP, epinephrine.

INTRODUCTION

Hemostasis is a physiological mechanism that drives immediate reaction to the vascular injury that accompanied by the activation of clotting factors and thrombocytes results in the formation of the clot/thrombus to prevent the blood loss¹. It composed of four major events such as, Vasoconstriction, activation of platelet (aggregation), formation of fibrin clot and fibrinolysis/dissolution of the clot². Blood coagulation cascade consists of a series of zymogens that can be converted by limited proteolysis leading to the generation of thrombin³ along with the activated platelets. Hence, coagulation cascade is a highly regulated physiological phenomenon. In other hand hyper activation of said mechanism results in the formation of unusual clot in the arteries and veins leads to thrombotic disorders. It mainly includes arterial thrombosis, atrial fibrillation, myocardial infarction/heart attack, unstable angina, deep vein thrombosis, pulmonary embolism and cerebral stroke⁵. Mortality and morbidity rate have been increasing worldwide due to thrombosis. Currently, several anticoagulant and antiplatelet agents have been used extensively to treat thrombosis; while life threatening side effects triggered by them limits their usage. Thus, in recent times, the natural products have been gaining much importance because of their least side effects. The several plant based drugs have been using to treat numerous medical emergencies. Bitter gourd one such *Momordica charantia* is an important source for several therapeutic efficiency, it is a store house for rich quantity of

macro, micro nutrients and secondary metabolites attributed to immense therapeutic applications for instance, it found to exhibit anticancer, antihelmintic, anti malarial, anti viral and cardio protective activities⁶⁻⁹. Recently, studies by Bhagyalakshmi Manjappa, *et al.*, 2014 reported on the anticoagulant and fibrinolytic effect of serine/metalloproteolytic activity from *Momordica charantia* seed extract¹⁰. However, the role of *Momordica charantia* extract on platelet function was least explored. Hence, the current study describes comparative effect of aqueous and ethanol extract of *Momordica charantia* seed on blood coagulation and platelet function.

MATERIALS AND METHODS

ADP type-I, ADP, epinephrine, collagen were purchased from Sigma Chemicals Company, St. Louis, USA. UNIPLASTIN, LIQUICELIN-E and FIBROQUANT were purchased from Tulip Diagnostics Pvt. Ltd., Goa, India. All other chemicals and reagents used were analytical grade. Human blood was collected from healthy adult volunteers with transcribed informed consent according to the procedures of Institutional Human Ethical Committee, University of Mysore, and Mysore.

All the experimentations were conducted in accordance with the ethical guidelines and were approved by the Institutional Human Ethical Committee. Conducting animal experiments were permitted by the Institutional Animal Ethical Committee

(UOM/IAEC/02/2016), Swiss Wister albino mice weighing 20–25 g from the central animal house facility, Department of Studies in Zoology, University of Mysore, and Mysore, India. The animal handlings were preceded in accordance with the guidelines of the Committee for the Purpose of monitoring and Supervision of Experiments on Animals (CPCSEA).

Preparation of aqueous extract of *Momordica charantia* seed (AEMCS) and protein estimation

Momordica charantia (bitter gourd) fruits were purchased from the local market of Tumkur and the seeds were collected from the fruit. The outer coat was removed from the seeds thoroughly chopped, homogenized using double distilled water and centrifuged at 5000 g for 20 min at 14°C. The sample obtained was stored at -20°C until it was used for further studies. Protein concentration was determined as described by Lowry, *et al.* (1951)¹¹ using bovine serum albumin (BSA) as standards.

Preparation of ethanol extracts of *Momordica charantia* seed (EEMCS)

Momordica charantia Seed 20 g was powdered and mixed with 100 ml of 80 % ethanol, the seed extract was filtered through Whatman filter paper and supernatant was collected and dried in a hot air oven at 60°C. The pellets obtained was dissolved in distilled water and stored at 4°C till further use.

Plasma recalcification time

The plasma recalcification time was determined according to the method of Quick, *et al.*, (1935)¹². Briefly, the Aqueous AEMCS and EEMCS (0–30 µg) was pre incubated with 0.2 ml of citrated human plasma in the presence of 10 mmol/l Tris-HCl (20 µl) buffer (pH 7.4) for 1 min at 37°C. To the pre incubated mixture, 20 µl of 0.25 mol/l CaCl₂ was added and the clotting time was recorded.

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

The method of Ardlie, *et al.* (1974)¹³ was employed. Nine volumes of human blood from healthy donors (who were non-smokers and non-medicated at least for the previous 15 days) in to one volume of acid citrate dextrose (93 mM sodium citrate, 7 mM citric acid and 140 mM glucose pH 6.5) followed by centrifugation at 90 g for 10 min at room temperature. The supernatant was called platelet rich plasma (PRP). The remaining blood was centrifuged at 500 g for 15 min and the supernatant obtained was the platelet poor plasma (PPP). The platelet concentration of PRP was adjusted to 3.1 x 10⁸ platelets/ml with PPP and maintained at 37°C was used within 2 h. All the above preparations were carried out using plastic (polypropylene tubes) wares or siliconized glassware.

Platelet aggregation

The turbid metric method of Born, (1962)¹⁴ was followed using a Chronology dual channel whole blood/optical lumi aggregation system (Model-700). Aliquots of PRP (0.45 ml) was pre-incubated with the AEMCS (0–75 µg) and EEMCS (0–75 µg) for

3 min 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 6 min. along with aliquots of PPP (0.45 ml). 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 according to the method of Boman, *et al.* (1957)¹⁵ using washed human erythrocytes. Briefly, packed human erythrocytes, egg yolk and PBS (1 : 1 : 8, v/v) were mixed; 1 ml of this was incubated independently with the various concentrations of AEMCS (0–250 µg) and EEMCS ((0–250 µg)) for 1 h at 37°C. The reaction was stopped by adding 9 ml of ice-cold PBS and centrifuged at 1000 g for 10 min at 4°C. The amount of hemoglobin released in the supernatant was measured at 540 nm. 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.*, (1987)¹⁶ was followed. Groups of five mice were injected separately into the right foot pads with different doses (0–200 µg) of AEMCS and EEMCS in 20 µl saline. The left foot pads received 20 ml saline alone served as controls. After 1 h, 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 x 100/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, *et al.*, (1969)¹⁷. A different concentration of AEMCS and EEMCS (0–200 µg) was injected (intra dermal) independently into the groups of five mice in 30 ml 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 3 h, the mice were anesthetized by diethyl ether inhalation and 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 10 mm 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.

Figure 1: Effect of AEMCS and EEMCS on plasma re calcification time

A: *Momordica charantia* (bitter gourd) seed. **B:** EEMCS and AEMCS both (0–30 µg) was pre incubated with 0.2 µl of citrated human plasma in the presence of 20 µl 10 µmol/lTris–HCl buffers (pH 7.4) for 1 min at 37°C. Twenty microliter of 0.25 mol/lCaCl₂ was added to the preincubated mixture and clotting time was recorded.

Figure 2: Effect of AEMCS and EEMCS on collagen induced platelet aggregation

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

Figure 3: Effect of AEMCS and EEMCS on ADP induced platelet aggregation.

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

Figure 4: Effect of AEMCS and EEMCS on Epinephrine induced platelet aggregation

Platelet Aggregation was initiated by adding Epinephrine as an agonist. **A and B: Aggregation trace of AEMCS and EEMCS** Trace 1 (Epinephrine 10 µM); Trace 2 (Epinephrine 10 µM + 25 µg sample); Trace 3 (Epinephrine 10 µM + 50 µg of sample); Trace 4 (Epinephrine 10 µM + 75 µg of sample), The values represent $\pm$ SD of three independent experiments, **C: Dose dependent platelet aggregation** **D: Platelet aggregation inhibition %**

Figure 5: Comparison of platelet aggregation inhibition efficiency of AEMCS and EEMCS.

C: Control, Coll: Collagen, ADP, EPI: Epinephrine. The values represent $\pm$ SD of three independent experiments.

Figure 6: Dose-dependent hemorrhagic activity of AEMCS and EEMCS

A and b: AEMCS and EEMCS : (A) Positive control venom (B) Saline, (C) 50 µg, (D) 100 µg and (E) 200 µg of AEMCS and EEMCS was injected independently into mice in a total volume of 50 µl intra-dermally respectively

RESULT AND DISCUSSION

The current study identifies the role of Aqueous and ethanol extract of *Momordica charantia* seed on blood coagulation cascade and platelet function. When AEMCS and EEMCS were analyzed for plasma recalcification time, only AEMCS enhanced the clot formation process of plasma from control 146s to 432s but EEMCS enhanced clotting time from control from 146 s to 160s (Figure 1). Suggesting that only AEMCS possess strong anticoagulant potential while, AEMCS have poor anticoagulation potential. The reason could be the active compounds in ethanolic extract namely terpenoids, flavonoids and phenolics (data not shown) did not interact with either intrinsic/extrinsic/common pathway coagulation factors, thus poor anti-coagulation was noticed. However, water extract is rich in proteins/enzymes/peptides have potential to interact with said pathways. Hence end up in strong anticoagulation. Anticoagulants have immense therapeutic applications in thrombosis. Anticoagulant and antiplatelet agents from earthworms, caterpillar, and venoms of snake, spider, and honey bees have been gaining much importance but yet require their validation¹⁸⁻²¹. Furthermore AEMCS and EEMCS were examined for anti-platelet activity using agonists such as Collagen, ADP and epinephrine with platelet-rich plasma (PRP) (Figure 2, Figure 3 and Figure 4). Both AEMCS and EEMCS inhibited the collagen, ADP and epinephrine induced platelet aggregation of PRP. AEMCS showed 65 %, 50 % and 85 % respectively for collagen, ADP and epinephrine induced platelet aggregation; however, 19 %, 91 % and 97 % platelet aggregation inhibition for EEMCS for the said agonists. Although, EEMCS showed strong antiplatelet potential for ADP and epinephrine it showed mild antiplatelet activity for collagen (19 %), while AEMCS (65 %). Except the said implication, AEMCS found to be the strong anticoagulant and moderate antiplatelet agent in comparison with EEMCS (Figure 5) Several agonists (collagen, epinephrine, ADP, Arachidonic acid, serotonin, norepinephrine, thromboxane A₂ and thrombin) activate platelets up on vascular injury^{22,23}. Activated platelets recruit additional platelets to the growing hemostatic plug by several feedback amplification loops by releasing platelet agonists such as ADP and thrombin stored in the α -granules^{19,23} under physiological condition. However, in pathological condition hyper activation of platelet is also a major contributing factor for thrombotic disorders²². Eptifibatide, derived from rattlesnake venom that inhibits glycoprotein IIb/IIIa receptor on platelets is currently being used in the treatment of thrombotic disorders^{17,18}. In addition, irreversible cyclo oxygenase 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)^{18,19,22,23}. Ethanolic extracts of *Acheranthus aspera* (leaves)²⁴, *Tridaxpro cumbens* (whole plant)²⁵, aqueous extract of *Abutilon indicum* (leaves)²⁶, *Acheranthus aspera* (whole plant)²⁷, Soshiho-tang²⁸, Cannabis and dicoumarin from *Viola yedoensis* Makino²⁹, Phenanthrenes and flavonoids from *Calanthea risanensis* (leaves), *Artocarpus communis* (root cortex)³⁰⁻³² found to exhibit anti platelet activity. AEMCS and EEMCS both did not cause RBC lysis, edema and hemorrhagic activities suggesting their nontoxic property (Figure 6).

CONCLUSION

In conclusion, this study for the first time attempted identifies anticoagulant and antiplatelet properties of AEMCS and EEMCS. Only AEMCS showed strong anticoagulant and mild anti platelet

activity; while, EEMCS showed only strong anti platelet activity with poor anticoagulation. Hence, further purification and biochemical characterization of active molecules from AEMCS and EEMCS through a light on better understanding their anticoagulant and antiplatelet potential.

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