Research Article

Oil Extract of *Momordica charantia* Seed (OEMCS) Exhibits Biphasic Effect on Blood Coagulation, Antiplatelet and Antioxidant Properties

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ABSTRACT

Aim: Extraction of Oil from Momordica charantia seed and its characterization. **Materials and Methods:** Oil was extracted from the dried seeds of Momordica charantia using Soxhlet apparatus with petroleum as a solvent at the temperature of 60°C for about 8 hr. Momordica charantia seed oil was characterized using GC-MS. anticoagulant, antiplatelet, and antioxidant assay toxicity studies were carried out. **Results:** The fatty acid profile obtained from GCMS suggested the presence of lipids at a fair percentage. (Saturated fats-38.67%, unsaturated fats-59.39% (MUFA-1.56% and PUFA-57.83). The iodine value of extracted fatty acid was found to be 66.7 with a refractive index of 1.493 at 40°C. OEMCS exhibited a biphasic effect on plasma recalcification time. OEMCS showed a strong procoagulant effect at volume 1 to 10 µg by decreasing the clotting time from control 291 to 33sec. However, as the volume of oil was increased above 10µg it drastically shifted from pro-coagulation to anticoagulation by increasing the clotting time from 194 sec to 267 sec. Furthermore, the effect of OEMCS oil on platelet aggregation was analyzed using agonists such as ADP and epinephrine. Interestingly, OEMCS oil showed 44% and 97% platelet aggregation inhibition for ADP and Epinephrine in platelet-rich plasma. The antioxidant property of oil extract was tested by DPPH and LPO assays, OEMCS oil showed bleaching of DPPH absorption, reflecting on proton donating ability, and thereby free radical scavenging ability was confirmed. OEMCS decreased by 80% lipid peroxidation when compared with NaNo2-treated PRP alone at the highest dose of 150µg. OEMCS did not cause hemolysis to RBC, hemorrhage, and edema in experimental animals revealing its non-toxic nature. **Conclusion:** OEMCS exhibits a biphasic effect on plasma coagulation but shows strong antiplatelet and antioxidant activities. Meanwhile, it revealed non-toxic properties, thus OEMCS may prove to be a good natural therapeutic agent in oxidative stress and cardiovascular disorders.

Keywords: Oil extract, Anticoagulant, Platelet rich plasma, Antiplatelet and Antioxidant.

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INTRODUCTION

Fats and oils are vital part of the human diet and play a prime role as high energy molecules. Currently, there is a high demand for the edible oil that have been extracted from various plants seeds.[1] Seeds of Wild plants have been recently explored as key source of unusual oils.[2] Saturated and unsaturated fatty acids of the seed oils significantly exhibit the nutritive, industrial and therapeutic values.[3] Seed oils exhibits long array of anti-inflammatory, fungicidal, insect repellent, wound healing, anti-hypertensive, anticarcinogenic, antimicrobial, antioxidant, antidiabetic and Cytotoxic properties.[4-10] *Momordica charantia* or bitter gourd is a revolutionary plant having immense therapeutic and industrial applications.[11] Several investigations documented that it is a good source of conjugated α-linolenic acids.[12,13]

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Hence, for diabetes and atherosclerosis bitter gourd has been a better folk medicinal.[14] Whereas, the seeds of bitter gourd stores huge quantity of conjugated linolenic acid.[15] Hence, the lipids profile of bitter gourd seed have been receiving exception attention due its high amount of polyunsaturated fatty acids (PUFAs) and other bioactive compounds.[16] Latest studies have reported that bitter gourd seed oil consists of 30–60% of α-eleostearic acid, a long-chain PUFA with conjugated double bonds (especially between 9 and 11/10 and 12 positions). The presence of said fatty acids in the bitter gourd responsible for potential health benefits namely anti-tumor, anti-atherosclerotic, anti-inflammatory, anti-oxidant, and serum lipid-lowering activities.[17] Some clinical studies suggested that an eicosapentaenoic acid (EPA) identified in bitter gourd showed anti-inflammatory and anti-cachexiogenic benefits.[18] In addition, Antimicrobial activity and *in vitro* antioxidant properties of Oil extract of *Momordica charantia* was also reported.[19] Despite said therapeutic application of *Momordica charantia* oil till date there is no reports available on the role of bitter gourd on coagulation, platelet function and oxidative stress. Hence in the present study oil was extracted from *Momordica charantia* Seed and its anticoagulant, antiplatelet, antioxidant and non-toxic properties were examined.

MATERIALS AND METHODS

Reagents

Analytical grade chemicals were used. Platelet- rich plasma (PRP) was obtained from healthy donors.

Preparation of Oil Extract from *Momordica charantia* Seed (OEMCS)

From local market of Tumkur Bitter gourd was purchased, seeds were obtained from fruit and dried. Seed coat was removed thoroughly crashed and oil was extract from Soxhlet method. In round bottom flask approximately 200ml of petroleum ether was poured. At the centre of the extractor 50g seed powder was placed in the thimble and Soxhlet was heated at 40-60°C. When solvent started boiling vapor ascends through the vertical tube into the condenser at the top and liquid condensate slowly dripped into the filter paper thimble in the centre. It was then removed from tube, dried in the oven, cooled in the desiccators and weighed again to determine the amount of oil extracted. Crude oil was collected and store at cool and dark place in a airtight brown container bottle for further usages.

Determination of Acid Value

25ml of diethyl ether and 25ml of ethanol was mixed in a 250ml beaker. The resulting mixture was titrated as for the Laboratory Handbook, 1997 protocol.

Determination of Iodine Value

The method specified by ISO 396[20] was used to determine Iodine value of *Momordica charantia* seed oil.

Determination of refractive index

A refractometer was used in this determination of refractive index of an oil by M.Z. Kyari, 2008 method.[21]

Plasma recalcification time

As for the method of Quick[22] Plasma recalcification time was determined. OEMCS (0-50µg) was pre-incubated with 0.2mL of citrated human plasma in the presence of 10mM Tris HCl (20µl) buffer pH7.4 for 1 min at 37°C. Then 0.25 M $CaCl_2$ (20µl) was added to the pre-incubated mixture and clotting time was recorded.

Preparation of platelet rich plasma (PRP)

PRP was prepared according to the method of Ardlie and Han.[23]

Platelet aggregation

Platelet aggregation was done according to the method.[24] Aliquots of PRP were pre-incubated with various concentrations of OEMCS(0-6µg) in 0.25ml reaction volume. The aggregation was initiated independently by the addition of agonists, such as ADP and epinephrine and followed for 6min.

Antioxidant Activity

DPPH (2, 2-diphenyl-1-picrlhydrazyl) Radical Scavenging Activity

In brief, 0.1mM solution of DPPH in ethanol was prepared. Different volume of OEMCS (0-25µg) was mixed with 100µl of DPPH. The mixture tubes were shaken vigorously and incubated for 30min at room temperature in the dark. The control was prepared as above without OEMCS, and ethanol was used for the baseline correction. The absorbance was read at 517nm in a UV spectrophotometer. Reference standard compound being used was ascorbic acid and experiments were performed in duplicates. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\text{Antioxidant activity \%} = \frac{\text{O.D of control} - \text{O.D of test sample}}{\text{O.D of Control}} \times 100$$

Lipid peroxidation

According to Ohakawa method[25] lipid peroxidation of OEMCS was measured. $NaNo_2$ (500µM) and OEMCS (0-150µg) was added to test tubes containing approximately 0-2mg of protein from lysate of PRP to all the tubes. To these tubes1.5ml 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 and reaction mixture was boiled for 45min at 45-60°C and content was centrifuged at 2000rpm for 10min. The obtained adducts were extracted into 1-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.

Direct Hemolytic Activity

Direct erythrocytes lysis was determined using washed human erythrocytes. Briefly, washed RBC and phosphate buffered saline (PBS) in the ratio of 1:9 v/v were mixed. From the mix 1ml suspension was incubated with OEMCS (0-150µg) for about 1hr at 37°C. After the incubation reaction was terminated using 9ml of ice-cold PBS and centrifuged at 1000g for 10min at 37°C. The released quantity of hemoglobin in the supernatant was read at 540nm. Activity was expressed as percentage of hemolysis against 100% lysis of cells due to addition of water (positive) and phosphate buffered saline taken as negative controls.

Edema Inducing Activity

The procedure of Vishwanath method was followed.[26] Groups of five mice were injected separately in to the right foot pads with different OEMCS (0-200µg). The left foot pads received 20µl saline alone served as controls. After 1hr mice were anaesthetized by diethyl ether inhalation. 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 × 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 method.[27] A different concentration of OEMCS (0-200µg) was injected (intradermal) independently into the groups of five mice in 30µl saline. Group receiving saline alone serves as negative control and group receiving venom (2 MHD) as positive control. After 3hr, mice were anaesthetized by diethyl ether inhalation. Dorsal patch of skin surface was carefully removed and observed for hemorrhage against saline injected control mice. The diameter of hemorrhagic spot on the inner surface of the skin was measured. The minimum hemorrhagic dose (MHD) was defined as the amount of the protein producing 10mm of hemorrhage in diameter.

RESULTS

Characterization of OEMCS

Oil was extracted from the dried seeds of *Momordica charantia* using Soxhlet apparatus with petroleum as a solvent at the temperature of 60°C for about 8 hr. *Momordica charantia* seed oil was characterized using GC-MS. Data obtained GC-MS suggested the presence of 20 different fatty acids having different retention time, which was compared with standards (C4-C24 methyl esters, Sigma 18919) (Figure 1). Out of the detected fatty acids about 38.67% of saturated fatty acids and was 59.39% of unsaturated fatty acids was identified. Interestingly, in case of saturated fatty acids, highest percentage of stearic Acid 36.50% and only 1.49% of palmitic acid was observed. On the other hand, polyunsaturated fatty acids (PUFA) content was about 57.83% and Monounsaturated fatty acids (MUFA) less than 1.56% was observed. In case of PUFAs 55.30% of Cis-5, 8, 11, 14, 17-Eicosapentaenoic Acid, 2.22% Linoleic Acid, 1.25% Oleic Acid and 0.26% linoelaidic Acid was observed (Table 1). The refractive index of the oil was found to be 1.493 at 40°C and iodine value and Saponification value of OEMCS was 66.7 and 168 respectively in Table 2.

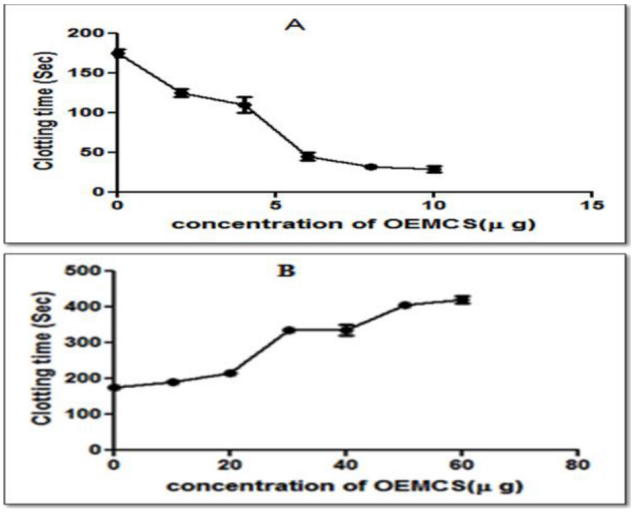

Figure 1: Plasma re-calcification time: (A) OEMCS(0-10µg) and (B) OEMCS(0-50µg) was pre-incubated with 0.2 ml of citrated human plasma in the presence of 20µl 10 Mm Tris–HCl buffer pH 7.4 for 1min at 37°C. 20µl of 0.25 M $CaCl_2$ was added to the pre-incubated mixture and clotting time was recorded.

Table 1: Fatty acid profile of OEMCS.

Carbonnumber	Name of fatty acid	Percentage
C8:0	Caprylic Acid	0.01
C13:0	Tridecanoic Acid	0.01
C14:0	Myrstic Acid	0.01
C14:1	Myristoleic Acid	0.02
C16:0	Palmitic Acid	1.49
C16:1	Palmitoleic Acid	0.01
C17:0	HeptaDecanoic Acid	0.08
C17:1	Cis 10- HeptaDecanoic Acid	0.01
C18:0	Stearic Acid	36.50
C18:1N9T	Elaidic Acid	0.02
C18: 1N9C	Oleic Acid	1.25
C18: 2N6T	Linoelaidic Acid	0.26
C18: 2N6C	Linoleic Acid	2.22
C18:3N6	Gamma Linolenic Acid	0.53
C20:0	Arachidic Acid	0.01
C20:1	Cis-11-Eicosenoic Acid	0.26
C18:3N3	LinolenicAcid	0.02
C22:0	Behenic Acid	0.03
C20:3N6	Cis-8,11,14- Eicosatrienoic Acid	0.01
C20:5N3	Cis-5,8,11,14,17-Eicosapentaenoic Acid	55.30
	Total Saturated Fat	38.67%
	Total Unsaturated Fat	59.39%
	MUFA	1.56%
	PUFA	57.83%

Table 2: Physical parameters of OEMCS.

Property	Value
Iodine value	66.7
Acid value	1.32
Saponification	168
Refractive index	1.493 at 40°C

OEMCS exhibits Biphasic effect on plasma coagulation

When OEMCS was studied for its effect on plasma coagulation cascade. Surprisingly, it exhibited biphasic effect on plasma recalcification time. OEMCS showed strong procoagulant effect at the volume 1to 10µg by decreasing the clotting time from control 194s to 33s (Figure 1A). However as the volume of oil was increased above 10-50µg, it drastically shifted from procoagulation to anticoagulation by increasing the clotting time from 194s to 467s (Figure 1B).

OEMCS Inhibits platelet aggregation

The effect of OEMCS on platelet aggregation was analyzed using agonists such as ADP and epinephrine

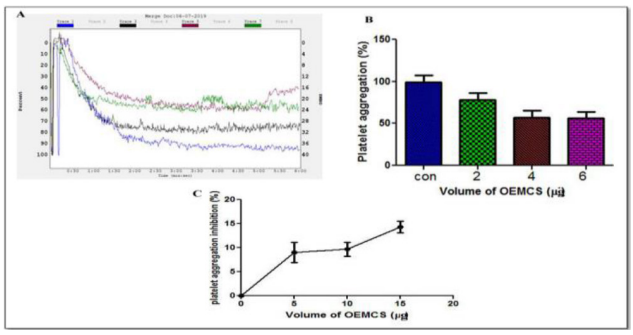

Figure 2: Platelet aggregation was initiated by adding ADP as an agonist.

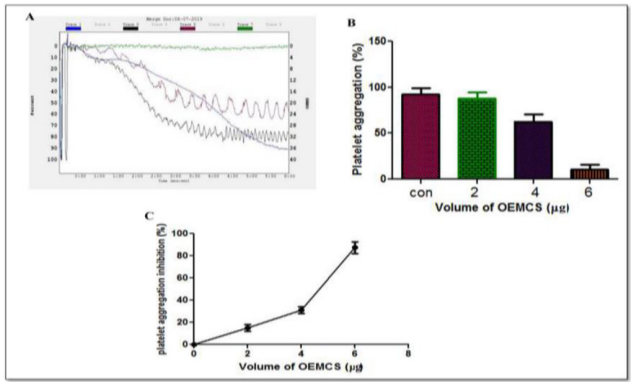

Figure 3: Platelet aggregation was initiated by adding Epinephrine as an agonist.

in platelet rich plasma. Interestingly, OEMCS showed 44% of ADP induced inhibition and 97% aggregation inhibition for agonist Epinephrine (Figure 2 and Figure 3. Both agonists induced platelet aggregation inhibition was at concentration of 0-6*µg OEMCS*.

(A)Traces of platelet aggregation: Trace 1 (ADP 10µM); Trace 2 (ADP 10µM +2µg of OEMCS); Trace 3 (ADP 10µM + 4µgof OEMCS); Trace 4 (ADP 10µM + 6µg of OEMCS). The values represent ± SD of three independent experiments. (B) Dose dependent platelet aggregation %. (C) Dose dependent platelet aggregation inhibition %.

Trace 1 (Epinephrine 5µM); Trace 2 (Epinephrine 5µM+2µg of OEMCS); Trace 3 (Epinephrine 5µM+ 4µg of OEMCS); Trace 4 (Epinephrine 5µM+6µg of OEMCS). The values represent ± SD of three independent experiments. (B) Dose dependent platelet aggregation %. (C) Dose dependent platelet aggregation inhibition %.

OEMCS exhibits antioxidant property

In vitro DPPH assay of OEMCS showed strong antioxidant property in a dose dependent manner. OEMCS effectively scavenged DPPH free radicals. The efficient radical scavenging activity was observed at the

concentration of 0-150µg *and the observed percentage was* found to be 80% that was compared with the positive control Vitamin C (Figure 4). Oxidative stress was also determined by non-enzymatic parameter in platelet rich plasma. Thus, the level of lipid peroxidation was measured in terms of malondialdehyde (MDA) levels and a remarkable increase was observed in $NaNo_2$ induced group. Interestingly, OEMCS decreased 80% lipid per oxidation when compared with $NaNo_2$ treated PRP alone at the highest dose of 150µg. however, OEMCS alone did not show alteration (Figure 5).

OEMCS is devoid of toxic property

OEMCS did not cause hemolysis to RBC (Figure 6), hemorrhage (Figure 6), and edema in experimental animals (data not shown), revealed its non-toxic nature. Packed human erythrocytes and phosphate buffered saline (PBS) incubated with different concentration of OEMCS (0-150µl) independently for 1hr at 37°C. +ve control: Water, –ve control: PBS for %of hemolysis.

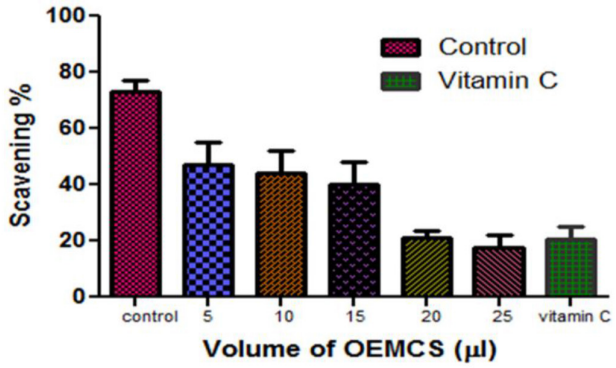

Figure 4: DPPH Radical Scavenging Activity: Dose dependent DPPH scavenging ability of OEMCS(0-25µg). -ve conrtol:DDPH alone and +ve control: Vitamin C,volume of OEMCS.

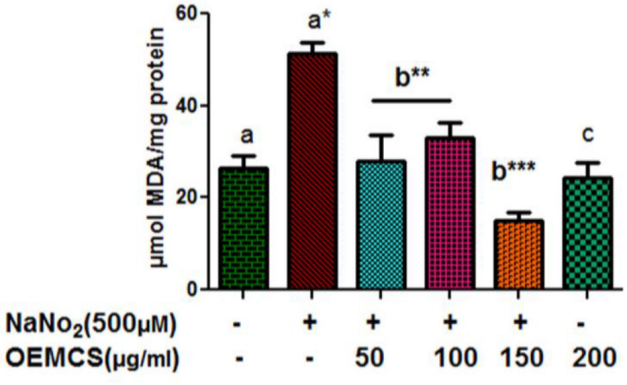

Figure 5: Effect of OEMCS on lipid peroxidation.

a: without treatment and sample a*: oxidative stress inducer $NaNo_2$ treated, from b**to b***: oxidative stress inducer and sample OEMCS (50-150µg) with different volume. C: with sample and without treatment. Each value represents the mean ± S.D. (n=3). *Significantly reduced stress in treated PRP when compared to $NaNo_2$.

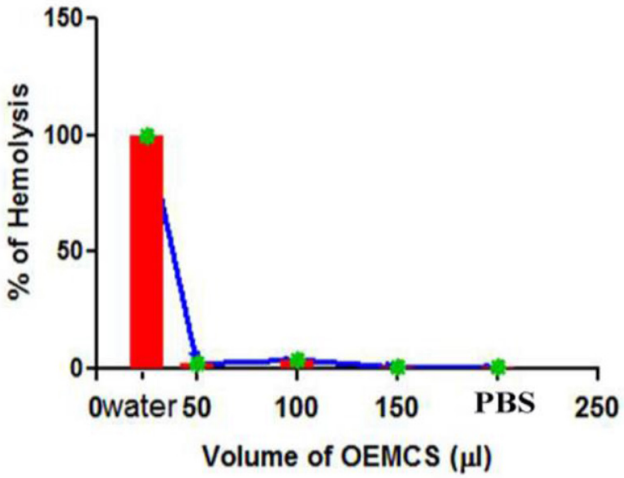

Figure 6: Direct Hemolytic assay.

The amount of hemoglobin released in the supernatant was measured at 540 nm.

DISCUSSION

Current study investigates the presence of fatty acids and their role on plasma coagulation, platelet aggregation and oxidative stress. The fatty acid composition of OEMCS was determined by comparing the relative retention times and the mass spectra of oil from the data library. The fatty acid profile obtained from GCMS suggested that the presence of lipids at a fair percentage. Saturated fat-38.67%, unsaturated fat-59.39% (MUFA-1.56% and PUFA-57.83). The iodine value of extracted fatty acid was found to be 66.7 with the refractive index of 1.493 at 40°C and saponification value was 168. Polyunsaturated fatty acids of oil from different sources exhibits vast beneficiary effects and PUFA known to play important roles in various physiological and pathological processes.[28] Recent studies have shown that someomega-3 (w-3) PUFAs, such as eicosapentaenoic acid (EPA) and dodecahexaenoic Acid (DHA)have protective effects on acute and chronic UV-induced changes.[29,30] Raw oil is used as an astringent in fungicidal lotion, insecticide and has shown moderate insect-repellent properties.[31] Anti-inflammatory and antioxidant potential of flaxseed was showed in animal models.[32] The oil from bergamot extract and Citrus essential oil showed hypolipidemic, hypoglycemic and anti-cancer properties.[33,34] *Momordica charantia seed* oil exhibits Cytotoxic effect in tumors cells.[9] Feeding of Bitter melon oil increases the number of apoptotic cells and reduce cell proliferation activity.[35] Anti-adiposity effect of bitter melon seed oil was reported.[36] OEMCS exhibited biphasic effect on plasma recalcification time. OEMC, showed strong procoagulant effect at the concentration 1to 10µg by

decreasing the clotting time from control 194s to 33sec. However as the concentration of oil was increased above 10µgit drastically shifted from procoagulation to anticoagulation by increasing the clotting time from 194sec to 400sec. This study may be explains OEMCS has dual functions in the coagulation system. It may act as a procoagulant to promote thrombus when bleeding occurs and also act as an anticoagulant while bleeding. During vascular injury blood loss will be prevented by hemostasis. However, the genetic and environmental factors may imbalance the hemostatic pathway that eventually leads to thrombosis.[37] Some compounds isolated from plants display hemostatic activity. Several plants extract exhibits pro-coagulant and anti-coagulant effect on blood hemostasis. For instance, latex of Jatropha curcas, garlic oil, Phenolic acids such as p-coumaric, syringic, vanillic, Caffeic and protocatechuic acids.[38-40] Hence OEMCS may prove to be a good hemostatic bioactive component. Furthermore, the effect of OEMCS on platelet aggregation was analyzed using agonists such as ADP and epinephrine. Interestingly, OEMCS in platelet rich plasma showed 44% for ADP and 97% for epinephrine platelet aggregation inhibition. This finding is in streak with a number of previous studies, Effects of polyunsaturated omega-3 fatty acid son antiplatelet therapy. Fish oil has been shown to reduce stimulated platelet aggregation and increase bleeding time.[36] Eicosapentaenoic acid on platelet function. High omega-3 fatty acid intake or a high plasma eicosapentaenoic concentration have been reported to exhibit reduced thrombogenicity by decreasing platelet aggregation and cardio protective effects. During hemostasis Platelet activation by several physiological agonists namely, collagen, ADP, thrombin, epinephrine and platelet activating factor play a crucial role in the primary hemostasis. However, similar to coagulation factors overexcited activation of the platelets due to genetic/environmental factors contributes equally for the thrombotic.[37] Hence from current exploration of OEMCS on platelet aggregation may primarily confirm to be betterment for thrombotic disorder. A number of scientific studies have revealed that the oil extracts from seeds of different plants showed strong antioxidant properties. Antioxidant properties of andiroba oil. [38] Sesquiterpene from the Essential Oil of *Aquilari acrassna*.[39] Essential oil from *Dracocephalum kotschyi* Boiss.[40]Accordingly antioxidant property of OEMCS Oil was tested by DPPH and LPO assays, OEMCS oil showed bleaching of DPPH absorption reflects on proton donating ability and there by free radical scavenging ability was confirmed. DPPH test clearly demonstrated the antioxidant activity of OEMCS. The observed antioxidant activity of an OEMCS attributed to the presence of great quantity fatty acids (MUFA and PUFA). Lipids and proteins oxidation is a key event that predicts the marked oxidative stress. Lipid peroxidation is a key marker to indicate the oxidative stress in cells. OEMCS suppressed the elevation of oxidative stress marker LPO. The mechanism oxidative stress arises from an imbalance between oxidants and antioxidants in favor of excessive generation of free radicals and this elevates biomolecules with consequent loss of its biological functions and/or homeostatic imbalances.[41] Increased oxidative stress enhance the accumulation of ROS/RNS can result in a number of deleterious effects such as lipid peroxidation, protein oxidation and DNA damage in turn leads to potential oxidative damage to cells and tissues.[42] Some of the pathological condition linked with the oxidative stress that includes diabetes, malaria, chronic renal failure, uremic syndrome, anemia and thalassemia. In this study, surprisingly OEMCS showed strong potency towards suppression of lipid peroxidation. Interestingly, OEMCS did not cause hemolysis when it was treated with RBC and did not cause hemorrhage and edema in experimental mice suggesting its non-toxic nature.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

OEMCS: Oil extracts of Momordica charantia seed; **GC-MS:** Gas chromatography –Mass spectroscopy; **MUFA:** Monounsaturated fatty acids; **PUFA:** Polyunsaturated fatty acids; **ADP:** Adenosine triphosphate; **PRP:** Platelet rich plasma; **DPPH:** 2,2-diphenylpicrylhydrazyl; **LPO:** Lipid peroxidation; **$NaNo_2$:** Sodium nitrite; **RBC:** Red blood cell; **DNA:** Deoxyribonucleic acid.

SUMMARY

Seed oil was extracted from *Momordica charantia* (Bitter gourd). The extracted oil was characterized by using GC-MS, and the fatty acid profile showed a high percentage of MUFA and PUFA. OEMCS exhibits a biphasic effect in coagulation assay at

low concentrations and showed procoagulant and at increased concentrations anticoagulant activity. Interestingly, OEMCS was demonstrated as a good antiplatelet agent by inhibiting ADP and epinephrine agonist-induced platelet aggregation in PRP. However antioxidant property of oil was observed in DPPH and LPO assays and also proved to be nontoxic in nature. In conclusion, the current study for the first time reports the biphasic effect on plasma coagulation, antiplatelet and antioxidant activities of oil of *Momordica charantia* seed. Thus OEMCS could be valuable candidate in the management of thrombotic disorders and pathological conditions of oxidative stress.

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