



## The Prediction of Milk Whey Extract (MWE) Bioactive Compounds Based on Proximate Analysis and Its Effects on Blood Coagulation: A New Approach

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### Abstract

The present work demonstrates the milk whey extraction, proximate analysis, and biochemical characterization of bioactive compounds in MWE. Effects of MWE on plasma coagulation and platelet aggregation. Proximate analysis of MWE was done according to AOAC. Whey protein banding was confirmed in 12%, 15%, and 18% SDS-PAGES. A quantitative analysis of bioactive compounds was done. The anticoagulant effects of MWE were tested using plasma recalcification time in both PRP and PPP, further confirmed by PT and APTT assays and in tail bleeding assays at concentrations of 0-100 µg. The non-toxic property of MWE was screened by edema, hemorrhage, and direct hemolytic activities. MWE proximate analysis showed the presence of both micro- and macronutrients. Qualitative analysis confirmed the presence of proteins and carbohydrates. A similar protein-banding pattern was observed in 12%, 15%, and 18% SDS-PAGE's in both reduced and non-reduced conditions. MWE prolonged the clotting time of human citrated plasma, both PRP and PPP, against the control 210 sec to 770 sec, suggesting its anticoagulant property. MWE delayed the clot progress of only the APTT ratio ( $8.99 \pm 0.09$ ), and PT was not altered, suggesting its role in an intrinsic pathway of blood coagulation. MWE exhibited antiplatelet activity in PRP against ADP and epinephrine-induced platelet aggregation. The percentage of inhibition was 74% and 100% for ADP and epinephrine, respectively. Interestingly, MWE exhibits nontoxic properties, it does not cause hemolysis,



### Article History

Received: 16 January 2023

Accepted: 15 March 2024


### Keywords

Antithrombotic;  
Antiplatelet;  
Cardiovascular Disease;  
Milk Whey Extract;  
Proximate Analysis,  
Nontoxic.

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Doi: <https://dx.doi.org/10.12944/CRNFSJ.12.1.35>

hemorrhage, or edema. Milk whey extraction and studies confirm bioactive compounds, and these exhibit antithrombotic, antiplatelet, and non-toxic properties. Hence, MWE not only acts as a good nutritive source but also may prove to be a therapeutic bioactive compound in the management of cardiovascular disease.

### Abbreviations

ADP	Adenosine diphosphate
APTT	Activated partial thromboplastin time
AOAC	Association of Official Agricultural Chemists
BSA	Bovine serum albumin
DAG	Diacylglycerol
FC	Folin-Ciocalteu's
g	Gram
h	Hour
ICP-OES	Inductively coupled plasma optical emission spectrometry
kDa	Kilo Dalton
L	Litre
MWE	Milk whey extract
mg	Milligram
ml	Millilitre
µg	Microgram
min	Minute
mM	Millimolar
mm	Millimetre
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PPP	Platelet-poor plasma
PRP	Platelet rich plasma
PRT	Plasma recalcification Time
PT	Prothrombin time
RBC	Red blood cells
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
Sec	Second
wt	Weight

### Introduction

The World Health Organization defines health as a state of complete physical, mental, and social well-being rather than only the absence of illness or incapacity. Generally, maintaining good health requires a balanced diet, regular exercise, and enough sleep. Due to risk factors like depression<sup>1-5</sup> and anxiety<sup>6-7</sup> lifestyle disorders such as obesity, hypertension, cardiovascular disease, and diabetes have recently shown high prevalence. According to the 2019–20 National Family Health Survey, 24% of men and 21% of women had hypertension.

Uncontrolled hypertension contributes to various cardiovascular issues, including thrombosis.<sup>8</sup> Many factors, including lifestyle, diet, environmental factors, and/or genetics, influence abnormal coagulation and may lead to thrombosis. Currently, many medications are available to treat thrombosis and dissolve blood clots, but the main drawbacks of these drugs are overbleeding, organ damage and hypersensitivity etc.<sup>9-10</sup> In the present scenario, research on natural products and their derivatives is gaining main attention due to the least side effects and least toxicity.<sup>11</sup> Generally, therapeutic bioactive compounds/proteins/peptides derived from different sources like plants, animals, microorganisms, etc. often modify the physiological functioning of cells.<sup>12</sup> According to the National Health and Nutrition Examination Survey (NHANES I), milk consumption may be regarded as a complete food because it contains essential nutrients like protein, vitamins, and minerals that can help fight a variety of diseases, including lowering the risk of hypertension and blood pressure.<sup>13-14</sup> One such natural source is milk, milk-derived products such as lactose, casein, yogurt, cheese, whey proteins, and so on. Recent studies on dairy and dairy products have focused on the nutritional and therapeutic properties of milk and its derivatives. In bovine milk, casein makes up around 80% of the total protein, and whey protein is about 20%. Casein has major components like  $\alpha_{s1}$  and  $\alpha_{s2}$ ,  $\beta$ ,  $\kappa$  (kappa) proteins, whereas, whey protein is a by-product of cheese manufacture, it contains essential amino acids as the quality of protein as alpha-lactalbumin ( $\alpha$ -LA), beta-lactoglobulin ( $\beta$ -LG), immunoglobulins (Igs), serum albumin, lactoferrin (LF), lactoperoxidase (LP), and protease-peptones.<sup>15-17</sup> Specifically, a milk-derived protein complex called whey is being promoted as a functional food with several health benefits. Different components of whey, including  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, lactoferrin, glycomacropetides, and immunoglobulins, exhibit a variety of immune-boosting properties. Many studies showed antihypertensive, antibacterial, antioxidant, anti-cancerous, immunomodulatory, antidiabetic,

antiviral, antimicrobial, and antithrombotic effects. Whey from milk helps control appetite, and hunger, and manage weight and obesity.<sup>18</sup> It also increases glutathione levels in the body, which supports the maintenance of the immune system's flexibility.<sup>19</sup> Hence, the study focused on the extraction of whey from bovine milk, proximate analysis, and investigating the antithrombotic, antiplatelet, and non-toxicity properties of MWE.

### Materials and Methods

The milk whey was obtained from Yelahanka, the mother dairy of the Karnataka Milk Federation (KMF), in Bangalore, Karnataka, India. Agappe Diagnostic Pvt., Kerala, India, PT and APTT reagents Fibrinogen from human plasma (Sigma Chemicals Co., St. Louis, USA) (India's Bangalore Genie Private Limited) Molecular weight indicators Unless otherwise noted, only analytical-grade compounds were utilized.

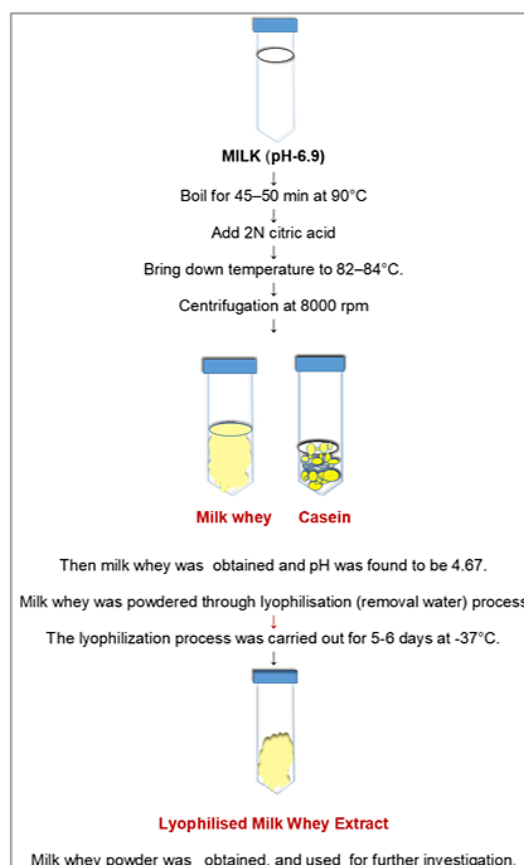
### Ethical Approval

All of the tests were approved by the Institutional Human Ethical Committee of Tumkur University and conducted under ethical guidelines. The animal research was authorized by Liveon Biolabs Private Limited's Institutional Animal Ethical Committee in Tumkur. Before handling the animals, the Committee for Monitoring and Supervision of Animal Experiments (CPCSEA) guidelines were followed. Sprague and Dawley female rats weighing 150–180 g was acquired from Liveon Bio Labs, Limited, and were given 6 days to acclimate in groups of 6 in polypropylene cages. The rats were fed a conventional diet of laboratory pellets and allowed unrestricted access to cow feed and tap water. The animals were kept in an animal housing with a temperature of  $27 \pm 2^\circ\text{C}$ , a 12 h light/12 h dark cycle, and humidity, light, and temperature levels ranging from 55 to 65%. Ethics permission for this experiment was granted by Liveon Biolabs Limited's Institutional Ani Mal Ethics Committee (LBLIAEC), Tumkur, under protocol number LBPL-IAEC-47-05-2019.

### Preparation of Milk Whey

Whey preparation at KM Fmother dairy. One batch of 1800 L of bovinemilk (pH 6.6) was heated for 45–50 min at  $90^\circ\text{C}$  before being added to 360 ml of 2N citric acid and allowed to cool to  $82\text{--}84^\circ\text{C}$ . Whey from milk that had been separated from casein was brought to

the lab. When the pH (digital pH meter MK VI) of the milk whey was found to be 4.7, it was lyophilized in (MAC, Macro Scientific Works PVT.LTD) to produce concentrated MWE. Prepared MWE was stored at  $-20^\circ\text{C}$  until it was used for further studies.



### Proximate Analysis of Milk Whey Extract

#### Moisture Content

A silica dish was baked dry and weighed ( $W_1$ ). A five-gram sample ( $W_2$ ) added, dried, and weighed. The dish then placed in an air oven (Thermo Fisher jacket-heating VT 6025 hot air oven) at  $105^\circ\text{C}$  to dry for 3 h, cooled, and weighed again. The dish was weighed after being placed back in the oven for 30 min and allowed to cool in the desiccator. This process was repeated until a consistent weight was reached ( $W_3$ ).<sup>20</sup> The dry matter content was calculated as per the below mentioned formula 1.

$$\text{Formula 1: Dry matter content} = \frac{(W_2 - W_3)}{(W_2 - W_1)} \times 100$$

**Ash Content**

The test material, labelled (W2), was weighed into a silica dish, ignited, cooled, and weighed (W1). Then placed in a muffle furnace at 500°C until fully ashed. After ashing, the dish containing the ash was removed from the furnace and kept in a desiccator to cool before weighing (W3).<sup>20</sup> The formula 2 below was used to compute the ash percentage.

Formula 2: % of Ash =  $(W3-W1/W2-W1) \times 100$

**Dietary Fiber**

A sample and digesting mixture were mixed in a 250 ml flat conical flask, refluxed for 45 min, and filtered. The residue was washed with 100 ml of boiling water, 50 ml of alcohol, and 50 ml of petroleum ether, and the sample filter paper was dried at 100°C to a constant weight. The residue was then ashed in a crucible at 600°C in a muffle furnace (Thermo Scientific CP1065058 Programmable muffle furnace, 1008cu in. 240VAC) for 4 h and then weighed again.<sup>20</sup>

**Estimation Fat Content (Soxhlet Extraction Method)**

A sample was weighed and placed in a flat bottom flask (W1) cleaned with petroleum ether, and submerged in the extractor (Glassco, Single Soxhlet Extractor, 1601.DNEU.02\*). The solvent was added, and the extraction process was carried out for 5 h. The solvent was removed by using a water bath heated to 60°C, the residue dried, and the flask and residue were weighed (W2).<sup>20</sup> The formula 3 below was used to compute the fat percentage.

Formula 3: % of Fat =  $(W2-W1/W1) \times 100$

**Estimation of Protein Content (Kjeldahl Method)**

A digesting tube (Kjeldahl digestion tubes) was filled with two grams (2 g) of sample and 20 ml of sulfuric acid. After being connected, the digester was given a 3 h run. The volume was increased to 100 ml by adding 50 ml of 40% NaOH and using distilled water.<sup>20</sup>

**Distillation**

5 ml of Borate (2%) was pipetted into a conical flask, followed by the addition of 3 drops of bromocresol and methylene indicator. 5 ml of digested sample

was added, followed by 20 ml of 40% NaOH to the distillation flask. 75 ml of the distillation was collected and titrated using 1M HCl solution.

**HCl Standardization**

A mixture of 15 ml of 40% NaOH solution and 5 ml of ammonium solution was pipetted and distilled. The freed ammonia was collected in a conical flask with 4 drops of mixed indicator and 5 ml of 2% boric acid. The usual 0.1M HCl was used to titrate the ammonia solution. The titration needed quantity of HCl served as the acid factor for the crude protein content estimates. The formula 4 below was used to compute the total carbohydrate percentage.

Formula 4:  $(A \times N \times F \times 14.007) / (\text{Weight of the sample taken} \times \text{Aliquot taken})$ .

Where: A = Volume of the acid used, N = Molarity of the acid, F = Factor 6.<sup>25</sup>

**Mineral Estimation (ICP-OES) and Vitamins**

The Milestone microwave burned 2 g of cheese on ash, 8 ml of 65% HNO<sub>3</sub>, and 2 ml of 30% H<sub>2</sub>O<sub>2</sub> for 15 min at 200°C, then added 50 ml of ultrapure water. All reagents and samples were analytical grade, using double-distilled water. All vitamins are estimated by HPLC method.<sup>20</sup>

**Estimating Carbohydrate Content (Nitrogen-Free Extract)**

The difference remaining after eliminating total crude protein, fat, ash, and crude fiber from the total dry matter was utilized to calculate the carbohydrate (nitrogen-free extract).<sup>20</sup>

Formula 5: % Carbohydrates =  $100 - (\text{moisture} + \text{protein} + \text{ash} + \text{fat} + \text{crude fiber})$

**Analysis of Bioactive Compounds of MWE Carbohydrate Test (Benedict's Test)**

After boiling the crude MWE with 2 ml of Benedict's reagent, a variety of green, red, and yellow colour showed that there were carbohydrates present.<sup>21</sup>

**Ninhydrin Test for Proteins**

When 2 ml of Ninhydrin 0.2% solution was added to the crude MWE while it was boiling, a violet colour emerged, indicating the presence of proteins.<sup>21</sup>

**Test for Phenols and Tannins**

The 2% solution of  $\text{FeCl}_3$  was dissolved in 2 ml of the crude extract. The presence of phenols and tannins was indicated by a blue-green colour.<sup>21</sup>

**Test of Flavonoids**

A few ml of lead acetate solution was combined with the crude MWE. The presence of flavonoids is shown by the production of a yellow precipitate.<sup>21</sup>

**Test for Saponins**

The crude MWE was combined with 5 ml of distilled water in a test tube and forcefully shaken. It was believed that the development of stable foam was a sign that saponins were present.<sup>21</sup>

**The Detection of Cardiac Glycosides (Test Keller-Kilani)**

A 2% solution of  $\text{FeCl}_3$  and 1-2 drops of glacial acetic acid were added to the crude MWE in a mixture of 2 ml. After that, the mixture was put into another test tube with 2 ml of concentrated  $\text{H}_2\text{SO}_4$ . A brown ring at interphase indicated the presence of cardiac glycosides.<sup>21</sup>

**Test for Steroid**

2 ml of chloroform and 2 ml of concentrated  $\text{H}_2\text{SO}_4$  were added side by side to the crude extract. Steroids were present because of the red colour and yellowish-green fluorescence that appeared.<sup>21</sup>

**Terpene Test**

The Salkowski test is used to determine whether terpenoids are present. A test tube is filled with 2 ml of the MWE, 2 ml of chloroform, and 3 ml of pure sulfuric acid ( $\text{H}_2\text{SO}_4$ ) to create a lower layer. An interface with a reddish-brown colour indicates the presence of terpenoids.<sup>21</sup>

**Polyphenol Test**

A 5% solution of lead acetate was diluted with a few drops of the crude extract. If polyphenols are present, a yellow precipitate will occur.<sup>21</sup>

**Estimating Protein**

Lowry's method was used to estimate total protein in BSA, with concentrations ranging from 0 to 200  $\mu\text{g}/\text{ml}$ . Add 5 ml of the acid solution, let it sit at room temperature (RT) for 10 min, then add 0.6 ml of the FC solution. Next, let each test tube allowed to settle at room temperature for 20 min. Take a 660nm

spectrophotometric reading (Thermo Nicolet iS50-Thermo Fisher Scientific, USA). The area under the curve for protein concentration was determined, the overall protein concentration was calculated.<sup>22</sup>

**SDS PAGE**

According to Laemmli,<sup>23</sup> 12%, 15%, and 18% MWE gel concentrations were used for SDS-PAGE, to put it briefly. To determine the protein molecular weight, the crude MWE stock was produced at a concentration of mg/ml under both reducing and non-reducing conditions. From 170 kDa to 9 kDa, molecular weight markers were utilized.

**Proteolytic Activity**

As indicated,<sup>24</sup> the proteolytic activity of 1 ml of fat-free casein (0.4 ml, 2% in 0.2M Tris-HCl buffer, pH 7.6) was assessed by incubating it with MWE (20-100 g) for 2 h and 30 min at 37°C (Isotherm forced convection lab incubator, Inlab Equipments(Madras) Pvt. Ltd). Undigested casein was precipitated and centrifuged (Remi, CB-30 BL, 20000 rpm, and cooling centrifuge) for 10 min. Folin-Ciocalteu's reagent and sodium carbonate were added to the supernatant, along with enzyme activity.

**In vitro and in vivo Antithrombotic Studies****Plasma Recalcification Time**

The plasma recalcification time was calculated. Briefly, the MWE (0-800  $\mu\text{g}$ ) concentrations were pre-incubated for 1 min at 37°C (Isotherm forced convection lab incubator, In lab Equipments (Madras)Pvt. Ltd) with 0.2 ml of citrated human plasma in the presence of 10  $\mu\text{l}$  of 10 mM Tris HCL buffer of pH 7.4. Then the pre-incubated mixture was then given 10  $\mu\text{l}$  of 0.25M  $\text{CaCl}_2$ , and the clotting time was noted.<sup>25</sup>

**Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT)**

MWE (5 mg/ml) and 100  $\mu\text{l}$  of normal citrated human plasma were pre-incubated for 1 min. Both APTT and PT were performed. 100  $\mu\text{l}$  of LIQUICELIN-E phospholipid precipitation from rabbit brain that had been stimulated for three min at 37°C (Isotherm forced convection lab incubator, Inlab Equipments (Madras) Pvt.Ltd) for APTT was added. By adding 100  $\mu\text{l}$  of 0.02 M  $\text{CaCl}_2$ , the clotting process was started, and the clotting time was recorded. 200  $\mu\text{l}$  of the PT reagent (UNIPLASTIN-rabbit brain thromboplastin) was added to start the PT



procedure. The visible dots duration was measured in seconds. The data of the control plasma incubated with the buffer for the same amount of time were used to determine the APTT ratio and international normalized ratio (INR) for PT at each point.<sup>26</sup>

#### **Tail Bleeding Assay**

The Denis method was used to measure the bleeding time in 1998.<sup>27</sup> A batch of 5 mice was given 30  $\mu$ l (20-100  $\mu$ g) MWE of PBS intravenously through the tail vein. Diethyl ether was used to anesthetize mice after 10 min, and the tip of each mouse was sharply cut off at a length of 3 mm. The tail was immediately immersed vertically in PBS that had been pre-warmed to 37°C (Isotherm forced convection lab incubator, Inlab Equipments (*Madras*) Pvt. Ltd). From the time the bleeding began until it stopped entirely, the bleeding time was tracked for 10 min or 600 sec.

#### **Whole Blood Clot Retraction Assay**

A whole blood clot lysis assay was carried out.<sup>28</sup> Four healthy volunteers of age 25-35 males, free of drug and alcohol use, sickness, and weight, provided blood for centrifugation tubes (500  $\mu$ l/tube), which were weighed and incubated for 45 min at 37°C (Isotherm forced convection lab incubator, Inlab Equipments(*Madras*)Pvt. Ltd). After forming a clot, serum was withdrawn, and tubes were weighed again. MWE (0 -75  $\mu$ g) was added and incubated for 90 min at 37°C (Isotherm forced convection lab incubator, Inlab Equipments (*Madras*) Pvt. Ltd). After the incubation period, the collected fluid was evacuated, and the percentage of clot lysis was calculated.

#### **Preparation of Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP)**

PRP and PPP was prepared according to the method of Ardlie *et al.* (1974).<sup>29</sup> The concentration of PRP was maintained adjusted to  $3.1 \times 10^8$  platelets/ml with PPP, and both maintained in 37 °C. PRP was used within 2 h for the aggregation assay. All the above preparations were carried out using plastic wares or siliconized glass wares.

#### **Aggregation of Platelets**

Platelet aggregation was done with the help of a chronological dual-channel whole blood/optical Lumi aggregation system (Mode-700) by method.<sup>30</sup> Aliquots of PRP were pre-incubated with a range of

MWE concentrations (10–30  $\mu$ l) in 0.25 ml reaction volume. When agonists like ADP and epinephrine were added, the aggregation was initiated and continued for 6 min.

#### **Toxicity Studies**

The mice utilized in this investigation weighed between 30 and 50 g and were fed. Mice were kept separately in polypropylene cages in a cold environment and kept at 23°C with a humidity level of 60% and on a dark/light cycle. All rats were fed with feeds and deionized distilled water in polypropylene bottles with stainless steel sippers. The mice were acclimated before a toxicity test was conducted. An amount of 5 mg/mL of crude MWE was made.

#### **Hemorrhagic Activity**

The hemorrhagic activity was measured in accordance<sup>31</sup> instructions. On the left and right sides of the dermis, mice received injections of MWE. On the mice's left and right sides, respectively, 3 units (375  $\mu$ g) of the crude milk whey sample and phosphate buffer were injected. The identical sample was injected into mice 2 in two units, 125  $\mu$ g on the left side and 250  $\mu$ g on right side. Diethyl ether was used to kill the mice, and the skin from the left and right sides was separated to check for toxicity. The dorsal portion of the skin was removed, and the inner surface of the hemorrhagic spot was measured in comparison to control mice, received 30  $\mu$ l saline injections. Skin inner surface hemorrhagic spot diameter was measured. The minimum hemorrhagic dose (MHD) was defined as the amount of the protein producing 10 mm of haemorrhage in diameter.

#### **Direct Haemolytic Assay**

Human erythrocytes thoroughly washed was used to assess the direct lysis activity. In a nutshell, washed RBC and phosphate-buffered saline (PBS) (1:9 v/v) were mixed. 1 ml suspension was individually treated with a concentration of MWE (0–150  $\mu$ g) for 1 h at 37°C. The reaction was terminated by adding 9 ml of ice-cold PBS and centrifuged at 1000 g for 10 min at 37°C (Isotherm forced convection lab incubator, Inlab Equipments (*Madras*) Pvt. Ltd). The released quantity of hemoglobin in the supernatant was read at 540 nm (Thermo Nicolet iS50-Thermo Fisher Scientific, USA). 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.

**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.

**Table 1: Proximate analysis of MWE**

<b>Milk Whey Extract (MWE)</b>								
Sl.No	Name of Nutrient	Percentage/Weight.	Sl.No	Minerals	mg/kg	Sl.No	Vitamins	mg/100g
1	Moisture	4.30±0.03	1	Iron	19.27±0.05	1	Vitamin A	236±0.01
3	Crude Ash	8.77±0.01	2	Zinc	11.34±0.03	2	Thiamine	0.03±0.01
4	Acid insoluble ash	0.14± 0.02	3	Calcium	2082.45±0.02	3	Riboflavin	0.01±0.00
5	Crude Fibre	1.60± 0.03	4	Magnesium	424.49±0.03	4	Vitamin C	0.02±0.01
6	Dietary fibre	6.70±0.03	5	Potassium	4924.55±0.04	5	Vitamin D	0.01±0.00
7	Carbohydrates	75.57±0.01	6	Sodium	1765.43±0.02	6	Folic Acid	0.01±0.00
8	Crude protein	10.60±0.02				7	Niacin	0.06±0.01
9	Crude Fat	0.72±0.04						

**Table 2: Qualitative analysis of bioactive compounds of MWE**

Sl. No	Test	result
1	Carbohydrates	+ve
2	Proteins	+ve
3	Tannins	-ve
4	Terpinoids	-ve
5	Anthocyanins	-ve
6	Plobatannins	-ve
7	Anthraquinones	-ve
8	Leucoanthocyanins	-ve
9	saponins	-ve
10	Steroids	-ve
11	Flavonoids	-ve
12	Polyphenols	-ve

Each point represents the mean±SD of three independent experiments, P< 0.03.

**Results and Discussion**

**Characterization of Bioactive Compounds of MWE**

Proximate analysis of MWE found to contain carbohydrates and proteins. Minerals and vitamin

analysis showed high concentrations of potassium, calcium and vitamin A respectively as shown in Table 1. An overview of the physicochemical makeup of the powdered MWE is shown in Table 1. Carbohydrates accounted up a significant percentage of the powder proximate composition (67.40%), followed by protein (10.60%), ash (8.77%), fat (0.72%), and moisture (4.30%). This information is in line with the study,<sup>32</sup> which showed comparable outcomes in terms of the amount of carbohydrates (67.40%), followed by protein (14.20%), ash (8.77%), fat (0.72%), and moisture (4.30%). The ash percentage was discovered to be 8.77%/w, which is comparable to the 8.52%<sup>32</sup> ash detected in goat milk whey and in line with the legislation's advice that a powdered products<sup>33</sup> ash content doesn't exceed 9.5%. Similar to GWP, MWE displayed a sufficient level of minerals and vitamins. Furthermore, this outcome is consistent with the findings of research by PALATNIK *et al.* (2015) and GOMES *et al.* (2013), which found that goat milk contains 0.55 g and 0.30 g, respectively.<sup>34-35</sup> Qualitative analysis of bioactive compounds of MWE (Table 2) showed negative for tannins, terpenoids, anthocyanins, Plobatannins, anthraquinones, and leucoanthocyanins and positive for protein and carbohydrates. Each point

represents the mean±SD of three independent experiments, P<0.01. Despite the MWE's anticancer, immunomodulatory, antiviral, antibacterial, and antidiabetic properties, the antithrombotic potential of the substance was investigated. According to Kamizake, Gonçalves, Zaia, & Zaia D.A, (2003),<sup>36</sup> bovine casein contains between 20 and 60 g/ml of protein, however, the protein content of MWE was measured using Lowry's method, and the findings revealed 56 g/ml.

**Analyzing the Proteins of MWE by SDS-PAGE**

The molecular weights and subunits of proteins in the MWE were determined using SDS-PAGE as shown if figure 1. In comparison to a report by Jung

*et al.* (2016)<sup>37</sup> that showed the 12% SDS-PAGE of goat milk protein with 5 protein bands, the result of the MWE's 12% SDS-PAGE showed 7 protein bands ranging from 170 kDa to 9 kDa, under reducing and non-reducing conditions, revealing the presence of monomeric and multimeric proteins, with 5 protein bands in 15% and 18% SDS-PAGE. Similar to Caillat *et al.* (2010)<sup>38</sup> several research reports showed proteins and novel peptides exhibiting good therapeutic properties other than casein/ enzyme by shiqi *et al.* (2020),<sup>39</sup> somaye *et al.* (2018).<sup>40</sup> In order to know the enzymatic property of MWE, the proteolytic activity was carried out for 50 µg, 100 µg, and 150 µg, and for higher concentrations, the results were negative and did not cause proteolytic activity.

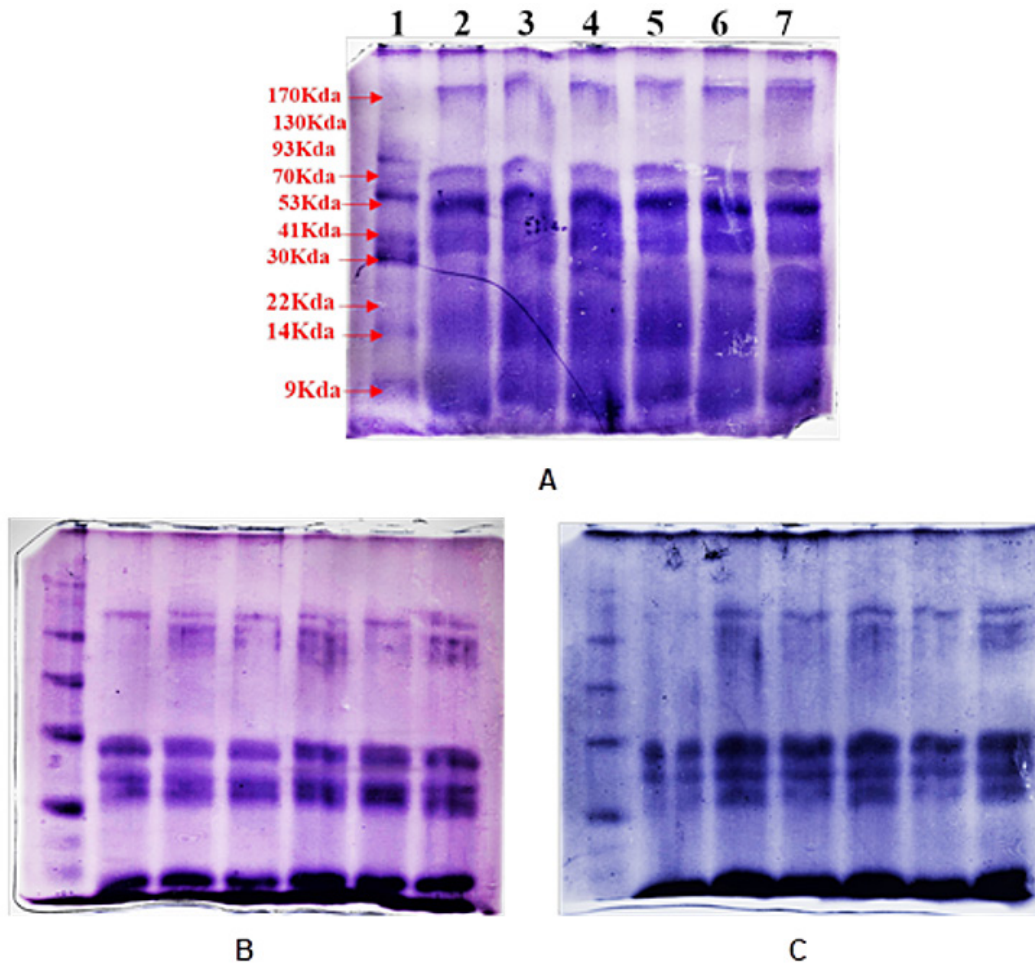


Figure 1. SDS-PAGE (A), 12% SDS-PAGE gel, (B) 15% SDS-PAGE, (C) 18% SDS-PAGE. Lane-1 Marker, lane-2(104.05µg) Non-reducing, lane-3(104.05µg) reducing, lane-4(112.84µg) Non-reducing, lane-5(112.84µg) reducing, lane-6(120.9µg) Non-reducing and lane-7(120.9µg) reducing

**Fig.1: SDS-PAGE of MWE**



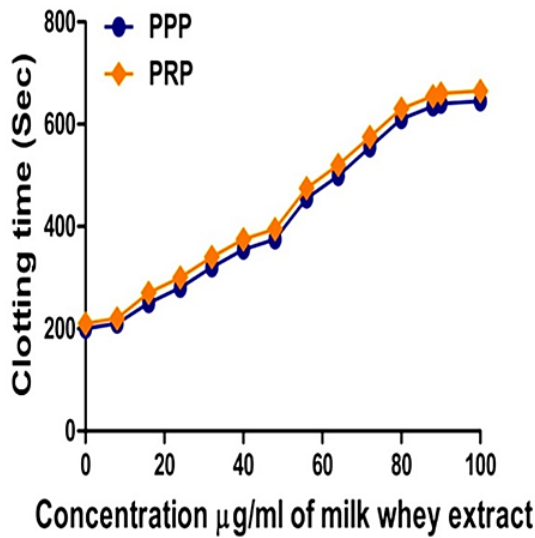


Fig. 2: Plasma recalcification time of MWE

**Antithrombotic Activity of MWE**

To determine the antithrombotic capabilities, *in vitro* plasma recalcification time, and *in-vivo* bleeding time were investigated. The study investigated the antithrombotic capabilities of MWE in human normal plasma. Figure 2 showed for both PPP, PRP, normal clotting time was 210 sec, and that the MWE demonstrated prolonged clotting time up to 770 sec and exhibits anticoagulant activities. Each point represents the mean±SD of three independent experiments, P<0.02. Many research showed natural products and their derivatives exhibits antithrombotic properties.<sup>41</sup> Additionally, Figure 3 represents MWE was verified by *in vivo* tail bleeding assay, the mice were injected with MWE solution in concentration gradient (0-100 μg) intravenously, which dramatically increased the bleeding time to 720 sec compare to control. Prolonged bleeding time indicates anticoagulant property of MWE. Further, the role of MWE in the intrinsic/ extrinsic pathway of the blood coagulation cascade was confirmed by measuring APTT and PT. surprisingly MWE prolonged clotting time of APTT only without altering PT indicating it interferes intrinsic pathway of the blood coagulation cascade. Each point represents the mean±SD of three independent experiments, P<0.01 From Marine creatures, herbal medicines, fermented food products like Japanese Natto and Korean Chungkook-Jang soy sauce, dung beetles, food-grade microorganisms, snake venoms, and earthworm secretions, several effective

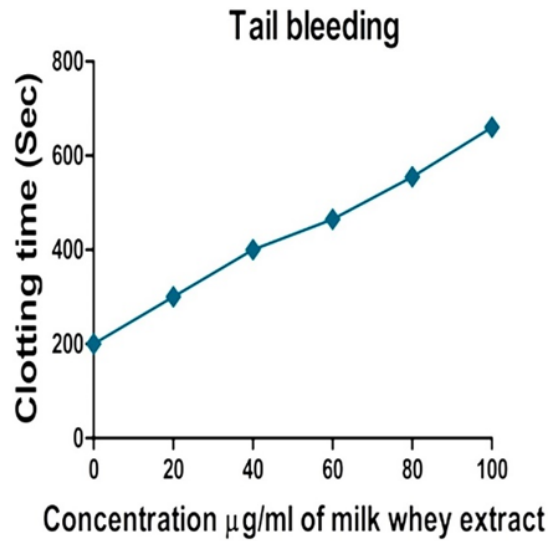


Fig. 3: *In vivo* tail bleeding assay of MWE

anticoagulant proteins/enzymes were identified by Mulenga A, *et al.* (2013).<sup>42</sup> Figure 4. Represents, the amount of retraction is gradually inhibited by pre-incubating MWE with whole blood. 90 % of the blood clot was destroyed when MWE (0–100μg) was incubated against the positive control streptokinase (600 U/ml). Each point represents the mean±SD of three independent experiments, P<0.01. Clot retraction assay confirms that MWE exhibits strong antithrombotic properties. All the above studies confirm antithrombotic properties of MWE. Several scientific reports showed milk and milk derived such as yogurt, cheese, etc. exhibit antithrombotic.<sup>43</sup>

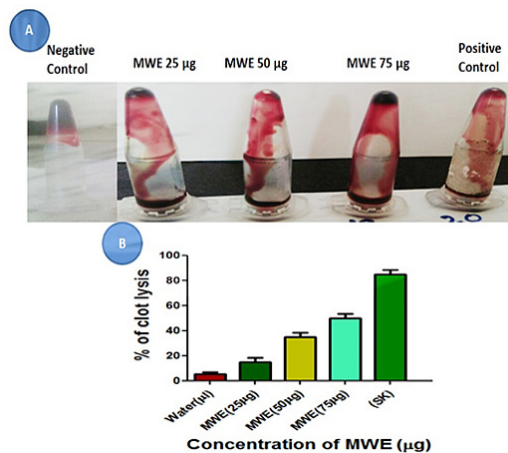


Fig. 4 (a) Whole blood clot lysis image (b) Percentage of whole blood clot lysis of MWE

**MWE Inhibits Platelet Aggregation**

The current study identified the effect of MWE on platelet aggregation was analysed using agonists such as ADP and epinephrine in PRP. Impressively, MWE at 30 µg concentration showed 74% of ADP induced inhibition (Figure 5) and 100% inhibition for epinephrine (Figure 6). Both agonists induced platelet aggregation inhibition at concentration of 0-30 µg of MWE. Figure 5. represents(A)Traces of platelet aggregation, (B) dose dependent platelet aggregation%, (C) dose dependent platelet aggregation inhibition%. Figure 6. represents(A) Traces of platelet aggregation,(B) Dose dependent platelet aggregation%, (C) dose dependent platelet aggregation inhibition%. The values represent

± SD of three independent experiments. During the clotting mechanism in primary haemostasis, activation of platelets takes place by many agonists like ADP, thrombin, collagen, DAG, epinephrine, and platelet-activating factor (PAF). In thrombotic disorders, hyperactivation of platelets is commonly observed, which is treated using Eptifibatide, a derivative from rattlesnake venom that inhibits glycoprotein IIb/IIIa receptor and so on Jung JL *et al.*(2013)<sup>44</sup> and Several platelet aggregation inhibitors have been identified from plants and animal sources.<sup>45-46</sup> Hence, aggregation results show that MWE proved to be a good therapeutic contender in the field of blood clotting /thrombotic disorders prevalence and treatments.

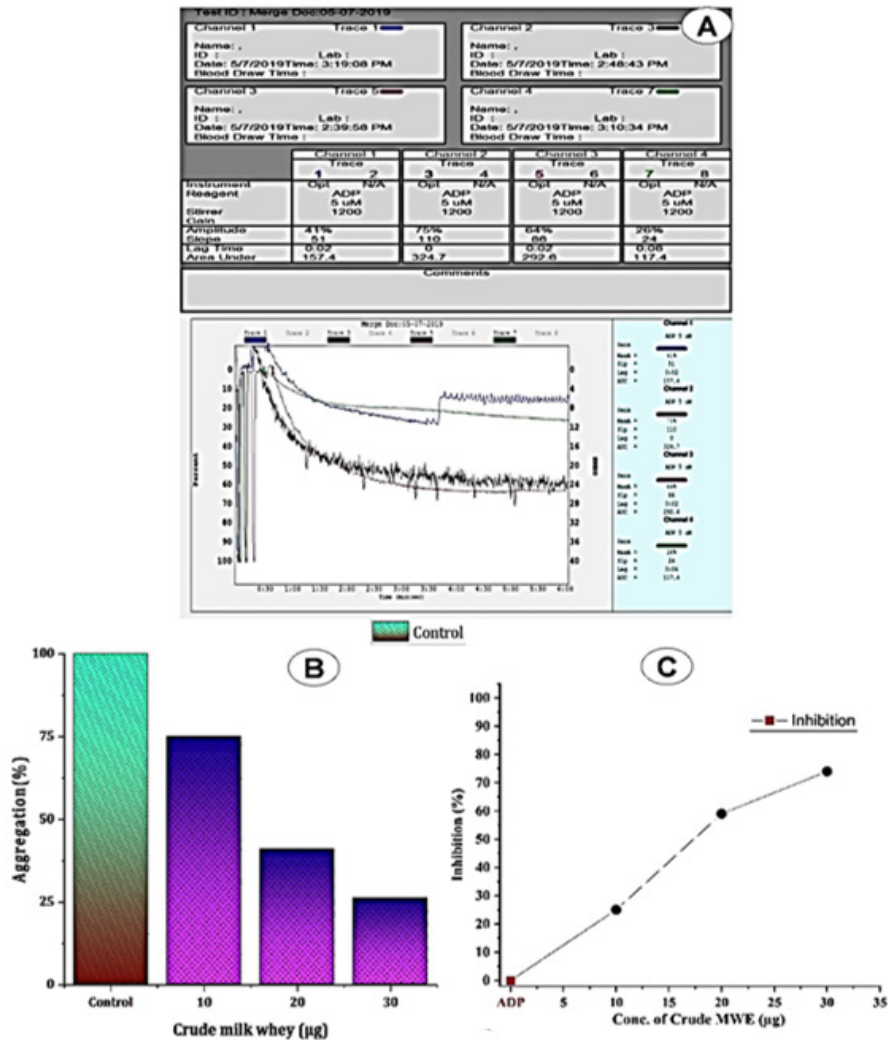


Fig. 5: Inhibition of ADP-induced platelet aggregation by MWE

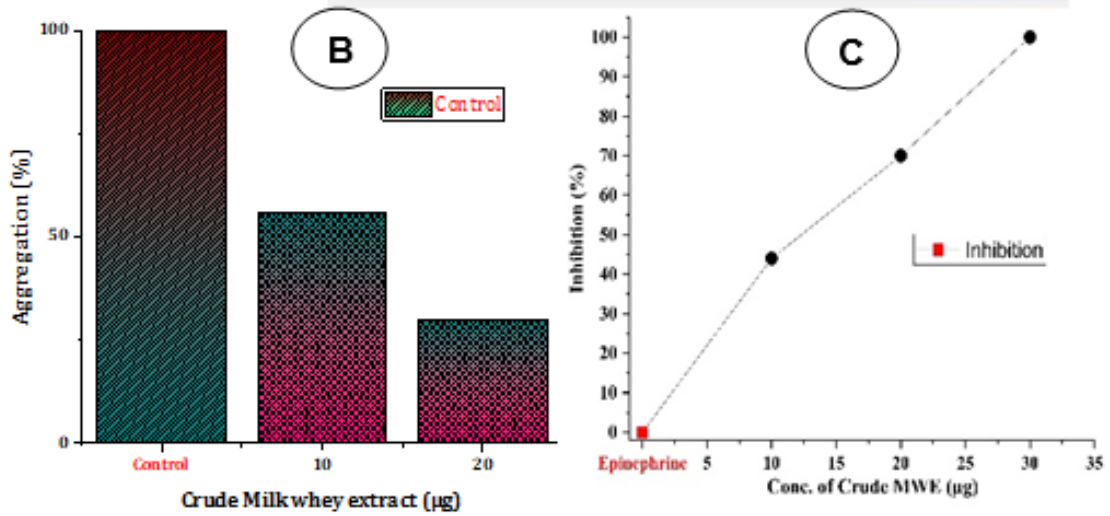
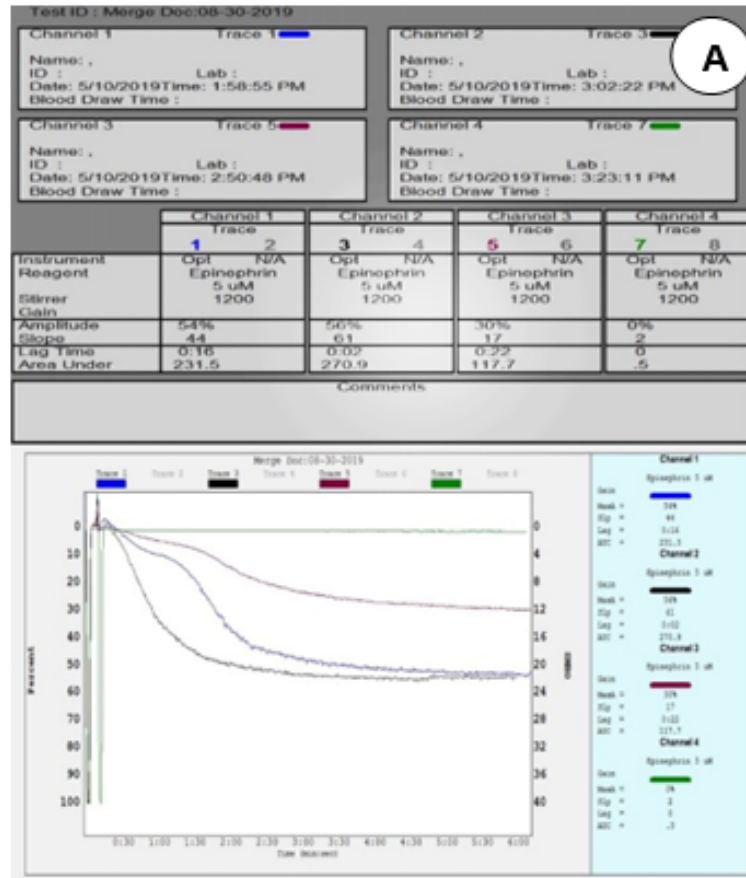
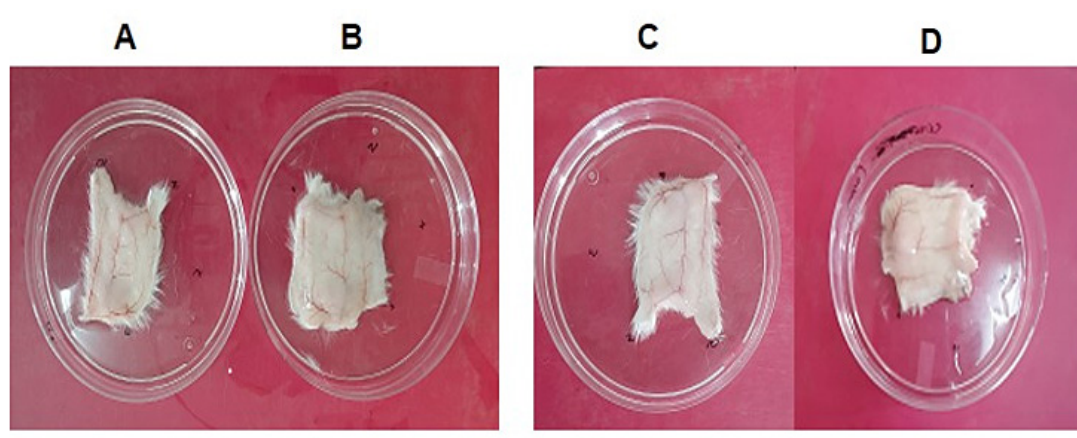
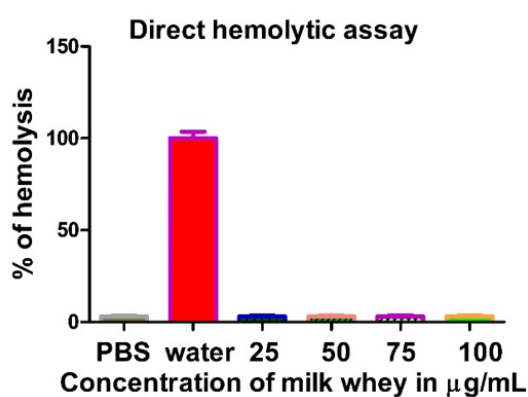


Fig. 6: Inhibition of Epinephrine-induced platelet aggregation by MWE



**Fig. 7: The dose-dependent hemorrhagic activity of MWE.**  
(A) Saline (B) 125 µg, (C) 250 µg, (D) 375 µg of MWE independently into mice in an of 50 µl intradermal. MWE :Milk whey extract



**Fig. 8: Direct hemolytic assay of MWE**

#### Nontoxic Property of MWE

MWE did not released haemoglobin by hemolysis of RBC showed in Figure 8. Packed RBC and PBS incubated with MWE concentration from 0-100 µg for 1 h at 37°C (Isotherm forced convection lab incubator, Inlab Equipments (*Madras*) Pvt. Ltd). Positive control: Water, negative control: PBS for percentage of hemolysis. The amount of hemoglobin released in the supernatant was measured at 540 nm(Thermo Nicolet iS50-Thermo Fisher Scientific, USA). Figure 7 showed MWE did not cause hemorrhage and edema was induced (data not revealed) in experimental mice up to the concentration of 100 mg. Interestingly, MWE did not cause hemolysis as shown in fig. 8 when it was treated with RBC and did not cause hemorrhage and

edema in experimental mice suggesting its non-toxic nature. The bioactive compounds found in MWE are thought to be interfering with the blood coagulation cascade based on the observations of all the aforementioned actions devoid of toxic properties.

#### Conclusion

In summary, proximate analysis of MWE results found to be higher content of macronutrients carbohydrates and proteins, and micronutrients like vitamin A, potassium and calcium. In this study, an attempt was made to explore the antithrombotic and antiplatelet activities of MWE. Purified compounds of MWE proved to be good nutraceutical molecules and can be fortified with the meal to treat and manage cardiovascular and other life style disorders. Further purification and characterization of these bioactive compounds/ proteins appear to be interesting.

#### Acknowledgment

B.M and A.T thank the University Grant Commission, New Delhi.

#### Funding source

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

#### Conflict of Interest

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

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