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Phytochemical Analysis, in vitro Anti-coagulant Activity of Different Solvent Fractions of Citrus medica Fruit Extract

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Abstract:

Background: Blood coagulation is an essential and tightly regulated process that swiftly forms clots. However, disruptions in blood coagulation are often observed in various disease conditions. This study focused on exploring the impact of partitioned solvent fractions of methanolic extract of citrus medica fruit anticoagulant using in vitro methods, seeking new therapeutic purposes for this plant. Dried and powdered fruit of citrus medica were extracted with 70% methanol, and the concentrated dried crude extract was subsequently subjected to liquid partitioning with petroleum ether, benzene, ethyl acetate, and butanol. Varying concentrations (2.5–10 mg/mL) of the fractions were tested in vitro on blood coagulation profile; clotting time (CT), prothrombin time (PT), and activated partial thromboplastin time (aPTT) and antioxidant potential. GCMS analysis of highest anticoagulant fraction was carried out.

Result: All fractions of citrus medica significantly ($P<0.05$) prolonged the clotting time, prothrombin and activated partial thromboplastin times. The highest prolongation effect was recorded with the butanol fraction at concentration of 7.5 mg/mL. From GCMS analysis data, fifteen compound present in butanol fraction to exhibit antioxidant and anticoagulant activity.

Conclusion: The study's findings highlight the potential of antioxidant and anticoagulant activity of citrus medica specifically its butanol fraction, as a promising and untapped source of bioactive molecules with therapeutic applications. It can be explored further for the development of new therapeutics targeting various health conditions. This discovery opens up exciting possibilities for harnessing the plant's bioactive molecules in the pursuit of novel therapeutic interventions.

Keywords: Blood coagulation, clotting time, prothrombin time, solvent partitioning, citrus medica

Introduction

Thrombosis ranks as the foremost global cause of mortality and stands as the most prevalent among non-communicable diseases targeted by the WHO for a reduction in incidence (source: WHO Fact Sheets, accessed on September 1, 2022). This condition arises from vascular blockages and typically manifests in clinical settings through instances such as myocardial infarction (occurring in 5 out of every 1000 individuals annually) (Fang & Alderman, 2002), stroke (with an annual incidence ranging from 1.3 to 4.1 per 1000 individuals) (Feigin VL et.al., 2003), or venous thromboembolism (abbreviated as VTE, affecting 1 to 3 per 1000 individuals annually) (Jonh AH et. al., 2016). To manage arterial thrombotic events like myocardial infarction and stroke, antiplatelet medications are primarily employed, while anticoagulants are used for prevention and treatment of VTE. As the name suggests, anticoagulants counteract the process of coagulation, which is also known as secondary hemostasis (Mackman et. al., 2020). Hemostasis is the body's natural mechanism for halting vascular disruptions and bleeding resulting from external injuries, achieved through the formation of blood clots in healthy individuals. However,

in pathological circumstances, coagulation initiates the formation of undesired intravascular blood clots, commonly referred to as thrombi (Ten Cate et. al., 2017).

The initial anticoagulant medications to counteract VTE, unfractionated heparin and warfarin, were stumbled upon unexpectedly during the early 20th century (Last, 2002). In subsequent years, these medications underwent enhancements to more effectively prevent or manage VTE. More recently, a new wave of anticoagulant drugs has been introduced. These drugs are specifically designed to target coagulation factor IIa (thrombin) and coagulation factor Xa (FXa), two pivotal enzymes in the coagulation process (Weitz et. al., 2017). Depending on the medical context, modern physicians possess the capability to prescribe varying anticoagulant medications tailored to the requirements of their patients. Nonetheless, despite the efficacy of anticoagulant drugs in VTE prevention, they Anticoagulant drugs still pose limitations in terms of flexibility and the risk of associated bleeding (Pollack et. al., 2020). Thus, there is an urgent need to develop a safe, effective, and affordable anti-thrombotic drug for clinical therapy. Recently, interest has grown in isolating thrombolytic agents and anti-thrombotic compounds from natural sources, including food, to offer safer and more effective alternatives (Lapikova et. al., 2008). Medicinal plants have historically been a valuable source of anticoagulant and anti-thrombotic molecules (Chaves et. al., 2010).

Citrus medica, commonly known as the citron, is a unique and ancient citrus fruit that has been cultivated and valued for its culinary, medicinal, and religious significance for centuries. Citrus is one of the most important commercial fruit crops grown in all continents of the world. Among the different species of citrus fruits, the Citrus medica is an important medicinal plant of the family Rutaceae (Rafiee et. al., 2007). Citrus medica traditionally used as an appetizer, carminative, refrigerant, stomachic, tonic, antispasmodic, expectorant, cardiotonic, and induration of the spleen tumors (Hartwell, 1982). Citrus medica peel is eaten raw with rice, also in remedy for dysentery (Bhuiyan et. al., 2009). Citrus medica is relevant to treatment of diabetes and Alzheimer's disease (Filomena et. al., 2007). The plant is reported to possess anthelmintic and repellent Activity (Bakare et. al., 2012), antimicrobial activity (Oliveira et. al., 2014), hypolipidemic activity, Hypoglycemic and antidiabetic activity (Sah et. al., 2011), insulin secretagogue activity (Peng et. al., 2009), anti-inflammatory (Yi et. al., 2010) and pain reducing activity in rats (Archana et. al., 2010), anti-implantation activity (Kachroo and Agrawal, 2011), antifertility activity (Patil et. al., 2013), Antiulcer activity (Nagaraju et. al., 2012), anti urolithiatic activity (Chavada et. al., 2012). The fruit juice exerts antimutagenicity and anticancer effect (Entezari et. al., 2009), antioxidant activity and cardio protective potential (Al-Yahya et. al., 2013).

Material and Method

Collection and preparation of powder:

Citrus medica fruit was sourced from the local area of Rajkot. Dr. Rutva Dave, Assistant Professor in the Department of Botany at H.&H.B. Kotak Institute of Science, Rajkot, Gujarat, India has verified its identification. The herbarium (BKMPC/01-03/2022) was preserved in the Department of Pharmacognosy at B.K. Mody Govt. Pharmacy College, Rajkot. Subsequently, the fruit was washed with water and left to air dry. Once dried, it was finely powdered using a mixed grinder (MG Livo MX-151, Maharaja mixer grinder) and sieved through a mesh (420). The resulting fine powder was stored in an airtight container for future use.

Preparation of fraction:

The powder underwent an exhaustive hydroalcoholic (30:70) extraction using the maceration method at room temperature for 24 hours. The resulting solutions were filtered through Whatman filter paper (No.42). Subsequently, the filtrates were recovered using a rotary film evaporator at 40°C, thoroughly dried, and stored in a sealed jar at 4°C for future use. For further partitioning, the crude extract (25 g) was subjected to successive solvent-solvent partitioning using solvents of increasing polarity - petroleum ether, benzene, ethyl acetate, butanol, and water (3×200 ml for each solvent type). The solvent portions of the respective fractions were evaporated, yielding the petroleum ether fraction (CMPF), benzene fraction (CMBF), ethyl acetate fraction (CMEAF), and butanol fraction (CMBUF), respectively. In each step of solvent fractionation, 20 ml of distilled water was added. The methanol-insoluble residues were designated as the water fraction (CMWF). All fractions were evaporated at a low temperature of 40°C and stored for subsequent use.

Preliminary phytochemical screening

The extracts underwent a preliminary phytochemical screening to assess the presence of flavonoids, tannins, phenolic compounds, alkaloids, glycosides, terpenoids, steroids, carbohydrates, and proteins. This screening was conducted using specific chemical tests corresponding to each compound (Khandelwal, 2009).

Total phenolic content

The total phenolic contents in the fractions were quantified using the modified Folin-Ciocalteu method (Kaur and Kapoor, 2002), with gallic acid serving as the standard. For this analysis, 200 µl of each fraction (1 mg/ml) was mixed with 2.8 ml of distilled water, 0.5 ml of Folin-Ciocalteu reagent, and 2.0 ml of 20% (w/v) sodium carbonate. The tubes were vortexed for 15 seconds and then left in the dark for 60 minutes for color development. Subsequently, the absorbance was measured at 650 nm using a UV spectrophotometer. The results were calculated and expressed as milligrams of Gallic Acid Equivalent (GAE) per gram of dry weight.

Total flavonoid content

The total flavonoids were assessed following the method outlined by Ordon-ez AAL (Ordon-ez et. al., 2002). For this, 0.5 ml of each fraction was mixed with 1.5 ml of methanol, 100 µl of 10% aluminum chloride, 100 µl of 1 M potassium acetate solution, and 2.8 ml of distilled water. After incubating the mixture at room temperature for 1.5 hours, the absorbance was measured at 420 nm. A standard curve was established using different concentrations of quercetin in methanol (20, 40, 60, 80, and 100 µg/ml), and the total flavonoids content was expressed as milligrams of Quercetin Equivalent (QUE) per gram of dry weight.

Determination of Total Antioxidant Capacity

The determination of the total antioxidant capacity was conducted using a method as described by Prieto with slight modifications (Prieto et. al., 1999). The total antioxidant capacity of the various fractions was assessed by combining 0.5 ml of each fraction (containing 500 µg of the respective fraction) with 3.0 ml of the Phosphomolybdenum reagent (composed of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 1% ammonium molybdate) in test tubes. The test tubes were then incubated at 95 °C for 10 minutes to allow the reaction to complete. After cooling to room temperature, the absorbance was measured at 695 nm using a spectrophotometer against a suitable blank. The final results were expressed as milligrams of Ascorbic Acid Equivalent (AAE).

In-vitro anticoagulant activity

Experimental animals

Sprague Dawley rats with a weight range of 180 to 220 grams were sourced from the animal house facility. These rats were housed in ventilated rooms maintained at a temperature of 24 ± 2 degrees Celsius, with a 12-hour light-dark cycle and a relative humidity of 54 ± 5%. They were provided with standard pellet food and had access to water ad libitum throughout the experimental period. The animals underwent a one-week habituation period. The experiments were conducted in accordance with the guidelines established by the CPCSEA and were approved by the IAEC (Institutional Animal Ethical Committee) (BKMGPC/IAEC28/RP91/2022) of B. K. Mody Government Pharmacy College, Rajkot, Gujarat, India.

Blood sampling and plasma preparation

Venous blood was directly collected from healthy subject and used for the clotting time measurement. Venous blood was collected in1:10 (v/v) 3.8% trisodium citrate for invitro anticoagulant assay. The citrated blood samples were then subjected to centrifugation at 6000 rpm for 10 minutes using a Compact Centrifuge to obtain platelet poor plasma (PPP) (Shah et. al., 1999). The plasma was separated and stored in the refrigerator at −4 °C until use.

Blood clotting time measurement

The measurement of in vitro clotting time was performed using a modified method based on Lee and White, as previously reported by (Osoniyi and Onajobi, 2003). Clotting tubes were prepared, containing 0.5 ml of various fractions of *Citrus medica* suspended in Normal Saline (NS) at concentrations ranging from 2.5 to 10 mg/mL. A

control tube with NS alone was also included. These tubes were then incubated in a water bath at 37 °C. After incubation, 0.5 ml of freshly drawn blood was carefully introduced into each of the incubated tubes by allowing it to flow down the side of the tube, while simultaneously starting a stopwatch. At 30-second intervals, the tubes were gently tilted to an angle of 45° to observe for the formation of a blood clot. The time taken for the first observation of clot formation was recorded, and the tilting process was continued at intervals until the tubes could be inverted without blood flowing out. The stopwatch was immediately stopped, and the time was recorded as the final clotting time.

Prothrombin time (PT) activity assay

The PT assays were conducted using commercially available reagent kits (PT (Prothrombin time)®, Robonik India Pvt. Ltd., India) following the recommended protocols provided by the manufacturer (Gao et. al., 2014). For each assay, 100 µl of plasma sample was mixed with 100 µl of various fractions (ranging from 50 to 500 µg/µl) that were diluted in normal saline. After a 5-minute pre-incubation period, 100 µl of PT assay reagent (consisting of rabbit brain extract and calcium chloride) pre-warmed at 37°C for 10 minutes was added, and the clotting time was recorded and measured. A vehicle control using normal saline was used for comparison. Each PT assay was performed in triplicate.

Activated prothrombin time (APTT) activity assay

APTT activity assays were conducted using commercially available reagent kits (APTT (Activated prothrombin time testing)®, Robon 6t ik India Pvt. Ltd., India) following the recommended protocols provided by the manufacturer (Gao et. al., 2014). For each assay, 100 µl of plasma was mixed with 100 µl of various fractions (ranging from 50 to 500 µg/µl) at 37°C. After a 5-minute pre-incubation period, pre-warmed APTT reagent was added to the mixture. The clotting time was measured after the addition of pre-warmed 50 µl of CaCl2 solution. Normal saline was used as a vehicle control. The APTT assay was performed in triplicate.

Gas chromatography-mass spectrometry (GC–MS) analysis.

The ethyl acetate fraction of *Citrus medica* underwent GC-MS analysis using the Toshvin GCMS-TQ8040 system equipped with a fused silica capillary column (30 m in length × 250 µm in diameter × 0.25 µm in thickness), packed with Elite-5MS. Pure helium gas (99.99%) was utilized as the carrier gas, flowing constantly at a rate of 1 mL/min. For GC-MS spectral detection, an electron ionization energy method with a high ionization energy of 70 eV (electron Volts) was adopted, with a scan time of 0.2 seconds and fragments ranging from 40 to 800 m/z. The injection quantity was set to 1 µL (split ratio 10:1), and the injector temperature was maintained at a constant 250 °C. The column oven temperature was initially set at 50 °C for 3 minutes, then increased at a rate of 10 °C per minute up to 280 °C, and finally raised to 300 °C for 10 minutes. The identification of phytochemicals in the test samples was accomplished by comparing their retention time (in minutes), peak area, peak height, and mass spectral patterns with those of authentic compounds stored in the National Institute of Standards and Technology (NIST) library 2008.

Static Analysis:

The data were presented as means ± SD based on three measurements (n=3). Statistical comparisons of the extended time among all extracts and the vehicle control (0.9% Normal Saline) were performed using one-way ANOVA (Multiple comparison) with GraphPad Prism version 6.0 (San Diego, California). A p-value greater than 0.01 was considered statistically significant.

Result:

Preliminary phytochemical screening

The initial screening of phytochemicals indicated that the fractions exhibited the existence of triterpenoids, glycosides, phenolic acids, flavonoids, sterols, and tannins (Table 1).

Table 1. Phytochemical screening of solvent fractions of *Citrus medica* fruit

Chemical Group	Name of Fractions				
	CMPF	CMBF	CMEAF	CMBUF	CMWF
Alkaloids	-	-	-	-	-
Carbohydrate	-	-	+	-	+
Glycosides	-	-	+	+	+
Triterpenes	+	+	+	+	+
Sterols	-	+	+	+	+
Flavonoids	-	+	+	+	-
Phenolics / Tannins	-	+	+	+	+
Saponins	-	-	+	+	+
Proteins	-	-	-	-	-

CMPF – Citrus medica Petroleum ether fraction, CMBF - Citrus medica benzene fraction, CMEAF - Citrus medica ethyl acetate fraction, and CMBUF - Citrus medica butanol fraction

Total phenolic content

The total phenolic content of the fractions was determined and expressed as milligrams of Gallic acid equivalents per gram of dry weight. The results are presented in table 2. Among the fractions, CMEAF exhibited the highest phenolic content (175.77 ± 2.58 mg/g), followed by CMBUF (96.89 ± 2.67 mg/g).

Table 2. Phenolic, flavonoid content and total antioxidant activity of fractions of *Citrus medica* fruit

Name of fractions	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QAE/g)	Total Antioxidant capacity (mg AAE/g)
CMPF	21.23 ± 2.97	09.54 ± 0.84	51.54 ± 4.75
CMBF	36.33 ± 2.74	10.26 ± 0.87	58.22 ± 5.21
CMEAF	175.77 ± 2.58	41.81 ± 0.91	188.42 ± 4.75*
CMBUF	96.89 ± 2.67	38.14 ± 0.88	139.65 ± 5.35*
CMWF	45.34 ± 2.82	12.22 ± 0.89	78.2 ± 4.11

Values expressed as mean ± SEM with n = 3.

Total flavonoid content

The total flavonoid content of the fractions was determined and expressed as milligrams of quercetin equivalents per gram of dry weight. The results are presented in table 2. Among the fractions, CMEAF exhibited the highest flavonoid content (41.81 ± 0.91 mg/g), followed by CMBUF (38.14 ± 0.88).

Total antioxidant capacity

The evaluation of total antioxidant capacity measures a sample's ability to donate electrons, thereby neutralizing the effects of free radicals such as ROS. Among the fractions, CMEAF showed the highest TAC at 188.42 ± 4.75 mg AAE, followed by CMBUF (139.65 ± 5.35 mg AAE), CMWF (78.2 ± 4.11 mg AAE), and CMBF (58.22 ± 5.21 mg AAE). Notably, the butanol, ethyl acetate, and aqueous fractions exhibited significant antioxidant activity during this test, as demonstrated in table 2. However, it is important to highlight that the aqueous fraction displayed a notably lower antioxidant activity compared to the ethyl acetate and butanol fractions.

Clotting time measurement

The fractions of *Citrus medica* fruit were found to have an impact on clotting time. The experimental results demonstrated that as the concentration of *C.medica* fractions increased, the clotting time was prolonged. The clotting time reached its maximum point at a concentration of 7.5 mg/mL. However, beyond this concentration, the clotting time decreased with further increases in concentration. These findings are depicted in Figure 1. The presence of anticoagulant(s) in *C.medica* is indicated by this result, suggesting that their optimal function occurs within a specific range of concentrations. At the tested concentration, all partitioned fractions of *C.medica* led to a significant increase in clotting time compared to the control samples. Among the tested fractions, the butanol, ethyl acetate, and aqueous fractions exhibited the longest clotting times (Figure 2). Notably, a significantly longer clotting time of the butanol fraction ($p > 0.001$) was observed at a concentration of 7.5 mg/ml compared to 10 mg/ml (Figure 3).

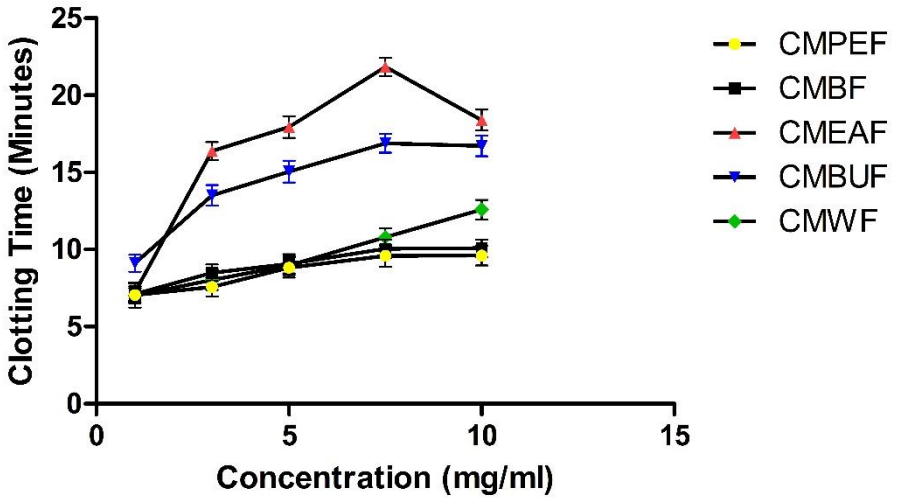

Figure 1: In vitro clotting time of varying concentrations of fractions of *Citrus medica* fruit

The fractions were suspended in normal saline. Results are expressed as mean ± SE values (n=3) for each concentration.

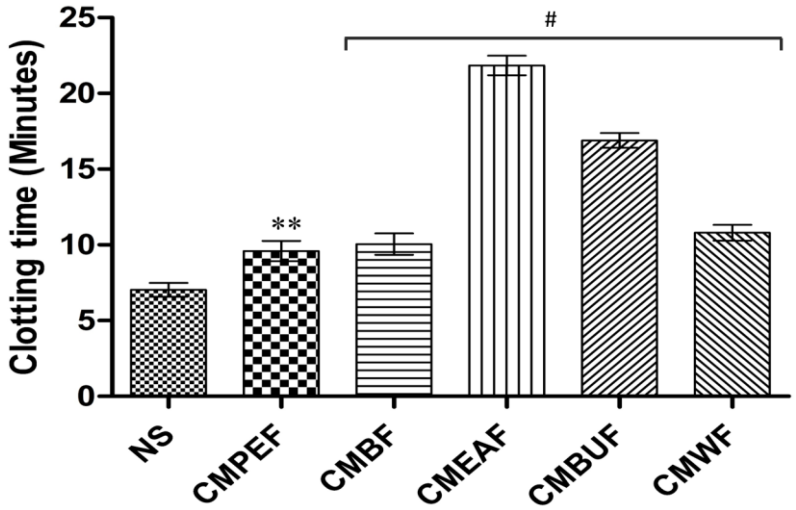

Figure 2: Mean clotting time of normal blood treated with different fractions at 7.5 mg/mL

Results are expressed as Mean ± SE values (n=3). All fractions were suspended in saline. *p<0.05, **p<0.01, #p<0.001 significant when compared with Normal saline (vehicle control).

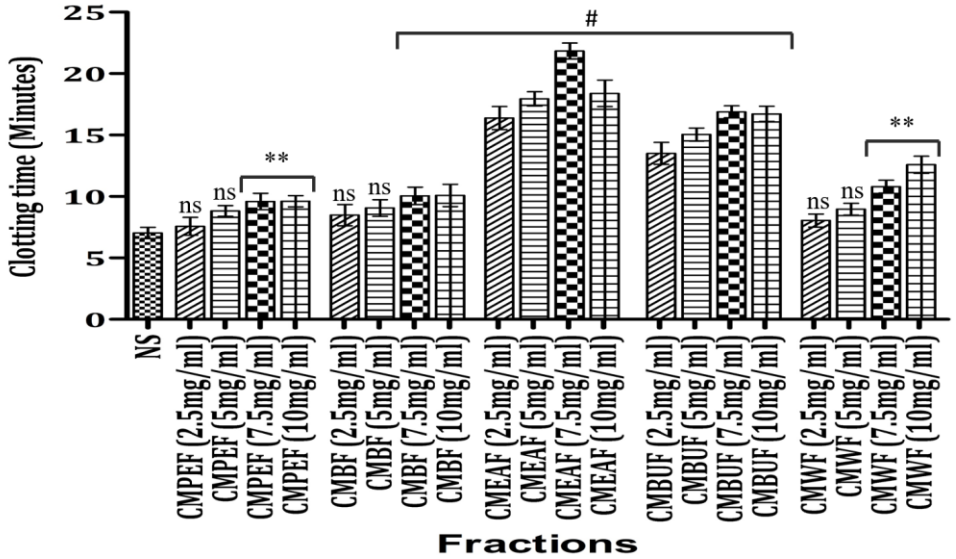

Figure 3: In vitro clotting time of normal blood treated with the fractions at 2.5 mg/mL, 5 mg/mL, 7.5 mg/mL & 10 mg/mL

Results are expressed as Mean ± SE values (n=3). All fractions were suspended in saline. *p<0.05, **p<0.01, ***p<0.005, #p<0.001 significant when compared with Normal saline (vehicle control).

PT assay

At a concentration of 7.5 mg/mL, all fractions of *C.medica* displayed a noteworthy (P > 0.01) increase in the Prothrombin Time (PT) compared to the control (Figure 4). The butanol fraction exhibited no significant difference in PT between the concentrations of 7.5 mg/mL and 10 mg/mL. However, other fractions showed significantly (P > 0.05) shorter PTs at a concentration of 10 mg/mL compared to 7.5 mg/ml.

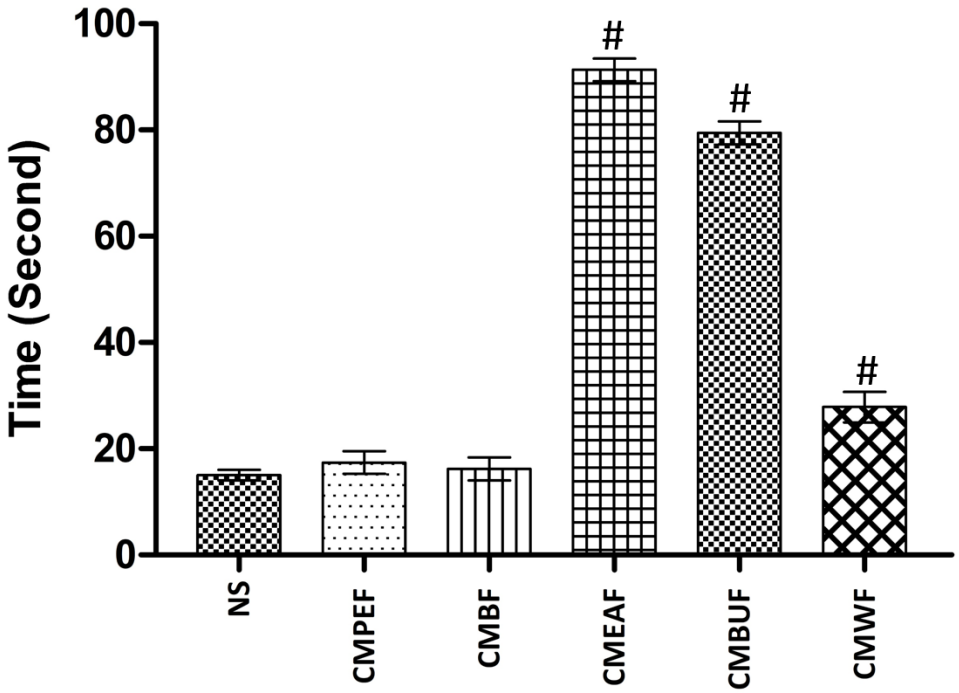

Figure 4: Prothrombin time of normal plasma treated with all fractions at 7.5 mg/mL

Results are expressed as Mean ± SE values (n=3). All fractions were suspended in saline. *p<0.05, **p<0.01, ***p<0.005, #p<0.001 significant when compared with Normal saline (vehicle control).

To further investigate the impact on PT, the fractions with the most significant effect on clotting time (butanol, Aqueous, and ethyl acetate fractions), representing the polar and non-polar fractions, were tested at concentrations of 2.5, 5, 7.5, and 10 mg/mL. A notable (P > 0.001) prolongation of PT was observed at all tested concentrations (2.5, 5, 7.5, & 10 mg/mL) compared to the control. The butanol fraction consistently demonstrated a significantly (P > 0.001) longer PT than the Aqueous fraction and ethyl acetate fraction at all concentrations. Additionally, the recorded PT was significantly (P > 0.05) longer at a concentration of 7.5 mg/mL compared to other concentrations (Figure 5).

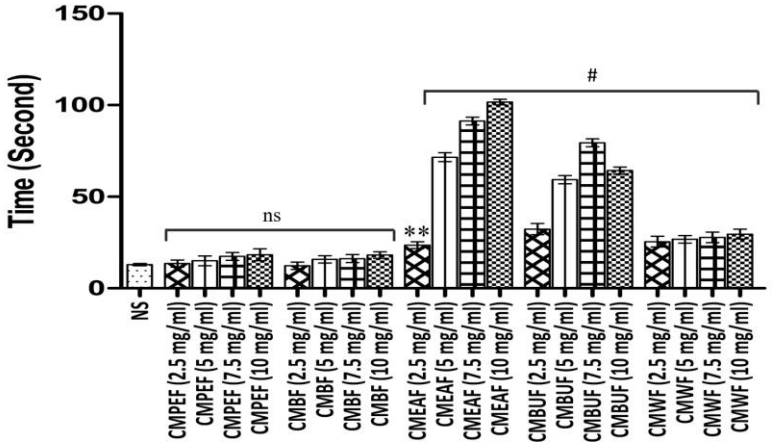

Figure 5: Prothrombin time of normal plasma treated with all fraction at 2.5, 5, 7.5 & 10 mg/mL

Results are expressed as Mean ± SE values (n=3). All fractions were suspended in saline. *p<0.05, **p<0.01, ***p<0.005, #p<0.001 significant when compared with Normal saline (vehicle control).

aPTT assay

All the fractions of *C.medica* demonstrated a significant (P > 0.001) increase in activated Partial Thromboplastin Time (aPTT) at all tested concentrations compared to the control. The measured aPTT times for the butanol and ethyl acetate fractions did not exhibit a significant difference between each other. However, both fractions displayed a notably (P > 0.001) longer aPTT at a concentration of 7.5 mg/mL (Figure 6).

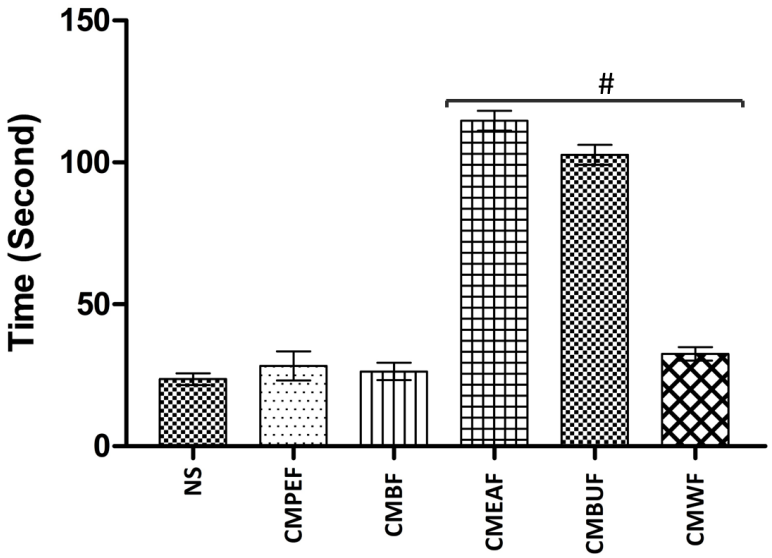

Figure 6: aPTT time of normal plasma treated with all fractions at 7.5 mg/mL

Results are expressed as Mean ± SE values (n=3). All fractions were suspended in saline. *p<0.05, **p<0.01, ***p<0.005, #p<0.001 significant when compared with Normal saline (vehicle control).

Longer aPTT times were observed for all the tested fractions at a concentration of 7.5 mg/mL in comparison to other concentrations (Figure 7). The butanol fraction exhibited the most significant prolongation effect on aPTT at the tested concentrations, with values of 70.66, 114.67, and 83.33 seconds at 5, 7.5, and 10 mg/mL, respectively.

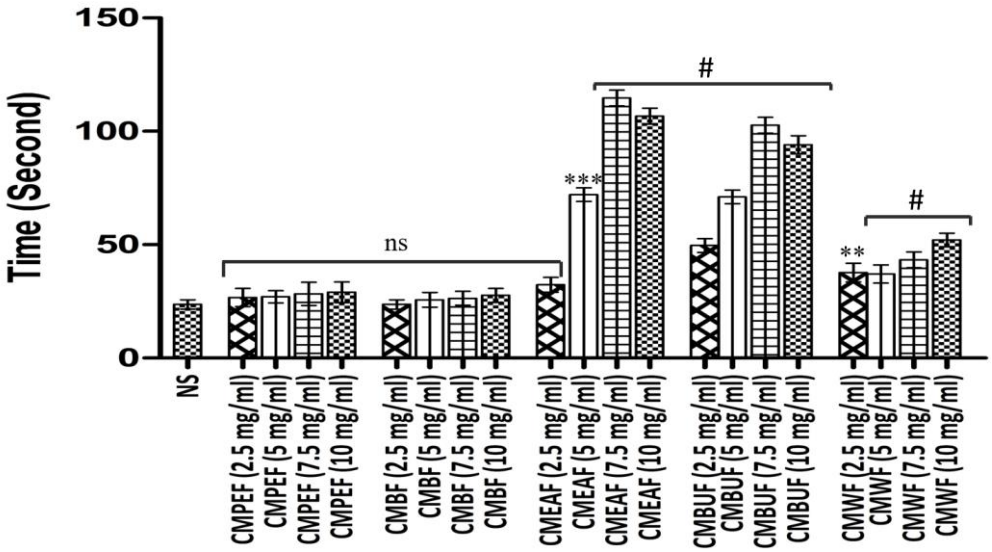

Figure 7: aPTT of normal plasma treated with all fraction at 2.5, 5, 7.5 & 10 mg/mL

Results are expressed as Mean ± SE values (n=3). All fractions were suspended in saline. *p<0.05, **p<0.01, ***p<0.005, #p<0.001 significant when compared with Normal saline (vehicle control).

Gas chromatography-mass spectrometry (GC–MS) analysis

The gas chromatography-mass spectrometry (GC-MS) analysis of the ethyl acetate fraction of *C.medica* revealed a total of 15 peaks (Figure 8). These peaks correspond to the bioactive compounds, which were identified by comparing their peak retention time, peak area (%), height (%), and mass spectral fragmentation patterns with those of known compounds listed in the National Institute of Standards and Technology (NIST) library. The analysis revealed the presence of 15 identified compounds in the ethyl acetate fraction of *C.medica* (Table 4).

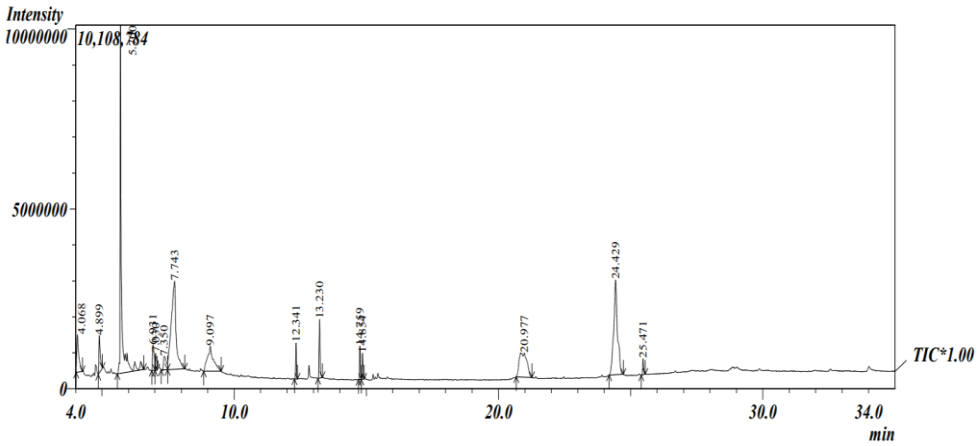

Figure 8: GCMS analysis of ethyl acetate fraction of *Citrus medica* fruit

Table 3 GCMS identified phytochemical components of the ethyl acetate fraction of *Citrus medica* fruit

S/N	Retention time	Name of compound	Molecular Formula	Peak Area (%)	Reported biological activity
1	4.068	Maltol	C6H6O3	3.95	antioxidant activity (Han et. al., 2015) that has been reported to ameliorate several diseases of the nervous system (Kang et. al., 2008), Diabetic Neuroprotective (Guo et. al., 2018), liver & kidney (Sha et. al., 2021), and anti-inflammatory activity (Ahn et. al., 2022)
2	4.899	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	C6H8O4	2.85	Antioxidant activity (Kim et. al., 2022), α-glucosidase inhibitability (Van Chen et. al., 2022), antifungal activity (Hamid et. al., 2016), antimutagenic activity (Berhow et. al., 2000) and also antitumor activity (Ban et. al., 2007), anti-plasmodial activity (Amlabu et. al., 2018)
3	5.7	5-Hydroxymethylfurfural	C6H6O3	21.21	antioxidant Activity (Zhao et. al., 2013) and antiproliferative activities, antioxidant, anti-ischemic, and anti-tyrosine enzyme effects, improving blood rheology and protective in hypoxic brain injury (Li et. al., 2011), anti-sickling (Abdulmalik et. al., 2005), anti-inflammatory (Yamada et. al.,2011), hepatoprotective (Ding et. al., 2010), antiarthritic (Feng et. al., 2011), anti-allergen (Alizadeh et. al., 2017)

4	6.931	Sulfurous acid, cyclohexylmethyl hexadecyl ester	C23H46O3S	2.31	Antioxidant activity (Soleha et. al., 2020)
5	7.03	Butane, 1,1'-[methylenebis(oxy)]bis[3-methyl- / Furazan-3-ol, 4-amino / 2-Pyrrolidinone, 3-hydroxy-3,4-dimethyl	C11H24O2	1.11	No activity reported
6	7.35	Pyrazole, 3-methylaminomethyl-5-propyl-	C8H15N3	1.99	No activity reported
7	7.743	Sucrose	C12H22O11	22.58	No activity reported
8	9.097	3-Deoxy-d-mannoic lactone	C6H10O5	7.7	No activity reported
9	12.341	Hexadecanoic acid, methyl ester	C17H34O2	1.36	Immunosuppressive (Saeidinia et. al., 2004), antioxidant and antibacterial activity (Ganesan et. al., 2022), Antifungal, anti-inflammatory, hypocholesterolaemia and cancer prevention activities (Kalpana et. al., 2012)
10	13.23	2H-1-Benzopyran-2-one, 5,7-dimethoxy-	C11H10O4	2.78	No activity reported
11	14.759	9,12-Octadecadienoic acid (Z,Z)-, methyl esteR	C19H34O2	1.42	anti-inflammatory, Antibacterial, Hypocholesterolemic and Hepatoprotective activities, Antioxidant and Antimicrobial properties (Rahman et. al., 2014), Anti-arthritic, Antihistamine (Henry et al., 2002)
12	14.854	8,11,14-Docosatrienoic acid, methyl ester	C23H40O2	1.29	Antitumor, Antioxidant and Anti-Inflammatory Activities (Chen et al., 2021)
13	20.977	Olean-12-en-3-ol, acetate, (3.beta.)-	C32H52O2	9.77	Antibacterial activity (Lazreg-Aref et al., 2012), Anti-inflammatory, antitumor, Antifungal, anti-diabetic, Anti-hyperlipidemic activities (Okoye et al., 2014),

					Antioxidative and antiviral activity (Parvez et al.,2018), Antiallergic, Antiulcer, Antinociceptive activity (Oliveira et al., 2004), Antiplatelet Activity (Aragão et al., 2007), Sedative, anticonvulsant and Anxiolytic activity (Aragão et al., 2009)
14	24.429	Lup-20(29)-en-3-ol, acetate, (3.beta.)-	C32H52O2	18.83	Antiprotozoal, Anti-inflammatory, Antitumor, Anti-prostate cancer, Anti-head and neck squamous cell carcinoma, Anti-melanoma, Cancer chemopreventive, Cardioprotective, Hepatoprotective, Antimicrobial, Antiurolithiatic, Antiallergic, antidiabetic, Anti-aging, snake venom Antiserum activity, Antifertility agents, Gastroprotective, protect in neurodegenerative disorder (Sharma et al., 2020), Antiplatelet Activity (Saputri et al., 2012)
15	25.471	Squalene	C30H50	0.85	Hypolipidemic, Hepatoprotective, Cardioprotective, Antioxidant, antitumor and Anti-toxicant activity (Muzalevskaya et al., 2015), Antimicrobial efficacy (Toh et al., 2023), Antidiabetic activity (Widyawati et al., 2023), Healing agent (Wołosik et al., 2013), Anti-cancer properties (Gunes et al., 2013), Cardioprotective and Antioxidant effect (Ibrahim et al., 2020),

	Anti-inflammatory activity (Lou-Bonafonte et al., 2018)

Discussion

Phytochemical screening

According to the existing literature, the bark of *C.medica* contains a range of phytochemical constituents. The preliminary phytochemical screening confirmed the presence of triterpenoids, glycosides, phenolic acids, flavonoids, sterols, and tannins in the fractions (Jaiswal et al., 2019).

Total phenolic & Flavonoid content

Phenolic compounds represent a diverse group of phytochemicals commonly found in various food and medicinal plants as secondary metabolites. These compounds possess redox properties that enable them to act as antioxidants (Soobrattee et al., 2005). Their ability to counteract free radicals is facilitated by the presence of hydroxyl groups, particularly 3-OH, making the total phenolic concentration a valuable parameter for quickly assessing antioxidant activity. Flavonoids, such as flavones, flavanols, and condensed tannins, are secondary metabolites found in plants. Their antioxidant activity relies on the presence of free hydroxyl (OH) groups, particularly 3-OH. Besides demonstrating antioxidant activity in laboratory tests, plant flavonoids also function as antioxidants in living organisms (Geetha et al., 2003). Research has indicated that the antioxidant properties of phenolic compounds are primarily attributed to their redox capabilities, hydrogen donation capacity, singlet oxygen quenching, and chain-breaking abilities (Rice-Evans et al., 1996). The significant presence of these compounds in methanolic, aqueous, and ethyl acetate fractions further motivated our investigation into their antioxidant potential. Hence, our current study suggests that the phenolic acids and flavonoids identified in the solvent fractions of *C.medica* may play a significant role in their antioxidant activity in in vitro environments.

Total Antioxidant activity

The assessment of total antioxidant activity is a crucial parameter utilized to evaluate the ability of a substance to combat oxidative stress and protect against oxidative damage. Numerous assays and techniques have been devised to quantify total antioxidant activity, and these have offered valuable insights into the potential health advantages of various compounds and substances (Agati et al., 2012).

Phenolic compounds, especially flavonoids, have been extensively studied for their antioxidative properties. These compounds possess redox capabilities that enable them to donate hydrogen atoms or electrons to free radicals, effectively neutralizing their harmful effects. The presence of hydroxyl (OH) groups in phenolic compounds is associated with their antioxidative activity, enhancing their ability to scavenge free radicals efficiently. The total phenolic concentration has been identified as a valuable indicator of a substance's antioxidative capacity, with higher concentrations often correlating with greater antioxidative activity (Agati et al., 2012).

Flavonoids, a specific subclass of phenolic compounds, are well-known for their antioxidative properties and are abundantly found in plant-based foods and beverages, such as fruits, vegetables, tea, and wine. They exhibit antioxidative activity both in vitro and in vivo, with mechanisms including free radical scavenging, inhibition of oxidative enzymes, chelation of metal ions, and modulation of cellular signaling pathways related to oxidative stress (Agati et al., 2012).

Numerous studies have explored the relationship between total antioxidant activity and health outcomes. A high dietary intake of antioxidant-rich foods, such as fruits and vegetables, has been linked to a reduced risk of chronic diseases, including cardiovascular diseases, certain cancers, and neurodegenerative disorders. However, it is important to acknowledge that while in vitro assays provide valuable information about antioxidant capacity, translating these findings to in vivo effects is complex. Factors like bioavailability, metabolism, and interactions with other molecules in the body can influence the overall antioxidative capacity observed in vivo (Fazal et al., 2023).

Overall, assessing total antioxidant activity is a crucial aspect of understanding the potential health benefits of various compounds and substances. Phenolic compounds, including flavonoids, play a significant role in contributing to this activity. Further research is essential to delve into the mechanisms of action, bioavailability, and specific health effects of different antioxidants to gain a comprehensive understanding of their impact on human health (Agati et al., 2012).

Anticoagulant Activity

A wide range of anticoagulant and procoagulant medications is commonly used to regulate blood coagulation in various medical conditions, such as cardiovascular disease, diabetes mellitus, and bleeding disorders. However, many of these drugs are associated with undesirable side effects. As a result, there is a need for the identification and development of new anticoagulant and procoagulant drugs that have fewer adverse effects. Polyphenols (Mirza et al., 2019), flavonoids (Guglielmonea et al., 2022), carotenoids (Melo et al., 2013), and polysaccharides (Amira M 2017) are known to exhibit multiple biological activities, including antioxidant, antiplatelet, anticoagulant, and antithrombotic effects. Researchers have demonstrated the anticoagulant activity of tannins, while some terpenoids are recognized for their antioxidant properties (Jing et al., 2014). Additionally, saponins are known to possess antiplatelet, anticoagulant, and fibrinolytic characteristics (Mosa et al., 2011).

The clotting parameters PT and aPTT are essential for assessing the clotting process. PT evaluates the activity of factors related to the extrinsic coagulation pathway, while aPTT measures factors associated with the intrinsic and common pathways. PT is commonly used to monitor the effectiveness of coumarin therapy (vitamin K antagonists), whereas aPTT is typically employed to evaluate the efficacy of heparin treatments (Davison C 2011). Abnormally prolonged PT and/or aPTT in clinical assessment indicate abnormalities in the activity of specific clotting factors. For example, if aPTT is prolonged while PT is normal, it suggests the need to analyse factors VIII, IX, and XI related to the contact pathways (Achneck et al., 2010). On the other hand, if both PT and aPTT are affected, it indicates issues with factors V, X, and prothrombin (factor II) of the common pathway. Thus, the elongated PT and aPTT resulting from treatment with *C.medica* imply the inhibition of factors V, X, and prothrombin in the common coagulation pathway. Further investigation is required to determine the specific mechanism of this inhibition.

However, one potential mechanism of action for the *C.medica* fractions could involve the direct inhibition of the common coagulation pathway. This inhibition may occur through multiple pathways, such as reducing the generation of thrombin (factor IIa) by inhibiting factor Xa and its cofactor Va, or by impeding the interaction between thrombin and fibrinogen, thereby preventing fibrin formation.

These observations suggest the presence of a protease inhibitor in the *C.medica*, which could effectively inhibit these proteases. Consequently, the conversion of zymogens into active factors Xa, Va, and thrombin would be prevented. Furthermore, the active component(s) of *C.medica* may activate the natural anticoagulant pathway by binding to Antithrombin III, inducing a conformational change that activates protein C (PC) into activated protein C (APC). Together with its cofactor (protein S), APC inhibits factors Va and VIIIa, which are crucial cofactors for the activation of factors Xa and IIa. Ultimately, this dual action inhibits the activation of factor Xa and thrombin in the common coagulation pathway, providing a potential explanation for the anticoagulant effects.

Heparin exerts its anticoagulant effects by binding to a specific site on Antithrombin, leading to a conformational change that exposes the binding site for inactivating factor Xa and thrombin (IIa) by Antithrombin about 1,000 times. This significantly enhances the anticoagulant activity of *C.medica* (Bryan et al., 2013). Warfarin, on the other hand, is a vitamin K antagonist that inhibits the activation or synthesis of vitamin K-dependent proteins involved in the coagulation pathway. These proteins include factors X, IX, VII, and prothrombin. Vitamin K is essential for the carboxylation of specific glutamic acid residues, which is necessary for the calcium-binding ability and physiological activation of these proteins (Truong et al., 2011).

Studies have suggested that all-trans retinoic acid exerts its anticoagulant effects by downregulating Tissue Factor and upregulating thrombomodulin expression, thereby increasing the antithrombotic potential of microvascular endothelial cells (Ghaffari et al., 2019). The anticoagulant activity of ethyl acetate and butanol fraction of

C.medica demonstrates a similar mechanism of action to heparin, with a narrow concentration range of activity peak indicating fast action and reversibility.

Conclusion

The study demonstrated that the ethyl acetate and butanol fractions of *C.medica* fruit possess high levels of phenolic and flavonoid content, which exhibit significant antioxidant and anticoagulant properties, offering potential for treating blood coagulation disorders. However, further in vivo studies are essential to gain a deeper understanding of the specific mechanisms through which the anticoagulant components of the plant exert their therapeutic effects.

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Author contributions

RK is the main contributor of the manuscript, writing and editing, and collecting data, editing, and submission/correspondence of the above research article. All authors read and approved the final manuscript.

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Not applicable.

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