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RESEARCH ARTICLE

GC-MS based metabolite profile, assessment of *in vitro* antioxidant and anticoagulant properties of *Melastoma malabathricum* L. extracts

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Abstract

Melastoma malabathricum L., commonly known as Indian Rhododendron is an herbaceous shrub, widely used in traditional medicine to treat diseases like diabetes, wound healing, and bacterial infections. This study highlights the metabolite profile, and antioxidant capacity along with the anticoagulant and *in vitro* thrombolytic properties of the leaf extract (MMLE) and flower extract (MMFE) of *M. malabathricum* L. GC-MS based metabolite profiling of the methanolic MMLE and MMFE established the presence of compounds like Squalene (12.63%), Catechol (6.04%), .beta.-Sitosterol (5.99%) in MMLE and 5-Hydroxymethylfurfural (17.97%), Diosgenin (10.09%), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (8.71%) in MMFE. Free radical scavenging activity assays affirmed the strong antioxidant properties of both MMLE and MMFE. Both MMLE and MMFE demonstrated better proteolytic specificity towards fibrinogen than casein. Further, electrophoretic analysis of fibrinogen degradation products revealed that MMLE exhibited αβ-fibrinogenase activity and degraded the γ-band of fibrinogen. However, MMFE demonstrated weak αβ-fibrinogenase activity with little effect on the γ-band of fibrinogen. MMLE exhibited dose-dependent anticoagulant properties and inhibited the prothrombin activation property of Factor Xa. However, MMFE showed no significant impact on the blood coagulation process despite its ability to inhibit Factor Xa. Both MMLE and MMFE had substantial *in vitro* clot (thrombus) lysis properties. The present study substantiates the presence of important metabolites with significant pharmacological roles. In addition, the study also highlights the potential effects of the plant extracts in blood coagulation processes which can be of therapeutic relevance in the field of cardiovascular drug discovery.

Keywords: *Melastoma malabathricum* L.; Metabolite Profiling; Antioxidant; Anticoagulant; Blood Coagulation; Thrombolytic.

1. Introduction

India has a long history of exploring a wide variety of bioresources, including several native medicinal herbs. Plant-based compounds are proven to be beneficial with preventative and therapeutic relevance for human health (Nyunt et al., 2019). Compared to synthetic pharmaceuticals, ethnic medicinal plants are considered to be the most reliable source of any form of drug against several life-threatening diseases. Approximately 80% of medications and one-third of people in affluent nations utilize traditional medicines made of substances derived from medicinal plants to deal with several ailments and diseases (Krishnaiah et al., 2011). *Melastoma malabathricum* L., a common evergreen shrub belonging to the Melastomaceae family, is native to the tropical and subtropical regions, especially in Southeast Asia, including India (Susanti et al., 2007; Amalia et al., 2019; Nyunt et al., 2019). Different parts of *M. malabathricum* L. have been reported to be used as a part of folklore medicines to treat various kinds of diseases (Khoo et al., 2014). Scientific exploration of *M. malabathricum* L. has elucidated numerous therapeutic aspects such as antidiabetic and antilipidaemic activity (Balamurugan et al., 2014), anticoagulant (Khoo et al., 2014), anti-bacterial, antioxidant and cytotoxic properties (Alwash et al., 2014). The present study explores the metabolite profile of *M. malabathricum* L., in addition to its antioxidant, anticoagulant and thrombolytic properties, to substantiate and provide validation of its use in folklore medicine. The study further aims to provide insight into the antioxidant and anticoagulant properties of *M. malabathricum* L.

2. Materials and methods

2.1. Materials

All chemicals and reagents were purchased from HiMedia, India. Aluminium chloride was purchased from Finar, India. DPPH and ABTS were obtained from SRL, India. Blood coagulation factors were purchased from Sigma or HiMedia, India. Liquicelin® and Uniplastin® were purchased from Tulip Diagnostics, India.

2.2. Collection of plant sample

Samples of *M. malabathricum* L. were collected from the localities of Dergaon (Lat: 21°41′ 60.00″N, Long: 93°58′ 0.001″E) and sent to the Botanical Survey of India, Shillong for its authentication and identification (Acc no. 99356).

2.3. Preparation of crude extracts

For the preparation of methanolic extracts, fresh leaves and flowers of *M. malabathricum* L. were collected, cleaned and shade-dried. The dried samples were ground into powder; 2.0 g of the dried powder of leaf and fruit was weighed and plunged in methanol in the ratio of 1:15 (w/v) for 72 hours. The mixture was filtered and concentrated using a rotary evaporator. The extracts were then stored at 4°C until further use. Aqueous extracts were prepared by maceration of fresh leaves and flowers using deionized water (autoclaved, pH 7.4) for 4 hours at 4 °C. The mixture was filtered and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was evaporated to dryness and used immediately.

2.4. GC-MS-based untargeted metabolite analysis of crude methanolic MMLE and MMFE

10 mg of the methanolic plant extracts of MMLE and MMFE was plunged in 1.0 ml of methanol (HPLC grade) for 24 h at room temperature with continuous shaking. The samples were then centrifuged at 5000 rpm, and the supernatant was filtered through regenerated cellulose (RC) syringe filter (0.45 µm) and transferred into glass vials for further analysis. The GC-MS analysis of MMLE and MMFE was performed on a Shimadzu GC2010 Plus - triple quadruple (TP-8030) GC-MS/MS system fitted with EB-5MS

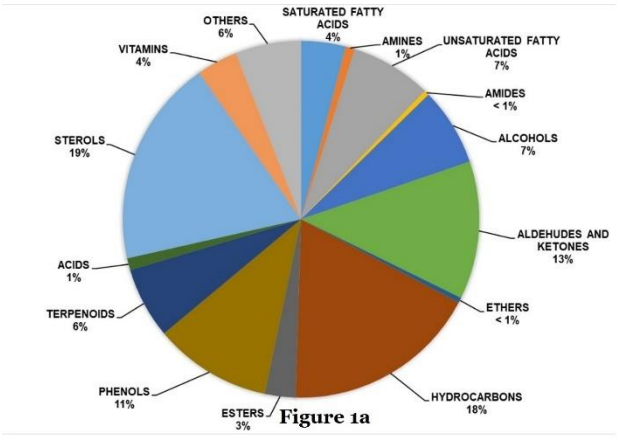


Figure 1. Graphical representation of the total metabolite profile of methanolic (a) MMLE, and (b) MMFE, identified by GC-MS. (c) Venn diagram representing common metabolites in MMLE and MMFE. The numerical values in the diagram represent the discriminatory metabolites (listed in Table 1) that are unique to MMLE and MMFE and, conversely, also shared between the extracts.

column. 1.0 µl sample was injected using Helium as carrier gas (1 ml/min) under split-less conditions. The oven temperature was programmed from 50 °C (isothermal for 2 min) with an increase of 3 °C/minute up to 150 °C followed by an increase of 4 °C/minute to 200 °C and ending with a 2 min isothermal at 200 °C. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV with an ion source temperature of 200 °C, and a continuous scan from 50 to 600 m/z was performed. The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST, USA) library. Noisy peaks and column bleed (silanes/siloxanes) were removed from the total ion chromatograms (TIC) before further studies (Dehingia et al., 2017; Juneja et al., 2020).

2.5. Determination of antioxidant activity

2.5.1. DPPH assay

DPPH assay was performed according to a previously standardized procedure with slight modifications (Herald et al., 2012). Briefly, 0.5 mg/ml of methanolic MMLE and MMFE was taken in a microtitre plate in varying concentrations (10-100 µl) in a final volume of 100 µl. 100 µl DPPH (0.1 mM) was added and the plate was incubated for 10 min in the dark and absorbance was calculated at 515 nm. DPPH % scavenging activity was calculated by using the following formula:

$$\text{DPPH \% scavenging activity} = \left[\frac{A_C - A_0}{A_C}\right] \times 100;$$

where, A_C is the absorbance of the control and A_0 is the absorbance of the sample. IC_{50} value was calculated using GraphPad Prism5.

2.5.2. ABTS assay

ABTS assay was performed following the protocol of Re et al (1999) with slight modifications. Briefly, 7 mM ABTS (1.0 ml) was mixed with 2.45 mM potassium persulphate (1.0 ml) for the preparation of $ABTS^{\cdot+}$. The mixture was incubated at room temperature in the dark for at least 16 hours. Thereafter, the absorbance of $ABTS^{\cdot+}$ solution was adjusted to 0.7 ± 0.1 with methanol at 734 nm. 50 µl and 100 µl of methanolic plant samples (0.01 mg/ml) were mixed with 100 µl of $ABTS^{\cdot+}$ solution and incubated at room temperature for 5 min. Thereafter, the absorbance was recorded at 734 nm using a plate reader (MultiSkan Sky High 1119500).

ABTS % scavenging activity was calculated by using the following formula:

$$\text{ABTS \% scavenging activity} = \left[\frac{A_C - A_0}{A_C}\right] \times 100;$$

where, A_C is the absorbance of the control and A_0 is the absorbance of the sample.

2.5.3. Reducing power assay

Reducing power assay was performed by following the protocol described by Krisnaiah et al (2011). Methanolic MMLE and MMFE of different concentrations (10-100 mg/ml) were mixed with 250 µl phosphate buffer (50 mM, pH 6.6) and 250 µl potassium ferricyanide (1% w/v). The mixture was incubated at 50 °C for 20 min. After the stipulated time, the reaction was stopped by adding 250 µl of 10% TCA (ice-cold) and centrifuged at 3000 rpm for 10 min. 100 µl of supernatant was mixed with equal volumes of deionized water and 20 µl of ferric chloride (0.1% w/v) solution, and absorbance was recorded at 700 nm in a plate reader (MultiSkan Sky High 1119500). Ascorbic acid (1 mg/ml) served as the standard.

2.6. Determination of total phenolic content (TPC)

The total phenolic content of the methanolic MMLE and MMFE was performed by the Folin –Ciocalteau method (Singleton, 1966). Briefly, 40 µl of plant extract (0.01 mg/ml) was added to 250 µl of freshly prepared Folin Ciocalteau reagent (1:10 v/v in deionized water) and 250 µl of sodium carbonate (7.5% w/v). The mixture was then incubated at 45 °C for 15 min. Absorbance was calculated in a plate reader at 765 nm. TPC has been expressed in terms of gallic acid equivalent (µg GAE /mg of extract).

2.7. Determination of total flavonoid content (TFC)

The total flavonoid content of the crude methanolic extracts was evaluated by the Aluminium chloride method (Lefahal et al., 2018). Briefly, to 250 µl of sample (0.1 mg/ml), 7.5 µl of 5% potassium acetate was added and incubated for 5 min. 15 µl of 10% aluminium chloride was added to the mixture. After 6 min, 50 µl of 1.0 M sodium hydroxide was added and centrifuged for 10 min at 5000 rpm. Absorbance was measured at 510 nm. TFC has been expressed in terms of Quercetin equivalent (µg of Quercetin/ mg of extract).

2.8. Determination of proteolytic activity of aqueous MMLE and MMFE

The quantitative proteolytic activity of the aqueous MMLE and MMFE on the substrate casein was performed by following a standard protocol (McDonald and Chen, 1965) with some modifications (Mukherjee et al., 2012). The specific activity was determined in terms of 'n' moles of tyrosine released per mg of extract per min per ml.

2.9. Fibrinogenolytic activity of aqueous MMLE and MMFE

Electrophoretic analysis of the fibrinogenolytic activity of MMLE and MMFE was studied by incubating 2.0 µg of aqueous MMLE and MMFE with 30 µl fibrinogen (2.5 mg/ml, pH 7.4) for different

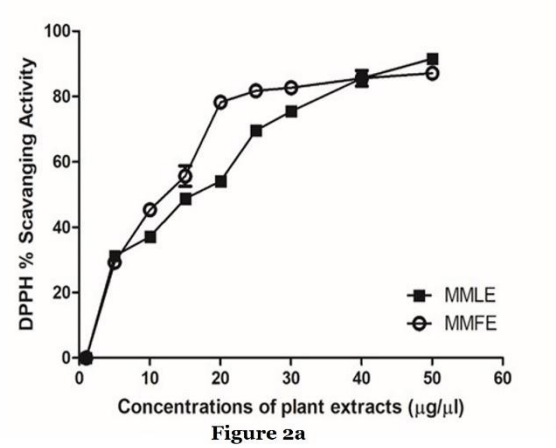


Figure 2a

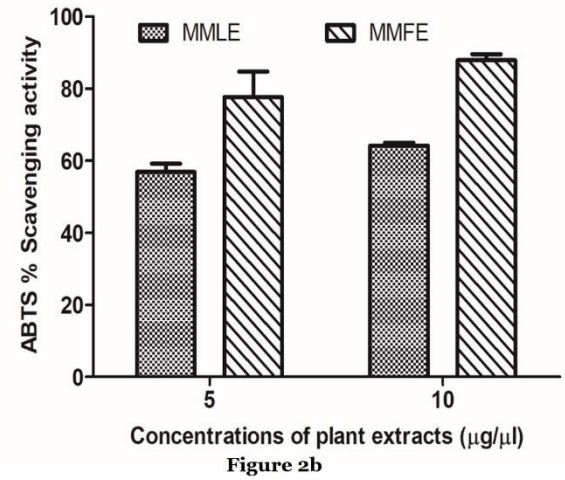


Figure 2b

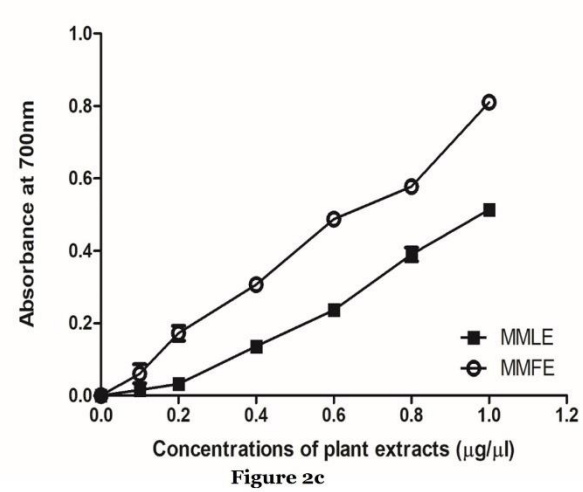


Figure 2c

Figure 2. Antioxidant capacity of MMLE and MMFE. (a) DPPH % scavenging activity of MMLE and MMFE at different concentrations (1.0 – 50.0 µg/µl). (b) Dose-dependent ABTS % scavenging activity of MMLE and MMFE (5.0 µg/µl and 10.0 µg/µl). (c) Free radical scavenging activity of MMLE and MMFE as calculated by reducing power assay at various concentrations (0.1 – 1.0 µg/µl).

time intervals (30 min to 6 h) at 37 °C. The fibrinogen degradation products were visualised by SDS PAGE (12 %). The quantitative fibrinogenolytic activity was performed according to a previously standardized protocol (Mukherjee et al., 2012). The fibrinogenolytic activity was expressed in terms of 'n' moles of tyrosine liberated per mg per min per ml.

2.10. *Determination of anticoagulant activity of aqueous MMLE and MMFE*

The plasma recalcification time test (clotting time, CT) was performed according to a previously standardized protocol (Thakur et al., 2015). Briefly, aqueous MMLE or MMFE (2.0 µg) was added to 300 µl of pre-warmed mammalian (goat) platelet-poor plasma (PPP) and incubated for 3 min at 37 °C. Thereafter, 40 µL of 250 mM $CaCl_2$ was added, and the time for the appearance of visible fibrin threads was recorded using a stopwatch. Similarly, the dose-dependent effect of MMLE and MMFE was also assessed (1.0 – 5.0 µg). One unit of anticoagulant activity has been defined as a 1.0-second increase in the clotting time of PPP as compared to the clotting time of normal PPP under identical assay conditions (Mukherjee et al., 2012; Thakur et al., 2015). Activated partial thromboplastin time (aPTT) test and Prothrombin time (PT) test were performed using commercially available Liquicline® and Uniplastin® following the manufacturer's protocol.

2.11. Determination of FXa inhibitory effects of aqueous MMLE and MMFE

To study the effects of plant extracts on prothrombin activation of FXa, 1.0 µg FXa was incubated with aqueous MMLE and MMFE for 30 min at 37 °C. To this, 12.0 µg prothrombin was added and incubated for 60 min at 37 °C. Prothrombin degradation products, if any, were analysed by 12% SDS-PAGE under reducing conditions. Prothrombin incubated with FXa or PBS (1X) served as the positive and negative control, respectively.

2.12. *Determination of in vitro clot (thrombus) lysis activity of aqueous MMLE and MMFE*

In vitro clot (thrombus) lysis activity of the aqueous extracts was assessed according to the previously standardized protocol (Gogoi et al., 2018), with slight modifications. To 1.0 ml of citrated mammalian (goat) blood, 100 µl of $CaCl_2$ (250 mM) was added. The clot was allowed to form at 37 °C for 1 h, and the dry weight of the clot was recorded. Thereafter, 5.0 µg of aqueous plant extract was added to the clot and incubated at 37 °C for 90 min. The supernatant was removed carefully, and the dry weight of the clot was recorded. For negative control, clots were incubated with PBS buffer (1X, pH 7.4). Clots incubated with streptokinase (1.0 µg/µl) served as the positive control. The *in vitro* clot lysing activity was expressed in terms of mg of blood clot lysed per µg of plant /streptokinase as compared to the negative control.

2.13. Statistical analyses

All the experiments were performed in triplicates and the data has been expressed as mean ± standard deviation or unless otherwise indicated. The statistical analysis of the data was performed by student's t-test using GraphPad Prism5. The value of $p \leq 0.05$ was considered as significant.

3. Results

3.1. GC-MS analysis of MMLE and MMFE

GC-MS-based metabolite profiling of the plant extracts revealed the presence of 108 and 107 metabolites in methanolic MMLE and MMFE, respectively. In MMLE, the percentage of sterols was found to be highest (19%), followed by hydrocarbons (18%), and aldehydes and ketones (13%) (Figure 1a). On the contrary, the percentage of aldehydes and ketones was reported to be significantly high (46%) in MMFE, followed by saturated fatty acids (13%) and terpenoids (13%) (Figure 1b). The list of total metabolites identified in MMLE and MMFE has been listed in Table 1a and Table 1b, respectively. Metabolites like squalene (steroid), catechol (phenol) and .beta.-sitosterol (steroid) are some of the major metabolites in MMLE (Table 1a) whereas 5-hydroxymethylfurfural (aldehyde), diosgenin (terpenoid), and 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (ketone) are some major metabolites in MMFE (Table 1b). Venn representation (Figure 1c) of the metabolite profile of the extracts revealed the presence of only four metabolites common in both MMLE and MMFE which has been listed in Table 1c.

3.2. Assessment of antioxidant assay

The antioxidant property of the plant extracts was studied by its ability to scavenge DPPH free radical by DPPH antioxidant assay, $ABTS^{\bullet+}$ radical by ABTS assay and reduction of potassium ferricyanide (Fe^{3+}), i.e., by reducing power assay. Both MMLE and MMFE could reduce the stable, purple-coloured DPPH radical into yellow-coloured DPPH-H. As shown in Figure 2a, MMFE showed

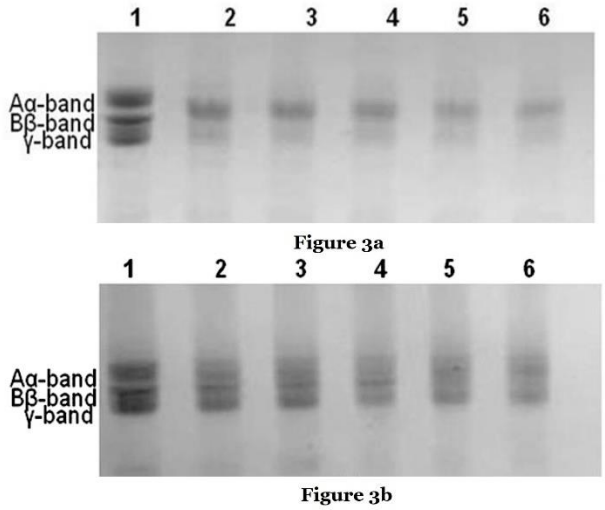


Figure 3. Time-dependent fibrinogenolytic activity of (a) MMLE, and (b) MMFE analysed by SDS PAGE (12 %) under reduced conditions. Lane 1, Control; Lane 2, 30 min; Lane 3, 1 h; Lane 4, 2 h; Lane 5, 4 h; Lane 6, 6 h.

higher DPPH scavenging activity as compared to MMLE. The ABTS percent scavenging activity of MMLE was 56.83 ± 4.08 at 5 µg/µl and 64.18 ± 1.41 at 10 µg/µl whereas the ABTS percent scavenging activity of MMFE was 77.24 ± 12.21 at 5µg/µl and 87.87 ± 2.86 at 10 µg/µl, respectively (Figure 2b). Reducing power assay of the methanolic MMLE and MMFE demonstrated a higher percentage of ferric reduction by MMFE as compared to that of MMLE (Figure 2c).

3.3. Assessment of TPC and TFC

The TPC of methanolic MMFE was observed to be 248.20 ± 21.34 µg GAE/mg of extract whereas that of MMLE was 215.80 ± 11.73 µg GAE/mg of extract. A higher concentration of flavonoids was determined in MMLE which accounted for 86.8 ± 10.90 µg QE/mg of extract. The TFC of MMFE was comparatively lower and was determined to be 51.7 ± 6.00 µg QE/mg of extract.

3.4. Assessment of proteolytic activity of aqueous MMLE and MMFE

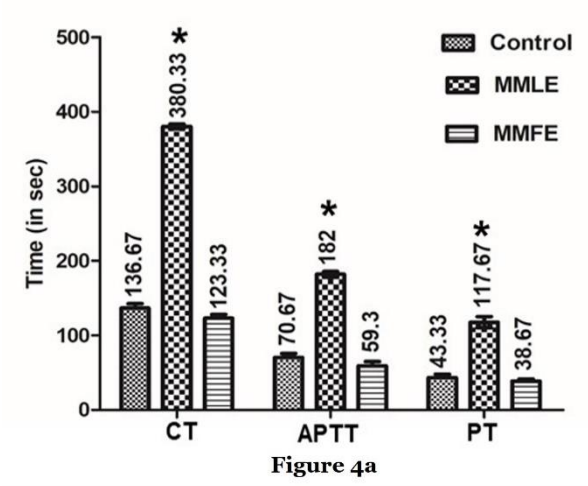
The specific activity of MMLE towards casein was calculated to be 354.01 ± 3.91 nM tyrosine released/mg/ml/min and that of MMFE was calculated to be 82.91 ± 0.98 nM tyrosine released/mg/ml/min, respectively. The qualitative fibrinogenolytic analysis of the plant samples as carried out by SDS-PAGE (12%) suggested that under physiological conditions, the aqueous MMLE hydrolysed the Aα- chain completely within 30 min and partial degradation of Bβ- chains of fibrinogen in a time-dependent manner (Figure 3a). Interestingly, MMLE could also degrade the γ- chain of fibrinogen within 30 min of incubation (Figure 3a). MMFE led to partial hydrolysis of Aα- chain of fibrinogen while the Bβ- chain of fibrinogen was observed to be hyrolysed only after 4 h incubation under physiological conditions (Figure 3b). The quantitative fibrinogenolytic activity of MMLE and MMFE accounted for $2.9x10^4$ ± 58.5 nM tyrosine released/mg/ml/min and $1.2x10^4$ ± 96.88 nM tyrosine released/mg/ml/min, respectively.

3.5. CT, PT and APTT of MMLE and MMFE

MMLE could significantly increase the CT, PT and APPT whereas MMFE did not have any significant effect on the CT, PTT and APTT (Figure 4a). Additionally, MMLE was observed to increase the CT in a dose-dependent manner (Figure 4b).

3.6. Determination of FXa inhibitory effects of aqueous MMLE and MMFE

Electrophoretic analysis of the effect of MMLE and MMFE on prothrombin activation property of factor Xa revealed that both MMLE (2.0 µg/µl, 5.0 µg/µl) and MMFE (2.0 µg/µl, 5.0 µg/µl) inhibited the activation of prothrombin by FXa to form thrombin (Figure 5).



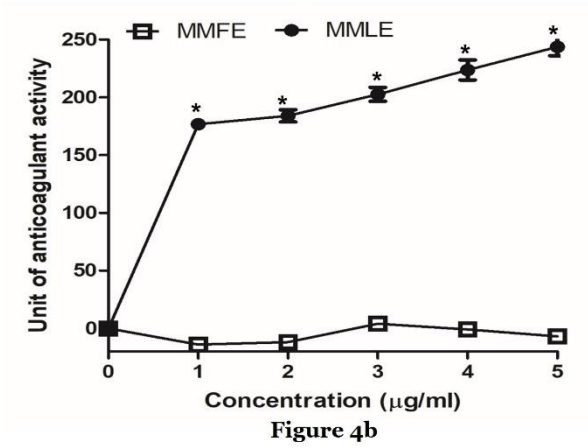


Figure 4. Anticoagulant activity of MMLE and MMFE. (a) Effect of aqueous extracts of MMLE (5.0 µg/µl) and MMFE (5.0 µg/µl) on plasma recalcification time (clotting time, CT), PT and APTT of

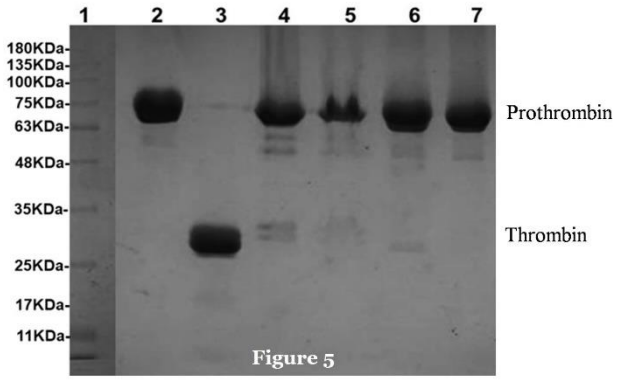


Figure 5. Inhibition of prothrombin activation property of FXa by aqueous MMLE and MMFE. The degradation products were separated by 12% SDS-PAGE under reduced conditions. Lane 1, protein molecular markers; Lane 2, prothrombin (PTH) (5.0 µg/µl) ; Lane 3, PTH (5.0 µg/µl) + Factor Xa (FXa) (0.1 µg/µl); Lane 4, [FXa (0.1 µg/µl) pre-incubated with aqueous MMLE (2 µg)] + PTH (5 µg/µl); Lane 5, [FXa (0.1 µg/µl) pre-incubated with aqueous MMLE (5.0 µg)] + PTH; Lane 6, [FXa (0.1 µg/µl) pre-incubated with aqueous MMFE (2.0 µg)] + PTH (5.0 µg/µl); Lane 5, [FXa (0.1 µg/µl) pre-incubated with aqueous MMFE (5.0 µg)] + PTH (5 µg/µl).

3.7. In vitro clot (thrombus) lysis activity of aqueous MMLE and MMFE

The *in vitro* clot lysis activity of aqueous MMLE, MMFE and Streptokinase is shown in Table 2. The thrombolytic activity of MMLE and MMFE (5.0 µg/µl) was found to be significant as

compared to that of Streptokinase at a concentration 1.0 µg/µl. MMLE showed better thrombolytic activity than MMFE.

4. Discussion

GC-MS metabolite profiling of ethanol extracts of leaf extracts of *M. malabathricum* previously reported the presence of compounds like phthalic acids, oleic acids and hexadecenoic acid, amongst others (Sophiya et al., 2021). In another study, the GC-MS profiling of ethanolic leaf extracts showed the presence of metabolites like pentadecanoic acid,14-methyl, methyl ester, 9-Octadecenoic acid methyl ester while acetone extracts showed the presence of metabolites like 1,4-Benzodiazepin2[1H,3H]-acetone one,7-chloro-1-ethyl-5-phenyl and ethyl acetate extracts depicted the presence of metabolites like Nonanedioic acid, dibutyl ester (Ismail et al., 2022). In the present study, the GC-MS-based metabolite profiling revealed the presence of several significant volatile metabolites with biological relevance in the methanolic MMLE and MMFE (Tables 3a-b). A significantly high amount of squalene was discovered in the methanolic leaf extracts of *M. malabathricum*, suggesting its possible involvement in biotherapy. The antioxidant and anti-tumour properties of squalene have been previously documented by Casuga et al (2016). Squalene is also reported to be a potent metabolite against cardiovascular disorders in a different study (Ibrahim et al., 2020). In addition, several other metabolites identified by GC-MS have been reported to be of biomedical relevance. The anti-cancer and anti-oxidant activities of beta-sitosterol have been previously reported extensively (Gupta et al., 2011; Khan et al., 2022). Other metabolites such as doconexent are known to have anti-inflammatory properties (Liang et al., 2022), while diosgenin is known to have beneficial effects against inflammation, cardiovascular and blood disorders, reproductive, and cancer chemotherapy (Patel et al., 2013). Diosgenin is also reported to be a potent drug candidate in the treatment of breast cancer (Yang and Wang, 2021). N-hexadecanoic acid has been reported to have numerous bioactivities such as anti-inflammatory, antioxidant, hypocholesterolemic nematicide, pesticide, anti-androgenic flavour, hemolytic activity as per previous reports (Abubakar and Majinda, 2016). It is suggested that high concentrations of these volatile compounds may contribute to higher antioxidant properties of MMLE and MMFE, together with the non-volatile phenolic compounds. Benzofuran, 2,3-dihydro-, Maltol, 7-Hexadecenal, (Z)-and beta-Sitosterol were found in common in both MMLE and MMFE.

The antioxidant capacity of a plant extract cannot be determined by a single test method because the principles of each of the antioxidant assays differ from each other not only in the case of the substrate used but also in the reaction conditions of the assays (Csepregi et al., 2016). Consequently, three antioxidant assays were conducted to test the free radical scavenging ability of MMLE and MMFE. The DPPH assay of MMLE and MMFE revealed a higher IC_{50} value of MMLE as compared to that of MMFE. A higher IC_{50} value of a plant sample suggests a lower antioxidant capacity (Brighente et al., 2007). The methanolic flower extracts of the plant show higher antioxidant activity as compared to those of the methanolic leaf extracts. Similar findings were also reported by Saravanan and Mahadeva (2015), who reported higher IC_{50} values of flower extracts as compared to the other plant parts. Significant antioxidant activities in the methanolic and aqueous leaf extracts had also been previously reported (Zakaria et al., 2011). Both the ABTS per cent scavenging assay as well as the reducing power assay of the methanolic plant extracts corresponded with the DPPH free radical scavenging assay and showed high antioxidant capacities of the flower extracts. TPC and TFC of the plant extracts revealed higher phenolic content in the methanolic flower extract and higher flavonoid content in the leaf extract. High phenolic content in the flower extracts may contribute to their high antioxidant activity. Similar positive correlations between TPC, TFC and the antioxidant capacity of plant species have been reported (Danladi et al., 2015).

Proteolytic enzymes or proteases are considered amongst the most important enzymes and are widely distributed among plants, animals and microbes. These enzymes play numerous vital physiological roles in diverse biological processes including regulation and prevention of unwanted protein degradation. The irreversible cleavage property of proteases makes them a crucial enzyme for the activation of protein molecules that are involved in important biological processes like blood coagulation, fibrinolysis, complement activation etc. (Dayanand, 2013). Aqueous MMLE and MMFE showed effective proteolytic activity towards casein, thus making it an ideal source of natural protease. The α- and β-fibrinogenase obtained from different plant sources are known to hydrolyze the Aα- and Bβ- subunits of fibrinogen. Though both MMLE and MMFE showed potent fibrinogenolytic activity, the electrophoretic analysis of fibrinogen degradation by MMLE and MMFE suggested their differential action on fibrinogen. The mechanism involved in fibrinogen degradation may be explored further to understand the differential effects of the plant extracts on haemostasis.

Further investigation highlighted the effects of MMLE and MMFE on the blood coagulation cascade. MMLE prolonged APTT, suggesting its inhibitory effects on one or more blood coagulation factors belonging to the intrinsic pathway. In addition, MMLE also showed prolonged effects on PT, indicating its inhibitory effect on the extrinsic factors of blood coagulation. In a study conducted by Manicum et al (2010), the aqueous leaf extract demonstrated potent anticoagulant activity based on activated partial thromboplastin time (APTT) assay. However, MMFE did not affect either the intrinsic or the extrinsic pathways of the blood coagulation cascades which might imply its procoagulant properties. Although the preliminary anticoagulant activity of leaf extracts of *M. malabathricum* L. was reported previously, the inhibitory effects of the leaf and flower extracts on the blood coagulation protein FXa have been studied for the first time. FXa belongs to both the intrinsic and extrinsic pathway of blood coagulation, and plays an important role in conversion of inactive prothrombin to active thrombin, ultimately forming blood clots (McCarty and Robinson, 2016). Hence, FXa may be considered as one of the most suitable blood coagulation factors for non-hemostatic therapies (McCarty and Robinson, 2016). MMLE and MMFE demonstrated potent inhibitory effects of prothrombin activation by FXa. Interestingly, though MMFE demonstrated FXa inhibitory effects, the impact of this inhibition was insignificant as MMFE did not show an overall anticoagulant effect in blood plasma. These observations need to be investigated further to understand the effect of MMFE on the blood coagulation process. The *in vitro* clot lysis activity of MMLE and MMFE confirmed the significant thrombolytic properties of the plant extracts. Higher clot lysis activity was observed in MMLE as compared to that of MMFE. In-depth studies on the thrombolytic activities of the plant may help characterize and quantify the compounds responsible for thrombolytic potency of the plant. Further extensive analysis of the plant for its anticoagulant and thrombolytic properties, and the detailed mechanisms involved in such processes may establish the effectiveness of the plant as an effective natural source of antithrombotic/ thrombolytic drugs.

5. Conclusion

The study highlighted the presence of significant metabolites with biomedical relevance in the leaf and flower extracts of *M. malabathricum* L., which is likely to contribute to the medicinal properties of the plant. In addition to demonstrating potent antioxidant capacity, the plant extracts also demonstrated noteworthy anticoagulant and *in vitro* thrombolytic properties. Further in-depth investigation of the metabolites of the plant may be beneficial in establishing its role in the preparation of novel drugs. Moreover, the role of the plant and its metabolites as potent anticancer and antithrombotic agents is warranted.

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Author contributions
PN- performed and analyzed the experiments, and prepared the manuscript. RT -conceptualized and designed the experiments, analyzed the data, and finalized the manuscript.

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Conflict of interest
The authors declare no conflict of interest.

Table 1a. Metabolites profile *Melastoma malabathricum* leaf extract (MMLE) screened and identified using Gas Chromatography – Mass Spectrometry (GC-MS).

Nature of Compounds	Name of Compounds	Area %	Retention Time
Saturated Fatty Acids	Undecanoic acid, 10-bromo-	0.38	32.29
	Methyl 12-oxo-9-dodecenoate	2.26	46.564
	Methyl (Z)-5,11,14,17-eicosatetraenoate	0.15	49.345
	Methyl 8,11,14,17-eicosatetraenoate	1.2	49.467
Unsaturated Fatty Acids	Doconexent	4.26	28.332
	Methyl 11-cyclohexylundecanoate	1.41	35.873
	Bis(tridecyl) phthalate	0.19	36.495
	Cyclopropanepropanoic acid, 3-[1-(methoxycarbonyl)ethenyl]-2,2-dimethyl-, methyl ester, trans-	0.14	36.927
	Cyclopentaneundecanoic acid	0.41	37.773
	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	0.87	39.337
Amines	1-Amino-2,6-dimethylpiperidine	0.64	12.688
	1,4-Methanocycloocta[d]pyridazine, 1,4,4a,5,6,9,10,10a-octahydro-11,11-dimethyl-, (1.alpha.,4.alpha.,4a.alpha.,10a.alpha.)-	0.18	26.342
Amides	1H-Pyrazole-1-carbothioamide, 3,5-dimethyl-	0.44	6.315
Alcohols	1-Hexen-4-ol, 1-chloro-3,5-dimethyl-	0.29	5.3
	1,2-Benzenediol, 3-methyl-	0.9	20.205
	1,4-Benzenediol, 2-methoxy-	0.22	23.238
	Bicyclo[3.1.1]hept-3-en-2-ol, 4,6,6-trimethyl-, [1S-(1.alpha.,2.beta.,5.alpha.)]-	0.15	29.353
	2,3,3a,4,5,6,7,7a-Octahydro-1H-cyclopenta[a]pentalen-7-ol	1.64	29.636
	4,6,10,10-Tetramethyl-5-oxatricyclo[4.4.0.0(1,4)]dec-2-en-7-ol	0.38	30.634
	2,4-Dimethoxybenzyl alcohol	0.51	35.016
	7-Tetracyclo[6.2.1.0(3.8)0(3.9)]undecanol, 4,4,11,11-tetramethyl-	1.07	44.301
	Androstenediol	0.21	52.559
	Androstane-3,17-diol, 17-methyl-, (3.beta.,5.alpha.,17.beta.)-	0.28	54.204
	Kauren-18-ol, acetate, (4.beta.)-	0.21	54.342
	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3.beta.,4.alpha.,5.alpha.)-	1.14	54.841
	Maltol	0.12	14.803
Aldehydes	Phenylglyoxal	0.31	13.391
	5-Ethylcyclopent-1-enecarboxaldehyde	0.13	18.58
	2-Isopropylidene-3-methylhexa-3,5-dienal	0.18	28.436
	Retinal	0.36	31.609
	7-Hexadecenal, (Z)-	0.23	35.591
Ketones	Tetrahydrocyclopenta[1,3]dioxin-4-one	0.39	11.876
	Cyclopentanone, 2-ethyl-	0.14	12.05
	4-Hepten-3-one, 4-methyl-	0.53	18.393
	Hydroquinone	0.33	19.832
	4-Cyclopentene-1,3-dione, 4-(3-methyl-2-butenyl)-	0.32	24.696
	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one	0.19	25.499
	3,3-Dimethyl-4-phenyl-4-penten-2-one	0.94	27.286
	3',5'-Dimethoxyacetophenone	0.44	27.575
	3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl)-	0.12	28.697
	1-Penten-3-one, 1-phenyl-	0.81	30.328
	Bicyclo[6.3.0]undec-1(8)-en-3-one, 2,2,5,5-tetramethyl-	0.14	33.572
	2-(3-Isopropyl-4-methyl-pent-3-en-1-ynyl)-2-methyl-cyclobutanone	0.24	33.75
	3-Buten-2-one, 3-methyl-4-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]heptan-1-yl)-	0.5	33.846
	Androst-5-en-17-one	0.18	41.503
	Benz[e]azulen-3(3aH)-one, 4,6a,7,8,9,10,10a,10b-octahydro-3a,8,10a-trihydroxy-5-(hydroxymethyl)-2,10-dimethyl-, [3aR-(3a.alpha.	0.16	46.685
	Androstan-3-one, 17-hydroxy-1,17-dimethyl-, (1.alpha.,5.alpha.,17.beta.)-	0.15	47.711
	Tetrahydroaraucarolone	0.57	52.926
	Androstan-17-one, 3-[(triethylsilyl)oxy]-, (3.alpha.,5.beta.)-	0.48	55.945
	Androstane-17-carboxylic acid, 11,18-epoxy-18-hydroxy-3-[(trimethylsilyl)oxy]-, .gamma.-lactone, (3.alpha.,5.beta.,11.beta.,17.	0.81	58.617
	17-Methoxy-4-methyl-d-homo-18-norandrosta-4,8,13,15,17-pentaen-3-one	2.91	58.798
	3,3'-Dimethyl-1,5',8'-trihydroxy-2,2'-binaphthalene-1',4',5,8-tetrone	0.69	59.201
Hydrocarbons	1,3-Butadiene-1-carboxylic acid	0.22	6.852
	Cyclobutane, 1,1,2,3,3-pentamethyl-	0.63	8.898
	1-Pentene, 2,4,4-trimethyl-	0.29	11.01
	Hexane, 2-methyl-4-methylene-	0.4	11.202
	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-	0.4	24.025
	Dispiro[2.1.2.4]undecane, 8-methylene-	2.16	24.106
	3.beta.,17.beta.-dihydroxyestr-4-ene	0.23	26.872
	Cyclopentane-3'-spirotricyclo[3.1.0.0(2,4)]hexane-6'-spirocyclopentane	1.56	28.174
	Bicyclo[4.1.0]heptane,-3-cyclopropyl,-7-hydroxymethyl, (cis)	0.39	28.998
	Biphenylene, 1,2,3,6,7,8,8a,8b-octahydro-4,5-dimethyl-	0.89	29.916
	4-Tetradecyne	3.1	34.029
	1,3,14,16-Nonadecatetraene	0.27	39.213
	1-Octadecyne	1.83	39.551
	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	3.54	53.224
	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-	1.38	55.019
	Tri(n-butoxy)-tert.-butoxysilane	0.27	54.445
Esters	Cyclopentanecarboxylic acid, ethenyl ester	0.17	9.643
	Carbamic acid, phenyl ester	0.69	10.65
	Phosphonic acid, methyl-, bis(trimethylsilyl) ester	0.14	19.394
	Butanoic acid, 2-methyloct-5-yn-4-yl ester	0.17	30.815
	2-Propenoic acid, 3-(4-hydroxyphenyl)-, methyl ester	0.24	31.995
	Undecanoic acid, 2-nonyl-, methyl ester	1.35	36.621
Ethers	Ether, 6-methylheptyl vinyl	0.15	25.799
	(-)-Isolongifolol, methyl ether	0.3	32.539

Phenols	Phenol, 2-methoxy-	1.14	14.047
	Catechol	6.04	17.453
	Phenol, 2,6-dimethoxy-	0.76	21.913
	2-Methoxy-4-vinylphenol	0.53	20.913
	Phenol, 2,5-bis(1,1-dimethylethyl)-	0.12	26.241
	Phenol, 3,4,5-trimethoxy-	0.14	28.58
	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	0.21	30.894
	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	1.26	31.769
	1,1'-Biphenyl, 2-formyl-4',5',6'-trimethoxy-	0.34	48.857
Terpenoids	cis-.alpha.-Bisabolene	0.23	25.061
	Andrographolide	1.84	30.404
	cis-Z-.alpha.-Bisabolene epoxide	0.54	33.695
	4a,7-Methano-4aH-naphth[1,8a-b]oxirene, octahydro-4,4,8,8-tetramethyl-	0.13	35.191
	10-Methyldodecan-5-olide	0.32	43.872
	Betulin	3.32	52.201
Sterols	.beta.-Sitosterol	5.99	48.483
	Squalene	12.63	51.351
Vitamins	.gamma.-Tocopherol	0.79	56.049
	Vitamin E	2.89	58.135
Acids	Cyclobutanecarboxylic acid, 2,7-dimethyloct-7-en-5-yn-4-yl ester	0.25	27.075
	2,2-Dimethyl-2-[2,4,6-trimethylphenyl]acetic acid	0.15	38.835
	Cholan-24-oic acid, 3,12-dioxo-, (5.beta.)-	0.38	57.911
	Methanesulfonic acid, 2-(3-hydroxy-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]	0.29	43.743
Others	Glycerin	0.16	5.18
	Benzofuran, 2,3-dihydro-	2.87	18.155
	1H-Cycloprop[e]azulene, 1a,2,3,4,4a,5,6,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1a.alpha.,4.alpha.,4a.beta.,7b.alpha.)]-	0.15	24.928
	(2,6,6-Trimethylcyclohex-1-enylmethanesulfonyl)benzene	0.23	26.668
	Benzene, (1,2-dimethoxyethyl)-	0.31	31.176
	1,1,6-trimethyl-3-methylene-2-(3,6,10,13,14-pentamethyl-3-ethenyl-pentadec-4-enye)cyclohexane	0.17	38.329
	Isoaromadendrene epoxide	0.32	50.757
	Bicyclo[5.2.0]nonane, 4-methylene-2,8,8-trimethyl-2-vinyl-	1.49	54.616
	1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]	0.19	24.611

Table 1b. Metabolites profile of *Melastoma malabathricum* flower extract (MMFE) screened and identified using Gas Chromatography – Mass Spectrometry (GC-MS).

Nature of Compounds	**Name of Compounds**	**Area %**	**Retention Time**
Saturated Fatty Acids	2-Furancarboxylic acid, cyclobutyl ester	0.07	9.545
	3-Methyl-butyric acid, 4-butyl-2-methyl-5-oxotetrahydrofuran-3-yl ester	0.33	11.407
	Hexanethioic acid, S-ethyl ester	0.63	11.07
	Nonanoic acid	0.22	15.391
	Glutaric acid, cyclohexylmethyl 1-naphthyl ester	1.21	16.836
	Furan-2-carboxylic acid, 5-ethyl-	0.23	19.175
	5-oxoheptanoic acid, 4,4-dimethyl-	0.01	21.77
	Tetradecanoic acid	0.32	27.782
	Pentadecanoic acid	0.1	29.94
	Hexadecanoic acid, methyl ester	0.24	31.321
	n-Hexadecanoic acid	5.79	32.073
	Heptadecanoic acid, 16-methyl-, methyl ester	0.07	35.279
	Octadecanoic acid	1.15	35.921
	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	1.27	41.965
	Octadecanoic acid, 2,3-dihydroxypropyl ester	1.33	45.193
	Methoxyacetic acid, 4-hexadecyl ester	0.07	37.019
	Dodecanoic acid	0.07	23.134
	Oxalic acid, 6-ethyloct-3-yl propyl ester	0.04	31.89
Unsaturated Fatty Acids	4-tert-Butylcyclohexyl methyl ethylphosphonate	0.2	9.325
	2-Octenoic acid, 4,5,7-trhydroxy	0.46	9.729
	Hepta-2,4-dienoic acid, methyl ester	0.18	12.161
	Benzoic acid	0.19	12.551
	Phthalic acid, decyl 2,7-dimethyloct-7-en-5-yn-4-yl ester	0.06	17.414
	Diethyl Phthalate	0.2	24.08
	Benzoic acid, 4-hydroxy-3,5-dimethoxy-, methyl ester	0.21	28.229
	1,2-Benzenedicarboxylic acid, diundecyl ester	0.19	32.215
	n-Propyl 9,12-octadecadienoate	0.12	34.694
	9,12-Octadecadienoic acid (Z,Z)-	2.67	35.434
	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	2	35.563
	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	0.09	38.909
Amines	Ethanamine, 2-methoxy-N-(2-methoxyethyl)-N-methyl-	0.28	5.103
	Tromethamine	0.09	6.67
	2-Buten-1-amine, N,N-diethyl-, (Z)-	0.34	7.696
	3-Aminopyrazine 1-oxide	0.12	6.74
Amide	2-Butenediamide, (E)-	0.22	5.886
	Hydrazinecarbothioamide, 2-cyclopentylidene-	0.04	21.61
	9-Octadecenamide, (Z)-	0.96	39.562
	13-Docosenamide, (Z)-	0.69	46.153
Alcohols	Benzyl alcohol	0.19	8.755
	Furaneol	0.46	9.398
	2-Propyl-1-pentanol, chlorodifluoroacetate	0.16	10.462
	1,2,3-Benzenetriol	6.45	18.82
	1-Octanol, 2,2-dimethyl-	0.05	22.495

	1,3-Propanediol, ethyl octadecyl ether	0.05	39.967
	1,3-Butanediol, 2TMS derivative	0.07	43.175
Aldehydes	2-Furancarboxaldehyde, 5-methyl-	2.56	6.907
	Benzeneacetaldehyde	0.61	9.021
	2,5-Furandicarboxaldehyde	1	9.957
	2-Nonenal, (E)-	0.04	12.27
	Benzaldehyde, 2,4-dimethyl-	0.06	13.963
	5-Hydroxymethylfurfural	17.97	14.445
	5-Acetoxymethyl-2-furaldehyde	0.57	16.571
	4-Hydroxy-2-methoxybenaldehyde	0.68	19.125
	7-Hexadecenal, (Z)-	0.86	44.82
Hydrocarbons	2-(1-Hydroxyethyl)-2-methyl-1,3-oxathiolane	0.27	14.822
	Dodecane, 4,6-dimethyl-	0.14	15.66
	2,6,10-Trimethyltridecane	0.06	16.055
	Decane, 1-iodo-	0.44	16.95
	Oxirane, octyl-	0.22	19.26
	5,5-Diethylpentadecane	0.17	21.706
	Nonane, 5-methyl-5-propyl-	0.1	23.33
	Eicosane	1.66	26.637
	11-Methyltricosane	0.19	28.02
	Heptadecane, 3-methyl-	0.21	31.275
	Tetrapentacontane	0.26	32.296
	Hexacontane	0.17	36.05
	Tetracosane	0.14	36.229
	Octacosane, 1-iodo-	0.03	28.142
	Acetate, [4-hydroxy-4-(1-methylethyl)-5-methyl-2-hexynyl] ester	0.53	26.58
	1-Hexene, 1-butoxy-2-ethyl-	0.24	7.529
	2-Nonene, (E)-	0.47	9.085
	Benzene, 1-chloro-4-methoxy-	1.05	19.017
	Neophytadiene	0.1	29.493
Ether	2,2'-Bifuran, 2,2',5,5'-tetrahydro-	0.05	5.558
	5-(Hydroxymethyl)-2-(dimethoxymethyl)furan	0.06	16.238
	Eicosyl isopropyl ether	0.07	36.509
Ester	dl-3,4-Dehydroproline methyl ester	0.03	6.407
	Oxalic acid, monomorpholide, dodecyl ester	0.37	7.808
	Glycine, N-(ethoxycarbonyl)-, ethyl ester	0.34	11.661
Ketones	4-Cyclopentene-1,3-dione	0.54	5.177
	Ethanone, 1-(2-furanyl)-	0.13	5.722
	2(5H)-Furanone	0.54	5.795
	1,2-Cyclopentanedione	0.36	6.009
	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	1.35	7.265
	2(3H)-Furanone, dihydro-4,4-dimethyl-5-(2-oxopropyl)-	0.12	8.204
	2,5-Furandione, 3-methyl-	0.14	8.37
	4-Hydroxy-6-methylhexahydropyrimidin-2-thione	1.76	8.921
	Furyl hydroxymethyl ketone	0.59	10.115
	1,3-Cyclohexanedione, 2-methyl-	0.61	10.64
	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	8.71	11.903
	2(1H)-Naphthalenone, 4a,5,8,8a-tetrahydro-1,1,4a-trimethyl-, trans-	0.05	19.345
	Pyrimidine-2,4(1H,3H)-dione, 6-hydroxy-5-methyliminomethyl-	2.93	26.143
	Androsta-1,4-dien-3-one, 17-hydroxy-17-methyl-, (17.alpha.)-	3.64	43.438
	Levetiracetam	0.27	11.465
	Maltol	0.53	10.97
Phenol	Phenol	0.59	7.385
	5-(3-Hydroxypropyl)-2,3-dimethoxyphenol	0.17	30.853
	2,4,5-Trihydroxypyrimidine	1.15	10.184
Sugars	d-Glycero-d-galacto-heptose	0.75	10.885
	Octyl-.beta.-D-glucopyranoside	0.04	23.935
Terpenoids	Diosgenin	10.09	43.622
	7-Ketodiosgenin acetate	2.58	44.99
Sterols	.beta.-Sitosterol	0.12	48.593
Others	Ethyl iso-allocholate	0.35	23.79
	Propargyl ethyl sulfide	0.04	7.94
	L-Glycine, N-ethoxycarbonyl-	0.39	9.13
	Benzofuran, 2,3-dihydro-	0.33	14.047

Table 1c. List of metabolites common in MMLE and MMFE

Nature of compound	Name of compound	Area % in MMLE	Area % in MMFE
Sterols	.beta.-Sitosterol	5.99	0.12
Others	Benzofuran, 2,3-dihydro-	2.87	0.33
Aldehyde	7-Hexadecenal, (Z)-	0.23	0.86
Alcohol	Maltol	0.12	0.53

Table 2. *In vitro* thrombolytic activity of aqueous MMLE and MMFE

Samples	mg of clot lysed μg^{-1} protein
Control	2.77±0.68
Streptokinase	730±13.75
MMLE	61.2±1.06*
MMFE	44.8±4.82*

*All values are represented as ±S.D. p-values ($p<0.05$) depict the significance difference as tested against control.

Table 3a. Metabolites in MMLE with biomedical relevance identified through GC MS analysis.

Name of the compound	Area %	Biomedical relevance
Squalene	12.63	Antioxidant, anti tumor (Casuga et al., 2016)
Catechol	6.04	Antimicrobial activity (Kocaçalışkan et al., 2006)
.beta.-Sitosterol	5.99	Anti-cancer, antioxidant (Khan et al., 2022; Gupta et al., 2011)
Doconexent	4.26	Anti-inflammatory (Liang et al., 2022)
Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	3.54	Antimicrobial activity (Habbab et al., 2016)
Betulin	3.32	Anti-tumor activity, anti-viral, anti-inflammatory (Hordyjewska et al., 2019)

Table 3b. Metabolites in MMFE with biomedical relevance identified through GC MS analysis.

Name of the compound	Area %	Biomedical relevance
5-Hydroxymethylfurfural	17.97	Antioxidant, antiproliferative (Zhao et al., 2013)
Diosgenin	10.09	Antioxidant, anti-cancer (Patel et al., 2013)
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	8.71	Antimicrobial, anti-inflammatory (Amala and Jeyaraj, 2014; Yu et al., 2013)
1,2,3-Benzenetriol	6.45	Antimicrobial, anti-fungal and antibiotic (Lima et al., 2016)
n-Hexadecanoic acid	5.79	Antioxidant,anti-inflammatory (Abubakar and Majinda, 2016)
Androsta-1,4-dien-3-one, 17-hydroxy-17-methyl-, (17.alpha.)-	3.64	-

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