A Comprehensive Study on Phytochemical Analysis, in Vitro Antioxidant, and Anti-Coagulant Activities of Phenolic-Rich Solvent Fractions of Tecomella Undulata Bark 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 *Tecomella undulata* bark anticoagulant and antioxidant activities using in vitro methods, seeking new therapeutic purposes for this plant. Dried and powdered bark of *Tecomella undulata* 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 *Tecomella undulata* bark 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, ten 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 *Tecomella undulata*, 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, *Tecomella undulata* bark

Introduction

Thrombosis underlies several vascular disorders [1], and abnormal lipid metabolism is associated with various cardiovascular and cerebrovascular conditions like high-fat hyperlipidemia, obesity, hypertension, atherosclerosis, coronary heart disease, myocardial infarction, and cerebral thrombosis [2]. The factors triggering these conditions include elevated levels of low-density lipoprotein-cholesterol (LDL-C), decreased levels of high-density lipoprotein-cholesterol (HDL-C), and increased free radicals and lipid peroxidation [2]. In the medical market, three primary types of anti-thrombotic drugs exist: anticoagulant drugs, anti-platelet drugs, and

thrombolytic drugs. However, these drugs often lead to adverse reactions and are expensive [3]. 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 [4]. Medicinal plants have historically been a valuable source of anticoagulant and anti-thrombotic molecules [5].

Furthermore, free radicals pose significant risks to cells and tissues, contributing to various diseases, including cancer, cardiovascular disease, and neurodegenerative disorders [6,7]. Antioxidants play a crucial role in neutralizing free radicals and mitigating oxidative stress. These antioxidants, such as vitamins, flavonoids, and phenolic compounds, help reduce oxidative damage [8,9].

High cholesterol levels, along with the generation of reactive oxygen species (ROS) and LDL oxidation, are pivotal in the development of coronary artery diseases (CAD) and atherosclerosis [6]. Atherosclerosis can lead to blood clot formation due to platelet aggregation and coagulation activation [10]. Therefore, plants with anticoagulant and antioxidant properties hold promise for potential use in medicine, particularly for treating atherosclerosis.

Tecomella undulata, a member of the Bignoniaceae family, is a well-known tree referred to as "ammora" in English or locally known as honey tree, desert teak, marwar teak, or white cedar. Its distribution spans across Arabia, southern Pakistan, and northwest India, thriving at elevations of up to 1200 meters. It is a very important medicinal plant of Rajasthan, India. The plant has significant therapeutic potential attributed to the presence of valuable secondary metabolites. Examinations of the plant components have resulted in the discovery of pharmacologically significant compounds, including Iridoid Glucoside [13], naphthoquinone [14,15], phytosterols, flavonoid glycoside, flavanol [16], fatty alcohol [17], fatty acid [18], and triterpenoids [19]. For an extended period, this plant has been employed as a remedy for syphilis, urinary disorders, spleen enlargement, gonorrhea, leukoderma, jaundice, and liver diseases [11,12]. However, the available literature shows limited research conducted on this particular plant. Notably, the plant exhibits valuable properties like antifungal and anti-termite effects [20], potential treatment for typhoid fever [21], analgesic and anti-inflammatory properties [22], antimicrobial activity [23,24], and a study has been conducted on its impact compared to the chlorpromazine drug in albino rats [25]. Additionally, it shows non-specific spasmolytic action [26]. The practices of Family Planning and Sex Disease Treatment have been documented in Samahni Valley, Pakistan [27]. Furthermore, this plant has shown potential in treating various health conditions, including hepatitis [28], syphilis, gonorrhea, conjunctivitis, blood purification, antidiabetic properties, and antioxidant effects [29,30]. Additionally, there is ongoing research on its potential as a drug to combat the spread of AIDS [31].

Material and Method

Collection and preparation of powder:

Tecomella undulata bark was sourced from the local area of Dabhoi, district: Baroda. Dr. Rutva Dave, Assistant Professor in the Department of Botany at H.&H.B. Kotak Institute of Science, Rajkot, 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 bark 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 (TUPF), benzene fraction (TUBF), ethyl acetate fraction

(TUEAF), and butanol fraction (TUBUF), respectively. In each step of solvent fractionation, 20 ml of distilled water was added. The methanol-insoluble residues were designated as the water fraction (TUWF). 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 [32].

Total phenolic content

The total phenolic contents in the fractions were quantified using the modified Folin-Ciocalteu method [33], 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 [34]. 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 [35]. 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).

ANTIOXIDANT ACTIVITY

DPPH radical scavenging assay

The DPPH radical scavenging activity was determined following Choi's method [36]. Various concentrations of fractions (25, 50, 75, 100, 150, 200, and 250 µg/ml) were mixed with 1.6 ml of methanolic DPPH solution (0.1 mM) in test tubes. The mixture was vigorously shaken and then kept in the dark at room temperature for 30 minutes. The spectrophotometric absorbance of the mixture was measured at 517 nm. Ascorbic acid served as the reference standard. The percentage of DPPH radical scavenging activity (% DRSA) was calculated using the equation:;

% DRSA = [(A0- A1)/ A0] X 100

Where A0 is the absorbance of the control and

A1 is the absorbance of the extracts/standard.

Superoxide radical scavenging activity:

The superoxide radical scavenging activity of the different fractions was assessed using a method based on Beauchamp and Fridovich (1971) with modifications [37]. The reaction mixture consisted of 100 µl of Ethylenediamine tetraacetic acid (EDTA) (0.1 M), 200 µl of NaCN, 50 µl of riboflavin (0.12 mM), 100 µl of

Nitroblue Tetrazolium NBT (1.5 mM), various concentrations of the fractions (25, 50, 75, 100, 150, 200, and 250 µg/ml), and phosphate buffer (67 mM, pH 7.8), making a total volume of 3 ml. The percentage inhibition was calculated. A parallel blank was run omitting sample with distilled water in the reaction mixture.

% Inhibition = $[(A_0 - (A_1 - A_2)/ A_0] \times 100$
Where A_0= absorbance without sample, A_1= absorbance with sample,
A_2= absorbance of sample omitting NBT

Nitric oxide radical scavenging activity:

Nitric oxide radicals were generated from sodium nitroprusside solution under physiological pH conditions [38]. To initiate the reaction, four milliliters of sodium nitroprusside (10 mM) were mixed with 1 ml of various fractions (25, 50, 75, 100, 150, 200, and 250 µg/ml) in PBS (pH 7.4). The mixture was then incubated at 25°C for 150 minutes. Following the incubation period, 0.5 ml of the solution was combined with 1.0 ml of 1.0% sulphanilic acid reagent (in 2% orthophosphoric acid), mixed thoroughly, and left to stand for 5 minutes for diazotization completion. Next, 1.0 ml of 0.1% NEDD (naphthylethylenediamine) was added to the solution, mixed, and allowed to stand for 30 minutes in the dark, leading to the formation of a pink-colored chromophore. The same procedure was repeated for various concentrations of ascorbic acid, used as the reference standard. Finally, the absorbance of these solutions was measured at 540 nm against the corresponding blank solution with PBS instead of the sample. The % of nitric oxide radical scavenging was calculated using equation;

% Scavenging = $[(A_0 - A_1)/ A_0] \times 100$
Where A_0 is the absorbance of the control, and
A_1 is the absorbance of the extracts/fractions/standard.

Metal ion chelating activity:

The capacity of the extracts to chelate iron ions was assessed following the method outlined by Gulcin [39]. Various concentrations of the fractions (25, 50, 75, 100, 150, 200, and 250 µg/ml) and ascorbic acid were introduced to 2.5 ml of a solution containing 2 mM FeCl3. To initiate the reaction, 0.2 ml of 5 mM ferrozine was added, and the volume of the mixture was adjusted to 4 ml with methanol. The mixture was vigorously shaken and allowed to stand at room temperature for 10 minutes for incubation. Following the incubation period, the spectrophotometric absorbance of the resulting color was measured at 562 nm. The percentage of inhibition was calculated using the appropriate formula:

% inhibition = $[A_0 - (A_1 - A_2)]/A_0 \times 100$
Where,
A_0 is the absorbance of the control, containing $FeCl_3$ and ferrozine only,
A_1 is the absorbance in the presence of the tested sample and
A_2 is the absorbance of the sample under identical conditions as A_1 with methanol instead of ferric chloride solution.
EDTA was used as a standard.

Reducing power

The determination of reducing power was carried out based on a previously described method with some modifications [40]. Fractions ranging from 2 µg/ml to 125 µg/ml were added to a reaction mixture comprising 2.5 ml of a solution containing 0.2 M phosphate buffer at pH 6.6 and 1% w/v potassium ferricyanide. The mixture was then incubated at 50°C for 20 minutes. The reaction was halted by adding a 10% w/v solution of trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 minutes to collect the supernatant. To the resulting 2.5 ml of supernatant, 2.5 ml of distilled water and 0.5 ml of ferric chloride solution (0.1%, w/v) were added and thoroughly mixed. The absorbance of the greenish-blue chromogen was measured at 700 nm. A higher absorbance value of the reaction mixture indicated a stronger reducing power. As a control, a parallel blank was run, replacing the sample with 2.5 ml of distilled water. The results were expressed as the percentage of activity exhibited by 0.2 µg/ml of ascorbic acid.

In-vitro anticoagulant activity

Blood sampling and plasma preparation

Blood anticoagulated with citrate was obtained from the Red Cross Blood Bank in Rajkot. 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) [41].

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 [42]. Clotting tubes were prepared, containing 0.5 ml of various fractions of *Tecomella undulata* 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 [43]. 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)®, Robonik India Pvt. Ltd., India) following the recommended protocols provided by the manufacturer [43]. 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 butanol fraction of *Tecomella undulata* 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 [44].

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 Tecomella undulata bark

Chemical Group	Name of Fractions				
	TUPF	TUBF	TUEAF	TUBUF	TUWF
Alkaloids	-	-	-	-	-
Carbohydrate	-	-	-	-	+
Glycosides	-	-	-	+	+
Triterpenes	+	+	+	+	+
Sterols	-	+	+	+	+
Flavonoids	-	+	+	+	-
Phenolics / Tannins	-	+	+	+	+
Saponins	-	-	+	+	+
Proteins	-	-	-	-	+

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, TUEAF exhibited the highest phenolic content (172.77 ± 2.38 mg/g), followed by TUBUF (106.89 ± 2.47).

Table 2. Phenolic, flavonoid content and total antioxidant activity of fractions of *Tecomella undulata* bark

Name of fractions	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QAE/g)	Total Antioxidant capacity (mg AAE/g)
TUPEF	39.23 ± 2.87	11.54 ± 0.74	71.54 ± 4.85
TUBF	46.33 ± 2.54	15.26 ± 0.83	78.22 ± 6.21
TUEAF	172.77 ± 2.38	42.11 ± 0.98	178.32 ± 5.85*
TUBUF	106.89 ± 2.47	36.24 ± 0.81	137.43 ± 4.51*
TUWF	45.34 ± 2.52	14.22 ± 0.79	91.2 ± 5.11

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, TUEAF exhibited the highest flavonoid content (42.11 ± 0.98 mg/g), followed by TUBUF (36.24 ± 0.81).

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, TUEAF showed the highest TAC at 178.32 ± 5.85 mg AAE, followed by TUBUF (137.43 ± 4.51 mg AAE), TUWF (91.2 ± 5.11 mg AAE), and TUBF (78.22 ± 6.21 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.

***IN VITRO* ANTIOXIDANT ACTIVITY**

DPPH free radical scavenging activity

The relative potency of the fractions was observed to be TUEAF < TUBUF < TUWF < TUBF < TUPEF, as shown in Figure 1. The corresponding IC50 values were determined as 100.29±2.11, 110.35±1.41, 133.97±2.62, 185.47±3.01, 159.62±2.3 µg/ml, respectively (Table 3). The results indicate that the TUEAF and TUBUF fractions demonstrate the highest effectiveness in scavenging DPPH radicals in vitro.

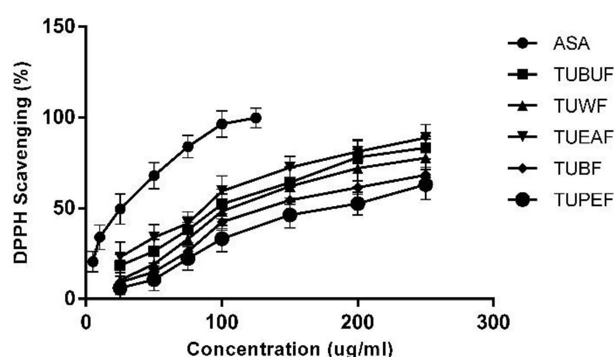

Figure 1: DPPH scavenging activity of fraction of *Tecomella undulata* bark

Values expressed as mean ± SEM with n = 3.

Table 3 IC_{50} values of fractions of *Tecomella undulata* bark on different *in vitro* antioxidant activity

Name of extracts	DPPH radical scavenging activity (ug/ml)	Superoxide anion scavenging (ug/ml)	Ferric ion chelating (ug/ml)	Nitric oxide radical scavenging (ug/ml)
TUPEF	185.47± 3.01	204.75± 2.1	206.77± 3.41	212.54± 3.39
TUBF	159.62± 2.3	186.38± 3.33	195.27± 5.41	208.22± 4.17
TUEAF	100.29± 2.11	137.07± 2.28	137.68± 4.32	150.56± 4.44
TUBUF	110.35± 1.41	133.39± 3.25	142.98± 3.49	162.48± 3.21
TUWF	133.97± 2.62	168.67± 2.27	178.49± 4.20	187.58± 2.61
Ascorbic Acid	32.73± 1.71	33.06± 2.31	56.54± 2.44	54.64± 2.78

Superoxide radical scavenging activity:

Figure 2 illustrates the patterns of fractions in their ability to quench superoxide anion. Table 3 presents the IC50 values for all fractions. TUBUF showed a higher IC50 value of 133.39±3.25 µg/ml, followed by TUEAF with an IC50 value of 137.07±2.28 µg/ml, when compared with the positive control ascorbic acid (IC50=33.06±2.31µg/ml).

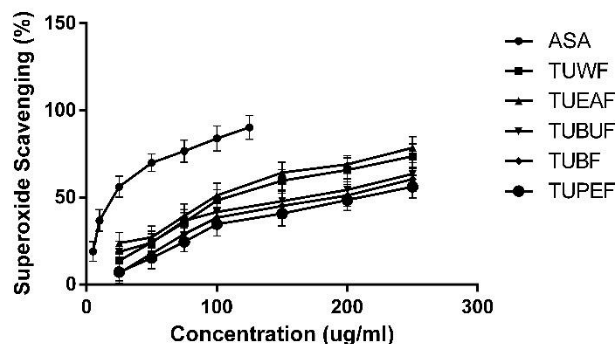

Figure 2: Superoxide scavenging activity of fraction of *Tecomella undulata* bark

Values expressed as mean ± SEM with n = 3.

Metal chelating activity:

The ferrous ion chelating ability of the different fractions was investigated using the ferrozine-Fe2+ complex method, as presented in Figure 3. All the fractions demonstrated a reasonable capacity to chelate iron (II) ions, showing a dose-dependent behaviour. The IC50 value for TUEAF was 137.68± 4.32 µg/ml, making it the fraction with the highest iron chelating efficacy among the tested fractions. The other fractions required higher concentrations to chelate iron, with IC50 values for TUWF, TUBF, and TUPEF measured at 178.49± 4.20 µg/ml, 195.27± 5.41 µg/ml, and 206.77± 3.41 µg/ml, respectively, as shown in Table 3. Ascorbic acid, used as the reference standard, exhibited the lowest IC50 value of 56.54± 2.44 µg/ml.

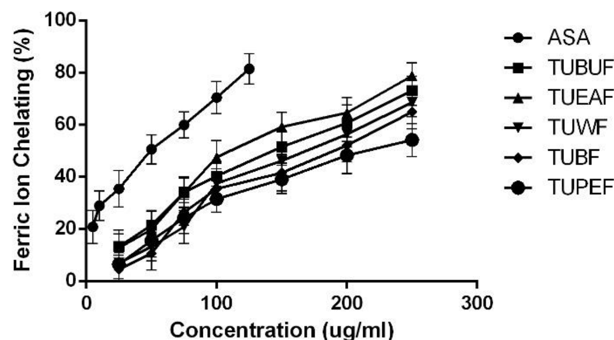

Figure 3: Ferric ion chelating activity of fraction of *Tecomella undulata* bark

Values expressed as mean ± SEM with n = 3.

Nitric oxide scavenging activity:

In the study (Figure 4), it was observed that the NO scavenging capacities of the fractions, as well as the positive control, increased in a dose-dependent manner. These findings were supported by the IC50 analysis data presented in Table 3. Among the fractions, TUEAF exhibited the highest NO scavenging ability, with an IC50 value of 150.56± 4.44 µg/ml, followed by TUBUF (IC50 = 162.48± 3.21 µg/ml), TUWF (IC50 = 187.588± 2.61 µg/ml), TUBF (IC50 = 208.22± 4.17 µg/ml), and TUPEF (IC50 = 212.54± 3.39 µg/ml). Notably, only TUEAF and TUBUF displayed reasonably better NO scavenging activity when compared to that of ascorbic acid (IC50 = 54.64± 2.78 µg/ml).

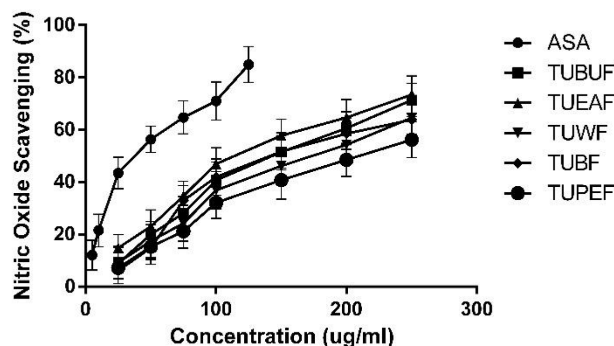

Figure 4: Nitric oxide scavenging activity of fraction of *Tecomella undulata* bark

Values expressed as mean ± SEM with n = 3.

Reducing power Activity

Figure 5 illustrates the reductive power of the fractions. The rise in absorbance signifies an augmentation in the reducing power activity. The outcomes revealed a concentration-dependent increase in the absorbance at 700 nm for the fractions.

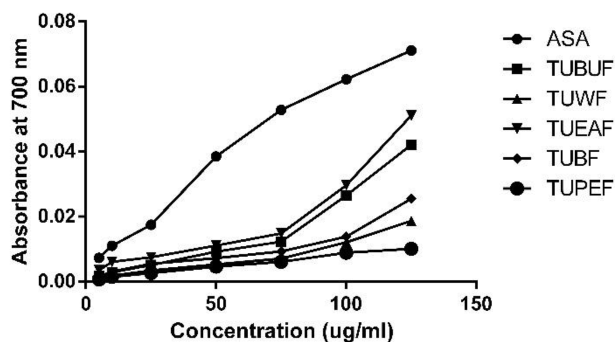

Figure 5: Reducing power activity of fraction of *Tecomella undulata* bark

Values expressed as mean ± SEM with n = 3.

***Tecomella undulata* bark fractions prolonged the Clotting time**

The fractions of *Tecomella undulata* bark were found to have an impact on clotting time. The experimental results demonstrated that as the concentration of *T.undulata* 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 6. The presence of anticoagulant(s) in *T.undulata* 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 *T.undulata* 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 7). 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 8).

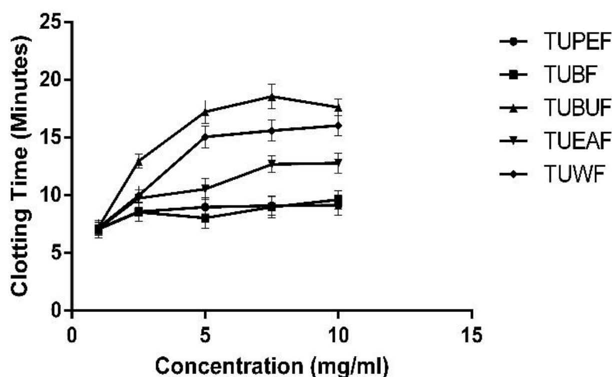

Figure 6: In vitro clotting time of varying concentrations of fractions of *Tecomella undulata* bark

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

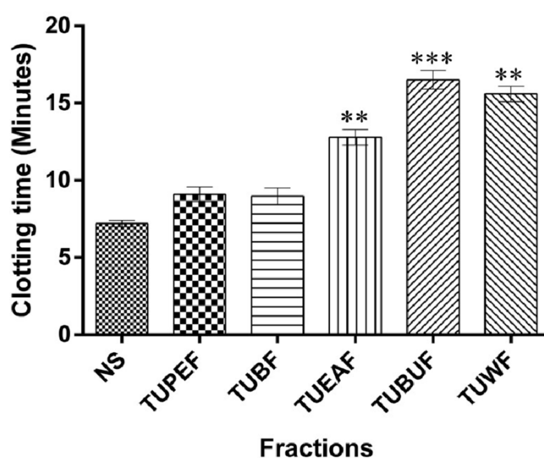

Figure 7: Mean clotting time of normal human blood treated with different fractions at 7.5 mg/mL

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).

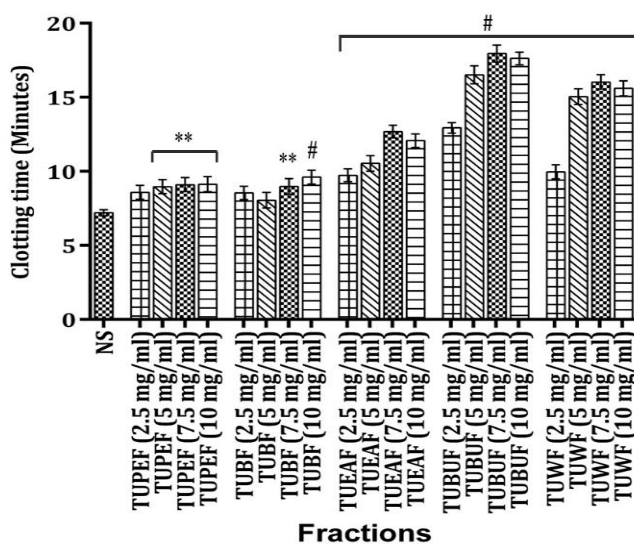

Figure 8: In vitro clotting time of normal human blood treated with the fractions at 2.5 mg/mL, 5 mg/mL, 7.5 mg/mL & 10 mg/mL

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 *T. undulata* displayed a noteworthy (P > 0.01) increase in the Prothrombin Time (PT) compared to the control (Figure 9). 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.

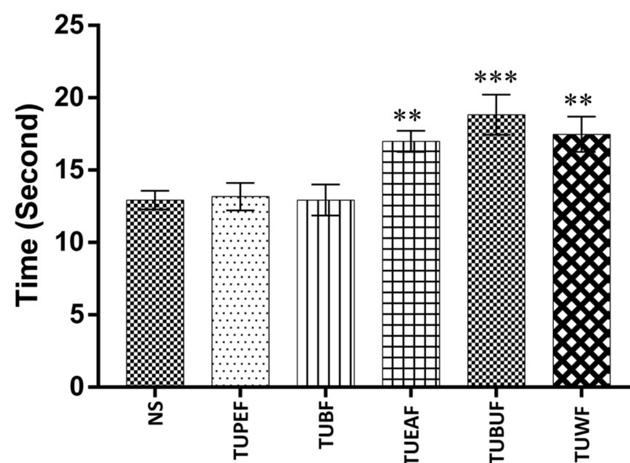

Figure 9: Prothrombin time of normal human plasma treated with all fractions at 7.5 mg/mL

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 10).

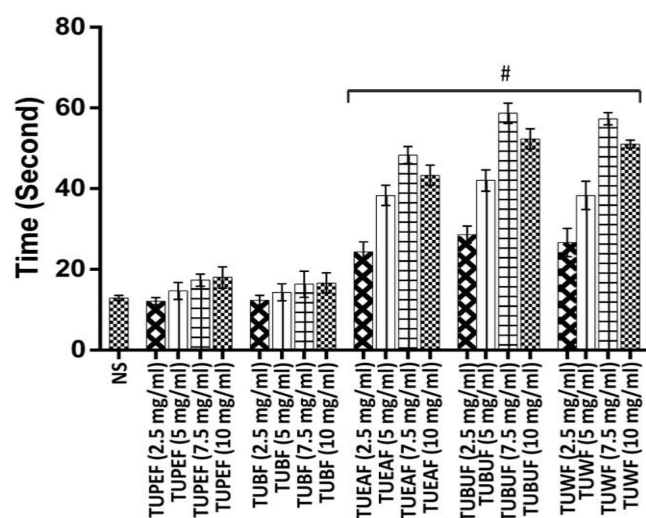

Figure 10: Prothrombin time of normal human plasma treated with all fraction at 2.5, 5, 7.5 & 10 mg/mL

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 *T. undulata* 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 11).

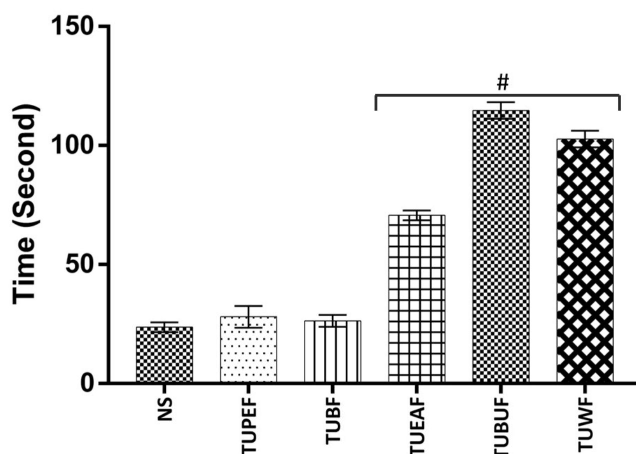

Figure 11: aPTT time of normal human plasma treated with all fractions at 7.5 mg/mL

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 12). 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.

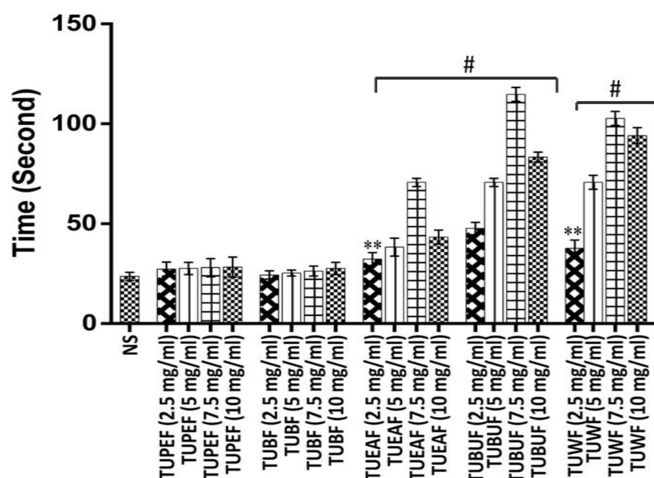

Figure 12: aPTT of normal human plasma treated with all fraction at 2.5, 5, 7.5 & 10 mg/mL

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 butanol fraction of *T.undulata* revealed a total of 10 peaks (Figure 14). 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 10 identified compounds in the butanol fraction of *T.undulata* (Table 4).

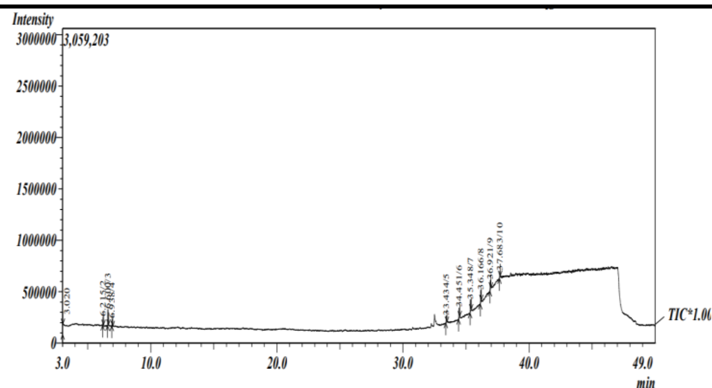

Figure 13: GCMS analysis of butanol fraction of Tecomella undulata bark

Table 4 GCMS identified phytochemical components of the butanol fraction of *Tecomella undulata* bark

S/N	Retention time	Name of compound	Molecular Formula	Peak Area (%)	Reported biological activity
1	3.02	2-Trifluoroacetoxydodecane	C14H25F3O2	6.95	Antioxidant [45]
2	6.215	Pentanedioic acid, 2-oxo-, dimethyl ester	C7H10O5	7.24	Antioxidant, antiplatelet, anticoagulant activity [46-47]
3	6.6	1-Octanol, 2,2-dimethyl-	C10H22O	13.7	Anti-inflammatory and analgesic activities [48], antimicrobial [49], antioxidant [50-51], cytotoxic activity [51]
4	6.938	3,3,6-Trimethyl-2,5-heptanedione	C10H18O2	7.14	Antimicrobial activity [52]
5	33.434	Pentacosane	C25H52	8.28	Antibacterial Activity [53-55], antifungal activity [56], Antiproliferative Activity [57], antioxidant activity [47], Insecticidal efficacy [58], Antibacterial Activity [59] Antidiabetic activity [60]
6	34.451	Tetracontane	C40H82	12.46	Antifungal activity [56], antioxidant activity [47], Antiproliferative Activity [57], Insecticidal efficacy [58], Antibacterial Activity [59], Anticholinesterase Activities [61], breast cancer [62]
7	35.348	Tetratetracontane	C44H90	14.09	Anticholinesterase Activities [61],

					Antimicrobial activity [63]
8	36.166	Triacontane, 1-iodo-	$C_{30}H_{61}I$	13.6	Insecticidal efficacy [58], Antibacterial Activity [59].
9	36.921	Octacosane, 1-iodo / Hexacosane, 1-iodo	$C_{28}H_{57}I$ / $C_{26}H_{53}I$	10.4	Anti-α-glucosidase & anti-cyclooxygenase activity [64]
10	37.683	Dotriacontane	$C_{32}H_{66}$	6.13	Antimicrobial, antioxidant, Antispasmodic, antibacterial, and Antiviral [65], Anticandidal Activity [66], Antifungal anti-inflammatory, cytotoxic activity [67], Anti-α-glucosidase and anti-cyclooxygenase activities [64]

Discussion

Phytochemical screening

According to the existing literature, the bark of *T.undulata* 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 [68].

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 [69]. 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 [70,71]. 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 [72]. 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 *T.undulata* bark 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 [73-75].

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 [73-75].

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 signalling pathways related to oxidative stress [73-75].

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 [49-51].

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 [73-75].

In-vitro antioxidant activity

DPPH radical scavenging assay

DPPH free radical scavenging activity refers to the capacity of a substance to neutralize or eliminate the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical. This activity serves as a common measure of a compound or sample's antioxidant potential, indicating its ability to combat oxidative stress and safeguard cells against damage caused by free radicals. A higher DPPH free radical scavenging activity signifies a more potent antioxidant capability [76-77]. Based on the results, it was observed that the ethyl acetate and methanol fractions demonstrated the most effective scavenging of DPPH radicals in vitro.

Superoxide radical scavenging activity:

Superoxide anions are reactive oxygen species (ROS) that play a significant role in causing cellular damage. These superoxide anions are considered primary ROS as they can generate reactive by-products through direct interactions with other molecules or catalysed processes involving metals or enzymes, including those produced within the mitochondria. Due to their harmful effects on the body, these ROS are associated with various diseases such as strokes, cancer, diabetes, liver damage, and neuronal lesions [78-79]. Considering the potential risks associated with superoxide anions, we evaluated the radical scavenging activity of the fractions obtained from *T. undulata.* The butanol and ethyl acetate fractions have demonstrated notable capacity in effectively quenching superoxide anions.

Metal chelating activity

The iron chelating effect plays a vital role in inhibiting the interaction between metals and lipids by forming insoluble metal complexes with ferrous ions. This effect is highly significant as it prevents the generation of hydroxyl radicals by impeding the interaction between iron and hydrogen peroxide. Consequently, it effectively hinders the decomposition of hydrogen peroxide and the formation of even more harmful free radicals [80]. Figure 4 clearly demonstrates the substantial iron chelating activity of all fractions. Figure 4 clearly demonstrates the substantial iron chelating activity of all fractions. The order of effectiveness was as follows: TUEAF < TUBUF <

TUWF < TUBF < TUPEF. All fractions exhibited a reasonable capacity to chelate iron (II) ions, with the efficacy being dose-dependent.

Nitric oxide scavenging activity:

The Griess reagent method was employed to investigate the scavenging activity of fractions against nitric oxide released by sodium nitroprusside [81]. The study (Figure 5.4B) revealed that the NO scavenging capacities of fractions and the positive control increased in a dose-dependent manner.

Reducing power Activity

The reducing power assay evaluates the electron-donating capacity of a sample, which is indicative of its ability to donate electrons. The results of this test were quantified as the reducing activity equivalent to ascorbic acid. The reducing power of compounds is believed to act as an inhibitor of chain reactions triggered by free radicals through the donation of electrons. This activity is mediated by redox reactions [82].

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 [83-84], flavonoids [82-88], carotenoids [83], and polysaccharides [89] 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 [90]. Additionally, saponins are known to possess antiplatelet, anticoagulant, and fibrinolytic characteristics [91].

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 [92]. 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 [93]. 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 *T.Undulata* 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 *T.undulata* 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 *T.undulata*, 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 *T.undulata* 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.

The treatment of thrombotic conditions often involves the use of common anticoagulant drugs such as heparin, warfarin, all-trans retinoic acid, and novel anticoagulants (NOACs). These drugs have different characteristics in terms of their duration of action and reversibility. Heparin is a fast-acting anticoagulant that can be reversed, while

warfarin has a slow and long-acting effect that is also reversible. All-trans retinoic acid acts slowly, and NOACs are fast-acting but cannot be reversed [94-95].

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). This significantly enhances the anticoagulant activity of *Tecomella undulata* Antithrombin by about 1,000 times [96]. 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 [97-98].

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 [99-100]. The anticoagulant activity of ethyl acetate and methanolic fraction of *T.undulata* demonstrates a similar mechanism of action to heparin, with a narrow concentration range of activity peak indicating fast action and reversibility.

The GC-MS analysis of the butanol fraction of *T.undulata* bark revealed the presence of various bioactive compounds with diverse biological activities. Notably, Pentanedioic acid, 2-oxo-, dimethyl ester, 2-Trifluoroacetoxydodecane, 1-Octanol, 2,2-dimethyl, Pentacosane, Tetracontane, and Dotriacontane were identified to have antioxidant activity. Additionally, Pentanedioic acid, 2-oxo-, dimethyl ester was reported to exhibit antiplatelet and anticoagulant activity [46-47]. Previous studies by Karthikeyan Mohanraj et al have reported several compounds in *T.undulata* bark, including Heptacosane, Tecoside, Lapachol, Dehydrotectol, Undulatoside A, 1-Triacontanol, 3,4-Dimethoxybenzoic acid, 1-Octacosanol, Triacontane, Nonacosane, Tecomelloside, Ferulic acid, beta-Sitosterol, Daucosterol, Rutin, and beta-Sitosterol-beta-D-glucoside, among others [101-103]. Research by Shao Shuai et al, Jun-Hui Choi et al, and Laibin Zhang et al has indicated that ferulic acid and its derivatives possess antithrombotic and anticoagulant activity [104-106]. Moreover, studies by Prachi S Salunkhe et al and Debananda Gogoi et al have reported that beta sitosterol exhibits antithrombotic and anticoagulant activity [107-108]. Lapachol has been found to inhibit vitamin K epoxide reductase and vitamin K quinone reductase [109]. Furthermore, 1-Triacontanol, 3,4-dihydroxybenzoic acid, and rutin have been associated with prolonged APTT and PT time [110-112]. The results of this investigation suggest that *T.undulata* contains bioactive compounds with anticoagulant properties, showing promising potential for the development of novel anticoagulant drugs.

Conclusion

The study demonstrated that the ethyl acetate and butanol fractions of *T.undulata* bark 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.

Acknowledgements

Authors are thankful to Head of Department, B.K. Modi Govt. Pharmacy College, Rajkot, India, for providing necessary facilities and Dr. Mital Manvar, Atmiya institute of pharmacy, Atmiya University, Rajkot, India, being source of encouragement in writing this research article.

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Abbreviations

LDL-C Low Density Lipoprotein-cholesterol

HDL-C High-density lipoprotein-cholesterol

ROS Reactive Oxygen Species

CAD Coronary Artery Diseases

TUPF Tecomella undulata petroleum ether fraction

TUBF Tecomella undulata benzene fraction

TUEAF Tecomella undulata ethyl acetate fraction

TUBUF Tecomella undulata butanol fraction

TUWF Tecomella undulata water fraction

GAE Gallic Acid Equivalent

QUE Quercetin Equivalent

DPPH 2,2-diphenyl-1-picrylhydrazyl

DRSA DPPH radical scavenging activity

EDTA Ethylenediamine tetraacetic acid

NBT Nitroblue Tetrazolium

NEDD Naphthylethylenediamine

PPP Platelet poor plasma

PT Prothrombin time

APTT Activated prothrombin time

GC–MS Gas chromatography-mass spectrometry

NS Normal Saline

NIST National Institute of Standards and Technology

Figure Legend

Figure 1: DPPH scavenging activity of fraction of *Tecomella undulata* bark

Figure 2: Superoxide scavenging activity of fraction of *Tecomella undulata* bark

Figure 3: Ferric ion chelating activity of fraction of *Tecomella undulata* bark

Figure 4: Nitric oxide scavenging activity of fraction of *Tecomella undulata* bark

Figure 5: Reducing power activity of fraction of *Tecomella undulata* bark

Figure 6: In vitro clotting time of varying concentrations of fractions of *Tecomella undulata* bark

Figure 7: Mean clotting time of normal human blood treated with different fractions at 7.5 mg/mL

Figure 8: In vitro clotting time of normal human blood treated with the fractions at 2.5 mg/mL, 5 mg/mL, 7.5 mg/mL & 10 mg/mL

Figure 9: Prothrombin time of normal human plasma treated with all fractions at 7.5 mg/mL

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

Figure 11: aPTT time of normal human plasma treated with all fractions at 7.5 mg/mL

Figure 12: aPTT of normal human plasma treated with all fraction at 2.5, 5, 7.5 & 10 mg/mL

Figure 13: GCMS analysis of butanol fraction of *Tecomella undulata* bark