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Phytochemical Evaluation and Bioactivity of *Polysiphonia subtilissima* Montagne

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The chemical profile and bioactivity of *Polysiphonia subtilissima*, a marine bioactive red alga from the east coast region of Odisha state of India is investigated. Qualitative tests of the extracts confirmed the presence of various primary and secondary metabolites. Moreover, the agar, carrageenan, fatty acids, flavonoids and polyphenols, employed DPPH scavenging, HRBC and optimized critical dilution to test the extracts for antioxidant, anti-inflammatory, antibacterial and anticoagulant effects were investigated. The methanolic extract showed total phenolic (3.14 mg), flavonoid (2.72 mg) and fatty acid (0.12 mg in ethylacetate and 0.1 mg in chloroform) and highest antioxidant activity (95%), at 515.5 nm. At 100 and 200 µg/mL doses, the aqueous extract had the highest anti-inflammatory activity (72.13% and 89.61%). Maximum bactericidal inhibitions of 73.7%, 77.6% and 58% were detected in the ethanolic fraction at 500 µg/mL. Water extract showed anticoagulant action with activated partial thrombo-plastin time (APTT) clotting durations of 139 s, prothrombin time (PT) of 70 s and thrombin time (TT) of 60 s.

Keywords: *Polysiphonia subtilissima*, Seaweeds, Biological activities.

INTRODUCTION

Seaweeds, also known as marine macroalgae, are multicellular algae that thrive in marine environments. They play a crucial role in marine ecosystems as both a home and food source for many different kinds of marine organisms [1]. According to their pigments and other distinguishing features, seaweeds fall into four primary groups: the prokaryotic Cianophyceae (blue green algae), eukaryotic red algae (Rhodophyta), brown algae (Phaeophyta) and the green algae (Chlorophyta) [2]. Seaweeds are present in many maritime ecosystems all over the world, in both intertidal and subtidal areas and their presence depends on elements like wave activity, salinity and the type of substrate [3]. India has a diverse coastline, spanning over 7,516.4 km, with diverse habitats and a rich biota [4-6]. Different regions of India's coastal line distribute different genera of seaweeds. The east coast, along the Bay of Bengal, is characterized by mangrove ecosystems and diverse seaweed flora. Gracilaria, ulva and enteromorpha species are among those present [7,8]. Seaweeds have traditionally been used as a repository for therapeutic compounds. These compounds possess a high concentration of bioactive molecules that have the potential for therapeutic use. Seaweed frequently contains vital components such as carbohydrates, proteins, minerals, vitamins, polyphenols, fatty acids and colour pigments [2,9,10]. Various extracts exhibited a wide range of promising bioactivities, including antioxidant, anticoagulant, antihypertensive, antilipidemic, antidiabetic, antibacterial, antifungal, antiviral, antiinflammatory, anticancer, hepatoprotective, *etc*. [5,6,11].

The goal of this study is to confirm and classify the presence of primary and secondary metabolites present in *Polysiphonia subtilissima* Montagne, a red algae species from the Rhodomelaceae family [12]. The objective is to assess the potential therapeutic properties of these compounds, which may provide a clue for their use as medicinal agents. Chilika lake, situated in the Puri, Khordha and Ganjam districts of Odisha state on the east coast of India, stands as Asia's largest brackish water lagoon and the second largest coastal lagoon globally. Chilika Lake, is considered as an assemblage of marine, brackish and freshwater ecosystems [13,14]. The algae were collected from the coastal belt of Chilika, Odisha state of India and a comprehensive and methodical qualitative chemical analysis was

conducted to discover the chemical class of both primary and secondary metabolites [15]. Quantitative estimations, which include agar, carrageenan, fatty acids, flavonoids and polyphenols, were performed. The extracts were analyzed for the bioactivity, taking into account the known types of bioactivities exhibited by red algae. Since 2021, we have been collecting samples at regular intervals. However, occasional difficulties in obtaining enough specimens have necessitated conducting some tests at a later time.

EXPERIMENTAL

All the chemical reagents and solvents required to perform the experiments were purchased from Himedia (India), Merck (India), SRL Pvt. Ltd., Lobogens, Loba Chemie (India) and used without further purification unless otherwise stated. Deionized water was procured from ECLEN, Ahmadabad, India and its total dissolved solids (TDS) was determined and found to be zero. The UV-2450, Shimadzu, Japan, was used for all UV-visible spectroscopic determinations.

Collection and authentication of crude *Polysiphonia subtilissima* Montagne: The specimen of *P. subtilissima* Montagne was obtained by scraping it off the rocks and boulders submerged in the shallow waters of Chilika Lake, the largest coastal lagoon in India. Chilika is located on the eastern coast of India, specifically between the latitudes 19º28′–19º54′ N and longitudes 85º5′–85º38′E. *Polysiphonia subtilissima* Montagne is extensively distributed in certain areas of Chilika, such as Kalijai, Pathara and Satpara, throughout the months of December, January and February. The likely reason for blooming in this season is the variation in the salinity gradient and water temperature during the winter season. A marine biologist was successful in identifying the red algae by studying its morphology. The sample was brought to the laboratory in plastic bags containing water to prevent evaporation, keeping in mind about water-to-specimen ratio [13-15]. Then, the sample was carefully rinsed with distilled water after being rinsed with tap water to eliminate any contaminants.

Preparation of algal extracts: Initially, petroleum ether was employed for the purpose of eliminating superficial fats, lipids, waxes and chlorophylls. Subsequently, the entire plant was desiccated by delicately compressing it between tissue papers and allowed to dry at a temperature below 30 ºC for 7 days. Following the drying process, the entire plant was pulverized into a coarse powder using a commercially available grinder and mixer. A total of 10 g of powdered samples were subjected to hot soxhletation extraction process to extract the active ingredients from the powdered sample. Extraction was performed using solvents with progressively higher polarity, including *n*-hexane, DCM, ethyl acetate, chloroform, water, methanol and ethanol. The extraction was carried out by preparing a 10 g thimble of coarse powder with 200 mL of solvent in each run. A continuous extraction for 4 h was optimized and carried out. Finally, the extracts were filtered and concentrated under a rotary vacuum evaporator at a ~ 50 ºC to save any thermolabile components, if any, present in the extracts [15].

Qualitative analysis of bioactive phytocomponents: All the extracts were analyzed for the presence of primary and secondary metabolites *i.e.*, presence of phytocomponents by implementing a series of general qualitative tests of the different extracts [15].

Detection of carrageenan (galactans): A 4 g of dry powdered sample was heated at 80 ºC in 200 mL of water, while being continuously stirred and then filtered. Added 200 mg of KCl in the filterate solution, centrifuged to isolate the precipitated carrageenan from the liquid fraction of the extract and finally dried for further evaluation [16].

Detection of agar: A 5 g sample was washed thoroughly to eliminate foreign substances and subsequently subjected to prolonged heating with water. The agar was solubilized in the water and the resulting solution was filtered to eliminate any remaining seaweed fragments. The aqueous extract was cooled to room temperature, resulting in the formation of a gelatinous mass,which confirmed the presence of agar. Further confirmation for the presence of agar was shown by the formation of a canary yellow colour when a small sample was heated in an alcoholic KOH solution. To provide additional evidence of the existence of agar, a 0.5 mL of conc. HCl was introduced into a 4 mL solution containing the extracted agar. The solution was subjected to heat in a water bath for 30 min, subsequently cooled to the ambient temperature and then separated into two equal parts. In the first part, 3 mL of 10% NaOH solution was mixed with equal amounts of Fehling's solutions A and B, then heated them using a water bath. In second part, a 10% solution containing $BaCl_2$ was introduced [17].

Quantitative analysis of bioactive phytocomponents : The quantitative determination of phytochemicals in the extract of red alga *Polysiphonia substilissima* Montagne was performed using conventional techniques [18].

Determination of total flavonoid content: The total flavonoid content was estimated using the $AlCl_3$ colorimetric assay. A total of 1 mL of algal extracts was diluted with 200 µL of distilled water separately, followed by the addition of 150 µL of $NaNO_2$ (5%) solution. This mixture was incubated for 5 min and then 150 µL of $AlCl_3$ (10%) solution was added and allowed to stand for 6 min. Then 2 mL of NaOH (4%) was added and made up to 5 mL with distilled water. The mixture was thoroughly shaken before being left to rest at room temperature for 15 min. The absorbance was measured at 510 nm. The total flavonoid content was expressed as mg QE/g extract on a dry weight basis [19].

Determination of total phenolic content: A range of standard solutions with varying concentrations (*e.g.* 10, 20, 30, 40, 50, 60, 70, 80 and 90 ppm) were prepared using 100 ppm stock solution of pure gallic acid dissolved in distilled water. The algal extract was subjected to methanol extraction to isolate the phenolic compounds present in it. The methanolic extract was filtered and then mixed with 0.1 mL of sample, the standard solution and 0.5 mL of Folin-Ciocalteu reagent. The mixture was left undisturbed for 5 min at room temperature to facilitate the development of colour. A 1.5 mL of solution containing 20% Na_2CO_3 was added to the reaction mixture and thoroughly shaked. The reaction mixture was left at ambient temperature in the absence of light for 30 min, which results in the formation of a blue colour [20]. Then, the absorbance of the reaction

mixture was measured at 760 nm using UV-Vis spectrophotometer. Similarly, the absorbance values for both the sample and the standard solutions were also recorded. A calibration curve was generated by plotting the absorbance values of the standard solutions against their respective concentrations. The total phen-olic content of the sample was evaluated using the calibration curve.

Gravimetric estimation of total fatty acid: Accurately weighed 2 g of algal powder was introduced to Soxhlet fat extraction equipment containing 30 mL of 2 N NaOH solution as a saponification reagent (to hydrolyze the triglycerides into glycerol and free fatty acids) and then refluxed for 2 h. Following saponification, the reaction mixture was acidified using HCl to release the unreacted fatty acids. The released fatty acids formed a solid, which was separated with *n*-hexane. The fatty acids undergo dissolution in the solvent, resulting in the formation of a solution. The solvent containing the fatty acids was transferred to a container and allowed to evaporate the solvent in a drying oven, resulting in the residual presence of the fatty acids [21]. The total fatty acid content was calculated using the following equation:

$$\text{Total fatty acid content (\%)} = \frac{\text{Weight of fatty acids}}{\text{Weight of sample}} \times 100$$

***In vitro* evaluation of bioactivity of the extracts**

Antioxidant activity (DPPH free redical scavenging assay): Methanolic extracts of samples were measured from 1 to 5 mg/mL in a test tube and 5 mL of 0.004% w/v solution of DPPH in methanol was added. The mixture was vortexed for 1 min and kept in the dark at room temperature for 30 min. The UV absorbance of all the sample solutions was measured at 515.5nm against blank samples of 80% w/v methanol [22]. The percentage scavenging effect was calculated using the following equation:

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the samples. Lower absorbance meant that the samples had more DPPH-free redical scavenging activity.

Anti-inflammatory activity: The HRBC method was employed to evaluate the *in vitro* anti-inflammatory activity. Human blood samples were collected and was combined with an equivalent amount of sterilized Alsevers solution. The solution was centrifuged at a speed of 3000 rpm resulting in the separation of the packed cells. The concentrated cells were rinsed with isosaline solution and then diluted to 10% suspension using isosaline. The HRBC suspension was utilized to assess the anti-inflammatory efficacy. Different concentrations of extract, reference sample and control were individually combined with 1 mL of phosphate buffer, 2 mL of hyposaline and 0.5 mL of HRBC solution. The assay mixtures were incubated at 37 ºC for 30 min and then centrifuged at 3,000 rpm. The liquid that remained after sedimentation was separated out and the amount of hemoglobin was determined at 560 nm wavelength. The proportion of hemolysis was calculated by considering the hemolysis observed in control as 100% [23].

$$\text{Protection (\%)} = 100 - \frac{\text{OD sample}}{\text{OD control}} \times 100$$

Antibacterial activity: In this work, the microdilution method as described by Beaulieu *et al.* [24] for determining the minimum inhibitory concentration (MIC) of aqueous extract was followed. Briefly, the aqueous extract was sterilized by filtration on a 0.2 µm polyethersulfone membrane. Two-fold serial dilutions of samples were prepared in duplicate by repeated transfer of 125 µL volumes of tryptic soy broth (TSB) in a 96-well flat-bottom microplate. Each well was inoculated with 50 µL of bacterial suspension at 1×10^5 cfu/mL. Bacterial strains *Escherichia coli* K12, *Micrococcus luteus* ATCC 9342 and *Staphylococcus aureus* ATCC 6571 were selected. The optical density was monitored at 600 nm every 10 min with a PowerWaveXS2 microplate spectrophotometer. The percentage of inhibition was calculated by following Cox *et al.* [25] method.

Anticoagulant activity: The anticoagulant activity of water, methanol and ethanol extracts was determined by measuring the intrinsic and/or common activated partial thromboplastin time (APTT), extrinsic (prothrombin time, PT) or common (thrombin time, TT) pathways of the coagulation cascade. All the assays were carried out using a semi-automated blood coagulometer and assay kits from AMR Health Care (Coimbatore, India), according to the manufacturer's instructions. Briefly, 90 µL of normal human plasma were mixed with 10 µL of 0.9% NaCl solution containing various sample concentrations for each assay. For APTT assay, 100 µL of APTT assay reagent was added to the mixture and then left ideally at 37 ºC for 3 min followed by the addition of 100 µL of 0.025 M $CaCl_2$, finally the clotting time (APTT) was recorded. To conduct TT and PT tests, the mixture was heated to 37 ºC for 2 min (PT) or 1 min (TT). After adding 200 µL of PT or 100 µL of TT test reagent, the clotting time was observed [26,27]. All assays used 0.9% NaCl solution as negative control.

RESULTS AND DISCUSSION

Phytochemical screening: The phytochemical analysis of various extracts was performed by the standard protocols and shows the presence of various phytochemical compounds as summarized in Table-1. The Molisch test confirmed that carbohydrates are present, whereas Benedict's test confirmed the presence of reducing sugars. Formation of yellow precipitate indicates the presence of reducing sugar in the concentration range of 0.5% to 1% [28]. When the extracts react with iodine, a polysaccharide which resembles floridean starch, appear as shown by absorption peaks at 525-530 nm. The aqueous extract exhibited a higher concentration of carbohydrates compared to the other extracts. The acrolein test revealed the presence of lipids in the ethylacetate and chloroform extracts. The extracts show positive responses in biuret and ninhydrin tests, indicating the presence of protein. The aqueous solution abundantly contained secondary metabolites such as polyphenols, glycosides, flavonoids, saponins, tannins and terpenoids. The methanolic extracts revealed the presence of steroids, polyphenols, alkaloids, flavonoids, saponins and tannins. Both alcoholic extracts contained almost identical secondary metabolites but varies in quan-

TABLE-1
PRELIMINARY QUALITATIVE PHYTOCHEMICAL SCREENING DATA OF THE DIFFERENT EXTRACTS OF *P. subtilissima*

Phyto constituents	Chemical tests	Solvent extracts						
		Water	Methanol	Ethanol	Ethyl acetate	Dichloro-methane	Chloroform	Hexane
Carbohydrates	Molisch reagent	+++	+	+	–	–	–	–
	Benedict test	++	–	–	–	–	–	–
	Fehling's test	++	+	+	–	–	–	–
Lipids	Acrolein test	–	–	–	+	–	+	–
Polyphenols	Ferric chloride test	++	+++	++	+	–	–	–
Steroids	Liebermann–Burchard test	–	++	+	–	–	–	–
Glycosides	Borntrager's test	+	+	+	–	–	–	–
	Keller-Kiliani test	+	+	+	–	–	–	–
Alkaloids	Dragendorff's test	–	++	+	+	–	+	–
	Mayer's test	–	+	+	–	–	–	–
	Hager's test	–	–	–	–	–	–	–
	Wagner's test							
Flavonoids	Shinoda test	+	+++	++	–	–	–	+
	Ferric chloride test	+	+++	++	+	–	–	+
	Sulfuric acid	+	–	+	+	–	–	–
Saponin	Foam test	+	–	–	–	–	–	–
	Steroidal saponin test	++	++	++	–	–	–	–
	Triterpenoidal saponin test	+++	++	++	–	–	–	–
Tannins	Ferric chloride test	+++	++	++	–	–	–	–
	Phenazone test	++	+	+	–	–	–	–
	Goldbeater's skin test	+++	+	+				
Triterpenoids	Tschugajeu test	++	–	–	–	–	–	–
	Briekorn and Brinar test	++	–	–	–	–	–	–
Proteins	Biuret test	++	–	–	–	–	–	–
	Ninhydrin test	++	–	–	–	–	–	–

+++: Intensely present, ++: Moderately present, +: Present, –: Absent

tities. Some flavonoids were found to be present in the non-polar extract *i.e.*, hexane.

It was also observed that certain chemical components were present during certain seasons but not during others. For example, protein content was higher during the monsoon season, while carbohydrates were more abundant in the pre-monsoon season. These variations in chemical composition depend on factors such as salinity and surface temperature of the water.

Presence of carrageenan and agar: The test confirms the presence of carrageenan and the characteristics of gel with a short texture and brittleness suggests that it contains more kappa carrageenan. The formation of a gelatinous mass, the formation of a canary yellow hue and the formation of crimson precipitate of cuprous oxide with Fehling A confirm the existence of agar and its reducing sugars in agar. The formation of a delicate white barium sulfate precipitate further confirmed the presence of sulfate in agar.

Total phenolic, flavonoid and fatty acid content: The highest total phenolics (3.14 ± 0.15 mg GAE/g dry weight) and flavonoids (2.72 ± 0.05 mg RUE/g dry weight) were found in methanol extract, while the highest total fatty acid (0.12 ± 0.43 mg CAE/g dry weight) was calculated in ethyl acetate extract (Table-2).

Antioxidant activity: The highest scavenging activity was observed in methanolic extract with 95.0% followed by ethanol 94.78%, water 91.22% and ethyl acetate 74.4%. The high antioxidant activity may be due to the presence of various types of phytochemicals like vitamins, polyphenols, phycocyanin, phycoerythrin, flavonoids, xanthones, coumarins, carotenoids, phenolic acid, tannins, anthocyanins and polyunsaturated fatty acids, which have a tendency to absorb, neutralize and quench singlet and triplet oxygen.

TABLE-2
COMPARATIVE RESULTS OF TOTAL PHENOLIC, FLAVONOID AND FATTY ACID CONTENT IN THREE DIFFERENT EXTRACTS OF *P. subtilissima*

Solvent extracts	Total phenolics (mg GAE/g dry wt.)	Total flavonoid (mg QUE/g dry wt.)	Total fatty acid (mg CAE/g dry wt.)
Water	1.89 ± 0.11 mg	1.11 ± 0.16 mg	–
Methanol	3.14 ± 0.15 mg	2.72 ± 0.05 mg	–
Ethanol	1.46 ± 0.32 mg	1.19 ± 0.9 mg	–
Ethyl acetate	1.38 ± 0.05 mg	–	0.12 ± 0.43 mg
DCM	–	–	–
Chloroform	–	–	0.1 ± 0.76 mg
Hexane	–	0.7 ± 0.66 mg	–

Values are mean of three analyses of the extract ± standard deviation (n=3) GAE: Gallic acid equivalent, QUE: Quercetin equivalent, CAE: Catechin equivalent.

Anti-inflammatory activity: The anti-inflammatory activity of three different solvent extracts was also evaluated and the results are shown in Table-3. At 200 µg/mL, aqueous extract contains 89.6.1% anti-inflammatory activity, whereas the methanolic extract had 65.3.9%. The preliminary qualitative analysis of the extracts showed that the methanolic extract had more flavonoids, polyphenols and tannins than the water extract, however, it is observed that the water extract was more

TABLE-3
ANTI-INFLAMMATORY ACTIVITY RESULTS
OF DIFFERENT EXTRACTS OF *P. subtilissima*

Groups	Concentration (µg/mL)	% Control protection means ± S.E.M
Control	–	–
Aqueous extract	100	72.13 ± 1.9
	200	89.6.1 ± 5.1
Methanolic extract	100	55.81 ± 2.8
	200	65.3.9 ± 5.6
Standard	100	87.62 ± 3.3
	200	91.12 ± 5.6

The results were expressed as mean ± S.E.M. [n = 3]

effective at reducing inflammation. There has been no mention of the ethanolic extract due to its negligible activity.

Antibacterial activity: The antibacterial study was carried out aginst three pathogenic strains of bacteria *viz*. *Escherichia coli*, *Staphylococcus aureus* and *Micrococcus luteus*. The ethanolic extract at 500 µg/mL concentration was most effective against *S. aureus* and *E. coli* inhibiting growth by 77.6% and 73.7%, respectively; but it was moderately effective against *M. luteus*, inhibiting growth by 58%. In case of water extract at 500 µg/mL concentration, the most effectiveness was against *S. aureus*, inhibiting 71.9% of bacterial growth followed by *E. coli* (inhibiting 68.6%). However, it was least effective against *M. luteus*, killing only 33.5% of bacteria. The methanolic extract could not exhibit any significant inhibition in any of the studied bacterial strains (Table-4).

Anticoagulant activity: The clotting time assay revealed enhanced anticlotting activity in the water fraction, with 139 s in APTT, 70 s in PT and 60 s in TT. The positive control was taken as heparin and the APTT was found to be 0.85 ± 0.85 µg/mL, the PT value was 13.66 ± 1.2 µg/mL and the TT value was 1.02 ± 0.5 µg/mL. The methanol and ethanol fractions were found to exhibit a little less effect than the water extracts. The clotting time of the negative control (0.9% NaCl) for APTT, PT and TT was found to be 39, 14 and 13 s, respectively. The methanolic fraction showed better results than the ethanolic fraction. Table-5 provides the calculated values for the time and concentration required to achieve different parameters like APTT, PT and TT.

Conclusion

Based on the results, this study supports the hypothesis that *Polysiphonia subtilissima* contains significant compounds with various bioactivities. The abundance of many primary and secondary metabolites, including carbohydrates, proteins, lipids, polyphenols, saponins, triterpenoids, flavonoids, tannins, steroids, alkaloids and glycosides, contribute to the versatility of this red alga. Various bioactivities were conducted using the insights obtained from the qualitative analysis. The bioactive chemicals have antioxidant, anti-inflammatory, antibacterial and anticoagulant properties. Due to the seasonal fluctuation of the chemical composition and its limited accessibility for collection, a comprehensive biological examination was not feasible.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

TABLE-4
ANTIBACTERIAL ACTIVITY RESULTS OF *P. subtilissima* IN DIFFERENT EXTRACTS

Concentrations (µg/mL)	Growth inhibition (expressed in %)								
	Escherichia coli K12			*Staphylococcus aureus* ATCC 6571			*Micrococcus luteus* ATCC 9342		
	Water	Ethanol	Methanol	Water	Ethanol	Methanol	Water	Ethanol	Methanol
500	68.6 ± 1.6	73.7 ± 2.3	22.06 ± 0.9	71.9 ± 1.2	77.6 ± 0.8	22.4 ± 3.2	33.55 ± 1.6	58 ± 2.5	–
250	23.78 ± 2.5	34.2 ± 0.5	10.05 ± 1.5	33.6 ± 0.5	33.77 ± 3.5	–	12.0 ± 3.7	24.9 ± 5.5	–
125	11.91 ± 0.5	18.6 ± 1.2	3.5 ± 2.5	16.58 ± 1.8	–	–	–	–	–

TABLE-5
ANTICOAGULANT ACTIVITY RESULTS OF THREE EXTRACTS OF *P. subtilissima*
(EXPRESSING CLOTTING TIME WITH RESPECT TO CONCENTRATION)

	Time (s)				Concentration (µg/mL)			
	Clotting time (negative control)	Maximum clotting time as measured in coagulometer			Heparin	Aqueous fraction	Methanolic fraction	Ethanolic fraction
		Water	Methanol	Ethanol				
Activated partial thromboplastin time (APTT)[a]	39	139	67	44	0.85 ± 0.85	2.5 ± 1.03	11.0 ± 2.15	35.0 ± 0.98
Prothrombin time (PT)[a]	14	70	42	17	13.66 ± 1.2	15.66 ± 0.89	35.5 ± 1.07	45.8 ± 0.23
Thrombin time (TT)[a]	13	60	19	15	1.02 ± 0.5	2.1 ± 2.5	9.5 ± 0.65	29.6 ± 0.55

[a]Concentration of the sample required to achieve a twofold increase in clotting time relative to the negative control (0.9% NaCl). Data shown as the mean ± SD, n = 3.

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