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Research Article

TO STUDY THE *IN VITRO* INHIBITION OF BLOOD PLATELET AGGREGATION BY ISOLATED COMPOUNDS FROM *CLAUSENA DENTATA* (WILLD.) ROEM.

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ABSTRACT

Objectives: The objectives of the study were to study the antiplatelet aggregation activity of compounds isolated from extracts of *Clausena dentata. Clausena* (*Rutaceae*) is a genus of about 23 species of unarmed trees and shrubs. The stem bark of *C. dentata* is used in veterinary medicine for the treatment of wounds and sprains. Even though *C. dentata* has a lot of potential medical uses, the study of pharmacological properties is very scarce.

Methods: The plant *C. dentata* was collected from Kadagaman, near Tiruvannamalai, Tamil Nadu, India, and authenticated by Centre for Advanced Study in Botany, University of Madras, Chennai. The dry powder of stem bark was extracted with hexane, chloroform, and methanol. The extracts were subjected to qualitative phytochemical screening and column chromatography. Four compounds were isolated. All the isolated compounds were subjected to adenosine diphosphate (ADP)-induced platelet aggregation and compared with aspirin.

Results: The isolated compounds from *C. dentata* and standard aspirin showed significant antiplatelet activity against ADP-induced platelet aggregation.

Conclusion: The compounds, 3-(1,1-dimethylallyl)xanthyletin, dentatin, nordentatin, and carbazole showed significant antiplatelet aggregation activity. Among the compounds, nordentatin showed more activity of antiplatelet aggregation.

Keywords: *Clausena dentata*, Coumarin, Dentatin, Adenosine diphosphate, Aspirin, Platelet.

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INTRODUCTION

The family *Rutaceae* consists of about 140 genera and 1300 species. These plants are aromatic trees, shrubs, and few herbs and are distributed throughout the warm and temperate regions of the world, being most abundant in South Africa and Australia. The aroma of the plant is due to the universal occurrence of lysigenous oil cavities in the leaves and other young organs. A number of plants of *Rutaceae* are of medicinal value and furnish several drugs and pharmaceutical products. Pilocarpine (used in the treatment of glaucoma) from *Pilocarpus jaborandi* and diosphenol (used in the treatment of diuretic) from *Barsoma betulina* are the best-known drugs. Essential "oil of bergamot" and "oil of Rue" used in perfumery and medicine, respectively, are extracted from *Citrus* species and *Ruta graveolens*. *Clausena* (*Rutaceae*) is a genus of about 23 species of unarmed trees and shrubs mainly grow in Indo-Malayan with a few in China, Africa, and Australia. Ten species are known to grow in India, of which five are of economic importance. The stem bark of *Clausena dentata* is used in veterinary medicine for the treatment of wounds and sprains [1]. The dried powdered rootstock is also used by the Kols, the tribes in Chotanagpur region, India, for decayed teeth. In Cambodia, the stem is considered bitter tonic and astringent [2]. The infusion is given for colic pain with diarrhea. *C. dentata* is used for digestion and as diuretic. Even though *C. dentata* has lot of potential medical uses, the study on chemistry of the plant is very scarce [3]. According to the World Health Organization, >80% of the total world's population depends on the traditional medicines to satisfy their primary health-care needs [4]. Considering the importance of the plant, the present study was undertaken with the following to study the antiplatelet aggregation activity of compound isolated from extracts *C. dentata*. Platelet aggregation causes major disease in humans. The medical plant possesses variety of phytochemicals which help human from escaping from various diseases [5]. Coumarins have a variety of bioactivities including anticoagulant, antimicrobial, vasodilator, anthelmintic, analgesic, anti-inflammatory, and hypothermic activities [6]. Coumarin is the parent molecule of warfarin, which acts as a Vitamin K antagonist. Warfarin is clinically useful anticoagulant and widely employed as rodenticide, whose discovery was based on the studies of the bleeding tendency of cattle suffering from "sweet clover diseases" [7]. The usefulness of coumarins and coumarin derivatives has been shown in various areas of analysis [8]. Areas where coumarins are widely used include estimation of enzymatic activity, for example, 7hydroxy coumarin derivatives as fluorogenic enzyme substrate [9]. 7amino4methyl coumarin, 4-methylumbelliferone used as intracellular ion indicators of pH and gas detection.

METHODS

Plant collection

The plant *C. dentata* (Willd.) Roem. was collected from Kadagaman, near Tiruvannamalai, Tamil Nadu, India, and the identification was confirmed as *C. dentata* at Centre for Advanced Study in Botany, University of Madras, Chennai. A voucher specimen of the plant has been deposited at the herbarium. The collected plant material was free from disease and also free of contamination of other plants.

Preparation of extracts

The dry powder of stem bark (2.5 kg) was first soaked, at room temperature, in hexane (1:4 w/v) for 24 h. The extract was suction filtered using Whatman filter paper. This was repeated for 2 more days and similar extracts were pooled together and concentrated at 40°C under reduced pressure using Buchi R153 Rotavapor [10,11]. The residual plant material was extracted successively with chloroform and methanol in the same manner as followed for hexane. All the concentrates were subjected to column chromatography to isolate the active principles. Four compounds were isolated and characterized by

the second author. The compounds are 3-(1,1-dimethylallyl)xanthyletin, dentatin, nordentatin, and carbazole.

Thin-layer chromatography (TLC)

Pre-coated silica gel TLC sheet (E. Merck) was used for TLC. The crude extracts were spotted at 2 cm from the edge of the sheet. The chromatogram was developed with a mixture of suitable solvent system and dried at room temperature. The spots were visualized with ultraviolet (UV) light at 254 and 346 nm. The dried TLC plates were then sprayed with 10% H_2SO_4 and heated at 110°C for 5 min. Alternatively, the developed TLC plates were placed in iodine chamber. The R_f values of the colored spots were recorded.

Qualitative phytochemical screening

The different qualitative chemical tests were performed for establishing profile of given extract for its chemical composition. The extracts were subjected to test for alkaloids, glycosides, carbohydrate, proteins and amino acids, phytosterols, fixed oils and fats, gums and mucilages, and volatile oil.

***In vitro* inhibition of blood platelet aggregation by isolated compounds**

Requirements

The study requires platelet-rich plasma (PRP), tyrode buffer solution, adenosine Diphosphate (ADP), spectrophotometer (Systronics UV visible 118) and aspirin.

Procedure

Platelet aggregation was studied with PRP and it was resuspended in Tyrode buffer containing $NaHCO_3$ (9.5 mM), glucose (5.5 mM), NaH_2PO_4 (0.5 mM), $MgCl_2$ (0.6 mM), and gelatin (0.25%) which were adjusted to pH 7.4 with 0.25 M HCl. To obtain aggregation in PRP, the platelet count was adjusted to 0.13×10^7 for each assay. The inducer ADP at the final concentration of 5 μM was used throughout the study. Platelet aggregation was recorded (absorbances) by spectrophotometrically at 600 nm for each concentration. *In vitro* testing for the isolated compounds for platelet aggregation was done by dissolving the components in 2% Tween 80. It was suspended in PRP and then incubated at 37°C for 1 min before treatment with the platelet aggregating agent [12]. The isolated compounds *in vitro* blood platelet inhibition activities were compared with aspirin.

RESULTS

TLC

The TLC profile of hexane, chloroform, and methanol extracts of *C. dentata* reveals that the presence of coumarin and alkaloids.

Qualitative phytochemical screening

Qualitative chemical tests revealed the presence of various phytochemicals in hexane, chloroform, and methanol extracts of *C. dentata* (Table 1). The methanol extract showed positive test for alkaloids. All the extracts contained carbohydrates, glycosides, amino acids, proteins, and volatile oils. Ferric chloride test showed the presence of phenolic compounds, in all the extracts. Saponins, phytosteroids, fixed oils, and fats were absent.

***In vitro* effect of ADP on platelets**

Different concentrations (1–5 μM) of ADP-induced platelet aggregation (Table 2) with a difference in absorbance value as compared to the control value. About 5 μM of ADP-induced more platelet aggregation

Table 1: Qualitative phytochemical screening of various extracts of *Clausena dentate*

S. No.	Phytochemical test	Hexane	Chloroform	Methanol
1.	Alkaloids			
	Mayer's reagent	–	–	+
	Wagner's reagent	–	–	+
	Hager's reagent	–	–	+
	Dragendorff's reagent	–	–	+
2.	Carbohydrates and glycosides			
	Molisch's test	+	+	+
	Fehling's test	+	+	+
	Barfoed's test	+	+	+
	Benedict's test	+	+	+
	Borntrager's test	+	+	+
	Legal's test	+	+	+
3.	Saponins			
	Foam test	–	–	–
4.	Proteins and amino acids			
	Millon's reagent	+	+	+
	Biuret reagent	+	+	+
	Ninhydrin reagent	+	+	+
5.	Phytosteroids			
	Liebermann–Burchard's test	–	–	–
6.	Fixed oils and fats			
	Spot test	–	–	–
	Saponification test	–	–	–
7.	Phenolic compounds and flavonoids			
	Ferric chloride test	+	+	+
	Gelatin test	+	–	–
	Lead acetate test	+	–	–
	Alkaline reagent	+	–	–
	Magnesium and hydrochloric acid Reduction	+	–	–
8.	Gums and mucilages			
	Alcohol 95% test	+	–	–
9.	Volatile oils			
	Steam distillation	+	+	+

–Negative, +Positive

than other concentrations of ADP. Therefore, the optimum dose of 5 μM was used throughout the study due to pronounced platelet aggregation.

In vitro* inhibition of blood platelet aggregation by isolated compounds from *C. dentata

The isolated compounds from *C. dentata* and standard aspiring showed significant antiplatelet activity against ADP-induced platelet aggregation (Table 3a-d).

DISCUSSION

***In vitro* inhibition of blood platelet aggregation**

Platelets or thrombocytes are small colorless, non-nucleated, and moderately retractile bodies. These formed elements of blood are considered to be the fragments of cytoplasm. Their diameter is 2.5 μg (2–4 μm) and the volume is 7.5 μm^3 (7–8 μm^3). Normal platelet count is 2.5 (2–4) lakhs/mm^3 of blood. Decrease in platelet count is called thrombocytopenia and it occurs in conditions such as acute infections, typhoid, smallpox, and tuberculosis. The increase in platelet count is called thrombocytosis. It occurs in conditions such as rheumatic fever, bone fractures, asphyxia, and hemorrhage.

Platelets play an important role in the process of atherothrombosis by adhering to the damaged regions (caused by reaction oxygen species of the endothelial surface). The activated platelets form platelets-to-platelets bond and bind to leukocytes, also bringing them into a complex process of plaque formation and growth [13]. Blood platelets are involved in hemostasis. The normal hemostatic system limits blood loss by precisely regulated interactions between components of the vessel wall, circulating blood platelets, and plasma proteins. Platelets can adhere to blood vessel walls, release bioactive compounds, and aggregate to each other. The well-established increase in these properties during arterial thrombosis and atherogenesis [14] provided the rationale for many drugs which inhibit platelet function [15] and also explains the considerable interest in the role of platelets and antiplatelet therapy in cardiovascular diseases. At the site of endothelial cell injury, they are triggered to various processes such as adhesion, shape change, secretion (release reaction), and aggregation which are collectively referred to as platelet activation. The aggregation is the final event of a multiple-step process. Several agonists, such as ADP, thrombin, collagen, and serotonin, induce the release of arachidonic acid after phospholipase activation through calcium mobilization [16].

Arachidonic acid is metabolized by cyclooxygenase to endoperoxide and subsequently to thromboxane A_2. These thromboxanes induce free cytoplasmic calcium levels and this mobilized calcium stimulates the release of the secretary granule contents including ADP, serotonin, and other related bioactive compounds for various biological processes. The antiplatelet therapy constitutes the best available tool for ameliorating the mechanisms related to atherogenesis and this drug has interestingly inhibited platelet aggregation [17]. Several drugs have been developed to block the different steps in platelet activation pathways; inhibition of platelet function by aspirin has been very well described [18]. Carbazole alkaloids – clausine-d and clausine-f – isolated from *C. excavata* showed significant antiplatelet aggregation activity.

When platelets come in contact with any wet surface or rough surface, these are activated and stick to the surface. The factors which cause adhesiveness are collagen, thrombin, ADP, thromboxane A_2, and calcium ions. The activated platelets group together and become sticky. The sticky nature is due to ADP and thromboxane A_2. Platelets are responsible for the formation of the intrinsic prothrombin activator. This substance is responsible for the onset of blood clotting. The cytoplasm of platelets contains the contractile protein known as thrombosthenin.

Table 2: Platelet aggregation induced by ADP

S. No.	Concentration of ADP in μM	Time (min)	Mean absorbance (nm)±standard deviation	t-test	95% confidence interval of the mean	
					Lower	Upper
1.	Control	1	0.273±0.001	39.192	0.034	0.030
		2	0.275±0.001	41.641	0.036	0.032
		3	0.275±0.001	48.990	0.042	0.038
		4	0.276±0.001	51.403	0.048	0.043
		5	0.277±0.001	64.000	0.045	0.041
2.	1 μM	1	0.305±0.001	39.192	0.034	0.030
		2	0.309±0.001	41.641	0.036	0.032
		3	0.314±0.001	48.990	0.042	0.038
		4	0.321±0.001	51.403	0.048	0.043
		5	0.323±0.001	64.000	0.045	0.041
3.	2 μM	1	0.323±0.001	61.237	0.052	0.048
		2	0.325±0.001	61.237	0.052	0.048
		3	0.346±0.001	88.182	0.074	0.070
		4	0.349±0.001	89.406	0.075	0.071
		5	0.350±0.001	110.000	0.075	0.071
4.	3 μM	1	0.341±0.001	83.285	0.070	0.066
		2	0.346±0.001	86.957	0.073	0.069
		3	0.355±0.001	99.204	0.083	0.079
		4	0.355±0.002	96.755	0.081	0.077
		5	0.357±0.002	97.980	0.082	0.078
5.	4 μM	1	0.351±0.001	118.000	0.081	0.077
		2	0.353±0.002	117.500	0.080	0.076
		3	0.355±0.001	77.476	0.085	0.079
		4	0.357±0.003	99.204	0.083	0.079
		5	0.357±0.002	121.000	0.083	0.079
6.	5 μM	1	0.354±0.002	99.204	0.083	0.079
		2	0.355±0.001	97.980	0.082	0.079
		3	0.355±0.003	92.223	0.084	0.079
		4	0.355±0.003	96.755	0.081	0.077
		5	0.357±0.001	97.980	0.082	0.078

All values are expressed as mean±standard deviation (n=3), $p<0.001$ compared to control (unpaired t-test). ADP: Adenosine diphosphate

This contractile protein is responsible for clot retraction. Platelets accelerate the processes of hemostasis as the phytochemical investigation revealed that *C. dentata* contained coumarins such as 3-(1,1dimethylallyl)xanthyletin, dentatin, nordentatin, and carbazole alkaloid. In the present investigation, the use of *C. dentata* for preventing coronary artery diseases was explored. The isolated compounds, (3-(1,1-dimethylallyl)xanthyletin, dentatin, nordentatin, and carbazole, showed significant antiplatelet aggregation activity. Antiplatelet aggregation activity was more in nordentatin which was comparable with standard drug aspirin. The prevention activity may be due to the presence of coumarin compound.

CONCLUSION

ADP-induced blood platelet aggregation study was carried out. The compounds, 3-(1,1-dimethylallyl)xanthyletin, dentatin, nordentatin, and carbazole, showed significant antiplatelet aggregation activity. Among the compounds, nordentatin showed more activity of antiplatelet aggregation.

Table 3a: Analysis of variance for the antiplatelet aggregation activity of isolated compounds

S. No.	Concentration of compounds (µg/mL)	Time (min)	Mean absorbance (nm)±standard deviation	F value	95% confidence interval mean	
					Lower	Upper
1.	Control	1	0.273±0.001	6716.44	0.270	0.275
		2	0.275±0.001	4893.79	0.272	0.277
		3	0.274±0.001	4170.50	0.271	0.276
		4	0.276±0.001	6924.00	0.273	0.278
		5	0.277±0.001	5100.66	0.279	0.279
2.	3-(1,1-dimethylallyl) xanthyletin (µg)					
	5	1	0.330±0.005	6716.44	0.328	0.331
		2	0.328±0.001	4893.79	0.325	0.330
		3	0.327±0.001	4179.50	0.324	0.329
		4	0.327±0.001	6924.00	0.327	0.327
		5	0.327±0.005	5100.66	0.326	0.329
	25	1	0.320±0.005	6188.71	0.274	0.279
		2	0.318±0.005	6173.71	0.315	0.318
		3	0.316±0.005	6171.57	0.313	0.318
		4	0.316±0.001	6513.21	0.314	0.319
		5	0.314±0.001	6703.11	0.313	0.319
	50	1	0.311±0.005	6611.27	0.270	0.275
		2	0.311±0.001	5740.57	0.308	0.313
		3	0.309±0.001	4749.33	0.308	0.311
		4	0.309±0.000	7297.50	0.309	0.309
		5	0.309±0.000	6162.42	0.309	0.309
	100	1	0.309±0.001	5191.45	0.306	0.311
		2	0.307±0.005	3539.22	0.303	0.311
		3	0.307±0.005	5662.79	0.305	0.308
		4	0.307±0.005	5811.45	0.305	0.308
		5	0.305±0.005	5722.83	0.303	0.306

Values are expressed as mean±standard deviation (n=3), p<0.05 compared to control and standard (aspirin), ANOVA

Table 3b: Analysis of variance for the antiplatelet aggregation activity of isolated compounds

S. No.	Concentration of compounds (µg/mL)	Time (min)	Mean absorbance (nm)±standard deviation	F value	95% confidence interval mean	
					Lower	Upper
3.	Dentatin (µg)					
	5	1	0.325±0.001	4845.33	0.322	0.327
		2	0.321±0.005	6297.77	0.320	0.323
		3	0.321±0.000	5776.14	0.321	0.321
		4	0.321±0.001	4492.00	0.323	0.320
		5	0.320±0.005	4933.83	0.318	0.321
	25	1	0.320±0.005	6444.44	0.318	0.321
		2	0.319±0.005	6447.33	0.317	0.320
		3	0.318±0.005	5198.72	0.317	0.320
		4	0.318±0.001	4632.00	0.315	0.320
		5	0.317±0.001	4522.66	0.314	0.319
	50	1	0.311±0.001	4951.45	0.308	0.313
		2	0.310±0.005	8032.73	0.309	0.312
		3	0.310±0.001	6113.14	0.308	0.311
		4	0.310±0.005	7300.00	0.310	0.310
		5	0.309±0.005	5392.33	0.307	0.310
	100	1	0.307±0.005	6904.44	0.305	0.308
		2	0.306±0.001	8487.73	0.306	0.306
		3	0.305±0.005	5658.83	0.304	0.307
		4	0.305±0.001	6644.00	0.305	0.305
		5	0.305±0.001	6541.85	0.305	0.305

Values are expressed as mean±standard deviation (n=3), p<0.05 compared to control and standard (aspirin), ANOVA

Table 3c: Analysis of variance for the antiplatelet aggregation activity of isolated compounds

S. No.	Concentration of compounds (µg/mL)	Time (min)	Mean absorbance (nm)±standard deviation	F value	95% confidence interval mean	
					Lower	Upper
4.	Nordentatin (µg)					
	5	1	0.314±0.005	6207.27	0.313	0.316
		2	0.313±0.001	4625.60	0.310	0.315
		3	0.313±0.001	5677.85	0.313	0.313
		4	0.312±0.001	6661.50	0.312	0.312
		5	0.312±0.001	5589.85	0.312	0.312
	25	1	0.311±0.001	7531.73	0.311	0.311
		2	0.310±0.001	7587.46	0.310	0.310
		3	0.309±0.001	5843.42	0.309	0.309
		4	0.307±0.005	5915.57	0.306	0.309
		5	0.307±0.001	6774.00	0.307	0.307
	50	1	0.305±0.005	6576.44	0.303	0.306
		2	0.299±0.005	8132.46	0.297	0.305
		3	0.298±0.005	6216.00	0.296	0.299
		4	0.298±0.001	7468.00	0.298	0.298
		5	0.297±0.005	5533.79	0.296	0.299
	100	1	0.293±0.001	5323.45	0.290	0.295
		2	0.292±0.005	7325.94	0.290	0.293
		3	0.291±0.005	5882.79	0.289	0.292
		4	0.290±0.005	6106.83	0.288	0.291
		5	0.290±0.001	6904.00	0.290	0.290

Values are expressed as mean ± standard deviation (n = 3), p<0.05 compared to control and standard (aspirin), ANOVA

Table 3d: Analysis of variance for the antiplatelet aggregation activity of isolated compounds

S. No.	Concentration of compounds (µg/mL)	Time (min)	Mean absorbance (nm)±standard deviation	F value	95% confidence interval mean	
					Lower	Upper
5.	Carbazole (CD-4) (µg)					
	5	1	0.347±0.005	8101.94	0.345	0.348
		2	0.347±0.001	9536.26	0.347	0.347
		3	0.346±0.001	6276.45	0.344	0.347
		4	0.346±0.001	8225.50	0.346	0.346
		5	0.346±0.001	6911.00	0.346	0.346
	25	1	0.345±0.001	6109.83	0.342	0.347
		2	0.342±0.005	7869.50	0.340	0.343
		3	0.341±0.001	4969.80	0.338	0.343
		4	0.340±0.001	8114.00	0.340	0.340
		5	0.340±0.001	7945.50	0.340	0.340
	50	1	0.342±0.001	4332.14	0.340	0.343
		2	0.340±0.001	4050.14	0.339	0.342
		3	0.338±0.001	3778.42	0.335	0.340
		4	0.337±0.001	3646.42	0.334	0.339
		5	0.334±0.005	3578.12	0.333	0.336
	100	1	0.355±0.005	6603.57	0.333	0.336
		2	0.332±0.002	2512.50	0.327	0.336
		3	0.330±0.005	6411.57	0.329	0.332
		4	0.329±0.005	6703.00	0.328	0.331
		5	0.327±0.007	6231.00	0.326	0.330

Values are expressed as mean ± standard deviation (n = 3), p<0.05 compared to control and standard (aspirin), ANOVA

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AUTHORS' CONTRIBUTIONS

Raju Kamaraj performed preparation and analysis of extract. Annamalai Maduram contributed to design of the experiment and manuscript writing.

CONFLICTS OF INTEREST

We declare that we have no conflicts of interest.

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