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Effect of flavonoid rich fraction from the leaves of *Clinacanthus nutans* on antiradical and anti-inflammatory properties

S Tamilselvi M D
Professor, Department of Gunapadam Marunthakkaviyal, Sri Sairam Siddha Medical College and Research Centre, Poonthadalam, West Tambaram, Chennai, Tamil Nadu, India

Abstract
To evaluate the antioxidant and anti-inflammatory activities of flavonoid rich fraction from the leaves of *Clinacanthus nutans*. In this framework, the in vitro antioxidant activity was demonstrated by ABTS radicals, reducing power, lipid peroxidation, nitric oxide scavenging activities and anti-inflammatory activity was evaluated first by studying inhibiting the denaturation of albumin and inhibition of lipoxygenase activities. Total phenolic and flavonoid content were found respectively (78.32 ± 2.45) mg gallic acid equivalent/g, and (43.21 ± 2.78) mg quercetin equivalent/g. The extract displayed significant scavenging activity of some radicals such as ABTS EC_{50} value 65.31µl/ml at 100 µl/ml. Flavonoid rich fraction from the leaves of *C. nutans* showed in vitro anti-inflammatory activity by inhibiting albumin denaturation EC_{50} value 69.32 µl/ml and inhibition of lipoxygenase EC_{50} value 83.65 µl/ml. Our results show that Flavonoid rich fraction from the leaves of *C. nutans* has good antioxidant activity and interesting anti-inflammatory properties. Flavonoid rich fraction from the leaves of *C. nutans* extract can be used to prevent oxidative and inflammatory processes.

Keywords: antioxidant, anti-inflammatory, *Clinacanthus nutans*

Introduction

Natural products, such as plant extract or pure compounds and also standardized extracts, deliver limitless chances for new drug discoveries because of the matchless chemical diversity they can afford. Rendering to the World Health Organization (WHO), more than 70% of the world's population trusts on traditional medicine for their prime healthcare necessities. This has taken the interest of many researchers to explore local medicinal plants for valuable medicinal traits. Several studies indicate that medicinal plants contain compounds like amini acid, polyphenol, flavonoid, tannnin, and other water soluble compounds. These compounds are substantial in beneficial application against human and animal pathogens, including bacteria, fungi and viruses (Selvakumar *et al.*, 2011) [13].

Accumulating scientific evidence suggests that over-production of reactive oxygen species (ROS) may be the root cause of chronic diseases such as cancer, cardiovascular diseases, neuro degeneration. Logically, dietary antioxidants, those that can mitigate the damaging effects of ROS, have been suggested to be beneficial for health promotion, although this is a hotly debated topic, as too little ROS activity may have undesirable health consequences (Zhang et al., 2015) [15]. However, the research on dietary antioxidants has been developing rapidly. This special issue collects a small sample of 6 articles, most of them mini reviews on dietary antioxidant and health. These articles provide timely updates on the respective topics covered. Among the plant-based polyphenolic antioxidants, proanthocyanidins, the oligomers of flavan-3-ol, are the most studied group of phytochemicals. (Liang et al., 2015) [8]. Due to their structural diversity and complexity, PACs from different fruits and edible plants have different bioactivities. For example, cranberry contains mainly A-type proanthocyanidins, which are believed to be responsible for its health benefits against urinary tract infection, while PACs found in okra are potent inhibitors of starch hydrolases (Pandey et al., 2012) [10].

Inflammation is a severe response by living tissue to any kind of injury. There can be four primary indicators of inflammation: pain, redness, heat or warmness and swelling. When there is injury to any part of the human body, the arterioles in the encircling tissue dilate. This gives a raised blood circulation towards the area (redness) (Burke *et al.*, 2005). Vasoactive chemicals also increase the permeability (increase pore size) of these arterioles which allows blood cells, chemical substance, blood protein s and fluid to accumulate in that region. This fluid accumulation causes swelling and may compress nerves in the area resulting in pain. In addition, prostaglandins, that might also result in 'irritation' of the nerves and further contribute to pain. Most people who take anti-inflammatory drugs have no side effects, or only minor types. When taken appropriately, the advantage usually far outweighs the possible harms. In particular many people have a short course of an anti-inflammatory for all sorts of painful conditions.

C. nutans is a perennial herb which can grow up to 1 m tall with pubescent branches and cylindrical, striate, and glabrescent stems. The leaves are simple, opposite, narrowly elliptic-oblong or lanceolate (2.5–13.0 cm long × 0.5–1.5 cm wide). This shrub is about 1 m tall, and stems cylindrical, striate and glabrescent. The petiole is 0.3–2.0 cm, sulcate, bifariously pubescent and leaf blade lanceolate-ovate, lanceolate or linear-lanceolate. The leaves are apex acute or acuminate and exsculptate; dentate or subentine margins. Both surfaces of leaves are pubescent when young then glabrescent. The leaf base are cuncate, obtuse rounded or truncate; often oblique. Petiole is 3–15 mm long. The flowers are sordidly yellow or greenish yellow and dense cymes at the top of branches and branchlets; always covered with 5-alpha cymules. The calyx of flower about 1 cm long with grandular-pubescent. Corolla is dull red with green base, about 3.0-4.2 cm. The stamen is exerted from the throat of corolla. The ovary is compressed into two cells and each cell has two ovules. The styles are filiform with shortly bidentate. Capsule is oblong basally wrapped into 4-seeded short stalk.

Materials and Methods

Plant collection

Clinacanthus nutans leaves was obtained from Herbal garden of Government Siddha Medical College, Arumbakkam, Chennai, Tamilnadu, India. A plant taxonomist authenticated the plant and samples were kept in the Medicinal Botany herbarium with voucher specimen numbers MB/GSMC-280/2021.

Phytochemical Studies

The chemical constituents in the leaves of *C. nutans* were classified qualitatively using phytochemical reagents according to procedures described by Trease and Evans, (1983) [14]. The leaves were screened for the presence of saponins, flavonoids, tannins, alkaloids, glycosides, and other constituents.

Extraction Procedure

The shade dried and powdered leaves of *C. nutans* (200 g) were boiled in methanol for 15 min. The methanolic extract was evaporated under reduced pressure using a rotavapor. The residue was resuspended in boiling water and successively exhausted with solvent of increasing polarity: diethyl ether (4 × 150 mL), ethyl acetate (5 × 150 mL), and n-butanol (4 × 150 mL). The extraction yielded (i) 375.4 mg of diethyl ether extract, (ii) then 1.2 g of ethyl acetate extract, and, finally, (iii) 3.1 g of butanolic extract.

ABTS (2, 2'-Azino-Bis-3-Ethyl Benzthiazoline-6-Sulphonic Acid) Radical Scavenging Assay

ABTS radical scavenging activity of flavonoid rich fraction of *C. nutans* was followed by Re et al. (1999). ABTS radical was newly prepared by addition 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to produce an absorbance of 0.70 at 734 nm and the same was used for the antioxidant activity. The final solution of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Ascorbic acid. Correspondingly, in the experiment group, 1 ml reaction mixture encompassed 950 µl of ABTS solution and 50 µl of different concentration of each extracts. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734 nm against distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound. ABTS Scavenging Effect (%) = $[(A_0 - A_1/A_0) \times 100]$ Where A_0 is the absorbance of the control reaction and A_1 is the absorbance of extract.

Ferric Reducing Power Assay

Reducing power was determined by the method described by Hazra *et al.* (2008) [5]. Different concentrations of the flavonoid rich fraction of *C. nutans* were mixed with 1.25 mL of 0.2 mol/L, pH 6.6 sodium phosphate buffer and 1.25 mL of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min. After incubation, the reaction mixture was acidified with 1.25 mL of trichloroacetic acid (10%) and centrifuged at 3 000 r/min for 10 min. Finally, 0.5 mL of freshly prepared $FeCl_3$ (0.1%) was added to this solution, and the absorbance was measured at 700 nm. Ascorbic acid at various concentrations was used as standard.

Inhibition of Lipid Peroxidation Activity

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa et al. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of flavonoid rich fraction of *C. nutans* in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard.

Inhibition of lipid peroxidation (%) by the each extracts was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

Inhibition of Albumin Denaturation

A solution of 0.2% (w/v) of egg albumin was prepared in a PBS (pH 6.4). A volume of 50 µL of the flavonoid rich fraction of *C. nutans* or standard at different concentrations was added to 5 mL of this stock solution. The test tubes were heated at 72 °C for 5 min and then cooled. The absorbance of these solutions was determined at 660 nm (Karthik et al., 2013) [7].

Lipoxygenase Inhibition Assay

Determination of LOX activity by spectrophotometric assay followed by Chen, and Gunathilake (2018) [4]. Inhibition experiments were run by measuring the loss of soybean 15-LOX activity (5µg) with 0.2µM linoleic acid (Sigma) as the substrate prepared in solubilized state in 0.2M borate buffer (pH 9.0). Inhibition studies in presence of various concentrations of extracts (25, 50, 75, 100 µg/mL) and reference compound *viz.*, quercetin was recorded at 234 nm using UV-Vis spectrophotometer (Deep Vision, DU 730). The inhibitory effect of the flavonoid rich fraction from the leaves of *C. nutans* was also expressed as percentage of enzyme activity inhibition. EC_{50} indicating the concentration required to inhibit 50 % LOX activity was also calculated. Values of hydroperoxide content and lipoxygenase activity were calculated from equation, Specific activity (LOX) =ΔA. V/ε.l.c

Statistical Analysis
All data were analysed using one-way ANOVA, and results presented as mean ± SEM. Graphical representations were done using bar chart or line graph. Level of significance was placed at value ≤ 0.05.

Result and Discussion
Phytochemical screening
The phytochemical screening of the *Clinacanthus nutans* leaves studied presently showed the presence of alkaloids, flavonoids, phenol, Terpenoids, glycosides and saponin, and absence of glycosides and Saponin (Table -1).

Table 1: Phytochemical screenings of *Clinacanthus nutans*

Sl. No.	Phytochemical Constituents	Observation	*C. nutans* extract
1	Alkaloids		
	-Dragendorff's test	Orange / red precipitate	+
	-Mayers test	Creampie ppt	+
2.	Flavonoids		
	-Alkalai Reagent	Intense yellow colour	+
	-Lead aceate test	Precipitate formed	+
3.	Glycosides		
	-Keller-Killiani test	Pink colour (Ammonia layers)	-
4.	Tannin		
	-$FeCl_3$ test	Blue-black colour	+
5.	Saponins		
	-Frothing test	Foam	-
6.	Terpenoids		
	-Salkowski test	Reddish brown colour ring formed in interface	+
7.	Polyphenols		
	-Ferrozine test	Raddish blue	+
8.	Anthocyanin		
	-Ammonia test	Pink color in ammonia layer	+

+ Positive activity; - Negative activity

Free Radical-Scavenging Ability Using Abts Assay by Flavonoid Rich Fraction from the Leaves of *C. nutans*
The radical scavenging ability was measured by ABTS assay as per given in table 4. The inhibition percentage of the ABTS radical activity was assessed on average and high free radical-scavenging values were found in flavonoid rich fraction from the leaves of *C. nutans.* In ABTS assay, inhibition percentage was high flavonoid rich fraction from the leaves of *C. nutans* 74.37% with EC_{50} value 65.31µl/ml. The pure ascorbic acid was lower activity (66.32 with EC_{50} value 71.32) (Table-2). Nevertheless, in present study, it is showed that these activities were mainly due to anthocyanin and flavonoids compounds. It is known that vitamin C (ascorbic acid) and carotenoids are chief source of discrepancy of antioxidant/ antiradical activities in plant materials (Jao and Ko, 2002) [6].

Table 2: Free radical-scavenging ability using ABTS assay of flavonoid rich fraction from the leaves of *C. nutans*

Different concentration of extract	Percentage of ABTS radical activity	
	Flavonoid rich fraction of *C. nutans*	Ascorbic acid (+ve control)
25 µl/ml	21.36±0.21	17.32±0.48
50 µl/ml	37.64±1.63	34.32±1.48
75 µl/ml	59.64±2.45	53.21±2.63
100 µl/ml	74.31±0.23	66.32±1.23
EC_{50} value	65.31	71.32

[a]Results are expressed as percentage inhibit of ABTS ability with respect to control. Each value represents the mean+SD of three experiments

Ferric Reducing Power
Ferric reducing power is a simple test of antioxidant capacity, and often used as indicator of antioxidant potential for a plant extract. In this test, antioxidant electron donation leads to the neutralization of the free radical. An increase in absorbance corresponds to an increase of the reducing power of the extract tested.

Flavonoid rich fraction from the leaves of *C. nutans* was characterized by higher ferric reducing power than other data reported for the same water extract of the plant EC_{50} 71.32±1.89 µg/mL (Fig-1). The antioxidant molecules present in the extract play a reductant role and cause the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Oliveira et al., 2009) [9].

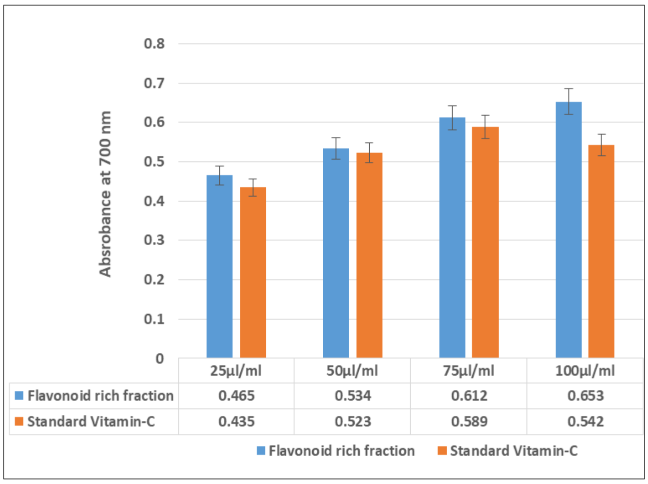

Fig 1: Ferric reducing power activity of flavonoid rich fraction from the leaves of *C. Nutans*

Inhibition of Lipid Peroxidation Activity of Flavonoid Rich Fraction from the Leaves of *C. nutans*

The flavonoid rich fraction from the leaves of *C. nutans* also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in flavonoid rich fraction from the leaves of *C. nutans* (71.34%) and lowest inhibition percentage of ascorbic acid was found in 64.32% (Table-3). As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Normally, the mechanism of phenolic compounds for antioxidant activity includes neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals (Govindappa et al., 2011) [3].

Table 3: Inhibition of lipid peroxidation activity of flavonoid rich fraction from the leaves of *C. nutans*

Different concentration of extract	Percentage of lipid peroxidation	
	Flavonoid rich fraction from the leaves of *C. nutans*	**Standard Vitamin-C**
25 µl/ml	23.54±0.28	19.64±1.78
50 µl/ml	41.32±2.48	36.21±1.35
75 µl/ml	57.34±1.45	51.34±2.12
100 µl/ml	71.34±0.56	64.32±0.87
EC_{50} value	68.32	72.34

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

Inhibition of Albumin Denaturation

Protein denaturation is involved in inflammation and plant extracts showing inhibition of denaturation are often tested for anti-inflammatory activity. For inhibiting thermally induced denaturation of albumin, the extract showed an astonishingly effect at different concentrations as shown in Table-. During the investigation of the activity of the plant extract on albumin denaturation we observed that flavonoid rich fraction from the leaves of *C. nutans* showed a greater protection comparatively to data observed (67.32 ±1.89)% at 100 µg/mL (Table-4) . According to the fact that proteins denaturation is the cause of inflammation and rheumatoid arthritis, the protection of albumin denaturation confirms and contributes to anti-inflammatory activity of flavonoid rich fraction from the leaves of *C. nutans* (Sakat et al., 2009) [11].

Table 4: Inhibition activity of albumin denaturation flavonoid rich fraction from *C. nutans*

Different Concentration of extract	Inhibition percentage of albumin denaturation	Diclofenac sodium (+ve control)
25 µl/ml	18.32±2.45	15.78±1.67
50 µl/ml	33.64±1.69	30.21±2.65
75 µl/ml	53.24±0.23	48.32±1.78
100 µl/ml	67.32±1.89	63.24±2.47
EC_{50} Value	69.32	73.65

Results are expressed as percentage inhibited albumin denaturation with respect to control. Each value represents the mean+SD of five experiments

Lipoxygenase inhibition activity of flavonoid rich fraction from the leaves of *c. nutans*

The inhibition of LOX using linoleic acid as substrate was determined for the anti-inflammatory activity of flavonoid rich fraction from the leaves of *C. nutans.* The flavonoid rich fraction at 100µl/ml concentration exhibited more inhibition than the other concentration. The inhibition percentage was above 61.23% at 100µl/ml (Table-5). The standard diclofenac sodium was showed 55.36% inhibition at 20 µg/mL. The flavonoid rich fraction was showed higher inhibition activity than positive control. Lipoxygenase catalyzes the addition of molecular oxygen to fatty acids containing a *cis*, *cis*-1, 4-pentadiene system. This reaction originates unsaturated fatty acid hydroperoxides. These products are further converted into others that play a key role in inflammatory processes (Sakat et al., 2012) [12]. Hence, flavonoid bioactive compound of *C. nutans* which are able to inhibit that enzyme can be considered as antioxidants and possessing anti-inflammatory properties.

Table 5: Inhibition activity of Lipoxygenase flavonoid rich fraction from *C. nutans*

Ethyl acetate extract Concentration	Inhibition percentage of LOX	Diclofenac sodium (+ve control)
25 µl/ml	16.34±0.78	14.32±2.56
50 µl/ml	31.25±0.89	26.34±1.49

75 µl/ml	46.34±1.58	42.36±2.41
100 µl/ml	61.23±0.24	55.36±1.69
EC_{50} Value	83.65	89.34

Results are expressed as percentage inhibited Lipoxygenase with respect to control. Each value represents the mean+SD of five experiments

Conclusion

This study demonstrated *in vitro* antioxidant and anti-inflammatory activities of flavonoid rich fraction from the leaves of *C. nutans*. Flavonoid rich fraction, through scavenging, chelating and reducing activities indicated in the performed tests, showed a good antioxidant activity. Furthermore, the protection of RBCs indicated a membrane stabilizing effect of the extract. These results lead to the conclusion that flavonoid rich fraction from the leaves of *C. nutans* has antioxidant and anti-inflammatory potential.

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Reference

1. Chen AO, Whitaker JR. Purification and characterization of a lipoxygenase from immature English peas. J. Agric. Food Chem,1986:34:203-211.
2. Cos P, Vlietinck AJ, Berghe, DV, Maes L. Anti-infective potential of natural products: How to develop a stronger in vitro ‘proof-of-concept’. J. Ethnopharmacol,2006:106:290-302.
3. Govindappa M, Naga SS, Poojashri MN, Sadananda TS, Chandrappa CP. Antimicrobial, antioxidant and in vitro anti-inflammatory activity of ethanol extract and active phytochemical screening of *Wedelia trilobata* (L.) Hitchc. J. Pharmacogn. Phytother,2011:3:43–51.
4. Gunathilake KDPP, Ranaweera KKDS, Rupasinghe HPV. Influence of Boiling, Steaming and Frying of Selected Leafy Vegetables on the In Vitro Anti-inflammation Associated Biological Activities. Plants (Basel),2018:7(1):16.
5. Hazra B, Biswas S, Mandal N. Antioxidant and free radical scavenging activity of *Spondias pinnata.* BMC Complement Altern Med,2008:8:63.
6. Jao CH, Ko WC. “1, 1- Dipheny1-2 picryl hydrazyl (DPPH) radical scavenging by protein hydrolysates from tuna cooking juice,” Fisheries Science,2002:68:430–435.
7. Karthik K, Kumar BR, Priya VR, Kumar SK, Rathore RS. Evaluation of anti-inflammatory activity of *Canthium parviflorum* by *in-vitro* method. Indian J Res. Pharm Biotechnol,2013:1(5):729-730.
8. Liang T, Zhang X, Xue W, Zhao S, Zhang X, Pei J. Curcumin induced human gastric cancer BGC-823 s apoptosis by ROS-mediated ASK1-MKK4-JNK stress signaling pathway. International Journal of Molecular Sciences,2014:15:15754–15765.
9. Oliveira I, Coelho V, Baltasar R, Pereira JA, Baptista P. Scavenging capacity of strawberry tree (*Arbutus unedo* L.) leaves on free radicals. Food Chem Toxicol,2009:47(7):1507-1511.
10. Pandey AK, Mishra AK, Mishra A. Antifungal and antioxidative potential of oil and extracts derived from leaves of Indian spice plant Cinnamomum tamala. Cellular and Molecular Biology,2012:58:142–147.
11. Sakat S, Tupe PN, Juvekar AR. *In-vitro* anti-inflammatory activity of aqueous and methanol extracts of *Erythrina indica* Lam leaves. Pharmacology online,2009:3:221-229.
12. Sakat S, Juvekar AR, Gambhire MN. In vitro antioxidant and anti-inflammatory activity of methanol extract of *Oxalis corniculata* Linn. Int. J. Pharm. Pharm. Sci,2010:2:146–155.
13. Selvakumar K, Madhan R, Srinivasan G, Baskar V. Antioxidant assays in pharmacological research,” Asian Journal of Pharmacy and Technology,2011:1(4):99–103.
14. Trease GE, Evans WC. Textbook Of Pharmacognosy, Ballieri Tindall, London, UK, 12th edition, 1983.
15. Zhang Q, Cheng G, Qiu H et al. The p53-inducible gene 3 involved in flavonoid-induced cytotoxicity through the reactive oxygen species-mediated mitochondrial apoptotic pathway in human hepatoma cells. Food & Function,2015:6:1518–1525.