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Isolation, Characterization and Microvascular Activity of Anthocyanins From *Ficus Racemosa* Fruits.

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ABSTRACT

Anthocyanins (ACN) are part of a large and wide spread group of plant constituents known collectively as flavanoids. In the present research work anthocyanins were extracted from *Ficus racemosa* fruit. Family: Moraceae, using acidified methanol (1% HCL in methanol). The extracted anthocyanins were isolated and purified using the Amberllite – XAD4 resin. Anthocyanins were characterized by means of Chromatographic and Spectral data obtained from HPLC and UV Spectroscopy. Two major pigments identified were Peonidin -3-glucoside and Pelargonidin -3-glucoside. Peaks for anthocyanins, which are present in very low amount, and in less concentration, can be verified by means of commercially available external standards. The samples were diluted in the mixture of methanol and formic acid just before the injection on the column and separated on Lichrospher-100 Reversed phase C18 end capped column (5 micron) 125mm x 4mm using gradient solvent system consisting of 5Mm of phosphoric acid and acetonitrile . DAD detector was employed at 520 nm for anthocyanins. An *Ficus racemosa* fruit anthocyanosides preparation (equivalent to 30% of anthocyanidins) demonstrated significant vasoprotective effect in rabbits, the skin capillary permeability increase, due to chloroform, was reduced after i.p. (25-100 mg/kg) anthocyanosides. Comparison was made between the action of the methanol extract and a known protective microvascular drug proxerutin (25 mg/kg). The mixed of anthocyanosides was more active that the proxerutin.

KEYWORDS: Anthocyanidins, *Ficus racemosa*, Vasoprotective activity.

INTRODUCTION

Anthocyanins are the colour pigments which are widely distributed in plants. The term 'Anthocyanin' is derived from Greek words. 'Antho' means flower and 'cyanin' means blue. It designates the blue pigment of flowers (1).They are mainly distributed among flowers, fruits and vegetables and are responsible for bright colours such as red, purple, magenta, orange (2) Anthocyanins possess ability to protect both large and small blood vessels from oxidative damage derives from a range of effects, including mitigating microvessel damage from high blood-sugar levels that cause complications in diabetics (3). By the same token, diabetic retinopathy, which damages eyesight, is caused by leaking, damaged capillaries (4). Anthocyanin pigments and associated flavonoids have demonstrated ability to protect a myriad of human diseases. Anthocyanin-rich mixtures of bioflavonoids may provide protection from DNA cleavage, estrogenic activity (altering development of hormone-dependent disease symptoms), enzyme inhibition, boosting production of cytokines (thus regulating immune responses), anti-inflammatory activity, lipid peroxidation, decreasing capillary permeability and fragility, and membrane strengthening (5). Anthocyanins help maintain micro capillary integrity by stabilizing capillary walls. Chemical structure (position, number,

and types of substitutions) of the individual anthocyanin molecule also has a bearing on the degree to which anthocyanins exert their bioactive properties (6) and the structure/function relationships also influence the intracellular localization of the pigments (7).

As a potential major component of our daily diet, more and more research has concentrated on their biological activities and possible health benefits in protecting against some chronic diseases such as cancer, cardio, cerebrovascular, atherosclerosis and diabetes (8). Some of these biological activities and protective functions are attributable to their high antioxidant capacities (9). One possible source of anthocyanin pigments is the fruit of *Ficus racemosa*, a highly productive plant, which provides fruits during long periods of the year and is a large deciduous tree distributed all over India mostly in the evergreen forest. All parts are cooling, sweet, acrid, vulnerary, anti-dysenteric useful in 'Kapha', vaginal diseases. The root is useful in hydrophobia. The bark is acrid, galactagogue, good for gravid uterus. The ripe fruit is sweet, useful in blood diseases, burning sensations, fatigue, urinary, discharges, leprosy, and intestinal worms. The bark is useful in asthma and piles. The leaves are astringent to the bowels and good for bronchitis, disease of kidney and spleen. The milk is aphrodisiac and vulnerary, useful in inflammations. The bark is given as astringent and as a wash for wounds. The root is useful in dysentery. The figs are given in haemoptysis and menorrhage. The various parts of the plant are used in small pox, muscular pain, adenitis, scabies, spermatorrhoea, orchitis, and failure of lactation (10). The hypoglycemic effect of *Ficus racemosa* leaves extract was studied in rats with streptozotocin induced Diabetes. The leaf extract possess significant hypoglycemic activity in hyperglycemic rats (11). Also, the β-sitosterol and lupeol were isolated and identified from bark & it shows a potential as a hypoglycemic agent (12). The Ethanolic extract of the leaves of *Ficus racemosa L,* were studied to check its potential as anti-inflammatory, analgesic and anti-pyretic activity of ethanolic extract used in African medicine (13). The objective of the present work is to isolate and characterize the anthocyanins pigments present in the *Ficus racemosa* fruit by Chromatographic and Spectral data. Here we also report the vascular protective action of anthocyanosides rich extracts from Ficus racemosa fruit which was compared to that of standard proxerotin.

MATERIALS AND METHOD

Fruit

Ficus racemosa fruits were chosen by intensive red colour. The fruits were harvested during May at ideal stage of ripeness from Hindustan Antibiotics Colony Plantation in Pune city (India). After harvesting the fruits were washed, packed in the polyethylene bags and stored in the dark at −15° for 1 day before the extraction of anthocyanin pigments. The plant material was authenticated at Botanical Survey of India (BSI), Pune and the voucher specimen given was RVS-1.

Chemicals

Peonidin-3-glucoside, Pelargonidin-3-glucoside, Cyanidin-3-glucoside, Maldivin-3-glucoside were obtained from National Chemical Laboratory (NCL), Pune. The solvents used were of HPLC grade, were obtained from Merck (India). Amberllite XAD4 resin was obtained from Thermax Chemical Ltd. (India). All other chemicals used were of analytical grade.

General Experimental Procedure.

Anthocyanin Extraction

The extraction was done using methanolic HCL (14). Nearly about 1 Kg of fruits of *Ficus racemosa* (Skin and Pulp) was blended in a food mill to produce a thick puree. It was then blended with methanolic HCL (1% HCL in mehanol) and kept overnight at −15° C covering with Aluminium foil and then filtered through the Buchner funnel (15). The filter cake residue was reextracted with acidified methanol (1% HCL) until a clear solution was obtained. The filtrates were combined and placed on a Buchi rotavapor at 35° C (25–30 min) until all residual methanol was evaporated and concentrated extract of *Ficus racemosa* fruit was obtained.

Anthocyanin Purification

The methanolic extract was passed through Chromatographic Column (25 × 0.5 cm) initially loaded with Amberllite XAD4 resin, which was previously hydrated in distilled water. After loading the concentrated extract of *Ficus Racemosa* fruit, anthocyanins and other phenolics were adsorbed onto the column. Sugars acids and other water soluble compounds were eluted with 3 volumes of distilled water. The adsorbed anthocyanins were then eluted with 0.1% HCL in methanol v/v. The methanolic extract was then concentrated using a Buchi rotavapor at 35° C and stored at −10° C in cold conditions (16).

HPLC Analysis

The HPLC apparatus used was Thermo Separation Products with DAD detector (190-600 nm)

Preparation of Standards

The anthocyanins standards were collected from NCL, Pune. The standards used for the present study are Cyanidin-3-glucoside chloride, Pelargonidin-3-glucoside chloride, Peonidin-3-glucoside chloride, Malvidin-3-glucoside chloride. Initially, solutions containing flavonoids were used to obtain retention time and spectral information for the identification of the anthocyanidins present in the fruits of *Ficus Racemosa*. These solutions were prepared by dissolving standard compound in methanol (1-2 mg/mL of each compound). After removing an aliquot for HPLC analysis, the standard solution was mixed with 5 mL of Reagent A (5mM H_3PO_4), and 10 mL 100% HPLC graded Acetonitrile. This reacted solution was also analyzed by HPLC. Two series of standard solutions were analyzed by HPLC to determine the quantity of compound versus chromatograph peak area information. The most concentrated solution in each series was precisely prepared from four solid standards. Specifically, 300-500 µg of each of the glycones was weighed to the nearest 0.1 µg. The amounts were combined, then dissolved and diluted to 5.00 mL with reagent A. Next, the concentrate was diluted 1.00 mL to 10.00 mL and 0.100 mL to 10.00 mL with Reagent A to produce two other solutions for the series. The standard solutions were analyzed by HPLC using 2, 10, and 20 µL injections.

Extraction and preparation of sample for HPLC and spectrophotometric analysis.

The anthocyanin pigments purified through Column Chromatography (5mL) were taken and then the volume was made up with 2% HCL/MeOH. The sample was filtered through 0.45 µL PTFE filter into an HPLC vial and then was analyzed by HPLC.

HPLC analysis of phenolics components in Ficus racemosa fruit

The LC system used in HPLC analysis is Thermo Seperation Products modular system equipped with an 1100 Series diode array detector and a HP Kayak computer with HPLC 3D ChemStation software for instrument operation and data analysis. The LC method uses the Lichrospher-100 Reversed phase C_{18} end capped column (5 micron) 125mm × 4mm (i.d) fitted with Reverse phase C_{18} column (2 micron) 4mm × 4mm (i.d) Lichrogaurd column (Merck) was used. The mobile phase is a run mobile phase A= 5mM H_3PO_4, pH = 2.5, B = 100% Acetonitrile (HPLC grade).The gradient elution system used is given in the table 1. The PH was adjusted to PH 2.5. Flow rate 1.3 mL min^{-1}. Injection volume 20µl. Analysis at 25°C (R.T). Solvents and Samples were filtered through 0.45 µL Millipore filter type HA. Detection was carried out at 530 nm (17)

Table 2: The gradient elution system for HPLC.

Min	% A	% B	Flow rate ml / min
0	90	10	0.7ml
20	80	20	0.7ml
30	50	50	0.7ml
35	25	75	0.7ml
40	25	75	0.7ml
41	90	10	0.7ml
50	90	10	0.7ml

Characterisation and quantification of phenolic compounds by HPLC.

Compounds were characterized on the basis of retention times and UV-VIS spectra of previously described standards and by using data of already published UV-VIS spectra (18–21). The ratio of the average absorbance in the 400-440 nm range versus the absorbance maximum in the visible range of spectrum (A400-440/ Avis max) was calculated from the recorded UV-VIS spectra and used as an indicator of glycoside structure of anthocyanins (19).

Experiment on microvascular permeability Animals used.

Adult, healthy, white rabbit weighing between 2.0-2.5 kg were used in these experiments. The animals were kept in an air-conditioned animal room at temperature of 25°C. The animals were given a commercial feed and allowed tap water.

Microvascular permeability.

The male rabbits (2 kg mean weight, 12 animals in each experimental group) were treated intraperitoneally with either NaCl (0.9%) (control group) or the extract (25, 50, 100 mg/kg). After 30 min three zones of depilated skin were irritiated with chloroform applied by means of a cotton tipped glass tube pressed lightly on the skin for 30 s. After a further 60 min, the histamine was administered intradermally, 0.8 µg to each of the three zones (i.e. 2.4 µg/rabbit), followed by immediate intravenous application of Evans blue (25 mg/kg, as 10% aqueous solution). The animals were killed 30 min after histamine administration and the skin zones (each 3 × 3cm) were accurately cut off and extracted with 4 mL formamide for 72 h at 45°C. The

Table 2: The Standards of Anthocyanins eluted in the HPLC System

Sr.No	Standard Anthocyanins	Elution time	Peak height	Peak area
1	Peonidin-3-glycoside chloride	16.3	17.252	142.289
2	Cyanidin-3-glycoside chloride	18.2	15.536	130.509
3	Pelargonidin-3-glycoside chloride	22.1	14.262	116.228
4	Maluidin-3-glycoside chloride	28.2	16. 216	135.321

Table 3: The Anthocyanins isolated from *Ficus racemosa* fruit eluted in the HPLC system

Sr.No	Standard Anthocyanins	Elution time	Peak Height	Peak area	Peak Area*	Conc. Mgm/100 gm
1	Peonidin-3-glycoside chloride	16.3	14.223	120.232	12.12	0.32+0.006
2	Pelargonidin-3-glycoside chloride	22.0	10.829	92.264	9.78	0.24+0.002

Table 4: Effect of the anthocyanosides-rich methanol extract of *Ficus racemosa* fruit on increased microvascular permeability induced by both chloroform and histamine in rabbits.

Treatment mg/kg	Inhibition (%)
0.9% NaCl	0
25	46.1
50	76.3
100	89.2
Proxerutin 25	45.8

absorption of the decanted supernatant was measured at 620 nm and the Evans blue content read from the calibration curve (22)

RESULTS

Commercially available standards of anthocyanins were initially chromatographed. The anthocyanin standards eluted between 10–40 min. Several standard co-eluted in this system which was subsequently modified through a number of steps to a gradient employing 5mM phosphoric acid (solvent A) and acetonitrile (Solvent B). The standards eluted were Peonidin-3-glucoside chloride (16.3), Cyanidin-3-glucoside chloride (18.2), Pelargonidin-3-glucoside chloride (22.1), Malvidin-3-glucoside chloride (28.2). The elution time and peak area is given in table 2. Individual peak identities were established by comparison to the profile obtained from the standards. Two peaks Peonidin-3-glucoside (16.3), and Pelargonidin-3-glucoside (22.0) were observed in the HPLC chromatogram of the anthocyanins extracted, isolated and purified from *Ficus racemosa* fruits which were matching to the standards of Peonidin-3-glucoside chloride (16.3), and Pelargonidin-3-glucoside chloride (22.1). The elution time and peak area is given in table 3.Thus the gradient elution solvent system produces the clear patterns for anthocyanins in reasonable time < 40 min. Thus standards were used to verify the identity of the peaks of isolated anthocyanins pigments from *Ficus racemosa* fruit.Extract from *Ficus racemosa* significantly inhibited capillary permeability in rabbits and showed a concentration dependent. The protective microvascular activity was measured as a counteracting effect on the leakage of Evans blue introduced intravenously. Proxerutin (25 mg/kg) a protective microvascular was used as reference drug in this study. The results are shown in Table 4. The extract of *Ficus racemosa* fruit 120 min after peritoneal treatment at a dose of 25 and 50 mg/kg produced an inhibitory effect of 46.1 and 76.3% respectively on the capillary

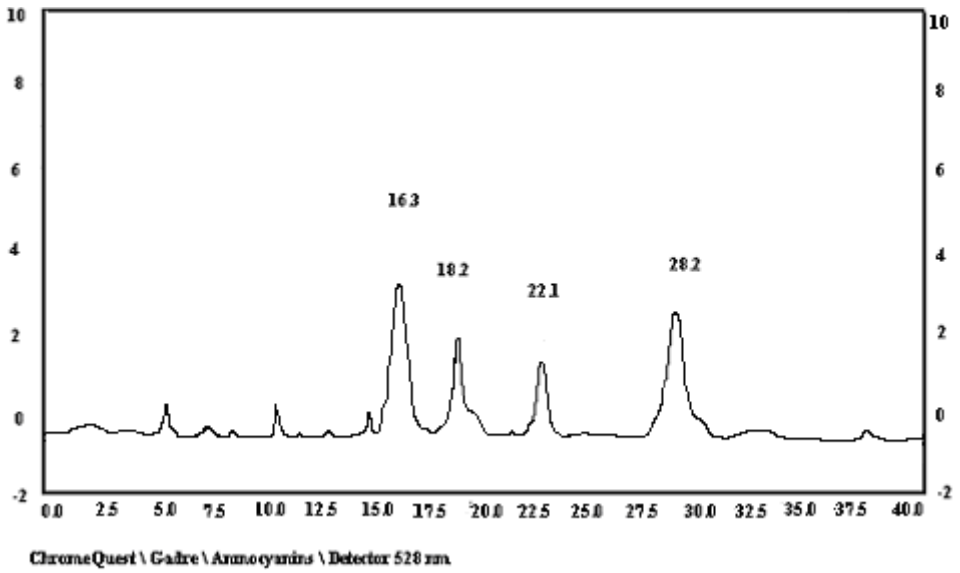

Figure 1: The HPLC Chromatogram of Standard Anthocyanins.

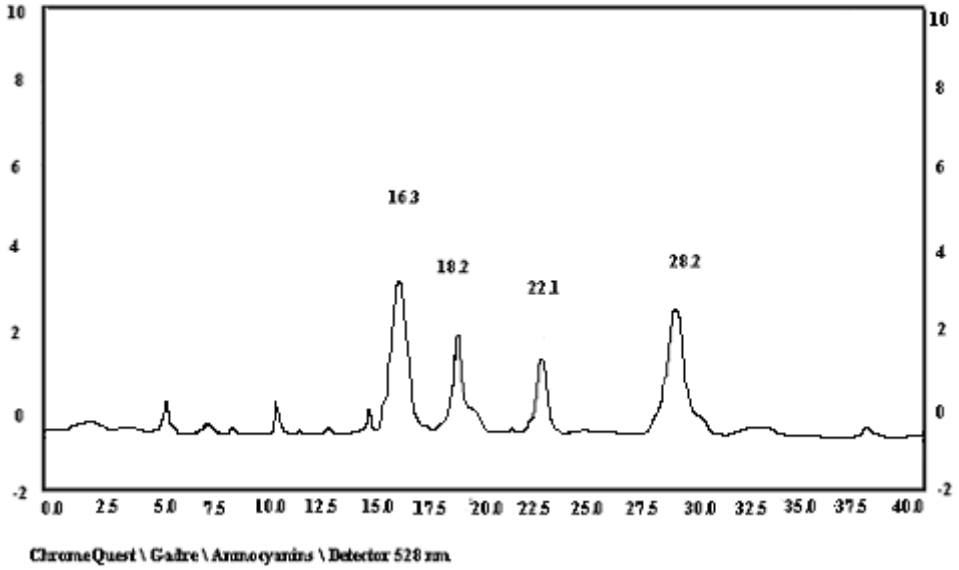

Figure 2: HPLC chromatogram of Anthocyanins Isolated from *Ficus racemosa*

permeability in rabbit skin increased by chloroform and histamine. This effect was more pronounced at 100 mg/kg (89.2% reduction).

DISCUSSION

Methanol extract of *Ficus racemosa* fruit have a composition of Peonidin-3-glycoside and Pelargonidin-3-glycoside as the major anthocyanins, and the Pelargonidin as minor anthocyanin. The results showed that the anthocyanosides-rich methanol extract from *Ficus racemosa* fruit produced a significant inhibitory effect on the capillary permeability in rabbit skin.

CONCLUSION

The HPLC analysis confirms the identity of anthocyanin pigments, isolated from the fruits of *Ficus racemosa*. Peonidin-3-glucoside and Pelargonidin-3-glucoside anthocyanins were identified in the concentrated extract of the fruit. The effect of methanol extract of *Ficus racemosa* fruit stems on the capillary permeability in rabbit skin is presented and also compared the effects of methanol extract with those of proxerutin. All the concentrations of extract assayed decreased the capillary permeability.

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