HPTLC FINGER PRINT ANALYSIS OF PHYTOCOMPOUNDS AND *IN VITRO* ANTIOXIDANT ACTIVITY OF *EUGENIA FLOCCOSA* BEDD.

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ABSTRACT

This paper has reported the preliminary phytochemical screening, HPTLC analysis of phytocompounds and *in vitro* antioxidant activities of ethanol extract of *Eugenia floccosa* leaves. This is the first report on the antioxidant activity of this plant. The preliminary phytochemical analysis showed the presence of alkaloids, coumarin, catechin, steroids, flavonoids, saponins, phenols, glycosides and terpenoids. HPTLC analysis also confirmed the presence of alkaloids, steroids, flavonoids, saponins, phenols, glycosides and terpenoids. The antioxidant activities of the leaves in ethanol extract are assessed using different models like DPPH, superoxide radical, hydroxyl radical and $ABTS^+$ cation radical and reducing power at different concentrations. The ethanol extract at 800µg/ml showed maximum scavenging activity. Results obtained revealed that, ethanol extract of leaves of *E. floccosa* possess highly antioxidant activity. Thus his study suggests that, *E. floccosa* plant can be used as a potent source of natural antioxidant.

Key words: *E. floccosa*, HPTLC analysis, antioxidant activity, DPPH, reducing power.

INTRODUCTION

A free radical is a molecule with one or more unpaired electrons in the outer orbital. Many of these free radicals are in the form of reactive oxygen and nitrogen species, these can occur due to oxidative stress brought about by the imbalance of the bodily antioxidant defense system and free-radical formation (Wong *et al*., 2000). Oxidative stress has been linked to cancer, aging, ischemic injury, inflammation and neurodegenerative diseases. Reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical, peroxyl radical and nitric oxide radical attack biological molecules such as lipids, proteins, enzymes, DNA and RNA, leading to cell or tissue injury associated with aging, antherosclerosis, carcinogenesis and may lead to the development of chronic diseases related to the cardio and cerebrovascular systems (Chen *et al*., 2005; Halliwell and Gutteridge, 1986). The most commonly used synthetic antioxidants presently used as butylatedhydroxyanisole (BHA), butylatedhydroxy toluene (BHT), propylgallate (PG) and tert butylatedhydroquinone. However, these synthetic antioxidants have side effects such as liver damage and carcinogenesis (Wichi, 1988). Free-radical scavengers are antioxidants which can provide protection to living organisms from damage caused by uncontrolled production of reactive oxygen species and subsequent lipid peroxidation, protein damage and DNA strand breaking (Ghosal *et al*., 1996).

In the past few years natural antioxidants have generated considerable interest in preventive medicine. The food industry also uses natural antioxidants as a replacement of conventional synthetic antioxidants in food by natural products that are considered to be promising and a safe source (Zupko *et al*., 2001). As a result of which, much attention has been directed towards the characterization of antioxidant properties of plant extracts /their functions and identification of the constituents responsible for those activities (Valentao *et al*., 2001; Haraguchi *et al*., 1996; Soares *et al*., 1997).

The present study aims to assess the antioxidant capacity of ethanol extract of *E. floccosa* leaf. Plant extracts were tested for phytochemical screening, HPTLC analysis and different free radical scavenging activities including the 1, 1-diphenyl picryl hydrazyl (DPPH), superoxide radical, $ABTS^+$ cation radical, hydroxyl radical and their reducing power capacity.

MATERIALS AND METHODS

PLANT MATERIAL AND PREPARATION OF PLANT EXTRACT

The leaves of *E. floccosa* were collected from upper Kothiyar, Agasthiarmalai Biosphere Reserve, Western Ghats, Tamil Nadu. The leaf samples were air dried and powdered. Required quantity of powder was weighed and transferred to stoppered flask and treated with the ethanol until the powder is fully immersed. The flask was shaken every hour for the first 6 hours and then it was kept aside and again shaken after 24 hours. This process was repeated for 3 days and then the extract was filtered. The extract was collected and evaporated to dryness by using a vacuum distillation unit. The final residue thus obtained was then subjected to HPTLC analysis and assessment of antioxidant activity. The extracts were subjected to qualitative test the identification of various phytochemical constituents as per standard procedures (Brindha *et al.,* 1981; Lala, 1993).

HPTLC ANALYSIS FOR PHYTOCOMPOUNDS

Test solution 2µl and 4 µl of standard solution was loaded as 6mm band length in the 4x10 silica gel $60F_{254}$ HPTLC plate using Hamilton syringe and Camag Linomat 5 instrument. Mobile phase was chloroform-methanol (9.9:0.1) for alkaloid, ethyl acetate- butanone- formic acid-water (5:3:1:1) for flavonoid, ethyl acetate-methanol-ethanol-water (8:1:1:1:0.4:0.8) for glycosides, chloroform-methanol-water (9:1:0:1) for saponin, ethyl acetate-methanol-acetic acid-water (10:2.2:1.1:2.6) for steroid and n-hexane-ethyl acetate (1:1) for terpenoid were used. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254nm and UV366nm. Finally, the plate was fixed in a scanner stage and scanning was done at 254nm for alkaloid, flavonoid, 366nm for glycosides, terpenoids 500nm for saponin and steroid. The peak table, peak display and peak densitogram were noted.

ANALYSES OF ANTIOXIDANTS

DPPH RADICAL SCAVENGING ACTIVITY

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the nonradical form DPPH-H Blois, 1958.

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method Blois (1958) Briefly, an 0.1mm solution of DPPH in methanol was prepared, and 1ml of this solution was added to 3 ml of the solution of all extracts in methanol at different concentration (50, 100, 200, 400, 800 µg/ml).The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbances were measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10UV: Thermo electron corporation).Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition)= $\left\{\left(\frac{Ao - A1}{Ao}\right) \times 100\right\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

SUPEROXIDE RADICAL SCAVENGING ACTIVITY

Superoxide anion scavenging activity was measured according to the method of Robak and Gryglewski (1988) with some modifications. All the solutions were prepared in 100mM phosphate buffer (pH 7.4)1ml of reduced Nicotinamide adenine dinucleotide (NADH, 468 µm) 3ml of plant extract of different concentration (50, 100, 200, 400, 800 µg/ml) were mixed. The reaction was initiated by adding 100ml of phenanzine methosulphate (PMS,60µm).the reaction mixture was incubated at 25°C for 5 min, followed by measurement of absorbance at 560nm.the percentage inhibition was calculated by using the following equation

Superoxide radical scavenging activity = $\left\{\left(\frac{Ao - A1}{Ao}\right) \times 100\right\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the test were performed in triplicates and the results were averaged

ANTIOXIDANT ACTIVITY BY RADICAL CATION (ABTS. +)

ABTS assay was based on the slightly modified method of Re *et al*. (1999). ABTS radical cation (ABTS.+) was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use . The ABTS.+ solution was diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. After addition of 100µL of sample or trolox standard to 3.9 mL of diluted ABTS.+ solution ,absorbance was measured at 734 nm by Genesis 10s UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC).

ABTS radical cation activity = $\left\{\left(\frac{Ao - A1}{Ao}\right) \times 100\right\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

HYDROXYL RADICAL SCAVENGING ACTIVITY

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell *et al.* (1987). Stock solutions of EDTA (1mM), FeCl3 (10mM), Ascorbic Acid (1mM), H_2O_2 (10mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1ml EDTA , 0.01ml of $FeCl_3$,0.1ml H_2O_2, 0.36ml of deoxyribose, 1.0ml of the extract of different concentration (50, 100, 200, 400, 800 µg/ml) dissolved in distilled water,0.33ml of phosphate buffer (50mM , pH 7.9), 0.1ml of ascorbic acid in sequence . The mixture was then incubated at 37^0c for 1 hour. 1 A 1.0ml portion of the incubated mixture was mixed with 1.0ml of 10%TCA 1.0ml of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation is calculated by using the following equation

Hydroxyl radical scavenging activity = $\left\{\left(\frac{Ao - A1}{Ao}\right) \times 100\right\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

REDUCING POWER

The reducing power of the extract was determined by the method of Singh *et al*, (2009). 1.0ml of solution containing 50, 100, 200, 400, 800 µg/ml of extract was mixed with sodium phosphate buffer (5.0 ml, 0.2 M, pH6.6) and potassium ferricyanide (5.0ml, 1.0%): The mixture was incubated at 50^oC for 20 minutes. Then 5ml of 10% trichloroacetic acid was added and centrifuged at 980gm (10 minutes at 5^oC) in a refrigerator centrifuge. The upper layer of the solution (5.0 ml) was diluted with 5.0ml of distilled water and ferric chloride and absorbance read at 700nm. The experiment was performed thrice and results were averaged.

RESULTS AND DISCUSSION

PHYTOCHEMICAL AND HPTLC ANALYSIS

The phytochemical analysis of ethanol extract of *E. floccosa* leaf showed the presence of alkaloid, catechin, coumarin, tannin, saponin, steroid, flavonoid, phenol, sugar, glycoside, xanthoprotein and fixed oil. The HPTLC results were tabulated in peak tables (Tables 1-6). Peak display and peak densitogram were noted (Figures 1-18). The HPTLC analysis showed the presence of alkaloids, steroids, terpenoids, glycosides, flavonoids and saponins in the ethanol extract of *E. floccosa* leaf.

PHYTOCHEMICAL STUDIES

Presence or absence of certain important compounds in an extract is determined by colour reactions of the compounds with specific chemicals which act as dyes. This procedure is a simple preliminary pre-requisite before going for detailed phytochemical investigation. Various tests have been conducted qualitatively to find out the presence or absence of bioactive compounds. Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemcial screening;

chemo profiling and marker compound analysis using modern analytical techniques. In the last two decades, HPTLC has emerged as an important tool for the qualitative, semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations. An HPTLC method is fast, precise, sensitive and reproducible with good recoveries for standardization of herbal drugs.

In the present study, the preliminary phytochemical study on *Eugenia floccosa* leaf have revealed the presence of alkaloid, coumarin, catechin, flavonoid, phenol, saponin, steroid, glycoside, terpenoid, sugar and xanthoprotein. HPTLC investigations also confirmed the presence of alkaloids, glycosides, flavonoids, steroids, terpenoids and saponins, which could made the plant useful for treating different ailments as having a potential of providing useful drugs of human use. This is because; the pharmacological activity of any plant is usually traced to a particular compound.

Therapeutically terpenoids exert wide spectrum of activities such as antiseptic, stimulant, diuretic, anthelmintic, analgesic and counter-irritant. Many tannin containing drugs are used in medicine as astringent. They are used in the treatment of burns as they precipitate the proteins of exposed tissues to form a protective covering. They are also medically used as healing agents in inflammation, leucorrhoea, gonorrhoea, burns, piles and antidote. Tannins have been found to have antiviral, antibacterial, antiparasitic effects, antiinflammatory, antiulcer and antioxidant property for possible therapeutic applications. It was also reported that, certain tannins were able to inhibit HIV replication selectively and was also used as diuretic (Akiyama *et al*., 2001; Lv *et al*., 2004; Kolodziej and Kiderlen, 2005).

Saponins, a group of natural products occur in the leaf extract of *E. floccosa*. In plants, the presence of steroidal saponins like, cardiac glycosides appear to be confined to many families and these saponins have great pharmaceutical importance because of their relationship to compounds such as the sex hormones, cortisones, diuretic steroids, vitamin D etc., (Evans and Saunders, 2001). From plant sapogenins a synthetic steroid is prepared and to treat a wide variety of diseases such as rheumatoid arthritis, collagen disorders, allergic and asthmatic conditions (Claus, 1956). Saponin reduces the uptake of certain nutrients including glucose and cholesterol at the gut through intra-luminal physicochemical interactions. Hence, it has been reported to have hypocholesterolemic effect and thus may aid lessening metabolic burden that would have been placed in the liver (Price *et al.,* 1987).

Several authors reported that flavonoids, sterols/terpenoids, phenolic acids are known to be bioactive antidiabetic principles (Oliver-Bever, 1986; Rhemann and Zaman, 1989). Flavonoids are known to regenerate the damaged beta cells in the alloxan induced diabetic rats (Chakravarthy *et al.,* 1980). Flavonoids act as insulin secretagogues (Geetha *et al.,* 1994). Most of the plants have been found to contain substances like glycosides, alkaloids, terpenoids, flavonoids etc, which are frequently implicated as having antidiabetic effects (Loew and Kaszhin, 2002).

ANTIOXIDANT ACTIVITY

Oxidative stress has been implicated in the pathology of many diseases and conditions including diabetes, cardiovascular disease, inflammatory conditions, cancer and ageing (Marx, 1987). Antioxidants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and by many other mechanisms and thus prevent disease (Evans and Miller, 1997).

DPPH is a relatively stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH. From the present results it may be postulated that, *E. floccosa* leaf extract reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles (Sreejayan and Rao, 1996). DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution loses colour stochiometrically depending on the number of electrons taken up (Evans and Miller, 1997). The results of the DPPH scavenging activity of *E. floccosa* leaf extract are shown in Figure 19. The scavenging ability of ethanol extracts was comparable to ascorbic acid.

Superoxide anion is an oxygen-centered radical with selective reactivity. This species is produced by a number of enzyme systems in auto-oxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen.

Table 1: HPTLC Peak table for alkaloids assigned in *Eugenia floccosa*

Track	Peak	Rf	Height	Area	Assigned substance
A	1	0.03	329.7	4668.1	Unknown
A	2	0.10	38.4	693.6	Unknown
A	3	0.20	14.2	214.9	Unknown
A	4	0.23	28.5	790.1	Unknown
A	5	0.45	26.5	755.4	Unknown
A	6	0.62	39.7	1555.4	Alkaloid 1
A	7	0.79	305.7	13989.3	Unknown
A	8	0.84	167.8	8308.8	Unknown
PIP	1	0.51	381.9	12279.9	Piperine 1 standard
PIP	2	0.57	291.1	10297.8	Piperine 2 standard

Table 2: HPTLC Peak table for steroids assigned in *Eugenia floccosa*

Track	Peak	Rf	Height	Area	Assigned substance
A	1	0.02	284.8	7173.3	Unknown
A	2	0.11	148.3	4567.4	Steroid 1
A	3	0.18	258.7	10176.5	Steroid 2
A	4	0.25	211.7	12316.9	Steroid 3
A	5	0.34	95.7	2025.1	Unknown
A	6	0.50	110.4	2003.5	Unknown
A	7	0.56	120.7	3089.0	Unknown
A	8	0.62	111.5	5720.9	Unknown
A	9	0.73	74.0	3403.5	Unknown
A	10	0.86	33.9	894.0	Steroid 4
A	11	0.92	36.2	507.2	Unknown
A	12	0.96	273.3	8856.6	Unknown
SOLA	1	0.62	271.2	7410.9	Solasodine standard

Table 3: HPTLC Peak table for terpenoids assigned in *Eugenia floccose*

Track	Peak	Rf	Height	Area	Assigned substance
A	1	0.05	328.3	8373.5	Unknown
A	2	0.09	227.5	6412.5	Terpenoid 1
A	3	0.16	169.9	5117.1	Terpenoid 2
A	4	0.21	175.9	13804.9	Terpenoid 3
A	5	0.32	120.2	5833.1	Terpenoid 4
A	6	0.39	81.0	2916.5	Unknown
A	7	0.53	277.1	11202.9	Unknown
A	8	0.59	124.5	4696.9	Terpenoid 5
A	9	0.69	253.0	12836.0	Unknown
A	10	0.74	264.6	9331.7	Unknown
A	11	0.88	19.8	611.8	Terpenoid 6
SOL	1	0.68	84.3	3021.7	Solanesol standard

It can also reduce certain iron complexes such as cytochrome (Gulcin *et al.*, 2005). The present study showed potent superoxide radical scavenging activity for *E. floccosa* leaf extract. Superoxide radical scavenging activity of ethanol extract of *E. floccosa* leaf extract is presented in figure 20. The superoxide radical scavenging activity of *E. floccosa* leaf extract was comparable to ascorbic acid.

Table 4: HPTLC Peak table for glycosides assigned in *Eugenia floccosa*

Track	Peak	Rf	Height	Area	Assigned substance
A	1	0.17	17.7	238.2	Unknown
A	2	0.34	19.2	172.6	Unknown
A	3	0.54	97.2	2093.6	Glycoside 1
A	4	0.59	36.9	554.1	Unknown
A	5	0.62	53.8	1729.2	Glycoside 2
A	6	0.66	48.3	661.3	Unknown
A	7	0.69	80.0	2196.9	Glycoside 3
A	8	0.74	17.9	249.0	Glycoside 4
A	9	0.77	25.2	469.7	Unknown
A	10	0.88	157.2	4359.0	Glycoside 5
STE	1	0.34	21.0	498.2	Stevioside standard

Table 5: HPTLC Peak table for flavonoids assigned in *Eugenia floccosa*

Track	Peak	Rf	Height	Area	Assigned substance
A	1	0.08	11.0	222.0	Unknown
A	2	0.19	27.1	1043.8	Unknown
A	3	0.25	38.8	1329.7	Unknown
A	4	0.36	33.8	723.5	Flavonoid 1
A	5	0.39	25.6	475.7	Flavonoid 2
A	6	0.48	38.8	998	Flavonoid 3
A	7	0.59	58.3	2483.9	Flavonoid 4
A	8	0.65	96.1	4813.4	Flavonoid 5
A	9	0.74	65.4	2419.7	Flavonoid 6
A	10	0.81	49.8	1785.2	Unknown
A	11	0.86	99.2	3655.1	Unknown
A	12	0.96	266	10569.7	Unknown
RUT	1	0.27	388.6	10192.6	Rutin standard

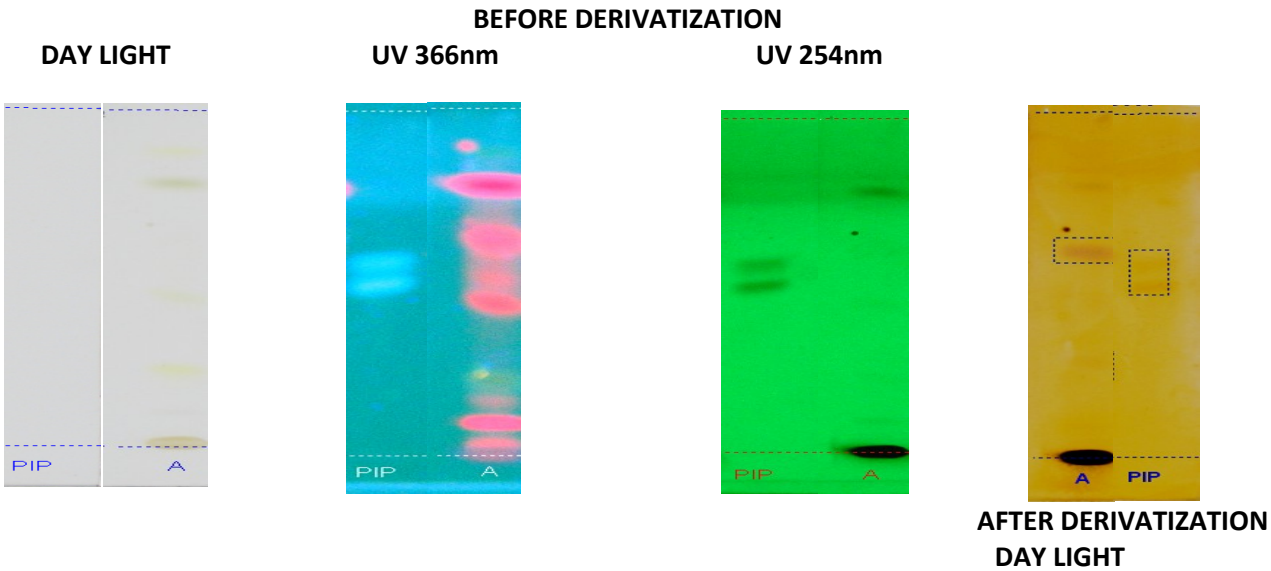


Figure 1: HPTLC chromatogram for alkaloids

Table 6: HPTLC Peak table for saponins assigned in *Eugenia floccosa*

Track	Peak	Rf	Height	Area	Assigned substance
A	1	0.08	12.9	132.7	Unknown
A	2	0.51	23.7	636.9	Unknown
A	3	0.70	69.7	2091.3	Saponin 1
A	4	0.86	30.7	1130.4	Saponin 2
A	5	0.96	53.8	1786.3	Unknown
SAP	1	0.55	55.5	1575.3	Saponin standard

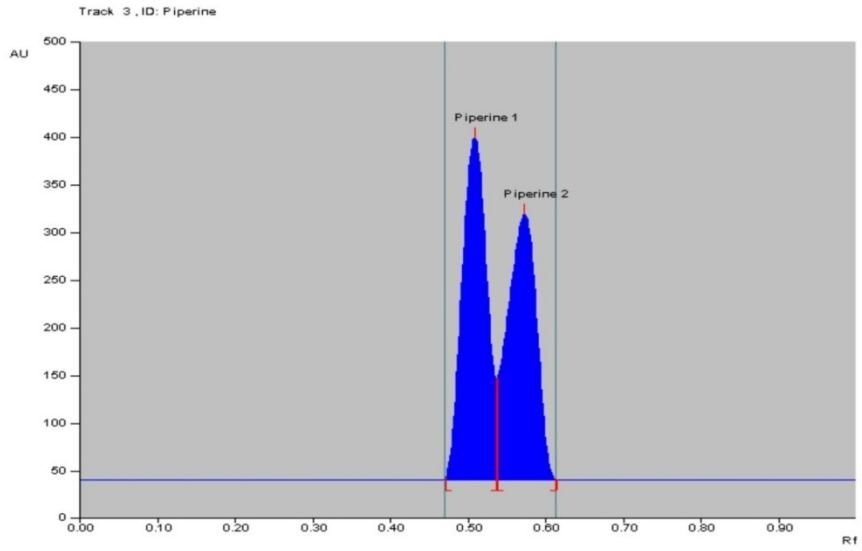


Figure 2: Track PIP-Piperine 1 and Piperine 2 standard densitogram display (scanned at 254 nm)

BEFORE DERIVATIZATION

DAY LIGHT **UV 366nm** **UV 254nm**

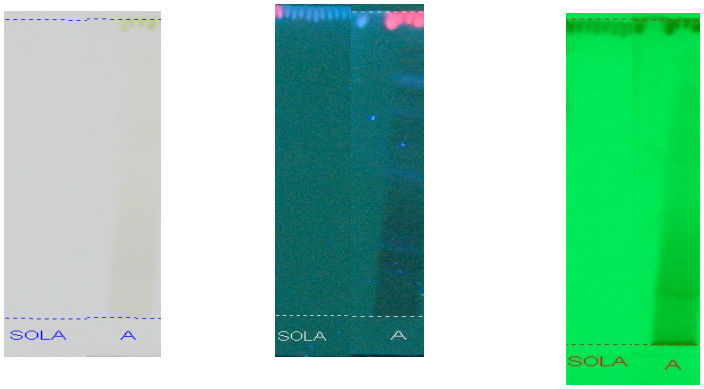
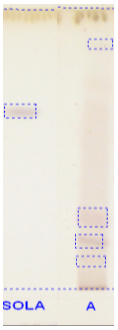


Figure 3: Track A *Eugenia floccosa* leaf ethanol extract peak densitogram display (scanned at 254 nm)



AFTER DERIVATIZATION
DAY LIGHT

Figure 4: HPTLC chromatogram for steroids

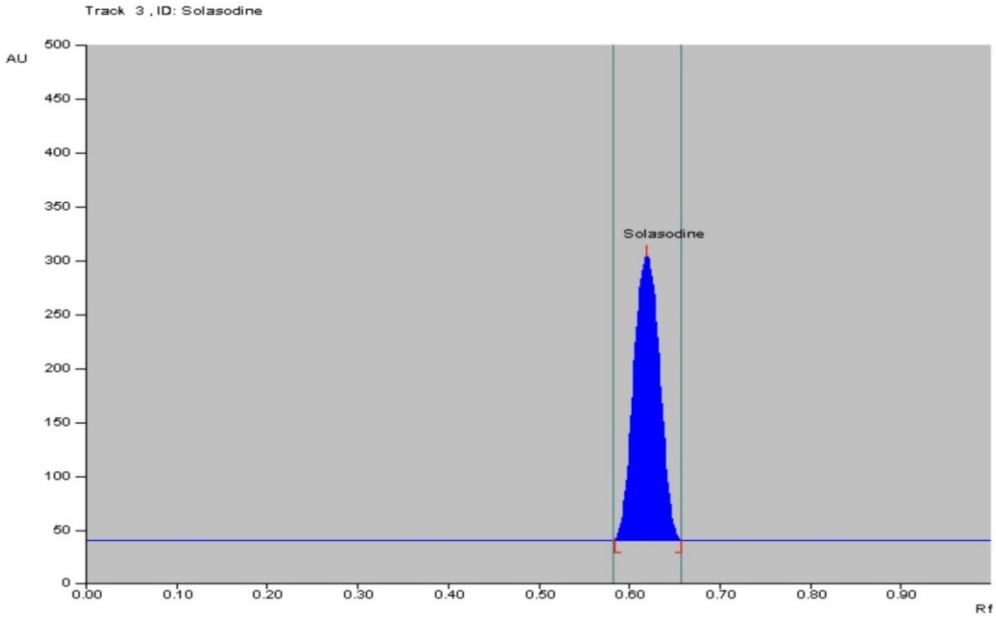


Figure 5: Track SOLA – Solasodine standard densitogram display (scanned at 254 nm)

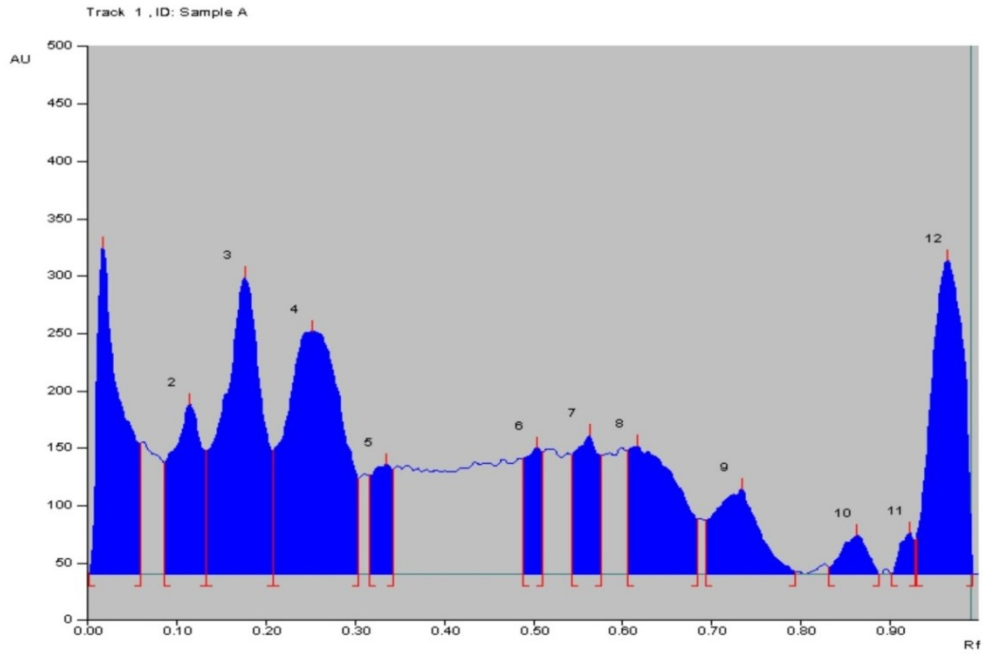
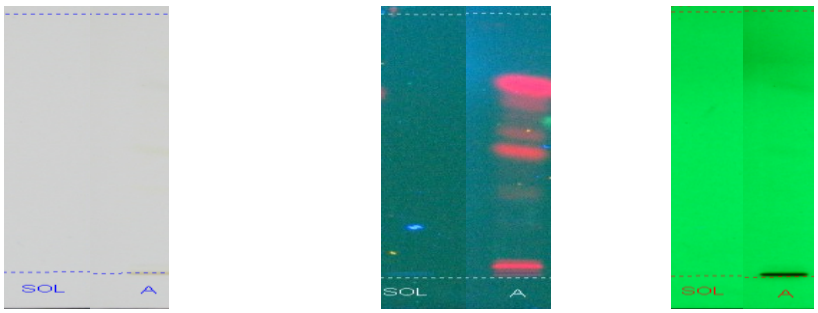


Figure 6: Track A *Eugenia floccosa* leaf ethanol extract peak densitogram display (scanned at 254 nm)

BEFORE DERIVATIZATION

DAY LIGHT **UV 366nm** **UV 254nm**



AFTER DERIVATIZATION

DAY LIGHT **UV 366nm**

Figure 7: HPTLC chromatogram for terpenoids

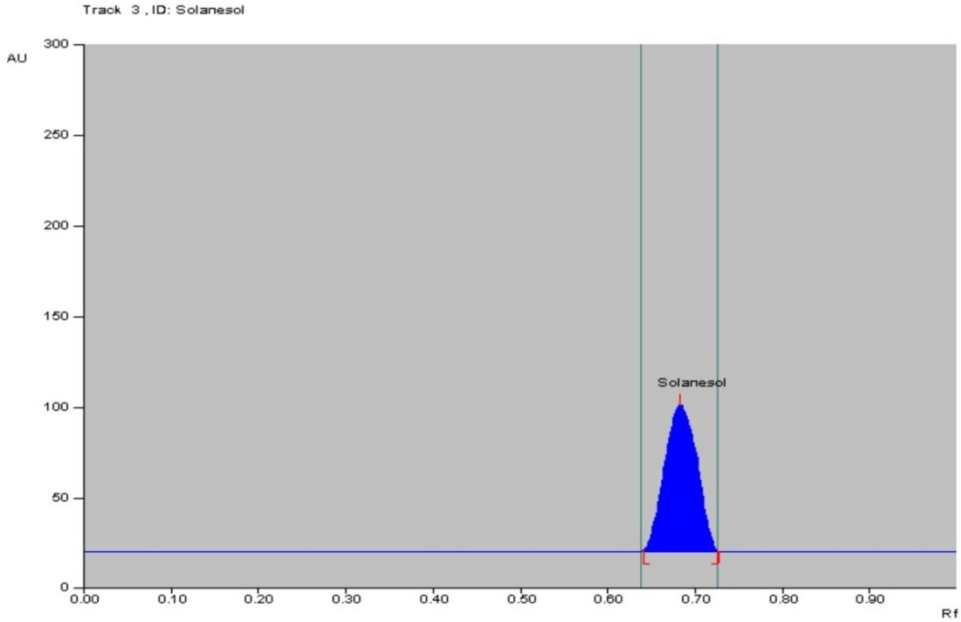


Figure 8: Track SOL – Solanesool standard densitogram display (scanned at 254 nm)
SOL – Solanesool standard densitogram display (scanned at 254 nm)

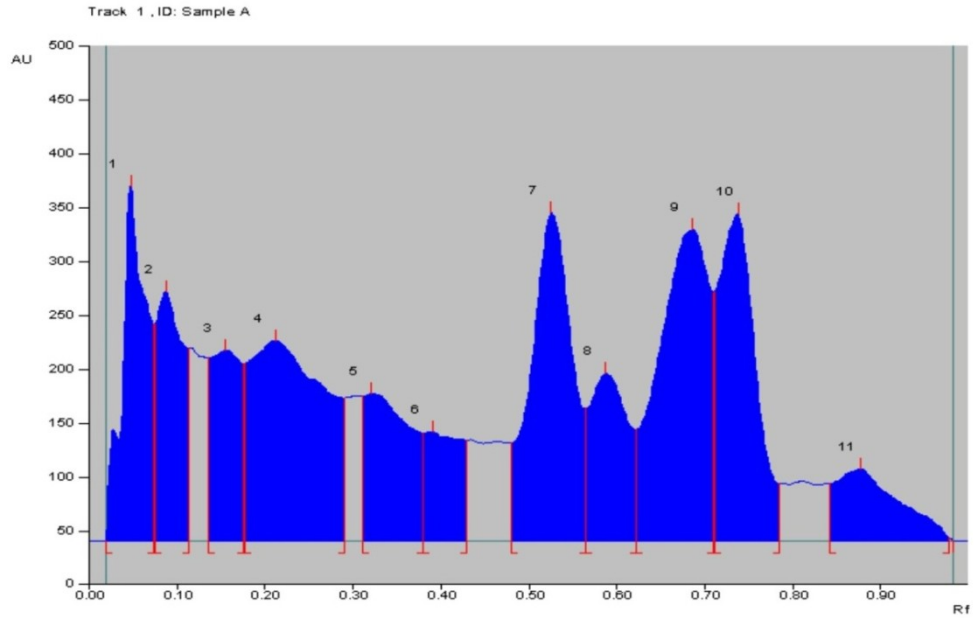


Figure 9: Track A *Eugenia floccosa* leaf ethanol extract peak densitogram display (scanned at 254 nm)

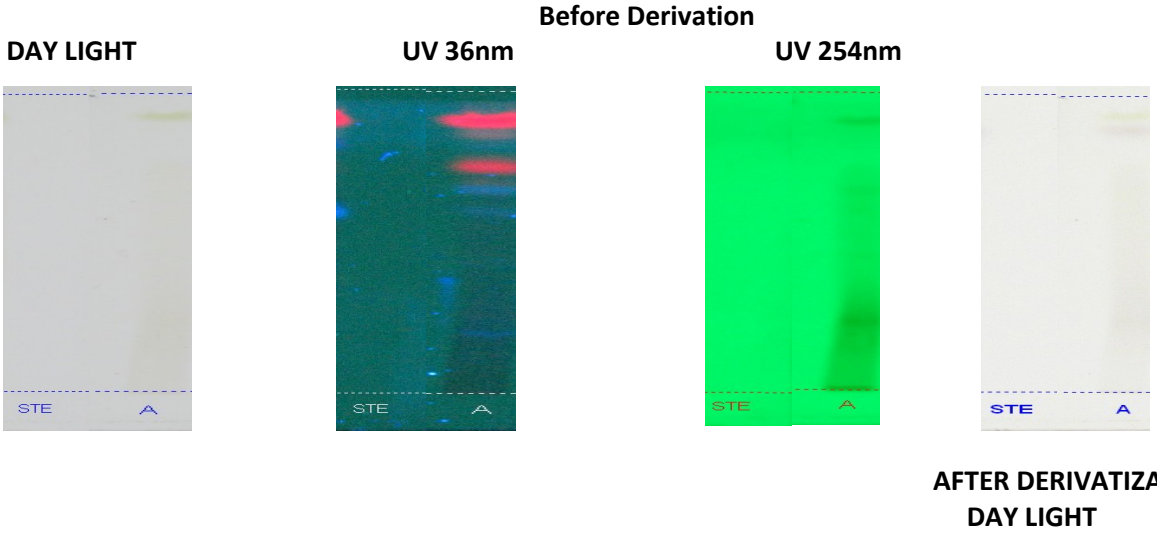


Figure 10: HPTLC chromatogram for glycosides

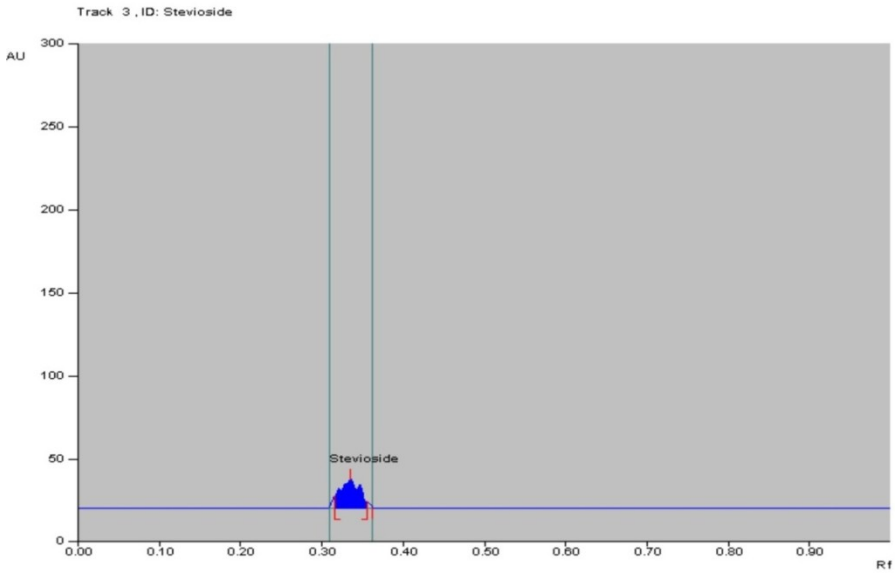


Figure 11 Track STE – Stevioside standard densitogram display (scanned at 254 nm)

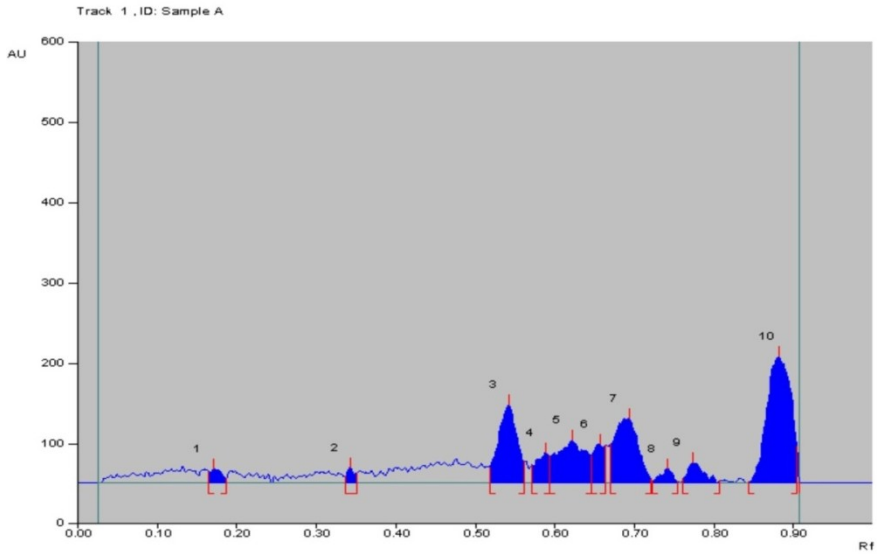


Figure 12: Track A *Eugenia floccosa* leaf ethanol extract peak densitogram display (scanned at 254 nm)

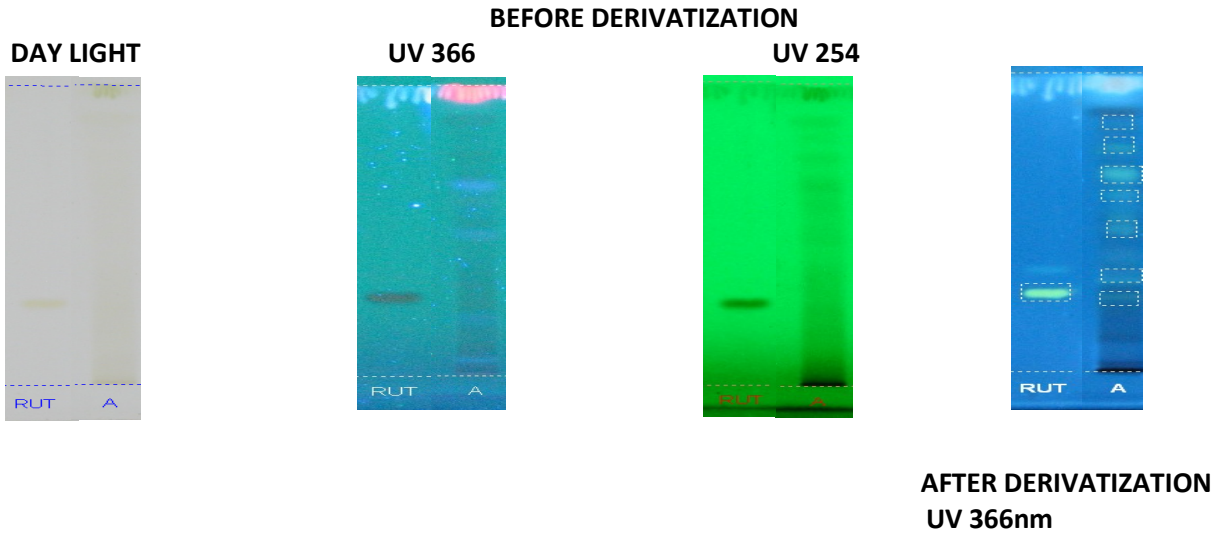


Figure 13: HPTLC chromatogram for flavonoids

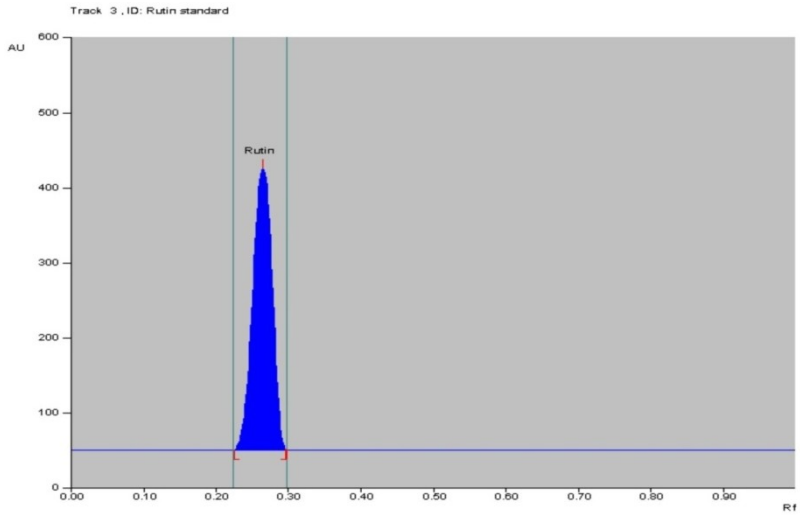


Figure 14: Track RUT – Rutin standard densitogram display (scanned at 254 nm)

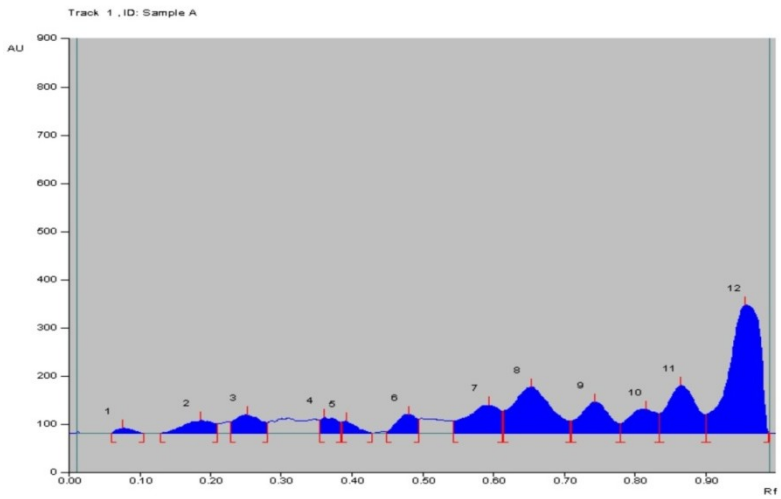


Figure 15: Track A *Eugenia floccosa* leaf ethanol extract peak densitogram display (scanned at 254 nm)

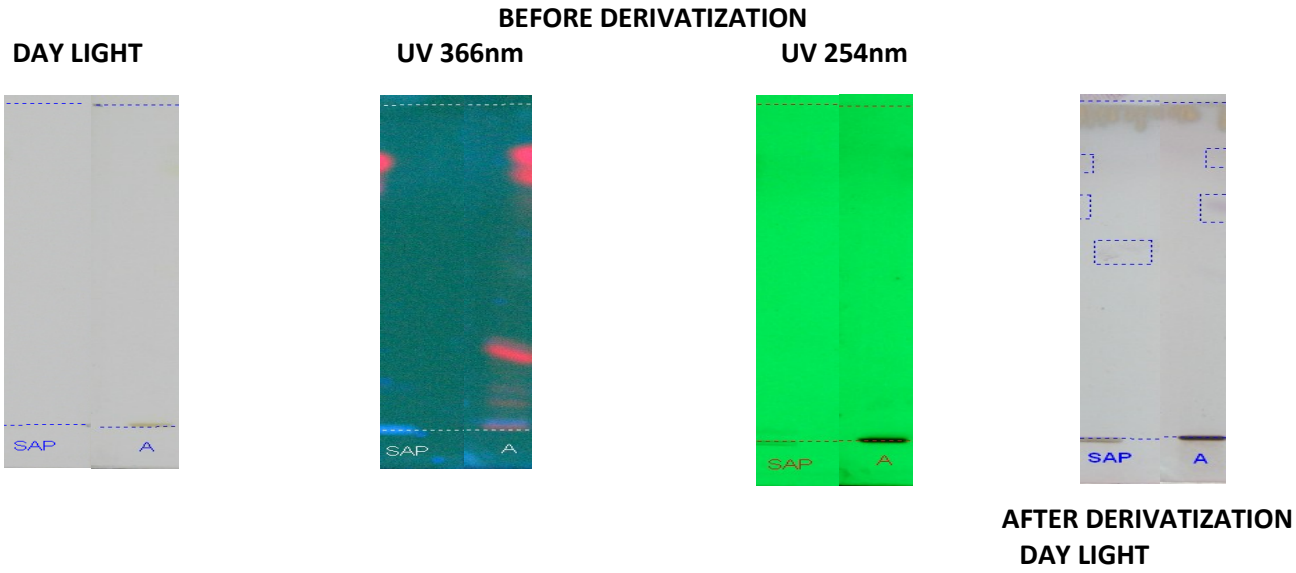


Figure 16: HPTLC chromatogram for saponins

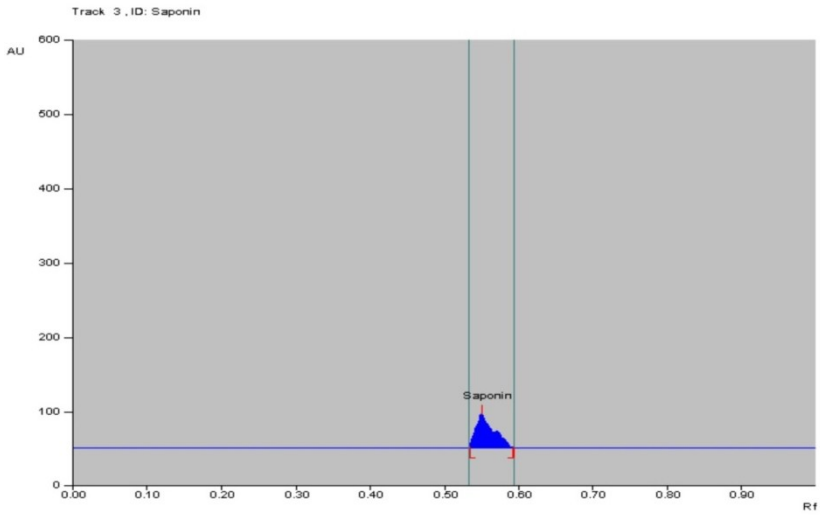


Figure 17: Track SAP – Saponins standard densitogram display (scanned at 254 nm)

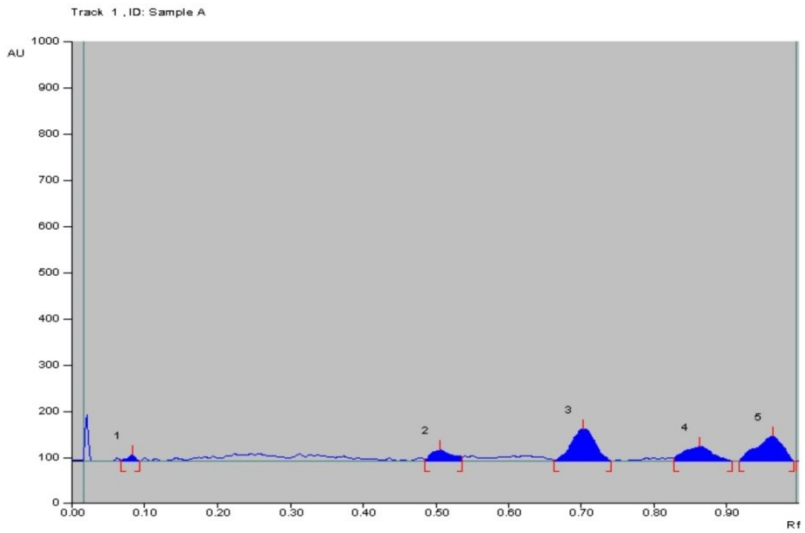


Figure 18: Track A *Eugenia floccosa* leaf ethanol extract peak densitogram display (scanned at 254 nm)

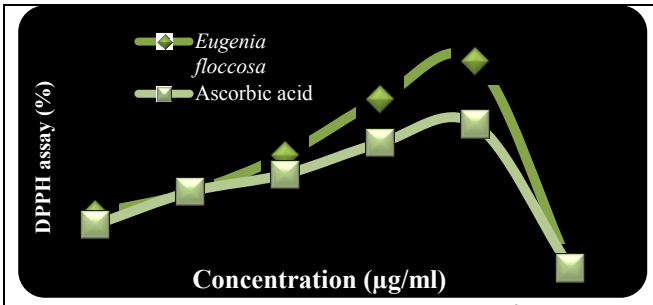


Fig 19: DPPH radical scavenging activity of methanol extract of *E. floccosa*

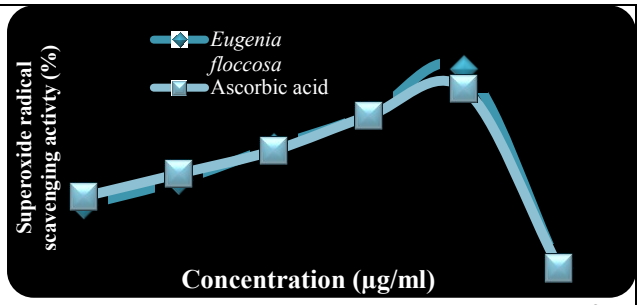


Fig 20: Superoxide radical scavenging activity of methanol extract of *E. floccosa*

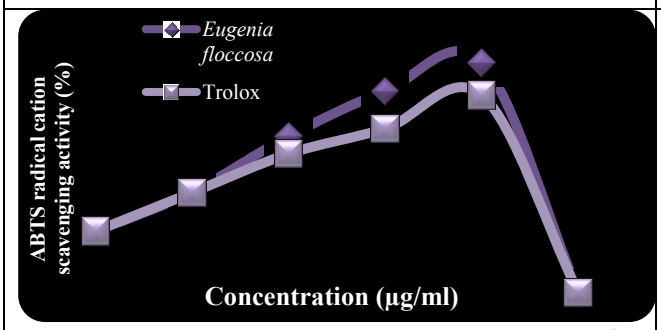


Fig 21: ABTS radical cation scavenging activity of methanol extract of *E. floccosa*

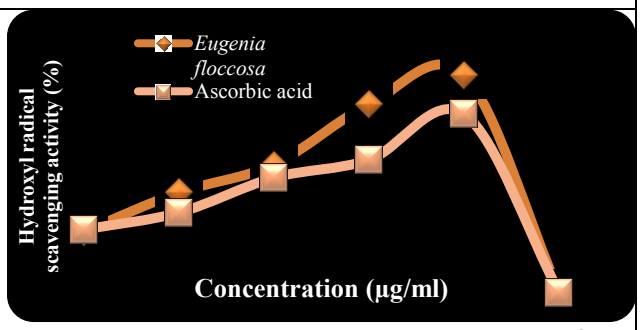


Fig 22: Hydroxyl radical scavenging activity of methanol extract of *E. floccosa*

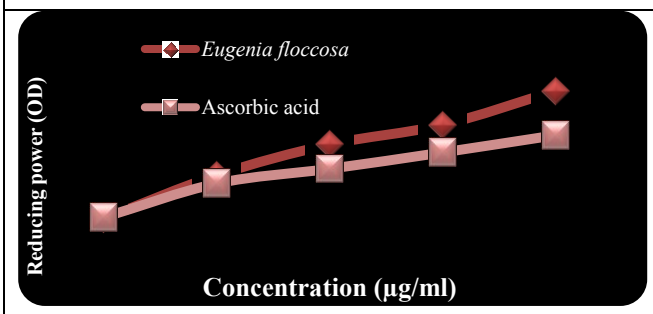


Fig 23: Reducing power ability of methanol extract of *E. floccosa*

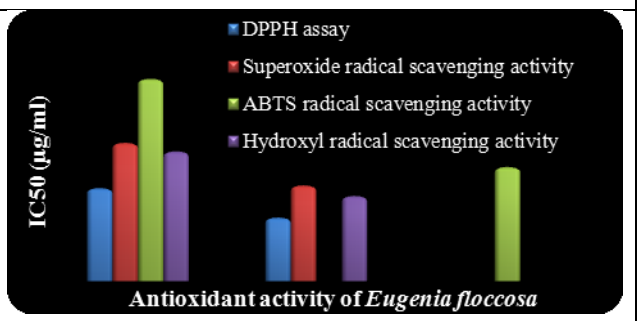


Fig 24: IC_{50} values of methanol extract of *E. floccosa*

The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^+$, which has a characteristic long, wave length absorption spectrum (Sreejaeyan and Rao, 1996). The ABTS chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. It is a decolorization assay, thus the radical cation is performed prior to addition of antioxidant test system, rather than the generation of the radical taking place continually in the presence of antioxidant. The results obtained imply the activity of the extract either by inhibiting or scavenging the $ABTS^+$ radicals since both inhibition and scavenging properties of antioxidants towards $ABTS^+$ radicals have been reported earlier (Youdim and Joseph, 2001).

The free radical scavenging ability of the ethanol extract from *E. floccosa* leaf was also determined using ABTS radical cation and presented in figure 21. The percentage scavenging activity and IC_{50} of the investigated extract at 1 min of the reaction time was calculated. The highest percentage activity at 72.55% (800µg/ml) was found for ethanol extract of *E. floccosa* leaf.

Hydroxyl radical is the most reactive among ROS and it bears the shortest half-life compared with other ROS. Figure 22 shows the hydroxyl radical scavenging activity of ethanol extract of *E. floccosa* leaf and compared with ascorbic acid. It was observed that ethanol extract of *E. floccosa* leaf had higher activity than that of ascorbic acid.

At a concentration of 800 µg/ml, the scavenging activity of ethanol extract of *E. floccosa* leaf reached 68.56% while at the same concentration, that of the ascorbic acid was 56.29%.

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Oktay *et al*., 2003). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995).

Allhorm *et al* (2005) recently reported that, reducing property can be a novel antioxidation defense mechanism; this is possibly through the ability of the antioxidant compound to reduce transition metals. Reduced metals such as Fe(II) or Cu(I) rapidly react with lipid hydroperoxides, leading to the formation of reactive lipid radicals and conversion of the reduced metal to its oxidized form (Gogvadze *et al*., 2003).

As shown in figure 23, the reducing power of ethanol extract of *E. floccosa* increased with increase in concentration. At a concentration of 800 µg/ml, reducing power of ethanol extract of *E. floccosa* leaf was 0.871% while at the same concentration, that of the ascorbic acid was 0.678%.

The IC_{50} values of *E. floccosa* leaf extract and standard ascorbic acid for DPPH, hydroxyl, superoxide radical scavenging activity and trolox for ABTS radical cation scavenging activity were found to be 31.45 µg/ml and 21.48 µg/ml; 43.84 µg/ml and 28.76 µg/ml; 46.74 µg/ml and 32.34 µg/ml and 68.32 µg/ml and 38.56 µg/ml respectively (Figure 24).

The present study reveals that, the leaf of *E. floccosa* exhibits satisfactory scavenging effects in all the radical scavenging assays. This is the first report on the antioxidant property of this plant. It is reported that, flavonoids are natural products which have been shown to possess various biological properties related to antioxidant mechanisms (Shirwaikar *et al*., 2004). Thus, in the present study, the antioxidant potential of *E. floccosa* may be attributed to the presence of flavonoids and the other constituents present therein which was confirmed by phytochemical and HPTLC analysis. This further showed the capacity of the extracts to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical-related pathological damage.

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