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# TLC separation and antioxidant activity of flavonoids from *Carissa bispinosa*, *Ficus sycomorus*, and *Grewia bicolor* fruits

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## Abstract

**Background:** *Carissa bispinosa*, *Ficus sycomorus*, and *Grewia bicolor* are edible fruit plants that grow in the wild. The plants produce yellow-, red-, and purple-colored fruits and thus can be good sources of flavonoids for fighting oxidative reactions in humans, food, and the pharmaceutical industry. The present study aimed at isolating flavonoids from *C. bispinosa*, *F. sycomorus*, and *G. bicolor* fruits and determining their antioxidant activity using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2-azino-bis 3-ethylbenz-thiaz-oline-6-sulfonic acid (ABTS) model radical assays.

**Methods:** Analytical and preparative thin-layer chromatography was used to isolate flavonoids from the fruits using methanol/chloroform/hexane (7:2:1, v/v/v) as a mobile phase system. The ABTS and DPPH radical scavenging methods were used to test for the antioxidant activity of the samples, using quercetin and catechin as reference standards.

**Results:** Thin-layer chromatographic profiling revealed two different types of flavonoids from each plant. *C. bispinosa* yielded two flavonoid bands,  $R_f$  values 0.11 and 0.38; *G. bicolor* yielded two flavonoid bands,  $R_f$  values 0.63 and 0.81; and *F. sycomorus* also yielded two types of flavonoids,  $R_f$  values 0.094 and 0.81. All the extracted flavonoids exhibited significant antioxidant activity of over 80% at a concentration of 200 mg/L. The order of radical scavenging activity for the 200-mg/L samples is *G. bicolor*  $R_f$  (0.81) > *C. bispinosa*  $R_f$  (0.113) > *F. sycomorus*  $R_f$  (0.094) > *F. sycomorus*  $R_f$  (0.047) > *C. bispinosa*  $R_f$  (0.38) > *G. bicolor*  $R_f$  (0.63). *G. bicolor* ( $R_f = 0.81$ ) exhibited antioxidant activity that was superior to that of catechin.

**Conclusion:** The present study results show that *C. bispinosa*, *F. sycomorus*, and *G. bicolor* contain different flavonoid types with significant antioxidant activity of over 80% at a concentration of 200 mg/L. Therefore, the fruits can be used as a source of natural antioxidants which can be used as nutraceuticals to promote health, as preservatives to delay peroxidation of foods, and as flavoring for packed foods.

**Keywords:** Thin-layer chromatography, Flavonoids, Antioxidant activity, *Carissa bispinosa*, *Ficus sycomorus*, *Grewia bicolor*

## Background

The induction of many chronic and degenerative diseases is a direct result of oxidative stress caused by free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS), which cause oxidative damage of amino acids, lipids, proteins, and DNA [1, 2]. The degenerative diseases include ischemic heart disease, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases, and aging. Among the phytochemicals present in natural plant foods, antioxidants have

attracted attention among researchers since they protect the human body against oxidative stress and therefore prevent chronic non-communicable ailments [3]. Natural antioxidants are believed to be safer than synthetic ones. The later, such as BHT and BHA, are now being restricted by legislation because of possible toxic and carcinogenic effects, [4–6] and TBHQ has been banned in Japan and some European countries [7]. On the other hand, the antioxidants from plants are not only non-carcinogenic but reportedly have anti-carcinogenic effects [6].

Plants have been shown to be rich sources of flavonoids, which are molecules that can act as antioxidants.

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It has been noted that the protection afforded by the consumption of plant products such as fruits, vegetables, and legumes is mostly associated with the presence of flavonoids. Several researches have been done on separation and characterization of flavonoids in fruits, leaves, bark, roots, and seeds. However, no documented research on *Carissa bispinosa*, *Ficus sycomorus*, and *Grewia bicolor* flavonoid content and their antioxidant activity is available and this provides the rationale behind this study. This study may also give impetus to the sustainable use of the plants. The information obtained can then be used to professionally plan human diet that takes into account recommendations to use the traditional fruits in mainstream diet. The fruits are readily available in a number of areas globally and are only used by people as food supplements especially during food shortage times.

## Methods

### Sample preparation

Ripened and unripened *C. bispinosa*, *F. sycomorus*, and *G. bicolor* fruits were gathered from a forest in Mberengwa district, Zimbabwe, in the months of February and March of 2016. The samples were shade dried; however, *C. bispinosa* took a long time to dry. The dried *F. sycomorus* and *G. bicolor* fruits were ground to a powder using a grinder. The *C. bispinosa* was ground to a thin paste using a mortar and pestle. The dry powders were stored in polythene bags and kept in a cupboard prior to liquid-solid extraction. The *C. bispinosa* paste was put in polythene bag and stored in a fridge at 4 °C prior to liquid-solid extraction.

## Methods

### Instruments

A Mettler Toledo digital analytical balance AB204-S (4d.p) was used to measure the masses of samples during sample preparation. The same balance was used to measure masses of reagents during the preparation of solutions. A Labotec horizontal shaker was used to agitate the ethanol-fruit sample mixtures during liquid-solid extraction to optimize the extraction. A KnF Neuberger vacuum suction pump was used to enhance filtration to separate the liquid sample from the solid residue during sample preparation. A CE Serial No. 15 102295 Vilber Lourmat UV-Visualization detector set at 365 nm was used to visualize developed thin-layer chromatograms. A Genesys 10s UV-Vis Spectrophotometer was used to measure the absorbance of flavonoid containing fractions obtained from preparative thin-layer chromatography (TLC) and the standards used. A Thermo Scientific iD1 FT-IR spectrophotometer was used for generating spectra of sample extracts to confirm flavonoid presence.

### Chemicals and reagents

Analytical grade solvents were used in all liquid-solid extraction, and Folin-Ciocalteu reagents were purchased from SkyLabs, Johannesburg, South Africa. Hydrophobic-lipophilic balanced (HLB) sorbent, TLC plates (analytical and preparative), 2, 2-azinobis 3-ethyl benzthioazoline-6-sulphonic acid (ABTS), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), catechin, and quercetin were purchased from Sigma Aldrich, Darmstadt, Germany.

### Solvent extraction

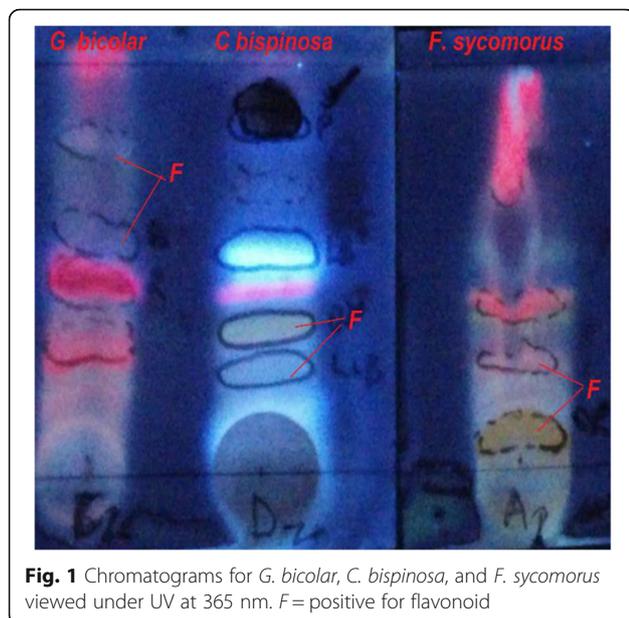
Ten grams of powdered samples of each of *F. sycomorus* and *G. bicolor* and 10 g paste of *C. bispinosa* were weighed and mixed with 20 mL of analytical grade absolute ethanol in a 50-mL volumetric flask. The samples were shaken for 30 min on a Labotec horizontal shaker. The samples were then filtered using Whatman No. 1 filter paper and placed in reagent bottles. The solvent maceration protocols were repeated three times for each set and the collected filtrates were combined.

### Solid phase extraction

The solid phase extraction was done according to the method of Mumin et al. [8] with minor modifications. Each set was conditioned by passing 5 mL of absolute ethanol through 0.4 g of the hydrophobic-lipophilic balanced sorbent by gravity in an SPE cartridge. Equilibration was achieved by passing 5 mL of distilled water through the sorbent. This was followed by loading the extract through the cartridge again under gravity. To maximize sample clean-up, 1 ml of acetone was used to wash the sorbent. Finally, 5 mL of absolute ethanol were used to elute the samples. The collected samples were stored in glass vials.

### Analytical thin-layer chromatography

TLC was done according to the method of Lihua et al. [9] with minor modifications. A 10 × 1.5 cm TLC plate was activated by heating at 100 °C for 10 min and allowed to cool to room temperature. Pencil lines were drawn 1.5 cm from one edge of the plates. Extract samples were spotted using thin capillary pipettes onto the pencil line. The plates were placed in a development chamber with a trial solvent. The solvent front was allowed to travel until about 1 cm from the top end. The TLC plates were removed and solvent front was marked using a soft pencil. They were air dried and then sprayed with a fine spray of 1% ethanolic aluminum chloride solution, left to dry and then visualized under UV light at 365 nm. The chromatograms were marked and retention factors were calculated and recorded. The resultant chromatograms were captured on camera (Fig. 1). The methanol/chloroform/hexane (7:2:1, v/v/v) produced the best separation of the spots.



**Fig. 1** Chromatograms for *G. bicolor*, *C. bispinosa*, and *F. sycomorus* viewed under UV at 365 nm. F = positive for flavonoid

#### Preparative thin-layer chromatography

Pre-coated thick silica gel on glass TLC plates measuring 20 × 20 cm was used. The methanol/chloroform/hexane (7:2:1, v/v/v) mobile phase solvent system was used. Each of the ethanol extracts from the three fruit samples was deposited as a concentrated band 1.5 cm from the edge of its respective TLC plate and allowed to dry. The plates, with dried samples, were gently lowered into the development tank, closed and left to develop. The plates were removed from the development chamber when the solvent front had traveled three quarters of the plates' length. The position of the solvent front was immediately marked with a soft pencil. The retention factor ( $R_f$ ) values of the different bands were then calculated using the equation:

$$R_f = \frac{\text{Distance of spot from origin}}{\text{Distance of solvent front from origin}}$$

Using the method reported by Mittal [10] with modifications, the bands that tested positive for the flavonoids, in the analytical TLC, were scratched off, mixed with 5 ml of absolute ethanol, allowed to stand for 10 min, and then filtered with Whatman No. 1 filter paper and collected in glass vials.

#### Preparation of standard solutions

0.01 g of each of catechin and quercetin standards was dissolved in 50 ml of methanol to make 200 mg/L stock solution. The 200 mg/L solution was serially diluted to give solutions of 100, 50, and 10 mg/L for each of the standards.

#### Preparation of sample solutions for ABTS assay

The recovered solutions of bands that tested positive for flavonoids were serially diluted to produce solutions of sample concentrations of 200, 100, 50, 10, and 5 mg/L of extract and tested for antioxidant activity.

#### ABTS<sup>•+</sup> radical generation

The ABTS<sup>•+</sup> radical was generated by reaction of 0.0021 M (1.057 g) ABTS and 0.000426 M (0.12 g) potassium persulfate solutions in 250 ml of distilled water. The mixture was left to stand in a dark cupboard for 16 h to allow for the generation of a stable monocationic ABTS radical that absorbs at 734 nm. After 16 h, the absorbance of the ABTS radical was measured.

#### ABTS assay of the samples and standards

0.5 ml of sample or standard was mixed with 1.0 ml of ABTS<sup>•+</sup> solution and left to stand for 6 min. Absorbance of the samples or standards was read after 6 min at 734 nm. The percentage antioxidant scavenging capacity was calculated using the following equation:

$$\% \text{Antioxidant scavenging capacity} = \frac{A_c - A_s}{A_c} \times 100$$

where  $A_c$  is absorbance of control and  $A_s$  is absorbance of the sample. The percentage total antioxidant scavenging activity for the standards and samples were plotted against the concentration in milligrams per liter of standard or sample used.

#### DPPH radical scavenging assay

The free radical scavenging activity of the flavonoid bands of each of the three fruits was also estimated using the 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) standard method as described by Afroz et al. [6], with minor modifications. DPPH is a stable free radical that strongly absorbs at 517 nm because of the presence of its odd electron. In the presence of a free radical scavenging antioxidant, the odd electron of DPPH will be paired up, thus decreasing the intensity of the absorption at 517 nm. One milliliter of the extract solution was mixed with 1.5 mL of 0.003% DPPH in methanol at concentrations of 62.50, 125, 250, 500, 1000, and 2000 mg/L, and the percentage of DPPH inhibition was calculated using the following equation:

$$\% \text{DPPH Inhibition} = \frac{A_{\text{dpph}} - A_s}{A_{\text{dpph}}} \times 100$$

where  $A_{\text{dpph}}$  = absorbance of DPPH in the absence of the extract and  $A_s$  = absorbance of DPPH in the presence of either the extract or the standard. The DPPH scavenging activity was expressed as the concentration of the extract required to decrease the DPPH absorbance by 50%

( $IC_{50}$ ).  $IC_{50}$  was graphically determined by plotting the percentage inhibition of DPPH radical against concentration of extract using linear regression. The  $IC_{50}$  value was calculated from the linearly regressed line.

### Statistical analysis

The analysis of variance (ANOVA) was conducted through the use of a General Treatment Structure (in randomized blocks) for both ABTS and DPPH analyses. The data was analyzed in GenStat 7 (Version 7.2.0.220). The blocking factor was the sample while the concentration of the sample was considered as the treatment. Each experiment was replicated three times giving a total of 96 experimental units for ABTS. For DPPH, each experiment was replicated three times giving a total of 126 experimental units. The results of the experiments were presented in graphical form. Student's *t* test, linear regression analyses (performed, in SPSS Version 16.0.2007), and Excel graphing were employed in order to explore the relationships between the variables.

## Results

### Analytical TLC for fruit extracts

Analytical TLC of all three fruit samples, after spraying with 1% ethanolic aluminum chloride, showed four spots under UV light at 365 nm. This indicates the presence of four different compounds and the  $R_f$  values were calculated and recorded (Table 1). The  $R_f$  values ranged from 0.094 to 0.88 for the three fruit extracts. *F. sycamoros* gave four spots at  $R_f$  values of 0.094, 0.47, 0.56, and 0.88; *C. bispinosa* had spots at  $R_f$  values of 0.11, 0.38, 0.69, and 0.84; and *G. bicolar* had spots at  $R_f$  values of 0.25, 0.47, 0.63, and 0.81 (Table 1).

### Preparative TLC for the fruit extracts

Preparative TLC was carried out using a methanol/chloroform/hexane (7:2:1, v/v/v) solvent system. The flavonoid containing bands were scrapped off using surgical blades, dissolved in ethanol, and filtered using Whatman No. 42 filter paper. The filtered solutions were recovered on a rotor vapor and then dissolved again in 12 ml of ethanol. Serial dilutions of the recovered solutions were made and their antioxidant activity was tested using the ABTS and DPPH free radical scavenging assays. Quercetin and catechin standards were used for comparison.

### Evaluation of antioxidant assay

Results of the antioxidant activity as determined by ABTS and DPPH antiradical activity are shown in Figs. 2 and 3.

### ABTS radical scavenging assay

Figure 2 shows plots of percentage ABTS antiradical activity of flavonoid extracts and selected standards

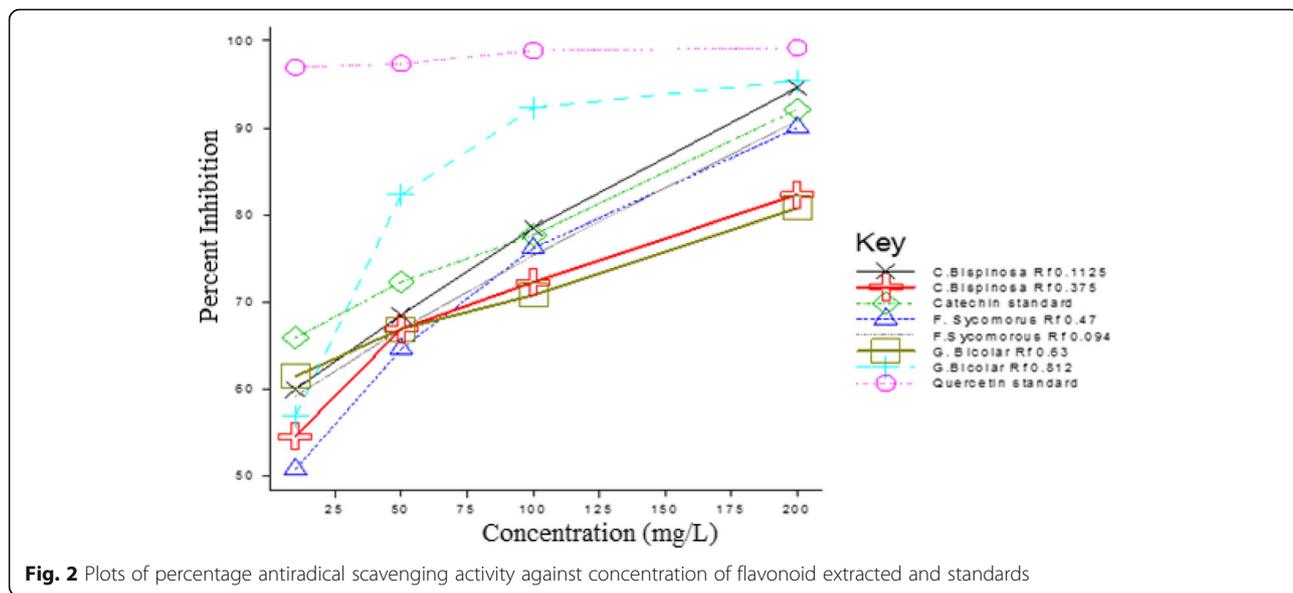
**Table 1** Flavonoid analysis using 1% ethanolic aluminum chloride revealing reagent

Fruit name	$R_f$ value	Test for flavonoids
<i>G. bicolar</i>	0.25	Negative
	0.47	Negative
	0.63	Positive
	0.81	Positive
<i>C. bispinosa</i>	0.11	Positive
	0.38	Positive
	0.69	Negative
	0.84	Negative
<i>F. sycamoros</i>	0.094	Positive
	0.47	Positive
	0.56	Negative
	0.88	Negative

against their concentration. The results show that none of the identified flavonoid extracts revealed an antioxidant activity which surpassed that of quercetin standard. *G. bicolar* ( $R_f=0.81$ ) revealed the greatest antioxidant activity surpassing that of the catechin standard from concentrations around 25 mg/L up to the 200 mg/L range (ANOVA;  $p=0.01$ ). Other flavonoid fractions such as *F. sycamoros* ( $R_f=0.094$  and 0.47), *C. bispinosa* ( $R_f=0.38$ ), and *G. bicolar* ( $R_f=0.63$ ) revealed antioxidant scavenging activity inferior to that of catechin standard at all concentration levels ( $p=0.00$ ). On the other hand, the *C. bispinosa* flavonoid fraction ( $R_f=0.11$ ) demonstrated antioxidant activity inferior to that of catechin standard at concentrations lower than 95 mg/L, but then equaled and surpassed that of catechin standard at concentrations in excess of 95 mg/L. Furthermore, the catechin standard demonstrated superior antioxidant scavenging activity over all flavonoid extracts at 10 mg/L. *C. bispinosa*, ( $R_f=0.11$ ) and *G. bicolar* ( $R_f=0.63$ ) have comparable antioxidant scavenging activity at concentrations between 25 and 50 mg/L, but *C. bispinosa* ( $R_f=0.11$ ) then surpasses *G. bicolar* ( $R_f=0.63$ ) from concentrations greater than 50 up to 200 mg/L. *C. bispinosa* ( $R_f=0.38$ ) and *G. bicolar* ( $R_f=0.63$ ) also demonstrate comparable antioxidant activity at concentrations of between 45 and 55 mg/L, but *C. bispinosa* revealed better antioxidant activity in concentrations in excess of 55 up to 200 mg/L.

### DPPH radical scavenging assay

Figure 3 shows plots of percentage DPPH antiradical activity of flavonoid extracts. Quercetin was used as a positive reference. The results show that percentage inhibition varies linearly with concentration from

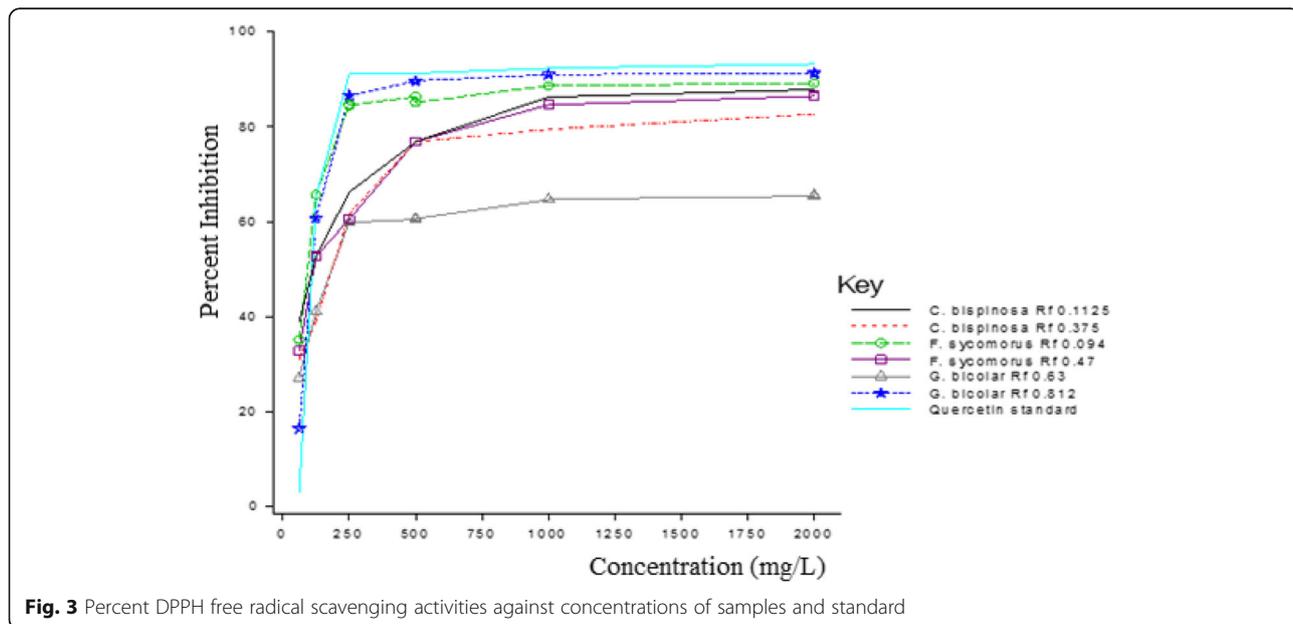


**Fig. 2** Plots of percentage antiradical scavenging activity against concentration of flavonoid extracted and standards

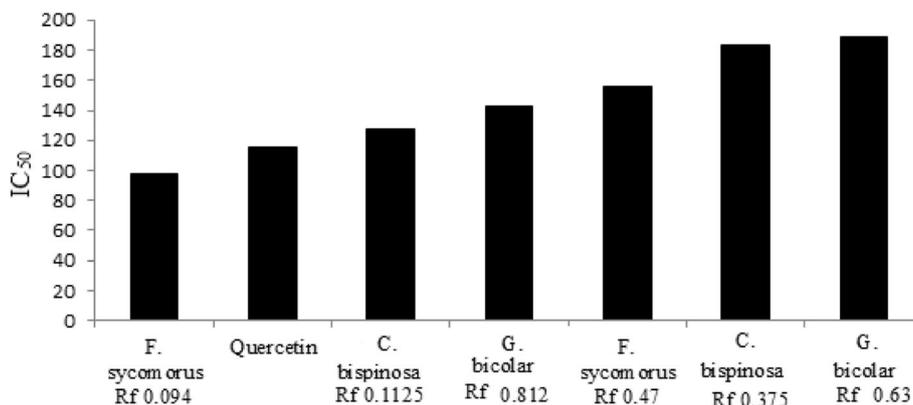
0 to 250 mg/L. The results in the linear range were statistically analyzed in order to determine the  $IC_{50}$  values for the sample and standard. The  $IC_{50}$  values were used to reflect antiradical scavenging ability. Basically,  $IC_{50}$  is the capability of an antioxidant and necessary concentration to reach 50% of scavenging DPPH free radical scavenging capability. This means that a small  $IC_{50}$  value means higher free radical scavenging capability while large  $IC_{50}$  means relatively low scavenging capability. The  $IC_{50}$  values were concentration dependent and varied according to the sample. Figure 4 is a graphical representation of how  $IC_{50}$  values varied with the sample.

**Discussions**

The study investigated antioxidant activity of flavonoids in three wild fruits *C. bispinosa*, *F. sycomotorus*, and *G. bicolor fruits*. Results from analytical TLC shows that each fruit consist of two different types of flavonoids. Sample bands showed orange-yellow, yellow-green, and dirty blue colors under UV light (Fig. 1) indicating the presence of flavonoids in the fruit extract. The orange-yellow is indicative of the presence of flavonol glycosides [11, 12]. The vivid yellow-green (brown) color maybe due to presence of flavone glycoside biflavonols and unusually substituted flavones [12]. The blue bands could be due to the presence of 5-deoxyisoflavones and



**Fig. 3** Percent DPPH free radical scavenging activities against concentrations of samples and standard



**Fig. 4** Plot of  $IC_{50}$  values versus flavonoid sample bands for the three fruits

7, 8-dihydroxy-flavanones [12, 13]. Furthermore, the blue bands could be due to the presence of anthocyanidins-3-glycosides. The blue band is mostly due to anthocyanidins-3, 5-diglycosides [12]. In the current study, a total of two flavonoid spots were discovered per fruit extract and none of these spots had similar retention values suggesting different flavonoid types (Fig. 1). The results show that *G. bicolar* contained flavonoids at  $R_f$  values of 0.63 and 0.81; *C. bispinosa* contained flavonoids at  $R_f$  values of 0.11 and 0.38; and the third fruit, *F. sycomorus*, had flavonoids at  $R_f$  values of 0.094 and 0.47. *G. bicolar* ( $R_f = 0.812$ ), maybe related in structure to flavonols, especially quercetin 3-O-rutinoside, whose mean  $R_f$  value is quoted as 0.9 [12, 13]. Crude fruit extracts of *F. sycomorus* showed antimicrobial activity in previous studies [14]. In folk role medicine, *F. sycomorus* is used as herbal medicine to treat fungal infections, jaundice, dysentery, cough, diarrhea, skin infection, stomach disorders, liver disease, epilepsy, tuberculosis, lactation disorders, helminthiasis, infertility, and sterility [15, 16].

The identified flavonoids were separated by preparative TLC and their antioxidant activity determined using the lipophilic and hydrophilic model radicals DPPH and ABTS respectively.

From the ABTS radical assay, quercetin standard has an antioxidant activity that was significantly greater than catechin and all samples at 200 mg/L concentration (ANOVA;  $p = 0.01$ ). On the other hand, the flavonoid at  $R_f$  value of 0.81 in *G. bicolar* has an ABTS $\cdot^+$  scavenging activity greater than catechin at concentration of 200 mg/L. The order of radical scavenging activity for the 200-mg/L samples is *G. bicolar*  $R_f$  (0.81) > *C. bispinosa*  $R_f$  (0.11) > *F. sycomorus*  $R_f$  (0.094) > *F. sycomorus*  $R_f$  (0.047) > *C. bispinosa*  $R_f$  (0.38) > *G. bicolar*  $R_f$  (0.63). All the bands gave antioxidant activity greater than 80%. The linear regressions analyses showed perfect positive correlation between percentage inhibition

and concentration, in the concentration range studied ( $R^2 = 0.99 \pm 0.002$ ).

DPPH scavenging assay revealed that the antiradical scavenging order was *F. sycomorus* ( $R_f = 0.094$ ,  $IC_{50} = 98.13$ ) > *C. bispinosa* ( $R_f = 0.11$ ,  $IC_{50} = 127.50$ ) > *G. bicolar* ( $R_f = 0.81$ ,  $IC_{50} = 142.94$ ) > *F. sycomorus* ( $R_f = 0.47$ ,  $IC_{50} = 155.83$ ) > *C. bispinosa* ( $R_f = 0.38$ ,  $IC_{50} = 183.38$ ) > *G. bicolar* ( $R_f = 0.63$ ,  $IC_{50} = 188.5$ ). The seeming discord in the antiradical scavenging ordering, between ABTS and DPPH assays, can be explained by observing that at very low concentration *F. sycomorus* ( $R_f = 0.09$ ) has better antioxidant activity than both *G. bicolar* ( $R_f = 0.81$ ) and *C. bispinosa* ( $R_f = 0.11$ ), in the ABTS assay at concentrations lower than 10 mg/L. The differences in antioxidant activity of samples suggest that the flavonoids in the studied samples were of a different nature. According to Re et al. [17], suppressed antioxidant activity may be due to glycosylation of the 3-OH and at positions 4' and 7-OH. Use of at least two antioxidant scavenging assays which complement each other reduces bias and helps to make quality deductions [18]. The protocols of the present study did not look at the actual structures of the flavonoids isolated from the fruits. Thus, the study needs to be extended in future to concentrate on the full structural elucidation of the flavonoids so that the differences in antioxidant activity observed in the present study can be better understood.

## Conclusion

This research has shown that *C. bispinosa*, *F. sycomorus*, and *G. bicolar* contain different flavonoid types with significant antioxidant activity of over 80% at a concentration of 200 mg/L. The fruits contain flavonoids that produced significant antioxidant activity comparable to that of quercetin and catechin. Hence, *F. sycomorus*, *C. bispinosa*, and *G. bicolar* can be used as a source of natural antioxidants which can be used as nutraceuticals to promote health, as preservatives to delay peroxidation of foods, and as flavoring for packed foods.

**Abbreviations**

ABTS: 2, 2-Azinobis 3-ethyl benzthioazoline-6-sulphonic acid; DPPH: 1, 1-Diphenyl-2-picryl-hydrazyl; HLB: Hydrophobic-lipophilic balanced; IC<sub>50</sub>: Half maximal free radical inhibitory concentration; TLC: Thin-layer chromatography

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**Availability of data and materials**

All the generated and analyzed data are included in this published article.

**Authors' contributions**

PD and MM collected the samples and performed the experimental work and statistical analysis. PD and LG managed the literature and wrote the first and final draft. All the authors read and approved the final draft.

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Competing interests**

The authors declare that they have no competing interests.

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