Published online in http://ijam.co.in ISSN No: 0976-5921

International Journal of Ayurvedic Medicine, Vol 14 (4), 2023; 945-951

Pharmacognostic Evaluation and Quantification of Quercetin in Muntingia Calabura Leaves and Fruits Using HPLC Method

Research Article

Soumya Sanikop[1], Kalpana Patil[1]*, Sachin Gudasi[1], Sathgowda Patil[2]

1. Department of Pharmacognosy, 2. Department of Pharmacology,
KLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi. Karnataka. India.

Abstract

Objective: To evaluate the pharmacognostic character of *Muntingia Calabura* and estimation of quercetin in flavonoid rich leaves and fruit fractions. Methods: Pharmacognostic studies were carried out in terms of morphological, microscopic characters, physicochemical parameters and HPLC fingerprint analysis of leaf and fruit extracts of *Muntingia calabura* samples using standard methods. Further, the extracts were used to analyze total phenol, flavonoid contents and estimated antioxidant activities using DPPH. Results: Fruit size. and color of *Muntingia calabura* were the distinguishing morphological characters observed in the present study. The detailed powder microscopy of leaf confirmed the presence of fibers, trichomes, vascular bundle and xylem vessels. Fruits with specified epicarp, reticulate xylem vessels, starch grains, endosperm, oil globules and parenchymatous tissue. Physicochemical parameters like ash values, and extractive values were determined. Preliminary phytochemical screening showed the presence of alkaloids, glycosides, steroids, carbohydrates and flavonoids. Using HPLC profiling, standard quercetin was found to have a retention time of 7.313 while flavonoid-rich leaf and fruit had retention times of 7.245 and 7.210, respectively. Conclusion: Based on the results of the research, it was discovered that *Muntingia calabura*, fruit and leaves contain a variety of secondary metabolites with strong antioxidant ability. The morphological and histological traits of *Muntingia calabura*, leaves and fruit can be witnessed under a microscope. Using HPLC profiling for quantification of quercetin in flavonoid rich fraction of leaves and fruits. The quantity of quercetin in the sample was found to be 4.231µg/ml for leaves fraction and 1.953µg/ml for fruit fraction. Methanol:0.1% Formic acid in ratio of 70:30 was selected as optimized mobile phase. The retention time was found to be 7.31 (Quercetin), 7.24 (leaves fraction) and 7.21 min (fruits fraction) respectively. This method offers a reliable and accurate means of quantifying quercetin in plant samples.

Keywords: *Muntingia Calabura,* Powder microscopy, Physico-chemical analysis, Quercetin, HPLC.

Introduction

Synthetic drugs or modern medicine has been used widely for the treatment of various diseases (1,2); however, this system of medicine comes with side effects and are not economical and thus people have started to give interest in herbal medicine/ green medicine since they are available easily and are economical and they have very minimal adverse effects or no harmful effects on the human body (3). Indians have been using herbal medicine to cure and treat several diseases and conditions and have a decade-old history with healing systems like Siddha, Unani & Ayurveda(4).

Since these herbal plants are used as medicine the purity and quality play a crucial role, since medicinal plants are high in demand the availability is decreased and this results in either adulteration or substitution. The majority of the time the active medicinal properties from these plants are removed and are sold without the main phytoconstituents(5). The therapeutic result and efficacy of an herbal plant are due to the presence of active phytochemical constituents present in them, the adulteration and mixing of similar-looking parts of plants will ultimately result in a decrease or no therapeutic. Value (6,7).

Along with the adulteration wrong identification of the particular plant is also a common error and thus standardization and pharmacognostic. studies of plants such as identification of different phytoconstituents, anatomical, morphological studies play a crucial role(8). Due to the presence of various phyto-constituents standardization of a particular medicinal plant becomes a complex task. However, the microscopic and macroscopic of the plant is the initial. step in determining the purity of a plant. Chromatographic techniques such as HPTLC. & HPLC. are also routinely involved in establishing the quality of herbal plants (9,10).

According to the WHO estimation around 80% of the populations belonging to developing countries. use herbal medicine/ green medicine for the treating several diseases and conditions(11,12). WHO define traditional medicine as “The health practices, approaches,

*** Corresponding Author:**
Kalpana Patil
Professor and Head, Department of Pharmacognosy, KLE College of Pharmacy, KLE Academy of Higher Education and Research, Nehru Nagar, 590010, Belagavi, Karnataka, India.
Email Id: kalpanapatil@klepharm.edu

knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination, to treat, diagnose and prevent illness or maintain well-being (13).

Muntingia calabura is commonly known as. Jamaican cherry & Japanese cherry belongs to the family of Muntingiaceae. It is grown majorly in Asian countries in warm. areas and is also cultivated as road side. tree in Malaysia. According to the previous literatures the plant is used as traditional plants for treating limited human illness. The leaves of the plants are boiled and are used.to treat the ulcers of gastric region, reduce swelling of glands of prostate, while the flowers and bark are used as antiseptic. In countries like Mexico the plant is used to treat ulcers of mouth and measles, whereas it is used to reduce stomach pain and treat cold in country Philippine. Apart from being used for treating several human ailments, the plant is used to make tea, jam and eaten raw as fruit (14,15).

According to the literature survey several studies have been carried out on the *Muntingia calabura*, and it tends to possess anti-inflammatory, anti-pyretic, anti-diabetic, antibacterial, antiulcer, antinociceptive and antioxidant activity(16).

Materials and methods

Plant collection & authentication

Muntingia calabura L. plant leaves and fruits were collected from July to August 2020 from natural habitats from the surrounding Belagavi, Karnataka, India. The plant was subjected for Authentication at the Indian Council of Medical Research Belagavi, Karnataka, India by the botanist Dr. Harsha Hegde with specimen no: RMRC-1586.

Chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH), was purchased from Sigma, (Sigma Aldrich GmbH, Steinheim, Germany), HCL, phloroglucinol, Glycerin, petroleum ether, ethanol, chloroform was purchased from fisher scientific and other reagents like Methanol HPLC graded was purchased from S D fine chem limited.

Macroscopic evaluation of *Muntingia calabura* leaves & fruits (17)

The leaves and fruits of *Muntingia calabura* were exposed to organoleptic assessment. Leaves and fruits were investigated for their morphological characteristics. Morphological parameters such as tone, smell, size, shape, and taste were concentrated with help of sensory organs.

Microscopic evaluation of *Muntingia calabura* leaves & fruits (18)

The transverse section of leaves and fruits of *Muntingia calabura* was examined with help of a microscope (trinocular microscope). Standard histological examinations were also performed on the crude powdered plant material. The thin segment of leaf and fruit of *Muntingia calabura* was dissected with a sharp edge and kept in phloroglucinol and HCl for 1-2min. The thin segment of leaf and fruit of *Muntingia calabura* transferred on a clean and dry glass slide with help of a brush.2-3 drops of glycerin water were placed on it and covered with a coverslip. Then observed under the trinocular microscope.

Physicochemical evaluation

Physicochemical parameters like percentage of loss on drying (LOD), acid insoluble ash, total ash and water-soluble ash were examined as per the guidance of Indian Pharmacopeia.

Preparation of Extract

Hydroalcoholic extraction of leaves and fruits were done by maceration process using water and ethanol as solvent. The extraction process was the same for both leaves and fruits of *Muntingia calabura.* The collected leaves and fruits were dried at room temperature. Dried fruits and leaves were coarsely powdered and subjected to a maceration process for 5 days. Then the mixture from a maceration was filtered and the filtrate was concentrated under vacuum using Rotary Evaporator the obtained concentrated extract was dried and stored in an airtight container."

Preparation of Flavonoid Fractionation (19)

By using the Cos et al. method, the extract of *Muntingia calabura*'s leaves and fruits was fractionated. Dichloromethane and 5% w/v citric acid were mixed in a 1:1 ratio to dissolve the hydroalcoholic extract. A separating funnel was used to separate the two layers, and the separated layers were then concentrated in a Rotary Evaporator under vacuum. 90 percent Methanol and 1:1 petroleum ether was used to dissolve the dichloromethane layer. With the use of a separating funnel, both layers were separated. Then fractions 1 and 2 were found. (Fraction 1 was the petroleum ether layer, which includes wax and lipids. The methanol layer in fraction 2 comprises phenolics, terpenes, and sterols. Add 10% ammonium hydroxide to the concentrated aqueous layer to maintained pH at 9. Dichloromethane was mixed with aqueous layer then obtained fraction 3 and fraction 4 (Fraction 3 was dichloromethane it contains alkaloids, Fraction 4 was aqueous layer it contains salts, flavonoids).

Preliminary phytochemical analysis (20)

Preliminary phytochemical screening of the extract for the determination of the presence of secondary metabolites like Flavonoids, Alkaloids, Saponins, Triterpenoids, Steroids, Tannins, Glycosides and Phenolics by standard methods.

Quantification of Quercetin in flavonoid rich fraction of leaves and fruits by using HPLC technique (21,22)

Analysis of marker compound by UV-Spectroscopy

UV Shimadzu 1800u UV-Visible double beam spectrophotometer equipped with matched 10mm quartz cuvettes. AR grade Methanol was used in. the study.

Stock solution preparation

A stock solution of Standard Quercetin was prepared by dissolving10 mg standard Quercetin in 10ml of methanol to obtained y1000µg/ml (primary stock solution). 1ml from the primary stock solution was diluted in. 10ml of methanol to obtain 100µg/ml secondary stock solution. Similarly, both flavonoid rich fraction of leaves and fruits were prepared.

Preparation of working standard

From the above primary stock solution of standard Quercetin, serial dilutions of 2,4,6,8 and 10 µg/ml were prepared, to get the standard calibration curve of standard Quercetin.

High-performance liquid chromatography (HPLC)

Chromatographic conditions

HPLC (Agilent technologies1220 Infinity II LC), manual sampler, Standard Quercetin were analyzed using a UV detector. The information gathered on the Open lab solution administrator system and ZORB AX – C18(2) Column (250 mm × 4.6 mm, 5µm) was used. The volume of injection was 10µl at room temperature and the flow rate was 1min/ml. The samples were identified at 370 nm with a duration of 10 minutes and the mobile phase (Methanol**:**0.1% Formic acid in the ratio of 70:30) was filtered through a 0.45m Millipore membrane nylon filter using a solvent filtering device.

Stock solution preparation

A stock solution of Standard Quercetin was prepared by dissolving 10 mg standard Quercetin in 10ml of methanol to obtained 1000µg/ml (primary stock solution) 1ml from the primary stock solution was diluted in 10ml of methanol to obtain 100µg/ml secondary stock solution. Similarly, a fraction of leaves and fruits were prepared.

Preparation of working standard

From the above primary stock solution of standard Quercetin, serial dilutions of 5, 10, 20, 40 and 80 µg/ml were prepared, to get the standard calibration curve of standard Quercetin.

Antioxidant activity by DPPH scavenging technique(23)

The antioxidant activity is performed using the UV-Spectroscopy method and the reagent DPPH (2,2-diphenyl-1-picrylhydrazyl). The hydroalcoholic extract of Muntingia calabura was produced as a stock solution at a concentration of 1 mg/mL. 4mL of DPPH was added to 1mL of test sample. concentrations of varied strengths, control was prepared without sample. Then the absorbance was measured at 517nm. Vitamin C was used as standard.

The percentage scavenging was calculated using the formula.

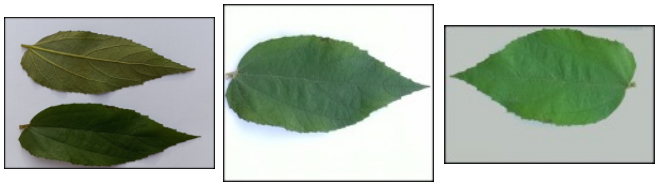
[(Control-Test)/Control] x 100.

Results and Discussion

Macroscopic evaluation of *Muntingia calabura* leaves

Macroscopic evaluation of *Muntingia calabura* leaves was carried out. upper surface of leaves was dark green and lower surface light green in color, alternative, Asymmetric petiole, Lanceolate, and Compound. The size of leaves was 4-15cm long and 1-6cm in width with a toothed margin. Leaves were covered with short hair. Elliptical shape, oblique base, acuminate apex was present. (Figure no.1)

Figure 1: Macroscopic evaluation of Muntingia calabura leaves



Macroscopic evaluation of *Muntingia calabura* fruits

Macroscopic evaluation of *Muntingia calabura* fruit was studied. Color of fruit was red and fruity smell. Taste was sweet. The size of fruit was small about 1-2cm length,1.5cmwide and round shape with soft texture. (Figure no.2).

Figure 2: Macroscopic evaluation of *Muntingia calabura* fruits

Microscopic evaluation of *Muntingia calabura* leaves

The midrib's transverse section was confirming the presence of the upper and lower epidermis. Collenchyma's were present underneath the upper epidermis and above the lower epidermis. The xylem was in the center, while the phloem was on the periphery. vascular bundle, Fibers, Trichomes were present. Stomata was present in epidermis. (Figure 3) and for fruit (figure 4).

Figure 3: Microscopic evaluation of *Muntingia calabura* leaves

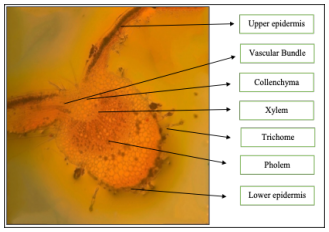
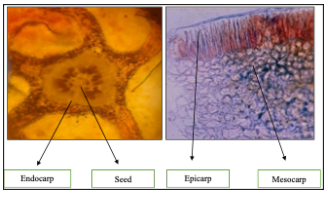


Figure 4: Microscopic evaluation of *Muntingia calabura* fruits



Powder microscopy of leaves and fruits

Powdered leaves and fruits of *Muntingia calabura* under microscopic investigation was confirming the presence of fibers, trichomes, vascular bundle, Xylem vessel parenchymatous tissue. (Figure 5 & 6)

Figure 5: Powder microscopy of leaves

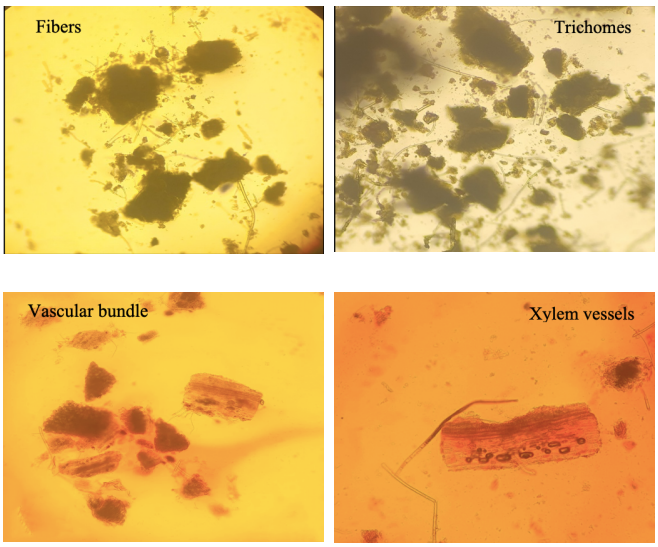
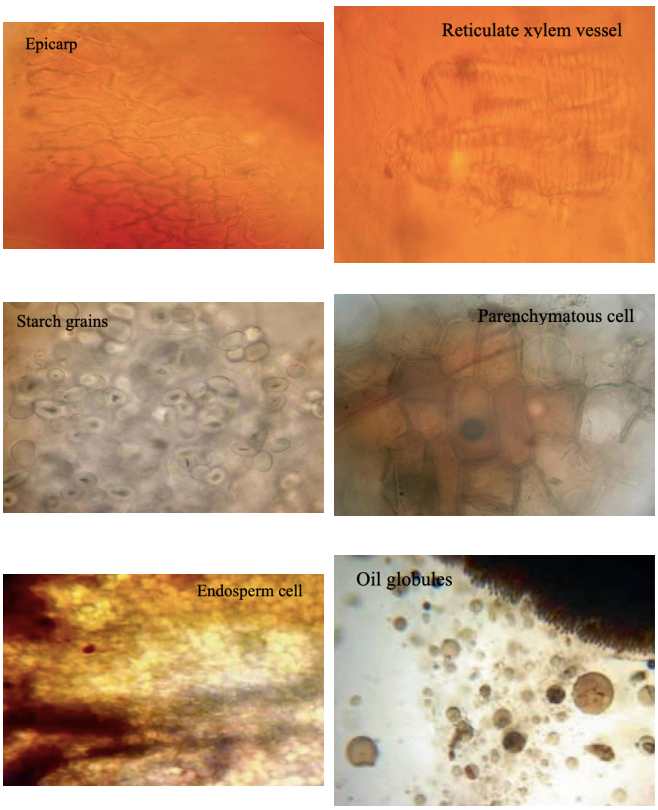


Figure 6: Powder microscopy of fruits



Physicochemical evaluation of leaves and fruits

Muntingia calabura leaves and fruits were subjected to "Physico-chemical parameters such as total ash, acid-insoluble ash, water-soluble ash, water-soluble extractive value, Alcohol soluble extractive value, and loss on drying" to examine the purity and quality of the drug by standard procedure. (Table no.1).

Table 1: Physicochemical evaluation of leaves and fruits of *Muntingia calabura*

Parameters	Values (%w/w) leaves	Values (%w/w) fruits
Total ash	9.5± 0.63	5.5± 0.86
Acid Insoluble ash	9± 0.93	0.5± 0.34
Water soluble ash	3.5± 1.12	2.5± 0.68
Alcohol soluble extractive	15.2± 0.74	36± 0.43
Water soluble extractive	17.6± 0.27	48.8± 0.89
Loss on drying	3.2±0.58	4.8±0.38

Preliminary phytochemical analysis

Phytochemical investigation ethanolic extract: by carrying out the different chemical test for the particular class of compounds it shows the presence of Steroids, Alkaloids, Flavonoids, Tannins, Triterpenoids, and Carbohydrates. (Table no.2)

Table 2: Preliminary phytochemical screening results of *Muntingia calabura*

Metabolites	M C Leaves	Metabolites	M C Fruit
Carbohydrates	+	Carbohydrates	+
Alkaloids	+	Alkaloids	+
Flavonoids	+	Flavonoids	-
Tannins	+	Tannins	+
Steroids	+	Steroids	+
Glycosides	+	Glycosides	-
Phenols	+	Phenols	+

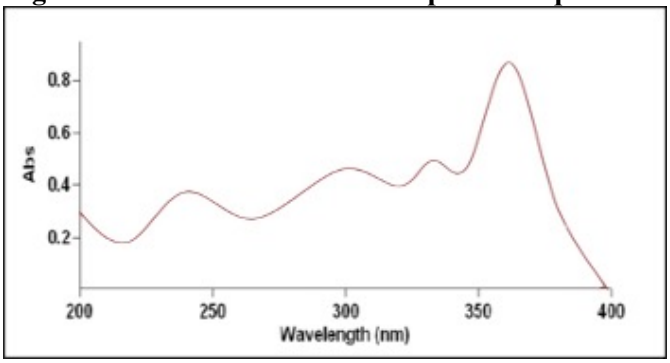
(+): the presence; (-): the absence

Quantification of Quercetin in flavonoid rich fraction of leaves and fruits by using HPLC technique:

Development and optimization of the method

The quantification of quercetin was accomplished using a new, reliable, and eco-friendly RP-HPLC technique. The UV spectra of marker in methanolic solution (100 µg/mL) were tested in the 200-400 nm range to determine the optimal wavelength. Quercetin has the maximum UV absorption at 370 nm, respectively. (Figure no.7)

Figure 7: Maximum absorbance spectra of quercetin



Flavonoid rich fraction of leaves, fruits and standard quercetin were analyzed with the below mentioned chromatographic conditions. (Table no.3).

Table 3: Chromatographic conditions

Test condition	Results
Elution	Isocratic
Detection wavelength	370nm
Mobile phase	Methanol:0.1%Formic acid (70:30)
Column	Agilent 5TC-C18(2)250*4.6nm
Retention time	7.3 min (quercetin), 7.2 min (leaves & fruits)
Flow rate	1ml/min
Run time	10min

HPLC technique is considered as convenient and sensitive method for the analysis of the natural compound. When the standard quercetin solution was injected five times, the peak's retention period remained constant. The standard quercetin's nature and solubility indicate that the mobile phase and stationary phase chosen were ideal. Methanol**:**0.1% Formic acid in ratio of 70:30 was selected as optimized mobile phase. The retention time was found to be 7.31 (Quercetin), 7.24 (leaves fraction) and 7.21 min (fruits fraction) respectively. It was evident from Fig. 8, 9 and 10. The standard Quercetin and both flavonoid fractions showed excellent peak characteristics with minimal tailing.

Figure 8: Chromatogram of Standard quercetin 10 µg/ml at 370nm

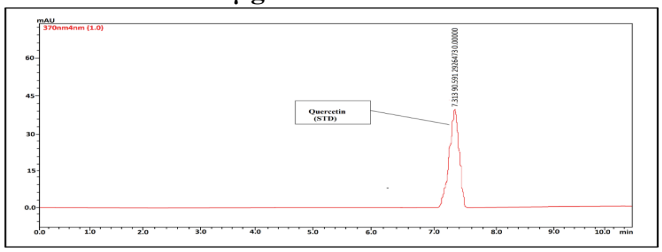


Figure 9: Chromatogram of flavonoid rich fraction of leaves 10 µg/ml at 370nm

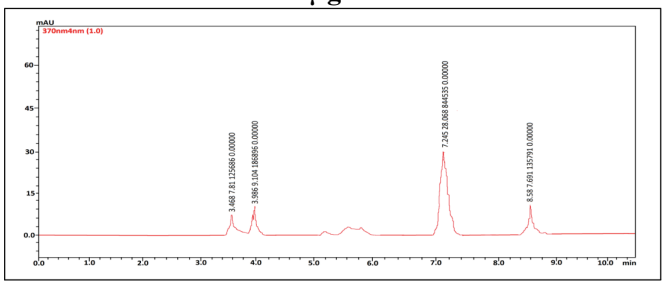
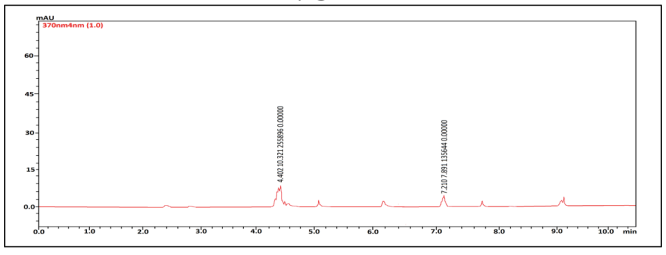


Figure 10: Chromatogram of flavonoid rich fraction of fruit10 µg/ml at 370nm



Quantification of standard quercetin in flavonoid rich Fraction of leaves and fruits

The developed HPLC technique was utilised to quantify quercetin in flavonoid rich Fraction of leaves and fruits. 1mg/ml of sample was prepared. A peak area of 844535 (leaves fraction) and 135644 (fruits fraction) was obtained after injecting 10 µl of the above-mentioned solution for standard marker estimation. The quantity of quercetin in the sample was estimated using the linearity equation, and it was found to be 4.231 µg/ml for the leaves fraction and 1.953 µg/ml for the fruit fraction (Table 4).

Table 4: Quantification data of quercetin in flavonoid rich leaves and fruit fractions

Samples	Retention time	Peak area	Amount estimated
Standard Quercetin	7.313 min	2926473	10 µg/ml
Flavonoid rich fraction of leaves	7.245 min	844535	4.231 µg/ml
Flavonoid rich fraction of fruits	7.210 min	135644	1.953 µg/ml

Antioxidant activity by DPPH scavenging technique

In the present study, the different concentrations the flavonoid rich fraction of leaves and fruits of *Muntingia calabura* were evaluated for their antioxidant activity. The antioxidant capacity of the Flavonoid rich leaves and fruit fraction were found to be 309µg/ml and 436.76µg/ml was compared with Ascorbic acid IC_{50} value at 173.99 µg/mL as the standard antioxidant. (Table no.5 and Figure 11,12).

Table 5: Antioxidant activity of samples

Name of compound	IC_{50} µg/ml.
Ascorbic acid	173.99
Flavonoid rich leaf fraction	309.82
Flavonoid rich fruit fraction	436.76

Figure 11: DPPH free radical scavenging activity of flavonoid rich leaf fraction

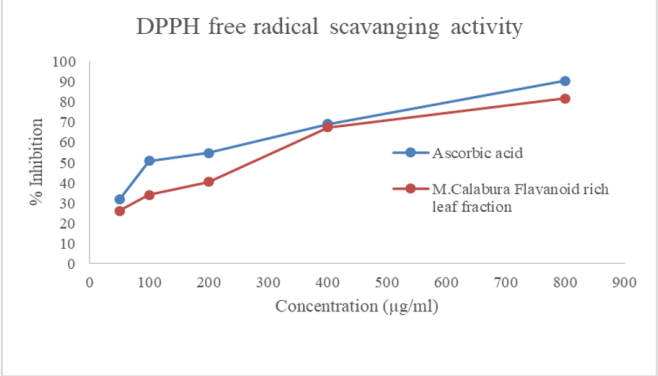
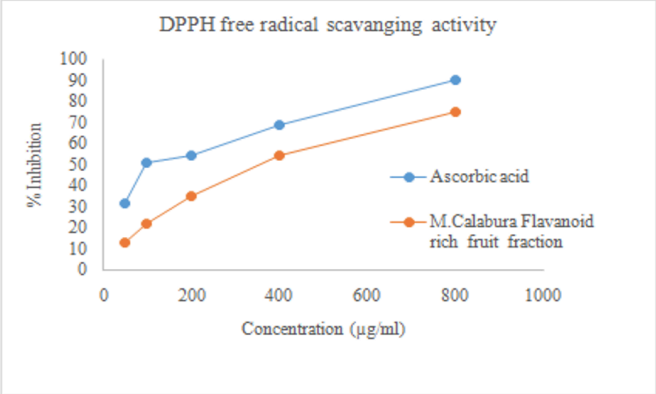


Figure 12: DPPH free radical scavenging activity of flavonoid rich fruit fraction



Conclusion

The micromorphology, phytochemical analysis, and HPLC analysis of the leaf and fruit of *Muntingia calabura*'s was carried out. Some of the pharmacognostic characteristics that are crucial for determining the correct species of the plant and for differentiating between closely related species of the same genus include the determination. of cell structural organisation and examination of the tissues system. The ethanolic extracts of *Muntingia calabura*'s leaves and fruits underwent a preliminary phytochemical investigation, which revealed the existence of a number of secondary metabolites. In addition, HPLC profiling revealed that whereas flavonoid-rich leaf and fruit had retention durations of 7.245 and 7.210, respectively, standard quercetin had a retention time of 7.313.

Declaration of Competing Interest: The authors declare no conflict of interest.

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