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Phytochemical and Antioxidant Studies on Leaf Extracts of *Muntingia calabura* L.

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Abstract
Medicinal plants act as a storehouse of ingredients which can be exploited for its therapeutic values. Hence, they serve as an asset for pharmaceutical industries. The present investigation aims to describe the qualitative and quantitative phytochemical analysis and *in vitro* antioxidant activity of *Muntingia calabura* L. leaf extracts. The phytochemical analysis revealed the presence of phenolics, flavonoids, steroids, terpenoids and alkaloids in EEMC, MEMC and AEMC. The total phenolic and flavonoid content was found to be higher in MEMC. The antioxidant activity of various leaf extracts of *Muntingia calabura* L. was determined mainly by DPPH, $ABTS^+$ and FRAP assays. In all the three antioxidant assays MEMC exhibited a greater potential to scavenge free radicals at its lower concentration. *Muntingia calabura* L. acts as an excellent source of biomolecules with highest free radical scavenging potential and hence very effective against oxidative stress related disorders.

Keywords
Muntingia calabura; Antioxidant; Phytochemicals; Free radical.

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1. INTRODUCTION
Plant based traditional system of medicine still provides health benefits for the mankind (1). Active pharmaceutical compounds isolated from plants possessing significant medicinal properties can be used in drug development and synthesis (2). Plant offers a good source of nutrients in spite of its therapeutic values (3). Plants played a significant role in human culture development worldwide. Since historical times, people use medicinal plants very frequently for various ailments. Phytomedicine are safe because they are either in combined or pooled form of more than one molecule.
Phytochemicals are naturally occurring; biologically active chemical compounds present in plants which provide health benefits for humankind (4). Many known and unknown phytochemicals are found in plants which are meant to protect the plant from disease and damage. Phytochemicals can offer a protective role against various human diseases (5). Phytochemicals are not an inevitable component for the sustenance of human life but have an importance in disease prevention. The most common cause of many diseases is the oxidative cell damage by free radicals (6). Oxidative stress related diseases are very common now a day. So, it is very essential to neutralize the free radicals generated by reactive oxygen species. Antioxidants can reduce the oxidative stress in cells by scavenging the free radicals (7). Plants acts as reservoir of natural antioxidants (8). It is very much essential to understand the biomolecules from plants that are

responsible for disease prevention and its antioxidant efficacy. *Muntingia calabura* L. is a medium sized evergreen tree native to American continent and is locally known as Jamaican cherry tree (9). In Peruvian folklore medicine, it is documented that *M.calabura* L. possesses so many medicinal values (10). The current study aimed to report the phytochemical composition of *Muntingia calabura* L. leaf samples and also to evaluate its antioxidant efficacy.

2. MATERIALS AND METHODS

2.1. Plant material

Muntingia calabura L. leaf samples were collected during March – April 2018 from kottayam district, Kerala, India. The plant material was authenticated by a taxonomist and the specimen is maintained in the institute.

2.2. Preparation of various extracts.

Plant leaf powder (40g) was extracted with 400 mL of different solvents with increasing polarities in soxhlet apparatus. The solvents used are petroleum ether, chloroform, ethyl acetate, methanol and water. The solvent was evaporated using a rotary evaporator and obtained the dried petroleum ether extract (PEMC), chloroform extract (CEMC), ethyl acetate extract (EEMC), Methanolic extract (MEMC)and aqueous extracts (AEMC).

2.3. Qualitative phytochemical analysis

The qualitative phytochemical analysis of various extracts of *Muntingia calabura* L. leaf samples were carried out by the method described by Evans (11).

2.4. Quantitative phytochemical analysis

2.4.1. Determination of total phenolics

The EEMC, MEMC and AEMC were taken to quantify the total phenolic content by the method described by Singleton and Rossi with slight modifications (12).To the 100 ml crude extract (1 mg/ml) add 0.2 mlof Folin-Ciocalteu reagent, 2 ml distilled water and 1 ml 15% Na_2CO_3. This reagent mixture was taken for measuring its absorbance at 765 nm using UV-Visible spectrophotometer (T60U, PG Instruments Limited, UK) after an incubation period of 2 h at room temperature. The values obtained were compared with Gallic acid standard and were expressed in mg equivalents of Gallic acid (mg GAE) per g dry weight of extract.

2.4.2. Determination of total flavonoids

Total flavonoid content of EEMC, MEMC and AEMC was determined using quercetin as standard by the procedure followed by Chang et al with minute modifications (13). A calibration curve of quercetin was prepared in the range of 10-100 µg/ml. Add 10% aluminum chloride (0.1 ml), 1 M potassium acetate (0.1 ml), 80% methanol (1.5 ml) and distilled water (2.8 ml) to the extract (0.5 ml) and standard (0.5 ml) kept in different test tubes and shaken well. A blank was also prepared in the same manner but the difference is that 0.5 ml of distilled water was used instead of the sample or standard and the aluminum chloride used in the assay was replaced by distilled water. All tubes were kept for an incubation period of 30 min at room temperature. Using UV-Visible spectrophotometer, the absorbance was read at 415 nm. The flavonoid concentration was expressed in mg quercetin equivalent (QE) per gram of extract.

2.5. *In vitro* antioxidant assays

2.5.1 DPPH radical scavenging assay

The DPPH radical scavenging assay was done by the method adopted by Sanchez-Moreno et al (14). 10 ml of test sample at different concentrations (0-200 µg/mL) were added to 190 ml DPPH (150 mM) which was prepared in methanol solution. The solutions were shaken well and kept at 37^0Cfor 20 min. The solvent used for the assay was taken as blank.Due to the quenching of DPPH free radicals there will be a decrease in absorbance of test samples. Absorbance at 517 nm using UV-Visible spectrophotometer was documented. Inhibition percentage was calculated as an indication of radical scavenging activity.

2.5.2. ABTS cation free radical-scavenging activity

Free radical scavenging potential of *Muntingia calabura* leaf extracts by means of $ABTS^+$ radical scavenging activity was evaluated by the method followed by Re et al (15), with minor modifications. ABTS is a colourless compound which is transformed into its blue–green counterpart $ABTS^+$ by losing an electron by nitrogen molecule on the parent compound. A stock solution of ABTS mixed with 2.45 mM potassium persulfate and allows the mixture to stand in the dark at room temperature for 12–16 h which generates $ABTS^+$. Different concentrations of *Muntingia calabura* leaf extracts (1.562–100 µg/ml) were taken to react with 180 µl of $ABTS^+$ solution and kept for 12 min in dark at room temperature. The absorbance of the sample extracts was monitored at 734 nm.

2.5.3. FRAP Assay

FRAP assay was followed to estimate the reducing potential of *Muntingia calabura* by method described by Benzie and Strain (16). The FRAP assay depends on the principle that the ferricyanide (Fe^{3+}) got reduced in accordance with the antioxidant potential of the extract. 2.5ml of 10 mM TPTZ solution in 40 mM HCl, 2.5ml of 20 mM $Fecl_3$. $6H_2O$ and 25ml of 300 mM acetate buffer (pH 3.6) constitutes the FRAP reagent. To 30µl of the extract 900 µl FRAP reagent and 70 µl water were added. The reaction mixture was kept at 37 ^{0}C for a 30 min time period. The absorbance of the reaction mixture was taken at 593nm and IC_{50} values were calculated.

3. RESULTS

3.1. Qualitative phytochemical analysis

The phytochemical analysis showed the presence of various phytoconstituents like alkaloids, phenolics, flavonoids, steroids, terpenoids etc., in ethyl acetate, methanol and aqueous extracts (Table 1). Phytochemical screening revealed the presence of phenolics and flavonoids as its principal components.

Phytochemicals	PEMC	CEMC	EEMC	MEMC	AEMC
Alkaloids	++-	++-	++--	+--	---
Flavanoids	---	---	+++	+++	+++
Phenolics	---	++-	+++	+++	+++
Tannins	---	---	---	+++	+--
Cardiac glycosides	---	---	---	---	---
Triterpenoids	---	---	+--	++-	++-
Steroids	+--	+--	++-	++-	+++
Saponins	---	---	---	---	+++

Table 1. Qualitative phytochemical analysis of various extracts of *Muntingia calabura* leaf. Key words: **+** indicates presence; **-** indicates absence.

Sample	Total phenolic content (mg of gallic acid/g Dry weight of the sample)	Total flavonoid content (mg of quercetin/g Dry weight of the sample)
EEMC	51.27± 0.42	22.38± 0.12
MEMC	82.56± 0.25	31.45± 0.27
AEMC	11.23± 0.68	16.59± 0.15

Table 2. Quantitative phytochemical analysis *Muntingia calabura* leaf extracts

3.2. Quantitative phytochemical analysis

The ethyl acetate, methanol and aqueous extract were found to contain rich amount of bioactive molecules like phenolics and flavonoids. These phytochemical agents present in the ethyl acetate, methanol and aqueous extracts of *Muntingia calabura* leaf samples were quantified and was shown in Table 2. The total phenolic content of EEMC is 51.27± 0.42, MEMC is 82.56 ± 0.25 and AEMC is 11.23 ± 0.68 mg equivalents of gallic acid/ g dry weight of the sample. The total flavonoid content of EEMC is 22.38 ± 0.12, MEMC is 31.45 ± 0.27 and AEMC is 16.59 ± 0.15 expressed as mg equivalents of Quercetin/g dry weight of the sample.

3.3. *In vitro* antioxidant assays

In vitro antioxidant activities of various extracts were analyzed using three different assays. The MEMC exhibited its highest potential to scavenge free radicals at its lowest concentration in all the three antioxidant assays.

3.3.1. DPPH assay

The DPPH radical scavenging activities of various extracts of *Muntingia calabura* were studied and were shown in Figure 1. IC_{50} values of all the extracts were estimated and it was noted that all the extracts of *Muntingia calabura* exhibited a significant DPPH radical scavenging activity. Among all the extracts studied, MEMC exhibited highest DPPH radical quenching activity with an IC_{50} value of 76.43±0.01 µg/ml. The IC_{50} values of other extracts are EEMC having 97.99 ± 0.02 µg/ml**,** AEMC having 180.6 ± 0.08 µg/ml, CEMC having 361.1 ± 0.04 µg/ml, PEMC having 418.2 ± 0.06 µg/ml. The IC_{50} value of standard ascorbic acid was observed as 54.38 µg/ml.

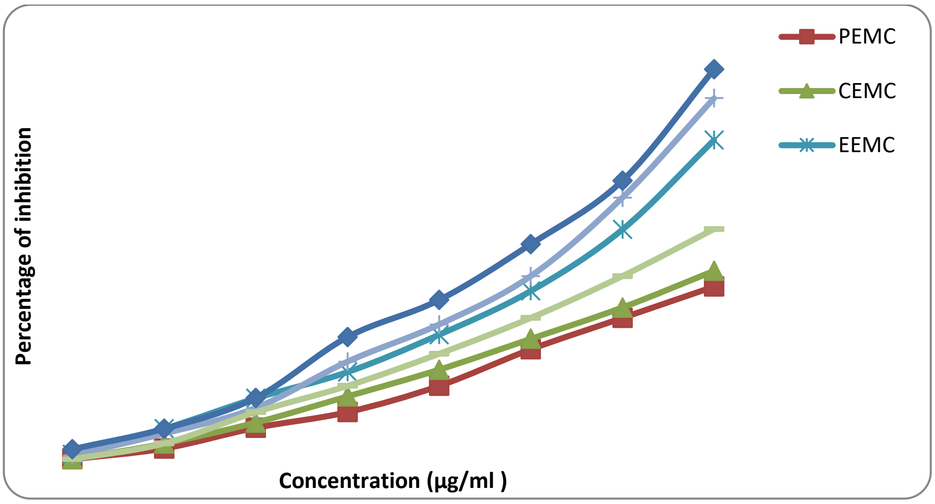

Figure 1. DPPH assay of various extracts of *Muntingia calabura* leaf

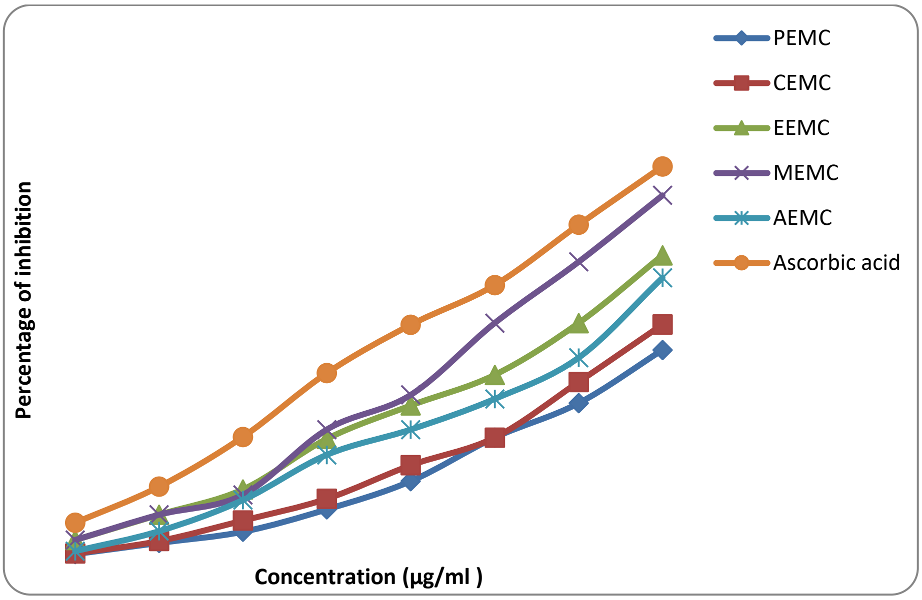

Figure 2. ABTS $^{+}$assay of various extracts of *Muntingia calabura* leaf

3.3.2. ABTS^{+} assay

ABTS assay relies on the principle that the measure of extracts needed to quench 50% of ABTS$^{\cdot+}$ radicals by electron donation. The IC_{50} value of MEMC is found to be 61.67±0.03 µg/ml in ABTS assay which showed highest activity than the other extracts. The IC_{50} value of EEMC was 113.07 ± 0.08 µg/ml, AEMC was 152.09 ± 0.07 µg/ml, CEMC was 314.30 ± 0.07 µg/ml, and PEMC was 396.01 ± 0.03 µg/ml, which also showed a potential to scavenge ABTS$^{\cdot+}$ radicals against the standard ascorbic acid with the IC_{50} value 37.45 µg/ml.

3.3.3. FRAP assay

FRAP assay is one of the powerful *in vitro* antioxidant assays which focuses on the reduction of ferric iron (III) into ferrous iron (II). Among all the leaf extracts of *Muntingia calabura* MEMC showed a maximum activity in FRAP assay and its IC_{50} value was found to be 82.42±0.03 µg/ml (Figure 3). The IC_{50} values of other extracts were 127.11 ± 0.02 µg/ml (EEMC), 132.44 ± 0.02 µg/ml (AEMC), 205.33 ± 0.07 µg/ml (CEMC), and 247.71 ± 0.03 µg/ml (PEMC) which was shown in Figure 3. In this assay the ferrous sulphate was taken as the standard (IC_{50} value 41.26 µg/ml).

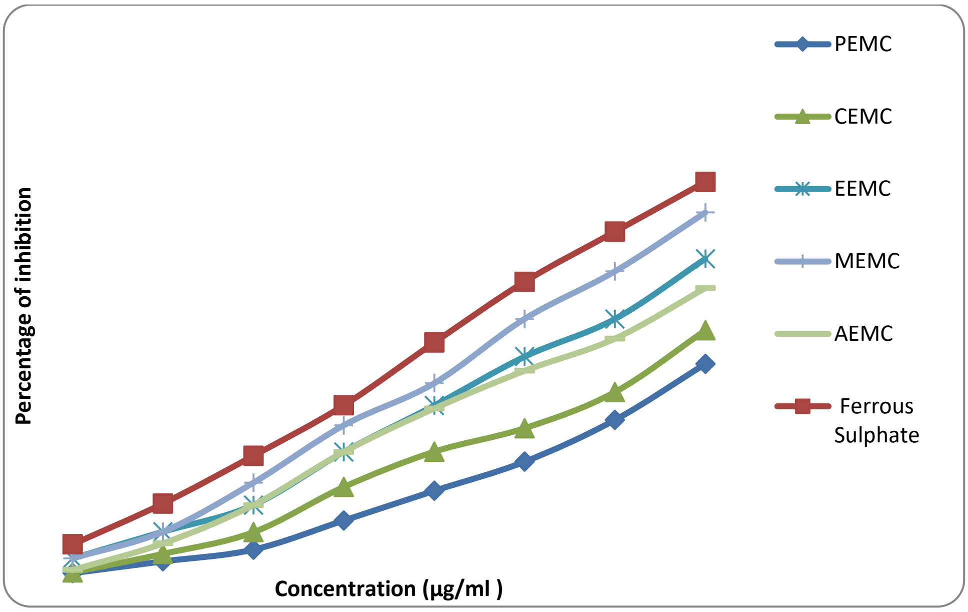

Figure 3. FRAP assay of various extracts of *Muntingia calabura* leaf

4. DISCUSSION

Medicinal plants played a central role in human and community health. Frequent usage of herbal medicines by a large population is possibly due to its efficacy, safety and cost effectiveness. Plant derived compounds have a significant role in novel drug development. The medicinal value of a plant was offered by chemical substances present in it, which elicits a definite physiological action on the human body (17). The most important bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds.

Muntingia calabura L. is a shrub widely grown as roadside tree and its leaves have well documented medicinal uses. In Peruvian folk remedies, the antiseptic property of *Muntingia calabura* L. leaves and its effectiveness in treating swellings of lower extremities are well reported. In South America the leaf decoctions of *Muntingia calabura* L. were used to reduce gastric ulcers (18).

The different extracts derived from the leaves of *Muntingia calabura* L. revealed the presence of various phyto constituents such as alkaloids, flavonoids, steroids, terpenoids etc in ethyl acetate, methanol and aqueous extracts. The total flavonoid and phenolic content in ethyl acetate, methanol and aqueous extracts were quantified and it was found that a conspicuous amount of secondary metabolites were present in it. The phenolics and flavones primarily act as antioxidants and therefore it is advantageous to determine the amount present in those extracts. These plant secondary metabolites have well documented medicinal and physiological actions.

The process of oxidation is an important step in various biological processes of living organisms for releasing energy. This causes the generation of free radicals. Higher level of free radicals, radical derivatives and non-radical reactive species can damage all the cellular components in our body leading to oxidative stress related disorders and hence they are hazardous (19). The reactive oxygen species is the key factor behind various diseases like cancer, diabetes, heart diseases and they damage the body organs like eyes, lungs, brain, kidney, heart, pancreas, etc (20). Antioxidants on the other hand protect our body from free radical damage by giving up their electrons and it can also stimulate wound healing (21). Thus, antioxidants act as a panacea for various diseases. Therefore, it is worthwhile to evaluate the antioxidant efficacy of *Muntingia calabura* L. leaf extracts. The *in vitro* antioxidant evaluation of various extracts revealed its capability to scavenge free radical at a lower concentration. The Methanolic extract of *Muntingia calabura* L. leaves exhibited higher antioxidant activity. The higher antioxidant efficacy of MEMC may be due to the rich phytochemicals present in it. The antioxidant properties of flavonoids are due to the chelating of metal ions, such as iron and copper. It has been recognized that, phenols and flavonoids showed antioxidant activity and their effects on human nutrition and health are considerable.

CONCLUSION

The phytochemical screening of *Muntingia calabura* L. leaf extracts provides evidence that it contains some important bioactive molecules which exhibits a strong antioxidant potential. Different extracts exhibited variations in their antioxidant potential and that may be due to the differences in their chemical composition. From the present study it was noted that the antioxidant potential increases with an increase in the sample concentration. Thus, we can conclude that *Muntingia calabura* L. leaves are a new alternative source for free radical scavengers and hence this plant will pave a way for the development of new drugs against oxidative stress related diseases.

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Compliance with ethical standards

Ethical statement: This article does not contain any studies with human participants or animals performed by any of the authors

Conflict of interest:

The authors declare that they have no conflict of interest.

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