

Antioxidant potentials of the pod extract of *Caesalpinia pulcherrima* Swartz (Fabeace) and the theoretical evaluation of the antioxidant property of the isolated compounds

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Abstract

Various diseases associated with oxidative stress have necessitated the need to investigate plants for antioxidant agents. This study aims to investigate the antioxidant potential of the extract and fractions of *C. pulcherrima* and estimate the quantum chemical properties of polyphenolic compounds isolated from its most active antioxidant fraction. Methanolic extract and partition fractions of the pods of the plant were assayed for their antioxidant activity using four models: 1,1-diphenyl-2-picrylhydrazyl radical scavenging, ferric reducing antioxidant power, total antioxidant capacity and hydroxyl radical scavenging activity. Total phenolic and flavonoid contents of the extract and fractions were also investigated. Furthermore, the quantum chemical properties of two polyphenolic compounds were calculated to predict the antioxidant potential.

The extract exhibited good antioxidant activity with $IC_{50} = 45.63 \mu\text{g/ml}$ comparable to ascorbic acid of $IC_{50} = 37.94 \mu\text{g/ml}$, high reducing power with value at 376.74 ± 6.78 , total antioxidant capacity obtained at ascorbic acid equivalence of $383 \pm 8.16 \text{ mgAAE/g}$ and potent hydroxyl radical scavenging activity with value at $580.97 \mu\text{g/ml}$ with ascorbic acid at $30.7 \mu\text{g/ml}$. The total phenol and flavonoid contents values at 540.53 ± 3.71 and 347.87 ± 7.13 respectively. Furthermore, the results obtained from the quantum chemical calculations reveals polyphenolic compounds; 5(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol and 3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5, 7-pentahydroxy flavonol as promising antioxidant agents. The study concluded that *C. pulcherrima* pods have good antioxidant activity. Also, the polyphenolic compounds are among the chemical constituents responsible for the antioxidant activity obtained for the extract and ethyl acetate fraction.

Keywords: *Caesalpinia pulcherrima*; Pods; Extract; Antioxidant

1. Introduction

Oxidative stress is characterised by increased production of reactive oxygen and nitrogen species (RONS) and reduced antioxidant capacity in the cardiovascular, renal and central nervous systems [1-2] These excessive amounts can lead to cellular damage and apoptosis, contributing to several acute and chronic pathological diseases such as cancer, stroke, myocardial infarction, diabetes, acute and chronic kidney disease, neurodegenerative diseases, macular degeneration and biliary diseases [3-5]. Several synthetic drugs are currently used as antioxidant agents. These drugs are presented

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with several side effects. Medicinal plants are known to produce antioxidant compounds that have lesser side effects. These antioxidant agents include flavonoids, tannins, stilbenes, coumarins, lignin, lignans and phenolic acids [6].

Caesalpinia pulcherrima (Fabaceae/Leguminosae) is a shrub rich in a variety of phytoconstituents including diterpenoids, flavonoids, peltogynoids, steroids, glycosides and more [7-9]. In folk medicine, extracts from various parts of the plant are used as stimulant, emenagogue, abortifacient and in the treatment of fever, ulcer, asthma, tumors, and skin diseases [9-10]. Pharmacologically, the plant has been reported to possess antimicrobial, analgesic, anti-inflammatory, anthelmintic, antimalarial, antiulcer, cytotoxic, antioxidant, antiviral, anticancer, immunosuppressive, anti-diabetic, antimicrobial activities and vasorelaxing effect [7, 9-10]. This study was aimed at investigating the antioxidant capacity of the extract and fractions of *C. pulcherrima* pod. It further predicted the antioxidant potentials of the polyphenolic compounds previously isolated from the ethyl acetate fraction [11] by calculating their electronic properties.

2. Material and methods

2.1. Plant materials and extraction

The pods of *C. pulcherrima* were collected at Obafemi Awolowo University campus, Ile-Ife, Osun State, Nigeria. It was identified and authenticated by Mr. Ademorriyo at the herbarium of the Department of Botany, Obafemi Awolowo University, Ile-Ife. The voucher specimen (IFE 17513) was prepared and deposited at the same herbarium. The pods were peeled to remove the seeds and air dried for 30 days. The air dried pods were ground to powder and 6 kg of the powdered pods was exhaustively extracted with methanol and concentrated *in vacuo* to obtain its methanolic extract with 9.6 % w/w yield.

The extract (160.55 g) was suspended in 500 mL of methanol/water (1:1) and successively partitioned with *n*-hexane (9×500 mL), DCM (3×500 mL) and ethyl acetate (16×500 mL) to obtain their corresponding *n*-hexane (28.13 g), DCM (47.75 g), ethyl acetate (19.65 g) and aqueous methanol (58.57 g) fractions.

2.2. Antioxidant assays of the crude extract and the fractions

2.2.1. Total Flavonoid Content

The total flavonoid content of the extract and the fractions was determined according to the method of Zhishen [12] as described by Miliauskas [13]. To 0.2 ml of the extract/fractions, 0.4 ml of distilled water was added. This was followed by the addition of 0.1 ml of 5 % (w/v) sodium nitrate. After 5 minutes, 0.1 ml of 10 % (w/v) aluminum chloride and 0.2 ml of 0.1 M sodium hydroxide solution were added and the volume made up to 2.5 ml with distilled water. The absorbance was then measured against the blank at 500 nm.

2.2.2. Total Phenolic Content

The total phenolic content of the extract and the fractions was determined using the Folin-Ciocalteu's method of Singleton and Rossi, [14] as described by Gulcin [15]. To 0.1 ml of 5 mg/ml of the extract/fractions was added to 0.9 ml of distilled water. 0.2 ml of 10 % Folin reagent was added. The resulting mixture was vortexed. After 5 minutes, 1 ml of 7 % Na₂CO₃ solution was then added to the mixture. The solution was diluted to 2.5 ml with distilled water and then incubated for 90 minutes at room temperature. The absorbance at 750 nm was then measured against the reagent blank. Standard preparation was done by preparing a stock solution of Gallic acid 100 µl/ml, aliquots of 0.2, 0.4, 0.6, 0.8 and 1 ml were taken and made up to a total volume of 1 ml.

2.2.3. DPPH scavenging assay

DPPH (2,2- diphenyl-1-picrylhydrazyl) free radical scavenging activity of the extract/fractions was determined using the method of Blois [16] as described by Brace [17] in which 1 ml of DPPH solution (0.3 mM DPPH dissolved in methanol) was prepared. To 1 ml each of the extract/fractions and methanol, 1 ml of DPPH was added. The mixture was vortexed and allowed to react at room temperature in a dark chamber for 30 minutes. The absorbance was measured at 517 nm against DPPH control (1 ml of methanol) as a blank using UV-VIS spectrophotometer.

2.2.4. Total Antioxidant Capacity Assay

The total antioxidant capacity was carried out according to Prieto [18]. Typically, 0.3 ml extract/fractions were mixed with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 minutes. Then the absorbance of the solution was

measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in place of the extract/fractions was used as a blank. The antioxidant activity was expressed as ascorbic acid equivalent (mg AAE/g extract) which served as positive control.

2.2.5. Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay was carried out according to method described by Benzie and Strain, [19]. A 300 mmol/L acetate buffer (pH 3.6), 10 mmol/L of 2, 4, 6-tri-(2-pyridyl)-1, 3, 5-triazine and 20 mmol/L of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ were mixed together in the ratio 10:1:1 respectively to give the FRAP working reagent. An aliquot (50 μl) of the extract/fractions at 5 mg/ml and 50 μl of standard solution of ascorbic acid was added to 1 ml of FRAP reagent. Absorbance measurement was taken at 593 nm wavelength exactly 10 minutes after mixing, against reagent blank containing 50 μl of distilled water.

2.2.6. Hydroxyl Radical Scavenging Assay

The ability of the extract to scavenge the hydroxyl radical generated by Fenton reaction was measured according to the modified method of Chung [20]. The Fenton reaction mixture containing 200 μl of 10 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 200 μl of 10 mM EDTA and 200 μl of 10 mM 2-deoxyribose was mixed with 1.2 mL of 0.1 M phosphate buffer (pH 7.4) containing 200 μl of the extract/fractions. Thereafter, 200 μl of 10 mM H_2O_2 was added to the mixture and incubated for 4 hours at 370 °C. About 1 ml of 2.8 % TCA and 1 ml of 1 % TBA were added and placed in boiling water bath for 10 minutes. The resultant mixture was allowed to cool to room temperature and absorbance was recorded at 532 nm in a UV-VIS spectrophotometer.

2.3. Theoretical modeling and optimization studies

DFT analysis of 5(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol and 3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5, 7-pentahydroxyflavonol previously isolated from the ethyl acetate fraction of *C. pulcherrima* pods [11] was carried out using Spartan 14 programme containing functional B3LYP (Lee-Yang Parr exchange correlation functional method). Also, 6-31G basis set was selected for the DFT study [21]. During the calculations, the values of the frontier orbital energies were computed from the most established conformation of the compounds.

3. Results and discussion

3.1. Antioxidant activity of the extract and the fractions

Total phenolic content and total flavonoid content of the extract of pods of *C. pulcherrima* were estimated. It was observed that the crude pod extract of *C. pulcherrima* contained high amount of total phenol and flavonoids (Table 1). The results obtained in this study are in accordance with the findings of other researchers who have reported high phenolic and flavonoid content in the pods of *C. pulcherrima* [22-23]. The antioxidant property of polyphenolic compounds has been reported [24-25]. A correlation between antioxidant activity and the phenolic content has been documented [23, 26]. Plant extracts with high phenolic content also show high flavonoid content [27] as evident in this study.

Table 1 Antioxidant activity of the extract and the fractions of the pod of *C. pulcherrima*

Extract/ Fractions	TPC (mg GAE/g)	TFC (Rutin Equivalent mg/g)	DPPH IC ₅₀ ($\mu\text{g}/\text{ml}$)	TAC (AAE mg/g)	FRAP (AAE mg/g)	HRSA IC ₅₀ ($\mu\text{g}/\text{ml}$)
CPA	540.53 $\pm$ 3.71	347.87 $\pm$ 7.13	45.63	383 $\pm$ 8.16	376.74 $\pm$ 6.73	580.97
<i>n</i> -hexane	422.5 $\pm$ 30.67 ^a	419.23 $\pm$ 12.38 ^a	56.32	253 $\pm$ 50.62 ^a	310.44 $\pm$ 7.35 ^a	2441
DCM	453.21 $\pm$ 7.21 ^a	414.51 $\pm$ 28.97 ^a	56.23	285 $\pm$ 9.80 ^a	376.34 $\pm$ 0.92 ^b	1382.58
EtOAc	407.87 $\pm$ 0.67 ^a	450.13 $\pm$ 10.40 ^a	48.87	357 $\pm$ 1.63 ^b	387.62 $\pm$ 2.30 ^b	1094.5
Aqueous MeOH	428.53 $\pm$ 7.51 ^a	326.4 $\pm$ 10.89 ^b	57.31	293 $\pm$ 3.27 ^a	377.77 $\pm$ 1.62 ^b	931.93

Data show the mean $\pm$ SEM ; TPC: total phenol content; TFC: total flavonoid content; DPPH: 1,1-diphenyl-2-picrylhydrazyl; TAC: Total antioxidant capacity; FRAP: Ferric reducing antioxidant power; HRSA: hydroxyl radical scavenging assay; IC₅₀: Concentration needed to give 50% activity; Values with different superscripts within columns are significantly different ($p > 0.05$, one-way analysis of variance followed by the Bonferroni t-test); CPA: extract of *C. pulcherrima* pod; DCM: dichloromethane; EtOAc: ethyl acetate; MEOH: methanol.

Several *in vitro* model systems have been used for assessing the antioxidant activity of various plant extracts. This study employed DPPH-radical scavenging, Total antioxidant capacity (TAC), Ferric Reducing Antioxidant Power (FRAP) and Hydroxyl Radical Scavenging antioxidant assays to evaluate the antioxidant potential of the crude extract and the fractions of the pods of *C. pulcherrima*.

All tested fractions exhibited appreciable and concentration dependent scavenging properties against the DPPH radical. The order of the fractions of *C. pulcherrima* from the strongest to the weakest DPPH radical scavenging activity was ethyl acetate (IC₅₀ 48.87 µg/ml), dichloromethane (IC₅₀ 56.23 µg/ml), *n*-hexane (IC₅₀ 56.32 µg/ml) and aqueous methanol (IC₅₀ 57.31 µg/ml). Smaller IC₅₀ value corresponds to higher and stronger antioxidant activity [28].

The total antioxidant capacity of fractions of *C. pulcherrima* was also evaluated. The ethyl acetate fraction gave the highest total antioxidant capacity. Total antioxidant capacity of the fractions of *C. pulcherrima* from the highest to the lowest was ethyl acetate, aqueous methanol, dichloromethane and *n*-hexane (Table 1). The evaluation of the total antioxidant capacity (TAC) may be an appropriate tool to determine the additive antioxidant properties of plant foods [29].

Ferric reducing antioxidant power (FRAP) of *C. pulcherrima* fractions was evaluated. Ethyl acetate fraction possessed the strongest ferric reducing antioxidant power of the four fractions of *C. pulcherrima* followed by the aqueous methanol, dichloromethane and *n*-hexane fractions. Ferric Reducing power assay method is an electron transfer based total antioxidant assay that is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe³⁺) to form potassium ferrocyanide (Fe²⁺), which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. The reducing ability of a compound generally depends on the presence of reductones (antioxidants), which exert its antioxidant activity by breaking the free radical chain by donating a hydrogen atom [30]. Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action [31].

The hydroxyl radical scavenging activity of fractions of *C. pulcherrima* was estimated. The ethyl acetate fraction exhibited the highest hydroxyl radical scavenging properties when compared to other fractions. The order of hydroxyl radical scavenging ability of fractions of *C. pulcherrima* from the highest to the lowest was *n*-hexane, dichloromethane, ethyl acetate and aqueous methanol (Table 1). All fractions scavenge hydroxyl radicals in a concentration dependent manner. The scavenging of the hydroxyl radicals may be due to the presence of hydrogen donating ability of the fractions.

3.2. Molecular modeling and quantum chemical calculations

Frontier molecular orbital analysis via computational method is of great importance as it gives information on the electronic properties of compounds and further insight on the reactivity and nature of the compounds [32]. Frontier molecular orbitals analysis often deals with electronic transitions as well as reactivity of molecules. The frontier molecular orbitals refer to the LUMO, which is largely related to the electron accepting ability of a compound and the HOMO which is concerned with its electron donating capacity. The energy difference between the frontier molecular orbital also known as energy gap is used to predict the reactivity and stability of compounds. Compounds with large energy gaps exhibit low reactivity as well as high kinetic stability while compounds with low energy gaps exhibit high reactivity and low kinetic stability [33]. The energy gap ΔE for 3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5, 7-pentahydroxyflavonol and 5(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol is 3.53 eV and 5.08 eV respectively. This suggests that 3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5, 7-pentahydroxyflavonol is likely to be more stable and less reactive than 5(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol.

Figure 1 shows that for 3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5, 7-pentahydroxyflavonol, the HOMO is distributed along the aromatic system as well as the hydroxyl groups on the aromatic system. The LUMO is localized over the other ring. The third ring neither participates in the LUMO and HOMO. For 5(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol, the LUMO and HOMO are distributed along the π network of both aromatic rings.

The quantum descriptors namely chemical hardness, electrophilicity, chemical potential are excellent tools in giving a vivid description of the reactivity and stability of the compounds. These descriptors were calculated using the formulas below; the results are displayed in Table 1.

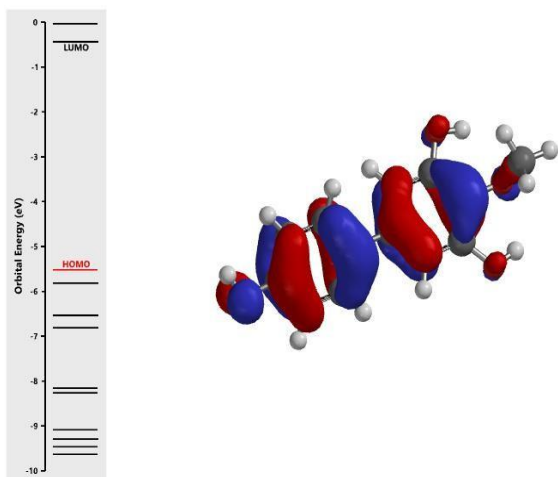
$$\Delta E = E_{LUMO} - E_{HOMO} \dots\dots\dots(1)$$

$$\eta = \frac{1}{2}(E_{LUMO} - E_{HOMO}) \dots\dots\dots (2)$$

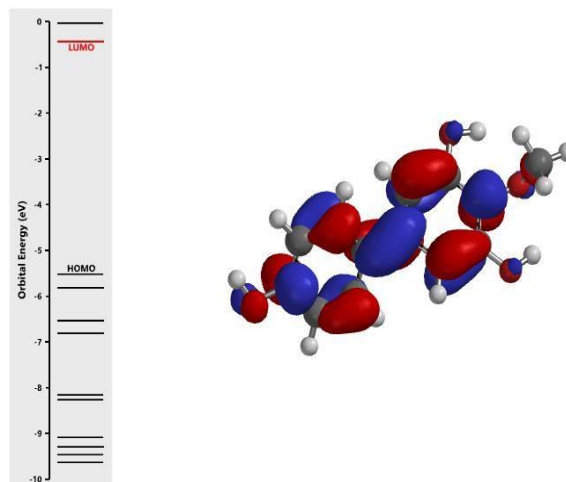
$$\mu = \frac{1}{2}(E_{HOMO} + E_{LUMO}) \dots\dots\dots (3)$$

$$\omega = \frac{\mu^2}{2\eta} \dots\dots\dots (4)$$

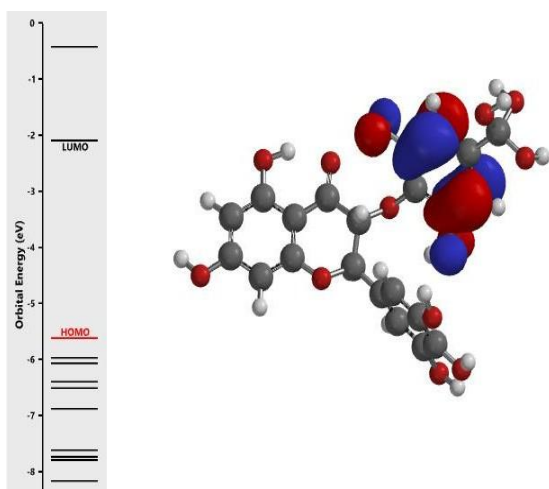
The chemical hardness which describes the resistance exhibited by a molecule to exchange electron density with its surrounding is high in molecules that possess large energy gaps, such molecules are said to be hard molecules, while molecules with lower energy gaps often possess low values for chemical hardness [34]. They are often referred to as soft molecules. From table 1, 5(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol has a higher value for hardness than 3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5, 7-pentahydroxyflavonol, hence it's said to be harder than 3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5, 7-pentahydroxyflavonol and is likely to offer more resistance with respect to a change in electron density.



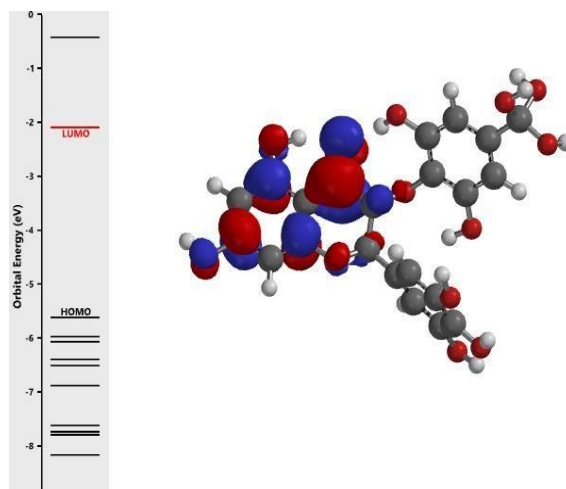
5(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol
(HOMO)



5(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol
(LUMO)



3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5,
7-pentahydroxyflavonol (HOMO)



3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5,
7-pentahydroxyflavonol (LUMO)

Chemical potential is the measure of alteration in energy of a molecule with respect to the electron number at a fixed potential. It is strongly linked to the electrophilicity index of a molecule, which is the energy stabilization of a molecule on acquiring an extra amount of electron density from its environment [34]. Strong electrophiles often possess high chemical hardness and low chemical potential while weak electrophiles possess low chemical hardness and high chemical potential. In effect it means that strong electrophile possess high electrophilicity index values while weak electrophiles possess low electrophilicity index values [33]. Compounds with electrophilicity index values above 0.8 are

referred to as strong electrophiles [34]. Hence 3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5, 7-pentahydroxyflavonol and 5(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol are strong electrophiles. Molecular electrostatic potential is an important molecular descriptor that is usually used in investigating the chemical reactivity of molecules. It is a three dimensional plot of electrostatic potential over electron density of molecular systems. It indicates the position, shape and size of positive, negative and neutral electrostatic potentials using a colour grading scheme [35]. It aids understanding of system physicochemical properties in relation to their molecular structure and help to identify reactive sites of nucleophilic and electrophilic attack during bonding interactions [36]. Blue regions are the more electropositive regions, as they lack electrons while the red regions are electron rich centers; hence they are likely to be probable sites for electrophilic attack. From figure 2, in both compounds low electrostatic potential regions are observed around the oxygen of the hydroxyl groups while a high electrostatic potential is found on hydrogen atoms. This suggests that the oxygen atoms are suitable for electrophilic interactions while the hydrogen atoms are suitable for nucleophilic interactions [35]. In both cases oxygen atoms are likely to act as electron donors as the electron rich regions are located on them.

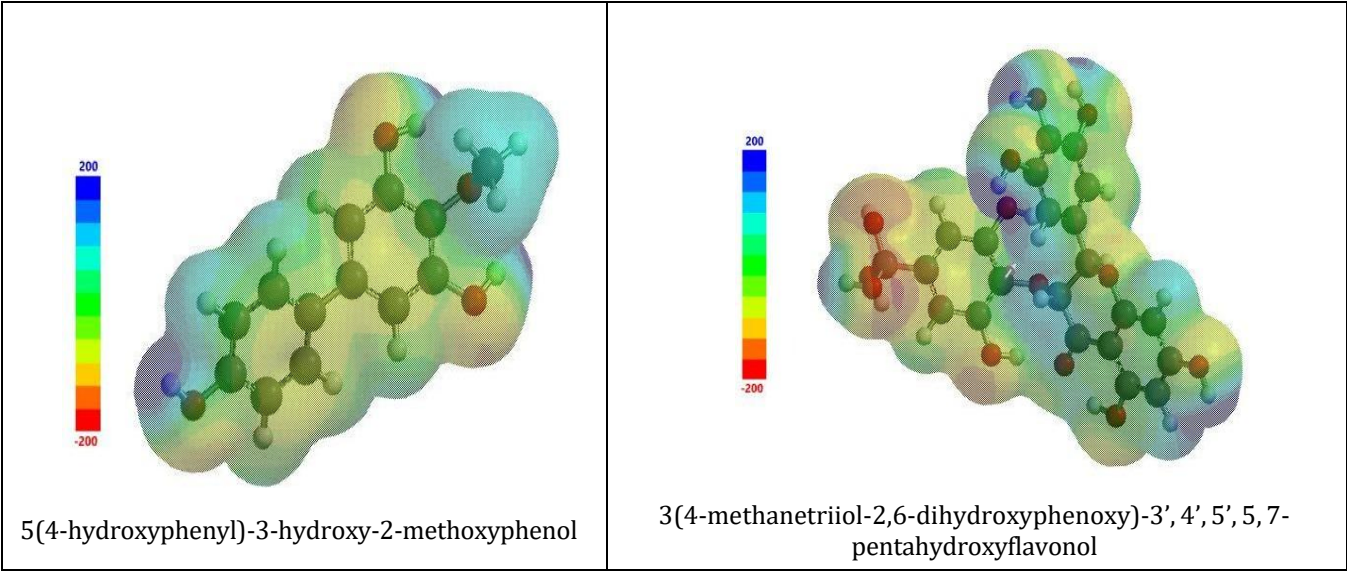


Table 1 Quantum descriptors of compounds

Compounds	E_{HOMO}	E_{LUMO}	ΔE	η (chemical hardness)	μ (chemical potential)	ω (electrophilicity index)
3(4-methanetriol-2,6-dihydroxyphenoxy)-3', 4', 5', 5, 7-pentahydroxyflavonol	-5.62	-2.09	3.53	1.77	-3.8	4.21
5(4-hydroxyphenyl)-3-hydroxy-2-methoxyphenol	-5.52	-0.44	5.08	2.54	-2.98	1.75

4. Conclusion

The crude extract as well as the fractions demonstrated appreciable antioxidant property based on the antioxidant methods employed in this study. Of all the fractions tested, ethyl acetate fraction had the highest DPPH radical scavenging activity, the strongest ferric reducing power and the highest total antioxidant capacity. This could be attributed to its higher total phenolic and total flavonoid content of 540.53 ± 3.71 mgGAE/g and 347.87 ± 7.13 mg/g rutin equivalent respectively. Therefore, the results of this study indicate that antioxidant property of *C. pulcherrima* may be one of the mechanisms by which this plant exerts its effectiveness in several free radical mediated diseases including diabetes as reported by Faloye [11]. The density functional theory studies strongly predicts that the isolated compounds are strong electrophiles. However, they containing electron rich oxygen centers which are likely to be involved in their exhibition of antioxidant activity.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

Authors declare no conflict of interest.

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