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Isolation of Bioactive Phytochemicals in Leaves of *Combretum dolichopentalum* and their Hydrogen Peroxide Scavenging Potentials

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Abstract

This study elucidated the bioactive constituents of leaves of *Combretum dolichopentalum*. The quantitative phytochemical analyses on the leaves of the plant revealed the presence of alkaloids (14.24 ± 2.24 %), flavonoids (17.00 ± 2.00 %), tannins (6.09 ± 0.32 %), saponin (4.19 ± 0.69 %), cyanogenic glycosides (2.89 ± 0.22 %), oxalate (2.56 ± 0.56 %) and phytate (0.10 ± 0.01 %). Further evaluation of the crude plant extract using gas chromatography-flam ionization detector (GC-FID) indicated presence and concentration of specific phytochemicals such as spartein, anthocyanin, lunamarine, epicatechin, rutin, and kaempferol. The free radical scavenging potential of flavonoid, saponins, alkaloid, and tannin precipitated from the plants showed increased scavenging abilities with increasing extract concentration. However flavonoid compared to saponins, alkaloid and tannin showed better scavenging activity with an IC_{50} of 36.10 mg/ml. These results indicate that *C. dolichopentalum* is endowed with phytoconstituent that has strong antioxidant potencies necessary to provide therapeutic effects.

Keywords: *Combretum dolichopenta;* Gas chromatography; Spartein; Kaempferol; Rutin

Introduction

The use of medicinal plants as fundamental components of the African traditional health care system is perhaps the oldest and the most assorted of all the therapeutic systems [1]. In many parts of Africa, medicinal plants are the most easily accessible and affordable healthcare resources available to the local communities. Medicinal plants are used and marketed worldwide as herbal drugs or as single active ingredients over centuries. Besides their popular consumption to treat and cure human illness, plant derived natural products play important roles as a source of pharmacological tools to enable the understanding of the biochemical pathways and the etiology of diseases [2]. Plants are sources of potential therapeutic agents against various diseases due to their biodiversity and presence of a wide array of bioactive phytochemicals and secondary metabolites [3]. The use of medicinal plants in the management of diseases is an important alternative therapy widely employed in developing countries. Several investigations have yielded compounds with properties useful for the development of modern synthetic drugs for the management of several diseases [4]. Currently, it is estimated that 80% of metabolites/plant extracts used as drugs and sold worldwide are derived from natural products and that over 100 new natural product-based lead drugs are in clinical development [5,6]. Due to growing drug discovery from natural products, researchers and pharmaceutical industries have increasing interest in traditional health practices used around the world. This interest has been rekindled for decades due to systemic demonstration that plants are the richest source of drugs for traditional system of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [7]. The pharmaceutical effects of plant are due to the presence of phytoconstituents called phytochemicals. Phytochemicals are biological active, naturally occurring secondary compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients [8].

Phytochemicals have biological properties, such as antioxidant activity [9], antimicrobial effect [10,11], and are associated with a lower incidence of heart disease, ischemic stroke, and other chronic diseases [12-16]. Phytochemicals can detoxify substances that cause cancer, by neutralizing free radicals, inhibiting enzymes that activate carcinogens and also activate enzymes that detoxify carcinogens [17,18].

Combretum dolichopentalum is used in treating disease conditions of the alimentary tract is used for the treatment of stomach ache, gastro intestinal disorders, such as dysentery, passage of bloody stool, diarrhea and stomach ulcer and reconditioning of the uterus after parturition by mother in Ibo ethnomedicine especially in Ezinihitte Mbaise and other Mbaise ethnic nationality of Imo State. The leaves are cooked until the fluid content turns red, and is prepared as soup for drinking [19]. According to the free radical theory of ageing, senescence and a variety of degenerative diseases associated with it are attributed to the deleterious attack of oxygen free radicals on cellular constituents, including connective tissues, chromosomes and mitochondrial DNA [20-22]. Unsaturated fatty acids of cellular membranes are biomolecules most susceptible to oxidative damage in cells, and this sensitivity increases as a function of their double bonds. Lipid peroxidation is mainly initiated by hydrogen abstractions from unsaturated fatty acids by oxygen centred radicals followed by the formation of hydroperoxides. Degradation of hydroperoxides results in a variety of derivatives including various carbonyl products [20-23]. Such unsaturated carbonyls include enals, dienals, trienals, hydroxylenals, 2-ketoaldehydes, deoxyosones and various reductions that are very reactive and toxic to almost all cellular and extracellular biomolecules [20,24,25]. Therefore this research is targeted at providing information on the phytoconstituents of leaves of *C. dolichopentalum* and their radical scavenging capacity.

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Materials and Method

Plant sample

Fresh leaves *of C. dolichopentalum* were harvested from farms in Obinze in Owerri West Local Government Area of Imo state. The plant was authenticated by Mr Ozioko, of the Bioresource Development and Conservation Program (BDCP), Research Centre, university of Nigeria Nsukka, Enugu State Nigeria.

Phytochemical analyses

Quantitative phytochemical screening and isolation was done using the procedures outlined by Harborne [26], Trease and Evans [27], Obadoni and Ochuko [28].

Phytochemical analyses using gas chromatography fitted with flame ionization detector (GC-FID) was also carried out on the plant to reveal specific phytochemicals. Briefly; the dried sample (20 g) was soaked for 72 hours in ethyl acetate. The filtrate was concentrated under reduced pressure, using rotary evaporator at a maximum temperature of 45°C to yield 1 g crude extracts. The ethyl acetate extract (1 g) was subjected to Thin Layer Chromatography (TLC), eluted with ethylacetate. The pure samples from the TLC were dissolved in ethyl acetate and 1 microliter was subjected to GC analysis for phytochemical determination.

Fixed settings

Instrument: Buck 530 gas chromatograph equipped with an on-column automatic injector, flame ionization detector, HP 88 capillary column (100 m x 0.25 µm film thickness,) CA, USA. Detector temperature was set at 250°C and both injectors temperatures were held at 220°C. The integrator chart speed was maintained at 2 cm/min and oven temperature was set at180°C and the GC was allow to warm up. While it was warming, instrument final temperature was set to 220°C and was allowed to run for 45 mins and ramped for 15 mins at a rate of 0°C/min. The analysis was started by the injection of 1.0 µl sample onto column A using the appropriate injection technique.

Alkaloid extraction: Alkaloid was extracted as described by Obadoni and Ochuko [28]. Briefly; Fifty grams (50 g) of the sample was weighed into a 1000 ml beaker and 500 ml of 29% acetic acid in ethanol was added and allowed to stand for 6 hrs. This was filtered and the filtrate concentrated on a rotary evaporator to one quarter of the original volume. The alkaloid was precipitated out using concentrated ammonium hydroxide which was added drop by drop until precipitate was complete. The solution was allowed to settle and the precipitation was collected by filtration using Whatman No. 1 filter paper. The precipitate obtained was tested for the presence of alkaloid using standard methods [26,27].

Saponin extraction: Saponin was extracted as described by Obadoni and Ochuko [28]. Briefly; Fifty grams (50 g) of the sample was weighed into a 1000 ml beaker and 500 ml of 20% ethanol was added and stirred using a glass rod. The mixture was heated over water bath for 4 hrs with continuous stirring while the temperature was maintained at 55°C. The mixture was filtered and the residue was re-extracted with 500 ml of 20% ethanol. The combined extract was reduced to 40 ml on a rotary evaporator. The concentrated extract was transferred into a 250 ml separation funnel and 50 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. This process was repeated thrice and finally 60 ml of n-butanol was added. The mixture was washed twice with a 10 ml of 5% sodium chloride. The remaining solution was heated over water bath and the residue dried to constant weight. The precipitate obtained was tested for presence of saponin using standard methods [26,27].

Flavonoid extraction: Flavonoid was extracted as described Boham and Kocipai [29]. Briefly; Fifty grams (50 g) of the plant sample were extracted repeatedly with 500 ml of 80% aqueous methanol at room temperature. The solution obtained was filtered with Whatman No. 45 filter paper. The combined filtrate was concentrated on a rotary evaporator. The precipitate obtained was tested for presence of flavonoid using standard methods [26,27].

Tannin extraction: Tannin was extracted by the method described by the International Oenological Codex, [30]. Briefly; Fifty grams (50 g) of the plant sample was extracted with 500 ml of water. The aqueous extract was extracted thrice with ethyl acetate to eliminate neutral substances. The extract was brought to pH 2 by the addition of concentrated HCl and re-extracted with ethyl acetate. The extract was concentrated on a rotary evaporator. The extract was tested for the presence of tannin using standard methods [26,27].

Radical scavenging/antioxidant studies

Hydrogen peroxide scavenging activity by flavonoid, alkaloids, saponins and tannins isolated from leaves of *C. dolichopentalum* was estimated by the method of Ruch et al. [31]. Each isolate (flavonoid, alkaloids, saponins and tannins) from *C. dolichopentalum* was dissolved in distilled water at various concentrations, mixed with 0.1 M phosphate buffer (pH 7.4) and 0.6 ml of 4 mM hydrogen peroxide solution prepared with the same buffer and incubated for 10 mins. The absorbance of each reaction mixture was recorded at 230 nm against blank solution containing the plant extract without H_2O_2. The amount of H_2O_2 inhibited by the isolates (flavonoid, alkaloids, saponins and tannins) were calculated using the following equation:

$$H_2O_2\text{-scavenging capacity} = [(\underline{Abscontrol-Abs_{sample}}) / (Abs_{control})] \times 100$$

Where: $Abs_{control}$ is the absorbance of H_2O_2 radical+methanol;

Abs_{sample} is the absorbance of H_2O_2 radical+sample isolate or standard.

Statistical Analyses

Data obtained were presented in mean ± standard deviation and simple percentages of triplicate determination

Results

Quantitative phytochemical screening (Table 1) of *C. dolichopentalum* leaves indicates the presence of flavonoid, alkaloid, saponins, tannins, cyanogenic glycosides, oxalate, and phytate. The analyses carried out on the sample revealed high concentrations of flavonoids (17.00 ± 2.24%), alkaloids (14.24 ± 4%), saponins (4.19 ± 0.69%), and tannins (6.09 ± 0.32%). Results of GC-FID (Table 2 and Figure 1) shows the presence of flavonoids like kaempferol (25.1564 µg/ml), Rutin (263.249 µg/ml); Anthrocyanin (0.5854 µg/ml), and epicatechin (6.5163 µg/ml). Other phytochemicals identified where Lunamarine (5.9893 µg/ml), Oxalate (1.179 µg/ml), Tannin (16.0476 µg/ml) and alkaloids like Sparteine (0.0021µg/ml). Result (Table 3 and Figure 2) showed that Flavonoid, saponins, alkaloid, and tannin scavenging abilities increased with increase extract concentration. However Flavonoid compared to saponins, alkaloid and tannin showed better scavenging activity with a 50% inhibition concentration (IC_{50}) of 36.10 mg/ml, R^2 of 0.94. Saponin showed IC_{50} of 126.25 mg/ml, R^2 of 0.97, while alkaloid had IC_{50} of 61.18

mg/ml, R^2 of 0.94 and tannin had IC_{50} of 55.56 mg/ml, R^2 of 0.97.

Discussion

Phytochemicals provide health benefits further than those attributed to macronutrients and micronutrients [8]. Appreciable amount of saponins, flavonoids, and tannins were observed in the leaves of *C. dolichopentalum*. Saponins are known to exert anticholesterolemic and hypoglycaemic effect through intra-lumenal physiochemical interaction or other yet unidentified activities [32]. Saponins have also been observed to protect plants from protozoans and molluscs and also act as antifungal and antiviral agents [33,34].

Phytochemicals (Quantitative)	% Concentration
Alkaloids	14.24 ± 2.24
Flavonoids	17.00 ± 2.00
Tannins	6.09 ± 0.32
Saponins	4.19 ± 0.69
Cyanogenic Glycosides	2.89 ± 0.22
Oxalate	2.56 ± 0.56
Phytate	0.10 ± 0.01

Values are mean ± standard deviation of triplicate determinations

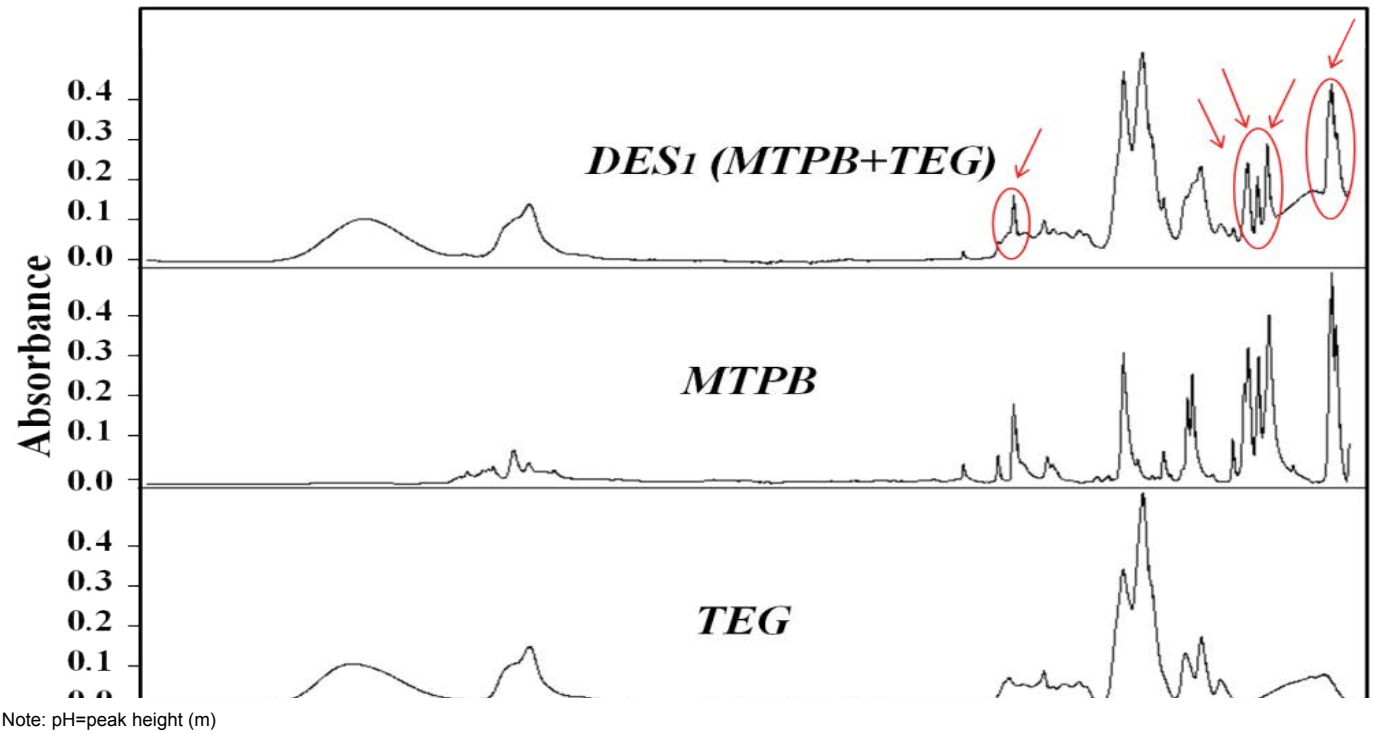
Table 1: Phytochemical composition of *C. dolichopentalum* leaves.

Name of Phytochemical (µg/ml)	Retention (min)	Area (m^2)	Peak Height (m)	Concentration (µg/ml)
Spartein	0.206	3284.7296	214.708	0.0021
Oxalate	5.726	6278.2144	356.512	1.179
Anthrocyanin	10.266	2928.024	166.381	0.5854
Tannin	14.496	3543.6292	201.37	16.0476
Lunamarine	19.033	3618.428	204.623	5.9893
Epicatechin	32.55	3220.2098	183.068	6.5163
Rutin	37.41	4478.5715	254.444	65.2853
Rutin	41.91	13580.3106	758.567	197.9637
Kaempferol	41.91	5322.8864	302.256	25.1564
Total		46255.0035		318.725

Table 2: Identification of phytochemical content of *C. dolichopentalum* leaves using gas chromatography.

The leaves of *C. dolichopentalum* contain flavonoids of the type; kaempferol, rutin, anthocyanin and epicatechin in varying concentration (Table 2 and Figure 1). At cellular levels, flavonoids have been found to exert a variety of biological effects [35], presumably mediated by specific interaction with molecular targets [36-38]. The capacity of flavonoids to act as antioxidant depends on their molecular structure. The position of OH groups and other features are important for flavonoids antioxidant and free radical scavenging activities.

Kaempferol is under consideration as a possible cancer treatment [39-41], because it reduces the resistance of cancer cells to anti-cancer drugs such as Vinblastine and paclitaxel [42]. This is to say that extract from *C. dolichopentalum* could function as an anticancer drug. Anthocyanins has been reported to be involved in the improvement of vision, inhibition of nitric oxide product, induction of apoptosis, decreased platelet aggregation, and neuroprotective effects [43]. A recent study has reported anthocyanin to have outstanding anti-sickling effects [44]. Rutin, also called rutinoside, quercetin-3-O-rutinoside and sophorin, is the glycoside between the flavonol quercetin and the dissacharide rutinoside. Rutin has been reported to contribute to antimicrobial [45], and antioxidant [46] properties of plant. Furthermore, it has been shown to inhibit *in vitro,* the vascular endothelial growth factor [47] in sub-toxic concentrations, so acts as an inhibitor of angiogenesis. This finding may have potential relevance for the control and management of some cancers.



Note: pH=peak height (m)

Figure 1: Chromatogram of phytochemicals in he leaves of *C. dolichopentalum.*

Concentration (mg/ml)	% inhibition by isolates			
	Flavonoid	Saponin	Tannin	Alkaloid
0	0	0	0	0
5	2.67 ± 0.12	3.51 ± 0.48	9.27 ± 1.02	1.19 ± 0.12
10	13.37 ± 2.33	7.02 ± 0.78	12.25 ± 2.02	12.30 ± 1.20
15	28.09 ± 3.60	8.77 ± 0.42	23.84 ± 1.22	13.09 ± 0.98
20	31.43 ± 3.23	14.47 ± 2.02	28.48 ± 2.22	24.60 ± 2.04
25	43.48 ± 4.33	18.42 ± 0.45	30.13 ± 2.01	28.97 ± 1.34
IC_{50}	36.1	126.25	55.56	61.18
r^2	0.94	0.97	0.966	0.94

Table 3: Summary of hydrogen peroxide radical scavenging activity of major phytochemicals from *C. dolichopentalum*.

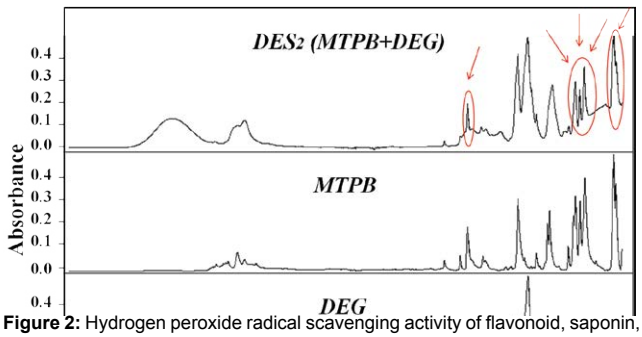


Figure 2: Hydrogen peroxide radical scavenging activity of flavonoid, saponin, alkaloid and tannin extracted from *C. dolichopentalum*.

The physiological effects of alkaloids have made them important compounds in medicine. They have been used as pain killers (Morphine), stimulants (Caffeine), muscle relaxers (Cocaine), tranquilizers (Curare), anti-cancer (Vincristine, Vinplastine), anaesthetics (Cocaine), anti-arrythmias (Quanidine), Vaso-constrictors (Ergonovine, Ephedrine), antimalarial (Quinine), poisons (Tobocurarine, coniine, strychinine), pupil expander (Atropine) and hallucinogenic drugs (mescaline) [48-51]. Our screening showed that *C. dolichopentalum* leaves contain alkaloids such as spartein. Sparteine is a quinolizidine alkaloid which has been reported to exhibit antiarrhythmic properties. Spartein was once used as a uterus contracting drug, but now abandoned because of its side effect. Spartein has also been reported to have hypotensive and CNS depressant properties and furthermore, are hypoglycemic and thus *C. dolichopentalum* can be exploited for anti-diabetic drugs [52]. Lunamarine is a quinoline alkaloid. Lunamarine has shown anticancer [53], anti-estrogenic [54], immunomodulatry [55], and anti-amoebic activity particularly against *Entamoeba histolycal* [56]. This indicates the use of *C. dolichopentalum* for the treatment of stomach ache, gastro intestinal disorders, such as dysentery, passage of bloody stool, and diarrhea.

Tannic acid was indentified in *C. dolichopentalum* leaves in high concentrations. Tannins are known to tar the outermost layer of the mucosa [57] and thereby render it less permeable and more resistant to chemical and mechanical injury or irritation. According to [57], tannins are used as astringent or antidote for various poisons and as a tropical haemostatic. Tannins have antioxidant, antimicrobial [58], anticancer [59], activities. Tannic acid is reported to have antihypertensive, antidiarrheal, anti-asthmatic, cardioprotective, antidiabetic, anti-cataractogenic, tumour inhibition, anti-inflamatory, and anti-adipogenic [59], and hepatoprotective [60] activities.

The leaves of *C. dolichopentalum* showed low concentration of phytate and oxalate. Phytate is a hexaphosphate ester of inisitol that is widely distributed in vegetables. It is considered an anti-nutrient because of the possibility of its interference with proteolytic digestion, in addition to the fact that the phosphorus in it is not nutritionally available to monogastric animals [61]. It is considered an antinutritional factor because it complexes with nutritionally essential divalent cations like Ca^{2+}, Fe^{2+}, Mg^{2+} and Zn^{2+}, thus rendering them unavailable from the diet. It is therefore advisable to use the leaves with mineral supplements. Results also showed that phytate in the plant leaves was higher than that found in *Sphenostylis stenocarpa* 0.42%; *Citrullus colocythis*, 0.64%, *Pentochethra macrophylla*, 0.36%; *Muanna flagellipes*, 0.33% [62]. Oxalate like phytate, has the ability to bind some divalent metal ions such as Ca^{2+} and Mg^{2+}, thereby interfering with their metabolism. Ingestion of an excessive amount of oxalate could cause muscular weakness or paralysis, hypocalcaemia, development of urinary calculi, blockage of the renal tubules by calcium oxalate crystals and gastrointestinal irritation.

Plant phytochemicals have antioxidant activities [8,9], exhibited by neutralizing reactive oxygen species, inhibiting and/or activating enzymes systems for free radical scavenging. Hydrogen peroxide (H_2O_2) is an important reactive oxygen species, because of its ability to penetrate biological membranes and its ability to form hydroxyl radicals in cells. It reacts with Fe^{++} or Cu^{++} ions [63] in a reaction called Fenton-Haber Weiss reaction. The results of the hydrogen peroxide scavenging ability of the phytochemical extract of *C. dolichopentalum* leaves were remarkable. The flavonoid, saponins, alkaloid and tannins precipitated from leaves of *C. dolichopentalum* scavenged H_2O_2 in a dose dependent manner. Also, the scavenging ability of flavonoid was higher compared to saponins, alkaloid and tannin from *C. dolichopentalum*. Polyhydroxylated compounds like flavonoids are known to possess high antioxidant activity. This activity could be due to flavonoid ability to absorb, neutralize and scavenge free radicals [64]. The presence of hydroxyl groups attached to the aromatic ring structures of flavonoid also confers it radical scavenging ability.

Kaempferol- a subfamily of flavonoid has been reported to scavenge H_2O_2 [40]. These effects indicate the possibility of *C. dolichopentalum* to minimize oxidative damage to some vital tissues and organs when used therapeutically. The low level of scavenging ability of alkaloid compared to flavonoid and tannin precipitated from *C. dolichopentalum* may be due to the solubility of alkaloid in the test medium and substrate used may influence the ability of a compound to scavenge different radicals [65]. A dose response relationship exist when changes in dose produce consistent, non random changes in effect either in the magnitude of the effects or in the percent of individuals responding at a particular level of effect.

According to the A.J. Clarke occupation theory, the intensity of drug action is proportional to the occupancy of the receptors or the concentration of the drug-receptor complexes [66,67]. But there are drugs that do not act through specific receptors either *in vivo* or *in vitro;* example includes anaesthetics and antioxidant which can be local or general. Antioxidants including those found in phytochemicals are made up of subfamilies with different structures. It is this structure that confers radical scavenging abilities on them. For instance, flavonoids are made up kaempferol, luteolin, apigenin, isorhamnetin, resveratrol, quercetin, epicatechin etc., with different potencies in scavenging radicals. Thus at different concentrations, a particular subfamily with less free- radical scavenging potentials, can actively engage in radical scavenging exercise resulting in a lower % inhibition.

Conclusion

The presence of bioactive flavonoids, alkaloids, saponin and tannins with pharmacological properties suggest that *C. dolichopentalum* possess antioxidant, anti-inflammatory, antiarrythmic, anticancer, and hypotensive properties. The presence of lunamarine confers anti amoebic activities in the plant, and this may be why natives of Mbaise and Ogwa of Imo state in Nigeria, use this plant to treat gastrointestinal disorders. Furthermore, the ability of the plant to scavenge hydrogen peroxide which can subsequently form hydroxyl radical in a fenton-Haber weiss reaction *in vitro,* suggest that *C. dolichopentalum* may as well possess the ability, to scavenge different radicals, especially the flavonoid extract from the plant.

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