IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 10, Issue 5 Ver. I (Sep - Oct. 2015), PP 68-74
www.iosrjournals.org

Combined Phytochemicals From Gongronema latifolium and Ocimum Gratissimum leaves Extracts Potentiate In Vitro Free Radical Scavenging.

*Itoro. Friday Usoh[1], Henry Dan Akpan[1] and EdetOkon Akpanyung[1]
[1]*Department of Biochemistry, Faculty of Basic Medical Sciences, University of Uyo, Nigeria.*

Abstract: *This study evaluated the phytochemical constituents of ethanolic leaves extracts of Gongronemalatifolium (GL) and Ocimumgratissimum (OG) against 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) radicals. The results of phytochemical investigation of the two leaves extracts unveiled the presence of alkaloids, tannins, flavonoids, polyphenols and saponins. The extracts of OG and GL singly and in combination possessed the potency to quench 50% free radicals significantly ($P<0.05$) at the inhibitory concentrations (IC_{50} in µg/ml) of 199.48, 598.68 and 399.20 respectively for combined (GLOG), GL and OG in DPPH scavenging test, and 193.20, 376.44 and, 376.72 for GLOG, GL and OG respectively in NO scavenging test when compared to ascorbic acid (standard). The significant antioxidant scavenging activity observed in combined extracts as compared to single extracts can be attributed to the different synergistic mechanisms exhibited by different polyphenolic compounds (tocopherols, flavonoids and other organic acids) present in these leaves extracts.*

Key words:*Gongronemalatifolium, Ocimumgratissimum, DPPH, nitric oxide, phytochemicals, antioxidants, free radicals*

I. Introduction

Oxidative stress results from an imbalance between formation and neutralization of prooxidant [1]. As a key player in several diseases such as cancer, diabetes mellitus, atherosclerosis, cardiovascular diseases, ageing, neurodegenerative and inflammatory diseases [2][3][4], oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation [5][6][7]. Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's populations, especially in the developing world [8].

Medicinal plants are a good source of minerals and vitamins and ethno-botanical reports offer information on their medicinal properties like antidiabetic[9], anticarcinogenic[10], hypolipidemic[11] and antibacterial activity[12]. They are particularly rich in antioxidants which work by significantly slowing or preventing the oxidative stress or damage caused by free radicals such as superoxide radical, hydroxyl radical and non-free radical species such as H_2O_2 and singlet oxygen which are associated with cellular and metabolic injury. Medicinal plants also contain varying amount of phytochemicals like vitamin C, flavonoids and carotenoids. Phytochemicals are natural compounds which are present in plants. These natural compounds work with nutrients and dietary fibres to protect animals and man against diseases. Studies have demonstrated that phytochemicals in common fruits and vegetables can have complementary and overlapping mechanisms of action, including scavenging of oxidative agents, stimulation of the immune system, hormone metabolism, antibacterial and antiviral effects [13]. The leafy vegetables are chlorophyll rich has been proven to help build red blood cells and help to decrease the risk of heart disease, stroke, and certain cancers [14]. In contrast to synthetic pharmaceuticals based upon single chemicals, many phytomedicines exert their beneficial effects through the additives or synergistic action, eliminating the problematic side effects associated with the predominance of a single xenobiotic compound in the body [15]. The beneficial medicinal effects of plant materials typically result from the combinations of secondary metabolites present in the plants.

Gongronem alatifolium Bent Hook, locally called "utasi" by the Efiks, Ibibios and Quas; ''utazi" by the Igbos and "arokeke" by the Yorubas in Nigeria is an herbaceous shrub, with yellow flowers that yields characteristics milky exudates when cut. It is traditionally used as food and its extracts in the treatment of malaria, diabetes, hypertension, and as laxative. The use of crude leaf extract of this shrub in maintaining a healthy blood sugar levels has been reported [16]. Some phytochemicals such as B-sistosterol, lupenyl esters, pregnane ester, glycosides, essential oils and saponins are associated with parts of this herb [17][18].

Ocimum gratissimum (Labiatae), popularly known as "scent leaf" is used as a condiment and spice in most parts of the World including Nigeria for preparation of different dishes. It is also used widely in folk medicine for the treatment of several ailments including fever, cough, respiratory disorders, epilepsy and

dermatitis [19][20]. Studies have shown that the leaf extract of *O. gratissimum* contain potent bioactive components (essential oils) and phytochemicals, which possess antioxidant [21] and antimicrobial [22][23] properties among others.

Objective behind the present study was to carry out the quantitative phytochemical analysis and evaluate 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and nitric oxide (NO) free radical scavenging activities of single and combined leaves extracts of *Gongronema latifolium* (GL) and *Ocimum gratissimum*(OG).

II. Materials and methods

2.1 Collection and Preparation of Plant Materials

Fresh but matured leaves of *Gongronema latifolium* and *Ocimum gratissimum* were collected from Atimbo, Akpabuyo Local Government Area of Cross River State. They were both identified and authenticated in the Department of Botany, University of Calabar, Calabar. 500g each of *Gongronema latifolium* and *Ocimum gratissimum* were thoroughly washed with clean tap water to remove dust particles and debris and shade dried.

The dried plant materials were separately ground into powder with KENWOOD electric blender (KENWOOD LTD. ENGLAND). The powdered samples were each soaked in 80% ethanol and kept in a glass container with a plastic screwed cap and kept in a refegirator for 48 hours at 4°C. They were filtered using a cheese material and afterwards WhatMan No.1 filter paper. The filtrates were separately concentrated invacuo at 37-40°C using a rotary evaporator. The concentrates were allowed open in a water bath (40°C) for complete ethanol removal. The dried extracts were refrigerated at 2-8°C until required for use. The concentration of the extract was determined by drying a known volume and measuring the dry weight.

2.2 Quantitative Phytochemical Analysis

Flavonoid determination was carried out by employing the ethyl acetate gravimetric method [24]. A measured weight of the sample was hydrolyzed by boiling in 100ml of 2M HCL solution for 30minutes. It was allowed to cool and then filtered. The filtrate was treated with 20ml ethyl acetate and the flavonoid precipitate was recovered by filtration with weighed filter paper. After drying in the oven at 100^{o}C for an hour, it was calculated as follows:

$$\% \text{ Flavonoid} = \frac{100\ (W_2 - W_1)}{W}$$

Where, W_1 = weight of sample; W_2 = weight of empty filter paper; W_3 = weight of paper + flavonoid precipitate.

Tannin was determined using the colorimetric method of Trease and Evans [25]. One gramme of the processed sample was mixed with 50ml of distilled water and shaken, then allowed to stand for an hour at room temperature. The mixture was filtered through Whatman filter paper no. 42. An aliquot of the filtrate was treated with 3ml of 0.1m $FeCl_3$ solution in 0.1N HCL solution. 1ml of 0.008 potassium ferrocyanide solution was added to it. Meanwhile, standard tannin solution (tannic acid) was prepared and diluted approximately. 1ml of the standard solution was equally treated with F_3Cl_3 and ferrocyanide solution. Absorbance of the developed blue colored solution was measured in a spectrophotometer against reagent blank. Where necessary, the sample solution was diluted before spectrophotometric reading.

The formula below was used to calculate the tannin content:

$$\%\text{Tannin} = \frac{100 \times A,}{W} \times C \times \frac{V,}{A_s} \times \frac{D}{V,}$$

Where, W=weight of sample; A_u =Absorbance of sample; A_s = Absorbance of standard tannin solution; C= Concentration of standard tannin solution; V_f =total extract volume; V_a = volume of extract analyzed; T= titre value of sample; D=Dilution factor where applicable.

Alkaloid was determined by the alkaline precipitation gravimetric method [24]. Five grammes of the sample were soaked in 100mls of 10% acetic acid solution in ethanol. It was filtered and the filtrate (i.e. extract) was reduced to 1/4th of the volume by evaporation. The concentrated extract was treated with drop wise addition of concentrated aqueous ammonia (NH_4OH) until in excess. The precipitated alkaloid was recovered by filtration using a weighed filter paper. It was washed with 1% NH_4OH solution and dried in the oven at 100^{0}C for 30mins. After cooling in the desiccator, it was re-weighed. The formula below was used to calculate the alkaloid content:

$$\%\text{Alkaloid} = 100 \times \frac{(W_2 - W_1)}{W}$$

Where, W=weight of sample; W_1 =weight of empty filter paper; W_2=weight of paper + alkaloid precipitate (dry).

Saponin was determined using the method of Trease and Evans [25] was used. Saponin in the sample was first extracted by boiling 5g of the sample in 100mls of 20% ethanol solution for 30mins. It was filtered and the filtrate treated with 30ml of ethyl ether in a separating funnel. The ether layer was discarded while the aqueous layer was measured. The aqueous layer returned to the separating funnel and treated with 30mls of normal butanol to extract the saponins. The butanol extract was washed twice with 20mls of distilled water each time. It was then washed with 100mls of 50% NaCL solution and then removed into a weighed evaporating dish. It was evaporated to dryness (100^0C for 30mins), cooled in a desiccator and re-weighed. Saponin content was calculated thus:

$$\%\text{Saponin} = \frac{W_2 - W_1 \text{ x } 100}{W}$$

Where, W=weight of sample; W_1 = weight of empty dish; W_2 =weight of dish + dry extract.

Polyphenol was determined using the method of Trease and Evans [25]. 0.2g sample was mixed with 10ml of methanol and shaken for 30mins in a mechanized shaker then filtered. 1ml of the filtrate was mixed with equal volume of folingocalfeau reagent in a flask. 2ml of 20% Na_2CO_3 solution was added to it. 1ml of standard phenol solution was equally treated. The absorbance of the developed blue coloration was measured in a spectrophotometer at 620nm against reagent blank. The formula below was used to calculate phenol content.

$$\%\text{ phenol} = \frac{100}{W} \text{ x } \frac{au}{as} \text{ x c x } \frac{vt}{va}$$

Where, W=weight of sample; au=absorbance of sample; as=absorbance in std phenol solution; c= concentration in std phenol solution; vt=total volume of extract analyzed

2.3 In vitro Antioxidant Assay

The free radical scavenging assays at varying concentrations of the two leaves extracts (table 1) were measured using DPPH and NO.

2.3.1 1, 1-diphenyl- 2-picrylhydrazyl (DPPH) Radical Scavenging Assay.

The DPPH is a stable free radical and is widely used to assess the radical-scavenging activity of antioxidant compounds. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH [26][27]. This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple colour is monitored at 517nm. The free radical scavenging activity can be measured by using 1, 1-diphenyl-2-picryl-hydrazyl by the method of McCune and Johns [28]. The reaction mixture (3.0ml) consisted of 1.0 ml of DPPH (0.3mM) in methanol, 1.0 ml of the extract at different concentrations (50- 800µg/ml) and 1.0ml of methanol. It was incubated for 25 min in the dark. Blank solutions were prepared with each test sample solution (2.0 ml) and 1.0ml of methanol. L-ascorbic acid was used as positive control. Thereafter, the absorbance of the assay mixture was measured at 517nm against each blank. Lower absorbance of the reaction mixture indicated higher radical scavenging activity.The percentage of inhibition can be calculated using the formula:

$$\text{Inhibition (\%)} = \frac{(A_{Contol} - A_{Test}) \text{ x } 100}{A_{Control}}$$

Where $A_{Control}$ is the absorbance of control and A_{Test} is the absorbance of test.

2.3.2 Nitric oxide radical (NO) scavenging assay

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiologic pH interacted with oxygen to produce nitrite ions, which were measured using the Griess reaction [29]. 3.0 ml of 10 mM sodium nitroprusside in phosphate buffer was added to 2.0ml of extract and reference compound in different concentrations (50- 800µg/ml). The resulting solutions were then incubated at 25°c for 60 min. A similar procedure was repeated with methanol as blank, which served as control. To 5.0ml of the incubated sample, 5.0ml of Griess reagent (1% Sulphanilamide, 0.1% naphthyethylenediaminedihydrochloride in 2% H_3PO_3) were added. The absorbance of the chromophore (purple azo dye) formed was measured at 540nm.Percent inhibition of the nitric oxide generated was measured by comparing the absorbance values of control and test preparations.Percentage nitric oxide scavenging activity was calculated using following equation:

$$(A_{Control} - A_{Test}) \text{ x } 100$$

$$\% \text{ Scavenging} = \frac{}{A_{Control}}$$

Where $A_{Control}$ is the absorbance of control and A_{Test} is the absorbance of test.

Table 1. Concentrations (µg/ml) of extracts of GL, OG, GLOG and standard used for freeradical scavenging test

Concentrations (µg/ml) of extracts and standard			
GL	OG	Combined (GLOG)	Standard (Vitamin C)
50	50	25+25	50
200	200	100+100	200
400	400	200+200	400
600	600	300+300	600
800	800	400+400	800

2.4 Statistical analysis

The results were reported as means±SD from six repeated determinations and evaluated with the analysis of student's t-test. Differences were considered to be statistically significant at $P<0.05$.

III. Results

Table 2. Quantitative phytochemical composition of leaves of G. latifolium and O. gratissimum

	Flavonoids (%)	Tannins (%)	Saponins (%)	Polyphenols (%)	Alkaloids (%)
GL	0.80 ±0.01	0.69 ±0.00	2.12 ±0.01	1.97 ±0.02	2.01 ±0.04
OG	1.90 ±0.01*	0.79 ±0.01*	0.67 ±0.01*	0.68 ±0.03*	2.82 ±0.02*

Values are expressed as mean ± SEM; n = 4; *p<0.05 vs GL.

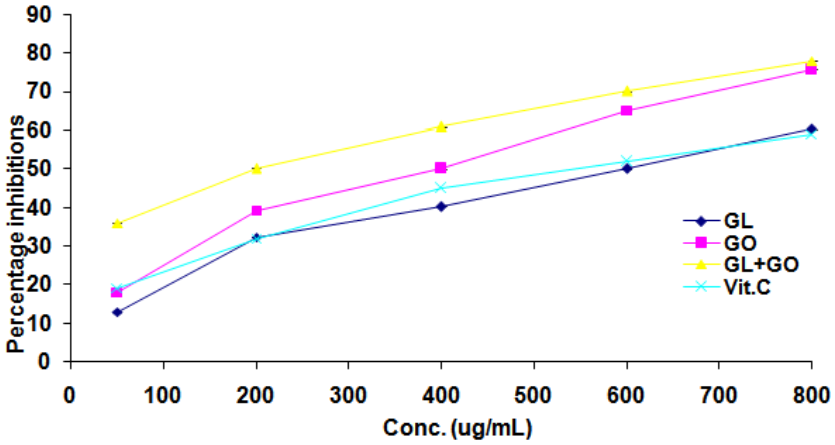

Fig.1 Percentage inhibitions of different concentrations of the leaves extracts and ascorbic acid in the DPPH scavenging activity.
Values are mean ± SEM, n = 6.

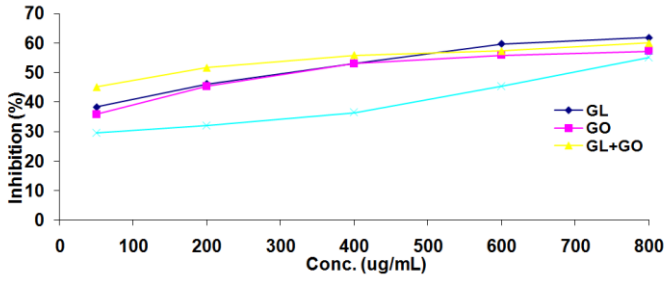

Fig. 2 Percentage inhibitions at different concentrations of the leaves extracts and ascorbic acid in the nitric oxide scavenging activity.
Values are mean ± SEM, n = 6.

The quantitative phytochemical compositions (table2) revealed the presence of flavonoids, tannins, saponins, polyphenols and alkaloids in the leaves extracts of *G. latifolium* (GL)and *O. gratissimum* (OG).From the results, OG leaves were significantly ($p<0.05$) richer in flavonoids (1.90±0.01), tannins (0.79±0.01), saponins (0.68 ±0.03) and alkaloids (2.82 ±0.02) than GL. The percentage of alkaloids (2.82 ± 0.02) in OG is significantly ($P<0.05$) higher than that of GL (2.01 ±0.04). The leaves extracts of GL exhibited a higher percentage of polyphenol (1.97 ± 0.02) and saponins (2.12 ±0.01) compared to OG.

The results of percentage inhibition of different concentrations (50, 200, 400, 600, 800µg/ml) of the extracts of *Ocimum gratissimum* (OG) and *Gongronema latifolium* (GL) (singly and in combination) in the DPPH and nitric oxide (NO) scavenging activity test are presented in figures 1 and 2 respectively, It was generally observed that the concentrations of the extracts of the two plants were inversely proportional to the absorbance while the absorbance showed direct proportionality to the percentage inhibition of nitric oxide and DPPH radicals. Also from the results, the DPPH and nitric oxide free radical scavenging activities of single GL, OG, combined GL and OG and ascorbic acid (a standard anti-oxidant drug) exhibited a dose dependent inhibitory effect at varying concentrations. Significant inhibition at different concentrations was observed in combined extracts group when compared to ascorbic acid. In DPPH and NO scavenging tests, the percentage inhibitions of ascorbic acid were remarkably lower than that of OG and GL at all extract concentrations but higher at 50µg/ml in DPPH scavenging test. It was observed that the DPPH and nitric oxide scavenging potency of the combined (GLOG) extracts was higher (yellow line graph) than that of single GL (deep blue line graph) and OG (pink line graph) extracts and standard (sky blue graph), and that of single extracts higher than that of standard. This was evidently shown in 50% inhibitory concentrations (IC_{50}) of 199.48µg/ml for GLOG, 598.68µg/ml for GL and 399.20µg/ml for OG in DPPH scavenging test and 193.20µg/ml for GLOG, 376.44µg/ml for GL and 376.72µg/ml for OG in nitric oxide scavenging test.

IV. Discussion

The presence study was conducted on quantitative phytochemical analyses of *Gongronem alatifolium* (GL) and *Ocimum gratissimum* (OG) and their combined in vitro DPPH and NO radical scavenging activities.The presence of phytochemicals in the leaves extracts of these plants revealed that these bioactive compounds may be responsible for the medicinal value of GL and OG. Alkaloids are known to play some metabolic role and also to control development in the living system. The presence of saponins in the leaves of these two plants may corroborate to the use of the leaves in lowering plasma cholesterol level and therefore serving as a potential remedy for the management of atherosclerosis and other related disorder like diabetes and obesity [30]. Saponins can cause the leakage of proteins and degradation of cell wall enzymes from the cell [31]. It content makes the leaves an important an important source of detergents, surface active agents used in industrial applications and also possesses beneficial health effect [32]. Flavonoids have antioxidants property and are effective on endothelial function hence reducing the oxidation of LDL [33]. Flavonoids have been used against the cancer causing tumors and it inhibits the promotion of growth and progression of tumors [34]. Phenols are important in regulation of plant growth, development and disease resistance, and when mixed with the flavonoids compounds in plants are reported to show multiple activities like antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory among others [35][36]. Tannins which are present in GL and OG leaves are bioactive compounds known to have potential antiviral activity [33], inhibit pathogenic fungi as well as potential prophylactic and therapeutic effect against cancer cells, but via different mechanism [37]. Tannins contribute to various medicinal properties such as antimicrobial, anti-inflammatory and astringent activity [38]. Nutritionally, tannins have been observed to form complexation with protein thereby preventing protein absorption.

DPPH and Nitric oxide (NO) radicals were found to be dose-dependently inhibited by the extracts of OG and GL singly and in combination. NO is generated at physiological pH from sodium nitropruside. Also, the bleaching of DPPH by the leaves extracts of OG and GL was measured in order to determine the free radical quenching capacity of the extract. The stable free radicals, produced by DPPH and NO were scavenged by the extracts in a dose- dependent manner. Additionally, It has been established that antioxidant effect of plant products is mainly due to radical-scavenging activity of phenolic compounds such as flavonoids, polyphenols, tannins, and phenolic terpenes[39][40][41]. The high phenolic contents of medicinal plants are also known to possess high anti-inflammatory [42] and anti-carcinogenic activities [41] and are of great value in decreasing the risk of many human diseases. Many studies have shown that many polyphenols (due to their redox properties) contribute significantly to the antioxidant activity [43][44]and do not only scavenge, adsorb and neutralize free radicals effectively but also play an important role in quenching singlet and triplet oxygen or decomposing peroxides [45]. In this study, the highest DPPH and NO scavenging activities were significant in combined extracts (GLOG) compared to single extracts.

V. Conclusion

From the study, the leaves extracts of GL and OG possessed in vitro free radical antioxidant quenching potency with the combined extracts exhibiting the highest inhibitory effect. The capacity of these extracts to mop up 50% nitric oxide and DPPH free radicals might not be unconnected with the synergistic contribution of antioxidant phytochemicals (flavonoids, polyphenols, etc) and vitamins (A, E and C) present in the two leave extracts. This justifies the medicinal use of the plants as food and for treatment of various maladies [46].

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