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COMPARATIVE AND SYNERGISTIC ANTIOXIDANT PROPERTIES OF *CARICA PAPAYA* AND *AZADARICHTA INDICA* LEAVES

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ABSTRACT

Comparative and synergistic antioxidant properties of *Carica papaya* leaf (CPL) and *Azadarichta indica* leaf (AIL), which are popularly used as medicinal plants were evaluated by analyzing the individual leaves and their 1:1 (mass/mass) homogenous combination (CPL+AIL) concurrently. The antioxidant properties measured were the levels of total phenol, tannin, total flavonoid, total carotenoid, vitamin C, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging ability, trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP). Of the two plants, AIL had significantly higher ($P < 0.05$) total phenol and tannin contents, as well as significantly higher ($P < 0.05$) DPPH scavenging ability, TEAC and FRAP, while CPL had significantly higher ($P < 0.05$) total flavonoid, total carotenoid and vitamin C contents. The combination (CPL+AIL) showed a synergy in the total phenol, tannin, total flavonoid, and total carotenoid contents, and in the DPPH free radical scavenging ability. The DPPH scavenging ability of the leaves increased in a dose-dependent manner, having IC_{50} in the order of APL< CPL+AIL< CPL [APL (0.50 mg/ml), CPL+AIL (0.61 mg/ml), CPL (0.93 mg/ml)]. The combination further showed an additive synergistic interaction in the TEAC and FRAP in the order of CPL+AIL > APL > CPL. However, there was a negative synergistic interaction in the vitamin C content. The study therefore concludes that while AIL has better antioxidant activities than CPL, the combination of CPL and AIL produces synergistic antioxidative interactions, which could be explored in their medicinal uses.

INTRODUCTION: Oxidative stress which occurs when the oxidative burden of the body exceeds its antioxidant capacity plays an important role in the pathogenesis of various degenerative diseases such as cancer, coronary heart disease and Alzheimer's disease [1, 2, 3, 4] and aging [5]. The inhibition of such free radical-mediated pathophysiology has become a central focus for research efforts designed to prevent or ameliorate tissue injury, and a number of studies have been performed to discover antioxidants from natural products or medicinal plants for the prevention or treatment of free radical-induced diseases [6]. The most likely and practical way to fight against degenerative diseases is to improve the body antioxidant status [7].

In living systems, free-radicals are generated as part of the body's normal metabolic process, and the free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, by bacterial leucocytes, through xanthine oxidase activity, atmospheric pollutants, and from transitional metal catalysts, drugs and xenobiotics [8].

Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability. Flavonoids and phenolic compounds widely distributed in plants have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory, and anti-carcinogenic. They were also suggested to be a potential iron chelator. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties [9].

Carica papaya Linn. (Pawpaw) is a plant that belongs to the family of *Caricaceae*. It is a herbaceous succulent plant with self-supporting stems [10]. Different parts of the plant are attributed with different medicinal values. For example, in African traditional medicine, the green leaves of papaya combined with leaves of *Azadirachta indica*, *Cymbopogon citratus*, *Psidium guajava* and stem bark of *Alstonia boonei* are boiled together and the hot infusion is drunk as one wine glass-full thrice daily in the treatment of malaria. Its fresh leaves are also efficacious in the treatment of gonorrhea, syphilis and amoebic dysentery [11].

Azadirachta indica A. Juss (neem) is well known as one of the most versatile medicinal plants having a wide spectrum of biological activity. All parts of the plant have been used for medicinal purposes including fruits, seeds, leaves, roots and barks [12]. The hypoglycemic actions of its leaves, stem bark and seeds have been articulated in a review by Biswas et al. [12], and Ebong et al. [13] indicated recently in their studies, the relative antidiabetic efficacy of its extracts when combined with that of *V. amygdalina*, over and above the individual extracts.

Medicinal plants in combination are shown to interact synergistically with high efficacy and have a broader spectrum of action [14]. In the practice of traditional medicine in Africa, the parts of the plant used are often prepared as decoction, concoction or simply soaked [15]; in which case different plants that are considered to exert similar curative effects are combined. Therefore, the objective of this study is to compare the antioxidant properties of *C. papaya* and *A. indica* leaves and evaluate their synergistic antioxidant potentials *in vitro*.

MATERIALS AND METHODS:

1. **Samples collection and preparation:** Fresh leaf samples of *C. papaya* and *A. indica* were collected from the two plants in Akingbele village in Ibadan, Nigeria, in February 2012. The leaves were authenticated at Department of Botany and Microbiology, University of Ibadan, Nigeria. Thereafter, the leaves were sorted, air-dried, milled into a fine particle size, put in air-tight bottles and stored in the refrigerator for subsequent analyses. At the point of analysis, a 1:1 (mass/mass) homogenous mixture of the milled leaves of *C. papaya* and *A. indica* was prepared by homogenizing 25g of each together.

   All the chemicals used for analysis were of analytical grade.

2. **Preparation of extract:** Methanolic leaf extract was prepared following the method of Chan et al. [16], by adding 25 mL of methanol to 0.5g of sample contained in a covered 50 mL centrifuge tube, and shaking continuously for 1 h at room temperature. The mixture was centrifuged at 3,000 rpm for 10 min, and then the supernatant was collected and store at -4^{o}C until analysis.

3. **Determination of total phenol content:** The total phenol content of samples methanolic extracts was determined according to the Folin–Ciocalteu method reported by Chan *et al.,* [16]. Briefly, 300 µL of extract was dispensed into test tube (in triplicates). To this was added 1.5 mL of Folin–Ciocalteu reagent (diluted 10 times with distilled water), followed by 1.2 mL of Na_2CO_3 solution (7.5% w/v).

   The reaction mixture was mixed, allowed to stand for 30 min at room temperature before the absorbance was measured at 765 nm against a blank prepared by dispensing 300 µL of distilled instead of sample extract. TPC was expressed as gallic acid equivalent (GAE) in mg/g material. The calibration equation for gallic acid was Y = 0.0645x – 0.0034 (R^2 = 0.9998).

4. **Determination of tannin content:** Tannin content of samples was determined according to the method of Padmaja [17] as follows. Sample (0.1g) was extracted with 5 mL of acidified methanol (1% HCl in methanol) at room temperature for 15 minutes. The mixture was centrifuged at 3,000rpm for 20minutes. 0.1 mL of the supernatant was added with 7.5 ml of distilled water, 0.5 ml of Folin-Denis reagent, 1 ml of 35% sodium carbonate solution and diluted to 10 ml with distilled water. The mixture was shaken well, kept at room temperature for 30 min and absorbance was measured at 760 nm. Blank was prepared with water instead of the sample. Tannin content was expressed as tannic acid equivalent (TAE) in mg/g material. The calibration equation for tannic acid was Y = 0.0695x + 0.0175 (R^2 = 0.9978).

5. **Determination of total flavonoid content:** Total flavonoid content was determined using aluminum chloride method as reported by kale et al. [18]. 0.5 mL of methanolic extract was dispensed into test tube, followed by 1.5 mL of methanol, 0.1 mL of aluminum chloride (10%), 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. The reaction mixture was mixed, allowed to stand at room temperature for 30 minutes, before absorbance was read at 514 nm. TFC was expressed as quercetin equivalent (QE) in mg/g material. The calibration equation for quercetin was Y = 0.0395x – 0.0055 (R^2 = 0.9988).

6. **Determination of total carotenoid content:** Total carotenoid content of samples was determined according the method of Rodriguez-Amaya [19]. Briefly, 0.2 – 0.3g of sample was transferred into a mortar and a small amount (0.5g) of celite was added. The mixture was ground with 20 ml of cold acetone and filtered with suction through a Buckner funnel with filter paper. Then the mortar, pestle, funnel and residue were washed with small amount of acetone, receiving the washings in the suction flask through the funnel. The residue was further macerated with cold acetone, filtered into the same suction flask repeatedly until the residue became colourless. After extraction, the acetone extract was partitioned with petroleum ether by putting 5 mL of distilled water and 10 mL of petroleum into a 250 mL separatory funnel, followed by slowly transferring the acetone extract in to the same funnel through the walls. The mixture was washed repeatedly with distilled water until acetone was completely washed and drained off. Thereafter, the total carotenoid was separated from other pigments in the ether extract by passing it through an alumina (Activity III) column, and eluting the total carotenoid with ether to a final volume of 25 mL. The absorbance of the extract was read at 450nm. To avoid the degradation of the total carotenoid, analysis was carried out under subdued light and the extract was collected into a vial wrapped with aluminum foil.

7. **Determination of vitamin C content:** The vitamin C content of the aqueous extract was determined using the method reported by Benderitter et al. [20]. Briefly, 75 µL DNPH (2 g dinitrophenyl hydrazine, 230 mg thiourea and 270 mg $CuSO_4.5H_2O$ in 100 mL of 5M H_2SO_4) was added to 500 µL reaction mixture (300 µL appropriate dilution of hydrophilic extract with 100 µL of 13.3% trichloroacetic acid and distilled water). The reaction mixture was subsequently incubated for 3 h at 37^oC, then 0.5 mL of 65% H_2SO_4 (v/v) was added to the medium, and the absorbance was measured at 520 nm, and the vitamin C content of the sample was subsequently calculated from the calibration curve prepared with ascorbic acid standard. The calibration equation for ascorbic acid was Y = 0.0139x + 0.0104 (R^2 = 0.9974).

8. **Estimation of DPPH free-radical-scavenging ability:** The free-radical-scavenging ability of the methanolic extracts against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was evaluated as described by Cervato et al. [21] with slight modification. Briefly, appropriate dilution of the extracts (1 mL) was mixed with 3 mL of 60µM methanolic solution of DPPH radicals; the mixture was left in the dark for 30 min before the absorbance was taken at 517 nm. The decrease in absorbance of $DPPH^{\cdot}$ on addition of test samples in relation to the control was used to calculate the percentage inhibition (%Inh.) following the equation: %Inh. = $[(A517_{control} – A517_{sample}) \div A517_{control}] \times 100$.

The IC_{50}, which stands for the concentration of extract required for 50% scavenging activity, was calculated from the dose-inhibition linear regression curve of each extract.

9. **Estimation of ABTS* radical-scavenging ability:** The ABTS* radical-scavenging ability of both extracts were determined according to the method described by Sellappan and Akoh [22]. The ABTS* radical was generated by incubating equal volume of a 7 mM ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM) in the dark for 16 h at room temperature and adjusting the absorbance at 734 nm to 0.7 ± 0.02 with 95% ethanol. Then 0.2 mL appropriate dilution of the extract was added to 2.0 mL ABTS* solution and the absorbance was measured at 734 nm after 15 min. The trolox equivalent antioxidant capacity (TEAC) was subsequently calculated. The calibration equation for TEAC was Y = -0.0505x + 0.1954 (R^2 = 0.9902).

10. **Determination of ferric reducing antioxidant power (FRAP):** The reducing property of the methanolic extracts was determined by assessing the ability of the extract to reduce $FeCl_3$ solution as described by Oyaizu [23]. A 2.5 mL aliquot was mixed with 2.5 mL of 200mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50^{o}C for 20 min. and then 2.5 mL of 10% trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. Then 5 mL supernatant was mixed with an equal volume of water and 1 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric reducing antioxidant property was subsequently calculated.

11. **Statistical analysis:** Experimental results were reported as Mean ± standard deviation of triplicate parallel measurements. Analysis of variance (ANOVA) was carried out for the results using SAS [24] at 95% confidence level. Significant differences between means were determined by Tukey Honest Significant Difference test.

RESULTS AND DISCUSSION: Although there are reports in part on the antioxidant properties of *Carica papaya* and *Azadarichta indica* leaves [25, 26], no reports exist on their comparative and synergistic antioxidative properties. This study therefore investigated the comparative and synergistic antioxidative properties of *C. papaya* and *A. indica* leaves popularly used as medicinal plants.

The antioxidant phytochemicals contents of the leaves are presented in **table 1**. The total phenol and tannin contents of CPL, AIL and CPL+AIL were significantly different ($\rho < 0.05$) in the order of AIL > CPL+AIL > CPL. This clearly showed that relative to CPL with total phenol and tannin contents of 8.98 ± 0.05 mg/g and 9.31 ± 0.30 mg/g respectively, AIL with total phenol and tannin contents of 13.32 ± 0.19 mg/g and 14.85 ± 0.38 mg/g respectively, is a better source of phenol and tannin. These total phenol and tannin levels of AIL obtained in this study are higher than the 6.3mg/g and 3.5 mg/g reported for tannin and total phenol, respectively, by Atangwho et al. [27]. However, the combination of the two (CPL+AIL) produced a synergistic effect, having total phenol content of 11.05 ± 0.01 mg/g and tannin content of 11.28 ± 0.38 mg/g.

TABLE 1: TOTAL PHENOL, TANNIN, TOTAL FLAVONOID, TOTAL CAROTENOID AND VITAMIN C CONTENT OF CPL, AIL AND CPL+AIL (IN MG/G DRY WEIGHT).

Phytochemical	CPL	AIL	CPL+AIL
Total phenol	8.98 ± 0.05^{C}	13.32 ± 0.19^{A}	11.05 ± 0.01^{B}
Tannin	9.31 ± 0.30^{C}	14.85 ± 0.38^{A}	11.28 ± 0.38^{B}
Total flavonoid	5.24 ± 0.11^{A}	2.69 ± 0.08^{C}	3.29 ± 0.16^{B}
Total carotenoid	3.38 ± 0.02^{A}	0.20 ± 0.01^{C}	0.43 ± 0.04^{B}
Vitamin C	1.44 ± 0.03^{A}	1.13 ± 0.02^{B}	0.77 ± 0.01^{C}

Data represent the mean ± standard deviation of triplicate readings. Values with the same uppercase superscript letter along the same row are not significantly different (P>0.05).

Plant-derived phenolic compounds are well known to exhibit antioxidant activity through a variety of mechanisms, including free radical scavenging, lipid peroxidation and chelating of metal ions [28]. They were reported to eliminate radicals due to their hydroxyl groups [29].

In addition to these antioxidant effects, phenols were reported to inhibit alpha-amylase, sucrase, as well as the action of sodium glucose-transporter 1 (SGLUT-1) of the intestinal brush border, hence their antidiabetic action [30].

Similarly, the levels of total flavonoid and total carotenoid in CPL, AIL and CPL+AIL (table 1) were significantly different ($\rho < 0.05$), but in the order of CPL > CPL+AIL > AIL. This also indicated that the combination of the two leaves had a synergistic effect, although CPL proved to be a richer source of total flavonoid and total carotenoid than AIL. Flavonoids have antioxidant activity and could therefore lower cellular oxidative stress [31], which has been implicated in the pathogenesis of various neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [32]. The high flavonoid content of the leaves may have contributed to their medicinal properties.

Carotenoids are lipid-soluble antioxidant, which have been credited with health-promoting effects including immune-enhancement and reduction of the risk of developing degenerative diseases such as cancer, cardiovascular diseases (CVD), cataract and macular degeneration [33, 34]. These activities have been attributed to an antioxidant property, specifically to the ability to quench singlet oxygen and interact with free radicals [35].

There was a negative synergistic interaction in the vitamin C content of the combination (CPL+AIL). While CPL had significantly higher ($\rho < 0.05$) vitamin C content than AIL, the vitamin C content of AIL was significantly higher ($\rho < 0.05$) than that of CPL+AIL (CPL > AIL > CPL+AIL). The reason for this negative synergy is not clear, but it may be due to the chemical nature and reactivity of the compounds present in the mixture, which may have affected the vitamin C level. As a water-soluble antioxidant, vitamin C is in a unique position to 'scavenge' aqueous peroxyl radicals before these destructive substances damage the lipids [36].

DPPH, a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of extracts of the leaves and their combination. As antioxidants donate protons to this radical, the absorption decreases.

The decrease in absorption is taken as a measure of the extent of radical scavenging. The concentration-response curves for inhibition of DPPH free-radical by the extracts are shown in **Fig. 1**. CPL, AIL and CPL+AIL all showed free radical scavenging activity in a dose-dependent manner in the order of AIL > CPL+AIL >CPL. The IC_{50} value for each extract, defined as the concentration of extract causing 50 per cent inhibition of DPPH absorbance, is shown in **table 2**.

Relative to CPL with IC_{50} of 0.93 mg/ml, AIL had IC_{50} of 0.50 mg/ml. Since IC_{50} is a measure of inhibitory concentration, a lower IC_{50} value is a reflection of greater antioxidant activity of the sample. Hence AIL displayed a higher free radical scavenging ability than CPL. However, the IC_{50} of CPL+AIL (0.61 mg/ml) is an indication of a synergistic interaction in the free radical scavenging ability of the combination of the two plants.

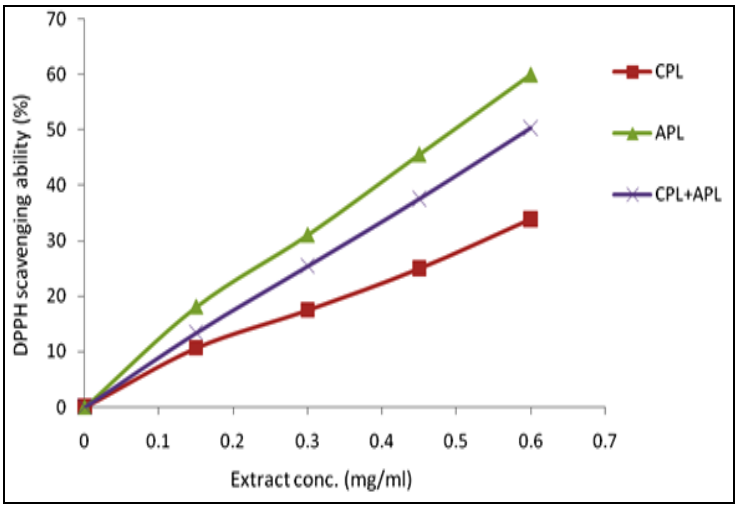

FIGURE 1. CONCENTRATION-RESPONSE CURVES FOR INHIBITION OF DPPH FREE-RADICAL BY CPL, APL AND CPL+APL EXTRACTS

TABLE 2: DPPH IC_{50}, TROLOX EQUIVALENT ANTIOXIDANT CAPACITY (TEAC) AND FERRIC REDUCING ANTIOXIDANT POWER (FRAP) OF CPL, AIL AND CPL+AIL

Antioxidant activity	CPL	AIL	CPL+AIL
DPPH IC_{50} (mg/ml)	0.93	0.50	0.61
TEAC (mM TE/g)	0.53 ± 0.13^{C}	0.78 ± 0.09^{B}	0.93 ± 0.07^{A}
FRAP(mg GAE/g)	6.82 ± 0.19^{B}	8.32 ± 0.06^{A}	8.55 ± 0.15^{A}

Data for FRAP and TEAC represent the mean ± standard deviation of triplicate readings.

TE is Trolox equivalent. Values with the same uppercase superscript letter along the same row are not significantly different (P>0.05).

ABTS***** scavenging ability reported as the Trolox equivalent antioxidant capacity (TEAC) is presented in table 2. The results revealed that the ABTS***** scavenging ability of CPL, AIL and CPL+AIL were significantly different ($\rho < 0.05$) in the order of CPL+AIL > AIL > CPL. This is a clear indication that AIL, with TEAC of 0.78 ± 0.09 mM TEAC/g, had a better ABTS***** scavenging ability than CPL with TEAC of 0.53 ± 0.13 mM TEAC/g. Interestingly, the combination of the two leaves (CPL+APL) had the highest ABTS***** scavenging ability (0.93 ± 0.07 mM TEAC/g), which reflects an additive synergistic interaction of CPL and AIL.

The ABTS assay is based on the inhibition of the absorbance of the radical cation ABTS***** which has a characteristic long wavelength absorption spectrum. ABTS radicals are more reactive than DPPH radicals and unlike the reactions with DPPH radical which involve H atom transfer; the reactions with ABTS radicals involve electron transfer process [9].

The reducing power reported as gallic acid equivalents, GAE (table 2) revealed that CPL, AIL and CPL+AIL were all able to reduce Fe(III) to Fe(II). However, while there was no significant difference ($\rho > 0.05$) in the reducing power of CPL+AIL and AIL, their reducing powers (8.55 ± 0.15 mg GAE/g and 8.32 ± 0.06 mg GAE/g, respectively) were significantly higher ($\rho < 0.05$) than that of CPL (6.82 ± 0.19 mg GEA/g). This indicates that AIL extract is a better electron and/or hydrogen donor than CPL extract.

The higher reducing power of AIL compared to CPL extract is attributable to its higher total phenol content, which also reflected in its higher DPPH free radical scavenging ability and TEAC. This is in agreement with the findings of Manaharan [37], who reported a positive correlation ($R^2 = 0.9218$) between total phenolic content and antioxidant activity. There was a slight additive synergistic effect in the reducing power of the combination (CPL+AIL); however this was not as prominent as in the TEAC.

CONCLUSION: In this study higher total phenolic content and antioxidant activities (DPPH and ABTS radical-scavenging ability, and reducing power), were observed for *Azadarichta indica* leaf (AIL) compared to *Carica papaya* leaf (CPL); this is an indication that if used individually, *A. indica* leaf could be more effective in ameliorating oxidative problems in diseased conditions than *C. papaya* leaf. Interestingly, the combination of *C. papaya* leaf and *A. indica* leaf (CPL+AIL) displayed three types of synergistic interactions – synergy, additive synergy and negative synergy, in their antioxidant properties with synergy being the most prominent.

This study, being the first to report synergistic interactions in the antioxidant properties of *C. papaya* and *A. indica* leaves, confirmed that medicinal plants in combination interact synergistically with high efficacy and have a broader spectrum of action.

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