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ANTIOXIDANT ACTIVITIES OF ETHANOL LEAF EXTRACTS OF PSIDIUM GUAJAVA L., TERMINALIA CATTAPA L. AND THEIR COMBINED FORMULATION

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ABSTRACT

Free radicals in the body are the cause of many chronic and debilitating conditions in humans by acting as oxidants in the body. This research was aimed at assessing the antioxidant potential of the leaf extracts of *Psidium guajava, Terminalia catappa* and a combination of both. The leaves of both plants were independently extracted with ethanol. Equal portions of the extracts of both species were also combined to obtain a formulation of ratio 1:1. The extracts were subjected to in vitro assays including DPPH (2, 2-Diphenyl-1-picrylhydrazyl) and nitric oxide scavenging activities, while in vivo antioxidant assays were evaluated by measuring superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and glutathione peroxidase (GPx) levels in Wistar rats following oral administration of 200, 400 and 800 mg/kg b.wt. respectively of the 1:1 extract formulation. The three extracts demonstrated dose dependent DPPH scavenging activity, although the 1:1 extract formulation was shown to possess lower free radical scavenging activity compared to the individual plant extracts. At the highest dose, the 1:1 extract formulation was shown to have exhibited a higher nitric oxide scavenging activity than the control, gallic acid. The 200 mg/kg b.wt. of 1:1 extracts formulation possess the highest antioxidant activity in the SOD and MDA assays. The study supports that ethanol leaf extracts of P. guajava and T. catappa possess potent antioxidant properties. However, additional work is required to obtain a more effective formulation from a combination of both extracts.

Keywords: Antioxidant, in vivo, Psidium guajava, Terminalia cattapa, Formulation, Leaf extracts

INTRODUCTION

The presence of free radicals in the body is the cause of many chronic and debilitating conditions in humans such as cardiovascular diseases, neurodegenerative diseases, cancer, aging, atherosclerosis, diabetes etc. These free radicals act as oxidants in the body, oxidizing nucleic acids, proteins, lipids or DNA and can initiate degenerative diseases (Yeshiwas and Mekonnen, 2018). Examples of free radicals are; hydrogen peroxide (H_2O_2), superoxide anion O_2^-, hydroxyl radical (OH^-) and lipid peroxyl. They can be generated as a result of metabolic activities in body tissues or disease conditions as well as introduced into the body through external sources such as foods, drugs, UV exposure and polluted environments. There is the presence of endogenous (substances/enzymes in the body) and exogenous substances collectively called antioxidants which counteracts the effect of free radicals and maintains balance. Endogenous enzymes include; catalase, superoxide dismutase and glutathione peroxidase/reductase. Exogenous substances such as C and E vitamins, β-carotene, uric acid, polyphenols, flavonoids, flavones and flavonols are also effective (Qian and Nihorimbere, 2004; Yeshiwas and Mekonnen, 2018).

Phytochemicals also possess antioxidant activities and play a protective role against free radicals (Zhang *et al*., 2015). Crude extract of *Curvularia* sp., an endophytic fungus isolated from the leaves of *Piliostigma thonningii*, demonstrated antioxidant activities (Ogbiko *et al.,* 2021). Antioxidant phytochemicals includes; polyphenols, flavonoids, quercetin, carotenoids, ascorbic acid (vitamin C), folic acid, vitamin E etc. An ideal antioxidant should readily absorb and mop up free radicals, chelate redox metals at physiologically relevant levels (Krishnaveni, 2014).

Psidium guajava L. commonly called guava is a member of the family Myrtaceae. The plant possesses pharmacologically important metabolites which contribute to its biological activities as anti-diabetic, anti-ulcer, anti-diarrhoeal, antimicrobial, anti-oxidant, cardioprotective effects, hepatoprotective, antipyretic, spasmolytic, immunomodulatory and contractile effect (Das *et al*., 2008; Rishika and Sharma, 2012; Jayakumari *et al*., 2012; Uduak *et al*., 2012).

Terminalia catappa L., commonly called almond fruit, belongs to the family Combretaceae. Some biological profile of the plant extract includes; antioxidant, anticancer, anti-HIV reverse transcriptase, hepato-protective effect, anti-inflammatory, anti-hepatitis, anti-diabetic, aphrodisiac and antiulcer activities (Annegowda *et al*., 2010; Bharath *et al*., 2014; Silva *et al*., 2015). *T. catappa* have been reported to contain substantially large proportion of active compounds such as polyphenols (flavonoids in particular), essential minerals (Cu, Zn, Fe and Mn), essential fatty acids and alkaloids (Douati *et al*., 2017).

Although both *P. guajava* and *T. cattapa* extracts have been reported to possess antioxidant capacities, this research was aimed at assessing the antioxidant potential of a combination of the leaf extracts of both *P. guajava* and *T. cattapa*.

MATERIALS AND METHODS

Collection of Plant Material

Fresh leaves of *Psidium guajava* and *Terminalia cattapa* were collected from mature trees of the individual plant species found within and around the University of Benin environment. They were authenticated at the Department of Plant Biology and Biotechnology, University of Benin. The *P. guajava* and *T. cattapa* specimens were assigned voucher numbers of UBHP378 and UBHT258 respectively and deposited in the herbarium.

Experimental Animals

A total number of 20 young male and female Albino Wistar rats were obtained from the Animal Unit of the Department of Animal and Environmental Biology, Faculty of Life Sciences, University of Benin, Benin City. The animals were housed in plastic cages in the Animal Unit of the Department of Animal and Environmental Biology, Faculty of Life Sciences, University of Benin, Benin City. The animals weighed between 170 to 240g.

They were kept and maintained under standard laboratory condition of room temperature, humidity and under twelve hour dark and light cycle. An acclimatization period of seven days to the new condition was allowed before the commencement of the experiment. They were fed with standard pellet diet and clean water, and the cages cleaned daily. The animal experimental procedures were in accordance with the ethical recommendations of Faculty of Life Sciences, University of Benin.

Extraction and Formulation

The leaves of *Psidium guajava* and *Terminalia cattapa* were air-dried under shade at room temperature. The shade dried, coarsely powdered leaves were extracted with absolute ethanol (99%) from Farmatrends Nigeria Ltd.

In addition to the respective extracts of *P. guajava* and *T. cattapa* obtained above, a 1:1 formulation was also prepared by combining both extracts in equal proportion, ratio 1:1.

Animal Experimental Protocol

Animal handling and use for *in vivo* antioxidant study was according to the OECD guidelines-407 (OECD, 2008). Twenty (20) albino rats randomised into 4 groups of 5 animals each were used. Group 1 rats received 0.5 ml/100 g b.wt. of distilled water orally, while doses of the extract formulation were administered orally to animals in Groups 2, 3 and 4 at 200, 400 and 800 mg/kg b.wt., respectively. The treatments were administered once daily for a period of 28 days. After the 28th day, the animals were sacrificed under mild chloroform anaesthesia and blood samples immediately collected through direct cardiac puncture with 21G needle attached to 5 mL syringe.

***In vitro* Anti-Oxidant Activity Study**

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) Photometric Assay

Scavenging activity on DPPH free radicals by the extract were assessed according to the method reported by Gyamfi *et al*. (1999) with modifications (Awah *et al*., 2010). The individual plant extracts and the 1:1 combination were prepared in different concentrations of 10, 50, 100, 200 and 400 µg/mL, respectively. A 2 ml solution of each concentration was separately diluted two-fold in methanol and mixed vigorously with 1.0 ml of 0.3 mM DPPH in methanol. It was allowed to stand at room temperature in the dark for 25 min. Blank solutions were prepared with each test sample solution (2.0 ml) and 1.0 ml of methanol, while the negative control was 1.0 ml of 0.3 mM DPPH solution plus 2.0 ml of methanol. L-ascorbic acid was used as the positive control. Thereafter, the absorbance of the assay mixture was measured at 518 nm against each blank with a UV-visible spectrophotometer.

The antioxidant activity was calculated as follows:

% antioxidant activity= 100 − [{(absorbance of sample − absorbance of blank) × 100} × (absorbance of control^{-1})].

Nitric Oxide Radical Scavenging Assay

The nitric oxide radical scavenging assay was according to the method of Panda *et al*. (2009). The extracts were prepared from a 10 mg/mL ethanol crude extract. These were then serially diluted with distilled water to make concentrations of 10, 50, 100, 200 and 400 µg/mL of the extracts (*P. guajava, T. catappa* and 1:1 extract formulation) and the standard gallic acid. These were stored at 4 °C for later use. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the ethanol extracts (10 − 400 µg/mL) and incubated at 25 °C for 180 mins. The extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. The colour tubes contained ethanol extracts at the same concentrations with no sodium nitroprusside. A volume of 150 µL of the reaction mixture was transferred to a 96-well plate. The absorbance was measured at 546 nm

The percentage antioxidant activity calculated as follows:

% antioxidant activity (AA) = 100 − [{(absorbance of sample − absorbance of blank)× 100} × (absorbance of control^{-1})].

***In Vivo* Anti-Oxidant Activity Study**

The blood sample obtained through direct cardiac puncture was allowed to clot for 30 minutes and then centrifuged at 2500 rpm for 15 minutes and serum was harvested (Yesufu *et al*., 2010). The harvested serum was then subjected to the following *in vivo* antioxidant tests (they were analysed in triplicates in each groups)

Malondialdehyde (MDA) Assay

Assay is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA); forming a MDA-TBA2 adduct (red colour complex) that absorbs light at 535 nm (Vershney and Kale, 1990).

Superoxide Dismutase (SOD) Assay

Principle of Reaction: Adrenaline auto-oxidizes rapidly in aqueous solution to adrenochrome, whose concentration can be determined at 420 nm using a spectrophotometer. The auto-oxidation of adrenaline depends on the presence of superoxide anions. The enzyme SOD inhibits the auto-oxidation of adrenaline by catalysing the breakdown of superoxide anion. The degree of inhibition is thus a reflection of the activity of SOD, and is determined at one unit of the enzyme activity (Misra and Fridovich, 1972).

$$2O_2^- + 2H \xrightarrow{SOD} H_2O_2 + O_2$$

Solutions were mixed properly and read at an absorbance of 420 nm.

Catalase (CAT) Assay

Catalase functions to catalyse the decomposition of hydrogen peroxide to water and oxygen (Cohen *et al*., 1970).

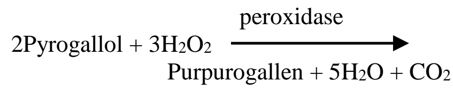
$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Absorbance was read at 480 nm between 30 – 60 seconds.

Glutathione Peroxidase (GPx) Assay

The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and reduce free hydrogen peroxide to water.

The reaction mixture consisted of 10 ml of buffered pyrogallol (0.05 M pyrogallol in 0.1 M phosphate buffer (pH 7.0) and 5 ml of 1% H_2O_2. To this was added 0.5 ml of tissue homogenate and absorbance change was measured at 430 nm for every 30 seconds for a minute (Flohe and Gunzler, 1984).



$$2\text{Pyrogallol} + 3H_2O_2 \xrightarrow{\text{peroxidase}} \text{Purpurogallen} + 5H_2O + CO_2$$

Statistical Analyses

Data were presented as mean ± standard error of mean (SEM) and one way analysis of variance (ANOVA) was used to compare the means. To establish significant differences between groups, the Turkey Multiple Comparison Test was used. Values were considered statistically significant if P value is less than 0.05 (P<0.05). Statistical analysis was carried out using Graphpad Prism 5.01.

RESULTS

DPPH scavenging activity of the different extracts shows a concentration dependent scavenging activity. However, amongst the three different extracts tested, *T. catappa* has the highest (77.96%) DPPH scavenging activity, which was comparable to the control (78.87%), ascorbic acid (Fig. 1).

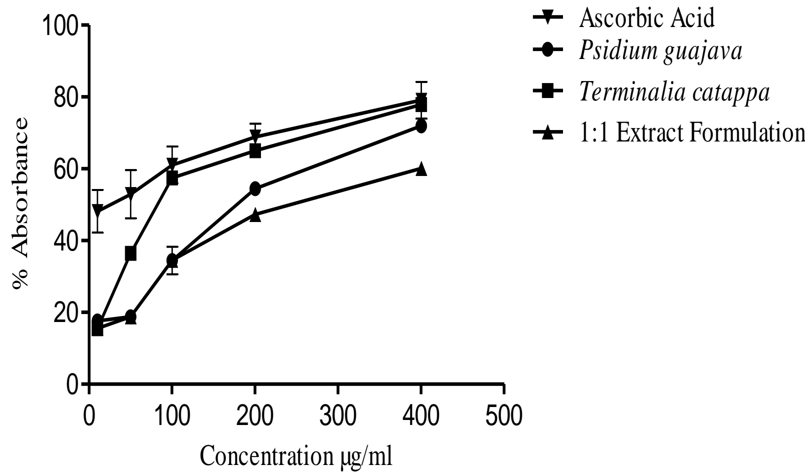


Fig. 1: DPPH scavenging activity of ethanolic leaf extracts of *P. guajava*, *T. catappa* and 1:1 extract formulation

Nitric Oxide Radical Scavenging Activity

Nitric oxide scavenging activity shows the activities of the extracts were concentration dependent. All three extracts (*P. guajava*, *T. catappa* and 1:1 extract formulation) demonstrated significantly (P<0.001) higher scavenging activities (82.11%, 67.23% and 71.97% respectively) than 57.12% obtained in gallic acid (control). *P. guajava* has the highest scavenging activity (Fig. 2).

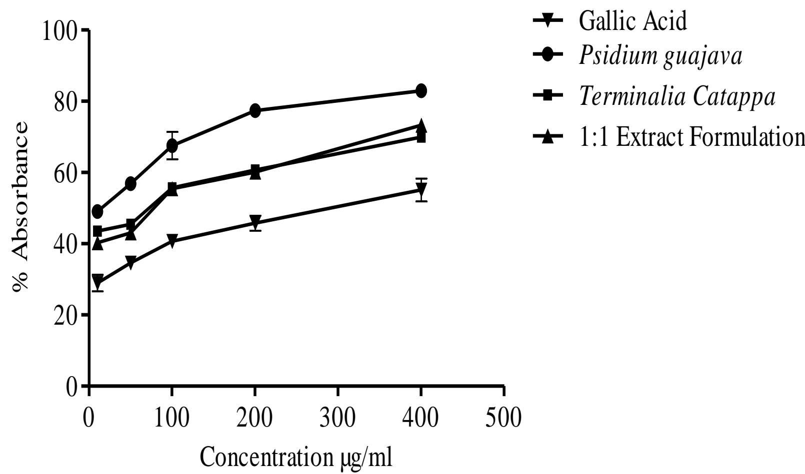


Fig. 2: Nitric Oxide scavenging activity of ethanolic leaf extracts of *P. guajava*, *T. catappa* and 1:1 extract formulation

Table reveals the 1:1 extract formulation treated groups exhibited a dose dependent decrease in antioxidant potential in the malondialdehyde (MDA) assay model. The 200 mg treated group possess the highest absorbance, significantly higher than the control ($P<0.001$). All doses of the extract also have significantly higher activities in superoxide dismutase and glutathione peroxidase assays. The extract, however lowers catalase activity significantly ($P<0.001$) when compared with control.

The 1:1 extract formulation treated groups demonstrate a dose dependent increase in the antioxidant potential in the glutathione peroxidise (GPx) antioxidant assay. At $P<0.001$ the 200 mg treated group shows a significantly lower absorbance than the control. There is no significant difference ($P>0.05$) between the control and 400 mg as well as the 800 mg treated groups.

Table 1: *In vivo* antioxidant activity of 1:1 ethanolic extract formulation of *P. guajava* and *T. catappa* leaves in Albino Wistar rats

Treatment	Activity			
	MDA $\times 10^3$ (Abs at 535 nm)	SOD (Abs at 420 nm)	CAT (Abs at 480 nm)	GPx (Abs at 430 nm)
Control (Distilled H_2O)	0.78±0.01	0.32±0.01	0.17±0.006	131.86±1.58
200 mg/kg Extract	1.40±0.04***	0. 70±0.00***	0.12±0.000***	96.11±2.65***
400 mg/kg Extract	0.70±0.03	0.39±0.02*	0.13±0.005	129.90±1.83
800 mg/kg Extract	0.64±0.02*	0.41±0.02**	0.13±0.000*	130.82±0.96

Values are mean±SEM: n=3; *=P<0.05; **= P<0.01; ***= P<0.001 for Turkey's test.

DISCUSSION

Plants are able to effect biological changes which have therapeutic value through anti-oxidation mechanism in living organisms. Reduced glutathione (GPx) concentrations, as well as modifications in catalase (CAT) and superoxide dismutase (SOD) activities are the most frequently used markers in tissues or in blood (Khaki *et al*., 2010), lipid peroxidation (MDA) inclusive. An alteration of anti-oxidative balance might result in tissue injury or damage, which is a contributing factor in chronic disease progression.

In vitro anti-oxidant study showed that the *Psidium guajava* and *Terminalia catappa* herbal extracts formulation possess some degree of antioxidant potentials. In the DPPH scavenging activity assay, the 1:1 extract formulation was shown to possess the least antioxidant activity. However this antioxidation potential is dose dependent and increases with increasing extract formulation concentration. The ascorbic acid used as the control had the highest antioxidation activity, even higher than the *P. guajava* leaf extract. These report does not support the work of Narendra *et al*. (2010) and Irondi *et al*. (2016) using the leaves of *P. guajava*. They demonstrated that the leaf extract of *P. guajava* possess significantly higher DPPH scavenging activity than ascorbic acid. In the nitric oxide scavenging assay, at the highest dose the 1:1 extract formulation exhibited a higher nitric oxide scavenging activity than gallic acid which was used as the control. Venkatachalam *et al*. (2012), also demonstrated that the leaf of *P. guajava* possess free radical scavenging activities using DPPH and nitric oxide scavenging assay.

The combination of *P. guajava* and *T. catappa* extracts in a 1:1 formulation was found to demonstrate free radical scavenging activities *in vivo* different from the control group. In the CAT and GPx assays, the antioxidant activities of the 1:1 extract treated groups were found to be lower than the control. The 200 mg/kg b.wt. of 1:1 extracts formulation nevertheless possess the highest antioxidant activity in the superoxide dismutase (SOD) and lipid peroxidation (MDA) assays. This observation is in agreement with previous report of anti-oxidative property of *P. guajava* (Neeraj *et al*., 2012; Punniyakotti *et al*., 2019).

P. guajava as *T. catappa* contains flavonoids and phenolic compounds. These phytochemicals are believed to be responsible for their antioxidant activities (Ogunlana and Ogunlana, 2008; Ravi *et al*., 2012; Krishnaveni, 2014; Omenna 2015; Yeshiwas and Mekonnen, 2018). There is a linear relationship between antioxidant potency, free radical-scavenging ability and the content of phenolic compounds of guava leaf extracts (Hui-Yin and Gow-Chin, 2007).) Flavonoids are known to be powerful antioxidant polyphenolic compounds as a result of their ability to inhibit peroxidation of polyunsaturated fatty acids in cell membranes (Torell *et al*., 1986; Faure *et al*., 1991). It is therefore likely that the anti-oxidant activities observed in the present study are attributable to flavonoids and phenols contained in each of the extracts.

CONCLUSION

The study revalidates the potent antioxidant potentials of *P. guajava* and *T. catappa* leaves. The 1:1 combination of the extracts did not yield a more effective outcome compared to the individual plants. However, further research is required to determine more potent formulations from other combinations of these plants.

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