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In vitro Assessment of the Free Radical Scavenging Activity of Psidium Guajava

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Abstract: Observable significant revisit of ancient approach to prophylaxis and anaphylaxis (herbaltherapy), though with modern dimensions of study envelopes our world of research today. Reports on the medicinal use of parts of *Psidium guajava* (Myrtaceae), including leaves and stem barks have been reported in, and beyond Africa. Air dried leaves of *P. guajava* were powdered and extracted with 95%v/v methanol by maceration, and the extract concentrated at 40°C using Rotary evaporator. The weight of the extracted plant material was recorded for yield calculations. *In vitro* assessment of the ability of the extract to scavenge the Reactive Oxygen Species (ROS), hydrogen peroxide, superoxide and the synthetic radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was determined with reference to the synthetic antioxidant Buthylated hydroxyanisole (BHA). Plant extract showed concentration- dependent scavenging activity on all reactive species used. Scavenging activity of plant extract on hydrogen peroxide and superoxide was more than that of BHA on same. However, BHA showed greater DPPH scavenging activity than plant extract.

Key words: *Psidium guajava*, BHA, DPPH, Superoxide, Hydrogen peroxide, ROS.

INTRODUCTION

Free radicals are associated with various physiological and pathological events such as inflammation, aging, mutagenicity and carcinogenicity. Simply defined, the term free radicals refer to any chemical species (capable of independent existence) possessing one or more unpaired electrons, an unpaired electron being one that is alone in an orbital. When paired in orbital, (the two electrons in an orbital have different spin directions), electrons are more stable. Varying reactivities notwithstanding, radicals, free radicals inclusive, have been known to be generally less stable than non-radicals[1]. Reactive oxygen species (ROS) capable of damaging DNA, proteins, carbohydrates and lipids are generated in aerobic organisms. These ROS include superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and single molecular oxygen. The deleterious reactions triggered by these ROS are controlled by a system of enzymic and non-enzymic antioxidants which eliminate pro-oxidants and scavenge free radicals[2]. Radical reactions are generally chain reactions. Most free radicals are extremely reactive and this probably explains why they ordinarily exist only in low concentration of the order 10^{-5} to 10^{-9}.

The radicals are generated at the initiation step, then they react in a series of propagation steps in which the number of the free radicals is conserved and termination ensures the destruction of the radicals. Oxygen is essential and central in free radical pathology due to its physicochemical properties such as water solubility and relatively high electron negativity[1]. Free radicals, especially the oxygen radical, superoxide, when formed could lead to the formation of other radicals. In fact, the toxicity of $O_2^{\cdot}$ in living organisms is due to its conversion into $OH^{\cdot}$ and reactive radical –metal complexes. Superoxide and hydrogen peroxide are converted into $OH^{\cdot}$ and other reactive radical complexes through the iron-catalyzed Haber- Weiss reaction or the superoxide driven Fenton reaction[3-5].

The uptake of one electron by molecular oxygen results in the formation of the superoxide anion radical. Superoxide anion radical owes its reactivity to the following factors[6].

- It is a strong base and can therefore abstract protons from a variety of compounds.
- It is a potent reducing agent. It can reduce quinines to semiquinones and transition metal ions into their reduced forms.

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- It is a nucleophile, hence may readily interact with a number of electrophiles.

Though a weak one, it is an oxidant. It may initiate the oxidation of molecules like ascorbic acid or epinephrine following hydrogen abstraction due to its basicity.

Hydrogen peroxide is the second intermediate produced during the stepwise one-electron reduction of molecular oxygen. It may also be generated directly during a two-electron reduction of molecular oxygen. Hydrogen peroxide is a stable molecule. In fact, it can act as both oxidizing and reducing agent. Hydrogen peroxide can generate hydroxyl radicals by an interaction with transition metal ions or a reaction with highly reactive oxidizing agents like NO and NO_2. The hydroxyl radical generated is a highly reactive oxidizing agent; it can abstract hydrogen atom from any hydrogen-carbon bond and partake in any addition reactions with aromatic systems at a reaction rate close to diffusion[6].

Notable research has been carried out on various parts of *P. guajava*. Permatoprotective activity of the leaf extract, molecular action mechanism against apoptosis by aqueous extract from budding leaves elucidated with human umbilical vein endothelial cell (HUVEC) model, hydrophilic and lipophilic antioxidant activities of fruits, cardio protective effects of extracts against ischemia-reperfusion injury in perfused rat hearts, studies on antimutagenic effects in *Salmonella typhimurium*, antibacterial activity of extracts against food-borne pathogens and spoilage bacteria and a number of other dimensions to the study of the applications and medicinal uses of *P. guajava* have all been reported[7-20]. In this study, the mode of antioxidant action of the ethanolic extract of *P. guajava* leaves was probed. *In vitro* methods of assessment were used to determine the scavenging activity of the extract on hydrogen peroxide, superoxide and DPPH radicals.

MATERIALS AND METHODS

Extraction of Plant Materials. P. guajava leaves were collected i*n situ*, authenticated and deposited at the herbarium, of the Botany Department, University of Ibadan, Oyo State, Nigeria. Leaf samples were air-dried in shade and powdered. 150g of powdered plant leaves was extracted in 900ml, 95% v/v methanol by maceration for 48 hours. After decantation of crude extract, filtration and concentration were carried out using Rotary Evaporator, and the weight of concentrated dried leaves obtained was recorded for the calculation of yield.

Determination of Hydrogen Peroxide (H_2O_2) Scavenging Activity of Plant Extract. Hydrogen Peroxide scavenging activity of plant extract was determined using a modification of the method of Ruch *et al*[21], by Gow Chin Yen and Hui-Yin Chen[22]. 4mM solution of H_2O_2 was prepared in phosphate - buffered saline (PBS, pH 7.4). H_2O_2 concentration was determined spectrophotometrically from absorbance at 230nm using molar absorptivity $81M^{-1}cm^{-1}$. 20 - 400µg plant extract corresponding to 0.05, 0.10, 0.15, 0.20, 0.25ml of 1mg/ml plant extract stock solution in 4ml distilled water were added to 0.6ml hydrogen peroxide-PBS solution. Absorbance of H_2O_2 at 230nm was determined 10 minutes later against a blank solution containing plant extract in PBS without H_2O_2. 20-400µg Buthylated hydroxyanisole was added in place of plant extract in 4ml distilled water and the solution wad added to 0.6ml H_2O_2 solution in PBS. Absorbance was determined 10 minutes later against a blank solution similar to that above.

Superoxide Scavenging Activity of Plant Extract. The effect of plant extract on superoxide generated in a non-enzymic system was measured spectrophotometrically[22]. The reaction mixture consisted of (10-1000µg) dilutions of plant extract made to 1ml with distilled water, 1ml, 60µM phenazine methosulphate (PMS), in phosphate buffer (0.1M, pH7.4) and 150µM, 1ml nitrroblue tetrazolium (NBT) in phosphate buffer. Incubation at ambient temperature followed for 5minutes, and the resultant colour was read spectrophotometrically at 560nm against a blank. The effect of Buthylated Hydroxytoluene (BHA) was also determined by replacing plant extract with 1ml BHA (10-1000µg) in methanol in the reaction mixture.

Determination of the Effect of Plant Extract on 1,1-diphenyl-2-picrylhydrazyl (Dpph) Radical. 1mM DPPH solution was prepared by dissolving 31.54mg DPPH in 95% v/v methanol and made up to 50ml with same. DPPH scavenging activity was assessed using the method of Hatano *et al*[23] as modified by Gow-Chin Yen and Hui-Yin Chen[22]. 200-1000µg corresponding to 0.2, 0.4, 0.6, 0.8, 1.0ml (1mg/ml) plant extract made up to 4ml with distilled water. 1ml, 1mM DPPH was added to each test tube, shaken and left to stand at room temperature for 30 minutes. Absorbance of the resulting solution was measured spectrophotometrically at 517nm. The effect of BHA on DPPH was also assessed for comparism with that of plant extract. Methanolic dilutions (0.2, 0.4, 0.6, 0.8, 1.0ml) of 1mg/ml BHA was made to 4ml with distilled water. 1ml DPPH radical (1nM) was added to each tube, andsame procedure as in DPPH scavenging experiment was followed.

RESULTS AND DISCUSSION

19.681g of extract was obtained from 150g of powdered leaves after concentration and drying of extracts. Percentage yield was calculated to be 13.12%. Results of the scavenging activity of plant extracts on hydrogen peroxide are shown in Table 1 and Figure 1.

Percentage free radical scavenging activity was calculated using the formula:

$$\% \text{ SA} = \frac{(A_C - A_E) \times 100}{A_C}$$

Where A_C = Absorbance of control
A_E = Absorbance of control
% SA = Percentage scavenging activity

It could be seen from Table 1 that plant extract scavenged H_2O_2 more effectively than BHA. Both BHA and plant extract scavenged H_2O_2 in concentration-dependent manner. Superoxide anion scavenging data shown in Table 2 and corroborated by Figure 2 showed an interesting trend. Even at concentrations as low as 0.05mg/ml, where BHA had less than 6% efficiency, plant extract mopped up more than 60% superoxide anion *in vitro*. Similarly while the percentage hydrogen peroxide activity of plant extract was 32.69% at a minimum concentration of 0.01mg/ml, which of BHA was 21.15% at same concentration. Plant extract and BHA scavenged 73.08% and 53.85% respectively at a maximum concentration of 0.05mg/ml. From Figure 3 (percentage DPPH scavenging activity), a trend that is removed from the one observed for hydrogen peroxide and superoxide anion above could be easily seen. At minimum concentration of 0.04mg/ml, plant extract and BHA scavenged 35.59% and 56.41% DPPH respectively. 61.10% and 82.06% DPPH scavenging activity at a maximum concentration of 0.16mg/ml was observed respectively for plant extract and BHA. Though both plant extract and BHA scavenged DPPH in concentration-dependent manner, BHA displayed better DPPH scavenging efficiency over plant extract. This may have been due to BHA's possession of a methoxy group which increases the accessibility of the radical centre of DPPH to BHA[24].

Statistical analysis (test of significance) of the data obtained from the free radical scavenging activity of plant extract and BHA using T-test (Paired two-sample for means) showed that the difference between the free radical scavenging activities of plant extract and BHA on the natural ROS used, was significant ($P<0.05$). However, a non significant difference in DPPH scavenging activity was seen between the two at same confidence limit. In other words, the plant extract's

Table 1: Hydrogen peroxide Scavenging Activity

Conc. of extracts(mg/ml)	Absorbance (230nm) *P. guajava*	BHA
0.0109	0.035±0.002	0.041±0.001
0.0217	0.032±0.002	0.036±0.002
0.0326	0.029±0.001	0.035±0.001
0.0435	0.018±0.003	0.028±0.001
0.0543	0.014±0.002	0.024±0.003

Absorbance of control = 0.052±0.001

Table 2: Superoxide scavenging activity of plant extract

Conc. of extracts (mg/ml)	Absorbance (560nm) P. guajava	BHA
0.05	0.063±0.002	0.178±0.002
0.1	0.058±0.003	0.171±0.002
0.15	0.038±0.002	0.170±0.002
0.2	0.014±0.002	0.146±0.005
0.25	0.011±0.000	0.142±0.001

Absorbance of control = 0.189±0.003

Table 3: Dpph Scavenging Activity of Plant Extract

Conc. of extracts mg/ml)	Absorbance (517nm) P. guajava	BHA
0.04	0.467±0.009	0.316±0.012
0.08	0.406±0.005	0.302±0.002
0.12	0.313±0.002	0.158±0.031
0.16	0.282±0.026	0.126±0.001
0.04	0.467±0.009	0.316±0.012

Absorbance of control = 0.725±0.012

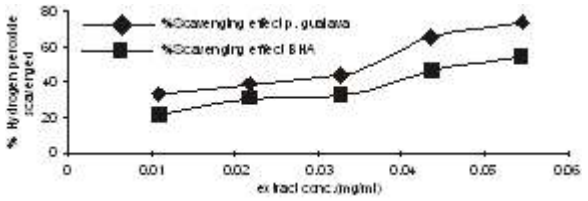

Fig. 1: Percentage Hydrogen Peroxide Scavenging Actinity.

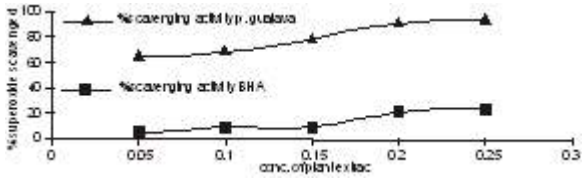

Fig. 2: Percentage Scavenging Activity of Extract on Superoxide Anion.

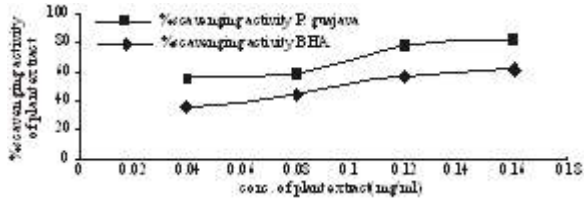

Fig. 3: percentage DPPHscavenging actinity of plant extact

superoxide radical and hydrogen peroxide scavenging activities were significantly higher than that of BHA. Although BHA's DPPH scavenging activity is higher

than that of plant extract, the difference in the DPPH scavenging activity of the two was not significant ($P<0.05$).

Discussion: Hydrogen peroxide only initiates lipid peroxidation weakly[25]. However, its ability to produce active oxygen species is due to its ability to generate highly reactive hydroxyl radical through the Fenton reaction[26]. The ability of plant extract to scavenge H_2O_2 could also reflect its ability to inhibit the formation of hydroxyl radical in vivo. Indirect stimulation of lipid oxidation by superoxide as a result of superoxide and hydrogen peroxide act as precursors of singlet oxygen and hydroxyl radical[27]. Since according to Harber Weiss reaction, both hydrogen peroxide and superoxide radical are required in the presence of metal catalyst for the formation of hydroxyl radical which is the dreaded free radical responsible in combination with molecular oxygen for cellular damages and oxidative degradation of macromolecules, it is therefore not illogical to presume that the marked ability of plant extract to scavenge both hydrogen peroxide and superoxide anion to a remarkable extent would culminate in remarkable hydroxyl radical formation, hence protection of macromolecules from oxidative damage. *In vivo* studies are however required to confirm this presumption.

BHA's better efficiency at scavenging DPPH may have been due to its possession of a methoxy group which increases the accessibility of the radical centre of DPPH to BHA[28].

Extrapolating from Figures 1 to 3, the IC_{50} of *P. guajava* scavenging activity on hydrogen peroxide is 0.037mg/ml while the extract's IC_{50} for its scavenging activity on both $SO_2^{\cdot-}$ and DPPH could not be determined within the extract concentrations used for the experiment. The minimum plant extract concentration (0.05mg/ml) used for $SO_2^{\cdot-}$ scavenging had above the 50% scavenging effect, required to be measured for IC_{50} determination. Similarly, the maximum plant extract concentration used for DPPH scavenging experiment did not scavenge up to 50% DPPH. Conversely, only the IC_{50} for BHA's superoxide scavenging activity could not be determined from the range of concentration of BHA used for the experiment. IC_{50} for hydrogen peroxide scavenging activity of BHA was 0.049mg/ml, while that of its DPPH scavenging activity was 0.10mg/ml.These results agree with previous works which obtained an IC_{50} a concentration above the maximum extract concentration used in present work for DPPH radical scavenging activity of *P. guajava* extract[29,30].

This research provides information which could trigger further research in the direction of partial or full isolation and characterization of the constituents of leaf extract of *P. guajava* in order to decipher the specific phytochemical constituent(s) responsible for the free radical scavenging activity of the plant. Where successful, the phytochemical(s) could be packaged in the appropriate dose(s) for the prevention of the onset of carcinogenesis, delay in the process of ageing, as well as the prevention of other free radical-induced health conditions.

Conclusion: Guarded inferences about the mechanism of antioxidant action of the methanolic extract of *Psidium guajava* leaves can be made from the present data. The remarkable mop-up potential of hydrogen peroxide and superoxide by the plant leaves could present a possible amelioration for the damages connected with hydroxyl radical to macromolecules by the following mechanisms among others:

- Scavenging hydrogen peroxide
- Scavenging superoxide anion radical
- Inhibiting the formation of hydroxyl radical from both hydrogen peroxide and superoxide as powered by Harber-Weiss reaction.

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