Guava (*Psidium guajava* Linn.) stem bark extracts: Toxicity and Free radical scavenging activity

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Abstract: Free radicals and reactive oxygen species (ROS) are associated with various physiological and pathological situations. They are capable of damaging DNA, proteins, carbohydrates and lipids which are generally in aerobic organisms. Therefore, this study was aimed at determining the lethal dose (LC_{50}) and investigating the effect of solvents of different polarity on free radical scavenging activity of Nigerian specie of *Psidium guajava* stem bark using UV-Visible Spectrophotometer. The crude methanol extract of *P. guajava* (CMEPG) stem bark was partitioned using hexane (HEFPG), ethyl acetate (EAFPG), butanol (BUFPG) and water (AQFPG). Free radical scavenging activities of CMEPG and the partitioned extracts were tested on 1, 1-Diphenyl-2-Picrylhydrazyl radical (DPPH) and hydroxyl radical generated from Hydrogen Peroxide (H_2O_2). Their activities were compared with known antioxidant standards; ascorbic acid (ASCAD), butylatedhydroxyanisole (BHA) and alpha-tocopherol (α-TOCO). The highest percentage inhibition was observed in HEFPG (97.39%) and BUFPG (96.50%) at 1.0 mg/ml in the DPPH test. BUFPG also scavenged hydroxyl radical generated from H_2O_2 better than CMEPG and other extracts. CMEPG had a percentage inhibition of 98.91% which is comparable to α−TOCO having 99.86% inhibition at 0.1 mg/ml. Brine shrimp lethality test was carried out to investigate the toxicity of *P. guajava* to lower animals and the result revealed that HEFPG was the most toxic with LC_{50} value of 41.7170 µg/ml while AQFPG with LC_{50} greater than 1,000 was non-toxic. Thus, *Psidium guajava* grown in Nigeria is a promising antioxidant agent.
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1. Introduction

Plant-derived drugs remain an important resource, especially in developing countries, in combating diseases. Approximately 60-80% of the world's population still relies on traditional medicines for the treatment of common illnesses (Oliver-Bever, 1986, Burkill, 1997, WHO, 2002). There has also been growing interest in the involvement of reactive oxygen species (ROS) in several pathological situations. Oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA (Deoxyribose Nucleic Acid) mutation, which can further initiate or propagate the development of diseases, such as cancer, liver injury and cardiovascular disease. Human bodies are exposed to free radicals from external sources such as radiation from the sun or X-rays, ozone and nitrous oxide, heavy metals like Mercury, Lead, smoke, alcohol, saturated fats, air pollution from cigarette smoke and other chemicals and pollutants. Our bodies also produce free radicals during essential activities, such as energy production and immunity. Stress is also another factor which produces adrenaline-related products, which not only restricts blood flow to our skin, but also generates potent, destructive free radicals. Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and treatment of diseases associated with oxidants (Paolo *et al*., 1991, Feskanich *et al*., 2000, Gordon, 1996, Halliwell, 1996, Lim *et al*., 2006).

P. guajava is a common plant because the fruit is edible and different part of the plant has enjoyed many medicinal applications as antibacterial, anti-inflammatory, anti-malaria, haemostatic, antispasmodic, as a tonic, anti-diarrhoeal, antidiabetic amongst others (Abdelrahim *et al*., 2002, Dutta *et al*., 2000, Rabe and Staden, 1997, Muruganandan *et al*., 2001, Olajide *et al*., 1999, Sen *et al*, 1995, Ticzon, 1997, Conway, 2001). Also much biological work has been carried out on *P. guajava* and many compounds of medicinal importance have been isolated (Arima and Danno, 2002, Begum *et al*., 2002, Michael *et al*., 2002, Morton, 1987, Gnan and Demello, 1999, Pranee *et al*., 1999, Hernandez, 1980, Ali *et al*., 1996, Lutete *et al*., 1994). Guava contains lycopene which according to some researchers may play a role in the prevention of different forms of cancer,

cardiovascular disease, cataracts and exercise-induced asthma (Clinton, 1998, Giovannucci, 1999; Lu *et al*., 2001; Chandrika, 2008). Fasola et al, (2011) reported the chemical composition, toxicity and antioxidant activities of essential oils of stem bark of Nigerian species of Guava. Also phytochemical investigation of the plant for haematological indices in albino Swiss Rats has also been reported (Fasola et al, 2012). The aims of the study however are to test and compare the free radical scavenging potentials of methanol, hexane, ethyl acetate, butanol and aqueous extracts obtained from the stem bark of *P. guajava* using two radical scavenging methods not yet reported in literature for this plant: effects on 1, 1-Diphenyl-2-Picrylhydrazyl radical (DPPH) and hydroxyl radical inhibition. DPPH radical gives strong absorption at 517 nm (deep violet colour) in visible spectroscopy. The absorption vanishes or is decolourized as the electron becomes paired off in the presence of a free radical scavenger. Spectrophotometric determination of the scavenging effect of the extracts on hydrogen peroxide was carried out at 285 nm. These two tests show the ability of the fractions as a proton donor or as a hydroxyl radical scavenger respectively. This antioxidant activity was compared with three known antioxidants standards ─ ascorbic acid, butylated hydroxyanisole and alpha-Tocopherol. The toxicity study of the extracts was carried out using Brine shrimp larvae (nauplii). Brine shrimp lethality test is a fast, accurate bench top bioassay for elementary toxicity investigations of natural products (Meyer *et al,* 1980, Dvorak *et al,* 1999).

2 Materials and methods

2.1. Chemicals and Reagents:

Sodium chloride, copper sulphate pentahydrate, ferric chloride, conc. Tetraoxosulphate (VI) acid, conc. HCl, ammonia solution, hexane, ethyl acetate, methanol, butanol, chloroform, hydrochloric acid, naphthol, bismuth nitrate, potassium iodide, sodium hydroxide, copper acetate, NaOH, sodium potassium tartarate, potassium chloride, glacial acetic acid, disodium hydrogen phosphate, and dihydrogen potassium phosphate were all BDH general purpose chemicals and solvents were distilled prior to use. Dimethylsulphoxide (M&B, England), hydrogen peroxide and silica gel 60 - 120 microns (Merck, Germany) and 1, 1 - diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, butylatedhydroxyanisole (BHA) and α-tocopherol were obtained from Sigma Chemical Co (St Louis, MO). Brine shrimp larvae eggs were obtained from Ocean Star International, Inc. Company, USA.

2.2. Equipment and Apparatus:

Soxhlet apparatus, Rotavapor RII0 (Buchi, England), Mettler analytical balance H80 (UK), Water Bath (Gallenkamp), pH meter (Jenway model), UV-Visible spectrophotometer (Unico1200 & Perkin Elmer lambda 25 models).

2.3. Plant collection and identification

Psidium guajava stem bark was collected in August 2009 behind Tedder Hall at the University of Ibadan, Oyo State, Nigeria and authenticated at the Herbarium of the Department of Botany and Microbiology of the institution. The stem bark was air dried. The dried sample was ground with a milling machine in the Wood and extraction laboratory of the Department of Chemistry of the same institution.

2.4. Reference Standards:

Ascorbic acid, butylatedhydroxyanisole (BHA) and α-tocopherol for antioxidant activity.

2.5. Sample preparation

The stem bark of *P. guajava* was weighed and air-dried for 4 weeks until the weight was constant and then pulverized using mill machine. The pulverized samples were weighed and kept for further analysis.

2.6. Extraction and Fractionalization of the Plant Sample

The ground sample (3 kg) was extracted with methanol (7.5 L) using Soxhlet apparatus. The crude methanol extract was concentrated with the aid of a Bucchi rotavapor and stored in a desiccator prior to further analysis. The extract was partitioned using hexane (non-polar solvent), ethyl acetate (moderately polar solvent), butanol (polar solvent) and aqueous fraction. Thereafter, toxicity test using Brine shrimp lethality assay and free radical scavenging activity screening were carried out on the fractions using the following spectrophotometric experiments; scavenging effect on 1,1-Diphenyl-2-Picrylhydrazyl radical (DPPH) and scavenging effect on hydroxyl radical generated from hydrogen peroxide.

2.7. Toxicity analysis: Brine shrimp lethality test

Toxicity to lower organisms was done using the brine shrimp lethality test (BST) (Meyer *et al*., 1982). The shrimp's eggs (Plate 1a) were hatched in sea water for 48 h at room temperature. The nauplii (harvested shrimps) (Plate 1b) were attracted to one side of the vials with a light source. Solutions of the extracts were made in DMSO, at varying concentrations (1000, 100, and 10 µg/ml) and incubated in triplicate vials with the brine shrimp larvae. Ten brine shrimp larvae were placed in each

of the triplicate vials. Control brine shrimp larvae were placed in a mixture of sea water and DMSO only. After 24 h the vials were examined against a lighted background and the average number of larvae that survived in each vial was determined. The concentration at fifty percent mortality of the larvae (LC_{50}) was determined using the Finney computer programme (Falope *et al*, 1993, Oloyede *et al*, 2010).

PLATE 1a: Brine Shrimp Eggs

PLATE 1b: A Brine Shrimp Larva

2.8. Antioxidant activities of extracts from *P. guajava* stem bark

2.8.1. Scavenging Effect on 1,1-Diphenyl-2-Picrylhydrazyl radical (DPPH)

The ability to scavenge DPPH free radical was carried out at room temperature. DPPH (3.94 mg) was dissolved in methanol (100 ml) to give 100 µm solutions. The fractions (0.5 ml) were added to 3.0 ml of the methanol solution of DPPH at varying doses (1.0 mg/ml to 0.0625 mg/ml). The decrease in absorption at 517 nm of DPPH was measured 10 minutes later. The actual decrease in absorption was measured against that of the control and the percentage inhibition was also calculated. The same experiment was carried out on butylatedhydroxylanisole (BHA), ascorbic acid and α-tocopherol which are known antioxidants. All test and analysis were run in triplicates and the results obtained were averaged (Hatano *et al,* 1988). The actual decrease in absorption induced by the test compound was calculated by subtracting that of the control and the percentage inhibition was determined (Gulcin *et al*, 2002, Mutee *et al*, 2010 and Oloyede *et al,* 2010)

2.8.2. Scavenging Effect on Hydrogen Peroxide (H_2O_2)

Hydroxyl radical scavenging effect of *P. guajava* fractions was carried out at 285 nm. A solution of 2 mM hydrogen peroxide was prepared in phosphate buffered-saline (PBS) pH 7.4. The fractions at concentrations 0.1 - 0.0065 mg/ml were added to the H_2O_2 solution. Decrease in absorbance of H_2O_2 at 285 nm was determined spectrophotometrically 10 minutes later against a blank solution containing the test extract in PBS without H_2O_2. All tests were run in triplicates and averaged (Soares *et al*, 1997, Oloyede and Farombi, 2010). The same experiment was carried out on the following antioxidant standards; butylatedhydroxyanisole (BHA), ascorbic acid and α-tocopherol.

3. Results and discussion

3.1. Brine Shrimp toxicity test

Toxicity analysis revealed that some of the fractions were toxic at varied degrees. The decreasing order of toxicity of the extracts of *P. guajava* as seen in Table 1 is HEFPG > BUFPG > EAFPG > AQFPG. HEFPG has LC_{50} of 41.717 µg/ml which indicates that it is the most toxic. It has been established by other workers that secondary metabolites from plants which are active medicinally are most times toxic to Brine shrimp larvae *Artermia silina* nauplii which is a living organism with no advance nervous system (Aiyelaagbe, *et al*, 2009; Oloyede *et al*, 2010). However, AQFPG was not toxic to the larvae (Table 1) as the LC_{50} was greater than 1000 µg/ml.

3.2. Antioxidant Activity

3.2.1 Scavenging effects on DPPH

An antioxidant which can donate an electron to DPPH will decolorize the purple color with a decrease in absorption at 517 nm in a UV spectrophotometer. The degree of reduction in absorbance measurement is indicative of the free radical scavenging (antioxidant) ability of the extract (Soares *et al*, 1997, Ayoola *et al*., 2008, Ramzi *et al*., 2008). Table 1 shows the result of the analysis.

TABLE 1: Toxicity of Extracts on Brine Shrimp larvae

Plant	Part use	Fraction	LC_{50} (µg/ml)	Upper Limit	Lower Limit
P. guajava	Stem bark	HEFPG	41.7170	210.3242	0.0084
		EAFPG	590.9066	4551.2680	7.0588
		BUFPG	172.0463	324.1597	65.6696
		AQFPG	1492.9190	8964.6080	397.1169

* LC_{50} = the lethal concentration to half of the test organisms, LC_{50} < 1,000 = toxic, HEFPG = Hexane Fraction of *P. guajava,* EAFPG = Ethyl Acetate Fraction of *P. guajava,* BUFPG = Butanol Fraction of *P. guajava,* AQFPG = Aqueous Fraction of *P. guajava*

TABLE 2: The Free Radical Scavenging Activities of *P. guajava* on DPPH at 517 nm*

CONC (mg/ml)	CMEPG	HEFPG	EAFPG	BUFPG	AQFPG	ASCAD	BHA	α−TOCO
	ABSORBANCE (517 nm)							
1.000	0.0507 ± 0.001^{d}	0.0551 ± 0.000^{a}	0.0551 ± 0.000^{a}	0.0281 ± 0.002^{e}	0.0666 ± 0.001^{c}	0.0884 ± 0.008^{e}	0.0335 ± 0.001^{d}	0.6967 ± 0.001^{d}
0.500	0.0578 ± 0.000^{d}	0.0555 ± 0.004^{d}	0.0600 ± 0.001^{a}	0.1364 ± 0.004^{d}	0.0687 ± 0.003^{c}	0.3495 ± 0.007^{d}	0.0492 ± 0.001^{c}	0.7017 ± 0.001^{c}
0.250	0.0636 ± 0.000^{c}	0.0990 ± 0.006^{c}	0.0625 ± 0.000^{a}	0.4027 ± 0.012^{c}	0.0696 ± 0.001^{c}	0.3878 ± 0.008^{c}	0.0529 ± 0.001^{b}	0.7028 ± 0.001^{c}
0.125	0.3893 ± 0.004^{b}	0.2575 ± 0.009^{b}	0.0831 ± 0.002^{a}	0.4720 ± 0.010^{b}	0.2675 ± 0.018^{b}	0.4702 ± 0.004^{b}	0.0560 ± 0.002^{b}	0.7072 ± 0.001^{b}
0.0625	0.4096 ± 0.009^{a}	0.4462 ± 0.006^{a}	0.0842 ± 0.062^{a}	0.4980 ± 0.009^{a}	0.3657 ± 0.018^{a}	0.5214 ± 0.010^{a}	0.0654 ± 0.003^{a}	0.7148 ± 0.002^{a}

*Absorbance values are Mean ± S.D. The superscripts are the level of significance ($p<0.05$), CMEPG = Crude Methanol Extract of *Psidium guajava,* HEFPG = Hexane Fraction of *Psidium guajava,* EAFPG = Ethyl Acetate Fraction of *Psidium guajava,* BUFPG = Butanol Fraction of *Psidium guajava,* AQFPG = Aqueous Fraction of *Psidium guajava,* ASCAD = Ascobic Acid, BHA = Butylatedhydroxylanisole, α-TOCO = α-Tocopherol.

Butanol fraction of *P. guajava* has the lowest absorption value (0.0281 ± 0.002) which is lower than BHA with an absorbance value of 0.0335 ± 0.001, the lowest among the standards. Also, the percentage inhibition generally decreases as the concentration decreases (Fig 1). The highest percentage inhibition (97.39%) was observed in HEFPG. CMEPG at 1.0 mg/ml had percentage inhibition of 92.17% and 36.75% at the lowest concentration (0.0625 mg/ml). There was no significant difference in the percentage inhibition of EAFPG and AQFPG at all the concentrations. A 96.50% inhibition was recorded for BUFPG at 1.0 mg/ml and 38.06% at 0.0625 mg/ml. The highest percentage inhibition (91.87%) was recorded for BHA at 0.0625 mg/ml. The lowest percentage inhibition (13.35%) at 1.0 mg/ml and 11.09% at 0.0625 mg/ml was recorded for α−TOCO (Fig. 1). ASCAD did not inhibit DPPH effectively. BHA however, gave the highest inhibition among the standards used in the experiment. The extracts from *P. guajava* showed promising free radical scavenging activity especially the highly polar butanol fraction. It can therefore be inferred that the mechanism involved in free radical scavenging chemistry defers.

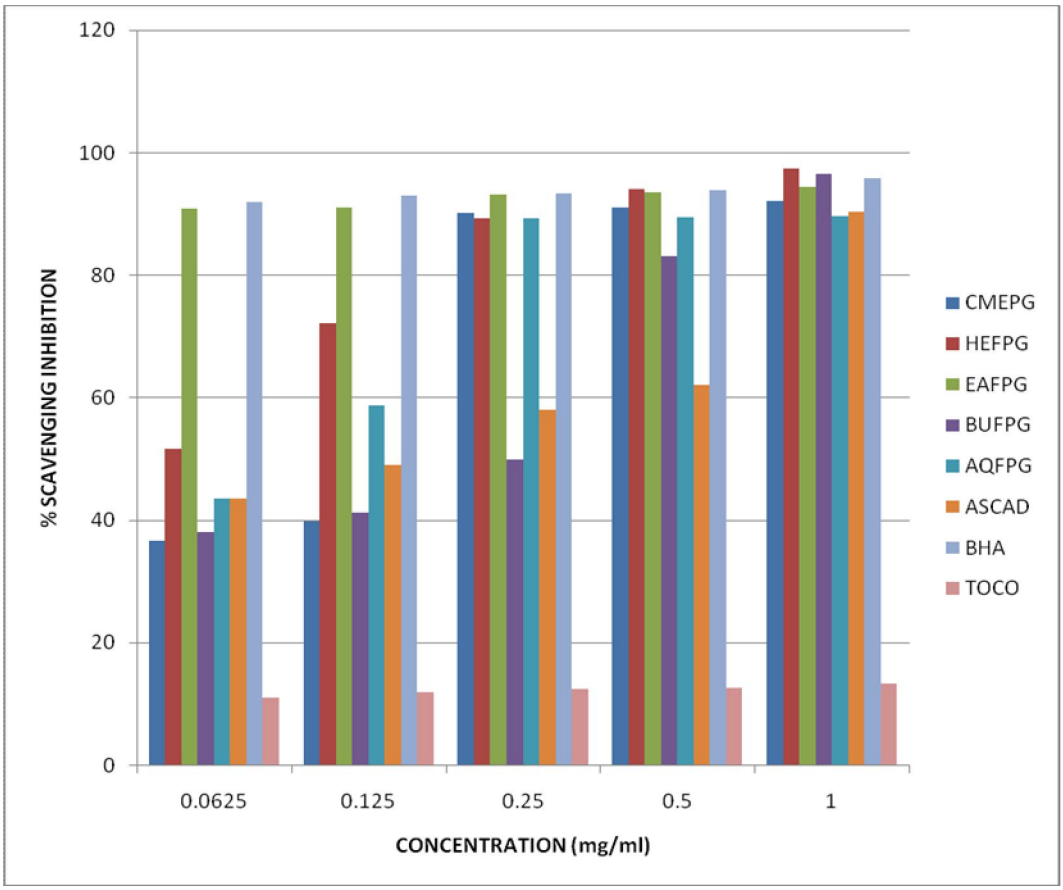

Figure 1: Percentage inhibition of *P. guajava* extracts and standards on DPPH

3.2.2. Scavenging effects on Hydrogen peroxide (H_2O_2)

The scavenging activities of *P. guajava* extracts and antioxidants; ascorbic acid (ASCAD), butylatedhydroxyanisole (BHA) and α-tocopherol on H_2O_2 were measured in triplicates after 10 min of incubation at 285 nm. The analysis as seen in Table 2 showed that at concentration of 0.1 - 0.00625 mg/ml, all the extracts CMEPG, HEFPG, EAFPG, BUFPG and AQFPG scavenged hydroxyl radical as there was decrease in absorption at 285 nm; the absorption increases as the concentration is decreased. The activities were high and comparable to the standards; BHA and α–TOCO. ASCAD has the lowest percentage inhibition at 0.0125 and 0.00625 mg/ml unlike the other samples (Fig 2). CMEPG at 0.1 mg/ml had a percentage inhibition of 98.91% while α-TOCO had the highest percentage inhibition (99.86%). The free radical-scavenging effect increases as the concentration is increased. ASCAD at 0.00625 mg/ml had 19.79% inhibition unlike BHA which had 98.81% (Fig. 2). This result revealed that *P. guajava* was able to scavenge hydroxyl radical. Hydroxyl radical ($^{\bullet}OH$) initiates the process of lipid peroxidation by abstracting hydrogen atom from fatty acid side chains. However, antioxidants interfere with the chain reaction by donating hydrogen atoms to peroxyl radicals, stopping them from abstracting hydrogen and terminating the dangerous chain reactions (Halliwell and Gutteridge 1984, Slater, 1984).

TABLE 3: Free Radical Scavenging Activities of *P. guajava* extracts on H_2O_2 at 285 nm*

CONC (mg/ml)	CMEPG	HEFPG	EAFPG	BUFPG	AQFPG	ASCAD	BHA	α–TOCO
	ABSORBANCE (285 nm)							
0.1000	0.0411 ± 0.014^{e}	0.0495 ± 0.002^{e}	0.0256 ± 0.005^{e}	0.0352 ± 0.002^{e}	0.0583 ± 0.006^{e}	0.1952 ± 0.001^{d}	0.0513 ± 0.001^{e}	0.0054 ± 0.001^{e}
0.0500	0.0928 ± 0.005^{d}	0.2107 ± 0.006^{d}	0.1161 ± 0.002^{d}	0.0613 ± 0.001^{d}	0.2601 ± 0.004^{d}	0.2078 ± 0.003^{d}	0.0561 ± 0.002^{d}	0.0456 ± 0.004^{d}
0.0250	0.2131 ± 0.004^{c}	0.2398 ± 0.002^{c}	0.2438 ± 0.001^{c}	0.0820 ± 0.002^{c}	0.3271 ± 0.007^{c}	1.1611 ± 0.008^{c}	0.0684 ± 0.001^{c}	0.1228 ± 0.003^{c}
0.01250	0.3275 ± 0.003^{b}	0.2576 ± 0.006^{b}	0.4398 ± 0.001^{b}	0.0961 ± 0.006^{b}	0.6438 ± 0.003^{b}	2.7043 ± 0.004^{b}	0.0963 ± 0.001^{b}	0.1740 ± 0.004^{b}
0.00625	1.0284 ± 0.007^{a}	0.3091 ± 0.006^{a}	0.8911 ± 0.002^{a}	0.2584 ± 0.007^{a}	1.3234 ± 0.001^{a}	3.0234 ± 0.062^{a}	0.1202 ± 0.000^{a}	0.5045 ± 0.003^{a}

*Absorbance values are Mean ± S.D. The superscripts are the level of significance ($p<0.05$) CMEPG = Crude Methanol Extract of *P. guajava,* HEFPG = Hexane Fraction of *P. guajava,* EAFPG = Ethyl Acetate Fraction of *P. guajava,* BUFPG = Butanol Fraction of . *guajava,* AQFPG = Aqueous Fraction of *P. guajava,* ASCAD = Ascobic Acid, BHA = Butylatedhydroxylanisole, α-TOCO = α -Tocopherol.

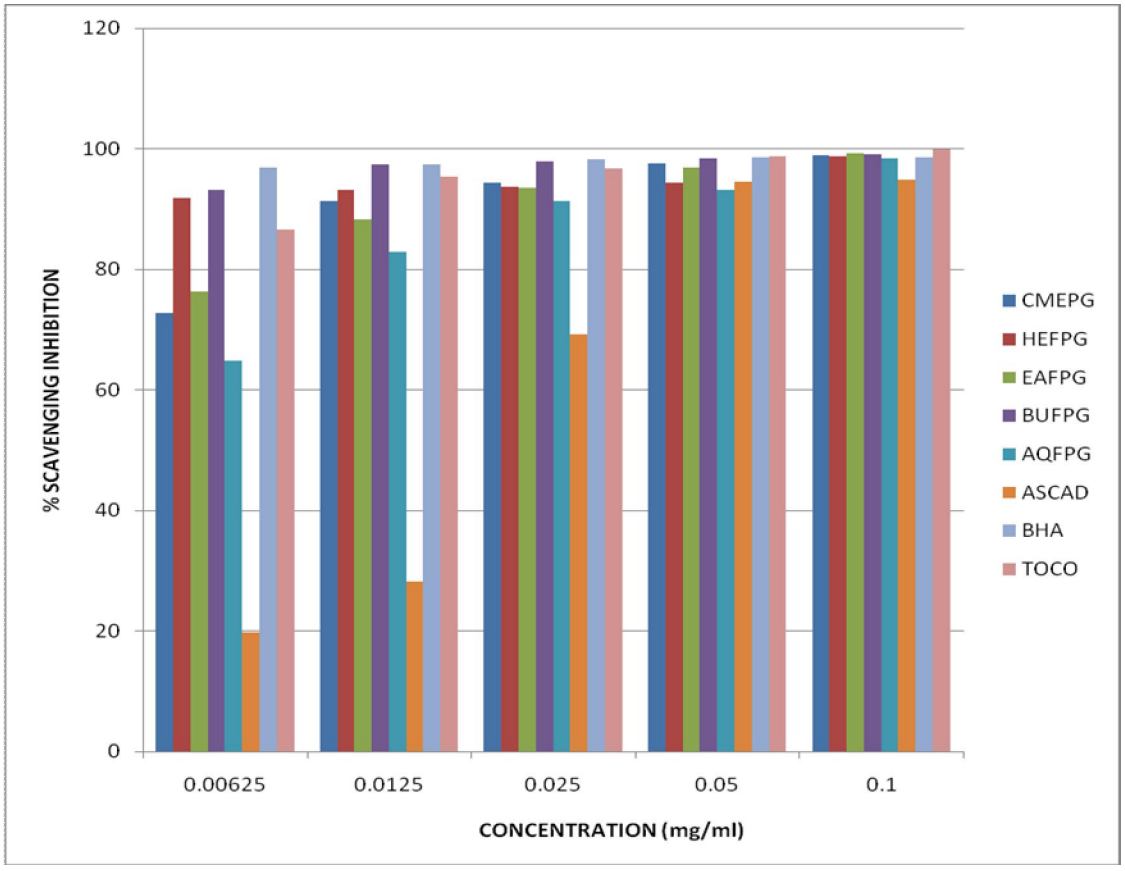

Figure 2: Percentage inhibition of *P. guajava* extracts and standards on H_2O_2

4. Conclusion

The medicinal importance of *P. guajava*, a plant with edible fruit has again been proven in the present study. The plant stem bark extracts was capable of donating proton in the reaction involving DPPH (1,1 Diphenyl-2-Picrylhydrazyl) as well as scavenge hydroxyl radical generated from H_2O_2 (hydrogen peroxide) in a reaction involving Ultraviolet-visible spectrophotometry. Polarity of the solvent used for extraction has effect on the plant activity as the polar methanol extract was the most effective. The free radical-scavenging assay has been efficiently used as an adequate parameter for selecting medicinal plants with promising antioxidant properties. The toxicity of this plant using Brine shrimp larvae makes it a valuable entity in the therapy of diseases involving cell or tumour growth but the use at high dose should be properly monitored.

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