ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Comparison of *In-Vitro* Antioxidant Activities and Total Phenolic Contents in Water and Methanol Extracts of Stems Bark of *Spondias mombin*

Boni ANR[1*], Ahua KM[1], Kouassi K[1], Yapi H[1], Djaman AJ[1,2], and Nguessan JD[1]

[1]Laboratory of Biochemical Pharmacodynamy, Félix Houphouët-Boigny University of Abidjan, Côte d'Ivoire, 22 BP 582 Abidjan 22
[2]Pasteur Institute of Côte d'Ivoire

ABSTRACT

This study was undertaken to evaluate the antioxidant and free radical scavenging activities of aqueous (AESPM) and methanolic (MESPM) extracts of *Spondias mombin* (*Anacardiaceae*). Antioxidant activities were measured by ferric thiocyanate assay and thiobarbituric acid method. Free radical scavenging activities using 1, 1-diphenyl-2-picryl-hydrazil (DPPH) reducing power, ferric reducing antioxidant power (FRAP), total phenolics, flavonoids and flavonols were also evaluated. So, MESPM showed the highest level of total phenolic, flavonoid and flavonol contents compared to AESPM. In DPPH scavenging assay, the IC_{50} values of MESPM (5.83 ± 0.88 µg/mL) and the reference standard ascorbic acid (4.56 ± 0.22 µg/mL) were comparable. The methanolic extract of *S. mombin* also showed strong reducing power, FRAP activity and antioxidant activity with FTC method compared to the aqueous extract. However, there are no statistically difference ($p>0.05$) between antioxidant activities of MESPM, AESPM and ascorbic acid by TBA method. These results suggest that *S. mombin* may act as a chemopreventative agent, providing antioxidant properties and offering effective protection from free radicals.

Keywords: *Spondias mombin*, free radicals, antioxidant, phenolic compounds.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Oxygen is essential for the survival of all on this earth. During the process of oxygen utilization in normal physiological and metabolic processes approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals. Free radicals are types of Reactive Oxygen Species (ROS), which include all highly reactive, oxygen-containing molecules. Types of ROS include the hydroxyl radical, the super oxide anion radical, singlet oxygen, nitric oxide radical, hypochlorite radical and various lipid peroxides [1]. At high levels, free radicals and oxidants generate oxidative stress, a deleterious process that can damage important cellular components especially proteins, nucleic acids and polyunsatured fatty acids in cell membranes and plasma lipoproteins [2]. So, oxidative stress plays a major part in the development of chronic and degenerative diseases such as diabetes, rheumatic disorders, aging, cancer, cardiovascular and neurodegenerative disorders [3, 4].

The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced *in situ*, or externally supplied through foods and/or supplements. These antioxidants act as free radical scavengers by preventing and repairing damages caused by ROS, and therefore can enhance the immune defense and lower the risk of cancer and degenerative diseases [5].

Recently, interest has increased considerably in finding natural antioxidants for use in foods or medicinal materials to replace synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) etc. which are being restricted due to their side effects such as carcinogenicity [6]. Traditional medicine is widespread and plants still presents a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several anti-inflammatory, digestive, anti-necrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or anti-radical scavenging mechanism as part of their activity [7, 8].

In order to contribute to the search for new natural antioxidants, the aqueous and methanolic extracts of stems bark of *Spondias mombin* were investigated for antioxidant properties using *in vitro* standard methods and thus justify the traditional use of this plant. Phenolics content of the extracts were also determinated.

MATERIAL AND METHODS

Chemicals

Potassium ferricyanide, trichloroacetic acid (TCA), Folin-Ciocalteu's phenol reagent, gallic acid and ascorbic acid, aluminium chloride, sodium acetate, Sodium carbonate were obtained from Merck Co. (Germany). 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), ferric chloride, 2, 4, 6-tripyriyl-s-triazine (TPTZ), thiobarbituric acid (TBA) were purchased from Sigma Chemical Co (St Louis, USA). All other chemicals were of analytical grade.

Plant materials

The stem bark of *Spondias mombin* was collected in February 2013 from Afféry (Region of Adzopé, south of Côte d'Ivoire). The plant was identified by her vernacular name and later authenticated by Professor Aké Assi Laurent of Laboratory of Botany, University Felix Houphouët Boigny (Côte d'Ivoire). A voucher specimen of the plant n° 15778 was enregistered in herbarium of National Floristic Center (University Félix Houphouët Boigny, Côte d'Ivoire).The collected plant material was air dried in darkness at room temperature (20°C). Stem bark were cut up and stored in tight-seal dark containers until needed.

Preparation of plant extracts

One hundred grams (100g) of washed, air dried powdered stem of the plants were extracted with 1.5 L of distilled water at room temperature for 48 hours with stirring at interval. The aqueous solution obtained were filtered using a Buckner funnel and Whatman N° 1 and concentrated to dryness at 40°C using a rotary evaporator under reduced pressure. The dried extracts were weighed and then stored at 4°C for subsequent analysis. The preparation of the methanolic extract used the same procedure except that ground plant material was in this case shaken in methanol. The aqueous and methanol extracts of stems bark of *Spondias mombin* were named respectively AESPM and MESPM.

Total phenolics content

Total phenolics content of plant extracts were determined by the modified Folin-Ciocalteu method [9]. An aliquot (0.5 mL of 0.1g/mL) of the extracts was mixed with 5 mL Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 mL (75 g/L) of sodium carbonate. The tubes were vortexed for 15 secondes and allowed to stand for 30 min at 40°C for colour development. Absorbance was then measured at 765 nm using the UV-VIS spectrophotometer. Samples of extract were evaluated at the final concentration of 0.1 mg/mL. A calibration curve was performed in parallel under the same operating conditions using gallic acid as a positive control. The results are expressed as mg gallic acid equivalent per gram of dry extract (mg GAE/g).

Total flavonoids content

Total flavonoids in the plant extracts were determined using aluminium chloride colorimetric method [10]. Each plant extract (0.5 mL of 0.1 g/mL) in the methanol was separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After 30 min at room temperature, the absorbance of the reaction mixture was measured at 415 nm using spectrophotometer. The same procedure was repeated for the standard solution of quercetin and the calibration line was constructed. Flavonoids content in extracts were expressed in terms of quercetin equivalent per gram of dry extract (mg QE/g).

Flavonols content

Total flavonols in the plant extracts were estimated using the method of Kumaran and Karunakaran [11]. To 2 mL of sample, 2 mL of 2 % $AlCl_3$ ethanol and 3 mL (50 g/L) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 hours at 20 ^{o}C. Extract samples were evaluated at final concentration of 0.1 mg/mL. Total flavonols content was expressed in term of quercetin equivalent per gram of dry extract (mg QE/g).

DPPH radical scavenging assay

The effect of extracts on DPPH radical was determined using the method of Koleva et al [12]. Different concentrations of each plant extract were added at an equal volume to methanolic solution of DPPH (100 µM). The mixture was allowed to react at room temperature in the dark for 15 min. The absorbance was then measured spectrophotometrically at 517 nm. The experiment was repeated for three times. Ascorbic acid was used as standard control. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH inhibition (\%)} = 100 - [(A_1/A_0) \times 100]$$

Where A_1 is the absorbance of DPPH radical without sample extract and A_0 is the absorbance of DPPH radical with sample extract.

IC_{50} values denote the concentration of sample which is required to scavenge 50% of DPPH radicals.

Ferric reducing antioxidant power (FRAP) assay

A modified method of Benzie and Strain [13] was adopted for the FRAP assay. The stock solutions included 300 mM acetate buffer (3.1 g CH_3COONa and 16 mL CH_3COOH), pH 3.6, 10 Mm TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL $FeCl_3.6H_2O$. The temperature of the solution was raised to 37^{o}C before using. Plant extracts (150µL) were allowed to react with 2850 µL of FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results are expressed in µM Fe (II)/g dry mass and compared with that ascorbic acid.

Reducing power assay

The reducing antioxidant power of plant extracts was determined by the method of Oyaizu [14]. Different concentrations of plant extracts (0-100 µg/mL) in 1 ml of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50^{o}C for 20 min. Then, 2.5 mL of trichloroacetic acid (10%) was added to mixture, which was then centrifuged for 10 min at 3000

rpm. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). The absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer. Increased absorbance of the reaction mixture indicates increase in reducing power.

Inhibition of lipid peroxidation by Ferric thiocyanate (FTC) method

The antioxidant activities of plant extracts were determined according to the FTC method [15] with slightly modifications. Four milligrams of each extract samples were dissolved in 4 mL ethanol (99.5 %) and kept in dark bottle (d = 40 mm, t = 75 mm). Each mixture was mixed with 4.1 mL linoleic acid (2.5% in ethanol 99.5%), 8 mL phosphate buffer (0.02 M, pH 7.0) and 3.9 mL distilled water to make up the volume to 20 mL. Ascorbic acid was used as a positive control while an another bottle without sample was used as a negative control. The mixture was incubated at 40- 45^{o}C. After incubation, 9.7 mL ethanol (75 %) and 0.1mL NH4SCN (30%, as a colour reagent) was added to 0.1 mL of the mixture. Precisely 3 min after the addition of 0.1 ml of $FeCl_2$ (0.002 M) in HCl 3.5% to the reaction mixture, the absorbance of the resulting red colour was measured at 500 nm using spectrophotometer every 24 h until a day after the absorbance of the control reached maximum value (day seven). The inhibition of lipid peroxidation was calculated as follows:

$$\% \text{ Inhibition} = 100 - [(A_1/A_0) \times 100]$$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample extracts.

Thiobarbituric acid (TBA) test

The TBA test was conducted according to the combined method of Kikuzaki and Nakatani [16] and Ottolenghi [17]. A milliliter of sample from the previous FTC method was added with 2 mL of trichloroacetic acid and 2 mL of thiobarbituric acid solution. This mixture was then placed in a boiling water bath at 100^{o}C for 10 min. After cooling, it was centrifuged at 3000 rpm for 20 min and absorbance of the supernatant was then measured at 532 nm using UV-Vis spectrophotometer.

Statistical analysis

Results are expressed as mean ± S.E.M. of three determinants. Comparisons among the groups were tested by one-way ANOVA using Graph Pad Prism, Version5.0 (Graph Pad Software, San Diego, CA, USA). P values < 0.05 were considered significant.

ISSN: 0975-8585

RESULTS

Total phenolic, flavonoid and flavonol contents

The yield of extract obtained from 100 g of dry plant material was measured for each extract (Table 1). The highest yield of solid residue was obtained using methanol as extraction solvents. Methanolic extract (MESPM) and aqueous extract (AESPM) of stems bark of *Spondias mombin* were studied for their contents of total phenolic, flavonoid and flavonol. Table 2 showed that highest phenolic compounds was observed in MESPM compared to AESPM.

Table 1: yield of different extracts of *Spondias mombin*

Extracts	Yield (g/100g of sample)
AESPM	8.73
MESPM	12.58

AESPM: Aqueous extract of *Spondias mombin,* **MESPM**: Methanolic extract of *Sponias mombin*

Table 2: Polyphenol contents of different extracts of stems bark of *S. mombin*

Phenolics	AESPM	MESPM
Total polyphenol[a]	183.5 ± 1.89	343.5 ± 6.44
Flavonoids[b]	7.48 ± 0.19	11.28 ± 0.45
Flavonols[c]	15.64 ± 1.04	31.73 ± 0.54

Values are expressed as mean ± SEM. (n = 3).[a] Expressed as mg gallic acid/g of dry plant, [b]Expressed as mg quercetin/g of dry plant, [c]Expressed as mg quercetin/g of dry plant

DPPH radical scavenging activity

S. mombin extracts were investigated for their radical scavenging activity and the results are shown in Figure 1. All of the extracts tested possess radical scavenging activity. This activity increased with increasing concentration of the sample extract. These activities were dose dependent. The IC_{50} calculated are reported in Table 3. MESPM showed dominant activity with lowest IC_{50} (5.83±0.88µg/ml) compared to AESPM ($p<0.05$) which was quite similar with ascorbic acid (4.56 ± 0.22 µg/ml) ($p>0.05$). MESPM with high level of phenolic, flavonoids contents and highest amount of flavonols showed the best radical scavenging activity.

Table 3: IC_{50} values of different extracts for free radical scavenging activity by DPPH radical

Extracts	IC_{50} (µg/ml)
AESPM	10.33 ± 1.09**
MESPM	5.83 ± 0.88
Ascorbic acid	4.56 ± 0.22

Values are expressed as mean ± SEM. (n = 3).**$p<0.01$, when compared with ascorbic acid used as reference.

ISSN: 0975-8585

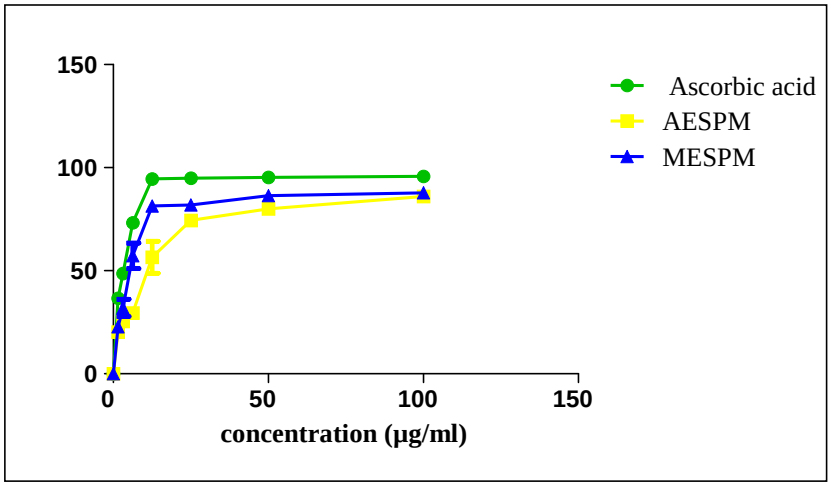

Figure 1: Free radical scavenging activity of different extracts of Stems bark *S. mombin* on DPPH radical

Reducing power assay

The reducing power of plant extracts are summarized in Figure 2. From the figure, reducing power increased with an increased in extracts concentration. The data show that MESPM extract was found to be more active than AESPM extract but less than the reference molecule which is ascorbic acid.

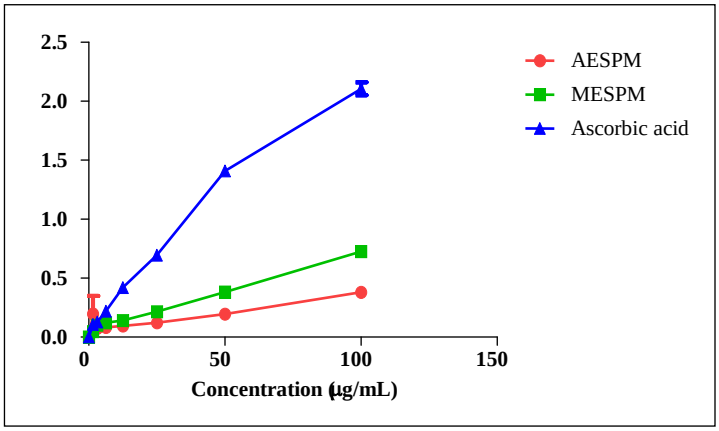

Figure 2: Reducing power of different extracts of stems bark of *S. mombin*

Ferric Reducing Antioxidant Power (FRAP) assay

The results of FRAP assay are showed in Table 4. The range of the extracts was range of 266.0 – 2056.10 µmol Fe (II)/g. the FRAP values of different extracts of stems bark of *S. mombin* were significantly lower than that ascorbic acid ($p<0.001$) but the FRAP values of MESPM (456.8 ±16.92 µmol Fe (II)/g) was so significantly higher than AESPM values (266.02 ± 4.69 µmol Fe (II)/g) ($p<0.01$).

ISSN: 0975-8585

Table 4: Ferric reducing antioxidant property (FRAP) of different extracts of stems bark of *S. mombin*

Extracts	FRAP (µmol Fe(II)/g)
AESPM	266.0 ± 4.69***
MESPM	456.8 ± 16.92***
Ascorbic acid	2056.10 ± 48.56

Values are expressed as mean ± SEM. (n = 3). ***p<0.001, when compared with ascorbic acid used as reference

Inhibition of lipid peroxidation by Ferric thiocyanate (FTC) method

The hydroperoxides inhibitory activity of *S. mombin* extracts throughout the seven days of incubation period was shown in Figure 3. From the analysis, it shows that all samples had been oxidized when stored for seven days at 40-45°C.

After seven days storage, all samples exhibited good effect in inhibiting linoleic acid oxidation compared to control (water) ($p<0.05$). The result in figure 4 indicated that the inhibition percentage of lipid peroxidation of AESPM (56.21 ± 1.22 %) was significantly lower ($p<0.01$) compared to reference compound ascorbic acid (72.87 ± 3.20 %). Also, MESPM inhibition percentage of lipid peroxidation had greater than that AESPM ($p<0.05$). However, antioxidant activities of MESPM (65.78 ± 2.00 %) and ascorbic acid were similar because there are no statistically difference between these values ($p>0.05$).

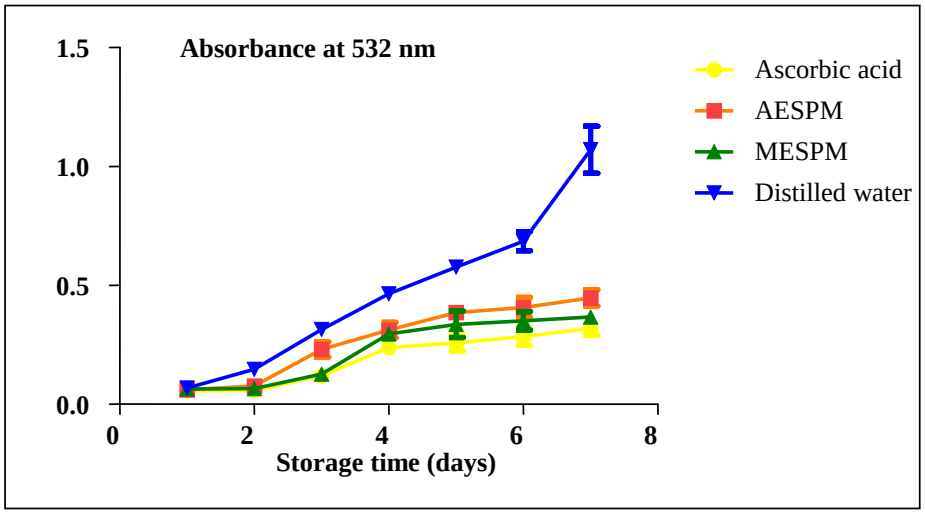

Figure 3: Antioxidant activity of different extracts of stems bark of *S. mombin* by FTC (Ferric thiocyanate) method

ISSN: 0975-8585

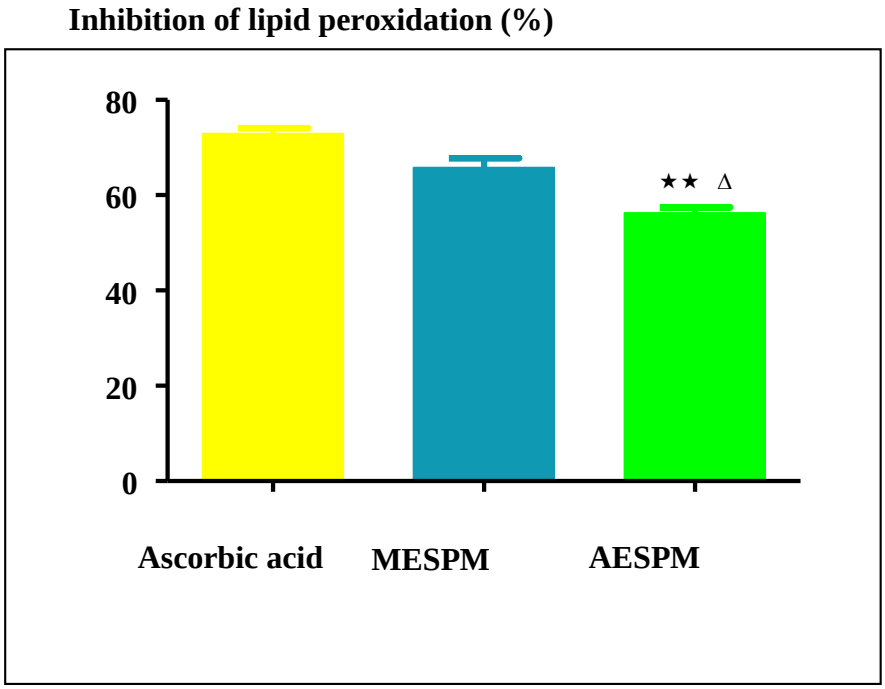

Figure 4: Antioxidant activity of different extracts of stem bark of *S. mombin* determined by FTC method after seven days of storage

*Values are expressed as mean ± SEM. (n = 3). ***p<0.01, when compared with ascorbic acid. Δ p<0.05, when compared with MESPM.*

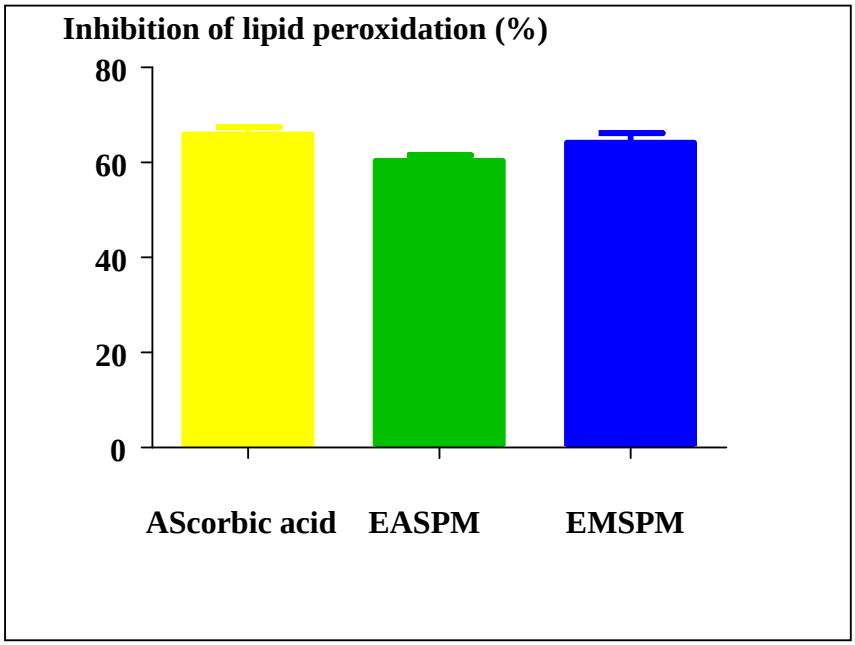

Figure 5: Antioxidant activity of different extracts of stems bark of *S. mombin* by TBA method

Values are expressed as mean ± SEM. (n = 3). p>0.05, when compared with ascorbic acid.

Thiobarbituric acid (TBA) test

Percentage inhibition of lipid peroxidation by TBA method of methanolic and aqueous extracts of *S. mombin* was determined and compared with ascorbic acid, the reference (Figure 5). The results of this study showed that inhibition percentage of lipid peroxidation of MESPM (64.20 1.95 ± 1.95 %) and AESPM (60.38 ± 1.13 %) were statistically no different from ascorbic acid, the reference (65.88 ± 1.55 %) with $p>0.05$.

DISCUSSION

Polyphenolic compounds are known to have antioxidant activity. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [18]. The results strongly suggest that phenolics are important components of this plant, and some of its pharmacological effects could be attributed to the presence of these valuable constituents.

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples. DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [19]. Usually, higher total phenol and flavonoids contents lead to better DPPH-scavenging activity [20].

The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing properties are generally associated with the presence of reductones, which exert antioxidant action by breaking the free radical chains, via hydrogen atom donation. Reductones are also reported to prevent peroxide formation, by reacting with certain precursors of peroxide [21].

The ferric reducing antioxidant power (FRAP assay) is widely used in the evaluation of the antioxidant component in polyphenols. The antioxidant potentials of the methanol and aqueous extracts of stem bark of *S. mombin* were estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). The ability to reduce Fe (III) may be attributed to hydrogen donation from phenolic compounds [22] which were present in *S. mombin.* Antioxidant activity increased proportionally to the polyphenol content. According to recent reports, a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species [23].

The FTC test measures the amount of peroxides during the initial stages of lipid peroxidation. Thus, a low absorbance value is indicative of high antioxidant activity by inhibition of hydroperoxide of tested sample. Results from the FTC test confirm earlier findings of this study, indicating that MESPM and AESPM might possess high levels of primary antioxidant compounds that are capable of suppressing hydroperoxide formation during the initial stages of

lipid peroxidation through radical-chain breaking mechanisms. These phenolic compounds may donate hydrogen and can terminate the free radical reaction chain by changing it to the stabile compounds [24].

FTC is used to measure the production of peroxide compound at the initial stage of oxidation while TBA test is used to measure the secondary product of oxidation such as aldehyde and ketone [25]. No statistically difference between MESPM, AESPM and ascorbic acid could be due to secondary product such as malonaldehyde which is not stable for a long period of time. It would be turned into alcohol and acid, which cannot be detected by a spectrophotometer [26].

CONCLUSION

Based on the results, it is possible to conclude that methanolic extract of *S. mombin* (MESPM) which contain the highest amount of phenolic compounds, flavonoids and flavonols, exhibited the greatest radical scavenging activities and antioxidant activities with most of the methods used compared to aqueous extract (AESPM). The obtained results might be considered sufficient to further studies for the isolation and identification of bioactive principles responsible for the antioxidant activity.

ACKNOWLEDGEMENTS

Authors are thankful to Professor Aké Assi Laurent (Laboratory of Botany, Training and Research Unit-Biosciences, Félix Houphouët Boigny University of Abidjan, Côte d'Ivoire) for botanical identification of the plant. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

REFERENCES

[1] Chandra MS, Balamurugan V, Thiripura S, Rekha R. JCPR 2012; 4: 197-202.
[2] Kasote DM, Hegd MV, Deshmukh KK. Am J Food Technol 2011; 6: 604- 612.
[3] Luximon-Ramma A, Bahorun T, Soobrattee MA, Aruoma OI. J Agric Food Chem 2002; 50: 5042-5047.
[4] Dasgupta N, De B. Food Chem 2007; 101: 471-474.
[5] Pham-Huy LA, He H, Pham- Huyc C. Int J Biomed Sci 2008; 4: 89-96.
[6] Gülçin I, Mshvildadze V, Gepdiremen A, Elias R. Phytomedicine 2006; 13: 343-351.
[7] Lin CC, Huang PC. Phytother Res 2002; 14: 489-494.
[8] Repetto M, Llesuy SF. Braz. J Med Biol Res 2002; 35: 523-534.
[9] Mc Donald S, Prenzler PD, Autolovich M, Robards K. Food Chem 2001; 73:73-84.
[10] Chang C, Yang M, Wen H, Chern J. J Food Drug Anal 2002; 10:70-76.
[11] Kumaran A, Karunakaran RJ. LWT 2007; 40: 344-52.
[12] Koleva II, Van Beek TA, Linssen JPH, De Groot A, Evstatieva LN. Phytochem Anal 2002; 13: 8-17.
[13] Benzie IFF, Strain JJ. Anal biochem 1996; 239: 70-76.

[14] Oyaizu M. Jpn J Nutr 1986; 44: 307-315.
[15] Osawa T, Namiki M. J Agric Food Chem 1981; 45: 735-739.
[16] Kikuzaki H, Nakatani N. J Food Sci 1993; 578: 1407-1410.
[17] Ottolenghi A. Arch Biochem Biophysics 1959; 79: 355-358.
[18] Zheng W, Wang SY. J Agric Food Chem 2001; 49: 5165- 5170.
[19] Dehpour AA, Ebrahimzadeh MA, Nabavi SF, Nabavi SM. Grasas Aceites 2009; 60: 405-412.
[20] Ebrahimzadeh MA, Bahramian F. Pak. J. Biol. Sci. 2009; 12: 413-419.
[21] Pin-Der- Duh, X. J Am Oil Chem Soc 1998; 75:455-461.
[22] Shimada K, Fujikawa K, Yahara K, Nakamura T. J Agric Food Chem 1992; 40: 945- 948.
[23] Oktay M, Gulcin I, Kufrevioglu OI. LWT 2003; 36: 263- 271.
[24] Amarowicz R, Naczk M, Shahidi F. J Agric Food Chem 2000; 48: 2755-2759.
[25] Farag RS, Badei AZMA, Hawed FM, El-Baroty GSA. J Am Oil Chem Soc 1989; 66: 793-799.
[26] Asmah R, Vijay K, Huzaimah AS. Towards Bridging Science and Herbal Industry. Kuala Lumpur: FRIM. 2000.