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Full Length Research Paper

Activity guided isolation and characterization of antioxidant and antibacterial agents from some local Nigerian plants

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This study aimed to present the activity guided fractionation, isolation and characterization of antioxidants and antibacterial agents from combined mixture of plants (*Vitex doniana*, *Diospyros mesipiliformis*, *Acacia polycantha*, *Pirinari macrophylla*, *Ficus sycomorus* and *Parkia biglobosa*) and that of *Pergularia tomentosa.* Combined Mixture of Plants (CMP) is used locally in ratio of 1:1 for the treatment of bacterial infections. The CMP and *P. tomentosa* were extracted with methanol separately; the residues obtained were also separately suspended in water and successively fractionated with hexane, ethylacetate and n-butanol. All the fractions obtained were screened for antimicrobial and antioxidant activities. For CMP, only the ethyl acetate fraction (EF) indicated marginal antibacterial activity with 8.0, 7.0 and 7.0 mm zone of inhibition against *Micrococcus luteus* (MTCC 2470), *Bacillus subtilis* (MTCC 121) and *Salmonella typhimurium,* respectively. Minimum inhibitory concentration (MIC) for the CMP was greater than 1000 for *M. luteus* and *S. typhimurium* and 87.5 µg/ml for *B. subtilis.* The CMP fraction was subjected to chromatographic separations which resulted in the isolation and characterization of five bioactive constituents, gallic acid, 3β-OH-α-amyrin, 5,7,3'.4',5'-pentahydroxy-3-O-glucophyranoside flavones (myricetin 3-O-β-rhamnopyranoside), 5,7,3',4' tetrahydroxy-3-O-glucopyranoside flavone (quercetin 3-O-β-rhamnopyranoside) and 3,5,7,3',4'-pentahydroxy flavones (quercetin). They were characterized with the help of ESI-MS, IR, ^{1}H C^{13}, HMBC/HSQC and COSY-NMR data. These compounds did not show antibacterial activity when tested separately but exhibited appreciable antioxidant activities in different manner. Chromatographic fractionation of hexane extract of *P. tomentosa* resulted in the isolation of lupeol acetate (LA) with marginal but selective activity against *M. luteus* and the activity is due to LA rather than the combined constituents. These findings suggest that the fractions of the extracts and pure compounds possess antibacterial and antioxidant properties.

Key words: Antioxidant properties, antibacterial activity, NMR data, *Pergularia tomentosa*, combined mixture of plants.

INTRODUCTION

Diseases caused by pathogenic bacteria and fungi present critical problem to human health and are one of the main causes of morbidity and mortality worldwide (WHO, 1998). Resistance to antibiotics and the occur-

rence of toxicity during prolonged treatment with present day drugs have been the reasons for extended search for newer drugs to treat microbial infections (Fostel and Lartey, 2000). Drug resistance is on the increase and there is need to search for other antimicrobial agents (Sharma and Kumar, 2006; Negi and Dave, 2010). Combination therapy is an alternative approach in the search for novel compounds with ability to deal with antibiotic resistant microorganisms. The combination can be of different plant extracts or plant extracts with standard antibiotics or chemicals. Studies have shown that plant extracts in combination of two or more are yielding effective antimicrobial activity against several microorganisms that even include drug resistant bacteria (Karmegam et al., 2008). Thus, interviews with traditional healers in Sokoto, Nigeria, indicated the use of the six plants in combination of 1:1 in the treatment of bacterial infections without any scientific validations. Plants have been used to treat infectious diseases due to their antimicrobial properties. This is due to the presence of various kinds of phytochemicals including phenolic compounds, alkaloids, terpenoids and essential oils (Lewis and Elvin-Lewis, 1995; Cowan, 1999).

Pergularia tomentosa (PS milk weed) is used in Northern Nigeria for tanning and treatment of skin diseases. Its isolated cardenolides have been shown to cause apoptotic cell death of Kaposi's sarcoma cells (Hamed et al., 2006). The roots have found applications in the treatment of bronchitis, constipation and skin diseases (Hammiche and Maize, 2006). It is well known that free radicals cause cell damage through mechanism of covalent binding and lipid peroxidation with subsequent tissue injury (Osawa et al., 1990). Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals. Antioxidant properties of certain flavonoids of plant origin have already been established (Di Carlo et al., 1999). *Ficus sycomorus* is used locally for antimicrobial treatment in Nigeria and has been reported to have antimicrobial activities (Hassan et al., 2007). In the present work, we evaluated the synergistic antibacterial properties of the combined mixture of plants (CMP) and isolated and characterized the bioactive principles of the CMP and *P. tomentosa*. The pure compounds were also screened for antibacterial and antioxidant properties. To the best of our knowledge these have not been reported so far. Therefore, it is worthwhile in this study to present the activity guided fractionation, isolation and characterization of antioxidants and antibacterial agents from the CMP and *P. tomentosa*.

MATERIALS AND METHODS

Plant material

The leaves, roots and stems of the selected plants were collected from the adjoining area of Usmanu Danfodiyo University (UDU), Sokoto, Nigeria. After proper taxonomic identification of all the plants (before combination) by the Taxonomist of Botany Unit (U.D.U.), the plant parts (leaf, root and stem) were open air-dried under the shade and pulverized into a moderately coarse powder.

Chemicals

DPPH, ascorbic acid, quercetin and $FeCl_3$ were purchased from Sigma Chemical Co. (St. Lois, MO, USA). Vanillin from BDH, Follin Ciocalteu's phenol reagent and sodium carbonate were from Merck Chemical supplies (Darmstadt, Germany). All the chemicals and solvents used were of analytical grade.

Microbial organisms

The microbial organisms used were available in the Molecular and Bio-prospection Unit, of Central Institute of Medicinal and Aromatic Plants, Lucknow, India. The bacterial isolates were maintained on nutrient agar medium.

General experimental procedures

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 (300 MHz). Column chromatography was performed with silica gel (60 to 120 mesh). TLCs were run on ready-made aluminum sheets (silica gel 60 F254, 0.25 mm, 20 × 20 cm, Merck, Germany) while preparative TLCs were run on glass plates (silica gel 60 F254, 0.5 mm, glass plates 20 × 20 cm) from Merck, Germany. Spots on the TLC plates were visualized by spraying with vanillin sulfuric acid and heating the plate in oven for 5 min at 100°C. Vacuum liquid chromatographic (VLC) separation was run over silica gel H (average particle size approximately 10 µm). The powdered leaf of *P. tomentosa* (PT) and the parts of the combined mixture of plants (1:1) were extracted with methanol separately and each residues obtained were dissolved in water separately and each were further fractionated with hexane, petroleum ether and n-butanol. The following fractions were obtained:

P. tomentosa (methanolic leaf) extract = 28.9 g → HF (g), EF (g), BF (g),
Combined mixture of plants = 40.0g → HF (g), EF (g), BF (g).

Isolation of bioactive compounds from the ethyl acetate fraction (EF) of combined mixture of plants (CMP)

The ethyl acetate fraction (EF) of combined mixture of plants that showed remarkable antibacterial and antioxidant activities was further fractionated. Eight grams (8 g) of this fraction was subjected to vacuum liquid chromatographic (VLC) separation over silica gel H (average particle size approximately 10 µm). Stepwise gradient elution was carried out with hexane, hexane-chloroform, chloroform, chloroform-methanol and methanol. A total of 249 fractions were collected. The fractions were pooled on the basis of their TLC profile as follows: Fractions 14 to 62 (270 mg), fractions 63 to 73 (308 mg), fractions 152 to 184 (325 mg), fractions 201 to 214 (524 mg), fractions 215 to 230 (495 mg), fractions 231 to 246 (1000 mg).

Isolation of bioactive compounds from the hexane fraction of *Pergularia tomentosa*

Separately, activity guided separation of hexane fraction of *P. tomentosa* which showed antibacterial activity was carried out. After series of chromatographic separation, a total of 114 ddfractions were collected. The fractions were pooled on the basis of their TLC

profiles and the hexane fraction resulted in the isolation of lupeol acetate (Figure 6) and its antibacterial activity was determined.

Antioxidant activity

Free radical scavenging activity

It was measured using the modified method of Blois (1985). DPPH (50 µL of 0.1 mM dissolved in methanol) was added to the tested compounds at different concentrations (1, 5, 10, 25, 50 and 100 µg) and 40 µL of Tris-HCl were also added. Equal volume of methanol, Tris-HCl and DPPH were added in the control test. The mixture was shaken vigorously and incubated at 37°C for 20 min. The absorbance at 517 nm was measured spectrophotometrically. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percentage of scavenging of DPPH was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{AO - A1}{AO} \times 100$$

Where, AO is the absorbance of the control reaction, A1 is the absorbance in the presence of the sample.

Total phenolics estimation

The amount of total phenolics was determined by Folin-Ciocalteu's colorimetric method (Wolf et al., 2003). Briefly, the concentration of the compounds (1, 5, 10, 25, 50 and 100 µg) were mixed with 50 µL of distilled water and 250 µL of Folin-Ciocalteu's reagent were added and mixed properly. A 250 µL of sodium carbonate was then added. The mixture was incubated at 37°C for 90 min and the absorbance was measured at 765 nm by a XPLORER XP2001 spectrophotometer. Gallic acid was used as a standard and total phenolics were expressed as grams of Gallic acid equivalent (g of GAE) per 100 g of fresh weight.

Nitric oxide scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction (Green et al., 1982; Marcocci et al., 1994). The reaction mixture containing 100 µL of sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and the compounds (1, 5, 10, 25, 50 and 100 µg) were incubated at room temperature for 30 min. After incubation, 50 µL of incubated reaction mixture were added to 100 µL of Griess reagent (1:1 sulfanilamide: naphthylethylene diaminehydrochloride). The absorbance of the chromophore formed was measured at 546 nm. The percentage of nitric oxide scavenging activity was calculated using the following equation:

$$\text{\% inhibition} = \frac{AO - A1}{AO} \times 100$$

Where, AO is the absorbance of the control reaction, A1 is the absorbance in the presence of the sample.

Total antioxidant capacity

The assay was based on the reduction of molybdenum (VI) to molybdenum (V) by the compounds and the subsequent formation of a green phosphate Mo (V) complex to acid pH (Priesto et al., 1999). Compounds (1, 5, 10, 25, 50 and 100 µg) were combined with 1 ml of total antioxidant capacity (TAC) reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95°C for 90 min and cooled down to room temperature. The absorbance was measured at 695 nm against reagent blank. The total antioxidant capacity was expressed as the number of equivalent of ascorbic acid (mg/g of dry mass).

Reducing power

The reducing power of the extract/compound was determined according to the method of Oyaizu (1986). Different concentrations of the compounds (1, 5, 10, 25, 50 and 100 µg) were mixed with 250 µL phosphate buffer (pH 6.6, 0.2 M) and 250 µL (1%) potassium ferricyanide. The mixture was incubated at 50°C for 20 min. A 250 µL of trichloroacetic acid (10%) was added to the mixture and centrifuged at 5000 rpm for 3 min. Then 250 µL of the supernatant was mixed with 250 µL of distilled water and 50 µL of $FeCl_3$ (0.1%). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated reducing power.

Total flavonoids

Estimation of total flavonoids was done according to the method of Ordon Ez et al. (2006). To 50 µL of the compounds (1, 5, 10, 25, 50 and 100 µg), 150 µL of methanol, 10 µL of $AlCl_3$, 10 µL of potassium acetate and 280 µL of distilled water were added. The mixture was incubated at room temperature (25 to 37°C) for 30 min. The absorbance of the reaction mixture was measured at 415 nm. A yellow color indicated the presence of flavonoids content. Total flavonoids content was calculated as quercetin (mg/g).

FRAP assay

The stock solution included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 ml $C_2H_4O_2$) pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-5-triazine) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$. The fresh working solution was prepared by mixing 2.5 ml acetate buffer, 2.5 ml TPTZ and 2.5 ml $FeCl_3$ (FRAP reagent). A 50 µL of the compound was added to 1.5 ml of FRAP reagent. The mixture was mixed and incubated at 37°C for 5 min. Absorbance was measured at 593 nm. Results were expressed as $FeSO_4$ equivalent (Benzie and Strain, 1996).

Disc diffusion assay

The CMP and *P. tomentosa* extracts and pure compounds were screened for antibacterial activity against the following organisms: *Staphylococcus aureus* (MTTC 96), *Staphylococcus aureus* (MTTC 2940), *Escherichia coli* (MTTC 739), *Micrococcus luteus* (MTCC 2470), *Bacillus subtilis* (MTCC 121), *Streptococcus mutants* (MTCC 890), *Raoultella planticola* (MTCC 530), *Klebsiella pneumoniae* and *Salmonella typhimurium*. Strains were grown overnight at 36°C in nutrient broth medium. Inoculums for the assays were prepared by diluting cell mass in 0.85% NaCl solution, adjusted to McFarland scale 0.5 (1.5×10^8 CFU/ml) and prepared nutrient agar plates were seeded with 1.5×10^8 CFU/ml suspensions of test bacteria. The antibacterial activity of culture was determined using disc diffusion assay according to the Clinical and Laboratory Standard Institute (CLSI, formerly NCCLS). Absorbent disc (5 mm) were impregnated with 5 µL of the CMP and *P. tomentosa* extracts (100

Table 1. Antibacterial activity of ethyl acetate fraction of combined mixture of plants and the isolated compounds.

Parameter	SA-96	SA-2940	ML	EC	BS	SM	STM	KP	RP
Plant extract/pure compounds/drugs									
CMP	-	-	8.0		7.0	-	7.0	-	-
73-91LPE	-	-	10.0		-	-	-	-	-
45-46 cmp	-	-	-	-	-	-	-	-	-
126-141	-	-	-	-	-	-	-	-	-
128-157 cmp	-	-	-	-	-	-	-	-	-
79-83	-	-	-	-	-	-	-	-	-
cp 87-112	-	-	-	-	-	-	-	-	-
Kanamycin	22	20	24	23	35	17	29	5	25
Ampicillin	23	24	25	5	32	5	25	5	5

Zone of inhibition are recorded in mm, - = no activity. SA = *Staphylococcus aureus* (MTTC 96 and MTTC 2940) and (MTTC 2940), EC = *Escherichia coli* (MTTC 739), ML= *Micrococcus luteus* (MTCC 2470), BS = *Bacillus subtilis* (MTCC 121), SM = *Streptococcus mutants* (MTCC 890), RP= *Raoultella planticola* (MTCC 530), KP = *Klebsiella pneumoniae* and STM = *Salmonella typhimurium.* CMP = combined mixture of plants (ethylacetate fractions), 73-91LPE (Figure 6), 45-46 cmp (Figure 2). 126-141 (Figure 4), 128-157 cmp (Figure 3), 79-83 (Figure 1), CP87-112 (Figure 5).

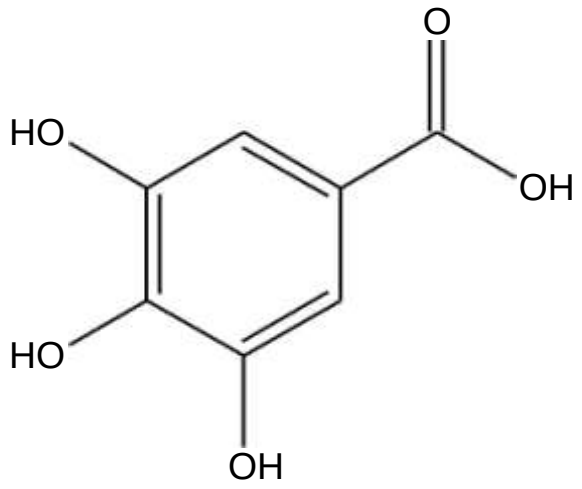

Figure 1. Fractions 79-83 = Gallic acid.

mg/ml) and pure compounds (10 mg/ml) and placed onto the surface of inoculated agar plates. Plates were incubated at 37°C for 24 h. Positive control discs of kanamycin and ampicillin was included. Antibacterial activity was expressed as the diameter of the inhibition zone (mm) produced by the extracts (Mellou et al., 2005).

Minimum inhibitory concentration

The CMP and *P. tomentosa* extracts that showed some activity were subjected to MIC test. MIC test was carried out according to the method of Ellof (1998), using Muller-Hinton Broth on a tissue culture test plate (96 wells). The stock solutions of extracts were transferred into the first well, and serial dilutions were performed in order to have concentrations in the range of 1000 to 7.81 µg/ml. Inoculums for the assays were prepared by diluting cell mass in 0.85% NaCl solution, adjusted to McFarland scale 0.5, added to all wells and incubated at 36°C for 24 h. MIC was defined as the lowest concentration of the extracts that inhibited visible growth.

RESULTS AND DISCUSSION

The results of antibacterial activity are presented in Table 1, which shows that only ethyl acetate fraction (EF) of combined mixture of plants (CMP) has marginal antibacterial activity with 8.0, 7.0 and 7.0 mm zone of inhibitions for *M. luteus*, *B. subtilis* and *S. typhimurium*, respectively. Minimum inhibitory concentration (MIC) for the CMP was greater than 1000 for *M. luteus* and *S. typhimurium* and 87.5 µg/ml for *B. subtilis.* Figures 1 to 5 did not show antibacterial activity. Lupeol acetate (Figure 6) was found to inhibit *M. luteus* (MTCC 2470) with inhibition zone of 10.0 mm. Lupeol acetate belongs to lupane type triterpenes and was reported to have antimicrobial activity (Prachayasittikul et al., 2010).

Our findings are consistent with the study on synergistic activity of six plants that showed activity against pathogenic bacteria by Karmegam et al. (2008). All the pure compounds isolated from the CMP extract did not show antibacterial activity. The antibacterial activity of the ethylacetate fraction of CMP was due to combination of all the constituents in the CMP rather than the individual compounds isolated. A series of chromatographic separation of ethylacetate fraction of CMP (as outlined in the experimental section) resulted in the isolation and characterization of bioactive constituents, Gallic acid (Figure 1), 3β-Hydroxy-α-amyrin (Figure 2), 5,7,3'.4',5'-pentahydroxy-3-O-glucophyranoside flavones (Figure 3), 5,7,3',4' tetrahydroxy-3-O-glucopyranoside flavones (Figure 4), 3,5,7,3',4'-pentahydroxy flavone (Figure 5). All the compounds isolated were characterized with the help of ESI-MS, IR, ^{1}H C^{13}, HMBC/HSQC and COSYNMR. Chemical analysis has indicated that some complex compounds elaborated by natural organisms may hardly be synthesized by chemical processes (Azas et al., 2002). However, the bacterial resistance to chemical

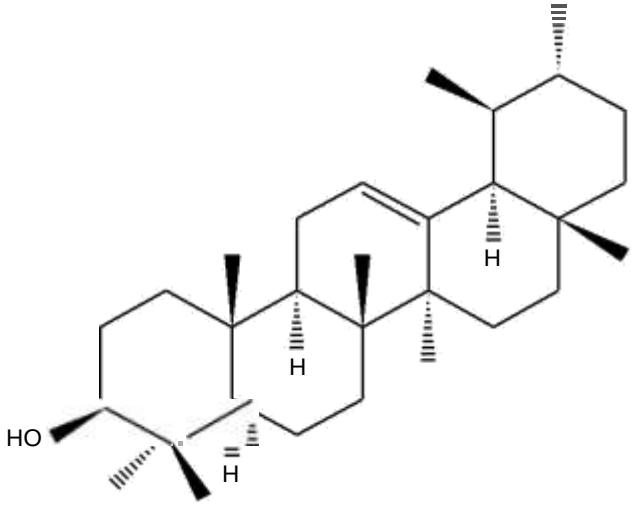

Figure 2. Fractions 45-46 cmp = 3β-Hydroxy-α-amyrin.

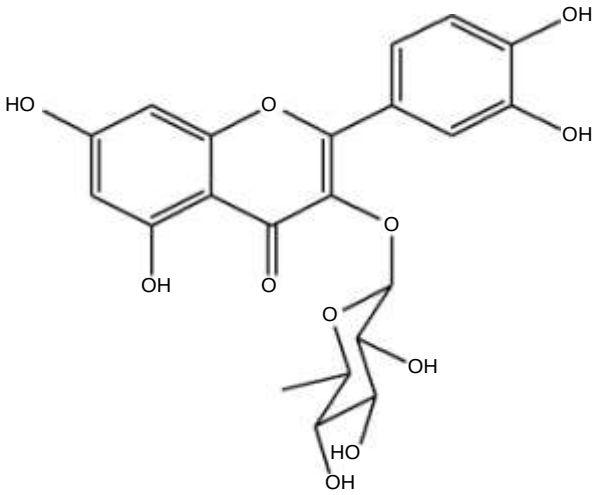

Figure 4. Fractions 126-141 = 5,7,3',4' tetrahydroxy-3-O-glucopyranoside flavone (quercetin- 3-O-β-rhamnopyranoside).

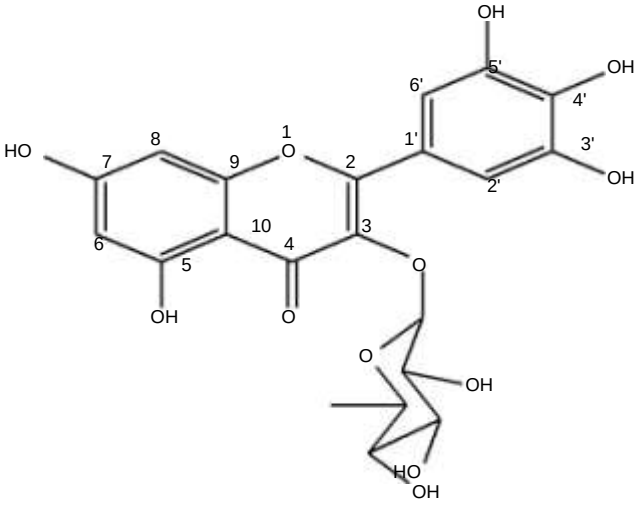

Figure 3. Fractions 128-157 cmp = 5,7,3',4',5'-pentahydroxy-3-O-glucophyranoside flavones (myricetin -3-O-β-rhamnopyranoside).

treatment still remained important. Natural products isolated from the plants in the present study may be potential sources of new antioxidant drugs.

Activity guided separation of hexane fraction of *P. tomentosa* was also carried out. After series of chromatographic separation, a total of 114 fractions were collected. The fractions were pooled on the basis of their TLC profile and the hexane fraction resulting in the isolation of bioactive constituent (Figure 6) lupeol acetate (LA). LA was screened for its antibacterial activity, which showed marginal but selective activity against *M. luteus* (Table 1). This confirms that the lack of antibacterial activity of hexane extract of *P. tomentosa* was due to combination of all the constituents rather than LA alone.

^{1}H NMR (CDCl $_3$, 300 MHz): δ 7.05 (2H, s, H-3 & H-7), ^{13}C NMR (CD_3OD, 300 MHz): 170.42 (C-1'), 121.93 (C-1), 110.30 (CH, C-2), 146.35 (C-3), 139.57 (C-4), 146.35 (C-5), 110.30 (CH, C-6)

^{1}H NMR δ 0.77 to 1.14 (24H, 6x ter-Me & 2x sec-Me) 1.3 (1H, d, J = 6.3Hz, 18α-H), 3.20 (1H, dd, J = 5.1, 1.02 3α-H) 5.20 (1H, t, J = 3.0, 12-H)

^{13}C NMR ($CDCl_3$, 300 MHz): 38.76 (CH_2, C-1), 27.25 (CH_2, C-2), 78.98 (CH, C-3), 38.76 (C-4), 55.16 (CH, C-5), 18.34 (CH_2, C-6), 32.91 (CH_2, C-7), 39.99 (CH, C-8), 47.69 (CH, C-9), 36.88 (C-10), 23.25 (CH_2, C-11), 124.39 (CH, C-12), 139.30 (C-13), 42.03 (C-14), 28.08 (CH_2, C-15), 26.60 (CH_2, C-16), 33.73 (C-17), 59.04 (CH, C-18), 39.59 (CH, C-19), 39.59 (CH, C-20), 31.23 (CH_2, C-21), 41.51 (CH_2, C-22), 28.08 (CH_3, C-23), 15.62 (CH_3, C-24), 15.62 (CH_3, C-25), 16.84 (CH_3, C-26), 23.25 (CH_3, C-27), 28.08 (CH_3, C-28), 17.46 (CH_3, C-29), 21.39 (CH_3, C-30).

^{1}H NMR (MeOD, 300 MHz): δ 6.13 (1H, d, J = 2.0 Hz, H-6), 6.29 (1H, d, J = 2.0 Hz, H-8'), 6.89 (2H, s, H-2' & H-6'), 5.25 (1H, d, J = 1.5 Hz, H-1''), 3.43 (1H, dd, J = 5.7, 9.0 Hz, H-4''), 3.73 (2H, m, H-2'' & H-3''), 4.17 (1H, m, H-5''), 0.89 (3H, d, J = 6.0 Hz, H-6'').

^{13}C NMR (MeOD, 300 MHz): δ158.41 (C-2), 136.24 (C-3), 179.57 (C-4), 163.05 (C-5), 99.81 (C-6), 165.83 (C-7), 94.72 (C-8), 159.37 (C-9), 105.79 (C-10), 121.85 (C-1'), 109.59 (C-2'), 146.43 (C-3'), 137.83 (C-4'), 146.75 (C-5'), 109.59 (C-6'), 103.56 (C-2''), 71.80 (C-3'') 73.27 (C-4'') 79.45 (C-5'') 72.00 (C-6''), 17.62 (C-7'').

^{1}H NMR (MeOD, 300 MHz): δ 6.19 (1H, d, J = 2.2 Hz, H-6), 6.38 (1H, d, J = 2.2 Hz, H-8), 6.88 (1H, d, J = 8.4 Hz,

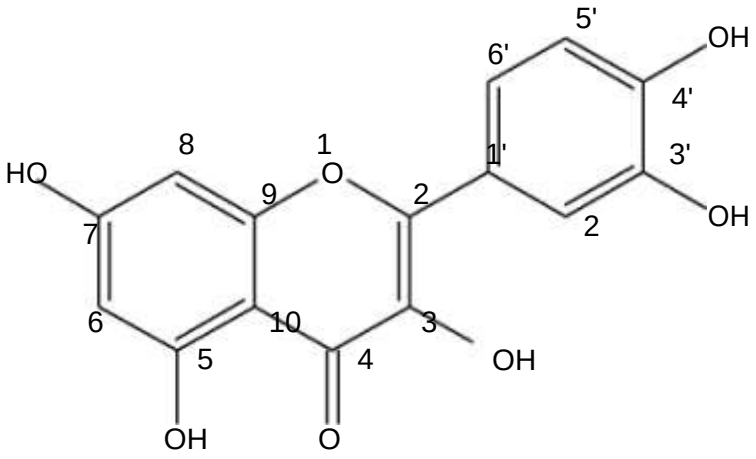

Figure 5. Fractions cp87-112 = 3,5,7,3',4'-pentahydroxy flavone (quercetin).

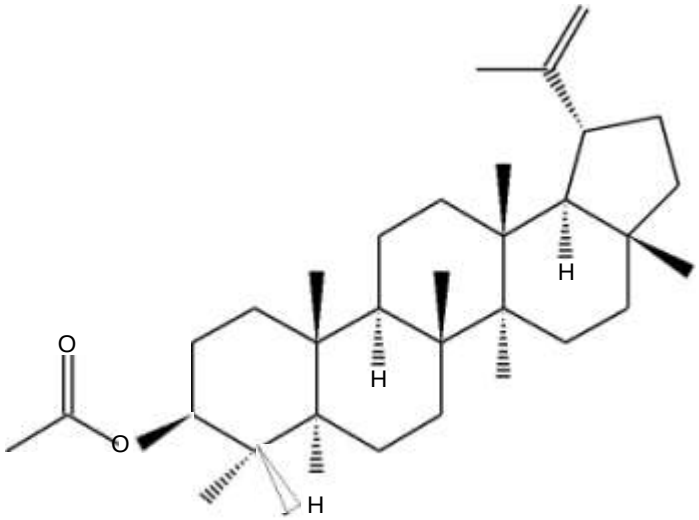

Figure 6. Fractions 73-91 LPE = Lupeol acetate.

H-5'), 7.30 (2H, m, H-2' & H-6'), 5.34 (1H, d, J = 1.5 Hz, H-1''), 3.90 (1H, dd, J = 1.5, 3.3 Hz, H-2''), 3.72 (1H, m, H-3''), 3.45 (1H, m, H-4''), 4.18 (1H, m, H-5''), 0.93 (3H, d, J = 6.0 Hz, H-6'').

^{13}C NMR (MeOD, 300 MHz): δ 158.26 (C-2), 132.25 (C-3), 177.36 (C-4), 162.48 (C-5), 99.29 (C-6), 165.60 (C-7), 94.36 (C-8), 159.25 (C-9), 103.56 (C-10), 123.35 (C-1'), 116.72 (C-2'), 147.81 (C-3'), 147.18 (C-4'), 116.72 (C-5'), 122.93 (C-6'), 103.56 (C-2''), 71.91 (C-3''), 73.27 (C-4''), 79.45 (C-5''), 72.03 (C-6''), 17.64 (C-7'').

^{1}H NMR (Py, 300 MHz): δ 6.74 (1H, d, J = 2.2 Hz, H-6), 6.78 (1H, d, J = 2.2 Hz, H-8), 8.63 (1H, s, H-2'), 8.10 (1H, d, J = 8.4 Hz, H-6'), 7.40 (1H, d, J = 8.4 Hz, H-5').

^{13}C NMR (Py, 300 MHz): δ 157.26 (C-2), 137.96 (C-3), 177.36 (C-4), 162.48 (C-5), 99.29 (C-6), 165.60 (C-7), 94.36 (C-8), 157.50 (C-9), 104.50 (C-10), 123.35 (C-1'), 116.72 (C-2'), 147.81 (C-3'), 147.18 (C-4'), 116.72 (C-5'), 121.12 (C-6').

Lupeol acetate: white needles (30 mg)

^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.69 (1H, *s*, H-29b), 4.57 (1H, *s*, H-29a), 4.47 (1H, *dd, J* = 4.4, 12.8 Hz, H-3), 2.05 (3H, *s*, H-2*l*), 1.69 (3H, *s*, H-30), 1.03 (3H, *s*, H-25) 0.94 (3H, *s*, H-28), 0.85 (3H, *s*, H-23), 0.84 (3H, *s*, H-24), 0.83 (3H, *s*, H-26), 0.79 (3H, *s*, H-27).

^{13}C NMR ($CDCl_3$, 300 MHz): δ 38.43 (CH_2,C-1), 27.83 (CH_2,C-2), 81.38 (CH,C-3), 38.78 (C-4), 55.77 (CH,C-5), 18.60 (CH_2,C-6), 34.60 (CH_2,C-7), 41.24 (CH,C-8), 50.73

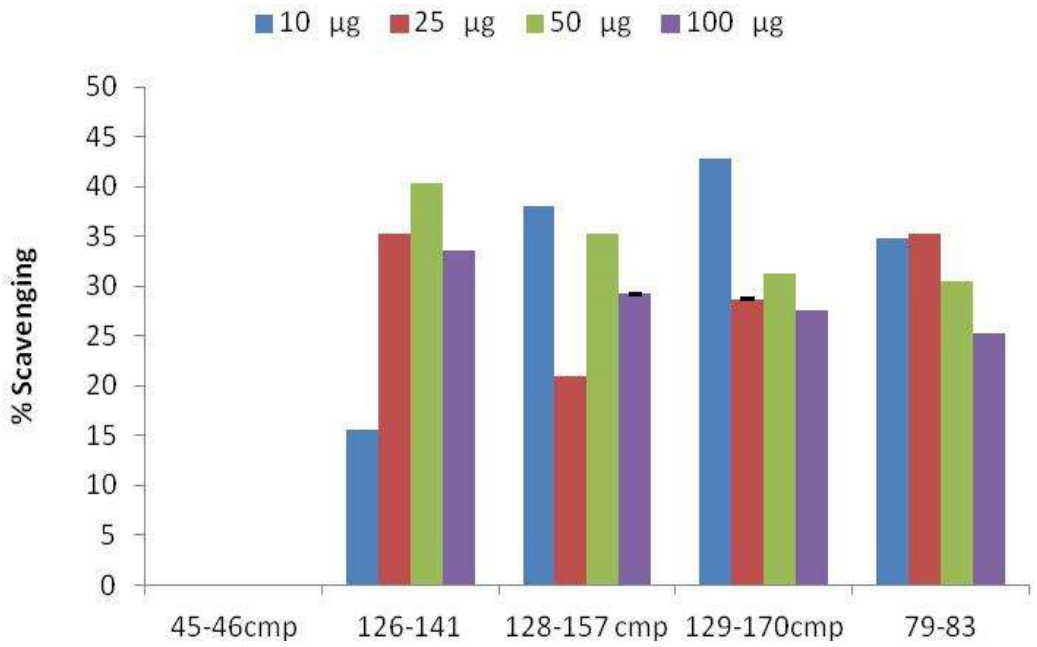

Figure 7. DPPH scavenging activity of pure compounds.

(CH,C-9), 37.48 (C-10), 21.33 (CH_2,C-11), 25.48 (CH_2,C-12), 38.19 (C-13), 43.22 (C-14), 27.83 (CH_2,C-15), 35.97 (CH_2,C-16), 43.40 (C-17), 48.67 (CH,C-18), 48.41 (CH,C-19), 151.37 (C-20), 30.11 (CH_2,C-21), 40.40 (CH_2,C-22), 28.35 (CH_3,C-23), 14.90 (CH_3,C-24), 16.59 (CH_3,C-25), 16.37 (CH_3,C-26), 14.54 (CH_3,C-27), 18.40 (CH_3,C-28), 109.77 (CH_2,C-29), 19.69 (CH_3,C-30), 171.45 (C-1'), 21.74 (C-2').

Results of antioxidant studies are presented in Figures 7 to 13. In Figure 7, the amount of DPPH reduced was quantified by measuring increases in absorbance at 517 nm. The DPPH scavenging ability of the tested compounds may be attributed to their hydrogen donating ability. Non DPPH scavenging activity was observed for 73 to 91 LPE (Figure 6) and 45 to 46 cmp fractions (Figure 2). The fractions 126 to 141 (Figure 4), 128 to 157 cmp (Figure 3), cp87 to 112 (Figure 5) and 79 to 83 (Figure 1) have showed appreciable DPPH scavenging activity in different manner and for Figure 1 the activity decreases at 50 and 100 µg; this indicates that at higher concentration the activity was inhibited. This order was reversed in 126 to 141 fractions (Figure 4), in which the DPPH scavenging activity increases with increasing concentration of the compound but reduction was observed at 100 µg. Hence, the DPPH scavenging activity of the compounds may be represented as:

cp87 - 112 > 126 - 141 > 128 - 157 cmp > 79 - 83

Nitric oxides (NO) are potent inhibitors of physiological processes such as smooth muscle relaxation, neuronal signaling, platelet aggregation and regulation of cell mediated toxicity (Hagerman et al., 1998). In Figure 8, a non NO scavenging activities were observed for fractions 73 to 91 LPE (Figure 6). The nitric oxide (NO) scavenging activity of all the compounds was low with maximum of 22% inhibition for fractions 126 to 141 (Figure 4) at 25 µg but with no activity at 50 and 100 µg. The compound 45 to 46 cmp (Figure 2) has little NO scavenging activity but the activity increased as the concentration increased; however, the activity dropped drastically at 100 µg.

Fractions 79 to 83 (Figure 1) did not show any activity. The compound 128 to 157 cmp (Figure 3) showed activity only at 10 µg, this shows that the NO scavenging activity of this compound was inhibited at concentration higher than 10 µg. The reducing power (Figure 9) of the compounds of fractions 126 to 141 (Figure 4), 128 to 157 cmp (Figure 3), and 79 to 83 (Figure 1) were found to increase in dose- dependent manner. Ferric reducing antioxidant power (FRAP) showed no activity in fractions cp87 to 112 (Figure 5) and 73 to 91 (Figure 6). However, 126 to 141 fractions (Figure 4) showed equal activity at 10 and 25 µg but there after increased dose - dependently. The 45 to 46 cmp fractions (Figure 2) did not show any activity. The ferric reducing antioxidant power (FRAP assay) is widely used in the evaluation of the antioxidant components of polyphenols (Luximan-Ramma et al., 2005). From the results, there is a relationship between total phenols and reducing power of the tested pure compounds. The FRAP assay (Figure 13) may be represented as:

79 - 83 > 128 - 157 cmp > 126 - 141.

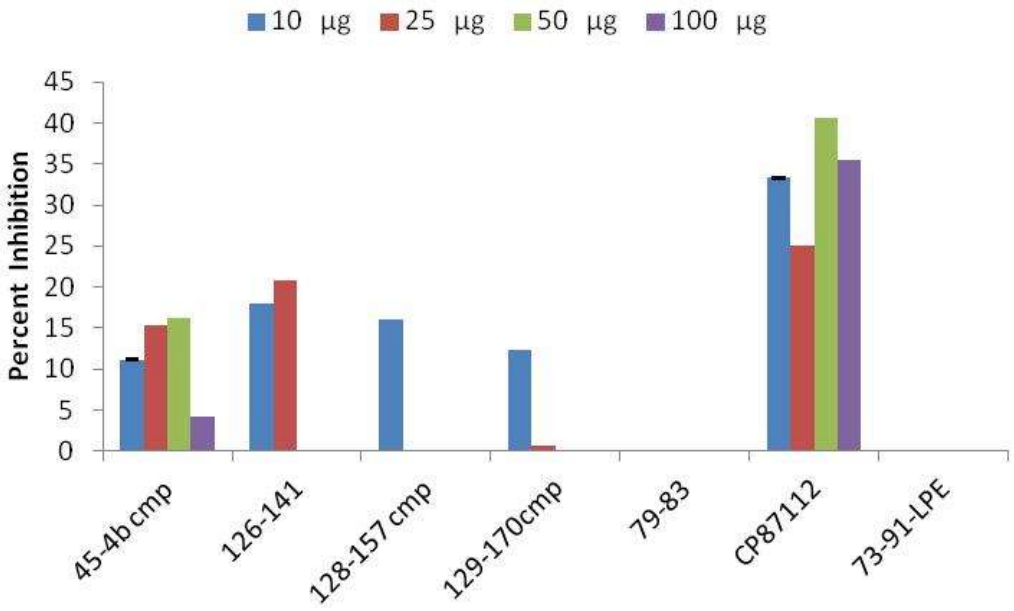

Figure 8. Nitric oxide scavenging activity of pure compounds.

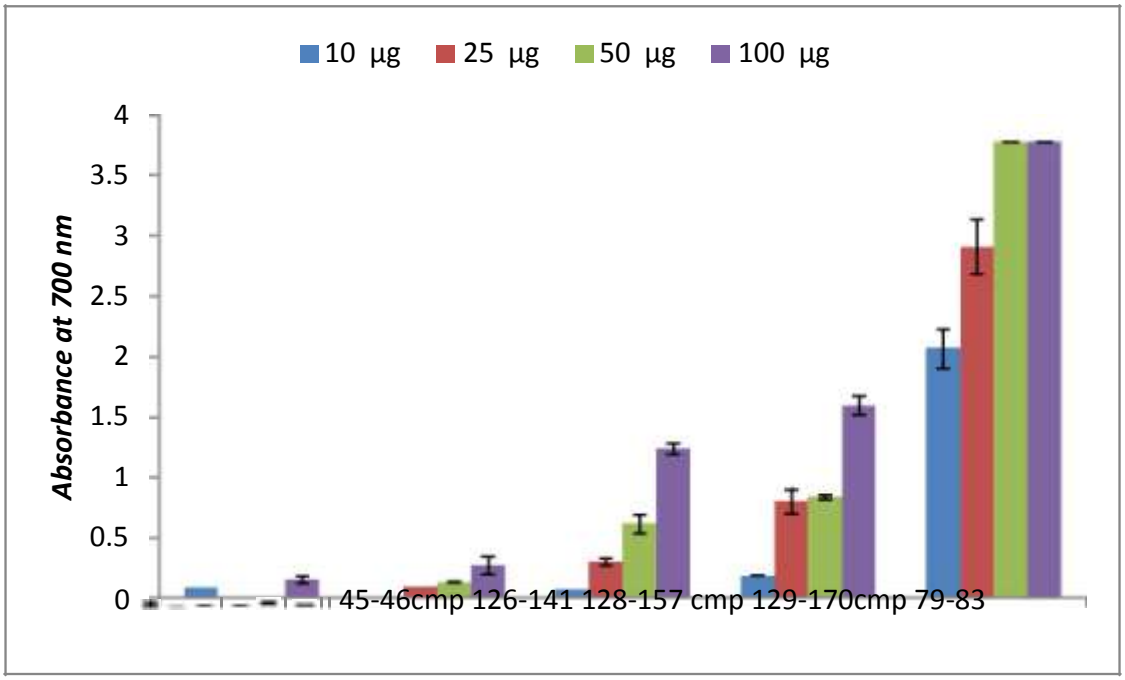

Figure 9. Reducing power ability of pure compounds.

In Figure 10, little amount of total flavonoids (TF) were observed for 73 to 91 LPE fractions (Figure 6). The total flavonoids contents were also found to increase in a dose- dependent manner for 128 to 157 cmp (Figure 3) and 79 to 83 (Figure 1) compounds. It dropped slightly at 100 µg for compound 126 to 141 (Figure 4) and at 50 to 100 µg for 45 to 46 cmp (Figure 2). The total phenolics (TP) were increased in dose dependant manner with exception of 45 to 46 cmp fractions (Figure 2) and 73 to 91 LPE (Figure 6) that showed little or no activity (Figure 11). The antioxidant activity of polyphenolic compounds is mainly due to their redox properties which play an important role in adsorbing to and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Zheng and Wang, 2001). Phytochemicals like polyphenols possess significant antioxidant

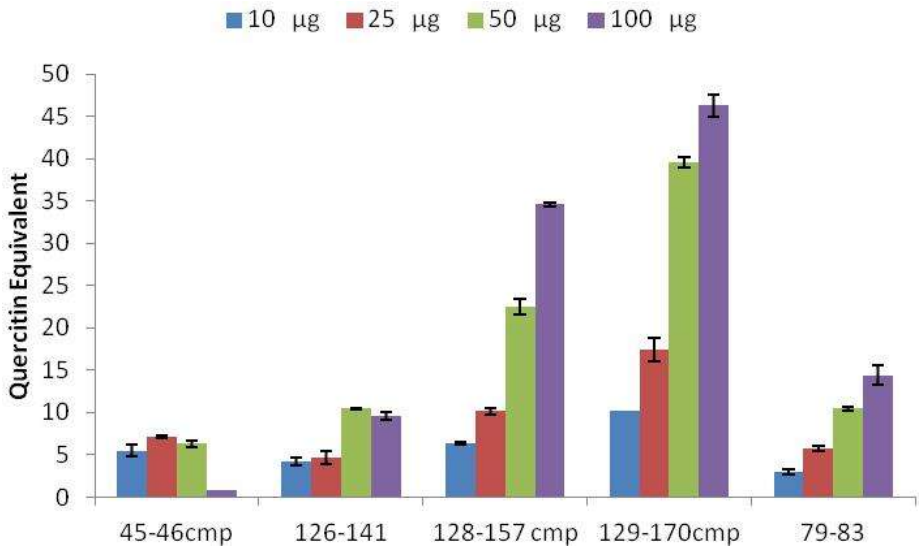

Figure 10. Total flavonoids capacity in terms of quercetin equivalent of pure compounds.

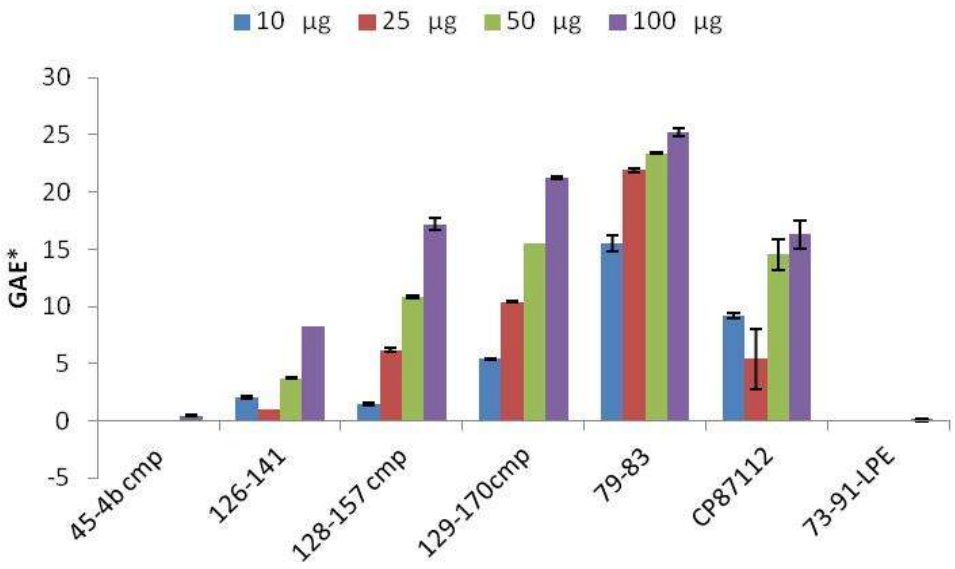

Figure 11. Total phenolics expressed in terms of Gallic acid equivalent (GAE*) of pure compounds.

capacities that are associated with lower mortality and rate of diseases (Anderson et al., 2001; Djeridane et al., 2006). The pharmacological effect demonstrated by the ethylacetate fraction of the combined plants mixture suggests that the phenolics have some pharmacological effects and could be attributed to these valuable constituents. All the results of total phenolic estimation were expressed as Gallic acid equivalent (Figure 11) and are represented as follows:

79 - 83>128 - 157 cmp > 126 - 141 > 45 - 46 cmp

For total antioxidant capacity (TAC), 73 to 91 LPE fractions (Figure 6) did not show any activity. TAC in 45 to 46 cmp fractions (Figure 2), 128 to 157 cmp (Figure 3) were found to increase in a dose- dependent manner but for 126 to 141 (Figure 4) it increases from dose 25 to 100 µg. However, 79 to 83 (Figure 1) showed only equal activity at 10 and 25 µg. All the results were expressed as ascorbate equivalent (Figure 12) and may also be represented as follows:

45 - 46 cmp > 128 - 157 cmp > 79 - 83 > 126 - 141

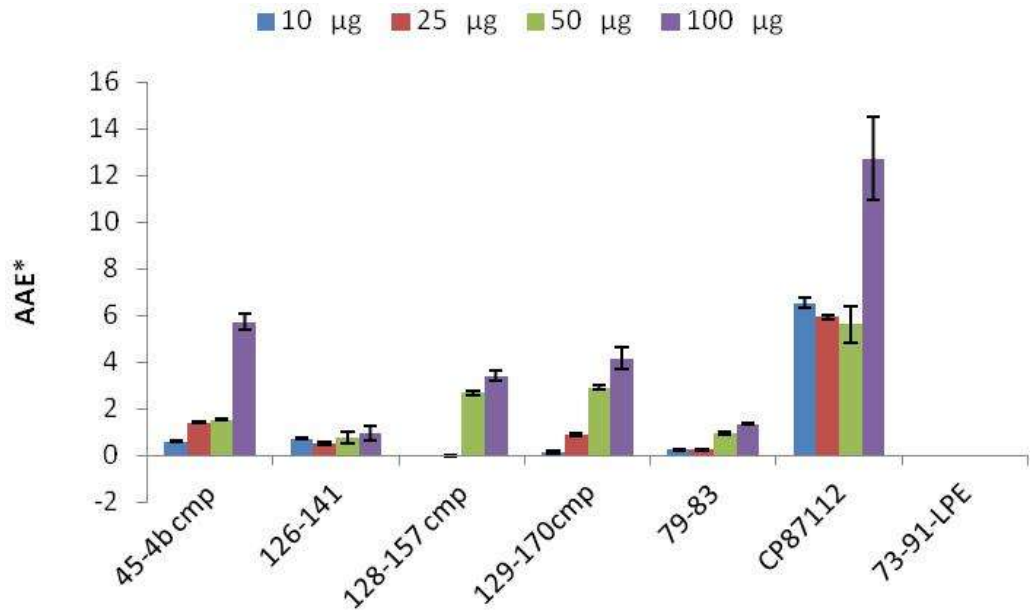

Figure 12. Total antioxidant capacity in terms of ascorbate equivalent (AAE*) of pure compounds.

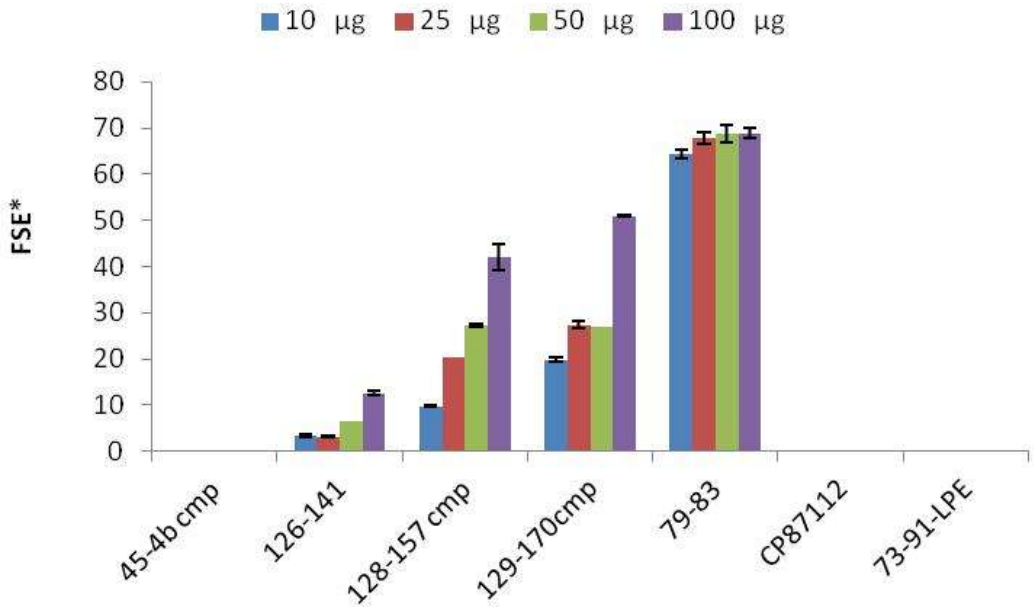

Figure 13. FRAP assay expressed in terms of ferrous sulphate equivalent (FSE*) of pure compounds.

The effects of the isolated compounds in the present study is due to their phenolic acids and flavonoids nature and have been demonstrated to exhibit antioxidant activity (Sliva et al., 2006; Kasture et al., 2009).

Conclusion

These findings, suggest that the extracts/pure compounds possess antibacterial and antioxidant properties. The pharmacological effects demonstrated by the extracts could be attributed to their phytocompounds. Further screenings for in vitro antimalarial and anticancer activities of the compounds isolated are recommended.

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