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-glucopyranoside flavones (myricetin 3-0-β-rhamnopyranoside), 5,7,3’4’ tetrahydroxy-3-O-glucopyranoside flavone (quercetin 3-0-β-rhamnopyranoside) and 3,5,7,3’4’-pentahydroxy flavones (quercetin). They were characterized with the help of ESI-MS, IR, 1H C NMR, 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 Larney, 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 Ficus sycomorus. 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 Ficus sycomorus.

MATERIALS AND METHODS

Plant material

The leaves, roots and stems of the selected plants were collected from the adjoining area of Usman 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 FeCl₃ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Vanillin from BDH, Folin 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 ¹H and ¹³C 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) 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 Ficus sycomorus which showed antibacterial activity was carried out. After series of chromatographic separation, a total of 114 dfractions 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; Marcacci 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 FeCl₃ (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 AlCl₃, 2.5 μL of potassium acetate and 280 μL of distilled water were added. The mixture was incubated at 50°C for 15 min. A 250 μL of trichloroacetic acid (10%) was added to the mixture and centrifuged at 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 C₂H₃NaO₃.3H₂O and 16 mL C₂H₃O₂) pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-5-triazine) solution in 40 mM HCl and 20 mM FeCl₃.3H₂O. The fresh working solution was prepared by mixing 2.5 ml acetate buffer, 2.5 ml TPTZ and 2.5 ml FeCl₃ (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 FeSO₄ 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 (MTCC 96), Staphylococcus aureus (MTCC 2940), Escherichia coli (MTCC 739), Micrococcus luteus (MTCC 2470), Bacillus subtilis (MTCC 121), Streptococcus mutans (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 × 10⁸ CFU/ml) and prepared nutrient agar plates were seeded with 1.5 × 10⁶ 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
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 syner-gistic 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-glucopyranoside 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, 1H C13, HMBC/HSQC and COSY/NMR. 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

**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).
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.

![Figure 2. Fractions 45-46 cmp = 3β-Hydroxy-α-amyrin.](image1)

![Figure 3. Fractions 128-157 cmp = 5,7,3',4'-pentahydroxy-3-O-glucopyranoside flavones (myricetin -3-O-β-rhamnopyranoside).](image2)

![Figure 4. Fractions 126-141 = 5,7,3',4'-tetrahydroxy-3-O-glucopyranoside flavone (quercetin -3-O-β-rhamnopyranoside).](image3)
Figure 5. Fractions cp87-112 = 3,5,7,3',4'-pentahydroxy flavone (quercetin).

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

$^{13}$C NMR (MeOD, 300 MHz): $\delta$ 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), 159.29 (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): $\delta$ 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').

$^1$H NMR (CDCl$_3$, 300 MHz): $\delta$ 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'), 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): $\delta$ 38.43 (CH$_2$C-1), 27.83 (CH$_2$C-2), 81.36 (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

Lupeol acetate: white needles (30 mg)
 However, the DPPH scavenging activity increased with increasing concentration of the compounds. The DPPH scavenging activity of pure compounds. The FRAP assay (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 (Figure 2) has little NO scavenging activity but the activity increased as the concentration increased; however, the activity dropped drastically at 100 μg. 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) did not show any activity. The compound 128 to 157 cmp (Figure 3) showed activity 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 (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.

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 (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.
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
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:

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:

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

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

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REFERENCES

Anderson KJ, Teuber SS, Gobelle A, Cremin P, Water house AL, Steinber FM (2001). Walnut polyphenolics inhibit in vitro human plasma and LDL oxidation. Biochemical and molecular action of nutrients. J. Nutr. 131:237-2842.

Azas N, Saulmocin N, Delmas F, Di Giorgio C, Gasquet M, Laget M, Timon-David P (2002). Synergistic in vitro antimalarial activity of plant extracts used as traditional herbal remedies in Mali. Parasitol. Res. 88:165-171.

Benzie IFF, Strain JJ (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power". The FRAP assay. Analyt. Biochem. 239:70-76.

Blos MS (1985). Antioxidant determination by use of stable free radicals. Nature 29:119-1200.

Cowan MM (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12:564-582.

Di Carlo G, Mascolo N, Izzo AA, Capasso F, (1999). Flavonoids: Old and new aspects of a class of natural therapeutic drugs. Life Sci. 65:337-353.

Djeridane A, Youssi M, Nadjiemi B, Boutassouna D, Stocker P, Vidal N (2006). Antioxidant activity of compounds. Food Chem. 97:654-660.

Ellor JN (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 64:711-713.

Fostel JM, Larney PA (2000). Emerging novel antifungal agents. Drug Discovery Today 5:25-32.

Green LC, Wagner DA, Glagowski J, Skipper PL, Wishnok JS, Tannenbaum S (1982). Analysis of nitrate, nitrite and [15N] nitrate in biological fluid. Analyt. Biochem. 126:131-138.

Hagerman AE, Riedl KM, Tones GA, Sorik KN, Ritchard N, Hartzf PW (1999). Spectrophotometric phenacyclidine and flavonoid components in extracts of Acacia fistula. J. Agric. Food Chem. 50:5042-5047.

Hassan SW, Lawal M, Muhammad BY, Umar RA, Bilbis LS, Farouk UZ, Ebbo AA (2007). Antifungal activity and phytochemical analysis of Ficus sycomorus stem bark extracts. J. Plant Sci. 2:209-215.

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Kasture VS, Katti SA, Mahajan D, Wagh R, Mohan M, Kasture SB (2009). Antioxidantand antiparkinson activity of gallic acid derivatives. Pharmacol. Online 1:385-395.

Lewis WH, Elvin-Lewis MP (1995). Medicinal plants as sources of new therapeutics. Ann. Missouri Bot. Gard. 82:16-24.

Liu Y, Pham D, Hammiche H, Maiza K (2006). Traditional medicine in central Sahara: A vision for all 2. Measuring health. World Health Organization, Geneva.

Negi BS, Dave BP (2010). Evaluation of in vitro antimicrobial activity from the leaves extract of some Algerian medicinal plants extracts containing phenolic Cassia fistula Linn. J. Pure Appl. Microbiol. 4:557-564.

Oraibi Z, Gomem JS, Boudjelfallah F, Azzine A, Garrab M, Oummou R (2004). Antioxidant activities of the brown fractions of Myrtus communis. Proces. Biochem. 39:259-264.

Osawa T, Iyi Y, and Takeuchi M (1986). Studies on product of browning reaction prepared from glucosamine. Japanese J. Nutr. 44:307-315.

Prachayasittikul V (2010). New bioactive triterpenoids and antimalarial activity of Diospyros rubra l. EXCLI J. 9:1-10.

Prieto P, Pineda M, Aguilar M (1999). Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolydenum complex: specific application to the determination of vitamin E. Anal. Biochem. 269:337-341.

Sharma L, Kumar A (2006). Antimicrobial activity of Ageratum conyzoides Linn a plant with extra medicinal value. Asian J. Exp. Sci. 20:41-46.

Silva S, Gomes L, Leitao F, Coelho AV, Bous LV (2006). Phenolic compounds and antioxidant activity of Olea europaea L. fruits and leaves. Food Sci. Technol. Int. 12:385-395.

Wolf K, Wu X, Liu RH (2003). Antioxidant activity of apple peels. J. Agric. Food Chem. 51:609-614.

World Health Organization (1998). The World Health Report. Life in the 21st Century. A vision for all 2. Measuring health. World Health Organization, Geneva.

Zheng W, Wang SY (2001). Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food Chem. 49:5165-5170.