

Full Length Research Paper

Comparative evaluation of antioxidant activity of crude extracts of *Commiphora* spp and formulated polyherbal tablets

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This study is planned to determine the antioxidant activity. Indian medicinal plants were selected to evaluate for free radical scavenging capacities and antioxidant activities using commonly accepted assays. They were extracted with ethanol to show the best antioxidant results. Flavonoids and phenolic were included and used as standards in this study. Each sample under assay condition showed a dose-dependent free radical scavenging effect of DPPH (1, 1-diphenyl-2-picryl hydrazyl radical) and a dose-dependent inhibitory effect of lipid peroxidation. Among plant extract of *Commiphora* species in family Burseraceae that is *Commiphora berryi*, *C. caudata* and *C. pubescens*, the antioxidant properties of three different species of *Commiphora* were formulated to polyherbal tablets and the *in-vitro* studies are evaluated by different methods like phosphomolybdate method, nitric oxide scavenging activity, superoxide anion radical scavenging activity, scavenging of hydrogen peroxide and lipid peroxidation inhibition. A positive result was observed in the antioxidant activity potential between the total flavonoid and phenolic content assay. The total phenolic contents following order EECB > EECP > EECC and PHTF-II ≈ PHT F-I respectively. Flavonoid content and other activities can be performed by ethanolic crude extracts of poly herbal tablet formulation. The entire sample respectively when analyzed at a concentration of 1000 µg/ml.

Key words: *Commiphora berryi*, *Commiphora caudata*, *Commiphora pubescens*, anti oxidant activity, *in vitro* studies.

INTRODUCTION

Plants contain a wide variety of free radical scavenging

molecules, such as flavonoids and phenols. Several methods have been developed to measure the free radical scavenging capacity (RSC), regardless of the individual compounds which contribute towards the total capacity of a plant product in scavenging free radicals (Larson et al., 1988). The methods are typically based on the inhibition of the accumulation of oxidized products, since the generation of free radical species is inhibited by the addition of antioxidants and this gives rise to a reduction of the end point by scavenging free radicals. The reliable method to determine RSC involves the measurement of the disappearance of free radicals, such as 2,2-diphenyl-1-picrylhydra-zyl radical (DPPH⁺).v The role of free radicals and active oxygen in the

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Abbreviation: DPPH, - 2, 2-diphenyl-1-picrylhydra-zyl; RSC, radical scavenging capacity; O₂, superoxide; H₂O₂, hydrogen peroxide; OH, hydroxyl; FeCl₃, ferric chloride; FeCl₂, ferrous chloride; A_o, absorbance of the control; A₁, absorbance of crude extracts and standards, T, sample; C, vehicle control; B, zero time control; CB, commiphora berryi; CC, commiphora caudate; CP, commiphora pubescens; PHT, poly herbal tablet; PHF; poly herbal formulation.

pathogenesis of human diseases including cancer, aging and atherosclerosis has been recognized (Halliwell et al., 1992).

Electron acceptors, such as molecular oxygen, react rapidly with free radicals to become radicals themselves, also referred to as reactive oxygen species (ROS). The ROS include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) (Grisham and McCord, 1986). Lipid peroxidation, which involves a series of free radical mediated chain reaction processes, is also associated with several types of biological damage. Therefore much attention has been focused on the use of antioxidants, especially natural antioxidants to inhibit lipid peroxidation and to protect from damage due to free radicals. A great number of aromatic, spicy, medicinal and other plants contain chemical compounds, exhibiting antioxidant properties. Sources of natural antioxidants are primarily, plant phenolics that may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Pratt and Hudson, 1990; Halliwell et al., 1994; Mitscher et al., 1996; Owen et al., 2000; Sala et al., 2002).

The aim of this work is to determine the total phenols content and antioxidative properties of ethanolic extracts of *C. berryi*, *C. caudata*, *C. pubescens* by phosphomolybdate method, DPPH, nitric oxide scavenging activity, superoxide anion radical scavenging activity, scavenging of hydrogen peroxide and lipid peroxidation inhibition and reducing power assays. Additionally, total flavonoid content in ethanol extract have been determined. The present paper deals with the free radical scavenging and antioxidant activities of ethanolic extract of *Commiphora* species plant was used for the polyherbal formulation of the ethanolic extract.

MATERIALS AND METHODS

Reagents

Aerosil, dicalcium phosphate, sodium starch glycolate, micro crystalline cellulose, nitric oxide, superoxide, DPPH, hydrogen peroxide, ethanol, methanol, ammonium chloride and potassium acetate.

Sample collection

The green leaves of *C. berryi*, *C. caudata*, *C. pubescens* were collected from Tuticorin, Tirunelveli and Pudukottai districts in Tamil Nadu during the month of December. The garbling process refers to the separation of that portion of the plant to be used from other parts of the plant, dirt and other extraneous matter. Although there are machines that perform garbling, usually garbling is performed by hand. After removing all such unwanted adhered materials; the collected materials were then spread over trays and dried under shade, with regular sifting of collected plant materials everyday to avoid growth of fungus. Approximately 50 g of leaves were ground using a grinder (blender 80115) for 20s. The unfermented *Commiphora* species leaves and barks were kept in the oven at

40°C and put in a desiccator for at least 24 h prior to analysis. The freshly collected plants were then authenticated by the botanists from Botanical Survey of India, Coimbatore, Tamil Nadu, India and a voucher specimen of all the plants were submitted in our laboratory.

Soxhlet extraction

Such shade dried bark/leaves/aerial portions of plant were ground in grinder to powder and then subjected for extraction (Pulok et al., 2002). The powdered bark of known quantity was taken in a soxhlet apparatus and extracted with absolute ethanol. The material was extracted continuously for 72 h. The crude ethanol extract was then concentrated by distilling off the solvent under reduced pressure/vacuum and subjected for further studies (Pulok et al., 2002).

Formulation of polyherbal tablets

The selected plants were extracted with ethanol exhaustively and the crude extracts were concentrated and dried under vacuum. The vacuum dried/concentrated crude herbal extracts were either semisolid or soft in its consistency and may have less stability on storage. Therefore, formulation of the crude extracts as polyherbal tablets with suitable excipients was evaluated (Robert and Pulok, 2003). The ideal recipe used for the formulation of polyherbal tablets is as follows:

Manufacturing method

The crude ethanol extracts of *C. berryi*, *C. caudata* and *C. pubescens* obtained was concentrated and then dried to a semisolid mass under vacuum. The dried crude extracts were then granulated by wet granulation method which is one of the most widely used processes of agglomeration of pharmaceutical powders in pharmaceutical industry. Wet granulation process involves wet massing of the dried extracts blended with a granulating liquid, wet sizing followed by drying under controlled atmosphere and temperature conditions. Dicalcium phosphate is a white, odorless, tasteless powder for increasing the bulk of the preparation. The crude extract blend material has to be passed through sieves of suitable size to form granules of coarse size and fine powder. Other required excipients such as disintegrating agent, glidant, lubricant etc may be selected suitably for the formulation of polyherbal tablets based upon the preformulation studies. The formulation is shown in Table 1 (sodium starch glycolate, micro crystalline cellulose and aerosil).

Characterization in nutrients and photochemicals

Phenolic components

Determination of total phenols content: The level of total phenols in the crude extracts was determined as μg of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol graph. Total soluble phenolic components present in the crude extracts of the formulated polyherbal tablets were determined with Folin Ciocalteu reagent using pyrocatechol as a standard (Chang et al., 2002). 1 ml of extract solution was taken in a test tube to which 0.2 ml of Folin Ciocalteu reagent (1:2 in distilled water) was added.

After 20 min, 2 ml of purified water and 1 ml of 15% sodium

Table 1. Recipe of polyherbal tablet formulations.

Polyherbal tablets – formulation - I		
Ingredients	Weight taken (mg)	Concentration (%)
<i>Commiphora berryi</i>	113	18.08
<i>Commiphora caudata</i>	113	18.08
<i>Commiphora pubescens</i>	113	18.08
Aerosil	15.9	2.41
Dicalcium phosphate	241.49	38.70
Sodium starch glycolate	20.09	3.22
Micro crystalline cellulose	10.04	1.61
Total	624.72	100.18
Polyherbal tablets – formulation – II		
Ingredients	Weight taken (mg)	Concentration (%)
<i>Commiphora berryi</i>	113	18.08
<i>Commiphora caudata</i>	113	18.08
<i>Commiphora pubescens</i>	113	18.08
Aerosil	15.9	2.41
Mannitol	241.49	38.70
Sodium starch glycolate	20.09	3.22
Lactose	10.04	1.61
Total	624.72	100.18

for 30 min then absorbance was measured at 765 nm. Carbonate solution was added. The solution was allowed to stand overnight. The concentration of total phenolic compounds in the crude extracts was determined as μg of pyrocatechol equivalent by using an equation that was obtained from standard pyrocatechol graph.

Flavonoids content

Total flavonoid content was measured by the aluminium chloride colorimetric assay (Oyaizu, 1986). An aliquot (1 ml of 1:10 g/ml) of extracts of the formulated polyherbal tablets or standard solution of quercetin was mixed with 3 ml of methanol, 0.2 ml of 10% aluminium chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water and it was kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg/ml in methanol. The concentration of flavonoids was expressed in terms of $\mu\text{g}/\text{ml}$ of sample by using an equation that was obtained from standard quercetin graph.

Reducing power ability

The reductive potential of the extract was determined according to the method of (Oyaizu et al., 1986). The different concentrations of the measurement of the reductive ability 1 ml extract diluted with distilled water at different concentrations, 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% (W/V) potassium ferricyanide was added and incubated at 50°C for 30 min. After that 2.5 ml of trichloroacetic acid (10%) were added to the mixture, which was then centrifuged for 10 min at 3000 g. 2.5 ml of supernatant was withdrawn and then mixed with 2.5 ml water and 0.5 ml of freshly prepared 0.1% FeCl_3 . The absorbance was measured at 700 nm.

Evaluation of antioxidant capacity by phosphomolybdate method

The total antioxidant capacity of the extracts of the formulated polyherbal tablets was determined with phosphomolybdenum using α -tocopherol as a standard. An aliquot of 0.1 ml of plant extracts (100 μg) solution was combined with 1 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were then capped and incubated in a boiling water bath at 95°C for 90 min. Then the samples were cooled to room temperature and the absorbance was measured at 695 nm against a blank solution prepared in the same conditions by replacing the sample with 0.1 ml of methanol. The total antioxidant capacity was expressed as μg equivalents of α -tocopherol.

Nitric oxide scavenging activity

Nitric oxide radical scavenging activity was determined according to the method reported (Kim, 2001) with slight modification. Sodium nitroprusside, a nitric oxide (NO) donor was used to assess the ability of extracts of the formulated polyherbal tablets to inhibit NO release. Scavenger of nitric oxide competes with oxygen, leading to reduce the production of nitrite ions. Different concentrations (50, 100, 200, 400, 800 and 1000 $\mu\text{g}/\text{ml}$) of the extracts of the formulated polyherbal tablets were prepared in 100 ml, to which 0.1489 g of sodium nitroprusside (final conc. 5mM) was added and incubated at room temperature. At different time intervals, 5.6 ml of reaction mixture was taken out and 0.2 ml of Griess reagent A (1% sulfanilamide in 5% phosphoric acid) was added, and kept at 30°C for 10 min. After incubation, 0.2 ml of Griess reagent B (0.1% N-1-naphthylethylenediamine dihydrochloride) was added and kept at 30°C for 20 min. After incubation, absorbance was measured at 542 nm against blank. Quercetin was used as standard. The nitric

oxide radicals scavenging activity was calculated according to the following equation:

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

Superoxide anion radical scavenging activity

Superoxide anion radical ($O_2^{\bullet -}$) scavenging activity was assayed by the modified irradiated riboflavin/EDTA/nitroblue tetrazolium (NBT) system (Blois, 1958). The reaction mixture contained 150 μL of 0.03 mM riboflavin, 1 mM EDTA, 0.06 mM methionine and 0.03 mM NBT solution in 50 mM potassium phosphate buffer (pH 7.8) and 10 μL of methanolic solutions of various concentrations of test compounds (extracts the formulated polyherbal tablets) in a 96-well plate. The photoinduced reactions were performed in an aluminum foil-lined box with two 20 W fluorescent lamps. The reactant was illuminated at 25°C for 7 min. The photochemically reduced riboflavin generated $O_2^{\bullet -}$, which reduced NBT to form blue formazan. The unilluminated reaction mixture used as a blank. Reduction in absorbance of NBT was measured at 570 nm before and after irradiation using a microplate reader. The effective relative concentration IC_{50} is the concentration of sample that inhibits 50% of NBT reduction. L-ascorbic acid was used as positive control. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percent inhibition of superoxide anion generation was calculated using the following formula:

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

DPPH radical scavenging assay

The hydrogen donating ability of extracts of *C. berryi*, *C. caudata*, *C. pubescens* and the formulated polyherbal tablets were examined (Ruch et al., 1989) in the presence of DPPH stable radical. Sample stock solution (1 mg/ml) was diluted to final concentration of 50, 100, 200, 400, 800 and 1000 $\mu\text{g/ml}$ in ethanol. 1 ml of 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solution of different concentration and allowed to react at room temperature. After 30 min the absorbance values at 517 nm were converted into percentage inhibition of antioxidant activity using the following formula:

$$\% \text{ Inhibition of antioxidant activity} = 100 - \left[\frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{control}}} \times 100 \right]$$

Ethanol (1.0 ml), solution of plant extracts of the formulated polyherbal tablets (2.5 ml) and DPPH solution (1 ml, 0.3 mM) was used as the test solution, DPPH solution (1 ml, 0.3 mM), ethanol (2.5 ml) was used as a negative control. Standard ascorbic acid solution was used as positive controls. The IC_{50} values were calculated by linear regression of plots where the abscissa represented the concentration of tested plant extract and the ordinate represent the average percent of antioxidant activity. Experiment was repeated in triplicate.

Lipid peroxidation inhibition

The liver was dissected out from the sacrificed animals were chilled immediately by immersion in ice-cold normal saline. The liver was perfused with ice-cold normal saline through the portal vein before

homogenization. The liver homogenate was prepared in a ratio of 1 g of wet tissue to 9 ml of 0.86% NaCl by use of a glass homogenizer. The mixture was centrifuged for 10 min at 3000 X g and the supernatant was transferred and used as 10% rat liver homogenate. Lipid peroxidation was assessed by measuring methane diacarbonylic aldehyde (MDA) in rat liver homogenate applying a modified thiobarbituric acid (TBA) method (Amrit et al., 2006). Reaction was initiated by the addition of 4 mM FeCl_2 (30 μL) into a mixture of 6 mM ascorbic acid (60 μL), 5% liver homogenate (4.0 mg protein/ml, 600 μL) and 30 μL of sample solution (extracts of the formulated polyherbal tablets) at various concentration (50, 100, 200, 400, 800 and 1000 $\mu\text{g/ml}$). The reaction mixture was incubated at 37°C for 30 min. After incubation, the reaction was stopped by the addition of 20% trifluoroacetic acid (TFA, 500 μL) and then centrifuged at 3500 X g for 10 min. The reaction supernatant (1 ml) was mixed with 0.67% (w/v) TBA (500 μL), and then heated in boiling water bath for 10 min. After cooling, the absorbance of the solution was determined at 532 nm. It was calculated as follows:

$$[1 - (T - B) / (C - B)] \times 100\%$$

In which T, C and B are absorbance values at 532 nm.

Correlation analysis

The correlation between the amount of phenolic and flavonoid content of the crude extracts and the in-vitro anti-oxidant activity assays was carried out by performing linear regression analysis by using MS excel.

RESULTS

Total phenolic content

The total phenolic components in the crude extracts of the formulated polyherbal tablets were determined spectrophotometrically by the Folin-Ciocalteu method. By comparing the absorbance values of the extracts of the formulated polyherbal tablets and standard solutions at various concentrations after reaction with Folin-Ciocalteu reagent; the results of the colorimetric analysis for the estimation of total phenolics in terms of pyrocatechol equivalents ($\mu\text{g/ml}$ pyrocatechol equivalent) are given (Standard curve equation: $y = 0.0156x + 0.0033$, $r^2 = 0.996$ for ascorbic acid). The total phenol content in the crude extracts was found to be linear based on the concentration of the extract used for the determination ($y = 0.0598x + 14.793$, $r^2 = 0.8879$ for CB; $y = 0.0542x + 10.551$, $r^2 = 0.9084$ for CC; $y = 0.0577x + 12.709$, $r^2 = 0.9008$ for CP and $y = 0.1652x + 3.391$, $r^2 = 0.9046$ for PHF). As revealed by the data, the total phenolics contents of CB, CC, CP, PHT F-I and PHT F-II were found to be 69.46, 59.37, 65.28, 191.83 and 194.11 PE $\mu\text{g/ml}$ of sample respectively when analyzed at a concentration of 1000 $\mu\text{g/ml}$ shown in Table 2. Overall, the order of the phenolic contents of the crude extracts and in the formulated tablets were found to be in the following order CB > CP > CC and PHTF-II > PHT F-I

Table 2. Phenolic content.

Name	0	50	100	200	400	800	1000
<i>Commiphora berryi</i>	0	17.81±0.42 ^a	24.27±0.31 ^b	35.18±0.95 ^c	48.35±0.39 ^d	61.05±0.89 ^e	69.05±0.41 ^f
<i>Commiphora caudate</i>	0	10.15±0.21 ^a	18.14±0.37 ^b	29.11±0.36 ^c	39.19±1.07 ^d	54.14±1.07 ^e	59.31±0.46 ^f
<i>Commiphora pubescens</i>	0	13.1±0.52 ^a	21.61±0.41 ^b	32.44±0.99 ^c	44.59±0.68 ^d	58.25±0.67 ^e	65.27±0.68 ^f

The total phenolics contents of CB, CC, CP, PHT F-I and PHT F-II were found to be 69.46, 59.37, 65.28, 191.83 and 194.11 PE µg/ml of sample respectively when analyzed at a concentration of 1000 µg/ml. Overall, the order of the phenolic contents of the crude extracts and in the formulated tablets were found to be in the following order CB > CP > CC and PHTF-II > PHT F-I respectively.

Table 3. Flavonoid content.

Name	0	50	100	200	400	800	1000
<i>Commiphora berryi</i>	0	0.14±0.01 ^a	0.19±0.02 ^b	0.31±0.02 ^c	0.57±0.02 ^d	0.85±0.03 ^e	1.09±0.06 ^f
<i>Commiphora caudate</i>	0	0.84±0.04 ^e	0.12±0.03 ^a	0.24±0.01 ^b	0.48±0.02 ^c	0.75±0.02 ^d	0.84±0.01 ^e
<i>Commiphora pubescens</i>	0	0.94±0.08 ^e	0.14±0.01 ^a	0.26±0.01 ^b	0.49±0.02 ^c	0.78±0.01 ^d	0.92±0.02 ^e

The total flavonoid contents of CB, CC, CP and PHF was found to be 1.09, 1.87, 2.22, 3.1 and 5.18 QE µg/ml of sample respectively when analyzed at a concentration of 1000 µg/ml. Overall, the order of the flavonoid contents of the test samples was found to be in the following order of CP > CC > CB and PHTF-II > PHT F-I respectively.

respectively.

Total flavonoid content

The results of the colorimetric analysis for the estimation of total flavonoids in terms of quercetin equivalents (µg/ml quercetin equivalent) are given (Standard curve equation: $y = 1.0396x + 0.018$, $r^2 = 0.999$ for quercetin). The total flavonoid content in the crude extracts was found to be linear based on the concentration of the extract used for the determination ($y = 0.001x + 0.0842$, $r^2 = 0.9819$ for CB; $y = 0.0014x + 0.5825$, $r^2 = 0.78279$ for CC; $y = 0.0017x + 0.606$, $r^2 = 0.8294$ for CP and $y = 0.0021x + 0.9557$, $r^2 = 0.9084$ for PHF). As revealed by the data, the total flavonoid contents of CB, CC, CP and PHF was found to be 1.09, 1.87, 2.22, 3.1 and 5.18 QE µg/ml of sample respectively when analyzed at a concentration of 1000 µg/ml shown in Table 3. Overall, the order of the flavonoid contents of the test samples was found to be in the following order of CP > CC > CB and PHTF-II > PHT F-I respectively.

Reducing ability

For the measurements of the reducing ability, "Fe³⁺– Fe²⁺ transformation" in the presence of extracts of the formulated polyherbal tablets were found and reported. The reducing power of extracts of the formulated polyherbal tablets and BHT was found to increase with increasing concentrations. The reductive capabilities of extract were compared with BHT. It is observed that the

reducing powers of the crude extracts and BHT on Fe³⁺ were concentration dependent. The reducing powers increased with increasing concentration of BHT, CB, CC, CP and extracts of PHT F. For instance, the absorbance at 700 nm was found to be 0.25, 0.40, 0.45, 0.48, 0.55 and 0.59 for BHT at the doses of 50, 100, 200, 400, 800 and 1000 µg/ml. The reducing power observed was found to be in the order of 0.89, 0.78, 0.84, 0.97 and 1.07 respectively for CB, CC, CP, PHT F-1 and PHT F-11 at the dose of 1000 µg/ml in Table 4. The reducing power was found to be in the order of CB > CP > CC for crude extracts and PHT F-11 > PHT F-11 for the polyherbal formulations respectively shown in Figure 1. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). The antioxidant activity has been attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and radical scavenging (Oyaizu, 1986).

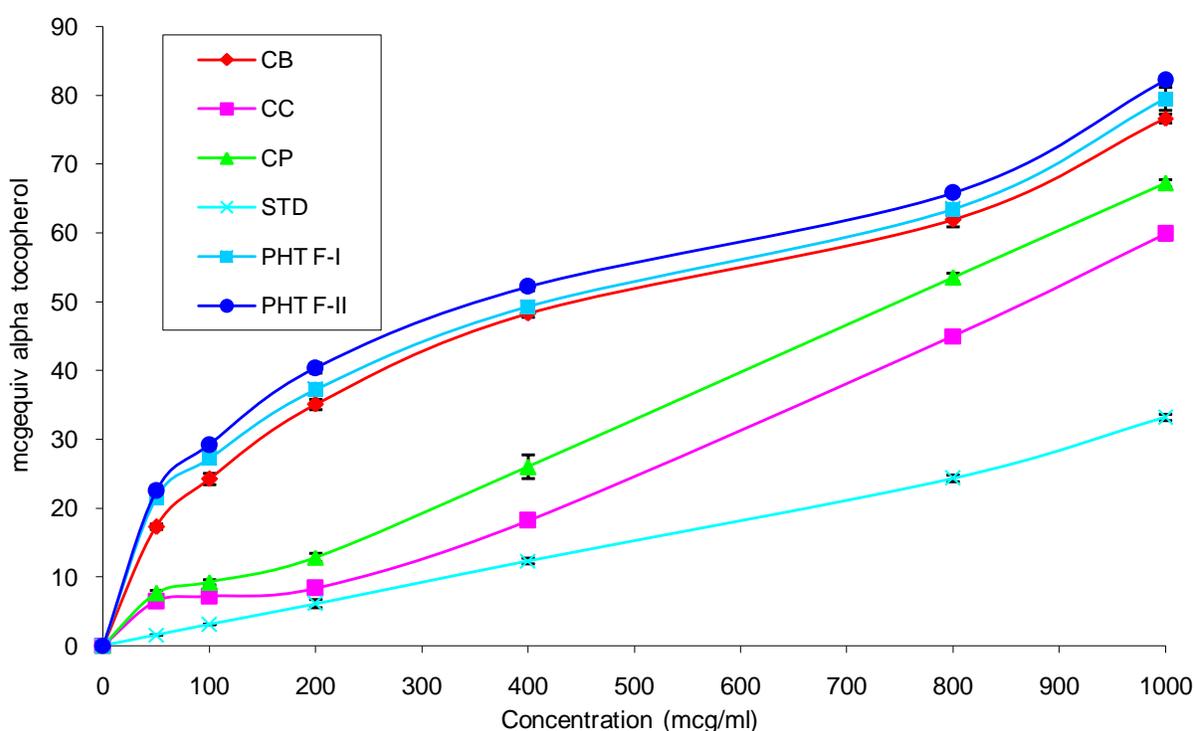
Total antioxidant capacity

All the crude extract tested possessed significant dose dependent antioxidant activity and displayed good linear correlation ($y = 0.0647x + 14.024$; $r^2 = 0.9138$ for CB; $y = 0.0574x - 0.2025$; $r^2 = 0.9826$ for CC; $y = 0.065x + 1.5065$; $r^2 = 0.9956$ for CP; $y = 0.0648x + 16.092$; $r^2 = 0.9011$ for PHF-I; $y = 0.0664x + 17.532$; $r^2 = 0.8873$ for PHF-II respectively) was shown in Figure 2. Among the extracts tested in Table 5, CB, CC, CP, PHF-I and PHF-II

Table 4. Reducing power ability.

Name	0	50	100	200	400	800	1000
<i>Commiphora berryi</i>	0	0.64±0.05 ^b	0.74±0.04 ^b	0.74±0.04 ^b	0.79±0.03 ^b	0.81±0.01 ^b	0.89±0.01 ^b
<i>Commiphora caudate</i>	0	0.41±0.02 ^c	0.56±0.01 ^{b,c}	0.56±0.01 ^c	0.59±0.01 ^c	0.68±0.01 ^c	0.78±0.01 ^{b,c}
<i>Commiphora pubiscens</i>	0	0.54±0.02 ^d	0.68±0.02 ^{c,d}	0.68±0.02 ^d	0.72±0.02 ^d	0.77±0.03 ^c	0.84±0.02 ^{c,d}
Standard	0	0.25±0.01 ^a	0.44±0.02 ^a	0.44±0.02 ^a	0.48±0.01 ^a	0.55±0.02 ^a	0.59±0.01 ^a
PHT F-1	0	0.67±0.02 ^e	0.82±0.02 ^d	0.82±0.02 ^e	0.83±0.03 ^e	0.87±0.03 ^c	0.97±0.04 ^d
PHT-F2	0	0.69±0.05 ^f	0.86±0.03 ^e	0.86±0.03 ^f	0.87±0.06 ^f	0.93±0.02 ^d	1.07±0.06 ^e

The reducing power observed was found to be in the order of 0.89, 0.78, 0.84, 0.97 and 1.07 respectively for CB, CC, CP, PHT F-1 and PHT F-11 at the dose of 1000 µg/ml. The reducing power was found to be in the order of CB > CP > CC for crude extracts and PHT F-11 > PHT F-1 for the polyherbal formulations respectively.

**Figure 1.** Antioxidant capacity by phosphomolybdate method.

contains 76.59, 59.83, 67.19, 79.46 and 82.16 µg α-tocopherol equivalent/mg respectively when analyzed at a concentration of 1000 µg/ml.

Nitric oxide scavenging assay

The IC₅₀ value of CB, CC, CP, PHT F-I and PHT F-II was found to be 1515.15, 2173.91, 1666.66, 1470.58 and 1470.58 µg/ml and 1394.23 µg/mL whereas the IC₅₀ value of Curcumin was found to be 1785.71 µg/ml shown in Table 6. The comparative ability of extracts to quench NO radicals is indicated in Figure 3. Nitric oxide (NO) is

an essential bioregulatory molecule required for several physiological processes like neural signal transmission, immune response, cardiovascular dilation and blood pressure (Messina and Messina, 1996). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer, and other pathological conditions (Moncada et al., 1991). Oxygen reacts with the excess nitric oxide to generate nitrite and peroxy nitrite anions, which act as free radicals (Cotran et al., 1999). It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities (Hagerman et al., 1998). In the

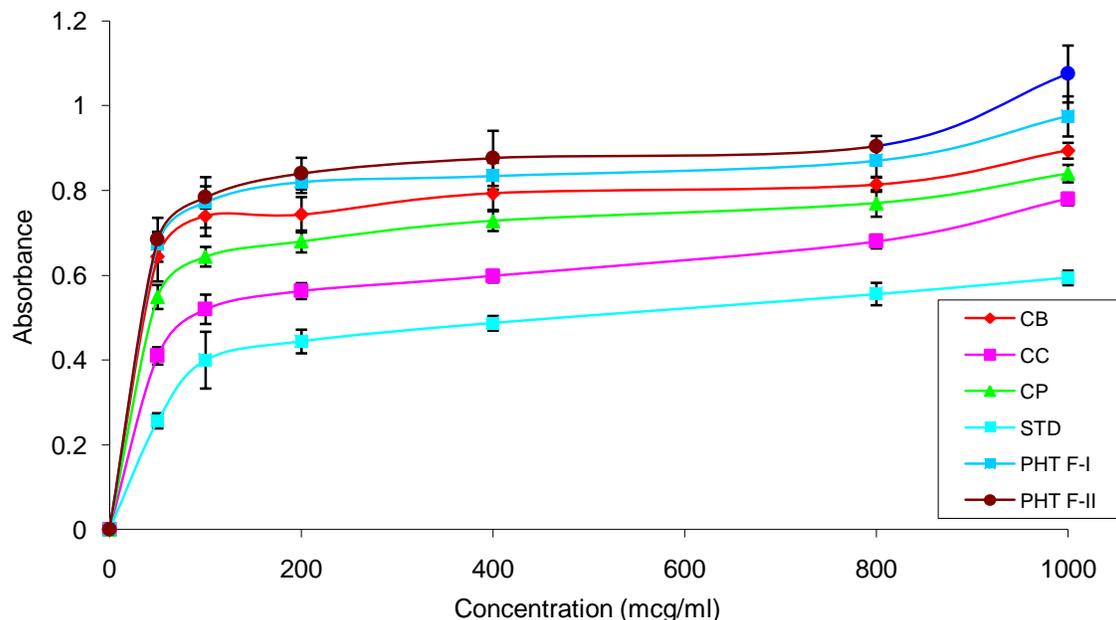


Figure 2. Reducing power ability.

Table 5. Antioxidant capacity by phosphomolybdate method.

Name	0	50	100	200	400	800	1000
<i>Commiphora berryi</i>	0	17.4±0.39 ^b	24.01±0.81 ^b	35.53±0.75 ^a	48.2±0.53 ^b	61.47±1.04 ^b	75.68±0.63 ^b
<i>Commiphora caudate</i>	0	6.59±0.46 ^c	7.16±0.10 ^c	8.4±0.58 ^b	18.4±0.41 ^c	44.75±0.52 ^c	59.85±0.87 ^c
<i>Commiphora pubiscens</i>	0	7.61±0.41 ^d	9.21±0.37 ^d	12.43±0.61 ^c	26.16±1.72 ^d	53.33±0.61 ^d	67.58±0.52 ^d
Standard	0	1.53±0.03 ^a	3.03±0.04 ^a	6.16±0.59 ^a	12.55±0.41 ^a	24.77±0.48 ^a	33.46±0.45 ^a
PHT F-1	0	21.6±0.78 ^e	27.47±0.49 ^e	37.47±0.49 ^d	49.07±0.60 ^e	63.07±0.35 ^e	79.07±1.67 ^e
PHT-F2	0	22.3±0.75 ^f	29.37±0.56 ^f	40.47±0.72 ^e	52.03±0.60 ^f	65.03±0.45 ^f	82.39±0.57 ^f

The extracts tested, the CB, CC, CP, PHF-I and PHF-II contains 76.59, 59.83, 67.19, 79.46 and 82.16 µg α-tocopherol equivalent/mg respectively when analyzed at a concentration of 1000 µg/ml. The antioxidant activity was found to be in the order of CB > CP > CC for crude extracts and PHT F-1 > PHT F-1I for the polyherbal formulations respectively.

Table 6. Nitric oxide scavenging activity.

Name	0	50	100	200	400	800	1000
<i>Commiphora berryi</i>	0	1.64±0.16 ^b	7.2±0.19 ^b	14.3±0.13 ^b	21.6±0.17 ^b	26.32±0.14 ^b	29.2±0.45 ^b
<i>Commiphora caudate</i>	0	0.952±0.009 ^c	2.36±0.06 ^c	6.6±0.08 ^c	13.1±0.09 ^c	19.4±0.09 ^c	21.9±0.16 ^c
<i>commiphora pubiscens</i>	0	1.93±0.86 ^d	4.4±0.18 ^d	10±0.35 ^d	17±0.85 ^d	24.5±0.12 ^d	27.6±0.16 ^d
Standard	0	1.48±0.12 ^a	2.75±0.07 ^a	8.6±0.12 ^a	15.4±0.14 ^a	23.2±0.31 ^a	25.6±0.30 ^a
PHT F-1	0	2.14±0.2 ^e	4.6±0.27 ^e	7.9±0.16 ^e	17.2±1.0 ^e	26.9±0.17 ^e	31.4±0.11 ^e
PHT F-1	0	2.32±0.13 ^f		8.7±0.15 ^f	17.7±0.28 ^f	28±0.22 ^f	32.6±0.13 ^f

The extract competes with oxygen to react with nitric oxide and thus inhibits generation of the anions and counteracts the formation of NO. The extracts tested, the CB, CC, CP, PHF-I and PHF-II contains 29.2, 21.9 and 27.6, respectively when analyzed at a concentration of 1000 µg/ml. The NO scavenging assay was found to be in the order of CP > CB > CC for crude extracts and PHT F-1 > PHT F-1I for the polyherbal formulations respectively.

present study, the extract competes with oxygen to react with nitric oxide and thus inhibits generation of the anions

and counteracts the formation of NO. This property of the extracts could be utilized in certain pathological

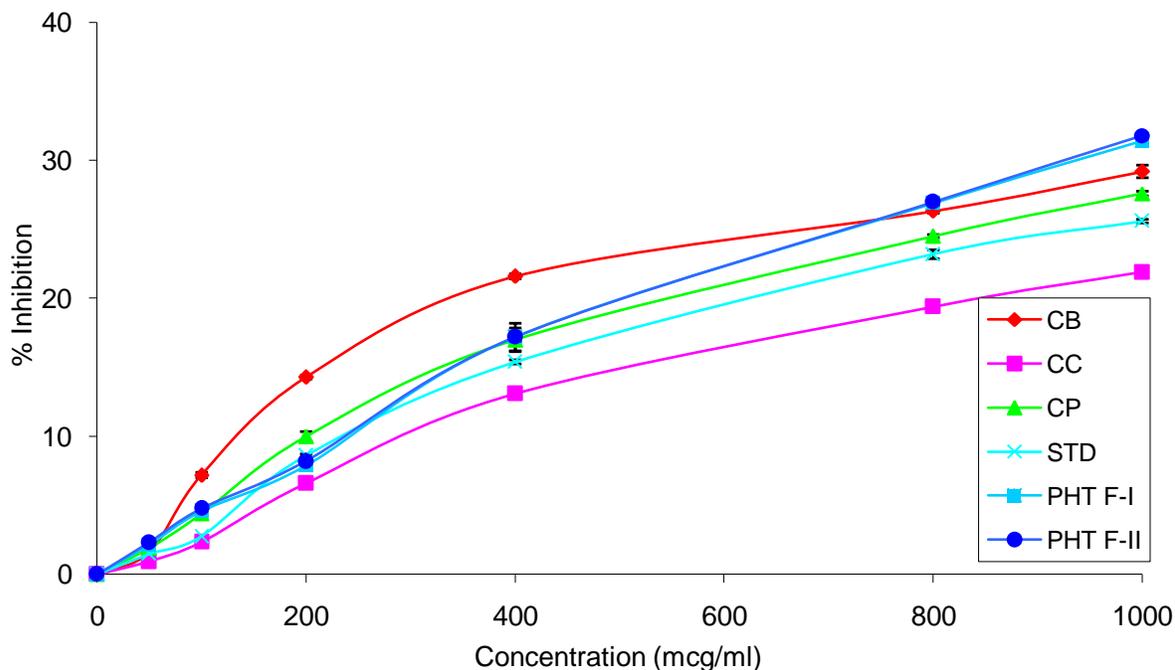


Figure 3. Nitric oxide scavenging activity.

Table 7. Superoxide radical scavenging assay.

Name	0	50	100	200	400	800	1000
<i>Commiphora berryi</i>	0	2.86±0.34 ^b	8.5±0.08 ^b	19.3±0.31 ^b	35.9±0.53 ^b	45.76±0.41 ^b	50.8±0.41 ^b
<i>Commiphora caudate</i>	0	1.56±0.08 ^c	4.26±0.07 ^c	8.7±0.18 ^c	21.8±0.28 ^c	36±0.18 ^c	40.9±0.23 ^c
<i>Commiphora pubiscens</i>	0	2.45±0.11 ^d	4.6±0.27 ^d	12.65±0.22 ^d	29.4±0.27 ^d	42±0.29 ^d	47.8±0.33 ^d
Standard	0	2.1±0.31 ^a	5.15±0.22 ^a	10.6±0.23 ^a	26±0.57 ^a	39.5±0.46 ^a	43.2±0.25 ^a
PHT F-1	0	3.2±0.09 ^e	6.47±0.13 ^e	13.2±0.13 ^e	23.2±0.63 ^e	46.18±0.60 ^e	57.3±0.53 ^e
PHT-F2	0	3.56±0.15 ^f	6.83±0.10 ^f	13.47±0.14 ^f	25.7±0.24 ^f	50.4±0.33 ^f	59.3±0.39 ^f

The superoxide radical scavenging activity of the tested compounds followed the order: PHT F-II < PHT F-I < CB < CP < CC < ascorbic acid.

conditions where excess generation of NO creates problems and may also help to arrest the chain of reactions initiated by excess generation of NO that is detrimental to human health. Eight isolated compounds from the oleogum resin of *Commiphora wightii* reported to inhibit NO production in lipopolysaccharide induced murine macrophages J774.1 significantly when evaluated *in-vitro* (Meselhy, 2003).

Superoxide radical scavenging assay

The extract inhibited the formation of the blue formazan, and the percent inhibition was proportional to the concentration with an IC₅₀ value of 877.19, 1063.82, 961.53, 866.55 and 819.67 for CB, CC, CP, PHT F-I and PHT F-II

respectively and 1063.82 µg/ml for ascorbic acid respectively shown in Table 7. The percent inhibition of superoxide radical generation by EECB and PECB compared to ascorbic acid. The superoxide radical scavenging activity of the tested compounds followed the order: PHT F-II < PHT F-I < CB < CP < CC < ascorbic acid shown in Figure 4. Superoxides are produced from molecular oxygen due to oxidative enzymes of body as well as through nonenzymatic reaction such as auto-oxidation by catecholamines (Sainani et al., 1997). These results indicated that the tested extract had a noticeable effect on scavenging superoxide when compared with ascorbic acid, which was used as a positive control. Superoxide anion is a free radical generated by one electron transfer and plays an important role in the formation of other reactive oxygen species such as

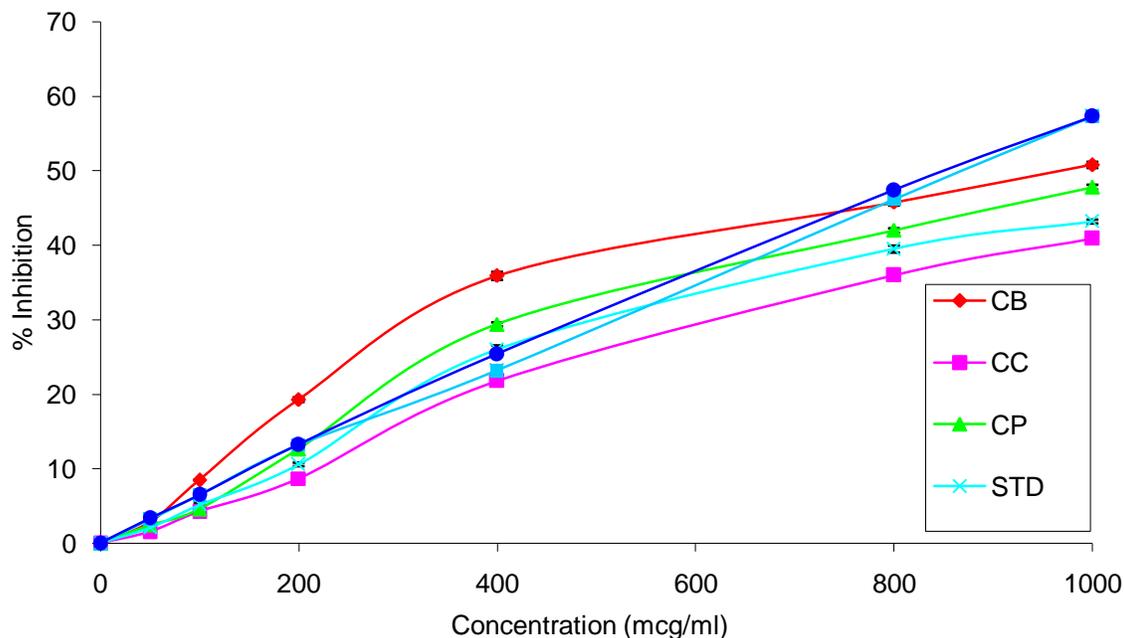


Figure 4. Superoxide radical scavenging assay.

hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Stief, 2003). It can also react with nitric oxide and form peroxynitrite, which can generate toxic radicals such as hydroxyl radical (Halliwell, 1997). A polyherbal formulation containing *Commiphora mukul* as an ingredient was reported to scavenge superoxide and hydroxyl radicals and thus prevents atherogenesis (Mary and Babu, 2003). Quenching of singlet molecular oxygen by *Commiphora myrrha* extracts and menthofuran was reported (Racine and Auffray, 2005).

DPPH

Antioxidants react with DPPH, which is a stable free radical, and convert it to 1, 1-diphenyl-2-(2,4,6-trinitrophenyl)hydrazine. The solution therefore loses colour stoichiometrically depending on the number of electrons taken up (Blois, 1958). The degree of discoloration indicates the scavenging potentials of the antioxidant compounds. CB, CC, CP, PHT F-I and PHT F-II reduced DPPH to a yellow coloured product in a concentration dependent manner confirms the radical-scavenging activity. The dose of CB, CC, CP, PHT F-I, PHT F-II and ascorbic acid required to reduce the absorbance of DPPH control solution by 50% (IC_{50}) at 30 min was determined by scavenging the DPPH radicals at various doses of crude extracts and ascorbic acid. The radical-scavenging activity decreased in the following order: ascorbic acid > CP > CC > CB > PHT F-II > PHT F-I shown in Figure 5. The IC_{50} values obtained were

tabulated in Table 8. Effectiveness in antioxidant properties inversely correlated with IC_{50} value. From the results, it may be postulated that both the plant extracts have hydrogen donors thus scavenging the free radical DPPH. Proton radical-scavenging action may be one of the predominant mechanisms for oxidation. DPPH has a proton free radical and shows characteristic absorption at 517 nm (purple). When it encounters proton radical scavenger, its purple color fades rapidly (Yamagushi et al., 1998). Moderate DPPH radical scavenging activity compared against the activity of gallic acid and catechin was observed for fractions isolated from ethyl acetate extract of *C. wightii* was reported (Zhu et al., 2001). Significant anti-oxidant activity (ABTS assay) was observed and reported for the stem extracts of *C. tenuipetiolata* ($IC_{50} = 5.10 \mu\text{g/ml}$), *C. neglecta* ($IC_{50} = 7.28 \mu\text{g/ml}$) and *C. mollis* ($IC_{50} = 8.82 \mu\text{g/ml}$). Extracts generally exhibited poor anti-oxidant activity in the DPPH assay, with the exception of *C. schimperi* (stem), *C. neglecta* (stem), *C. tenuipetiolata* (stem and leaf), and *C. edulis* (stem), with IC_{50} values ranging between 7.31 and 10.81 $\mu\text{g/ml}$ (Paraskevava et al., 2008).

H₂O₂ radical scavenging assay

The ability of the crude extracts of *C. berryi* to scavenge hydrogen peroxide was determined according to the method (Ruch et al., 1989). The results obtained were displayed in Figure 6, compared against the results obtained for α -tocopherol which is used as standard.

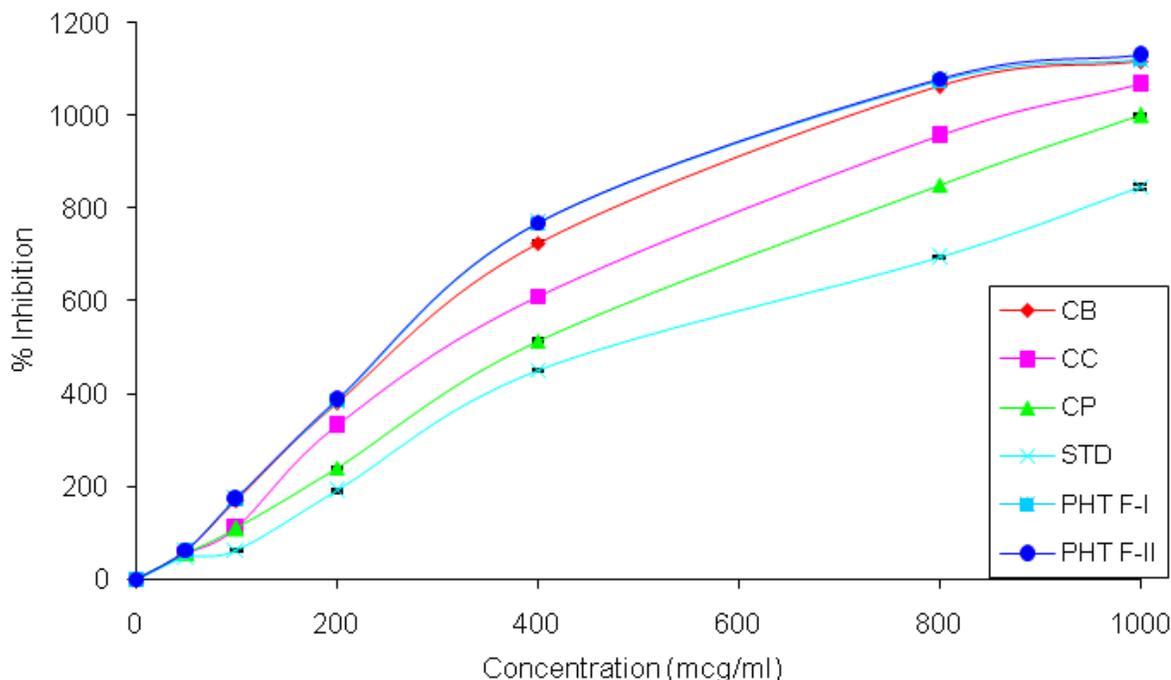


Figure 5. 2, 2-diphenyl-1-picrylhydra-zyl radical assay.

Table 8. 2, 2-diphenyl-1-picrylhydra-zyl radical assay.

Name	0	50	100	200	400	800	1000
<i>Commiphora berryi</i>	0	62.06±2.83 ^b	170.81±0.99 ^b	381.1±1.22 ^b	726.31±3.35 ^b	1063.03±1.86 ^b	1116.08±4.32 ^b
<i>Commiphora caudate</i>	0	55.59±2.67 ^c	109.71±2.02 ^c	331.65±1.3 ^c	611±2.78 ^c	956.16±1.60 ^c	1066.45±2.92 ^c
<i>Commiphora pubiscens</i>	0	56.51±1.16 ^d	109.88±1.98 ^d	240.48±0.96 ^d	515±2.95 ^d	851.48±0.78 ^d	1000.6±4.52 ^d
Standard	0	48.54±2.90 ^a	64.8±1.32 ^a	190.71±3.86 ^a	452.13±3.18 ^a	694.06±1.17 ^a	845.7±4.64 ^a
PHT F-1	0	63.26±0.70 ^e	172.28±1.70 ^e	382.35±5.18 ^e	765.68±4.11 ^e	1073.01±0.97 ^e	1120.86±1.5 ^e
PHT-F2	0	64.06±0.86 ^f	173.66±0.67 ^f	440.6±1.31 ^f	827.93±4.19 ^f	1089.91±2.5 ^f	1160.41±1.44 ^f

The dose of CB, CC, CP, PHT F-I, PHT F-II and ascorbic acid required to reduce the absorbance of DPPH control solution by 50% (IC₅₀) at 30 min was determined by scavenging the DPPH radicals at various doses of crude extracts and ascorbic acid. The radical-scavenging activity decreased in the following order: ascorbic acid > CP > CC > CB > PHT F-II > PHT F-I. The IC₅₀ values obtained.

From the results it was revealed that the crude extracts and the formulated polyherbal tablets were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Crude ethanol extracts of CB, CC, CP, PHT F-I AND PHT F-II (1000 µg/ml) exhibited 86.19, 80.19, 94.76, 98.38 and 101.48% hydrogen peroxide scavenging activity, respectively, while, α-tocopherol (1000 µg/ml), 242.86% activity shown in Table 9. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H₂O₂ is very important throughout biological systems (Halliwell, 1997). Scavenging of H₂O₂ by both the extracts of *C. berryi* may be attributed to their phenolics, which can donate electrons to H₂O₂, may thus neutralize it to water

(Halliwell et al., 1985). The differences in H₂O₂ scavenging capacities between the two extracts may be attributed to the difference in the structural features of their active components, which determine their electron donating abilities (Wettasinghe et al., 2000).

Lipid peroxidation

Lipid peroxidation has been implicated in the pathogenesis of number of diseases and clinical conditions. Malondialdehyde and other aldehydes have been identified as products of lipid peroxidation that reacts with the thiobarbituric acid to give pink coloured species. The aldehyde products are responsible for DNA damage,

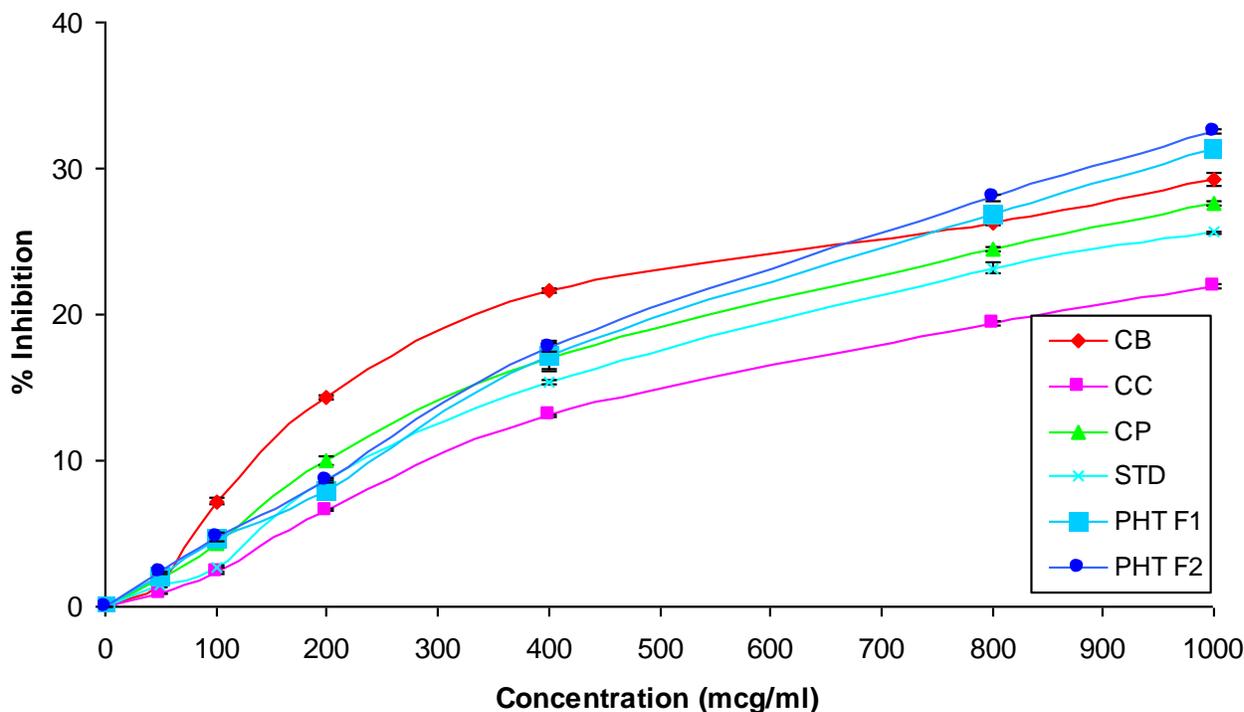


Figure 6. Hydrogen peroxide inhibition.

Table 9. Hydrogen peroxide.

Name	0	50	100	200	400	800	1000
<i>Commiphora berryi</i>	0	20.77±0.95 ^b	28.57±1.10 ^b	45.45±1.78 ^b	57.4±0.83 ^b	71.16±1.43 ^b	86.19±0.3 ^b
<i>Commiphora caudate</i>	0	4.75±0.86 ^c	25.63±1 ^c	42.86±0.89 ^c	52.19±0.96 ^c	66.48±0.67 ^c	80.08±1.39 ^c
<i>Commiphora pubiscens</i>	0	5.98±0.84 ^d	30.23±1.42 ^d	48.8±0.40 ^d	65.3±0.58 ^d	81.3±0.76 ^d	94.33±0.37 ^d
Standard	0	45.75±1.22 ^a	58.56±1.09 ^a	69.97±1.19 ^a	88.45±1.002 ^a	216.93±3.65 ^a	242.77±3.52 ^a
PHT F-1	0	22.59±0.90 ^e	32.85±1.12 ^e	50.19±1.2 ^e	68.48±1.16 ^e	85.62±0.63 ^e	98.13±0.17 ^e
PHT-F2	0	24.88±0.87 ^f	35.67±1.5 ^f	54.84±1.09 ^f	70.16±0.76 ^f	88.80±0.48 ^f	101.2±3.57 ^f

The crude ethanol extracts of CB, CC, CP, PHT F-I AND PHT F-II (1000 µg/ml) exhibited 86.19, 80.19, 94.76, 98.38 and 101.48% hydrogen peroxide scavenging activity, respectively, while, α-tocopherol (1000 µg/ml), 242.86% activity.

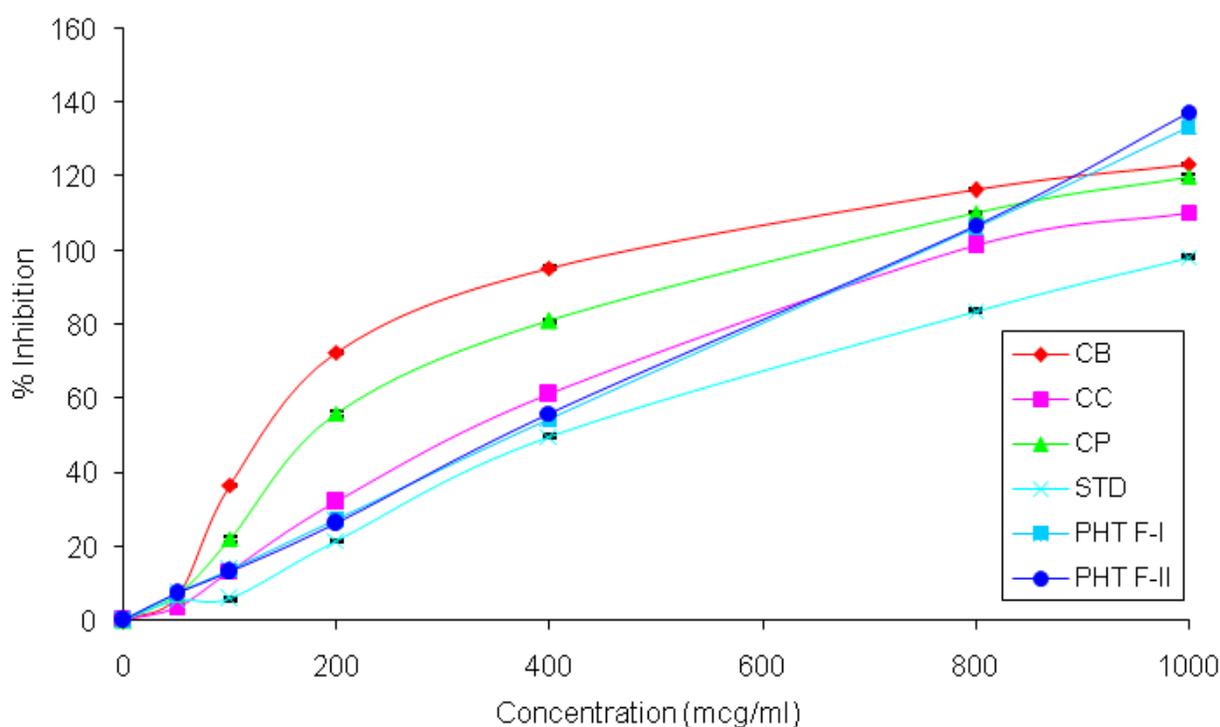
generation of cancer and aging related diseases. The decrease in the concentration of the malondialdehyde with the increase in the concentration of crude extract indicates the potential antioxidant role (Wiseman and Halliwell, 1996). Lipid peroxidation may cause peroxidative tissue damage in inflammation, cancer, aging, ulcer, cirrhosis, and atherosclerosis (Didem et al., 2007). Three compounds isolated through bioactivity guided fractionation from the hexane soluble portion of methanol extract of gum resin collected from gum guggul was reported to show significant lipid peroxidation (Francis et al., 2004). Lipid peroxidation was significantly reduced in the presence of the crude extracts of *C. berryi*, comparable to the standard compound α-tocopherol. The

extract inhibited lipid peroxidation, and the percent inhibition was found proportional to the concentration with an IC₅₀ value of 340.13, 416.66, 364.96, 375.93, 340.13 and 490.19 µg/ml for α-tocopherol respectively the percent inhibition of lipid peroxidation by CB, CC, CP, PHT F-I and PHT F-II compared to α-tocopherol shown in Table 10. The lipid peroxidation activity of the tested compounds followed the order: CB, PHT F-II < CP < PHT F-I < CC < α-tocopherol Figure 7. The essential oil of *C. myrrha* provides the best protection against squalene peroxidation. These results demonstrate that squalene peroxidation during solar exposure is mainly because of singlet oxygen and not due to free radical attack. This suggests that sun care cosmetics should make use not

Table 10. Lipid peroxide assay.

Name	0	50	100	200	400	800	1000
<i>Commiphora berryi</i>	0	6.67±0.22 ^a	36.5±0.21 ^b	72.4±0.28 ^b	95.5±0.31 ^b	116.4±0.23 ^b	123.4±0.34 ^b
<i>Commiphora caudate</i>	0	3.66±0.24 ^b	13.4±0.13 ^c	32±0.52 ^c	61.1±0.46 ^c	101.4±0.32 ^c	110.5±0.27 ^c
<i>Commiphora pubescens</i>	0	7.2±0.3 ^c	22±0.52 ^d	56±0.64 ^d	81±0.40 ^d	110.5±0.27 ^d	120±0.73 ^d
Standard	0	5.42±0.24 ^a	5.9±0.57 ^a	21.5±0.19 ^a	49.9±0.33 ^a	83.7±0.53 ^a	98.2±0.45 ^a
PHT F-1	0	7.3±0.09 ^d	13.7±0.44 ^e	27.3±0.41 ^e	54.6±0.24 ^e	106.2±0.42 ^e	133.4±0.36 ^e
PHT-F2	0	7.67±0.52 ^e	14.2±0.61 ^f	35.2±0.18 ^f	66.9±0.49 ^f	112±0.41 ^f	147.6±0.25 ^f

The extract inhibited lipid peroxidation, and the percent inhibition was found proportional to the concentration with an IC₅₀ value of 340.13, 416.66, 364.96, 375.93, 340.13 and 490.19 µg/ml for α-tocopherol respectively the percent inhibition of lipid peroxidation by CB, CC, CP, PHT F-I and PHT F-II compared to α-tocopherol. The lipid peroxidation activity of the tested compounds followed the order: CB, PHT F-II < CP < PHT F-I < CC < α-tocopherol

**Figure 7.** Lipid peroxidation inhibition.

only of free radical scavengers but also of singlet oxygen quenchers (Auffray, 2007).

Correlation analysis of antioxidant parameters

The correlation coefficient obtained after linear regression analysis between the amount of phenolic and flavonoid content of the crude extracts and the in-vitro anti-oxidant activity assays were tabulated. The result indicates a strong association between antioxidative activities and phenolic/flavonoid compounds, suggesting that phenolic/flavonoid compounds are probably responsible for the

antioxidative activities of crude extracts of *C. berryi*, *C. caudata* and *C. pubescens*. Many studies have revealed that the phenolic contents in plants are related to their antioxidant activities, and the antioxidant activities of phenolic compounds are probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Chang et al., 2002) or may be related to substitution of hydroxyl groups in the aromatic rings of phenolics, thus contributing to their hydrogen donating ability (Brand-Williams et al., 1995) The aforesaid results demonstrated that the free radical scavenging effect of the crude extracts of plants correlates closely with their

corresponding phenolic and flavonoid contents.

DISCUSSION

The phytochemical tests indicated the presence of carbohydrate, phytosteroids, proteins, gums; phenolic compounds and tannins in ethanol extract of *C. berryi* (Gowrishankar et al., 2004). Phenolic compounds are a class of antioxidant agents which act as free radical terminators (Shahidi and Wanasundara, 1992). The key role of phenolic compounds as scavengers of free radical and antioxidants is emphasized in several reports (Moller et al., 1999). It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process (Wiseman et al., 1996). It has been revealed that active oxygen species such as OH and O₂ are thought to be agents that cause oxidative damage, and much attention has been focused on active oxygen scavenging agents such as α -tocopherol and natural phenolics like flavonoids and tannins in preventing cell damage. Consequently the antioxidant activities of plant/herb extracts are often explained with respect to their total phenolics and flavonoids contents, with good correlation. Incidentally the extracts also showed the highest phenolic and flavonoid content. Phenolic content in the aforementioned plants was well correlated with its antioxidant potential ($P < 0.001$), whereas flavonoid content in the aforementioned plants was less correlated. The presence and absence of correlation between chemical constituents and antioxidant potential give new insight into defenses against free radical mechanisms in plants. The total phenolic contents of the crude extracts and the formulated tablets were found to be in the following order EECEB > EECP > EECC and PHTF-II \approx PHT F-I respectively. The order of the flavonoid contents of the test samples was found to be in the following order of EECP > EECC > EECEB and PHTF-II \approx PHT F-I respectively. Among the extracts tested for the total antioxidant capability, the EECEB, EECC, EECP, PHT F-I and PHT F-II contains 76.59, 59.83, 67.19, 79.46 and 82.16 μ g α -tocopherol equivalent/mg respectively when analyzed at a concentration of 1000 μ g/ml. The reducing power was found to be in the order of EECEB > EECP > EECC for crude extracts and PHT F-II \approx PHT F-I for the polyherbal formulations respectively.

The IC₅₀ value of NO scavenging assay for EECEB, EECC, EECP, PHT F-I and PHT F-II was found to be 1515.15, 2173.91, 1666.66, 1470.58 μ g/ml and 1394.23 μ g/ml whereas the IC₅₀ value of Curcumin was found to be 1785.71 μ g/ml. The superoxide radical scavenging activity of the tested compounds followed the order: PHT F-II \approx PHT F-I < EECEB < EECP < EECC < ascorbic acid. The DPPH radical-scavenging activity decreased in the

following order: ascorbic acid > EECP > EECC > EECEB > PHT F-II \approx PHT F-I. The lipid peroxidation activity of the tested compounds followed the order: EECEB, PHT F-II < EECP < PHT F-I < EECC < α -tocopherol. Crude ethanol extracts of EECEB, EECC, EECP, PHT F-I and PHT F-II (1000 μ g/ml) exhibited 86.19, 80.19, 94.76, 98.38 and 101.48% hydrogen peroxide scavenging activity, respectively, while, α -tocopherol (1000 μ g/ml) showed 242.86% activity. All the crude extracts tested possessed significant dose dependent antioxidant activity and displayed good linear correlation. The antioxidant activity has been attributed to various mechanisms, among which may be due to the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity and radical scavenging. The aforesaid results demonstrated that, the free radical scavenging effect of the crude extracts of plants correlates closely very well with their corresponding phenolic and flavonoid contents.

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