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Research Article

EVALUATION OF THE ANTIOXIDANT POTENTIAL OF A NEWLY DEVELOPED POLYHERBAL FORMULATION FOR ANTIOBESITY

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ABSTRACT

The antioxidant properties of the developed polyherbal formulation for antiobesity were evaluated for its probable free radical mechanisms by the methods, such as the DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay, ABTS (2, 2'-azinobis-3-ethyl-benzothiozoline-6-sulphonic acid) and Superoxide radical scavenging assays. Herbal drugs such as *Phyllanthus emblica (fruits), Macrotyloma uniflorum* (seeds), *Plumbago zeylanica (roots)*, *Curcuma longa (rhizomes)* were used for the preparation of the polyherbal formulation (PHF). Different combinations of PHF were prepared using *Macrotyloma uniflorum* as the base material and were labelled as MPhPl (For 1), MPhCur (For 2), MPlCur (For 3), and MphPlCur (PHF4, Developed formulation). Their standardized aqueous extracts were compared for the antioxidant potential.The results showed that PHF4 was most active in the DPPH (IC_{50} value 7.46±0.04µg/ml), and ABTS assay (IC_{50} value 0.199± 0.004µg/ml) when compared to the standards Quercetin (IC_{50} value 10.25±1.45µg/ml) and trolox (IC_{50} value 0.250±0.007µg/ml) used respectively. Different combinations of PHF were prepared using *Macrotyloma uniflorum* as the base material and were labelled as MPhPl (For 1), MPhCur (For 2), MPlCur (For 3), and MphPlCur (PHF4, Developed formulation). Their standardized aqueous extracts were compared for the antioxidant potential.The results showed that PHF4 was most active in the DPPH (IC_{50} value 7.46±0.04µg/ml), and ABTS assay (IC_{50} value 0.199± 0.004µg/ml) when compared to the standards Quercetin (IC_{50} value 10.25±1.45µg/ml) and trolox (IC_{50} value 0.250±0.007µg/ml) used respectively. The extracts were also investigated for the total phenolic content, total flavonoids, and total tannin content. The present studies suggest that the developed formulation has potent antioxidant activity and those phenolic compounds such as tannins and flavonoids present possibly suggest for the antioxidant action.

Keywords: Antioxidant assays; 2, 2′-azinobis (3-ethylbenzothiazoline-6 sulfonic acid) (ABTS) assay; 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, Superoxide radical scavenging assay.

INTRODUCTION

An antioxidant is "a molecule capable of inhibiting the oxidation of other molecules. Low levels of antioxidants or inhibition of the antioxidant enzymes, cause oxidative stree and may damage or kill cells."[8]

Free radicals result in oxidative stress contributes to more than hundred disorders in humans including artheroscelorosis, arthritis, gastritis, cancer, diabetes mellitus[35], etc. Natural antioxidants such as vitamin E (α-tocopherol), vitamin C, and polyphenols/flavonoids are known to be used to treat and prevent the diseases [6,32].

Recent research associated with natural antioxidants has increased in various fields, including food chemistry, food biology, natural plant chemistry, medicinal plants. Flavonoids possess antioxidant activity [16], can scavenge free radicals,superoxide anions, inhibit or kill many bacterial strains, important viral enzymes such as reverse transcriptase and protease [13,10,25], influence the metabolism of drugs [5,13] and are also found to be antigenic when bound to plasma proteins[12].

The wide spectrum of components found in Indian gooseberry (*Phyllanthus emblica* L.,syn *Emblica officinalis* Gaertn.) are alkaloids, ascorbic acid, tannins, trigalloylglucose, flavonoids (such as, quercetin 3-b-D-glucopyranoside, kaempferol 3-b-Dglucopyranoside, isocorilagin, quercetin and kaempferol)[37], coumarins, triterpenes, steroids, furanolactones [9], phyllemblic acid [26].

Curcuminoid is the main component in Turmeric (*Curcuma longa* L) which is responsible for their major biological effects. Curcumin, the major component in curcuminoid that influence to increase the antioxidant defense mechanisms [15, 7].

Active constituents found in horse gram (*Macrotyloma uniflorum* (Lam.) Verdc .are polyphenols including dietary tannins, Kaempferol-3-O-ß-D glucoside, ß-sitosterol, sigmasterol, and phenolic acids (ferulic, vanillic, Þ-coumeric, caffeic acids, etc.,) which have been proved for their antioxidant mechanism [17].

Chitranone, Difuranonaphthoquinones, Plumbagin [33, 19, 21, 1, 28] and flavonoids like 2-(2, 4-Dihydroxy-phenyl)-3, 6, 8- trihydroxy-chromen-4-one are the bioactive compounds found in Chitraka (*Plumbago zeylanica* Linn Syn. /*Plumbago rosea* Linn.

Condensing the importance of this area, the polyherbal formulation consisting of *Phyllanthus emblica, Macrotyloma uniflorum*, *Plumbago zeylanica*, *and Curcuma longa* was developed and tested for the antioxidant activity. *In vitro* antioxidant activity was done by free radical scavenging assays like DPPH assay [3], Super oxide assay [22], and ABTS assay [24].

MATERIALS AND METHODS

Preparation of PolyHerbal Formulations

The dried parts of *Phyllanthus emblica (fruits), Macrotyloma uniflorum* (seeds), *Plumbago zeylanica (roots)*, *Curcuma longa (rhizomes)* were collected from different parts of Coimbatore. The plant materials were identified and authenticated by Dr.S.Rajan, Field Botanist, Survey of Medicinal Plants and Collection Unit, Central Council for Research in Homeopathy Department of AYUSH, Emerald.

The herbal drugs were cleaned by hand sorting .The individual drugs were pulverized with a mixer grinder and sieved (through mesh 40) to obtain a fine powder. Each of the powders was taken in equal quantities (by weight) and was thoroughly mixed to get a homogenous formulation of four different combinations (with *Macrotyloma uniflorum* as the base material for each of the formulation). The formulations were labelled as MPhPl (For 1), MPhCur (For 2), MPlCur (For 3), and MphPlCur (PHF4, Developed formulation) .They were stored in air-tight bottles for future use.

Chemicals

2,2-diphenyl-1-picrylhydrazyl(DPPH) [RM 2798, Himedia, India],Quercetin [Sisco Research Laboratories Pvt. Ltd, Mumbai, India], 2,2,-azinobis (3-ethylbenzoline-6-sulfonic acid (ABTS), Ammonium persulphateAPS) [Rankem,India], Nitroblue tetrazolium (NBT) [94060,S.d.fine Chemicals, India], phenazine ethasulphate (PMS) [5165, Loba Chemie,India], Nicotinamide adenine dinucleotide (NADH)[RM 393Himedia, India], *Folin Ciocalteau's* reagent [Merck, Mumbai, India].

Qualitative Analysis

1. Preliminary phytochemical testing

Equal portions by weight of all ingredients were homogenously mixed and subjected to Soxhlet extraction in refluxing distilled water .The extraction was continued for 48 hours using distilled water four times of weight of the crude drug mixture. The aqueous extract was further concentrated to a semi-solid substance under reduced pressure in a rotary evaporator at 45-50° C. The extract was qualitatively tested for the presence of different phytoconstituents such as alkaloids, amino acids, reducing sugars, tannins, saponins, steroids, terpenoids, flavonoids as per the standard methods described by standard methods [11, 34, 18].

2. TLC of Isolated tannic acid [23, 36, 11]

The dry powders of *Phyllanthus emblica* (fruits), *Macrotyloma uniflorum*, PHF4 were extracted with acetone under reflux. After complete extraction, solvent was recovered under conventional distillation unit. The extract was purified with ethly acetate by refluxing under slow stirring (25-30 rpm). The ethyl acetate fraction was filtered and the residue was collected. The residual paste obtained was dissolved in 3-4 times double distilled water with stirring at 30 rpm. The solution was passed through a Whatman filter paper and a clear solution was collected, allowed to stand for 30 min and finally spray dried. The powder tannic acids obtained from the two samples were subjected to qualitative chromatographic studies.

For thin layer chromatographic studies of tannic acid, precoated silica gel 60 F_{254} aluminium plates of size 20x20cm were used. TLC on silica gel with CH_3Cl: ethyl acetate: formic acid (5: 4:1 v/v/v) as final mobile phase was performed to improve the separation and identification of tannic acid in the samples. Ascending development of the plates was performed at room temperature (25°C ±2 °C) with the same solvent system in a Camag twin trough chamber, previously saturated with the mobile phase for 30 min. The average development time was 15min. After the development, the plates were air dried and the spots were detected. The colour and R_f values were recorded using UV $_{254\ nm}$ and UV$_{366\ nm}$.The detection limit of the samples was 5µg.

Determination of total phenolic content

Total phenolic content was determined using *Folin-Ciocalteau* (FC) reagent according to the method of Singleton and Rossi [29]. Briefly, the formulation extract (0.1 ml) was mixed with 0.75 ml of FC reagent (previously diluted 1000-fold with distilled water) and incubated for 5 min at 22°C, then 0.06% Na_2CO_3 solution was added. After incubation at 22°C for 90 min, the absorbance was measured at 725 nm. Standard of 100µg/ml was prepared and a standard curve of absorbance versus concentration of gallic acid (5-80µg/ml) was plotted. The phenolic content was determined from a gallic acid standard curve. All tests were performed six times.

Determination of total flavonoid content

The total flavonoid content was determined with aluminium chloride ($AlCl_3$) according to a known method [38] using quercetin as the standard. The plant extract (0.1 ml) was added to 0.3 ml distilled water followed by $NaNO_2$ (0.03 ml, 5%). After 5 min at 25°C, $AlCl_3$ (0.03 ml, 10%) was added. After a further 5 min, the reaction mixture was treated with 0.2 ml 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. All tests were performed six times. The flavonoid content was calculated from a quercetin standard curve and expressed as µg of quercetin per milligram.

Quantitative estimation of tannins

The total tannins were estimated by the Folin Dennis method [27]. 0.5g of the powdered material was weighed and transferred to a 250 ml conical flask. 75 ml of water was added and boiled for 30 min. The supernatant was collected after centrifuging at 2,000 rpm for 20 min. The volume was made up to 100ml in a volumetric flask. 1 ml of the sample extracts were transferred and added to 75 ml of water. 5ml of Folin-Denis reagent was added to 10ml of sodium carbonate solution and diluted to 100ml with water. The absorbance was read at 700nm after 30 min. Water was used as a blank instead of the sample. The calibration curve was obtained for standard tannic acid in the concentrations of 0 to 100µg/ml and was presented in the standard graph .The quantity of the tannins present in the aqueous extract of the samples was calculated as % mg equivalent of Tannic acid. All tests were performed six times.

Free –radical scavenging activity

DPPH Assay

The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH radical method [3] . The DPPH solution (0.1mM) in ethanol was prepared and 1.0mg/ml of this solution was added to 3.0 ml of extract solution (or standard) in solvent at different concentrations (10-50 µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture showed higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation.

$$\% \text{ inhibition} = \text{control} - \text{test}/\text{control} \times 100$$

Where 'control' was the absorbance of the control reaction and 'test' was the absorbance in presence of extract. The mean values were calculated from six experiments. The positive controls were those using the standard solution quercetin.

ABTS Radical Scavenging Activity

The ABTS radical scavenging activity of the extract was measured by [24]. ABTS radical cation (ABTS·+) was produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium persulphate and the mixture was allowed to stand in dark at room temperature for 12-16 hrs before use. Different concentrations (10-50 µg/ml) of extract or standard (0.5 ml) were added to 0.3 ml of ABTS solution and the final volume was made up with solvent to make 1 ml. The absorbance was read at 745nm and the % inhibition was calculated. The experiment was performed six times.

$$\% \text{ inhibition} = \text{control} - \text{test}/\text{control} \times 100$$

Superoxide Anion Scavenging Activity Assay

The scavenging activity of extract towards superoxide anion radicals was measured by the method of Nishimiki, 1972 [22]. About 1ml of nitro blue tetrazolium solution (156 µM in 100 mM phosphate buffer, pH 7.4), 1 ml nicotine amide adenine dinucleotide solution (468 µM in 100 mM phosphate buffer, pH 7.4) and 0.1ml of different concentrations of extract and standard in water were mixed. The reaction was started by adding 100 µl of phenazine methosulphate (PMS) solution (60 µM) in 100 mM phosphate buffer, (pH 7.4) to the mixture. The reaction mixture was incubated at room temperature for 5 min and the absorbance at 560 nm was measured against reagent blank in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation.

$$\% \text{ Inhibition} = \text{control} - \text{test}/\text{control} \times 100$$

Where 'control' was the absorbance of the control (without extract) and 'test' was the absorbance in the presence of the extract. The experiment was repeated six times.

Statistical Analysis

All data were expressed as the mean values ± standard deviation .All measurements were replicated six times. The IC_{50} values were calculated by the formula Y = 100*A1/(X + A1), where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration (linear regression analysis, $^{***}p < 0.0001$ was considered significant) using InStat3 software. The IC_{50} values were also compared by paired t tests and $^{***}p < 0.001$ vs 0 µg/ml was considered significant.

RESULTS AND DISCUSSION

The qualitative study reported the presence of tannins, flavonoids, phenols, alkaloids, glycosides, sterols in the aqueous extract of the developed polyherbal formulation (PHF4). TLC analysis of tannic

acid which was isolated from *Phyllanthus emblica* indicated the presence of six clear purple spots of R_f values of 0.14, 0.42, 0.57, 0.85, and 0.94. *Macrotyloma uniflorum* extract showed four purple spots of R_f values 0.42, 0.78, 0.85, and 0.94. Two prominent dark purple spots of R_f values 0.85 and 0.94 were shown in the extract of PHF4. The results of TLC analysis of tannic acid on silica gel layer with the mobile phase (5:4:1 v/v/v) is presented in Fig 1and Table 1.

In the estimation of phenolic content, absorbance (at 725nm) of Gallic and aqueous extract of PHF4 in different concentrations (mg/ml) was linear. The phenolic content was expressed as gallic acid equivalents (GAE) detected in PHF4 (100 mg) and yield obtained was 90.37 ± 0.004 mg/ml gallic acid-equivalent (y= r^2 =0.996) (Fig 2).The total flavonoid concentration detected was 66.7± 0.4µg/mg (r^2 =0.998) and expressed as µg/mg quercetin equivalent in the sample (Fig 3). The quantity of Tannins present in the sample was found to be 2.01±1.02 % mg equivalent of Tannic acid (r^2 =0.999) (Fig 4). Tannic acid is known to suppress hydroxyl radical formation [2]. It was reported that the polyphenolic nature of tannic acid, it's relatively hydrophobic "core" and hydrophilic "shell" are the features responsible for its antioxidant action[14].

The results obtained in the present study showed that aqueous extract of PHF4 is a potent source of natural antioxidant and this suggests that phenolic compounds present in the four herbal drugs in combination provide substantial antioxidant activity.

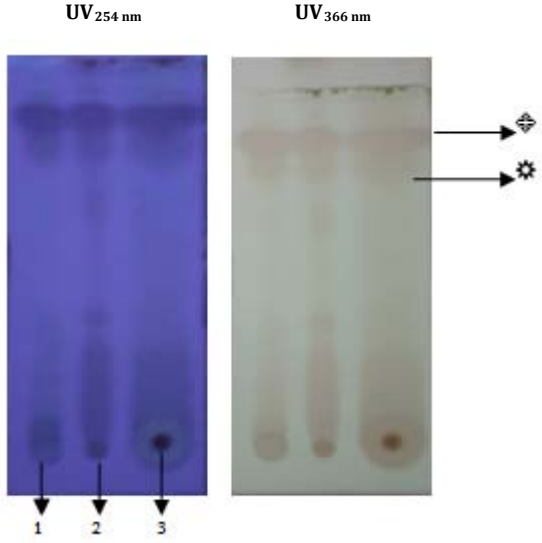

Fig. 1: TLC of isolated tannic acid in *Phyllanthus emblica* (1), *Macrotyloma uniflorum* (2), and PHF4 (3). R_f value of 0.94; R_f value of 0.85

Table 1: TLC profile of tannic acid *Phyllanthus emblica, Macrotyloma uniflorum*, and PHF4

S. No.	Samples	R_f of Sample	Inference	Solvent system
1.	*Phyllanthus emblica*	0.14, 0.42, 0.57, 0.85, and 0.94.	Dark purple spots	CH_3Cl:ethyl acetate:formic acid (5: 4:1 v/v/v)
2.	*Macrotyloma uniflorum*	0.42, 0.78, 0.85, and 0.94	Dark purple spots	
3.	PHF4	0.85 and 0.94.	Dark purple spots	

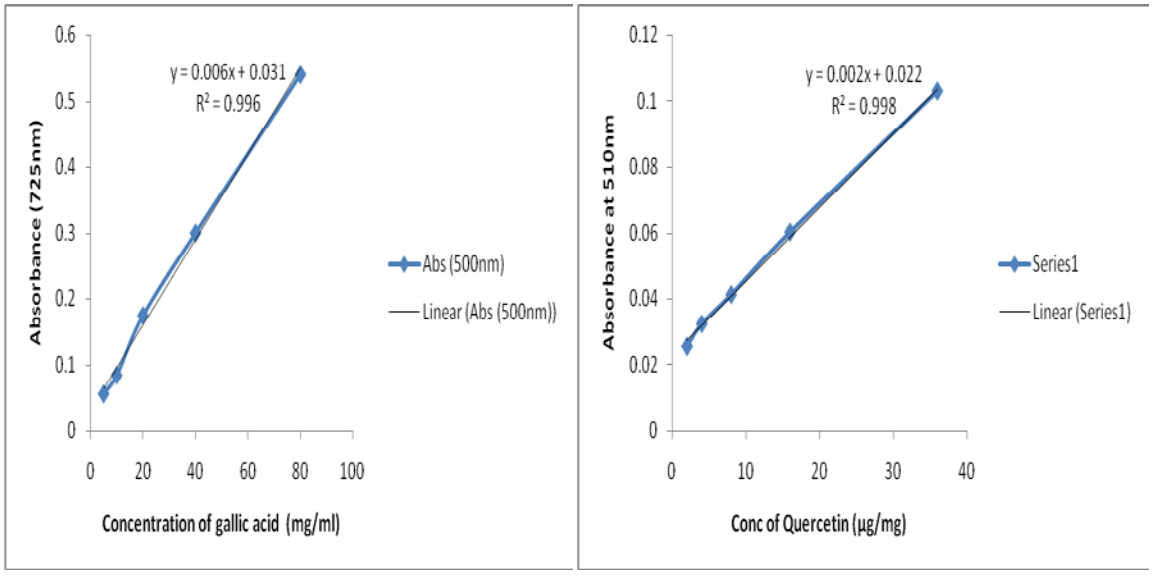

Fig. 2: Determination of total phenolic content and Figure 3: Determination of total flavonoid content of the final formulation (PHF) (All data are expressed as mean ± S.D. (n = 6))

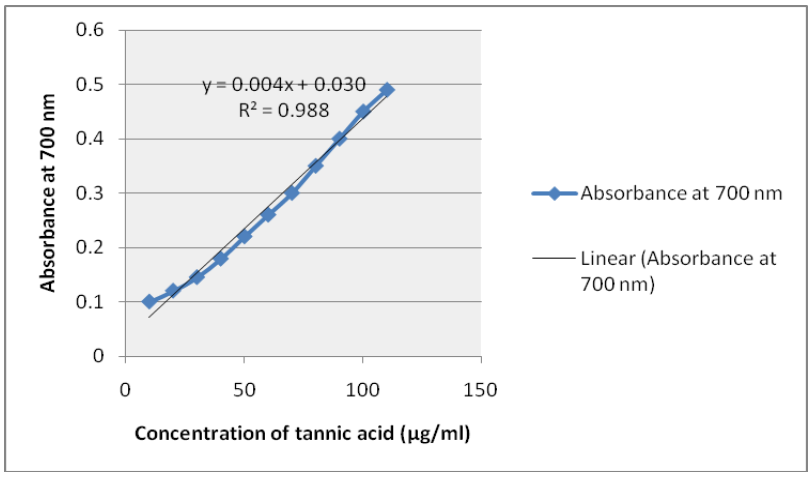

Fig. 4: Total tannic acid in PHF 4 (All data are expressed as mean ± S.D. (n = 6))

All the four formulations were evaluated for antioxidant activity by using the DPPH, ABTS, and superoxide models. Results showed that in comparison to individual formulations, PHF4 was superior antioxidant in all the models tested.

The antioxidant activity was assessed based on the scavenging activity of the stable DPPH free radical. The IC_{50} value for each sample was determined graphically by plotting the percentage disappearance of DPPH as a function of the sample concentration (Fig 4).

The IC_{50} values obtained for the combination of formulations were significant ($p< 0.0001$) for free radicals. IC_{50} for MPhPlCur was 7.46 ±0.04µg/ml (y= 0.69x+ 56.92, r^2= 0.9996) which was lower than the positive quercetin (IC_{50} = 10.25 ±1.45µg/ml). Free radical scavenging activity on DPPH assay for the three formulations were in the order of MPlCur > MPhCur > MPhPl (7.85 ±1.35µg/ml, y= 0.77x+ 54.07, r^2= 0.9995; 8.62±1.25µg/ml, y= 0.73x+ 46.63, r^2= 0.9965; 9.53 ±0.07µg/ml, y= 0.85x+ 40.42; r^2= 0.9977 respectively).

The effect of quercetin and kaempferol on DPPH radical scavenging is well-known. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [30]. Radical scavenging activity of flavonoids toward DPPH radicals is dependent on the number of hydroxyl groups in the B ring. The greater the number of hydroxyl groups in the B ring, the greater the radical-scavenging potency of flavonoids. However, the C_2-C_3 double bond apparently does not contribute to the hydrogen-donating ability of flavonoids in the absence of a polyhydroxylated structure in the B ring. These hydroxyl groups help to stabilize the aryloxyl radical after hydrogen donation in the process of scavenging the free radicals [4].

In ABTS model MPhPlCur exhibited IC_{50} value of 0.199±0.004 µg/ml (y= 0.01x+ 49.13, r^2= 0.9985) (IC_{50} value of standard compound, trolox is 0.250±0.007 µg/ml). MPhPl, MPlCur and MPhCur showed IC_{50}values of 0.233±0.005µg/ml (y= 0.01x+ 51.86, r^2= 0.9966); 0.282 ±0.004µg/ml (y= 0.01x+55.16, r^2= 0.9967); 0.316 ±0.007µg/ml (y= 0.01x+ 56.84, r^2= 0.9983) respectively (Figs 5 & 6).

In superoxide model, IC_{50} values of scavenging activity of increasing order was shown as follows: MPhCur (9.27±0.02µg/ml, y= 1.04 x+ 43.27, r^2= 0.9965) > PHF4 (12.5± 0.25µg/ml, y= 1.46 x+ 25.97, r^2= 0.9970) > MPhPl (13.5 ±0.05µg/ml, y= 1.05 x+ 27.04, r^2= 0.9970) > MPlCur (15.62 ±1.35µg/ml, y= 1.42x+ 16.89; r^2= 0.9971) respectively (Fig 7). IC_{50} value of standard quercetin was found to be 13.06±1.35 µg/ml.

Free radical scavenging assays using synthetic radicals like $DPPH^{\cdot}$ (1,1-diphenyl-2-picrylhydrazyl),$ABTS^{\cdot+}$(2,2'-azinobis-3-ethyl-benzothiozoline - 6-sulphonic acid), or biological radicals such as superoxide radical anions offer an easy and rapid way to screen herbal drugs, food and beverages for *in vitro* antioxidant activity. Antioxidants, such as phenolic compounds, can play a protective role to inactivate harmful reactive oxygen species [6,31].The antioxidant activity of phenolic compounds is mainly attributed to their redox properties, which allow them to act as reducing agents, hydrogen donor and quenchers of singlet oxygen [24,20].

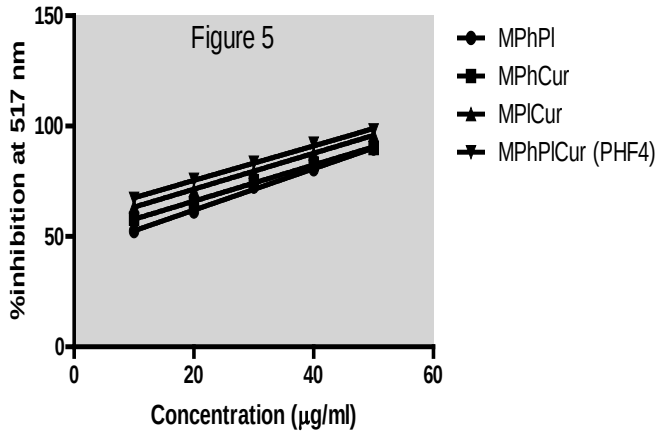

Fig. 5: DPPH free radical scavenging assay: Effect of different formulations on DPPH free radicals with reference to standard compound (quercetin, IC_{50} = 10.25±1.45µg/ml).

The percentage inhibition was plotted against the concentration of sample. All data are expressed as mean ± S.D. (n = 6). $^{**}p < 0.001$ vs 0 µg/ml and $^{***}p < 0.0001$ for significant differences between means determined by linear regression.

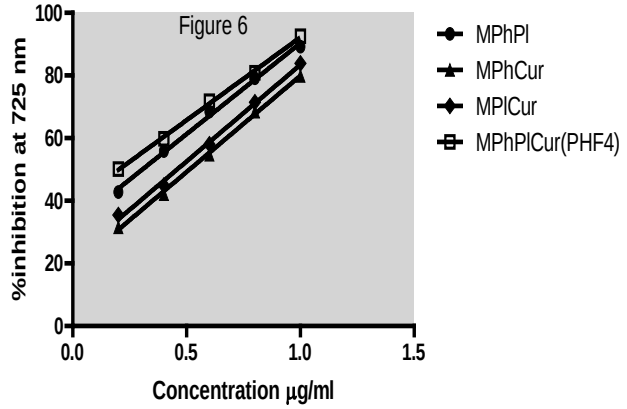

Fig. 6: ABTS radical scavenging assay: Effect of different formulations on decolourisation of ABTS radical cation (Reference compound, trolox, IC_{50} = 0.250±0.007µg/ml) respectively.

The percentage inhibition was plotted against the concentration of sample. All data are expressed as mean ± S.D. (n = 6). $^{**}p < 0.001$ vs 0 µg/ml and $^{***}p < 0.0001$ for significant differences between means determined by linear regression

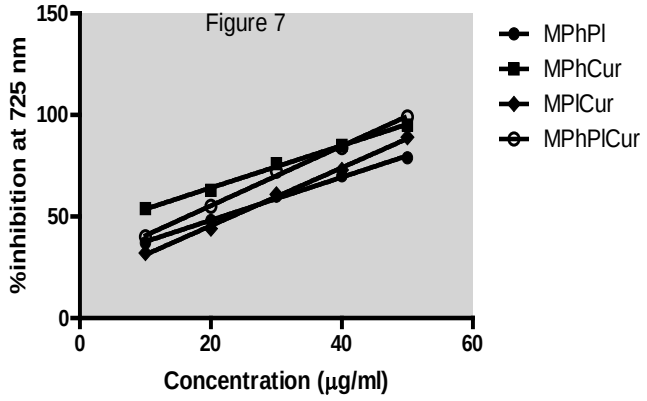

Fig. 7: Superoxide radical scavenging assay. Scavenging effect of different formulations extracts on superoxide radical.

The data represent the percentage superoxide radical inhibition. All data are expressed as mean ± S.D. (n = 6). $^{**}p < 0.001$ vs 0 µg/ml. The IC_{50} value of the standard (Quercetin) is 13.06 ± 1.35 µg/ml. $^{***}p < 0.0001$for significant differences between means determined by linear regression.

CONCLUSION

This study affirms the *in vitro* antioxidant potential of aqueous extract of the developed polyherbal formulation, with results comparable or significantly higher to those of the standard compounds such as Quercetin and Trolox.

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