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A COMPARISON OF *in vitro* FREE RADICAL SCAVENGING ACTIVITY OF FRESH AND DRY RHIZOMES OF *Curcuma longa*

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ABSTRACT

In the present study, the free radical scavenging activity of fresh and dry rhizomes of *Curcuma longa* was compared. The fresh and dry rhizomes of *Curcuma longa* were serially extracted into different solvents (petroleum ether, benzene, chloroform, ethyl acetate, methanol and water) of increasing polarity using a Soxhlet apparatus. The free radical quenching activity of the extracts were characterized against a battery of free radicals such as DPPH (1,1'-diphenyl-2-picryl hydrazyl), H_2O_2 (hydrogen peroxide), ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid), SO (superoxide), NO (nitric oxide) and $OH^{\bullet}$ (hydroxyl), generated *in vitro.* The results obtained in the present study indicate that both fresh and dry rhizomes of *Curcuma longa* are more potent in scavenging free radicals in an effective way at a concentration of 10µg/ml.

KEYWORDS: Free radical scavenging - extracts - fresh and dry rhizomes - *Curcuma longa*.

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INTRODUCTION

Reactive oxygen species (ROS) are formed as a result of the metabolism of oxygen within cells for energy generation in the process of respiration[13]. ROS are effectively scavenged by the antioxidant system present in the body. Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before the vital molecules get damaged. If free radicals are not scavenged properly, excessive accumulation of ROS leads to oxidative stress, which, in turn, will cause cellular damage via peroxidation of lipid membrane, proteins, and nucleic acids[20]. Reactive oxygen species, such as hydroxyl radical, hydrogen peroxide and superoxide anions, are produced as by products in aerobic organisms and have been implicated in the pathology of a vast variety of human diseases including cancer, atherosclerosis, diabetes mellitus, hypertension, AIDS and aging[16]. Therefore, antioxidant activity is important in the free radical theory of aging and associated diseases. Traditional medicine worldwide is being re-evaluated by extensive research on different plant species and their therapeutic principles. Plants produce antioxidants to control the oxidative stress caused by oxygen, and can represent a source of new compounds with antioxidant activity[4]. *Curcuma longa* (turmeric) is one of the main species belonging to the family of the Zingiberaceae, used to enhance the colour, aroma and flavour of the food in most regions of Southern Asia. Turmeric has long been known to play a significant role in Ayurveda, Chinese medicines and traditional household treatments[15]. It has been used in traditional medicine for the treatment of various external or internal inflammatory conditions such as arthritis, colitis and hepatitis[2]. The main active components of turmeric are curcuminoids and essential oil. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a phenolic natural product isolated from the rhizome of *Curcuma longa*. It has been shown that curcumin exhibits anti-inflammatory and antimutagenic properties in addition to anticarcinogenic activity[11]. The study focused on the effect of loss in moisture content due to shade drying. The aim of the present study was to compare the *in vitro* free radical scavenging activity of fresh and dry rhizomes of *Curcuma longa*.

MATERIALS AND METHODS

1. Plant collection and preparation of the extract

The rhizomes were collected from Bhavani, Erode district, Tamil Nadu. The fresh rhizomes were shade dried, powdered with mechanical grinder and stored in an air tight container. The fresh rhizomes (15g) and dry rhizome powder (15g) were extracted serially into a series of solvents of increasing polarity from non polar to polar such as petroleum ether, benzene, chloroform, ethyl acetate and methanol in a Soxhlet apparatus at 60°C - 80°C. The final residue was extracted with distilled water. The solvent was evaporated to dryness and the resulting residue was dissolved in DMSO at a final concentration of 20mg/5µl. The concentration used in the present study was 10µg.

2. Reagents and Chemicals

All chemicals and reagents used were of analytical grade. DPPH (1,1'-diphenyl-2-picryl hydrazyl), ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid), were purchased from Himedia. H_2O_2 was obtained from Rankem.

3. DPPH free radical scavenging activity[19]

The different solvent extracts and aqueous extract (0.01ml) were added to the methanolic solution of 0.4mM DPPH and methanol. The mixture was then allowed to stand at room temperature for 15 minutes in the dark. DPPH in methanol solution was used as a positive control and methanol alone acted as blank. After incubation, the conversion of purple colour to yellow colour was read at 518nm in a spectrophotometer (Genesys 10-S, USA). The per cent inhibition was calculated using the formula,

$$\% \text{ Scavenging} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Sample)}}{\text{Absorbance (Control)}} \times 100$$

4. ABTS scavenging assay[32]

ABTS solution was a mixture of 7mM ABTS and 2.45mM ammonium per sulphate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours in order to generate ABTS radical cations (ABTS+) before use.The extracts (0.01ml) were added to the ABTS solution (0.3ml) and the final volume of each was made up to 1ml with ethanol. The absorbance was read at 745nm and the percentage inhibition was calculated.

5. Hydrogen Peroxide Scavenging Activity[29]

A solution of H_2O_2 (40mM) was prepared in 0.1M phosphate buffer (pH 7.4). To 0.01ml of the extract, 2.99 ml of the H_2O_2 in phosphate buffer was added. After 10 minutes, the absorbance of the reaction mixture was recorded at 230nm in a spectrophotometer. A solution containing a phosphate buffer was used as a blank. A solution containing H_2O_2 in phosphate buffer was used as a control.

6. Superoxide Scavenging Activity[37]

Superoxide anions were generated in samples that contained 0.01ml of the rhizome extracts (10 µg), 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.64ml of 0.067M phosphate buffer (pH 7.6). DMSO was used as a control for the rhizome extracts. The initial optical density was measured at 560nm in a spectrophotometer. The tubes were incubated in white light for 30 minutes. The absorbance was measured again at 560nm. The difference in absorbance before and after illumination was indicative of superoxide anion scavenging activity.

7. Measurement of Nitric Oxide Scavenging Activity[10]

The reaction was initiated by adding 2.0ml of sodium nitroprusside, 0.5ml of PBS, 0.5ml of rhizome extracts (10µg) and incubated at 25 (C for 30 minutes. Griess reagent (0.5ml) was added and incubated for another 30 minutes. Control tubes were prepared without the extracts. The absorbance of pink coloured chromogen was read at 546nm against the reagent blank, in a spectrophotometer (Genesys 10-S, USA).

8. Measurement of Hydroxyl Radical Scavenging Activity

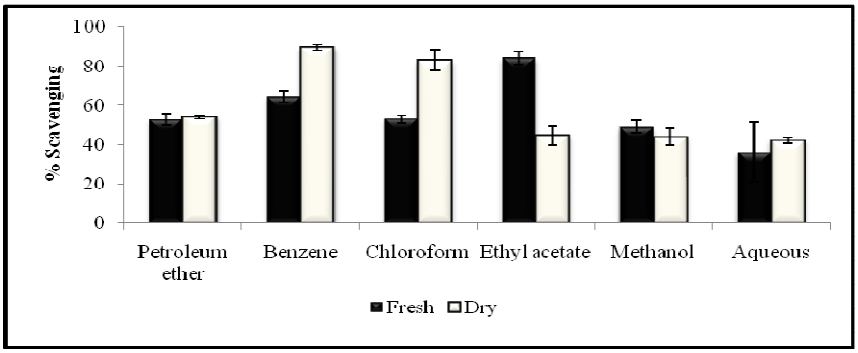
The extent of hydroxyl radical scavenging from Fenton reaction was quantified using 2'-deoxyribose oxidative degradation[9]. The reaction mixture contained 0.1ml of deoxyribose, 0.1ml of $FeCl_3$, 0.1ml of EDTA, 0.1ml of H_2O_2, 0.1ml of ascorbate, 0.1ml of KH_2PO_4-KOH buffer and 0.01ml of rhizome extracts in a final volume of 1.0ml. The mixture was incubated at 37 (C for 1 hour. At the end of the incubation period, 1.0 ml of thiobarbuturic acid was added and heated at 95°C for 20 minutes to develop the colour. After cooling, the thiobarbuturic reacting substances (TBARS) formation was measured spectrophotometrically (Genesys 10-S, USA) at 532nm against an appropriate blank. The hydroxyl radical scavenging activity was determined by comparing the absorbance of the control with that of the sample. The per cent TBARS production for positive control (H_2O_2) was fixed at 100% and the relative per cent TBARS was calculated for the extract treated groups.

RESULTS

1. DPPH free radical scavenging assay

The extent of fresh and dry rhizome extracts to scavenge DPPH in an *in vitro* system is represented in Figure 1. The maximum scavenging of DPPH was exhibited by the benzene extract of dry rhizome, followed closely by the ethyl acetate extract of fresh rhizome and by the chloroform extract of dry rhizome. The DPPH scavenging ability was lower in the aqueous extract of both the rhizomes.

Figure1
DPPH scavenging activity

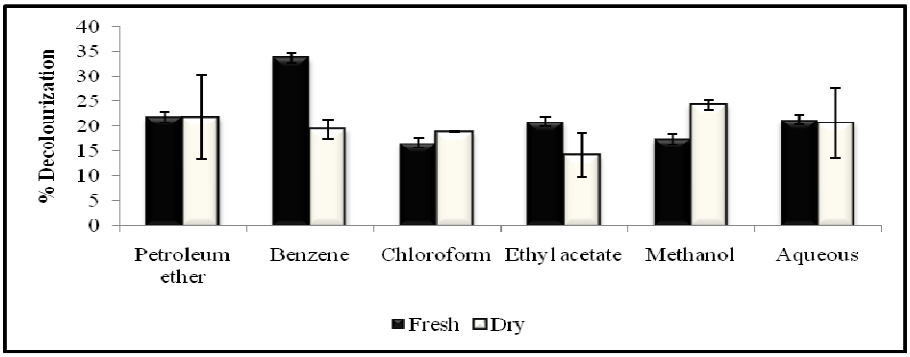


The values are mean ± SD.

2. ABTS free radical scavenging assay

Figure 2 shows the percent decolourization of ABTS *in vitro* by the fresh and dry rhizome extracts of *Curcuma longa*. The results of the study showed that the maximum extent of decolourization was mediated by the benzene extract of fresh rhizome, followed by the methanol extract of dry rhizome, while this was found to be low in ethyl acetate extract of dry rhizome.

Figure 2
ABTS scavenging activity

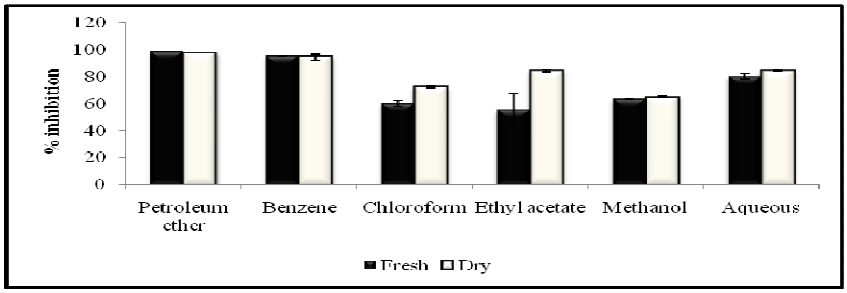


The values are mean ± SD.

3. Hydrogen Peroxide free radical scavenging assay

The results of the fresh and dry rhizome extracts of *Curcuma longa* against H_2O_2 is depicted in Figure 3. It revealed that the petroleum ether extract of both fresh and dry rhizomes exhibited the maximum scavenging activity, followed closely by the benzene extract of both fresh and dry rhizomes of *Curcuma longa*. There was significant activity in the other extracts also.

Figure 3
H_2O_2 scavenging activity

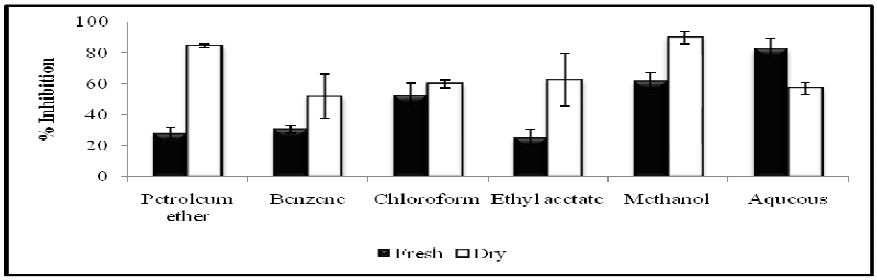


The values are mean ± SD.

4. Superoxide scavenging assay

The superoxide scavenging activity of fresh and dry rhizomes of *Curcuma longa* is represented in Figure 4. The maximum extent of superoxide generation *in vitro* was inhibited by the methanol and petroleum ether extract of dry rhizome followed closely by the aqueous extract of fresh rhizome.

Figure 4
Superoxide scavenging activity

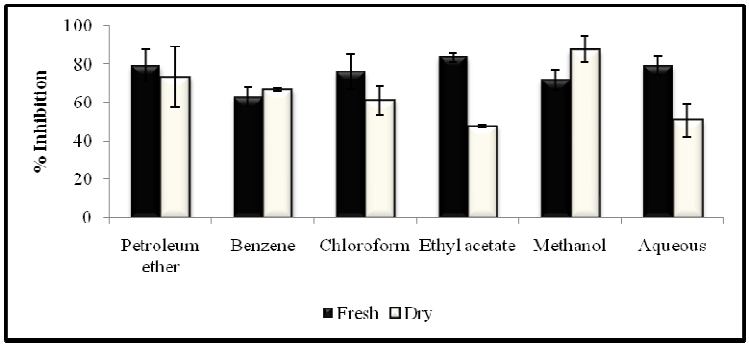


The values are mean ± SD.

5. Nitric oxide scavenging assay

The nitric oxide scavenging activity of the fresh and dry rhizomes of *Curcuma longa* is depicted in Figure 5. The methanol extract of dry rhizome and the ethyl acetate extract of fresh rhizome exhibited maximum nitric oxide scavenging activity.

Figure 5
Nitric oxide scavenging activity

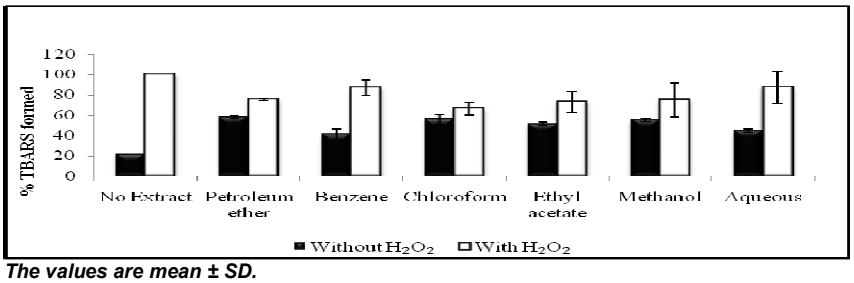


The values are mean ± SD.

6. Hydroxyl radical scavenging assay

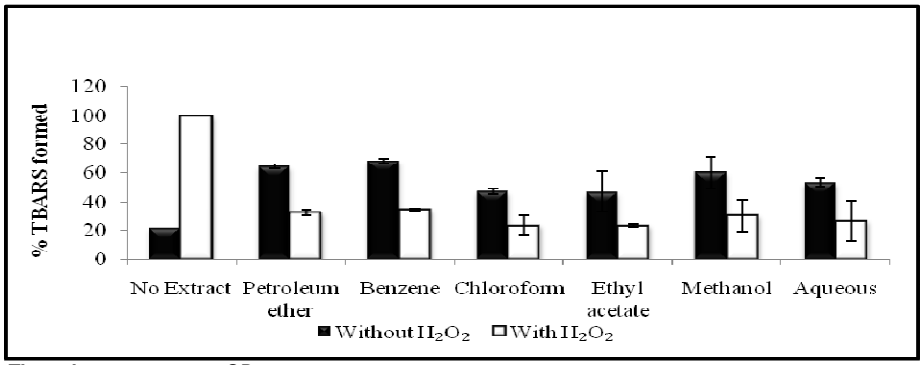
The hydroxyl radical scavenging activities of fresh and dry rhizomes are represented in Figures 6 and 7. Chloroform extract of fresh rhizome and ethyl acetate extract of dry rhizome exhibited minimum TBARS formation when compared to the other extracts.

Figure 6
Hydroxyl radical scavenging activity of fresh rhizome



The values are mean ± SD.

Figure 7
Hydroxyl radical scavenging activity of dry rhizome



The values are mean ± SD.

DISCUSSION

Plants with antioxidant activities have been reported to possess free radical scavenging activity[23]. Various factors like stereo-selectivity of the radicals, solubility of the extract in different testing systems, the polarity of the solvent and the functional groups present in the bioactive compounds have been reported to affect the capacity of the extracts to react and quench different radicals[1]. The major constituents such as ar-turmerone and alpha-turmerone present in the essential oil of rhizomes (*Curcuma longa* Linn.) have been reported to have higher antioxidant properties. The amount of major components present in the dry rhizome was considerably less than that of fresh rhizomes, which was suggested to be responsible for the highest antioxidant activity in fresh rhizome[33]. The present study focused on the effect of various extracts of fresh and dry rhizomes of *Curcuma longa* against both synthetic (DPPH, ABTS) and natural (H_2O_2, super oxide, nitric oxide and hydroxyl) free radicals. The antioxidant activity of leaf extracts of Angolan *Cymbopogon citratus* prepared with different solvents (water, methanol and ethanol) showed different levels of antioxidant activity[34]. Thus, it became essential to compare the antioxidant activity of the plant extracts in solvents of different polarity. The model system of scavenging DPPH free radicals is a simple and acceptable method to evaluate the scavenging activity of antioxidants. In the present study, a benzene extract of dry rhizome and ethyl acetate extract of fresh rhizome were shown to have potent scavenging DPPH free radicals, suggesting the presence of compounds that are capable of donating hydrogen to the free radicals. Ethyl acetate soluble fraction of curcumin, demethoxycurcumin and bisdemethoxycurcumin showed DPPH scavenging activity[7, 35]. These compounds may get fractionated into the ethyl acetate extract of fresh rhizome which is responsible for the DPPH scavenging activity. Similar results were obtained for different extracts of *Dregea volubilis*[4] and ethanolic extracts of the two varieties of the fruit of *Zizyphus Mauritania* (Boroi) [3] which scavenged DPPH free radical effectively *in vitro.* ABTS, a protonated radical, has characteristic absorbance maxima at 734nm, which decreases with the scavenging of proton radicals[18]. In the ABTS radical scavenging assay, benzene extract of fresh rhizome showed significantly higher scavenging activity, followed by the methanol extract when compared to other extracts. Our results were in accordance with the extent of ABTS scavenging reported for the Vayasthapana Rasayana formulation at a concentration of 100µg/ml[21]. Our results also agree with the ABTS radical scavenging activity of five different extracts of rhizoma *Cimicifugae*[14]. Hydrogen peroxide is an oxidant that is being continuously generated in living tissues during various metabolic processes. The detoxification of H_2O_2 is important, as otherwise it will generate extremely reactive oxygen species including hydroxyl free radical. From the results, petroleum ether extract of both fresh and dry rhizomes exhibited maximum H_2O_2 scavenging activity. Induction with H_2O_2 was

significantly suppressed by curcumin in a dose dependent manner[6]. Similar results were observed in the seed and leaf extracts of *A. moschatus*[12] and aqueous extracts of roots of *W. somnifera*[26] which were capable of scavenging H_2O_2 in a concentration dependent manner. The production of superoxide anion radicals ($O_2^{\bullet-}$) is an important factor in the killing of bacteria by phagocytes such as monocytes, macrophages, eosinophils and neutrophills. In the present study, methanol and petroleum ether extracts of dry rhizome, followed closely by the aqueous extract of fresh rhizome, showed maximum scavenging activity when compared to the other extracts. Curcumin exhibited superoxide anions and hydroxyl radical scavenging activity[27, 28]. The turmeric anti-oxidant protein (TAP) present in the aqueous extract of turmeric[30] may responsible for quenching of free radicals. Our results are supported by other studies, wherein the methanolic extract of *Moringa* peregrine[8] and stilbenoids from *R. emodi* [5] exhibited maximum superoxide scavenging activity. Nitric oxide is produced by macrophages, and can be converted into peroxynitrites, which will cause diverse chemical reactions in a biological system. In the nitric oxide scavenging assay, the amount of nitric oxide generated from the decomposition of sodium nitroprusside at physiologic pH was scavenged by all the extracts, where methanol extract of dry rhizome and ethyl acetate extract of fresh rhizome exhibited maximum activity. Curcumin reduced the amount of nitrite formed by the reaction between oxygen and nitric oxide generated from sodium nitroprusside[36]. Our results also coincide with that of methanolic extract of soybean *meal*[24] and methanolic extract of *C. tinctorious* and its isolated constituent dehydroabietylamine, which exhibited maximum nitric oxide scavenging activity with increase in concentration[25]. The chloroform extract of the fresh rhizome showed minimum TBARS formation, reflecting maximum hydroxyl scavenging activity. Similar results were reported in the aqueous extract of the root of *Cassia sieberiana*[22] and the essential oil from *Wedelia chinensis* (Osbeck) [17], which exhibited maximum hydroxyl radical scavenging activity. Methanolic extract of *A. vulgaris* was shown to have considerable scavenging activity against the radicals generated *in vitro* like DPPH, ABTS, hydrogen peroxide and hydroxyl[31]. In the present study, all the extracts had scavenged the free radicals in an effective manner. This showed that the curcumin and the turmeric antioxidant protein present in the extracts played a vital role in scavenging both synthetic and natural free radicals.

CONCLUSION

Based on the results of the study, it is clear that all of the extracts of both fresh and dry rhizomes have powerful *in vitro* antioxidant activity against various free radicals. From the above analysis, the possible mechanism of antioxidant activity of all the extracts includes reductive ability, hydrogen donating ability and scavenging of free radicals which may be due to the presence of phytoconstituents such as curcumin and TAP present in the extract of *C. longa*. Further studies are needed to confirm the *in vivo* potential of the formulation in the management of various age-related human diseases resulting from oxidative stress.

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