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Antioxidant Potential and Total Phenolic Content of Essential Oil and Various Extracts from *Alpinia malaccensis* (Burm.f.) Roscoe

SONALI SETHI, OM PRAKASH* and A.K. PANT

Department of Chemistry, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar-263 145, India

*Corresponding author: E-mail: oporgchem@gmail.com

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The aim of the present study was to evaluate antioxidant assays of essential oil and different extracts from *Alpinia malaccensis* (Zingiberaceae) rhizomes. The essential oil was found to possess higher phenolic contents (50.30 µg/mL) than the extracts. The antioxidant assay of essential oil and extracts evaluated by different methods revealed good to moderate antioxidant potential with different IC_{50} values *viz.* (160.62-230.34 µg/mL) in Fe^{3+} reducing power, (97.68-242.70 µg/mL) in Fe^{2+} metal chelating ability, (66.40-151.25 µg/mL) in DPPH, (61.81-136.16 µg/mL) in OH radical, (68.68-133.46 µg/mL) in NO radical and (58.33-199.87 µg/mL) in superoxide anion scavenging activities respectively in comparison to the standard antioxidants, butylated hydroxyl toluene, catechin, gallic acid and ascorbic acid. Based on these observations it can be concluded that this important herb can be a good source to develop a safe, ecofriendly and sustainable natural antioxidant and food preservative.

Keywords: *Alpinia malaccensis*, Rhizomes, Essential oil, Antioxidant activity, Total phenols.

INTRODUCTION

Reactive oxygen species and reactive nitrogen species are both produced in human body in a regulated manner in cells, which help in maintaining homeostasis for healthy tissues and also for signalling molecules. Most of the cells produce superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) on the demand of body[1]. Every cell possess enzymatic protective mechanisms such as superoxide dismutase (SOD), glutathione peroxidase *etc*, against any harmful effects of free radicals. α-Tocopherol (vitamin-E) and vitamin C, prevents the propagation of free radical reactions in cell membranes of human body. Other non-enzymatic antioxidants include carotenoids, flavonoids and related polyphenols, α-lipoic acid, glutathione *etc*[2].

Over production of free radicals in the body cause several human diseases and ageing. Several pathological and neurological disorders leading oxidative stress are also caused by free radicals[3]. The antioxidants are substances that neutralize/quench free radicals or their actions[2]. Herbal plant extracts contains many antioxidants such as flavonoids, tannins, polyphenols and many other biologically active compounds that possess potent power in the reduction of several diseases and improve health effects, these phytoantioxidants are preferred over the synthetic antioxidants because are safe, versatile and possess bioregulatory properties[4,5].

The genus *Alpinia* (Zingiberaceae) is the largest, widespread throughout tropical areas of East and South India. It possess remarkable biological activities[6]. *A. malaccensis* is a perennial plant growing widely from subtropical to tropical areas where the rhizomes are frequently used for the treatment of abdominal pain[7].

The search of literature revealed the analysis of antioxidant activity of polar extracts of rhizomes of *A. malaccensis*[8]. In continuation to our research on Zingiberaceae, the present investigation was performed to evaluate the antioxidant activity, total phenolic content of *A. malaccensis* rhizome essential oil and extracts prepared in organic solvents with different polarity.

EXPERIMENTAL

Fresh rhizomes of *A. malaccensis* collected from Tarai region of Kumaun hills in India was identified by Dr. D.S. Rawat (Plant taxonomist), Department of Biological Science, G.B. Pant University of Agriculture and Technology, Pantnagar, India. The voucher specimen has been deposited in Department of Botany, for future reference.

Extraction of essential oils: Fresh rhizomes (1 kg) were subjected to hydro distillation in Clevenger's apparatus for 8 h. Extraction of distillate by diethyl ether followed by drying over anhydrous Na_2SO_4 and removal of solvent yielded 0.04 %

of oil. The oil was designated as *Alpinia malaccensis* rhizome essential oil (AMREO) for its further use.

Preparation of extracts: 1.5 kg, shade dried, powdered rhizomes were extracted by successive soaking for 7 days each in organic solvents of different polarity, like petroleum ether, hexane, dichloromethane. The extracts were filtered and concentrated using rotary evaporator. The yields of different extracts *viz*; *Alpinia malaccensis,* rhizome petroleum ether extract (AMRPE), *A. malaccensis,* rhizome hexane extract (AMRHE), *A. malaccensis* and rhizome dichloromethane extract (AMRDE) were found 0.94, 0.83 and 0.82 % respectively. All the extracts were stored at 4 °C for further analysis and biological activity determinations.

Antioxidant activity: Radical-scavenging activity: The essential oil and all the extracts were studied for their radical scavenging potential using various methods as under:

DPPH radical scavenging activity: To study the scavenging ability of the antioxidants by this method, a standard protocol was followed[9]. In brief different amounts of the tested sample (50-250 µg/mL) were added to 5 mL of a 0.004 % methanolic solution of DPPH. Finally the absorbance was read against a blank at 515 nm after 0.5 h of incubation in the dark. All the observations were taken as triplicate. Butylated hydroxyl toluene (BHT), catechin and gallic acid were used as the standard antioxidant. Inhibition of free radical by DPPH in percent (IC %) was calculated by using the equation, IC % = $(A_0 - A_t/A_0) \times 100$, where A_0 and A_t are the absorbance values of the control and test sample respectively. Per cent inhibition was plotted against concentrations and the equation for the line was used to obtain the IC_{50} value.

Hydroxyl radical scavenging activity: This activity was evaluated using the method as described earlier[10]. 60 µL of $FeSO_4 \cdot 7H_2O$ (1 mM) was added to 90 µL of aqueous 1,10-phenanthroline (1 mM), 2.4 mL of 0.2 M phosphate buffer (pH 7.8) was added to the above mixture, followed by the addition of 150 µL of hydrogen peroxide (0.17 mM) and 1.5 mL of different concentrations of sample (50-250 µg/mL). The absorbance of the mixture was read at 560 nm against blank after 5 min. Ascorbic acid was used as the standard. The % inhibition was calculated as, % hydroxyl radical scavenging capacity (IC %) = $[(A_0 - A_t)/A_0] \times 100$, where A_0 and A_t are the absorbance values of the control and the test sample respectively.

Nitric oxide radical scavenging activity: The nitric oxide scavenging activity of oil and extracts was determined by using a reported method[11]. 2 mL of sodium nitroprusside (10 mM) in phosphate buffer saline (PBS) pH 7.4 was mixed with different concentration of sample (50-250 µg/mL) and incubated at 25 °C for 2.5 h. To the above sample 1 mL of Griess reagent (1 % sulphanilamide, 0.1 % naphthylethylenediamine dichloride and 2 mL orthophosphoric acid) was added. As a result pink colour was obtained and the absorbance was read at 546 nm. The % inhibition was calculated by the equation. % Nitric oxide scavenging capacity (IC %) = $(A_0 - A_t/A_0) \times 100$ where A_0 and A_t are the absorbance values of the control sample and the test sample respectively. The percent inhibition was plotted against concentration and the equation for the line was used to obtain the IC_{50} value.

Superoxide anion scavenging activity: Superoxide anion scavenging activity of oil and extracts was determined according to the reported method[12] with slight modifications. In brief 1 mL of nitroblue tetrazolium (NBT) solution (100 µM of NBT in 100 mmol/L phosphate buffer, pH 7.4), 1 mL of NADH (468 µmol in 100 mM/L phosphate buffer, pH 7.4) solution and varying concentration of test samples (50-250 µg/mL) were mixed well. The reaction was started by the addition of 100 µL of phenazine methosulfate solution (PMS) (60 mM of 100 mM/L phosphate buffer, pH 7.4). The reaction mixture was incubated at 30 °C for 15 min. The absorbance was measured at 560 nm in a spectrophotometer. All the readings were taken in triplicate and ascorbic acid was used as the standard. The % inhibition was calculated by the equation. % superoxide scavenging capacity (IC %) = $(A_0 - A_t/A_0) \times 100$ where A_0 and A_t are the absorbance values of the control sample and the test sample respectively. Per cent inhibition was plotted against concentration and the equation for the line was used to obtain the IC_{50} value.

Reducing power: The reducing power of essential oil and various extracts was determined by the method reported earlier[9]. Varying concentrations of tested sample (50-250 µg/mL) were mixed with 2.5 mL of the phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1 % potassium ferricyanide. The mixtures were incubated at 50 °C for 20 min. After incubation, 2.5 mL of 10 % trichloroacetic acid was added to the mixtures, followed by centrifugation at 650xg for 10 min. The upper layer (5 mL) was mixed with 5 mL of distilled H_2O and 1 mL of 0.1 % ferric chloride and absorbance of the resulting solution were measured at 700 nm using spectrophotometer. All the readings were taken in triplicate and butylated hydroxyl toluene, catechin and gallic acid were taken as the standard. The reducing power (RP) of samples was calculated by the following formula: RP (%) = $(A_0 - A_t) \times 100$; where: A_0 and A_t are the absorbance values of the control sample and the test sample respectively. Per cent inhibition was plotted against concentration and the equation for the line was used to obtain the RP_{50} value.

Metal chelating ability: The chelation of Fe^{2+} by essential oil and extracts were evaluated by using the method developed earlier[9]. Different concentration of tested sample (50- 250 µg/mL) were first mixed with 1 mL of methanol and 3.7 mL of deionized water. The resulting mixture was allowed to react with 0.1 mL of $FeCl_2$ (2 mM) and 04.2 mL of ferrozine (5 mM) for 10 min at room temperature. Then, the absorbance was measured at 562 nm. All the readings were taken in triplicate and EDTA (0.01 mM), citric acid (0.025 M) were taken as standard. The metal-chelating ability of the tested sample, expressed as percentage were calculated according to the formula IC (%) = $[(A_0 - A_t/A_0] \times 100$, where A_0 and A_t are the absorbance values of the control sample and the test sample respectively. The percent of chelating ability was plotted against concentration and the equation for the line was used to obtain the IC_{50} value.

Total phenols: The total phenolic content of the oil and various extracts was estimated by using the Folin-Ciocalteu reagent using gallic acid as a standard by a previously described method[13]. 0.5 mL of the oil or the extract solutions were mixed

with 1 mL of Folin-Ciocalteu reagent, 1 mL of aqueous solution of 7 % sodium carbonate and 5 mL of distilled water. The reaction mixture was mixed thoroughly and was allowed to stand for 30 min. The absorbance was read at 765 nm. The same procedure was also applied to the standard solutions of gallic acid. The calibration equation for gallic acid obtained was $y = 0.011x + 0.031$ ($R^2 = 0.998$) where y is the absorbance and x is the concentration of gallic acid in µg/mL. All tests were carried out in triplicate and the obtained results were the mean values and the standard deviation.

Statistical analysis: Experiments were performed in triplicate and data analyzed are mean ± SE subjected to one way ANOVA by using SPSS 16 (Statistical Package for the Social Science). Means are separated by the Tukey's multiple range test when analysis of variance (ANOVA) was significant ($p < 0.05$). Pearson correlation test was used to assess correlations between means.

RESULTS AND DISCUSSION

DPPH radical scavenging activity: To investigate the scavenging power of natural compounds DPPH which itself is a free radical is frequently employed. Compounds possessing antioxidant power donate the proton to DPPH resulting in the discolouration of deep violet colour. *Alpinia malaccensis* rhizome essential oil and all the extracts exhibited strong to good DPPH radical scavenging activity in a dose dependent manner. The lower IC_{50} values indicated maximum antioxidant potential of essential oil/extracts. The radical scavenging potential of AMREO and extracts in term of IC_{50} values revealed the order *viz*; AMREO (IC_{50} = 66.40 µg/mL) > AMRDE (IC_{50} = 115.70 µg/mL) > AMRHE (IC_{50} = 125.33 µg/mL) > AMRPE (IC_{50} = 151.25 µg/mL). The IC_{50} of all the standards were lower than the samples and the order was found butylated hydroxyl toluene (IC_{50} = 33.81 µg/mL) > catechin (IC_{50} = 42.99 µg/mL) > gallic acid (IC_{50} = 49.27µg/mL). Based on this observation it can be inferred that the antioxidant power of AMREO and extracts might be attributed to their hydrogen donating ability to DPPH free radical. The IC_{50} values of AMREO and all the extracts have been recorded in Table-1.

Hydroxyl radical scavenging activity: Perusal of Table-1 reveals that AMREO and all the extracts scavenge the OH free radical as function of amounts in a selected dose levels in comparison to the standard antioxidant ascorbic acid. The results obtained in AMREO and extract for their good anti-oxidant potential in terms of IC_{50} (µg/mL) values were 136.16, 121.52, 103.92, 61.81 for AMRPE, AMRHE, AMRDE and AMREO respectively. IC_{50} of ascorbic acid was observed 44.36 µg/mL.

Nitric oxide radical scavenging activity: Through their nitric oxide scavenging ability AMREO and all the extracts exhibited different degree of antioxidant activity as indicated by IC_{50} values obtained from these samples. However the order of nitric oxide scavenging activity was AMREO (IC_{50} = 68.68 µg/mL) > AMRDE (IC_{50} = 104.80 µg/mL) > AMRHE (IC_{50} = 120.18 µg/mL > AMRPE (IC_{50} = 133.46 µg/mL) whereas the IC_{50} of ascorbic acid was 62.03 µg/mL lower than all the samples (Table-1)

Super oxide anion scavenging activity: Oxidative enzymes of body and non-enzymatic reaction react with oxygen and leads to the production of superoxides[14]. *Alpinia malaccensis* rhizome essential oil and different extracts were found to scavenge the superoxides with different rates in terms of different IC_{50} values for the samples as presented in Table-1. AMRPE, AMRHE, AMRDE and AMREO were able to scavenge the superoxide anion with an IC_{50} of 199.87, 138.99, 124.25, 58.33 µg/mL respectively. The IC_{50} ascorbic acid, the standard antioxidant was obtained 32.28 µg/mL.

Reducing power: In this assay, ferric ions (Fe^{3+}) are reduced to ferrous ions (Fe^{2+}) with change in color. The intensity of color depends on the reducing potential of compounds present in the reaction medium and subsequently related to the antioxidant activity[15]. In present study the Fe^{3+} to Fe^{2+} reducing activity to exhibit the antioxidant ability in terms of their RP_{50} values of AMREO and different extract was obtained in the order of AMREO (RP_{50} = 160.62 µg/mL) > AMRDE (RP_{50} = 200.32 µg/mL) > AMRHE (RP_{50} = 213.37 µg/mL) > AMRPE (RP_{50} = 230.34 µg/mL). However the RP_{50} of the standards were, butylated hydroxyl toluene (RP_{50} = 117.88 µg/mL) >

TABLE-1
ANTIOXIDANT ACTIVITY OF ESSENTIAL OIL AND VARIOUS EXTRACTS OF *A. malaccensis* USING FIVE DIFFERENT TESTING METHOD

Sample/ Standard	Assay						
	DPPH scavenging activity/IC_{50} (µg/mL)	•OH scavenging activity/IC_{50} (µg/mL)	Nitric oxide radical scavenging activity/ IC_{50} (µg/mL)	Superoxide anion scavenging activity/IC_{50} (µg/mL)	Reducing power ability/ RP_{50} (µg/mL)	Metal chelating ability/IC_{50} (µg/mL)	Total phenols (µg/mL)
AMRPE	151.25 ± 1.5^{g}	136.16 ± 0.0^{e}	133.46 ± 0.5^{e}	199.87 ± 1.8^{e}	230.34 ± 0.6^{g}	242.70 ± 2.1^{f}	23.50 ± 1.4^{a}
AMRHE	125.33 ± 0.4^{f}	121.52 ± 0.5^{d}	120.18 ± 0.3^{d}	138.99 ± 1.9^{d}	213 ± 3.4^{f}	179.47 ± 1.0^{e}	34.12 ± 0.1^{b}
AMRDE	115.70 ± 4.1^{e}	103.92 ± 1.4^{c}	104.80 ± 0.2^{c}	124.25 ± 1.3^{c}	200.32 ± 0.1^{e}	144.87 ± 2.5^{d}	35.80 ± 0.2^{c}
AMREO	66.40 ± 1.8^{d}	61.81 ± 2.5^{b}	68.68 ± 0.3^{b}	58.33 ± 1.3^{b}	160.62 ± 0.4^{d}	97.68 ± 1.0^{c}	50.30 ± 0.3^{d}
BHT	33.81 ± 0.4^{a}	NA	NA	NA	117.88 ± 0.3^{a}	NA	NA
Catechin	42.99 ± 2.7^{b}	NA	NA	NA	143.91 ± 0.6^{b}	NA	NA
Gallic acid	49.27 ± 1.1^{c}	NA	NA	NA	151.47 ± 0.6^{c}	NA	NA
Ascorbic acid	32.46 ± 0.2^{a}	44.36 ± 4.5^{a}	62.03 ± 0.3^{a}	32.28 ± 1.2^{a}	115.63 ± 0.2^{a}	NA	NA
EDTA	NA	NA	NA	NA	NA	47.72 ± 2.4^{a}	NA
Citric acid	NA	NA	NA	NA	NA	61.76 ± 0.4^{b}	NA

NA = Not applicable; Values are means of three replicates ± SD. Within a column, mean values followed by the same letter are not significantly different according to Tukey's test ($p < 0.05$).

TABLE-2
CORRELATION OF TOTAL PHENOLS WITH IC_{50} VALUES

Phenols	Total phenols	Correlation coefficient (R)					
		IC_{50} (µg/mL)					RP_{50} (µg/mL)
		DPPH scavenging	•OH scavenging	Nitric oxide radical scavenging activity	Super oxide anion scavenging activity	Metal chelating ability	Reducing power ability
Total phenols	01	-0.993**	-0.997**	-0.979**	-0.994**	-0.966**	-0.986**

*Significant at $\alpha = 0.05$, **Significant at $\alpha = 0.01$

catechin (RP_{50} = 143.91 µg/mL) > gallic acid (RP_{50} = 151.47 µg/mL). A dose dependency was observed in oil and extracts (Table-1).

Metal chelating activity: Fe^{2+} ion form complex with ferrozine in presence of competitor complexing agent the equilibrium between Fe^{2+} ion and ferrozine is disturbed and indicated by decrease in colour intensity thus indicating its antioxidant activity. In the present study different extracts and AMREO demonstrated different degree of binding capacity for Fe^{2+} ion expressed their ability as a per oxidation protector. *Alpinia malaccensis* rhizome essential oil were found to have significantly higher Fe^{2+} chelating abilities followed by extracts. The IC_{50} values for AMREO and extracts for their anti oxidant potentiality was obtained as AMREO (IC_{50} = 97.68 µg/mL) > AMRDE (IC_{50} = 144.87 µg/mL) > AMRHE (IC_{50} = 179.47 µg/mL) > AMRPE (IC_{50} = 242.70 µg/mL).

Total phenols: The total phenols in all the extracts were obtained in the range of 23.50 to 50.30 µg/mL as given in Table-1. The highest phenolic content was observed in the AMREO followed by AMRDE, AMRHE and AMRPE. The correlation of the phenol with antioxidant activity has already been reported earlier[16]. The antioxidant activity of AMREO and different extracts from present study might be possibly due to the presence of phenols and hydrogen donating molecules in the oil and extracts. In present study the total phenols were correlated with IC_{50}/RP_{50} values of different methods used for the determination of antioxidant assay in essential oil and extracts. The results obtained and recorded in Table-2 showed negative correlation of total phenols with the IC_{50} values of DPPH radical scavenging, metal chelating ability, superoxides anion scavenging activity, OH radical scavenging activity, nitric oxide radical scavenging activity and reducing power ability at $\alpha = 0.01$ or 0.05 (level of significance).

We have already reported the antioxidant activity, total phenolic content and hepatoprotective activity of methanolic extract of rhizomes of *A. malaccensis*[17]. To the best of our knowledge the study on antioxidant activity on *A. malaccensis* essential oil and extracts prepared in non-polar solvents is being reported first time from Uttarakhand region of India. However the antioxidant activity of polar extract and essential oil has already been reported[8,18]. Based on the results reported and obtained in present study, it can be concluded that the herb *A. malaccensis* can be used as natural oxidant and a possible substitute of harmful synthetic food preservatives and antioxidants, besides its proper documentation in traditional system.

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