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Original Article

ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITY OF THE MIXTURE OF ETHANOLIC EXTRACTS OF *ALPINIA SPECIOSA* AND *ALPINIA CALCARATA* RHIZOME

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ABSTRACT

Objective: The present study was aimed to investigate the antioxidant and free radipotentials of the mixture ethanolic extract of rhizomes of *Alpinia speciosa* and *Alpinia calcarata*.

Methods: The ethanolic mixture extract of rhizomes was prepared in the ratio of 50:50 of *Alpinia speciosa* and *Alpinia calcarata* was subjected to the analysis of phytochemicals, total phenolics and flavonoid contents and free radical scavenging activities such as DPPH, Nitric oxide radical, Superoxide radical, Hydroxyl radical, Ferric reducing antioxidant power, reducing power and Metal ion-chelating activities were determined.

Results: The rhizome mixture extract showed total antioxidant content, good flavonoid and moderate amounts of phenolics content. In DPPH, Nitric oxide and Superoxide radical scavenging activity, IC_{50} was found 136.22±3.44 µg/ml, 53.67±0.47µg/ml, 74.20±1.48µg/ml respectively with the standard ascorbic acid [3.61±0.20 µg/ml, 49.06±0.18 µg/ml and 39.81±0.22 µg/ml]. For the Hydroxyl radical scavenging activities, the IC_{50} values were 42.02±2.51 µg/ml with the standard Rutin [17.15±0.02 µg/ml]. The Ferric reducing antioxidant power was found 497.32±7.49 mmol/Fe (II)g with the standard Ascorbic acid 1956.20±74.08 mmol/Fe (II)g. The Metal chelating activity was found 96.23±0.39 mg EDTA/g sample with the standard ascorbic acid 205.86±0.53 mg EDTA/g sample. The reducing power was determined, the concentrations 20 µg/ml absorbance of the rhizome mixture was 0.05 and 100 µg/ml absorbance 0.14 with the standard ascorbic acid was absorbance 0.54 and 2.0.

Conclusion: The results of present study showed that mixture ethanolic extract of rhizomes of *Alpinia speciosa* and *Alpinia calcarata* possesses high potential antioxidant activity and could be a potential source a natural antioxidant that could have great importance as therapeutic agents in preventing oxidative stress-related degenerative diseases.

Keywords: Antioxidants, Free radicals, *Alpinia speciosa, Alpinia calcarata*, Total Phenolics, and flavonoids, DPPH

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INTRODUCTION

It is increasingly being realized that many of today's diseases are due to the "oxidative stress" that results from an imbalance between formation and neutralization of pro-oxidants. Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation. These changes contribute to cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases [1]. All human cells protect themselves against free radical damage by enzymes such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherol, and glutathione. Sometimes these protective mechanisms are disrupted by various pathological processes, and antioxidant supplements are vital to combat oxidative damage. Recently much attention has been directed towards the development of ethnomedicines with strong antioxidant properties but low cytotoxicities.

Several medicinal plants (Rasayana) have also been extensively used in the Indian traditional (Ayurveda) system of medicine for the treatment of a number of diseases. Some of these plants have shown potent antioxidant activity [2 and 3]. However, the majority of plants have not yet been screened for such activity. One such plant that is being used is *Alpinia speciosa*. The genus *Alpinia*, comprising more than 230 species, belongs to the family, Zingiberaceae (Ginger family).

Alpinia speciosa (Zingiberaceae) is a native of China, Japan, Indo-China, Cambodia, Thailand, Taiwan, Vietnam and Malaysia [4, 5]. It is economically important since most of these are used as spices, food additives and flavoring agent [6]. It is also used as food and herbal medicine. Even though the plant used as a digestive, spleen, and liver tonic, various *Alpinia speciosa* were also used in the treatment of dyspepsia, gastralgia, sea sickness, rheumatism, catarrhal afflictions and for abdominal colic pains. In addition, the rhizomes also possess ulcer curing property [7].

Alpinia calcarata Rosc. (Zingiberaceae) locally known as Heenaraththa, is an important rhizomatous medicinal herb which grows in the wet zone of Sri Lanka. It is often cultivated in gardens in Eastern and Southern India for its white flowers. The rhizomes of *Alpinia calcarata* have long been used in the indigenous system of medicine. The extract from the rhizome is used as an expectorant for curing bronchitis and asthma. It stimulates digestion, purifies blood and improves the voice and also shows minor anti-inflammatory activity, but nowadays the herb is accredited with anti-tubercular properties and found good in rheumatism, a stomach disorder. Many of which have been used as traditional medicines in the treatment of various ailments or for general healthcare [8, 9].

Based on traditional uses we selected this plants for the study. The purpose of the present study was to evaluate antioxidant and free radical scavenging activities of the mixture ethanolic extracts of rhizomes (*Alpinia speciosa* and *Alpinia calcarata*).

MATERIALS AND METHODS

Collection of plant material

The rhizomes of the *Alpinia speciosa* and *Alpinia calcarata* were collected from the Salem district, Tamilnadu, India. Authenticated by ABS Botanical Conservation Research and Training Centre, Kaaripatti-636106, Salem, Tamil Nadu, India.

Chemicals

Polyvinylpolypyrrolidone (PVPP), aluminium trichloride ($Alcl_3$), sodium hydroxide (NaOH), 1,1-diphenyl,2-picryl hydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine(TPTZ), hydrochloric acid (HCL), iron

(III)chloride hexahydrate ($Fecl_3.6H_2O$), ethylene diamine tetraacetic acid (EDTA),nitro blue tetrazolium chloride (NBT), trichloroacetic acid (TCA) were obtained from Merck, Mumbai, India, sodium nitrite ($NaNO_2$) was obtained from Qualigens Fine Chemicals, Mumbai, India. Ferrous sulfate ($Feso_4$) were obtained from Hi-Media Laboratories Pvt. Ltd, Mumbai, India.

Preparation of extracts

Fresh rhizomes of *Alpinia speciosa* and *Alpinia calcarata* were washed thoroughly under running tap water followed by sterile distilled water and dried under shade. The shade dried material was ground into coarse powder using a mechanical grinder (Panasonic make). This coarse powder was sieved by 1 mm pore size sieve. The powder was stored in airtight containers at room temperature till further use.

Soxhlet extraction

Exhaustive soxhlet extraction was performed using a classical soxhlet apparatus with accurately weighed combined of each 10 g of the rhizomes powder. Successive extraction of dried coarse powder of rhizome was carried out with solvents in increasing order of polarity viz. Hexane, Petroleum ether, Chloroform, Ethyl acetate, Ethanol, and Water. The extraction was done for 48 h and finally all the extracts were evaporated under vacuum. The solvents were evaporated under reduce pressure to get semisolid masses. These extracts were sealed in airtight containers and stored at [-4℃] for further use.

Phytochemical screening

The ethanolic extract of *Alpinia calcarata* and *Alpinia speciosa* in 50:50 ratio was subjected to the qualitative phytochemical screening for the presence of chemical constituents. Phytochemical test were carried out adopting standards procedure [10].

Determinations of total phenolic contents

The total phenolic content of mixture ethanolic extract of rhizomes was determined by Folin-ciocalteu method. Using the same extract, the tannins were estimated after treatment with polyvinyl-polypyrrolidone (PVPP). The amount of total phenolics were calculated as the tannic acid equivalents (TAE) via, Siddhuraju and Becker, 2003[11] and Siddhuraju and Manian, 2007[12].

Determination of total flavonoid content

The total flavonoid content was determined according to the method described by Zhishen *et al.*, 1999 [13]. 0.5 ml aliquot of the mixture ethanolic extract of rhizomes (2 mg/2 ml) was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, and then 2 ml of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5 ml, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. The absorbance of the mixture was determined at 510 nm versus water blank. The analysis was performed in triplicate and the results were expressed as rutin equivalent.

Total antioxidant activities

Free radical scavenging activity on DPPH

The DPPH radical scavenging activity of mixture ethanolic extract of Rhizomes was measured according to the method of Blois (1958) **[**14]. IC_{50} values of the extract, i.e., the concentration of extract necessary to decrease the initial concentration of DPPH by 50% was calculated.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was used to estimate the reducing capacity of the sample, according to the method of Benzie and Strain, 1996**[**15]. The FRAP reagent contained 2.5 ml of a 10 mM TPTZ solution in 40 mM HCl, 2.5 ml of 20 mM FeCl3.6H2O and 25 ml of 300 mM acetate buffer (pH3.6). It was freshly prepared and warmed at 37 °C. 900µl FRAP reagent was mixed with 90µl water and 10µl of the ethanolic mixture extract of rhizomes. The reaction mixture was incubated at 37 °C for 30 min and the absorbance was measured at 593 nm.

Hydroxyl radical scavenging activity

The scavenging activity of mixture ethanolic extract of rhizomes on hydroxyl radical was measured according to the method of Klein *et al.*, 1991 **[**16]. Different concentrations of the ethanolic mixture extract of rhizomes (100-500µg) were added with 1 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90 ° C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1 ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0g of ammonium acetate, 3.0 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1L with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as a control. The intensity of the color formed was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity was calculated as follows:

% HRSA=1-(difference in absorbance of sample/difference in absorbance of blank) × 100

Nitric oxide radical scavenging activity

The nitric oxide scavenging activity of mixture ethanolic extract of rhizomes along with the reference standard ascorbic acid was measured according to the method of Sreejayan and Rao, 1997[17]. The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Superoxide radical scavenging activity

Superoxide radicals were generated by a modified method of Beauchamp and Fridovich, 1971[18]. The assay was based on the capacity of the mixture ethanolic extract of rhizomes to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (100-500µg) of ethanolic mixture extract of rhizomes. Reaction was started by illuminating the reaction mixture with ethanolic mixture extract of rhizomes for 90 seconds. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as:

% Inhibition = (control OD-sample OD/control OD) x 100

Reducing power

The reducing power of ethanolic mixture extract of rhizomes was determined by the method reported by Siddhuraju *et al.*, 2002 **[**19]. 100-500µg of extracts was taken in 1 ml of phosphate buffer and 5 ml of 0.2M phosphate buffer (pH 6.6) was added. To this, 5 ml of 1% potassium ferricyanide solution was added and the mixture was incubated at 50 °C for 20 min. After the incubation, 5 ml of 10% TCA was added. The content was then centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5 ml) was mixed with 5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Then the absorbance of the reaction mixture was read spectroscopically at 700 nm.

Chelating capacity

Chelating property of ethanolic mixture extract of rhizomes was assessed by bipyridyl assay by Yamaguchi *et al.*, 2000**[**20]. The reaction mixture contained 0.25 ml of ethanolic mixture extract of rhizomes having the concentration of 1 mg, 0.25 ml of 1 mM FeSO4 solution, 1 ml of 0.2 M Tris-HCL buffer (pH 7.4), 1 ml of 2,2' bipyridyl solution (0.1% in 0.2 M Tris-HCl), 0.4 ml of 10% hydroxylamine-HCl and 2.5 ml of ethanol. The final volume was made up to 5 ml with deionized water and the absorbance was determined at 522 nm. The chelating activity of the extracts was evaluated using EDTA as

standard. The results were expressed as mg EDTA equivalent/g sample extracts.

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($P<0.05$) using Statistical (Statsoft Inc., Tulsa, USA). Values expressed are means of three replicate determinations±standard deviations.

RESULTS

Phytochemical investigation

Preliminary phytochemical screening of ethanolic mixture extract of rhizomes was carried out to reveal the different primary and secondary metabolites. Phytochemical screening of rhizomes mixture had revealed the presence of flavonoids, alkaloids, phenols, steroids, tannins, proteins and carbohydrates as given in table 1.

Table 1: Phytochemical test for ethanolic extract in *Alpinia* mixtures

S. No.	Test	Ethanolic mixture extract of rhizomes
1.	Carbohydrates	
	a) Fehling's test	+
	b) Benedict's test	+
	c) Molisch's test	+
2.	Proteins	
	a) Million's test	+
	b) Biuret's test	+
3.	Aminoacids	
	a) Ninhydrin test	+
4.	Steroids	
	a) Salkowki's test	+
5.	Thiols	-
6.	Alkaloids	
	a) Dragendorff's test	-
	b) Wagner's test	+
	c) Hager's test	-
7.	Flavanoids	
	a) Alkaline reagent test	+
	b) NH_4OH test	+
	c) Mg turning test	+
	d) Zn test	+
8.	Phenols	
	a) Ferric chloride test	+
9	Saponins	
	a) Foam test	-
10.	Cardiac Glycosides	
	a) Legal's test	-
	b) Keller killani test	+
11.	Tannins	-

(+) = Present,(-) =Negative

Table 2: Total phenolics and flavonoid content

Sample	Total phenolics (mg TA/g sample)	Total flavonoid (mg Ru/g sample)
Rhizome mixture (RM)	0.87±0.12	36.49±14.22

Each value represents mean±SD (n = 3). TA-Tannic acid equivalent, Ru-Rutin equivalent

Total phenolic and flavonoid contents

Phenolic compounds are a class of antioxidant agents, which act as free radical terminators. Total phenols were measured by Folin-Ciocalteu reagent in terms of Tannic acid equivalent. The total phenolic in ethanolic mixture extract of rhizomes was found to be 0.87±0.12 mg/g of extract as given in table 2.

Total Flavonoids compounds in ethanolic mixture extract of rhizomes were also measured spectrophotometrically by using the aluminium chloride colorimetric assay. The amount of total flavonoid content was found to be 36.49±14.22 mg/g of extract calculated as rutin equivalent as given in table 2.

DPPH radical scavenging activity

The rhizome extract was tested for the DPPH free radical scavenging ability, the ethanolic mixture extract of rhizomes and standard ascorbic acid was found to be 136.22±3.44µg/ml and 3.61±0.20 µg/ml respectively.

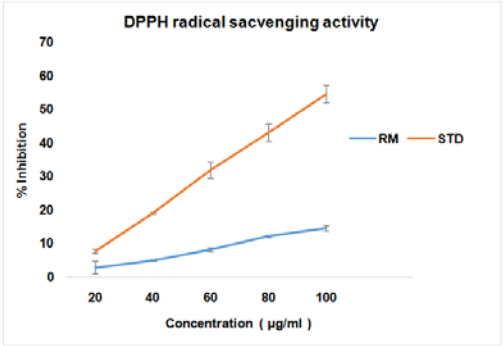

Fig. 1: DPPH radical scavenging activity of ethanolic mixture extract of rhizomes

Values are mean of three independent analysis±SD of triplicates (n = 3). RM-Rhizome mixture STD-Ascorbic acid

The IC_{50} value of the extract was less than that of the standard. At 20µg/ml, the minimum percentage inhibition of the rhizome mixture was 2.8% and 100µg/ml, the maximum percentage inhibition was 14.59% whereas that of standard Ascorbic acid at 20µg/ml, the minimum percentage inhibition 7.75µg/ml and 100µg/ml, the maximum percentage inhibition at 54.62%. The results are presented in fig. 1.

Ferric reducing antioxidant power (FRAP)

The mixture ethanolic extract of rhizomes was comparable to that of standard ascorbic acid. At 60µg/ml, the percentage scavenging of the rhizome extract was 497.32±7.49 mmol Fe (II)/g whereas that of ascorbic acid was 1956.20±74.08 mmol Fe (II)/g (table 3). The higher the FRAP value, the greater is the reducing power of the tested compound.

Table 3: Ferric reducing antioxidant power assay [FRAP]

Sample	FRAP (m mol Fe(II)/g sample)
Rhizome mixture (RM)	497.32±7.49
Ascorbic acid (STD)	1956.20±74.08

Values are mean of three independent analysis±SD of triplicates (n = 3).

Hydroxyl radical scavenging

The IC_{50} values of the rhizome mixture and standard rutin were noted to be 42.02±2.51µg/ml and 17.15±0.02µg/ml respectively. At 20µg/ml the minimum percentage inhibition of the rhizome extract was 2.27% and 100µg/ml the maximum percentage inhibition was 37.94% whereas that of standard rutin at 20µg/ml was noted to be 38.7% and 100µg/ml was found to be 68.56%. The results are shown in fig. 2.

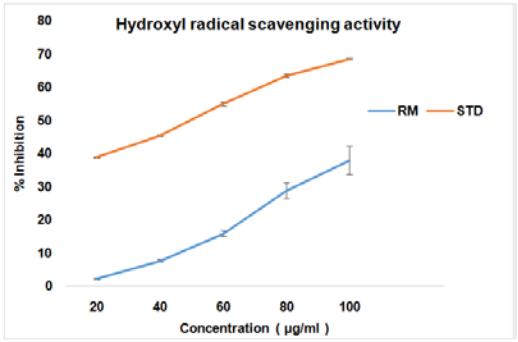

Fig. 2: Hydroxyl radical scavenging activity of mixture ethanolic extract of rhizomes

Values are mean of three independent analysis±SD of triplicates (n = 3). RM-Rhizome mixture and Standard-Rutin

Nitric oxide radical scavenging activity

The mixture ethanolic extract of rhizomes and the standard ascorbic acid caused a moderate dose-dependent inhibition of nitric oxide with an IC_{50} of 53.67±0.47µg/ml and 49.06±0.18µg/ml.

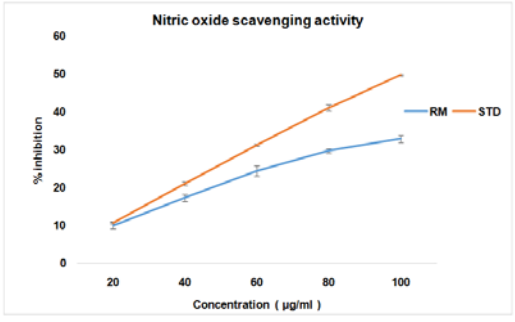

Fig. 3: Nitric oxide scavenging activity of ethanolic mixture extract of rhizomes

Values are mean of three independent analysis±SD of triplicates (n = 3). RM-Rhizome mixture and Standard-Ascorbic acid

The rhizome mixture inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. At 20µg/ml, the percentage inhibition of the rhizome extract was noted to be 10.07% and 100µg/ml, the percentage inhibition was 32.97% whereas that of standard Ascorbic acid was noted to be 10.80% and 49.77% as illustrated in fig. 3.

Superoxide radical scavenging activity

The IC_{50} values of the ethanolic mixture extract of rhizomes and ascorbic acid on superoxide scavenging activity were 74.20±1.48 µg/ml and 39.81±0.22 µg/ml respectively. At 20µg/ml, the percentage inhibition of the rhizome mixture was 5.56% and 100µg/ml, the percentage inhibition was 20.61% whereas that of standard ascorbic acid was noted to be 10.42% and 52.59% as mentioned in fig. 4.

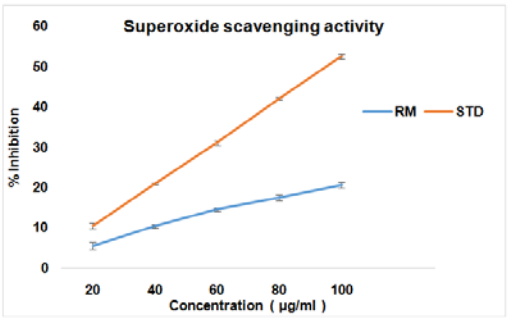

Fig. 4: Superoxide scavenging activity of ethanolic mixture extract of rhizomes

Values are mean of three independent analysis±SD of triplicates (n = 3). RM-Rhizome mixture and Standard-Ascorbic acid

Reducing power

The table 4 shows the reductive capabilities of ethanolic mixture extract of rhizomes and ascorbic acid. At 20µg/ml, the absorbance of the rhizome mixture was 0.05 and 100µg/ml, the absorbance was 0.14 whereas that of standard ascorbic acid was absorbance at 700 nm noted to be 0.54 and 2.0.

Metal chelating activity

The activity of mixture ethanolic extract of rhizomes on chelating the ferrous ions is shown in table 5. The rhizome mixture extracts and ascorbic acid equivalent were found to be 96.23±0.39 mg/g and 205.86±0.53 mg/g respectively.

DISCUSSION

Free radicals are the major cause of various chronic and degenerative diseases in the living systems. The vast amounts of synthetic molecules are available for free radical scavenging antioxidants, but adverse side effects are associated with these

compounds. An alternative solution for this problem is to consume the naturally available antioxidants from the medicinal plants because they are having lower side effects and comparatively safe [21]. Many of the naturally occurring antioxidant compounds like phenols, flavonoids were isolated from various medicinal plants [22].

Table 4: Reducing power

Sample	Concentration (µg)	Absorbance at 700 nm
Rhizome mixture (RM)	20	0.05±0.004
	40	0.07±0.003
	60	0.09±0.003
	80	0.11±0.009
	100	0.14±0.006
Ascorbic acid (STD)	20	0.54±0.003
	40	0.84±0.001
	60	1.23±0.001
	80	1.67±0.002
	100	2.00±0.002

Values are mean of three independent analysis±SD of triplicates (n = 3).

Table 5: Metal chelating activity

Sample	Metal chelating activity (mg EDTA/g sample)
Rhizome mixture (RM)	96.23±0.39
Ascorbic acid (STD)	205.86±0.53

Values are mean of three independent analysis±SD of triplicates (n = 3).

Isolation of pharmacologically active components from the medicinal plant is the long and tedious process. Therefore the phytochemical screening is necessary to eliminate unnecessary separation procedures. This method is performed to allow localization and targeted isolation of new or useful constituents with potential activities and also this procedure enables recognition of known metabolites in extracts or at the earliest stages of separation and is significant for inexpensive [23]. In this study, the preliminary phytochemical screening ascertained the presence of including carbohydrate, alkaloids, flavonoids, glycosides, steroids, tannins and saponin which confirmed that, ethanolic rhizome mixture extract of *Alpinia speciosa* and *Alpinia calcatara* have pharmacologically active components. Plant phenolic compounds exhibit remarkable antioxidant activity by scavenging dangerous free radicals like super oxide anion, hydrogen peroxide, hydroxyl radical and nitric oxide generated during normal metabolic processes. Phenolic compounds are plant secondary metabolites with diverse beneficial biological activities such as anti-inflammatory, anti-allergic, antibacterial, anti-atherosclerotic, anti-carcinogenic activity, anti-mutagenic, anti-tumor, antiviral effects [24].

Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infections and degenerative diseases. The presence of antioxidants leads to the disappearance of these radical chromogens, the most widely used ones are DPPH, FRAP, hydroxyl radicals, nitric oxide radicals, superoxide radicals, reducing power and metal chelating activity, etc. [25].

DPPH is a stable free radical that possesses a characteristic absorption maximum at 517 nm, which is diminished in the presence of a compound (i.e. antioxidants) capable of reducing it to its hydrazine form by hydrogen/electron donation. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation [26]. In this assay, results are expressed as the ratio percentage of the absorbance decrease of DPPH radical solution in the presence of extract at 517 nm to the absorbance of DPPH radical solution at the same wavelength. Ethanolic mixture extract of rhizomes exhibited strong free radical scavenging activity on DPPH assay.

DPPH is a stable free radical that accepts an electron of hydrogen radical to become a stable diamagnetic molecule. The reduction in DPPH radical was determined by the decrease in its absorbance at 517 nm induced by antioxidants. From the result obtained it may be postulated that mixture ethanolic extract of rhizomes reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles. DPPH radicals react with suitable reducing agents; the electrons become paired off and the solution losses colour stoichometrically depending on the number of electrons taken up [27]. It was found that the DPPH radical scavenging activity was reduced by the hydrogen donating ability. The results revealed that the DPPH radical scavenging activity might be attributed to the electron donating ability.

The DPPH assay method is based on the reduction of DPPH, a stable free radical. With the odd electron, the free radical DPPH gives a maximum absorption at 517 nm by visible spectroscopy (purple colour). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, e. g., a free radical-scavenging antioxidant. The absorption strength is decreased, and the resulting decolorization (yellow colour) is stoichiometric with respect to the number of electrons captured [28]. This reaction has been widely used to investigate the ability of plant extracts and fractions and/or pure compounds of those, to act as free radical scavengers or hydrogen donors.

FRAP assay, non-enzymatic antioxidants react with pro-oxidants and inactive them. In a redox reaction, antioxidants act as 'reductants'. In this context, the antioxidant power can be referred to as 'reducing ability'. In this FRAP assay, an easily reducible oxidant, Fe III is used in excess. Thus there is a reduction of Fe III-TPTZ complex by antioxidant [29].

Antioxidative activity has been proposed to be related to reducing power. Therefore, the antioxidant potential of mixture ethanolic extract of rhizomes was estimated for their ability to reduce TPTZ–Fe (III) complex to TPTZ–Fe (II) along with standard ascorbic acid. FRAP assay was used by several authors for the assessment of antioxidant activity of various food product samples. Halvorsen *et al.*, 2006[30] suggested most of the secondary metabolites are redox-active compounds that will be picked up by the FRAP assay.

The highly reactive hydroxyl radical can cause oxidative damage to DNA, lipids and proteins. Hydroxyl radicals are highly strong reactive oxygen species, and there is no specific enzyme to defense against them in the human body. Therefore it is important to discover some chemicals with good scavenging capacity on these reactive oxygen species. In this study the hydroxyl radical

scavenging ability of ethanolic mixture extract of rhizomes was compared with rutin showed more pronounced hydroxyl radical scavenging activity in a dose-dependent manner [31].

The nitric oxide scavenging assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. In the present study, the ethanolic mixture extract of rhizomes scavenged the nitric oxide radical and/or inhibited the nitrite formation but at much lower levels when compared with standard antioxidant Ascorbic acid. The extract inhibits nitric oxide formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis. The polyphenolic compounds present in ethanolic mixture extract of rhizomes might be responsible for the observed scavenging activity. NO• scavenging activity of flavonoids and phenolic compounds are already well known [32] (Madson *et al.*, 2000).

Superoxide is a highly reactive molecule that can react with many substrates produced in various metabolic processes including phagocytosis. It can cause the oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess superoxide dismutase enzyme, which catalyzes the breakdown of superoxide radical [33]. Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive oxygen species. Photochemical reduction of flavins generates $O2^{-}$, which reduces NBT, resulting in the formation of formazan [34]. The ethanolic mixture extracts of rhizome were found to be efficient scavengers of superoxide radical generated in PMS–NADH–NBTsystem *in vitro* and their activities are incomparable to that of ascorbic acid. From this experiment using ethanolic mixture extract of rhizomes of; it is noted that the inhibition of the formation of formazan and also the percentage inhibition are directly proportional to the concentration of the rhizome extracts. In our study, the results indicate that the ethanolic mixture extracts of rhizomes shows a moderate dose-dependent increase in the superoxide scavenging as compared to the standard ascorbic acid which renders it as a potent superoxide scavenger.

The reducing power increases with increasing concentration. Among the extract; however, their reducing power was inferior to standard. Polyphenolic contents of the ethanolic mixture extract of rhizomes appear to function as electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. A similar observation in terms of dose-dependent activity between the polyphenolic constituents in terms of dose-dependent and reducing power has been reported for several plant extract [35].

The metal ion chelating activity increased with increasing concentration of ethanolic mixture extract of rhizomes. This chelating agent may serve as a secondary antioxidant because it reduces the redox potential thereby stabilizing the oxidized form of the metal ions [36]. Though the ethanolic mixture extract of rhizomes did not exhibit a strong antioxidant effect as that of the standard antioxidant ascorbic acid, it did have an activity that reveals ethanolic mixture extract of rhizomes as a potential electron donor which can react with the free radicals and convert them to a stable product.

CONCLUSION

The finding of this study supports the view that the ethanolic mixture extract of *Alpinia speciosa* and *Alpinia calcatara* are promising sources of potential antioxidant and may be efficient as preventive agents in some diseases and can be considered as a natural herbal source in the pharmaceutical industry.

CONFLICT OF INTERESTS

Declared none

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