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A Comparative Study of Polyphenolic Composition and *In-vitro* Antioxidant Activity of *Illicium verum* Extracted by Microwave and Soxhlet Extraction Techniques

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ABSTRACT

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Star anise (Illicium verum) was extracted with ethanol using microwave - assisted extraction and soxhlet extraction. Total phenolic content of microwave – assisted extract (MSA) and conventional extract of star anise (SSA) reached 271.2 mg/g and 289 mg/g dry weight, respectively, expressed as gallic acid equivalents, which were quantified using Folin–Ciocalteau reagent. Subsequently, total flavonoids content of MSA and SSA extracts were found to be 14.64 mg/g and 13.98 mg/g dry weight, respectively, expressed as catechin equivalents ($P < 0.05$). Antioxidant properties of two extracts were investigated employing various established systems in vitro including 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, ABTS assay, total antioxidant activity, hydroxyl radical assay, nitric oxide radical scavenging and total reducing power. MSA and SSA showed excellent antioxidant in all test systems compared to standard ascorbic acid and the antioxidant activities of MSA were all superior to those of SSA. The results obtained in this study clearly indicate that star anise has a significant potential to use as a natural anti-oxidant agent.

Keywords: Illicium verum; microwave assisted extract; antioxidant activity; total flavonoids content; total phenolic content.

INTRODUCTION

Many extraction techniques have been used for obtaining bioactive compounds from plant origins nowadays. Conventional extraction is usually performed at reflux temperature for several hours, and this method is very time consuming and requires relatively large quantities of solvents[1]. Over the last decade, the demand for new extraction techniques has encouraged the development of alternative extraction techniques. Microwave-assisted extraction is the simplest and the most economical technique for extraction of many plant derived compounds [2]. Microwave assisted extraction can reduce both the extraction time and solvent consumption compared to conventional methods, as demonstrated by the drastically accelerated removal of a variety of compounds from solid matrices [3]. Furthermore, it has the potential to improve extraction quality. Many studies have been undertaken to investigate bioactive compounds of plants deal with microwave-assisted extraction [2-4]. China is very rich in endogenous medicinal and aromatic plants that are used as natural health care products in traditional medicine. A number of these plants have been investigated for their biological activities and antioxidant principles [5-7].

Illicium verum, commonly called Star anise, star aniseed, or Chinese star anise is a spice that closely resembles anise in flavor, obtained from the star-shaped pericarp of *Illicium verum* belongs to family *Schisandraceae* and a small native evergreen tree of southwest China. The star shaped fruits are harvested just before ripening. Star anise has been used in a tea as a remedy for rheumatism, and the seeds are sometimes chewed after meals to aid digestion. As a warm and moving herb, star anise is used to assist in relieving cold-stagnation in the middle jiao, according to Traditional Chinese medicine. The techniques of fluorescent microscopy and gas chromatography[8] and the combined technology of TLC with HPLC-MS/MS [9] were employed to distinguish the species.

GC and GC-MS analysis of fruit volatile oil of star anise (*Illicium verum* Hook) showed the presence of 25 components which account for the 99.9% of the total amount. The major components were trans-anethole (94.37%), methyl chevicol (1.82%) and cis-anethole (1.59%). 15 Components were identified from its acetone extract accounting for 80.27% of the total amount. trans-Anethole (51.81%) was found as a major component along with linoleic acid (11.6%), 1-(4-methoxyphenyl)-prop-2-one (6.71%), foeniculin (5.29%) and palmitic acid (1.47%)[10]. Star anise (*Illicium verum*) was assessed for its anti-carcinogenic potential in *N*-nitrosodiethylamine (NDEA) initiated and phenobarbital (PB) promoted hepato-carcinogenesis [11]. Ethanol extract, volatile oil, and water extract of the fruits of *Illicium verum* (star anise) have been assessed for free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical[12]. Star-anise powder and its ethanol/water (80:20) soluble fraction showed strong antioxygenic activity in refined sunflower oil while the petroleum ether fraction exhibited marginal antioxygenic activity and the water-

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soluble fraction was practically devoid of any activity in sunflower oil[13].

MATERIALS AND METHODS

Chemicals:

Folin ciocalteu Reagent, Sodium carbonate, Ammonium peroxysulphate, Ascorbic acid, Sodium Nitroprusside, Sodium dihydrogen phosphate, Orthophosphoric acid, Ethanol L.R. grade, Ferric chloride, Thiobarbituric acid, Acetic acid, EDTA were purchased from Merck Pvt Limited, Mumbai. Gallic acid, DPPH, 2 deoxyribose, ABTS were purchased from Himedia, Mumbai. Sulfanilamide, Naphthyl ethylenediamine dihydrochloride were purchased from Loba Cheme Pvt. Limited, Mumbai.

Plant Material:

The dried fruits of *Illicium verum* were purchased from local market of Berhampur, Ganjam District, Odisha, India. Further taxonomic identification was conducted by Dr. A. K. Panigrahi, Professor, Department of Botany, Berhampur University, Berhampur, Orissa, India. A voucher specimen of the plant (RIPS/H/0111) has been deposited in the herbarium at the department of botany, Berhampur University.

Microwave and soxhlet extraction of *Illicium verum:*

Five grams of *Illicium verum* fruit powder were put into a 250 ml extraction vessel and extracted with varied amount of solvent (50 ml- 150 ml) under different microwave extraction conditions, microwave power at 140-320 W, ethanol concentration (65–99.9%, v/v), irradiation time (5–30 min) and ratio of solvent to material (10–30 ml/g). The Soxhlet extraction was with 5.0g powder of *Illicium verum* with 150 ml 95% ethanol 2 hours. The mixtures were filtered and the solvent was removed. Extracts were finally dried in a vacuum drier and dry extracts were stored in a freezer until use.

Determination of Total Phenol content:

Total phenolic content was determined by Folin-ciocalteau reagent method in which gallic acid was used as a standard phenolic compound[14]. 5 ml of the reagent was mixed with 1 ml of gallic acid at different concentrations and 3 minutes later 4 ml of 2% sodium carbonate was added to each of the solutions. Blue color developed after thirty minutes was read at 760 nm.

Similarly 50 µg/ml and 100 µg/ml concentrations of ethanolic extract were treated in the similar manner as that of the standard gallic acid. The concentrations of total phenols were expressed as mg/g of dry extract[15]. All the determinations were performed in triplicate. Total content of phenolic compounds were expressed as Gallic acid equivalents (GAE) calculated by the following formula:

$$C = c.\ V/m$$

Where: C- total content of phenolic compounds, mg/g plant extract, in GAE;

c- the concentration of gallic acid established from the calibration curve, mg/ml;

V- the volume of extract, ml;

— the weight of pure plant extract

Scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH assay):

The free radical-scavenging activity of *Illicium verum* ethanol extract was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH. In this assay, the purple chromogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine[16]. The scavenging capacity is generally evaluated in organic media by monitoring the absorbance decrease at 515–528nm until the absorbance remains constant. 0.1 mM solution of DPPH in ethanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution in ethanol at different concentrations (1–35 µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. Ascorbic acid was used as the reference compound. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical-scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the following formula:

$$\% \text{ inhibition} = (A_0 - A_t)/A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_t was the absorbance in the presence of the extract. All the tests were performed in triplicate and the graph was plotted with the mean values.

Scavenging of nitric oxide radical:

Nitric oxide radical (NO) has a pivotal role in the regulation of diverse physiological and pathophysiological processes. Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide[17] which interacts with oxygen to produce nitric ions that can be estimated by using Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide. Sodium nitroprusside (5 mM) in phosphate buffer saline (PBS) was mixed with 3.0 ml of different concentrations (5–5000 µg/ml) of *Illicium verum* ethanol extract and incubated at 25°C for 180 min. The samples were added to Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthyl ethylenediamine dihydrochloride). The absorbance of the chromaphore formed during the diazotization of nitrite with

sulphanilamide and subsequent coupling with napthyl ethylenediamme was read at 546nm and referred to the absorbance of standard solutions of ascorbic acid treated in the same way with Griess reagent as a positive control. The percentage of inhibition was measured similar to that of DPPH assay.

Scavenging of ABTS radical:

The ABTS assay was employed to measure the antioxidant activity of the bark extract. ABTS was dissolved in de-ionised water to 7mM concentration, and Ammonium persulphate added to a concentration of 2.45mM. The reaction mixture was left to stand at room temperature overnight (12 to 16 h) in the dark before usage. 0.5 ml of ethanol extract (0.1-20 µg/ml) was diluted with 0.3 ml ABTS solution and made up to the volume with ethanol. Absorbance was measured spectrophotometrically at 745nm. The assay was performed at least in triplicates. Fresh stocks of ABTS solution were prepared every five days due to self-degradation of the radical. The assay was first carried out on ascorbic acid, which served as a standard. The percentage of inhibition was measured similar to that of DPPH assay.

Scavenging of hydroxyl radical:

The hydroxyl radical scavenging capacity was measured using modified method as described previously[18]. Stock solutions of EDTA (1 mM), $FeCl_3$ (0.2mM), ascorbic acid (1mM), H_2O_2 (10mM) and deoxyribose (28mM) were prepared in distilled de-ionized water. The assay was performed by adding 0.1 ml EDTA, 0.1 ml of $FeCl_3$, 0.1 ml of deoxyribose, 0.5 ml of extract (0.1 – 1000µg/ml) each dissolved in ethanol and 0.1 ml of ascorbic acid in sequence. The mixture was then incubated at 37°C for 1 h. The above reaction mixture was treated with dodecyl sulphate 8.1%, 0.2 ml, thiobarbituric acid 0.8%, 1.5 ml, and acetic acid 20%, 1.5 ml and pH 3.5 and kept in oil bath maintained at 95°C for 1 hour and the absorbance was measured at 532 nm. The hydroxyl radical- scavenging activity of the extract was reported as the percentage of inhibition of deoxyribose degradation and was calculated according to the formula using ascorbic acid as a positive control.

Test of total antioxidant activity:

The total antioxidant activity was measured using a modified version of the method[19]. Sample solution (0.3 ml, 0.5 mg/ml) in 95% ethanol was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95 ºC. The total antioxidant capacity was determined using measuring the absorbance at 695 nm each 30 min after the mixture had cooled to room temperature. The control was prepared as above, without sample. The antioxidant activity is expressed as the absorbance of samples measured at 695 nm. A higher absorbance indicated a higher total antioxidant capacity. Ascorbic acid was used for comparison. All tests were performed in triplicate and mean values were centered.

Test of reducing power:

One milliliter of various ethanol extract solution (0.2, 0.5, 0.8, and 1.2 mg/ml) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%)[20]. After the mixture was incubated at 50 ºC for 20 min, trichloroacetic acid (2.5 ml, 10%) was added, and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%), and then the absorbance was measured at 700 nm against a blank. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used for comparison. All tests were performed in triplicate and mean values were centered.

Determination of total flavonoids content:

The total flavonoids contents of ethanol extracts were measure[21]. Extract solution (1 ml, 0.1 mg/ml) was placed in a 10 ml volumetric flask and then 5 ml of distilled water was added followed by $NaNO_2$ solution (0.3 ml, 5%). After 5 min, $AlCl_3$ solution (0.6 ml, 10%) was added. After another 5 min, sodium hydroxide solution (2 ml, 1 M) was added and volume was made up with distilled water. The solution was mixed thoroughly and absorbance was measured at 510 nm. Total flavonoids amounts were expressed as micrograms of catechin equivalents per gram dry matter. All tests were performed in triplicate and mean values were centered.

Statistical Analysis:

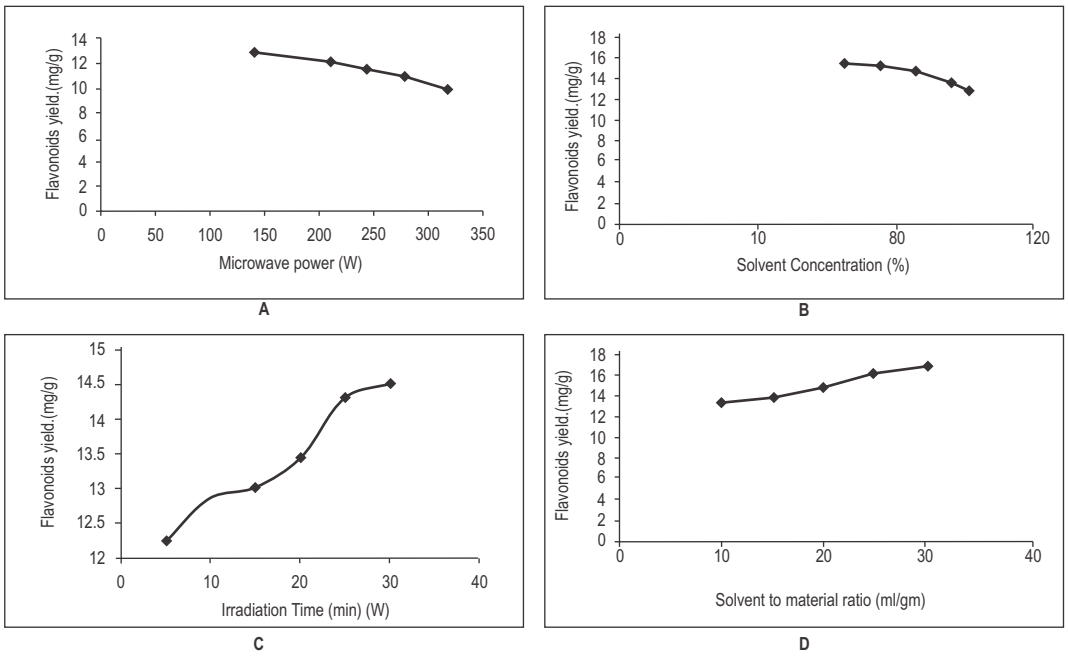
The data were subjected to one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Bartlett's test for equal variance test ($P < 0.05$) using the Graph pad prism 4 software. Difference was considered significant when P-value was < 0.05.

RESULTS AND DISCUSSION

Microwave and Soxhlet extraction of *Illicium verum:*

Figure 1 shows that the yield of flavonoids is affected by many factors. In general, the extraction efficiency was higher at lower microwave power 140W. The yield increased with the decrease of ethanol concentration significantly when the ethanol volume percentage in the solvent was lower than 65% (v/v). Further the increase of duration of microwave radiation or solvent ratio can give increased content of flavonoids significantly. Over exposure in the microwave may cause the loss of flavonoids. The optimum conditions of microwave-assisted extraction were obtained by dual extraction with 65% ethanol 30 ml/g material at 140W for 30 min. The contents of

Fig. 1: Effect of different factors on flavonoids yield.



A) Microwave power (95% ethanol, solvent ratio 10 ml/g, 78 ºC, 10 min, two cycles);
B) Ethanol concentration (140W, solvent ratio 10 ml/g, 78 ºC, 10 min, two cycles);
C) Duration of irradiation (140W, 95% ethanol, solvent ratio 10 ml/g, 78 ºC, two cycles);
E) solvent ratio (140W, 95% ethanol, 78 ºC, 10 min, two cycles).

flavonoids under the optimal extraction conditions were 14.83 mg/g for microwave extract of *Illicium verum* fruit or star anise fruit (MSA) while that of soxhlet or conventional extract of *Illicium verum* fruit or star anise fruit (SSA) was 13.98 mg/g dry weight of extract.

Total Phenol content:

The content of phenolic compounds (mg/g) in ethanol extract of MSA and SSA was found to be 271.2±0.93 mg/g and 289±0.78 mg/g plant extract and expressed in gallic acid equivalents (Table 1). These results suggest that the higher levels of antioxidant activity were due to the presence of phenolic components. The same relationship was also observed between phenolic and antioxidant activity in roseship extracts[22]. The interests of phenolics are increasing in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food[23]. Therefore, it would be valuable to determine the total phenolic content of the plant extracts.

Table 1: Comparison of total phenolic content, total flavonoids content and free radical scavenging activities of MSA and SSA and ASC A

Sample	Total phenolic content[a]	Total flavonoid content (mg/g)[b]	DPPH Ic_{50} (µg/ml)[c]	ABTS IC_{50} (µg/ml)[d]	Hydroxyl radical IC_{50} (µg/ml)[e]	Nitric oxide assay IC_{50} (µg/ml)[f]
MSA	271.2±0.93	14.64	0.508	0.714	18.65	266.09
SSA	289±0.78	13.98	0.534	0.726	18.71	267.80
Ascorbic acid			0.460	0.492	18.46	259.36

Values in the same column followed by different letters within each column are significantly different ($p < 0.05$).
a Milligrams of gallic acid per 1 g (dry weight) of extract.
b Milligrams of catechin per 1 g (dry weight) of extract.
c The effective concentration at which DPPH radicals were scavenged by 50%.
d The effective concentration at which ABTS radicals were scavenged by 50%.
e The effective concentration at which hydroxyl radicals were scavenged by 50%.
f The effective concentration at which Nitric oxide radicals were scavenged by 50%.

Inhibition of DPPH radical:

The DPPH radical is a stable organic free radical with an absorption maximum band around 515–528 nm and thus, it is a useful reagent for evaluation of antioxidant activity of compounds[24]. In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants[25]. It has been documented that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), reduce and decolorize 1, 1-diphenyl-2-picrylhydrazine by their hydrogen donating capabilities.

The ethanol extract of *Illicium verum* demonstrated a concentration dependent scavenging activity by quenching DPPH radicals. The concentration of *Illicium verum* needed for 50% inhibition (IC_{50}) was found to be 0.508 µg/ml and 0.534 µg/ml for MSA and SSA respectively, whereas 0.460 µg/ml (Table 1) was needed for ascorbic acid (ASC A). The different concentrations of ethanolic extract of *Illicium verum* (1-35 µg/ml) showed antioxidant activities in a dose dependent manner (57.09% ± 0.21- 94.56% ± 0.16) on the DPPH radical scavenging assay. A higher DPPH radical scavenging activity is associated with a lower IC_{50} value. The results were also found to be statistically significant (Table 2).

Inhibition of Nitric oxide radical:

Nitric oxide plays an important role in various types of inflammatory processes in the animal body. Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities[26]. In the present study the crude ethanol extract of the *Illicium verum* was checked for its inhibitory effect on nitric oxide production. Nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be inhibited by *Illicium verum*. *Illicium verum* extract at varied concentrations showed remarkable inhibitory effect of nitric oxide radical-scavenging activity. Results showed the percentage of inhibition in a dose dependent manner. The various concentrations of extract (50 – 5000 µg/ml) showed the percentage of inhibition in a dose dependent manner (20.97%±0.43–91.24%±0.28)). The concentration of *Illicium verum* fruit extract needed for 50% inhibition (IC_{50}) was found to be 266.09 µg / ml for MSA and 267.80 µg/ml for SSA, whereas 259.36 µg/ml was needed for ascorbic acid (Table 1).

Inhibition of ABTS radical:

The reduction capability of ABTS radical was determined by the decrease in its absorbance at 745 nm which is induced by antioxidants. The ethanol extract of *Illicium verum* fruit at quantities of (0.1 - 20 µg/ml) scavenged the ABTS radicals in a dose dependent manner. Ascorbic acid at a concentration of (0.1-20µg/ml) also found to produce dose dependent inhibition of ABTS radicals. The various concentrations of extract 0.1 – 20 µg/ml showed 5.73±0.47–75.94±0.67 for MSA, 4.84±0.31-73.41±0.09 for SSA and 9.65±0.45-94.93±0.17 for ascorbic acid, percentage inhibition on ABTS radical scavenging activity. Results showed the percentage of inhibition in a dose dependent manner. The quantity of *Illicium verum* fruit extract required to produce 50% inhibition of ABTS radical were 0.714 µg/ml and 0.726 µg/ml for MSA and SSA respectively. Similar effect was produced by ascorbic acid nearly at concentration 0.492µg/ml (Table 1).

Inhibition of Hydroxy radical:

Several *In-vitro* methodologies for determination of HO scavenging capacity are available mostly based on Fe^{3+} EDTA + H_2O_2 + ascorbic acid system to generate a constant flux of HO radicals. Those radicals attack the sugar 2-deoxy-d-ribose (used as target), degrading it into a series of fragments, some or all of which react upon heating with thiobarbituric acid at low pH to give a pink chromogen. If a HO scavenger is added to the reaction mixture, it will compete with deoxyribose for HO radicals, inhibiting the degradation of the target species. The hydroxyl radical is an extremely reactive free radical

Table 2: Effect of ethanolic bark extract of *Illicium verum* (P values), on different radical scavenging activities

Sample	DPPH radical scavenging activity	Hydroxy radical scavenging activity	Nitric oxide radical scavenging activity	ABTS radical scavenging activity
	BTEV	BTEV	BTEV	BTEV
MSA	<0.000057	< 0.000043	<0.00008	0.00008
SSA	<0.000071	< 0.000032	<0.000074	0.00007
	P < 0.05P	< 0.05P	< 0.05P	< 0.05

MSA: Microwave extract of *Illicim verum;* SSA: Soxhlet extract of *Illicium verum*; BTEV: Bartlett's test for equal Variance

formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells[27] Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity[28]. Among the reactive oxygen species, the hydroxyl radical is the most reactive and induces severe damage to the adjacent biomolecules[29]. The potentially reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins Ferric EDTA incubated with H_2O_2 and ascorbic acid during which hydroxy radicals were formed in the free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that on heating with TBA form a pink chromogen. When ethanolic extract of *Illicium verum* fruit and the reference compound, ascorbic acid, added to the reaction mixture they removed hydroxyl radicals from the sugar and prevented degradation. The IC_{50} value of ethanolic extract of *Illicium verum* fruit on hydroxyl radical were found to be 18.65 µg/ml, 18.71 µg/ml and 18.46 µg/ml for MSA, SSA and ascorbic acid respectively (Table 1). The various concentrations of extract 0.1–1000 µg/ml showed 15.96±0.29–94.87±0.07 for MSA, 15.34±0.03-94.69±0.08 for SSA and 17.85±0.23-95.96±0.53 for ascorbic acid, percentage inhibition on hydroxyl radical scavenging activity. Results showed the percentage of inhibition in a dose dependent manner. The ability of the above mentioned extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen species, thus reducing the rate of the chain reaction.

Total antioxidant capacity assay:

In order to examine the total antioxidant capacity, the Mo (VI) to Mo (V) reduction in the presence of the extracts was investigated. Ascorbic acid was also used for comparison purpose. In this assay, a green phosphate/Mo (V) complex will be formed in the condition of acid pH and could be monitored at 695 nm with a spectrophotometer[30]. According to the results, MSA and SSA had significant activities. All effects increased with increasing reaction time and increasing concentration. Apparently that total antioxidant activity decreased as follows: ASC A > SSA > MSA

Reducing power assay:

Measurement of reducing potential can reflect some aspects of antioxidant activity in the extracts. In this assay, the presence of reductants (antioxidants) in the samples would result in the reduction of the Fe^{3+}/ferricyanide complex to its ferrous form and amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm[31]. Higher absorbance value indicates higher reducing power. The concentrations of samples measured were 0.2, 0.4, 0.6, 0.8 and 1.2 mg/ml. The absorbances of MSA at 700 nm were 0.175, 0.269, 0.383, 0.414 and 0.523, respectively, while those of SSA were 0.315, 0.410, 0.514, 0.571 and 0.628, respectively.

CONCLUSION

Free radicals particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in the pathogenesis of several chronical and degenerative diseases such as inflammation, cardiovascular diseases, neurodegenerative diseases, cancer and aging related disorders. It was concluded that the ethanol extract of *Illicium verum* fruit containing total phenolic compounds and were capable of inhibiting, quenching free radicals to terminate the radical chain reaction, and acts as reducing agents. A significant and linear relationship was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity. The results of the present study would certainly help to ascertain the potency of the crude extracts from *Illicium verum* as potential source of natural antioxidants.

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