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Dinesha R

Adichunchanagiri Biotechnology and
Cancer Research Institute, B.G.
Nagara-571 448, Mandya District,
Karnataka, India
Email: r.dinesha@gmail.com

Thammannagowda SS

Adichunchanagiri Biotechnology and
Cancer Research Institute, B.G.
Nagara-571 448, Mandya District,
Karnataka, India,
Email: sstgabcricri@gmail.com

Shwetha KL

Adichunchanagiri Biotechnology and
Cancer Research Institute, B.G.
Nagara-571 448, Mandya District,
Karnataka, India,
Email: shwethaklgowda@gmail.com

Prabhu MSL

Adichunchanagiri Biotechnology and
Cancer Research Institute, B.G.
Nagara-571 448, Mandya District,
Karnataka, India,
Email: prabhulingam6@gmail.com

Madhu CS

Adichunchanagiri Biotechnology and
Cancer Research Institute, B.G.
Nagara-571 448, Mandya District,
Karnataka, India,
Email: madhucs89@gmail.com

Leela Srinivas

Adichunchanagiri Biotechnology and
Cancer Research Institute, B.G.
Nagara-571 448, Mandya District,
Karnataka, India,
Email: directorabcricri@gmail.com

Correspondence**Leela Srinivas**

Adichunchanagiri Biotechnology
and Cancer Research Institute, B.G.
Nagara-571 448, Mandya District,
Karnataka, India,
Email: directorabcricri@gmail.com

The antioxidant and DNA protectant activities of Star Anise (*Illicium verum*) aqueous extracts

Dinesha R, Thammannagowda SS, Shwetha KL, Prabhu MSL, Madhu CS, Leela Srinivas

Abstract

This study aims to determine the antioxidant activity of the aqueous extract of Star Anise (*Illicium verum*) against H₂O₂ induced DNA damage and human peripheral lymphocyte cell death. The antioxidant activities of the above evaluated by Lipid peroxide inhibitory activity, Hydroxyl radical scavenging activity, DPPH (1,1-diphenyl-2-picrylhydrazyl) activity and superoxide radical scavenging activity. The DNA damage protectant activity was evaluated by submarine gel electrophoresis and the H₂O₂ (100 μM) induced cell death in human peripheral lymphocytes protectant activity was evaluated by cell viability test. Standard antioxidants like BHA, Curcumin and α-tocopherol were used as positive control. The ambient temperature water extract and boiling water extract of Star anise showed effective antioxidant activity. The protection provided by Star Anise extracts against cell death induced by H₂O₂ was promising and the same in DNA damage also. The present study reveals that, the extracts exhibited effective prevention ability against H₂O₂ induced cell death and DNA protection. The above protectant activities of extracts of Star Anise may due to the presence of polyphenols, proteins and flavonoids in Star Anise extracts.

Keywords: Star Anise, antioxidant, DNA damage, H₂O₂.

1. Introduction

Free radicals such as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) produced during the normal metabolism can damage the cells resulting in lipid peroxidation, alteration of protein and nucleic acid structures [1, 2]. The deteriorated activities of antioxidant enzymes with increased levels of oxidative DNA damage have been reported in several diseases [3, 4, 5]. Antioxidants that prevent or neutralize free radicals are key elements to combat fatigue and in the treatment of several diseases [6, 7, 8]. Thus, the evaluation of extracts of spices preparations having antioxidant properties requires understanding of its potential to scavenge reactive oxygen species and enhance antioxidant defense in the body [9, 10, 11]. Spices are the alternate sources of antioxidants, which used in time immemorial [12, 13]. The present study evaluated the antioxidant properties of Star Anise (*Illicium verum*) based on its ability to scavenge free radicals, ability to reduce iron, lipoxigenase activity and hydrogen peroxide-induced lipid peroxidation. In addition to the above, the ability of reducing/preventing cytotoxic and DNA protectant activity were also studied. Star Anise (*Illicium verum*) is a dark brown coloured fruit originally distributed in tropical areas of Asia. It is widely used as herbal medicine, spice, and cosmetics. The fruits are commonly used as a spice and pharmaceutical treatment for flatulence and spasmodic pain. The paste/powder of Star anise is employed topically to treat rheumatism and nostalgia, and is also used as an antiseptic [14]. The aim of the present study was to investigate the antioxidant activity of ambient temperature water extract and boiling water extracts of Star Anise. Earlier studies in our laboratory proved that, antioxidant proteins isolated, purified and characterized from dietary components, such as Turmeric (*Curcuma longa*), Curry leaves (*Murraya Koenigii*) effectively inhibited ROS-induced lipid peroxidation and DNA damage [15, 16, 17, 18, 19, 20].

2. Materials and Methods

Star anise (*Illicium verum*) was obtained from local market, Mysore, Karnataka state, India. BHA (Butylated Hydroxy Anisole), TBA (Thiobarbituric Acid), Curcumin was purchased from Sigma Aldrich chemicals (St Louis, MO, USA). Ferrous sulphate, Ascorbic acid, DPPH (2,2-Diphenyl-2-picrylhydrazyl), NBT (Nitro blue tetrazolium), DMSO (Dimethyl sulphoxide), Bovine serum albumin(BSA), Calf thymus DNA, linolenic acid and Gallic acid from Himedia Private Ltd India. Hydrogen peroxide was purchased from SRL, India. All other chemicals unless otherwise mentioned were of analytic grade and procured from Merck India Pvt. Ltd., of Darmstadt, Germany. Solvents were distilled before use.

3. Preparation of ambient water and boiling water extracts:

One gram of the dried Star Anise (*Illicium verum*) fruit powder was vortexed with 100 ml of ambient temperature double distilled water and boiling double distilled water respectively, vortexed for two hours at room temperature. The solution was homogenized and the resultant suspension was centrifuged at 10,000 rpm in refrigerated centrifuge (Kubota co., Osaka Japan) for 20 mins at 4 °C. The supernatants were filtered using Whatman No. 1 filter paper, followed by 0.22 µm Sartorius microbial filter. Then the extracts were freeze dried and lyophilized and stored at -20 °C for further studies. The efficacies of the extracts reported here were quantified based on the dry weight of the whole extract per volume of assay solution.

4. Proximate analysis

The proximate analysis such as protein, total sugars, polyphenols, flavonoids, ascorbic acid, α -tocopherol were done for both ambient temperature water extract (ATWE) and boiling water extracts (BWE).

4.1 Estimation of Protein content

The protein content of the both extracts was estimated according to Bradford's method [21] using bovine serum albumin as standard. Aliquots of different extracts were made up to 0.1ml with distilled water and 0.9 ml of Bradford's reagent was added. The total protein content was calculated using the standard curve.

4.2 Estimation of total sugar

The total sugar concentration of both extracts of Star anise was estimated by Dubois method [22] and Dextrose used as standard. Different aliquots of extracts were made up to 1ml with distilled water followed by the addition of 1ml of 5% phenol and 5 ml of concentrated sulphuric acid. Orange colour developed was read at 520 nm. The sugar concentration was calculated accordingly.

4.3 Determination of total phenolic compounds

Total content of phenolic was determined by using Gallic acid as standard according to the method of Folin-ciocalteu reaction [23] with minor modification. The star anise extracts and the standard Gallic acid were dissolved in 0.5 ml of water and were mixed with 500 µl of 50% Folin-ciocalteu reagent. The mixture was then allowed to stand for 10min followed by the addition of 1.0 ml of 20% Sodium carbonate. Incubated at 37 °C, 10 min and the absorbance of the supernatant were read at 730 nm. The total phenolics content was expressed as Gallic acid equivalents in milligrams per gram of powder.

4.4 Estimation of flavonoids

Total flavonoid content was determined colorimetrically [24]. Standard calibration curve was prepared using Quercetin. 10mg of Quercetin was dissolved in 80% ethanol and then diluted solutions were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm by using proper controls. The total flavonoid content was calculated accordingly.

4.5 Estimation of Ascorbic acid

Ascorbate was determined according to the method of Sadasivam and Manickam [25]. Ascorbic acid was first dehydrogenated by bromination. The dehydroascorbic acid was then reacted with 2, 4-dinitrophenyl hydrazine to form osazone and dissolved in sulphuric acid to give an orange red colour solution, which was measured photometrically at 540 nm. Dehydroascorbate alone reacts with 2, 4-dinitrophenyl hydrazine quantitatively and not the other reducing substances present in the extract. The total ascorbic acid content in the different extracts of Star Anise was expressed in mg per gram of dry sample.

4.6 Estimation of reducing sugars

The content of reducing sugars in star anise extracts was determined according to the method of Miller [26], using dextrose as the standard. The solution (0-2000 µl) containing various concentration of dextrose was added to series of tubes and the volume made up to 2 ml with distilled water. 2 ml of 2,4-dinitrosalicylic acid reagent was added to each tube. The mixture was then allowed to stand for 15 minutes followed by the addition of 1.0 ml of potassium sodium tartrate. Finally the volume was made up to 10 ml with distilled water. Reducing sugar was measured spectrophotometrically at 540 nm. The total reducing sugars content of Star Anise was expressed as dextrose equivalents in mg per gram of dry sample.

5. Antioxidant activity

Antioxidant activity of aqueous and boiling water extract of star anise was studied in different model system to study their antioxidant activities.

5.1 Lipid peroxidation inhibition activity of extracts of Star Anise

A simple spectrophotometric assay was done for evaluating antioxidant activity was based on the inhibition of peroxidation in Linoleic acid [27]. An assessment of oxidation was achieved by measurement of thiobarbituric acid reactive substances [28]. 100 µl of Linoleic acid was subjected to peroxidation by 10:100 µmol of ferrous sulphate and ascorbic acid [29] in final volume of 1 ml of Tris buffered saline (20 mM, pH 7.4, 150 mM NaCl). The reaction mixture was treated with or without ATWE and BWE of star anise (25 µg), BHA (400 µM), α -tocopherol (400 µM) and Curcumin (400 µM). The contents were incubated for 1 hour at 37 °C. The reaction was terminated by the addition of 10 µl of 5% phenol and 1 ml of 1% trichloroacetic acid (TCA). To each system 1 ml of 1% thiobarbituric acid (TBA) was added, the contents were kept in a boiling water bath for 15 min, cooled and centrifuged at 6,000 rpm for 10 min. The absorbance of supernatants was measured colorimetrically at 535 nm. Appropriate blanks were included for each measurement. The negative control without any test sample was considered as 100%

peroxidation. The control was without any antioxidant or test sample (Figure -1).

5.2 Hydroxyl radical scavenging activity of extracts of Star Anise

The hydroxyl radical scavenging activity of extracts of Star Anise was done [30] with minor modifications. The reaction mixture containing FeCl₃ (100 μM), EDTA (104 μM), H₂O₂ (1 mM) and 2-deoxy- D-ribose (2.8 mM) were mixed with or without ATWE and BWE of star anise (25 μg) in 1 ml final reaction volume made with potassium phosphate buffer (20 mM pH 7.4) and incubated for one hour at 37 °C. BHA and Curcumin (400 μM) were used as positive control. The mixture was heated at 95 °C in water bath for 15 min followed by the addition of 1 ml each of TCA (2.8%) and TBA (0.5% TBA in 0.025 M NaOH containing 0.02% BHA). Finally the reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. Absorbance of supernatant was measured at 532 nm using the negative control without any antioxidant was considered 100% oxidation. The percentage hydroxyl radical scavenging activity of Star Anise was determined.

5.3 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity was assessed according to the method of Shimada *et al.* [31]. The α-tocopherol at various concentrations ranging from 0 to 100 μM was mixed in 1 ml of freshly prepared 0.5 mM DPPH ethanolic solution and 2 ml of 0.1 M acetate buffer pH 5.5. The resulting solutions were then incubated at 37 °C for 30 min and measured calorimetrically at 517 nm. α-tocopherol (400 μM) was used as positive control under the same assay conditions. Negative control was without any inhibitor or ATWE and BWE of Star anise. Lower absorbance at 517 nm represents higher DPPH scavenging activity. The % DPPH radical scavenging activity of star anise extract was calculated (Figure-3).

5.4 Superoxide scavenging activity by alkaline DMSO method

The Superoxide radical (O₂^{•-}) scavenging activity of Star Anise was measured according to the method of Lee *et al.* [32] with minor modifications. The reaction mixture containing 100 μl of 30 mM EDTA (pH 7.4), 10 μl of 30 mM hypoxanthine in 50 mM NaOH, and 200 μl of 1.42 mM nitro blue tetrazolium (NBT) with or without ATWE and BWE of star anise and SOD serving as positive control at various concentrations ranging from 50-300 μg. After the solution was pre-incubated at ambient temperature for 3 min, 100 μl of xanthine oxidase solution (0.5U/ml) was added to the mixture and incubated for one hour at 37 °C, and the volume was made up to 3 ml with 20 mM phosphate buffer (pH 7.4). The solution was incubated at room temperature for 20 minutes, and absorbance was measured at 560 nm. Appropriate controls were included to rule out the artifacts induced reaction. The control was without any inhibitor. Inhibitory effect of star anise on superoxide radicals was calculated as follows.

$$\% \text{ Superoxide radical scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

5.5 Protective effect of extracts of Star Anise on H₂O₂ induced DNA damage

The DNA damage induced by H₂O₂ was analyzed on 0.6% submarine agarose gel according to the method of Sultan *et al.* [33] with minor modifications. Calf thymus DNA (10 mg) was mixed well in one ml of 20 mM Potassium phosphate buffer, at pH 7.4,

150 mM NaCl and store at 4 °C. The sheared 15 μg of calf thymus DNA was treated with 1mM of H₂O₂ with or without ATWE and BWE of Star Anise and Curcumin (400 μM) in 100 μl potassium phosphate buffer (20 mM, pH 7.4). The reaction mixture was mixed with 10 μl of sample loading buffer (0.5% bromophenol blue, 50% glycerol in water) and then the reaction mixture was incubated at 37 °C for 30 min and then placed on ice for 10 min to stop the reaction. 20μl of the reaction mixture (15 μg of DNA) was run on 0.6% agarose with ethidium bromide (1 μg/ml). The electrophoresis was carried out using 20 mM TBE buffer (40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, 18 mM NaCl, pH 8) at 60 V for 3 h and DNA was visualized under a UV transilluminator.

6. Statistical analysis

Statistical analysis was done using student's t-test and a p<0.05 was considered as statistically significant when compared with controls and results refer to mean ± SD.

7. Results and discussion

Table 1: Proximate analysis of extracts of ambient temperature water extract and boiling water extract of Star Anise.

Contents	Ambient temperature water extract of Star Anise	Boiling water extract of Star Anise
Total protein (mg/g)	1.23±0.07	1.34±0.09
Total sugars (mg/g)	54.2±4.92	50.2±3.21
Total polyphenols (mg/g)	7.31±0.61	9.28±0.52
Flavonoids (mg/g)	0.65±0.02	0.57±0.04
Ascorbic acid (mg/g)	5.16±0.41	Nil
α-tocopherol (mg/g)	0.51±0.12	0.84±0.53
Reducing sugars (mg/g)	1.63±0.09	1.14±0.08

(Values are represented in mg/g dry weight of extracts)

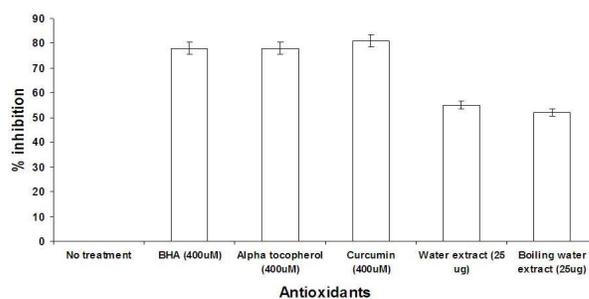


Fig 1: Lipid peroxidation inhibition activity of Star Anise extracts

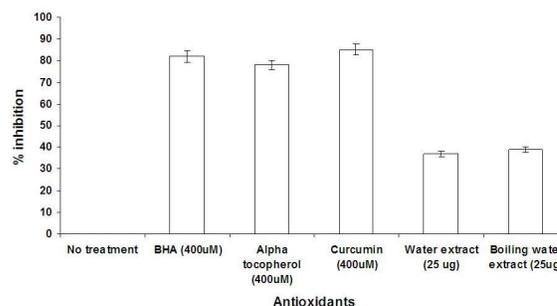


Fig 2: Hydroxyl radical scavenging activity of Star Anise

Linolenic acid (300 μg) + ferrous sulphate + ascorbic acid (10:100 μmol) ± ATWE and BWE of Star Anise (25μg) in 500 μl of TBS (10 mM Tris, pH 7.4, 0.15 M saline), incubating at 37 °C for 60 min. Lipid peroxidation was measured by TBARS.

Values are means \pm SD of triplicates.

The Hydroxyl radical scavenging activity of ATWE and BWE of Star Anise and standard antioxidants. Deoxy-D-ribose (2.8 mM) + ferric chloride + ascorbic acid (100:100 μ M) + H₂O₂ (1 mM) + EDTA \pm ATWE/BWE of Star Anise (25 μ g) in 1.0 ml Phosphate buffer (0.02 M, pH 7.4), incubating at 37 °C for 60 min. Values are means \pm SD of triplicates.

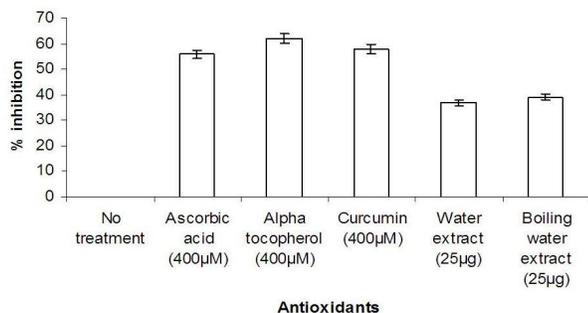


Fig 3: DPPH radical scavenging activity of Star Anise extracts

DPPH (0.5mM) + with or without ATWE and BWE of Star Anise (25 μ g)/ α -tocopherol/Ascorbic acid/Curcumin (400 μ M). Mixture incubated at 37 °C for 30 min and the absorbance read at 517 nm using spectrophotometer. Values are means \pm SD of triplicates.

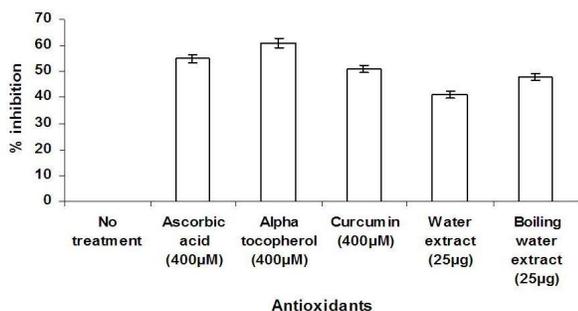


Fig 4: Superoxide scavenging activity of Star Anise extracts:

Hypoxanthine (30 mM) + Nitro blue tetrazolium (NBT) (1.42 mM) + with or without ATWE and BWE of Star Anise (25 μ g)/ α -tocopherol/Ascorbic acid/Curcumin (400 μ M). Mixture incubated at 37°C for 20 min and the absorbance read at 560 nm. Values are means \pm SD of triplicates.

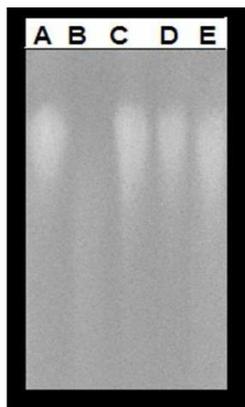


Fig 5: Inhibition of Hydrogen peroxide induced DNA degradation by extracts of

Star Anise.

Lane A: Calf thymus DNA untreated;
 Lane B: Calf thymus DNA As lane (A) + H₂O₂
 Lane C: Calf thymus DNA + H₂O₂ As lane (B) + ATWE of Star Anise (15 μ g)
 Lane D: Calf thymus DNA + H₂O₂ As lane (B) + BWE of Star Anise (15 μ g)
 Lane E: Calf thymus DNA + H₂O₂ As lane (B) + Curcumin (100 μ g)

7.1 Proximate Analysis

As shown in Table-1, the ambient temperature water extract of Star Anise contains sugar, traces of ascorbic acid and contains low amount of protein, whereas the boiling water extract contains more protein and polyphenols when compared to ambient temperature water extract of Star Anise. It was noticed that, there was no traces of ascorbic acid in boiling water extract.

7.2 Lipid peroxide inhibition assay

The lipid peroxidation assay that determines the production of MDA and related lipid peroxides in Linolenic acid was carried out. In the assay, ferrous sulfate: ascorbate system induced TBA reactive substances are produced. The inhibitory effect of ATWE and BWE of Star Anise against ferrous sulfate and ascorbic acid-induced lipid peroxidation was estimated. The ATWE and BWE of Star Anise showed the highest inhibition of lipid peroxidation, of about 55% and 52% at 25 μ g concentration respectively. It was compared with standard antioxidants, such as BHA, α -tocopherol and Curcumin which inhibited lipid peroxidation by 78% and 81% at 400 μ M concentration, respectively (Figure -1).

7.3 Hydroxyl radical-scavenging assay:

Hydroxyl radicals are most reactive and initiate to cell damage. Herein, we studied the effect of the extracts of Star Anise on hydroxyl radicals generated by Fe³⁺ ions by finding the degree of deoxyribose degradation, an indicator of TBA and Malondialdehyde (MDA) adduct formation. Ambient water extract and the boiling water extract of Star anise (25 μ g) exhibited 37% and 39% inhibition against MDA formation by scavenging the OH \cdot radicals. The scavenging potential of Star Anise extracts were compared with known antioxidants, such as BHA, α -tocopherol and Curcumin. Each of these antioxidants inhibited hydroxyl radicals by 82%, 78% and 85% at 400 μ M/80 μ g and concentration (Figure -2).

7.4 DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay

As shown in Figure-3, a direct approach with DPPH radicals - a stable radical used to evaluate the antioxidant activity of extracts of Star Anise. The ATWE & BWE of Star anise evidenced 37% & 39% scavenging activity at 25 μ g concentration of protein. The protein of Star anise exhibited a statistically significant DPPH radical-scavenging activity when compared to the group without any inhibitor. At the concentration of 400 μ M, standard antioxidants like Ascorbic acid, α -tocopherol and Curcumin showed 56%, 62% and 58% DPPH radicals respectively.

7.5 Superoxide scavenging activity by the extracts of Star Anise:

Superoxide anions are the most common free radicals in vivo and are generated in a variety of biological systems and the concentration of superoxide anions increases under conditions of

oxidative stress (Lee *et al.*, 2002)^[32]. The NBT assay was carried out to investigate the superoxide scavenging activity of extracts of Star anise. As shown in Figure-4, the superoxide scavenging activity of ATWE, BWE of Star Anise (25 µg) was found to be 41% and 48% respectively. Ascorbic acid, α-tocopherol and Curcumin were used as the positive control which offered 55%, 61% and 51% scavenging activity at the concentration of 400 µM.

7.6 Protective effect of extracts of Star anise on H₂O₂ induced DNA damage

As shown in Figure-5, the calf thymus DNA was treated with H₂O₂ for 30 min, extensive DNA fragmentation due to oxidation by hydroxyl radicals was seen on agarose gel as shown in the figure-5, by the enhanced mobility lane B as compared to the untreated DNA (lane A). The lane C and lane D shows that, DNA treated with H₂O₂ along with Aqueous extract of Star Anise and boiling water extract of Star Anise (each 15 µg) protected DNA damage as assessed by the similar mobility of the DNA (lane E) which is treated with Curcumin (100 µg) in comparison to the untreated DNA (lane A). The effectiveness of both the extracts of Star Anise to prevent H₂O₂ induced DNA damage was associated to its hydroxyl radical scavenging activity and also lipid peroxidation inhibition activity as observed by Deoxyribose assay and TBARS assay. This suggests that the ATWE & BWE of Star anise could prevent H₂O₂ mediated oxidative DNA damage.

8. Conclusion

The above results showed that, the both ATWE & BWE of Star anise extracts are rich with Carbohydrates and polyphenols. It was also proved that the aqueous extracts of Star Anise acts as an antioxidant at a dosage of 25 µg and it provide protection to DNA against peroxides. Its antioxidants activities could be mainly due to the high content of polyphenols and carbohydrates along with combined effect of all phytochemicals.

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