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ANTIOXIDANT ACTIVITIES AND GC-MS ANALYSIS OF DRIED FRUITS OF *ILLICIUM VERUM* HOOK.F.

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ABSTRACT

Spices are flavoured, aromatic substances used commonly as condiments and preservatives. In recent years, antioxidant properties of spices are well explored by scientific community due to its natural origin. Antioxidant properties are related to the efficiency of a substance to protect the food substances containing lipids and oils from oxidative deterioration. They control the rancidity process and retard the formation of toxic oxidation products. Due to natural origin of spices and increasing attention towards green consumerism, use of spices as an antioxidant alternative is an emerging research area. The antioxidant compounds such as flavonoids, polyphenolics, terpenoids, lignans, sulfides, carotenoids, saponins, coumarins, plant sterols, curcumins, and phthalides present in spices are mainly responsible for their antioxidant properties. Spices come from different parts of a plant other than the leaves while herbs come from leaves of a plant. Spices and herbs can be classified into various groups based on flavour/taste, taxonomy or part of the plant where they came from. Research studies were carried out for evaluating the antioxidant and anti-inflammatory activities of ethanol extract of dried fruits of *Illicium verum.* Antioxidant activities such as DPPH$^{\cdot}$ radical, ABTS$^{\bullet+}$ radical cation, phosphomolybdenum reduction and Fe^{3+} reduction, also haemolytic inhibition method were carried out for ethanol extract of dried fruits of *Illicium verum*. 2,2'-Bipyridine,6,6'-dimethyl-, Flavone, Phenol,2,6-bis(1,1-dimethylethyl)-4-ethyl-, 1-Hexacosene, Octadecanoic acid,3-oxo,methyl ester were the active compounds that were detected by GC-MS analysis.

KEYWORDS: DPPH$^{\cdot}$ radical, ABTS$^{\bullet+}$ radical cation, anti-inflammatory, GC-MS, 2,2'-Bipyridine,6,6'-dimethyl-.

INTRODUCTION

Illicium verum belonging to the Magnoliaceae family, commonly known as Chinese star anise is one of the flavors used in China 5 spices, cultivated in mountainous region especially in Lanson province, Cochin, China (Southern China), and Vietnam.[1,2] The *Illicium verum* fruits are capsule like aggregate with star-shaped five to 10 pointed boat shaped section about on eight averages (Figure 1). Each arm is a seed pod. The fruits have tough skin and rust colored outer portion and seeds with high oil content.[3] Star anise is one of many spices that contain bioactive compounds as well as a number of phenolic and flavonoid compounds, having antioxidant, preservative, and antimicrobial properties.[4] Star anise has carminative, antispasmodic, antiseptic, antimicrobial, antidiarrheal activities and is used to treat colics and as a tranquilizer. Researchers attribute these effects to the presence of two coumarin derivatives: 7-hydroxycoumarin and 7-methoxycoumarin.[5] In addition, it is the major source of shikimic acid, a primary ingredient in the anti-flu drug (Tamiflu).[6]

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism.[7,8] In general, free radicals are very short lived, with half lives in milli, micro or nanoseconds. The most common reactive oxygen species (ROS) include superoxide (O_2-) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO-) radicals, and reactive hydroxyl (OH$^{\cdot}$) radicals. The nitrogen derived free radicals are nitric oxide (NO$^{\cdot}$) and peroxynitrite anion (ONOO-). Under physiological conditions, ROS formation and elimination are delicately balanced. However, enhanced activity of oxidant enzymes and/or reduced activity of antioxidant enzymes lead to oxidative stress. Majority of the diseases/disorders are mainly linked to oxidative stress produced due to free radicals.[9,10] Free radicals are atoms, molecules or ions with unpaired electrons that are highly unstable, short lived and active towards chemical reactions with other molecules. They may be derived from oxygen, nitrogen and sulphur.[11,12] Internally, free radicals are produced as a normal part of metabolism within the mitochondria, through xanthine oxidase, peroxisomes, inflammation processes, phagocytosis, arachidonate pathways, ischemia and physical exercise.

Free radicals derived from oxygen and nitrogen are known as reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively. Formation of ROS and RNS in the cells can occur by enzymatic and/or non-enzymatic reactions. ROS and RNS are involved in many physiological activities and function as cellular signaling agents. Activation of phagocytes produces ROS in amounts enough to kill intruding bacteria.[13] Antioxidants are substances that neutralize free radicals or their actions.[14] The antioxidants acting in the defense systems act at different levels such as preventive, radical scavenging, repair and de novo, and the fourth line of defense, i.e., the adaptation. Free radicals have different types of reaction mechanisms. They can react with surrounding molecules by (a) Electron donation, reducing radicals, and electron acceptance, oxidizing radicals, (b) Hydrogen abstraction, (c) Addition reactions, (d) Self-annihilation reactions, and (e) by disproportionation.[15]

Dried fruits of *Illicium verum*

Fig.1: Habitat of dried fruits of *Illicium verum.*

MATERIALS AND METHODS

Extraction process

The dried fruits of *Illicium verum* were collected from Indian herbal market, Chennai, Tamil Nadu, India.

The dried fruits of *Illicium verum* were cleaned, macerated and soaked in ethanol for 72 hours. The brown coloured supernatant (extract) was filtered and condensed in room temperature devoid of heat supply, which yields glassy brown extract.[16,17]

Determination of total phenols and flavonoids

Qualitative analysis for the ethanol extract of dried fruits of *Illicium verum* was performed in order to find the derivatives of various phytochemicals.[16] Total phenolic content of the ethanol extract of dried fruits of *Illicium verum* was determined following the methodology[18] and was estimated as gallic acid equivalent (GAE/mg of extract). Total flavonoid content of the ethanol extract of dried fruits of *Illicium verum* was assessed by aluminium chloride reagent method[19] and was estimated as quercetin equivalent (QE/mg of extract).

In vitro antioxidant activities

DPPH˙ radical scavenging activity

The radical scavenging activity of ethanol extract of dried fruits of *Illicium verum* (20-120 µg/mL) was carried out by the reduction DPPH˙ free radical method.[20] The decrease in absorbance was measured at 517 nm using UV-Vis spectrophotometer. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated as:

$$\% \text{ of DPPH}^{\cdot} \text{ radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

ABTS$^{\bullet+}$ (2,2-azinobis (3-ethylbenzo thiazoline-6-sulfonic acid) radical cation scavenging activity

The ethanol extract of dried fruits of *Illicium verum* (2-12 µg/mL) from the stock solution was pipetted and the assay was performed according to the method.[21] The absorbance was measured at 734 nm using UV-Vis spectrophotometer. Ascorbic acid was used as the standard reference. The percentage of inhibition was calculated as:

$$\% \text{ of } ABTS^{\bullet+} \text{ radical cation inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

Phosphomolybdenum reduction activity

The antioxidant capacity of ethanol extract of dried fruits of *Illicium verum* (20-120 µg/mL) was assessed as described.[22] The absorbance of the coloured complex was measured at 695 nm using UV-Vis spectrophotometer. Ascorbic acid was used as the standard reference. The percentage of reduction was calculated as:

$$\% \text{ of Phosphomolybdenum reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] \times 100$$

Ferric (Fe^{3+}) reducing power activity

The reducing power of ethanol extract of dried fruits of *Illicium verum* (20-120 µg/mL) was determined by slightly modified method.[23] The absorbance was measured at 700 nm using UV-Vis spectrophotometer. Ascorbic acid was used as the standard reference. The percentage of reduction was calculated as:

$$\% \text{ of } Fe^{3+} \text{ reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] \times 100$$

Anti-inflammatory activity by Heat induced haemolysis

Varying concentrations (20-120 µg/mL) of ethanol extract of dried fruits of *Illicium verum* was carried out by the haemolytic inhibition method.[24] The absorbance was taken at 560 nm. Diclofenac was used as a standard reference. The percentage of inhibition was calculated as:

$$\% \text{ of haemolytic inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] \times 100$$

Gas Chromatography–Mass Spectrometry (GC–MS) analysis

For GC-MS analysis, the ethanol extract of dried fruits of *Illicium verum* were injected into a HP-5 column (30 m X 0.25 mm i.d with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. Following chromatographic conditions were used: Helium as carrier gas, flow rate of 1 mL/min; and the injector was operated at 200°C and column oven temperature was programmed as 50-250°C at a rate of 10°C/min injection mode.[25] Following MS conditions were used: ionization voltage of 70 eV; ion source temperature of 250°C; interface temperature of 250°C; mass range of 50-600 mass units

RESULTS AND DISCUSSION

Phytochemical analysis provides a definitive profile of the various bioactive compounds such as alkaloids, glycosides, steroids, etc. (Table 1) which may be responsible for the antioxidant and anti-inflammatory activities for the ethanol extract of dried fruits of *Illicium verum* with respect to their phenols and flavonoids content.

Table 1: Phytochemical analysis of ethanol extract of dried fruits of *Illicium verum*.

S. No	Phytochemicals	Tests	Results
1	Alkaloids	Mayer's test	+
		(b) Hager's test	+
2	Phenols	Ferric chloride test	+
3	Tannins	Lead acetate test	-
4	Flavonoids	Sodium hydroxide test	+
5	Glycosides	Legal's test	+
6	Steroids	Libermann-Burchard test	+
7	Terpenoids	Salkowski test	+
8	Saponins	Foam test	-
Quantitative Estimations			
1	Phenols	104.13±0.28 GAE/mg	
2	Flavonoids	43.58±0.16 QE/mg	

Phenolic compounds are ubiquitously distributed throughout the plant kingdom.[26,27] Phenolic phytochemicals are known to exhibit several health beneficial activities such as antioxidant, anti-inflammatory, antihepatotoxic, antitumor, and antimicrobial.[28] The wide spectrum of bioactivities displayed by phenolic compounds isolated from different foods or food products has dictated a demand for accurate determination of phenolic compounds in different food matrices.[29]

DPPH˙ radical and ABTS$^{\bullet+}$ radical cation scavenging activities of ethanol extract of dried fruits of *Illicium verum*

Evaluation of antioxidant activity by DPPH method is the best screening option for herbal based drugs. DPPH˙ (1,1-Diphenyl-2-picrylhydrazyl) is a stable nitrogen centered free radical which has an unpaired valence electron at one atom of nitrogen bridge.[30] The ability of ethanol extract of dried fruits of *Illicium verum* to scavenge free radicals formed was assessed using 1,1-diphenyl-2-picryl hydrazyl radical (DPPH). The ethanol extract of dried fruits of *Illicium verum* demonstrated high capacity for scavenging free radicals by reducing the stable DPPH (1,1-diphenyl-2- picryl hydrazyl) radical to the yellow coloured 1,1-diphenyl-2-picryl hydrazine and the reducing capacity increased with increasing concentration of the extract. The maximum DPPH˙ radical scavenging activity of ethanol extract of dried fruits of *Illicium verum* was 69.42±0.17% at 120 µg/mL concentration. The IC_{50} value for the ethanol extract of dried fruits of *Illicium verum* was found to be 52.97 µg/mL concentration respectively (Figure 2) and was compared with standard (Ascorbic acid, IC_{50} = 40.79 µg/mL concentration).

ABTS$^{\bullet+}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate and ABTS$^{\bullet+}$ radical cation gets reduced in the presence of ethanol extract of dried fruits of *Illicium verum* and the remaining radical cation concentration was then quantified at 734 nm. It can be prepared using $K_2S_2O_8$ as an oxidant. The blue-green colour of ABTS solution is formed by the loss of an electron by the nitrogen atom of ABTS (2, 2-azinobis (3ethylbenzothiazolin-6-sulfonic acid)). The decolourization of the solution takes place in the presence of hydrogen donating antioxidant (nitrogen atom quenches the hydrogen atom.[31] The maximum ABTS$^{\bullet+}$ radical cation scavenging activity of ethanol extract of dried fruits of *Illicium verum* was 87.35±0.36% at 12 µg/mL concentration (Figure 2) and the IC_{50} value for the ethanol extract of dried fruits of *Illicium verum* was found to be as 3.72 µg/mL concentration respectively, which was compared with standard ascorbic acid (IC_{50} = 2.37 µg/mL concentration).

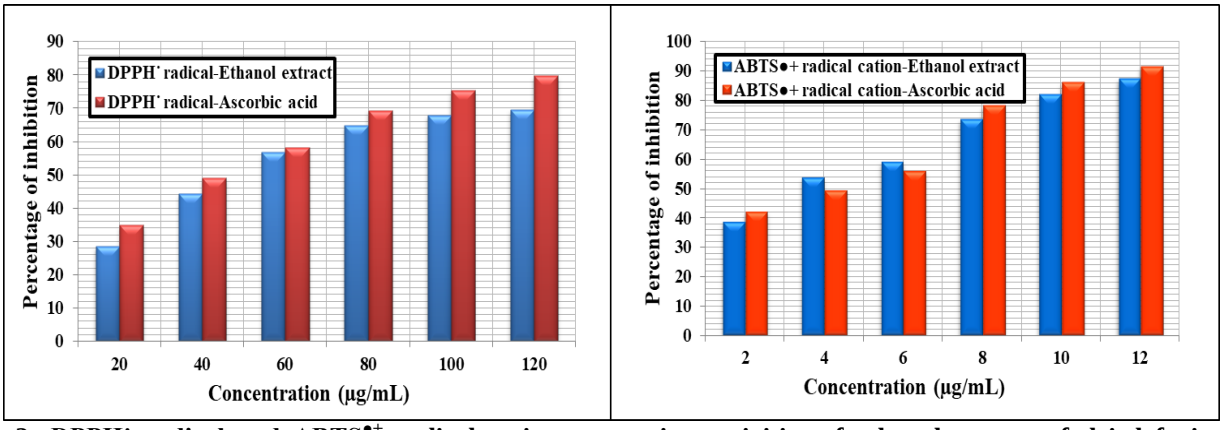


Fig.2: DPPH˙ radical and ABTS$^{\bullet+}$ radical cation scavenging activities of ethanol extract of dried fruits of *Illicium verum*.

Phosphomolybdenum reduction and Ferric (Fe^{3+}) reducing power activities of ethanol extract of dried fruits of *Illicium verum*

The total antioxidant activity of ethanol extract of dried fruits of *Illicium verum* was measured spectrophotometrically by phosphomolybdenum reduction method, which is based on the reduction of Mo (VI) to Mo (V) by the formation of green phosphate/Mo (V) complex at acidic pH, with a maximum absorption at 695 nm.[32] The maximum phosphomolybdenum reduction of ethanol extract of dried fruits of *Illicium verum* was 84.19±0.28% at 120 µg/mL concentration with the RC_{50} value of 62.27 µg/mL concentration respectively (Figure 3). It was compared with the standard ascorbic acid (RC_{50} = 57.61 µg/mL).

The reducing power of Fe^{3+} to Fe^{2+} by ethanol extract of dried fruits of *Illicium verum* was studied and showed reduction ability in a dose dependent manner. The maximum reduction of ethanol extract of dried fruits of *Illicium verum* was 53.14±0.12% at 120 µg/mL concentration (Figure 3). Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action.[33] The RC_{50} value for the ethanol extract of dried fruits of *Illicium verum* was found to be 69.26 µg/mL concentration respectively and was compared with the standard (42.58 µg/mL concentration) Ascorbic acid.

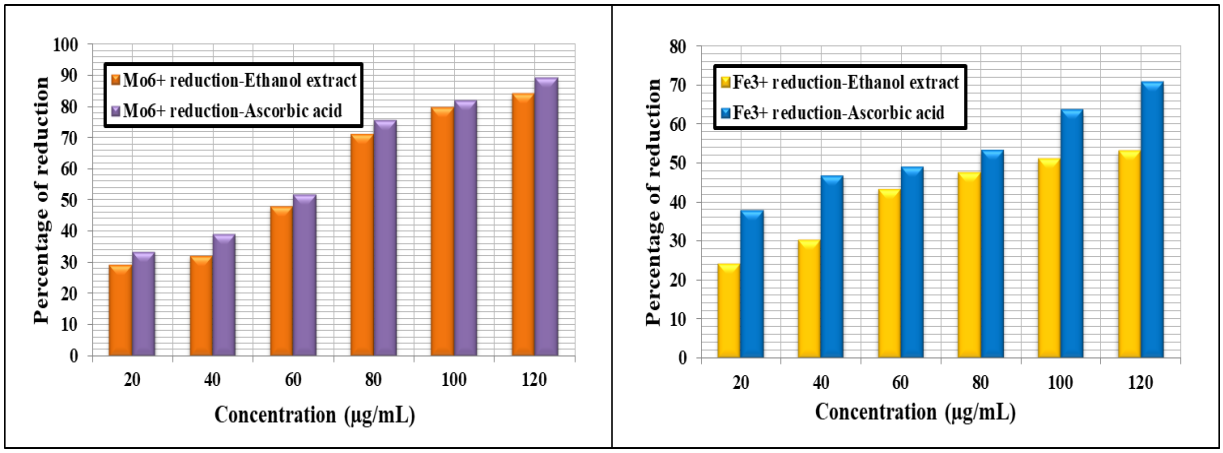


Fig.3: Phosphomolybdenum and Ferric reducing power activities of ethanol extract of dried fruits of *Illicium verum*.

Anti-inflammatory activity by Heat induced haemolysis

Haemolysis is due to red blood cells destruction which resulted from lysis of membrane lipid bilayer. Erythrocytes are considered as major target for the free radicals owing to the presence of both high membrane concentration of poly unsaturated fatty acids (PUFA) and the oxygen transport associated with redox active haemoglobin molecules, which potent promoters of activated oxygen species. The erythrocyte model has been widely used as the direct indication of toxicity of injectable formulations as well as general indication of membrane toxicity.[34] The maximum haemolytic inhibition for the ethanol extract of dried fruits of *Illicium verum* was 58.37±0.43% at 120 µg/mL concentration (Figure 4) with the IC_{50} value of 69.70 µg/mL and was compared with the standard (55.95 µg/mL concentration) Diclofenac.

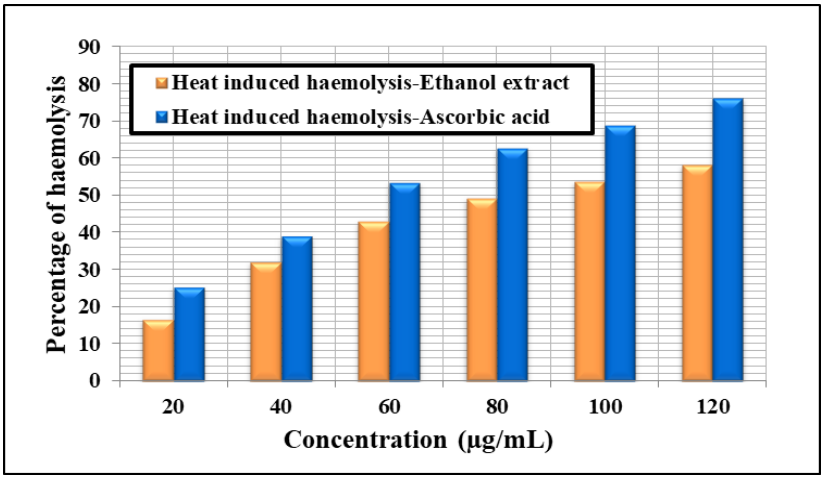


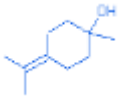
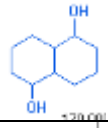
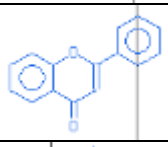
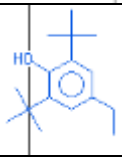
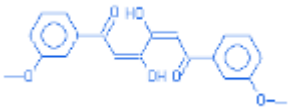
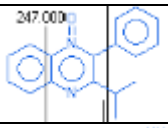
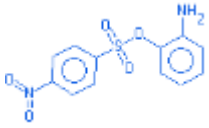
Fig.4: Heat induced haemolysis of ethanol extract of dried fruits of *Illicium verum*.

Gas Chromatography–Mass Spectrometry (GC–MS) analysis

In the present study chemical constituents have been identified from ethanol extract of dried fruits of *Illicium verum* by GC-MS analysis. GC-MS chromatogram of ethanol extract of dried fruits of *Illicium verum* showed 12 peaks indicating the presence of phytochemical constituents. On comparison of the mass spectra of the constituents with the NIST library the phytoconstituents were characterized and identified (Table 2 and Figure 5). The compound Quinoxaline,2-isopropyl-3-phenyl-,4-oxide are used as antimicrobial, antitubercular, antiviral

antiprotozoan, chronic and metabolic diseases, chronic inflammation and anti-glutameric activities (Table 3). Elaidic acid, isopropyl ester were used as an anti-inflammatory, hypocholesterolemic, cancer preventive and hepatoprotective, anticoronary, antiacne, insectifuge, antieczemic activities. These bioactive compounds justify the use of dried fruits of *Illicium verum* for the isolation of individual phytoconstituents by chromatographical methods, subjecting to pharmacological activity evaluation.

Table 2: GC-MS analysis of ethanol extract of dried fruits of *Illicium verum*.

S. No	Retention Time	Compound Name	Compound Structure	Molecular weight (g/moL)	Molecular Formula
1	13.55	Cyclohexanol, 1-methyl-4-(1-methylethylidene)-		154	$C_{10}H_{18}O$
2	15.85	1,5-Naphthalenediol, decahydro-		170.56	$C_{10}H_{18}O_2$
3	17.22	2,2'-Bipyridine,6,6'-dimethyl-		184.53	$C_{12}H_{12}N_2$
4	18.08	Flavone		222	$C_{15}H_{10}O_2$
5	19.05	Phenol,2,6-bis(1,1-dimethylethyl)-4-ethyl-		234.44	$C_{16}H_{26}O$
6	20.65	Z,Z-8,10-Hexadecadien-1-ol acetate		280	$C_{18}H_{32}O_2$
7	28.38	3,4-Dihydroxy-1,6-bis-(3-methoxy-phenyl)-hexa-2,4-diene-1,6-dione		353.92	$C_{20}H_{18}O_6$
8	31.37	1-Hexacosene		364	$C_{26}H_{52}$
9	23.48	Octadecanoic acid,3-oxo,methyl ester		312.18	$C_{19}H_{36}O_3$
10	19.97	Quinoxaline,2-isopropyl-3-phenyl-,4-oxide		263.23	$C_{17}H_{16}N_2O$
11	22.05	Benzenesulfonic acid,4-nitro-,2-aminophenyl ester		294.14	$C_{12}H_{11}N_3O_5S$
12	25.87	Elaidic acid, isopropyl ester		324.10	$C_{21}H_{40}O_2$

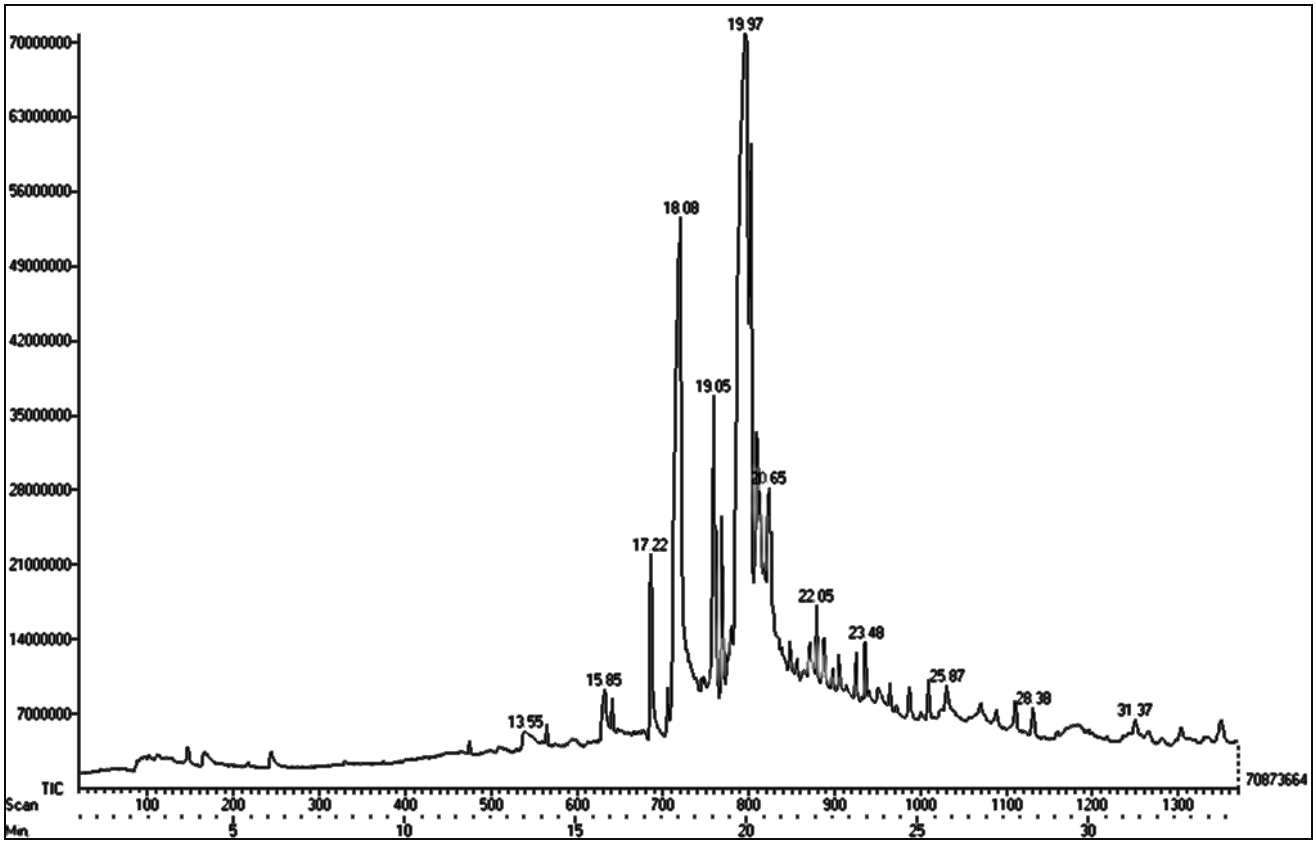


Fig.5: GC-MS Chromatogram of ethanol extract of dried fruits of *Illicium verum.*

Table 3: Pharmacological activities of ethanol extract of dried fruits of *Illicium verum.*

S. No	Compound Name	RT	Molecular weight	Molecular formula	Pharmacological activity[35,36,37]
1	Flavone	18.08	222	$C_{15}H_{10}O_2$	Production of Reactive Oxygen Species (ROS) can be reduced by flavonoids Relevance of plant defense mode of action is highly possible by flavonoids Formation of oxygen radicals can be prevented by flavonoids thereby inhibiting the enzyme activity
2	Phenol,2,6-bis(1,1-dimethylethyl)-4-ethyl-	19.05	234.44	$C_{16}H_{26}O$	Antimicrobial activity Antioxidant activity Antimalarial activity Immuno-modulatory effect
3	Quinoxaline,2-isopropyl-3-phenyl-, 4-oxide	19.97	263.23	$C_{17}H_{16}N_2O$	Antimicrobial activity Antitubercular activity Antiviral activity Antiprotozoan activity Chronic and metabolic disease bioactivity Chronic inflammation Anti glutameric activity
4	Elaidic acid, isopropyl ester	25.87	324.10	$C_{21}H_{40}O_2$	Anti-inflammatory activity Hypocholesterolemic activity Cancer preventive and Hepato-protective activity Anti-coronary activity Anti-acne activity Anti-eczemic activity Insectifuge

CONCLUSION

Star anise fruits could be considered as good source of natural compounds with potent antioxidant and anti-inflammatory activities. The results underline that the ethanol extract of dried fruits of *Illicium verum* possess free radical scavenging ability and definitive reducing power effect. Significant relationship between the antioxidant capability and the total phenol and flavonoid content was found, indicating as an effective source of natural antioxidants. In this connection, spices and herbs have been used for thousands of years for flavour, aroma, as colouring in foods and as preservatives. They contain powerful antioxidants that have been proven to be effective in inhibiting lipid oxidation or slowing down the onset of rancidity in foods. Antioxidants from spices and herbs possess desirable properties such as being natural, non-GMO and having clean label ingredients (i.e., can be listed as spice or herb or flavouring).

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