In vitro antioxidant, hepatoprotective potential and chemical profiling of *Syzygium aromaticum* using HPLC and GC-MS

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Abstract: The objective of the present study was to evaluate the *in vitro* antioxidant and hepatoprotective potential of *Syzygium aromaticum* (clove) against CCl_4-induced hepatotoxicity using rat liver slice culture (LSC) model. Antioxidant activity in terms of DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) of different concentrations of *S. aromaticum* was in the range of 41.01-90.33% and 138.15-595.63 Fe (II) mg/mL, respectively. Plasmid pBR322 DNA protection activity was observed with all three concentrations of *S. aromaticum* against H_2O_2 induced oxidative damage, as no strand breaks were observed. Chemical profiling through HPLC confirmed the presence of six major phenolic acids and 13 volatile bioactive compounds were identified though GC-MS. Significant hepatoprotection ($p<0.05$) was observed in liver slice culture (LSC) as liver slices treated with various concentrations of *S. aromaticum* extract presented very low percentage cytotoxicity (7.35-16.16%) as compared to the CCl_4 treated liver slices (75.58 %). The hepatoprotective potential of *S. aromaticum* may be due to the presence of bioactive components as confirmed by HPLC and GC-MS. The results of present study support the use of *S. aromaticum* in the formation of potential hepatoprotective drugs against various liver diseases.

Keywords: *S. aromaticum*, antioxidant, hepatoprotective, liver slice culture, GC-MS.

INTRODUCTION

The liver is a chief organ for the metabolism, regulating the synthesis, secretion, excretion and detoxification of numerous endogenous and exogenous substances such as xenobiotics (Juza and Pauli, 2014; Tajiri and Shimizu, 2013). Hepatic drug biotransformation results in the production of free radicals which reacts promptly through several reaction mechanism to the nearby molecules. Electron sharing and capturing, hydrogen abstraction which ultimately leads to lipid per oxidation, DNA strand breaks, mutation of gene expression and oxidation of proteins are among various reactions of free radicals (Uccello *et al*., 2012). Since the liver is involved in various biochemical reactions, the liver cells are prone to attack and necrosis by the free radicals (Cichoż-Lach and Michalak, 2014). Experimental evidence shows the association of free radicals with the onset of liver diseases and several other ailments including cancer, stroke, atherosclerosis, arthritis, asthma and other age related diseases (Jaeschke, 2011).

The management of liver diseases is still a challenge to modern medicine because the traditional or synthetic treatments choices available for liver diseases are controversial due to their high prices and serious side effects (Kumar *et al*., 2011). Therefore, use of herbal medicines is progressively rising among the general public. Despite the fact that bioactive constituents of most herbal medicines are not identified, they are appreciably effective, inexpensive and have minor side effects (Asadi-Samani *et al*., 2015). The pathological and detrimental action of free radicals can be reversed by the use of antioxidants from herbal drugs. These antioxidants scavenge the free radicals and restore the normal physiological system. The antioxidants are usually derived from natural sources like medicinal plants or they are synthetically prepared. Owing to potential carcinogenicity, synthetically prepared antioxidants are not preferred as compared to natural ones (Carocho and Ferreira, 2013). Therefore, natural antioxidants from plant origin are being extensively investigated for their protective effects against free radicals induced diseases and current research emphasize on the use of natural antioxidants. Natural antioxidants and bioactive compounds from herbal extracts can be used to treat the liver diseases and significantly accelerate recovery process (Yuan *et al*., 2016; Moradi *et al*., 2016). In search of an effective and reliable hepatoprotective drug, a number of medicinal plants have been explored until now using *in vitro* and *in vivo* experimental models (Asadi-Samani *et al*., 2015). On the other hand, chemical toxins (paracetamol, carbon tetrachloride, thioacetamide and galactosamine) are frequently used to induce hepatic injury in experimental models including both *in vitro* and *in vivo* conditions. Among these, the most frequently used hepatotoxin is carbon tetrachloride (CCl_4) to induce hepatic injury in animal models (Bhoumik *et al*., 2016).

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Chemical profiling is the initial step to standardize the traditional medicine and natural products (Feng *et al*., 2016). The most useful chromatographic approaches for chemical profiling of bioactive compounds include thin layer chromatography, HPLC, LC-MS and GC-MS (Liang *et al*., 2014). From plant sources, phenolic compounds can be identified and quantified through HPLC which is the most preferred and consistent technique (Khoddami *et al*., 2013). Chemical profiling in terms of separation and identification of bioactive constituents from both plants and non-plant samples can be achieved effectively through gas chromatography-mass spectrometry (GC-MS) (Feng *et al*., 2016). Moreover, the isolation and identification of individual bioactive constituents from medicinal plants can be carried out though GC-MS (Strehmel *et al*., 2014).

Syzygium aromaticum (Clove) is among the most valuable spices which are being used for centuries to preserve the food and enhance its flavor. Moreover, numerous medicinal uses of *S. aromaticum* has been investigated and identified till date (Cortés-Rojas *et al*., 2014). *S. aromaticum* contains many bioactive compounds including gallic acid, eugenol and eugenol acetate which make it one of the richest source of phenolic compounds (El-Hadary and Hassanien, 2016). Various biologically active constituents are present in *S. aromaticum* and the most frequently reported biological activities of *S. aromaticum* are antimicrobial (El-Maati *et al*., 2016), anti-inflammatory, antioxidant (Sultana *et al*., 2014), antiulcerogenic (Issac *et al*., 2015), hepatoprotective (El-Hadary and Hassanien, 2015), Antihyperglycemic, hypolipidemic, neuroprotective, and platelet aggregation inhibition effects (Milind and Deepa, 2011; Cortés-Rojas *et al*., 2014).

The objective of present study was to evaluate the *in vitro* antioxidant and hepatoprotective activity of *Syzygium aromaticum* using liver slice culture (LSC) model against CCl_4 induced hepatotoxicity. This is the first paper reporting *in vitro* hepatoprotective activity of *S. aromaticum*. Hepatoprotective activity of *S. aromaticum* have been evaluated in experimental animals using CCl_4-induced hepatotoxicity models in most of the previous reports. Liver slice culture (LSC) system has been used in the present study for the assessment of hepatoprotective activity of *S. aromaticum* against the cytotoxic effects of CCl_4 in the liver slices. Moreover, chemical profiling of *S. aromaticum* was performed through HPLC and GC-MS to confirm the presence of bioactive components responsible for its hepatoprotective activity.

MATERIALS AND METHODS

Collection of sample

The fresh, healthy and mature buds of *S. aromaticum* were washed, dried, and finely ground into powder form for extraction. Voucher specimen of *S. aromaticum* was prepared and deposited in the Herbarium, Department of Botany, University of Agriculture, Faisalabad. The voucher number was 1512-1-16, given from Herbarium, Department of Botany, University of Agriculture, Faisalabad.

Reagents and standards

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical, standard silymarin, 2, 4, 6-tripyridyl-S-triazine (TPTZ), and reference chemicals were purchased from Sigma Aldrich Co. (Fluka, USA). Plasmid DNA (pBR322) was obtained from Oxoid Ltd. (Hampshire, UK). Glacial acetic acid, sodium acetate (CH_3COONa), Ferric Chloride ($FeCl_3$), Ferrous sulphate ($FeSO_4.\ 7H_2O$), Sodium phosphate ($NaHPO_4$), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2), hydrochloric acid (HCl), agarose, glucose, potassium chloride (KCl), magnesium sulphate ($MgSO_4$), sodium chloride (NaCl), mono potassium phosphate (KH_2PO_4), calcium chloride ($CaCl_2$), ascorbic acid, carbon tetrachloride (CCl_4), HPLC grade methanol and absolute ethanol were purchased from Merck (Germany).

Extraction of bioactive compounds

Finely ground buds of *S. aromaticum* (30g) were macerated in absolute ethanol (150mL) and extracted by shaking in an orbital shaker for 72 hours at room temperature. The extract was vacuum dried at 45°C in a vacuum rotary evaporator (Rotavapor, Buchi R-215, Switzerland). The solvent-free, crude concentrated extract was stored at -4ºC for further analyses (Sultana *et al*., 2014).

DPPH free radical scavenging assay

Different concentrations of *S. aromaticum* were prepared as 100, 500 and 1000µg/mL in dimethyl sulfoxide (DMSO) and assessed for their ability to scavenge free radicals by using spectrophotometric assay as described by Hakkim *et al*. (2016). Reaction was started by mixing each concentration (5µL) with 100mM methanolic DPPH solution (195µL) and absorbance was recorded at 517 nm, subsequent to incubation of 30 min in a dark place at room temperature. DPPH inhibition percentage was calculated as follows:

$$Inhibition\ (\%) = \left(1 - \frac{As}{Ac}\right) \times 100$$

Where "Ac" is the absorbance of only DPPH solution whereas, "As" corresponds to the sample absorbance with DPPH. All the experiments were done in triplicates and values were expressed as Mean ± S.D.

Ferric reducing antioxidant power (FRAP) assay

The antioxidant power of *S. aromaticum* extract at different concentrations (100, 500 and 1000µg/mL) was determined using FRAP assay following the method described by Rabeta and Faraniza (2013). FRAP reagent

was prepared by mixing 300mM acetate buffer (pH 3.6), 10mM 2, 4, 6-tripyridyl-S-triazine (TPTZ) (dissolved in 40mM of HCl) and 20mM $FeCl_3.H_2O$ in a ratio of 10:1:1. A total of 200 µL of each concentration of *S. aromaticum* was added to 2800 µL of FRAP reagent and incubated for a period of 30 minutes at 37º C in water bath. After 30 min, increase in the absorbance was measured at 593 nm and FRAP reagent was used as blank. Standard curve was plotted using series of stock solution of aqueous solution of $FeSO_4.7H_2O$. The results were expressed as ferrous equivalent Fe (II) mg/g of *S. aromaticum*. All the experiments were done in triplicates and values were expressed as Mean ± S.D.

DNA protection assay

The DNA protective activity of *S. aromaticum* against H_2O_2-induced DNA damage was evaluated through the method described by Riaz *et al*. (2012). Plasmid pBR322 DNA (0.5µg/3µL) was mixed with *S. aromaticum* at three different concentrations (100, 500 and 1000µg/mL). Fenton reaction was started by adding 30% H_2O_2 (4µL) and 2mM $FeSO_4$ (3µL) into the reaction mixture. The total volume of reaction mixture was made up to 15 µL using sodium phosphate buffer (pH 7.4). Untreated DNA, DNA treated with 30% H_2O_2, DNA treated with 2mM $FeSO_4$ and DNA treated with both 30% H_2O_2 and 2mM $FeSO_4$ were simultaneously run as controls. The comparative difference in the migration of oxidized, untreated and DNA treated with different concentrations of *S. aromaticum* was observed on horizontal agarose gel electrophoresis (Major Science, USA). After staining the gel with ethidium bromide, it was documented through Syngene model Gene Genius unit (Syngene, Cambridge, UK). Ability of *S. aromaticum* to protect the DNA damage induced by H_2O_2 was measured by comparing the DNA bands of controls and oxidized DNA treated with different concentrations (100, 500 and 1000µg/mL) of *S. aromaticum*. Experiments were carried out in the dark, in order to avoid the photo-excitation of samples.

Chemical profiling of syzygium aromaticum

Chemical profiling of bioactive constituents present in *Syzygium aromaticum* was carried out through HPLC and GC-MS analysis.

High performance liquid chromatography (HPLC) analysis

The HPLC analysis of *S. aromaticum* extract was carried out on Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan). Sample for HPLC analysis was prepared according to the method described by Dek *et al*. (2011). *S. aromaticum* extract (50mg) was dissolved and homogenized in methanol (24mL) with subsequent addition of distilled water (24mL) and 6M HCl (10mL). This mixture was then incubated at 95ºC for 2h in an oven. Prior to HPLC analysis, the mixture was filtered using a membrane filter (0.45µm). Separation was achieved on a C18 column (25 cm× 4.6 mm, 5µm). The mobile phase was a mixture of 6% aqueous acetic acid and acetonitrile with gradient elution program (0-15min = 15% acetonitrile, 15-30=45% acetonitrile, 30-45min = 100% acetonitrile) with a flow rate of 1mL/min at room temperature. For HPLC analysis, 10µL of the sample was injected automatically into the HPLC system. Analysis was done on HPLC system equipped with UV-Vis detector at 280nm. Various standard phenolic acids and flavonoids were run for quantification.

Gas chromatography mass spectrometry analysis

Prior to GC-MS analysis, *S. aromaticum* extract was dissolved in HPLC grade methanol from which 0.2µL of diluted sample was automatically injected. GC-MS analysis was performed using an Agilent Technologies 7890A GC, equipped with a HP-5 MS capillary column (30m × 0.25mm; film thickness, 0.25µm) and a mass spectrometer 5975C (Agilent Technologies, USA) as detector. Mass Spectrum were taken with a mass range of m/z 45-500 at 70 eV. Carrier gas, Helium was used with a 1mL/min flow rate. Injector and detector temperature were both set at 250°C. Whereas, the temperature program of column was 60°C for 0 min, with 10°C per min gradual increased to 300° C at 10° C/min and held for 5 minutes with total run time of 29 minutes. The bioactive constituents of *S. aromaticum* were identified through the National Institute of Standards and Technology Standard Reference Database (NIST, Gaithersburg, MD, USA) (Ho *et al*., 2014).

In Vitro hepatoprotective activity

In vitro hepatoprotective activity of various concentrations (50, 100, 200, 400, 600, 800 and 1000µg/mL) of *S. aromaticum* extract was evaluated through Liver Slice Culture (LSC) assay. LSC was maintained according to the protocol developed by Wormser *et al*. (1990) and modified by Rajoapdhye and Upadhye (2016**)**.

Liver Slice Culture

For the assessment of the hepatoprotective activity, albino rats (6–7 weeks old, weight 200-250g) of either sex were used for the preparation of liver slices. The rats were dissected after decapitation, lobes of liver were detached and moved to pre-warmed Kreb's Ringer Hepes (KRH) medium, pH 7.4 (2.5mM Hepes, 118mM NaCl, 1.5mM KH_2PO_4, 2.85mM KCl,1.18mM $MgSO_4$, 2.5mM $CaCl_2$, 4.0mM glucose and 5mM β-hydroxy butyrate). Thin liver slices were prepared after cutting the liver with sharp blade. The liver slices were transferred to capped Erlenmeyer flask and washed with KRH medium (10mL) after every 10 min during the period of 1h incubation. For a single experiment, 20-22 liver slices individually weighing 4-6 mg and collectively weighing 100-120 mg were used. Individual experimental systems containing liver slices were pre-incubated on a shaker water bath at 37 °C for an hour in capped test tubes containing 2mL

KRH medium. After pre-incubation, 2mL fresh KRH medium was added to test tubes containing liver slices before treatment with 15.5 mM CCl_4 and *S. aromaticum* extract at different concentrations (50, 100, 200, 400, 600, 800 and 1000 µg/mL).

Experiment design

For further respective treatments, the liver slices were divided into twelve (12) different sets. Set 1, Background control, KRH medium only; set 2, Low control, liver slices incubated in KRH medium; set 3, High control, liver slices incubated in 15.5mM CCl_4; set 4, liver slices incubated in 15.5mM CCl_4 + *S. aromaticum* extract (50 µg/mL); set 5, liver slices incubated in 15.5mM CCl_4 + *S. aromaticum* extract (100µg/mL); set 6, liver slices incubated in 15.5mM CCl_4 + *S. aromaticum* extract (200 µg/mL); set 7, liver slices incubated in 15.5mM CCl_4 + *S. aromaticum* extract (400µg/mL); set 8, liver slices incubated in 15.5mM CCl_4 + *S. aromaticum* extract (600 µg/mL); set 9, liver slices incubated in 15.5mM CCl_4 + *S. aromaticum* extract (800µg/mL); set 10, liver slices incubated in 15.5mM CCl_4 + *S. aromaticum* extract (1000 µg/mL); set 11, liver slices incubated in 15.5mM CCl_4 + standard silymarin (100µg/mL); set 12, liver slices incubated in 15.5mM CCl_4 + 10mM ascorbic acid. After the respective treatments, all the liver slice cultures were incubated for 2 h at 37°C in shaker water bath. After the final incubation, culture medium from liver slices was collected and lactate dehydrogenase (LDH) was estimated as a cytotoxicity marker, using a commercial kit (BioVision Inc., San Francisco). Standard silymarin (100 µg/mL) and Ascorbic acid (10mM) were used as standards. Percentage cytotoxicity was calculated (in terms of LDH leakage from the liver slice culture) in the presence and absence of *S. aromaticum* extract using following formula. Respective treatments showing minimum percentage cytotoxicity were considered more hepatoprotective against CCl_4-induced hepatotoxicity.

$$Cytotoxicity\ (\%) = \left(\frac{Test\ Sample_{Abs} - Low\ Control_{Abs}}{High\ Control_{Abs} - Low\ Control_{Abs}}\right) \times 100$$

Where,

Low $Control_{Abs}$ = Absorbance of culture medium collected from liver slices incubated with KRH medium only

High $Control_{Abs}$= Absorbance of culture medium collected from liver slices incubated with 15.5 mM CCl_4 only

Test $Sample_{Abs}$ = Absorbance of culture medium collected from liver slices incubated with different concentrations of *S. aromaticum* extract and 15.5 mM CCl_4

STATISTICAL ANALYSIS

All values were expressed as mean ± S.D. Statistical analysis was performed using Graphpad prism (version 7.0) by one-way ANOVA followed by Tukey's multiple comparison test for *in vitro* hepatoprotective activity. $P < 0.05$ was considered statistically significant.

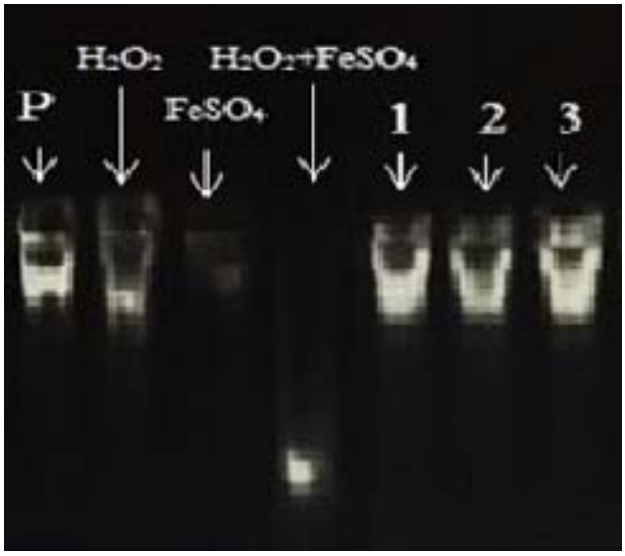
RESULTS

DPPH radical scavenging assay

The DPPH free radical scavenging activity was measured in term of percentage inhibition of DPPH radical at 100, 500 and 1000µg/mL concentrations of *S. aromaticum*. Percentage inhibition varied from 41.01± 2.00 to 90.33± 1.53. *S. aromaticum* extract showed better antioxidant potential (90.33±1.53) at a concentration of 1000µg/mL as compare to standard ascorbic acid (86.16±3.04) and Silymarin (77.67±1.53) by DPPH scavenging assay method. Moreover, *S. aromaticum* extract showed DPPH radical scavenging activity in a dose-dependent manner i.e. with increase in the concentration of extract, antioxidant activity also increased gradually (table 1).

Ferric reducing antioxidant power (FRAP) Assay

It was observed that the ferric reducing ability of *S. aromaticum* increased in a concentration dependent manner at different concentrations with values of 138.15 ±4.54, 429. 46±10.25 and 595.63 ± 14.63 Fe (II) mg/g at 100, 500 and 1000µg/mL respectively (table 1). Standard ascorbic acid and silymarin presented FRAP values as 672.61±4.61 and 680.06±3.70 Fe (II) mg/g respectively.



Untreated pBR322 DNA (P), DNA treated with 30 % H_2O_2, DNA treated with 2mM $FeSO_4$, DNA treated with both H_2O_2 and $FeSO_4$

Lane 1-3: pBR322 DNA + 30 % H_2O_2+ *S. aromaticum* (100 µg/mL), (500 µg/mL) and (1000 µg/mL)

Fig. 1: Pattern of Agarose gel electrophoresis of pBR322 plasmid DNA treated with 30% H_2O_2 and different concentrations of *S. aromaticum*

DNA protection assay

The DNA protective effect of *S. aromaticum* extract at three different concentrations (100, 500 and 1000 µg/mL)

Table 1: DPPH Radical Scavenging Activity and FRAP value of different concentrations of *S. aromaticum* extract

Test Sample	DPPH[M]	FRAP[N]
S. aromaticum (100 µg/mL)	41.01 ± 2.00[c]	138.15 ± 4.54[c]
S. aromaticum (500 µg/mL)	73.33 ± 1.53[b]	429. 46 ± 10.25[b]
S. aromaticum (1000 µg/mL)	90.33 ± 1.53[a]	595.63 ± 14.63[a]

[M] Values are (Percentage Inhibition of DPPH) mean ± SD, [N] Values are given as (Fe (II) mg/g extract), calculated as $FeSO_4.7H_2O$ equivalents, Different letters in superscript within the same column indicate significant differences ($p<0.05$) among different concentrations of *S. aromaticum*.

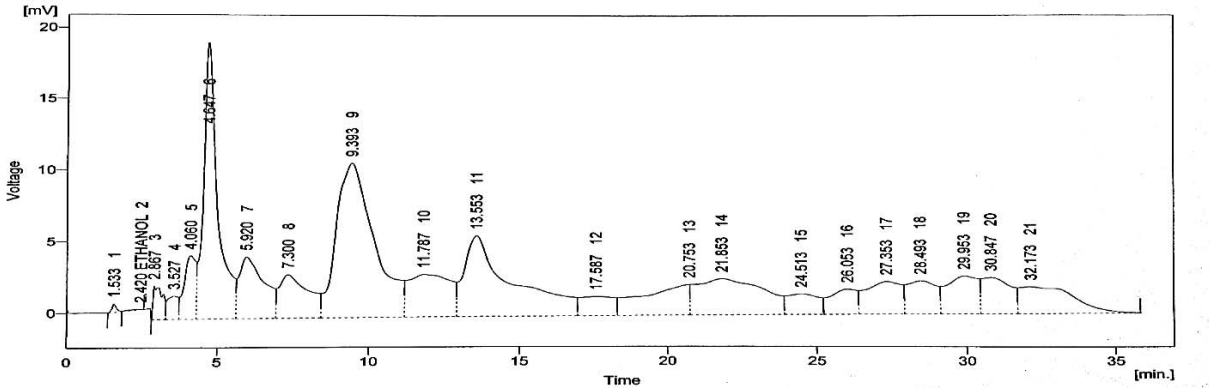


Fig. 2: Representative HPLC chromatogram of *Syzygium aromaticum*

on H_2O_2-induced DNA damage along with controls has been presented in fig. 1. Hydroxyl radicals are generated as a result of Fenton reaction induced by 30% H_2O_2 and 2mM $FeSO_4$, which induce the oxidative damage ultimately leading to opening of circular plasmid pBR322 DNA due to strand breaks. All three concentrations of *S. aromaticum* extract showed plasmid pBR322 DNA protection against damage induced by H_2O_2 (fig. 1, Lane 1-3) in comparison to the DNA treated with 30% H_2O_2, 2 mM $FeSO_4$ or with both. The DNA band corresponding to positive controls exhibited DNA disintegration and strand breaks.

Chemical profiling of Syzygium aromaticum

HPLC analysis of Syzygium aromaticum

The chromatogram obtained though HPLC analysis of *Syzygium aromaticum* is shown in fig. 2. Six major phenolic acids and flavonoids were identified and quantified in the HPLC analysis including vanillic acid, gallic acid, ferulic acid, p-coumaric acid, sinapic acid and quercetin with values of 38.81mg/L, 22.53mg/L, 28.48 mg/L, 1.34mg/L, 1.37mg/L and 2.59mg/L respectively . HPLC chromatogram of *Syzygium aromaticum* has been presented in fig. 2.

GC-MS analysis of Syzygium aromaticum

Through GC-MS, thirteen (13) bioactive compounds were identified in the *Syzygium aromaticum* extract. table 2 presents the details of identified bioactive compounds. GC-MS chromatogram of *S. aromaticum* is shown in fig. 3.

In vitro hepatoprotective activity

Hepatotoxicity was induced by the addition of CCl_4, which is cytotoxic to the liver slices and releases LDH in the culture medium. Hepatotoxic effect of CCl_4 was confirmed by the significant increased ($p<0.05$) in the percentage cytotoxicity (in terms of LDH leakage) of high control (75.58±3.54) as compare to low control (2.34± 1.34). Various concentrations of *S. aromaticum* extract (50, 100, 200, 400, 600, 800 and 1000µg/mL) showed hepatoprotective effect against CCl_4 induced hepatotoxicity, presenting significantly ($p<0.05$) lower percentage cytotoxicity ranging from 7.35±0.49 to 16.16 ±0.27 as compared to the high control (75.58±3.54). The hepatoprotective activity of *S. aromaticum* extract was comparable with silymarin (5.53±0.40) and ascorbic acid (11.47±0.45) which were used as standard (fig. 4).

DISCUSSION

Nature has blessed us with numerous plants with medicinal and economical significance which not only provide us food but also serve as Phytomedicine. Medicinal plants are significantly important because they are potentially used as food as well as they serve as ingredients of traditional medicine (Moradi *et al*., 2016). Primary sources of natural antioxidants are fruits, vegetables and medicinal plants and scientific evidence advocates the use of antioxidants to reduce the risk of chronic diseases including hepatic and cardiac diseases and cancer (Kumar *et al*., 2011). For centuries, spices including clove, thyme, mint, cinnamon and oregano have been used as food preservatives and owing to their antioxidant and antimicrobial properties they also serve as medicinal plants (Kumar *et al*., 2012). Among the other spices, *S. aromaticum* (clove) has attracted the attention of researchers particularly because it is a potent antioxidant and antimicrobial agent (Cortés-Rojas *et al*., 2014).

Table 2: Bioactive compounds identified by GC-MS

S. No.	Retention Time	Name of the compounds	Molecular formula	Molecular weight	Area (%)
1.	9.360	2-methoxy-4-(2-propenyl)-, (Z)- Phenol (Eugenol)	$C_{10}H_{12}O_2$	164	2.102
2.	9.489	Copaene	$C_{15}H_{24}$	204	0.840
3.	9.850	Benzene, 1,2-dimethoxy-4-(2-propenyl)- (Methyl eugenol)	$C_{11}H_{14}O_2$	178	1.024
4.	11.347	Nephthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-(δ-Cadinene)	$C_{15}H_{24}$	204	1.274
5.	13.472	2',3',4' Trimethoxy acetophenone	$C_{11}H_{14}O_4$	210	9.367
6.	13.583	Alph-Tetralone, 8-fluoro-5,6-dimethoxy-	$C_{12}H_{13}FO_3$	224	1.079
7.	14.065	2H-1-Benzopyran, 6,7-dimethoxy-2,2-dimethyl (Precocene II)	$C_{13}H_{16}O_3$	220	2.830
8.	15.897	4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-methyl- (Eugenin)	$C_{11}H_{10}O_4$	206	32.264
9.	16.706	1,4-dimethoxy-6,7,8,9-tetrahydro-5-benzocycloheptenone	$C_{13}H_{16}O_3$	220	13.422
10.	16.852	1,4-Nephthalenedione, 5,8-dihydroxy-2-methoxy	$C_{11}H_8O_5$	220	5.281
11.	16.998	Phenanthrene, 1-methyl-7-(1-methylethyl)-(Retene)	$C_{18}H_{18}$	234	2.183
12.	17.730	1,4-Dimethoxy-6,7,8,9-tetrahydro-5-benzocycloheptenone	$C_{13}H_{16}O_3$	220	15.648
13.	18.246	2,4-Diamino-5,6,7,8-tetrahydro-9H-cyclohepta[4,5]thieno[2,3-d]pyrimidine	$C_{11}H_{14}N_4S$	234	12.686

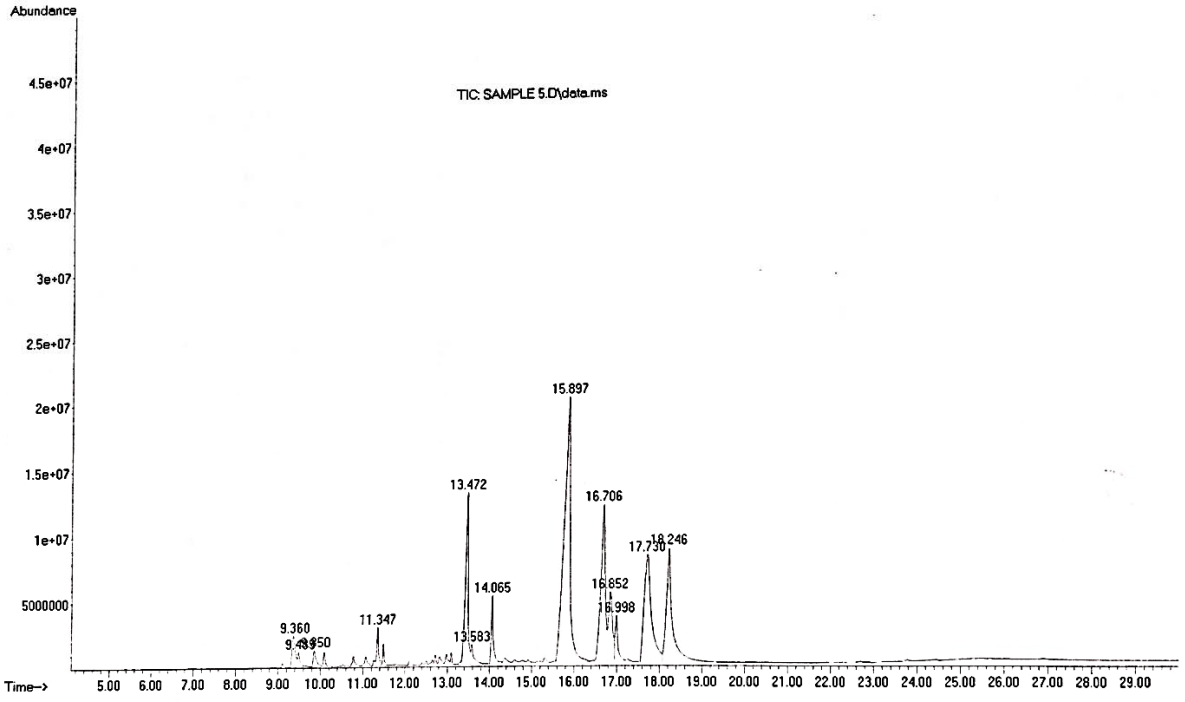


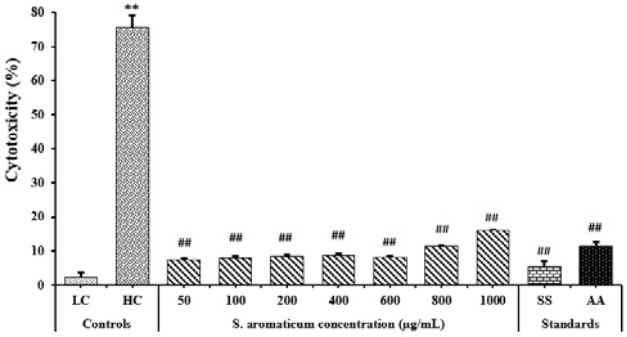
Fig. 3: GC-MS chromatogram of *Syzygium aromaticum*

In order to calculate the nutritional value of food, plants and their bioactive compounds, antioxidant activity is an important parameter. Recent research has considerably focused to find natural antioxidant to replace the synthetic ones with harmful side effects like carcinogenesis. *In vitro* antioxidant activity of *S. aromaticum* extract was assessed through the DPPH free radical scavenging and FRAP (Ferric Reducing Antioxidant Power) assay. Based on DPPH and FRAP tests, *S. aromaticum* presented higher antioxidant activities at higher concentration of the extract. Thereby, exhibiting a concentration dependent antioxidant activity. Furthermore, *S. aromaticum* exhibited the higher antioxidant activities as compared to standard silymarin and ascorbic acid. According to DPPH

assay, the free radical scavenging activity increased from 41.01% to 90.33% with increase in concentration from 100 to 1000µg/mL (table 1). Regarding FRAP test, the higher ferric reducing power was observed (595.63± 14.63) at higher concentration (1000µg/mL) of *S. aromaticum*. Antioxidant potential of plant extracts with dose dependent response has also been supported by other studies (Soni and Sosa, 2013; Yadav *et al*., 2014) and in present study same trend was observed. With gradual increase in the phenolic acids, flavonoids and other plant secondary metabolites, increase in the antioxidant potential of medicinal plants has been supported by many studies (Soni and Sosa, 2013). Free radicals cause oxidative damage and cause a number of acute and chronic disorders including cancer, diabetes, immunosuppression, atherosclerosis, coronary heart diseases, neurodegeneration and aging (Nimse and Pal, 2015). In the management and prevention of these diseases, antioxidants play a significant role through their radical scavenging ability. The health-promoting effect of secondary metabolites from medicinal plants such as phenolic acids, terpenoids and flavonoids is mainly due to their antioxidant activity which is due to their counteraction against the free radicals. Ingestion of naturally occurring antioxidants has found to be inversely correlated with morbidity and mortality arising from degenerative diseases in various epidemiological studies. In fact, an inverse association has been demonstrated between the consumption of fruits and vegetables and age-related diseases like cancer and coronary heart diseases (Vijayalakshmi and Ruckmani, 2016; Pantavos *et al*., 2015).

S. aromaticum exhibited prominent DNA protection on all the three concentrations (100, 500 and 1000 µg/mL) (fig. 1, Lane 1-3). In a previous study, dose-dependent DNA protection was observed at different concentrations of *S. marianum* and potential protection was observed at 10 µL/mL (Ali *et al*., 2015). However, it is generally estimated that DNA protective effect of medicinal plants mainly arise from its antioxidant activity through the inhibition of enzymes which are involved in the initiation reactions of DNA strand breaks (Guha *et al.*, 2011). DNA protective effect of *S. aromaticum* against H_2O_2-induced DNA damage was observed with all the concentrations even at low concentration of 100µg/mL. For the purpose of prevention from cancer and other diseases induced by free radical, this property can be capitalized using *S. aromaticum* in the development of drugs.

Currently, HPLC is the most consistent and reliable analytical technique to identify the phenolic compounds present in medicinal plants (Khoddami *et al*., 2013). HPLC Chromatogram revealed the presence of some noteworthy phenolic acids and flavonoids including Quercetin, gallic acid and ferulic acid in *Syzygium aromaticum* extract, which have strong antioxidant activity. Gallic acid (GA) presents a critical role in various biological and pharmacological activities due to its strong antioxidant effect (Güleç and Demirel, 2016). Ferulic acid protects against CCl_4-induced acute hepatic injury by reducing the oxidative damage and inflammatory signaling pathways (Paiva *et al.*, 2013). Individual bioactive components of *S. aromaticum* extract were isolated and identified through GC-MS. Among the bioactive constituent of *S. aromaticum*, eugenol has been reported to be responsible for most of the pharmaceutical effects, such as anti-giardiasis, gastroprotective and hepatoprotective activity (Anbu and Anuradha, 2012; El-Hadary and Hassanien, 2016). Gallic acid and copaene, although not predominant in *S. aromaticum*, reported to have pharmacological effects such as antimicrobial, anticancer and antitumor activity (Türkez *et al*., 2014).



LC, Low Control; HC, High Control; AA, ascorbic acid; SS, Standard Silymarin ** indicates significance ($p<0.05$) compared to the low control and ## indicates significance ($p<0.05$) compared to the high control (one way ANOVA, Tukey's multiple comparison test). Values are presented as the mean ± S.D (n=3)

Fig. 4: Percentage Cytotoxicity (in terms of LDH release) presented by different concentrations of *S. aromaticum* against CCl_4-induced hepatotoxicity

Liver Slice Culture (LSC) is an appropriate *in vitro* model, for the screening and experimental evaluation of hepatotoxic and hepatoprotective agents. LSC retain the specific architecture of liver tissues and maintain the cell identity, diversity and functional heterogeny. Due to this reason, it closely resembles *in vivo* situations and can be used as an alternative approach to assess the hepatoprotective potential (Naik *et al*., 2004; Rajopadhye and Upadhye, 2016; Fernando and soysa, 2016). Employing this model CCl_4-induced hepatoxicity was confirmed by assessing the leakage of LDH by liver slices into the culture medium. In fact, LDH is chiefly present in the cytosol of liver cells and leaked out whenever the hepatic cells are lysed by hepatotoxin or any other cytotoxic compound. Numerous research reports have demonstrated that CCl_4-induced hepatotoxicity is principally due to its free radical metabolite $CCl_3^{\cdot}$, which alkylate the proteins and macromolecules such as poly-unsaturated fatty acids and induce lipid per oxidation

(Olusola and Noghayin, 2015). The particular target of this oxidative damage are the polyunsaturated fatty acids which are present largely in the cell membranes of hepatocytes, ultimately leading to cellular homeostasis through disintegration of membrane (Mnaa *et al*., 2014). Treatment of liver slices with different concentrations of *S. aromaticum* prior to CCl_4 exposure, prevented these cytological changes and protected the hepatocytes (fig. 4). The hepatoprotective effect of *S. aromaticum* (clove) could be due to the presence of potential antioxidant and hepatoprotective bioactive compounds including gallic acid, ferulic acid, eugenol and copaene as identified by HPLC and GC-MS. The hepatoprotective effect of *S. aromaticum* is evidenced from another study by Adefegha *et al*. (2014) which demonstrated the protective activity of clove extract against CCl_4 induced hepatotoxicity in rats. The overall hepatoprotective activity of *S. aromaticum* is possibly due to its property to act as antioxidant by counteracting free radicals thus protecting the hepatocellular membrane against oxidative stress.

CONCLUSION

The findings of the present study suggest that the *S. aromaticum* is a potential antioxidant and hepatoprotective agent. Bioactive compounds present in the *S. aromaticum* extract may possibly contribute to its strong antioxidant and hepatoprotective effect therefore justifying its use in traditional medicinal system to prevent and manage various liver diseases. However, in order to evaluate the holistic and synergistic effect of *S. aromaticum*, further *in vivo* work including clinical trials is required.

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