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RESEARCH ARTICLE

Assessment of Chemical Constituents of *Allium sativum* Essential Oil Extracted by using Hydrodistillation Technique and their Pharmacological Potential

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Abstract

The current study was based on identification of chemical components and pharmacological potential of *Allium sativum* essential oil. The pharmacological properties mainly antioxidant, anti-inflammatory and anti-diabetic potential of *Allium sativum* Essential Oil (ASEO) were also studied. *Allium sativum* is a traditional medicinal plant of the *Alliaceae* family. The extracted essential oil from fresh bulbs of *Allium sativum* through hydro distillation process was further analyzed by GC-MS. To determine the potential of *Allium sativum* essential oil for reducing inflammation and preventing diabetes, evaluation of antioxidant assays, protein denaturation and glucose absorption by yeast cells were studied. The results explained the presence of several organosulfur compounds that were identified by using different combinations of non-polar solvents mainly represented as Diallyldisulfide, Methyl Allyl Disulfide, Methyl Allyl Trisulfide, Diallyltrisulfide and Diallyltetrasulfide. The antioxidant potential of ASEO expressed as IC_{50} values of DPPH, ABTS, β-carotene assay, Nitric Oxide, superoxide, hydroxyl scavenging and metal chelating were 5.93 mg/ml, 2.1 mg/ml, 7.94 mg/ml, 5.49 µg/ml, 11.55 µg/ml, 7.66 µg/ml and 10.60 µg/ml respectively. Further, total phenolic level, total flavonoid level, FRAP assay and total antioxidant capacity were evaluated and they were as follows: 4.99 ± 0.41 mg GAE/g, 98.46 ± 0.16 mg QE/g, 4.99 ± 0.26 mg/g and 15.80 ± 0.14 mg/g. Furthermore, the anti-inflammatory assay was assessed by considering the protein denaturation method which exhibits IC_{50} value 3.53µg/ml. In addition to this, anti-diabetic potential was assessed by considering glucose absorption by yeast cells at various glucose concentrations (5mM, 10mM, 25mM) which indicated that the ASEO enhanced glucose uptake via the yeast cell membrane. Upregulation of glucose was reported with sequential elevations in the ASEO concentration. Based on the above results, it was revealed that *Allium sativum* essential oil exhibits reasonable antioxidant, anti-inflammatory and anti-diabetic activity that could be considered as an alternative therapeutic agent in inflammatory diseases. However, future research is required to isolate prominent functional components to determine the observed potential in preclinical studies.

Keywords: Anti-diabetic, Anti-inflammatory, Antioxidant, *Allium sativum* Essential Oil (ASEO), GC-MS, Hydro-distillation

1. Introduction

To maintain a healthier life, the existence of homeostasis between pro-oxidant and antioxidant ratios limit the extent of the risk of chronic diseases. Free radicals mainly oxygen and nitrogen are constantly produced during cellular metabolism in biological system[1]. They are highly reactive intermediates; however, the biological system can efficiently detoxify them within seconds. At the extreme level, imbalance in redox status-imposed

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toxicity via production of free radicals that ultimately damaged the biomolecules and results in oxidative stress. It may dramatically increase the risk factors associated with multiple metabolic syndromes[2]. To overcome the accumulation of risk factors, several ways are identified to prevent metabolic syndromes such as stop smoking, eat healthy diet, exercise, avoid alcohol and excessive sun exposure[3].

Numerous evidence suggests that oxidative stress and inflammation can be responsible for chronic diseases including cancer, diabetes, autoimmune diseases, arthritis, asthma etc[4]. However, self-destruction triggered by inflammation, if uncontrolled and unresolved leads to pathological conditions, prompts the necessity to develop safe and effective anti-inflammatory agents[5]. Natural therapeutics for chronic diseases can be an alternative approach due to their safety, cultural approval and fewer side effects than synthetic drugs. This can be novel opportunistic way to overpower the oxidative stress which in turn protect humans from health disorders[6].

Allium sativum L., commonly named as garlic belongs to the *Alliaceae* family. In past decades, garlic was either used in traditional medicine or as raw vegetable for culinary purposes. Edible plants are consumed as spices in flavoring and seasoning throughout the world because of their rich nutritional characteristics. The chemical composition of garlic includes organosulfur compounds, enzymes, amino acids, fatty acids, carbohydrates, water, proteins, fibers and trace minerals[7]. Furthermore, it is a major source of secondary metabolites including flavonoids, phenolics and terpenoids. Besides this, organosulfur compounds are produced through the metabolism of allicin formed by the conversion of alliin through the enzyme allinase during the crushing of garlic bulbs[8]. Its protective mechanism elevates the level of antioxidant enzymes and prevents chromosomal damage by DNA repair mechanisms. It is available in various forms such as garlic extract, powdered form and essential oil which benefits human health. "Essential oil" confers the essence of plant extract, hydrophobic, complex mixture of volatile components mainly terpenes and non-terpenic compounds. It can promote antimutagenicity, detoxification, cell cycle arrest, apoptosis, antiviral, antimicrobial, anticancer, neuroprotective, analgesic and immunomodulation properties[9]. So, keeping the above beneficial properties of garlic in mind, the essential oil from fresh bulbs of *Allium sativum* was extracted by hydrodistillation technique and then the various constituents were determined through GC-MS using different solvent approaches. The extracted oil was further checked for its antioxidant, anti-inflammatory and anti-diabetic potential.

2. Materials and Methods

2.1 Reagents Used

In the present study, chemicals used were DPPH (2,2-Diphenylpicrylhydrazyl), sodium sulphate, ascorbic acid, quercetin, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate, trolox, $FeCl_3.6H_2O$, TPTZ (2,4,6-tripyridyl-s-triazine), sodium acetate, $FeSo_4.7H_2O$, NADH, phenazinemethosulfate (PMS), nitrobluetetrazolium (NBT), β-carotene, linoleic acid, tween-40, Sodium Nitroprusside, Thiobarbituric Acid (TBA), sulphanilic acid reagent, N-(1-naphthylethylenediamine dihydrochloride), EDTA, $FeCl_3$ anhydrous, 2-deoxyribose, trichloroacetic acid (TCA), Sodium Hydroxide, gallic acid, tris-Hcl, hydroxylamine HCl, KH_2PO_4, KOH, Na_2HPo_4 , NaH_2Po_4, 2,2-bipyridyl, folin-ciocalteau reagent, sodium carbonate, ammonium molybdate, sodium nitrite, aluminium chloride, methanol, acetic acid, chloroform, H_2So_4, orthophosphoric acid. All chemicals were purchased from Himedia, Merck, SRL and were of analytical grade.

2.2 Procurement of Sample

Fresh bulbs of *Allium sativum* were collected from the premises of Banasthali Vidyapith, Rajasthan. The bulbs were properly cleaned and peeled before performing hydrodistillation. Then, the bulbs were chopped and blended with water to prepare fine paste which was then heated in a round bottom flask.

2.3 Sample Preparation

2.3.1 Hydrodistillation Technique

The essential oil was extracted in a clevenger apparatus (ASGI), attached to flask containing garlic extract, heated for 4-5 h until no more essential oil was obtained. This generates condensed vapour which was then separated through an oil-water separator. The low pressure of water pipe was continuously switched on to cool down the condenser temperature. After this, the extracted oil was dried over anhydrous sodium sulphate and stored in a vial at 4° C for further examination. Extraction was done in

triplicates and oil yield (w/w) was calculated depending on the fresh weight of sample[10].

$$\text{Oil yield (\%)} = \frac{\text{Volume of essential oil obtained (ml)} \times 100}{\text{Weight of sample (g)}}$$

2.4 GC-MS (Gas Chromatography Mass Spectrometry) Analysis

The chemical composition of *Allium sativum* Essential Oil (ASEO) was determined by GC-MS analysis. An electron ionization system 0eV was considered in this method. Helium gas was considered as carrier gas at a constant flow rate of 2ml/min. Ion source and MS transfer line temperatures were adjusted at 280°C and 250°C temperature. The initial oven temperature was maintained at 50°C for 1 min and then further increased to 300°C at a rate of 2°C/min followed by maintaining at 300°C for 4 min. The maximum oven temperature was maintained at 350°C. Diluted sample of 1µl was injected in spilt fewer modes. The percentage of oil components was identified as percentage by peak area normalization. Components of essential oil were identified on the basis of retention time in column relative to computer matching of electron ionization mass spectra using the NIST library for the GC-MS system[11].

2.5 FT-IR (Fourier-Transform Infrared Spectroscopy) Analysis

A Bruker Alpha FTIR spectrophotometer was considered for the identification of functional groups in ASEO. The oil drop was placed in such a way so that it covers the ATR crystal for infrared spectrometry analysis. Standards such as diallyl disulfide (DADS), Diallyltrisulfide (DATS) and Allyl Sulfide (AS) were used as reference compounds to compare their spectra with sample. The data was recorded in absorbance v/s wavelength. IR spectra were recorded in the range 700 – 4000 cm^{-1}. Thirteen spectra for the sample were obtained at room temperature. OPUS software was considered to perform data analysis[12].

2.6 Phytochemical Analysis

2.6.1 Total Flavonoid Level

The level of flavonoids in ASEO was estimated by the aluminium chloride colorimetric method[13]. The reaction tubes contained various concentrations of ASEO and 300 µl of Sodium Nitrite (5%), which were then incubated for 5 min. After this, 0.6 ml of aluminium chloride (10%) was added and further incubated for 6 min. Then, added 4ml of sodium hydroxide (1M) and adjusted the volume of tubes upto 10ml with distilled water. Absorbance was taken at 510nm and standard calibration curve of quercetin was prepared to estimate flavonoid content. The result was calculated as mg of quercetin equivalent (QE/g) of plant material. All the tests were run in triplicates and represented as mean ± SD.

2.6.2 Total Phenolics Level

The phenolics level in ASEO was identified by the Folin-Ciocalteu Reagent (FCR) with slightly modified method[14]. According to this, various concentrations of ASEO were well mixed with 5ml of FCR (1:10) and kept at room temperature for 5min. Further, added 4 ml of sodium carbonate (8%) in each test tube and kept in the dark for 30 min. The OD was taken at 765 nm in UV-VIS spectrophotometer against blank. Gallic acid was taken as reference compound. The concentration of TPC was calculated as mg of gallic acid equivalent per gram by the equation obtained from the gallic acid calibration curve. The quantification of phenolic constituents in ASEO was run in triplicates and results were represented as mean ± SD.

2.7 Level of Total Antioxidant Capacity

The level of TAC (Total Antioxidant Capacity) was detected by the phosphomolybdenum method[15]. In this protocol, preparation of TAC chemical was done by mixing H_2SO_4 (0.6 mol/l), ammonium molybdate (4 mmol/l), orthophosphoric acid (28 mmol/l) in a ratio of 1:1:1. Different concentrations of ASEO were mixed with TAC reagent and tubes were placed in water bath for 90 min at 95°C. After this, tubes were allowed to cool down at RT (room temperature) and OD was taken at 765 nm. Ascorbic acid was taken as standard compound. TAC reagent without sample was taken as blank. The results were represented as mg of ascorbic acid equivalent per g of plant material. The entire test was run in triplicates and represented as mean ± SD.

2.8 FRAP (Ferric Ion Reducing Antioxidant Power) Assay

This experiment was conducted to evaluate the free radical scavenging capacity of ASEO which was conducted spectrophotometrically[16]. It depends on the reducing ability of antioxidants to convert colorless

complex (Fe^{3+}-TPTZ) into blue colored complex (Fe^{2+}-TPTZ). In this assay, the FRAP working solution was prepared by the addition of stock solution of 300 mM acetate buffer, 10 mM TPTZ(2,4,6-tripyridyl-s-triazine) in 40 mM HCl, 20 mM $FeCl_3.6H_2O$ in the ratio 10:1:1 at room temperature. During the experiment, a freshly prepared working solution was always preferred to obtain better results. Various dilutions of ASEO were dissolved with 1.5 ml of FRAP working reagent and kept for 5 min at RT (room temperature). The reading was taken at 594 nm using UV-VIS spectrophotometer. FRAP reagent was used as blank. All the tests were determined in triplicate. $FeSo_4.7H_2O$ was considered as standard compound and the standard curve of $FeSo_4.7H_2O$ was compared with the absorbance of test sample.

2.9 Identification of Antioxidant Potential

2.9.1 DPPH Assay

It is a simple and sensitive test used to measure the scavenging potential of ASEO against DPPH free radicals[17]. In this assay, 0.1 mM of DPPH solution was prepared in methanol. Further, DPPH solution (4 ml) was added to various concentrations of ASEO and incubated the tubes in dark light for 30 min at 25ºC. The reading was taken at 515 nm against methanol as blank in UV-VIS spectrophotometer. The % inhibition was measured by difference in concentrations of control and test samples. Lower the absorbance at high concentration of essential oil implies greater free radical radical activity. The entire test was run in triplicate. Ascorbic acid was considered as standard compound. The % inhibition of free radicals by antioxidants was determined by the below mentioned formula:

$$\% \text{ inhibition} = \frac{A_{control} - A_{test} \times 100}{A_{control}}$$

$A_{control} \rightarrow$ Absorbance of control sample.

$A_{test} \rightarrow$ Absorbance of test sample.

The IC_{50} value was measured by the regression equation of graph plotted between percentage (%) inhibition and sample concentration.

2.9.2 ABTS Assay

In this method, the antioxidant potential of ASEO was evaluated by its free radical scavenging activity towards ABTS radical cation[18]. A stock solution of ABTS was prepared at a concentration of 7 mM ABTS in water. ABTS cation formation occurs by mixing ABTS stock solution with potassium persulfate ($K_2Cr_2O_7$) at 2.45 mM concentration in the ratio 1:0.5. Before starting the experiment, this mixture was incubated for 12 – 16 h at room temperature in dark light which results in incomplete ABTS oxidation and gives blue green color solution. Then, the absorbance of the solution was maintained at 0.705 ± 0.05 at 734 nm by diluting the ABTS solution with ethanol which can be considered as working solution. Different concentrations of ASEO were taken in test tubes and then 2 ml of working ABTS was added. The tubes were kept for 5 - 6 min at 30ºC and reading was taken at 734 nm after initial mixing. Trolox was used as standard compound. The entire test was run in triplicate.

2.9.3 Superoxide Radical (O^{2-}) Scavenging Activity

In this experiment, various concentrations (20 -100 µg/ml) of ASEO were dissolved with 1ml of NADH at 144µM concentration and 1ml of NBT at 677 µM. In addition to this, 0.1ml of PMS (60µM) was added to the various test tubes and incubated at 30º C for 2-3 min. The absorbance was taken at 560 nm in UV-VIS spectrophotometer. Ascorbic acid was used as standard compound and the assay was performed same as for the test samples. All the tests were run in triplicate. The decline in absorbance with rise in sample concentration and standard explains the upregulation in scavenging activity[19].

2.9.4 β-Carotene Bleaching Activity

The antioxidant assay was analyzed by the inhibitory action of ASEO against conjugated diene hydroperoxides arising from linoleic acid oxidation[20]. In this assay, β-carotene and linoleic acid stock solution was prepared using 0.5mg of powdered β-carotene dissolved in chloroform (1ml). Further, 20mg of linoleic acid and 200 mg of Tween 40 were added to this mixture. Chloroform was completely evaporated from the mixture through rotatory evaporator under vaccum at 60 rpm and 40ºC for 10–15 min. Then, added 100 ml distilled water with continuous shaking for 4-5 min to obtain an emulsion. ASEO extract of 350 µl was dissolved in methanol and to it added 2.5 ml of emulsion and then recorded the initial absorbance at 470 nm. After that, incubated the test tubes at 50ºC in a water bath for 1h and recorded the OD at an interval of 10 min. Ascorbic acid was considered as reference compound. Blank was prepared by similar procedure as that of stock solution of

linoleic acid emulsion without using β-carotene. All the tests were run in triplicate.

2.9.5 Nitric Oxide Scavenging Assay

The activity was carried out according to "Griess-Ilosvay reaction" protocol with slight modifications[21]. In this experiment, the reaction mixture contained 3ml of sodium nitroprusside (10 mM) dissolved in PBS (0.1M, pH = 7.4) and various dilutions of ASEO and standard were incubated at 25°C for 2.5 h. Then, added 1 ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) to 0.5ml of reaction mixture and incubated the tubes for 5min to complete the diazotization reaction. Then, added 1 ml of napthyl ethylene diaminedihydrochloride (1%) with proper mixing and incubated the resulting mixture for 30 min at 25°C. Pink color was developed in reaction tubes and the OD was taken at 564 nm in UV-VIS spectrophotometer. Ascorbic acid was considered as reference compound.

2.9.6 Hydroxyl Radical Scavenging Activity

In this experiment, hydroxyl radicals were produced by the ascorbic acid-iron EDTA-H_2O_2 system (fenton reaction). The reaction mixture contained 0.1ml of EDTA (1 mmol/l), 0.1 ml of $Fecl_3$ (200 µmol/l), 0.1 ml of 2-deoxyribose (28 mmol/l) in KH_2PO_4-KOH buffer, 0.1 ml H_2O_2 (1 mmol/l), 0.1 ml ascorbic acid(1mmol/l) and various concentrations of ASEO (5-25 µg/ml). The tubes were then incubated for 1 h at 37°C. After that, 1 ml of TBA (1 % in 0.2 % NaOH) and TCA (2%) were added by proper mixing and incubated at 100°C for 20 min. The reading was taken at 532 nm in UV-VIS spectrophotometer. Quercetin was considered as reference compound[17].

2.9.7 Metal Chelating Assay

This assay was performed by the ferrous ion (Fe^{2+}) chelation method[22]. It is mainly based on Fe^{2+} chelation in the reaction mixture by antioxidants present in the essential oil. The reaction tubes contained 0.5 ml of $FeSo_4.7\ H_2O$ (10 mmol/l), 1ml of Tris-Hcl (0.2 mol/l), 1ml of 2,2-bipyridyl (0.1 % in 0.2 mol/l HCl), 0.4 ml of Hydroxylamine HCl (10 %), 2 ml ethanol and the volume was build upto 10 ml with distilled water. Various concentrations of ASEO (10-50 µg/ml) and EDTA (1-5 ug/ml) as standard compound were used to reduce free radical concentrations. Then, incubated the reaction tubes for 1-2 min and reading was taken at 522 nm in UV-VIS spectrophotometer.

2.10 Anti-Inflammatory Potential (*In Vitro*)

2.10.1 Denaturation of Protein

In this protocol, various concentrations of ASEO and Sodium Diclofenac (considered as reference compound) were mixed with 1 % Bovine Serum Albumin (BSA). Further, reaction mixtures were incubated at 37°C in a BOD incubator for at least 15 min which was followed by incubation at 70°C for 5 min in a water bath. The tubes were then cooled down and absorbance was taken at 660 nm. Bovine serum albumin with water was considered as control[23]. The % inhibition was determined by the formula explained in DPPH activity.

2.11 Antidiabetic Potential

This assay was considered by observing the uptake of glucose by yeast cell suspension. Baker's yeast, commercially available, was dissolved with distilled water to make 1% suspension. At room temperature of 25°C, the suspension was kept overnight. Further, on next day, the suspension was centrifuged at 4200 rpm for 5 min. After centrifugation, collected and diluted the supernatant in a 1:9 ratio with the addition of distilled water to prepare a 10% yeast cell suspension. About 1-5 mg of ASEO was dissolved in methanol and supplemented with different concentrations (5 mM, 10 mM and 25 mM) of 1 ml of glucose solution. The mixture was kept for 10 min at 37°C. Then, 0.1 ml of cell suspension was added to the tubes containing glucose and ASEO, vortexed and further incubated for 60 min at 37°C. After incubation, the tubes were centrifuged at 3800 rpm for 5 min and glucose concentration was determined at 520 nm spectrophotometrically. All the reagents except ASEO were used as control. Metronidazole was used as a reference compound. The % increase in glucose absorption was measured by below mentioned formula:

$$\%\ \text{percent increase in glucose absorption} = \frac{\left(A_{control} - A_{test}\right) \times 100}{A_{control}}$$

2.12 Statistical Analysis

The entire tests were run in triplicate and data was tabulated as mean±SD. The regression equation of graph (% inhibition v/s sample concentration) was considered to determine IC_{50} value. One-way ANOVA was used to measure the p-value. P<0.05 was considered to be statistically significant. This was performed by SPSS software.

3. Results

3.1 Oil Yield and Chemical Constituents of Essential Oil

Essential oil extraction from *Allium sativum* bulbs was done by hydrodistillation and oil yield was 0.2 % which was further analyzed by GC-MS. The color of the essential oil was pale yellow with a gastric odor. The constituents of essential oil were dominated by allylpolysulfides mainly diallyldisulfide, trisulfide methyl-2-propenyl (methyl allyl trisulfide), trisulfide di-2-propenyl (diallyltrisulfide), tetrasulfide di-2-propenyl(diallyltetrasulfide), 2-vinyl-4H-1,3-dithiine, E-1-allyl-2-(prop-1-en-yl) disulfane. The percentage may vary depending on the solvent systems used during the experiment, as shown in Table 1. The higher percentage of compounds depends on the solvent system such as diallyl disulfide (35.84%) in 90% methanol, methylallyltrisulfide (44.24%) in 70% hexane, E-1-allyl-2-(prop-1-en-yl) disulfane (8.46%) in 90% methanol, diallyltetrasulfide (2.27%), methyl allyl disulfide (1.40%) and 2-vinyl-4H-1,3-dithiine (0.55%) in 80% acetone.

Table 1. Identification of chemical constituents in *Allium sativum* essential oil extracted through hydrodistillation using different solvent system

S.No.	Solvents	Compound	RT	Area %
1.	10:90(Hex:met)	Diallyldisulfide (E)-1-Allyl-2-(prop-1-en-yl) disulfane Methyl allyltrisulfide Diallyltrisulfide	13.08 14.28 16.29 26.24	28.78 6.03 24.61 36.45
2.	50:50(Hex:met)	Diallyldisulfide (E)-1-Allyl-2-(prop-1-en-yl) disulfane Methyl allyltrisulfide Diallyltrisulfide	13.08 14.31 16.31 26.29	33.85 6.54 29.12 24.64
3.	90:10(Hex:met)	Diallyldisulfide (E)-1-Allyl-2-(prop-1-en-1-yl)disulfane Methyl allyltrisulfide Diallyltrisulfide	13.08 14.28 16.29 26.22	26.54 5.71 27.38 36.32
4.	60% acetone	Diallyldisulfide E)-1-Allyl-2-(prop-1-en-yl) disulfane Methyl allyltrisulfide Diallyltrisulfide	13.03 14.23 16.24 26.17	29.83 7.46 26.20 29.33
5.	70% acetone	Diallyldisulfide E)-1-Allyl-2-(prop-1-en-yl) disulfane Methyl allyltrisulfide Diallyltrisulfide	13.03 14.23 16.24 26.14	25.46 6.45 22.57 33.75
6.	80% acetone	Diallyldisulfide E)-1-Allyl-2-(prop-1-en-yl) disulfane Methyl allyltrisulfide 2-vinyl-4H-1,3-dithiins Diallyltrisulfide 1-Allyl-3-propyltrisulfane (E)-1-Allyl-3-(prop-1-en-1-yl) trisulfane Methyl allyl disulfide Diallyltetrasulfide	12.96 14.16 16.16 20.56 26.12 26.89 27.82 30.82 40.31	18.16 5.57 23.63 0.55 44.99 0.30 0.45 1.40 2.27

Table 1 to be continued...

S.No.	Solvents	Compound	RT	Area %
7.	60% methanol	Diallyldisulfide E)-1-Allyl-2-(prop-1-en-yl) disulfane Methyl allyltrisulfide Diallyltrisulfide	13.08 14.28 16.29 26.24	28.49 6.20 26.86 34.38
8.	90% methanol	Diallyldisulfide E)-1-Allyl-2-(prop-1-en-yl) disulfane Methyl allyltrisulfide Diallyltrisulfide	13.11 14.33 16.34 26.42	35.84 8.46 29.78 18.67
9.	100% methanol	Diallyldisulfide (E)-1-Allyl-2-(prop-1-en-yl)disulfane Methyl allyltrisulfide 2-Vinyl-4H-1,3-dithiine Diallyltrisulfide	13.08 14.28 16.29 20.71 26.24	23.98 5.55 26.19 0.39 40.85
10.	Acetone:Hexane(50:50)	Diallyldisulfide (E)-1-Allyl-2-(prop-1-en-1-yl)disulfane Methyl allyltrisulfide Diallyltrisulfide	13.03 14.21 16.24 26.17	27.54 4.61 32.87 31.91
11.	Acetone: Methanol (50:50)	Diallyldisulfide (E)-1-Allyl-2-(prop-1-en-1-yl)disulfane Methyl allyltrisulfide Diallyltrisulfide	13.01 14.23 16.24 26.17	32.01 8.29 21.45 32.45
12.	Hexane (70%)	Diallyldisulfide (E)-1-Allyl-2-(prop-1-en-1-yl)disulfane Methyl allyltrisulfide Diallyltrisulfide	13.01 14.21 16.19 26.12	26.51 7.71 44.24 16.84

RT = Retention time, Area % = Quantification based on GC-MS Peak area normalization

From these results, we reached on the conclusion that the majority of allyl compounds are soluble in medium polar solvents whereas other allyl compounds such as methyl allyltrisulfide are more soluble in non-polar solvents. The major components of essential oil obtained from hydrodistillation are diallyltrisulfide, methylallyltrisulfide and diallyl disulfide. Such a proportion of organosulfur compounds can be considered better for scavenging free radicals which was confirmed by antioxidant assays. The data is depicted in Table 1.

3.2 Identification of Functional Groups

The FT-IR spectra of ASEO was noticed in 700 – 4000 cm^{-1} range. Absorption of IR light by the sample at different wavelengths was observed. The wave number indicated the chemical bond stretching and the higher peak revealed a large amount of chemical functional groups, whereas the lower peak is indicative of a limited amount of functional groups. The FT-IR spectra of DADS represented a sequence of higher absorption peaks at 720, 914, 1073, 1214 cm^{-1} which were associated to C-O stretching vibration from the –C-O-H group, C-S-C group vibrational mode and =C-H bend, C-O and C-O-C vibration, CH_2-CH_2=CH_2 vibration of DADS respectively. Further, lower peaks were associated at 860, 1422, 1512, 1634 represented a series of vibrations at =C-H bending vibration, C-S vibration, C=O stretching and C=C stretching vibration respectively. The vibrations of C=O stretching, C-H stretching vibration were considered at the absorption peaks of 1839, 2977, 3080.

The FT-IR spectra of DATS represented a sequence of higher absorption peaks at 719, 914, 983,1216 cm^{-1} which were associated to C-O stretching vibration from the –C-O-H group, C-S-C group vibrational mode =C-H bend and CH_2-CH_2=CH_2. Further, lower peaks were associated at 857, 1074, 1634 represented a series of vibrations at =C-H bending vibration, C-O and C-O-C vibration, C=O stretching and C=C stretching vibration respectively. The vibrations of -CH_2-CH_2=CH-group, C–O stretching vibration from C–O–H group, C–S vibration, vibration of

-CH_2-CH_2=CH-group, C=O stretching, C-H stretching vibration were considered at absorption peaks of 1293, 751, 1399, 1422, 1843, 2977, 3080.

Furthermore, allylsulfide represented a sequence of higher absorption peaks at 752, 912, 987 cm^{-1} which were associated to C-O stretching vibration from the –C-O-H group, C-S-C group vibrational mode and =C-H bend of allyl sulfide respectively. Further, lower peaks were attributed at 1222, 1425, 1634 represented a series of vibration at -CH_2-CH_2=CH-group, vibration of C–S and -CH_2-CH_2=CH-group, C=C stretching vibration and C=O stretching respectively. The vibrations of C-O and C-O-C vibration, C=O stretching, C–H stretching vibration were considered at absorption peaks of 1073, 1837, 2910, 2977, 3078.

The wavenumber of standards mainly DADS, DATS, AS were compared to determine the existence of compounds in ASEO. The peaks of ASEO were attributed to 719, 915, 984, 1216 cm^{-1} which were associated to C-O stretching vibration from the –C-O-H group, C-S-C group vibrational mode and =C-H bend, CH_2-CH_2=CH_2 vibration of allyl sulfide respectively. Further, lower peaks were attributed at 859, 1420, 1634 represented a series of vibrations at =C-H bending vibration, C–S vibration and vibration of -CH_2-CH_2=CH-group, C=C stretching vibration and C=O stretching respectively. The absorption peaks of vibration of C-O and C-O-C vibration, vibration of -CH_2-CH_2=CH-group, C=O stretching, C–H stretching vibration were considered at absorption peaks of 1074, 1295, 1842, 2911, 2975, 3079. The result obtained from FT-IR spectra revealed that most of the peaks in ASEO resembled the peaks related to standard compounds. This showed that ASEO contain these organosulfur compounds. The data is depicted in Table 2.

3.3 Quantitative Analysis of Phytochemicals

As, most of volatile compounds were identified by GC-MS analysis whereas secondary metabolites such as phenols, flavonoids in ASEO are also present but in less amount. The quantitative estimation was done as mg of gallic acid or quercetin equivalent, which was measured by the regression equation values of phenol (y = 0.697x - 0.354) and flavonoid (y = 0.027x + 0.021). The phenolic and flavonoid content in *Allium sativum* essential oil was

Table 2. FTIR analysis of *Allium sativum* essential oil which was compared with wavenumber of standard compounds such as Diallyl disulfide, Diallyltrisulfide, Allyl sulfide

Standard/sample						
	Diallyl disulfide		**Diallyltrisulfide**	**Allyl sulfide**	***Allium sativum* essential oil**	**Vibration modes**
S.no.	**Wavenumber(cm^{1})**					
1.	710-760	720	719, 751	752	719	C–O stretching vibration from C–O–H group
2.	855-860	860	857	-	859	=C-H bending vibration
3.	900-1000	914,985	914,983	912,987	915,984	Vibrational stretches of C-S-C group and =C-H bend.
4.	1060-1080	1073	1074	1073	1074	C-O and C-O-C vibration
5.	1200-1300	1214	1216,1293	1222	1216, 1295	Vibration of -CH_2-CH_2=CH-group.
6.	1360-1450	1422	1399,1422	1425	1420	C–S vibration, vibration of -CH_2-CH_2=CH-group.
7.	1500-1550	1512	–	–	–	C=O stretching
8	1634	1634	1634	1634	1634	C=C stretching vibration and C=O stretching
9.	1830-1850	1839	1843	1837	1842	C=O stretching
10.	2900-3000	2977	2977	2910	2911,2975	C–H stretching vibration
11.	3000-3100	3080	3080	3078	3079	C–H stretching vibration

found to be 4.99 ± 0.41 µg GAE/mg oil and 98.46 ± 0.16 µg QE/mg oil.

3.4 Level of Total Antioxidant Capacity

The total antioxidant capacity of ASEO was calculated by the regression equation (y = 0.225x + 0.104) of the standard curve. The TAC was found to be 4.99 ± 0.26 µM AAE.

3.5 FRAP Assay

The regression equation of $FeSO_4$ was y = 0.092x + 0.091, which was further used to analyze the sample values. The FRAP assay value of ASEO was 15.80 ± 0.14 µM Fe^{2+}/ L.

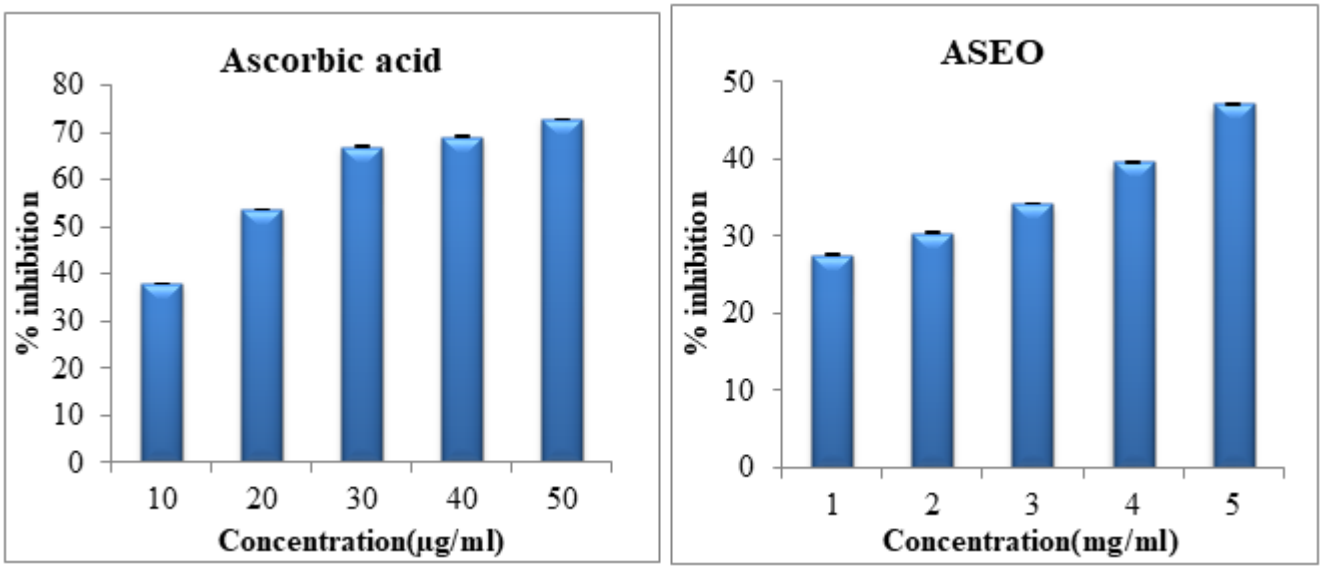
3.6 Antioxidant Assay

3.6.1 DPPH Assay

The antioxidant reaction with free radicals terminates the oxidation process, which results in the prevention of radical formation. The color of DPPH solution changed from purple to yellow in the presence of ASEO and ascorbic acid. The DPPH activity protocol generally depends on the reduction of DPPH solution via the electron donating characteristic of antioxidants. To interpret the outcomes of concentration dependency in this assay, various dilutions of ASEO were considered and their inhibition concentrations were calculated as follows: 27.54, 30.41, 34.26, 39.61 and 47.17 % respectively. To compare the results, ascorbic acid was considered as standard compound and the inhibition concentrations recorded were 38.09, 53.79, 67.00, 69.22 and 72.89 respectively. The regression equations of sample and standard were y = 4.846x + 21.26 and y = 8.502x + 34.68. The IC_{50} values of the sample and standard predicted as 5.93 ± 0.1 mg/ml and 1.801 ± 0.1 µg/ml respectively (Figure 1(a) and 1(b); Table 3).

3.6.2 ABTS Assay

This assay depicts the reduction of the blue-green color of ABTS cation radical solution prepared by mixing the ABTS with potassium persulfate for 12-16 h in dark condition. The addition of ASEO and Trolox as reference compound changes the blue-green color of the solution into a colorless solution within 5 - 6 min. An increase in the concentration of trolox as well as ASEO decreases the color of the solution, which was represented by decrease in absorbance due to an increase in antioxidant concentration. Inhibition concentrations of various dilutions of the sample were 31.41, 50.72, 66.27, 76.30 and 85.40. Beside this, inhibition concentrations of the standard were 10.89, 14.32, 31.21, 65.14 and 78.58. The regression equations of sample and standard were y = 13.35x + 21.95 and y = 18.61x -15.82 .The IC_{50} values of ASEO and standard were 2.10 ± 0.01 mg/ml and 3.53 ± 0.02 µg/ml (Figure 2(a) and 2(b); Table 3).

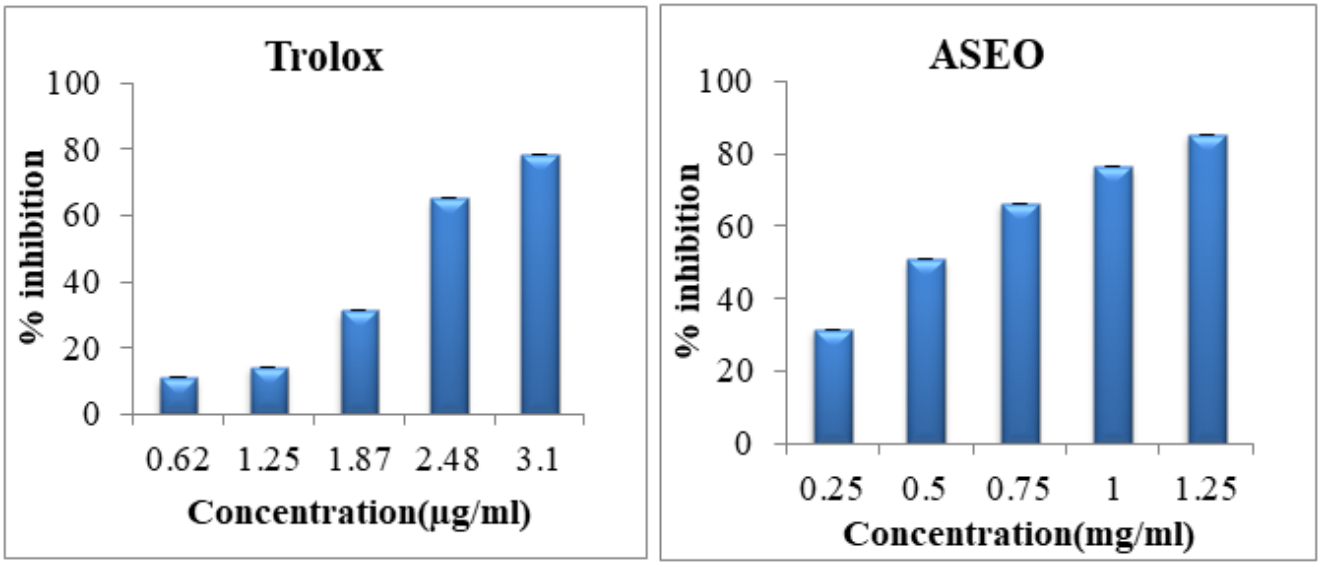


(1a) **(1b)**

Figure 1. DPPH scavenging activity of standard compound, ascorbic acid (Figure 1a) and *Allium sativum* essential oil (Figure 1b). Data expressed as percentage inhibition and significance level of the standard is p<0.05 and sample is p>0.05.

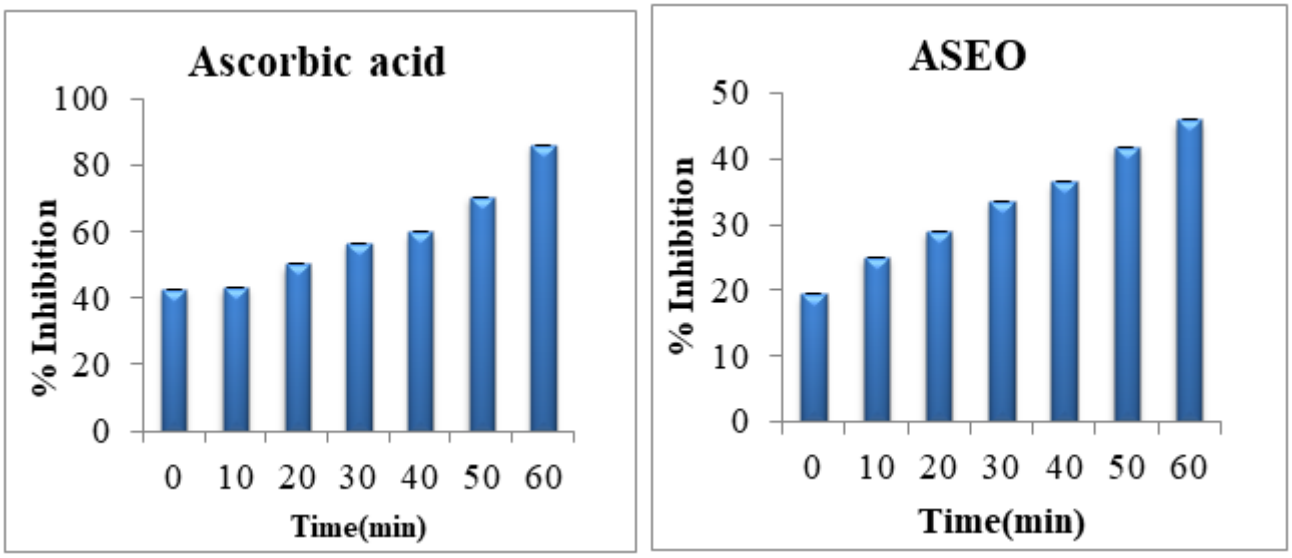
3.6.3 β-carotene Bleaching Activity

The assay is related to the reduction of color intensity of β-carotene at 470 nm for every 10 min for 1h. It determines the ability of antioxidants to inhibit lipid peroxidation during initiation as well as elongation. Initially, in the first half of incubation, the scavenging activity of the standard reaches to 56.7 % whereas the sample inhibits 33.6 %. After incubation of 1 hour, the scavenging rate increased to 86.14% and 45.93% in both the standard and sample respectively. The regression equation values for standard and sample were as follows: y = 6.933x + 30.8 and y = 4.301x + 15.83 respectively. The results showed



(2a) **(2b)**

Figure 2. ABTS scavenging assay of standard compound, Trolox (Figure 2a) and *Allium sativum* essential oil (Figure 2b). Data expressed as percentage inhibition and significance level of the standard is $p<0.05$ and sample is $p<0.05$.



(3a) **(3b)**

Figure 3. β-carotene bleaching assay of standard compound, ascorbic acid (Figure 3a) and *Allium sativum* essential oil (Figure 3b). Data expressed as percentage inhibition and significance level of the standard is $p<0.05$ and sample is $p<0.05$.

the ASEO significantly inhibits bleaching by β-carotene when compared to standard compound. The IC_{50} values of standard and sample were 2.77 ± 0.1 µg/ml and 7.94 ± 0.01 mg/ml (Figure 3(a) and 3(b); Table 3).

3.6.4 Scavenging of Nitric Oxide

Excessive Nitric Oxide (NO) concentration is linked to numerous inflammatory disorders. Increased levels of nitric oxide cause macromolecule damage that leads to cellular injury. NO scavenging activity is based on the production of nitric oxide ions from sodium nitroprusside, which further react with oxygen to generate nitrite ions. The addition of antioxidants against oxygen molecules reduces the generation of nitrite ions in solution, which can be detected at 546 nm. At 100 **µg/ml, the** percentage scavenging activity of the standard and sample were 62.11 % and 43.75 % respectively. The regression equations of the standard and sample were y = 7.501x + 27.45 and y = 5.001x + 22.54 respectively. The IC_{50} values of the NO scavenging assay of the standard and sample were 3.01 ± 0.01 and 5.49 ± 0.001 µg/ml respectively (Figure 4, Table 3).

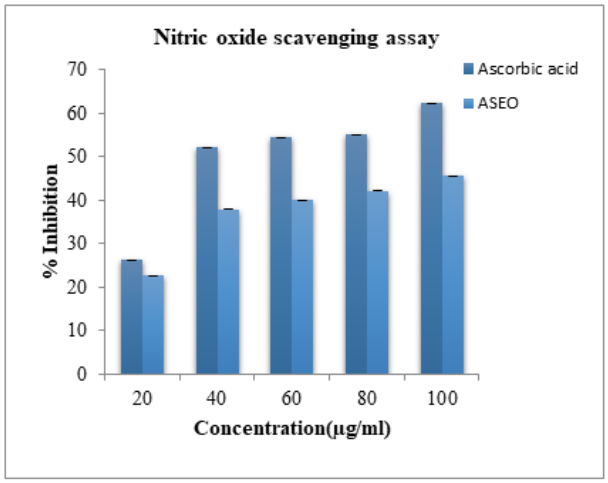
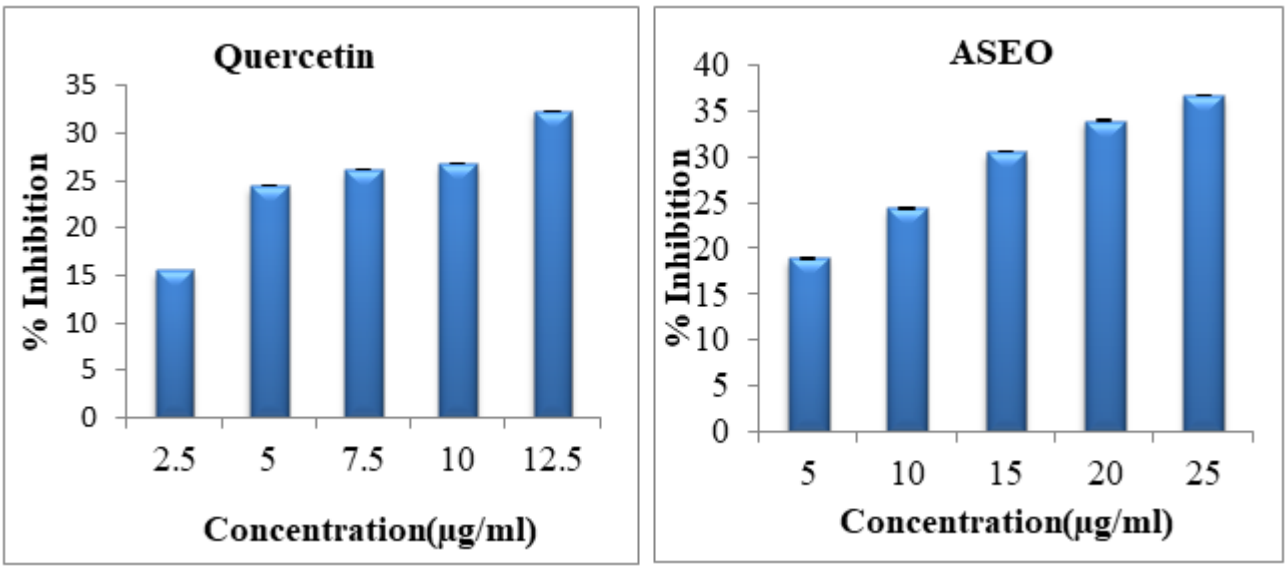


Figure 4. NO scavenging assay of standard compound (ascorbic acid) and *Allium sativum* essential oil. Data expressed as percentage inhibition and significance level of the standard and sample is insignificant (p>0.05).

3.6.5 Scavenging of Hydroxyl Radical

Hydroxyl radical (highly reactive species) cause serious destruction to biological molecules. It binds nucleotides and causes DNA damage, leading to inflammatory disorders such as carcinogenesis, mutagenesis and cytotoxicity. The capacity to scavenge hydroxyl free radicals depends on the antioxidants in the sample and standard. The % scavenging potential of the standard was 15.55, 24.42, 26.15, 26.74 and 32.36 %. Similarly, the percentage scavenging activity of the sample was 18.88, 24.36, 30.52, 33.97 and 36.72 %. The regression equations of standard and sample were y = 3.594x + 14.26 and y = 4.529x + 15.30. The IC_{50} values of the standard and sample



(5a) **(5b)**

Figure 5. Hydroxyl scavenging assay of standard compound (quercetin) (Figure 5a) and *Allium sativum* essential oil (Figure 5b). Data expressed as percentage inhibition and significance level of the standard is p<0.05 and sample is p>0.05.

were 9.94 ± 0.01 µg/ml and 7.66 ± 0.01 µg/ml respectively (Figure 5(a) and 5(b); Table 3).

3.6.6 Superoxide Scavenging Assay

Superoxide free radicals accelerate DNA damage by targeting essential biomolecules, which ultimately leads to cellular damage. In this assay, the superoxide scavenging potential of ASEO and the standard compound was analyzed using PMS-NADH-PMS system. The results explained that there is a decrease in absorbance by increasing the antioxidant concentrations. At 100 µg/ml, inhibition concentrations of standard and sample were 68.8% and 41.02%. The regression equations of standard and sample were y = 5.173x + 43.44 and y = 1.318x + 34.788 respectively. Further, the IC_{50} values of the standard and sample were 1.27 ± 0.01 and 11.55 ± 0.002 µg/ml respectively (Figure 6, Table 3).

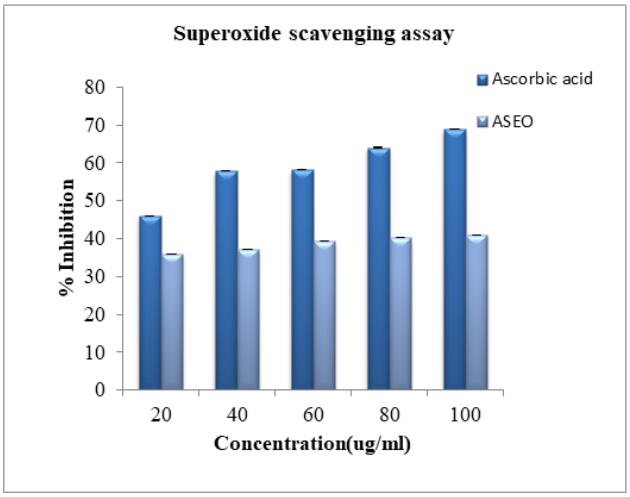
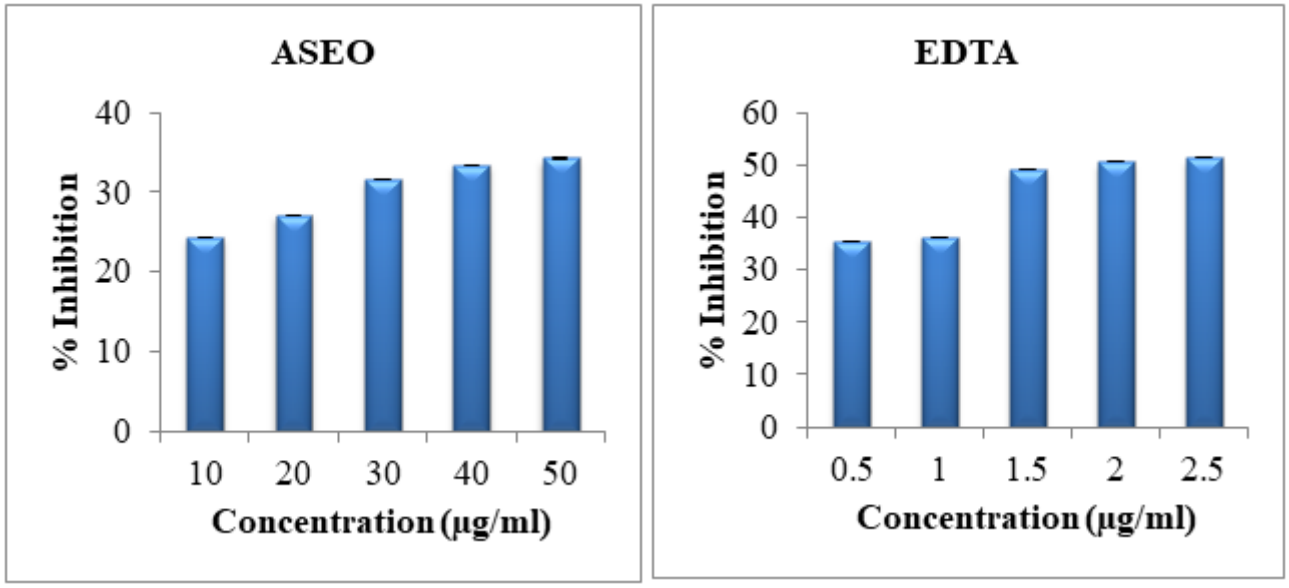


Figure 6. Superoxide scavenging assay of standard compound, ascorbic acid and *Allium sativum* essential oil. Data expressed as percentage inhibition and significance level of the standard is p<0.05 and sample is p<0.05.

3.6.7 Metal Chelation Assay

The prediction of results can be elucidated by reducing the color of reaction tubes with increasing the concentration of ASEO and EDTA. The % inhibition of ASEO was 24.39, 27.08, 31.65, 33.45, 34.25, whereas the inhibition concentrations of EDTA were 35.22, 36.29, 49.14, 50.56, 51.43. The regression equations of standard and sample were y = 4.6686x + 30.529 and y = 2.6092x + 22.338 respectively. The IC_{50} values of EDTA and sample were 4.17 ± 0.01 and 10.60 ± 0.03 µg/ml respectively (Figure 7(a) and 7(b), Table 3).



(7a) **(7b)**

Figure 7. Metal chelation assay of *Allium sativum* essential oil (Figure 7a) and standard compound, EDTA (Figure 7b). Data expressed as percentage inhibition and significance level of the sample is p>0.05 and standard is p<0.05.

Table 3. The table showed the antioxidant potential based on IC_{50} values of *Allium sativum* essential oil and standard compound

Sample/standard	DPPH	ABTS	Nitric oxide	Superoxide Scavenging	Metal chelating	β-carotene	Hydroxyl Scavenging
Allium sativum essential oil	5.93 ± 0.1 mg/ml	2.10 ± 0.01 mg/ml	5.49 ± 0.001 µg/ml	11.55 ± 0.002 µg/ml	10.60 ± 0.03 µg/ml	7.94 ± 0.01 mg/ml	7.66 ± 0.01 µg/ml
Ascorbic acid	1.801 ± 0.1 µg/ml	–	3.01 ± 0.01 µg/ml	1.27 ± 0.01 µg/ml	–	2.77 ± 0.1 µg/ml	–
Trolox	–	3.53± 0.02 µg/ml	–	–	–	–	–
EDTA	–	–	–	–	4.17±0.01 µg/ml	–	–
Quercetin	–	–	–	–	–	–	9.94 ± 0.01 µg/ml

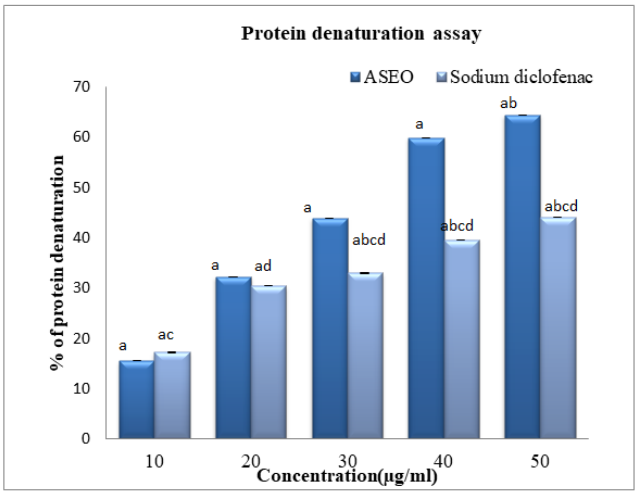


Figure 8. Protein denaturation assay of ASEO and sodium diclofenac (reference drug). The error bars shows mean±SE of triplicates. The bars represent alphabetical letters which explains different p values (a = $p<0.001$, b = $p<0.01$, c = $p<0.05$, d = $p>0.05$).

3.7 Effects of ASEO on *In Vitro* Anti-inflammatory Potential

Allium sativum essential oil showed significant anti-inflammatory potential in comparison to the standard drug, sodium diclofenac. The present investigation revealed concentration dependent inhibition of protein denaturation in the presence of ASEO and sodium diclofenac within a concentration range of 10-50 µg/ml. From observing the result pattern, it can be considered that the response of sodium diclofenac against protein denaturation was found to be less in comparison to ASEO. This was further observed by considering the IC_{50} value of sample at 3.53 µg/ml which is less than the standard drug (5.71 µg/ml). Therefore, the outcome of the assay revealed that ASEO possesses anti-inflammatory potential, which could be further used as natural drug development tool for inflammatory disorders and for other industrial products (Figure 8).

3.8 Impact of ASEO on Glucose Absorption by Yeast Cells

Allium sativum essential oil initiated the glucose absorption through the membrane of yeast cells. Uptake of glucose at different concentration ranges (5, 10 and 25 mM) was compared to the standard drug, metronidazole (Figure 9). The consequence of standard drug on the uptake of glucose at 25 mM glucose concentration was more in comparison to ASEO (Figure 9(c)). In the case of ASEO, the uptake of glucose at 1 mg/ml was > 50 % which was increased to 80 % when 5 mg/ml of ASEO was used (Figure 9(a)). It means that an increase in ASEO concentration enhanced yeast cells capacity towards glucose uptake. Further, Figure 9(b) and 9(c) also showed a increase in glucose absorption with a gradual rise in sample concentration by yeast cells. There is an inverse relationship to glucose concentration when the glucose absorption by yeast cells is compared at various glucose concentrations for the same amount of ASEO.

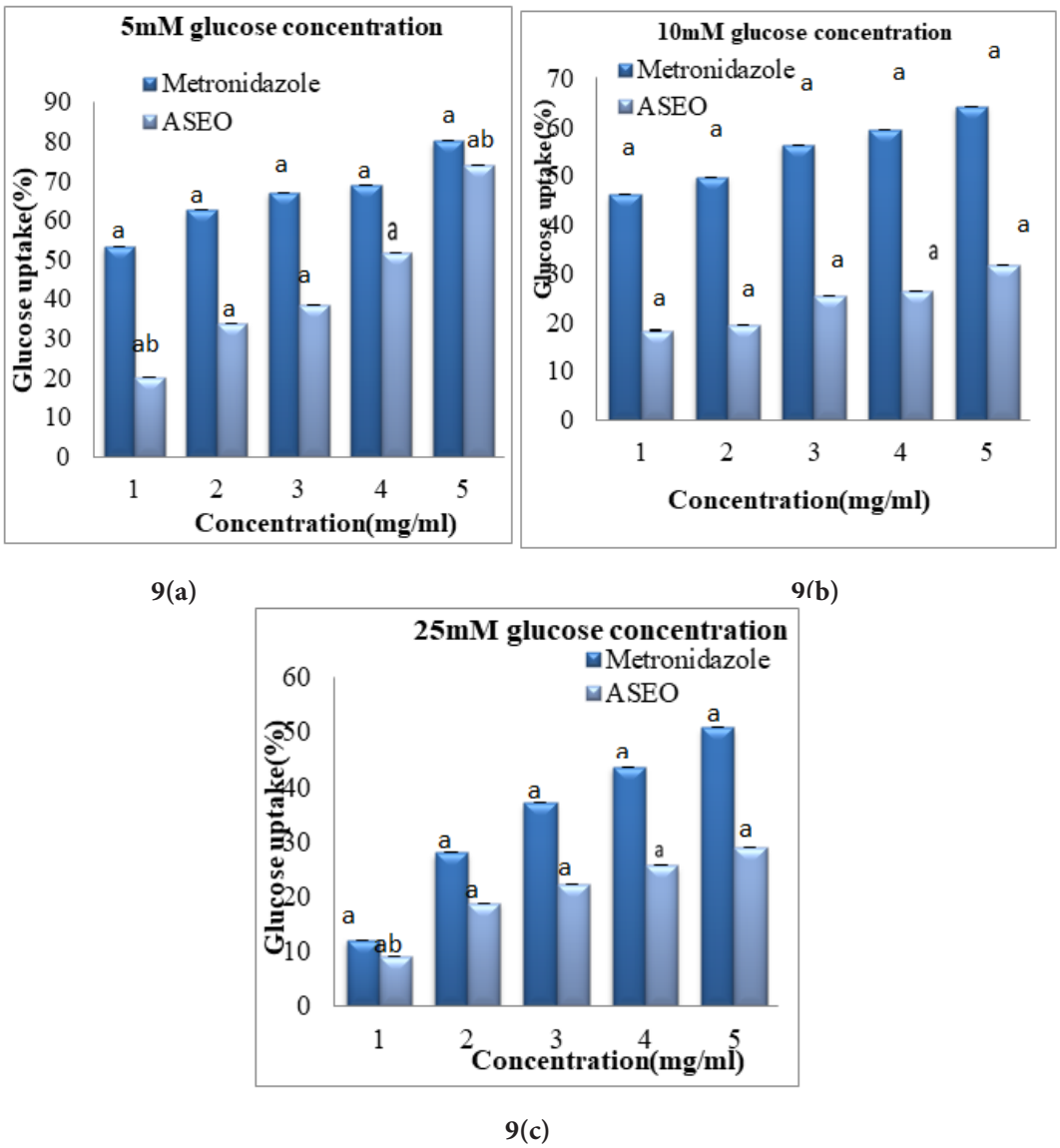


Figure 9. Glucose absorption by yeast cells at 5mM (Figure 9a), 10mM (Figure 9b), 25mM (Figure 9c) glucose concentration in the presence of ASEO. ASEO = *Allium sativum* essential oil. The error bars shows mean±SE of triplicates. The bars represent alphabetical letters which explains different p values (a = p<0.001, b = p<0.01, c = p<0.05, d = p>0.05).

4. Discussion

Garlic is the most common natural product consumed for medicinal and culinary purposes since ancient times. The antioxidant, anti-inflammatory and anti-diabetic potential of ASEO extracted from the fresh bulbs of *Allium sativum* may be due to the presence of organosulfur components. Antioxidant potential and identification of chemical components of *Allium sativum* essential oil are in agreement with the study conducted earlier[25]. These organosulfur compounds are known to function as antioxidants that protect tissues from oxidative stress and inflammatory conditions. In the current study, it was noticed that the constituents present in the essential oil of garlic can effectively scavenge the reactive oxygen species mainly hydroxyl, superoxide, Nitric Oxide radicals in a concentration-dependent manner. The body's response to infections, irritations, or other ailments is commonly known as inflammation. As a result, it affects a number of illnesses, including arthritis, cancer, diabetes, and asthma. This can be overcome via assessing the anti-inflammatory

activity of ASEO which is performed by the *in vitro* protein denaturation assay. Protein denaturation is one of the major cause of inflammatory disorders due to auto antigens production in certain arthritic diseases[26]. So, there is a need to explore the agents that can reduce protein destruction. This could be justifiable for the production of anti-inflammatory drug. An increase in protein denaturation inhibition with respect to an increase in sample concentration concluded protein stabilization by ASEO and the reference drug. From IC_{50} values, it can be assumed that ASEO is more effective than sodium diclofenac.

Furthermore, the anti-diabetic potential was performed by the glucose absorption through yeast cell membrane. Glucose transfer via the yeast cell membrane by means of facilitated diffusion rather than the phosphotransferase enzyme system. Factors responsible for glucose absorption by yeast cells involve internal glucose concentration or subsequent glucose metabolism. When interior sugar is converted into various other by-products, the glucose concentration will automatically reduce and upregulate the glucose absorption by yeast cells. Based on this consideration, it can be possible that ASEO enhanced glucose absorption through yeast cells which may be due to both facilitated diffusion and increased glucose metabolism. This may be due to binding of ASEO with glucose and its transfer across cell membranes for further metabolism.

5. Conclusion

The current study concluded that ASEO exhibits immense anti-inflammatory, anti-diabetic and anti-oxidant potential. The experimental data explained the medicinal potential of ASEO for counteracting ailments mainly inflammation and diabetes. Apart from this, further examinations are still needed which should be focused mainly on the isolation of bioactive compounds that account for remarkable effects and determining the mechanisms related to the anti-diabetic and anti-inflammatory potential of *Allium sativum* in preclinical studies.

6. Acknowledgements

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