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Hydrogen peroxide scavenging capacity and phytochemical analysis of *Medicago sativa* (*Alfalfa*) leaves

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Abstract
This research work was carried out to evaluate the hydrogen peroxide scavenging capacity and phytochemical analysis of *Medicago sativa* leaves. The leaves were found to have high antioxidant activities. *Medicago sativa* extract caused inhibition of hydrogen peroxide which was concentration dependent. From the analysis, it was discovered that at every concentration, the methanol extract of the leaves showed better scavenging capacity than the standard used. The phytochemicals from the leaves of *Medicago sativa* (Linn.) were extracted with ethanol and subjected to GC/MS analysis. The chromatogram depicted five peaks indicating the presence of five compounds. 1,1-dimethoxy-2-methylpropane (59.781%), 1,3-dimethyl Benzene (16.688%), Ethanol, 2-(2-butoxyethoxy) acetate (2.788%), N-(4-Methylbenzenesulfonyl)-2-methylazetidin-3-one (1.921%). Brief analysis of the compounds reveals the rich pharmacological potential of this part of the plant and thus justify the use of the Leaves of this plant in the treatment of various diseases.

Keywords: Hydrogen peroxide, phytochemicals, scavenging

1. Introduction
Medicago sativa (Linn.) popularly known as *Alfalfa* is a genus of the family Leguminosae, it has long been used by Herbalists to treat variety of ailments in Turkey, China, Iraq, India and America. According to reports, Alfalfa has recorded many health benefits, it has the ability to lower cholesterol, report showed that 160 grams of *Alfalfa* seeds per day could decrease total blood cholesterol levels. This may be as a result of its high content of saponins, which are plant compounds known to lower cholesterol levels. It serves as an antioxidant, antiulcer, antimicrobial, hypolipidemic, antidiabetic, anti-inflammatory, anti-asthmatic and diuretic agent. It is also used in the treatment of stroke, heart disease, diabetes, cancer, and menopausal symptoms in women [1]. *M. sativa* is used traditionally to improve the memory and as a rejuvenator. It cures kidney pain, cough, sore muscles and problems related to central nervous system (CNS) [2]. In India, herbalists use *M. sativa* to treat ulcers, arthritis pain and fluid retention. Early Americans used *M. sativa* to treat arthritis, boils, cancer, scurvy, urinary and bowel problems. In Turkey it is used as cardiotonic and to treat scurvy and arthritis [3, 1].
Alfalfa extract improves blood sugar control, it lowers blood sugar levels by increasing the release of insulin from the pancreas. *Alfalfa* has been reported to contain high plant compound called phytoestrogens, which are chemically similar to the hormone estrogen. Phytoestrogens have several benefits which include easing menopausal symptoms that are caused by decreased levels of estrogen. *M. sativa* can be used as an alternative natural carotenoid instead of the synthetic apo-ester in goldfish diets [4]. A compound isolated from *M. sativa* known as *Medicagenic* acid exhibited significant nematicidal activity against the plant-parasitic nematode *Xiphinema index* [1]. A study showed that *M. sativa* or *alfalfa* has the ability to reduce cell death and DNA damage caused by free radicals. It does this by both lowering the production of free radicals and improving the body's ability to fight them [6]. Antioxidants are known to reduce the number of free radicals in our system that can cause cell damage and disease. Foods protect us from free radical damage, which is responsible for many of the effects of aging on both the body and mind [1, 7].
High levels of oxidative stress affect every organ and system in the body and have been linked with many diseases from Alzheimer's disease, cancer and heart disease to accelerated aging, asthma, diabetes, etc.
Oxidative stress is believed to lead to the development of the most prevalent chronic diseases and disorders killing adults today, especially heart disease, cancer and diabetes. The body use antioxidants to lessen the impact of free radicals throughour diets [1]. However, *Alfalfa* may cause uterine stimulation or contractions. Therefore, it should be avoided during pregnancy. In spite of the long traditional use of *M. sativa* for treatment of various ailments, many of the claims have not been scientifically evaluated to ascertain its traditional claims.

2. Methodology
2.1 Extraction of Plant Materials / Preparation
The leaves of *Medicago sativa* were pounded with mortar and pestle, soaked in ethanol and methanol in different beakers for 48 hours and filtered. The filtrates were concentrated with rotary evaporator at 40^{o}C and were used for GC- MS and antioxidant analyses respectively.

2.2 Hydrogen Peroxide Scavenging Activity of *Medicago sativa* L.
Method of [9] was modified and used to carry out the scavenging activity of extracts of the leaves of *Medicago sativa* towards hydrogen peroxide radicals. Solution of hydrogen peroxide (40 Mm) was prepared in phosphate buffer pH 7.4 and its concentration was determined by using UV spectrophotometer at absorbance of 560 nm. 0.1mg/ml of the extract was added to hydrogen peroxide solution and absorbance measured at 560 nm using UV spectrophoto-meter against a blank solution

containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extract and standard compound was calculated using the given formula:
Where,
= Absorbance of the extract at 560 nm
= Absorbance of the blank at 560 nm
The experiment was repeated in triplicate.

Calculation of IC_{50}

The methanolic extracts of *Medicago sativa* at various concentrations (0.625-1mg/ml) were taken for the study and IC_{50} values which shows 50% inhibition was calculated using regression analysis in MS excel.

Statistical analysis

All experimental measurements were carried out in triplicate and were expressed as average of three analyses ±standard deviation. Statistical analyses were performed by one sample t-test and p-values were done by one way ANOVA. The p-value<0.05 were regarded as significant.

2.3 GC/ MS Analysis

The instrument used for the analysis of Gas chromatography was GC-MS Shimadzu QP 2010, Japan gas chromatography 5890-11 with a fused GC column (OV- 101) coated with polymethyl silicon (0.25 nm x 50m). This was performed using the conditions as follows: Temperature programming from 80-200ºC held at 80 ºC for 1 minute, rate 5 ºC/ min and at 200 ºC for 20 mins. FID temperature 300 ºC, injection temperature 250 ºC, carrier gas nitrogen at a flow of 1ml /min, split ratio 1:75. The column length was 30 m with a diameter of 0.25 mm and the flow rate of 50 ml/min. the elutes were automatically passed into a mass spectrometer with a dictator voltage set at 1.5 kv and sampling rate of 0.2 sec. The mass spectrum was also equipped with a computer fed mass spectra data bank. Hermle Z 233 M-Z centrifuge Germany was used. Reagents and solvents like ethanol, chloroform, diethyl ether, and hexane were all analytical grade and were procured from Merck, Germany.

The database of National Institute Standard and Technique (NIST) was used for interpretation of mass spectrum. Then the phytochemicals were identified based on the hits returned after comparing the unknown peak value and chromatogram from GC MS against the known chromatogram, peak value from the NIST Library database. Subsequently, the details about their molecular formula, molecular weight, structure were also obtained [10, 11].

3. Results and Discussion

Table 1 shows hydrogen peroxide scavenging activity of the methanol extract and standard. The regressions coefficient R^2 of 0.8737 and 0.9178 for the methanol extract and standard respectively suggest that *Medicago sativa* extract caused inhibition of hydrogen peroxide which was concentration dependent. At a concentration of 1mg/ml, the scavenging percentages were 68.7 and 67.0 for methanol extract and standard respectively. For both standard and extracts, P values were significant ($p < 0.05$). The methanol extract showed better scavenging capacity than Ascorbic acid which was used as the standard. This was supported by their IC_{50} values, which for the extract was found to be 0.197 mg/ml compared to standard ascorbic acid 0.211 mg/ml. The presence of different phytochemicals in the plant extract of which tannins have a higher percentage could be an explanation for the higher antioxidant activity of the plant extract than the standard used in this study [12].

Table 1: Percentage Inhibition of concentrations of *M Sativa* in comparison with Ascorbic acid

Concentration (mg/ml)	% Inhibition of *M. Sativa*	% Inhibition of Ascorbic acid
0.0625	28.7	25.0
0.125	30.3	28.7
0.25	59.3	54.7
0.50	66.0	61.0
1.0	68.7	67.0

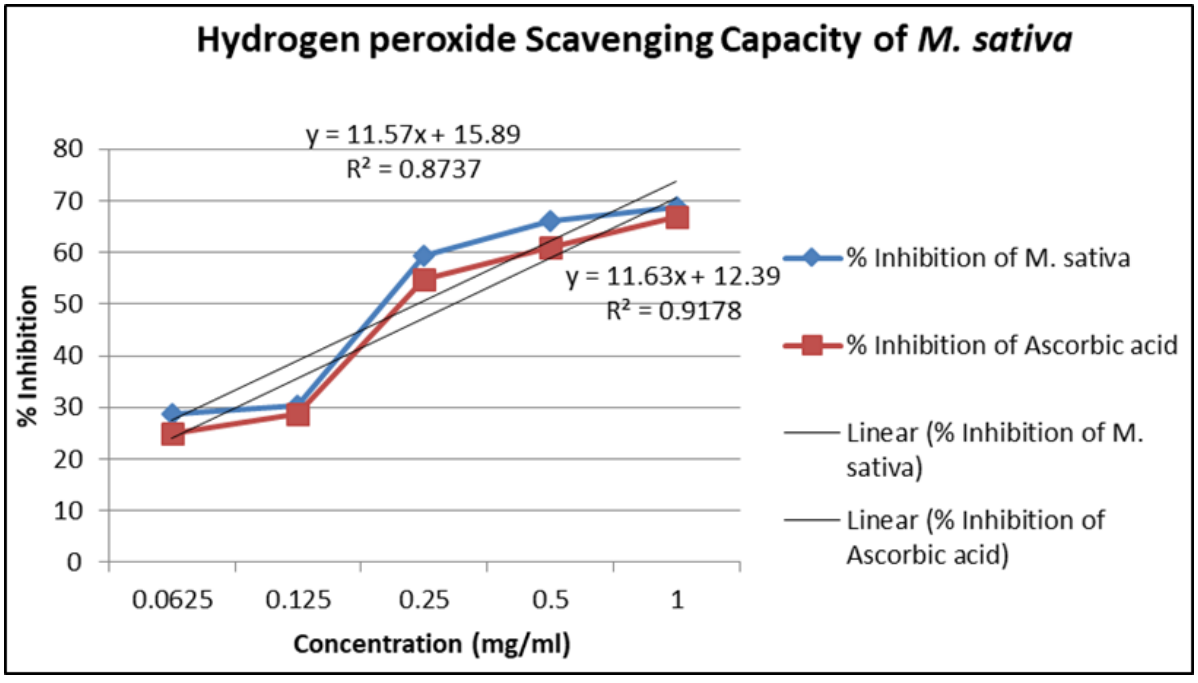

Fig 1: Graph of hydrogen peroxide scavenging capacity of *M. sativa*

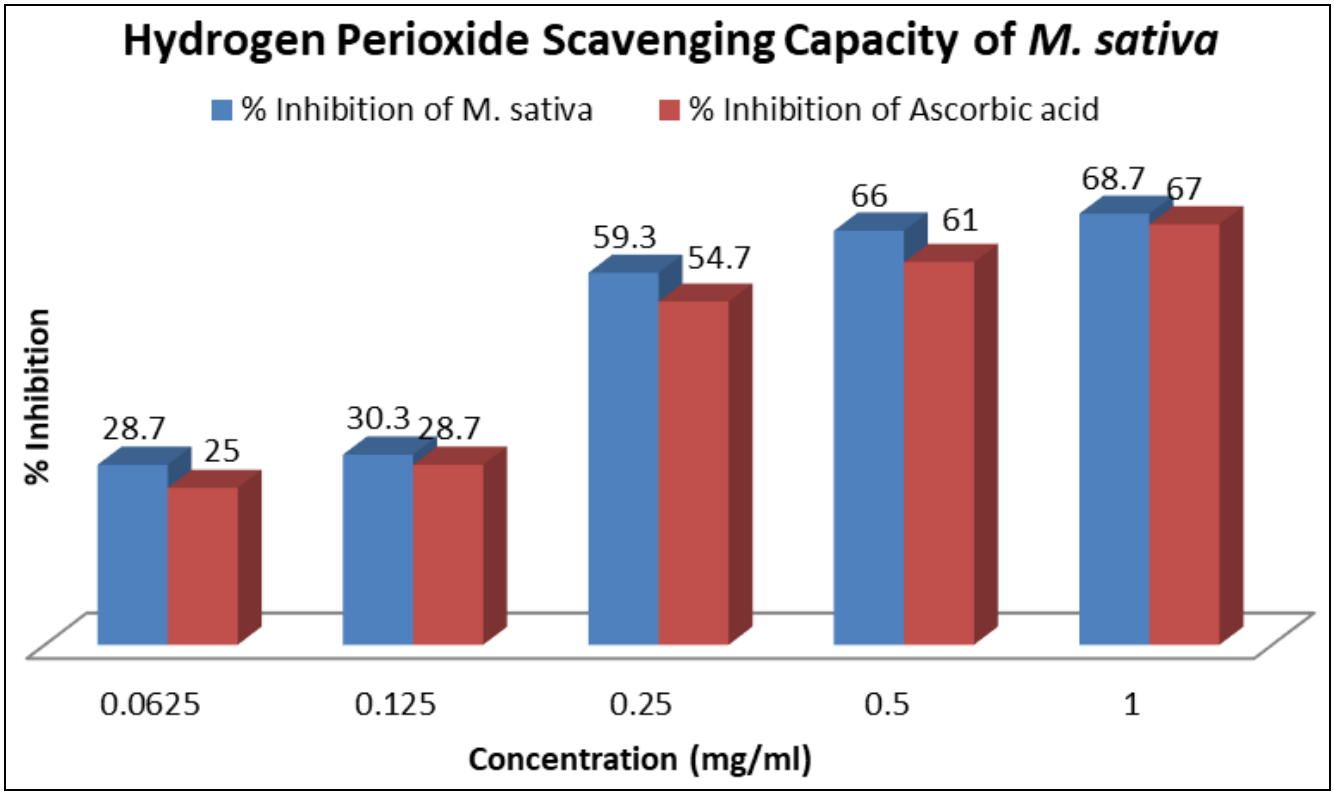

Fig 2: Bar chart showing Hydrogen peroxide scavenging capacity of *M. sativa,* compared with ascorbic acid

Table 2: The list of phytochemicals identified with respect to the chromatogram obtained from GC-MS analysis of ethanol extract of the leave of *Medicago sativa*

SN	RT	Component	Formula	MW	%
1	2.914	Propane, 1,1-dimethoxy-2-methyl-	$C_6H_{14}O_2$	118	59.781
2	3.157	Benzene, 1,3-dimethyl-	C_8H_{10}	106	16.688
3	4.416	Benzene, 1,3-dimethyl-	C_8H_{11}	107	18.822
4	4.877	Ethanol, 2-(2-butoxyethoxy)-, acetate	$C_{10}H_{20}O_4$	204	2.788
5	5.322	N-(4-Methylbenzenesulfonyl)-2-methylazetidin-3-one	$C_{11}H_{13}NO_3S$	239	1.921

The ethanol extracts of the leaves of *Medicago sativa* contain rich phytochemical compounds. The chromatogram of the GC/MS analysis showed five compounds. The individual names of compounds identified with respect to their individual peak value were listed in table 2 with their peak number, retention time molecular formula, molecular mass and percentage of area are listed in detail in table 2. The compounds are; 1, 1-dimethoxy-2-methylpropane 59.781 %, 1, 3-dimethyl Benzene 16.688 %, Ethanol, 2-(2-butoxyethoxy) acetate 2.788 %, N-(4-Methylbenzenesul-fonyl)-2-methylazetidin-3-one 1.921 %. The first compound is 1, 1-dimethoxy-2-methylpropane and it constituted the bulk of the oil with the highest percentage (59.781 %). Analysis of the compound showed that the compound can be used as an additive. It is used as a flavouring agent in food [13]. Compound 2 *is* an aromatic hydrocarbon, 1, 3-dimethyl Benzene also known as *meta*-xylene. The major use of *meta*-xylene is in the production of isophthalic acid which is used as a copolymerizing monomer to alter the properties of polyethylene terephthalate. M*eta*-Xylene is also used as a raw material in the manufacture of 2, 4-and 2, 6-xylidine as well as a range of smaller-volume chemicals [14].

Conclusion

Free radicals are known to be the cause of many pathological conditions such as inflammation, metabolic disorders, cells ageing, atherosclerosis and carcinogenesis. Free radicals involved Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). The ROS are involved in more than one hundred diseases including, malaria, acquired immunodeficiency syndrome, diabetes, anaemia and cardiovascular diseases. The leaves of *Medicago sativa* have been found to be good antioxidants from the result of the analysis. The presence of a wide range of chemical compounds indicates that the plant could lead the way for the development of novel agents having good biological activity. Many chemical compounds are present in the plant but isolation of active constituents by using various appropriate chromatographic techniques should be carried out. Further studies can be carried out using different extraction methods.

Below are the GC/MS analysis showing the structures of the compounds with their fragmentation patterns.

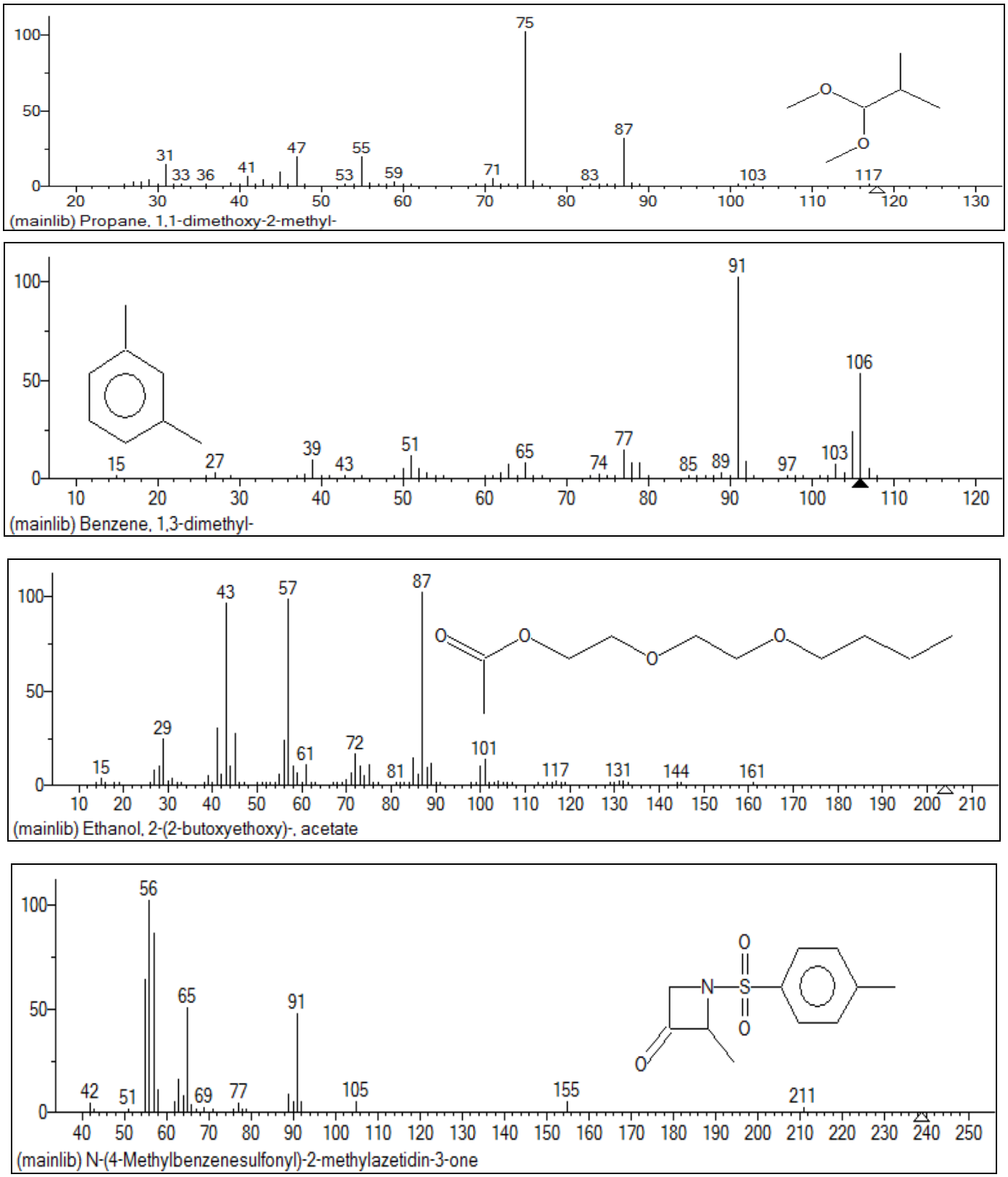

Fig 4: GC-MS Analysis of the leaves of *Medicago sativa*

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