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EVALUATION OF IN VITRO ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF METHANOL EXTRACT OF STANDARDIZED LACTUCA SATIVA L. LEAVES

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Abstract:

Objective: The aim of present work was to standardize and evaluate the antimicrobial and antioxidant activities of L. sativa leaves. Method: Physiochemical, phytochemical, and TLC technique was used for the standardization of leaves. A methanol extract of leaves was evaluated for antimicrobial activity by using agar well diffusion method and antioxidant assay by DPPH and FRAP assay. Results: Physicochemical such as loss on drying (7.19%), total ash (5.2%), acid insoluble ash (4.1%), water soluble ash (0.9%), hexane soluble (2.4%), chloroform (7.9%), methane soluble (16.2%), and water-soluble (12.6%) extractive value were identified. The bioactive secondary metabolites such as alkaloids, phenols and tannins, phytosterols and triterpenoids, saponins, anthraquinones glycosides, and cardiac glycosides were identified. RP-TLC and normal TLC studies of the methanol extract were carried out and the observations will help in identification of crude powder. Total phenolic and flavonoid content of the methanol extract was found to be 53.64 ± 0.27mg GAE/g and 16.55 ± 0.49mg QE/g using Folin-Ciocalteu and AlCl3 colorimetric method respectively. The antibacterial activity of the extract was exhibited against S. aureus, S. aureus (CI), B. subtilis, and E. coli at concentration 20mg/ml. The zone of inhibition ranged from 8.2-13.8 mm, MIC's ranged from 6.25 to 25 mg/ml and MBC of 50 and <50 mg/ml. In the DPPH assay, the IC50 value of methanol extract and ascorbic acid were 210.57 µg/ml and 31.92 µg/ml respectively. Conclusions: These results clearly indicate that L. sativa is to be a natural source of antioxidant and it could be alternative antibiotics.

Keywords: Lactuca sativa, leaves, standardization, antimicrobial, antioxidants.

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INTRODUCTION:

The leaves of *Lactuca sativa* L, F. Asteraceae is an important vegetable throughout the world because of its fast growth and commercial value and mainly used as a fresh salad food [1]. It is an annual plant apart from the leaves the other parts like stem and seeds are also used. The region of the Middle East (Egypt and Iran) is considered a Centre of lettuce origin. Many wild Lactuca species occur between the Euphrates and Tigris Rivers [2]. It is especially important as a commercial crop in Asian, American and European continents, where China, the U.S., Spain, Italy, India, and Japan are among the world's major producers, recently China is one of the leading countries, produced approximately half of the total production of lettuce [3]. It is most often used for salads in Saudi recipe or all over the world, although it is also seen in other kinds of food, such as soups, sandwiches, and wraps [4]. The leaves of Lettuce popularly known as Khas is widely consumed in Saudi Arabia and other parts of the world. Lettuce is a common vegetable in Saudi Arabia, being cultivated on more than 4000 ha of land and production and consumption are steadily rising [5]. It is not only rich in vitamins A and C which support skin health and immune system and mineral potassium which keep healthy bone but also contains dietary fiber and other minerals, which make it a very nutritious source of food [6]. It is an economically important vegetable for the salad next to tomato. Fresh leaves of Lettuce collected from South Africa is reported for the total phenols, flavonoids, β-carotene, ascorbic acid, and antioxidant property. Red lettuce varieties are recommend for the salad due to the high content of total phenols and flavonoid compounds [7]. Intake of lettuce is reported to improve health benefits, dietary fiber content that helps in digestion, β-carotene and lutein reducing the risk of cancers, heart disease and stroke, phenolic compounds and Vitamin A (β-carotene) and C are responsible for antioxidant properties [8]. Among the different types of lettuce, the utmost common ones are Romaine, Iceberg and lose leaf and generally consume freshly because after long storage the active compounds present in it start decreasing [9]. Reactive oxygen species (ROS), causing damage to proteins, lipids, and DNA, have been associated with several health conditions and minimizing the oxidative damage is one of the most important approaches to the initial prevention of oxidative stress-related health problems [10]. The fruits and vegetables are a good source of the natural antimicrobial agents and used to protect from several infectious disease conditions [11]. To the best of our knowledge there are no published reports regarding the physicochemical, phytochemicals and in vitro antimicrobials and antioxidant activity of Saudi Arab cultivated green lettuce leaves to confirm its medicinal properties. The objective of this investigation was to standardize the leaves powder using physicochemical parameters like ash content, extractive value, loss on drying and its extract using RP-TLC and preliminary phytochemical analysis and was to quantify the bioactive compounds like total phenolic and flavonoid contents and was to evaluate the antimicrobials and antioxidant properties.

MATERIALS AND METHODS:

Materials:

Chemicals and Bacterial stains

All the chemical and reagents procured were of analytical grade, TLC was procured from Merck while the other AR Grade material like glacial acetic acid, ethanol, potassium iodide, lead acetate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), Ascorbic acid, potassium ferricyanide were from Sigma Aldrich. Mueller-Hinton agar (Oxoid, Basingstoke, England) and *Staphylococcus aureus* (ATCC 29213), *Staphylococcus aureus* (Clinical isolated), *Bacillus subtilis* (ATCC 10400) and *Escherichia coli* (ATCC 10536) were obtained from College of Pharmacy, Microbiology Research Lab, Prince Sattam Bin Abdulaziz University, Al-Kharj Saudi Arabia.

Collection and identification of plant material

The leaves of *L. sativa* L was collected during the month of December 2017 from the Local mall of Al-Kharj, Saudi Arabia. The collected plant was then authenticated by Dr. M. Atiqur Rahman, from College of Pharmacy, Medicinal, Aromatic and Poisonous Plants Research Center, King Saud University, Riyadh. A voucher specimen (PSAU-CPH-6-2017) is maintained in the herbarium of College of Pharmacy, Prince Sattam Bin Abdulaziz University. The leaves were washed under running tap water to remove the surface dirt and the leaves were air dried under shade. The dried leaves were powdered and used for physicochemical standardization and for the preparation of methanol extract.

Methods

Preparation of methanol extract

A mass of 100 g of powdered plant material was extracted with 90% methanol (500 ml) in Soxhlet apparatus for 5h and it was filtered using filter paper (Whatman no 2). The alcoholic crude extract was freed from solvent using rotary vacuum evaporator and then air dried. The resulting methanol extract was used for the TLC identification and phytochemicals

screening, phenolic and flavonoid contents, antimicrobials, and antioxidant activities.

Physicochemical and Fluorescence analysis of powder

Physicochemical parameters such as percentage of ash (total, water-soluble and acid insoluble) values, the percentage of moisture content, and the percentage of extractive (hexane, ethyl acetate, methanol, and water) values were performed according to the official methods [12]. Fluorescence analysis was carried out using ultraviolet light at wavelengths of 254 and 366 nm [13]. About 100 mg of *L. sativa* fine powdered drug was placed on a glass slide and observed in daylight and under UV at 366, 254nm. Slide containing powder was treated with 2 ml 1 of 1N NaOH (Methanol), 1N NaOH (Aqueous), picric acid, acetic acid, H2SO4 (Conc.), HNO3 (Conc.), HCl (Conc.), FeCl3 Solution, KOH solution (Methanol), KOH solution (Aqueous), Ammonia solution, iodine solution, AgNO3 solution, and lead acetate solutions and these were observed in daylight and under UV light.

Standardization of a crude extract

Thin layer chromatographic identification and phytochemicals screening were used for the standardization of crude extract. For the TLC identification methanol extract of *L. sativa* was made on a pre-coated silica gel RP-18 modified silica gel coated by fluorescent indicators F254s and normal TLC 60 F 254 plates (Merck). The solvent system, methanol: water (8:2) was used for reverse phase TLC plate and hexane: ethyl acetate (7.5: 2.5) was used for the normal TLC plate [14]. The spots on the plates were observed and calculated for the retention factor (Rf) values. Screening of the crude extract of *L. sativa* for the various biologically active secondary metabolites was performed by using reported methods [15-16]. The presence of the active compounds was graded as high Present (+++), present (++), and low present (+), and absent (-).

Quantification of total phenolic and flavonoid contents

Accurately 50 mg of the extract was dissolved in 50 ml methanol and sonicated for 45 minutes at 1,000 ×g for 10 min. The clear supernatant was collected and stored in an amber bottle for analysis. The total phenolic and flavonoids content of crude extract of *L. sativa* was determined with Folin-Ciocalteu and Aluminium chloride colourimetric method respectively [17-18].

Phenolic content

The phenolic content of the crude extracts was quantified using Gallic acid calibration curve. For the phenolic content, 0.2 ml samples were mixed with distilled water (0.6 ml) and 0.2 ml Folin-Ciocalteu reagent (1:1). After 10 min, 1 ml of sodium carbonate solution (7.5% w/v) was transferred to the mixture and it was then diluted up to 3 ml with water. To prepare a calibration curve of gallic acid, 0.5ml aliquots of 6.25, 12.5, 25, 50, 100, 150, and 200 µg/ml of gallic acid solutions were mixed with 0.2 ml Folin–Ciocalteu reagent (1:1) and 1 ml (7.5%) sodium carbonate. The reaction mixture was kept in the dark at 25°C for 30 min and the phenolic content was determined at 765 nm against reagent blank by UV Spectrophotometer 1650 Shimadzu, Japan. All measurements were carried out in triplicate. The phenolic content was calculated as gallic acid equivalents GAE/g of dry plant material on the basis of a standard curve of gallic acid ($y = 0.0105x - 0.0455$; $R2 = 0.9946$).

Flavonoids content

The flavonoids content of the crude extracts was quantified using quercetin calibration curve. To prepare a calibration curve of quercetin, from the stock solution (1000µg/ml), standard solutions (10, 20, 40, 80 and 100µg/ml) were prepared using methanol. Accurately 1ml of extract or standard quercetin solutions was separately added in a 10ml volumetric flask, containing 4ml water and 0.3ml of NaNO3 (5%). After 5min, 0.3ml of AlCl3 (10%) was added to the mixture. The reaction mixture was kept in the dark at 25°C for 5 min and then add 2ml of sodium hydroxide (IM). After 5 min incubation, the reaction mixture was vortex and total flavonoids content was determined at 510 nm against reagent blank by UV Spectrophotometer 1650 Shimadzu, Japan. All measurements were carried out in triplicate. The flavonoids content was calculated as mg quercetin equivalent (mg QE)/g of dried extract on the basis of a standard curve of quercetin ($y = 0.0091x + 0.0195$; $R2 = 0.9963$).

In vitro antioxidant activity

Scavenging activity

Free Radical scavenging (FRS) action of *L. sativa* extract against the stable DPPH was measured using UV-spectrophotometer [19]. The DPPH solution (1 mmol/l) was prepared in methanol and stored in an amber coloured. Different concentration (10-1000 µg/ml) of sample or standard was prepared in methanol. One milliliter of each dilution was mixed with One milliliter of DPPH solution and these were incubated at 30 °C in darkness for 30 min. Inhibition of DPPH radical by the methanol extract was measured at 517 nm against the control (DPPH

solution: methanol, 1:1). The experiment was performed in triplicate.

Reducing power capacity

Reducing power (RP) capacity of *L. sativa* was measured using $FeCl_3$ method [20]. Different concentrations of the extract and standard ascorbic acid (250-2000 µg/ml) was prepared in methanol. Accurately, 1ml of each dilution was mixed with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and these were incubated at 50 °C for 30 min. The reaction mixtures were then centrifuged after addition of 2.5ml Trichloroacetic acid (10%). Accurately 2.5ml supernatant was removed from each test tube and mixed with 0.5ml $FeCl_3$ (0.1%). The absorbance was measured at 700 nm against the blank solution. All the experiments were performed in triplicate.

Antimicrobial activity

Inhibition of Zone

The methanol extract 20mg/ml and standard drugs (Ampicillin, 10µg/ml) were prepared and used for the antibacterial activity. Four bacteria strains, *S. aureus*, *S. aureus* (CI*), *B. subtilis*, and *E. coli* were used for the antimicrobial study. All strains were subcultures using Mueller-Hinton agar (MHA, Oxoid, Basingstoke, England) plates at 37°C. Antibacterial activities of *L. sativa* against four selected microorganisms were investigated by the agar disk diffusion method [21]. The methanol extract of *L. sativa* was dissolved in dimethyl sulfoxide (DMSO), filter and stored at 4°C. The four holes (About. 5mm diameter and 3mm height, each) were made in each solidified Petri plates containing MHA and bacterial strain using sterile borer. Accurately 30µl of sample and standard was added to the hole and incubate at 37°C for 24h. The determination of antibacterial activity was determined by measuring the diameters of inhibitory zones.

MIC and MBC

The agar media, MHA (Mueller Hinton Agar) and NA (Nutrient Agar) were used for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) respectively using agar dilution method [22]). The minimum inhibitory concentration (MIC) value of the *L. sativa* extract was determined as the lowest concentration required for the inhibition of bacterial growth and the minimum bactericidal concentration (MBC) was determined as the lowest concentration of an antibacterial agent required to kill particular microorganisms.

Statistical Analysis

Results were expressed as mean ± SD. Linear regression (y=mx +c) analysis was used to calculate the IC50 value. MS office Excel software 2007 was used for the plotting of graphs. The values of the correlation coefficient, intercept, slope and standard errors were obtained by nonlinear and linear regression analysis.

RESULTS:

Physicochemical and fluorescence analysis of powder

The study of physicochemical standardization such as total ash (5.2%), acid insoluble ash (4.1%) and water-soluble (0.9%), the loss on drying (7.19%) and hexane (2.4%), chloroform (7.9%), methanol (16.2%) and water-soluble (12.6%) extractive values revealed the presence of inorganic and inorganic compounds and reported in Table 1. Results of the fluorescence analysis of *L. sativa* was reported in Table 2. The powdered samples were treated with various solvents or reagents as 1N NaOH (water and methanol), Picric acid, Acetic acid, H2SO4, HNO3, 1N HCl, FeCl3, KOH solution (water and methanol), Ammonia solution, iodine solution, silver nitrate, and lead acetate solution. Various colour like yellow, brown, golden, green, black, olive colour and orange were observed.

Standardization of a crude extract

For the RP-TLC, several solvent systems using methanol and water and acetonitrile with water were tried but the mixture of methanol and water (8:2) was showed the presence of characteristic spot. Several solvent systems, such as Hexane: ethyl acetate (8:2 & 7.5:2.5), Hexane: ethyl acetate (7.5: 2.5), Chloroform: Methanol (9:1 & 7:3, Toluene: Ethyl acetate: Formic acid, (8: 2: 0.5) and Methanol: Chloroform (6:4) was tested on the normal TLC plates but TLC analysis using Hexane: Ethyl acetate (7.5:2.5) showed the presence of characteristic spots (Figure 1). The methanol extract of *L. sativa* showed single distinct spot (Rf=0.87) on the RP-TLC plate after spraying with Ninhydrin reagent, and 1, 2, 2, and 5 distinct spots with visual, short UV, long UV, and Ninhydrin-H2SO4, respectively on the normal 60 F 254 plates plate. The Rf values of the spots were presented in Table 3. The results found from the phytochemical screening of *L. sativa* methanol extract indicated the presence of alkaloids, glycosides, saponins, steroids and triterpenoids, phenols, tannins, and flavonoids (Table 4).

Total phenolic and flavonoids content evaluation

The result of total phenolic and flavonoid contents of the *L. sativa* extract was shown in Table 5 and a

gallic acid standard curve and quercetin standard curve were showed in Figure 2 and Figure 3 respectively. The total phenolic content of the methanolic extract of *L. sativa* was estimated by FCR and tabulated in gallic acid equivalents (GAE) and it was calculated from the linear regression equation of standard curve (y = 0.0105x + 0.0455, R2= 0.9946). The results showed that the methanolic extract contains 53.64±0.27 mg GAE/g. The total flavonoid content of the methanolic extract was determined using $AlCl_3$ colourimetric method and it was calculated from the linear regression equation of the standard curve of quercetin (y = 0.0091x + 0.0195; R2=0.9963) and expressed as quercetin equivalent per gram of plant extract and it was found to contain 16.55±0.49 mg QE/g.

In vitro antioxidant activity

The result of percentage inhibition of DPPH free radical by the methanol extract of *L. sativa* was shown in (Figure 4) and it was directly proportional to the concentration of methanolic extract. At 1000µg/ml, the extract showed 82.06%; whereas positive standards, ascorbic acid exhibited 86.4% respectively. The IC50 values of methanol extract and ascorbic acid were: 210.57µg/ml and 31.92µg/ml respectively (Table 5). The reducing power of the *L. sativa* was evaluated and it was compared with ascorbic acid and the results were given in Figure 5. At 2000µg/ml, the methanolic extract showed 0.279a, whereas the ascorbic acid showed 1.901a.

Antimicrobial studies

The results of the zone of inhibition, MIC and MBC, was of the antibacterial activity of the methanol extract of *L. sativa* was shown in Table 6. The methanol extract had good inhibitory action on *S. aureus* ATCC (10.6mm), *S. aureus* clinical isolated (8.2mm) *B. subtilis* (12.4mm) and *E. coli* (13.8mm) but less when compared to that of the standard Ampicillin. In the present study, the MIC values were ranged from 6.25 to 25 mg/ml and the MBC values were ranged from equal and more than 50 mg/ml. The lowest MIC was obtained for *E. coli* ATCC and the highest MIC was obtained for *S. aureus* (C.I*).

DISCUSSION:

Physicochemical and fluorescence analysis of powder

Due to the wide use of *L. sativa* in homemade remedy or traditional medicines, standardization becomes an important measure for ensuring the quality, purity, and authenticity of the crude drugs. The physicochemical study could be a valuable source of information which is commonly helpful in the evaluation of purity and quality of a crude drug. Deterioration of the plant material based on the amount of water present in powder material. If the water content is more, the powder can be easily deteriorated due to the presence of microorganisms. The ash value of plant material indicated the extent of minerals and earthy materials associated with the powder material [23]. The loss on drying and ash contents are the important tools to measure the presence of volatile and foreign materials. The extractive values not only give an idea about the judgment of exhausted or adulterated but also chemical constituents present in the powder drug [24]. In the present result, the extractive value of methanol was highest followed by water. Fluorescence studies are a rapid and substitute method for the clarification of confusing specimen. When physical and chemical methods are insufficient, the fluorescence studies not only a useful tool for the identification of adulterants but also an important tool for the determination of constituents and gives an idea about the nature of chemicals present in the plant [25].

Standardization of a crude extract

TLC fingerprints analysis may serve as useful data for the standardization of raw plant material and may be an alternative technique, for the analysis of crude plant extracts [26]. The present finding of Reverse phase TLC and normal TLC may be a useful parameter for the quality control of studies on *L. sativa* methanol extract. The qualitative phytochemical analysis made for the methanol extract of this plant revealed the presence of alkaloids, carbohydrates, phenols, tannins, steroids, flavonoids, cardiac glycosides, anthraquinone glycosides, and saponins. These leaves secondary metabolites are reported to have many biologically and therapeutically active drugs [27], so the methanol extract of this plant is exhibited in many medicinal properties.

Total phenolic and flavonoids content evaluation

Investigations of the composition of polyphenols in lettuce have demonstrated the presence of comparatively high content of sugar-conjugated quercetin in the leaves of lettuce [28-30]. The study of phenolic and flavonoids compounds is one of the best methods for the rapid identification of natural antioxidant source [31]. Phenolic and flavonoids compounds have healing properties by neutralizing the free radicals produced in living cells during the metabolic process. Plants those have a high amount of these compounds showed good antioxidants activity in both in vitro as well as in vivo models and the antioxidants activities of these compounds totally based on the free OH particularly 3OH [32]. These

compounds are known to reveal a wide range of biological activities including antimicrobial and antioxidant properties [33]. The present study of *L. sativa* methanol extract revealed the presence of a good amount of phenolic and flavonoids and may be a good source of antioxidant and antimicrobial agents.

In vitro antioxidant activity

DPPH free radical scavenging potential is one of the simple and fastest and most acceptable methods for the exploration of natural and synthetic compounds [34]. In this method 2,2,-diphenyl-1-picrylhydrazyl reacts with an antioxidant, changing the colour intensity proportionally to the antioxidant activity [35]. The DPPH radical scavenging abilities of the *L. sativa* methanol extract was less than those of ascorbic acid, the study showed that the extract has the proton-donating ability and could be a good source of antioxidant. Antioxidant (or Reducing power) capacity of the methanolic extracts of *L. sativa* was concentration dependent. The presence of antioxidant compounds in the extract causes the reduction of ferric (Fe3+) to ferrous (Fe2+) form Perl's Prussian blue [36]. The methanol extract of *L. sativa* had phenolic and flavonoids, and the correspondence referred to the fact that phenolic and flavonoids possess high antioxidant potential [37].

Antimicrobial activity

Antimicrobial control by phenolic and flavonoids are well known [38]. In the present work, we explore the use of *L. sativa* methanol extract, as antimicrobials agents and it was found that the phenolic compounds may be responsible for their antibacterial activity. The previous studies have been reported that phenolic compounds have high antimicrobial activity against both Gram-positive and Gram-negative bacteria [39]. In the previous studies Ismail and Mirza, reported that the efficacy of leaves extracts against analgesic, anti-inflammatory, anti-depressant, and anti-coagulant and concluded that leaves of this plant can use as an herbal drug due to its multiple effects [40]. Results obtained in the present study indicate that *L. Sativa* methanol extract exhibited antibacterial activity against both *S. aureus*, *B. subtilis* and *E. coli*, so it can be used as a natural antimicrobial agent of plant origins [41].

CONCLUSIONS:

The present study showed that the methanol extract of *L. sativa* contains a significant amount of phenolic and flavonoids and thus, can be suggested that these compounds are responsible for the antioxidant and antimicrobial activity. There is close relationship between total phenolic and flavonoids contents, and antioxidant activity [42-43] and antimicrobial activity [44-45] of lettuce and other salad leaves. Antioxidant properties of *L. sativa* leaf may play a role in preventing ailments such as cancer, gastrointestinal ailments, joint pain, atherosclerosis, cardiac ailments, wound healing, diabetics and other degenerative diseases [46]. Antimicrobial properties of *L. sativa* leaf may play a role in preventing the disease caused by *S. aureus*, *B. subtilis*, and *E. coli*. Based on the present finding, *L. sativa* leaves possess the abilities to be a good candidate in the search for a natural antioxidant and antimicrobial agent against both degenerative and infectious diseases.

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Conflicts of interest

The authors declare there is no conflict of interest.

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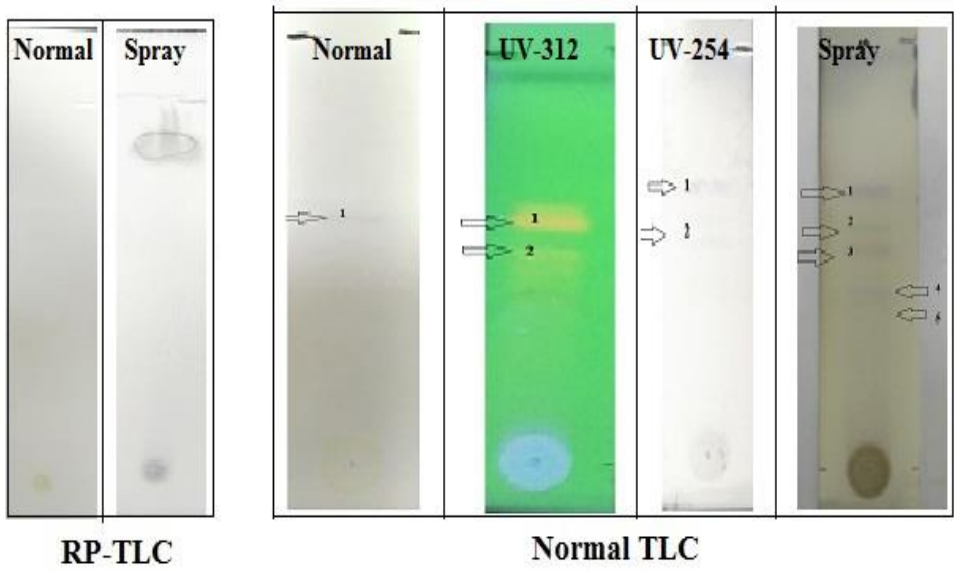

Figure 1: TLC of Methanol extract of *L. sativa* using RP-TLC and Normal TLC plates

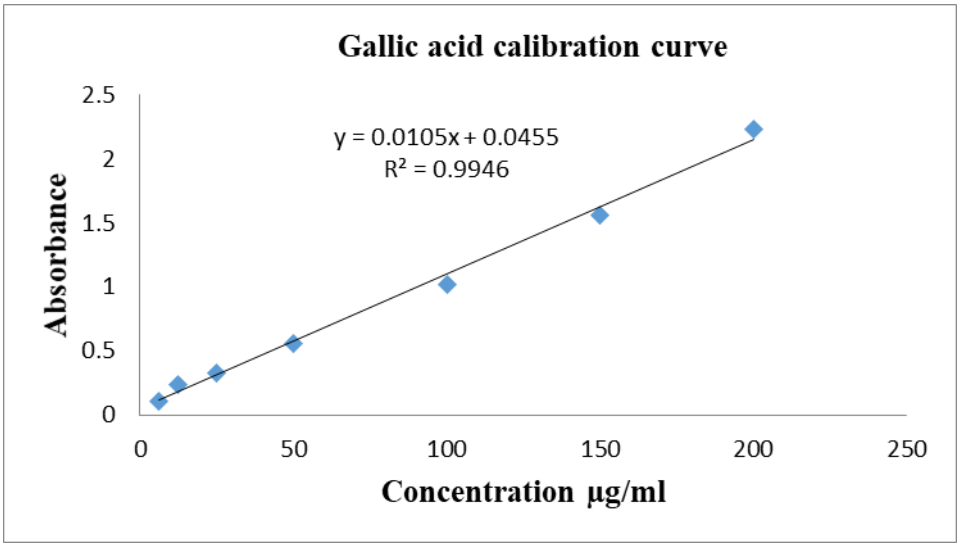

Figure 2: Standard calibration curve of Gallic acid (Total phenols), concentration y = 0.0105x +0.0455; R^2 = 0.9946. Each point represents the mean of triplicate determinations

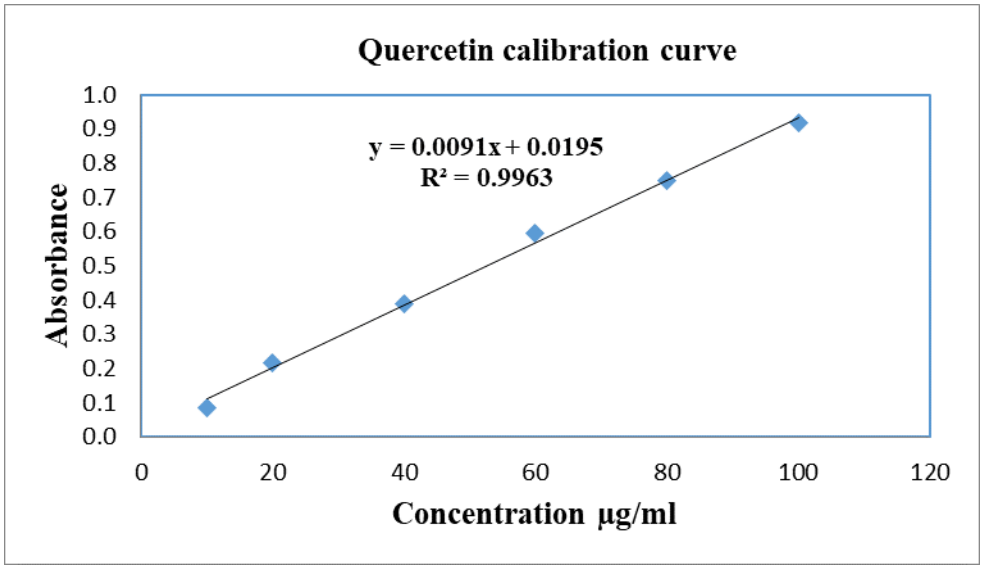

Figure 3: Standard calibration curve of quercetin (Total Flavonoids), concentration y = 0.0091+ 0.0195; R^2 = 0.9963. Each point represents the mean of triplicate determinations.

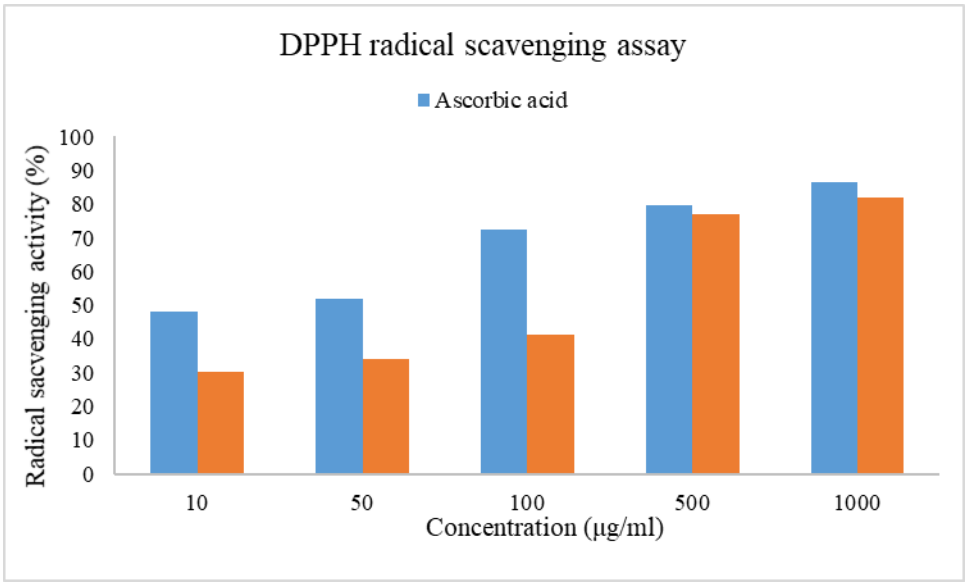

Figure 4: DPPH radical scavenging assay of methanol extract of *L. sativa*. Values are means of triplicate determinations ($n = 3$).

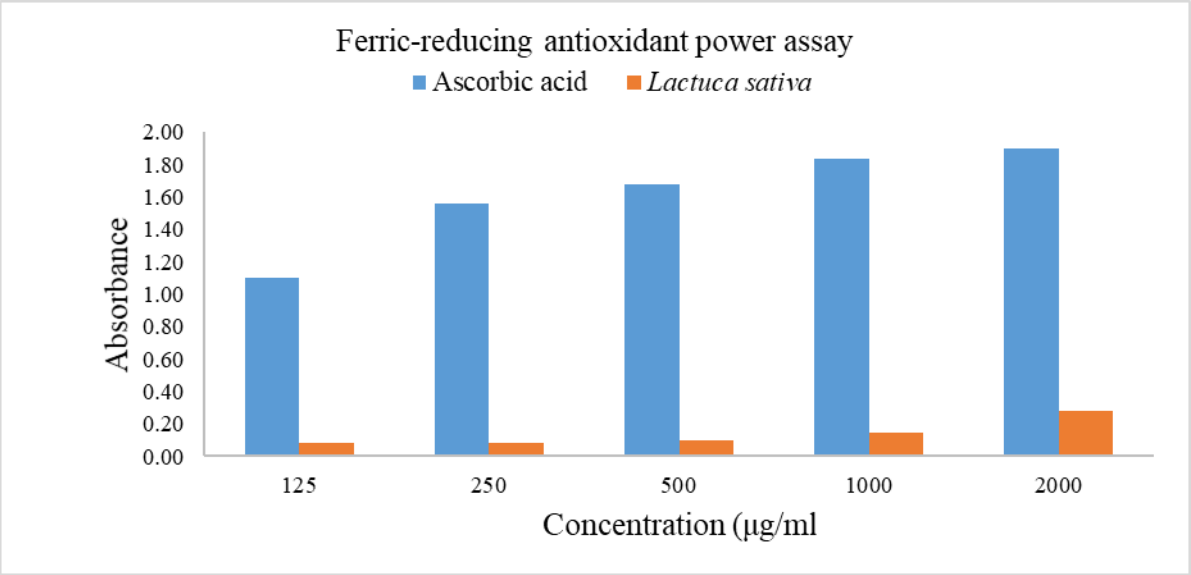

Figure 5: Ferric Chloride reducing power activity of methanol extract of *L. sativa (dry).* Values are means of triplicate determinations ($n = 3$) ± standard deviation.

Table 1: Physicochemical analysis of *L. Sativa* leaves powder

Parameters	**Value obtained on dry weight powder (% w/w)***
Loss on drying	7.19
Total Ash (%w/w)	5.2
Acid insoluble Ash (%w/w)	4.1
Water soluble Ash (%w/w)	0.9
Hexane/Yellow green	2.4
Chloroform/ Green	7.9
Methanol/Deep green	16.2
Water/Light yellow	12.6

*Average of three reading

Table 2: Fluorescence analysis of powder of *L. Sativa* leaves

Reagents	Normal light	Under UV Light	
		Short, 254nm	Long, 365nm
1N NaOH (Methanol)	Yellow	Yellow	Yellow
1N NaOH (Aqueous)	Green	Golden	Brown
Picric acid	Pastel green	Brown	Brown gray
Acetic acid	Olive green	Light ivory	Pearl white
H2SO4 (Conc.)	Brown	Brown olive	Brown
HNO3 (Conc.)	Pastel green	Green	Green
HCl (Conc.)	Green	Chroma Green	Green
FeCl3 Solution	Green	Brown grey	Black olive
KOH solution (Methanol)	Pale green	Black olive	Brown olive
KOH solution (Aqueous)	Brown yellow	Black olive	Black olive
Ammonia solution	May green	Black olive	Brown olive
Iodine solution	Yellow orange	Black	Black olive
AgNO3 solution	Brown gray	Brown gray	Brown olive
Lead acetate solution	Pastel green	Brown olive	May green

Where, NaOH (Sodium hydroxide), H_2SO_4 (Sulfuric acid), HNO_3 (Nitric acid), HCl (Hydrochloric acid), $FeCl_3$ (Ferric chloride), KOH (Potassium hydroxide), $AgNO_3$ (Silver nitrate) and UV-Ultraviolet

Table 3: TLC study of *L. Sativa* leaves methanol extract

TLC	Solvent system	Condition of Plate	No*	Rf Value
Reverse Phase	Methanol : Water (8:2)	Normal and UV-light	-	-
		Spraying (Ninhydrin)	1	0.87
Normal	Hexane : Ethyl acetate (7.5 : 2.5)	Normal light	1	0.56 (Green)
		UV light (312nm)	2	0.56 (Yellow) 0.41 (Green)
		UV light (254nm)	2	0.56 (Black) 0.41 (Black)
		Spraying (Ninhydrin)	5	0.66 (Violet), 0.56 (Green) 0.52 (Violet), 0.41, 0.37

Number of spots*

Table 4: Phytochemical analysis of *L. Sativa* leaves methanol extract

Secondary metabolites	Results
Alkaloids (Mayer's and Wagner's)	(+/++)
Carbohydrates (Molisch, Fehling test)	(+++/++)
Phenols and Tannins (1% and 10%, Ferric Chloride)	(+/+)
Steroids and Triterpenoids (Salkowski and Libermann)	(+/-)
Flavonoids (Lead acetate)	(++)
Anthraquinonoid glycoside (*Borntrager's* test)	+
Cardiac glycoside (Killer Killani and Baljets)	(+/+)
Saponins (Foam test)	(+++)

Key= (++: high Present, ++: present, +: low present, -: absent)

Table 5: Total phenolic and flavonoids content of *L. Sativa* leaves methanol extract

Contents of Phenolic and Flavonoids and IC50 value of *L. Sativa* and ascorbic acid	(Average of three reading ± SEM)
Total Phenolic contents (mg GAE/g dry extract)	53.64 ± 0.27*
Total Flavonoids Contents (mg QE/g, dry extract)	16.55 ± 0.49*
IC50 (*L. Sativa*)	210.57 µg/ml**
IC50 (Ascorbic acid)	31.92 µg/ml**

***Mean of three reading ± SEM and **Average of three reading.**

Table 6: Zone of inhibition, MIC, and MBC of bacterial agents of *L. Sativa* leaves methanol extract

Bacterial and Fungal strains	**Zone of inhibition (mm*)**		**MIC (mg/ml)**	**MBC (mg/ml)**
	Extract (50mg/ml)	Standard***		
S. aureus ATCC	10.6	23.5	12.5	50
S. aureus (C.I**)	8.2	18.24	25	>50
B. subtilis ATCC	12.4	21.8	12.5	>50
E. coli ATCC	13.8	23.4	6.25	50

(mm*= millimetre**, ****CI =clinically isolated Munich, ***Amp= Ampicillin (10µg/ml**)**, and Mean of three reading (n=3)