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Extraction, Screening, and Characterization of Bioactive Compounds from *Moringa oleifera*: Extends Life-span of *Caenorhabditis elegans*.

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Abstract

Moringa oleifera is the most commonly cultivated species of the Moringaceae family. It has high nutritional value and a remarkable range of therapeutic uses. Every plant part is beneficial in many ways including medicinal uses. The present study aimed to investigate the presence of various phytochemicals and bioactive compounds from *M. oleifera* flowers and leaves and to check its effect on lifespan extension of *Caenorhabditis elegans.* Initial phytochemical screening of flowers and leaves methanolic extracts estimated the presence of flavonoids, alkaloids, tannins, and vitamin C. Further quantitative analysis revealed a considerable concentration of phenols, vitamin C, and flavonoids. The plant extracts also showed *in vitro* antioxidant activity through DPPH, FRAP, and reducing power assay. TLC analysis was performed for the optimization of a specific solvent system suitable for the separation of various compounds. The characterization performed by UV absorption, GC-MS and FTIR revealed the presence of palmitic acid, linolenic acid, and linoleic acid. Moreover, the *in vivo* life-span assay was carried out on *C. elegans*. *M. oleifera* extracts were applied to *C. elegans* to investigate its anti-aging potential. The extracts were applied to N2 wild-type worms in different concentrations such as 10µg/ml, 20µg/ml, and 40µg/ml. Treatment with plant extracts showed an increase in lifespan from 2 to 8 days. The effect of Moringa on *C. elegans* proved its antiaging potential and the compounds characterized have potential antioxidant activities that can be further purified and used in pharmaceuticals.

Keywords *Moringa oleifera*, bioactive compounds, antioxidant activity, TLC/GC-MS/FTIR, *Caenorhabditis elegans.*

Introduction:

For several centuries, a variety of plants have been used globally not only as nutritional supplements but also as conventional treatments for numerous diseases. The *M. oleifera* (Moringa or drumstick tree) is one of these nutritional plants which is rapidly growing, deciduous, drought resistant, a small or middle-sized tree, and about 8-10m in height. It is native to India and widely cultivated in the tropical & subtropical regions of Asia and Africa [1].

Moringa tree is highly nutritious with a wide range of pharmacological properties. The leaves of Moringa are a significant source of protein, calcium and potassium, vitamin C, and β-carotene. It also acts as a substantial source of natural antioxidant compounds such as phenolics, flavonoids, ascorbic acid, and carotenoids [2, 3]. Its flowers have been reported to be rich in flavonoids, alkaloids, waxes, Ca^{2+},

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and K^+ [4, 5]. It has been demonstrated that various parts of *M. oleifera* such as leaf, stem, fruit, flowers, seed and root generate different biological activities [6], including analgesic [7], antipyretic [8], anti-atherosclerotic [9], immune-boosting [10], anti-cardiovascular disease [11], antiviral [12], antioxidant [13,14], antimicrobial [15], anti-inflammatory [16,17] and anti-tumor effects [12,18].

A variety of techniques can be used to determine and estimate the presence of various phytochemical compounds. The most commonly used technique for the recovery of bioactive compounds from plants is solvent extraction using various solvents of specific polarity [19]. Screening and characterization of isolated compounds can be done by various techniques such as TLC, HPLC, GC-MS, and FTIR [20, 21, and 22].

It has been demonstrated that if reactive free radicals are decreased or the concentration of antioxidant substances is increased, the lifespan of an organism can be increased [23, 24]. The most widely used animal model for life span studies is *C. elegans*. It is readily available, easy to culture in the laboratory, has a short lifespan, its genome is fully sequenced and 40% of genes are associated with human diseases [25, 26].

This study aimed to evaluate the bioactive compounds present in flower and leaves extracts of *M. oleifera* as well as to investigate its effect on lifespan extension of *C. elegans*.

Materials and Methods

Procurement of experimental plant

The experimental plant *M. oleifera* was collected from the botanical garden of the institute.

Preparation of the plant extracts

Leaves and flowers of *M. oleifera* were washed in the distilled water by 2-3 times and dried in the hot air oven at 55°C for 2-3 days till it gets moistureless. Leaves and flowers were crushed to form a dry powder. The powdered samples were dissolved in 80% ethanol and 80% methanol. Then centrifugation was done and the supernatant was collected and allowed to dry. The dried samples were dissolved in different solvents such as ethanol, methanol, distilled water, and DMSO. Thus, the extracts obtained were used for various analyses.

Phytochemical screening of extracts

The methanol extract was used for preliminary phytochemical analysis using standard protocols. The following qualitative tests were done as follows [27]:

Test for alkaloids

Hager’s Test: Plant extract was treated with few drops of Hager’s reagent (saturated picric acid solution). The formation of a yellow precipitate indicates the presence of alkaloids.

Test for flavonoids

Ferric chloride test: Plant extract was treated with few drops of Ferric chloride solution. The appearance of blackish-red color indicates the presence of flavonoids.

Lead acetate test: Plant extract was treated with few drops of lead acetate (10%) solution. The formation of a yellow precipitate indicates the presence of flavonoids.

Test for tannins

Gelatin Test: Plant extract was treated with gelatin solution. The appearance of a white precipitate indicates the presence of tannins.

Test for vitamin C

DNPH Test: Plant extract was treated with Dinitrophenyl-hydrazine dissolved in concentrated sulphuric acid. The formation of a yellow precipitate indicates the presence of vitamin C.

Determination of bioactive constituents

The freshly prepared extracts were subjected to standard phytochemical analysis for different constituents such as phenols,

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vitamin C, and flavonoids.

Estimation of total phenols

The amount of total phenol was determined by Folin-Ciocalteu's reagent (FCR) method [28]. Different aliquots of plant extract (10µl to 60µl) were taken into test tubes and diluted FCR was added up to 2.5ml. The mixture was incubated at room temperature for 15 min. Then 2ml of sodium carbonate solution was added and further incubated for 30 min at room temperature and the absorbance was measured at 650 nm. Total phenol values are expressed in terms of tannic acid equivalent (mg/g of the extract).

Estimation of vitamin C

The amount of vitamin C (Ascorbic acid) was determined colorimetrically. Different aliquots of the sample extract were taken and distilled water was added up to 3ml. Then 2ml of DNPH reagent was added followed by 1-2 drops of thiourea. The content was mixed properly and incubated at 37°C for 3 hours. After incubation, orange-red colored osazone crystals were formed. Then 7ml of 80% sulphuric acid was added to dissolve the crystals. The absorbance was measured at 540nm.

Estimation of flavonoids

The total flavonoid concentration was determined by adding 1 gm of samples were mixed with Ethanol: HCl (85:15) and incubated at 4°C overnight. The next day, the mixture was centrifuged at 2500 rpm and the supernatant was collected. The absorbance was measured at 535nm.

Reducing power assay

The reducing power assay was determined by the Fe^{3+} - Fe^{2+} transformation in the presence of the extracts. 1 ml of plant extract solution was mixed with 2.5ml potassium ferricyanide and incubated at 50°C for 20 minutes. After incubation 2.5ml of trichloroacetic acid was added and centrifuged at 3000 rpm for 10 minutes. The supernatant (~2.5ml) was collected and mixed with 2.5ml of distilled water and 0.5ml of ferric chloride. The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated greater reducing power. Vitamin C was used as a positive control.

Free radical scavenging assays

DPPH (2, 2-Diphenyl-1-picrylhydrazyl) assay

The antioxidant capacity of methanolic extract was measured by adopting the method proposed by Brand-Williams & Basel with some slight modification [29]. Methanolic extract of samples (200µL) was taken in tubes. To the tubes 2.8ml of DPPH (0.025g/l of methanol) was added and incubated in dark for 30 minutes. The absorbance was measured against methanol without DPPH and results were expressed as percentage inhibition of DPPH radical.

FRAP (Ferric reducing antioxidant power) assay

This method was given by Benzine & Strain [30]. For the assay 100µl of methanolic extract was taken in a test tube. Then 2600µl of FRAP reagent and 300µl of distilled water were added. Incubation was done at 37°C for some time. The absorbance was measured at 593nm. The ferric reducing activity was expressed in mM $FeSO_4.7H_2O$ (0.25-25mM).

H_2O_2 (Hydrogen peroxide) scavenging assay

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch et al. [31]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). The different concentration of extract (5- 20 µl) was added to 0.6 ml of H_2O_2 solution (43 mM). The absorbance value of the reaction mixture was recorded at 230nm. A blank solution contains sodium phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of crude extract and standard compounds were calculated using the following equation,

$$\% \text{ scavenged } [H_2O_2] = [(A_C - A_S) / A_C] \times 100$$

Where A_C = absorbance of the control and A_S = absorbance of the sample.

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Isolation and characterization of bioactive compounds

TLC (Thin Layer Chromatography)

Thin-layer chromatography (TLC) was carried out to isolate the components mainly present in the plant extracts. The analysis was performed on pre-coated 20×20 cm (0.25mm thick) TLC plates F_{254} silica gel 60. There were 20 different standards used for the analysis. Each standard solution (concentration 1mg/ml) along with samples was applied as spots onto TLC sheets. Thirteen different mobile phases were selected (according to their polarity) for the optimization of the appropriate solvent system. The chromatographic chamber was previously saturated with the appropriate mobile phase. The plates were developed at room temperature in a chamber. After drying, the plate was visualized under a UV transilluminator. Then the R_f (retention factor) values were counted for samples and compared with the R_f values of standards. The standards and solvent systems used are listed in (Table 1).

Determination of compounds by UV absorption

Samples were dissolved in different solvents such as distilled water, methanol, ethanol, DMSO. The whole spectrum of samples using respective solvents as a blank was taken. Comparison of the absorbance peaks of samples was done with the λ_{max} of reference compounds [32].

Standard curve of isolated compounds from TLC

After TLC analysis some spots of samples were carefully scraped out from silica gel. It was then dissolved into 1ml of an appropriate solvent. Centrifugation was done at 2000rpm for 10 min to remove silica. The solution of standards (1mg/ml) was prepared. The absorbance of aliquots of standard solution (2-10µl) and samples was measured at the appropriate wavelength. Then the standard curve was plotted and the concentration of isolated compounds was estimated.

GC-MS (Gas Chromatography-Mass Spectrometry)

Gas chromatography coupled to mass spectrometry is a versatile tool to separate, quantify and identify unknown (volatile) organic compounds and permanent gases. The analysis was performed on Perkin Elmer (Turbomass mass spectrometer) using PE-5ms column of 30m length and Helium as a carrier gas. Chromatographic conditions were optimized (Injection temperature - 220°C, injection volume - 1µl, column temperature - 80°C to 280°C at the rate of 10°C per minute). The scanning range of MS was 20 to 620 amu (atomic mass unit). The analysis data obtained was compared with the standard library.

FTIR (Fourier Transformed Infrared Spectroscopy)

It is a method for the identification of the functional group of unknown compounds present in the samples. FTIR was performed on Perkin Elmer (spectrum GS). For the analysis, the sample (2mg) was crushed with KBr (200mg) and a pellet was formed with the help of mechanical pressure. The formed pellet was observed at the different coming wavelengths in the FTIR instrument. The analysis data obtained was compared with the standard IR chart.

Life-span assay on Caenorhabditis elegans

Source of C. elegans

Wild-type *C. elegans* worms and *Escherichia coli* OP50 (food source) were ordered Caenorhabditis Genetics Center (CGC).

Growth conditions and diet

Nematode *C. elegans* (N2 wild-type) were maintained at 20°C on standard nematode growth medium (NGM) seeded with grown *E. coli* OP50 culture as a food source.

Synchronization of worms

Lifespan assay was done with the synchronized worms. Synchronization is the

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Table 1: List of different standards and solvent systems used in the TLC analysis

Standards	gallic acid, tannic acid, salicylic acid, ellagic acid, phenol, resorcinol, ascorbic acid, chlorogenic acid, pyrogallol, α-naphthol, rutin, morin, catechol, picric acid, caffeic acid, myricetin, p-nitrophenol, quercetin, naringin, kaempferol
Solvent systems	Water Ethyl acetate Chloroform Ethyl acetate: isopropanol: water Toluene:ethyl acetate:formic acid:methanol Ethyl acetate: formic acid Toluene:ethyl acetate:acetic acid Toluene:acetone:formic acid n-hexane: ethyl acetate: formic acid n-hexane: ethyl acetate: acetic acid Petroleum ether: ethyl acetate: formic acid Butanol: ammonium hydroxide: water Butanol: methanol

Table 2: Presence of phytochemical constituent revealed by a phytochemical screening of *M. oleifera* flowers and leaves

Sr. No.	Test	Methanolic extract of M. oleifera	
		Flowers	Leaves
1	Alkaloids	++	++
2	Flavonoids	++	++
3	Tannins	++	++
4	Vitamin C	++	++

(++ indicates the presence of constituent).

process to arrest all the worms at one stage of the life cycle. Synchronization of worms was achieved via bleaching that kills the worms by dissolving the outer cuticle and releasing the eggs from the guts of gravid hermaphrodites. Synchronization was done by two different protocols.

Protocol 1: Worms were allowed to grow on NGM for 2-3 days. After sufficient eggs/adults were seen, 5ml of M9 buffer was poured on the plate to dislodge the worms. The worms were then centrifuged at 2500rpm for 10 minutes. After centrifugation, the remaining M9 buffer was removed and 15ml 20% alkaline hypochlorite solution was added and mixed. After that, the centrifugation was done for one minute at the maximum speed. Then the supernatant was removed and this step was performed again. The fresh M9 buffer (7ml) was added and incubated at 20°C overnight.

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Protocol 2: From the starved L1 larvae plate the worms were transferred onto *E. coli* OP50 containing NGM plates and allowed to grow for about 2 days at 25°C. The worms were collected by washing off the plate using sterile water and centrifuged at 1200rpm for 2 minutes. This step was repeated after supernatant removal. Then the bleach/NaOH solution was added and incubated at room temperature for 5 minutes. As the worms get dissolved, M9 buffer was added followed by S-complete media with repeated centrifugation and subsequent removal of the supernatant. The worms were re-suspended in S-complete media along with carbenicillin and repeatedly examined under a microscope. Then worm suspension (120µl) was transferred to an NGM plate containing OP50 and incubated at 20°C until worms were at the L1 stage.

Life-span assay at 20°C and 25°C

Lifespan assay was performed at 20°C & 25°C on NGM plates in duplicates. Worms that synchronized at the L4 stage were transferred to the plates containing plant extract of different concentrations (10µg, 20 µg, and 40 µg) and culture of OP50. The control was prepared with OP50 without extract. All plates were supplemented with FUdR (5-fluoro-2'-deoxyuridine) which inhibits the progeny development. At the time of assay, the different concentrations of extract were prepared and added to the experimental plates. Animals were scored every day for survival. The worm which did not respond to repeated touching was scored as dead. The experiment was terminated when all worms were scored as dead.

Result and Discussion:

Phytochemical screening of extracts

Investigations on the phytochemical screening of *M. oleifera* aqueous methanol extracts revealed the presence of alkaloids, flavonoids, tannins, and vitamin C in both leaves & flowers (Table 2).

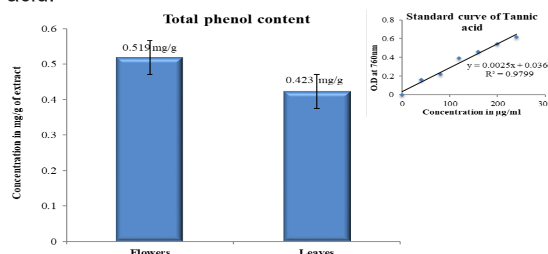
Determination of bioactive constituents

Total phenolic content

The plant extract is composed of a complex mixture of phenolics differing in the number and arrangement of both hydroxy and methoxy groups on the aromatic rings of phenolic acids. Plant phenolics are a major group of compounds that have primary antioxidation or free radicals scavenging activities.

In the present study, the total phenol content of flowers and leaves of *M. oleifera* was found to be 25.95 mg/g and 22.26 mg/g of extract respectively, in terms of tannic acid equivalent. It was found that phenol content is higher in flowers than in leaves (Graph 1).

Graph 1: Total Phenol content present in M. oleifera flowers and leaves equivalent to tannic acid. The graph at the upright corner displays the standard of Tannic acid.

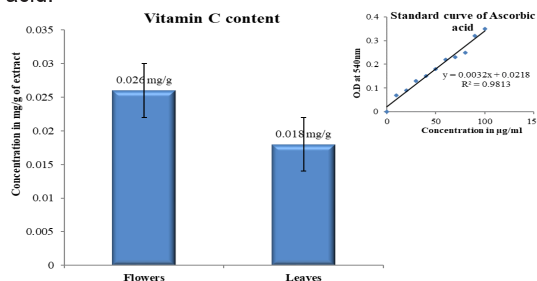


Vitamin C content

The presence of vitamin C is directly associated with the antioxidant activity of the plant depending on its concentration. Vitamin C was estimated with the help of a standard calibration curve of ascorbic acid.

In the present study, the vitamin C content of flowers and leaves of *M oleifera* was found to be 1.31 mg/g and 0.942 mg/g of extract, respectively. The high vitamin C content corresponds to high antioxidant activity in flowers as compared to leaves (Graph 2).

Graph 2: Vitamin C content obtained in M. oleifera flowers and leaves equivalent to ascorbic acid. The graph at the upright corner displays the standard of Ascorbic acid.



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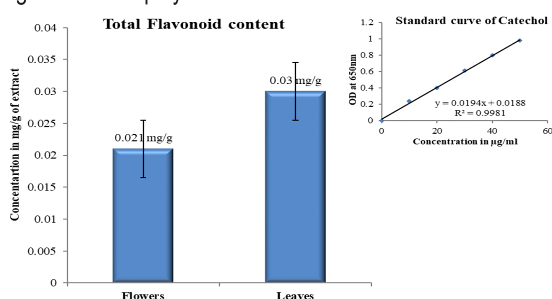
Total flavonoid content

Flavonoids are potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage. The concentration of flavonoids estimated by the colorimetric method was 1.80 mg/g in *M. oleifera* flowers and 4.64 mg/ml in the case of leaves (Graph 3).

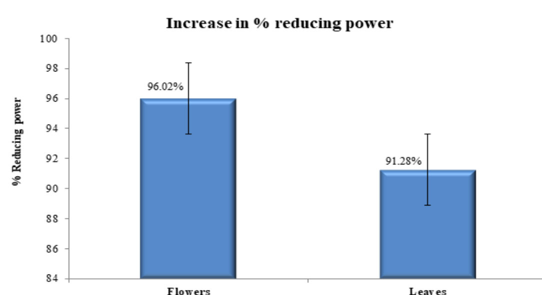
Graph 3: Total flavonoids content present in M. oleifera flowers and leaves equivalent to catechol. The graph at the upright corner displays the standard of Catechol.



Reducing capacity assessment

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. *M. oleifera* plant extract shows high reducing power due to their activity as an electron donor and thereby halting the radical chain to more stable products. The reducing power of *M. oleifera* leaves and flowers was found to be 96.02% and 91.28%, respectively (Graph 4). The reducing power of plant extract increased gradually in a concentration-dependent manner.

Graph 4: Shows % increase in reducing the power of both flowers and leaves of M. oleifera.

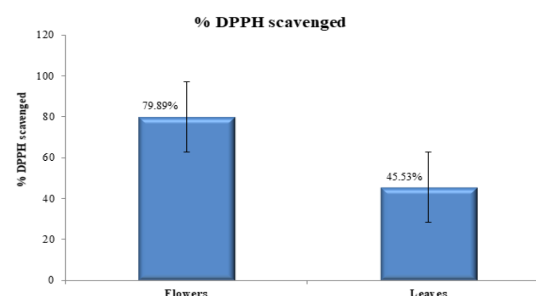


Free radical scavenging activity

DPPH scavenging activity

DPPH assay is usually employed to evaluate the ability of antioxidants to scavenge free radicals. The change in absorbance was measured at 517 nm, as the reaction between antioxidants and DPPH progresses, the absorbance decreases. The scavenging effect of antioxidants is influenced by their concentration and type of radical. The DPPH scavenging activity was found to be more in flowers than leaves, i.e. 75.89% and 45.53% respectively (Graph 5).

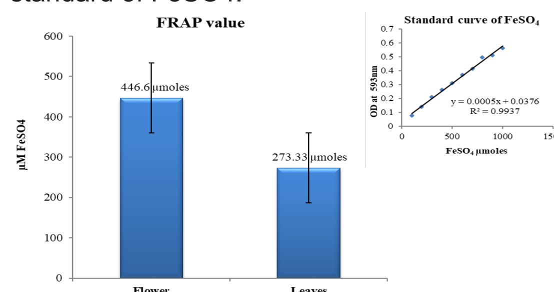
Graph 5: Shows DPPH scavenging capacity of both flowers and leaves of M. oleifera which indicates its free radical scavenging capacity.



FRAP assay

FRAP value gives higher antioxidant capacity as its value is based on reducing ferric ion, where antioxidants work as a reducing agent. Higher FRAP values indicate higher antioxidant activity. The FRAP value obtained in

Graph 6: Ferric reducing power capacity of M. oleifera flowers and leaves equivalent to FeSo4. The graph at the upright corner displays the standard of FeSO4.



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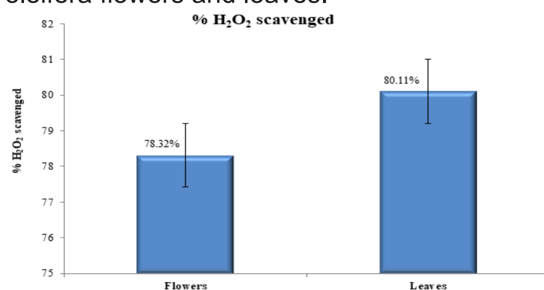
flowers and leaves was 446.6 µmoles and 273.3 µmoles, respectively. These values indicate that the antioxidant capacity is higher in flowers than in leaves (Graph 6).

Hydrogen peroxide (H_2O_2) scavenging activity

Hydrogen peroxide has strong oxidizing properties. It can be formed *in vivo* by many oxidizing enzymes, such as superoxide dismutase, and can cross cellular membranes and may slowly oxidize several intracellular compounds. Hydrogen peroxide scavenging activity of *M. oleifera* was found to be 78.32% in flower extract and 80.11% in leaves extract (Graph 7).

Graph 7: Shows H2O2 scavenging activity of M. oleifera flowers and leaves.



Isolation and characterization of bioactive compounds

TLC (Thin Layer Chromatography)

In TLC analysis different solvent systems were used to separate the compounds from the sample and their R_F values were compared with the standards. In our study, the most suitable TLC solvent system for analysis was shown to be toluene: ethyl acetate: acetic acid. In this solvent system, the samples were separated accurately, it may be because the polarity of the solvent system was appropriate for the separation of the samples. Another solvent system found to be appropriate was butanol: ammonium hydroxide: water. The result shows that the compounds like gallic acid, pyrogallol, catechol, resorcinol, salicylic acid, picric acid, p-nitrophenol, morin, and kaempferol may present in flower and catechol, resorcinol, salicylic acid, gallic acid, quercetin, chlorogenic acid, morin, and caffeic acid may present in leaves (Table 3).

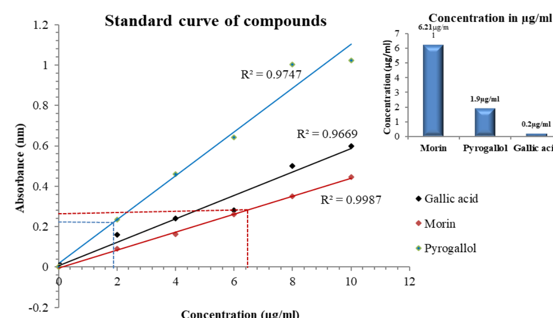
UV absorption

The powdered extracts were dissolved in different solvents like distilled water, ethanol, methanol, DMSO. As every compound absorbs at a specific wavelength, their UV spectra were recorded between 200-1100 nm. The absorption of the maximum peaks was compared with the standard λ_{max} of the reference compounds [32]. As shown in the comparative data some of the compounds were obtained in both the samples (Table 4).

Quantification of compounds by standard curve

The standard curves were prepared using various standards such as Gallic acid, Pyrogallol, and Morin. The concentration of compounds was obtained by plotting the graph of concentration versus OD. The concentration obtained of Gallic acid, Pyrogallol, and Morin was 0.2µg/ml, 1.9µg/ml, and 6.21µg/ml, respectively (Graph 8).

Graph 8: Standard curves of Gallic acid, Pyrogallol, and Morin. Their absorbance was measure at 273nm, 540nm, and 360nm, respectively. The graph at the upright corner displays a comparison of the compound's concentration in M. oleifera extract.



Identification of compounds by GC-MS (Gas Chromatography-Mass Spectrometry)

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Table 3: The result obtained from TLC analysis by comparing the RF values of sample spots with standards.

Solvent system	Presence of Possible Compounds	
	M. oleifera (flower)	M. oleifera (leaves)
Ethyl acetate: isopropanol: water	Gallic acid	-
Toluene: ethyl acetate: formic acid	Gallic acid, catechol, resorcinol, quercetin	Catechol, gallic acid, resorcinol, quercetin
Toluene: ethyl acetate: acetic acid	Gallic acid, Pyrogallol, Catechol, Resorcinol, salicylic acid, picric acid, p-nitrophenol, morin	Catechol, resorcinol, salicylic acid, gallic acid, quercetin
Toluene: acetone: formic acid	Gallic acid, resorcinol, picric acid, pyrogallol, catechol, quercetin, salicylic acid	Resorcinol, picric acid, quercetin, salicylic acid
n-hexane: ethyl acetate: formic acid	Resorcinol	-
n-hexane: ethyl acetate: acetic acid	Gallic acid, Pyrogallol, catechol, p-nitrophenol, resorcinol, picric acid, ascorbic acid	-
Butanol: ammonium hydroxide: water	Morin	Chlorogenic acid, morin, caffeic acid
Butanol: methanol	Kaempferol, myricetin	Kaempferol, myricetin

Table 4: List of compounds identified in different solvent extracts by comparing the maximum absorbance of the sample with reference compounds with the help of UV- visible spectroscopy**.**

Plant extracts	Compounds identified	
	In M. oleifera flowers	In M. oleifera leaves
Distilled water extract	Nobiletin, p- coumarate, Ferulate, Apigenin, Kaempferol, Daidzein	p- coumaroylquinic acid
Methanolic extract	Nobiletin, p- coumaric acid, Apigenin, Glycitin, Gallic acid, Quercetin, Catechin, Rutin, Resorcinol, Palmitic acid, Linoleic acid	Apigenin, Palmitic acid, Linolenyl alcohol
Ethanolic extract	Tangeretin, Gallic acid, Glycitin, p-coumaric acid, Naringenin	Myricetin, Quercetin
DMSO extract	Tangeretin, Ferulic acid, Salicylic acid, p- coumarate, Nobiletin	Ferulic acid, p- coumarate, Salicylic acid

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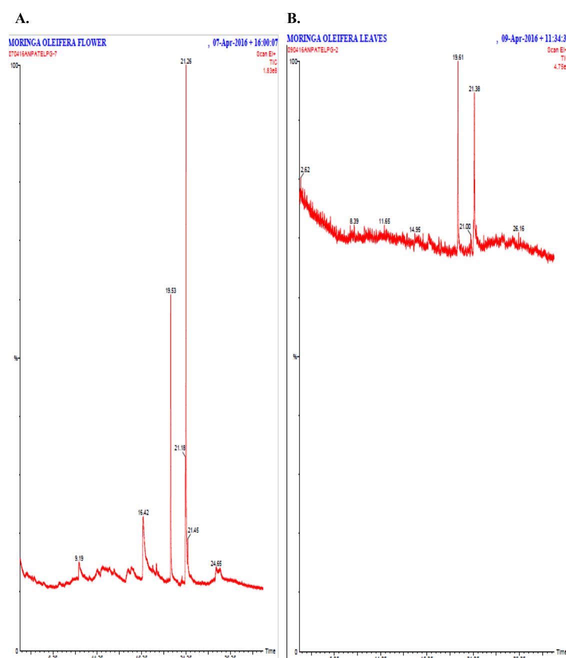

Figure 1: A.) Gas chromatogram of *M. oleifera* flowers, B.) Gas chromatogram of *M. oleifera* leaves.

Gas chromatography is used for the further confirmation of the compound present in the crude. It is used to get the detailed structure of analytes based on mass to charge ratio and can also separate volatile and non-volatile compounds. In GC analysis, two sharp peaks for the *M. oleifera* flower were observed at 21.26 and 19.53 cm (Fig 1 A), while for *M. oleifera* leaves it was at 19.61 and 21.38 cm (Fig 1 B). Mass Spectroscopy for both flowers and leaves was performed for further insight.

The MS analysis of the peak (21.76 cm) obtained in GC corresponds to the molecular weight of 280 and it probably shows the presence of Linoelaidic acid (Fig 2 A). MS of the GC peak of flowers (19.176 cm) corresponds to the molecular weight of 256 and hinting the presence of N-Hexadecanoic acid (Fig 2 B).

MS of the GC peak for leaves (19.61 cm) corresponds to the molecular weight of 256 indicates the presence of is N-Hexadecanoic

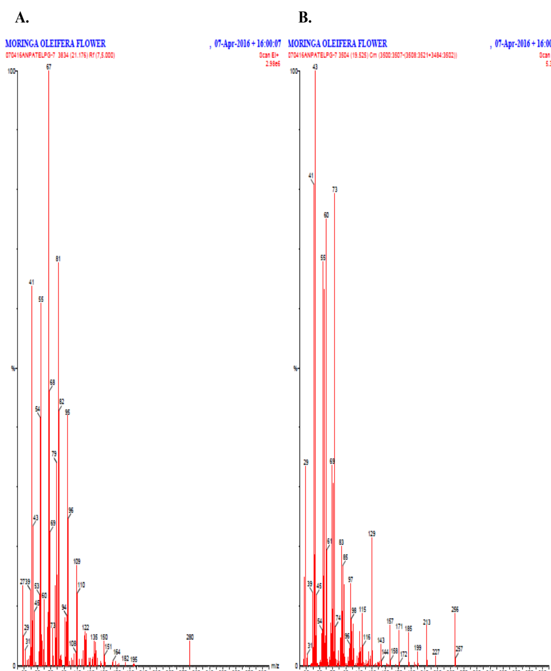

Figure 2: A.) Mass spectra of *M. oleifera* flowers obtained at 21.17 GC peak, B.) Mass spectra of *M. oleifera* flowers obtained at 19.17 GC peak.

acid (IUPAC) and also known as Palmitic acid (Fig 3 A). MS of the GC peak for leaves (21.38 cm) corresponds to the molecular weight of 278 and suggesting the presence of 9,12,15-octadecatrienoic acid,(zzz)(IUPAC) and commonly known as the Linolenyl alcohol (Fig 3 B).

Identification of functional group by Fourier Transformed Infrared spectroscopy (FTIR)

FTIR is valuable for the characterization and identification of the compounds or the functional groups (chemical bonds) present in an unknown mixture of plant extract. The spectrum of an unknown compound can be identified by comparison to a library of known compounds. FTIR data of *M. oleifera* flowers shows the different range of IR spectrum, which matches with the known standard functional group (ranges) (Fig 4 A). From this analysis, the functional groups like Alcohol, Phenol, Amine, Alkyl, Carboxylic acid, Ether, Methyl were

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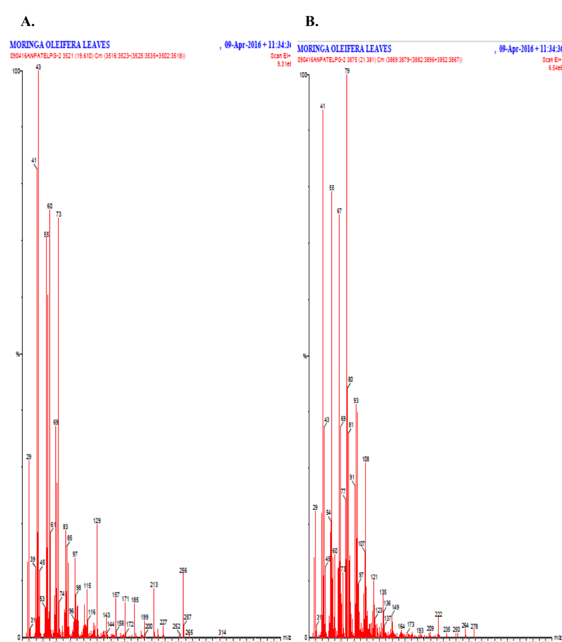

Figure 3: A.) Mass spectra of *M. oleifera* leaves obtained at 19.61 GC peak, B.) Obtained at 21.38 GC peak.

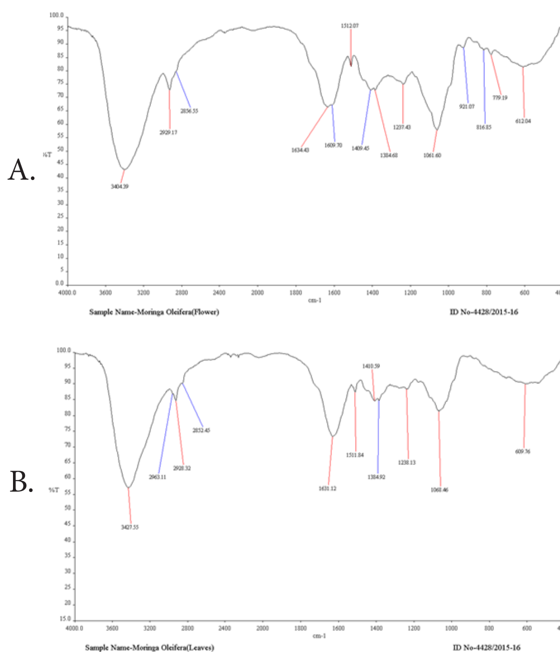

Figure 4: A.) FTIR spectra of M. oleifera flowers, B.) FTIR spectra of *M. oleifera* leaves.

obtained (Table 5).

FTIR data of *M. oleifera* leaves shows the different range of IR spectrum, which matches with the known standard functional group (Fig 4 B). From this analysis, functional groups like Amine, Alkyl, Methyl, Nitro, and Ether were obtained (Table 6).

Life-span assay on C. elegans

Synchronization of worms

The synchronized worms were obtained with the help of bleaching, as only eggs can survive during bleaching, and worms of the different stages were eliminated. Synchronization was necessary for the lifespan assay to calculate the mean increase in the survival rate of the *C. elegans* after feeding with the extract. All the worms of the L1 stage were grown for 28 hours to arrest them at the L4 stage (Fig 5).

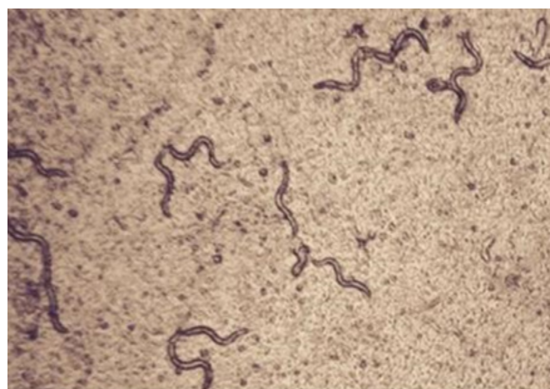

Figure 5: Synchronized worms at the L4 stage of the life cycle.

Life-span assay at 20°C

The normal lifespan of *C. elegans* at 20°C is 2-3 weeks. The effect of *M. oleifera* methanolic flower and leaves extracts on the lifespan of *C. elegans* was checked. Lifespan assay was done with synchronized worms. The synchronized worms at the L4 stage were transferred to the plates containing plant extract

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Table 5: Obtained FTIR spectra of *M. oleifera* flowers compared with the standard IR chart.

Sample peaks (Wavenumber cm^{-1})	Bond	Type of bond	Specific type of bond	Reference peaks (Wavenumber cm^{-1})	Appearance
3404	O-H	Alcohols, phenols	High concentration	3200-3400	Broad
	N-H	Primary amines	Any	3400-3500	Strong
2929	C-H	Alkyl	Methylene	2925	Medium to strong
	N-H	Ammonium ions	Any	2400-3200	Multiple broad peaks
2856	N-H	Ammonium ions	Any	2400-3200	Multiple broad peaks
	C-H	Alkyl	Methylene	2850	Medium to strong
1609	C=O	Carboxylic acid / derivatives	Carboxylate(salts)	1550-1610	-
			Amino acid		
			Zwitter ions		
	N-H	Primary amines	Any	1560-1640	Strong
1384	C-H	Alkyl	Methyl	1380	Weak
	N-O	Nitro compounds	Aliphatic	1380	Weaker
1237	C-O	Ethers	Aromatic	1220-1260	-
1061	C-O	Alcohols	Primary	1040-1060	Strong/ broad
	C-X	Fluoroealkanes	Ordinary	1000-1200	-
816	C-H	Vinyl	Trisubstituted alkenes	800-840	Strong to medium
	C-H	Aromatic	Para-disubstituted benzene	800-860	Strong
779	C-H	Aromatic	Meta-disubstituted benzene	750-800	Strong
612	C-X	Chloroalkanes	Any	540-760	Weak to medium

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Table 6: Obtained FTIR spectra of M. oleifera leaves compared with the standard IR chart.

Sample peaks (Wavenumber cm^{-1})	Bond	Type of bond	Specific type of bond	Reference peaks (Wavenumber cm^{-1})	Appearance
3427	N-H	Primary amines	Any	3400-3500	Strong
2963	C-H	Alkyl	Methyl	2960	Medium to strong
2928	C-H	Alkyl	Methylene	2925	Medium to strong
	N-H	Any	Any	2400-3200	Multiple broad peaks
2852	C-H	Alkyl	Methylene	2850	Medium to strong
	N-H	Ammonium ions	Any	2400-3200	Multiple broad peaks
1631	N-H	Primary amines	Any	1560-1640	Strong
	C-N	C=N	Any	1615-1700	Similar conjugation effects to C=O
1384	C-H	Alkyl	Methyl	1380	Weak
	N-O	Nitro compound	Aliphatic	1380	Weaker
1238	C-O	Ethers	Aromatic	1220-1260	-
1068	C-N	Aliphatic amines	Any	1020-1200	Often over-lapped
609	C-X	flouroalkanes	ordinary	1000-1100	-

of different concentrations (10µg, 20 µg, and 40 µg) and culture of OP50 was provided as food. The control was prepared with OP50 without extract. Animals were scored every day for survival. The worm which did not respond to repeated touching was scored as dead. The lifespan increase of *M. oleifera* flowers obtained at 40µg was highest i.e. 8 days as compared to 10µg and 20µg which were 4 and 6 days, respectively. In the case of *M. oleifera* leaves the increase in the survival rate obtained was 2, 4, and 8 days at 10µg, 20µg, and 40µg, respectively (Graph 9 A and B). The increase in life span might be because of the presence of potent antioxidant compounds in *M. oleifera* which can reduce the harmful free radicals produced during metabolic processes.

Life-span assay at 25°C

The survival rate of *C. elegans* at 25˚C is lower than that of 20˚C as the lifecycle of *C. elegans* is temperature-dependent. The effect of *M. oleifera* extracts on the lifespan of *C. elegans* was checked. The life-span assay was done with synchronized worms. The synchronized worms at the L4 stage were transferred to the plates containing plant extract of different concentrations (10µg, 20 µg, and 40 µg) and culture of OP50 was provided as food. The control was prepared with OP50 without extract.

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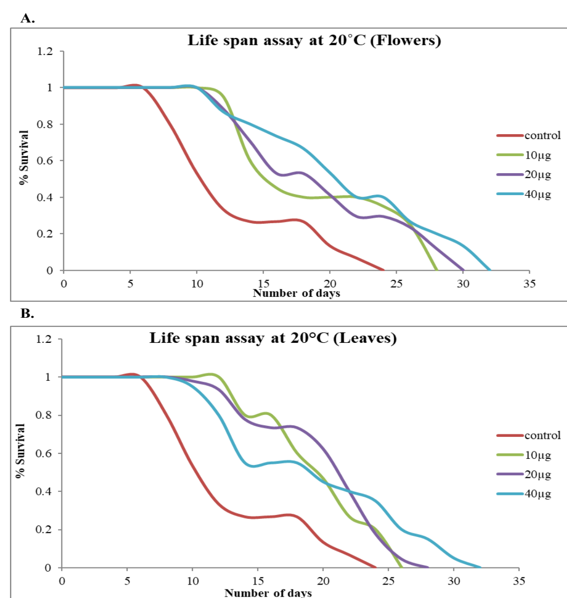
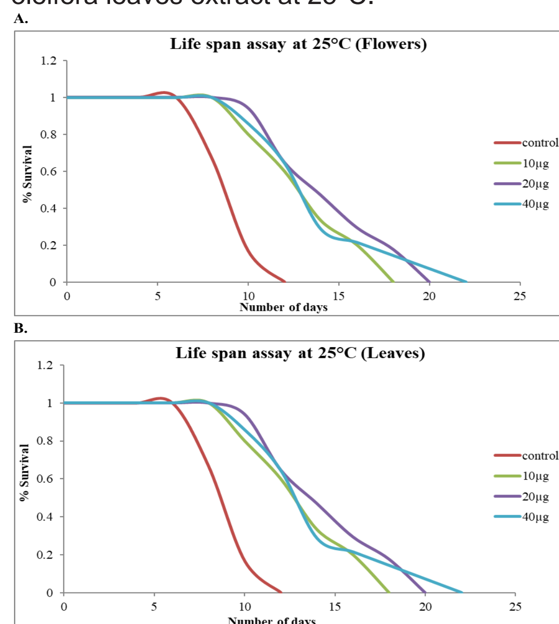
Graph 9: Increase in percentage survival of C. elegans at different concentrations (10, 20, and 40µg) of A.) M. oleifera flower extract and B.) M. oleifera leaves extract at 20°C.



Graph 10: Increase in percentage survival of C. elegans at different concentrations (10, 20, and 40µg) of A.) M. oleifera flower extract and B.) M. oleifera leaves extract at 25°C.



Animals were scored every day for survival. The worm which did not respond to repeated touching was scored as dead. The life span was increased, as observed in *M. oleifera* flowers and leaves which were 6, 8, and 10 days at 10µg, 20µg and 40µg, respectively (Graph 10 A and B). The extended lifespan shows that even under stress conditions the compounds present in *M. oleifera* can exhibit the potent anti-aging effect.

The primary phytochemical analysis has confirmed the presence of alkaloids, flavonoids, tannins, and vitamin C in both leaves & flowers. These compounds are known to be biologically active and linked with the various activities of the *M. oleifera* plant. Many previous reports demonstrated the phytochemicals as a potent antioxidant [33, 34, 35, and 36] and possess anti-inflammatory [37] activities that make them pharmacologically important to prevent and even cure various diseases [38, 39]. The quantitative analysis indicated the presence of a good amount of phytochemicals such as phenols, flavonoids, and vitamin C. The results of antioxidant assays proved the correlation of antioxidant and free radical scavenging capacity with phytochemical concentration. Further, in TLC analysis the most suitable solvent system was found to be toluene: ethyl acetate: acetic acid and butanol: ammonium hydroxide: water, which might be because its polarity was complemented with the compounds present in the sample. The use of standard compounds in TLC gave an idea about the presence of some of the important bioactive compounds. In the GC-MS analysis the compounds like Linoelaidic acid, N-Hexadecanoic acid were identified in flowers; and N-Hexadecanoic acid and Linolenyl alcohol were identified in leaves. These compounds are also reported to have antioxidant and other pharmacological properties. Additionally, the FTIR analyzed the presence of several functional groups that are an important part of phytochemicals such as phenols and flavonoids. Moreover, the *M. oleifera* flowers and leaves were applied to

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C. elegans to check whether its life span has affected under the treatment of extracts. As increased oxidative stress has a direct impact on aging and age-related diseases, the antioxidant proved to be beneficial for delaying aging. The antioxidant properties of Moringa made a positive effect on the aging of *C. elegans* and its life span was improved in normal and even in stress conditions. As the study on *C. elegans* applies directly to humans, Moringa may be one of the pharmaceutically important herbs, the compounds of which may be isolated and used in the modern medicinal system against various diseases.

Conclusion

Based on the results of the present study, it can be concluded that *M. oleifera* flowers and leaves have the potential to act as a source of useful drugs because of the presence of various phytochemical components such as phenols, vitamin C, tannins, and flavonoids. Thin-layer chromatography, UV absorption, GC-MS, and FTIR were carried out to the characterization of unknown compounds present in the plant extracts. The characterization results revealed the presence of palmitic acid, linolenic acid, and linoleic acid. These compounds containing potent anti-oxidative activities are now maximally used in the pharmacological industries for the production of medicines. *In vivo,* life-span assays were carried out on *C. elegans* and the life span was extended. As it has a genome similarity of 65% with humans, the increase in the lifespan of *C. elegans* indirectly serves for the production of novel medicine to cure the diseases caused by oxidative damage. Based on promising activities of extracts of *M. oleifera* flowers and leaves, it could be subjected to isolate most active components for drug development therapy.

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