ORIGINAL RESEARCH PAPER
Volume - 11 | Issue - 11 | November - 2022 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

ASSESSMENT OF NUTRITIONAL, ANTIOXIDANT AND ANTIFUNGAL POTENTIAL OF DIFFERENT EXTRACTS OF LEAVES OF MORINGA OLEIFERA (LAM.) WITH ITS CHEMICAL PROFILING.

Chemistry

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ABSTRACT

Five compounds, namely, 17-tritriacontanone, 1-hentriacontanol, Ethyl heptadecanoate, 4-[2'-O-acetyl-α-L-rhamnosyloxy] benzyl isothiocyanate, and 4,8,12,16-tetramethyl heptadecan-4-olide were isolated from the methanolic extract of leaves of *Moringa oleifera.* These compounds were characterized by using various spectroscopic techniques 1HNMR, FT-IR, LC-MS and GC-MS. Out of these five compounds, 17-tritriacontanone, 1-hentriacontanol and ethyl heptadecanoate were first time reported while 4-[2'-O-acetyl-α-L-rhamnosyloxy] benzyl isothiocyanate and 4,8,12,16-tetramethyl heptadecan-4-olide were already reported compounds. Different solvent fractions of leaves of *Moringa oleifera* were investigated for their phytochemical composition by determining total phenolics content, total flavonoid content, total alkaloid content, and mineral content, DPPH free radical scavenging activity and antifungal activity. Correlation studies between various phytochemicals and DPPH free radical scavenging activity were also carried out.

KEYWORDS

Moringa oleifera, phytochemicals, phenolics, antioxidant, minerals.

INTRODUCTION:

Moringa oleifera is commonly known as horseradish tree, drumstick tree, miracle tree, ben oil tree, Mother's best friend, etc (FAROOQ *et al*., 2007). It belongs to the family Moringaceae (SAINI, 2016). It is a small to medium-sized tree that grows up to a height of 10-12m. Leaves are alternate, oddly tripinnate, compounded, and triangular. They are 20-70cm long and are spirally arranged on the twig. *Moringa oleifera* is known as 'Miracle Vegetable' as almost all the parts of this plant are used in medicines and functional food (SIDDHURAJU and BECKER, 2003). It is a natural and whole source of vitamins, minerals, proteins, and antioxidants (MOYO *et al*., 2011). Three non-government organizations, namely Trees for Life, Church World Service, and Educational Concerns for Hunger Organizations, have prescribed *Moringa* as "Natural nutrition for Tropics". Leaves of *Moringa* have a great potential for those who are suffering from malnutrition as they contain Vitamin A four times of carrot, Vitamin C seven times of oranges, Calcium four times of milk, Potassium three times of banana, iron twenty-five times of spinach, proteins two times of milk and a good balance of all the essential amino acids (GANATRA *et al*., 2012). *Moringa* can be used as a complement to modern medicine as it can boost the immune system. Now days, it is being examined as a bio-enhancer of drugs and nutrients because of its production of compounds with antibiotic activity. Its leaves are antihelmentic, aphrodisiac and cure hallucinations, dry tumors, hiccough and asthma (FUGLIE, 2005). They are applied as poultice to sores and in the treatment of anaemia and menstrual irregularities. Because of isothiocyanates, they act as antidiabetic and anticancerous agents. The presence of flavonoids in leaves make them a potent source of antioxidants (JUNG, 2014). Leaves of this plant have shown various biological activities but it is yet to draw the attention for its use in agriculture. Keeping all these in mind it was thought of interest to investigate this plant for determination of its bioactive components as well as its efficacy for antifungal activity and nutritional potential.

MATERIALS AND METHODS:

Chemicals:

allic acid, aluminium chloride, bismuth nitrate, disodium sulphide, butylated hydroxy anisole (BHA), sodium hydroxide, sodium silicate, Neseller's reagent, 2,4-dinitrophenol (DNP), ammonium molybdate, ammonium vandate and potassium iodide were purchased from CDH, Daryaganj, New Delhi, India. Folin-ciocalteau reagent, thiourea, catechin and 1,1-diphenyl-2-picryl hydrazyl (DPPH) were obtained from Himedia Laboratories Pvt. Ltd., Nashik, Mumbai, India. All other chemicals and solvents used in the study were of analytical grade and purchased from CDH or SD Fine Chem Limited, Mumbai, India.

Plant Material:

Leaves of *Moringa oleifera* were procured from the campus of Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India, in month of January. All collected plant samples were washed thoroughly with water and shadow-dried for 10-15 days. These samples were then crushed with hands and stored in air-tight containers for further use. ***Preparation of plant extract/ fractions:*** Leaves of *Moringa oleifera* were extracted with hot methanol by soxhlet method for 8 hours. The process was repeated thrice and the respective extractives were pooled together. The obtained extractives were evaporated on a rotary evaporator to give a crude extract which was divided into two parts. One part was further fractionated into different solvents viz hexane, benzene, chloroform, ethyl acetate, acetone, and water (Fig 1). Each obtained fraction was evaporated to give a crude mass and stored in a refrigerator till use.

Column Chromatography:

The other part of the above-obtained methanol extract was mixed with silica gel 60-120 mesh size and subjected to column chromatography. A glass column of 1000×40 mm size was packed with a slurry of silica gel (60-120 mesh size) in hexane. A portion of the methanolic extract of leaves was introduced onto the column and eluted with solvents of increasing polarity. The elutropic series of solvents used were hexane, benzene, ethyl acetate, methanol, and their mixtures. Each eluate obtained was monitored by using Thin Layer Chromatography plates. The column chromatography afforded five compounds labeled as 1 to 5.

Identification of compounds:

Melting points were determined with a Ganson Electrical Melting Point apparatus. IR spectra were recorded with a Perkin Elmer Spectrum RX-I FTIR. ^{1}HNMR spectra of the isolated compounds were recorded on Sophisticated multinuclear FT NMR Spectrophotometer model Avance-II (Bruker 400MHz). LC-MS were recorded with a Waters Micromass Q-ToF Micro Mass spectrometer. It is equipped with electron spray ionization (ESI) and atmospheric pressure chemical ionization (APcI) source with a mass range of 4000 amu in quadruple and 20000 amu in ToF. The GC-MS of selected samples was recorded with Thermo Scientific TSQ 8000 Gas Chromatograph- Mass Spectrometer with triple quadruple and 2.1100amu mass range.

Nutritional Potential:

Determination of Total Phenolics Content (TPC):

Folin-Ciocalteau method (SINGLETON and ROSSI, 1965) was used to determine the total phenolics content of various solvent fractions of *Moringa oleifera* leaves. Gallic acid (2.5-50µg/ml) was used as a reference standard for plotting the calibration curve. The total phenolics content was calculated as mean±Standard Deviation (S.D.) and expressed as mg gallic acid equivalent (GAE)/g of dry extract.

Determination of Total Flavonoids Content (TFC):

The TFC of various solvent fractions of leaves of *Moringa oleifera* was estimated by aluminium chloride colorimetric assay (MARINOVA *et al*., 2005). Catechin was used as reference standard to construct the calibration curve. The TFC was calculated as mean±S.D. and expressed as mg catechin equivalent (CE)/g of dry extract.

Determination of Total Alkaloids Content (TAC):

The total alkaloids content was determined by the spectrophotometric method of Dragendorff's reagent method (SREEVIDYA and MEHROTRA, 2003). The total alkaloids were calculated from a linear regression equation obtained from the calibration plot of bismuth nitrate and expressed as mg bismuth nitrate equivalent (BNE)/g of dry extract.

Determination of Mineral Content:

Minerals (Fe, Cu, Zn and Mn) were determined by using Atomic Absorption Spectrophotometer. The mineral content was determined by using the following formula and expressed as mg/100g of the extract.

Mineral content = $(Abs_{Sample} - Abs_{Blank}) \times$ Dilution factor.

Nitrogen content was determined by the colorimetric (Nessler's reagent) method (LINDNER, 1944). The absorbance of the samples was read at 440nm using spectronic20 UV Visible spectrophotometer against a blank. Nitrogen concentration was determined by using a standard curve of ammonium sulfate and expressed in mg/100g.

Phosphorus content was also determined by Vanadomolybdo – phosphoric yellow color method (OLIVER and FUNNELL, 1961). The Phosphorus content (mg/100g) was determined using the standard plot of phosphorus chloride. Potassium content in the acid digest of plant samples was determined by taking a reading on Flame Photometer against a reagent blank.

DPPH Free Radical Scavenging Activity:

The free radical scavenging activity of different solvent fractions of *Moringa* leaves and standard butylated hydroxy anisole (BHA) was evaluated by using DPPH (2,2'-diphenyl-1-picrylhydrazyl) method (KATAYAMA, 2002). The decrease in absorbance was measured at 517nm using UV- Visible spectrophotometer against a blank prepared similarly without sample. The percentage antioxidant activity was calculated by using the equation:

$\%DPPH^*_{sc} = \{(A_{cont} - A_{samp})/A_{cont}\} \times 100$ where A_{cont} is the absorbance of control and A_{samp} is the absorbance of sample.

Antifungal Activity:

The antifungal activity of different extracts/ fractions of leaves of *Moringa oleifera* was investigated using two fungal species i.e. *Rhizoctonia solani* and *Fusarium oxysporum*. The fungal isolates were allowed to grow on Potato Dextrose Agar(PDA) (TUITE, 1969) at 25±2ºC until they sporulated. A 4-6 day old culture of each fungus was used for testing antifungal activity. Poisoned Food Technique (GROVER and MOORE, 1962) was used to determine the antifungal activity of different solvent fractions and extract of *Moringa oleifera* leaves. The fungal growth inhibition (%) was calculated by using the following formula:

$$\% \text{ inhibition} = \frac{C - T}{C} \times 100$$

Where C = mycelia growth in control plate, T = mycelia growth in treated plate

Statistical Analysis:

All the experimental measurements were carried out in triplicate and results were presented as mean± standard deviation. One-way and two-way analysis of variance (ANOVA) was carried out to assess any significant differences between the means ($p<0.05$) in Online Statistical Analysis (OPSTAT), CCS HAU, Hisar. EC50 values of antifungal activity and IC50 values of antioxidant activity were calculated using SPSS Statistics 19 software and regression analysis in Microsoft Excel, respectively. All other measurements and calculations were carried out in Microsoft Excel 2007. To find out the relationship between various phytochemicals and antioxidant activity, correlation was calculated by using OPSTAT.

RESULTS:

Isolation and characterization of compounds:

Compound 1: **^{1}H NMR (δ, CDCl$_3$)** 0.88 (t, J=8.0 Hz, 6H, 2× -C**H**$_3$), 1.25 (br, J=20.0 Hz, 52H, 26× -C**H**$_2$), 1.63 (m, J=8.0 Hz, 4H, 2× -C**H**$_2$), 2.34 (t, J=4.0 Hz, 4H, 2× -CH$_2$CO). IR ν_{max}^{KBr} (cm-1); 2917, 2849, 1708, 1472, 1463,729, 719. **LC-MS (m/z, % intensity)** 485(88.53), 437(3.43), 409(22.46), 387(11.23), 338(100), 337(17.37), 301(32.15), 274(18.56), 239(18.56), 188(8.86), 162(6.15), 148(35.58), 132 (36.64), 104(5.21).

Compound 2: 1H NMR (δ, CDCl3) 0.86 (t, J=8.0 Hz, 3H, 1× -C**H3**), 1.25 (br, J=8.0 Hz, 56H, 28× -C**H2**), 1.55(m, J=8.0 Hz, 2H, 1× -C**H2**), 3.64(t, J=8.0 Hz, 2H, 1× -CH2-OH). IR ν_{max}^{KBr} (cm-1); 3308, 2955, 2917, 2849, 1473, 1463, 1063, 730, 719. LC-MS (m/z, % intensity) 415(12.11), 351(7.11), 338(25.71), 301(6.74), 274(5.87), 188(11.23), 132(35.95).

Compound 3: **^{1}H NMR (δ, CDCl$_3$)** 0.88(m, J=8.0Hz, 6H, 2× -C**H**$_3$), 1.25(br, 24H, 12× -C**H**$_2$), 1.63(m, J=8.0Hz, 2H, 1× -C**H**$_2$CH$_2$COO), 2.23(m, 2H, 1× -C**H**$_2$COO), 3.56 (q, 2H, 1× -OC**H**$_2$). ν_{max}^{KBr} (cm-1); 2917, 2849, 1716, 1462, 1377, 1215, 1048, 758, 719. LC-MS (m/z, % intensity) 301(100), 279(14.21), 267(10.54), 243(17.35), 223(5.65), 202(89.23), 163(6.41), 149(11.63), 107(10.51), 85(33.38).

Compound 4: **^{1}H NMR (δ, CDCl$_3$)** 1.42 (m, J=4.0Hz, 3H, 1× -C**H**$_3$), 1.63(m, J=16.0Hz, 1H, 1× -C$_5$-**H**), 2.16(s, J=4.0Hz, 3H, 1× -OCOC**H**$_3$), 2.32 (t, J=8.0Hz, 1H, 1× -C$_4$-**H**), 3.96 (m, J=12.0Hz, 2H, 1× -C$_3$-**H** and 1× -C$_2$-**H**), 4.07 (m, J=8.0Hz, 1H, 1× -C$_1$-**H**), 6.80 (d, J=8.0Hz, 2H, 1× -C$_7$-**H**$_2$), 7.11(m, J=8.0Hz, 2H, 1× -C$_2$-**H** and 1× -C$_6$-**H**), 7.36 (m, 2H, 1× -C$_3$-**H** and 1× -C$_5$-**H**). **IR** ν_{max}^{KBr} (cm-1); 3418, 2916, 2849, 1732, 1716, 1510, 1462, 1248, 1182. LC-MS (m/z, % intensity) 363(6.90), 353(2.46), 333(6.21), 316(30.36), 301(100), 279(11.78), 243(15.25), 202(83.34), 163(3.50), 149(8.60).

Compound 5: **^{1}H NMR (δ, CDCl$_3$)** 0.86 (m, 3H, 1× -C**H**$_3$), 1.08 (m, J=4.0Hz, 6H, 2× -C**H**$_3$), 1.24-1.67 (m, 18H, 9× -C**H**$_2$), 1.94 (m, J=8.0Hz, 4H, 2× -C**H**$_2$), 2.13-2.26(m, J=8.0Hz,6H, 2-C**H**$_3$)ν_{max}^{KBr} IR (cm-1); 2919, 2850, 1714, 1667, 1651, 1462, 1377, 1260, 1087, 759. LC-MS (m/z, % intensity) 323(12.85), 307(8.35), 289(4.10), 253(3.94), 191(22.56), 188(10.85), 180 (4.86), 107(100).

Nutritional Potential:

Total Phenolics Content:

Folin-ciocalteau reagent method (SINGLETON and ROSSI, 1965) was used to determine the total phenolics content of various solvent fractions of *Moringa oleifera*.. A perusal of Fig 3 showed that acetone fraction of leaves of *M. oleifera* contained the highest amount of total phenols, i.e., 52.00±0.13 mg GAEg^{-1}. Ethyl acetate, water, and methanol extract had moderate amount of total phenolics i.e., 20.33±0.07, 30.54±0.26, and 24.71±0.19 mg GAEg^{-1}, respectively. While hexane (6.44±0.13 mg GAEg^{-1}), benzene (5.10±0.16 mg GAEg^{-1}), and chloroform (7.00±0.06 mg GAEg^{-1}) fractions of leaves of drumstick tree contained a meager amount of total phenols.

Total Flavonoids Content:

Total Flavonoids content was determined by using aluminium chloride colorimetric assay (MARINOVA *et al*., 2005). A study of the data presented in Fig 2 revealed that the amount of total flavonoids content was maximum in ethyl acetate fraction of leaves of *M. oleifera* followed by methanol extract and acetone fraction containing 63.00±0.33 and 51.00±0.33 mg CEg^{-1} of flavonoids. Water fraction contained moderate amount of total flavonoids i.e., 27.33±0.33 mg CEg^{-1}, while hexane, benzene, and chloroform fractions contained a very low amount of total flavonoids with 4.56±0.19, 5.44±0.39, and 12.00±0.33 mg CEg^{-1} respectively.

Total Alkaloids Content:

A study of the data presented in Fig 2 revealed that among various fractions of leaves, methanol fraction contained the highest amount of total alkaloids. The amount of total alkaloids in various extract/ fractions of leaves of *Moringa oleifera* varied from 25.50±1.00 to 97.83±0.76 mg BNEg^{-1}. Various extract/ fractions of leaves contained total alkaloids in the order: methanol (97.83±0.76 mg BNEg^{-1}) > chloroform (87.67±0.76 mg BNEg^{-1}) > benzene (81.86±0.66 mg BNEg^{-1}) > water (79.00±0.50 mg BNEg-^{1}) > ethyl acetate (66.00±0.50 mg BNEg^{-1}) > acetone (56.33±0.76 mg BNEg^{-1}) > hexane (25.50±1.00 mg BNEg^{-1}).

Mineral Content:

The present study results (Table 1) showed that iron content was found to be minimum in hexane fraction i.e., 4.45±0.03 mg/100g, and maximum in chloroform fraction i.e., 30.11±0.33 mg/100g. The concentration of Copper ranged from 0.20±0.02 mg/100g in hexane fraction to 4.01±0.58 mg/100g in ethyl acetate fraction. Various extract/ fractions of leaves of *Moringa oleifera* contained zinc content in the following order: acetone (457.10±1.55 mg/100g) > methanol (237.80±0.58 mg/100g) > ethyl acetate (234.80±0.78 mg/100g) > water (224.10±1.45 mg/100g) > chloroform (134.10±0.03 mg/100g) >

hexane (78.10±0.78 mg/100g) > benzene (39.10±0.33 mg/100g). For Manganese, the trend followed by leaves of *M. oleifera* was: water (2.33±0.10 mg/100g) > hexane (1.62±0.02 mg/100g) > acetone (1.18±0.04 mg/100g) > methanol (1.03±0.01 mg/100g) > ethyl acetate (0.60±0.02 mg/100g) > chloroform (0.52±0.03 mg/100g) > benzene (0.38±0.01 mg/100g). The maximum nitrogen concentration was found in acetone fraction (1162.50±0.08 mg/100g) while minimum in hexane fraction (387.50±1.16 mg/100g). Moderate amounts of Nitrogen were found in ethyl acetate (1050.00±0.01 mg/100g), methanol (900.00±0.34 mg/100g), chloroform (850.00±0.78 mg/100g), and water (800.00±0.04 mg/100g) fractions of leaves of *M. oleifera*. Comparatively, low amounts were found in benzene fraction (612.50±0.45 mg/100g). For Phosphorus, trend followed by various fractions of leaves of *M. oleifera* was: chloroform (312.50±0.02 mg/100g) > methanol (293.75±2.58 mg/100g) > water (225.00±0.34 mg/100g) > benzene (206.20±0.67 mg/100g) > ethyl acetate (187.50±0.56 mg/100g) > hexane (137.50±0.01 mg/100g) > acetone (112.50±0.67 mg/100g). Potassium content (Table 1) was found to be very high as compared to other minerals. The respective order for various extract/ fractions of leaves of *M. oleifera* was: water (1575.00±1.23 mg/100g) > methanol (1387.50±1.55 mg/100g) > acetone (185.30±0.34 mg/100g) > hexane (57.30±0.34 mg/100g) > ethyl acetate (25.10±0.04 mg/100g). Differences in mineral composition of a plant could result from variation in edaphic factors and agro-climatic conditions.

DPPH Free Radical Scavenging Activity:

The data presented in Fig 4 showed free radical scavenging activity of various extract/ fractions of leaves of *Moringa oleifera* at 100, 200, 400, 600, 800, and 1000 µg/ml concentrations. Benzene and chloroform fractions of *Moringa* leaves had not shown any activity up to the highest tested concentration. Maximum antioxidant activity was demonstrated by acetone fraction with 98.35±0.28% inhibition at 600 µg/ml concentration and 186.96µg/ml IC_{50} value. Similar activity was shown by ethyl acetate and water fractions and methanol extract with 286.33, 195.32, and 247.55 µg/ml IC_{50} values. The lowest activity was exhibited by hexane fraction with 43.01±0.27% inhibition at 1000 µg/ml concentration. Critical difference for antioxidant activity was calculated for various extract/ fractions of leaves and concentrations. Interaction of the compounds and concentrations was statistically significant with a value of 0.643 for concentration × compound.

Antifungal Activity:

Antifungal activity of various extract/ fractions of leaves of *Moringa oleifera* at different test concentrations against *Rhizoctonia solani* and *Fusarium oxysporum* are presented in Fig 5 and Fig 6. The activity data revealed that leaves extract/ fractions of *M. oleifera* were more active against *R. solani* than *F. oxysporum*. However, all the concentrations were found to be statistically different. Chloroform fraction exhibited the highest activity at 2000 µg/ml concentration with 82.75±0.34% growth inhibition against *R. solani* and 114.65 µg/ml EC_{50} value. *F. oxysporum* was found to be the most sensitive for ethyl acetate fraction of leaves of *M. oleifera* with 30.39±0.34% growth inhibition at 2000 µg/ml concentration. Very good activity was shown by methanol, acetone, chloroform, and ethyl acetate fractions of leaves of drumstick tree against *R. solani* at all the tested concentrations. Moderate activity was exhibited by benzene and acetone fractions of leaves against *F. ooxysporum*. The lowest antifungal activity was shown by benzene and water fractions at 250 µg/ml concentration with 6.47±1.18% and 4.51±0.34% growth inhibition against *R. solani* and *F. oxysporum,* respectively. In terms of EC_{50} value (Fig 7), chloroform fraction was the most active with 114.65 µg/ml EC_{50} value. Critical difference values for antifungal activity of various extract/ fractions of leaves of *Moringa oleifera* were calculated. Interaction of compounds and concentrations was statistically significant with a value 1.352 against *R. solani* and 1.529 against *F. oxysporum* for concentration × compound.

DISCUSSION:

Isolation and characterization of compounds:

Compound 1 (17-tritriacontanone):

Compound 1 was obtained on elution with pure hexane as a white solid (15mg) with a melting point of 76-78ºC. Its R_f value was found to be 0.62 in benzene: hexane (3:7). The appearance of the absorption band at 1708 cm^{-1} confirmed the presence of the carbonyl group. Other absorption bands appeared at 2917, 2849, 1472, 1463, 729, and 719 cm^{-1}. LC-MS analysis suggested the molecular mass of the compound to be 468, having molecular formula $C_{33}H_{66}O$. Thus, based on the above information, the compound could be characterized as 17-tritriacontanone and assigned the structure given in Fig 2. This is the first time report of isolation and characterization of 17-tritriacontanone from *Moringa oleifera* leaves.

Compound 2 (1-hentriacontanol):

It was obtained on elution with benzene: hexane (1:19) as shiny white needles. The purity of the compound was confirmed by TLC showing a single spot with R_f value 0.19 in benzene: hexane (3:7) on development with iodine. The melting point of the compound was observed to be 82-84ºC (literature m.p. 87ºC (HEILBRON, 1965). LC-MS data showed M^+ peak as molecular mass of compound 2 to be 452 with the molecular formula $C_{31}H_{64}O$. The IR indicated the presence of absorption bands at 3308 (-OH), 2955 (-CH_2), and 2917 (-CH_2). All the above discussed spectral data helped to identify the compound 2 as 1-hentriacontanol (Fig 2). This is the first report of isolation and characterization of 1-hentriacontanol from leaves of *Moringa oleifera*.

Compound 3 (Ethyl heptadecanoate):

It was obtained on elution with benzene: hexane (1:9) as a solid (30mg). It was recrystallized from benzene. The presence of ester was determined by a positive hydroxamic acid test. The purity of the compound was confirmed based on its behavior on the TLC plate as a deep yellow spot (R_f = 0.24) with iodine as a developing phase. The melting point of the compound was observed to be 25-26ºC (literature m.p. 24.7ºC (KNOTHE and DUNN, 2009). LC-MS data showed $[M+3]^+$ peak at 301, suggesting its molecular formula to be $C_{19}H_{38}O_2$. The IR spectra indicated the presence of absorption bands 2917 and 2849 (>CH_2) and 1716 cm^{-1} (>C=O). All the above discussed spectral data were in agreement with the literature data of ethyl heptadecanoate. So, this compound is identified as ethyl heptadecanoate (Fig 2). This is the first time report of isolation and characterization of ethyl heptadecanoate from *Moringa oleifera* leaves.

Compound 4 (4-[2'-O-acetyl-α-L-rhamnosyloxy] benzyl isothiocyanate:

Compound 4 was obtained as a viscous oily liquid (33mg) on elution with benzene: hexane (1:1). Its R_f value was found to be 0.22 in ethyl acetate: benzene (1:19). The purity of the compound was checked on a TLC plate. It was obtained as a single, deep yellow spot when developed with iodine as a developing phase on TLC. LC-MS analysis of the compound indicated its molecular formula and molecular mass to be $C_{16}H_{19}NO_6S$ and 353, respectively. The IR spectra of compound 4 gave absorption bands at 3418, 1732 and 1716 cm^{-1} indicating hydroxyl and carbonyl groups. All the above discussed spectral data agreed with the literature of 4-[2'-O-acetyl-α-L-rhamnosyloxy]benzyl isothiocyanate (ZAHIRAH *et al*., 2018). So, compound 4 was identified as 4-[2'-O-acetyl-α-L-rhamnosyloxy]benzyl isothiocyanate (Fig 2).

Compound 5 (4,8,12,16-tetramethyl heptadecan-4-olide):

Compound 5 was obtained on elution with ethyl acetate: benzene (1:14) as a viscous oily liquid (25mg). The purity of the compound was checked on TLC as a single spot when placed in iodine. Its R_f value was found to be 0.35 in ethyl acetate: benzene (1:1). The molecular formula $C_{21}H_{40}O_2$ and molecular mass 324 were deduced from its LC-MS analysis. The IR spectra of the compound MOL-V gave absorption peaks at 1714 and 1667 cm^{-1} indicating the presence of carbonyl group in it. Thus, based on the above information, compound 5 was identified as 4,8,12,16-tetramethyl heptadecan-4-olide (Fig 2). It is an already reported compound from the leaves and seeds of *Moringa oleifera*.

Nutritional Potential:

Plant phenolics are the most abundant secondary metabolites possessing one or more aromatic rings with hydroxyl groups. These include phenolic acids, flavonoids, tannins, stilbenes, and lignans. Because of their ability to scavenge free radicals and active oxygen species, they act as high-level antioxidants. In addition, they exhibit considerable free radical-scavenging activities (through their reactivity as hydrogen-donating or electron-donating agents) and metal ion-chelating properties (DAI and MUMPER, 2010). Folin-ciocalteau reagent method was used to determine the total phenolics content of various solvent fractions of *Moringa oleifera*. This method is based on the transfer of electrons in an alkaline medium from phenolic compounds to phosphomolybdic/ phosphotungstic acid complexes to form blue colored complex whose intensity was determined spectrophotometrically at 725nm (SINGLETON and

ROSSI, 1965). The differences in the amount of total phenolics content in various solvent fractions might be attributed to the availability of different extractable compounds and the effectiveness of different solvents to dissolve endogenous/ phenolic compounds (DAI and MUMPER, 2010. Phenolic compounds are more soluble in polar solvents because of the presence of hydroxyl group in their structure (ARYAL *et al*., 2019). In addition to solvent efficiency on extractable phenols, the differences in phenolics content may be due to geographical conditions and agro-climatic differences. Environmental factors such as growing season, soil type, temperature, seed variety, and agronomic practices have also significantly affected the phytochemical compositions (ZUBAIR, 2011).

Flavonoids are probably the most important class of natural phenolics and have the ability to donate electrons or hydrogen atoms readily, so they can directly scavenge reactive oxygen species. They are also known to act as an antioxidant, both as radical scavengers and as metal chelators (KUMAR and PANDEY, 2013). As a basic quantitative determination, the flavonoids content was determined by using aluminium chloride in a colorimetric assay. Its basic principle is that aluminium chloride forms acid-stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols (PEKAL AND PYRZYNSKA). In addition, it also forms acid-labile complexes with o-dihydroxyl groups in A or B ring of flavonoids. The differences in the amount of total flavonoids content might be attributed to the availability of various extractable phytochemicals in different solvents. Some biological, environmental, and seasonal variations and genetic diversity (ARYAL, 2019) also affects the flavonoids content present in Moringa leaves. Alkaloids are nitrogen-containing organic compounds of plant origin. Most of the alkaloids have complex chemical structures of multiple ring systems. These compounds are known to possess many pharmacological properties. The alkaloid content was determined using Dragendorff's reagent method, in which alkaloids are precipitated as $(BiI_3)(Alk{\cdot}HI)$ by Dragendorff's reagent. Bismuth forms a yellow bismuth complex $\{Bi[CS(NH_2)_3]\}(NO_3)_3$ in nitric acid medium with thiourea, whose intensity is measured at 435nm. The bismuth from the alkaloidal complex is completely released by disodium sulphide (PAVLOV, 2009). Alkaloids are secondary metabolite compounds that shows a wide range of biological activities. The major property of alkaloids is their basic nature which causes them to decompose in presence of oxygen on exposure to heat and light. The free form of basic alkaloids is generally soluble in organic solvent whereas its salts are highly soluble in water (SALAMAH and NINGSIH, 2017).

Antioxidant Activity:

Antioxidants affect lipid oxidation at different stages due to differences in their mode of action (RICHARD, 1997). Oxidation of lipids is a very complex process resulting in a great variety of oxidation products. Many factors, particularly temperature, light, and initiators (metal enzymes), influence the oxidation process and resulting products. For this reason, different methods are needed for monitoring oxidation processes to assess primary or secondary oxidation changes and the efficiency of antioxidants. Phenolic acids account for about one-third of total daily intake, while flavonoids account for about remaining two-thirds of a regular human diet. Due to the chemical diversity of phenolic compounds and the complexity of composition in plant samples, it isn't easy to study each phenolic antioxidant individually (BONOLI, 2004). Moreover, the study of the total antioxidant power of a complex is often more valuable because of the cooperative action of antioxidants. In the present study, the antioxidant activity was evaluated by the DPPH method. 2,2'-diphenyl-1-picrylhydrazyl radical is one of the few stable and commercially available organic Nitrogen radical (DPPH) whose alcoholic solutions have a characteristic absorption maximum at 517 nm. When an electron or hydrogen atom donating antioxidant (AH) is added to DPPH, a decrease in absorbance at 517nm occurs due to the formation of the non-radical form DPPH-H which does not absorb at 517nm (MOLYNEUX, 2004). This reaction is measured by de-coloration assay, where the decrease in absorbance at 517nm produced by the addition of the antioxidant to the DPPH in methanol or ethanol is measured. DPPH +AH → DPPH-H+A The difference in the free radical scavenging activities of different solvent fractions is due to difference in polarity of extraction solvents. DPPH is only soluble in organic solvents, which is an important limitation of this method. Also the reaction of DPPH with antioxidants depend on the chemical structure of antioxidants present in various extract/ fractions (ULEWICZ-MAGULSKA AND WESOLOWSKI, 2019).

Antifungal Activity:

Various extract/ fractions of all parts of *Moringa oleifera* were screened *in-vitro* for antifungal activity against two phytopathogenic fungi i.e., *Rhizoctonia solani* and *Fusarium oxysporum*. The poisoned food technique determined the activity at four different test concentrations, i.e., 250, 500, 1000, and 2000 µg/ml concentrations**.** This difference in antifungal activity of various extract/ fractions of *Moringa oleifera* might be due to presence of different compounds in different extracts since different extract/ fractions used in this study vary in terms of their polarity.

The relationship between various phytoconstituents, i.e. total phenolics content, total flavonoid content, total alkaloid content, and antioxidant activity, is explained by finding correlation between phytochemicals and their activity. Irrespective of different solvents, there was a good and positive correlation between total phenolics, flavonoids, alkaloids, and antioxidant activity in terms of IC_{50} value. In the present study, phenols and flavonoids of various fractions were significantly and positively correlated with antioxidant activity. These results indicated that different phenolic substances had different degrees of contribution to the overall antioxidant activity. Furthermore, the mixture of phenolic substances in the extract may have a synergistic effect, which is affected by varying test conditions.

Some fractions were also having a non-significant correlation between total phenolics, total flavonoids, and antioxidant activities. The lack of correlation could be due to different responses of different phenolic compounds in other solvent systems. Some research workers who investigated different crops also reported that there might not be a significant correlation between flavonoids content and antioxidant activity (PAL *et al*., 2011, ACHARRYA *et al*., 2012, MEDA *et al*., 2013).

Conflict of Interest: The authors declare no conflict of interest.

Acknowledgements:

The authors are thankful to SAIF, CIL and UCIM, Panjab University, Chandigarh for providing facilities for spectral analysis. In addition, we gratefully acknowledge the Department of Plant Pathology, CCS HAU, for their kind assistance in antifungal activity.

Table 1. Mineral content (mg/100g) of various fractions of leaves of Moringa oleifera

Sr. No.	Extract/ Fractions	Iron (Fe)	Copper (Cu)	Zinc (Zn)	Manganese (Mn)	Nitrogen (N)	Phosphorus (P)	Potassium (K)
1.	Hexane	4.45±0.03	0.20±0.02	78.10±0.78	1.62±0.02	387.50±1.16	137.50±0.01	57.30±0.34
2.	Benzene	20.29±0.04	1.02±0.03	39.10±0.33	0.38±0.01	612.50±0.45	206.20±0.67	ND
3.	Chloroform	30.11±0.33	3.42±0.67	134.10±0.03	0.52±0.03	850.00±0.78	312.50±0.02	ND
4.	Ethyl acetate	16.40±1.16	4.01±0.58	234.80±0.78	0.60±0.02	1050.00±0.01	187.50±0.56	25.10±0.04
5.	Acetone	20.10±1.34	1.85±0.08	457.10±1.55	1.18±0.04	1162.50±0.08	112.50±0.67	185.30±0.34
6.	Water	25.69±0.15	2.53±0.88	224.10±1.45	2.33±0.10	800.00±0.04	225.00±0.34	1575.00±1.23
7.	Methanol	22.76±0.96	0.62±0.02	237.80±0.58	1.03±0.01	900.00±0.34	293.75±2.58	1387.50±1.55
SE(d)		0.632						
CD at 5%		1.369						
CV%		3.877						

Mineral content was expressed in mg/100g, All values are mean ± SD, Values are mean of three replicates (n=3), ND means not detected

Table 2. Antioxidant activity IC50 values of leaves of *Moringa oleifera*

Sr. No.	Extract/ Fractions	Regression Equation	IC50 value (µg/ml)
1.	Hexane	y = 2E-05x2 + 0.0109x + 9.5525	1175.47
2.	Benzene	ND	ND
3.	Chloroform	ND	ND
4.	Ethyl acetate	y = -0.0001x2 + 0.2215x - 5.2235	286.33
5.	Acetone	y = -0.0002x2 + 0.2801x + 4.6236	186.96
6.	Water	y = -0.0002x2 + 0.2764x + 3.6442	195.32
7.	Methanol	y = -6E-05x2 + 0.1829x + 8.4007	247.55
8.	BHA	y = -0.008x2 + 1.3156x + 33.976	13.25

ND means not detected

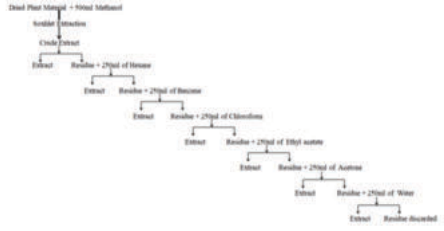

Figure 1. Schematic representation of sequential extraction of fractions from the leaves of Moringa oleifera.
At every step the residue was dried at room temperature prior to extraction with next solvent.

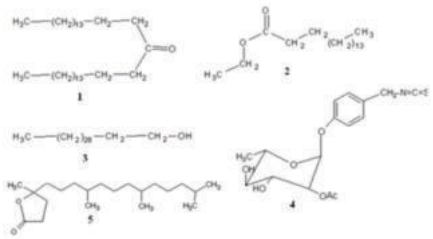

Figure 2.Chemical structures of compounds isolated from leaves of Moringa oleifera

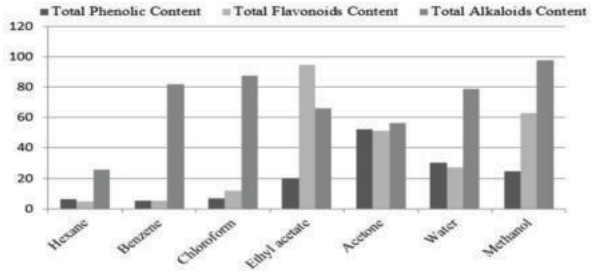

Figure 3. Phytochemical composition of different extract/ fractions of Moringa oleifera leaves.
All values are mean of three replicates. Total phenolics content is expressed in mg GAEg-1 (milligrams of gallic acid equivalent per gram). Total flavonoids content is expressed in mg CEg-1 (milligrams of catechin equivalent per gram). Total alkaloids content is expressed in mg BNEg-1 (milligrams of bismuth nitrate equivalent per gram).

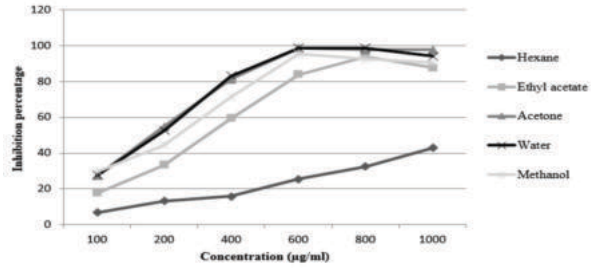

Figure 4. DPPH free radical scavenging activity of various extract/ fractions of leaves of Moringa oleifera

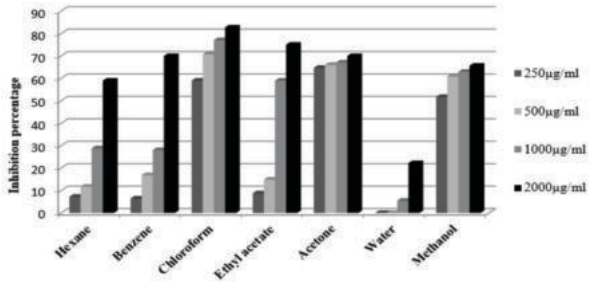

Figure 5. Antifungal activity of various extract/ fractions of leaves of Moringa oleifera against Rhizoctonia solani

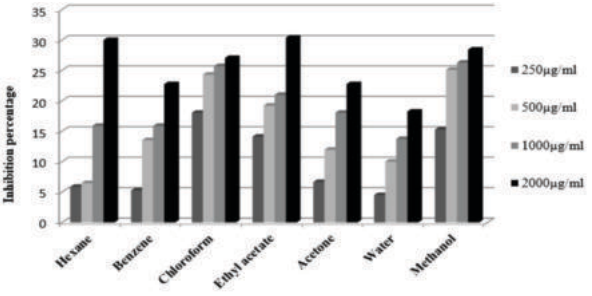

Figure 6. Antifungal activity of various extract/ fractions of leaves of Moringa oleifera against Fusarium oxysporum

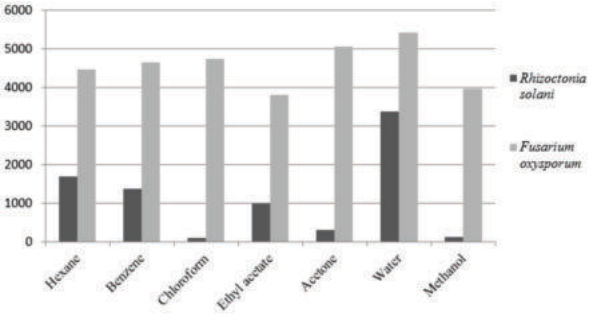

Figure 7. EC50 values of different extract/ fractions of leaves of *Moringa oleifera*

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