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Phytochemical Investigations and Antifungal Activity of Bark of *Moringa oleifera* (Lam.)

JYOTI PUNIA*, RAJVIR SINGH and SUMAN

Department of Chemistry and Biochemistry, College of Basic Sciences and Humanities, Chaudhary Charan Singh Haryana Agricultural University, Hisar-125 004, India

*Corresponding author: E-mail: jyotipunia11@gmail.com

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Phytochemical study of methanolic extract of bark of *Moringa oleifera* afforded three compounds *viz*., 2,11-dihydroxy-12-methoxy lactone, β-sitosterol and xylitol. Out of these, two compounds were new and reported for the first time from *Moringa oleifera* bark namely, 2,11-dihydroxy-12-methoxy lactone and xylitol. Also the methanolic extract was fractionated using solvents of different polarity. The obtained fractions and methanolic extract were then evaluated for their antifungal activity by poisoned food technique at 250, 500, 1000 and 2000 µg/mL concentrations against two phytopathogenic fungi. All the fractions of bark had shown maximum antifungal activity at 2000 µg/mL concentration against *R. solani* and *F. oxysporum*. All the extract/fractions were also more toxic against *Rhizoctonia solani* than *Fusarium oxysporum*.

Keywords: *Moringa oleifera*, Antifungal activity, Isolation, Phytochemicals, *Rhizoctonia solani*, *Fusarium oxysporum*.

INTRODUCTION

Plants are natural factories that continuously synthesize compounds in themselves. These compounds possess numerous biological and pharmacological activities which form a basis for production of numerous drugs based on natural sources. In recent years, a significant interest in natural products as a potential source of medicines has been developed among the researchers in the world. *Moringa oleifera* is also a store house of natural products. It is a small to medium sized tree which grows up to a height of 10-12 m. It is known by a number of names like drumstick tree, Mother's best friend, Miracle vegetable, *etc.* [1]. The bark is whitish grey, soft corky and gummy. When wounded the bark exudes a gum which is initially white in colour but change to reddish brown on exposure. The corky bark yields a coarse fiber that is utilized for making mats, paper and cordage. The stem exudes a mucilaginous gum that is used in leather tanning and calcico printing [2]. The bark of *Moringa* too has an excellent biosorbent property for removal of heavy metal ions from waste water or effluents [3]. The bark of *Moringa oleifera* is aphrodisiac and abortifacient and can be used to cure dental caries, common cold, epilepsy, headache and ulcer. Barks are boiled in water and soaked in alcohol to obtain soft drinks and infusions that can be used to treat stomach ailments, poor vision, joint pain, diabetes, hypertension and uterine disorders [4]. Root bark is used as antiviral, anti-inflammatory and analgesic. It is used to treat heart complaints, eye diseases, tridosha fevers, inflammation, dyspepsia and enlargement of spleen [5]. According to The Ayurvedic Pharmacopoeia of India, the dried root bark is used in the treatment of goiter, glycosuria and lipid disorders while leaf, seed, stem bark and root bark in internal abscess and pain. Root juice is employed in cardiac tonic and is epileptic and used for nervous debility, asthma, enlarged liver and spleen, deep seated inflammation and as diuretic in calculus affection. Decoction is used as a gargle in hoarseness and sore throat. Roots and fruits of Moringa oleifera (Lam.) also have antiparalytic activity [6].

EXPERIMENTAL

All the solvents used were of analytical grade. Melting points were determined with a Ganson Electrical Melting Point apparatus. ^{1}H NMR spectra of the isolated compounds were recorded on Sophisticated multinuclear FT NMR spectrophotometer model Avance-II (Bruker 400 MHz). $CDCl_3$ and DMSO were used as solvents. Chemical shifts were recorded in δ (ppm) using tetramethyl silane (TMS) as an internal standard. IR spectra were recorded with a Perkin Elmer spectrum RX-I

FTIR. It has a resolution of 1 cm^{-1} and scan range of 4000 to 250 cm^{-1}. 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 having mass range of 4000 amu in quadruple and 20000 amu in ToF.

Bark of *Moringa oleifera* was collected from the campus of Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India. These were washed thoroughly with water to remove dust, shadow dried and chopped into small pieces. These were then kept in air tight containers for further use.

Extraction and fractionation: The shadow dried chopped pieces of bark of drumstick tree were extracted with hot methanol by refluxing method for 8 h. 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. This extract was further divided into two parts. One major part was mixed with silica gel (60-120 mesh size) and used to fill the column. The remaining part was further fractionated with different polarity solvents *viz*. hexane, benzene, chloroform, ethyl acetate, acetone and water. Each obtained fraction was evaporated to give a crude mass and stored in a refrigerator till use. These fractions and methanolic extract were used for determination of antifungal activity.

Preliminary phytochemical screening: The freshly prepared methanolic extract of bark of *Moringa oleifera* was subjected to qualitative chemical tests using standard methods. This helps in the identification of various classes of bioactive chemical constituents.

Column chromatography: The methanolic extract of bark of *Moringa oleifera* was mixed with silica gel 60-120 mesh size and subjected to column chromatography. A glass column of 1000 mm × 40 mm size was packed with slurry of silica gel (60-120 mesh size) in hexane. A portion of the methanolic extract of bark was introduced onto the column and eluted with solvents of increasing polarity. The elutropic series of solvents used was hexane, benzene, ethyl acetate, methanol and their mixtures. Each eluate obtained was monitored by using thin layer chromatography plates. The column chromatography afforded three new compounds labelled as **I** to **III**.

Compound **I** was obtained on elution with pure benzene as a pale yellow solid, 47 mg, melting point 68-72 °C. It was crystallized from ethyl acetate:benzene (1:9). The presence of ester was confirmed by hydroxamic acid test of the compound. The molecular formula $C_{21}H_{32}O_6$ was deduced from *m/z* 384 by its LC-MS. ^{1}H NMR (δ, $CDCl_3$): 0.88 (t, *J* = 8.0 Hz, 3H, 1×-C**H**$_3$), 1.25 (br, 9H, 3×-C**H**$_3$), 2.37 (d, *J* = 8.0 Hz, 2H, 1×-C**H**$_2$-C=O), 3.65 (m, *J* = 4.0 Hz, 3H, –OC**H**$_3$), 4.11 (m, *J* = 8.0 Hz, 1H, -C**H**OH), 4.23 (m, *J* = 8.0 Hz, 2H, 2×-CHO**H**). IR (KBr, ν_{max}, cm^{-1}): 3243, 2917, 2849, 1731, 1510, 1470, 1178, 1047, 720. LC-MS (*m/z*, % intensity) 409.28 (100), 387.31 (40.10), 384.91 (3.48), 316.3 (34.10), 301.24 (53.33), 279.25 (8.24), 205.16 (13.88), 203.25 (6.20), 202.24 (40).

Compound **II** was obtained on elution with ethyl acetate: benzene (1:1) and crystallized out from ethyl acetate, 35 mg, melting point 135-137 °C. It gave green colour with Liebermann Burchard's reaction indicating the presence of steroid. The LC-MS and elemental analysis suggests the molecular formula and mass to be $C_{29}H_{45}O$ and 412. ^{1}H NMR (δ, DMSO): 0.67(s, *J* = 8.0 Hz, 3H, 1×C_{18}-C**H**$_3$), 0.79(d, *J* = 4.0 Hz, 6H, 1×C_{26}-C**H**$_3$ and 1×C_{27}-C**H**$_3$), 0.83 (t, *J* = 8.0 Hz, 3H, 1×C_{29}-C**H**$_3$), 0.90(d, *J* = 4.0 Hz, 3H, 1×C_{21}-C**H**$_3$), 1.24 (s, 3H, 1×C_{19}-C**H**$_3$), 1.40-3.18 (m, 29H, 11× -C**H**$_2$ and 7× -C**H**), 5.12(m, 1H, C**H**-OH), 5.32 (br, *J* = 4.0 Hz, 1H, =C**H**). IR (KBr, ν_{max}, cm^{-1}): 3400, 2934, 2869, 1463, 1367, 1074, 925. LC-MS (*m/z*, % intensity): 409 (10.56), 375 (3.78), 301 (20.83), 243(16.05), 154 (7.35), 123 (23.46), 106 (100).

Compound **III** was isolated on elution with methanol:ethyl acetate (1:9) and crystallized out from ethyl acetate to get brown solid, 47 mg, melting point 272-274 °C. It gave positive Molisch test which ensures the presence of sugar. Its R_f value was found to be 0.88 in toluene:ethyl acetate:acetic acid (5:4:1). The molecular formula $C_5H_{12}O_5$ was deduced from *m/z* 147 by its LC-MS. ^{1}H NMR (δ, DMSO): 2.89 (m, *J* = 4.0 Hz, 2H, 1×C_2-C**H** and C_4-C**H**), 3.12 (m, *J* = 4.0 Hz, 1H, 1×C_3-C**H**), 3.69 (s, *J* = 4.0 Hz, 1H, 1×C_3-O**H**), 4.51 (m, *J* = 4.0 Hz, 4H, 1×C_1-C**H**$_2$ and 1×C_5-C**H**$_2$), 4.79 (m, *J* = 8.0 Hz, 2H, 1×C_1-O**H** and 1×C_5-O**H**), 5.17 (d, *J* = 4.0 Hz, 2H, 1×C_2-O**H** and C_4-O**H**). IR (KBr, ν_{max}, cm^{-1}): 3392, 3242, 2918, 2849, 1445, 1195, 1050. LC-MS (*m/z*, % intensity) 235.1 (30), 176.1 (17), 147.1 (75), 104.1 (45), 84.1 (78), 66.1 (100).

Test organism: The antifungal activity of different extracts/fractions of bark of *Moringa oleifera* was investigated using two phytopathogenic fungi *i.e. Rhizoctonia solani* and *Fusarium oxysporum* which were obtained from the Department of Plant Pathology, College of Agriculture, Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India. The fungal isolates were allowed to grow on Potato Dextrose Agar [7] (PDA) at 25 ± 2 °C until they sporulated. A 4-6 day old culture of each fungus was used for testing antifungal activity.

Bioassay: Poisoned food technique [8] was used for determination of antifungal activity of different solvent fractions and extract of *Moringa oleifera* bark. Two sets were maintained-one for the treatment and another for control. The treatment set at different concentrations *viz*. 250, 500, 1000 and 2000 µg/mL was prepared by mixing the required quantity (25, 50, 100 and 200 mg, respectively) in 1 mL of DMSO and then added pre-sterilized PDA. In control set, 1 mL DMSO was mixed with PDA. These treatments and control were then poured in pre-sterilized Petri plates and allowed to solidify at room temperature. After solidification, mycelia disc of 5 mm diameter cut out from 4-6 days old culture of test fungi were aseptically placed in Petri plates of different treatment and control sets. The Petri plates were then wrapped with para film along the rim to prevent contamination. The inoculated plates were then inverted and incubated at 25 ± 2 °C and the observations were recorded when the control plate got completely filled with test fungus. Colony diameter was determined by measuring the average radial growth of each plate. The data recorded in each case was mean of three replicates. 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.

The concentration of plant extract/fractions producing 50 % growth inhibition (EC_{50}) was calculated using SPSS Statistics 19 software.

Data 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. EC_{50} values of antifungal activity were calculated using SPSS Statistics 19 software. All other measurements and calculations were carried out in Microsoft Excel 2007.

RESULTS AND DISCUSSION

Preliminary phytochemical screening: Phytochemical investigation of the methanolic extract of bark of drumstick tree (Table-1) showed the presence of saponins, carbohydrates, anthraquinone glycosides, alkaloids, flavonoids, terpenoids, phytosterols, proteins and amino acids while tannins and cardiac glycosides were found absent.

Isolation and characterization of isolated compounds

Compound I (2,11-dihydroxy-12-methoxy lactone): Fractions 1-30 were separated by silica gel column chromatography on elution with pure benzene. Purity of the compound was checked on TLC plate as a single deep yellow spot by using iodine as a developing agent. The compound was obtained as pale yellow solid (47 mg). Its R_f value was found to be 0.48. LC-MS data showed that the molecular mass of the compound **I** to be 380 with molecular formula $C_{21}H_{32}O_6$. The IR spectra of the compound indicated the presence of hydroxyl, carbonyl and methylene groups at frequencies 3243, 1731 and 2917, 2849, 1047 and 720 cm^{-1}, respectively.

The ^1H NMR spectra of the compound **I** in $CDCl_3$ exhibited a triplet at 0.88 δ (J = 8.0 Hz) for methyl group. Intense resonance related to three methyl groups was found at 1.25 δ. A doublet peak at 2.37 δ with coupling constant J = 8.0 Hz was assignable to protons alpha to carbonyl groups. A multiplet integrating for methoxy protons appeared at 3.65 δ. Another multiplet centred at 4.11 δ for one proton was assignable to methine group attached to –OH functionality which itself appeared as a multiplet at 4.23 δ with J value 8.0 Hz. Thus, based on the above information, the compound I could be characterized as 2,11-dihydroxy-12-methoxy lactone. This is probably the first report of isolation and characterization of 2,11-dihydroxy-12-methoxy lactone from bark of *Moringa oleifera*.

Compound II (β-sitosterol): The compound was obtained on elution with ethyl acetate: benzene (1:1) and crystallized out from ethyl acetate, 35 mg, melting point 135-137 °C (literature m.p. 136-137 °C [9]). It responded to Liebermann-Burchard reaction and gave green colour indicating the presence of steroid. The IR spectra of this compound showed a peak at 3400 cm^{-1} indicating the presence of –OH group in the compound. The LC-MS and elemental analysis suggests the molecular formula and mass to be $C_{29}H_{45}O$ and 412.

The ^1H NMR spectra of the compound in DMSO exhibited a singlet at 0.67 δ for three protons which was assignable to methyl group present at C_{18} position. A doublet centered at 0.79 δ with J = 4.0 Hz integrating for six protons indicated two methyl groups positioned at C_{26} and C_{27}. A doublet centered at 0.90 δ with J = 4.0 Hz integrating three protons suggested the presence of a methyl group at C_{21} and a triplet at 0.83 δ (J = 8.0 Hz) representing three protons was assignable to methyl group at C_{29} position. A singlet at 1.24 δ representing three protons could be due to methyl group at C_{19} position. Appearance of a multiplet in the range of 1.40-3.18 δ representing twenty nine protons hinted the presence of seven methines and eleven methylenes. Another multiplet centered at 5.12 δ integrating for one proton could be of a proton positioned at alpha to hydroxyl group. A broad signal at 5.32 δ for one proton was assigned to an olefinic proton. A complete agreement of the data of compound II with the literature data of β-sitosterol established the identity of the compound II to be β-sitosterol.

Compound III (xylitol): It was obtained as brown solid (47 mg) on elution with methanol:ethyl acetate (1:9) and recyrstallized from ethyl acetate, melting point 272-274 °C. Its R_f value was found to be 0.88 in toluene: ethyl acetate: acetic acid (5:4:1). It responded to Molisch test, formation of purple colour at the junction of two liquids which indicate the presence of carbohydrates. Absorption at 3392, 3242, 2918, 2849, 1445, 1195 and 1050 cm^{-1} indicated the presence of hydroxyl and methylene groups. LC-MS of the compound III provides information regarding molecular mass to be 152.

The ^1H NMR spectrum of compound III in DMSO has shown a multiplet at 2.89 δ integrating for two protons of C_2-CH and C_4-CH. Another multiplet at 3.12 δ could be of proton at C_3 position. A singlet appeared at 3.69 δ having J value 4.0 Hz integrating for one proton confirmed the presence of OH group at C_3 position. There was a multiplet for four protons at

TABLE-1
PRELIMINARY PHYTOCHEMICAL SCREENING OF METHANOLIC EXTRACT OF BARK OF *Moringa oleifera*

S. No.	Phytochemicals tested	Name of the test	Results
1	Saponins	Frothing test	Positive (Presence)
2	Tannins	Ferric chloride test	Negative (Absence)
3	Carbohydrates	Fehling's test, Tollen's reagent test	Positive (Presence)
4	Cardiac glycosides	Keller-Killiani test	Negative (Absence)
5	Anthraquinone glycosides	Hydroxyanthraquinine test	Positive (Presence)
6	Alkaloids	Hager's test	Positive (Presence)
7	Flavonoids	Alkaline reagent test	Positive (Presence)
8	Terpenoids	Salkowski test	Positive (Presence)
9	Phytosterols	Liebermann- Burchard's test	Positive (Presence)
10	Protein	Biuret test	Positive (Presence)
11	Amino acids	Millon's test	Positive (Presence)

4.51 δ indicating the presence of $–CH_2$ groups at C_1 and C_5 positions. Another –OH functionalities appeared at 4.79 δ (C_1 and C_5) and 5.17 δ (C_2 and C_4) with J value 8.0 Hz and 4.0 Hz. The spectral and literature data helped us to identify compound that it could be xylitol (Fig. 1).

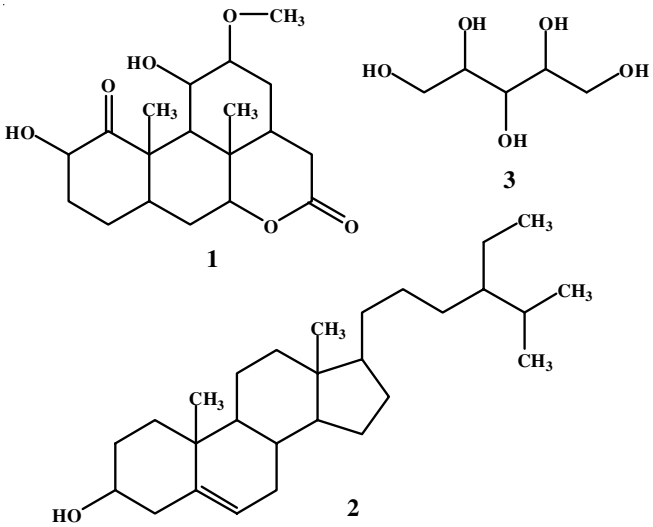

Fig. 1. Chemical structures of compounds isolated from bark of *Moringa oleifera*

Antifungal activity of bark of *Moringa oleifera*: A study of the data presented in Table-2 depicts that acetone and ethyl acetate fractions of bark of drumstick tree had shown maximum antifungal activity at 2000 µg/mL concentration *i.e.* 63.92 ± 1.22 % and 63.53 ± 0.59 %, respectively against *Rhizoctonia solani*. It was followed by hexane having 60.59 ± 0.59 % growth inhibition at 2000 µg/mL concentration. Moderate activity was shown by benzene fraction and methanol extract having 51.57 ± 0.68 % and 52.94 ± 0.83 % growth inhibition, respectively at 2000 µg/mL concentration. Chloroform and water fractions inhibited 45.49 ± 0.34 % and 42.92 ± 0.59 % mycelial growth of *R. solani* at 2000 µg/mL concentration, respectively. Among all the tested fractions, acetone fraction had the lowest value of EC_{50} *i.e.* 1562.18 µg/mL and hence was the most active fraction.

The data presented in Table-3 shows that the bark of *Moringa oleifera* had very low antifungal activity against *Fusarium oxysporum* at all the tested concentrations. Water fraction possessed maximum activity *i.e.* 23.73 ± 0.34 % at 2000 µg/mL concentration with EC_{50} value 4239.00 µg/mL. Chloroform and ethyl acetate fractions exhibited moderate activity having 18.04 ± 0.34 % and 19.41 ± 0.59 % growth inhibition at 2000 µg/mL concentration against *F. oxysporum*, respectively. Acetone fraction also inhibited 16.27 ± 0.34 % mycelial growth of the

TABLE-2
ANTIFUNGAL ACTIVITY (%) AND EC_{50} VALUES (µg/mL) OF VARIOUS EXTRACT/FRACTIONS OF BARK OF *Moringa oleifera* AGAINST *Rhizoctonia solani*

S. No.	Extract/Fractions	Growth inhibition (%)				EC_{50} (µg/mL)
		250 µg/mL	500 µg/mL	1000 µg/mL	2000 µg/mL	
1	Hexane	7.45 ± 0.34	16.08 ± 0.68	30.98 ± 0.90	60.59 ± 0.59	1586.99
2	Benzene	4.71 ± 0.59	9.22 ± 1.48	17.45 ± 1.48	51.57 ± 0.68	2200.98
3	Chloroform	5.49 ± 0.34	8.63 ± 0.90	17.45 ± 1.48	45.49 ± 0.34	2663.55
4	Ethyl acetate	6.08 ± 0.59	8.82 ± 0.59	20.59 ± 0.59	63.53 ± 0.59	1704.26
5	Acetone	3.92 ± 0.68	11.76 ± 0.59	26.86 ± 0.34	63.92 ± 1.22	1562.18
6	Water	4.31 ± 0.34	8.82 ± 0.59	12.55 ± 0.34	42.92 ± 0.59	3007.25
7	Methanol	3.92 ± 0.34	14.90 ± 0.34	28.04 ± 0.34	52.94 ± 0.83	1859.38
Factors		SE (d)	CD at 5 %			
Concentration		0.228	0.459			
Compound		0.302	0.607			
Conc. × Compound		0.604	1.213			

All the values are mean ± S.D.; Mean of three replicates was taken (n = 3); µg/mL means microgram per millilitre; EC_{50} means inhibition concentration at which 50 % of the growth is inhibited.

TABLE-3
ANTIFUNGAL ACTIVITY (%) AND EC_{50} VALUES (µg/mL) OF VARIOUS EXTRACT/FRACTIONS OF BARK OF *Moringa oleifera* AGAINST *Fusarium oxysporum*

S. No.	Extract/Fractions	Growth inhibition (%)				EC_{50} (µg/mL)
		250 µg/mL	500 µg/mL	1000 µg/mL	2000 µg/mL	
1	Hexane	5.10 ± 0.68	6.27 ± 0.68	12.35 ± 0.59	15.49 ± 0.34	6190.91
2	Benzene	5.10 ± 0.34	9.22 ± 0.34	12.35 ± 0.59	15.49 ± 0.34	6067.87
3	Chloroform	6.27 ± 0.68	11.57 ± 0.34	16.27 ± 0.34	18.04 ± 0.34	6471.81
4	Ethyl acetate	4.12 ± 0.59	11.96 ± 0.34	16.27 ± 0.34	19.41 ± 0.59	6101.03
5	Acetone	4.51 ± 0.34	9.80 ± 0.34	13.92 ± 0.34	16.27 ± 0.34	7184.56
6	Water	4.51 ± 0.34	7.84 ± 0.34	20.98 ± 0.34	23.73 ± 0.34	4239.00
7	Methanol	3.73 ± 0.34	8.04 ± 0.34	9.80 ± 0.34	14.51 ± 0.34	6548.81
Factors		SE (d)	CD at 5 %			
Concentration		0.133	0.267			
Compound		0.176	0.354			
Conc. × Compound		0.352	0.707			

All the values are mean ± S.D.; Mean of three replicates was taken (n = 3); µg/mL means microgram per millilitre; EC_{50} means inhibition concentration at which 50 % of the growth is inhibited.

tested fungi at 2000 µg/mL concentration. Hexane and benzene fractions have shown 15.49 ± 0.34 % antifungal activity at 2000 µg/mL concentration while methanol extract has shown the lowest activity *i.e.* 14.51 ± 0.34 at the same concentration. All the bark extract/fractions of *Moringa oleifera* were found to be more active against *Rhizoctonia solani* than *Fusarium oxysporum* (Figs. 2 and 3). All the fractions of bark had shown maximum antifungal activity at 2000 µg/mL concentration against *R. solani* and *F. oxysporum*.

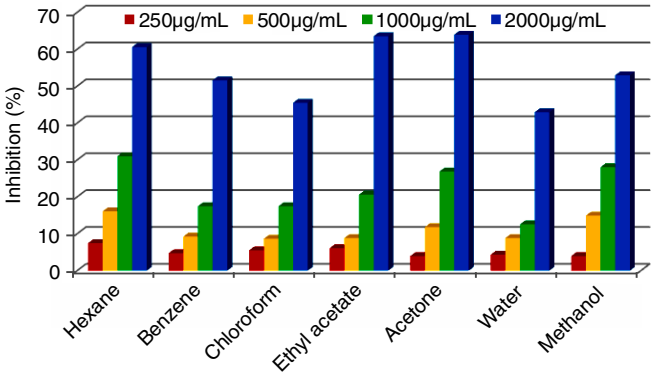

Fig. 2. Comparative analysis of antifungal activity of different extract/ fractions of bark of *Moringa oleifera* against *Rhizoctonia solani*

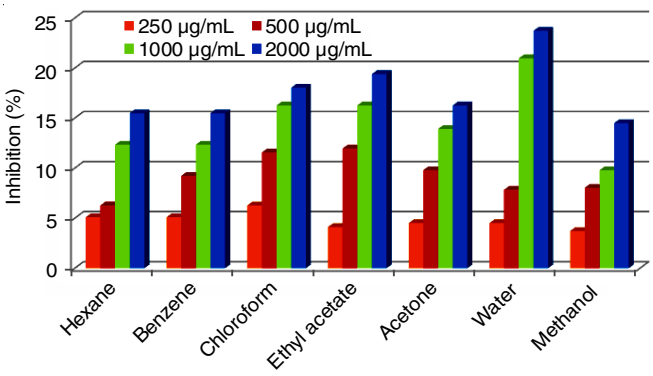

Fig. 3. Comparative analysis of antifungal activity of different extract/ fractions of bark of *Moringa oleifera* against *Fusarium oxysporum*

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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