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MORINGA OLEIFERA LEAVES EXTRACT: A POTENT ELICITOR FOR GERMINATION OF *ALFALFA SPROUT* WITH HIGH ANTIOXIDANT ACTIVITY

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ABSTRACT

Elicitation is an efficient tool to enhance/induce both chemical, and physiological changes which may subsequently influence the biological activity. This may stimulate both defense and stress-induced responses in many important medicinal plants and foods. Elicitors from different origins could be used either alone or with hydroponic sprays during growth or right before harvest. A better knowledge on the effect of certain compounds on biosynthetic pathways in responding to specific treatments with elicitors, would be a very useful way to augment the production of secondary metabolites or produce new metabolites. This will help in production a high quality, healthy, and useful medicinal plants and foods. *Moringa oleifera* (MO) leaves extract contains a several active constituents; alkaloids, carotenoids, glucosinolates, polyphenols, tannins and saponins and considered as a good biotic elicitor. It was used in this study to enhance both phenolic and antioxidant contents in germinated alfalfa sprout. In order to determine the ideal concentration for maximum elicitor response, various dilutions were tested (0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625g/L). Germination of alfalfa seeds in continuous light and soaking seeds in 0.0625, 0.03125g/L MO extract before germination significantly increases the levels of total phenolics and their antioxidant activity. The maximum amount of flavonoids were exudate after 8 hours of germination. The optimal concentration to elicit maximum phenolic levels was further used to study the biological activities.

KEYWORDS

Elicitors, *Moringa oleifera*, Alfalfa Sprout, Antioxidant and Total phenolics.

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INTRODUCTION

The liver research laboratory (FAB-Lab, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt) presented several approaches for a better utilization of natural products as potential therapeutic agents; anti-herpes (Badria *et al,* 2003)[1], immunomodulatory (Mikhaeil *et al,* 2003), schistosomicidal drug (Badria *et al,* 2001)[2],

antimutagens (Badria, 1994)[3], and colon cancer therapy (Ibrahim *et al,* 2014)[4].

However, there are number of examples which deal with enzymes as drug targets involved in the designing of enzyme inhibitors from commonly available natural products, such as; potential cataract therapy with differential inhibitory activity on aldose reductase (Elimam *et al,* 2017)[5], tyrosinase inhibitors for hyperpigmentation (Badria, 2001)[6].

Later, modulation of different biological activities via semi-synthesis of commonly available natural products was extensively studied by Badria's group including the followings; potent topoisomerase inhibitors (Abdel Bar *et al,* 2009)7, LTA4H inhibitor as potential colorectal cancer therapy (El-Naggar *et al,* 2017)[8], breast cancer inhibitors (Abdel Bar *et al,* 2010)[9], chemo-sensitization of cisplatin-resistant ovarian cancer by cucurbitacin B (El-Senduny *et al,* 2016)[10], and acetylcholinesterase inhibitors as a selective anti-Alzheimer agent (Abdel Bar *et al,* 2019)[11].

Optimizing the constituents of medicinal plants and foods would be a costly approach to enhance nutrition and disease control. Therefore, using certain treatments, such as precursor application and elicitor feeding could be utilized as a great and versatile alternative to improve chemical composition in plants and subsequently, increase its nutritional and therapeutic values either as a food, pharmaceuticals or drugs.

The use of elicitors as a tool to enhance the phytochemical content in plants, applied alone or in combinations at selected time points of the vegetable growth, should not be confused with those administered during the plant production cycle or pre-harvest, such as conventional fertilization (Baenas *et al,* 2014)[12]. *Moringa oleifera* leaves, which is the used natural biotic elicitor in this study, contain diverse bioactive chemical components (Dangi *et al,* 2002)[13] as presented in Figure No.1.

In 2004, Randhir *et al*,[14] showed the stimulation of phenolics, antioxidant and antimicrobial activities in dark germinated mung bean sprouts in response to peptide and phytochemical elicitors. In this study, *Moringa oleifera* was used as elicitor to the germinated alfalfa seeds to increase the phenolic, flavonoid contents and antioxidant activity, with a comparison between germination in light and dark conditions.

MATERIAL AND METHODS

Plant materials

Moringa olifera leaves were collected from the farm of Faculty of Pharmacy, Mansoura University, Egypt in November 2017 and were identified by Prof. Zain El-abdeen Abd El-Hameed Mohamed, Agricultural Botany Department, Faculty of Agriculture, Mansoura University. Dried seeds of alfalfa (*Medicago sativa.* L) were purchased from local market in Egypt.

MO leaves extraction

MO extract was prepared by soaking 500 gram of the crushed fresh MO leaves in 2L of 70% methanol for three days. Extractions were carried out three times and the organic solvents were removed at 50^{o}C using a rotary vacuum evaporator.

Treatment of alfalfa seeds and germination in dark and light

Dried seeds of alfalfa were soaked in distilled water for the control and in distilled water in presence of MO leaves extract for the treatments with different concentrations. The MO extract dilutions tested were 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625g/L (0.5g was dissolved in 40ml 90 % ethanol and complete to 1L with dist. water). Ten grams of seeds were placed in 200 ml of the soak solution in 250ml conical flasks. The flasks were then placed under aeration for 24 hours. The pre-soaked seeds were washed in distilled water and germinated in flats lined with moist paper towels. The flats were covered with aluminum foil and the seeds were germinated in the continuous dark and light at room temperature in the range of 22 ± 2^{o}C. The germinating seeds were kept moist with distilled water and sprouts were collected after 3 days of growth for analysis (Randhir R. *et al,* 2004)[15].

Extraction of seeds and elicited sprouts

The ground seeds and sprouts were extracted by shaking with 70% methanol overnight. Extractions were carried out twice and the organic solvents were removed at 50^0 C using a rotary vacuum evaporator.

Determination of total phenolic content in elicited seeds.

This method was carried out according to (Tega *et al*, 1984)[16]. Briefly, one milligram of the extract was dissolved in 1ml of MeOH/ H_2O (6: 4) containing 0.3 % HCl. To 100µl of the extract and 100µl Folin-Ciocalteau regent (10 % v/v), 2 ml sodium carbonate (2% w/v) were added and mixed completely. After 30 minutes, the absorbance of the solution was measured at 750nm. Quantitation was based on the standard curve (Figure No.2) of gallic acid (0-50µg/ml), dissolved in methanol/water (6:4) containing 0.3 % HCl. Phenolic content was expressed as µg/mg extract of gallic acid equivalent (GAE).

Determination of total flavonoid content in elicited seeds

The total flavonoid content was estimated using the $AlCl_3$ method as described by (Lamaison and Carnat, 1990)[17]. Briefly, 1ml of methanolic extract solution (10mg/ml) was added to 1ml of 2% methanolic $AlCl_3$, $6H_2O$. The absorbance was measured 10 minutes later at 450 nm. Flavonoid content was expressed in µg quercetin/10mg extract by comparison with standard quercetin treated in the same conditions.

Determination of antioxidant capacity in elicited seeds

Total anti-oxidant capacity was carried out using ABTS assay as described by (Lissi *et al,* 1999)[18]. Briefly, the reaction mixture was consisted of 2 ml of ABTS solution (60µM) and 3ml of MnO_2 solution (25mg/ml), all prepared in phosphate buffer (pH 7, 0.1M). The mixture was shaken, centrifuged, and decanted. The absorbance ($A_{control}$) of the resulting green-blue solution ($ABTS^+$ radical solution) was recorded at 750nm. The absorbance (A_{test}) was measured upon the addition of 100µl of 1mg/ml solution of the test sample in spectroscopic grade MeOH/buffer (1:1 v/v) to the ABTS solution. The decrease in absorbance is expressed as % inhibition which is calculated from the following equation:

$$\% \text{ inhibition} = \frac{A_{control} - A_{test}}{A_{control}} X\ 100$$

Ascorbic acid 100µl (2mM) solution was used as standard antioxidant (positive control). Blank sample was run using solvent without ABTS.

Analysis of alfalfa seeds exudate during germination for production of phenolics and flavonoids contents by Thin Layer chromatography (TLC)

Ten grams of alfalfa (*Medicago sativa* L.) seed, surfaces were sterilized 3 minutes with 70% ethanol, rinsed with sterile water and imbibed in sterile, aerated water. Imbibing solutions were changed after 1, 2, 3, 4, and 24 hours, 3, 4, 5, 6, 7, 8 and 9 days to remove seed-derived compounds. At the end of 24 hours, seeds were placed on a glass bottel containing 50ml distiled water. Containers were maintained under an 16/8 h light/dark, 25/20°C. Solutions containing root exudate were changed every 24 hours for 9 days and partitioned with ethyl acetate. After being dried, the ethyl-acetate-soluble fraction was dissolved in absolute methanol (Hartwig *et al.,* 1990). Then, TLC plate using solvent system (7.5 methylene chloride: 2.5 methanol) and $FeCl_3$ for phenolics.

Twenty-four flasks containing 5 g of alfalfa (*Medicago sativa L.*) seeds, surface sterilized for 3 min with 70% ethanol, rinsed with dist. water and imbibed in 20ml dist., aerated water. Imbibing solutions were removed after time interval 1 hour for 24 hr. The imbibing solutions were analyzed for their total flavonoid content as previously mentioned using $AlCl_3$ method.

RESULTS AND DISCUSSION

This study was to designed to investigate the effect of germination of alfalfa seeds in the continuous dark and light using different concentration of MO leaves extract on the phenolic, flavonoid contents and antioxidant activity of the alfalfa sprouts.

Alfalfa sprouts weights were estimated for 3 days of continuous light and dark germination as shown in Figure No.2.

Results in Table No.1 revealed that the yield in light is always more than that in dark conditions, especially at concentration (0.0625) of MO extract. These observations are consistent with that of (Sen and Chawan, 1970)[19], which confirms that seeds germination is preferred in light.

The total phenolic content of alfalfa sprouts was estimated for 3 days of continuous light and dark germination. The different MO extract concentrations tested were 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.015625g/L.

For all treatments and control higher phenolic levels was observed during continuous light germination at concentration 0.03125 and 0.015625 of MO extract (Table No.2), this level was approximately 119.7 % and 108.4788 % higher than control on the same day, respectively. In addition, this level was approximately 15.76871 % higher than control in the dark.

The total flavonoid content of alfalfa sprouts was estimated for 3 days of continuous light and dark germination. For all treatments and control higher flavonoid levels was observed during continuous light germination at concentrations 0.0625, 0.03125 and 0.015625 of MO extract (Table No.3), this level was approximately 14.19 %, 11.47 % and 9.36 % higher than control on the same day, respectively.

The changes in the flavonoid content in alfalfa seeds during germination are presented in Figure No.4, whereas the maximum amount of flavonoids were exudated after 8 hours of germination. The release of flavonoids in into root exudates have been reported in alfalfa and it was found that these flavonoids could induce nodulation (nod) genes in *Rhizobium meliloti* bacteria required in the formation of nitrogen fixing root nodules (Hartwig *et al,* 1991)[20].

The antioxidant activity in elicited alfalfa sprout extract was substantially improved over control when seeds germinated in continuous light at 0.0625 and 0.03125 concentrations of MO extract (Table No.4). It was observable that there is no anti-oxidant activity detected for those germinated in the dark.

Germination of alfalfa seeds in continuous light and soaking seeds in 0.0625, 0.03125g/L MO extract before germination significantly increases the level of phenolic content and antioxidant activity. The antioxidant activity of the different extracts could be correlated to their total polyphenol concentration (Yi *et al,* 2006)[21].

Table No.1: The weight (grams) of fresh sprouts after three days of germination in light and dark conditions

S.No	10 g seed/ tray	Weight of the fresh sprout after 3 days in Dark (g)	Weight of the sprout after 3 days in Light (g)
1	Control (water only)	66.47	72.8
2	(0.5 g/L) MO extract	46.24	62.88
3	(0.25 g/L) MO extract	53.42	68.69
4	(0.125 g/L) MO extract	59.55	66.14
5	(0.0625 g/L) MO extract	64.01	68
6	(0.03125 g/L) MO extract	60.66	64
7	(0.015625 g/L) MO extract	50.55	65.88

Table No.2: Total phenolic content (TPC), (µg/1mg MO extract) of seed and sprouts

S.No		TPC (µg/1mg) in light	TPC (µg/1mg) in dark
1	Control (water only)	11.13889	21.13889
2	(0.5 g/L) MO extract	18.5	14.88889
3	(0.25 g/L) MO extract	21.69444	11.55556
4	(0.125 g/L) MO extract	17.52778	11.55556
5	(0.0625 g/L) MO extract	19.05556	15.02778
6	(0.03125 g/L) MO extract	24.47222	16.41667
7	(0.015625 g/L) MO extract	23.22222	14.47222
8	Seed TPC	36.55556	

Table No.3: Total flavonoid content (TVC), (µg/10 mg extract) of seed and sprouts

S.No		TVC (µg/10mg) in light	TVC (µg/10mg) in dark
1	Control (water only)	8.879357	5.206434
2	(0.5 g/L) MO extract	6.761394	6.841823
3	(0.25 g/L) MO extract	7.163539	5.849866
4	(0.125 g/L) MO extract	8.477212	5.50134
5	(0.0625 g/L) MO extract	10.13941	5.849866
6	(0.03125 g/L) MO extract	9.898123	4.536193
7	(0.015625 g/L) MO extract	9.710456	5.072386
8	Seed	39.79088	

Table No.4: Antioxidant activity of selected seed and sprout using ABTS assay

S.No		Antioxidant activity in light	Antioxidant activity in dark
1	Control (water only)	-	-
2	(0.5 g/L) MO extract	4.44	-
3	(0.25 g/L) MO extract	-	-
4	(0.125 g/L) MO extract	23.33	-
5	(0.0625 g/L) MO extract	68.89	-
6	(0.03125 g/L) MO extract	32.22	-
7	(0.015625 g/L) MO extract	1.11	-
8	Seed	93.33	

(-) refers to the extracts with no antioxidant activity.

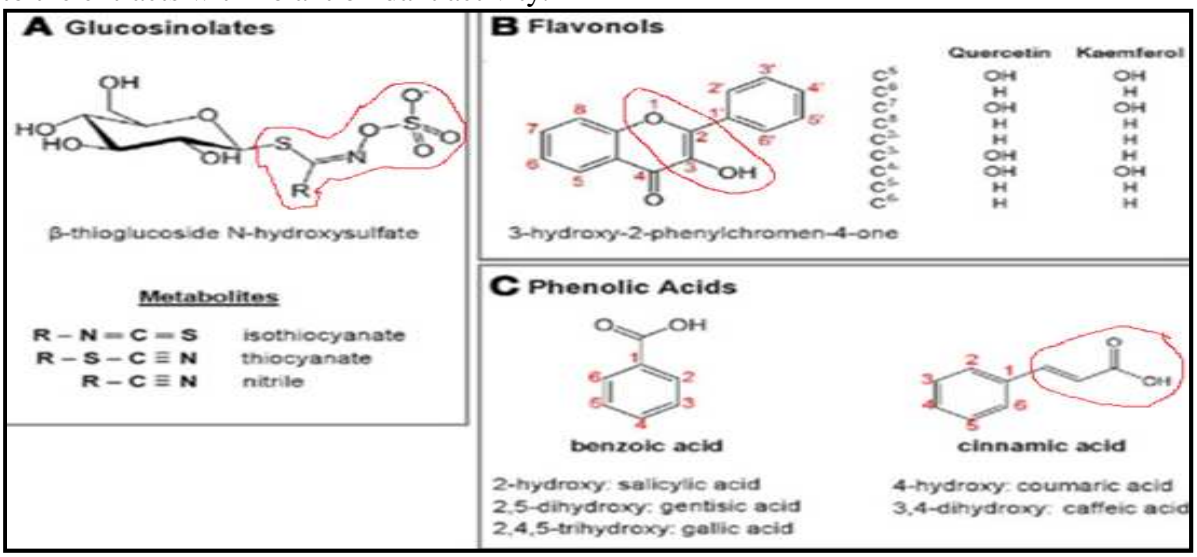

Figure No.1: Some bioactive compounds in *M. oleifera* leaves, www.pubmed.ncbi.nlm.nih.org/

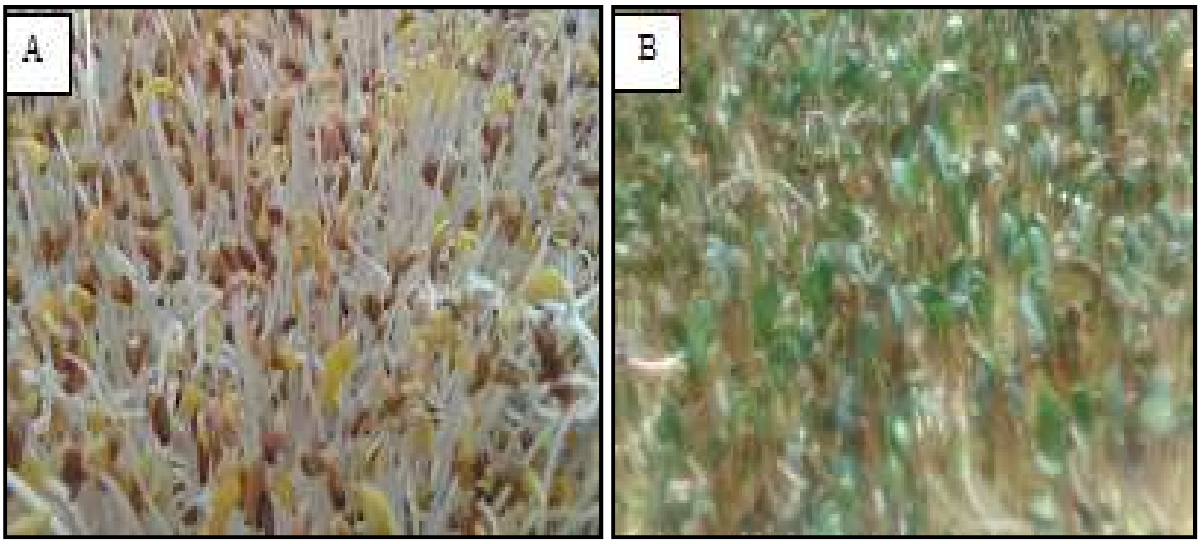

Figure No.2: (A) Alfalfa sprout in dark, (B) Alfalfa sprout in light, after 3 days of growth

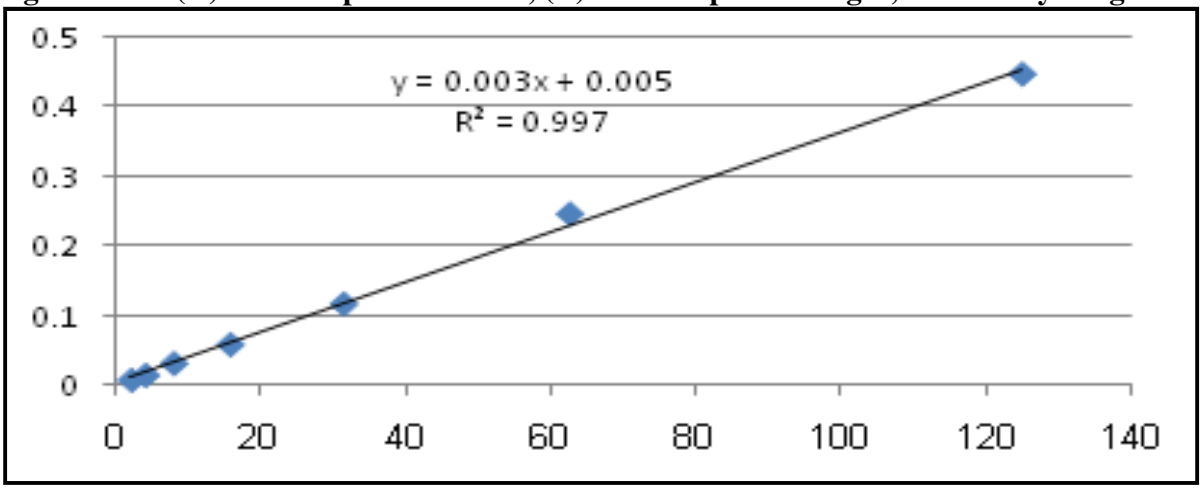

Figure No.3: Gallic acid standard curve showing absorbance (Y-axis) and concentration (µg/ml) on (X-axis)

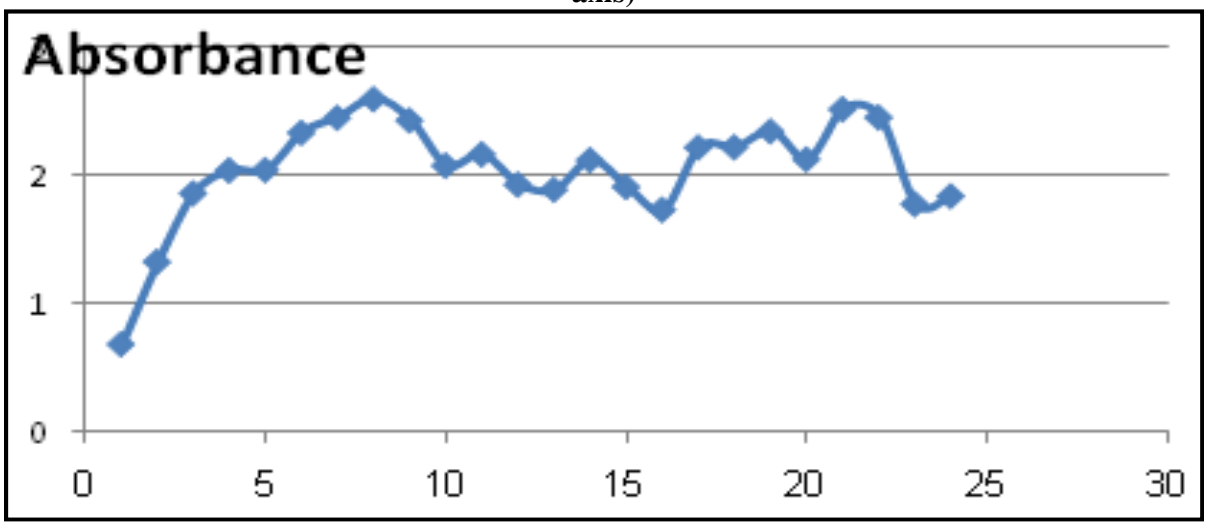

Figure No.4: The change of the flavonoid contents during germination of alfalfa seeds

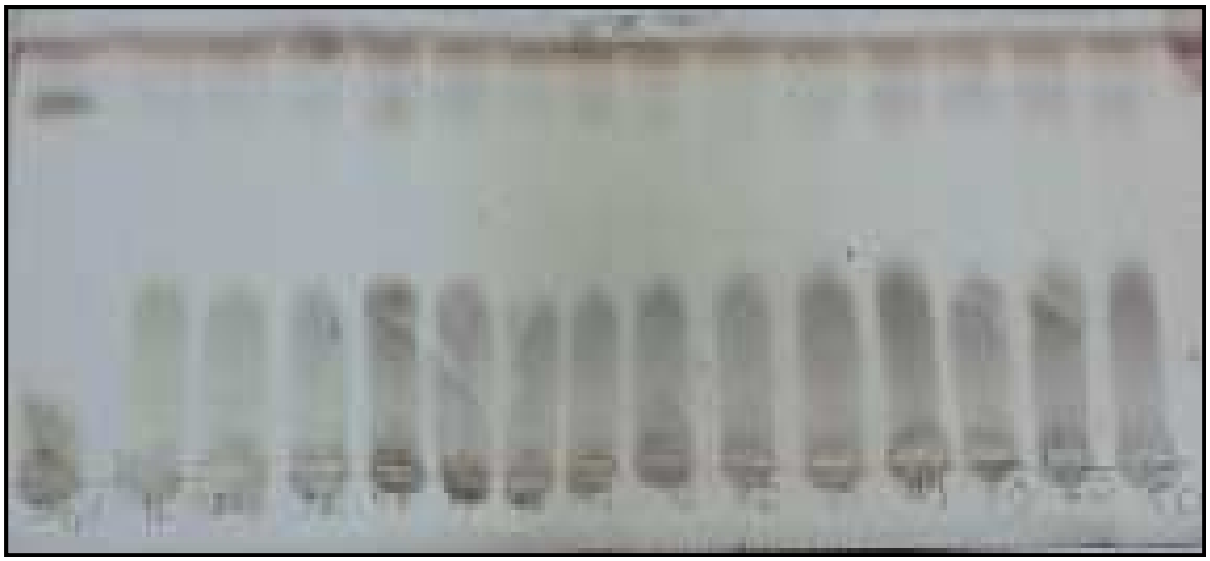

Figure No.5: Photograph of the TLC plate alfalfa seed and the elicited sprouts extracts with different concentrations of MO extract in light and dark, using solvent system (7.5 methylene chloride: 2.5 methanol)

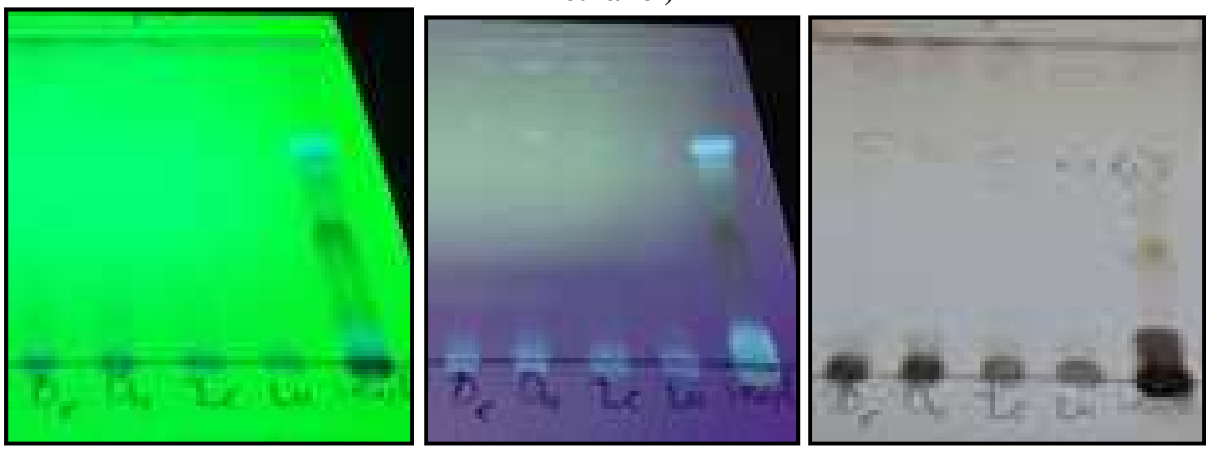

Figure No.6: Photograph of the TLC plate the alfalfa seed and the elicited sprouts extracts with different concentrations of MO extract in light and dark, using solvent system (9 ethyl acetate: 1 methanol)

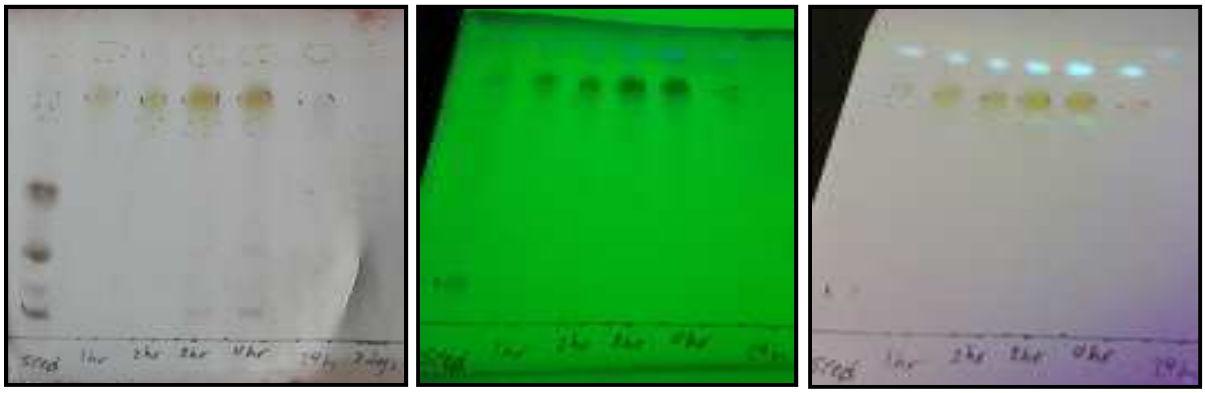

Figure No.7: Photograph of the TLC plate of alfalfa seeds during germination through after 1, 2, 3, 4, 24 hours and 2 days using solvent system (n-butanol: water: acetic acid (4:1:1)

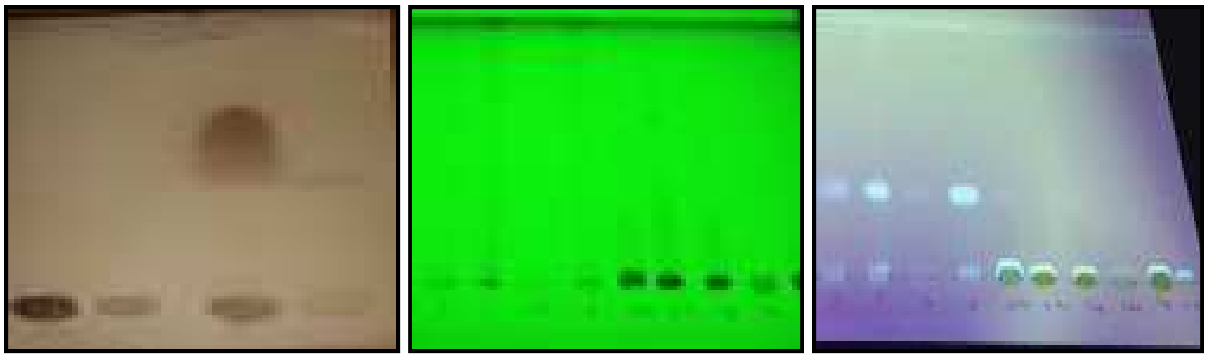

Figure No.8: Photograph of the TLC plate of alfalfa seeds during germination throughout 9 days using solvent system (Pet. ether: ethyl acetate 8:2) – methylene chloride: methanol (9:1)

CONCLUSION

Germination of alfalfa seeds in continuous light and soaking seeds in 0.0625, 0.03125g/L MO extract before germination significantly increases the level of phenolic content and antioxidant activity. Therefore, MO extract could be used as a potent elicitor from biotic origin, to induce secondary metabolites production. This could help in production a high quality, healthy, and useful medicinal plants and foods.

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CONFLICT OF INTEREST

There is no conflict of interest.

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