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ROLE OF PROTEIN KINASE R IN INDUCING DNA DAMAGE IN HCV PATIENTS

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The hepatitis C virus (HCV) may be a major blood borne pathogen of human. There are approximately 120-130 million or 3% of the total world population that are infected with HCV. According to World Health Organization (WHO), there are approximately three to four million new cases of infection every year (Mohd *et al*., 2013). HCV is considered a major community health issue, since the virus is the etiological reason of chronic hepatitis that recurrently evolvement to a cirrhosis and hepatocellular carcinoma (HCC) (Hauri *et al*., 2004). Protein kinase RNA regulating (*PKR*) is a double stranded RNA (ds-RNA) stimulated protein kinase that stimulate cellular apoptosis pathways (Deb *et al.,* 2001; Onuki *et al*., 2004). *PKR* is present in cell as inactive form and exchange to active state by very low concentration of ds-RNAs. Most natural

ds-RNA activators of PKR are created in cell infected with virus as viral replication or transcription products (Terada *et al*., 2004; Garcia *et al*., 2006). PKR is an integral part of mechanism of human innate immune reaction, which is the cell's first line of defense against viral infection (Toroney *et al*., 2010). The molecular mechanisms regulating function of *PKR* in normally cells divided are basically anonymous. PKR is associated with the control of HCV replication (Gale and Foy, 2005). Particular viruses such as hepatitis viruses progress several strategies to *PKR* down-regulation and overawed the host defense mechanism against virus replication (Yan *et al*., 2007). Many DNA tumour viruses promote cellular revolution through inactivating the vital tumor suppressor protein *p53***.** In dissimilarity, it is not known *p53* function is interrupted by HCV, unique, RNA virus oncogene that is the foremost infectious foundation of liver cancer in many world regions (Mitchell *et al*., 2017). *PKR* is *a p53* object gene and shows a vital portion in the tumor-suppressor purpose of *p53*. Stimulation of *p53* by geno-toxic stress changes a significant level of *PKR* expression, resulting in translational inhibition and cell apoptosis. *P53* stimulation convinced by DNA damage assists cell apoptosis by stimulating *PKR* (Agami and Bernards, 2000).

This study aim was to quantify *PKR* and *P53* genes expression in chronic HCV infected patients and indicate the vital significance of these genes in the HCV disease progression.

MATERIALS AND METHODS

Patients

This study was conducted on 50 cases that divided into two groups as follows Group one (GI): included 30 patients with chronic HCV and positive PCR collected from AL-Menoufya liver institutes. Group two (GII): included 20 normal, completely healthy patients considered as the control, they were selected from outpatient clinics of internal department, AL-Menoufya University Hospital. All cases were collected in the period from April to September 2019.

The inclusion criteria of all patients' samples were HCV antibody positive using a second-generation enzyme-linked immune-sorbent assay (ELISA) and positive serum HCV RNA by quantitative polymerase chain reaction (PCR); not under medication and were negative hepatitis B virus infection. Beside investigations needed to fulfill the selection criteria, all cases included in this study were exposed to the following:

1. Full history and clinical examination

2. For complete blood cell count (CBC) and molecular categorization of *PKR* and *P53* genes expression, whole blood sample from each case were used.

3. Serum samples were separated; where one was used for testing liver functions; Alfa Feto-protein (AFP) level and

hepatitis markers which were done using COBAS INTEGRA 800 chemistry auto analyzer (Roche Diagnostics Ltd., CH-6343 Rotkreuz, Switzerland) and therefore the emain aliquots serum sample were stored at -80°C.

4. Quantification of *PKR*-RNA and *P53*-RNA genes was finished using RT- PCR supported SYPR GREEN after mRNA extraction from blood fresh sample. Quantification of mRNA was designed by using the arithmetic formula: ($2^{-\Delta ct.}$), during which ΔCT is the difference between the CT of a given objective complementary DNA (cDNA) and an endogenous reference cDNA. Thus, this value yields the amount of the normalized objective to an endogenous reference.

- ELISA for HBcAb and HBsAg was finished for all cases to exclude the presence of hepatitis B viral etiology of liver disease.

Statistical analysis

Statistical analysis was completed by means of the Statistical Package for the Social Sciences (SPSS software version 25, Chicago, Illinois). The devices recycled for statistical comparisons were analyzed by the Student's t-test for parametric data and Mann-Whitney test for non-parametric data. Regression and correlation were finished by spearman's coefficient method. Receiver Operating Characteristic (ROC) curve was used for detecting the cut off value, Kappa agreement to test the agreement. The cut-off value for significance was at a P-value less than 0.05.

RESULTS AND DISCUSSION

This study was conducted on the samples from 50 patients who were categorized into two groups. The age of the studied testers ranged from 21-75 years with a mean age of 47.8±14.3. Sex distribution in studied patients (GI) was 11 (36.7%) females and 19 (63.3%) males, while in the control cases (GII) number of females was six (30%) and number of males was 14 (70%). No significant difference was observed between the two studied groups as regards the distribution of age and sex.

There wasn't significant difference between the two studied groups regards the CBC results (Table 1), this approve with (El-dahshan *et al*., 2018). But the other parameters such as alkaline phosphatase (ALP); Gamma-glutamyl transferase (GGT) and also AFP showed highly significant difference between the two groups (Table 2). This disagreed with McPherson *et al*., (2011).This may due to the difference in the HCV genotype while they used samples collected from patients infected with HCV genotype 1 and other infected with HCV genotype 3. While There is a highly significant difference in levels of ALP in GI compared with G2 (P-value less than 0.001), while the value of ALP concentration in GI ranged from 34-70 IU/L with mean ±SD (52.4±9.15) but it ranged from 25-45 IU/L with mean ±SD (31.6±6.05) in group II.

Furthermore a highly significant

difference in GGT levels was observed in GI compared with GII (P-value less than 0.001), while the median value of GGT concentration was 50 IU/L with Inter-Quartile range (IQR) of 45-75 IU/L in group I, but it was 31.5 IU/L with IQR of 25-35 IU/L in group II.

The median value of serum level of AFP was 3.0 ng/ml with IQR of 2.20-9.00 ng/ml in group I but it was 0.07 mg/dl with IQR of 0.01-0.20 ng/ml in group II.

The liver functions show highly significant difference in G1 compared with G2 that was shown in Table (3). This disagreed with El-dahshan *et al.,* (2018), who compared between 2 groups infected with chronic HCV before treatment and during follow up. The results before treatment showed no significant difference but the results during follow up showed significant value for several parameters (p value <0.001), namely Alanine aminotransferase (ALT), but different parameters showed no significant difference such as Aspartate aminotransferase (AST); total bilirubin (T BIL), direct bilirubin (D BIL), albumin (ALB) and AFP. In all HCV cases compared with the controls, very highly significant difference was observed in levels of T BIL (p-value less than 0.001) but ALB level show significant result (p equal to 0.007).

A highly significant difference in levels of ALT in group I was observed as compared with group II (P-value less than 0.001), while in group I the median value of ALT concentration was 55.0 IU/L with (IQR) of 45.0-65.0 IU/L, but it was 24.00 IU/L with IQR of 20.0-25.0 IU/L in group II.

Moreover a highly significant difference in median AST levels was observed in G1 compared with G2 (P-value less than 0.001),while the median value of AST concentration was 55.0 IU/L with IQR of 48.0-65.0 IU/L in GI, but it was 23.00 IU/L with IQR of 20-25 IU/L in GII (Table 3).

In group I, the median value of T BIL was 1.15 mg/dl with IQR of 1.0-1.3 mg/dl, and it was 0.4 mg/dl with IQR of 0.3-0.55 mg/dl in group II.

Also a significant elevated level of ALB in G1 as compared with G2 was observed but not as in other liver functions, in which the mean ±SD of ALB concentration in G1 was 3.54±0.73, while in G2 mean ±SD was 3.96±0.28.

No significant differences were observed between the two studied patients for *p53* gene expression. This finding was concordant to that found by Loguercio *et al.,* (2003).

No significant differences were observed between the two studied groups according to *PKR* gene expression (p value 0.094) as that showed in Table (5). This is in concordance with other study (Chen *et al*., 2004).

When correlation occurred between two markers and different parameters in two groups, the

values showed that ALB was high significantly correlated with two markers (*PKR* and *P53*) (p value <0.001*).

There was a negative correlation between *PKR* gene expression and *P53* gene expression and other parameters (Age, ALT, AST, BIL, AFP, PLT, Hemoglobin Hb, GGT and ALP) (p value ≥0.05) (Table 6), this finding agreed with Mohamed *et al.,* (2012). But this differs from that the recent studies observed that a positive correlation was found between AST but not ALT and the degree of inflammation in chronic hepatitis patients. This difference is due to the degree of inflammatory activity, while higher AST level was related with higher grades histology activity index (HAI) of HCV related chronic hepatitis (Hung *et al.,* 2008).

The results revealed that the significance of *PKR* wasn't detected in chronic HCV patients. Others recounted a significant increase after the HCC progression indicating the involvement of HCV in the process of hepato-carcinogenesis (De-Mitri *et al.,* 2007; Koike 2007).

There was a negative relationship between *PKR* gene expression for predict cases and the control. ROC curve appeared in Fig. (1).

Also there was a significant negative correlation between *P53* gene expression for predict cases and the control. ROC curve appeared in Fig. (2). *P53* considered critical in cell-cycle arrest and apoptosis after DNA damage; alterations in its function may accelerate the progression from chronic liver disease to HCC (Kumar *et al*., 2011). The infection with HCV may cause the loss of normal *p53*-mediated DNA damage responses and may have relevance to HCC origination in persons infected with chronic HCV. But this was dependent on the viral impact and also *PKR* expression, this was agreement with Mitchell *et al*., (2017).

Though, there wasn't a significantly *PKR* and *p53* genes expression in patients with chronic liver disease and normal cases in this current study. But Fig. (3) show highly sensitivity for the two genes level.

Moreover, there was a sensitive cut-off for *PKR* level which can be used in exclusion of HCV patients into low risk and high risk groups for progress of tumour. Some authors support our findings as they suggested that *PKR* was not acting as a classical tumour suppressor protein but a potential growth stimulus (Hiasa *et al.,* 2003**).**

Furthermore other studies revealed that expression of *PKR* gene was lower in HCC cases with HBV than in HCV infection (Tamada *et al*., 2002). These conclusions may reflect the difference in viral effect on *PKR* gene expression and recommend that *PKR* might have a tumour promoting action in some cancer cells.

SUMMERY

PKR is a *p53* target gene and acts an imperative role in the tumor-suppressor role of *p53*. Initiation of *p53* by genotoxic tension prompts a significant level of *PKR* expression, that ensuing in translational embarrassment and cell apoptosis. The current study aims to evaluate prognostic influence of *PKR* gene expression in chronic HCV patients; Correlate *PKR* and *P53* gene expression to liver function tests and also shows the role of *PKR* in patients infected with HCV. We concluded that *PKR* was established to be an independent prognostic issue indicating the vital biological significance of this gene in the HCV disease process. In spite of the restriction of this study related to sample size, it paved the way for further future studies using more samples. Further investigations on a larger scale *via* well-standardized performances and more samples are recommended to validate these results of ROC curve or define a suitable one.

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Table (1): Comparison between the two studied groups according to CBC.

	Patients (n = 30)	Normal (n = 20)	p
PLT($\times 10^3$) Min. – Max.	120.0 – 460.0	160.0 – 340.0	0.211
Median (IQR)	222.50(190.0 – 320.0)	194.0(185.0 – 226.50)	
Hb Min. – Max.	9.90 – 16.0	10.40 – 16.0	0.338
Mean ± SD.	13.41 ± 1.81	13.94 ± 1.95	
TLC ($\times 10^3$) Min. – Max.	4.0 – 9.0	4.0 – 9.0	0.658
Mean ± SD.	6.60 ± 1.59	6.40 ± 1.50	

P: p value for relating between the studied groups. PLT: platelets;

Hb: Hemoglobin; TLC: T lymphocyte cell.

Table (2): Comparison between the two studied groups according to different parameters.

	Patients (n = 30)	Normal (n = 20)	Test of Sig.	P
ALP Min. – Max.	34.0 – 70.0	25.0 – 45.0	t=8.931*	<0.001*
Mean ± SD.	52.40 ± 9.15	31.60 ± 6.05		
Median (IQR)	50.0(45.0 – 59.0)	30.0(26.50 – 34.50)		
AFP Min. – Max.	1.0 – 15.0	0.01 – 0.40	U=0.0*	<0.001*
Mean ± SD.	4.91 ± 4.08	0.10 ± 0.11		
Median (IQR)	3.0(2.20 – 9.0)	0.07(0.01 – 0.20)		
GGT Min. – Max.	35.0 – 194.0	20.0 – 45.0	U=22.0*	<0.001*
Mean ± SD.	64.23 ± 33.94	32.15 ± 7.33		
Median (IQR)	50.0(45.0 – 75.0)	31.50(25.0 – 35.0)		

t: Student t-test. U: Mann Whitney test.

P; p value for relating between the studied groups. *: Statistically significant at $p \leq 0.05$.

Table (3): Comparison between the two studied groups according to liver functions.

	Patients (n = 30)	Normal (n = 20)	p
Direct bilirubin Min. – Max. Median (IQR)	0.20 – 2.20 0.50(0.40 – 0.80)	0.03 – 0.30 0.10(0.10 – 0.20)	<0.001*
Total bilirubin Min. – Max. Median (IQR)	0.80 – 3.0 1.15(1.0 – 1.30)	0.10 – 0.90 0.40(0.30 – 0.55)	<0.001*
ALB Min. – Max. Mean ± SD.	2.10 – 4.90 3.54 ± 0.73	3.50 – 4.40 3.96 ± 0.28	0.007*
AST Min. – Max. Median (IQR)	35.0 – 90.0 55.0(48.0 – 65.0)	16.0 – 40.0 23.0(20.0 – 25.0)	<0.001*
ALT Min. – Max. Mean ± SD.	35.0 – 89.0 57.07 ± 14.20	16.0 – 34.0 23.50 ± 4.78	<0.001*

P: p value for relating between the studied groups; *: Statistically significant at $p \leq 0.05$.

Table (4): Comparison between the two studied groups according to *P53* gene.

P53	Patients (n = 30)	Normal (n = 20)	U	p
Min. – Max.	0.13 – 1.87	0.05 – 5.66		
Mean ± SD.	0.59 ± 0.46	1.11 ± 1.29	217.0	0.100
Median (IQR)	0.44(0.22 – 0.81)	0.71(0.47 – 1.23)		

U: Mann Whitney test. P: p value for matching between the studied groups.

Table (5): Comparison between the two studied groups according to *PKR* gene.

PKR	Patients (n = 30)	Normal (n = 20)	U	p
Min. – Max.	0.08 – 2.14	0.0 – 2.0		
Mean ± SD.	0.66 ± 0.52	0.48 ± 0.56	215.50	0.094
Median (IQR)	0.56(0.25 – 1.0)	0.32(0.02 – 0.71)		

U: Mann Whitney test. P: p value for relating between the studied groups.

Table (6): Correlation between the two markers (*PKR* and *P53*) and different parameters in patients (n= 30).

	PKR		P53	
	r_s	p	r_s	P
Age (years)	0.288	0.123	0.334	0.071
PLT	0.077	0.685	0.195	0.302
Hb	-0.200	0.289	-0.158	0.404
TLC	0.058	0.761	0.167	0.378
ALP	0.181	0.337	0.068	0.720
AFP	0.332	0.073	0.253	0.177
GGT	0.138	0.468	-0.076	0.688
Direct bilirubin	0.234	0.213	0.044	0.816
Total bilirubin	0.105	0.580	-0.222	0.238
ALB	-0.675	<0.001*	-0.639	<0.001*
AST	0.208	0.270	0.166	0.381
ALT	0.348	0.059	0.230	0.222

r_s: Spearman coefficient; n: number of patients; *: Statistically significant at $p \leq 0.05$.

Table (7): Agreement (sensitivity, specificity) for *PKR* to predict cases (vs control)

	AUC	P	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
PKR	0.641	0.094	76 – 0.806	0.125	90.0	35.0	67.50	70.0

AUC: Area Under a Curve; p value: Probability value; CI: Confidence Intervals;
NPV: value of Negative predictive PPV: value of Positive predictive.

Table (8): Agreement (sensitivity, specificity) for *P53* to predict cases (vs control)

	AUC	P	95% C.I	Cut off	Sensitivity	Specificity	PPV	NPV
P53	0.638	0.100	0.471–0.806	≤1.072	90.0	40.0	69.2	72.7

AUC: Area Under a Curve; p value: Probability value; CI: Confidence Intervals;
NPV: value of Negative predictive PPV: value of Positive predictive.

Table (9): Agreement (sensitivity, specificity) for *P53* and *PKR* to predict cases (vs control).

	AUC	P	95% C.I	Sensitivity	Specificity	PPV	NPV
P53+ PKR	0.635	0.109	0.462 – 0.808	93.33	50.0	73.7	83.3

AUC: Area Under a Curve; p value: Probability value; CI: Confidence Intervals;
NPV: value of Negative predictive PPV: value of Positive predictive.

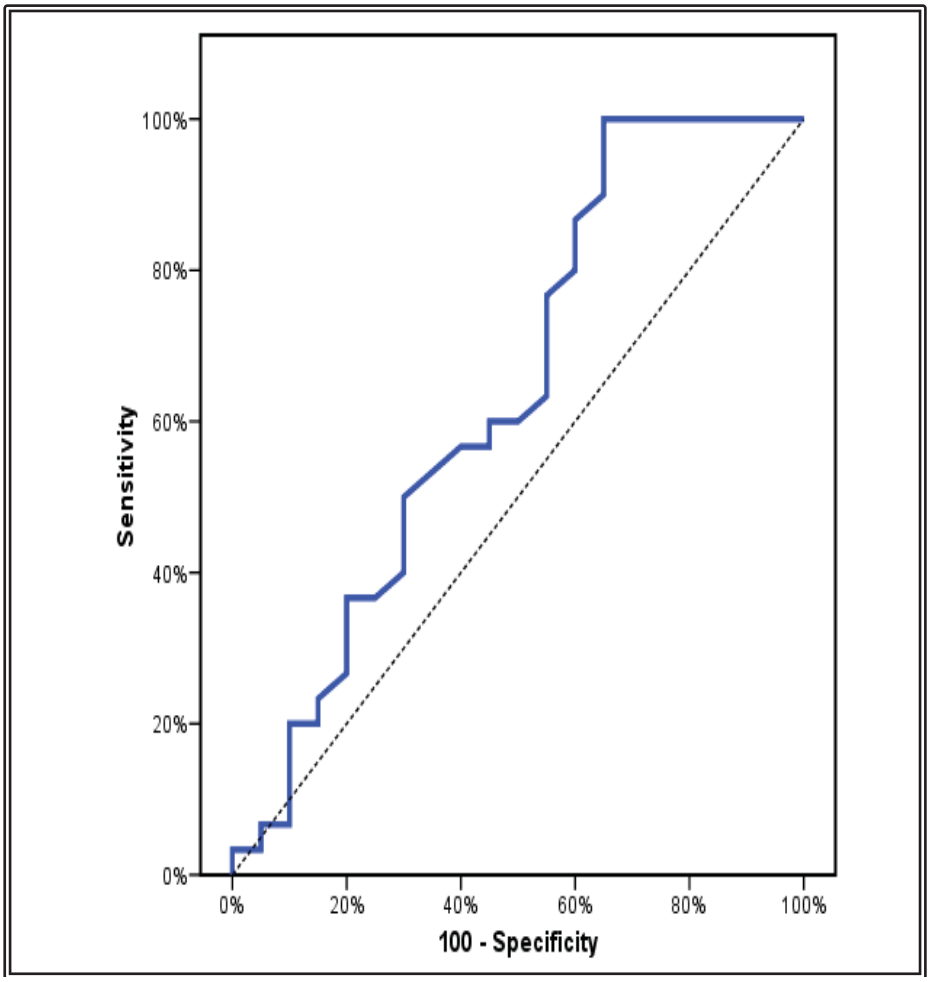

Fig. (1): ROC curve for *PKR* gene expression to predict cases (vs control).

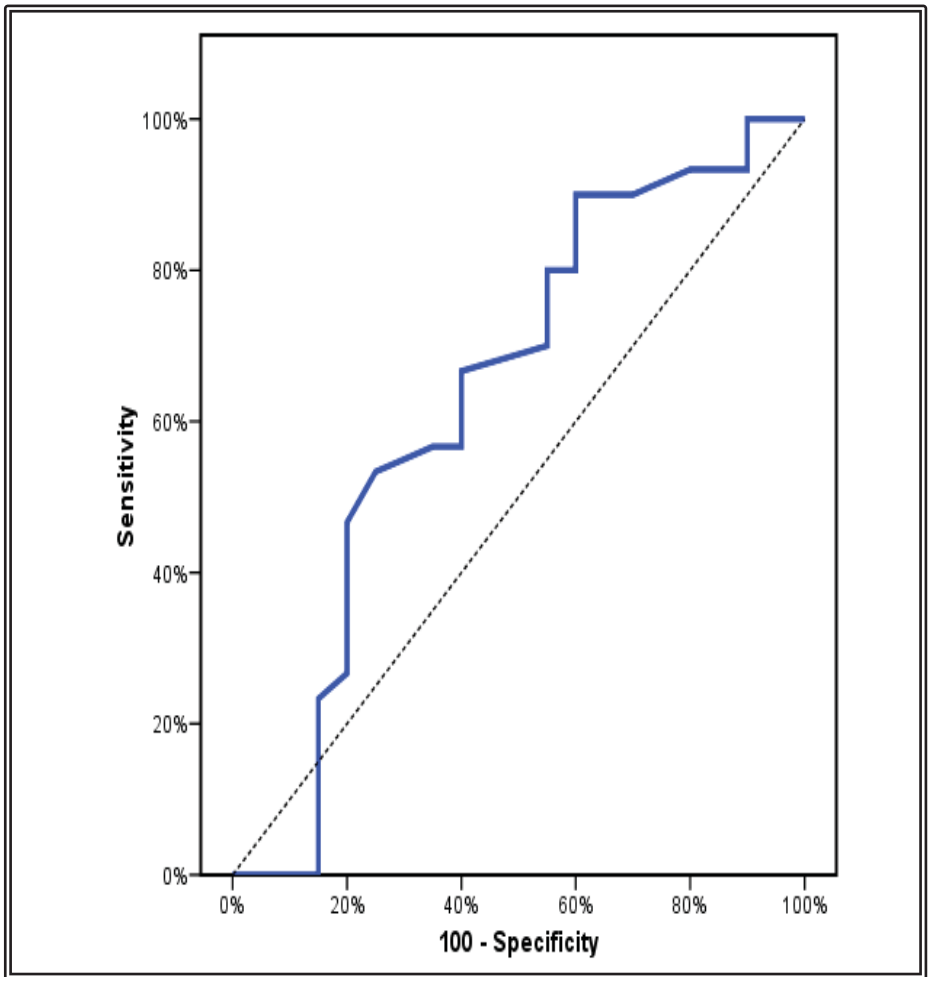

Fig. (2): ROC curve for *P53* gene expression to predict cases (vs control).

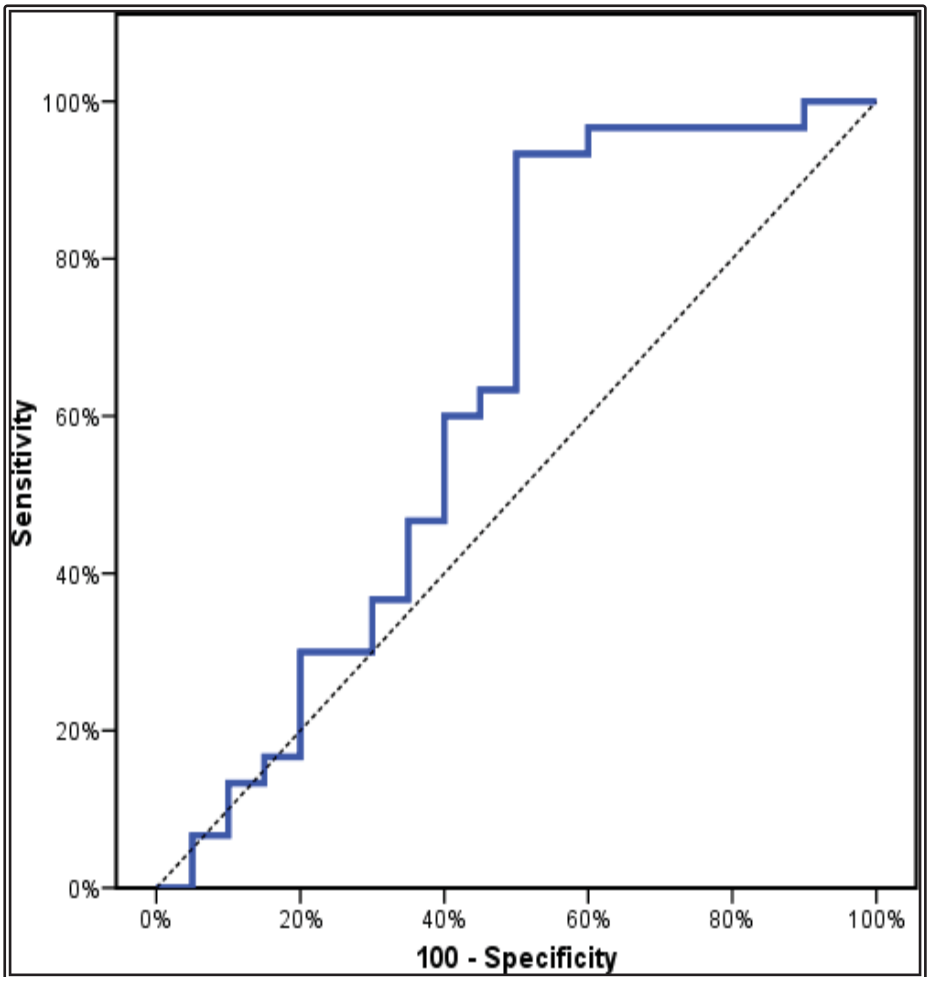

Fig. (3): ROC curve for *P53* and *PKR* genes expression to predict cases vs. control.

ISOLATION AND CHARACTERIZATION OF HALOPHILIC-ENDOPHYTES FROM EL-HAMRA OASIS, EL-NATRUN VALLEY, EGYPT. I: IMPACT OF ENDOPHYTIC BACTERIAL ISOLATES ON TOMATO (*LYCOPERSICON ESCULENTUM*) GROWTH PROMOTION UNDER GREENHOUSE CONDITIONS.

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Dehydration-inducing stress conditions are significant problems affecting plant distribution, survival, and productivity worldwide. The severity of dehydration-inducing conditions in a given area is affected by different environmental factors as in high temperatures and increased evaporation/precipitation ratio and affected by the soil drainage system and poor water management in cultivated lands (Egamberdiyeva, 2007). Therefore, finding an efficient, low cost, easily adaptable method for managing dehydration-inducing stress conditions in plants is a major challenge, where minor improvement could have a measurable economic impact (Venkateswarlu and Shanker, 2009).

The majority of cultivated plant species are classified as glycophytes, while halophytic species are primarily found in hypersaline environments, and their growth is severely affected upon removal of NaCl from their environment (Surve *et al*., 2012; Solomon and Viswalingam, 2013). Two distinctive classes of microorganisms are found in a given salinity-affected soil; the first are halophytic microorganisms living in the root-growing zone and requires at least 0.2 M NaCl concentration and cannot grow in the lack of salt. The second is halo-tolerant microorganisms that grow in the absence of salt and the presence of comparably high salt concentrations (Ara *et al*., 2013).

Plant growth-promoting bacteria (PGPB) are soil-borne bacteria that form a symbiotic relationship with plants (Díaz-Zorita and Fernández -Canigia, 2009); they tend to facilitate the growth of plant

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Web Site (*www.esg.net.eg*)

either by colonizing the rhizosphere and endosphere of plants and activate various pathways within plants to facilitate salt-withstanding mechanisms, directly/indirectly (Islam *et al*., 2016). Plant growth-promotion affects plants 1) directly through facilitating nutrient acquisition (phosphorus solubilization, nitrogen fixation), and production of plant growth hormones (Odoh, 2017), or 2) indirectly through siderophore and hydrocyanic (HCN) production and defensive action against biotic pathogens (Goswami *et al*., 2014). Therefore, it could be used in bio-inoculants in agricultural practices to promote sustainable agricultural production (Mei and Flinn, 2010).

The area of El-Natrun Valley, Al-Bahira governorate, is an area known for its hypersaline soda lakes. Salinity reaches up to 5.0 M NaCl, with a pH range of 8.5-11 and temperatures reaching mid-50s during summer, a rich ecosystem for isolation of alkaliphilic, haloalkaliphilic thermo-alkaliphilic microorganisms (Mesbah *et al*., 2007). To our knowledge, most of the available studies on halophilic bacterial isolation and characterization from high-saline lakes in Egypt focused only on phylogenetic analysis of the different classes with limited information on their agricultural potentials (Oren, 2002; Vahed *et al*., 2011).

Keywords: Endophytes, Halophytic bacteria, IAA production, Growth characteristics, Tomato**,** 16S rDNA.

MATERIALS AND METHODS

Sampling site: Hypersaline soda lakes of El-Hamra, located in an extended depression approximately 90 km northwest of Cairo; that area is part of El-Natrun Valley, which is occupies approximately 60 x10 km (Taher, 1999) Fig. (1).

Samples collection: Different plant tissues from four plant species growing in the area (*Phragmites australis*, *Tamarixnilotica*, *Juncus rigidus* and *Halocnemum strobilaceum*) were collected from sites surrounding the lake in triplicates; the samples were placed in sterile containers. All samples were collected in June 2016-17, transported on ice and processed within 2-4 h after collection.

Isolation of halo-endophytic bacteria from plant tissues: All collected plant tissues (roots, stems and leaves) were carefully cleaned with tap water for 30 secs, then rinsing in sterile ddH_2O for 1-2 mins. In laminar flow-hood, surface sterilization was performed by rinsing in 70% ethanol for 1 min, followed by drenched in 20% Clorox solution (1% sodium hypochlorite) for 5 mins. The plant tissues were rinsed 5-6 times with sterile ddH_2O. The samples were allowed to surface dry for 5-10 mins before aseptically dissected into small sections and placed on solid LB medium (Cat # L1704.0500, Duchefa Biochem, Netherland) supplemented with 10% NaCl. The plates were incubated for 48 hr at 28±2 °C. To obtain pure bacterial isolates, bacterial colonies that were morphologically distinct were selected and cultivated repeatedly on fresh solid LB

medium with 10% NaCl. Some uncut disinfected-surface tissues and the last rinsing water were also inoculated onto separate solid LB plates as a control to validate the effectiveness of the surface sterilization procedure. All purified isolates were stored at –80 ºC for further use.

Morphological studies of the isolates: All purified bacterial isolates were characterized morphologically as colony color, form, elevation, margin, etc. Gram staining and morphological studies of cells under a microscope were also performed (data not shown).

Growth characterization of the isolates: Growth of the isolates were determined at different pH (4, 7, and 10), temperature (30, 40, and 55 °C), and NaCl concentrations (5, 10, 15, and 20 %; the cultures were inoculated in LB broth pH 7.0 in a shaker incubator 100 rpm at 30°C for 48 hr.). Bacterial growth was measured at 600 nm wavelength (Table 1).

Molecular analysis of the isolates: All purified isolates were identified using sequences of 16S rDNA. Bacterial-DNA isolation was performed following the procedure by Sambrook *et al.* (2009); amplification of 16S ribosomal-RNA amplification was conducted using 27 F and 1492 R primers (27F **AGAGTTTGATCMTGGCTCAG** and 1492R **CGGTTACCTTGTTACGACTT**). PCR was carried out in 25.0 µL reactions and PCR conditions were as follow: denaturation at 94°C for 5 min, then 35 cycles of 94°C for 1 min, 65°C for 90 sec and 72°C for 1 min, then extending cycle for 7 min at 72°C. Amplified products were gel-separated and visualized using 1.0% agarose gel which stained by ethidium bromide- (0.5 µg/mL). PCR-products were purified using QIAquick Kit (Qiagen, Netherlands), and were sequenced. The sequences were revealed using the basic local alignment search tool (BLAST) and comparisons with the GenBank nucleotide database http://www.ncbi.nlm.nih.gov. Once the sequences were identified, they submitted to the NCBI (accession no. KY608807 - KY608843). Sequences were initially aligned with CLUSTAL Omega method, by MEGA7 software phylogenetic trees were constructed (Kumar *et al.*, 2016) with 1000 bootstrap replications, and evolutionary distances were determined using the p-distance method.

IAA quantification: Following a modified procedure by Patten and Glick, (2002), IAA quantification was conducted using 20 µL aliquots of overnight bacterial growth cultures were used to inoculate 5 ml LB medium fortified with 0.1% tryptophan and incubated in the dark for 24 hr at 30 °C; the cultures were centrifuged (5,500×g, 10 min) and 1.0 ml of supernatant was used for quantification of IAA by mixing with 4.0 ml Salkowski's reagent (150 ml H2SO4, 250 ml ddH_2O and 7.5 ml of 0.5 mol FeCl3•6H_2O; Glickmann and Dessaux, 1994); the reactions were incubated for 30 min at room temperature, then light absorption was measured at 535

nm. **Greenhouse experiment:** Tomato seeds Cv. Castle Rock was germinated in 96-well foam trays filled with soil mixture (1 peat moss:1 washed sand) in the greenhouse. Thirty days post-germination, uniformly growing seedlings were transplanted into 25 cm pots filled with the soil mixture. Five bacterial isolates (HEP3B1, HEP4A2, HEP4A4, HEP1B3, and HEP1B2; Accession no. KY608812, KY608814, KY608816, KY608809, and KY608808, respectively) were inoculated tomato plants separately using different approaches. The plants divided into five groups (1st did not receive any foliage or soil application, 2nd group received IAA spray application at a concentration of 1 mg/L, 3rd group received 50 ml bacterial foliage-spray application, 4th and 5th groups received 10 and 50 ml direct soil application, respectively). A complete randomized block design was used with three replicas (each replica consisted of 3 plants). A week post transplanting into the final pots, the plants were divided into four greenhouse benches (block), and different growth characteristic measurements were recorded (plant height at 37 51 and 65 days post-germination (dpg), as well as above-ground fresh and dry weight at the end of the experiment). The bacterial cultures were prepared as follows, a single colony from each of the chosen bacterial isolates was placed in 5 ml LB liquid medium, fortified with 0.1% tryptophan, and placed in a shaker incubator for 48 hr. The next day, 1 ml of the culture "1 x 107 CFU/ml" was used to inoculate 1-liter LB-tryptone medium, followed by 48 hr incubation in a 30 ºC shaker incubator at 100 rpm. The greenhouse experiment was terminated 65 days post-germination, and the following parameters (plant heights above ground fresh and dry weight) were recorded immediately in the greenhouse and dry weight (60 °C oven for 72 hrs.).

Statistical analysis: Statistical analysis and variance analysis were performed using ASSISTAT software version 7.6 beta by Professor Francisco de Assis Santos e Silva (http://www.assistat.com).

RESULTS AND DISCUSSION

Endophytic bacteria isolation from plants growing in the Hamra Oasis, El-Natrun Valley. The area of El-Natrun Valley is located in the arid region of the northern part of Egypt. It is situated below sea level and consist of several lakes and water feed is provided from underground water from the Nile River, which touches surrounding grass swamp areas (Ali *et al*., 2013), mimicking an estuarine environment (Gutierrez *et al*., 2009a) and making it a rich source for isolating halophytic microorganisms. Different plant species are flourishing under these harsh conditions, including *P. australis, T. nilotica, J. rigidus, and H. strobilaceum*.

A total of 37 isolates were purified from the tissue of *P. australis, T. nilotica, J. rigidus, and H. strobilaceum* (Table (1); Figs. 2A B C and D). The different isolates were then identified using 16S ribosomal DNA sequencing. Then the sequences were defined using a basic alignment search tool (BLAST, http:// www.ncbi.nlm.nih.gov) and comparisons

with the GenBank nucleotide databank, and once the sequences were identified and submitted to the NCBI (accession no. KY608807 to KY608843; Table (1); Fig. (1). Based on the resulted sequences, the different isolates were classified as *Vibrio sp. V. parahaemolyticus*, *V. alginolyticus*, *Bacillus sp.*, *B. pumilus*, *B. sonorensis*, *B. lichenformis*, *B. safensis*, *B. subtilis*, *B. mojavensis*, *B.aerius*, *B. flexus*, *B. axarquiensis*, *B. tequilensis*, *Staphylococcus epidermidis*, and *S. succinus* (Fig. 1).

A phylogenetic tree of endophytic bacterial isolates based on 16S nucleotide sequences was constructed by the neighbor-joining method and evolutionary studies performed in MEGA 7. The sequences' alignment was done with CLUSTALW; bootstrap values were calculated from 1000 re-sampling, with genetic distances shown on scale bars. The tree displayed two major branches, I and II; the 1st is divided into six separate clades (Clade A to F), and while the 2nd consisted only of *Vibrio sp*. *Staphylococcus* specimens we separated into a single clade (A). *Bacillus flexus* did not share enough similarities with the rest of the used samples in this study, so it was separated alone in a clade B. *Bacillus safensis* and *B. pumilus* share genetic similarities to be in the same clade C separated from the rest *Bacillus sp*. Interestingly *B.aerius* had high similarities to *B. pumilus* HEP6C1 and differed from the rest, so resulted in being in clade D. In clade E, there were many similar species of *Bacillus* (*mojavensis*, *axarquiensis*, *subtilis*, and *tequilensis*). Finally, clade F contained all *B. licheniformis* specimens along with *B. sonorensis* HEP3B1.

Morphological and physiological characterization of the different endophytic isolates.

No growth was achieved in all isolated bacteria under acidic conditions, pH 4 (therefore, omitted from Table 1). The numbers present in Table (1) under pH 7 and 10 represent bacterial growth (O.D. ≈ 600) 48 hr post-inoculation. All bacterial isolates grow well at 5% NaCl concentration, equivalent to 0.85 mol NaCl (except for isolates HEP9A2), while no growth was observed under higher NaCl concentrations (10, 15, and 20%; Table 1).

Quantification of IAA production in the different endophytic isolates.

A preliminary screening of all isolates had been performed following the procedure of Bric *et al*., (1991) using a thin nitrocellulose membrane, which indicates that the isolates we had in our hands are indeed IAA-producing bacteria (data not shown). Measurement of IAA isolates-production was conducted a colorimetric method using Salkowski reagent (Gordon and Weber, 1951; Patten and Glick, 1996). The red color was visible within a few mins, yet all samples were measured 30 mins post-incubation in the dark. Bacterial production of IAA is supported in nature *via* the presence of tryptophan from root-exudates, decaying plant cells, or the addition of organic fertilizer (Arkhipchenko *et al*., 2006). Therefore, for IAA quantification, and based upon previous work by

different groups (Patten and Glick, 2002; Swain *et al*., 2007), which indicated that tryptophan added to the bacterial growth medium caused an increase in IAA production (different classes of rhizosphere bacteria), therefore in the present work, LB medium was fortified with 0.1% L-tryptophan was used. Different endophytic bacterial isolates have been found to possess the ability of plant's phytohormone production, thus facilitating plant growth, as in gibberellins (GA3; Khan *et al*., 2014), abscisic acid (ABA; Shahzad *et al*., 2017), and indole acetic acid (IAA; Ali *et al*., 2017). Vendan *et al.,* (2010) confirmed the isolation of more than 50 bacterial endophytes (belonging to *Firmicutes*, *Actinobacteria*, α-*Proteobacteria* γ-*Proteobacteria* families) from ginseng that reported IAA-producing activity. Miliūtė and Buzaitė, (2011) reported that 18 bacterial endophytes were isolated from apple buds, and most of the isolates showed IAA production activity. Sorty *et al.,* (2016) also reported the isolation of different IAA-producing endophytes (belonging to different genera) from *Psoralea corylifolia* L. (a weed). They concluded that the isolates' cell-free extract was able to enhance the germination of wheat seed and seedlings under saline stress *via* IAA production.

In plants, IAA acts as a signaling molecule and affects genes' expression under different conditions (Cassán *et al*., 2014; Egamberdieva *et al.,* 2015). The IAA production and, therefore, plant-growth depends on the IAA gradient in a delicate balance with other growth hormones, resulting in inhibition, stimulation, and differentiation of tissue. Low IAA levels trigger root elongation while a higher-level causes laterals and adventitious root formation (Ghosh *et al.*, 2013). Related-root colonizing phytohormones of rhizobacteria and endophytes effectively colonized and supplied additional IAA for plant growth and development (Sukumar *et al*., 2013). Because of all this, in the present work, we concentrated on identifying isolates based on their ability to produce IAA and found that some of the highest IAA-producing isolates (HEP3B1, HEP4A2, and HEP4A4) belong to *the Bacillus* genus while the other two (HEP1B3, and HEP1B2) belongs to *Vibrio*.

Bacillus sp. is characterized in numerous reports for their IAA production in large quantities (Zhao *et al*., 2011; Bibi *et al*., 2018), yet surprisingly, two isolates are isolated belong to Vibrio sp. (Gutierrez *et al*., (2009b) and later, Kerkar *et al*., (2012) reported the isolation of highly producing IAA Vibrio sp. Gutierrez *et al*. (2009b) reported for the first time, the isolation of 8 Vibrio type strains and five additional species-level clades as IAA-producing from the rhizosphere of Spartina alterniflora and Juncus roemerianus plants. In this study, one of the plants used for the isolation of endophytes belongs to the Juncus genus (J. rigidus) growing in the Hamra Oasis's surrounded environment. At the same time, Kerkar *et al*., (2012) reported the isolation of high IAA-producing Vibrio diazotrophicus from biofilms attached to

the saltern area. Based on the results provided in Table (1), the highest IAA-producing strains were chosen (HEP3B1, HEP4A2, HEP4A4, HEP1B3, and HEP1B2; (Table 1) for further greenhouse studies.

Treatment of tomato plants under greenhouse conditions causes a significant increase in plant height and dry weight. The following isolates (HEP3B1, HEP4A2, HEP4A4, HEP1B3, and HEP1B2) were used separately in a greenhouse experiment to study their effect on promoting the growth of tomato plants under non-stressed terms (Figs. 2 E and F). Different application methods were used (50 ml bacterial foliage-application, 10- and 50 ml direct bacterial application to soil surface), along with two controls (no-application at all, and 50 ml foliage application of 1.0 mg/L IAA solution; Table (2).

The effect of each of the five bacterial isolates for promoting the growth of tomato plants growth (plant height, fresh and dry weight; Figs. 3A, B, and C, respectively) was analyzed. HEP1B3 and HEP4A4 strains cause an increase in plant height significantly compared to other strains and non-inoculated controls (Figure 3A). All tested bacterial strains caused a significant accumulation of above-ground fresh weight, compared to non-inoculated controls (except those inoculated with HEP1B2 strain; Figure 3B). Tomato plants inoculated with the different bacterial strains showed a significant increase in dry weight than non-inoculated controls (Fig. 3C).

The bacterial application method was also tested (foliage spraying vs. direct application to the soil surface at 10 and 50 ml). Results indicate that direct soil application (at 10 and 50 ml) caused a significant increase in tomato plant height (Figure 4A), as well as in fresh and dry weight accumulation (Figs. 4B and C). Although foliage application did not cause a significant increase in plant height compared to non-inoculated controls (Fig. 4A), significant differences were observed at fresh and dry weight levels (Figs. 4B and C, respectively). In general, regardless of the application method used, all the bacterial was separately inoculated-tomato plants resulted in an increment in above-ground fresh and dry weight compared to non-bacterial inoculated controls (Figs. 4B and C, respectively).

Figure (5) summarizes the effect of bacterial treatment on tomato plants growing under greenhouse conditions. It shows that bacterial inoculants were significantly taller (Fig. 5A), accompanied by an accumulation of higher fresh and dry weight non-inoculated controls (Figs. 5B and C, respectively). Our results also indicated that endophytes-inoculated tomato plants showed an increase in fresh (13.2 up to 43.4 %) and dry weight (42.4 up to 52.6%) under non-stressed conditions compared to non-inoculated controls. Previous work using different *Pseudomonas sp.* to examine the effectiveness of different inoculation methods on tomato plant

growth-promotion revealed that the soaking of tomato seedling roots and tomato seed coating had statistically similar results improving plant growth to other methods used (Adesemoye and Ugoji, 2006).

Plant growth promotion due to IAA-production by endophytic bacteria has been reported by different workers (Yasmin *et al*., 2009; Adesemoye and Egamberdeiva, 2013) on potato promotion and tomato plants upon inoculation with endophytic growth-promoting bacteria. Yasmin *et al*. (2009) concluded that improvement in fresh weight and potato yield was connected to IAA production. While Adesemoye and Egamberdeiva, (2013) tested three isolates of *Pseudomonas* (*P. putida*, *P. chlororaphis,* and *P. extremorientalis*) with tomato plant growing under salinated soil, and reported a fresh weight stimulation of 26-28%, coupled with a 22% increase in tomato fruit yield compared to the non-inoculated plants.

Final conclusion. In a world where extensive usage of fertilizers and chemicals is necessary to accommodate the world's growing population, any new non-traditional method is encouraged to maintain highly productive agriculture. In the present study, we investigated the possibility of using halophytic endophytic bacterial strains isolated from salinity tolerant species growing in Egypt's saline area to promote and induce growth characteristic of a commercially important crop in Egypt growing under non-stressed greenhouse conditions. Inoculation of tomato plants with different bacterial strains increased plant height, accompanied by an increase in fresh and dry weight. This approach could provide an environmentally benign technique to increase crop productivity, yet further investigations to evaluate the performance of different endophytic microorganisms-inoculated plant under dehydration-inducing stress conditions is still to follow to identify agronomically-important endophytic microorganisms for commercial crops in Egypt.

SUMMARY

Thirty-seven endophytic bacterial isolates were isolated from tissues of four salt-tolerant plant species (*Phragmites australis*, *Tamarix nilotica*, *Juncus rigidus*, and *Halocnemum strobilaceum*) growing on shores of the El-Hamra Oasis (hypersaline soda lakes, with salinity level, reaches up to 5.0 M NaCl), EL-Natrun Valley, Egypt. Isolates were determined by different morphological, physiological, and molecular characters. Sequencing data of 16S rRNA declare that the 37 isolates belong to 3 genera *Bacillus*, *Staphylococcus*, and *Vibrio* (accessions no. Ky608807 - KY608843). All isolates produced Indol-3-acetic acid (IAA) when allowed to grow in LB media fortified with 0.1% L-tryptophan. The highest five potent isolates "IAA- producer" were selected and used to inoculate tomato (*Lycopersicon esculentum*) plants under greenhouse conditions. Based on plant growth characteristics that were measured at 37, 51, and 65 days post-germination; we found that 10

or 50 ml (1 x 107 CFU/ml) direct addition of bacterial culture to soil surface caused a significant increase in tomato plants' height (above-ground fresh and dry weight) compared to direct foliage spraying. When pooled together, all bacterial treatment caused a significant increase in tomato height (ranging from 4.9 up to 25.8%), fresh weight (13.2 up to 43.4%), and dry weight (42.37 up to 51.58%, with an average of 46.73%), compared to non-bacterial inoculated controls (non-treated, or IAA-sprayed) 65 days post-germination.

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Authors' Contribution: Both authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Data Availability:

All datasets analyzed in the study are included in the manuscript and presented as tables and figures.

Ethics Statement: This article does not contain any studies with human participants or animals performed by any of the authors.

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Table (1): List of the endophytic bacterial strains isolated from tissues of different plant species that grow in the region around the Hamra Oasis, Natrun Valley (*Phragmites australis, Tamarixnilotica, Juncus rigidus and Halocnemumstrobilaceum*). The table also shows their growth characteristic under different pH, temperatures, NaCl concentration, as well as IAA production.

Code	Scientific name	Accession	O.D. at pH 10 30°C 40°C 55°C	O.D. at pH 7 30°C 40°C 55°C	NaCl (%) 5 10 15 20	IAA (ug/100 ml culture)	Plant Species, tissue
HEP1 B1	*V. parahaemolyticus*	KY608807	- - -	3.05 2.84 -	++ - - -	10.77	*T. nilotica*, Root
HEP1 B2*	*Vibrio sp*	KY608808	- - -	2.94 2.67 -	++ - - -	20.00	*T. nilotica*, Root
HEP1 B3*	*V. alginolyticus*	KY608809	1.45 0.46 -	3.00 2.74 -	++ - - -	31.00	*T. nilotica*, Root
HEP3 A1	*B. pumilus*	KY608810	1.79 1.01 -	2.57 1.96 -	++ - - -	9.52	*P. australis*, Root
HEP3 A2	*Bacillus sp*	KY608811	2.43 1.40 -	2.34 1.07 -	++ - - -	9.17	*P. australis*, Root
HEP3 B1*	*B. sonorensis*	KY608812	2.73 2.05 -	2.71 1.87 -	++ - - -	22.60	*P. australis*, Root
HEP3 B2	*B. lichenformis*	KY608813	2.90 2.10 -	2.43 2.07 -	++ - - -	5.52	*P. australis*, Root
HEP4 A2*	*Bacillus sp*	KY608814	2.40 0.99 -	1.81 2.68 -	++ - - -	43.52	*P. australis*, leaves
HEP4 A3	*B. safensis*	KY608815	2.64 0.90 -	1.89 2.91 -	++ - - -	11.05	*P. australis*, leaves
HEP4 A4*	*B. pumilus*	KY608816	2.73 1.27 -	3.00 2.91 -	++ - - -	43.58	*P. australis*, leaves
HEP4 C2	*B. pumilus*	KY608817	2.69 1.81 -	1.53 1.68 -	++ - - -	7.35	*P. australis*, leaves
HEP5 A2	*B. licheniformis*	KY608818	1.57 0.58 -	1.68 1.35 -	++ - - -	7.02	*P. australis*, Root

Table(1): Cont'

HEP5 A3	*Bacillus sp*	KY608 819	1.39	0.58	-	2.09	1.97	-	++	-	-	12.20	*P. australis*, Root
HEP5 A4	*B. licheniformis*	KY608 820	0.85	0.12	-	1.94	2.30	-	++	-	-	9.80	*P. australis*, Root
HEP5 B1	*B. licheniformis*	KY608 821	2.83	1.29	-	2.78	2.74	-	++	-	-	9.30	*P. australis*, Root
HEP5 B2	*B. licheniformis*	KY608 822	1.69	0.33	-	2.09	2.84	-	++	-	-	8.95	*P. australis*, Root
HEP5 C2	*B. licheniformis*	KY608 823	1.28	0.70	-	1.93	2.67	-	++	-	-	5.85	*P. australis*, Root
HEP6 B1	*B. subtilis*	KY608 824	1.09	0.21	-	1.63	0.36	-	++	-	-	5.52	*P. australis*, leaves
HEP6 B11	*B. mojavensis*	KY608 825	0.64	0.11	-	0.85	0.29	-	++	-	-	11.47	*P. australis*, leaves
HEP6 B2	*B. subtilis*	KY608 826	0.47	-	-	1.67	0.48	-	++	-	-	9.05	*P. australis*, leaves
HEP6 C1	*B. pumilus*	KY608 827	2.08	1.77	-	2.05	1.69	-	++	-	-	7.80	*P. australis*, leaves
HEP7 A2	*B. aerius*	KY608 828	2.90	2.53	-	1.79	1.48	-	++	-	-	5.67	*P. australis*, leaves
HEP7 A4	*B. pumilus*	KY608 829	2.30	1.34	-	2.59	2.10	-	++	-	-	5.75	*P. australis*, leaves
HEP8 B1	*B. flexus*	KY608 830	0.67	-	-	1.87	1.79	-	++	-	-	11.47	*J. rigidus*, shoots
HEP8 B2	*S. epidermidis*	KY608 831	0.90	0.43	-	1.98	2.03	-	++	-	-	9.02	*J. rigidus*, shoots
HEP9 A2	*B. safensis*	KY608 832	2.01	1.48	-	2.35	2.94	-	-	-	-	6.02	*J. rigidus*, shoots
HEP9 A4	*B. safensis*	KY608 833	2.40	2.03	-	2.87	3.01	-	++	-	-	12.20	*J. rigidus*, shoots

Table(1): Cont'

HEP9B1	*B. safensis*	KY608834	2.48 2.00 -	1.98 1.60 -	++ - - -	10.10	*J. rigidus*, shoots
HEP10A1	*B. subtilis*	KY608835	0.75 - -	1.99 2.64 -	++ - - -	7.35	*H. strobilaceum*
HEP10A4	*B. axarquiensis*	KY608836	0.68 - -	1.97 1.76 -	++ - - -	6.75	*H. strobilaceum*
HEP11A1	*B. subtilis*	KY608837	0.34 0.13 -	2.68 1.80 -	++ - - -	9.05	*H. strobilaceum*
HEP11A2	*B. subtilis*	KY608838	0.45 0.11 -	2.91 1.96 -	++ - - -	16.00	*H. strobilaceum*
HEP11A3	*B. licheniformis*	KY608839	1.30 0.72 -	2.78 1.68 -	++ - - -	11.47	*H. strobilaceum*
HEP13A2	*B. subtilis*	KY608840	0.74 - -	2.06 1.45 -	++ - - -	7.20	*J. rigidus*, shoots
HEP13A4	*S. succinus*	KY608841	1.08 0.50 -	1.04 0.53 -	++ - - -	5.53	*J. rigidus*, shoots
HEP15A3	*B. tequilensis*	KY608842	1.18 - -	1.10 1.02 -	++ - - -	10.52	*J. rigidus*, Root
HEP15A4	*S. succinus*	KY608843	1.11 0.65 -	2.10 1.99 -	++ - - -	5.85	*J. rigidus*, Root

Table (2): Measurements of plants height (37, 51, and 65 dpg), and above-ground fresh and dry weight (at 65 dpg) of tomato plants treated with 5 endophytic bacterial isolates.

TREATMENT	LENGTH T1	LENGTH T2	LENGTH T3	FRESH WEIGHT	DRY WEIGHT
CONTROL	35.10 ± 2.85	44.17 ± 4.67	55.67 ± 2.42	14.48 ± 0.23	3.98 ± 0.12
IAA SPRAY	33.87 ± 4.58	45.47 ± 2.37	60.67 ± 3.39	17.05 ± 0.26	4.94 ± 0.13
HEP4A2 SPRAY	30.33 ± 4.91	40.33 ± 2.67	53.33 ± 1.26	21.83 ± 1.92	5.72 ± 0.27
HEP4A2 (10ML)	31.33 ± 0.67	44.00 ± 3.05	60.01 ± 2.89	19.37 ± 1.11	5.81 ± 0.03
HEP4A2 (50 ML)	31.67 ± 1.45	49.33 ± 2.60	61.67 ± 1.67	21.11 ± 0.67	5.89 ± 0.14
HEP1B3 SPRAY	42.67 ± 4.70	58.33 ± 4.33	69.51 ± 1.69	16.07 ± 0.46	5.95 ± 0.15
HEP1B3 (10ML)	49.00 ± 1.01	65.51 ± 2.89	78.67 ± 3.21	20.34 ± 1.23	5.82 ± 0.41
HEP1B3 (50 ML)	33.00 ± 2.10	44.67 ± 2.09	62.01 ± 2.11	18.34 ± 0.51	6.05 ± 0.58
HEP4A4 SPRAY	36.00 ± 4.58	41.67 ± 1.67	57.01 ± 2.33	20.3 7± 0.31	5.69 ± 0.17
HEP4A4 (10 ML)	31.67 ± 2.40	51.67 ± 3.67	70.20 ± 1.01	18.99 ± 2.83	5.79 ± 0.25
HEP4A4 (50 ML)	40.33 ± 2.85	53.00 ± 1.00	70.33 ± 2.73	18.51 ± 1.57	5.72 ± 0.11
HEP1B2 SPRAY	31.00 ± 2.08	44.00 ± 3.79	52.67 ± 3.71	19.04 ± 0.75	6.28 ± 0.30
HEP1B2 (10 ML)	30.00 ± 1.15	40.33 ± 2.34	54.00 ± 3.05	15.91 ± 2.02	5.95 ± 0.63
HEP1B2 (50 ML)	26.33 ± 2.18	39.00 ± 2.89	50.67 ± 0.67	14.25 ± 1.55	5.89 ± 0.19
HEP3B1 SPRAY	40.67 ± 2.90	50.67 ± 0.67	58.67 ± 2.67	16.11 ± 1.36	5.78 ± 0.03
HEP3B1 (10 ML)	38.33 ± 1.67	50.00 ± 2.89	64.33 ± 3.48	19.18 ± 1.52	5.68 ± 0.16
HEP3B1 (50 ML)	36.00 ± 2.58	51.33 ± 3.81	65.00 ± 3.64	20.91 ± 1.49	5.54 ± 0.05

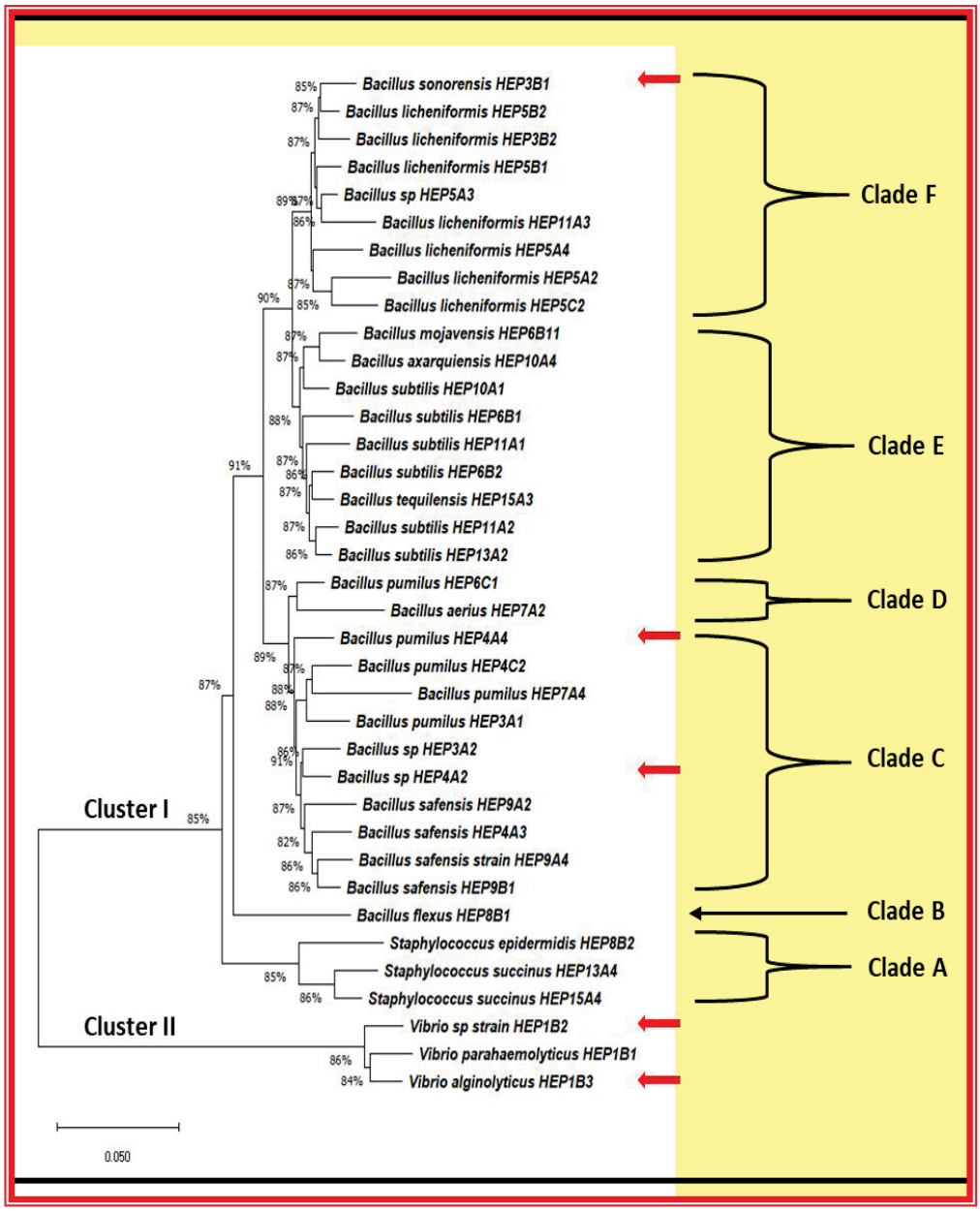

Fig. (1): Phylogenetic of endophytic bacterial isolates distribution based on 16S rDNA gene sequence from wild plants grown in the surroundings El-Hamra Oasis, EL-Natrun Valley. The phylogenetic relationships were inferred using the neighbor-joining approach from the *16 S* rRNA gene, and an evolutionary analysis was performed in MEGA 7. Alignment of the sequences was done with CLUSTALW, bootstrap values were calculated from 1000 re-sampling, with genetic distances shown on scale bars.

Fig. (2): Pictures representing El-Hamra oasis, AL-Natrun valley (A) with the distribution of different halophytic plants (B, C, and D) in the area. E and (F) representing part of the greenhouse experiments 37 days post germination and just before application of the different bacterial strains in the greenhouse.

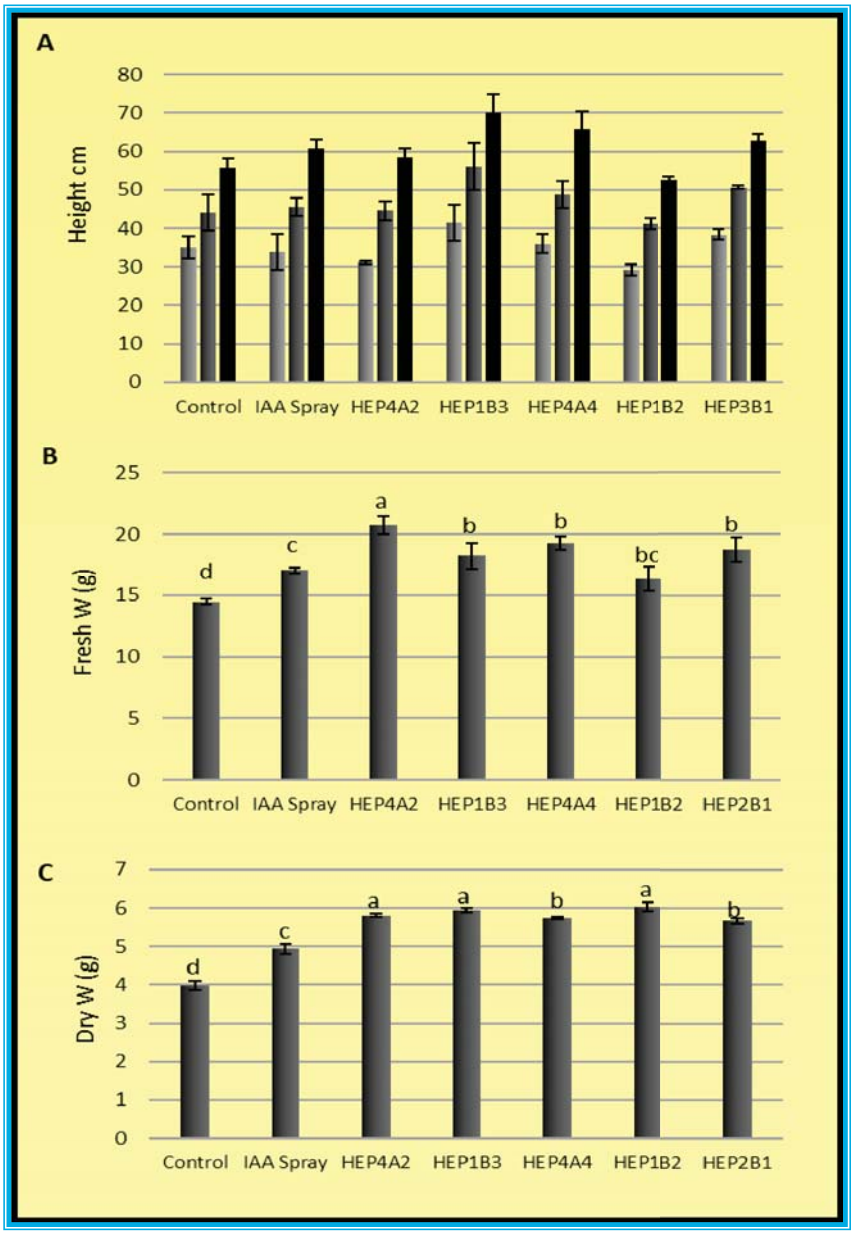

Fig. (3): Impact of endophytic bacterial isolates on tomato plants growing under greenhouse conditions on plant height (A), above ground fresh weight (B) and dry weight (C). Bars are the plant height ± standard error. Bars with same letter are not significantly different at $P \leq 0.05$.

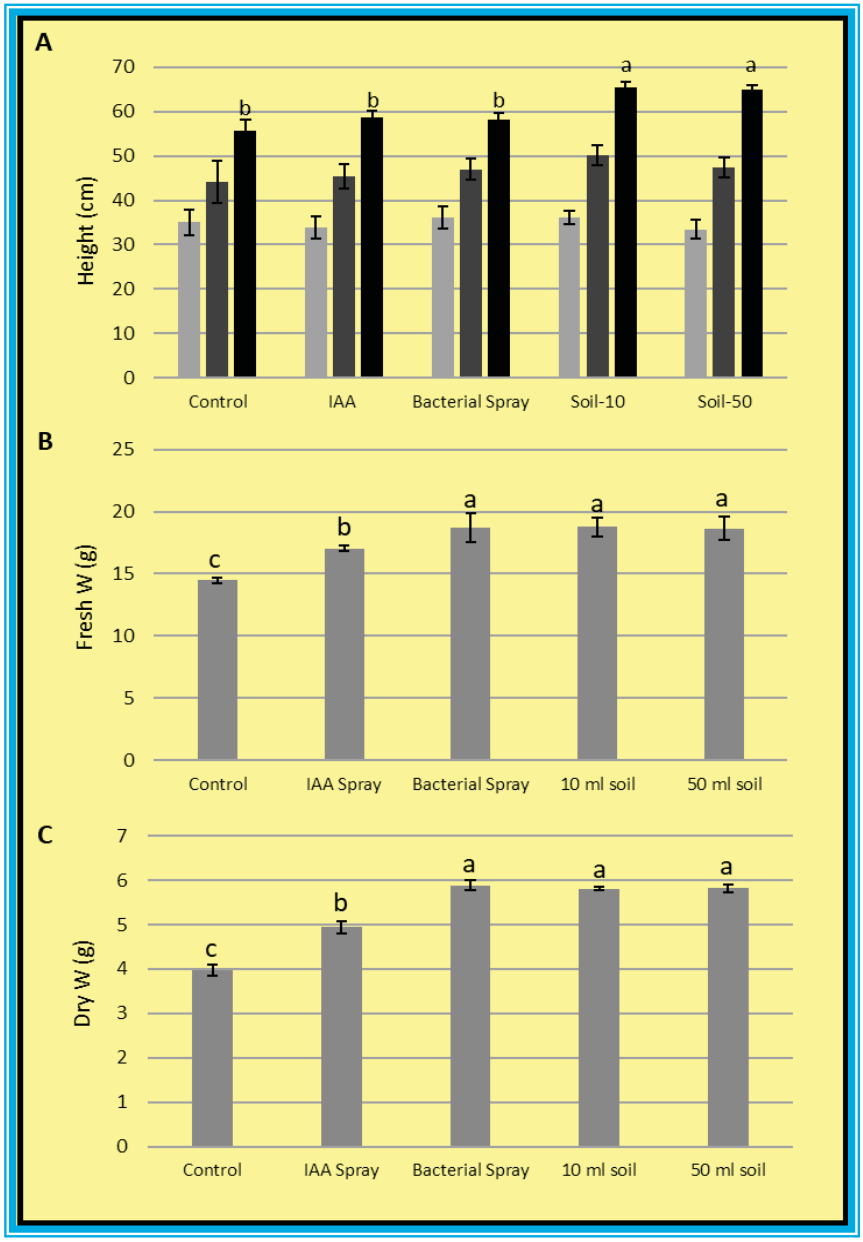

Fig. (4): The effect of different application methods on plant height (A), above ground fresh weight (B) and dry weight (C) of tomato plants growing under greenhouse conditions. Bars represents average plant height ± standard error. Bars with the same letter are not significantly different at $P \leq 0.05$.

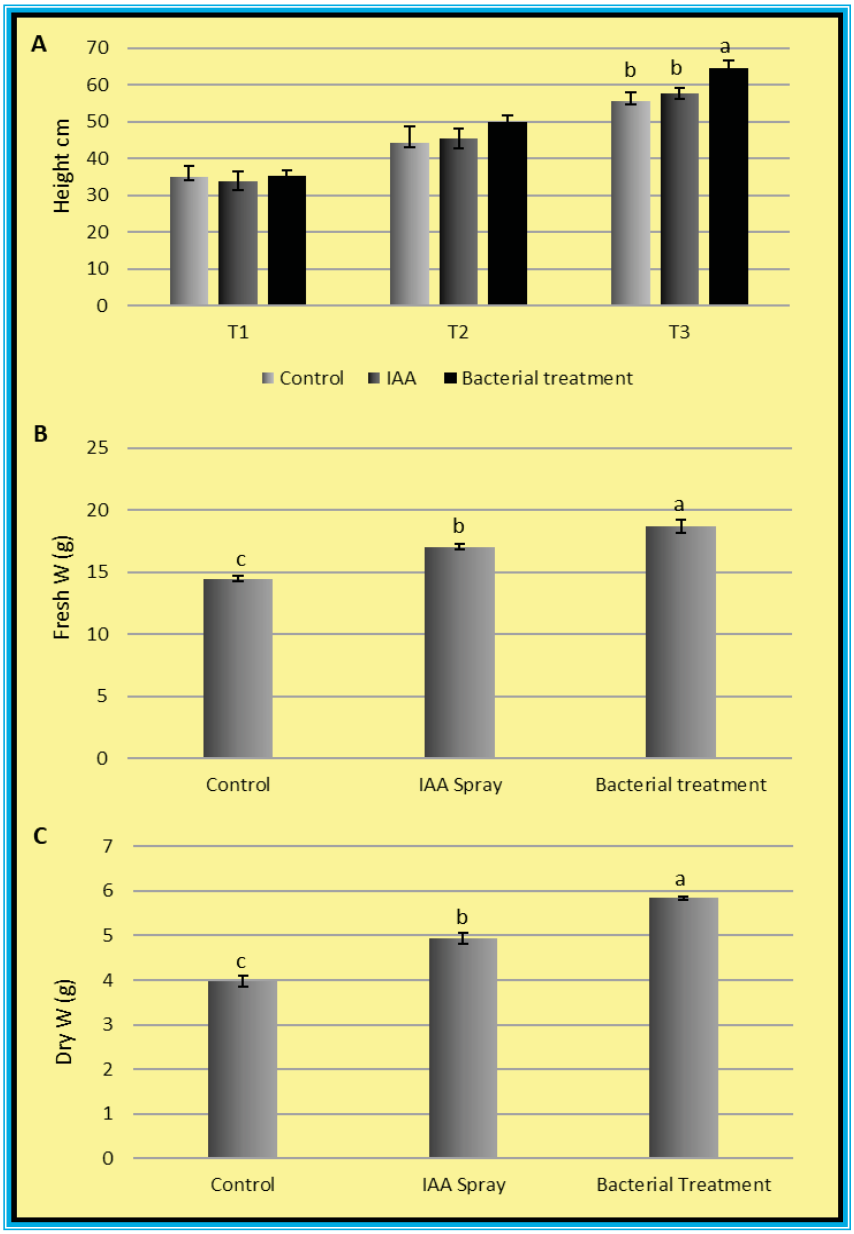

Fig. (5): Endophytic bacterial isolates effect vs. non-inoculated controls (non-treated, and IAA spray) on plants height (A), above ground fresh weight (B) and dry weight (C). (A) bacterial treatment causes a significant rise in plant height compared with IAA and un-treated plants. (B) bacterial treatment had also significant effect on increasing fresh (B) and dry weight (C) of tomato plants compared to non-bacterial treated controls. Bars represent average plant height ± standard error. Bars with same letter are not significantly different at $P \leq 0.05$.

MOLECULAR IDENTIFICATION AND PHYLOGENETIC RELATIONSHIPS OF *Origanum syriacum* L. AND *Origanum vulgare* L. USING CHLOROPLAST *rbcL* AND *matK* BARCODES GENES

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Genus *Origanum* is considered as an important multipurpose aromatic perennial herb used in folk medicine and as food additive since ancient times which belongs to the family *Lamiaceae* or *Labiatae*, tribe *Mentheae* subfamily *Nepetoideae*. It is comprises 43 species and 18 hybrids widely distributed in Eurasia and North Africa (Ietswaart, 1980; Duman *et al*., 1998). Ietswaart (1980) identified six subspecies within *O. vulgare* based on differences in indumentums, number of sessile glands on leaves, bracts and calyces, and in size and color of bracts and flowers. *Lamiaceae* family contains about 236 genera having 6900 to 7200 species (Harley *et al*., 2004). *Origanum vulgare* L. is a perennial aromatic herb, widely naturally distributed all over Euroasia and North Africa and is one of the most traded and consumed spice (Kokkini, 1997). *Origanum vulgare* L. is used as medicinal plant because of the essential oils produced in the aerial parts (Skoula and Harborne, 2002). Thymol and carvacrol as a major constituent of its essential oil were isolated by Baser *et al*., (2003). It is especially antimicrobial, economic importance and specific biological characters (Asdal *et al*., 2006) and antioxidant properties (Mastelic *et al*., 2008). In Egypt, *Origanum syriacum* L. subsp. *Sinaicum* (Boiss.), commonly known as 'Syrian marjoram' is an aromatic, herbaceous and perennial plant growing wild in the Sinai desert of Egypt (Tackholm, 1974). It is endemic species grow in mountainous areas of Saint Katherine, south of Sinai in dry rocky habitats, also have a vernacular local name as "Za'atar Katherine" or Bardaqwish and from the conservation point of view, it is an endangered plant (Tackholm, 1974; Boulos, 2002). *Origanum syriacum* L. species, as one of wild plant species, is endangered and has limited distribution. There are many challenges affecting both conservation and the distribution of wild plants in SKP (Saint Katherine Protectorate) such as; feral donkeys, plant over collection, tourist intrusions (e.g., tres-

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Web Site (*www.esg.net.eg*)

passing beyond trail borders and collection of firewood during camping), overgrazing, collection for scientific research, urbanization and settlement expansion and quarries as recorded by Guenther *et al*., (2005), Assi (2007), Hatab (2009) and Khafagi *et al*., (2013). The taxonomy of *Origanum* was found to be rather complex and nearly all of the sections are afflicted with some kind of taxonomic uncertainties (Lukas, 2010).

For molecular characterization and identification of the biological species based on using DNA sequence data were widely used for DNA barcoding in the last decades. Several international organizations, including iBOL (the International Barcode of Life Project) http:// www.ibol.org/, CBOL (the Consortium for the Barcode of Life) and ECBOL (the European Consortium for the Barcode of Life), have applied in large-scale DNA barcoding projects aiming to identify and classify all life on earth. Recently, the Consortium for the Barcode of Life **(**CBOL**)** Plant Working Group (CBOL 2009 and 2010) proposed two other chloroplast regions, the protein coding *rbcL* and *matK*, as a 2-locus combination barcode. DNA barcoding techniques play an important role in the identification of polymorphic plant species having a problematic taxonomic identity for the biodiversity investigation (Ajmal *et al*., 2014). DNA barcoding technique in plants with high effective and robust conserved regions is a characterizing using a partial DNA sequence from a standard and agreed-upon position in the genome of all species (http://barcoding.si.edu/DNABarCoding.htm). For instance, barcoding strategies have been deployed for the verification of plant products from several medicinal plants (Asahina *et al*., 2010; Xue and Li, 2011). Large data information gathered from two chloroplast chDNA barcodes (*rbcL* and *matK*) at molecular level used a universal barcode system far beyond taxonomic studies of land plants. Some investigators have chosen a combination of two regions (*matK* and *rbcL*) as a satisfactory compromise that best meets the DNA barcoding criteria (Hebert, 2003; Chase *et al*., 2005; De Vere *et al*., 2012 and; Fazekas *et al*., 2008& 2012). Using four candidate barcode regions (*rpo*B, *rbcL*, *matK* and *trnH-psbA* to evaluate the utility of using markers plant DNA barcodes in 64 species specimens, encompassing six different genera (i.e. *Mentha*, *Ocimum*, O*riganum*, *Salvia*, *Thymus* and *Rosmarinus*) to reduce cost and time for species identification (De Mattia *et al*., 2011 & 2012). Using one region barcodes from *rbcL*, *matK*, *psbA-trnH* loci of 14 *Labiatae* species were identified and analyzed with species identification from Pakistan (Schori and Showalter, 2011). Universal primers were used for amplification of *matK* and *rbcL* Loci in 2 different plant species (covering 14 families) from Saudi Arabia (Bafeel *et al*., 2011). To classify between 36 samples of *Thymus* spp. the core barcode regions (*matK* and *rbcL*) and the plastid intergenic spacer *trnH-psbA* were compared (Federici *et al*., 2013). Three cpDNA loci (*matK*, *rbcL* and *trnH-psbA*) as single region or as multi-region barcodes based on CBOL were used and analyzed for medicinal

plants of the *Labiatae* (*Lamiaceae*) family (Theodoridis *et al*., 2012). DNA barcoding as part from authentication of traded medicinal plants, also finds application in biodiversity monitoring, conservation impact assessment, monitoring of illegal trading, forensic botany, etc. (Nithaniyal *et al*., 2014; Ferri *et al*., 2015 and Mishra *et al*., 2016). For more than a decade, other several applications has been widely tested in the DNA barcoding, molecular systematics, identification at molecular level and community phylogenetic of some medicinal plants (Vohra and Khera, 2013; Techen *et al*., 2014; Zhou *et al*., 2014; Parveen *et al*., 2016; Bezeng *et al*., 2017 and Chen *et al.,* 2019).

The importance of *Origanum* species is due to active ingredients and essential oils which confer their medicinal, culinary and pharmaceutical properties. Moreover, threatens such as over-collecting of plants and human constructions in plants natural habitats which put them in their way to extinction. Therefore, the current investigation was carried out to identify and authenticate the endemic wild *Origanum* species, *Origanum syriacum* L. subsp. *sinaicum* (Boiss.) in Egypt and compare them with cultivated species; *Origanum vulgare* L. using two chloroplast genes (*rbcL* and *matK* genes) as the most important DNA barcode at the molecular level. In addition, to distinguish between the two *Origanum* species under study and with the other available species on NCBI database to fulfill a strict conservation plan and maintenance of the studied species. Phylogenetic relationship analysis and homology modeling of both sequences (*matK* + *rbcL* genes) using Basic Local Alignment Search Tool (BLASTn) and MEGA 7 software program to apply comparative sequences between of them.

MATERIALS AND METHODS

Plant materials collection

The two *Origanum* species available in Egypt; *Origanum syriacum* L. subsp. *sinaicum* (Boiss.) (Ecotype wild species) and *Origanum vulgare* L. (cultivated species) were collected for the present investigation. The fresh young leaves were collected as bulk for each species in spring of 2018, transferred into liquid nitrogen, and kept frozen at - 80 °C till use. The *Origanum* species chosen for the present study were wild type *Origanum syriacum* L. subsp. *sinaicum* (Boiss.) which was collected from the mountains of Saint Catherine Protectorate (SKP), South Sinai Governorate, Egypt and the other is cultivated species; *Origanum vulgare* L. which was collected from private farms at Kirdasa region, Giza Governorate, Egypt.

DNA extraction, primers design and PCR amplification

Genomic DNAs were extracted from 100 mg frozen tissue and loaded in 2.0 ml eppendorf tubes with one 5 mm stainless steel bead. Samples were ground using Tissue Lyser II (Qiagen, Ltd.) until the material became powder (frequency 20

Hz x 2 x ~ 30 sec) following the procedure described by DNeasy Plant Mini Kit (Qiagen Inc., Cat.no.69104, USA), this was performed following the manufacturer's instruction. The concentrations and quality of the genomic DNA samples were estimated on spectrophotometer ND-2000 (Nanodrop, USA). Finally, all the genomic DNA samples were diluted to a final concentration of 40 ng/ µl with TE buffer (10 mM Tris-HC1, pH 8.0 and 1 m M EDTA), then stored at -20°C for further use. DNA fragments were amplified *via* standard polymerase chain reaction (PCR). The entire coding plastid *rbcLa* (first part of *rbcL* gene ~ 700bp or less) was amplified using the primer pairs *rbcLa*_1Fwd (5′-ATG TCA CCA CAA ACA GAG ACT AAA GC-3′) and *rbcLa*_599Rev (5′-GTA AAA TCA AGT CCA CCR CG-3′) and PCR fragment a 599 bp of the *rbcL* gene as previously described by Levin *et al*., (2003) and Kress and Erickson, (2007). For *matK* primer design, 13 ORF full length sequence *matK* genes from different species belong to *Lamiaceae* family were retrievable from the National Center for Biotechnology Information (NCBI) database (GenBank). Accession numbers GU381790.1 (*Thymus pulegioides*), AY840173.1 (*Thymus serpyllum*), GU381789.1 (*Thymus caespititius*), GU381791.1 (*Thymus broussonetii* subsp. *hannonis*), GU381792.1 (*Thymus vulgaris*), GU381802.1 (*Origanum vulgare*), GU381799.1 (*Origanum elongatum*), GU381798.1 (*Origanum dictamnus*), GU381797.1 (*Origanum rotundifolium*), GU381801.1 (*Origanum microphyllum*), GU381800.1 (*Origanum dayi*), AY840165.1 (*Origanum vulgare*), and MG256495.1 (*Mentha spicata*) were used for multiple sequences alignment of nucleotide (BLSTn) to design specific primers pair of *matK* gene. The entire coding plastid maturase fragment (*matK*) was amplified ~ 884 bp or less using the primer pairs *matK*_466Fwd (5`- GTC CAT GTG GAA ATC TTG ATT C -3`) and *matK*_1349Rev (5`- CGT ACA GTA CTT TTG TGT TTA CG -3`) and PCR fragment ~ 884 bp or less 850 bp according to CBOL Plant Working Group (2009). Phusion® *Taq*, the High-Fidelity DNA polymerase (Thermo Scientific, Product codes: F-530L, 500 Unit) was used. For *rbcl* and *matK* master mix: The amplification reaction was carried out in 25 µl reaction volume contains; 2 µl DNA, 5 µl 5X Phusion HF buffer, 0.5 µl 10mM dNTP mix, 1.25 µl Fwd. primer (10 µM), 1.25 µl Rev. primer (10 µM), 0.25 µl Phusion DNA polymerase (Thermo Scientific, Product codes: F-530L, 500 Unit), 14.75 µl DEPC H_2O and was spin for 15 Sec. The reaction mixture was incubated in a Perkin-Elmer thermo cycler 9700. PCR program for *rbcL* and *matK* regions, the temperature profile in different cycles was as follows: an initial strand separation cycle at 98°C for 3 min followed by 35 cycles comprised of a denaturation step at 98°C for 30 seconds, an annealing step at 55°C for 30 seconds and an extension step at 72°C for 45 seconds. The final cycle

was a polymerization cycle for 7 min at 72°C. PCR amplifications samples were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TAE buffer at 95 volts. A 1Kbp DNA ladder was used as DNA standard size marker. PCR products were visualized on UV light using a Gel Documentation System (BIO-RAD 2000). PCR product were purification or fast cleanup from the agarose gel for sequence up to 10 µg can bind to each QIA quick column by using QIA quick gel extraction kit (Qiagen, Cat. No. 28704-28706).

Sequence editing, alignment and phylogenetic inference

Sequencing chromatograms of obtained two *rbcL* and two *matK* regions were analyzed by Macrogen, Seoul, south of Korea and translated into amino acid sequences by the *ExPASy* online program (https://web.expasy.org/translate) for each studied two *Origanum* species. All nucleotide sequences of *rbcL* and *matK* gene, Open Reading Frame (ORF) were searched in NCBI database, The National Center for Biotechnology Information GenBank Database, (http://www.ncbi.nlm.nih.gov). The homology searches were performed with Basic Local Alignment Search Tool of several sequences (BLASTn online program) on the basis of their homologies with sequences published in DDBJ/EMBL/GenBank database which are available using NCBI database (Altschul *et al*., 1990).

RESULTS AND DISCUSSION

Molecular description

Successful application for isolation with high quality and pure molecular size of DNA is quite a challenge to reduce degraded DNA of plant material especially in medicinal plants. DNA concentration were obtained using nanodrop spectrophotometer observes and ratio of 260 and 280 nm were (i.e., 27–35 ng mL-1). PCR based amplification of conserved regions (*matK and rbcL*) is primarily required to establish DNA barcodes for species identification. With both universal primers *rbcL* and *matK*, good results of PCR amplification were observed as about 700 or less 600 bp for the first part of partial fragment length of *rbcL* gene and about 900-850 bp for partial *matK* gene in two *Origanum* species (*Origanum syriacum* L. subsp. *Sinaicum* (Boiss.), (Ecotype of wild species) and *Origanum vulgare* L., respectively. The single fragment was purified from agarose gel and sequenced. Specific single fragment was obtained for each sample, size and reading sequence were determined by Macrogen analysis, and then aligned by BLASTn web sites to identify sequences similarities. The nucleotide sequences isolated in this paper have been deposited in the GenBank database

NCBI (National Center for Biotechnology Information) by BankIt, online from website (https://www.ncbi.nlm.nih.gov/ WebSub/) after it was processed by email (gb-admin@ncbi.nlm.nih.gov). Two *rbcL* gene sequences were available to DDBJ/EMBL/GenBank database with accession no. MT679150.1/QNJ99666 from *Origanum vulgare* L. (528bp) which predicted to encode a protein of 176 amino acids and with GenBank accession no. MT679151.1/QNJ99667 from *Origanum syriacum* L. subsp. *Sinaicum* (532 bp), which predicted to encode a protein of 177 amino acids as shown in Fig. (1.A). In the same context, the two sequenced *matK* genes sequences were available with GenBank accession no. MT679152.1/QNJ99668 from *Origanum vulgare* L. (745 bp) which predicted to encode a protein of 248 amino acids and accession no. MT679153.1/QNJ99669 from *Origanum syriacum* L. subsp. *Sinaicum* (775bp), which predicted to encode a protein of 258 amino acids as shown in Fig. (1. B).

Molecular phylogenetic analysis based on sequences of *rbcL* gene

Sequence homology of each nucleotide of the chloroplast 60 *rbcL* gene searches (Code 1 to 2) for *Origanum* species (current studies), (Code 3 to 7) for *Origanum* genus, (Code 8 to 10) for *Conradina* genus, (Code 11 to13) for *Dicerandra* genus, (Code 14 to 16) for *Monarda* genus, (Code 17 to 19) for *Pycnanthemum* genus, (Code 20) for *Bystropogon* genus, (Code 21 to 23) for *Clinopodium* genus, (Code 24 to 26) for *Satureja* genus, (Code 27 to 29) for *Mentha* genus, (Code 30 to 35) for *Thymus* genus, (Code 36 to 38) for *Agastache* genus, (Code 39 to 41) for *Dracocephalum* genus, (Code 42 to 44) for *Nepeta* genus, (Code 45 to 46) for *Prunella* genus, (Code 47 to 49) for *Lepechinia* genus, (Code 50 to 52) for *Rosmarinus* genus, (Code 53 to 55) for *Salvia* genus and (Code 56 to 60) for 5 different species; *Linum usitatissimum* (MG946893.1), *Arabidopsis thaliana* (AB917053.1), *Glycine max* (Z95552.1), *Chenopodium album* (JX848451.1) and *Triticum aestivum* (AY328025.1) as out group of the family were tested by top-scoring hits through NCBI database using Basic Local Alignment Search Tool (BLASTn) as shown in Table (1). Multiple Sequence Alignments (MSA) of 60 *rbcL* gene was carried out between sequences of selected nucleotide sequences of 17 different genera belonging to the family *Lamiaceae* or *Labiatae* available in GenBank databases, will be discussed briefly. The results revealed that *Origanum vulgare* L. in this investigation was closely related with high identity and similarity of 99.62% with 5 accessions of *Origanum* genus and less than 99.62 - 96.97% with 49 accessions from 17 different genera belonging to the family *Lamiaceae* with E-value = Zero. On the other hand, it showed low similarities with the other 5 different species (89.22%,

89.77%, 90.55% and 89.20%) with *Linum usitatissimum* (MG946893.1), *Arabidopsis thaliana* (AB917053.1), *Glycine max* (Z95552.1) and *Chenopodium album* (JX848451.1), respectively with E-value = Zero and 87.31% *Triticum aestivum* (AY328025.1) with E-value = 2e-176 as out group of the family *Lamiaceae* or *Labiatae*. The Multiple Sequence Alignments (MSA) of nucleotide partial *rbcL* gene from *Origanum syriacum* L. subsp. *sinaicum* (current study) exhibited high identity and similarity of 100.00% to 5 accessions with E-value = Zero. Moreover, *Origanum syriacum* subsp. *sinaicum* showed similarity less than 100.00-97.37% with 49 accessions from 17 different genera belonging to the family *Lamiaceae* with E-value = Zero. On the other hand, it showed low similarities with the other 5 different species (89.29%, 90.21% 90.99% and 89.66%) with *Linum usitatissimum* (MG946893.1), *Arabidopsis thaliana* (AB917053.1), *Glycine max* (Z95552.1) and *Chenopodium album* (JX848451.1), respectively with E-value = Zero and 87.38% *Triticum aestivum* (AY328025.1) with E-value = 5e-178 as out group of the family *Lamiaceae* or *Labiatae*. Based on these results, phylogenetic relationships analyses enable us to check the closest of species from 17 different genera belonging to the family *Lamiaceae* were conducted in MEGA 7.0 software program by Maximum Likelihood (ML) tree with the highest log likelihood (-2836.15) is shown (Kumar *et al*., 2016). The phylogenetic tree was constructed based on the 60 amino acid sequences encoded from the *rbcL* gene that have the highest percentages of similarity with species from 17 different genera, showing closely related species clustering together from the family *Lamiaceae* and they showed low percentages of similarities towards relatively distantly related species scattering with 5 different species from families as out group of the family as shown in Fig. (2).

Molecular phylogenetic analysis based on sequences of maturase gene (*matK*)

Sequence homology of each nucleotide of the chloroplast 60 maturase gene (*matK*) searches (Code 1 to 2) for *Origanum* species (current studies), (Code 3 to 6) for *Origanum* genus, (Code 7 to 10) for *Conradina* genus, (Code 11 to13) for *Dicerandra* genus, (Code 14 to 17) for *Monarda* genus, (Code 18 to 20) for *Pycnanthemum* genus, (Code 21-23) for *Bystropogon* genus, (Code 24 to 26) for *Clinopodium* genus, (Code 27 to 29) for *Satureja* genus, (Code 30 to 32) for *Mentha* genus, (Code 33 to 36) for *Thymus* genus, (Code 37 to 39) for *Agastache* genus, (Code 40 to 42) for *Dracocephalum* genus, (Code 43 to 46) for *Nepeta* genus, (no. record) for genus *Lepechinia*, (Code 47 to 49) for *Prunella* genus, (Code 50 to 53) for *Rosmarinus* genus, (Code 54 to 55) for *Salvia* genus and (Code 56 to 60) for 5 different species with *Linum usitatissimum* (HM544115.1), *Arabidopsis thaliana* (KM892769.1), *Glycine max* (EF550007.1), *Chenopodium album* (KX133100.1) and *Triticum aestivum* (AF164405.1) as out group of the family

were tested by top-scoring hits through NCBI database using Basic Local Alignment Search Tool (BLASTn) as shown in Table (2). The Multiple Sequence Alignments (MSA) of 60 maturase gene (*matK*) were carried out between sequences of selected nucleotide sequence with 17 different genera belonging to the family *Lamiaceae* or *Labiatae* available in GenBank databases, which will be discussed briefly. The results revealed that *Origanum vulgare* L. in this investigation was closely related with high identity and similarity 100.00% with four accessions of *Origanum* genus and less than 100.00% - 92.73% with 40 accessions with 17 different genus belonging to the family *Lamiaceae* with E-value = Zero. On the other hand, it showed low similarities with the other 5 different species with 71.02% *Linum usitatissimum* (HM544115.1) with E-value = 1e-76, 75.68% *Arabidopsis thaliana* (KM892769.1) with E-value = 2e-134, 74.46% *Glycine max* (EF550007.1) with E-value = 3e-136 and 76.85% *Chenopodium album* (KX133100.1) with E-value = 6e-85, 70.99% *Triticum aestivum* (AF164405.1) with E-value = 1e-102 as out group of the family *Lamiaceae* or *Labiatae*. The Multiple Sequence Alignments (MSA) of nucleotide partial maturase gene (*matK*) from *Origanum syriacum* L. subsp. *sinaicum* (current study) exhibited high identity and similarity of 100.00% to 4 accessions with E-value = Zero. Moreover, *Origanum syriacum* L. subsp. *sinaicum* showed similarity less than 100.00-92.73% with 50 accessions of 17 different genera belonging to the family *Lamiaceae* with E-value = Zero. On the other hand, it showed low similarities with the other five different species with 71.02% *Linum usitatissimum* (HM544115.1) with E-value =1e-76, 75.68 % *Arabidopsis thaliana* (KM892769.1) with E-value =2e-134, 74.64% *Glycine max* (EF550007.1) with E-value = 4e 142 and 76.81% *Chenopodium album* (KX133100.1) with E-value = 2e-168 and 71.76% *Triticum aestivum* (AF164405.1) with E-value = 2e-114 as out group of the family *Lamiaceae* or *Labiatae*. Based on these results, phylogenetic relationships analyses enable us to check the closest of species from 17 different genera belonging to the family *Lamiaceae* were conducted in MEGA 7.0 software program by Maximum Likelihood (ML) tree with the highest log likelihood (-5692.60) is shown (Kumar *et al*., 2016). The phylogenetic tree was constructed based on the 60 amino acid sequences encoded from the *matK* gene have the highest percentages of similarity with species from 17 different genera, showing closely related species clustering together from the family *Lamiaceae* and they showed low percentages of similarities towards relatively distantly related species scattering with 5 different species from families as out group of the family as shown in Fig. (3).

Primer universality and species identification are two crucial criterions for an ideal DNA barcode. The two DNA barcodes showed high rates of amplification and sequencing successes, among which *rbc*L and *matK* genes had the best performance of universality. Phylogenetic

analysis using tree-based method is an important approach to determine the DNA region and evaluate its ability to verify whether it can identify and detect species-specific clusters of species from the same genus. In this study, ML analysis produced phylogenetic tree with better resolution for all tested barcodes. Maximum likelihood tree analysis was performed to evaluate the discriminatory power of the *rbcL* and *matK* genes.

Our data analyses were in agreement for used two plastid markers as the most rapidly evolving plastid coding regions and it consistently showed high levels of discrimination among angiosperm species (CBOL Plant Working Group, 2009). and Theodoridis *et al*., (2012) tested three cpDNA regions (*matK*, *rbcL*, *trnH-psbA*) that were proposed by previous studies in *Labiatae* species. The efficacy of a DNA barcoding approach as clear evidences to the recognition of commercial spices within the family *Lamiaceae*. Fazekas *et al*., (2008) and (2012) examined the suitability of different leading candidate markers and proposed the two-locus combination of *matK* and *rbcL* as the core plant barcode could be important used supplementary marker in appropriate cases. Other authors, Bafeel *et al*., (2011) used universal *matK* primer for *matK* as a barcode. The efficacy of a DNA barcoding approach as clear evidences to the recognition of commercial spices within the family *Lamiaceae* (De Mattia *et al*., 2011). Schori and Showalter (2011) analyzed 14 species from *Labiatae* in Pakistan and found that the *rbcL*, *matK*, *psbA-trnH* loci, could serve as single-region barcodes depending on plant to be identified, one region was preferred over the other to aid in species identification. Meanwhile, Bafeel *et al*., (2012) tested the potential of the *rbcL* marker for the identification of wild plants belonging to diverse families of arid regions. Recently, Federici *et al*., (2013) showed clear amplification and sequencing 36 samples of *Thymus* spp. using the molecular analysis of the core barcode regions (*matK* and *rbcL*) and the plastid intergenic spacer *trnH-psbA*. For herbal plant identification, *matK*, *rbcL*, *trnH-psbA*, ITS, trnL-F, 5S-rRNA and 18S-rRNA have been used as successful DNA barcodes by Mishra *et al*., (2016). While, Parveen *et al*., (2016) proposed that DNA barcoding as a means to identify herbal ingredients and to detect adulteration. However, general barcoding techniques using universal primers have been shown to provide mixed results with regard to data accuracy. More recently, Skuza *et al*., (2019) observed that nucleotide sequences had a high variability within *matK* and *rbcL* regions and the *matK* region is suitable for differentiation and discrimination between the studied species in the genus *Secale*. In this study, our results indicated that two plastid regions (*rbcL* and *matK*) could be a better choice for barcoding with excellent primer universality. They could also help to understand the relationships of co-occurring species and the species assembly within community when combining more information including species abundance and the functional traits of all the species in the future.

SUMMARY

Genus *Origanum* is one of the most species-rich as medicinal plants and pharmaceutically in the family *Lamiaceae* for several multipurpose used, and thus it is an endangered plant that needs a strict conservation plan. This genus contains many plants with medical uses, and thus an objective identification method is urgently needed. DNA barcoding is a sample fast technique at molecular level in the field of identification, authentication, classification and differentiation between two *Origanum* species and with other species. The current investigation was conducted to identify, discriminate and authenticate *Origanum vulgare* L. and *Origanum syriacum* L. subsp. *sinaicum* using two chloroplast genes (coding sequences) as the most common DNA barcodes, ribulose 1, 5-biphosphate carboxylase large subunit (*rbcL*) and maturase K (*matK*) genes. The partial sequence length of *rbcL* gene of two *Origanum* species were 528bp and 532bp with *Origanum vulgare* L. (MT679150.1/QNJ99666), and *Origanum syriacum* L. subsp. *sinaicum* (MT679151.1/QNJ99667), and similarly with *matK* gene were 745bp and 775 bp with *Origanum vulgare* L. (MT679152.1/QNJ99668), and *Origanum syriacum* L., subsp. *Sinaicum* (MT679153.1/QNJ99669), respectively. The alignments of the sequence chloroplast genes (*rbcL* and *matK*) were able to distinguish two *Origanum* species under study with high similarities and to the closely related species of *Origanum* genus, other 17 genera belonging to family *Lamiaceae* and take them away from five plant species from different families as out group of the family *Lamiaceae*. The obtained results revealed that *rbcL* and *matK* genes nucleotide sequence isolated from the two *Origanum* species in this investigation showed high similarities and closely related to NCBI recoded 17 genera belonging to the family *Lamiaceae*. Furthermore, a phylogenetic tree analyses were constructed based on amino acid sequence of 60 *rbcL* and 60 *matK* genes using MEGA 7 program by Maximum Likelihood (ML) method with the highest log likelihood (-2836.15) for *rbcL* gene and (-5692.60) for *matK* gene.

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Table (1): Homology of nucleotide sequences for 60 selected accession lists and its related Seq1: *Origanum vulgare* L. and Seq2: *Origanum syriacum* L. subsp. *sinaicum* for chloroplast, *rbcL* gene sequenced in this study, BLASTn top hits against GenBank database, similarity score and GenBank accession no.

Family *Lamiaceae* Tribe: *Mentheae*	Code	Scientific name of chloroplast *rbcL* gene (length bp)	GenBank Accession No.	Similarity% (bp)	
				**Origanum vulgare*	**O. syriacum* subsp. *sinaicum*
1. Genus *Origanum*	1	**Origanum vulgare* (528bp)	MT679150.1	----	99.62
	2	**O. syriacum* subsp. *sinaicum* (532bp)	MT679151.1	99.62	----
	3	*Origanum sipyleum*	HQ902792.1	99.61	100.00
	4	*Origanum vulgare*	MG224341.1	99.62	100.00
	5	*Origanum majorana*	KX783936.1	99.62	100.00
	6	*Origanum laevigatum*	MF349689.1	99.62	100.00
	7	*Origanum vulgare*	MF694999.1	99.62	100.00
2. Genus *Conradina*	8	*Conradina glabra*	KY765542.1	99.37	99.58
	9	*Conradina grandiflora*	MG582632.1	99.23	99.23
	10	*Conradina verticillata*	MH749055.1	99.24	99.62
3. Genus *Dicerandra*	11	*Dicerandra thinicola*	MG582633.1	98.84	99.23
	12	*Dicerandra immaculate*	KY765541.1	98.93	99.15
	13	*Dicerandra frutescens*	MG592699.1	98.84	99.23
4. Genus *Monarda*	14	*Monarda punctate*	MK526204.1	99.22	99.61
	15	*Monarda punctate*	KY627431.1	99.23	99.62
	16	*Monarda fistulosa*	MF349566.1	99.24	99.62
5. Genus *Pycnanthemum*	17	*Pycnanthemum incanum*	MG221861.1	99.23	99.62
	18	*Pycnanthemum tenuifolium*	MG223821.1	99.24	99.62
	19	*Pycnanthemum virginianum*	MG224042.1	99.24	99.62
6. Genus *Bystropogon*	20	*Bystropogon origanifolius*	KJ595607.1	99.43	99.81
7. Genus *Clinopodium*	21	*Clinopodium vulgare*	HQ590041.1	99.43	99.81
	22	*Clinopodium chinense*	FJ513146.1	99.43	99.81
	23	*Clinopodium repens*	MH116120.1	99.43	99.81

Table (1): Cont''.

8. Genus *Satureja*	24	*Satureja pilosa subsp. origanita*	KR063652.1	99.05	99.44
	25	*Satureja Montana*	MF349309.1	99.05	99.44
	26	*Satureja hortensis*	MG224422.1	99.05	99.44
9. Genus *Mentha*	27	*Mentha spicata*	KY400629.1	99.43	99.81
	28	*Mentha Canadensis*	KC473279.1	99.43	99.81
	29	*Mentha pulegium*	KY656718.1	99.43	99.81
10. Genus *Thymus*	30	*Thymus serpyllum*	KF997486.1	99.05	99.43
	31	*Thymus pulegioides*	JN892334.1	99.03	99.42
	32	*Thymus praecox*	MG221401.1	99.01	99.41
	33	*Thymus drucei*	MG221346.1	99.05	99.44
	34	*Thymus vulgaris*	Z37471.1	98.86	99.25
	35	*Thymus vulgaris* (II)	Z37472.1	99.24	99.62
11. Genus *Agastache*	36	*Agastache rugosa*	FJ513154.1	97.92	98.31
	37	*Agastache foeniculum*	MG222708.1	97.92	98.31
	38	*Agastache nepetoides*	MG222557.1	97.92	98.31
12. Genus *Dracocephalum*	39	*Dracocephalum moldavica*	HM590077.1	96.97	97.37
	40	*Dracocephalum rupestre*	HQ839685.1	97.16	97.56
	41	*Dracocephalum ruyschiana*	KF307354.1	97.54	97.93
13. Genus *Nepeta*	42	*Nepeta cataria*	MG946943.1	97.35	97.74
	43	*Nepeta bracteata*	MH998002.1	96.97	97.37
	44	*Nepeta cataria*	MN601459.1	97.35	97.74
14. Genus *Prunella*	45	*Prunella vulgaris*	MH116337.1	98.11	98.12
	46	*Prunella grandiflora*	FR865137.1	97.28	97.28
15. Genus *Lepechinia*	47	*Lepechinia chamaedryoides*	AY570387.1	98.11	98.50
	48	*Lepechinia fragrans*	AY570388.1	98.11	98.50
	49	*Lepechinia calycin*	AY570386.1	98.11	98.50
16. Genus *Rosmarinus*	50	*Rosmarinus officinalis*	HE963635.1	98.62	99.02
	51	*Rosmarinus officinalis*	HQ619754.1	98.67	99.06
	52	*Rosmarinus officinalis*	KM360960.1	98.86	99.25
17. Genus *Salvia*	53	*Salvia deserta*	JQ933991.1	99.05	99.44
	54	*Salvia fruticosa*	HM590078.1	99.05	99.44
	55	*Salvia officinalis*	JQ934010.1	99.05	99.44
Family *linaceae*	56	*Linum usitatissimum* (Out group)	MG946893.1	89.22	89.29
Family *Brassicaceae*	57	*Arabidopsis thaliana* (Out group)	AB917053.1	89.77	90.20
Family *Fabaceae*	58	*Glycine max*(Out group)	Z95552.1	90.59	90.99
Family *Amaranthaceae*	59	*Chenopodium album* (Out group)	JX848451.1	89.20	89.66
Family *Poaceae*	60	*Triticum aestivum* (Out group)	AY328025.1	87.31	87.38

Note:* *Origanum vulgare* L. and * *Origanum syriacum* L. subsp. *sinaicum* were used as current study.

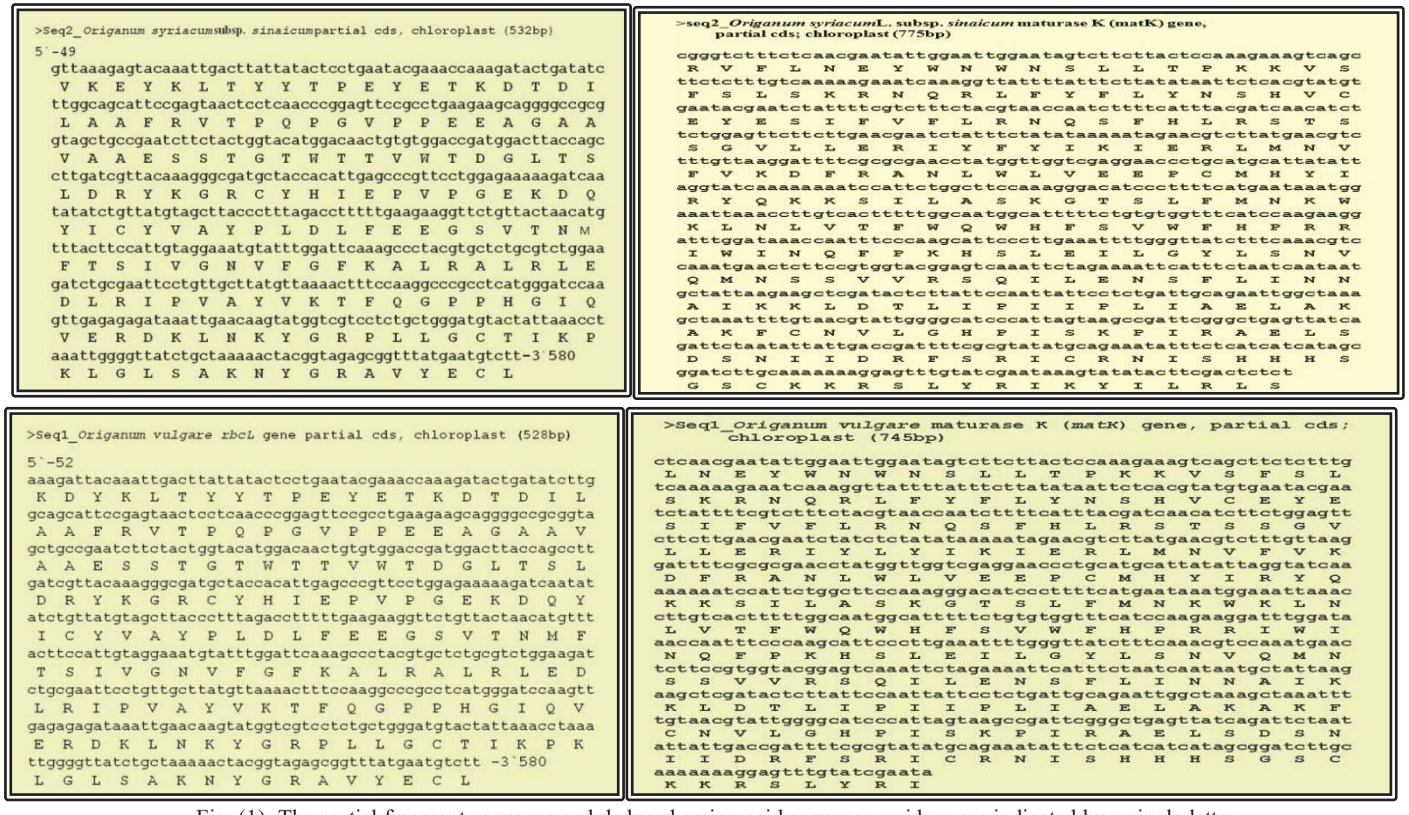
Table (2): Homology of nucleotide sequences for 60 selected accession lists and its related Seq1: *Origanum vulgare* L. and Seq2: *Origanum syriacum* L. subsp. *sinaicum* for chloroplast maturase gene (*matK*) sequenced in this study, BLAST top hits against GenBank database, similarity score and accession no.

Family: *Lamiaceae* Tribe: *Mentheae*	Code	Scientific name of chloroplast *matK* gene (length bp)	GenBank Accession No.	Similarity% (bp)	
				**Origanum vulgare*	**O. syriacum* subsp. *sinaicum*
1- Genus *Origanum*	1	**Origanum vulgare* (745bp)	MT679152.1	---	100.00
	2	**O. syriacum* subsp. *sinaicum* (775bp)	MT679153.1	100.00	---
	3	*Origanum laevigatum*	MF350147.1	100.00	100.00
	4	*Origanum majorana*	MN167195.1	99.87	99.87
	5	*Origanum vulgare*	MN167194.1	100.00	100.00
	6	*Origanum vulgare*	MK520369.1	100.00	100.00
2. Genus *Conradina*	7	*Conradina grandiflora*	KY607200.1	97.31	97.31
	8	*Conradina verticillata*	MH748917.1	97.58	97.61
	9	*Conradina glabra*	KY607199.1	97.14	97.14
	10	*Conradina canescens*	KJ772673.1	97.24	97.24
3. Genus *Dicerandra*	11	*Dicerandra christmanii*	KY607212.1	97.16	97.16
	12	*Dicerandra cornutissima*	KY607213.1	96.84	96.84
	13	*Dicerandra immaculata*	KY607214.1	97.14	97.14
4. Genus *Monarda*	14	*Monarda fistulosa var. mollis*	KT176605.1	97.58	97.68
	15	*Monarda didyma*	MG224897.1	97.49	97.52
	16	*Monarda clinopodia*	KP642819.1	97.36	97.40
	17	*Monarda fistulosa*	MF350057.1	97.58	97.64
5. Genus *Pycnan.*	18	*Pycnanthemum albescens*	MF350277.1	97.62	97.65
	19	*Pycnanthemum virginianum*	MG225271.1	97.64	97.66
	20	*Pycnanthemum albescens*	MH748968.1	97.71	97.74
6. Genus *Bystropogon*	21	*Bystropogon canariensis*	GU381726.1	98.07	97.98
	22	*Bystropogon origanifolius*	GU381727.1	98.02	98.09
	23	*Bystropogon origanifolius*	GU381728.1	98.07	98.14
7-Genus *Clinopodium*	24	*Clinopodium vulgare*	KJ592905.1	97.79	97.81
	25	*Clinopodium macrostemum*	MK601827.1	97.45	97.55
	26	*Clinopodium wardii*	KX526681.1	97.79	97.82

Table (2): Cont''.

8. Genus *Satureja*	27	*Satureja horvatii*	KX954592.1	98.39	98.44
	28	*Satureja subspicata*	KX954591.1	98.39	98.44
	29	*Satureja Montana*	MF350242.1	98.31	98.34
9. Genus *Mentha*	30	*Mentha Canadensis*	JN407140.1	98.39	98.45
	31	*Mentha spicata*	MN167204.1	98.52	98.58
	32	*Mentha suaveolens*	LC126645.1	98.52	98.45
10. Genus *Thymus*	33	*Thymus decussatus*	MN972469.1	99.31	98.53
	34	*Thymus longicaulis*	HE819415.1	99.13	98.31
	35	*Thymus vulgaris*	HE819430.1	99.27	98.45
	36	*Thymus serpyllum*	MF350183.1	99.33	99.35
11. Genus *Agastache*	37	*Agastache nepetoides*	MK509382.1	95.38	95.44
	38	*Agastache scrophulariifolia*	MK509383.1	95.37	95.43
	39	*Agastache foeniculum*	AY840146.1	94.54	94.56
12. Genus *Dracocephalum*	40	*Dracocephalum parviflorum*	MK520021.1	93.29	93.37
	41	*Dracocephalum tanguticum*	MF786820.1	92.73	92.73
	42	*Dracocephalum forrestii*	MF786791.1	93.36	93.36
13. Genus *Nepeta*	43	*Nepeta x faassenii*	MF349917.1	93.96	94.04
	44	*Nepeta italic*	HQ902725.1	94.63	94.55
	45	*Nepeta cataria*	MG224812.1	93.69	93.67
	46	*Nepeta bracteata*	MG946967.1	93.56	93.47
14. Genus *Prunella*	47	*Prunella vulgaris*	KX676737.1	95.55	94.85
	48	*Prunella vulgaris*	KP402374.1	95.44	95.44
	49	*Prunella vulgaris*	MF158707.1	95.20	94.56
15. Genus *Lepechinia*	----	No record	----	----	----
16. Genus *Rosmarinus*	50	*Salvia rosmarinus*	MF694874.1	95.34	94.61
	51	*Salvia rosmarinus*	KX783771.1	95.32	94.50
	52	*Rosmarinus officinalis*	KP172065.1	95.41	94.55
	53	*Rosmarinus officinalis*	FR719112.1	95.34	94.51
17. Genus *Salvia*	54	*Salvia rosmarinus*	MF349943.1	95.34	94.70
	55	*Salvia officinalis*	KC473367.1	95.57	95.58
Family *linaceae*	56	*Linum usitatissimum*	HM544115.1	71.02	71.02
Family *Brassicaceae*	57	*Arabidopsis thaliana*	KM892769.1	75.68	75.68
Family *Fabaceae*	58	*Glycine max*	EF550007.1	74.46	74.64
Family *Amaranthaceae*	59	*Chenopodium album*	KX133100.1	76.85	76.81
Family *Poaceae*	60	*Triticum aestivum*	AF164405.1	70.99	71.76

Note:* *Origanum vulgare* and * *Origanum syriacum* subsp. *sinaicum* were used as current study.



(A) **(B)**

Fig. (1): The partial fragment sequence and deduced amino acid sequence residues are indicated by a single letter code used by (https://web.expasy.org/translate/).

(A): *rbcL* gene from *Origanum vulgare* and *Origanum syriacum* subsp. *sinaicum* (528 and 532 bp) with G enBank accession no. MT679150.1/QNJ99666 and MT679151.1/QNJ99667, respectively.

(B): *matK* gene from *Origanum vulgare* L. and *Origanum syriacum* L. subsp. *sinaicum* (745 and 775bp) with GenBank accession no. MT679152.1/QNJ99668 and MT679153.1/QNJ99669, respectively.

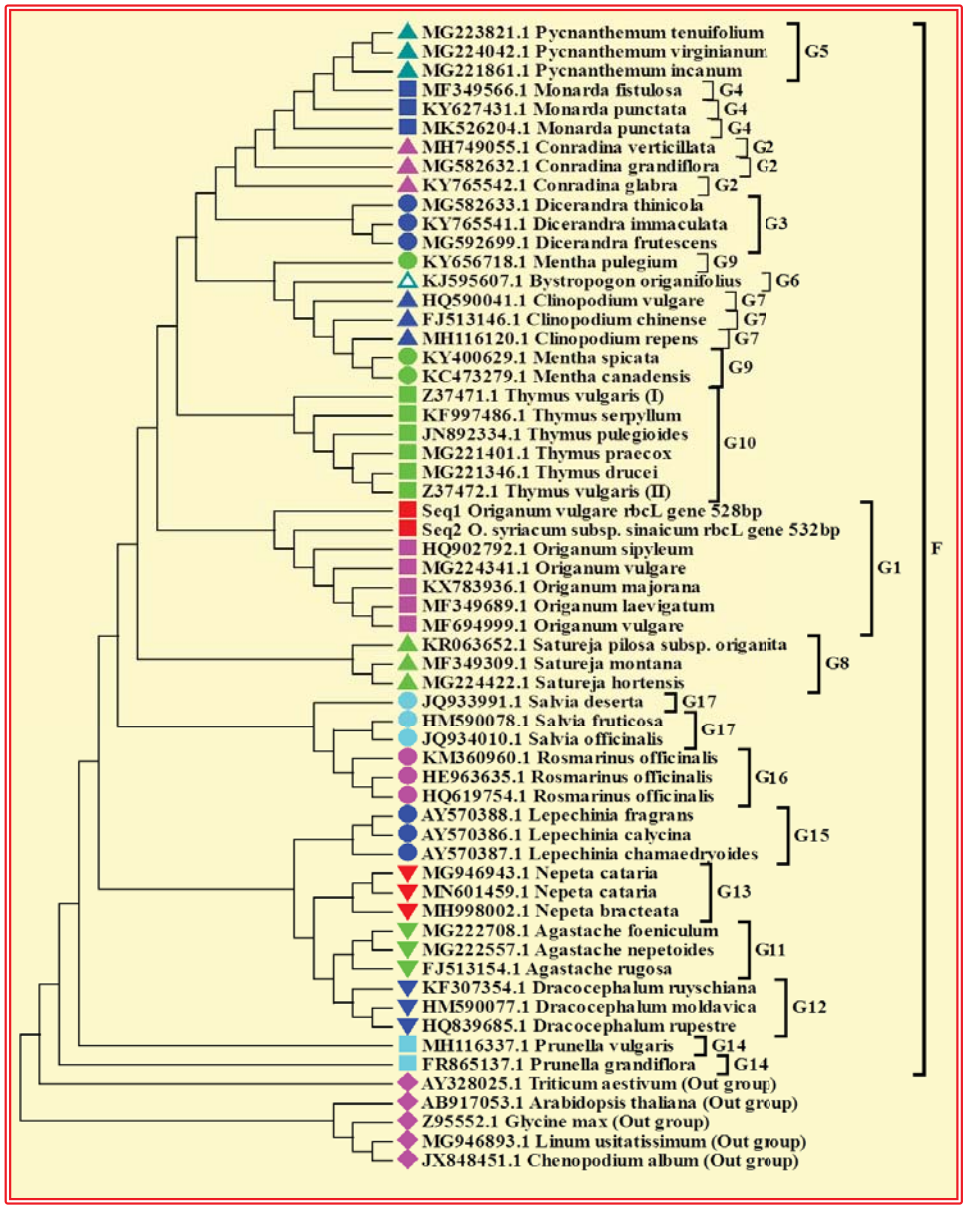


Fig. (2): Molecular phylogenetic analyses involved 60 amino acid sequences of *rbcL* gene were conducted in MEGA 7.0 software program by Maximum Likelihood method. The tree with the highest log likelihood (-2836.15) is shown. Note: G1: *Origanum* species, G2: *Conradina* species, G3: *Dicerandra* species, G4: *Monarda* species, G5: *Pycnant.* Species, G6: *Bystropogon* species, G7: *Clinopodium* species, G8: *Satureja* species, G9: *Mentha* species, G10: *Thymus* species, G11: *Agastache* species,G12: *Dracocephalum* species, G13: *Nepeta* species, G14: *Prunella* species, G15: *Lepechinia* species, G16: *Rosmarinus* species, G17: *Salvia* species and 4 different species from different family as out group. F: family *Lamiaceae.* Seq1: *Origanum vulgare* L. and Seq2: *Origanum syriacum* L. subsp. *sinaicum* were used as current study.

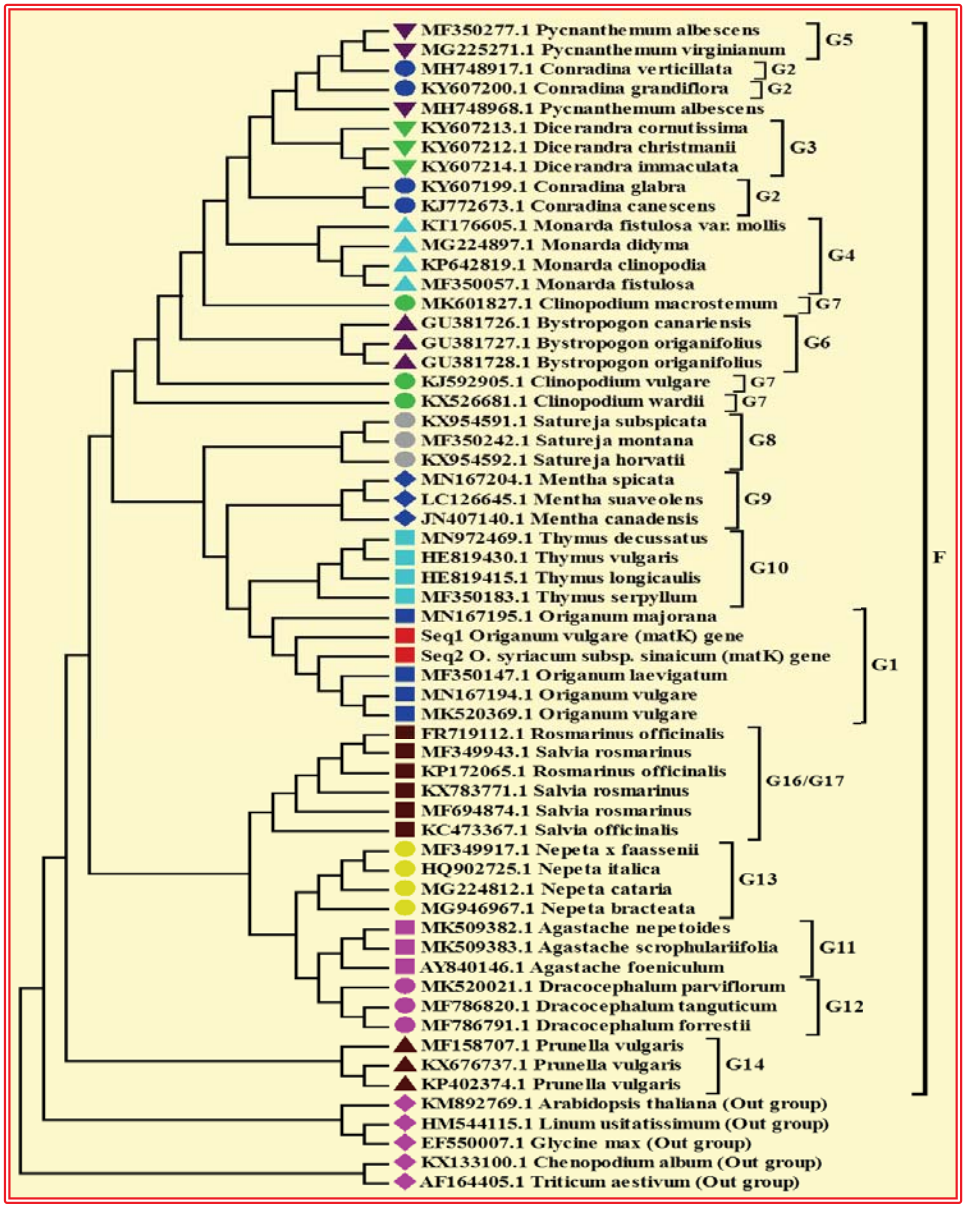


Fig.(3): Molecular phylogenetic analysis involved 60 amino acid sequences of maturase gene (*matK*) were conducted in MEGA 7.0 software program by Maximum Likelihood method. The tree with the highest log likelihood (-5692.60) is shown. Note: G1: *Origanum* species, G2: *Conradina* species, G3: *Dicerandra* species, G4: *Monarda* species, G5: *Pycnant.* Species, G6: *Bystropogon* species, G7: *Clinopodium* species, G8: *Satureja* species, G9: *Mentha* species, G10: *Thymus* species, G11: *Agastache* species,G12: *Dracocephalum* species, G13: *Nepeta* species, G14: *Prunella* species, G15: *Lepechinia* species (no. record), G16: *Rosmarinus* species, G17: *Salvia* species and 4 different species from different family as out group. F: family *Lamiaceae.* Seq1: *Origanum vulgare* L. and Seq2: *Origanum syriacum* L. subsp. *sinaicum* were used as current study.

PHOSPHATE SOLUBILIZING AND BIOCONTROL POTENTIAL OF AN *Aspergillus niger* ISOLATE FROM EGYPTIAN SOIL

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Phosphorus (P) is one of the most essential plant nutrients that significantly influence overall plant growth (Wang *et al.,* 2009). It has an influence on different key metabolic processes, like development and division of plant cells, energy transportation, signal transduction, biosynthesis of macromolecular, photosynthesis, and plant respiration (Shenoy and Kalagudi, 2005; Ahemad *et al.,* 2009; Khan *et al.,* 2009). The environment does not supply plants with soluble P. Primary and secondary minerals and/or organic compounds are therefore mainly the source of P. The concentration of P in the soil solution is much lower compared to other nutrients and ranges from 0,001 to 1 mg / l (Brady and Weil, 2002). P-mineral compounds generally contain aluminum (Al), iron (Fe), manganese (Mn), and calcium (Ca) and differ from soil to soil. For example, P forms a complex in acid soils with Al, Fe, and Mn, while it reacts very strongly with Ca in alkaline soils. In all conditions, however, the types of P soil compounds are determined primarily through soil pH and soil mineral type and concentrations (Khan *et al.,* 2014).

The P of phosphatic fertilizers or manure reacts extremely strongly with soil components and then becomes unavailable to plants. The insoluble kinds of P like tricalcium phosphate (Ca_3PO_4), aluminum phosphate (Al_3PO_4), iron phosphate (Fe_3PO_4), etc. these forms can be converted into soluble Phosphate by Phosphate solubilizing organisms in different soil types (Gupta *et al.,* 2007; Song *et al.,* 2008; Khan *et al.,* 2013; Sharma *et al.,* 2013). Generally, soil microorganisms are more efficient in supplying P by solubilization to plants from both inorganic and organic sources. (Toro, 2007; Wani *et al.,* 2007) and mineralization of complex Phosphate compounds (Ponmurugan and Gopi, 2006).

Plants encounter multiple biotic threats and adverse environmental factors. Pests, parasites, and pathogens have been identified since ancient times as the cause of biotic stress in plants. Two types of

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fungal parasites occur: Biotrophic fungi are certain fungi that require nutritional supplies from living plants and nectrotrophs which kill the plant using toxins (Glazebrook, 2005). In combination with bacteria, they can cause, among other symptoms, vascular wilts, leaf spots, and cankers and infect various parts of a plant (Gimenez *et al.,* 2018). Treating plant fungal infection with chemical pesticides harms human health and the environment. Therefore, an environment-friendly and cost-effective alternative are always required. Using fungi as biological control agents (BCAs) is an attractive treatment to substitute dangerous pesticides and deliver a wide field application potential (Das and Abdulhameed, 2020).

Built on their wide scope of disease prevention and increase in yield, fungal biological control agents are gaining much acceptance (Kaur and Reddy, 2017). Non-pathogenic and saprophytic fungi that inhabit soil can support several crops as they not only encourage development but also guard them against diseases (Pandya and Saraf, 2010) examples of such fungi include the genera *Aspergillus*, *Trichoderma*, *Penicillium Verticillium,* and others. Isolates of *Aspergillus*, *Paecilomyces,* and *Penicillium* are known to have an antagonistic effect on soil-borne pathogens in the rhizosphere of crops (Noveriza and Quimio, 2004).

Our research aims to help crop producers to increase their production by the introduction of novel fungal treatment with dual function to solubilize phosphate in the soil and to protect plants from soil-borne pathogens. The research will focus on screening soil for phosphate solubilizing fungi and the evaluation of their abilities. The obtained fungi could be used in the production of ecofriendly biofertilizers. Using such fertilizers could increase the profits of crop growers, reduce the use of harmful chemical fertilizers, reduce greenhouse gases emission, improving soil quality, and reduce production costs.

MATERIALS AND METHODS

Collection of rhizosphere soil sample

The soil sample was collected from the Agricultural Genetic Engineering Research Institute field near the sorghum rhizosphere. The sample comprised of soil adhering to the roots of sorghum. The sample was carefully shaken in plastic bags to separate the soil from the sorghum roots, and immediately transferred to a cooler until arrival at the laboratory. The sample was stored at 4ºC for further analysis.

Isolation of phosphate-solubilizing fungi (PSF)

Nearly 10 g of soil sample was moved to a sterilized Erlenmeyer flask having 90 mL of sterile distilled water. The sample was shaken at 120 rpm for 60 min. Then, a series of 10-fold dilutions of the suspension were prepared and 300 µL was plated on Pikovskaya's agar from each dilution. Pikovskaya's agar was made from 10 g glucose, 0.2 g NaCl, 0.2 g KCl, 0.5 g $(NH_4)_2SO_4$, 0.1 g

$MgSO_4 \cdot 7H_2O$, 0.0001g $FeSO_4 \cdot 7H_2O$, 0.0001g $MnSO_4 \cdot 7H_2O$, 0.5 g yeast extract, and 18 g agar in 1000 mL of distilled water, and 0.5% tricalcium phosphate as recalcitrant P source (Pikovskaya, 1948). The phosphate solubilizing fungi (PSF) was identified after eight days of incubation at 25ºC by the existence of a clear halo zone around fungal colonies. The experiment was repeated three times. Identified PSF was purified by repeated culturing on potato dextrose agar (PDA) at 25ºC. Microscopic examination was performed with an Olympus-FSX100 Inverted Microscope.

DNA extraction from fungi

DNA extraction of fungi was performed following the method of Aamir *et al.,* (2015). The genomic DNA was extracted from fungal cultures grown in liquid PDA for five days at 25ºC. Fungal mass was obtained by filtering the culture broth by 10 ml syringes encompassing glass wool to retain the fungal mass and let the broth pass through. The fungal mass was put in a 2ml tube containing sterile glass beads and lysis buffer (50mM EDTA, 100 mM Tris HCl [pH8.0], 3% SDS). Homogenization of fungal mass was accomplished using the Tissue Lyser II homogenizer (Qiagen, Germany). The fungal homogenate was centrifuged at 13,000 rpm for 10 min and the supernatant was transferred to a new microcentrifuge tube. 2 of RNase A (10mg/ml) was added to the supernatant and incubated at 37ºC for 15 min. After that, an equal volume of phenol: chloroform: Isoamyl alcohol (25:24:1) was added followed by centrifugation at 13,000 rpm for 10 min. The upper layer was transferred to a fresh microcentrifuge tube. Next, an equal volume of 100% ethanol was added. After precipitation at -20ºC for 30 min, the tube was centrifuged at 13,000 rpm for 10 min to pellet down the DNA. The DNA pellet was washed with 70% ethanol followed by centrifugation at 12,000 rpm for 5 min. The DNA pellets were air-dried and dissolved in sterilized ddH_2O.

Amplification of fungi Internal Transcribed Spacer region (ITS)

The ITS region of the *nuc* rRNA gene was amplified with primers ITS1: 5′TCCGTAGGTGAACCTTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′ (Wang *et al.,* 2018). Primers obtained in the lyophilized state were centrifuged at 1000 rpm for 5 min, deionized nuclease-free ddH_2O was added by recommended volume described in the primers sheet to form a concentration of 100 µM, vortexed thoroughly until complete resuspension then primers dilution applied 1:10 to from a regular PCR working concentration of 10 µM. Polymerase chain reactions (PCR) were conducted using 20-µL reaction mixture containing 0.5 µL of each primer (10 pmol/µL), 1.0 µL of genomic DNA (10 ng/µL), 8 µL of 2 × PCR Master Mix buffer (0.05 µg/µL Taq polymerase, 4 mM $MgCl_2$, and 0.4 mM dNTPs), and 10 µL of ultrapure sterile water. PCR cycling conditions were as follows: one initial cycle of denaturation at 94ºC for 3 min, followed by 34 cycles of 94ºC for 30 s, 50ºC

for 30 s, and extension at 72ºC for 45 s, and a final extension for 5 min at 72ºC.

Purification of the PCR product

ITS PCR product was purified using the AxyPrep™ DNA Gel extraction kit (Cat. # AP-GX-50) according to manufactures instructions. PCR product was transferred into a 1.5 ml microfuge tube, then a three X sample volume of buffer DE-A was added. Subsequently, Half X buffer DE-A volume of buffer DE-B was added and mixed well. An AxyPrep column was placed into a 2 ml microfuge tube. Then, the mix was transferred into the column, centrifuged at 12,000 xg for 1 min. The filtrate was discarded from the 2 ml microfuge tube and the AxyPrep column was returned to the 2 ml microfuge tube and 500 µl of bufferW1 was added. The mix was centrifuged at 12,000 xg for 30 sec. The filtrate was discarded from the 2 ml microfuge tube. The AxyPrep column was returned to the 2 ml microfuge tube and 700 µl of buffer W2 was added, centrifuged at 12,000 ×g for 30 sec, then the filtrate was discarded from 2 ml microfuge tube. The AxyPrep column was placed back into the 2 ml microfuge tube, centrifuged at 12,000 xg for 1 min to remove residuals of previous solutions. The AxyPrep column was transferred into a clean 1.5 ml microfuge tube. 30 µl of prewarmed deionized water (65ºC) was added to the center of the membrane and incubated for 5 min at room temperature for the elution of DNA followed by centrifugation at 12,000 xg for 1 min. DNA was quantified using the absorption of light at 260 and 280 nm (A260/280) by Thermo Scientific™ NanoDrop 2000. The sample was sequenced in the forward direction by Macrogen Inc. (Korea).

Bioinformatic analysis of DNA sequence

The raw sequence was proofread and edited manually with BioEdit 7.0.9 (Hall, 1999). A homology search was performed using The Basic Local Alignment Search Tool (BLAST) against the National Center for Biotechnology Information (NCBI) database, USA (http://www.ncbi.nlm.nih.gov) on the DNA level. The edited sequence was aligned using Clustal W (Thompson *et al.,* 1994) and adjusted manually as required. Finally, a neighbor-joining (NJ) phylogenetic tree was constructed with the Kimura 2-parameter model to calculate sequence divergence and subjected to 1000 bootstrap replications using MEGA 6.0 (Tamura *et al.,* 2013), with gaps treated as complete deletions.

Antagonistic effect of phosphate solubilizing fungi

Phosphate solubilizing fungi isolate was checked for antagonistic effect against two pathogens belonging to the genus Fusarium (*Fusarium solani* and *Fusarium verticillioides*). The pathogens used in the present research were obtained from the Molecular Microbiology Lab, Agricultural Genetic Engineering Research Institute (AGERI)/ARC, Egypt. Two-disc plugs (0.5-cm diameter) of pathogen and antagonist (4 days old cul-

ture) were transferred respectively to a single potato dextrose agar (PDA) plate (9-cm diameter). The antagonist plug was placed on the one side of the plate (about 2 cm from the edge of the plate towards the center), while the pathogen plug was placed at the other side of the plate opposite to the antagonist plug leaving 5 cm between the two plugs. A plug of the PDA medium was used as a control treatment while the pathogen plug was placed on the other side. Three replications (two plates/replicate) for each treatment were made, and the plates were incubated at 28 ± 2ºC for 5 days.

RESULTS AND DISCUSSION

Isolation of phosphate solubilizing fungi (PSF)

The formation of halo zones around colonies on Pikovskaya's medium is an indication that the microorganism has the phosphate-solubilizing capability. In the present study, six fungal isolates were isolated from a sorghum field, only one showed phosphate solubilizing capacity by displaying a clear zone of dissolved phosphate in solid Pikovskaya's medium, which indicated that this isolate is phosphate solubilizing fungi (PSF) exhibited the desired P-solubilizing ability (Fig. 1). The fungal isolate was named AG-A01. Microscopic examination of fungi showed that it tends to form filaments (hyphae) and conidial heads of the organism to be globose and dark brown and the spores are carbon black/dark brown color.

Phosphate solubilizing fungi was reported recently as an alternative strategy for using rock phosphate (RP) in soil fertilization (Wang *et al.,* 2018). Rock phosphate has a direct application for applying phosphate to the soil, but its effectiveness is highly dependent on the soil type which soil PH is the most important factor (Sulbarán *et al.,* 2009). An alternative was the use of microorganisms capable of solubilizing rock phosphate and of releasing soluble phosphorous through organic acid production, sugar chelating, pH reduction, and enzyme production. Several reports have shown that certain microorganisms can solubilize insoluble phosphate rock and release soluble phosphate. Fungi Aspergillus was an effective phosphate solubilizer among the microbes (Wang *et al.,* 2018; Adhikari and Pandey, 2019).

Identification and phylogenetic analysis of the AG-A01 isolate

DNA was extracted from Fungi. Then, the amplification of the selected Internal Transcribed Spacer (ITS) region resulted in a band with approximately 600 bp (Fig. 2) which is the expected size according to the designed primers. THE purified PCR product was sent to sequencing and the resulted sequence was compared to sequences in the gene bank using The Basic Local Alignment Search Tool (BLAST) on the NCBI website. The isolate AG-A01 was identified as *Aspergillus niger* with similarity 100% with *Aspergillus niger* isolate WA-TKA small subunit ribosomal RNA gene. Phylogenetic tree analyses with bootstrapping of the partial sequence (398 bp) of the fungi ITS region

suggested that this isolate was more closely related to *Aspergillus niger* than any *Aspergillus* group (Fig. 3). The AG-A01 isolate groups with *Aspergillus niger* with strong statistical support and shares 100% nucleotide sequence identity.

Antagonism effect of AG-A01 strain

The antagonism test showed clearly that there was a significant reduction in mycelial growth of tested pathogens after the confrontation with *Aspergillus niger*-isolate AG-A01 (Fig. 4). The pathogens *Fusarium solani* and *Fusarium verticillioides* exhibited a similar reduction of mycelium which reached nearly 99%. This reduction could be explained for the ability of *Aspergillus niger* to grow faster than the two fungi and preventing even their spores to grow and multiple. The results of this test agreed with the findings of many reports illustrated the role of *Aspergillus* species in the biocontrol of plant pathogens. For instance, Anwer *et al.,* 2017 who obtained an isolate from *Aspergillus niger* that can soluble phosphate and act as a biocontrol agent. Ruangsanka, (2014) stated that *Aspergillus niger* isolate had the highest pathogen inhibition percentage (64%) against the devastating soil-borne pathogen *F. oxysporum*. *Aspergillus niger* was also reported as a potential biocontrol agent for controlling fusarium wilt of tomato (Sharma *et al.,* 2011). In conclusion, in the current study, we isolated an efficient phosphate solubilizing fungus which is also a strong biocontrol agent. Due to its several positive effects, the *Aspergillus niger* isolate has the potential to be developed as a commercial product which can increase crop nutrient uptake and, hence increasing their yield.

SUMMARY

Phosphorus (P) is one of the most important plant nutrients that greatly affect overall plant growth. P-mineral always presents in soil with the insoluble condition. Phosphate compounds generally contain aluminum (Al), iron (Fe), manganese (Mn), and calcium (Ca) which vary according to soil type. Phosphate solubilizing fungi play a major role in rising soil phosphate bioavailability for plants by realizing phosphate from its compounds. The present study was aimed to isolate and characterize phosphate solubilizing fungi from Egyptian soil using a solid Pikovskaya (PVK) medium. In total, 6 fungal isolates were able to grow on Pikovskaya (PVK) medium but only one isolate (AG-A01) showed phosphate-solubilizing capacity. DNA was extracted from the isolate followed by amplification of selected Internal Transcribed Spacer (ITS) region and DNA sequencing. After analyzing the DNA sequence, the isolate AG-A01 was identified as *Aspergillus niger*. The fungal isolate also displayed an antagonism effect against two plant pathogens (*Fusarium solani* and *Fusarium verticillioides*). Thus, we consider this fungal isolate as a promising tool for the development of an efficient bio-fertilizer for the plant which has the potential to protect plants from pathogens especially fusarium species.

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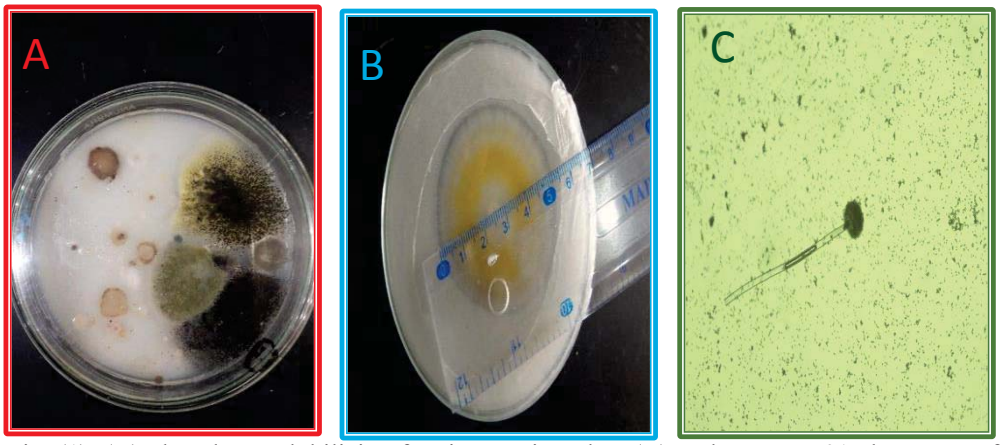

Fig. (1): (A) phosphate solubilizing fungi screening plate (B) Isolate AG-A01 clear zone of dissolved phosphate in solid Pikovskaya's medium (Picture from the back of the plate), (C) Microscopic Examination of fungal isolate.

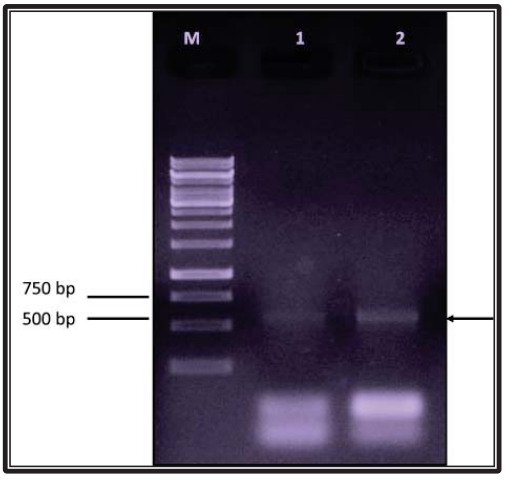

Fig. (2): PCR product of Fungi ITS region Lane 1 and 2 are the same.

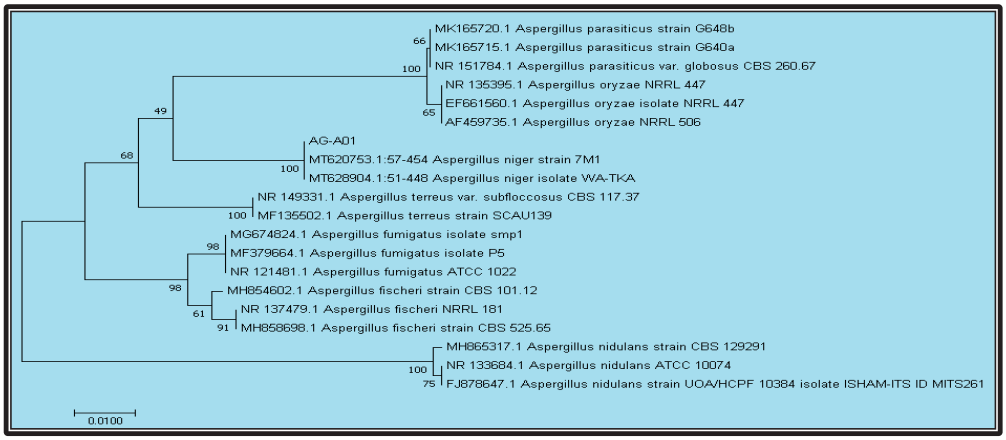

Fig. (3): Phylogenetic tree for ITS sequence of isolate AG-A01. The NJ phylogram was inferred from partial ITS sequence data. Bootstrap percentages of >70% derived from 1000 replicates are indicated at the nodes. Bar = 0.0050 substitutions per nucleotide position.

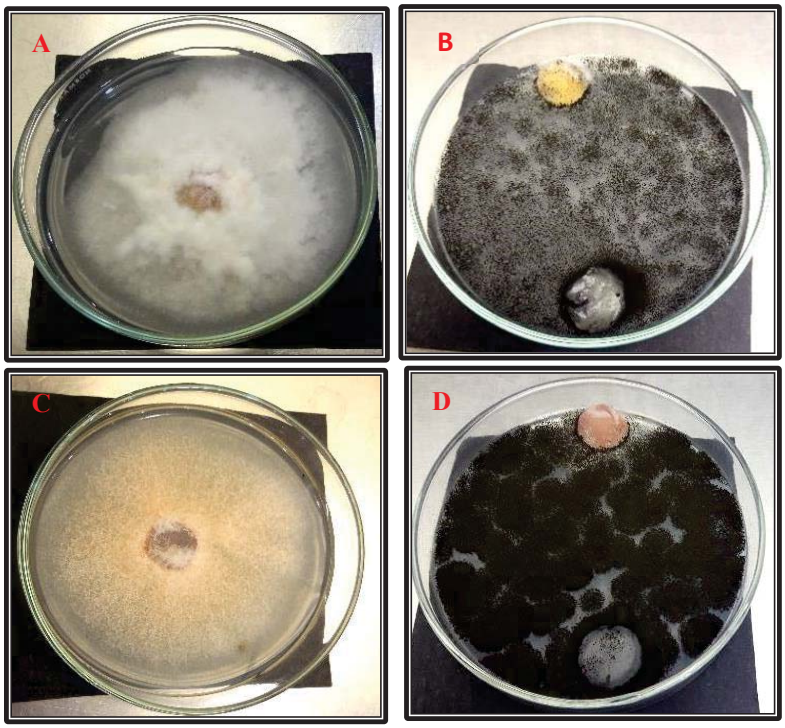

Fig. (4): Dual culture assay. (A) *Fusarium solani* growing on PDA (B) *Fusarium solani* and *Aspergillus niger* growing on PDA (C) *Fusarium verticillioides* growing on PDA (D) *Fusarium solani* and *Aspergillus niger* growing on PDA

AN APPROACH FOR IMPROVING STEVIOL GLYCOSIDES AND PRODUCTIVITY OF *stevia rebaudiana In vitro*

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Stevia rebaudiana is an important source of active natural compounds, especially steviol glycosides that are present mainly in the leaves (Modi and Kumar, 2018). Recently, stevia leaves has attracted economic interest as a new promising sweet-tasting calorie-free botanical food stuffs on the world market for their content of protein, antioxidant compounds, vitamins ,minerals, pigments, inorganic constituents and steviol glycosides that used as a sugar substitute or dietary supplements (Singh *et al.,* 2019). It has become well known not only for its sweetness but also for offering to biological properties and therapeutic benefits, where have shown activity in cancer prevention, as well as anti-diabetic, anti-obesity, anti-hyperglycemic, anti-hypertensive, anti-inflammatory, antibacterial or immunomodulatory, anti-diarrheal, and diuretic effect (Tadhani and Subhash 2006; Barriocanal *et al*., 2008 and Bernal *et al*., 2011). The leaves contain several steviol glycosides, which differ in chemical structure characteristics and sweetening properties up to 250-300 times sweetening potency that of sucrose. The major constituent being stevioside and rebaudioside A are constituting about 50% of the total glycosides and other minor amounts of rebaudiosides B, C, D, E, F, dulcoside A and steviolbioside (Bergs *et al*., 2012 and Prakash *et al*., 2014).

Hence, there is a need to replace sugar with low-calorie stevia sweeteners as a natural plant source which will improve of the products nutritional and the cultivation of *S. rebaudiana* Bertoni has successfully expanded to several countries (Sičžlabur *et al*., 2013 and Singh *et al*., 2019). Therefore, *in vitro* propagation or plant tissue culture appears as an alternative technique for rapid multiplication of stevia within a short period of time and can be improved a quality and quantity of planting (Das *et al*., 2005). Also, a large number of genetically similar or genetic improvement and disease-free plantlets can be obtained with higher levels of the sweetening compounds, larger foliar mass, and resistance to drought through direct organogenesis of *in vitro* propagation (Dhananjay and Deshpande, 2005).

Stevia rebaudiana can be propagated by two ways seeds and stem cuttings. But, seeds propagation have many problems like very small in size, low fer-

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Web Site (*www.esg.net.eg*)

tility, self-incompatibility, poor germination and heterogeneity, which subsequently change the level and structure of steviol glycoside (Sivaram and Mukundan, 2003 and Abdullateef *et al*., 2012). Also, propagation by stem cutting has many problems like low frequency of shoot occurrence and physical damage of the mother plant (Yadav *et al*., 2011). Therefore, *in vitro* culture was perfect useful tool for *S. rebaudiana* mass clonal propagation (Ramírez-Mosqueda and Iglesias-Andreu, 2015). Plant tissue culture as an important tool for the continuous production of active compounds including secondary metabolites and engineered molecules (Kadhimi *et al*., 2014). Similarly, in order to obtain homogeneous and highly vigorous plants in a short time, plant scientists are concentrating on developing optimal protocols for stevia species with industrial and medicinal importance. This can reduce the time, costs, rapid production, and scalability compared to those offered by alternative sources. The morphogenesis response seems to be highly dependent plant growth regulators used in the media, which is again cultivar and genotypic specific.

However, many studies examining the development of optimum protocols for the propagation in a short period (Depuydt and Hardtke, 2011; Razak *et al*., 2014 and Blinstrubienė *et al*., 2020) and total phenolic content and antioxidant activities (Roberts and Renwick, 2008*),* of *S. rebaudiana in vitro.* Also, some studies are focusing on the steviol glycosides content under the optimal light *in vitro* grown *S. rebaudiana* plants (Bondarev *et al*., 2001; and Ladygin *et al*., 2008) to improve physiology and medicinal properties in leaves. As the Joint FAO/WHO Expert Committee on Food Additive (JECFA) and Food and Drug Administration have approved rebaudioside-A with purity >95% and accepted daily intake (ADI) of stevioside up to 5.0 mg kg-1 body weight. Further research and development need to be carried out to improved stevia varieties with higher yield and quality through plant breeding methods and biotechnological approaches The purpose of this research was established an efficient regeneration protocol for enhance of steviol glycoside compounds as quality with improving productivity of stevia yield and evaluate the possible suitability of this technique to be applied to rapid production of selected plants.

MATERIAL AND METHODS

Explant source

Stevia rebaudiana plant namely Spanti was obtained from Sugar Crops Research Institute, Agricultural research center, (ARC). Under aseptic conditions in laminar airflow cabinet at Gene Transfer Laboratory (GTL), Agriculture Genetic Engineering Research Institute (AGERI), healthy young plants were collected from green house and washed under running tap water for 15 minutes. Thereafter, the explants were sterilized with 70 % (v/v) ethanol alcohol for a few second and then by 5% Clorox for 5 min. Explants were washed with sterile distilled water three

times, followed by 0.1% (w/v) mercuric chloride (HgC_{l2}) for another 5 minutes and rinsed with sterile distilled water three times.

***In vitro* studies**

The young leaves and hypocotyl as explants were cut into pieces of 2x2 cm then cultured on different MS medium on three stages to select the best media for callus, shoot and root formation. These stages were as follows:

1. **Callus induction stage**

The explants were cultured on MS medium (Murashige and Skoog, 1962) containing different concentration and combination of plant growth regulators (PGRs) Naphthaleneacetic acid (NAA), 2,4-Dicholorophenoxy acetic acid (2,4-D) and 6- Benzylaminopurine (BAP) as specified in Table (1). All media were with sucrose (30 gm/l) and solidified with agar (8 gm/1) then adjusted pH value to 5.75±0.05 before autoclaving at 121ºC for 20 min. Parameters callus biomass was investigated as described by Nosov (2011) on intervals for period of 30 days after culturing and incubating in growth chamber at 27±2ºC. The experiments were performed in triplicate.

2. **Differentiation shoot stage**

Callus was transferred on MS medium containing different concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5 or 3 mg/l)

3. **Root formation stage**

Differentiated shoots were transferred to MS supplemented with 1.0 mg/l of indole-3-butyric acid (IBA).

Effects of light and dark on productively and quality callus

From the above biomass, the best media were selected to be applied to get healthy callus with steviol glycosides. The cultures were divided into two groups Group was maintained under 18hrs light (18 hrs light/6 dark) and other group under dark conditions.

Extraction of steviol glycosides

One gram of callus from each culture medium was macerated in three times 20 ml of MeoH 80% using shaker (Type U850. US). Each maceration stage was constant for 30 minutes for complete extraction. Macerates were filtered and collected then completed to known volume with 80% MeoH. The extract was passed through active charcoal for clarifying and kept until further analysis.

Evaluate of total steviol glycoside

Steviol glycosides were analyzed according to (Vanek *et al*., 2001) using high-performance liquid chromatography (Agilent 1200, Agilent, Germany). The extract of steviol glycosides was separated on a reversed phase an Zorbax (NH_2) column (4.6x250 mm, 5 µm) with a mobile phase consisting of acetonitrile: water (84 : 70 v/v), pH 2.7 adjusted with H_3PO_4, at a flow rate 2 ml/min with ambient temperature (25ºC). Steviol glycosides were de-

tected at the wavelength of UV detector, 210 nm and compared with those obtained for pure standards of stevioside (ST), rebaudioside A (RA) and steviolbioside (stb) by means of retention time.

Acclimatization stage

Initially rooted plantlets were well washed in distilled water to remove the agar after pulled out gradually from the media. After sufficient roots were development, plantlets were subjected to hardening using two strategies, i.e. plastic pots with glass cover or Hoagland solution technique (hydroponic culture).

Hydroponic culture

As described by Hoagland and Arnon (1950), plants were transferred to Hoagland solution culture (company name). Topsin (0.5gm/l) was the fungicide that used to prevent fungal disses and air pump used for gas exchange.

Adaptation in soil

It is important to mentioned two important points, the first one that stevia plants which transplanted to a pots covered either with glass or plastic cover to keep humidity, and the second that acclimatization stage placed in growth room and then acclimatized plants transfer to green house after three weeks. *Stevia* plantlets were acclimatized by culturing plantlets in pots contain beat moss, sand and perlite (1:1:1). Topsin was added immediately after planting and regularly irrigated with topsine. Disper root® in a concentration of 0.5gm/l was used to stimulate root development. Plants were covered either by plastic phytacone or glass jars and incubated at growth room at 28ºC for one month.

Statistical Analysis

The treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% probability level according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Production of stevia is performed by stem cuttings which requires high laboratory potential or seeds that have a weak germination rate which limit the large-scale cultivation of stevia (Goettemoeller and Ching, 1999). Therefore, tissue culture is an alternative way for solving the limitation of stevia propagation. *In vitro* micro propagation from shoot tip and leaf explant has been reported (Uddin *et al*., 2006). Murmu *et al*., (2016) developed an appropriate and faster protocol for *Stevia rebaudiana* Bertoni clonal propagation through callus and shoot culture. Taak *et al*., (2020) optimize the best PGRs combination and concentration for shoot-induction, shoot-elongation and root-induction for stevia plant.

Callus formation

Two explants i.e., young leaves and hypocotyl segments of stevia were examined for their callus formation and proliferation ability on five different media No.1-5 (Table.1) Results showed that there were significant differences among all media with control; except medium contain only 2,4-D which young leaf explants failed to form any callus, while, the maximum callus initiation (100%) obtained from young leaf explants on 0.5

mg/l NAA. The lowest percentage of callus initiation (zero and 47.5%) obtained from hormone free medium and medium with 0.5 mg/l 2,4-D and 1.5 mg/l NAA, respectively (Fig.1a). Mathur and Shekhawat (2013) reported that development of callus didn't occur on medium without growth regulators (PGRs). While, Keshvari, *et al.,* (2018) showed that control without PGRs, had little calli which indicating the high reaction of stevia explants for callus production under aseptic *in vitro* conditions. In the case of callus developed from hypocotyl explants, the best callus formation (92.5%), was on media containing 2,4-D and NAA at concentration of 1.0 and 2.0 mg/l respectively. However, MS medium with only 2,4-D gave the lowest percentage of callus culture. (Fig.1b). Callus induction from hypocotyl explant reveled low response (30%) on the higher concentrations of 2,4-D (2 mg/l) and in the absence of NAA.

On the other hand to enhance callus formation from young leaf explants three other media were examined (No. 6-8, Table.1) in addition to the previous five media. Results showed that the young leaf explants couldn't develop callus in the absence of NAA. Medium containing 1.0 mg/l NAA revealed 95% regeneration efficiency and highest weight of callus (0.35gm) followed by medium with 1mg /l BAP and 1 mg /l 2,4-D (0.16 gm) after 4 weeks. Statistical analysis confirmed significant differences among all media under study (Fig. 2). Metry *et al.,* (2003) were established *in vitro* propagation system for *S. rebaudiana* using nodal cutting as an explant and MS medium supplemented with different combinations of PGRs. They found that callus formation obtained in the presence of auxin either NAA with BA or IAA with BA. Abd El-Motaleb *et al*., (2015) cultured leaves and nodal segment explants of *S. rebaudiana* plant on MS medium supplemented with different concentrations of 2,4-D (0.0, 0.5, 1.0, 2.0 and 4.0 mg/l), they found that the highest amount of callus was found on medium with 2.0 mg/l 2,4-D while, medium with 4.0 mg/l 2,4-D given the poorest callus, indicating that the higher concentration was not appropriate for callus initiation. Murmu *et al*. (2016) found that the best calluses induction on medium contain 2,4-D and NAA (100%) was (0.5 +1.5 mg/l) when using young, leaf and nodal segments explant. Taak *et al*., (2020) found that callus formation and maximum callus induction was achieved on medium containing 2 mg/l 2,4-D and 1 mg/l Kinetin with leaf disc explants as 80% of callus formation was achieved with leaf segments comparing to 60% with nodal, they also found that leaf discs were the most efficient for callus formation comparing with shoot tips segments.

Differentiation shoot stage

Obtained callus from different explants were cultured on different concentration of BAP in order to differentiate into shoots. Callus developed from hypocotyl failed to differentiate, while callus resulting from young leaf explants succeeded in differentiate and giving multiple shoots. Medium with 2.5 mg/l BAP re-

vealed highest percentage of callus differentiation (24%) among all treatments (Fig. 3), it was also the best in terms of the number of branches resulting from the callus. This data is agreement with the result has been previously obtained by Murmu *et al*., (2016) who found that optimal stevia shoot initiation was observed on MS medium supplemented with (0.5-2.0 mg/l) BAP.

Root formation

The obtained shoots were then transferred to MS medium with 1.0 mg/l IBA for root formation, all shoots were produced roots. Metry *et al*., (2003) found that MS medium supplemented with 0.01 mg/l IBA with 10 mg/l GA3 and STS was the best medium for stevia root formation. Verma *et al*., (2011) found that higher concentration of IBA (2.0 mg/l) gave highest root formation (69.76%) and early root initiation (7.1 days). Noordin *et al*., (2012) illustrated that increasing of IBA concentration gradually decreased root induction as 0.25 mg/l IBA showed the best effect in promoting root formation comparing with different concentrations of IBA ranging from 0, 0.25, 0.5, 1.0 and 1.25 mg/l. Our results is consistent with the results of Abdul Razak *et al*., (2014) and Murmu *et al*., (2016) that showed 1.0 mg/l IBA was the superior concentration for root induction in stevia plant. Moreover, Attaya (2017) showed that the optimum root growth root growth with (76.6 %) root formation was observed using 1.0 mg/l IBA on half strength MS medium. Regeneration steps start from the callus formation until reaching the root formation stage was illustrated in Fig. (4).

Identification and quantitative analysis of steviol glycoside compounds by HPLC in stevia leaves extract.

In this investigation, the different concentrations and combination of NAA, 2,4-D and BAP have positive effect on total biomass and steviol glycoside content in *S. rebaudiana cv. Spanti in vitro*. The combination with NAA, or BAP and 2,4-D effects in MS medium on steviol glycoside compounds in stevia callus under *in vitro* condition determined with HPLC analysis. The concentration of steviol glycosides were calculated as percent of total area while the concentration of stevioside (ST) and rebaudioside A (RA) were calculated as weigh percent of the callus. Results showed that the steviol glycosides were affected by both type and concentration of hormone in the medium. It can be concluded that steviol glycoside in supplemented medium were higher than that in mother leaves or MS free hormone (Fig. 5 and Table 2). The average ST and RA values in mother leaves were lower than in other media. This result is in agreement with Hsing *et al*., (1983), who reported that stevioside content in callus was 16.24% which it was two and four times higher than that in the leaf and flowers of the same plant. However, the steviol glycoside content in callus cultures might even be twice as high as in stevia leaves (Luwańska *et al.,* 2015 and Pandey *et al.,* 2016). According to Nazishb *et al.,* (2013), the addition of BA alone into the

MS-medium influences the steviosides content (30.89 µg/g-DW to 63.77 µg/g-DW). The range of stevioside content was from 8.38 to 49.21 mg /g the highest was in callus cultured on MS medium supplemented with 1 mg/l NAA followed by MS medium supplemented with 0.5 mg/l BAP and 1.5 mg/l 2,4-D and similar to that of MS medium supplemented with 1 mg/l BAP and 1 mg/l 2,4-D (Fig. 5). Such increase may be attributed to the combination of phytohormones *in vitro* culture. The studies of Bondarev *et al.,* (2003), Luwańska *et al.,* (2015) and Pazuki *et al.,* (2019) indicated that the presence of PGRs improve and regulate biochemical processes in plant, provide accurate growth and cell division *in vitro* culture and influenced on the amount of steviol glycoside (SvGls) profile when the addition of combination of these PGRs in media. Rebaudioside A as a high potency sweetener has a better quality of sweetness than stevioside which has a bitter aftertaste character that makes it difficult to use in many foods. Stevioside is 140 times sweeter than sucrose, while rebaudioside is 240 times sweeter (Prakash *et al*., 2008). It can be noticed that the a higher RA content (24.22 mg/gm) was obtained in MS medium containing of 1 mg/l BAP and 1 mg/l 2,4-D. The biosynthesis of steviol glycosides in stevia cell cultures depends to greater extent on genotypic of cell strain and composition or concentration of components of the nutrient medium (Bondarev *et al*., 2019 and Blinstrubienė *et al.,* 2020). It can be concluded that the differential of steviol glycoside concentration under all applied conditions may be related to metabolic shift to the methylerythritol phosphate pathway (MEP) in order to synthesize the chlorophyll phytophilic chain and then production of steviol glycosides, starting from the induction of ent-kaurenoic acid hydroxylase (KAH) activity in green stevia roots (Libik-Konieczny *et al.,* 2020). Blinstrubienė *et al.,* (2020) recorded variations in the steviol glycosides, bioactive compounds and antioxidant activity of the cellular compounds of callus from the leaf and stem explants when they studied that addition 2.0 µM NAA and 5.0 µM proline in media on their yield and quality characteristics. The literature also provides *In vitro* plant tissue culture offers a probability of generating plant material containing a great level of stable selected secondary metabolites as SGs (Ramakrishna and Ravishankar 2011; Hussain *et al.,* 2012 and Konieczny *et al*., 2020). Also, an increase in RA/ST ratio was summarized in Fig. (5). Tabulated data indicated that the RA / ST ratio was increased with increasing BAP concentration from 0.5 to 1mg/l in MS medium to 0.856 and 1.33 respectively and the lowest rate (0.12) was recorded in mother leaves while MS medium containing1mg/l NAA was recorded 0.435 .Development of *S. rebaudiana* variety with a higher of RA and a reduced stevioside is the main aim of plant breeders for the improvement and utilization of this source of natural sweeteners. Therefore, it can be selected to investigate the effects of photoperiod on steviol glycoside compounds in MS medium containing 1mg/l BA and 1mg/l 2,4-D and MS medium containing 1mg/l NAA.

Effect of photoperiod (light: dark) on steviol glycoside compounds

As the objective of the present study was to improve the quality of leaves, it was necessary to study the effect of photoperiod for 18 hrs light and 24 hrs dark on steviol glycoside compounds. From the suggested combination media two were selected to be exposed to 18 hrs light and 24 hrs dark photoperiod. Differences in steviol glycosides percent have been shown in Table (2) and Figs. (6 and 7). Light encouraged on production of high amount and different steviol glycosides component in callus. MS medium supplemented with 1 mg/l BAP and 1mg / l 2,4-D was more appropriate medium than MS for stevia callus production. Stevioside concentration as area % was considerably higher in MS medium supplemented with 1mg/l NAA under dark (86.76%), compared to the MS medium containing 1 mg/l BAP and 1mg /l 2,4-D (52.94%). Medium under light observed increase in area % of rebaudioside C , A and E than in under dark which showed more intense lingering sweetness than sucrose (Tao and Cho, 2020). Similar patterns of steviol glycoside increase for steviolbioside RA, and RE were found in MS medium supplemented with 1 mg/l BAP and 1mg /l 2,4-D (Fig.7) than 1mg/l NAA (Fig. 6). Light with an intensity of 8 Wt m-2 at 25 C and about 70% air humidity was shown to activate the SGs in callus about two times higher of SGs (Yoneda *et al*., 2018 and Bondarev *et al*., 2019). Libik-Konieczny *et al*., (2020) stated that culture conditions and the type of stress factor led to changes in the morphology and an increase in oxidative stress manifested as an enhancement in endogenous hydrogen peroxide concentration in the cultured samples under light or in the medium with the highest osmotic potential as well as the increase in the expression level of ent-kaurenoic acid hydroxylase, responsible for the redirection of metabolic route to steviol glygoside biosynthesis pathway. The approved measure of sweetness quality is the ratio of RA/ ST. Under the photoperiod, this media gave high RA /ST ratio in light (1.33) than in dark (0.94). Medium MS containing BAP at 1 mg/l and 2,4-D at 1 mg/l under 18 hrs light gave good results in composition and rates of steviol glycoside. Furthermore, the sweetness intensity rate was found to be about 1.43 times sweeter than MS medium containing NAA at 1mg/l as sucrose equivalent that it had the highest weight of callus.

3-Acclimatization stage

Two methods have been tested, and the rooted shoots were transferred into aquarium containing Hoagland solution and/or in pots containing soil. The plantlets that acclimatized under Hoagland solution couldn’t survive. On the other hand, the acclimatization under soil condition was successes as the plantlets survived with a percentage of 64%. It was observed that the thickness of stem played a role in acclimatization success. Results showed that the glass cover is better than plastic one as all plantlets under plastic

cover are died, while, the plantlets under glass cover survived (Fig. 8). Metry *et al*., (2003) illustrated that the percentage of stevia survived plantlets ranged from 20 to 80% and sand-peat moss was the best potting medium at 50% (v/v). Verma *et al*., (2011), optimize a protocol for *Stevia rebaudiana* Bertoni regeneration and acclimatization. They found that transfer plantlets to glass jar with polypropylene (PP) caps were better that plastic pots with polythene cover, as plantlets with glass jar appropriate relative humidity with high success during acclimatization. Razak *et al*., (2014) illustrate that during acclimatization, transparent polythene can improve the survival plants as it control the humidity and can help in adaptation of the plants into new environment. They also showed that the plastic covering was essential for the successful plant acclimatization.

Conclusion

An efficient regeneration protocol for *S. Rebaudiana* (Spanti) was established in this study. From the aforementioned results, it can be concluded that young leaves as explants, for callus induction, were better than hypocotyl. BAP with concentration 2.5 mg/l was best condition for stevia regeneration and 1.0 mg/l IBA was the best for successful rooting. Medium supplemented with 2,4-D and BAP gave good results in steviol glycoside components and increased in rebaudioside A content with high rate RA/ST. MS media supplement with NAA(1mg/l) was the best for callus weight, acclimatization and adaptation in soil**.**

SUMMARY

Nowadays, *Stevia rebaudiana* leaves have attracted economic interest as a natural non-nutritive, non-toxic, high-intensity sweeteners as well as its therapeutic benefits. The aim of the study was established an efficient regeneration protocol to improve stevia's sweetness with enhancing productivity of stevia leaves as well as to evaluate the possible suitability of this technique to be applied to rapid production. The young leaves and hypocotyl of *Stevia rebaudiana* namely Spanti explants were cultured on a MS medium supplemented with different concentrations and combination of NAA, 2,4-D and BAP as plant growth regulators. The results showed that leaf explants obtained highest callus formation (100%) on MS medium supplemented with 0.5 mg/l NAA while callus formation from hypocotyl explants was (92.5%) on MS medium contains 1.0 mg/l 2,4-D and 2.0 mg/l NAA. Callus derived hypocotyl failed to differentiate, while callus resulting from young leaf explants were differentiated into shoots on MS medium supplemented with 2.5 mg/l BAP. MS containing 1 mg/l BAP and1 mg/l 2,4D showed best results in steviol glycoside components and high rebaudioside A (RA) (24.22 mg/g callus) yield comparing with other used media under inoculation for 18 hrs light. Also this media gave the high rate of RA over stevioside (1.33) as an indicator of leaves quality with the sweetness intensity rate 1.43 times sweeter than MS medium containing 1mg/l NAA as sucrose equivalent

under the same experimental conditions. Medium with 1.0 mg/l IBA was suitable for rooting stevia shoots. Plantlets were acclimatized by 64% in the soil contain beat moss, sand and perlite (1:1:1) under controlled chamber at 28°C for one month which transferred to the green house.

Key words: Non-nutritive, callus formation, hypocotyl explants, steviol glycoside.

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Table (1): Different concentration and combination of PGRs for callus induction.

Medium No.	MS	NAA mg/l	BAP mg/l	2,4-D mg/l
Control	+	-	-	-
1	+	0.5	-	-
2	+	-	-	2.0
3	+	1.0	-	1.0
4	+	2.0	-	1.0
5	+	1.5	-	0.5
6	+	1.0	-	-
7	+	-	1.0	1.0
8	+	-	0.5	1.5

Table (2): Steviol glycoside compounds identified by HPLC in stevia callus and fresh leaves.

Compounds Concentration as Area%	Fresh leaves	Media				
		MS	MS + 1mg/l NAA		MS + 1 mg/l BA+ 1mg /l 2,4-D	
		Light	Light	Dark	Light	Dark
Steviolbioside	-	5.86	-	-	11.43	0
Stevioside	45.09	91.03	36.91	86.76	18.54	52.94
Rebaudioside C	33.00	-	40.05	-	0	22.39
Rebaudioside A	3.11	-	9.03	5.61	28.13	2.40
Rebaudioside E	4.60	-	4.51	6.64	22.81	-
Unkown coumpounds	3.86	3.860	2.12	-	15.37	-

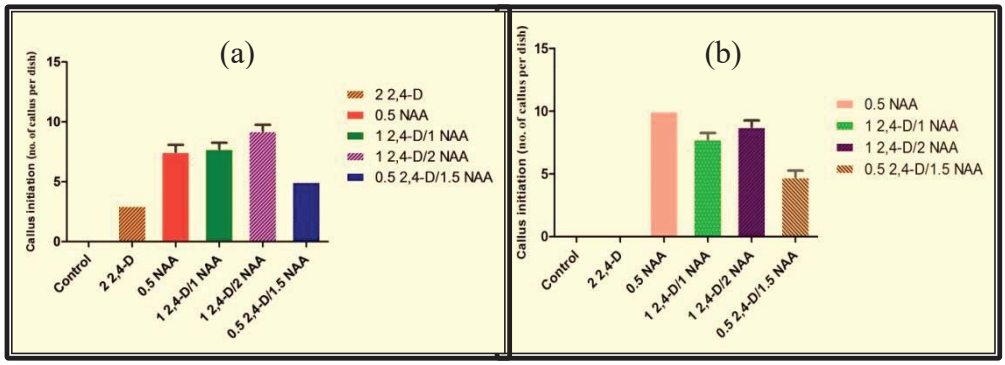

Fig. (1): Callus initiation from different plant growth regulators (a): leaf explants (b): hypocotyl explants.

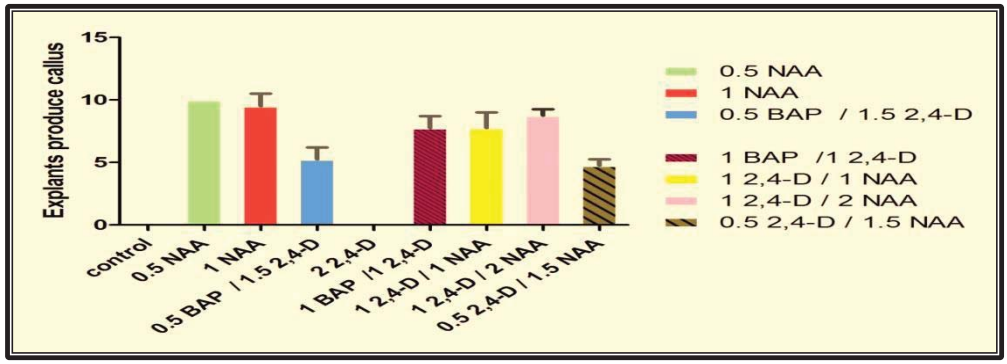

Fig. (2): Callus initiation using different growth regulators and young leaf explant.

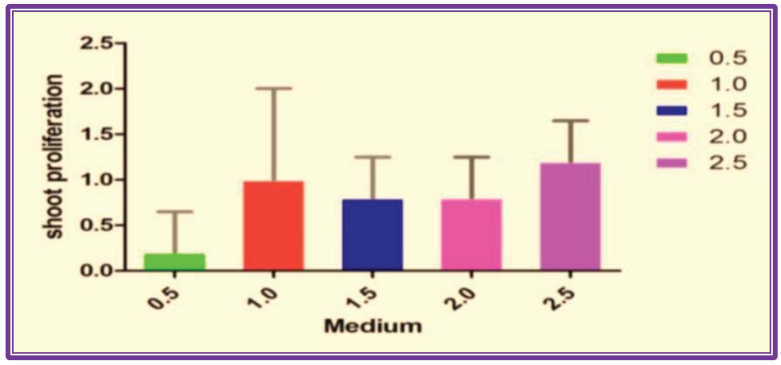

Fig. (3): Effect of BAP (mg/l) on shoot proliferation from calli explant regenerated from young leaf.

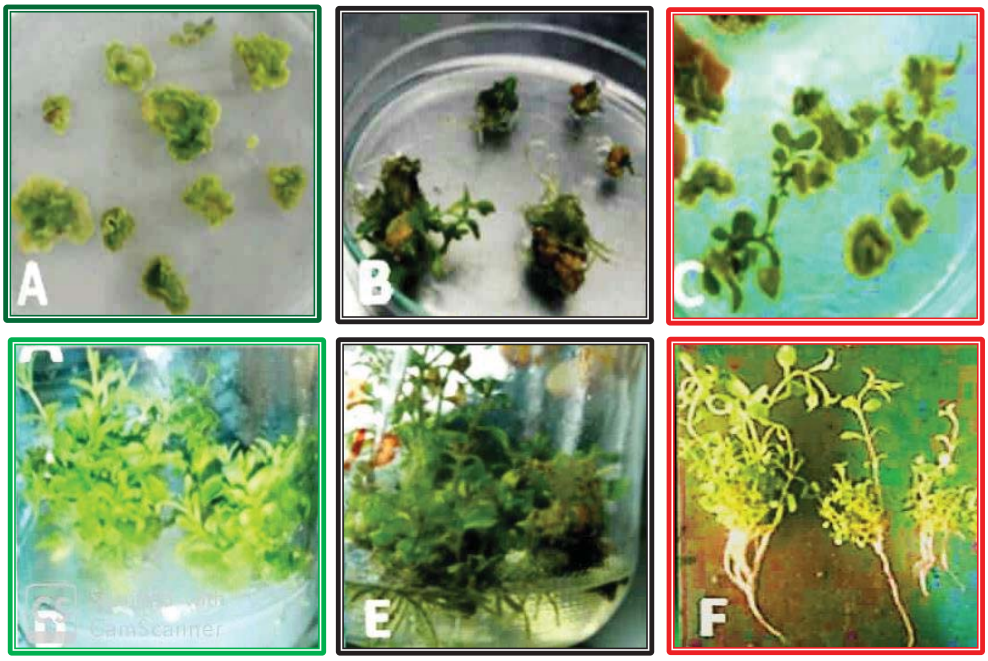

Fig. (4): Regeneration of *Stevia rebaudiana*.
A: Callus production after 3 weeks of leaf explants on MS medium with 1 mg/l NAA
B & C: Differentiated callus into shoot formation
D :Shoots regeneration from calli explant on MS supplemented with 2.5 mg/l BAP,
E :Root formation on MS with 1.0 mg/l IBA,
F :Will rooted stevia plants

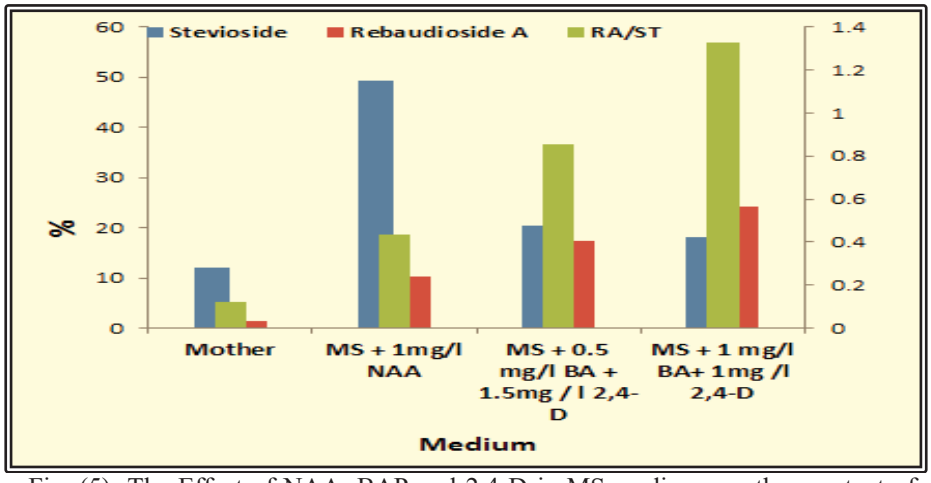

Fig. (5): The Effect of NAA, BAP and 2,4-D in MS medium on the content of stevioside(ST) ,rabudiosideA(RA) and RA/ST rate compounds.

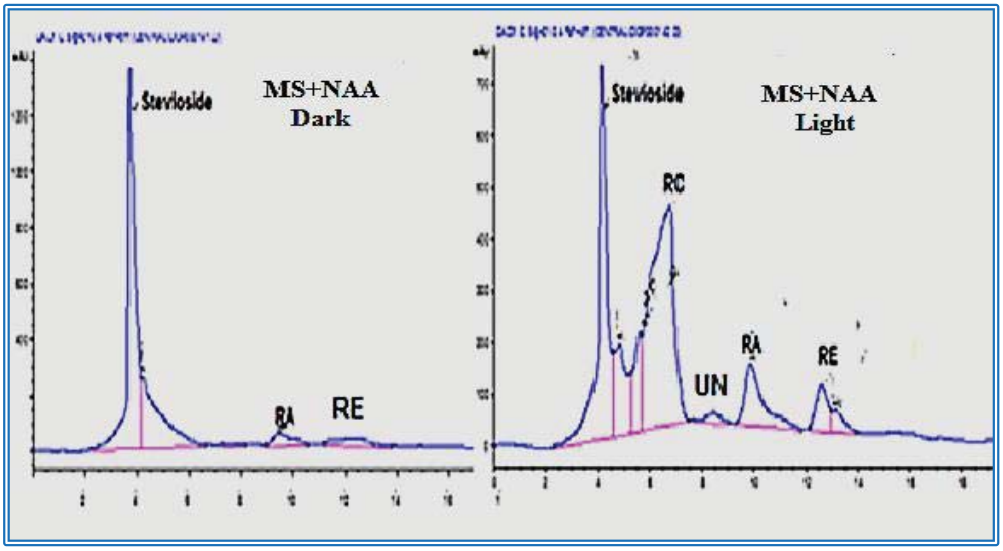

Fig (6): HPLC analysis of the steviol glycoside compounds in cultured callus on MS containing naphthaleneacetic acid (NAA 1mg/l).

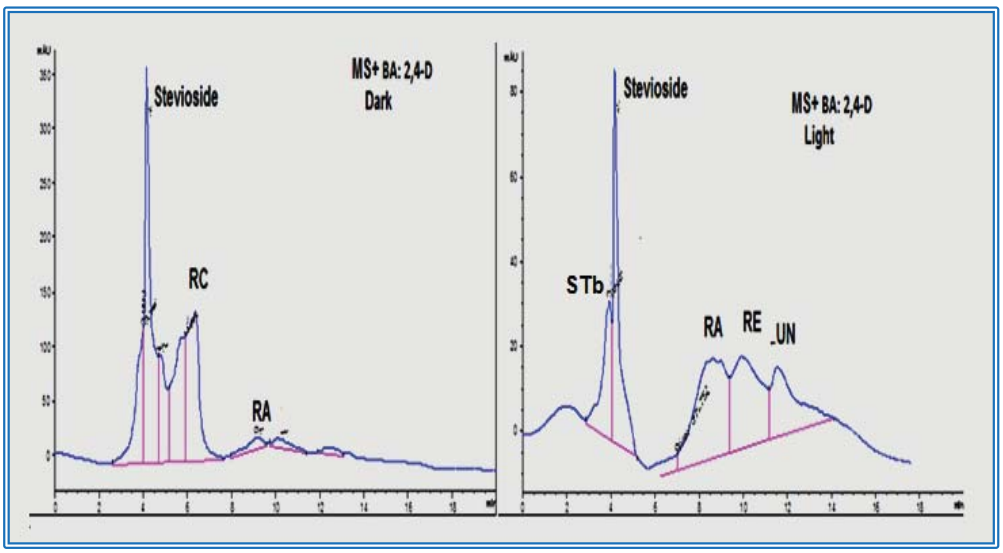

Fig .(7): HPLCanalysis of the steviol glycoside compounds in cultured callus on MS containing1mg/l benzylaminopurine (BAP):1mg/l 2,4-Dicholorophenoxyacetic acid (2,4-D).

Fig. (8): Acclimatization stages for stevia plantlets under soil condition.
A: stevia plantlets with glass cover under growth room conditions,
B&C: stevia plantlets will survive when transplant to green house after three weeks.
D&E&F: well acclimatized stevia plant in green house.

DNA BARCODING OF JOJOBA (*Simmondsia chinensis*) PLANTS CULTIVATED IN EGYPT USING *rbcL* GENE

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Jojoba (*Simmondsia chinensis*) is a dioecious shrub, native to the southwestern United States and northern Mexico, it is a nontraditional crop that can grow in arid and semi-arid areas, in addition, it is naturally adapted to saline soils and high-temperature environments (Al-Ani *et al*., 1972). Jojoba cultivated mainly for its seed oil, which contains liquid wax very similar to whale sperm in value. Seed oil is highly valued for their use in a wide range of pharmaceutical industry, also as biodegradable lubricants in the motor industry and as a biofuel product (Aburjai and Natsheh 2003; Baldwin, 1988). The use of jojoba in biofuel production has many advantages because the plant is non-edible, unlike corn or sugarcane, and can be irrigated with low quality water and cultivated in areas with high levels of drought, salinity and heat. The nature of the environment and weather in Egypt is suitable and encouraging for jojoba plant (Borlaug, 1985). Indeed, one of the main corners of the Egyptian economy is Agriculture; therefore, improving agricultural systems and irrigation strategies must be of great concern. Because of the above-mentioned reasons, the increase of jojoba cultivation is recommended. The identification and differentiation of male and female jojoba plants are very crucial during cultivation. Therefore, the use of traditional methods based on morphological criteria as well as the molecular methods is very important approaches. DNA barcoding is a tool for species identification that amplify a specific region of DNA and sequencing to create a global database of living organisms (Hebert *et al*., 2003). CBOL Plant Working Group (Consortium for the Barcode of Life: www.barcoding.si.edu) proposed portions of two coding regions from chloroplast genome (*rbcL* and *matK*) as a "core barcode" for plants in 2009 and to be supplemented with additional regions as required. In recent years DNA barcoding has been developing rapidly for a wide range of applications. Four plant DNA barcode markers, *rbcL* (Ribulose bisphosphate carboxylase), *matK* (MaturaseK),

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trnH-psbA (intergenic spacer region) and *ITS2* (internal transcribed spacer 2), have been developed and used. Most plant DNA barcodes are located in the chloroplast genome, either within coding sequences (such as *rbcL* and *matK*) or in intergenic regions (such as *trnH-psbA*) (Group, *et al*., 2009). However, some nuclear loci have been used as DNA barcodes, such as the internal transcribed spacer of the ribosomal DNA (*ITS*) (Bolson *et al*., 2015). Chase *et al*., (1993) employed *rbcL* as a marker for several early plant phylogenetic studies and soon this marker became popular in that field. The sequence analysis resulted from the studies of *rbcL* gene marker produced a large sequence database. Therefore, *rbcL* became an attractive candidate for molecular identification of plant species. Our aim here is to identify jojoba plants using *rbcL* DNA barcode.

MATERIALS AND METHODS

Plant materials

The analysis involved a number of one male and fifteen female plants (clones) growing at Go-green for Agricultural Investment and Development Company farm, Abo-Ghaleb, Giza, Egypt (GIADC).

DNA extraction, amplification and sequencing

Total DNA was extracted from fresh leaves using DNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. After purification of the DNA, *rbcL* DNA barcode region of chloroplast DNA was amplified *via* polymerase chain reactions (PCR). Primers used in the amplifications were

rbcLa-F:(5'ATGTCACCACAAACAGAGACTAAAGC-3') and rbcL724-R: (5'-TCGCATGTACCTGCAGTAGC-3').

For each PCR reaction, 1 µL (25 ng) of total DNA was included in a 50 µL reaction mixture containing 2 units Taq DNA polymerase (GoTaq, Promega), 1X buffer, 20 µM each primer, 0.2 mM dNTPs and ultra-pure water to a final volume of 50 µL. Amplification of the target region was conducted with a thermal cycler (Applied Biosystems, USA) under the following conditions: initial denaturation at 95℃ for 2 min, 35 cycles of 95ºC for 30 s, 55℃ for 30 s, and 72ºC for 1 min, followed by a final extension at 72ºC for 5 min. PCR products were examined on a 1.5% agarose gel in 1× TBE buffer, then visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000, USA). After that the PCR fragments were purified using EZ-10 spin column (Biobasic, USA) for the purification of PCR products. The purified fragments were sent to Macrogen Europe B.V. company (Netherlands) for sequencing.

Data analysis

The obtained sequence data were adjusted manually as needed for each sequence. The sequence data generated in this study were analyzed separately in the

GenBank (www. https://www.ncbi.nlm.nih.gov) for similarity search using Nucleotide BLAST database. The *rbcL* sequences were matched with the query sequences and available *rbcL* sequences of the examined plant species available in the GenBank. After that DNA sequences were aligned using multiple sequence alignment by MAFFT (https://www.genome.jp/tools-bin/mafft). All sequences were adjusted manually and submitted to GenBank, USA. Phylogenetic analyses were conducted with the same database. The phylogenetic trees were inferred with the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993).

RESULTS AND DISCUSSION

Cultivation of jojoba plants have spread in many areas of the world (Benzioni *et al*., 2005) including the Middle East. Few studies have been made on comparative analysis at the molecular genetic level to detect genetic distances among genotypes existing in different regions. Therefore, these studies for jojoba plants are an important task. This study was performed to investigate the possibility of using rbcL DNA barcode for identifying jojoba plants. The amplification of rbcL gene fragment was conducted using rbcLa-F and rbcL724-R primers to amplify approximately 550 bp fragment. As shown in Fig. (1), the amplification of *rbcL* yielded PCR products for all the tested samples. Amplification and sequencing success rate are the most important criteria to evaluate DNA barcoding for plant identification (CBOL Plant Working Group *et al*., 2009). The success rates of amplification and sequencing of *rbcL* fragment were 100%. Our results showed higher universality and success rates similar to those of Ibrahim *et al*., (2019) who conducted rbcL barcode for the identification of different Quinoa genotypes. While, Pei *et al*., (2015) performed *rbcL* barcode with 90%-100% success rate in the forest plants. While other studies showed a success rates up to 90% (Kang *et al*., 2017; Huang *et al*., 2015) for *rbcL* in the tropical forest plants. This study confirms that *rbcL* universality as DNA barcode could be obtained with *rbcL* primers from a wide range of plant species (e.g., Lahaye *et al*., 2008; Parmentier *et al*., 2013). Different candidate gene regions were used as potential barcodes for plants including *rbcL*, *matK*, *trnH-psbA* and *ITS2* (Kress & Erickson, 2007; Taberlet *et al*., 2007). However, the most used are *rbcL* and *matK* for plant DNA barcode. In our study we have used *rbcL* because many authors indicated that the success rate of identification is more than *matK* (Amandita *et al*., 20019; Kang *et al*., 2017; Khidir and Lawrence 1999). Also the use of *matK* as a barcode has been less interest mainly because universal primers are not available (Bafeel *et al*., 2011; Dong *et al*., 2015). Furthermore, Hollingsworth *et al*., (2011) indicated that *matK* still needs optimization in regard to primer combinations and needs to be adapted to specific taxonomic groups. The obtained sequences of the 16 jojoba samples were matched with the reference sequences in BLASTn (Altschul

et al., 1990). The query sequences were identified up to species level with 98 or 100% in either of the algorithms for all samples. Table (1) summarize the obtained results. Samples 1-12 and 16 have the same identity 99 and 100% with *rbcL* gene from different jojoba plants submitted in GenBank. While samples No. 13 and 14 produced 99 % and 98-99% similarity, respectively. On the other hand, sample No. 15 produced 98-99% similarity. Our results are consistent with those obtained by Ghareb *et al*., (2020) who carried out DNA barcoding using *rbcL* and *matK* genes for *Phlomis aurea* plant. The drawing of a phylogenetic tree represents the best hypothesis about how a set of species or other groups evolved from a common ancestor and how they are related to each other. Based on multiple sequence alignment, a phylogenetic relationship of tested samples using *rbcL* was constructed. As shown in Fig. (2), the pattern of branching in a phylogenetic tree reflects how species or other groups evolved from a series of common ancestors. There are two main branches from the common ancestor for all the tested clones. The produced pattern indicated that samples1-12 and 16 have the same cluster with the same branch and very related to each other. Also sample 13 is related to the same group but with a different branch with the same branch point. The other branch containing samples 14 and 15 which are closely related to each other but less related to the other samples. These results are expected due to the tested samples were jojoba clone plants from the same cultivated area as indicated before. As shown in Table (2), the obtained DNA sequence data for all samples were submitted to GenBank for providing the accession numbers for the nucleotide sequences. Definitely the increase of using plant DNA barcode will enrich the database with new sequences which will establish more the use of this technique in a wide range for the identification of different plants. In conclusion, our results support the claim that DNA barcoding in general can provide fast and reliable species identification, especially for the economically important plants.

SUMMARY

Precise identification of jojoba plant species using DNA barcode is very important because of the economic value of its seed oil. In this study, we selected 16 jojoba trees (clones) including one male and 15 female trees cultivated in the same farm to conduct this experiment. DNA samples were extracted from leaf tissues and subjected to PCR amplification using specific primers for *rbcL* gene fragment. The amplification was successful for all tested samples producing the same fragment with a size about 550 bp. Sequence analysis was performed for the purified products and consequently subjected to GenBank database analysis. The data analysis produced by BLASTn database revealed that the similarity search was from 98-100% with *rbcL* gene region form available DNA sequence in GenBank. Multiple sequence alignment was performed after confirmation of the DNA

sequence. The alignment clearly indicated the high similarity between all DNA sequences for the tested samples. Furthermore, a phylogenetic tree was produced from the alignment process which revealed the close relationship between the tested samples. The tree can be divided into two main branches one containing samples 1-13 and 16 while the other branch contains samples 14 and 15. These results indicate that the *rbcL* region is a good option for molecular identification of jojoba species. Finally, the obtained sequence data were submitted to GenBank to provide the accession numbers for the nucleotide sequences.

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Table (1): The homologous sequences best matching the *rbcL* sequences based on the BLASTn analysis.

Description	Accession No.	Idententity %	E value
GIADC_1 -12 and 16			
Simmondsia chinensis voucher 14CS9341 (KUN) plastid, complete genome	NC_040935.1	100.00%	0
Simmondsia chinensis voucher Yi14353 (KUN) plastid, complete genome	MK397929.1	100.00%	0
Simmondsia chinensis voucher 14CS9341 (KUN) plastid, complete genome	MK397898.1	100.00%	0
Simmondsia chinensis voucher Hosam00080 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast	JX571893.1	100.00%	0
Simmondsia chinensis ribulose-1,5-bisphosphate carboxylase oxygenase, large subunit (*rbcL*) gene, partial cds; chloroplast gene for chloroplast product	AF093732.1	99.39%	0
GIADC_13			
Simmondsia chinensis voucher 14CS9341 (KUN) plastid, complete genome	NC_040935.1	99.79%	0
Simmondsia chinensis voucher Yi14353 (KUN) plastid, complete genome	MK397929.1	99.79%	0
Simmondsia chinensis voucher 14CS9341 (KUN) plastid, complete genome	MK397898.1	99.79%	0
Simmondsia chinensis voucher Hosam00080 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast	JX571893.1	99.79%	0
Simmondsia chinensis ribulose-1,5-bisphosphate carboxylase oxygenase, large subunit (*rbcL*) gene, partial cds; chloroplast gene for chloroplast product	AF093732.1	99.58%	0
GIADC_14			
Simmondsia chinensis voucher 14CS9341 (KUN) plastid, complete genome	NC_040935.1	99.60%	0
Simmondsia chinensis voucher Yi14353 (KUN) plastid, complete genome	MK397929.1	99.60%	0
Simmondsia chinensis voucher 14CS9341 (KUN) plastid, complete genome	MK397898.1	99.60%	0
Simmondsia chinensis voucher Hosam00080 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast	JX571893.1	99.60%	0
Simmondsia chinensis ribulose-1,5-bisphosphate carboxylase oxygenase, large subunit (*rbcL*) gene, partial cds; chloroplast gene for chloroplast product	AF093732.1	98.99%	0

Table (1): Cont''

GIADC_15			
Simmondsia chinensis voucher 14CS9341 (KUN) plastid, complete genome	NC_040935.1	99.80%	0
Simmondsia chinensis voucher Yi14353 (KUN) plastid, complete genome	MK397929.1	99.80%	0
Simmondsia chinensis voucher 14CS9341 (KUN) plastid, complete genome	MK397898.1	99.80%	0
Simmondsia chinensis voucher Hosam00080 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast	JX571893.1	99.80%	0
Simmondsia chinensis ribulose-1,5-bisphosphate carboxylase oxygenase, large subunit (*rbcL*) gene, partial cds; chloroplast gene for chloroplast product	AF093732.1	99.19%	0

Table (2): Sample I.D. and Accession numbers provided by GenBank after submission of the DNA sequences.

Sample I.D.	Accession No.
GIADC_1	MT895755
GIADC_2	MT895758
GIADC_3	MT895757
GIADC_4	MT895756
GIADC_5	MT895746
GIADC_6	MT895747
GIADC_7	MT895749
GIADC_8	MT895745
GIADC_9	MT895754
GIADC_10	MT895751
GIADC_11	MT895753
GIADC_12	MT895752
GIADC_13	MT895759
GIADC_14	MT895744
GIADC_15	MT895748
GIADC_16	MT895750

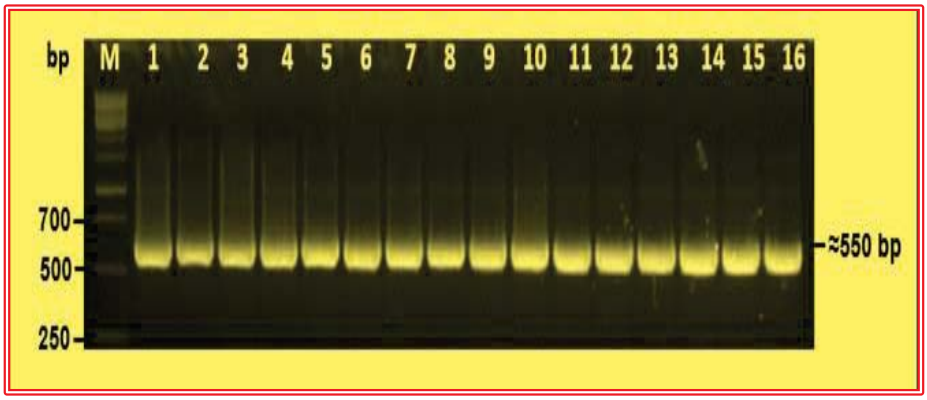

Fig.(1): PCR amplification of *rbcL* gene fragment for different jojoba plants. M: 1kb DNA ladder Marker. Lanes 1-16: Amplified fragments of *rbcL* gene for the tested samples.

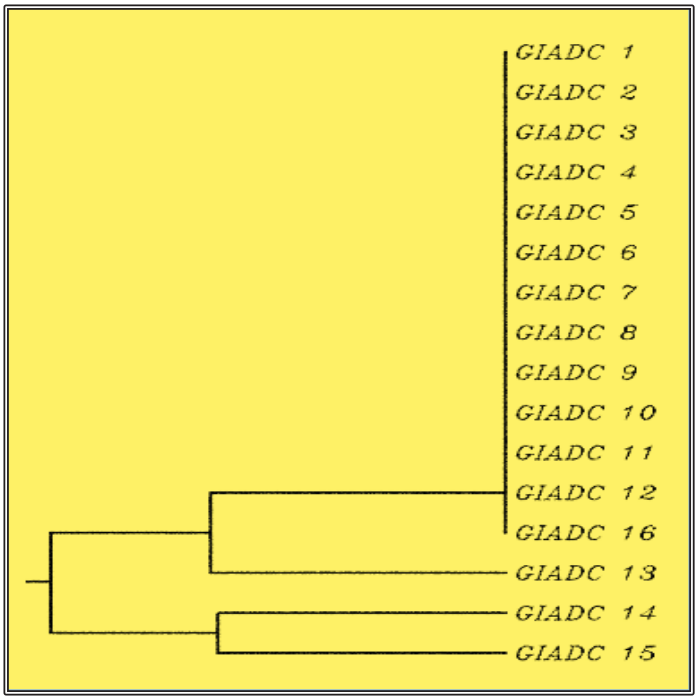

Fig. (2): Phylogenetic tree constructed by Neighbor joining method of the samples representing the selected jojoba plants based on *rbcL* gene sequences.

GENOTOXICITY OF ETHIDIUM BROMIDE IN ALBINO MICE TREATED WITH BACTERIAL PROBIOTIC

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Ethidium bromide (EtBr) is a synthetic chemical molecule of heterocyclic aromatic nature that follows the phenanthridine family. This drug can bind to nucleic acids through the intercalation process, in which the aromatic molecule is inserted between adjacent base pairs (Watkins, 1952). Genotoxicity of EtBr has been addressed in several studies, which confirmed that EtBr is a genotoxic agent, meaning that this substance can destroy DNA and RNA molecules, and many aspects of genetic toxicity. Interestingly, EtBr inhibited gene expression in *Escherichia coli;* this action has comforted its ability to bind to nuclear acids and act as nucleic acid- polymerases inhibitor (Waring, 1965).

Green Screen Assay, one of the genotoxicity tests, is performed in yeast and described as a sensitive and inexpensive assay. Implementation of this assay on ethidium bromide showed that EtBr is a genotoxic drug, and it has other effects, such as chromosomal changes, despite not being able to prove or denied its carcinogenicity (Cahill *et al.,* 2004). The genotoxicity of EtBr was confirmed using the luciferase enzyme activity, which in turn depends on the *p*53 gene. This test showed that EtBr is a strong genotoxin (Ohno *et al.,* 2005). EtBr also can destroy human chromosomes; thus, it caused apparent structural chromosomal changes, but according to the used dose (Wu *et al.,* 2006). Several studies have been conducted to detect EtBr mutagenicity, which was strongly confirmed (Prabhu *et al.,* 2010). Also, EtBr carcinogenicity was proven and was classified as a direct or a primary carcinogen that does not require activation by metabolism, but its action is directed toward nucleic acids. Furthermore, EtBr can inhibit communication between cells by affecting gap–junctions and being a genotoxic and carcinogenic agent (Na *et al.,* 1995). In contrast, some studies concluded that EtBr was an unknown carcinogenic agent (Sakai *et al.* 2010).

Probiotic is living microorganism added to animal and human food, giving a healthy and robust influence. They positively affect humans and animals' health by improving the balance of bacteria in the intestines when given in sufficient quantities (Hill *et al.,* 2014). Probiotics are

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often bacterial organisms that affiliate to some well-known genera, such as *Lactobacillus*, *Escherichia*, *Bifidobacterium*, *Enterococcus*, *Streptococcus*, and *Bacillus spp*., but some yeast break this general rule due to their ability to work as probiotic in some cases (Lukjancenko *et al.,* 2012). Despite that the lack of clinical trials supports probiotic safety for humanity (Slashinski *et al*., 2012), there is a general agreement for its health benefits for mammals. Many studies have pointed to the active role of probiotics in the face of chronic and acute diseases that afflict humans and animals, such as digestive system diseases, a widespread phenomenon among humans, respiratory system infections, female reproductive tract infection, allergy, cholesterol, and cancer (Mohania *et al.,* 2013). The administration of lactic acid bacteria (LAB) has been shown to effectively reduce DNA damage induced by chemical carcinogens (Li and Li, 2003). These anticancer effects of probiotics are inhibiting intestinal bacterial enzymes that convert procarcinogens to more proximal carcinogens (Nagpal *et al*., 2012).

The present study aimed to test the EtBr seriousness effect on researchers' health and confront these expected risks using probiotics intake. The current study was conducted by treating mice with different EtBr doses parallel with daily LAB-supplement to mice feed for one month. Histological examination and molecular studies were applied to mice's liver after completing a month of the experiment.

MATERIALS AND METHODS

Materials

A total of 49 male Swiss albino mice, *Mus musculus*, with bodyweight (b. w.) at 25-30g, were obtained from the Faculty of Medicine, Ain Shams University, Cairo, Egypt. Ethidium bromide was purchased from Fluka Biochem Co. (Cat. No. 46065), while LAB was obtained from the Dept. of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University. Commercial powdered milk (Nido) was purchased from the local market to be used as a probiotics carrier. It was mixed with liquid media contains Lactic acid bacteria, then the mixture was incubated at 37° C/ 24 hours and finally stored at 4ºC. Probiotics were added to mice food at ($24x10^7$ CFU/g).

Methods

Experimental design

Mice were randomly divided into seven groups; each one contained seven animals. Mice of groups one, two, and three respectively received high (60 mg/kg b. w.), median lethal (50 mg/kg b. w.), and low dose (40 mg/kg b. w.) of EtBr as drinking solutions in addition to a unique dose (24x107 CFU/g) of bacterial probiotics mixed with original food. Mice of group four received regular food and drink, representing control group. Mice of groups five, six, and seven received EtBr only as drinking solutions with different concentrations, representing three different EtBr doses (high, median lethal and

low dose, respectively). The duration of this experiment was one month of different daily treatments. Controlled experimental conditions were applied, as regular light/dark cycles were every 12 hours. Optimum temperature was set at 20-22 ºC with adequate ventilation, cleanliness, and proper nutrition (water and commercial standard diet pellet ad libitum).

Histological analysis

The histological observations due to the treatment with EtBr only and EtBr combined with Probiotics were examined in liver and testes tissues according to Bancroft *et al*., (1996); the main steps were: Fresh samples taken from the liver and testes of mice, representing different groups were fixed in 10 % formalin for 24 h; fixed samples were washed in tap water; Serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for dehydration; Specimens were cleared in xylene and embed in paraffin at 56 ºC in a hot air oven for 24 h; Paraffin bees wax tissue blocks were prepared; The blocks were sectioned by slide microtome at 4 µ thickness; Obtained tissue sections were collected on deparaffinized glass slides and stained by hematoxylin & eosin stain and examined using a light microscope.

Molecular genetics analysis

DNA fragmentation

DNA fragmentation analysis was done using a hypotonic solution (6% potassium chloride) to extract high-density nucleic acids (unpublished data). The main steps were: 0.5 g frozen liver was disrupted in 1.5 ml Eppendorf tube; One ml 6% KCl fresh prepared was added; Samples were vortexed for 10 min (to homogenize the sample); Homogenate samples were incubated at 55 ºC / 2 hours; Incubated samples were centrifuged at 1000 rpm / 15 min; Resulted supernatant (contained the DNA) was transferred to new sterile and DNase-free Eppendorf tube; Five µl DNA + 5 µl loading dye were mixed and electrophoresed on 2 % agarose gel (Johansson, 1972), 6 µl Gel Pilot Mid-Rang ladder 100 (Qiagen, Germany) was loaded in a separate well to provide fragment sizes of 2000, 1000, 750, 500, 250, and, 100 bp; The run was performed for one hour at 100 volts using an Applex submarine unit (10 cm x 8 cm). The amplicons were revealed using 1x EtBr on UV- trans-illuminator and photographed by Gel documentation system (SYNGENE, UK).

Quantitative PCR analysis

RNA extraction

Pure total RNA was isolated from frozen liver tissue (under sterilization conditions). According to the manufacturer's recommendations, reagents of ISOLATE II RNA Mini Kit (Bioline, Germany) were used. Concentrations of all samples were measured on a spectrophotometer; then, we made different concentrations equal by adding sterile water to complete the following steps correctly.

Complementary DNA (cDNA)

Extracted RNA was converted to cDNA by using the Tetro cDNA synthesis kit (Bioline, Germany). A two-step reverse transcriptase-polymerase chain reaction was applied according to recommendations submitted by the manufacturer. All reagents and samples were mixed well and briefly centrifuged, then kept on ice before use.

Real-Time PCR run

The RT reaction was amplified for cDNA of interest, a gene-specific primer of *p*53 was used to detect the *p53* mRNA and relatively quantify its amount; its sequences were 5' CCC AAA CTG CTA GCT CCC AT 3' as a forward primer (*p53* Mm-F1291), and 5' ACT ACT CAG AGA GGG GGC TG 3' as a reverse one (*p53* Mm-R1567), which were synthesized through Invitrogen, the UK for amplification of desired gene (*p53*). These primers were newly designed and based on GenBank accession (NM_ 001127233.1) using Geneious 8.1 software. Primer3 online tool was used to test hairpin formation and self-annealing of the designed primers and perform in silico PCR of those primers in the University of California, Santa Cruz database (http://genome.ucsa.edu), on the mouse genome. The expected molecular size of the in-silico PCR was 277 bp.

Stratagene MX3000 P (Agilent Technologies) machine was used to apply qPCR on representative samples, which consisted of 10 µl of 2 x Sensi FAST SYBR® No-Rox kit (Bioline, Germany), 0.5 µl *p53* reverse primer (*p53*Mm-R1567), 0.5 µl *p53* forward primer (*p53*Mm-F1291), 1 µl cDNA template and nuclease-free water (up to 20 µl). The conditions of the used program were: initial denaturation at 92 ºC / 2min, repeated 40 cycles of 92 ºC / 5 sec for denaturation phase, 56 ºC / 15 sec for annealing phase, and 72°C / 26 sec for extension phase. A disassociation test was performed from 95 ºC to 50 ºC at 10 min intervals.

Real-time PCR data analysis

The qPCR CT (Cycle Threshold) values were used to compare and estimate the relative expression level of *p53* in comparison to control. Excel 2013 was used to estimate Δ CT, as Δ CT target = CT control – CT treatment, $E = 10^{-(1/slope)}$, and Ratio $_{(test/calibrator)} = E^{CT(calibrator) - CT(test)}$. As the amount of PCR product, was doubled each cycle of exponential amplification resulted in a 2-fold increase in the number of copies; thus E was set to 2.

RESULTS AND DISCUSSION

Several criteria for studying the impact synthetic chemicals or microorganisms under test should be considered, such as the method of exposure, the duration and frequency of exposure, target organ, and the impact of substance (Klaassen, 2013). It was reported that touching or inhaling ethidium bromide leads to numerous risks on the object's genetic material (Harisha, 2005 and Garbett *et al.*, 2007). However, the question was, what would happen if the material has already reached the digestive system? The mice's

body was exposed to EtBr through the digestive tract (drinking) while probiotics were added to their diet to answer this question. Probiotics were used as a protective agent against the side effect of EtBr due to the recorded health benefits (Patten and Laws, 2015). Indeed, materials entering through the mouth are processed in the liver, and then the process of detoxification may obscure the full emergence of the real impact of EtBr. A single dose of probiotics was used while three different EtBr doses were applied every day for one month before the autopsy.

Histological and molecular analyses were applied to determine the effect of EtBr on the digestive system and the possible protective action of probiotics to reduce such effects**.**

Histological effects screening

Liver examination

The liver was affected in all treatments compared to the control group. Generally, a wide range of tissue changes emerged; fatty and degenerative changes, inflammation, hepatic veins dilation, and focal hemorrhage were the common histological signs of the treatments (Table 1 and Fig. 1).

Hepatotoxicity is a satisfactory infects the liver due to exposure to the influence of harmful chemicals, toxins, drugs, or any other factor. The detoxification job of the liver makes it the most vulnerable organ to be affected. The histological change degree depends on the duration of exposure, used dose, and the exposed object. Although the liver toxicity phenomenon is single multi-faceted, it has many histological manifestations such as portal hypertension, fatty liver, inflammation, etc. Many hepatotoxicity mechanisms have been clarified in various studies, which focused mainly on mitochondrial failure which in turn results in disable many vital processes in the liver cells finished with mentioned symptoms of hepatotoxicity (Navarro and Senior, 2006). In this regard, histological examination of affected organs is a powerful method to determine cytotoxicity, especially chemical-induced toxicity.

Administration of EtBr at a low dose

This treatment led to dilation and congestion of portal and central hepatic veins (plate 1, D). Furthermore, focal bleeding in hepatic parenchymal cells (plate 1, E). L dose of EtBr could cause severe alterations in hepatic cellular. Those manifestations reflect the occurrence of portal hypertension, which is considered the gateway to cirrhosis of the liver and many of its complications (de Franchis, 2010). Therefore, the proposed interpretation of this case was due to the dosage of ethidium bromide, which impacted the liver and was on its way to cause cirrhosis. The dose may lead to erratic hepatitis blood pressure, which explains the liver suffering from the detoxification of the substance being tested, despite the need for other supportive studies to obtain more explanation.

Administration of EtBr at a medium lethal dose

This treatment led to fatty changes (plate 1, F), the portal vein's congestion, and inflammatory cell infiltration in the portal area (plate 1, G). The appearance of fatty changes is a result of various causes as drugs, toxins, rapid weight loss, malnutrition, Insulin resistance, viral infection, pregnancy, oxidative stress, and genetics (Adams *et al.*, 2005) but also, many studies have shown that fat accumulation in the liver relates to metabolic disorders (Fabbrini and Magkos, 2015).

It is worth mentioning that the mechanisms of fat accumulation in the liver vary from one reason to another, but do not get out of one or more of the following events: increased fatty acids synthesis decreased oxidation of fatty acids, and presence of fatty acids in large quantities to the liver and disruption of triglyceride cycle (Treinen-Moslen, 2001). So, any altered pathway of this process, from which lipid β-oxidation leads effectively in an imbalance in the presence of fat in the liver. However, the existence of many pathways of lipid β-oxidation as peroxisomal β-oxidation and mitochondria β-oxidation, but the latter remains the most important and most influential, despite the emergence of some devastating diseases result of altered peroxisome (Bartlett and Eaton, 2004). In this connection, it can be explaining the emergence of a change in liver fat as a result of a direct impact of ethidium bromide on the mitochondria of liver cells (Stewart *et al.*, 2010), leading to an imbalance in the performance and function of this organelle, from which β-oxidation for lipids either increase or decrease.

Other manifestations were expected to appear associated with fat deposits, according to the "two-hit" hypothesis (Mehta *et al.*, 2002 and Bigorgne *et al.*, 2008); fatty acids accumulation in the hepatocellular "first hit" led to inflammation "second hit". Other liver injury symptoms appear after those two hits as fibrosis, cirrhosis, and liver carcinoma (not found in our histological results). Both environment and genetics contributed in "second hit" that could be explained simply by the action of adipose tissue of secreting specific cytokines that stimulate other mediated factors through specific pathways and trigger circulating lymphocytes to the liver leading to inflammation. It cannot overlook other factors' role in this mechanism as gut-derived bacterial endotoxin and oxidative stress for reactive oxygen species (ROS). In this setting, the mitochondrion is one of ROS's most important sources (Mehta *et al.*, 2002). By contrast, not in all cases the "second hit" was the inflammation, which would occur first, followed by steatosis (Tiniakos *et al.*, 2010). The appearance of the portal vein congestion reflected that EtBr at LD_{50} could cause hypertension, as shown at the treatment of L dose of EtBr (de Franchis, 2010).

Administration of EtBr at high dose

This treatment led to fatty change, and other degenerations appeared in the hepatocytes all over the hepatic paren-

chyma (plate 1, H) associated with focal hemorrhage (plate 1, I). Dilated and congested portal vein appeared with inflammatory cell infiltration in the portal area surrounding the dilated bile ducts (plate 1, J). According to the mentioned "two hits" theory, fat deposition "first hit" followed by inflammation "second hit" which was one of the causes of venous congestion (Kakar *et al.*, 2004). Thus, the existence of fat deposits in the hepatocytes led to a defect in its job performance, and then those symptoms occur, where a strong correlation was found between liver fat and abnormal manifestations of hepatic veins (Northup *et al.*, 2008).

Focal hemorrhage is a change in the hemostatic system attributed to a disruption in platelet production regulated by the liver. The platelets are responsible for keeping blood loss (Lisman *et al.*, 2002). Other studies have shown a relationship between those changes and liver failure in the performance of its functions because of chronic or acute diseases (Lisman *et al.*, 2010).

Inflammation is a defensive condition carried out by the Immune system when there is a detrimental inducer to a specific site in the body of an organism; immune cells of different types are blown special Kupffer cells sills around the injury site to secrete specific toxins to eliminate the injured and abnormal cells, but these toxins harmful to other healthy cells (Hall, 1996). The inflammations continued for many diseases that affect the liver special liver steatosis (Yin *et al*., 2007), so deposits of fats are often accompanied by inflammation.

The mechanism by which hepatic veins became abnormal may be due to alteration in blood flow regulated by ant thrombin III, protein S and protein C, and anticoagulant proteins produced by the liver. Any change in those anticoagulant proteins by an increase or decrease reflected directly on the veins and the case of passing blood, liver fat has not been lost in the events of change in these proteins' level, especially, Protein C (Northup *et al.*, 2008). Nevertheless, the question remains; why did specific changes associate in some doses of ethidium bromide without appearing in others? Moreover, why did other tissue changes be general with each dose? The answer needs many of the illustrations studies.

Administration of EtBr at low dose combined with probiotic bacteria

This treatment led to a degenerative change in the hepatocytes surrounding the dilated central vein (plate 1, K). Interestingly, liver tumors appeared by morphological examination (data not shown). The L+P group's liver appeared differently from the rest of previous transactions; lack of liver abnormality was detected. However, there were some adverse effects, such as degenerative change and dilated central vein. This means that new manifestations appeared while others' disappearances were already related to L treatment without probiotics.

Degeneration is an abnormal shape of liver cells, which increase in size with

almost completely lost vital functions, making it closer to die, so hepatocellular degeneration is the first step on the way to necrosis. This abnormal cellular manifestation occurred when the cell membrane was altered; then, permeability was affected. Subsequently, ion pumps were affected, followed by an influx of sodium and water, leading to ballooning degeneration. Another cause of degeneration was the toxic effects of bile acids on cytoplasmic components, leading to the gradual death of the cells and their degeneration (Dancygier and Schirmacher, 2010) that may explain the emergence of dilation in the biliary duct.

The appearance of liver tumors reflected the low gene expression of the *p53* gene known to be tumor suppressor gene (Isobe *et al.*, 1986), thus when functions of *p53* were affected and disappeared, tumors were accordingly formed (Desilet *et al.*, 2010). So probiotics were thought to inhibit *p53*, inducing tumors' appearance due to EtBr treatment, although probiotics are potent cancer treatment tools (James *et al.*, 2016). Appeared tumors represented a piece of evidence for EtBr carcinogenicity, by contrast to what was proven about unknown carcinogenicity of EtBr (Sakai *et al.*, 2010).

Administration of EtBr at median lethal dose combined with probiotic bacteria

This treatment led to inflammatory cell infiltration surrounding the adjacent hepatocytes' dilated central vein wall (plate 1, L). Furthermore, liver tumors appeared by morphological examination (data not shown). This means that not all manifestations appeared in this treatment than its counterpart (LD_{50}), while the new one appeared (tumors). The absence of some manifestations recorded in LD_{50} reflects probiotics' relative ability to coop with the EtBr effect but at a limited level. While the appearance of tumor referred to probiotic effect on gene expression of *p53* as discussed previously.

Administration of EtBr at high dose combined with probiotic bacteria

This treatment led to dilation in the portal vein (plate 1, M)**, i**n addition to the observation of liver tumors that appeared by morphological examination (data not shown). These results confirmed the previous interpretation; thus, dilation of portal vein reflected that EtBr could do its action although the presence of probiotics, which means that used probiotics could not completely coop with EtBr effect. Interestingly, tumors appearance resulted from *p53* inhibition, which may be caused by probiotics.

Probiotic bacteria cannot prove complete efficient in curbing the histological effects of EtBr, unlike expected, where all symptoms related to different used doses of EtBr did not disappear completely, but even new symptoms appeared in the L+P group (degeneration changes). This means that the cytotoxic effect of EtBr did not end at one hundred percent. However, it remains to a lesser extent, which means that used dose of LAB was unable to completely counter EtBr influence, this is supported by the incomplete

lack of the ability of fermented dairy products to resist colorectal cancer in mammals (Capurso *et al*., 2006), suggesting a relative ability of the resistance. However, they need confirmatory studies to rely on those microbes in formal treatment.

The general belief that probiotics are safe and their regular intake leads to general improving mammalian health, particularly resistance to a wide range of cancers (James *et al.,* 2016). However, some old evidence of probiotic ability to cause harm to some organisms, in particular some strains of LAB was reported (Mackay *et al*., 1999),

Control group of mice

This treatment led to the liver's regular appearance; thus, regular histological features were cleared in all hepatic cellular components. This normality resulted from receiving typical food and drinks. The aim of conducting this treatment was to obtain healthy animals used for comparison with treated animals, and then histological alteration, induced by tested material, became apparent.

Molecular studies analysis

Gene expression is a process that turns genotype to phenotype, so it is considered the secret key that must be studied well at its different levels when any change appears on the organism, particularly diseases. This brings us to the concept of Gene expression analysis, the study of the functional product of a gene in specific cell /tissue, is applied using various ways. A qPCR is a powerful tool in this regard, so it was used in the present study to measure the gene expression of *p53* gene.

DNA fragmentation analysis

Applying the DNA fragmentation procedure resulted in high-density nucleic acids. Electrophoresis of genomic DNA obtained by this technique appeared normal in all samples (Fig. 2). The photo illustrated the lack of direct effects of ethidium bromide and bacterial probiotics on the genetic material; therefore, there was no DNA fragmentation related to all treatments compared to the control group.

As for EtBr, these results contrasted with the expected; thus, the DNA intercalation process can destabilize the genetic material and negatively affect the chromosomal structure. So EtBr, an intercalator, can be regarded as a significantly contributed agent to cells' toxicity. In other words, it is well known that most intercalating agents have an inhibitory effect on DNA topoisomerases, which control the topography of the nucleic acid, leading to obstructing the correct pathway of gene expression in addition to causing abnormal chromosomal packaging. These effects can be completed by the occurrence of programmed cell death represented in DNA fragmentation (Wang *et al.,* 1997; Singer *et al*., 1999). That did not happen, where it did not produce any fragmentation in the genetic material by ethidium bromide. These discrepancies can be explained by the occurrence of another kind

of response, such as cell cycle arrest or DNA repair system enhancement (Woods and Vousden, 2001; Oren *et al.,* 2002). Otherwise, maybe more time was needed.

As for probiotics, DNA fragmentation analysis results were expected because it is well known that probiotics, especially LAB, have the anti-carcinogenic property (Li and Li, 2003). It was also proven that some strains of LAB could cope with chemical-induced genotoxicity in rats (Pool-Zobel *et al.,* 1996) and other mammals (Burns and Rowland, 2000). Many other studies have dealt with probiotics as living beneficent microbes play an active role in the resistance to the genetic material's negative changes (Madrigal-Santillán *et al.,* 2006; Corsetti *et al.,* 2008; Verdenelli *et al.,* 2010; Dominici *et al.,* 2011). Also, probiotic administration safety in a human was recorded, which indicates the absence of any undesirable effects on health; thus, genetic material was not affected (Van den Nieuwboer *et al.,* 2015).

Although not all studies showed probiotic force in responding to the health dangers, there are failures made by those microbes in this regard, such as the study in which products of fermented milk failed to prevent colorectal cancer (CRC) ultimately, where they succeeded in tests without the other (Capurso *et al.,* 2006). It is worth mentioning that the species but the strain of used probiotic showed highly specialized therapeutic. The experiment's dosage and conditions can control many of the obtained results in a clinical trial (Whorwell *et al.,* 2006). It is imperative to make entirely sure that each experiment has particular circumstances that control obtained results. However, there are essential criteria that must be met to achieve the desired goal of probiotics usage as probiotics can survive in the gastrointestinal tract and its inability to work as a pathogenic or toxic organism (Shalke, 2013).

Real-time PCR

After obtaining cDNA and applying q-PCR using a real-time PCR machine, obtained CT values were used to estimate ΔCT and Ratio of the *p53* gene expression, using subtractive equations. CT values of EtBr treatment groups were lower than CT values of EtBr with probiotics treatments, so all values (ΔCT and Ratio) estimated using CT values were different in the same way (Table 2 and Fig. 3).

Obtained CT values showed that all treatments led to alteration of *p53* gene expression, but at different levels than the control group. This means that EtBr, whether alone or with probiotic, could make an effective change even entered the cell, particularly hepatocellular. However, all doses of EtBr strongly affected gene expression of *p*53, but the high dose of EtBr was the most dose that resulted in high gene expression of *p*53; this means that this dose must be taken into account due to its significant influence on the health of mice because the high gene expression of *p53* means that the hepatic cell tried to coop with a form of cancer. There

was a relationship between the EtBr dose and the level of *p*53 expression; this was cleared from calculated Ratios.

Since alteration of *p53* gene expression is one of genotoxicity signs (Kirsch-Volders *et al.*, 2003), any agent that can cause induction of *p53* is considered genotoxin. This means that EtBr is a genotoxic agent due to obtained results, which reflected the induction of *p53* due to treatment with EtBr. Genotoxicity of EtBr may be due to its structure; thus, it contains a cationic compound (bromide), which has a high potential genotoxicity effect due to its covalent interaction with the negatively charged nucleic acids (Snyder and Arnone, 2002).

Many studies have an agreement with EtBr genotoxicity; thus, it was proven that EtBr adversely affected the transcription process of specific genes. It could directly interact with genetic material asserting its genotoxic effect (Waring, 1965). Another study showed EtBr genotoxicity depending on the presence of chromosomal aberrations induced with EtBr or not; using mammalian cells treated with EtBr, the obtained results showed that a few hours of treatment was sufficient to negatively affect the chromosomes (McGill *et al.,* 1974). Genotoxicity induced by chemicals has been indirectly measured in human cells using a wide range of tested chemicals *in vitro*. The activity of luciferase was appreciated; this enzyme was associated with *p*53*R2* response to genotoxins. EtBr could give a positive result in this assay (Ohno *et al.,* 2005). Like what was obtained about the EtBr ability to affect the centromere more heavily than chromosomes' arms (Wu *et al.,* 2006).

In contrast, EtBr cannot significantly induce *p53* after nearly three hours of treatment. So EtBr was regarded as an intercalating agent with no genotoxic effect, represented by the inability to induce *p53* (Nelson and Kastan, 1994). Since the actual act of *p53* is curbing tumors and cancers, it was more active in swelling. Increasing *p53* gene expression in the presence of EtBr meant that this chemical has a carcinogenic effect. However, this conclusion was the opposite of what was proven of unknown carcinogenicity of EtBr (Sakai *et al.*, 2010).

EtBr can stimulate *p53* in the treatments with EtBr only; otherwise, this effect was relatively decreased in the treatments of EtBr combined with LAB. This result means that used probiotic could slightly make a reverse effect, cleared by the obtained CT values. In other words, probiotic bacteria's presence led to a relative reduction of gene expression of *p53* compared to induced form in EtBr treatments. This observation can be returned to the anticarcinogenic of lactic acid bacteria, which may resist the harmful influence of EtBr, so less need for high production of *p*53. This hypothesis is supported by the disappearance of some of the histopathological symptoms resulting from EtBr. Several studies confirmed the antigenotoxic and anticarcinogenic effect of probiotics, especially LAB, which can reduce DNA damage caused by chemical

carcinogens in rats (Pool-Zobel *et al.,* 1996). LAB can decrease the level of specific bacterial enzymes (nitroreductase, glycosidase, azoreductase, and B-glucuronidase) and transform the pre-carcinogens into high active carcinogens (Li and Li, 2003). Interestingly, the anti-genotoxicity of probiotics was proven in human cells (Burns and Rowland, 2004).

The effect of yeast was tested in mice treated with aflatoxin. It was able to reduce micro-nucleated normochromic erythrocytes, in addition to its ability to change and modify the aflatoxin compound (Madrigal-Santillán *et al.,* 2006). While *Lactobacillus* extracted from cheese was able to discourage genotoxins (Corsetti *et al.,* 2008). Another evidence of probiotic efficacy was *Lactobacilli*'s ability to reduce the genotoxic effects of 4-NQO and turn it into a less toxic compound (Verdenelli *et al.,* 2010). On the other hand, the effect of four species of bifid bacteria has been tested in mice treated with some chemical genotoxins; the result showed the superior ability of these bacteria to reduce genotoxicity of tested chemicals (Dominici *et al.,* 2011).

Not all studies showed probiotic force in responding to the health dangers, but there are failures made by those microbes in this regard, such as the study in which products of fermented milk failed to prevent colorectal cancer (CRC) ultimately, where they succeeded in tests without the other (Capurso *et al.,* 2006). It is worth noting the participation of many factors in their influence on the results of such clinical trials, such as probiotic strain, dosage, and other trial conditions (Whorwell *et al.,* 2006). So another hypothesis is introduced, in which the low level of *p53* gene expression returns to the direct effect of probiotics; thus, LAB may inhibit *p53* itself that allows tumors to be formed (as described). However, it is imperative to ensure that each experiment has particular circumstances that control the obtained results. However, there are essential criteria that must be met to achieve the desired goal of probiotics usage as probiotics can survive in the gastrointestinal tract and its inability to work as a pathogenic or toxic organism (Shalke, 2013).

CONCLUSION

In the presence of EtBr, whether alone or with probiotic bacteria, *p53* was induced, reinforcing the idea that these microbes' presence did not confront the damage leading to the induction of gene. The lower gene expression in the presence of probiotics may be back to the direct effect of LAB on *p53*. The absence of fragmented DNA in all treatments indicates that EtBr has no genotoxic effect either alone or combined with LAB, although this interpretation is questionable due to *p53* induction. In contrast, histological examination results supported the cellular toxicity of EtBr and the incomplete ability of LAB in the face of that toxicity. The weak effort of probiotic bacteria may be due to the experiment's special conditions, so more supportive measures and confirmatory studies are required.

SUMMARY

Ethidium bromide (EtBr) is a nucleic acid intercalating agent used extensively as a fluorescent dye in molecular genetics laboratories. The current study aimed to determine the potential histo- and genotoxicity of EtBr and investigate the antigenotoxic effect of probiotic bacteria (Lactic Acid bacteria; LAB) on mammalian tissue (albino mice). Mice were randomly divided into seven groups, with seven different treatments. Different EtBr doses were used individually as drinking solutions with and without probiotic bacteria, which was introduced as a single dose as a food additive. After one month of the treatments, the liver was tested using histological assay, DNA fragmentation analysis, and quantitative RT-PCR technique. No significant genotoxic effect for EtBr on the liver was observed on histological examination and DNA fragmentation analysis. However, a considerable increase in the expression of the *p53* gene and correlated with the dosages increase. However, the *p*53 expression was altered upon applying the probiotics, while some histological changes were detected, but no DNA fragmentation was detected. The seriousness of EtBr on the organism's health is conditional with the applied doses. Probiotics were not luckier than EtBr; it did not provide expected health benefits. This unexpected action of probiotics may be due to the used dose and how to use it. Further analysis of both histological and molecular aspects using multiple controls and integrative experiments is required to explain probiotics' paradox effect versus the EtBr effect on mammalian cells and tissues.

Keywords: EtBr, Probiotic bacteria, genotoxicity, q-PCR, DNA fragmentation.

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Table (1): Histological signs appeared in different treatment and their relative degrees shown as presence (+, ++) or absent (-).

Treatment / histopathological signs	Congested hepatic veins	Dilated hepatic veins	Fatty Change	Degeneration change	Inflammatory infiltration	Hemorrhage	Tumor observation
L	+	+	-	-	+	+	-
$L.D_{50}$	+	-	+	-	+	-	-
H	+	+	++	+	++	++	-
L+P	-	+	-	++	-	-	+
$L.D_{50}$+P	-	+	-	-	+	-	+
H+P	-	+	-	-	-	-	+

L: low dose of EtBr, $L.D_{50}$: the median lethal dose of EtBr, H: high dose of EtBr, L+P: low dose of EtBr combined with LAB, $L.D_{50}$+P: the median lethal dose of EtBr combined with LAB, and H+P: high dose of EtBr combined with LAB

Table (2): The Ct values of Samples amplified with Real-time PCR.

Treatment	CT value	Δct	Ratio
L+P	28.34	-1.06	0.479
H+P	27.95	-0.67	0.628
$L.D_{50}$+P	27.15	0.13	1.094
C	27.28	0	1
H	25.56	1.72	3.294
L. D_{50}	26.58	0.7	1.624
L	26.26	1.02	2.028

L: low dose of EtBr, $L.D_{50}$: the median lethal dose of EtBr, H: high dose of EtBr, L+P: low dose of EtBr combined with LAB, $L.D_{50}$+P: the median lethal dose of EtBr combined with LAB, and H+P: high dose of EtBr combined with LAB.

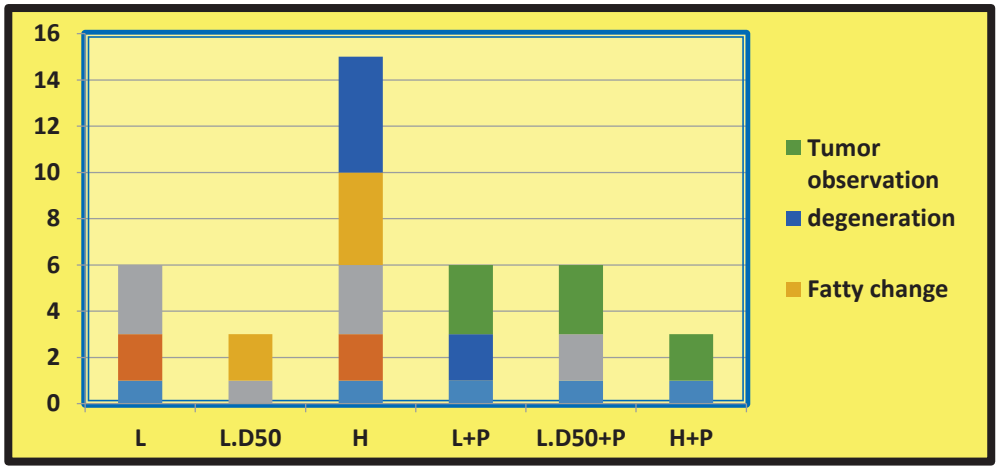
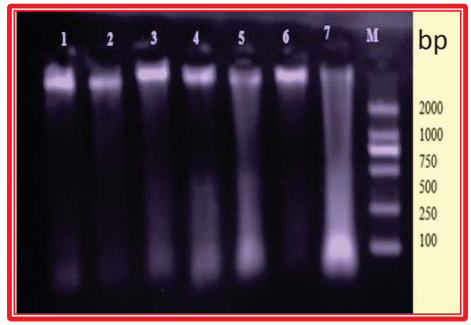


Fig. (1): Histogram showed different liver histological effects related to different doses of EtBr, whether alone (L, LD_{50}, and H) or combined with probiotics (L+P, LD_{50}+P, and H+P).

Fig. (2): Gel photo of DNA electrophoresis of samples obtained from treated and controlled mice, detecting DNA fragmentation. 1= high dose of EtBr (H), 2= median lethal dose of EtBr (LD_{50}), 3= Low dose of EtBr (L), 4= Negative control (C), 5= H+ Probiotic (H+P), 6= LD_{50}+Probotic (LD_{50}+P), 7= L+Probiotic (L+P).



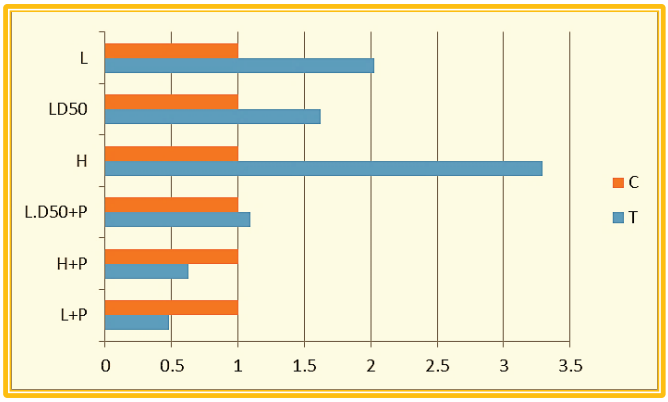


Fig. (3): Histogram showed the ratio of *p53* amplification obtained by real-time PCR.

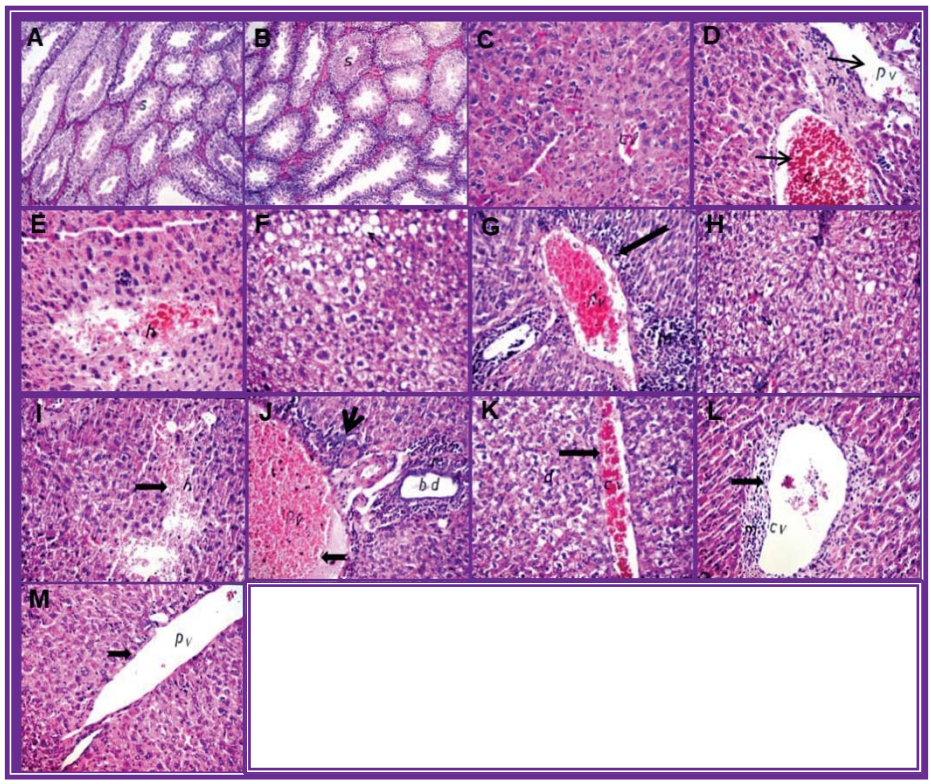


Plate (1): (A-B) Sections in tests of mice. (A) the negative control, showing the typical structure of testicles and spermatogonia (s). **(B)** a representative of the testicular tissue for all treatments, showing a typical structure of testicles and spermatogonia (s). **(C-M)** represents sections in the liver of mice (H & E X 400). **(C)** a section in the liver of negative control mice, **(D)** and **(E)** represent the low dose of EtBr. Arrows show dilation in hepatic veins, while (h) means hemorrhage. **(F)** and **(G)** median lethal dose of EtBr. In **(F)**, the arrow shows fatty change, while the arrow in **(G)** refers to the inflammatory cell's infiltration in the portal area. **(H), (I)** and **(J)** high dose of EtBr. **(H)** shows the fatty change and other degenerations in hepatocytes over the hepatic parenchyma**.** Arrow in **(I)** shows focal hemorrhage, while arrows in **(J)** show severe dilatation, congestion in the portal vein, and inflammatory cell infiltration in the portal area surrounding the dilated bile ducts. **(K)** the low dose of EtBr combined with a unique dose of probiotic bacteria, arrow shows dilated central vein. **(L)** the median lethal dose of EtBr combined with probiotic bacteria; arrow shows inflammatory cells infiltration surrounding the wall of the dilated central vein. **(M)** high dose of EtBr combined with probiotic bacteria; arrow shows dilatation in the portal vein.

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