

THE EFFECT OF CRUDE YEAST EXTRACT VERSUS ITS POLYSACCHARIDE FRACTION ON GROWTH, SEED YIELD, SILYMARIN CONTENT AND LEAF ANATOMY OF *Silybum marianum* (L.) GAERTN.

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ABSTRACT

For the aim of contrasting the effect of crude yeast extraction (CYE) with that of its polysaccharide fraction (YEPF) on growth, seed yield, silymarin content and leaf anatomy of *Silybum marianum* plants, CYE or YEPF was applied twice as a foliar spray at either 0 (as control), 3 or 6 gL⁻¹. Results indicated that CYE was more effective on plant growth whereas YEPF was more beneficial for the plant's reproductive processes, resulting in higher seed yield per plant compared with CYE.

Moreover, though both CYE and YEPF induced the accumulation of silymarin in plant organs, higher silymarin content in seeds, capitula, leaves and stem was recorded in response to the treatment with YEPF. In addition, the application of either CYE or YEPF at 6 gL⁻¹, preferably with the latter enhanced the photosynthetic and translocative components of the leaf tissues. The mechanism through which either CYE or YEPF affect plant growth, seed yield and silymarin accumulation was discussed. It could be concluded that utilization of yeast extract as an *in vivo* elicitor is feasible and that YEPF is preferred over CYE for enhancing seed yield and silymarin content in *S. marianum* plants.

INTRODUCTION

Silybum marianum (L.) Gaertn, a member of Asteraceae, commonly called as Milk thistle, grows natively in the Mediterranean area and is medicinally important as hepatoprotective (Parmoon et al., 2015). Various parts of the plant, particularly seeds, possess antioxidant, antifungal, immunomodulation potential and used as alternative medication for the treatment of various disorders. Seeds of the plant contain considerable amounts of flavonolignans (silymarin) with antihepatotoxic effects (Pradhan and Girish, 2006). Besides its antioxidant properties, silymarin has been reported to have anti-tumor (Roy et al., 2012), antiviral (Ramasamy and Agarwal, 2008) and anti-inflammatory as well as antifibrotic effects (Fuchs et al., 1997) effects.

Yeast extract (YE) had not only positive effects on plant growth and yield either in normal (Abou El-Yazied and Mady, 2012; Al-Tawaha and Ababneh, 2012; Mahmoud et al., 2015) and stress conditions (Amooaghaie et al., 2015), but also utilized to elicit the biosynthesis of secondary metabolites with medicinal value in various plant species. YE enhanced the accumulation of 6-methoxymellein in carrot cells (Guo and Ohta, 1994), polyphosphoinositol in *Cupressus lusitanica* (Zhao et al., 2004), galanthamine accumulation in *Lycoris chinensis* seedlings (Mu et al., 2009) and the alkaloid benzophenanthridine in *Eschscholtzia californica* suspension cell cultures (Cho et al., 2008). In addition, treatment of *S. marianum* suspension cultures with YE improved the production of silymarin and caused the release of silymarin to the culture medium at a level about three-fold higher than that of the control (Sanchez-Sampedro et al., 2005) and promoted the accumulation of silymarin in hairy root cultures of *S. marianum* (Hasanloo et al., 2008).

For using YE as an elicitor for *S. marianum* flavonolignan production, it is always added to either *in vitro* cell cultures (Sanchez-Sampedro et al., 2005) or hairy root cultures (Hasanloo et al., 2008). So, the effect of YE on elicitation *in vivo* is poorly-investigated and less-understood.

In its utilization as a biotic elicitor, crude YE was used (Sanchez-Sampedro et al., 2005; Hasanloo et al., 2008; Abraham et al., 2011; Ismail et al., 2013). In some other studies, the method of YE preparation was adjusted so that only the polysaccharide fraction of YE was isolated and acted as elicitor (Cakir and Ari, 2009; Mu et al., 2009; Ahmed et al., 2014). Whether the crude YE or only its polysaccharide fraction will do best as an elicitor for *S. marianum* flavonolignan production still unanswered question. So, the present study was conducted to: 1) Study the effect of *in vivo* YE-elicitation on flavonolignano production in *S. marianum*, along with its effects on growth attributes and yield of the plant, 2) Compare the elicitation effect of crude YE with its polysaccharide fraction.

MATERIALS AND METHODS

1- Plant material and cultivation conditions

Two field experiments were conducted at the Experimental Farm of the Agric. Bot. Dept., Fac. of Agric., Mansoura Univ. during the two successive growing seasons 2013/2014 and 2014/2015. Seeds of *Silybum marianum* (*S. marianum*) were obtained from medicinal and aromatic plants department, Horticulture Research Institute, Agric. Research Center, Giza, Egypt. Seeds were sown on 20th October during the two growing seasons in plots 3 x 3 m containing 6 equal-spaced rows. Seed hills were 30 cm apart containing 5 seeds/hill, which is then thinned to leave one plant per hill 25 days after sowing (DAS). Main physical and chemical characteristics of the experimental soil shown in Table (1) were estimated according to Hoddinott and Lamb (1990). All plots received the same tillage practices except for the tested treatments. Treatments comprised two levels 3, 6 g L⁻¹ from either crude yeast extract (CYE) or the polysaccharide fraction of the yeast extract (YEPF). The experiment was laid out in a randomized complete block design with three replications. Either CYE or YEPF was sprayed onto foliage using hand atomizer twice 40, 55 DAS at the rate of 75, 100 ml plant⁻¹ during the first, second application, respectively.

2- Preparation of YE

YE was prepared according to the method of Kobayashi et al. (1980). Dry yeast (*Saccharomyces cerevisiae*) powder at 3, 6 gL⁻¹ was shaken in a medium containing 150 g sucrose, 5 g KH₂PO₄, 2 g MgSO₄.7H₂O, 5 g CaCO₃ and 5 g peptone per 1 litre of distilled water and allowed to grow at 28°C for 3 days. The cells were then collected by centrifugation and washed. Four volumes of distilled water was added to the cell mass, then the mixture was kept at 37°C for 2 h with slow agitation to facilitate autolysis. The autolysate suspension was then subjected to centrifugation at 10,000 x g for 30 min, and the supernatant was used as the CYE.

3- Preparation of YEFP

For preparing the polysaccharide fraction of YE, the ethanol precipitation method as described by Hahn and Albersheim (1978) by dissolving the tested dose of

yeast in 50 ml of distilled water, then 50 ml of ethanol was added. The solution was permitted to precipitate for 3 d at 4°C in a refrigerator, then the supernatant was discarded. The remaining glutinous deposit was redissolved in 125 ml of distilled water and subjected to another cycle of ethanol precipitation. The final deposit was dissolved in 1 litre of distilled water and used as YEFP.

4- Recorded parameters and analyses

At 70 DAS, growth parameters presented in table (2) were estimated and leaf samples for the anatomical study were obtained. At 130 DAS, random samples from plant organs i.e. leaf, stem, capitulum and seeds were obtained for the determination of silymarin content. At the end of the experiment (160 DAS), yield and its components presented in table (3) were recorded.

Table (1)*. Mechanical and chemical analysis of the used soil (average of the two growing seasons).

CS %	FS %	S %	C %	CaCO ₃ %	OM %	TN %	AP ppm	EK ppm	TSS %
10.1	28.8	27.7	33.4	2.4	2.5	0.16	22.0	212.0	0.16

*CS, Coarse sand; FS, Fine sand; S, Silt; C, Clay; OM, Organic matter; TN, total N; AP, available P; EK, exchangeable K; TSS, total soluble solutes.

Table (2)*. Effect of crude yeast extract (CYE) and its polysaccharide fraction (YEFP) on growth parameters of *S. marianum* (average of the two growing seasons)..

	Plant height (cm)	No. of leaves	Leaf length (cm)	Leaf width (cm)	% of variegated areas	No. of prickles/ leaf lobe	Prickle length (mm)	Shoots FW g plant ⁻¹	Shoots PW g plant ⁻¹
Control	53.5 ^b	10.3 ^b	34.0 ^b	13.6 ^c	32.4 ^c	29.6 ^c	3.5	64.7 ^d	7.7 ^c
CYE 3gL ⁻¹	58.0 ^{ab}	12.0 ^{ab}	36.0 ^{ab}	15.0 ^b	35.0 ^b	40.8 ^{ab}	3.4	86.6 ^b	10.7 ^b
CYE 6 gL ⁻¹	62.7 ^a	13.2 ^a	42.5 ^a	19.4 ^a	36.2 ^{ab}	42.4 ^a	3.2	95.8 ^a	19.3 ^a
YEFP 3 gL ⁻¹	56.0 ^{ab}	10.0 ^b	32.0 ^b	16.7 ^{ab}	38.0 ^b	38.6 ^{ab}	3.6	74.0 ^c	9.7 ^b
YEFP 6 gL ⁻¹	54.0 ^b	9.7 ^b	28.7 ^b	13.8 ^c	40.2 ^a	42.7 ^a	3.8	68.6 ^{cd}	8.9 ^{bc}
Significance at P≤0.05	*	*	*	*	*	*	N.S.	*	*

* Different small letters within the same column indicate significant differences between means at P≤0.05 according to DMRT.

Table (3)*. Effects of crude yeast extract (CYE) and its polysaccharide fraction (YEFP) on seed yield and its components as well as seed protein and oil contents of *S. marianum* (average of the two growing seasons).

	No. of capitula/ plant	Capitulum diameter (cm)	No. of seeds/plant	Seed yield g plant ⁻¹	Seed oil content %	Seed protein content %
Control	4.2 ^c	3.9 ^b	236.5 ^c	22.3 ^c	26.4	22.7 ^b
CYE 3 gL ⁻¹	4.7 ^b	4.1 ^{ab}	317.0 ^b	26.0 ^{ab}	27.0	23.8 ^{ab}
CYE 6 gL ⁻¹	4.5 ^{bc}	4.2 ^{ab}	336.0 ^b	25.8 ^b	25.3	25.0 ^a
YE-PF 3 gL ⁻¹	4.8 ^b	4.4 ^a	387.8 ^a	28.4 ^a	27.8	21.2 ^{bc}
YE-PF 6 gL ⁻¹	5.6 ^a	4.5 ^a	352.0 ^{ab}	26.9 ^{ab}	28.3	21.8 ^{bc}
Significance at P≤0.05	*	*	*	*	N.S.	*

* Different small letters within the same column indicate significant differences between means at P≤0.05 according to DMRT.

- Extraction and determination of silymarin

A sample of 1 g powder of air-dried plant material was defatted by petroleum ether for 12 h at 50°C. The dried residue was subjected to a triple extraction with 50 ml methanol at 65°C for 8 h. Aliquots of methanol extracts were pooled and evaporated to

dryness. Samples were then re-dissolved in 10 ml of methanol and subjected to HPLC analysis using a phenomenex C-18 column (250 x 4.6 mm) eluted with MeOH-H₂O-AcOH at 40:60:5 ratio at a flow rate of 1 ml min⁻¹ and detection was done at 280 nm (Alikaridis et al., 2000). A mixture of flavonolignans comprising

silybin, silychristin and silydianin (Sigma Aldrich) was used as a reference standard.

- Determination of seed oil content

Seed oil content was determined using the soxhlet extraction method (AOAC, 2002). Oil was extracted from 20 g of seed powder in 300 ml of petroleum ether in a soxhlet apparatus at 60°C . After 5 h of reflux, the solvent was removed under reduced pressure in a rotary evaporator and oil residue was determined.

- Determination of seed total proteins

Seed total proteins content was determined spectrophotometrically at 595 nm using bovine Serum Albumin as a standard according to Bradford (1976).

- Leaf anatomy:

Leaf samples, the middle 1 cm from the leaf blade of the 3rd fully-mature leaf from plant top from 3 plants chosen randomly from each replicate were processed for the anatomical investigation according to O' Brien and Mc Cully (1981). Specimens were killed and fixed in 3.5% glutaraldehyde and post-fixed in 2.5% osmium tetroxide. Fixed samples were then washed in buffer (0.1M phosphate buffer, pH 7.0), dehydrated in a graded ethanol series, embedded in resin, semi-thin-sectioned (1-2 µm) using a Reichert microtome and stained with toluidine blue. Quantitative data were obtained using a calibrated eyepiece micrometer.

- Statistical analysis

Analysis of variance (ANOVA) was performed using the statistical package for Social Sciences (SPSS) version 16.0 software (SPSS, Inc, Chicago, IL, USA). Data are presented as pooled data of the two growing seasons. Duncan's Multiple Range Test was applied to determine significant differences between means when ANOVA was significant at P ≤0.05.

RESULTS

1- Effect of CYE and YEPF on growth parameters

Growth parameters of *S. marianum* were significantly affected by yeast extract (YE) treatments (Table 2). CYE at both levels increased fresh and dry

weight of shoots, whereas plant height, no. of leaves and leaf dimensions were increased only due to CYE at 6 gL⁻¹. YEPF had, generally, no significant effects on the estimated growth parameters. Data also show that both CYE and YEPF at both levels increased the percentage of leaf variegation and no. of prickles on the leaf margin. Increased leaf variegation was more evident in response to YEPF at 6 g L⁻¹. Prickle length was not significantly affected by treatments.

2- Effects of CYE and YEPF on flowering, yield as well as seed oil and protein content

CYE applied at both levels increased significantly no. of seeds/plant as well as seed yield/plant and promoted flowering through increasing no. of capitula and capitulum diameter (Table 3). YEPF increased significantly no. of capitula, capitulum diameter, no. of seeds and seed yield/plant. YEPF at 6 gL⁻¹ increased seed yield/plant by about 21% over control. Seed oil content was not significantly affected by treatments. On the other hand, protein content was significantly affected (Table 3). CYE at 6 gL⁻¹ significantly increased seed protein content, whereas the increase recorded at 3 gL⁻¹ and both levels of YEPF was insignificant.

3- Silymarin content:

Seeds contained the highest silymarin concentration, followed by the capitula (Table 4). Within vegetative organs, the leaf contained higher silymarin concentration (1.6 mg g⁻¹ DW) compared with the stem (1.1 mg g⁻¹ DW). Data also show that elicitation with CYE or YEPF increased silymarin content in seeds, capitula, leaves and stem. The increase was insignificant in leaves and seeds in response to CYE at 3 gL⁻¹. In inducing silymarin content, YEPF was more effective compared with CYE. Seed's silymarin content was increased by 38%, 49% and by 15%, 32% due to the treatment with YEPF and CYE at 3, 6 gL⁻¹, respectively. In addition, the higher level of either form, CYE or YEPF, was more effective in this respect.

Table (4)*. Effects of crude yeast extract (CYE) and its polysaccharide (YEPF) on silymarin content (mg g⁻¹ DW) in plant organs of *S. marianum* (average of the two growing seasons).

	Stem	Leaf	Capitulum	Seeds
Control	1.1 ^d	1.6 ^c	3.2 ^d	4.7 ^c
CYE 3 gL ⁻¹	1.8 ^c	2.2 ^{bc}	4.0 ^c	5.4 ^c
CYE 6 gL ⁻¹	2.6 ^{ab}	3.1 ^{ab}	5.2 ^{ab}	6.2 ^{ab}
YEPF 3 gL ⁻¹	3.4 ^a	3.6 ^a	5.4 ^{ab}	6.5 ^{ab}
YEPF 6 gL ⁻¹	3.1 ^{ab}	3.0 ^{ab}	6.0 ^a	7.0 ^a
Significance at P≤0.05	*	*	*	*

* Different small letters within the same column indicate significant differences between means at P≤0.05 according to DMRT.

4- Anatomical features of the leaf:

S. marianum leaf shows a dorsiventral structure (Fig. 1). Both abaxial and adaxial epiderms is unilayered. Mesophyll comprised a mostly 2-layered palisade tissue and a spongy tissue. The mid-vein vascular bundle shows the normal structure of a collateral bundle.

Both levels of YEPF increased blade thickness at mid-rib, mesophyll thickness, dimensions of the mid-rib vascular bundle (MRVB), no. of MRVB's xylem vessels and diameter of the MRVB's vessels. The enhancing effect of YEPF on leaf anatomical features was more pronounced at 6 g L⁻¹. CYE, at 6 g L⁻¹, increased the tested features of the leaf except vessel diameter and mesophyll thickness, whereas had no significant effects at 3 g L⁻¹.

DISCUSSION

The present study revealed a stimulating effect of crude yeast extract on growth attributes of *S. marianum* plants, in accordance with the results of previous studies (Nassar et al., 2011; Abbas, 2013). As a natural source of cytokinins, yeast extract (YE) stimulated cell division, synthesis of proteins, nucleic acids and chlorophylls, thereby increased plant growth (El-Sayed et al., 2002). Kedr and Farid (2000) reported that the positive effects of YE on plants is related to its capacity to induce the biosynthesis of plant growth hormones IAA and GA₃. This conclusion was supported by the results of a study by El-Tohamy et al. (2008) in which YE increased the level of plant growth substances IAA and GA₃, leading to an enhanced growth of bean plants.

Not only seed yield of *S. marianum* plants but also seed protein content was increased in response to the treatment with either form of YE according to the results of the present investigation. These results are in line with previous studies whose results indicated a stimulating effect of YE on seed yield and seed quality of different plant species. Treatment with YE resulted in enhanced seed yield of bean plants (Amer, 2004), protein content in soybean seeds (Al-Tawaha and Ababneh, 2012), and flowering as well as fruit set in olive trees (Mahmoud et al., 2015).

It is evident from the results of the present study that no. of seeds and seed yield/plant were higher in response to YEPF compared with CYE (Table 3), indicating a higher influential activity on the plant's reproductive processes. Higher reproductive induction by YEPF may be mediated through enhancing photosynthesis, translocation of photosynthetic products to sink or through other biochemical pathways. This conclusion is substantiated by the results of other studies. According to Hamasaki et al. (2014), YEPF not only increased fruit yield in tomato by reducing flower drop through enhancing calcium channels, but also increased individual fruit weight through promoting photosynthesis and/or translocation of photosynthesis products from leaves to fruits. In addition, YEPF-induced photosynthesis in *Lactuca sativa* was founded

on induction of photosystem II proteins, chlorophyll a-binding protein, ATP synthase and RuBisco (Kato et al., 2014). Silymarin content in seeds as well as in capitulum, leaf and stem were enhanced in response to the treatment with YE, preferentially in the YEPF form (Table 4). In accordance with the results of the present study, YE stimulated flavonolignans (Silymarin) production in hairy root cultures two-fold higher than the control cultures (Sanchez-Sampedro et al., 2005). They also found that this stimulation was accompanied with induced activity of lipoxygenase, which leads to induction of Jasmonate that constitutes an integral part of the YE-induced signal transduction pathway for the production of flavonolignans. The mechanism through which YE acts as an elicitor is speculative. The elicitation effect of YE may be due to its cations (Hasanloo et al., 2008). Other investigators postulated that YE-induced elicitation may be due to other components still unidentified (Srivastava and Srivastava, 2007). According to Sanchez-Sampedro et al. (2005), YE triggers the production of endogenous gasmonic acid and/or methyl jasmonate, which influence the production of secondary metabolites. Zhao and Davis (2005) postulated that a signal is produced by the elicitor and is perceived by a receptor on the plasma membrane and initiates a signal transduction network that regulates the expression of genes encoding for the synthesis of plant secondary metabolites.

According to Hamasaki et al. (2014), YEPF comprising mainly polysaccharides such as polymers of glucose (B-glucan) and mannose (mannoproteins). These B-glucan and mannoproteins were reported as microbe-associated molecular patterns (MAMPs) that activate plant metabolism (Obara et al., 2007). MAMPs induce plant defensive responses including the generation of reactive oxygen species, reactive nitrogen species, ethylene and expression of pathogenesis-related (PR) genes (He et al., 2007), which trigger the synthesis of secondary metabolites. In addition, He et al. (2007) postulated that MAMPs activate calcium-dependent protein kinases and mitogen-activated protein Kinase cascades that lead to transcriptional changes in numerous genes.

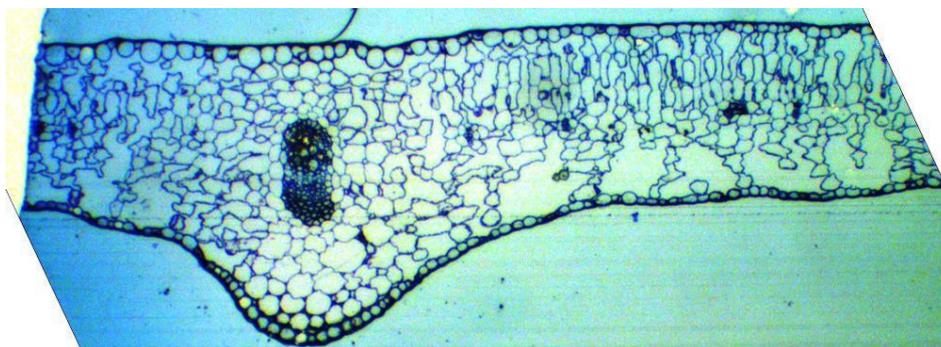
The anatomical characterization of the leaf of *S. marianum* reported here conforms with that previously published by Sidhu and Saini (2011) for the species. The present study revealed a stimulating effect of YE, in its YEPF form, on the leaf anatomical features, especially at 6 g L⁻¹ (Fig. 1, Table 5). This may provide a favourable environment for photosynthesis production and translocation leading to enhanced growth and yield. A stimulating effect of YE on the anatomical elaboration of kidney bean leaves was also reported (Nassar et al., 2011).

It could be concluded that utilization of YE as an *in vivo* elicitor is feasible and that YEPF is preferred over CYE for enhancing seed yield and silymarin content in *S. marianum* plants.

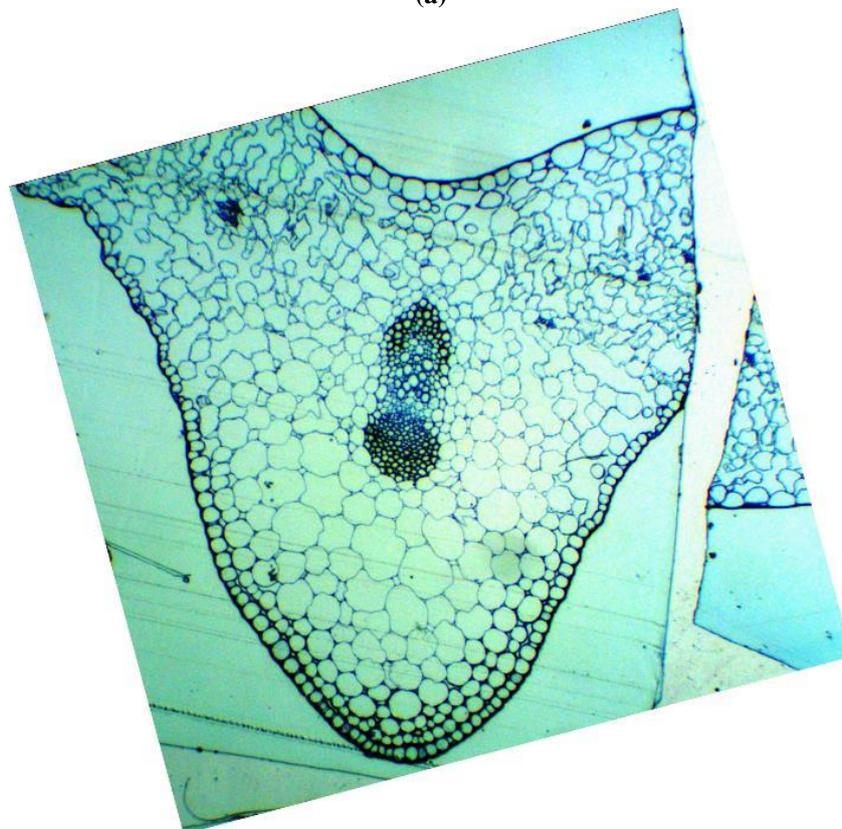
Table (5)*. Effects of crude yeast extract (CYE) and its polysaccharide fraction (YEPF) on anatomical features of the leaf of *S. marianum* plants (average of the two growing seasons).

	Blade thickness at mid-rib. (μ)	Mesophyll thickness (μ)	Mid-rib vascular bundle (MRVB) (μ)		No. of xylem vessels in MRVB	Vessel diameter in MRVB (μ)
			Length	Width		
Control	466.4 ^d	233.2 ^c	160.7 ^c	85.6 ^c	7.2 ^c	20.1 ^b
CYE 3 gL ⁻¹	497.8 ^{cd}	254.4 ^{bc}	195.8 ^{bc}	120.0 ^b	7.8 ^{bc}	19.8 ^b
CYE 6 gL ⁻¹	530.6 ^c	284.1 ^{bc}	232.0 ^{ab}	112.6 ^{ab}	8.4 ^b	21.2 ^b
YEPF 3 gL ⁻¹	603.0 ^b	307.4 ^{ab}	240.4 ^{ab}	127.2 ^a	10.3 ^{ab}	24.1 ^a
YEPF 6 gL ⁻¹	762.8 ^a	339.8 ^a	268.6 ^a	133.4 ^a	11.6 ^a	25.4 ^a
Significance at P \leq 0.05	*	*	*	*	*	*

* Different small letters within the same column indicate significant differences between means at P \leq 0.05 according to DMRT.



(a)



(b)

Fig (1): Cross section in *S. marianum* leaf blade as affected by YEPF at 6 gL⁻¹ (b) compared to untreated control (a); x 40.

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تأثير المستخلص الخام للخميرة مقارنة بجزء المستخلص المحتوى على السكريات العديدة على النمو، محصول البذور، محتوى السليمارين و التركيب التشريحي للورقة في نبات *Silybum marianum* (L.) Gaertn.
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أجريت الدراسة بهدف مقارنة تأثير المستخلص الخام للخميرة (CYE) مقارنة بجزء المستخلص المحتوى على السكريات العديدة (YEPF) على النمو، محصول البذور، محتوى السليمارين و التركيب التشريحي للورقة في نبات *Silybum marianum* (L.) Gaertn. عوملت النباتات بأى من صور المستخلص بطريقة الرش الورقى بمستويين هما ٣، ٦ جم/لتر وعوملت نباتات المقارنة بالرش بالماء المقطر. ولقد أوضحت النتائج أن CYE كان الأكثر تأثيراً في زيادة النمو الخضري بينما كان YEPF هو الأكثر فعالية في تحسين النمو الثمرى حيث كانت الزيادة في محصول البذور/النبات أعلى في حالة YEPF عنه في حالة CYE. ورغم زيادة محتوى السليمارين في أعضاء النبات نتيجة المعاملة بكل من CYE و YEPF، إلا أن الزيادة كانت أعلى تحت تأثير YEPF. ولقد أظهرت النتائج أيضاً أن YEPF هو الأفضل تأثيراً في تنشيط تكشف أنسجة البناء الضوئى و الأنسجة الناقلة في الورقة. ولقد تمت مناقشة آلية تأثير كل من CYE و YEPF على النمو، محصول البذور و محتوى السليمارين في النباتات. وعلى ضوء النتائج المتحصل عليها يمكن إستنتاج إمكانية استخدام مستخلص الخميرة كمحفز حيوى في ظروف النمو الطبيعية *in vivo* و أن إستخدام YEPF يفضل على CYE لزيادة محصول البذور و لتحفيز التخليق الحيوى للسليمارين في النباتات.