Enhancement of biochemical properties of natural sweetener of *Stevia rebaudiana* by using Abscisic acid and pacloputrazol.

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ABSTRACT

***Stevia Rebaudiana Bertoni* is a medicinal plant and commercially used as a non-caloric sweetener for diabetic patients. In the present study, a protocol was developed for *in vitro* propagation using 6-benzylamino purine (BAP) and Kinetin (Kin) for multiple shoot proliferation. For roots induction, we used Indole-3-butyric acid (IBA) and 1- Naphthaleneacetic acid (NAA). This study was conducted to investigate the antioxidant activity, phenolic compound and flavonoids in water extracts taken from leaves and stems, as well as products nutritive values (carbohydrates, protein, fat and crude fiber). The study also was conducted to produce low caloric sweeteners from *in vitro* leaves extract on quality and texture characteristics of food product (biscuit) At multiplication stage, was observed on Murashige and Skoog (MS) medium supplemented with 1.5 mg/l BA while, 1.0mg/l Kin induced the highest leaves number and longest shoots (55.33 and 7.83cm, respectively). The maximum number of roots (8.66 roots per explants) was obtained on a MS medium containing 2.0 mg/l IBA. At rooting stage, maximum number of roots was obtained with MS medium contained 2.0 mg/l IBA. The longest roots, leaves number, and plant length was recorded with MS contained 2.0mg/l NAA (10.00 cm, 25.80, and 10.66 cm, respectively). The well-rooted plantlets were successfully and acclimatized in the greenhouse with a survival rate of 96 %. Further, stevia leaves are a good source of carbohydrates(72.23% at 0.25 mg/l ABA), proteins (17.04 at 1mg/l ABA)and fats contents (12.45 at 1mg/l ABA). The highest content of crude fiber from the stem (39.31 at 0.25mg/l ABA). Better sensory characteristics scores and acceptability were given to biscuit by using 2.5and 5.0% leaves extract. Stevia as a sucrose replacement at (2.5 or 5.0%) had a better impact on Biscuit physical quality (Texture, color, taste, and general acceptability). So, stevia produces a variety of high potential natural-source and low-calorie sweetener. These results strongly suggest that due to its all favorable properties, stevia could be used in either food or cosmetic and pharmaceutical products.**

Key words: *Stevia rebaudiana* Bertoni, Non-caloric sweetener, *In vitro*, Baking, Rooting, .

1. INTRODUCTION

Stevia is a genus of approx. Two hundred species of herbs and shrubs from the family *Astraceae*. One of the representatives of the genus i1s sweetleaf *Stevia rebaudiana*, formerly called *Eupatorium rebaudianum* Bertoni **(Yadav, 2011).** Stevia is becoming a prevalent one among various medicinal plants for the treatment of different diseases over the world. **(Huxley, 1992). N**atural sweetener Stevia is a good source of proteins, carbohydrates, and fiber. It has both economic and medicinal importance. Stevia plant used as traditional herbal remedies for diabetes among many diseases around the world. The leaves have been known to contain 100 useful alkaloids, among other pharmacologically active compounds which can be used for the treatment of diabetes. It has particular importance to diabetic persons and diet conscious people, i.e., its products can be used as a substitute for artificial sweeteners for diabetic patients. Its other medicinal uses include regulating blood sugar, preventing hypertension and tooth decay as well as treatment of skin disorders. Stevia is helpful in weight and blood pressure management. Stevioside, the bioactive compound in its leaves, tastes about 300 times sweeter than sucrose and used as a sweetening agent in the industrial sector and is commercially relevant. Stevia is regenerated as a valuable natural sweetening agent because of its relatively good taste and chemical stability **(Yamazaki, 1991).** Products can be added to tea and coffee, cooked or baked goods, processed foods and beverages, fruit juices, tobacco products, pastries, chewing gum and sherbets. Stevia had been used for removing the bitter taste of medicinal plants **(Brandle and Rosa, 1992).** Conventionally, it is cultivated by seeds or stem cutting, but seed viability rate is poor. Due to poor seed viability and low germination rate, the common method of propagation by seed is restricted. Vegetative propagation by stem cuttings is also limited by the low number of individuals that can be obtained simultaneously from a single plant. Plant tissue culture is a suitable approach for micropropagation and the production of valuable secondary metabolites of plants. Propagation through tissue culture can be ideal as an alternative method to obtain a sufficient number of plants within a short period **(Ibrahim *et al.*, 2008).** The induction of multiple shoots from nodal segments of stevia was the highest in MS medium supplemented with 1.0 mg l-1 BA **(Nower, 2014).** Stevia plants are a good source of carbohydrates, protein, and crude fiber content. In this order, stevia leaves also contained on a dry weight basis for total soluble carbohydrates (**Khiraoui *et al.*,2017)**. **Hemada, *et al.*, 2016** recommended that sativa extract (as natural sweeteners) can be used as a sucrose substitute in different food products (cake, biscuit, and Jam). The aim of this study is the enhance and evaluates the biochemical characteristics by using abscisic acid and paclobutrazol on stevia rebaudiana. Also, using stevia extracts as high potential natural-source and low-calorie sweetener to make biscuits.

MATERIALS AND METHODS

Plant material and explants preparation:

Stevia plants were collected from the farm of the Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt. Shoot tips were cut and collected for sterilization described as **(Urbi and Zainuddin 2015).**

Culture medium and condition

(Murashige and Skoog 1962) MS basal medium with 30 gm /l of sucrose was used and solidified using 7.5 gm /l of agar. MS medium was supplemented with various concentrations of plant regulators such as 6-benzylamino purine (BAP), Kinetin (KN) for shoots production, Indole3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA) for roots induction. The pH of the media was adjusted to 5.75 – 5.80 before adding the agar and then autoclaved at 120°C for 20 min. All the cultures were incubated in a growth room at 25 ± 2°C with a photoperiod 16 h light and 8 h dark with 2000 lux of light intensity.

Shoot proliferation and root formation

For shoot proliferation, the explant (3 shoots/jar350 ml) were inoculated on the MS medium, contained different concentrations of BAP and KN at 0,0.5, 1.0, 1.50 and 2.0 mg/l. The cultures were kept in a growth chamber at 25 ± 2ºC, with 16 hours photoperiod and 2000 lux of light intensity. Data were recorded after three months (subcultures) as shoots number, leaves number, shoots length (cm), fresh weight, and dry weight. The regenerated isolated shoots were used as explants for root induction. Micro shoots of about 2-3 cm in length were excised and cultured on MS medium supplemented with IBA and NAA at 0.00, 0.50, 1.0, and 2.00 mg/l. Data recorded after one month as roots number, root length (cm), leaves number, and plant length (cm). The well-rooted plantlets were acclimatized in plant soil after two months.

Studying the effect of abscisic acid or paclobutrazol on *in vitro* shoots preservation and some chemical compositions.

In this experiment, the effect of added growth retardants: abscisic acid (ABA) or paclobutrazol (PBZ) at concentrations of 0.0, 0.25, 0.5, 0.75, 1 or 1.25 mg/l onto MS medium for preservation of shoots. Cultured shoots were incubated at 25ºC day and night temperature under 1000 lux light intensity. Shoots were cultured in 350 ml jars containing 60 ml of medium. The data were calculated three months month on shoots number; leaves number and shoot length (cm).

Chemical composition of raw material.

Moisture, ash, protein, fat, and crude fiber contents were determined according to the methods described by the **A.O.A.C. (2012).**

Total carbohydrates.

Total carbohydrates were calculated by differences according to **FAO (2003)** using the following equations: %Total carbohydrates = 100 – (moisture + ash + fat + protein).

Biscuits prepared with different levels of stevia extract.

From previous experiment explants were taken leaves and extracted described as **(Jayaraman, *et al.,* 2008)**. The Stevia extracts were used as sucrose replacement at different levels (0, 2.5, 5.0, 7.5 and 10.0 %), the amount of liquid (milk and/or water) in the standard recipe was modified to keep the amount of total liquids and sugar in balance Table (1). The substitution varies somewhat between different recipes biscuit for producing better characteristics. Biscuit were prepared as the procedures of **(Penfield and Camphell 1990)** with some modifications: the developed dough was kept in the refrigerator for 10 min., then flattened manually using wooden roller to about ½ cm. thickness and cut into spherical pieces and baked in oven at 180 ºC.

Table (1). Ingredients and methods used for biscuit preparation.

Ingredients	Treatments				
	Control	2.5 %	5%	7.5%	10%
Flour (g)	250	250	250	250	250
Sugar (g)	100	0	0	0	0
Stevia extract (ml)	0	60	60	60	60
Butter (g)	100	100	100	100	100
Ammonium bicarbonate (g)	0.33	0.33	0.33	0.33	0.33
Egg (whole, fresh) (g)	2	2	2	2	2
Vanilla	0.3	0.3	0.3	0.3	0.3

Organoleptic evaluation of biscuits

Texture, color, taste, odor, and overall acceptability were carried out by the aid of ten panelists. Judging score for each factor was as follows; 8 – 9 very good; 6 – 7 good; 4 – 5 fair; 2 – 3 poor and 0 – 1 very poor **(Molander, 1960).**

Data Analysis

The randomized factorial design was used, and data were subjected to analysis of variance.

Separation of means among treatments was determined using the L.S.D. test at 5% (Snedecor, and Cochran 1989).

RESULTS AND DISCUSSION

Effect of different cytokinin concentrations on the proliferation of *Stevia rebaudiana.*

In vitro multiplication of plants, the use of cytokinins in the multiplication medium is essential, this hormone enhancing cell division and *in vitro* proliferation of shoot tips. The highest shoots production was found in the MS medium supplemented with 1.5 mg/l BAP (72.67) after three subcultures of cultivation. Kin was less effective at inducing multiple shoots compared to BAP, while effective to formation the highest leaves number (55.33) at 1mg/l than other treatments, as shown in Table (2). At the same time, the lowest fresh and dry weight was recorded with all concentrations of Kin and control. *In vitro* propagation of *Stevia*, fresh and dry weight was affected by cytokinin concentration Fig. (1). The result was agreement according to **(Sivaram and Mukudan 2003);** the higher concentration of BA resulted in decreasing multiple shoots formation of stevia**. Tadhani *et al.*, (2006)** the maximum number of shoots were achieved on MS medium supplemented with 0.6 mg/l of BAP. However, the micro shoots inoculated in the MS medium supplemented with 1.0 mg/l Kin produced the longest shoots lengths (7.83cm) as shown in Table 1 and Fig. 1 **Das *et al.* (2011)** also demonstrated the longest shoots lengths of stevia when Kn was present in the medium. **Laribi *et al*., (2012)** showed that BAP (1mg /l) and IAA (0.25mg/l) combination was superior for multiple shoot bud induction (4.25 shoots). The highest shoot multiplication (7.33 shoots/explant) was found on MS medium supplemented with 1.0 mg/l BAP+ 0.5 mg/l Kin **(Singh *et al.*, 2017)**. **Thilakarathne, *et al.*, (2019)** showed that 1.0mg/l Kin resulted in the highest number of stevia shoots (11.8shoots).

Table 2: Effect of different concentrations of PGRs on proliferation of *Stevia rebaudiana* after three subcultures from cultured.

Cytokinin Conc. mg/l	Shoots no.	Leaves no.	Shoot length (cm)	Fresh weight (g)	Dry weight (g)
Control	35.00	17.33 CD	5.63 BC	2.81 C	0.34 B
0.50 mg/l BAP	45.66	18.00 C	2.60 E	7.61 B	0.82 B
1.0 mg/l BAP	65.66	17.33 CD	3.06 DE	7.61 B	0.92 B
1.5 mg/l BAP	72.667 A	11.67 D	2.86 DE	9.67 A	1.107 A
2 mg/l BA	44.00	12.67 CD	1.56 E	6.80 B	0.84 B
0.50 mg/l Kin	48.33	38.67 B	4.90 CD	0.52 C	0.06 d
1.0 mg/l Kin	56.66	55.33 A	7.83 A	0.89 C	0.08 d
1.5 mg/l Kin	64.33	52.00 A	7.06 A	0.80 C	0.06 d
2.0 mg/l Kin	48.33	36.67B	7.10 AB	0.99 C	0.17 c
L.S.D at 5%		**6.228**	**2.089**	**2.466**	**0.431**

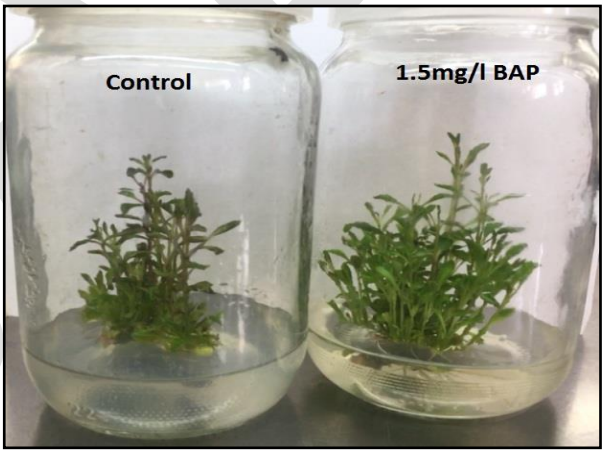

Fig. (1): *In vitro* propagation and development stevia shoots.

Effect of different concentrations of NAA and IBA, each alone, on root formations.

This experiment was carried out to determine the influence of various auxin concentrations on the growth and development during rooting stage estimated as root number, root length leaves number and plant length. Thus plantlets were cultured on MS basal nutrient medium supplemented with (0.0, 0.5, 1.0 and 2.0 mg/l IBA or NAA. The highest roots number was affected by auxins concentrations. IBA

concentrations stimulated root induction comparing to NAA concentrations as presented in **Table (3)** 2.0mg/l IBA recorded the best results for root number (8.66). Data also recorded the longest root; leaves number and plant length were obtained with 2.0mg/l NAA (10.00 cm, 25.80 and 10.66 cm, respectively) **Fig.(2).** Treatments of NAA surpassed the IBA treatments in increasing the root length, leaves number and plant length and gave survival 94% from adaptation in greenhouse **Fig. (3). Singh *et al.*, (2017)** showed that the root of stevia induction was performed on MS basal medium supplemented with different concentration of IAA, IBA and NAA.

Table (3): Effect of different concentrations of NAA and IBA on root induction after one month from cultures.

Auxin conc. mg/l		Roots no.	Roots length (cm)	Leaves no.	Plant length (cm)
IBA	**0.0**	4.00	4.00	11.00	8.33
	0.5	6.66	6.20	14.00	6.00
	1.0	6.66	8.16	17.66	9.00
	2.0	8.66	7.00	18.66	7.00
NAA	**0.5**	4.00	7.83	15.00	7.66
	1.0	5.00	8.16	20.00	8.66
	2.0	4.00	10.00	25.80	10.66
LSD at 5%		**1.373**	**1.463**	**3.061**	**1.415**

Fig. (2): *In vitro* induction and development stevia roots on MS medium supplemented with 2mg/l IBA.

Fig. (3): Development of stevia in greenhouse after two months.

Effect of different concentrations of ABA or PBZ on conservation of *Stevia* shoots.

Growth retardants add to the medium significantly influenced some morphological parameters at the conservation of shoots after three months from storage. ABA at 0.50mg/l increasing the shoots number, leaves number and shoot length (cm) than other treatments **Table (4).** At higher concentrations of paclobutrazol, it caused negative effects such as burned leaves and browning**. Pateli *et al.*, (2004)** reported that the effect of increasing paclobutrazol concentration on Epidendrum radicans caused a small increase in leaf thickness by 17-37 %. **Swamy and Smith (2005)** showed that the important role of ABA is to inhibit auxins, cytokinins, and gibberellins. These hormones enhance growth, cell division and cell elongation in plants Abscisic acid (ABA) at 2 mg/l affected to suppress shoot growth of mangosteen and longkong more than paclobutrazol **(Keatmetha, *et al.*, 2006).**

Table (4): Effect of different concentrations of ABA and PBZ on conservation of Stevia shoots

Growth retardants conc.mg/l		Shoots no.	Leaves no.	Shoot length (cm)
	C	10.33	23.00	5.17
ABA	**0.25**	6.33	23.33	4.80
	0.50	8.97	51.75	6.85
	0.75	7.33	45.00	5.73
	1.0	6.76	42.00	5.53
PBZ	**0.25**	5.33	30.33	5.30
	0.50	5.00	21.65	4.13
	0.75	4.00	12.65	3.60
	1.0	3.00	11.00	3.70
LSD at 5%		**1.077**	**6.970**	**1.268**

Fig. (4): Conservation of stevia on growth retardants after three months.

Effect of abscisic acid and paclobutrazol on carbohydrates content of stevia leaves and stems.

The proximate **carbohydrates** composition of dried *Stevia rebaudiana* leaves and stems is shown in Table 4.The highest value of carbohydrates content was achieved at 0.50, 0.75 and 1 mg/l PBZ than other treatments were (65.15, 66.44 or 66.00) respectively, as shown in Table 4. Leaves extract, recorded the highest value of carbohydrates compared to stems extract. From the same Table, there are highest significant differences on carbohydrates content with leaves and stems extract at 0.50 or 0.75 mg/l PBZ than other treatments. Carbohydrates are the main sources of energy and they are found as structural components of cellular elements **(Lemus-Modaca *et al.*, 2012**). Carbohydrate contents in dry leaves of sweetleaf ranged from 35.2 to 61.9 g·100 g/l product **(Abou-Arab *et al.*, 2010; Boonkaewwan *et al.*, 2006).** Stevia leaves are a good source of carbohydrates, protein, and crude fiber, which are the essential factors for the maintenance of health **(Khiraoui *et al.*, 2017)**.

Table (4): Effect of abscisic acid and paclobutrazol on carbohydrates content of leaves and stems of stevia plant.

Growth retardants conc.mg/l		Carbohydrates		Mean
		Leaves	Stem	
Control		56.59	44.67	**50.63^{e}**
ABA	**0.25**	65.77	47.95	**56.86^{c}**
	0.50	59.50	48.78	**54.14^{d}**
	0.75	58.99	46.05	**52.52^{de}**
	1.0	52.54	41.93	**47.24^{f}**
PBZ	**0.25**	72.23	51.73	**61.98^{b}**
	0.50	69.94	60.37	**65.15^{a}**
	0.75	69.11	63.76	**66.44^{a}**
	1.0	**68.09**	**63.91**	**66.00^{a}**
Mean		**63.64^{a}**	**52.13^{b}**	
LSD at 5% A= B= AxB=		**2.044 0.9638 2.891**		

Means within a column showing the same letters are not significantly different (P≥ 0.05).
Means within a row showing the same letters are not significantly different (P≥ 0.05).

Effect of abscisic acid and paclobutrazol on crude fiber content on dry basis in leaves and stems of stevia plant.

The results obtained by the biochemical analysis of dried stevia explants are presents in **Table 5,** show the effect of abscisic acid and paclobutrazol (mg/l) on crude fiber content in leaves and stems of stevia extract. There is a significant difference between control and other treatments. Stems extract, recorded the highest value of crude fiber than leaves extract. Interaction between explant type and concentration of ABA and PBZ has highest significant effect 39.31%. In this context, stevia crude fiber varies according to

(Gasmalla, *et al.*, 2014, Ruiz-Ruiz, *et al.*, 2015).

Table (5). Effect of abscisic acid and paclobutrazol on crude fiber content on dry basis in leaves and stems of *Stevia* extract.

Growth retardants conc.mg/l		Crude fiber		
		Leaves	Stem	Mean
Control		18.50	39.31	**28.90^{a}**
ABA	0.25	12.15	31.42	**21.79^{b}**
	0.50	10.40	28.86	**19.63^{c}**
	0.75	7.707	29.55	**18.63^{cd}**
	1.00	9.493	28.18	**18.84^{cd}**
PBZ	0.25	10.49	28.61	**19.55^{cd}**
	0.50	7.587	19.17	**13.38^{f}**
	0.75	13.45	19.70	**16.58^{e}**
	1.00	15.44	21.51	**18.47^{d}**
Mean		**11.69^{b}**	**27.37^{a}**	
LSD at 5% A= B= AxB=		1.122 0.5287 1.586		

Means within a column showing the same letters are not significantly different (P≥ 0.05).

Means within a row showing the same letters are not significantly different (P≥ 0.05).

Effect of abscisic acid and paclobutrazol on protein content on dry basis in leaves and stems of *Stevia* plant.

The chemical composition of stevia leaves or stems, changes depending on abscisic acid and paclobutrazol (mg/l) concentrations. Abscisic acid and paclobutrazol (mg/l) are enhancement of protein content in leaves and stems of stevia extract. There is a significant difference between treatments, the high value of protein content in stevia extract was achieved at 1 mg/l of ABA was 13.20% Table (6). Extract of stevia leaves showed the highest value (11.83%) of protein compared with stem extract. The highest values of protein content obtained with control and leaves extract at 0.75 or 1.0 mg/l ABA than other treatments. In this context, stevia crude fiber varies according to (**Pence *et al.*, 2005)** during culturing shoots with ABA, plants still engage in photosynthesis that causes the synthesis of storage proteins and lipids. These give rise to the best growth after recovery**.** Stevia has been found to be very impressive in protein content representing as a good source for the development of body structure and various physiological functions **(Chughtai *et al.*, 2019)**.

Table (6). Effect of abscisic acid and paclobutrazol on protein content on dry basis in leaves and stems of *Stevia* extract.

Growth retardants conc.mg/l		Protein		Mean
		Leaves	Stem	
Control		15.72	7.957	**11.84^{b}**
ABA	0.25	13.61	8.610	**11.11^{c}**
	0.50	12.54	6.900	**9.720^{d}**
	0.75	15.10	9.347	**12.23^{b}**
	1.00	15.04	11.36	**13.20^{a}**
PBZ	0.25	8.810	7.783	**8.297^{e}**
	0.50	10.93	6.953	**8.940^{e}**
	0.75	7.000	7.053	**7.027^{f}**
	1.00	7.730	6.973	**7.352^{f}**
Mean		**11.83^{a}**	**8.104^{b}**	
LSD at 5% A= B= AxB=		0.6822 0.3216 0.9647		

Means within a column showing the same letters are not significantly different (P≥ 0.05).
Means within a row showing the same letters are not significantly different (P≥ 0.05).

Effect of abscisic acid and paclobutrazol on fat content on dry basis in leaves and stems of *Stevia* extract.

Fats are the most concentrated form of energy for the body (37 kJ/g), helping in the absorption of fat-soluble vitamins (A, D, E, and K). The results are expressed in Table 7, show the effect of abscisic acid and paclobutrazol (mg/l) on fats content in leaves and stems of stevia extract. The highest value of fats content in stevia extract was achieved at 1 mg/l of ABA (10.01%) than other treatments. From the same Table, there was a significant difference between leaves and stems extract, and the leaves extract gives a good course of fats than stems extract. While there is a significant difference between ABA at (1.0 mg/l) 12.45% than all treatments.

Recently, **(Lemus-Mondaca, *et al.*, 2016)** have worked on the stevia dried from different techniques and investigated the fatty acid profile of stevia. Fat content in dry matter of stevia leaves amounts to 1.9 - 4.34 g/100 DW **(Abou-Arab *et al.*, 2010; Siddique *et al.*, 2014).**

Table (7). Effect of abscisic acid and paclobutrazol on fat content on a dry basis in leaves and stems of *Stevia* extract.

Growth retardants conc.mg/l		Fat		Mean
		Leaves	Stem	
Control		4.227	2.473	**3.350** ^{e}
ABA	**0.25**	4.283	4.470	**4.377**^{d}
	0.50	9.087	5.243	**7.165**^{b}
	0.75	8.173	6.100	**7.137** ^{b}
	1.0	12.447	7.580	**10.01** ^{a}
PBZ	**0.25**	4.597	9.087	**6.842** ^{b}
	0.50	5.937	8.173	**7.055**^{b}
	0.75	6.440	4.150	**5.295**^{c}
	1.0	5.763	3.263	**4.513** ^{cd}
Mean		**6.773**^{a}	**5.616**^{b}	
LSD at 5% A= B= AxB=		**0.9089 0.4284 1.285**		

Means within a column showing the same letters are not significantly different (P≥ 0.05).
Means within a row showing the same letters are not significantly different (P≥ 0.05).

Table (8). Effect of abscisic acid and paclobutrazol on ash content on a dry basis in leaves and stems of *Stevia* plant.

Growth retardants conc.mg/l		Ash		Mean
		Leaves	Stem	
Control		5.310	5.587	**5.448**^{c}
ABA	**0.25**	4.190	7.543	**5.867**^{bc}
	0.50	8.510	8.450	**8.48**
	0.75	7.770	9.850	**8.81**
	1.0	10.54	10.22	**10.38**
PBZ	**0.25**	5.327	6.153	**5.740**^{bc}
	0.50	6.460	7.223	**6.842**^{b}
	0.75	4.817	5.707	**5.262**^{c}
	1.0	2.973	4.007	**3.490**^{d}
Mean		**6.211**^{b}	**7.193**^{a}	
LSD at 5% A= B= AxB=		**1.314 0.6195 1.859**		

Means within a column showing the same letters are not significantly different (P≥ 0.05).
Means within a row showing the same letters are not significantly different (P≥ 0.05).

Effect of abscisic acid and paclobutrazol on ash content on a dry basis in leaves and stems of *Stevia* plant.

Chemical composition of dried stevia leaves and stems indicated in Table 8. From such data, it could be noticed that total ash was recorded the highest value of 10.38%, at 1.0 mg/l ABA than other treatments. From the same Table, there was a significant difference between leaves and stems extract, and the stems extract that gives the highest ash content than stems extract. ABA at 1.0 mg/l recorded a maximum of ash content with leaves and stems extract were (10.54 or 10.22, respectively) than other treatments. It is reviewed by **Marcinek and Krejpcio (2015)** the chemical composition of stevia leaves changes depending on the degree of their processing.

Preparation of biscuit using extraction of *Stevia.* Sensory properties.

Sensory properties of a food product are regarded as one of the most crucial attributes since they are most noticeable by the consumer **(Singham, *et al.*,2015).**Sensory evaluation refers to the scientific technique of invoking, computing, analysing, and interpreting the responses by the perception of senses (**Sidel and Stone 1993)**. The market success, a product must have the desired sensorial. As described in **Table 9** showed biscuit prepared with addition of stevia leaves extract, no significant differences was found among the prepared samples at (control, 2.5 or 5.0% of stevia leaves extract) compared to other treatments evaluated characteristics (texture, taste, odor and overall acceptability) except biscuit color of sensory evaluation, similar findings were observed by (**Yaseen, *et al.* 2005).** However, there are significant effect was observed on the texture, color, taste, odor and over all acceptability at 7.5 or 10% of stevia leaves extract, similar results agreement with (**Giri, and Rao, 2012)**. Sativa extract (as natural sweeteners) can be used as a sucrose

substitute in different food products (cake, biscuit, and Jam) **(Hemada, *et al.*, 2016)**.

Stevia leaves are an excellent source of diterpene glycosides stevioside, rebaudioside A-F, dulcoside, and steviolbioside, which are responsible for sweetness and have been utilized commercially for sugar substitution in foods, beverages, and medicines **(Gandhi *et al.*, 2018).** The leaves and extract of Stevia plant are used to sweeten different food **(Chughtai, *et al*., 2019).**

Table (9): Effect of *Stevia* on sensory properties of biscuit product.

Treatments	Texture	Color	Taste	Odor	Overall acceptability
control	8.63[a]	9.03[a]	8.59[a]	8.63[a]	8.69[a]
2.5g dried stevia/100 ml	8.52[a]	8.02[a]	8.54[a]	8.58[a]	8.54[a]
5g dried Stevia/100 ml	8.51a	7.16[c]	8.51[a]	8.56[a]	8.55[a]
7.5g dried stevia/100 ml	5.94[c]	6.31[d]	6.56[bc]	8.13[b]	6.75[c]
10g dried stevia/100 ml	5.44[d]	5.38[e]	6.59[c]	8.06[b]	6.25[d]
LSD at 0.05	**0.48**	**0.48**	**0.68**	**0.44**	**0.46**

Fig. (5): Biscuits prepared with different levels of *Stevia* extract.

CONCLUSION

Stevia leaves powder extract are high intensity nonnutritive natural health-promoting sweetener. Stevia has been found as a good source of nutritional constituents and functional properties for value addition.

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