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Comparative Analysis of Bioactive Compounds of *Balanites Aegyptiaca* L. Callus

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Abstract

Balanites (*Balanites aegyptiaca* L.), also known as desert palm or heglig, is a critically endangered plant and an endemic agroforestry species in Egypt. It is a member of the Balanitaceae family. *Balanites aegyptiaca* is a species with a wide range of applications in different countries, including medicinal, charcoal, pesticides, and forage, and *in vitro* callus production is critical for many applications in both basic and industrial research on this species. Plant tissue culture has numerous advantages for the potential synthesis of bioactive plant metabolites.

Culturing leaves on full strength Murashige and Skoog (MS) medium supplemented with 2.50 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) resulted in a green compact callus. Callus cultures were grown on MS media with various concentrations of 2, 4-D (1.00, 1.50, 2.00, 2.50, and 3.00 mg/L), as well as the cytokinins 6-benzylaminopurine (BAP) and Kinetin (6-furfurylaminopurine) (Kin) at 0.5 mg/L. The maximum fresh weight of callus was obtained using MS medium containing 2.5 mg/L 2,4-D plus 0.5 mg/L BAP, as well as the highest percentage of increase in fresh weight, which was 300 %.

Salicylic acid and chitosan were used as elicitors for secondary metabolites accumulation in callus culture. Chitosan (40 mg/L) increased quercetin 2.6 folds and kaempferol 8.5 folds rise after 45 days of culture. However salicylic acid enhanced the maximum accumulation of total phenols in (0.2 mg/L). The dramatic decrease in accumulation of coumarin may give rise to the consuming of such precursor in flavonoids biosynthetic pathways. Chitosan as an elicitor enhanced the biosynthesis of fucose sugar in (40 and 80 mg/L) and this may have antioxidant and anticancer properties.

The usefulness of salicylic acid and chitosan as elicitors for increasing *in vitro* production of secondary metabolites in plants is demonstrated in this work. These findings could point to the important role of plant callus culture which would play a potential role in the future of the phytopharmaceutical sector.

Keywords: Antioxidant capacity, *Balanites aegyptiaca*, chitosan, coumarin, elicitors, *in vitro* production, salicylic acid

1. Introduction:

The desert palm, or heglig, *Balanites aegyptiaca* (L.) Del., is a member of the Balanitaceae family (**Orwa et al., 2009**). It is a versatile evergreen prickly tree species that may be utilized as fodder, charcoal industry wood, timber, fuel wood, and many other raw materials (**Von Maydell, 1984**). The oil extracted from the tree's seeds is now used in a variety of sectors, including soap, shampoo, cream, herbal medicine, and even biodiesel production (**Charity et al., 2018; Linda et al., 2018; Naik and Balakrishna, 2018; Montasser et al., 2017**).

Balanites aegyptiaca (BA) is a wild plant that is popular in Egypt. It is regarded as the Date of the Desert. It includes 1.5% protein and 37% sugars, as well as 15% organic acids in the fruit mesocarp. The phytochemical composition of the various sections of the plant shows that, in addition to fatty acids and sterols, it contains high concentrations of saponins and moderate amounts of tannins, flavonoids, and cardiac glycosides **(Abdelaziz et al., 2020).**

Plants play an important role in the discovery of new medicinal products for drug development. Many African countries have recently conducted extensive research on medicinal plants for the treatment of various diseases and conditions, such as diabetes, malaria, anaemia, and cancer. Medicinal plants are more appealing as therapeutic agents in Sub-Saharan Africa due to their availability and lower cost when compared to'modern' pharmaceuticals (**Agbor et al., 2005**). Medicinal herbs are still a valuable source of pharmaceuticals that are safe, less poisonous, less expensive, readily available, and trustworthy all over the world.

Medicinal plants have long been utilized as traditional medicines and folk medicines around the

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world to cure and prevent a variety of human ailments. Medicinal plants, such as phenolics, flavonoids, stilbenes, tannins, terpenoids, and alkaloids, have therefore been recognised as key sources of novel biologically active secondary metabolites. For the generation of useful therapeutic molecules from plants, biotechnological technologies, particularly plant tissue culture, are critical. Because of the commercial value of these secondary metabolites, there has been a lot of interest in looking into ways to improve their production using tissue culture techniques (**Tiwari and Rana, 2015**).

As a medicinal plant, *Balanites aegyptiaca* has antimicrobial, antioxidant, anti-diabetic, antiasthmatic, and other properties. Pests, mollusks, and larvae were found to be toxic to them. In their callus culture, they have pharmacologically active substances such as flavonoids and saponins. Their anti-inflammatory properties have been known since antiquity. The plant has potentially useful applications in drug development and research (**Gajalakshmi et al., 2013**).

Secondary metabolite biosynthesis in plants can be induced by precursors and elicitors, which is influenced by environmental stresses (**Zhao et al., 2010**). Precursors are intermediates in secondary metabolite biosynthesis that, if not used at the proper time or concentration, can be harmful to the culture (**Gueven and Knorr, 2011**). The leaf, root, node, stem, petiole, shoot tip, embryo, and flower bud are all good candidates for callus culture. With young vegetative organs, callus induction is more effective. Explant source is one of the most important characteristics for successful long-term cell culture, according to (**Krul and Mowbray, 1984**). The type of explant also had an effect on callogenesis (**Zouzou et al., 2008**).

Coumarins are phenolic compounds with fused benzene and pyrone rings that possess anti-inflammatory, anticoagulant, antibacterial, anticancer, antioxidant, and neuroprotective properties (**Venugopala et al., 2013**).

Saponins, alkaloids, flavonoids, tannin, phenol derivatives, and terpenoids have been discovered in the phytochemical elements of medicinal plants such as *Balanites aegyptiaca*, and have been shown to have hypoglycemic and hypolipidemic effects (**Tanko et al., 2007**).

L-fucose is a 6-deoxy hexose with the L-configuration that can be found in a wide range of species. It is an endogenous hexose-deoxy-sugar that is one of the 8 necessary monosaccharides for humans. This uncommon sugar plays a role in a variety of biological processes. Several naturally occurring oligo- or polysaccharides contain L-fucose as a building block. For ages, humans have consumed a large amount of this monosaccharide. This sugar can be found as a free monosaccharide or as part of macromolecules that are covalently bonded to other sugars or proteins that humans can digest **(Schneider and Co-workers, 2017).**

Plant cell and tissue culture technology has a long history of being used to produce therapeutic compounds (**Rout et al., 2000; Verpoorte et al., 2002**). Researchers have attempted to utilize plant cell biosynthetic capacities for getting valuable chemicals and researching the metabolism of plants since plant cell and tissue culture arose as a specialty within plant biology (**Misawa, 1994; Verpoorte et al., 2002**). Interest in chemopreventive plant natural compounds has exploded in recent decades. Oxidative stress has been linked to the aetiology of a number of degenerative and aging-related disorders, and several researches have been conducted to find the most efficient antioxidants (**Halliwell, 1995; Aruoa, 2003; Soobrattee et al., 2005**).

Chitosan is a natural antibacterial compound that is safe and affordable and has been used in a variety of pharmaceuticals, foods, and cosmetics. Chitosan is also used in agriculture to promote plant development, increase crop yield, and prevent plant disease. Plants can respond to chitosan by producing more phytoalexin and reactive oxygen species (ROS) as a stress reaction. Low amounts of ROS operate as signal molecules, initiating a series of physiological processes ranging from gene expression to secondary metabolite formation, whereas high levels of ROS destroy membranes **(Kamalipourazad et al., 2016)**.

Plants' biological and biochemical processes are known to be affected by salicylic acid, which may play a vital role in controlling their development and production. salicylic acid (SA) may operate as a stressor or give protection against certain biotic and abiotic stresses, depending on its concentration **(Horváth et al., 2007).**

The goal of this work was to increase secondary metabolites production in callus culture of *Balanites aegyptiaca* utilizing (SA) and chitosan (CH) as bio-elicitors. Further, comparing the contents of bioactive ingredients between aerial parts for further future studies.

Experimental

Samples Collection

Balanites aegyptiaca was obtained from a wild population in New Valley, and healthy explants (leaves) were separated from the mother plant. The samples were collected in a clean nylon bag and then transported to the Desert Research Center's Plant Tissue Laboratory, Plant Tissue Culture Lab, Plant

Genetic Resources Department, Desert Research Center.

1. Collection of plant material and surface sterilization:

Healthy explants (leaf) of *Balanites aegyptiaca* were surface sterilized for 1 minute with 90 % clorox and then washed three times with sterilized distilled water. Washed explants were soaked in 10 % sodium hypochlorite (NaOCl) for 10 minutes containing a few drops of Tween-20 to break the surface tension of the water and facilitate the cleaning of external contaminants adhering to the surface of the leaves, followed by washed 3-4 times with sterile double distilled water.

2.1. Callus induction

Young leaves were sectioned into (1-2) cm sections and cultured on Murashige and Skoog's 1962 (MS) medium containing 3% sucrose and gelled with 2.7g/l phytagel supplemented with varying concentrations of 2, 4-D (2, 4-dichlorophenoxyacetic acid) at (0.0, 0.50, 1.00, 1.50, 2.00, 2.50, and 3.00 mg/l).

After 45 days, the percentage of callus fresh, dry weights, colour, and texture were measured.

3.1. Effect of subculture on callus growth

The callus cultures were grown on MS medium containing various concentrations of 2, 4-D (1.00, 1.50, 2.00, 2.50, and 3.00 mg/l) in combination with cytokinin BAP(6-benzylaminopurine) at (0.5 mg/l) or kin (Kinetin (6-furfurylaminopurine)) at (0.5 mg/l).

The percentage of callus freshn, dry weights, and callus texture were all measured

4.1. Elicitor preparation and treatment

Sigma-Aldrich purchased the chitosan (CH) and salicylic (SA) solutions. They were dissolved in distilled warm water and sterile filtered through a pre-filter (0.2 m pore size; Advantec). The sterilised SA and CH solutions at concentrations of (0.0, 0.2, 0.4, 0.8 mg/l) and (0.0, 20, 40, 80 mg/l), respectively, were added to callus culture flasks for 30 days incubation time.

Fresh, dry weights, color and callus texture were calculated in solid media (callus culture). After 45 days, the callus culture was harvested, filtered with filter paper, and liquid nitrogen freeze-dried. The samples were kept in the freezer until the biochemical analysis was performed.

4.2. Culture conditions:

Callus induction was performed in 350 mL glass jars with 40 mL of medium sealed with plastic caps.

The pH of the medium was adjusted to 5.8 before being gelled with phytagel and autoclaved for 20 minutes at 121°C and 15 lbs pressure. Cultures were incubated in total darkness for 7 days before being exposed to a 16-hour photoperiod provided by cool-white fluorescent lamps at a photon flux density of 40 µmol m^{-2} s^{-1} at 25 ± 2°C for 30 days.

Each glass jar was considered as an experimental unit, and the experiment was repeated at least three times with ten replicates for each treatment. The cultures were subcultured on fresh MS medium at 28-day intervals.

On the 30th day of induction, the induction rate (callus %), fresh weight, and dry weight were all recorded.

5. Biochemical and phytochemical analysis

5.1. Antioxidant capacity

According to **(Oktay et al., 2003),** momentarily, the following method was used to determine the scavenging effect of *Balanites aegyptiaca* callus extract against the synthetic radical DPPH (2,2-di-phenyl-1-picrylhydrazyl): about, 2 mL of a 0.004 % solution of DPPH in methanol was stirred with 0.5 mL of *Balanites aegyptiaca* callus methanolic extract. The reaction solution was vortexed and kept in the dark for 30 min at room temperature. The absorbance of the samples was read at 517 nm in the spectrophotometer. The scavenging rate was calculated using the following formula:

$$\mathbf{RSC\ \%= (A_{blank} - A_{sample}) / (A_{blank}) \times 100}$$

5.2. Total phenols

Briefly, dried callus samples were extracted with absolute ethanol at 45 °C, homogenized, filtered, then dryed. The dry film was re-suspended in absolute ethanol with a final volume of 20 mL. About 1 mL of ethanolic extract was taken and added to it with 10 ml of conc. HCl, boiled rapidly in a direct flame for 10 min, then placed in a boiling water bath for 10 min, cooled, and 1 mL of Folin-Ciocalteu reagent. After that, 5 mL of Na_2CO_3 20% was added and mixed, the volume was increased to 15 mL with distilled water, and after 30 min, the absorbance was read at 520 nm as Gallic acid against sample blank **(Snell and Snell, 1953).**

5.3. Total flavonoids

Flavonoids, are key bioactive chemicals occurring in plants. The aluminium chloride colorimetric test is commonly used to measure total flavonoid concentration against a flavonoid standard as quercetin and Kaempferol **(Shraim et al., 2021).** 1 mL refluxed ethanolic dry callus extract was dried in a water bath, added about 5 mL of 0.1M $AlCl_3$ (5g $AlCl_3$ at 100 mL methanol) and the developed yellow color was read at 445 nm as Quercetin and 266 as Kaempferol.

5.4. Determination of Coumarin by HPLC extraction and determination

The extraction of dried callus was performed by methanol 70% and determination was according to **Biswas et al., (2013)**. The HPLC system Thermo (Ultimate 3000) consisted of: pump, automatic sample injector, and associated DELL-compatible computer supported with Chromelion7 interpretation program.

A diode array detector DAD-3000 was used. The Thermo-hypersil reversed phase C18 column 2.5× 30cm was operated at 25º C. Mobile phase consists of distilled water (solvent A) and methanol (solvent B). The UV absorption spectra of the standards as well as the samples were recorded in the range of 230–400 nm. Standard and samples solutions and even the mobile phase were degassed and filtered through 0.45 µm filter membrane (Millipore).

The compounds were identified by comparing their retention time and UV absorption spectrum of the injected standards.

Inj. Vol: 20 µl
Column: RP- C18
Column size: 2.5× 30cm
Mobile phase: H_2O : Methanol with mixing ratio 75 :25
Flow rate: 1.0 ml/min
Temperature: 25°C
Detection: photo diode array (DAD)

5.5. Determination of sugars by HPLC extraction and determination

Carbohydrates are found in biological systems in both free (e.g., starch, cellulose) and conjugated (e.g., proteoglycans, glycoproteins, glycolipids) forms, serving as energy storage and structural support. Carbohydrates play a role in a variety of biological activities, such as cell recognition, development, interaction, and inflammation. Because of their complex structure and variability, carbohydrates are challenging to study.

The extraction of carbohydrates was firstly performed by methanol **Phillips and Williams (1982)**; **Langemeier and Rogers (1995).** The partial acid hydrolysis was done using 0.2 N H_2SO_4 by boiling solutions containing 0.1 g of defatted methanol extract in 12.5 ml of 4% w/w (4 g conc.H_2SO_4 + 96 ml distilled water), for a period of 3 hours at 100°C. Each sample was neutralized by adding 2 g of barium carbonate to the hydrolyzed solution. The neutralized hydrolysates were filtered to eliminate insoluble barium salts, and then HPLC was used to look for neutral sugar residues in the clear supernatants

The system Thermo (Ultimate 3000) consisted of: pump, automatic sample injector, and associated with computer compatible software supported with Chromelion7 interpretation program and diode array detector was used. The aminopropyl-bonded phase column (4gm high-performance carbohydrate column, Waters) was operated at 30ºC. The mobile phase was acetonitrile and water solution (75:25), isocratic mode. Sodium chloride was added (0.125%, w/v) to minimize the interference from NaCl. Sugar standards were dried at 60ºC in a vacuum oven overnight and then, dissolved in 60% ethanol. To assess recovery, callus extract various samples were injected with varied combinations of standard sugars (1 -5 ppm). Sugar was determined qualitatively using peak area measurements.

Inj. Vol: 20 µl
Column: Sugar-D
Column size: 4.6 mmI.D -250mm
Mobile phase: Acetonitrile: H_2O = 75:25
Flow rate: 1.0 ml/min
Temperature: 30°C
Detection: DAD 470 nm

6. Data analysis:

All of the experiments were completely random design. For statistical analysis, ANOVA was performed using the Costat software package. **Duncan (1955)** multiple range tests, as modified by **Snedecor and Cochran (1990)**, were used to test the significance of differences in treatment means at the 5% level. Means separated by the same letter are not statistically different at $P \leq 0.05$.

Results and discussion:

1. Callus induction

Callus was formed from *Balanites aegyptiaca* leaf segments, as shown in Table (1). The highest callus growth induction (68%) was achieved on MS medium supplemented with 2.5mg/L 2,4-D. The use of MS medium supplemented with 2.5mg/L 2,4-D, on the other hand, resulted in the highest mean fresh weight of callus (3.2 g) and green compact callus (Figure 1). These findings are supported by **Chapagain et al., (2006)**, who discovered that the rate of callus induction on inoculation explants ranged from 55 to 100 percent.

These findings were also in line with **Sharma et al., (2017)** and **Sen et al., (2014)**, who discovered that combining 2,4-D with BAP and NAA increased callus formation. Furthermore, **Ishaku et al., (2020)** discovered that 0.5 mg/L of the auxin NAA in the presence of 0.5 mg/L of the cytokinin BAP resulted in 100% callus development. The medium enriched with 2.00mg/L 2,4-D came in second, with a 2.7g major callus fresh weight and green compact callus.

On the other side, callus fresh weight was lower in MS medium lacking 2,4-D (control). Table (1) further shows that the presence of 2,4-D at less than 1.00 mg/L NAA was linked to the greeng friable callus shape. When 2,4-D was introduced to a medium with a concentration of more than 1.50 mg/L, a greenish compact callus resulted (Figure 1). The plant growth regulators and their concentrations influenced callus shape (colour and texture) (**Kumar and Nandi, 2015**).

2. Effect of subculture on callus growth

Table (2) and Fig. 2 show that after two months, 100% of Balanites aegyptiaca leaf sections produced green embryogenic callus on all MS media containing 2.5

mg/L 2,4-D in combination with BA, with the mean fresh weight of callus ranging from 1.3 to 4.50 g/jar. After two subcultures, MS medium containing 2.5 mg/L 2,4-D + 0.5 mg/L BAP produced the most fresh weight of callus and the highest percentage of increase in fresh weight, reaching 300 % (Figure 2), followed by 2.00 mg/L 2,4-D + 0.50 mg/L BAP, which produced 2.9 g of callus and 193.3 percent increase in fresh weight (Figure 2).

Table (1): Effect of MS medium containing 30 g/L sucrose, 2.7 g/L phytagel and different 2, 4-D concentrations on callus formation of *Balanites aegyptiaca*

2, 4-D conc. (mg/L)	Callus percentage	Fresh weight	Dry weight	Callus color	Callus texture
0.00	1^{g}	0.10^{g}	0.01^{g}	Green	friable
0.50	5^{f}	0.50^{f}	0.05^{f}	Green	friable
1.00	18^{e}	0.75^{d}	0.07^{e}	Green	friable
1.50	35^{d}	1.30^{c}	0.13^{d}	Green	compact
2.00	61^{b}	2.70^{b}	0.27^{b}	Green	compact
2.50	68^{a}	3.20^{a}	0.33^{a}	Green	compact
3.00	45^{c}	2.40^{c}	0.24^{c}	Green	compact

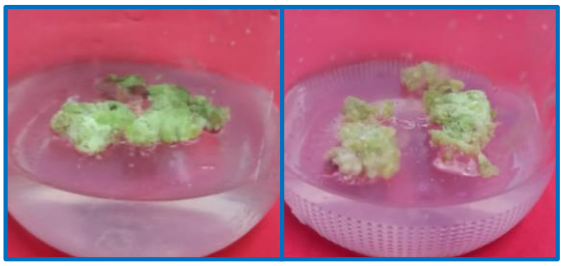

Figure (1): Callus of *Balanites aegyptiaca* induced from leaf explants on MS medium supplemented with 2.5 mg/L 2,4-D.

The medium containing 1 mg/L 2,4-D and 0.5 mg/L BAP had no significant effect on the mean fresh weight of callus (1.50 g/jar) when compared to the previous two media, but it did cause a significant reduction in the percentage of increase in fresh weight. On the other hand, the minimal response was obtained using MS medium supplemented with 1.00 mg/L 2,4-D + 0.50 mg/L Kin. The obtained results also revealed that the growth parameters for each BAP concentration increased as the 2,4-D concentration was increased.

Many recent articles on a variety of other plants may support the findings of this study. Regardless of climatic and geographical conditions, plant tissue culture techniques will provide continuous, sustainable, economical, and viable secondary metabolite production (**Chandran et al., 2020**). When compared to other procedures, *in vitro* callus induction is a simple and quick way to multiply cells, making it an efficient way to generate PDMCs on a large scale (**Kapoor et al., 2018**). Furthermore, the factors that cause callogenesis have been thoroughly studied, are highly consistent, and have been commercially used in tissue culture for other purposes for decades (**Ahmad et al., 2016**). According to the study, medicinal plant cell suspension cultures, which have the trait of fermentation with plant cell totipotency, could be a promising alternative "chemical factory" (**Yue et al., 2014**).

Table (2): The effect of different concentrations of plant growth regulators on callus induction of *Balanites aegyptiaca* after 45 days of culture

Growth regulators conc. (mg/L)			Callus percentage	Fresh weight	Dry weight
2, 4-D	BAP	Kin			
1.0	0.5	0.0	30^{f}	1.50^{h}	0.15^{g}
1.5	0.5	0.0	40^{e}	2.00^{f}	0.21^{e}
2.0	0.5	0.0	55^{b}	2.90^{b}	0.29^{b}
2.5	0.5	0.0	95^{a}	4.50^{a}	0.45^{a}
3.0	0.5	0.0	50^{c}	2.53^{d}	0.25^{d}
1.0	0.0	0.5	9^{i}	1.00^{j}	0.11^{i}
1.5	0.0	0.5	13^{h}	1.30^{i}	0.13^{h}
2.0	0.0	0.5	28^{g}	2.10^{e}	0.21^{e}
2.5	0.0	0.5	43^{d}	2.80^{c}	0.28^{c}
3.0	0.0	0.5	46^{e}	1.9^{g}	0.19^{f}

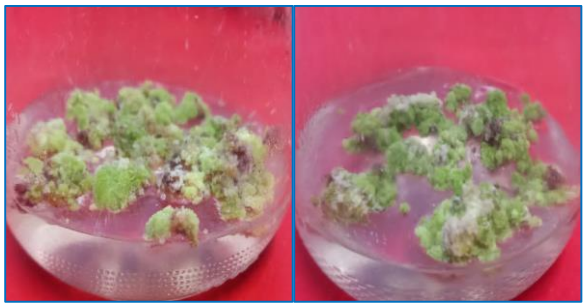

Figure (2): Callus of *Balanites aegyptiaca* induced on MS medium supplemented with 2.5 mg/L 2,4-D + 0.5 mg/L BA.

3. Effect of chitosan and salicylic acid on callus growth rate

In all stages of growth, the differences in coumarin production or accumulation between callus cultures were statistically significant in table (3).

For the elicitation treatment experiment, the plant growth regulators combination that induced the highest callus induction percentage and fresh weight was chosen. Table (3) shows the effect of adding chitosan (CH) at (0.0, 0.2, 0.4, 0.8 mg/L) and salicylic (SA) at (0.0, 20, 40, 80 mg/L) to the callus of Balanites aegyptiaca on coumarin accumulation percentage (mg/gm dry weight callus).

Table (3) reveal that the highest mean fresh weight of callus and the percentage of growth in fresh weight were achieved in suspension culture on the medium containing salicylic acid (0.2 mg/L), reaching 13.73 g/jar and 156%, respectively. Suspension culture had a concentration of 0.4 mg/L salicylic acid resulted in a high mean fresh weight of callus (11.03 g/jar), which was statistically different from the medium containing 0.2 mg/L salicylic acid, although the 125% increase in fresh weight of callus was lower. The medium supplemented with 0.8mg/L chitosan, on the other hand, produced the lowest callus formation.

The dry weight of callus changed dramatically depending on the chitosan and salicylic concentrations. On the medium containing 0.2 mg/L salicylic acid, the maximum dry weight of callus (1.43 g/g dry wt.) was seen, whereas the minimum dry weight of callus (0.22 g/g dry wt.) was observed on the medium containing 80 mg/L chitosan.

The effect of chitosan on *in vitro* cultures of *Morinda citrifolia* (L.) (Indian mulberry) (**Purwianingsih et al., 2019**) and *Ophiorrhiza mungos* var. *angustifolia* (Thw.) Hook. f. (Indian snake root) was also investigated (**Krishnan et al., 2018**). Chitosan was used as an elicitor to increase the anthraquinone content of M. citrifolia callus growing *in vitro*.

Table (3): Effect of different concentrations of chitosan and salicylic acid on callus growth rate of Balanites aegyptiaca on MS medium supplemented with 2.5 mg/L 2,4-D + 0.5 mg/L BA

Treatments	Callus fresh weight	Callus dry weight	Callus color	Callus texture
0.00	3.0^{d}	0.30^{d}	Green	compact
SA_1	6.33^{a}	0.61^{a}	Green	compact
SA_2	5.0^{b}	0.50^{b}	Green	compact
SA_3	3.38^{c}	0.38^{c}	Green	compact
CH_1	2.13^{f}	0.21^{f}	creamy	friable
CH_2	2.5^{e}	0.25^{e}	creamy	friable
CH_3	2.03f	0.20^{f}	creamy	friable

4. Effect of chitosan and salicylic acid on coumarin accumulation

Table (4) reveals that the highest value of coumarin was detected by leaf callus 7.66 mg/g dry weight. Regarding aerial parts of mother plant, the maximum record was introduced in case of fruit 5.35 mg/g dr.wt.

Balanites aegyptiaca contains alkaloids, flavonoids, terpenoids, and glycosides, all of which have antifungal activity, according to **Hussain et al., 2019**. Antibacterial activity of flavonoids, triterpenoids, steroids, and phenolic compounds against fungi has been demonstrated. The desert date's fruit, leaves, branches, and root contain bioactive metabolites such as flavonoids, alkaloids, tannins, and vitamins (**Farid et al., 2002; Maksoud and El Hadidi, 1988; Sagna et al., 2014**).

Our results are in line with (**Al Khateeb et al., 2017**) who found that the phenol accumulation of *in vitro*-grown *Rumex cyprius* Murb. (knotweed) under the influence of chitosan was also. The addition of chitosan to the growth medium did not significantly improve the phenol content; however, the antioxidant activity increased significantly with increasing chitosan concentration compared to the control. Furthermore, gallic acid was found only on *R. cyprius* plants grown in the presence of chitosan and not on control plants. On the contrary, chitosan increased the phenol content of *Brassica oleracea* var. *italica* (broccoli) while decreasing the antioxidant activity (**Carvacho et al., 2014**).

SA is a plant signal molecule that causes changes in plant metabolism at various levels in response to environmental stressors. Salicylic acid elicited growth-promoting responses in mice. SA increased the manufacturing of phenylpropanoid pathway defence chemicals, resulting in a buildup of coumarin-related substrates **(Dučaiová et al., 2013).**

5. Effect of chitosan and salicylic acid on antioxidant capacity, phenolics and flavonoids accumulation

Plant phenolic compounds are one of the most studied chemical families because of their incredible potential as pathogen defence, signalling molecules, and regulators of essential biochemical processes like antioxidant activity **(Karabourniotis et al., 2014).**

Flavonoids are phytochemicals that can be found in a wide range of plants, fruits, vegetables, and leaves, and they have medical chemistry applications. Flavonoids have a variety of therapeutic properties, including anticancer, antioxidant, anti-inflammatory, and antiviral effects. They're also neuro- and cardio-protective.

Table (4): Coumarin content as an active compounds identified by HPLC in *Balanites aegyptiaca* callus and plant parts treated by biochemical precursors

Treatments	Coumarin content by HPLC mg/g dr.wt
Fruit	5.35
Leaf	2.60
Stem	2.84
Leaf callus (control)	7.66
SA_1	1.73
SA_2	1.71
SA_3	3.51
CH_1	0.457

CH_2	0.395
CH_3	0.709

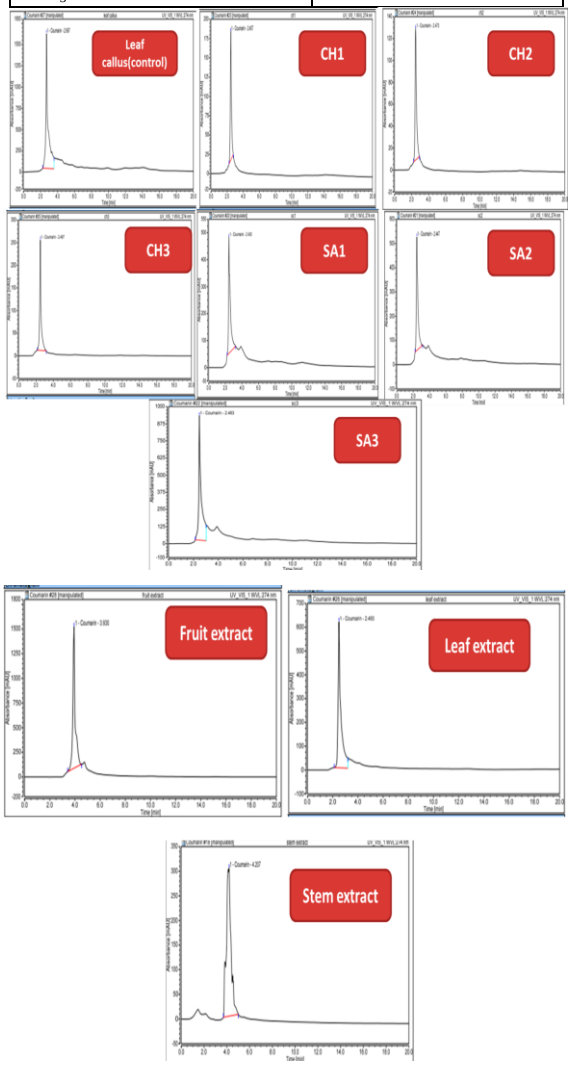

Figure (3): HPLC chromatogram of Coumarin content in *Balanites aegyptiaca* callus and plant parts treated by biochemical precursors.

The type of flavonoid, its (possible) mode of action, and bioavailability all influence these biological activities. These low-cost medicinal ingredients have significant biological activities, and their efficacy for a variety of diseases has been demonstrated (**Ullah et al., 2020).** In table (5), there was a significant effect of different treatments on total phenolics as gallic acid, antioxidant capacity and flavonoids.

The maximum antioxidant capacity of scavenging radicals was determined by fruit 86.07% followed by the stem 85.00%. The highest content of phenolics was determined also by fruits which gave the highest antioxidant content.

It is clear also from table (5) that, the highest content of quercetin was noticed in aerial part of stem and followed by callus treated chitosan 40 mg/L which also has high antioxidant capacity and kaempferol.

The result goes in some extent with **Hassanen et al., 2021** who worked on *Silybium marianum* callus fed with chitosan NPs. Likewise, **Chandra et al. (2015),** noticed that, Chitosan NPs increased the accumulation of flavonoids by 24% and phenolics by 20% in *Camellia sinensis* shoot culture. Further researches, Chitosan NPs foliar spray on *Momordica charantia* in concentrations of 10, 50 and 100 µM increased phenolics, anthocyanin and up-regulated antioxidant activity **(Sharifi-Rad et al., 2020)**. Results also were compatible with **Abo El-Fadl et al. (2022)** on avocado callus using chitosan NPs and some elicitors like salicylic acid, which increased secondary metabolites and antioxidant capacity.

Chitosan treatment effectively enhanced the overall amount of phenolic compounds in various plants, according to several authors. After elicitations, flavonoid and phenolic compound synthesis begins almost immediately. Overall, elicitors like chitosan are involved in signal transduction systems that cause secondary metabolic pathway enzymes like phenylalanine

ammonia-lyase (PAL) to express their genes. The total amount of phenols and flavonoids had a positive connection with PAL activity, indicating that chitosan treatment can regulate the accumulation of phenolic compounds by promoting PAL activity **(Pirbalouti et al., 2017).**

Flavonoids are water-soluble polyphenolic compounds that have anticancer, anti-inflammatory, and antidepressant properties. They are antioxidants and free radical scavengers. In the current study, a positive link was established between the contents of flavonoids and macro-elements, indicating that an increase in flavonoid level suggests an increase in the amount of plant nutrients **(Singh, 2016).** Chitosan150 mg/L increased isoflavonoid synthesis in *Pueraria candollei*, according to **(Udomsuk et al., 2011)**.

Table (5): some active compounds identified in Balanites aegyptiaca callus and plant parts treated by biochemical precursors

Treatments	Total phenols as Gallic acid mg/g dr. wt	Antioxidant capacity%	Total flavonoids as Quercetin mg/g dr.wt	Total flavonoids as Kaempferol mg/g dr.wt
Fruit	67.24 a	86.07 a	0.163 c	5.98 a
Leaf	32.18 b	80.14 c	0.141 e	4.39 b
stem	12.84 g	85.00 ab	0.232 a	6.01 a
Leaf callus (control)	14.99 d	74.92 d	0.079 h	0.783 h
SA_1	17.56 c	76.65 d	0.094 g	1.750 f
SA_2	8.15 h	75.27 d	0.0193 i	1.440 g
SA_3	14.48 e	76.04 d	0.094 g	2.89 d
CH_1	13.13 f	74.32 d	0.146 d	3.98 c
CH_2	9.61 g	82.35 bc	0.205 b	6.11 a

CH 3	7.72 i	73.58	0.104 f	2.03 e

6. Effect of chitosan and salicylic acid on sugars separated by HPLC

Table (6) clarified the effect of applied SA and CH on sugar contents as active ingredients. Also, the aerial parts of the plant were measured. The HPLC separated glucose, sucrose and fucose sugar. where, the highest content of glucose was determined in leaf callus which treated with SA 0.4 mg/L, followed by 0.2 mg/L. the maximum value of sucrose was found in leaf callus treated with 0.2 mg/L followed by 0.8 mg/L. the L-fucose sugar was detected only in the treated leaf callus extract with CH (40 and 80 mg/L). L-fucose has several potential applications in cosmetics, pharmaceuticals, and nutritional supplements **(Roca, 2015).**

Table (6) : Some sugars as an active compounds identified by HPLC in Balanites aegyptiaca callus and plant parts treated by biochemical precursors

Peak area %					
Treatments	Fucose	Sucrose	Glucose	Unknown	Unknown
Fruit	N.D	2.78	6.15	54.85	33.84
Leaf	N.D	2.43	12.18	36.66	48.73
Leaf callus (control)	N.D	2.43	14.53	35.49	47.55
SA 1	N.D	4.35	37.01	37.01	11.86
SA 2	N.D	N.D	57.03	42.97	-
SA 3	N.D	3.22	13.87	82.90	-
CH 1	N.D	2.65	18.77	23.53	19.12
CH 2	1.45	0.25	0.16	5.88	41.00
CH 3	0.27	0.12	0.04	18.04	13.44
N.D. not detected					

Conclusion:

According to our findings, salicylic acid and chitosan elicitors in callus development have a potential role in boosting secondary metabolite accumulation. Callus culture of Balanites aegyptiaca is one of the most effective methods for increasing coumarin accumulation in vitro. Salicylic acid, a precursor of coumarin, was found to have significant effects on improving coumarin production in Balanites callus cultures in this study. These insights could pave the way for the pharmaceutical sector, especially when performing suitable biological experiments. It is also concluded that every aerial part could be useful for a detected purpose according to the content of bioactive metabolites and will be used in *in vitro* studies, especially the stem part.

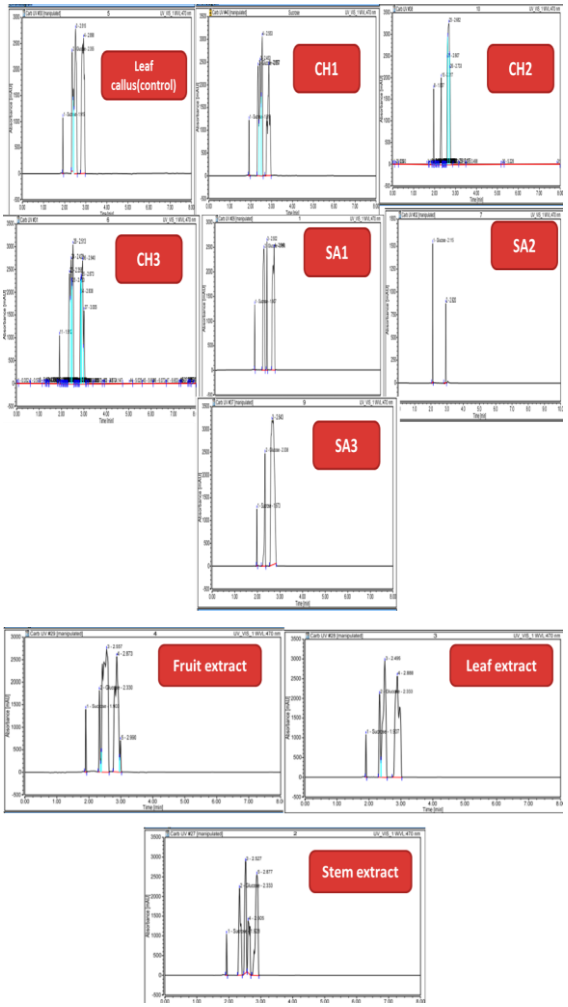

Figure (4): HPLC chromatogram of sugars content in *Balanites aegyptiaca* callus and plant parts treated by biochemical precursors

Conflicts of interest

"There are no conflicts to declare".

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