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Effect of salicylic acid and some plant extracts on growth and stimulation of cardiac glycosidic compounds of *Digitalis lanata* in vitro.

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ABSTRACT

The research aimed to investigate the effect of salicylic acid, and some plant extracts some factors on growth indicators and stimulating cardiac glycosides in digitalis leaves, in the Plant Tissue Culture Laboratory - College of Agricultural Engineering Sciences - University of Baghdad from 10-2-2022 to 6-1-2023. Branch cultures of the digitalis plant were obtained from planting the 1 cm length shoot tips, grown from sterilized seeds cultured in MS medium provided with 0.5 mg.L-1 of BA. After obtaining a sufficient number of vegetative branches by proliferating them more than once, they were cultured in MS medium provided with salicylic acid at the concentrations of 0, 1, 3, 5, and 7 mg.L-1, ginger extract at the concentrations of 0, 3, 5 and 7 g.L-1, and black pepper extract at the concentrations of 0, 3,5, and 7 g.L-1 as independent experiments. The results showed that salicylic acid concentration was superior 3mg.L-1 was significantly superior in the number of branches, number of leaves, fresh and dry weight, chlorophyll content, and the cardiac digoxin, reordering 2.67 branches.plant-1, 0.467cm, 9.67 leaves.plant-1, 1.328 g, 0.879 g., 1.376 mg, and 28.48 µg.g-1; and the treatment of ginger extract at a concentration of 5 gm l-1 and black pepper extract at a concentration of 3 gm l-1 was significantly superior separately in same traits, attaining 4.33 branches.plant-1, 14.43 leaves.plant-1, 1.567 g, 0.823 g, 1.497mg, and 29.32 µg.g-1 for ginger extract; and 2.133 branches.plant-1, 1,243 cm, 14.99 leaves.plant-1, 1.174 g, 0.659 g, 0.855 g.100 g-1fresh weight, and 29.66 µg.g-1 for black pepper extract, respectively.

تأثير حامض السالسلك وبعض المستخلصات النباتية في النمو وتحفيز المركبات الكلايكوسيدية القلبية لنبات الدجتالس الصوفي *Digitalis lanata* خارج الجسم الحي.

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المستخلص

نفذت التجربة بهدف اختبار تاثير حامض السالسيليك وبعض المستخلصات النباتية في المؤشرات الخضرية وتحفيز اهم مركبات الكلايكوسيدات القلبية لاوراق نبات الدجتالس، في مختبر زراعة الانسجة النباتية- كلية علوم الهندسة الزراعية – جامعة بغداد للمدة من ٢-١٠ - ٢٠٢٢ ولغاية ١-٦-٢٠٢٣ .تم الحصول على مزارع الافرع الخضرية لنبات الدجتالس من

زراعة اطراف الافرع بطول ١سم والتي تم الحصول عليها من البذور المعقمة والمزروعة في وسط MS المجهز بالـBA بالتركيز (٠.٥ ملغم .لتر ١-) ولحين الحصول على العدد الكافي من الافرع الخضرية زرعت في الوسط MS المجهز بحامض السالسيليك بالتراكيز (٠ ، ١ ، ٣ ، ٥ ، ٧ ملغم .لتر -١) و الوسط MS المجهز بمستخلص الزنجبيل بالتراكيز (٠ ، ٣ ، ٥ ، ٧ غم .لتر ١-) و الوسط MS المجهز بمستخلص الفلفل الاسود بالتراكيز (٠ ، ٣ ، ٥ ، ٧ غم .لتر -١) في تجارب مستقلة ، اظهرت نتائج تجربة حامض السالسيليك تفوق التركيز (٣ ملغم .لتر -١) معنويا في عدد الافرع وعدد الاوراق والوزن الرطب والجاف والكلوروفيل وdigoxin القلبية (٢.٦٧ فرع . نبات ١- ، ٩.٦٧ ورقة . نبات ١- ، ١.٣٢٨ غم ، ٠.٨٧٩ غم ١.٣٧٦ ملغم ٢٨.٤٨ مايكروغرام. غم-١ ، كما تفوقت معاملة مستخلص الزنجبيل بتركيز٥غم .لتر١- ومستخلص الفلفل الاسود بتركيز ٣غم . لتر ١- كلا على حدي معنوياً في معدل الصفات نفسها اذ بلغت ٤.٣٣فرع .نبات-١ ، ١٤.٤٣ ورقة . نبات-١ ، ١.٥٦٧غم ، ٠.٨٢٣غم ، ١.٤٩٧ ملغم .وزن طري ، ٢٩.٣٢ مايكروغرام.غم-١ ، ٢.١٣٣ فرع. نبات ١- ، ١.٢٤٣ سم ، ١٤.٩٩ ورقة. نبات ١- ، ١.١٧٤ غم ، ٠.٦٥٩ غم ، ٠.٨٥٥غم١٠٠. غم وزن طري ٢٩.٦٦ مايكروغرام. غم ١-).

الكلمات المفتاحية : الاكثار الدقيق ، مستخلص الزنجبيل، digoxin ، مستخلص الفلفل الاسود

INTRODUCTION

Medicinal plants and their active components are a crucial factor in the human struggle against disease after some medicines began to lose their preventive effectiveness against various diseases. Therefore, plants have become an indispensable source for treating diseases and a major component in preparing medicines (Al-Snafi2016; Marrelli.,2021) *Digitalis lanata* L. is a biennial flowering herbaceous plant and one of the most important medicinal plants. It grows naturally in eastern and Western Europe, central and western Asia, and northwestern Africa. Previously, the *Digitalis* genus was classified in the family Scrophulariaceae (Kreis.,2017 ; Verma et al.,2016; Ciocarlan.,2023)، but recent phylogenetic research has led to its reclassification in the plant family Plantaginaceae (Ravi et al.,2020 ;Jan et al.,2024). This plant is typically grown as an ornamental plant in gardens due to the beauty of its leaves in the first season and its flowers in the second. It can also be grown for medical purposes as it contains cardiac glycosides(Rana et al.,2022;Nikam et al.,2024). The group of glycosides is a significant component of the active materials found in medicinal plants, and they can account for most of the diverse types of known physiological effects. Glycosides are made up of two parts that are linked to each other (Cingoz et al.,2014; Gašić et al.,2023)، One part is known as the aglycone, the part that does not contain sugar molecules, which is the active part that represents the active substance and activates the enzyme action. The glycosides are classified into two types: cyanide and steroidal glycosides. The second type contains eight glycosides, the most important of which are cardiac glycosides, found in 11 plant families and more than 34 genera. Plantaginaceae is the most important plant family containing cardiac glycoside compounds, to whom the plant *Digitalis lanata* L. belongs (Almukhtar.,2022; de Swiet.,2023; Nikam.,2024).Cardiac glycoside compounds have been widely used as drugs to treat various heart diseases due to their high effectiveness and because some heart muscle failure cannot be controlled with other drugs, in addition to their indirect effects on the heart system, including regulating the heartbeat and enhancing the heart muscle contractile force and speed (Bala et al.,2021;Tomilova et al.,2022; El Gameel et al.,2023; Ibrahim.,2024)

Interest is increasingly turning towards plant tissue culture as an essential method for producing these compounds, as traditional agriculture cannot cope with the growing demand for these compounds, and traditional methods often face difficulties producing many natural materials (Tomilova.,2022; Nikam et al.,2024; Menezes.,2024) Some of these compounds are challenging to prepare in a lab, and biological manipulations may be necessary to manufacture and increase their production. Plant tissue culture allows cell growth control, making it easier to enhance product quality or speed up production by adding initiators or plant extracts to plant tissue cultures.(Karaś.,2020;Rednic,.2022;Tomilova,.2022). Due to the medical importance of the digitalis plant, it has become necessary to enhance its growth and increase its production of active compounds relying on tissue culture by adopting different systems and practices, including using salicylic acid (SA). It is a plant hormone with a phenolic nature traditionally extracted from the bark of willow plants(*salix sp.*), found as Colourless crystals that dissolve slowly in water (Sun et al.,2022; Shi et al.,2024; Abdulraheem et al.,2024).The stimulating effect that characterizes salicylic acid SA is due

to its role as a trigger to signal the appearance of secondary compounds in the biological pathway and as a mechanism for the plant's defense (Nazir et al.,2021;Das et al.,2022). Scientific research and studies conducted by several researchers, including Miclea et al. (2020), who examined some stress stimuli added to suspended cell cultures, refer to the role of salicylic acid in stimulating the biosynthesis of enzymes responsible for constructing plant secondary compounds. Batista and others (2019) also reported that adding salicylic acid to the medium increased the concentration of cardiac glycosides of the digitalis plant (Tomilova et al.,2022). Studies and scientific research also refer to the importance of natural plant extracts that are extracted from plants and added to the tissue culture media, including coconut milk in citrus cultures and some weed extracts, including mallow, in the tissue cultures media of cucumbers and potatoes (Ibrahim.,2022; Bhaskar.,2022) and licorice extract in tissue cultures of shrub roses (Al-Mamouri.,2009), as well as the extract of potato, banana, black pepper, and ginger These extracts stimulate the plant parts to grow, constitute roots, and increase vegetative branches, as well as the accumulation of medically active compounds(Saleem et al.,2022;Ozyigit.,2023;Shalaby et al.,2023). This study aims to induce the production of cardiac glycosides in the digitalis plant and stimulate its growth by adding salicylic acid and some plant extracts to the media culture in vitro.

MATERIALS AND METHODS

The study was conducted in the Plant Tissue Culture Laboratory of the Medicinal and Aromatic Plants Research Unit - College of Agricultural Engineering Sciences - University of Baghdad for the period from 2/10/2022 until 1/6/2023, using the seeds obtained from the American company Johnnys and included the following steps:

Seed disinfestation

The seeds were surface disinfested by soaking in a 1% commercial Clorox solution (containing 6% sodium hypochlorite) for 20 minutes and then rinsed 4-5 times with sterile distilled water. All the steps were performed under a laminar flow transfer hood (Al-Amery et al.,2023).

Seed germination

The disinfested seeds were cultured in a free medium of growth regulators and placed in a growth chamber under a photoperiod of 16 hours of light and 8 hours of darkness with a light intensity of 1000 lux and a temperature of 23 ± 2 °C For 4 weeks.

Proliferation stage

After 4 weeks of seed germination, the seedlings were chopped, and a single node was cultured in media provided with BA at the concentration of 0.5 $mg.L^{-1}$(Danial et al.,2019).

The resulting branches were then cultured in media of the same components, and this process was repeated more than once until reaching enough branches to enter the stage of increasing the stimulation of secondary compounds, by three treatments, first, using salicylic acid(SA) which has been prepared by dissolving 100 mg of SA in 100 ml of distilled water. Then, its effect was evaluated after culturing the shoots in MS media prepared with different salicylic acid concentrations (0, 1, 3, 5 and 7 $mg.L^{-1}$). The second way to increase plant compounds was to use the ginger extract, which has been prepared by drying it and grinding, and take 100 gm of the powder in 500 ml of distilled water and shake it intermittently. Then the solution was filtered, and the filtrate was collected in a glass watch and placed in an oven for drying at a temperature of 40 °C for two days until it dried completely and turned to a powder brown in color; after that, it was preserved and stored it in the refrigerator at 4°C until used (Al-Taie.,2012). Then, the shoots were planted in MS media containing different concentrations of ginger extract (0, 3, 5, and 7 $g.L^{-1}$). The third treatment included using black pepper extract, which prepared according to by Al-Taie (2012). The shoots were cultured in MS media prepared with different concentrations of black pepper extract (0, 3, 5, and 7 $g.L^{-1}$).

Cultures of each experiment were incubated separately in a growth chamber under a photoperiod of 16 h of light and 8 h of darkness, with a light intensity of 1000 lux and a temperature of 23 ± 2 °C, constituting 25 tubes, each of them contained one branch which is one replicate. Growth parameters, including branch length, number of branches, number of leaves, fresh weight, dry weight, and chlorophyll content, were recorded 4 weeks after culturing. The concentration of the digoxin compound was measured with an High performance liquid chromatography (HPLC) Germany-SYKAM type to determine the retention time and sample area for both the standard and sample solution, a C18 column (25cm×4.6mm×5µ) was used and the mobile phase (CH3CN: H2O: Orthophosphoric Acid) (40: 55: 5 mL) was pushed at a flow rate of 1.0 mL/min and the readings were measured at a wavelength of 214 nm and a temperature of 35 ◦C. (Ravi G. B. ,2019) and calculated according to the following formula(Ravi G. B. ,2019) (Fig 1.):

$$\textbf{Sample concentration} = \frac{\textbf{Standard concentration x sapmle area}}{\textbf{Standard area}} \textbf{x Dilution times}$$

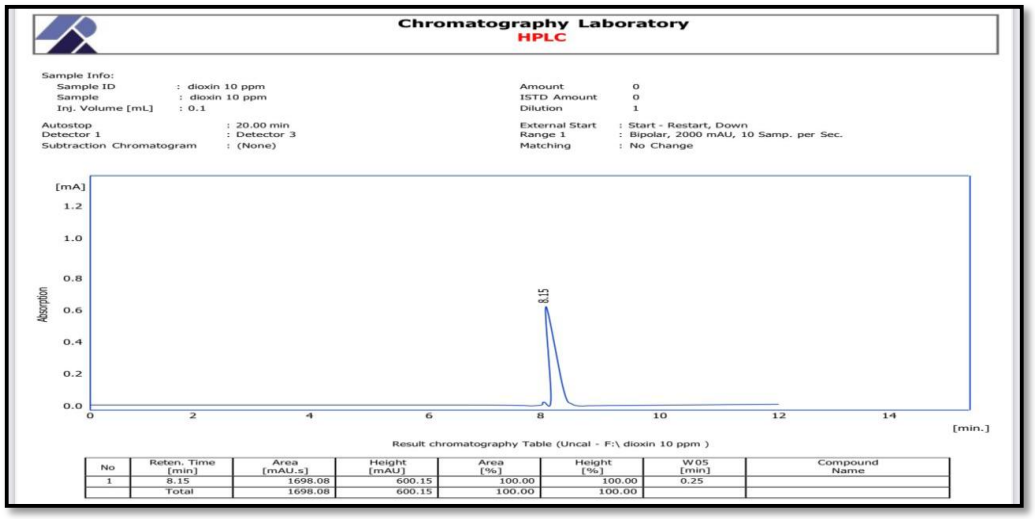

Figure 1. Standard curve of dioxin.

Statistical analysis

The experiment was implemented relying on the completely randomized design (CRD) as a factorial experiment, comprising ten replicates and statistically analyzed, using the software Ganstat(22.1), and the means were compared based on the least significant difference (LSD) at the probability level 0.05 (Al-Sahuki.,1990).

RESULT AND DISCUSSION

Effect of salicylic acid on the number and length of branches and number of leaves of digitalis plants:

The results in Table 1. showed that the demonstrated a significant superiority of the 3.0 mg.L^{-1} concentration to other concentrations, recording 2.67 branche.$plant^{-1}$ and 9.67 leaf.$plant^{-1}$, respectively, compared with the control treatment, which produced the lowest averages of the traits, recording 1.00 branche.$plant^{-1}$, 0.433 cm, and 4.00 leaves.$plant^{-1}$, respectively. Results in Table 2 illustrate that the different concentrations of salicylic acid significantly affected the averages of fresh and dry weight, chlorophyll, and digoxin content in the leaves, referring that the concentration of 3.0 mg.L^{-1} was significantly superior to all other concentrations, recording 1.328g, 0.879g, 1.376g.$100g^{-1}$ fresh weight and 28.48 µg.g^{-1}, respectively, compared with the control treatment, which recorded the lowest averages of 0.531g, 0.214g, and 0.127g.$100g^{-1}$ fresh weight, and19.72 µg.g^{-1}, respectively.(Fig 2.)

Table 1. Effect of salicylic acid in the number of branches, length, and leaves number of D.lanata.

Salicylic acid mg. L^{-1}	Branches Number	Branches long cm	Leaves Number Leves.$plant^{-1}$
0.0	1.00	0.433	4.00
1.0	2.00	1.333	9.37
3.0	2.67	0.467	9.67
5.0	1.67	0.747	5.67
7.0	1.33	1.667	6.57
LSD 0.05	0.81	0.667	1.559

Table 2. Effect of salicylic acid in the fresh and dry weights, chlorophyll, and digoxin in the leaves of the *D. lanata* .

Salicylic acid $mg.L^{-1}$	average fresh weight (gm)	average dry weight (gm)	chlorophyll $mg.100g^{-1}$ fish weight	Digoxin $\mu g.g^{-1}$
0.0	0.531	0.214	0.127	19.72
1.0	0.691	0.321	0.408	22.38
3.0	1.328	0.879	1.376	28.48
5.0	0.723	0.442	0.721	25.68
7.0	0.604	0.298	0.315	27.48
LSD 0.05	0.001	0.046	0.168	1.052

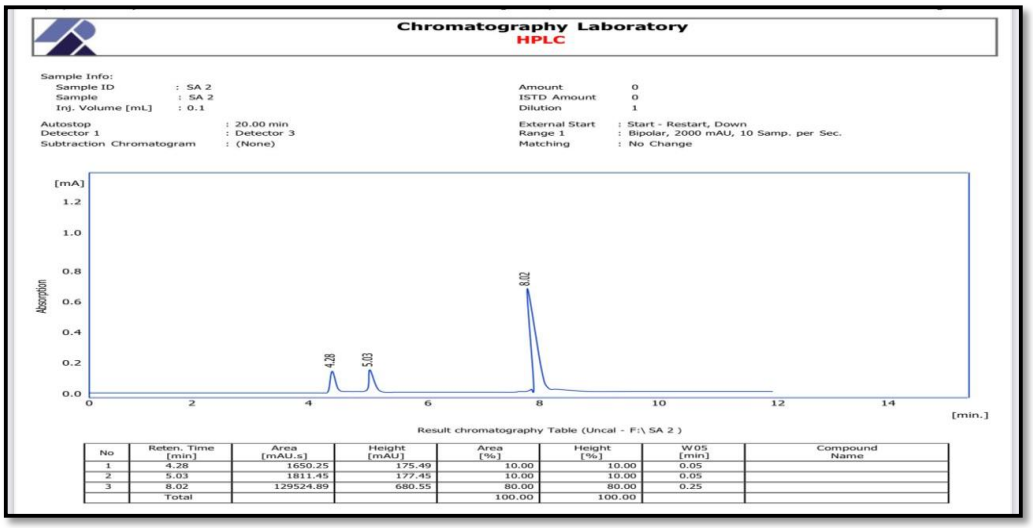

Figure 2. chromatogram of SA_2 ($3mg.L^{-1}$).

Effect of ginger extract on the number and length of branches and the number of leaves of digitalis plans

Results of Table 3 confirm that the different concentrations of ginger extract significantly affected the average number and length of proliferated branches and the number of leaves, as the concentration of $5g.L^{-1}$ was significantly superior to all other concentrations, recording 4.33 branches.$plant^{-1}$, 2.253 cm., and 14.43 lea.$plant^{-1}$, compared with the control treatment, which recorded the lowest rates of 1.00 branches.$plant^{-1}$, 1.667 cm, and 3.637 leaves.$plant^{-1}$, respectively.

Table 3. Effect of Ginger extract on the average number of branches, length of branches and number of leaves of D.lanata

Ginger extract gm.L^{-1}	Number of branches bran.plant^{-1}	long of branches (cm)	Number leaves lea.pant^{-1}
0.0	1.00	1.667	3.637
3.0	2.33	2.467	6.467
5.0	4.33	2.253	14.43
7.0	2.00	1.375	5.000
LSD 0.05	0.769	0.546	0.557

Effect of ginger extract on the fresh and dry weight, chlorophyll content, and cardiac digoxin in digitalis plant.

Results in Table 4 and figure 3 show that the ginger extract at different concentrations significantly affected the fresh and dry weight, chlorophyll content, and digoxin content in the leaves. The ginger extract concentration of 5g.L^{-1} was significantly superior to the other concentrations and recorded 2.133 g, 1.243 g, 14.99 mg.100 g^{-1}, and 29.32 µg.g^{-1}, compared with the control treatment, which recorded 0.352 g, 0.131 g, 0.188 g. 100 g^{-1}, and 22.71 µg.g^{-1} For the studied characteristics.

Table 4. Effect of Ginger extract on the average fresh and dry weights, chlorophyll, and digoxin compound in the leaves of the D. lanata

Ginger extract gm.L^{-1}	average fresh weight (gm)	average dry weight (gm)	chlorophyll mg.100 g^{-1}	Digoxin µg.g^{-1}
0.0	0.352	0.131	0.188	22.71
3.0	0.459	0.289	0.302	25.89
5.0	1.567	1.497	0.823	29.32
7.0	0.327	0.201	0.459	27.96
LSD 0.05	0.005	0.001	0.002	0.545

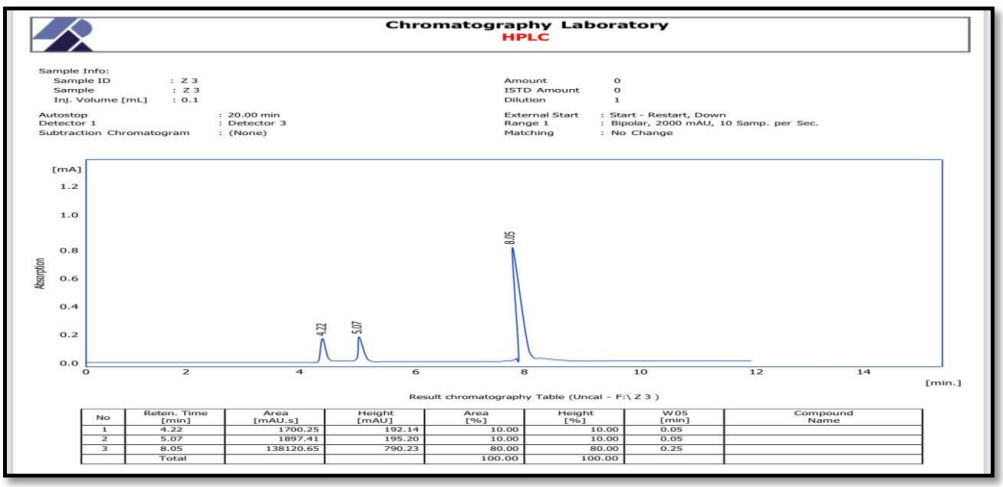

Figure 3. The chromatogram of **Ginger extract** (5g.L^{-1}).

Effect of the black pepper extract on the number and length of branches and the number of leaves of digitalis plans

Results in Table 5 demonstrate the significant effect of different concentrations of the black pepper extract on the number and length of the proliferated branches and the number of leaves. The concentration of 3 $g.L^{-1}$ of black pepper extract exhibited superiority to the other concentrations of these traits, recording 2.133 $branches.plant^{-1}$, 1.243 cm, and 14.99 $lea.plant^{-1}$, respectively; in comparison, the control treatment recorded the lowest value of the traits on an average of 1.00 $branches.plant^{-1}$, 0.666 cm, and 5.65 $lea.plant^{-1}$, respectively.

Table 5. Effect of Black Pepper Extract on the average number of branches, length of branches and number of leaves of *D.lanata.*

Black Pepper extract $gm.L^{-1}$	**Number of branches** $bran.plant^{-1}$	**long of branches (cm)**	**Number leaves** $lea.pant^{-1}$
0.0	1.00	0.666	5.65
3.0	2.133	1.243	14.99
5.0	1.001	1.253	6.67
7.0	1.247	1.624	6.38
LSD 0.05	0.518	0.054	2.267

From the results in Table 6, and figure 4,it is evident that the black pepper extract significantly affected the averages of the traits, plantlet fresh and dry weight, chlorophyll content, and digoxin content in the leaves. The black pepper extract concentration of 5 $g.L^{-1}$ was significantly superior to all other ones, recording 1.174 g, 0.659 g, 0.855 $mg.100\ g^{-1}$, and 29.66 $\mu g.g^{-1}$, respectively, compared with the control treatment, which recorded the lowest value of these traits, averaging 0.631 g., 0.232 g., 0.116 $mg.100\ g^{-1}$, and 22.06 $\mu g.g^{-1}$, respectively.

Table 6. Effect of Black Pepper Extract on the fresh and dry weights, chlorophyll, and digoxin in the leaves of the *D. lanata*

Black Pepper extract $gm.L^{-1}$	average fresh weight (gm)	average dry weight (gm)	Chlorophyll $mg.100\ g^{-1}$	Digoxin $\mu g.g^{-1}$
0.0	0.631	0.232	0.116	22.06
3.0	0.688	0.336	0.497	26.48
5.0	1.174	0.659	0.855	29.66
7.0	0.275	0.124	0.792	29.24
LSD 0.05	0.001	0.002	0.001	0.777

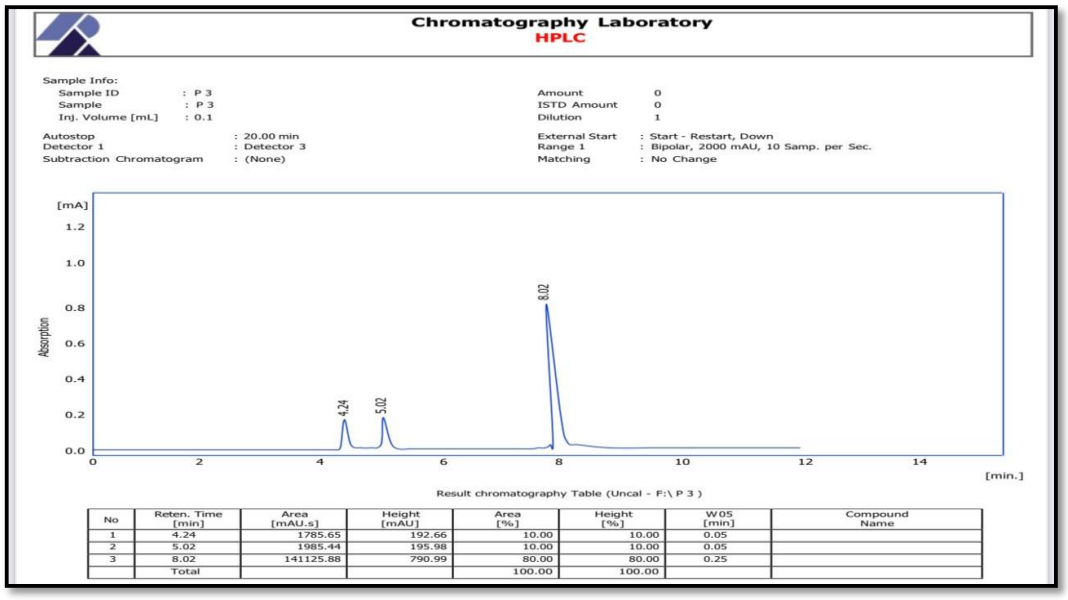

Fig 4. The chromatogram of black ginger extract (3g.L^{-1}).

Discussion

It is evident from the above that salicylic acid enhances vegetative traits by stimulating the production of endogenous hormones such as IAA, GA3, and cytokinins while preventing their breakdown and promoting their construction.(Abdulraheem et al.,2024, Nasseralla and Almukhtar.,2023), This was reflected in the increase in lateral branches (Table 1). Perhaps this is due to the synergistic effect produced by some phenolic compounds, and since salicylic acid is considered a widespread phenolic compound in plants, it participates directly in regulating the physiological processes with other growth regulators, including IAA, which has a role in the cell division, enlargement, and elongation, and thus this led to an increase in length of the branch (Table 1). These results are consistent with the findings of Al-Amery et al.,2023 and Jhundoo et al.,2024). It may also be due to salicylic acid's positive role in improving growth and the transpiration rate, regulating carbon assimilation, opening, and closing stomata, and absorbing and transporting nutrients within the plant. This opinion was consistent with the findings of Dias (2019), which refer to the effective role of growth regulators in increasing the number of branches within a brief period. Regarding the role of salicylic acid in improving physiological indicators, it had a role in increasing the chlorophyll content in the leaves (Table 2), which is due to stimulating the formation of the chlorophyll pigment by stimulating the formation of karna plates and the development of chloroplasts, in addition to stimulating the production of enzymes responsible for building photosynthesis pigments and inhibiting the activity of catabolic enzymes (Chavoushi et al.,2020;Gorni et al.,2020). Salicylic acid also increases secondary metabolite compounds (Table 2) through its stimulation of the gene expression and regulation of signal transduction during the gene expression process, in addition to its role in stimulating the activity of the proteins ADS, PAD4, ED5, and ICS, which are essential in the phenylpropanoid pathway (Hafeez et al.,2024; Arif et al.,2023) (Fig 2.)Differences in the concentration of secondary metabolite compounds may be due to genetic factors or environmental factors, including light, heat, humidity, type of plant part, and stage of plant growth, as well as other factors such as the pre-extraction physiological state (Kahveci et al.,2021).

Plant extracts contain elements or substances that can replace various growth regulators. Agricultural specialists refer to these compounds of plant origin as phytochemicals. Some of the most widely used plant extracts are ginger and black pepper (Pylak et al.,2019) (Fig 3.) . These extracts contain important secondary metabolites such as alkaloids, phenols, and glycosides and large quantities of potassium, phosphorus, iron, essential oils, proteins, fats, and other components that increase biological activities(Saleem et al.,2022;Shalaby et al.,2023). As a result, they promote

growth and increase the chlorophyll content in leaves by boosting the plant's photosynthesis rate, providing the necessary materials and energy for the growth and construction process(Fig 4.) . This, in turn, increases the secondary metabolite compounds within the plant, including cardiac glycosides (Polusa and Nirmala .,2003; Setyawati et al.,2021: Ei et al.,2024: Mirzapou et al.,2022).

CONCLUSIONS

Based on the experiment, we conclude that adding salicylic acid, ginger extract, and black pepper extract in separate experiments significantly affected growth indicators and the biochemical content in the leaves of the cardiac glycosides of the digitalis plant in vivo

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