Callus Induction and Enhancing the Production of Biomass and Pharmaceutical Components of *Thymus decussatus* as an Endangered Medicinal Plants in Egypt

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ABSTRACT:

Thymus decussatus is a perennial herbaceous endangered medicinal plant belongs to family *Lamiaceae*. *Thymus* species are considered the most popular herbs in Mediterranean region due to their medicinal and nutritional values. However, at the same time they are also threatened due to intensive harvesting. The protocol for inducing callus in *Thymus decussatus* was created and executed on Murashige and Skoog (MS) medium. This medium was supplemented with various concentrations of 6-Benzylaminopurine (BAP) in combination with Naphthalene acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D). The callus induction was performed using different types of explants derived from plants growing *in vitro*. The maximum callus induction percentage of 100%, was achieved by culturing stem segment explants on MS medium supplemented with 1.0 mg^{-1} BAP and 0.25 mgl^{-1} 2,4-D with callus fresh weight of 16.72 g and a dry weight of 0.37 g. The study investigated the impact of biotic (yeast extract) and abiotic (salicylic acid) elicitation on callus productivity and the accumulation of secondary metabolites. The best concentration of yeast extract (150 µm) that gave the highest callus fresh weight of 10.72 g and the best concentration of salicylic acid (250 µm) that gave the highest callus fresh weight of 15.01 g after 30 days of culturing. Salicylic acid (SA) showed superiority in promoting the development of callus, as evidenced by increased fresh and dry weight. Both elicitors gave positive results in enhancing secondary metabolites accumulation and increased the quantity of some phenolic compounds which have a significant biological effects and benefits for human health.

Keywords: callogenesis; Thyme; elicitor; secondary metabolites; salicylic acid; *lamiaceae*; yeast extract.

INTRODUCTION

Egypt has a significant biodiversity, with 529 therapeutic species, 60 endangered plants, and 13 pharmacopoeias (Boulos, 2009; Eissa *et al.*, 2014), with natural diversity ranging from deserts to coasts (the Mediterranean and Red coasts), Nile Delta, Nile River, Depressions, Oases, and Mountains (Zahran and Willis, 2008). The Sinai Peninsula, notably Saint Katherine Protectorate is the most viable source of traditional herbs in Egypt. Sinai plant species include unique metabolites with significant pharmacological effects (Batanouny *et al.*, 1999; Elshamy *et al.*, 2019). Lamiaceae is regarded an important family, featuring different aromatic plants comprised 236 genera and 6900-7200 species (Harley *et al.*, 2004; Heywood *et al.*, 2007).

Thymus genus is among the most significant aromatic plant with therapeutic properties (Stahl-Biskup and Sáez, 2002; Marin *et al.*, 2008). Also, the eighth-most abundant genus in Lamiaceae family. It offers a natural supply of monoterpene phenolic oils, oleoresins, fresh and dried plants (Lawrence and Tucker, 2002), and have been employed for numerous centuries in traditional medicine (Stahl-Biskup, 2002) because of their antibacterial, antiviral, antimicrobial, and antioxidative qualities (Reddy *et al.*, 2014). In recent decades, human activities have resulted in the consumption of conventional food supplements, overharvesting of fuel and pharmaceutical health products, constrained range distribution, a low rate of natural reproduction, mine overexploitation, and persistent overgrazing. *T. decussatus* is on the verge of extinction and is considered an extremely vulnerable species in Egypt (Jalili and Jamzad, 1999).

Secondary metabolites synthesized by many plant organs, including roots, stems, leaves, and other above ground structures, are utilized for the formulation of pharmaceuticals (Pan, 2014; Raomai *et al.*, 2015). Plant secondary metabolites are typically extracted from wild plants, a practice that often results in excessive utilization and thus endangers their survival (Song *et al.*, 2014). Besides, callus produced *in vitro* has the ability to synthesize secondary metabolites that are similar to those present in the parent plant so that, callus culture can provide sustainable therapeutic

compounds and could also be a new method by treating cultured cells as individual organisms instead of using the whole plant thus protecting it from extinction (Janarthanam *et al.*, 2010; Efferth, 2019). There are many attempts towards an efficient *in vitro* callus induction protocol for an endangered and valuable medicinal plant, (Kakalis *et al.*, 2023) recorded that different plant growth regulators, light conditions, and ascorbic acid supplements were assessed for optimizing callus induction from oregano leaves. Also, according to (Bakhtair *et al.*, 2016) callus induction of *T. persicus* was performed on MS medium supplemented with different concentrations of NAA and 2,4-D, alone or in combination with BAP and Kin.

Secondary metabolites synthesized and accumulated within plants act as defense mechanisms against biotic factors like herbivores, fungi, bacteria or viruses as well as abiotic factors such as UV radiation, drought stress, high/low temperature etc. (Kaur and Pati, 2018). Therefore there is need to increase the yield of secondary metabolite production in callus culture through specific elicitation since this is a powerful technique for enhancing biosynthesis and accumulation of secondary metabolites *in vitro* based tissue cultures of plants (Ramakrishna and Ravishankar, 2011; Wang and Wu, 2013). SA, also known as 2-hydroxybenzoic acid, it is a plant phenolic compound that an effective inducer which activates genes responsible for defence mechanism in plants. SA can considerably promote the synthesis of many different types of secondary compounds such alkaloids , terpenoids , phenolics among others phytoalexins too (Vlot *et al*., 2008). According to (Alvarez *et al.*, 2000) SA greatly enhanced production levels of alkaloids in the hairy root culture derived from *Brugmansia x candida* Pers. Similarly (Mendhulkar *et al.*, 2013) introduced term " salicylic acid " after finding its benefits towards total flavonoids content raised during suspension culture of *Andrographis paniculata* Burm.f. Also, yeast extract is a set of compounds that have a beneficial effect on plant growth, productivity, microelement composition, and the content of phytohormones and other plant metabolites (Naik and Al-Khayri, 2016; Halder *et al.*, 2019). According to Zhao *etal.,* 2014, elicitation with yeast polysaccharide effectively increased hairy root growth and Flavonoids (rutin and quercetin)production of Fagopyrum tataricum in aconcentration dependent manner by the stimulation of the phenylpropanoid pathway.

The aim here is therefore to develop an *in vitro* culture technique that will induce callus formation from different explant types of *T. decussatus* and *in vitro* stimulating secondary metabolite production using various elicitors which are alternative biotechnology methods for producing potent secondary metabolites required for future pharmacological investigations with *T. decussatus* as the plant material of choice where natural resources are scarce.

MATERIALS AND METHODS

Plant Materials:

Seeds of *T. decussatus* were collected from a wild populations in the mountain tops with in Saint Katherine Protectorate, South Sinai, Egypt and washed under tap water for an hour then soaked in 70% ethanol for 30 seconds. Seeds were surface sterilized with 0.75% commercial NaOCl solution for 15 minutes. All seeds were rinsed for 4-5 times with sterile double distilled water and cultured on MS medium **(**Murashige and Skoog, 1962) without any plant growth regulators (PGRs)for seed germination. After two months of germination the whole leaves were collected while the stems were cut in to sections of 1-1.5 cm-long segments. All parts were cultured on MS medium supplemented with different concentrations and combinations of (PGRs) for callus induction.

Nutrient medium and culture conditions:

The nutrient medium used in this study was MS culture medium supplemented with 3% sucrose, 0.5% phytagel, and pH was adjusted to 5.8 ± 2 using 1.0 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl). Then, all jars were closed with autoclavable polypropylene caps and autoclaved at a temperature of 121°C for 20 minutes under 1.1 kg/cm^2 . The laminar airflow chamber was exposed to ultraviolet (UV) light for 30 minutes to sterilize the surface of the working area.

Callogenesis:

The callus was induced from leaves and stem segments on MS medium supplemented with various concentrations of (BAP) (0.0, 0.5, 1.0, 2.0, 3.0 mgl^{-1}) in combination with different concentration of (NAA) (0.5, 1.0 ,2.0 mgl^{-1}) or with different concentration of 2.4-D (0.25, 0.5, 1.0 mgl^{-1}). Callus induction took place in 250 ml glass jars, containing 50 ml of the previous described medium, sealed with plastic caps. All the cultures were incubated in

a culture room maintained at a temperature of 25 ± 2°C under 16 h light and 8 h dark using cool-white fluorescent lamps (Philips, 58W, Holland). Results were recorded after 21 days of the third subculture (fresh, dry weight and morphological features of callus). The cultured tissues were carefully taken from the culture vessel and cleaned from phytagel particles that had adhered at the point of contact. After that, the tissue was placed in a pre-weighted petri dish and the weight was determined using a single pan digital balance of fresh weight of callus which presented as gram (g). The tissues were oven-dried at 50°C to a consistent weight on the same petri dishes for estimation of their dry weight as g. Percentage of callus induction frequency was detected according to the following equation of (Mostafiz and Wagiran, 2018).

Callus induction frequency (%) = (Number of explants induced callus / Number of explants cultured) × 100. Also, the colour, uniformity and texture of callus were recorded.

Elicitation:

Two elicitor types (yeast extract and salicylic acid) were used for the enhancement of productivity of biomass and secondary metabolites accumulation of callus culture. Yeast extract was dissolved in double sterile distilled water with different concentrations (0.0, 50, 100, 150, 200, 250 and 300 mgl^{-1}) and SA was dissolved in pure ethanol and were added with different concentrations (0.0, 50, 100, 150, 200, 250 and 300 µM) through a micro filter of 0.22 µ pore size to MS basal medium fortified with 1.0 mgl^{-1} BAP in combination with 0.25 mgl^{-1} 2,4-D. For elicitation purposes, equal fragments (1.0 g) after 2 months *in vitro* growing friable white callus were transferred to the previous medium fortified with different concentrations of yeast extract and SA separately. Increasing in callus growth represented by fresh and dry weight were recorded after 15 and 30 days of incubation under controlled conditions and compared to callus growth on control medium (without elicitors).

Preliminary phytochemical screening

The powdered samples of the produced calli of *T. decussatus* were screened for phytochemical constituents

Extraction of phenolic compounds

The callus extracts were prepared according to the method of (Castro *et al.*, 2016) . Briefly, 1.0 g of callus from each treatment was dried in an oven at 50°C for 24 hours, and then were soaked in 5 ml diethyl ether for 24 h. In order to prevent the evaporation of diethyl ether, the vials were kept closed and extraction was performed in a cold room. After 24 hours, the extracts were poured in to clean vials, and the leaves were rinsed with another 2.5 ml diethyl ether, which was added to the initial extracts. After that adding 1 ml of 80% methanol to the remaining solid material, the extracts were filtered (0.22 µm pore size) into clean vials and prepared for injection to HPLC instrument.

HPLC analysis

Phenolic compound contents of the callus was determined using high performance liquid chromatography (HPLC) instrument. HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using Zorbax Eclipse Plus C8 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 0.9 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0 –1 min (82% A); 1-11 min (75% A); 11-18 min (60% A); 18-22 min (82% A) ; 22-24 min (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 5.0 µl for each of the sample solutions. The column temperature was maintained at 40 °C.

Experimental design and statistical Analysis:

All experiments were conducted under controlled conditions with five replications. The statistical analysis was performed using one –way analysis of variance (ANOVA) by the general linear models (GLMs) approach in the Minitab 19 System. The least significant difference (LSD) approach was used for mean comparisons (Lesik, 2018).

RESULTS AND DISCUSSION

Effect of PGRs and explant type on callus induction

In this study, it was found that the selection between NAA or 2,4-D with BAP and choice of explants (leaf or stem segment) are essential considerations in callus induction experiments. Such conclusions may be drawn because different elements such as genotype, culture medium and its components, PGRs used as well as type of an explant all have their influence on the development systems of cell cultures as reported by (Pandey *et al.*, 2013; Abd El-Motaleb *et al.*, 2023).

Based on Table 1 the results showed that MS medium supplemented with BAP and NAA or 2,4-D were effective for inducing calli from stem segment explants. No callus was formed on control medium devoid of plant growth regulators (PGRs), indicating that a requirement for the presence of these substances in order to induce callusing from stem segments..

The highest callus induction frequency percentage resulted in MS medium supplemented with 1.0 mg l^{-1} BAP and 0.25 mg l^{-1} 2,4-D of stem nodal segment explants with fresh weight 16.72 g and dry weight 0.37 g and the formed callus was morphologically white, spongy, friable, and nodular (Table 1; Figure 1A), Concerning the callus obtained from leaf explants, the same medium also gave the maximum fresh weight of 12.25 g and dry weight of 0.27g, and were white brownish in colour, compact, friable, and had a nodular structure (Table 2; Figure 1B).

MS medium supplemented with 3.0 mgl^{-1} BAP and 2.0 mgl^{-1} NAA resulted in the lowest callus induction percentage (43.33%) for stem segment explants with fresh weight of 0.21 g and dry weight of 0.02 g , and morphologically white green with brownish colour and compact nodular texture (Table 1; Figure 2A). On the other hand, for leaf explant, MS medium supplemented with 2.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA resulted in the lowest callus induction percentage (33.33%) with fresh weight of 0.29 g, dry weight of 0.016 g, and morphologically green with brownish colour and compact nodular texture.(Table 2 : Figure 2F).

Both types of chosen plant tissue samples had the highest rate of callus formation, corroborating other studies conducted on various plant species, including *Mentha spicata* (Poovaiah *et al*., 2006) *Artemisia pallens* (Nathar and Yatoo, 2014), *Lavandula angustifolia* (Machado *et al*., 2014), *T. hyemalis* (Nordine *et al*., 2014) and *T. persicus* (Bakhtair *et al*., 2016). The production of calli and their characteristics (colour, texture, surface, and uniformity) varied Significantly varying based on the concentration and combination of PGRs. This result is similar with that obtained by (Abd El-Motaleb *et al*., 2023) who reported that the different levels of growth regulators in the media exhibited the morphological changes in calli and the nodal segments were more effective than leaf explants in terms of callus percentage, callus fresh and dry weight.

Callus produced from stem segment explants of *T. decussatus* cultured on MS medium supplemented with 1.0 mg l^{-1} BAP in combination with 0.25 mg l^{-1} of 2,4-D was superior than that produced from leaf explant cultured on the same medium composition (Figure 1A, B). This result agrees with (Bakhtair *et al*., 2016; Nasrat *et al*., 2022; Razavizadeh *et al*., 2019; Mahood *et al*., 2022). They found that the best explant type for callus induction was the stem segment. They also recorded that 2,4-D, is as a strong auxin, promoted callus formation more than other forms of auxin that agree with the results obtained by (Kakalis *et al*., 2023), who achieved maximum callogenesis rate (100%) from oregano leaves in MS medium with 0.5 mg l^{-1} 2,4-D + 3 mg l^{-1} BAP and harmony with the result obtained by (Abd El-Motaleb *et al*., 2023), observed that the combination of 0.25 mg l^{-1} BAP with 2 mg l^{-1} 2,4-D resulted in the highest callus percentage and fresh weight.

In general, the findings of this study strongly support and agree with those of (Pandey *et al*., 2013), who found significant differences in callus induction and plantlet regeneration in *Psoralea corylifolia* based on explant type and growth regulator composition of the culture medium. Also, this results are similarly consistent with those obtained by (Abd El- Motaleb *et al*., 2023), who showed that various quantities of (PGRs) in the medium caused morphological alterations in calli. Stem segments performed better than leaf explants in terms of callus percentage, fresh and dry weight, and initiation time. This result is in harmony with the results obtained by (Mahood *et al*., 2022). In contrast to the results reported by (Tokgoz and Altan, 2020; Kakalis *et al*., 2023), who achieved callus induction using leaf explants.

Effect of elicitation on callus growth

The impact of biotic elicitation, achieved by adding yeast extract (YE), and abiotic elicitation, achieved by adding SA, to the callus culture media may be observed in (Tables 3 , 4 and Figures 3, 4, and 5). These measurements specifically pertain to the growth of callus in terms of fresh and dry weight.

Elicitor type, dose, and treatment schedule all have a significant impact on callus growth and secondary metabolite generation. Table 3 shows that dose and incubation period had a synergistic effect on callus mass formation of *T. decussatus* plant. After 15 days of culture, the lowest concentration of YE (50 mgl^{-1}) had the

greatest impact on callus growth compared to the control medium and higher concentrations of YE.

Reaching the highest concentration of YE (300 mgl^{-1}), callus development gradually decreases to the minimum. After 15 days of culture, treatment with 50 mgl^{-1} YE resulted in the maximum callus growth, increasing fresh weight from 1.687 g to 4.02 g and dry weight from 0.167 g to 0.223 g. This value is markedly greater than all other treatments. After a period of 30 days, the experiment involved feeding with various concentrations of YE. The findings indicated that the treatment with YE led to a significant increase in callus growth. This was evident in the rise in fresh weight from 5.217 g in the control medium to 10.72 g, as well as the increase in dry weight from 0.23 g in the control medium to 0.337 g in callus treated with 150 mgl^{-1} YE (Figure 3).

Table (4) shows the influence of different SA concentrations and incubation times on callus growth of *T. decussatus*. The study found that higher concentrations of SA (200, 250 μM) had the greatest impact on callus growth after 15 days of culture, outperforming the control medium and other SA concentrations. The largest callus growth after 15 days of culture resulted from exposing it to 250 μM SA, which increased the fresh weight by 1.686 g to 2.63 g and dry weight by 0.166 g to 0.216 g; the all other treatments were much lower than this value.

After 30 days of culture, the results indicated that treatment with SA led to a significant increase in callus growth. This increase was accompanied by a notable rise in the fresh weight of callus, from 7.78 g in the control medium to 15.01 g. Additionally, the dry weight of calli treated with 250 μM SA increased from 0.336 g in the control medium to 0.47 g (Figure 4). Based on the findings, we can conclude that elicitor type (SA and yeast extract), concentration and elicitation period play important roles in inducing various responses linked to cell growth parameters (biomass yield). Furthermore, varying concentrations of elicitors play a significant role in cell development. However, a larger concentration of elicitors generates a hypersensitive response, leading to cell death, and an optimum level of elicitor was necessary for the induction, and this is in agreement with (Al-Khayri and Naik, 2020; Mahood *et al.*, 2022). It can be concluded that stem segment explants of *T. decussatus* showed the best results for callus induction *in vitro* when grown on MS medium with 1.0 mgl^{-1} BAP and 0.25 mgl^{-1} 2,4-D. The type of elicitor and incubation period influence culture growth, as measured by the fresh and dry weights of the calli. After 30 days of incubation, SA at 250 μM was more effective for callus biomass of *T. decussatus* than yeast extract.

Effect of biotic elicitation (YE) on productivity of callus phenolic compounds

Elicitation is one of the most effective and widely employed biotechnological tools for the induction of novel secondary metabolites or enhanced biosynthesis as well as accumulation of secondary metabolites *in vitro* plant tissue culture (Ramakrishna and Ravishankar, 2011; Wang and Wu, 2013)

For this investigation, the calli derived from the medium with the best elicitor concentration (150 mgl^{-1}) that gave the highest fresh and dry weight of callus were chosen and compared to callus on the control medium (without elicitor) and mother plant regarding the active constituents content.

Table 5 shows the influence of YE as a biotic elicitor on the concentration of secondary metabolites in callus of *T. decussatus*. The elicitation effect of YE gave positive results for chlorogenic acid and increased from 5.92 μg ml^{-1} in callus grown in control medium and 8.03 μg ml^{-1} in mother plant to 10.11 μg ml^{-1} with elicitor, ellagic acid from 0.67 μg ml^{-1} in callus grown in control medium and 1.25 μg ml^{-1} in mother plant to 2.76 μg ml^{-1} with elicitor, and daidzein from 0.62 μg ml^{-1} in callus grown in control medium and 1.31 μg ml^{-1} in mother plant to 4.34 μg $ml^{-1}$with elicitor (Graphs 1,3,4).

Chlorogenic acid, an ester of caffeine and (-)-quinic acid, serves as an intermediary in lignin production (Boerjan *et al.*, 2003). The name "chlorogenic acids" refers to a related polyphenol family of esters, containing hydroxycinnamic acids (e.g., caffeic, ferulic, and p-coumaric acid) and quinic acid (Clifford *et al.*, 2003).

Ellagic acid is a polyphenol present in many fruits and vegetables. Ellagic acid is an investigational medication being explored for the treatment of Follicular Lymphoma, the prevention of brain injury in intrauterine development limited newborns, the enhancement of cardiovascular function in adolescents, and the topical therapy of solar lentigines. Ellagic acid's medicinal action mostly includes antioxidant and anti-proliferative properties.

Daidzein is a naturally occurring isoflavone. Plants manufacture daidzein and other isoflavones through the phenylpropanoid pathway for signalling and defence against pathogens (Jung *et al.,* 2000). Recent research suggests that daidzein can be used to treat menopausal symptoms, osteoporosis, blood cholesterol, and reduce the incidence of hormone-related malignancies and heart disease. Puerarin and daidzein provide health benefits, however their low water solubility and poor bio availability limit their use (Wang *et al.,* 2022).

The current study found that YE increased the accumulation of several secondary metabolites in *T. decussatus* calli compared to the mother plant, such as chlorogenic acid, ellagic acid, and daidzein, which have amazing biological effects and benefits for human health. This outcome was consistent with (Cai *et al*., 2012; Ramirez-Estrada *et al*., 2016; Singh *et al*., 2018) who demonstrated that while the application of different stimulants to undifferentiated cells increased the production of secondary metabolites, this approach has several drawbacks. These include lower productivity compared to organ culture, genetic and biosynthetic instability over extended periods of cultivation, and inconsistent response to the same stimulant. Additionally, (Ramirez-Estrada *et al.,* 2016) found out that yeast or fungal elicitors are commonly used for inducing the production of secondary metabolites in plants especially when they are in hairy root or cell cultures.

Different studies have shown positive effects of YE on the amount of secondary metabolites synthesized. These researches indicate that YE can be used to improve plant and cell growth by increasing biomass. For instance, (Krstić-Milošević *et al*., 2017) applied biotic elicitor which is a YE leading to increased root growth and biomass production in *Gentiana dinarica.* Also, (Bayraktar *et al.,* 2016) used it for enhancing *Stevia rebaudiana* biomass) through promoting higher levels of growth.

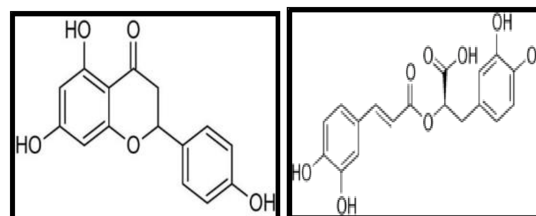
Effect of abiotic elicitation (SA) on productivity of callus phenolic compounds

SA is a derivative of phenolic called 2-hyroxy benzoic acid. It falls under the classification of plant hormones and it majorly controls the growth and development of plants (Raskin, 1992; Taguchi *et al*., 2001) . For this study, the calli derived from the medium with the best elicitor concentration (250 µM) that gave the highest fresh and dry weight of callus were chosen and compared to callus on the control medium (without elicitor) and mother plant regarding the active constituents content.

Table 6 presents findings on the effect of SA as an abiotic elicitor on secondary metabolites content in callus of *T. decussatus*. The elicitation effect of SA gave positive results and increased chlorogenic acid from 5.92 µg/ml in callus grown in control medium and 8.03 µg ml^{-1} in mother plant to 21.23 µg $ml^{-1}$with elicitor, naringenin increased from 0.18 µg/ml in mother plant and 0.38 µg ml^{-1} in callus grown in control medium to 0.96 µg ml^{-1} with elicitor and rosmarinic acid concentration increased significantly from 96.26 µg ml^{-1} found within callus grown in control medium and 465.78 µg ml^{-1} in mother plant to 815 µg ml^{-1} with elicitor, daidzein increased from 0.62 µg ml^{-1} in callus grown in control medium and 1.31 µg ml^{-1} in mother plant to 4.62 µg ml^{-1} with elicitor (Graghs 2,3,4).

Naringenin is a flavanone belonging to the flavonoid group of polyphenols (Felgines *et al.,* 2000). Naringenin has showed several biological effects, including anti-inflammatory, antioxidant, and skin healing (Wang *et al.,* 1999; Karuppagounder *et al*., 2016; Martinez *et al*., 2016; Al-Roujayee, 2017). It is utilized as a cosmetic ingredient and dietary supplement (Rebello *et al*., 2020).

Rosmarinic acid is a caffeic acid ester and a naturally-occurring phenolic components in a variety of plants belonging to the Lamiaceae family (Nadeem *et al.,* 2019). It possesses extraordinary biological benefits, including antiviral, antioxidant, antibacterial, anticancer, antidiabetic, anti-aging, cardioprotective, nephroprotective, hepatoprotective, antidepressant, antiallergic, and anti-inflammatory properties.



Naringenin Rosmarinic acid

The current study showed that SA stimulated the accumulation of some phenolic compounds in *T. decussatus* calli, which have remarkable biological effects especially rosmarinic acid which increased approximately two times it's accumulation in mother plant. This finding was consistent with (Mendoza *et al*., 2018), who found that SA (300

μM) and MeJA (3 μM) increased the amount of phenolics and flavonoids compounds, implying an inducer action of these elicitors in the phenylpropanoids metabolic pathway.Furthermore, the current study is consistent with the findings of (Figueroa Perez *et al.*, 2014; Kandoudi and Nemeth-Zamborine, 2022), who indicated that SA treatment of peppermint increased total phenolic compounds and flavonoids at all concentrations when compared to control.

CONCLUSION

Ultimately, the effectiveness of callus formation is contingent upon the growth regulators employed and the origin of the explant. The results of this study showed that *T. decussatus* explants cultivated *in vitro* exhibit varying responses based on the growth regulators used in the culture media and the source of the explants. In addition, the results demonstrated that 2,4-D is the most effective type of auxin for inducing callus formation when combined with BAP in the studied plant. The highest percentage of callus induction frequency (100%) was observed when stem segment explants of T. decussatus were cultured on MS medium supplemented with 1.0 mg l^{-1} BAP in combination with 0.25 mg l^{-1} 2,4-D. Finally the present work demonstrated a promising effect of yeast extract as a biotic elicitor and SA as an abiotic elicitor for the enhancement of the productivity of secondary metabolites content of *T. decussatus* callus derived from stem segment explants. From the results obtained in the present study we may recommended that, using callus as a modern biotechnological method for production of phytopharmaceutics.

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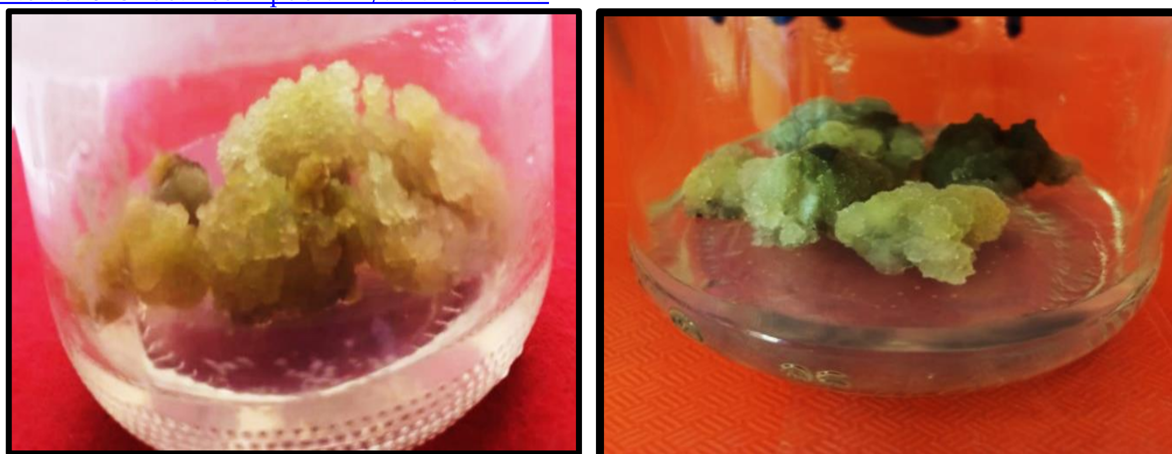


Figure 1: Callus formation of *T. decussatus* on MS medium fortified with 1.0 mgl^{-1} BAP +0.25mgl^{-1} 2,4-D A. from stem segment explant, B. from leaf explant

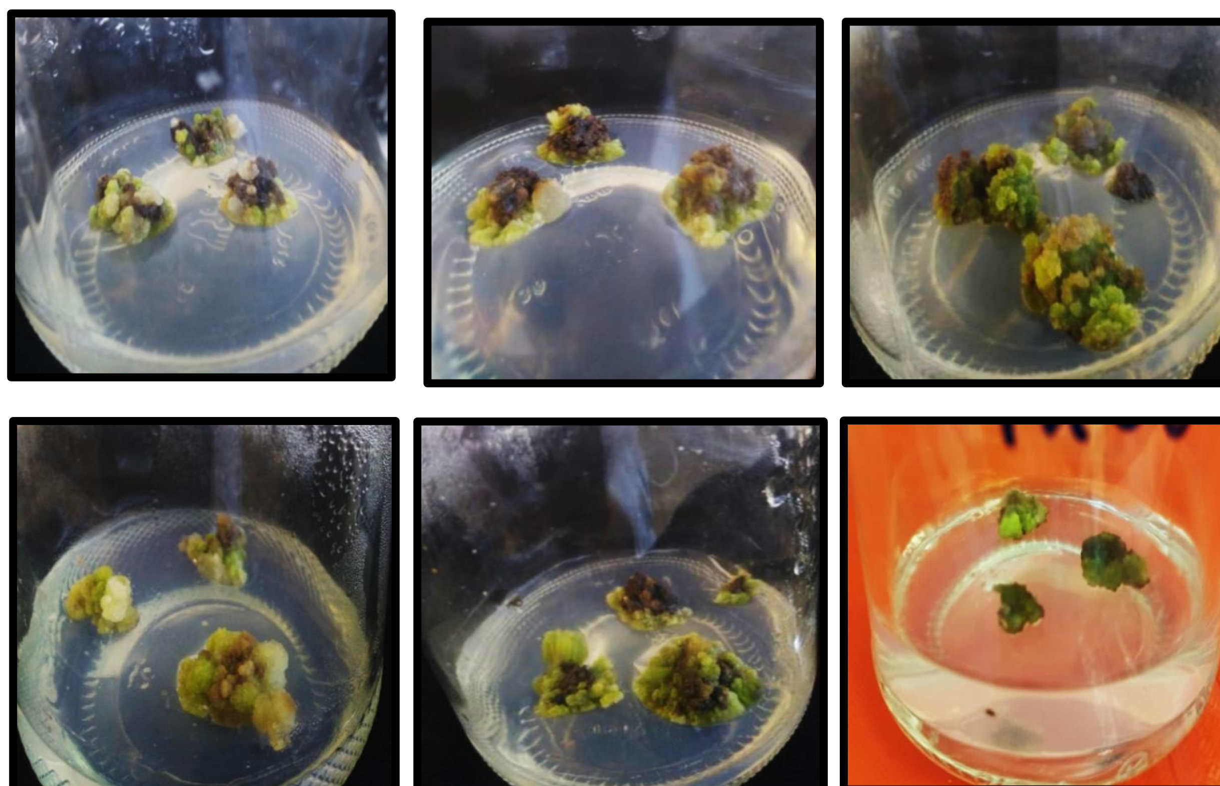


Figure 2: Callus formation of *T. decussatus* on MS medium fortified with A. 3.0 mgl^{-1} BAP+2.0 mgl^{-1} NAA; B. 1.0 mgl^{-1} BAP+1.0 mgl^{-1} NAA C. 1.0 mgl^{-1} BAP+2.0 mgl^{-1} NAA of stem nodal segment explants D. 2.0 mgl^{-1} BAP+0.5 mgl^{-1} NAA E. 0.5 mgl^{-1} BAP+2.0 mgl^{-1} NAA F. 2.0 mgl^{-1} BAP+1.0 mgl^{-1} NAA from leaf explants.

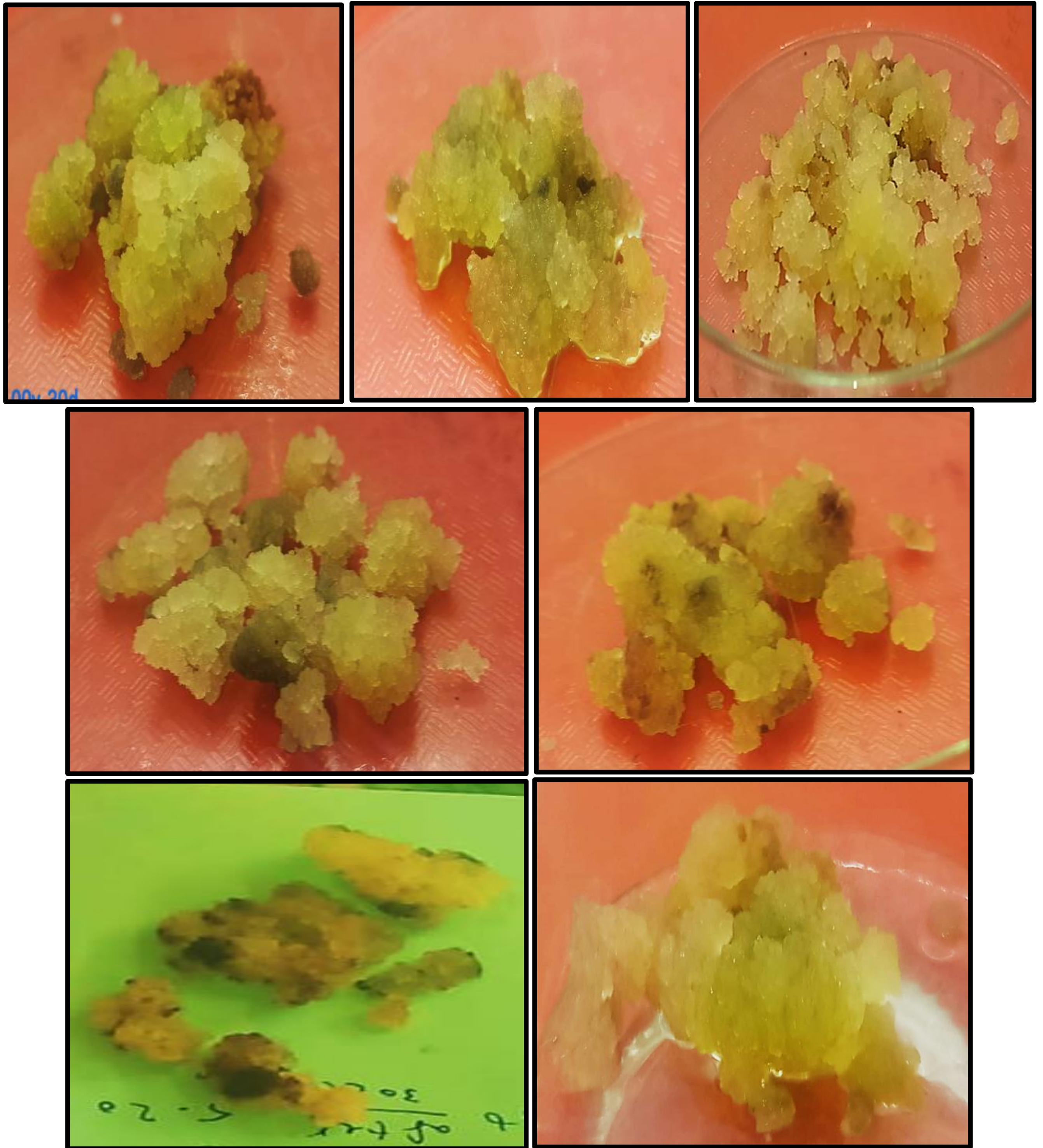


Figure 3: The effect of various concentrations of YE on callus growth of *T. decussatus* after 30 days of incubation. A. 50 B. 100 C. 150 D. 200 E. 250 F. 300 mgl^{-1} G. control.

Figure 4: The effect of various concentration of SA on callus growth of *T. decussatus* after 30 days of incubation . A. 50 μM B. 100 μM C. 150 μM D. 200 μM E. 250 μM F. 300 μM G. control.

Table 1: Effect of stem segment explant and various growth regulator treatments on fresh, dry weights and morphological character of *T. decussatus* calli.

PGRs treatment (mgl^{-1})	Callus induction frequency (%)	Fresh weight (g)	Dry weight (g)	Colour	Uniformi ty	Texture
Control(free MS medium)	0.00	0.00	0.00	-	-	-
(A)	66.67 ± 3.33^{cde}	0.36 ±0.22 ^{bc}	0.02 ±0.01^{b}	Brownish green	Patchy	Compact
(B)	83.33 ± 3.33^{abc}	0.81 ± 0.028 ^{bc}	0.05 ± 0.005 ^{b}	White green with brownish	Patchy	Compact friable
(C)	56.67 ±3.33^{def}	0.63 ± 0.09^{bc}	0.04 ± 0.01^{b}	Brownish green	Patchy	Compact friable
(D)	93.33 ± 6.67^{ab}	0.9± 0.38 ^{bc}	0.05 ± 0.02^{b}	Greenish white	Patchy	Compact
(E)	56.67 ±3.33^{def}	0.36 ± 0.05^{bc}	0.02 ± 0.002 ^{b}	Greenish white	Patchy	Spongy
(F)	53.33 ±3.33^{ef}	0.34± 0.08^{bc}	0.02 ± 0.004 ^{b}	green	Patchy	Compact friable
(G)	43.33 ± 3.33^{f}	0.21 ± 0.024^{c}	0.02 ± 0.00^{b}	White green with brownish	Patchy	Compact friable
(H)	100±0.00^{a}	16.72 ± 1.08^{a}	0.37 ± 0.018 ^{a}	White	Uniform	Spongy friable
(I)	76.67 ±3.33^{bcd}	2.207± 0.19^{b}	0.18± 0.00^{b}	Greenish white	Patchy	Compact friable
(J)	86.67 6.67^{abc}	1.33± 0.06^{bc}	0.18± 0.00^{b}	Greenish white	Patchy	Compact friable

Each value represents Mean±SE. Means that do not share a letter are significantly different. Results obtained after 21 days of third subculture.

(A) 0.5 BAP+ 2.0 NAA **(B)** 1.0 BAP+ 1.0 NAA **(C)** 1.0 BAP+ 2.0 NAA **(D**) 2.0 BAP+ 0.5 NAA (E) 2.0 BAP+1.0 NAA **(f)** 3.0 BAP+ 1.0 NAA **(G)** 3.0 BAP+ 2.0 NAA) **(H)** 1.0 BAP+ 0.25 (2,4 D) **(I**) 1.0 BAP+ 0. 5 (2,4 D) **(J)** 1.0 BAP+ 1.0 (2,4 D)

Table 2: Effect of leaf explant and various growth regulator treatments on fresh, dry weights and morphological character of T. decussatus calli.

Hormonal treatment (mgl-1)	Callus induction Frequency (%)	Fresh weight (g)	Dry weight (g)	Colour	Uniformity	Texture
Control (free MS medium)	0.00	0.00	0.00	-	-	-
(A)	46.67 ± 3.33c	0.49 ± 0.11^{b}	0.026 ± 0.007^{b}	White green with brownish	Patchy	Spongy friable
(B)	53.33 ± 3.33bc	0.60 ± 0.11 ^{b}	0.032± 0.003^{b}	White green with brownish	Patchy	Spongy friable
(C)	53.33 ± 3.33bc	1.003 ± 0.52^{b}	0.05 ± 0.03^{b}	White green with brownish	Patchy	Compact friable
(D)	73.33 ± 6.67ab	0.53 ± 0.13^{b}	0.028 ± 0.004^{b}	Greenish white	Patchy	Spongy
(E)	33.33 ±3.33c	0.29 ± 0.06^{b}	0.016 ± 0.00 ^{b}	Greenish white	Patchy	Compact
(F)	36.67 ± 3.33c	0.58 ± 0.13 ^{b}	0.025 ± 0.006^{b}	White green with brownish	Patchy	Compact friable
(G)	36.67 ± 3.33c	0.51 ±0.11 ^{b}	0.028 ±0.003^{bc}	Greenish white	Patchy	Compact
(H)	83.33 ± 3.33a	12.25 ± 1.11 ^{a}	0. 27 ± 0.032^{a}	Brownish white	Patchy	Compact
(I)	70 ± 5.77ab	1.81 ± 0.17^{b}	0.09 ± 0.01^{b}	Brownish white	Patchy	Spongy
(J)	86.67 ± 3.33a	1.147 ± 0.14^{b}	0.07333 ± 0.003 ^{bc}	Brownish white	Patchy	Spongy

Each value represents Mean±SE. Means that do not share a letter are significantly different. Results obtained after 21 days of third subculture.
(A)0.5 BAP+ 2.0NAA **(B)** 1.0 BAP+ 1.0 NAA **(C)** 1.0 BAP+ 2.0 NAA **(D)** 2.0 BAP+ 0.5 NAA (E) 2.0 BAP+1.0 NAA **(f)** 3.0 BAP+ 1.0 NAA **(G)** 3.0 BAP+ 2.0 NAA) **(H)** 1.0 BAP+ 0.25 (2,4 D) **(I)** 1.0 BAP+ 0. 5 (2,4 D) **(J)** 1.0 BAP+ 1.0 (2,4 D)

Table 3: Effect of different doses of YE as a biotic elicitor and incubation period on callus culture of *T. decussatus.*

YE conc. (mgl^{-1})	Harvesting time after 15 days		Harvesting time after 30days	
	Mean fresh weight (g) ± SE	Mean dry weight (g) ± SE	Mean fresh weight (g) ± SE	Mean dry weight (g) ± SE
0 (control)	1.687 ± 0.038^{d}	0.167 ± 0.012^{b}	5.217 ± 0.124^{c}	0.23 ± 0.006^{c}
50	4.02 ± 0.053^{a}	0.223 ± 0.007^{a}	7.78 ± 0.128^{b}	0.283 ± 0.003^{b}
100	3.51 ± 0.067b	0.213 ± 0.003^{a}	5.41 ± 0.232^{c}	0.207 ± 0.003^{cd}
150	2.343 ± 0.087^{c}	0.117 ± 0.009^{c}	10.72 ± 0.201^{a}	0.337 ± 0.003^{a}
200	1.73 ± 0.032^{d}	0.073 ± 0.003^{de}	3.787 ± 0.13^{d}	0.187 ± 0.003^{d}
250	1.696 ± 0.043^{d}	0.083 ± 0.003^{d}	3.817 ± 0.162^{d}	0.11 ± 0.006^{e}
300	1.483 ± 0.018^{d}	0.043 ± 0.003^{e}	3.607 ± 0.096^{d}	0.08 ± 0.006^{f}

Each value represents Mean±SE. Means that do not share a letter are significantly different. The initial weight of callus for all treatments is 1.0 g.

Table 4: Effect of different doses of SA as an abiotic elicitor and incubation period on callus culture of *T. decussatus*

SA conc. (µM)	Harvesting time after 15 days		Harvesting time after 30days	
	Mean fresh weight (g) ± SE	Mean dry weight (g) ± SE	Mean fresh weight (g) ± SE	Mean dry weight (g) ± SE
0 (control)	1.686 ± 0.037^{cd}	0.166 ± 0.012^{abc}	7.78 ± 0.128^{b}	0.336 ± 0.003^{b}
50	1.8 ± 0.044^{bc}	0.166 ± 0.009^{abc}	4.367 ± 0.766^{c}	0.216 ± 0.009^{c}
100	1.833 ± 0.049^{bc}	0.163 ± 0.007^{bc}	5.487 ± 0.221^{bc}	0.32 ± 0.006b
150	2.03 ± 0.061^{b}	0.203 ± 0.012^{ab}	7.473 ± 0.058^{bc}	0.33 ± 0.012^{b}
200	2.563 ± 0.041^{a}	0.193 ± 0.009^{ab}	8.73 ± 0.856^{b}	0.336± 0.018^{b}
250	2.63 ± 0.068^{a}	0.216 ± 0.018^{a}	15.01 ± 1.02^{a}	0.47 ± 0.031^{a}
300	1.483^{d} ± 0.027^{d}	0.14 ± 0.006c	6.2 ± 1.03^{bc}	0.233 ± 0.009^{c}

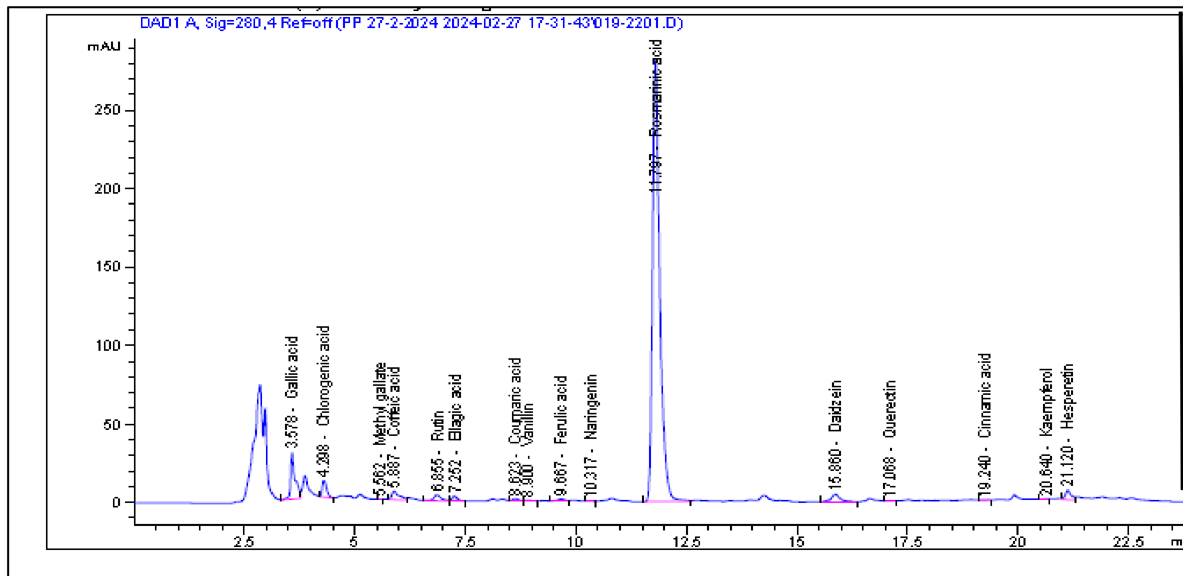
Each value represents Mean±SE. Means that do not share a letter are significantly different. The initial weight of callus for all treatments is 1.0 g

Table 5: Effect of biotic elicitor (YE) on the concentration of active constituents in callus of *T.decussatus*

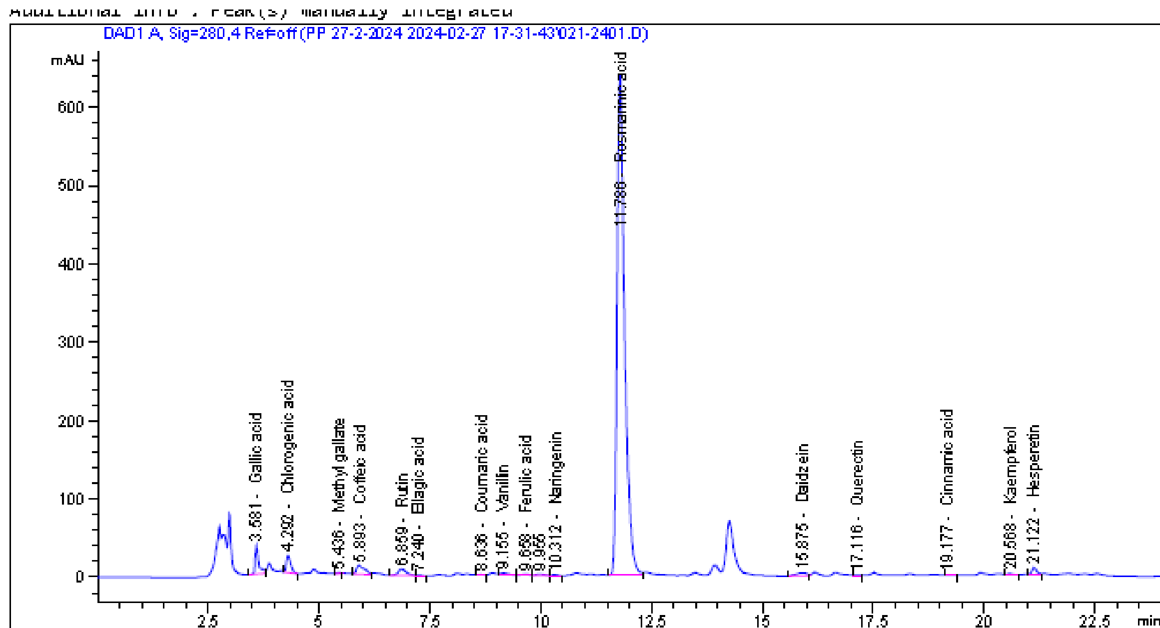
Active constituents	Mother plant			Control			Biotic elicitation(YE)		
	Area	Conc. ($\mu g ml^{-1}$)	Conc. ($\mu g\ g^{-1}$)	Area	Conc. ($\mu g ml^{-1}$)	Conc. ($\mu g\ g^{-1}$)	Area	Conc. ($\mu g ml^{-1}$)	Conc. ($\mu g\ g^{-1}$)
Gallic acid	386.61	33.30	666.08	153.44	13.22	264.36	202.68	17.46	349.19
Chlorogenic acid	57.91	8.03	160.70	42.64	5.92	118.31	72.86	10.11	202.17
Catechin	5.83	1.39	27.79	0.00	0.00	0.00	0.00	0.00	0.00
Methyl gallate	13.88	0.76	15.12	13.00	0.71	14.16	0.95	0.05	1.03
Coffeic acid	151.42	13.09	261.90	38.64	3.34	66.84	64.15	5.55	110.95
Syringic acid	42.89	3.09	61.74	12.03	0.87	17.32	0.00	0.00	0.00
Rutin	317.20	52.15	1042.90	24.58	4.04	80.82	44.15	7.26	145.16
Ellagic acid	12.28	1.25	24.99	6.54	0.67	13.32	27.16	2.76	55.28
Coumaric acid	36.74	1.33	26.64	3.10	0.11	2.25	16.83	0.61	12.21
Vanillin	104.41	4.14	82.90	4.86	0.19	3.86	6.37	0.25	5.06
Ferulic acid	56.44	3.40	68.02	6.17	0.37	7.44	17.02	1.03	20.51
Naringenin	1.84	0.18	3.54	3.98	0.38	7.65	1.32	0.13	2.53
Rosmarinic acid	4346.79	465.78	9315.58	898.37	96.26	1925.29	3224.39	345.51	6910.18
Daidzein	19.30	1.31	26.19	9.19	0.62	12.47	63.94	4.34	86.77
Querectin	33.36	2.09	41.81	3.08	0.19	3.86	3.44	0.22	4.31
Cinnamic acid	10.61	0.20	3.98	4.45	0.08	1.67	2.29	0.04	0.86
Kaempferol	11.53	0.88	17.55	4.18	0.32	6.36	3.43	0.26	5.21
Hesperetin	78.38	3.94	78.85	31.92	1.61	32.11	56.10	2.82	56.43

Table 6: Effect of abiotic elicitor (SA) on the concentration of active constituents in callus of *T. decussatus*

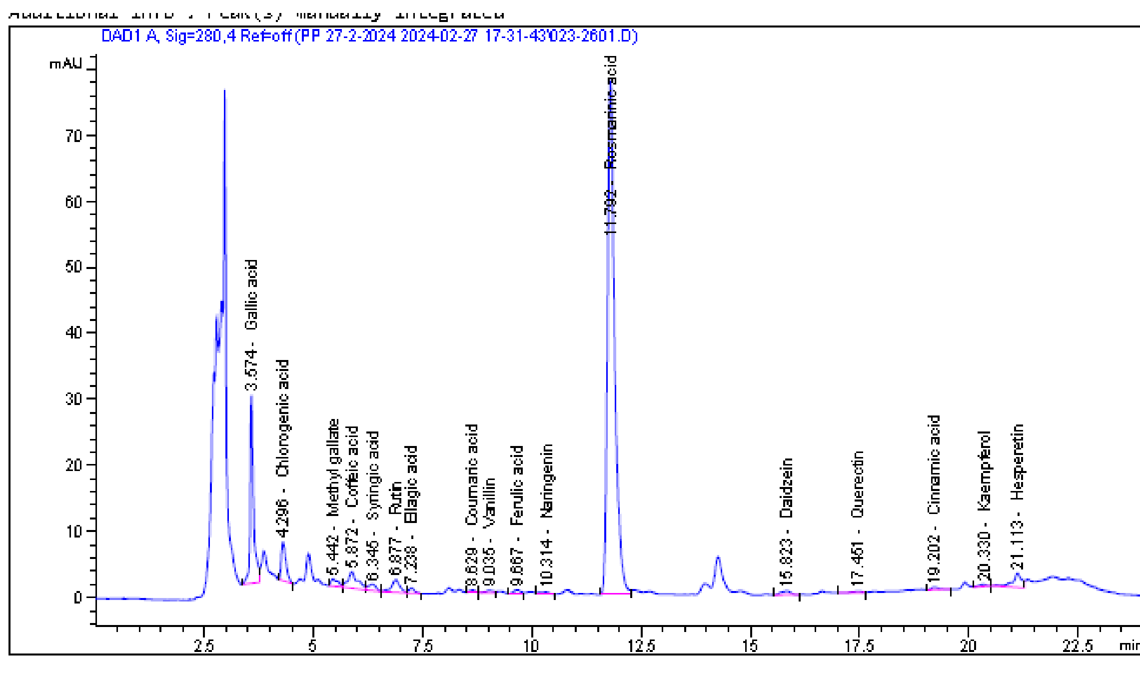
Active constituents	Mother plant			Control			Abiotic elicitation(SA)		
	Area	Conc. (μg ml^{-1})	Conc. (μg g^{-1})	Area	Conc. (μg ml^{-1})	Conc. (μg g^{-1})	Area	Conc. (μgml^{-1})	Conc. (μg g^{-1})
Gallic acid	386.61	33.30	666.08	153.44	13.22	264.36	208.63	17.97	359.43
Chlorogenic acid	57.91	8.03	160.70	42.64	5.92	118.31	152.98	21.23	424.51
Catechin	5.83	1.39	27.79	0.00	0.00	0.00	0.00	0.00	0.00
Methyl gallate	13.88	0.76	15.12	13.00	0.71	14.16	8.94	0.49	9.74
Coffeic acid	151.42	13.09	261.90	38.64	3.34	66.84	146.72	12.69	253.77
Syringic acid	42.89	3.09	61.74	12.03	0.87	17.32	0.00	0.00	0.00
Rutin	317.20	52.15	1042.90	24.58	4.04	80.82	110.12	18.10	362.08
Ellagic acid	12.28	1.25	24.99	6.54	0.67	13.32	6.27	0.64	12.75
Coumaric acid	36.74	1.33	26.64	3.10	0.11	2.25	8.20	0.30	5.94
Vanillin	104.41	4.14	82.90	4.86	0.19	3.86	37.96	1.51	30.14
Ferulic acid	56.44	3.40	68.02	6.17	0.37	7.44	14.10	0.85	17.00
Naringenin	1.84	0.18	3.54	3.98	0.38	7.65	9.98	0.96	19.19
Rosmarinic acid	4346.79	465.78	9315.58	898.37	96.26	1925.29	7612.22	815.69	16313.73
Daidzein	19.30	1.31	26.19	9.19	0.62	12.47	68.02	4.62	92.32
Querectin	33.36	2.09	41.81	3.08	0.19	3.86	4.38	0.27	5.48
Cinnamic acid	10.61	0.20	3.98	4.45	0.08	1.67	1.25	0.02	0.47
Kaempferol	11.53	0.88	17.55	4.18	0.32	6.36	13.22	1.01	20.11
Hesperetin	78.38	3.94	78.85	31.92	1.61	32.11	72.69	3.66	73.12



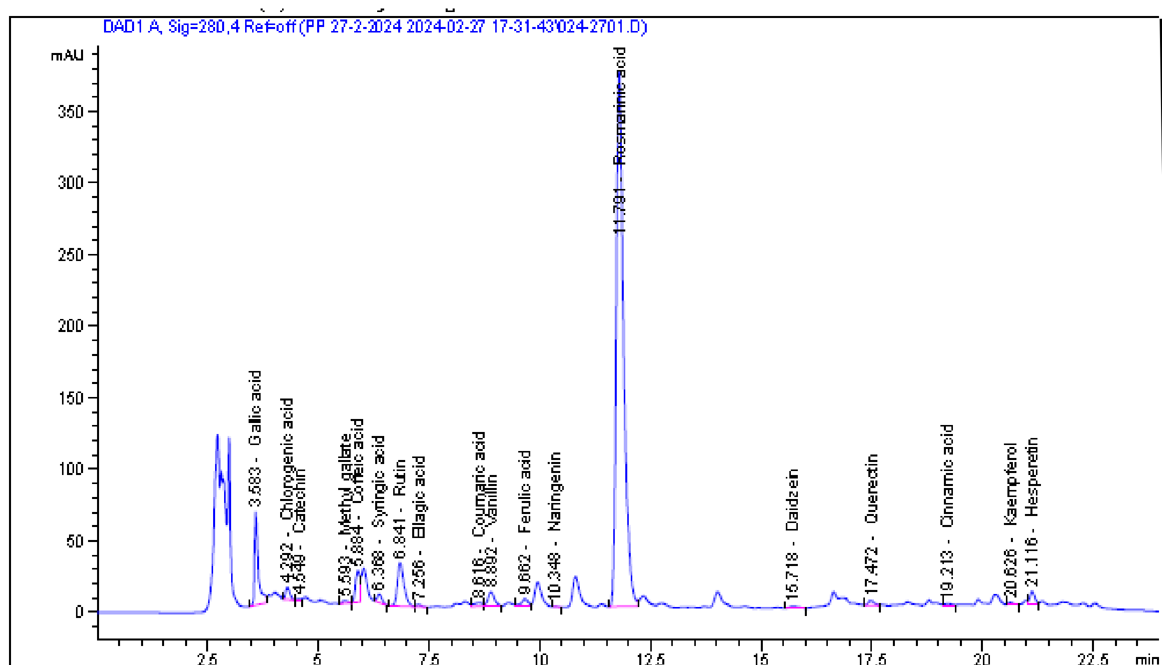
Graph 1: HPLC chromatogram showing the quantity of various phenolic compouds in callus of *T. decussatus* elicited with yeast extract.



Graph 2: HPLC chromatogram showing the quantity of various phenolic compouds in *T. decussatus* elicited with salicylic acid.



Graph 3: HPLC chromatogram showing the quantity of various phenolic compounds in callus of *T. decussatus* on control medium.



Graph 4: HPLC chromatogram showing the quantity of various phenolic compounds in mother plant of *T. decussatus*.

استحثاث الكالس وتعزيز إنتاجية الكتلة الحيوية والمكونات الصيدلانية من خلال استنباط نبات الزعتر المتصالب باعتباره من النباتات الطبية المهددة بالانقراض في مصر

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الملخص العربي:

استحثاث تكوين الكالس يعتمد بشكل اساسى على انواع وتركيزات منظمات النمو المستخدمة وكذلك على نوع المنفصل النباتى المستخدم ، فقد اوضحت نتائج الدراسة ان الزرعة المعملية للمنفصل النباتى لنبات الثايمس (الزعتر لمتصالب) على الاوساط الغذائية المختلفة قد اختلفت فى استجابتها لاستحثاث تكوين الكالس طبقا لانواع وتركيزات منظمات النمو النباتية المضافة الى بيئات الزراعة وكذلك نوع المنفصل النباتى ،وقد اوضحت النتائج ايضا ان افضل اوكسين استخدم لاستحثاث الكالس هو 2,4-D عند مزجة مع BAP ، وان اعلى نسبة مئوية لاستحثاث الكالس (100%) قد لوحظت على الاوساط الغذائية المزودة ب 1ملجرام /اللتر بنزيل ادينين مع 0.25 مللجرام /اللتر تو فور دى، ايضا اوضحت الدراسة التأثير الايجابى للمحفز الحيوى مستخلص الخميرة والغير حيوي السالسيلك اسيد وكذلك وقت المعاملة بهما على زيادة انتاجية الكتلة الحيوية للكالس و انتاجية المواد الفعالة.

الكلمات الاسترشادية: التولد، زعتر، المستخرج، المستقبلات الثانوية، حمض السالسيليك، العائلة الشفوية، مستخلص الخميرة.