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# International Journal of Secondary Metabolite

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Published at http://ijate.net/index.php/ijsm http://dergipark.gov.tr/ijsm **Research Article**

# Determination of Antioxidant Potential in the Leaf and Flower of *Paulownia tomentosa*

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**Abstract**: *Paulownia tomentosa*, which adapts to the land of Denizli, has a rapid growth feature. In this study, content analysis was carried out in order to determine the antioxidant content of the *Paulownia tomentosa* tree. According to this, the most much catechin was found in the general phenolic content of the plant (24035.90 µg/g in the leaf extract, 13837.14 µg/g in the flower extract). The least amount of chlorogenic acid was (34.863 µg/g in the leaf extract, 82.260 µg/g in the flower extract) found. β-carotene was obtained as 7716,00 µg/g in leaf content and 501,67 µg/g in flower contents. Our plant is a plant that contains total phenolic substances in leaf and flower extract. Due to its potential for impact; ethnobotany, is a plant species that can be used in phytotherapy, pharmacology, modern medical applications and animal feed industry.

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β-carotene

## 1. INTRODUCTION

*Paulownia tomentosa* tree is a species with rapid growth. *Paulownia* is a species of the family"Scrophulariaceae". The general spread area is China [1]. *Paulownia* can be planted within the scope of agriculture in many countries such as South Asia, Australia, Japan, Germany and Southern Europe [2]. Today, this tree approximately 2,4 million hectares of agricultural land is cultivated for various purposes [3]. *Paulownia* in needs high humidity and light. *Paulownia* prefers undamaged, alluvial soil and unsalted soil types [4,5]. These include: *Paulownia tomentosa, Paulownia elongata, Paulownia fortunei, Paulownia catalpifolia, Paulownia kawakamii, Paulownia farbesii, Paulownia albiphloea, Paulownia taiwaniana* [6,7]. For these species, the most studied and cultured species; *Paulownia elongata, Paulownia tomentosa* and *Paulownia fortunei* tree [8,9]. Its stem bark has been used in Chinese herbal medicine as a component of remedies for infectiousdiseases such as gonorrhea and erysipelas [10].

A wide variety of endergonic and exergonic compounds that are mutually interacting to neutralize free radical groups are called antioxidants [11,12]. Antioxidants have the property of slowing the oxidation of fats. Antioxidants are substances that neutralize free radicals in living

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things and prevent the cells from being affected or renew themselves [13]. Flavonoids are the polyphenol compound found in each plant and give color to plants. Most flavonoid groups show antioxidant properties in the human body. Neutralizes oxygen-containing, highly reactive molecules, preventing damage to cells [14,15].

There are many substances with antioxidant content. While some of these antioxidants can be produced in our body, others have to be taken from the outside. Research on the source and use of natural antioxidants has shown that some herbs and spices have more antioxidant content than synthetic antioxidants. The body produces these substances for defense purposes. The rapid increase in the side effects of artificial preservatives on health caused an increase in the demand for natural antimicrobial agents. As a result of this increase, researches on herbal substances and their protective effects have accelerated in recent years [16].

Phenolic compounds are substances which contain one or more hydroxyl groups attached to an aromatic ring [17, 18]. Phenolic compounds have an important effect on the interactions of plants with their environment [19]. Phenolic compounds contain more than 8000 flavonoid groups, most of which are found in leaves, flowers and roots in nature. These groups are thought to exist in the world of plants for many years [20, 21]. Vegetable, fruit, nuts, wine, coffee, tea, cocoa and medicinal plants have a high proportion of phenolic compounds [22, 23]. Phenolic acids are natural antioxidant substances found in plants. They provide the unique colors, fragrances and flavors of the plants. These compounds increase the nutritional value and quality of the foods they are present. Phenolic acids; ıt is used as preservative to extend the shelf life of foods. Besides these properties, plants with phenolic acid content are also used for the treatment of diseases.

β-carotene exhibits antioxidant properties and prevents the formation of free radicals by preventing the oxidation of unsaturated fats [24]. In addition to its antioxidant effect, one of the best proven tasks of carotenoids is that they have an important provitamin-A activity.

This study; It is aimed to detect antioxidant potential of leaf and flower parts of *Paulownia tomentosa*. For this purpose, content analysis of antioxidant properties, β carotene and phenolic substance contents of the parts of *Paulownia tomentosa* were performed.

## 2. MATERIALS and METHODS

### 2.1. Preparation of *Paulownia tomentosa* leaf and flower extract

*Paulownia tomentosa* leaves and flowers were collected from the campus garden of Pamukkale University in March-April 2017. After drying in the shade, leaves and flowers were pulverized. 1/10 of solvent and ground samples were extracted. 10 grams of milled specimen (leaf and flower) was prepared using 100 milliliters of methanol solvent [25]. It was then extracted with methanol solvent at 55 ºC in a water bath for 5-6 hours. The mixture obtained as a result of the extraction was filtered through filter paper (Whatman No: 1) and the solvents were removed from the rotary evaporator (IKA RV 10 USA). The extract was completely dried in the lyophilizer and the water was removed (Labconco Freezone 6 USA). The remaining extracts were stored at 4 ºC for use in subsequent studies [26].

### 2.2. Phenolic compound extraction analysis method

2 g samples were taken from *Paulownia tomentosa* leaves and flower extracts, 10 ml of % 96 ethanol was added and 2 minutes were mixed in the homogenizer. It was kept in a water bath at 45 °C for 1 night and then centrifuged at 4000 rpm for 5 minutes. The insoluble and sediment forming part, was removed by evaporation in the rotary evaporator until it was completely dry at 45 °C. Then the extracts were dissolved in 1 ml methanol and made ready for use in phenolic compound analysis [27]. 0,2 g extract sample was dissolved in mobile phase. Then sample was filtrated in 0,45 µm and was injected to the HPLC. HPLC System: Shimadzu Prominence Brand

HPLC. CBM: 20ACBM, Detector: DAD (SPD-M20A), Colon Furnace: CTO-10ASVp, Pump: LC20 AT. Autosampler: SIL 20ACHT, Computer Software: LC Solution, Mobile Phase: A: 3% Formic acid B: Methanol, Column: Zorbax Eclipse XDB-C18 (250 * 4,6 mm, 5 micron), Flow Rate: 0,8 mL / min [28].

### 2.3. Determination of DPPH free radical scavenging activity

To measure the DPPH radical scavenging activity, Braca et al. Modified method they used. The MeOH DPPH (Sigma) solution was prepared at the concentration 22 g·L-1. Tested extracts were dissolved in MeOH at different concentrations. A volume of test solution (0.2 mL) was mixed with DPPH solution (1.8 mL) and the absorbance of the mixture at 517 nm was measured each minute during the first 5 minutes of experiment and than each 5 minutes for the next 25 minutes. Using the recorded data, scavenging effect r was calculated [r = (1 - sample absorbance/control absorbance) × 100)] and the and the time course of activity increase was drew and the differences between the tested extracts were compared. The percentage of DPPH scavenging was compared to that of the calibrated Trolox standard. Results were expressed in terms of Trolox equivalent antioxidant capacity (µmol Trolox equivalents per 100 g dry weight of plant extract) [29].

### 2.4. Determination of total antioxidant capacity by β-carotene

Carotenoids are highly effective ROS (reactive oxygen species) sweepers. 10 g of flower and leaf samples were weighed and added to 100 ml of methanol. Methanol was evaporated, the residue was injected into the dissolved phase in the mobile phase.

β-carotene content was made using Shimadzu Prominence Brand HPLC (Tokyo, Japan) system. CBM: 20ACBM, Detector: DAD (SPD-M20A). Colon Furnace: CTO-10ASVp, Pump: LC20 AT, Autosampler: SIL 20ACHT, Computer Program: LC Solution, Mobile Phase: Methanol / ACN / THF (73/20/7), (v / v / v). Column: ODS 2 (100 * 4,6 mm, 5: µm), Flow Rate: 1 mL / min, Injection volume: 20: µL [30].

## 3. RESULT and DISCUSSION

### 3.1. Phenolic compound analysis findings

Phenolic substances are important antioxidant components. Substances in every organ of the plants show differences. In the flower extract, gallic acid (1150,302 µg/g), chlorogenic acid (82,260 µg/g), quercetin (124,579 µg/g), luteolin (144,570 µg/g) were higher than leaf extract. In the leaf extract; catechin (24035,90 µg/g), caffeic acid (1132,779 µg/g) and coumaric acid (140,695 µg/g) were obtained more than flower extract. The phenolic substance with the highest effect in the *Paulownia tomentosa* is the catechin component (Table 1). Standard chromotgram (Figure 1a) and *Paulownia tomentosa* flowers and leaf chromotgram (Figure 1b) are given graphs. This substance is more present in the leaf extract. Catechin is an important natural antioxidant source [31]. The presence of phenolic substance in *Paulownia tomentosa* leaf and flower extract indicates that it is a pharmacologically important source. Phenolic substance content analysis of methanolic extract of leaf and flower of *Paulownia tomentosa* was performed.

**Table 1.** Phenolic substance analysis results of samples

| Sample (µg/g) | Catechin | Gallic acid | Chlorogenic acid | Caffeic acid | Coumaric acid | Quercetin | Luteolin |
|---|---|---|---|---|---|---|---|
| Flower | 13837,14 | 1150,302 | 82,260 | 879,437 | 88,726 | 124,579 | 144,570 |
| Leaf | 24035,90 | 358,553 | 34,863 | 1132,779 | 140,695 | 58,961 | 102,166 |

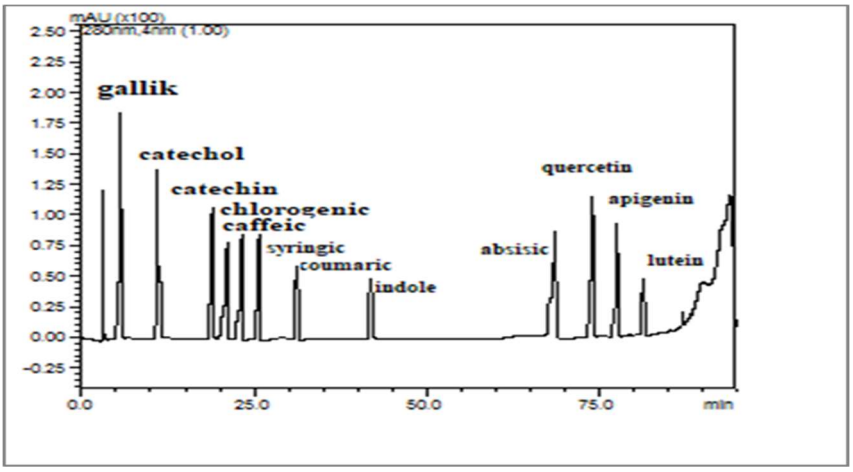


**Figure 1a.** Chromatogram for phenolic substance standard

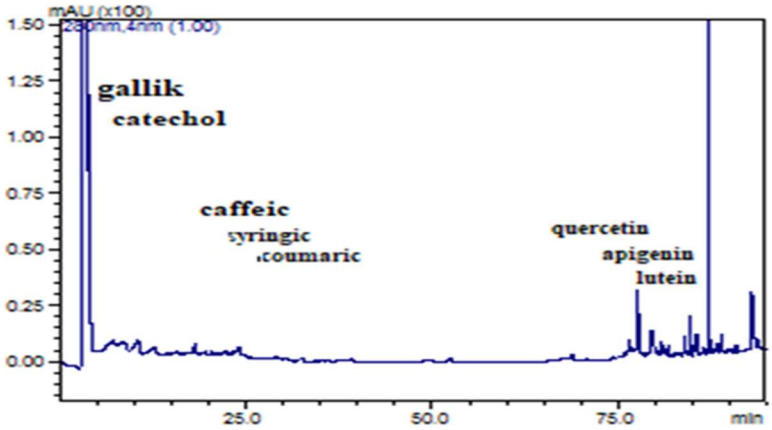


**Figure 1b.** Chromatogram of *Paulownia tomentosa* flowers and leaves

Phenolic substances are important antioxidant components. Thanks to these properties, they have an important place in pharmacology. Similar studies have shown that viscous secretion on the surface of *Paulownia tomentosa'*s unripe fruits has a radical inhibitory effect on the flavonones of methanol extract [32].

### 3.2. DPPH analysis findings

While the flower extract was found to be 223,280 µmolTE/g, the data were obtained as 1104,908 µmolTE/g in the leaf extract (Table 2). According to the results of DPPH activity analysis in *Paulownia tomentosa*'s leaf and flower extracts, the amount in the leaf extract is higher than the flower extract.

**Table 2.** *Paulownia tomentosa* leaf and flower DPPH analysis results

| Sample | DPPH activity analysis |
|---|---|
| Flower | 223,280 |
| Leaf | 1104,908 |

A study similar to that of *Paulownia tomentosa'*s fruit extracts was made. In the study, they obtained high antioxidant activity value in fruit extract. They used 3 different solvents in their study and obtained the best result with methanol solvent [33]. In our study, the highest value was obtained from leaf extract.

### 3.3. β -carotene analysis findings

According to Table 3, the amount of β-carotene in the leaf extract (7716,00 µg/g) of *Paulownia tomentosa* is higher than that of the flower extract (501,67 µg/g). β-carotene chromotgram standard (Figure 2a) and *Paulownia tomentosa* leaf and flower samples are given chromotgram graphs (Figure 2b). Carotene substances in the formation of the color of flowers and fruits and photosynthesis helps to have the task as a pigment [34]. The task of carotene on the leaf is to absorb the rays coming from the sun and turn it into chlorophyll. The majority of carotenoid substances in nature show antioxidant activity. According to these results; among the most obvious effects of β-carotene, which is ultra-violet absorbing feature which is more harmful than normal, has many various medical effects such as antioxidant effect, provitamin - A effect, anticancer effect [35].

**Table 3.** The amount of β-carotene found in the leaf and flower parts of the *Paulownia tomentosa*

| Sample (µg/g) | β-carotene concentration |
|---|---|
| Flower | 501,67 |
| Leaf | 7716,00 |

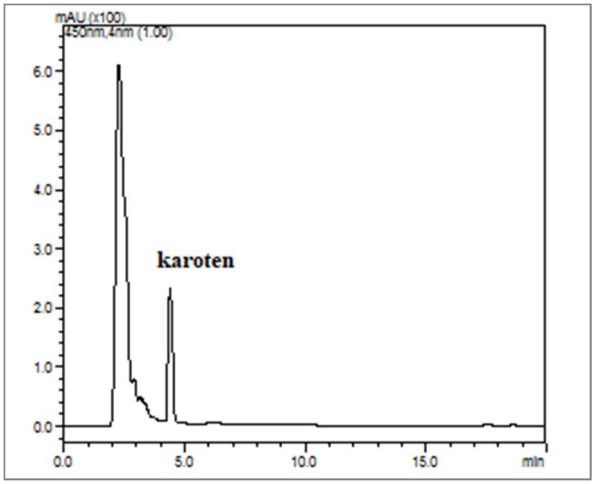


**Figure 2a.** β-carotene standard chromatogram

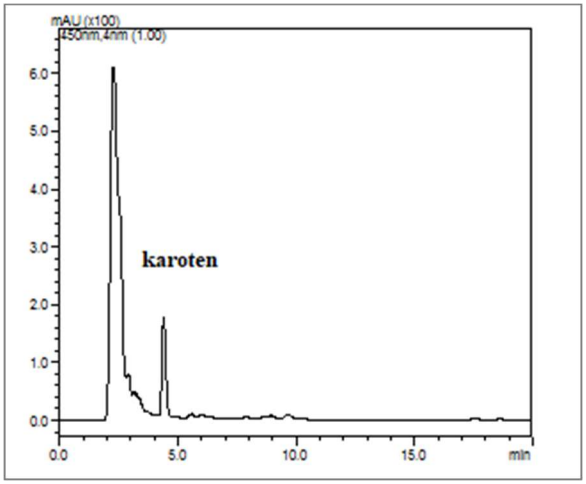


**Figure 2b**. Leaf and flower sample chromatogram

## 4. CONCLUSION

This study aims to show that *Paulownia tomentosa* can be used in pharmacology and alternative medicine because of its medical and aromatic potential. The reason for the presence of different amounts of phenolic substances in leaf and flower content; ıt is thought that the amount of catechin in the leaf is higher as it affects the storage places of the plant's chemical structure. The most common phenolic substance in the *Paulownia tomentosa* is the catechin component that it has stored in its leaves and flowers. Catechin, a colorless component; Antioxidant has been a natural antioxidant source due to its anti-carcinogenic and obesity prevention effect. Considering these characteristics, it is concluded that both the leaves and flowers of the *Paulownia tomentosa* are a rich source of antioxidants. Looking at these contents, plant can be used for medical purposes especially in the fight against many diseases, especially cancer. The phenolic substances and antioxidant contents of the leaf and flower extracts of *Paulownia tomentosa* have the potential to be as effective as synthetic antioxidants.

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**Conflict of Interests**

Authors declare that there is no conflict of interests.

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# Salt, Cold, and Drought Stress on Einkorn and Bread Wheat during Germination

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**Abstract**: Climate changes prompt salt, cold, and drought stresses especially during early crop growth stages. The damages during germination in wheat may even destroy whole crop. Here, 12 bread and 10 einkorn wheats entries were distressed under salt, cold and drought. Germination rate and germination power, coleoptile, shoot length, root length, shoot to root length ratio, root fresh and dry weight and root fresh to dry weight ratio were quantified under six salt, cold, drought stresses and one control. After ANOVA and LSD discriminated the entries, stress tolerance indices differentiated six tolerant and six susceptible entries. MANOVA, Pillai's Trace and Wilks' Lambda tests finalized the stress testing. Shoot and root length, root fresh and dry weight highly differed under salt, cold and drought. Bayraktar-2000 well tolerated salt, drought, salt-drought and salt-cold-drought; Gerek-79 salt, salt-drought, salt-cold-drought; Momtchil salt, cold and salt-cold-drought; İkizce-96 salt, drought and salt-drought. Einkorn Population 14 was susceptible to all stresses except cold and salt-cold; Population 15 to salt, salt-drought and salt-cold-drought; Population 11 to salt, drought, salt-drought and salt-cold-drought. These stresses sharply decreased shoot and root length, root fresh and root dry weight. The higher % decreases under salt, cold and drought were in shoot (59.72, 63.25 and 23.17) and root length (32.91, 51.77 and 34.69), root fresh (44.32, 49.11 and 38.88) and root dry weight (21.63, 42.14 and 41.97). Moreover, Pillai's trace and Wilks' Lambda tests differentiated both characters and entries ($P < 0.01$). In conclusion, Momtchil, Gerek-79, Bayraktar-2000, Populations 5, 6, and 1 are well endorsed against triple seedling stresses.

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Cold,
Drought,
Salt

## 1. INTRODUCTION

Wheat has been the main source of food in central - west Asia and Mediterranean basin since the beginning of agriculture [1-4]. It has been cultivated for more than ten thousand years and kept providing staple nutrition for humans since then. Today's global wheat production of

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about 670.8 million tons per year directly influences human survival and life quality by leading the production of various foods, including bread, pasta, noodles, cakes, and biscuits.

Einkorn (*T. monococcum* ssp. *monococcum* L.), the first primary cultivated wheat ancestor emerged around the Karacadağ Mountains in the southeastern Turkey [5]. It has survived as an animal feed [6] in Turkey, Italy, and Bulgaria [2] and has become a popular human food today because of its health supporting characteristics [7].

Stresses like salt, cold or drought applies a force on a unit wheat area as well [8] and restricts its growth and decreases yield [9-12]. Many abiotic factors may affect wheat, reduce productivity and produce stress responses, 31.56% by heat, 26.61% by drought, and 23.38% by salt. Salinity, drought or cold adversely worsens crop yield and quality, limits water absorption, decreases soil osmotic potential (Izadi et al., 2014), induces water deficit, and causes morphological, physiological and biochemical deteriorations, and finally, restricts yield. The salt stress, for example, affects the wheat crop 20-30% [13, 14], cold [15] and drought 100% [16] across the world.

Characterizing genetic resources with efficient screening tests, under *in vivo* and / or *in vitro* salt, cold and drought stresses during germination may identify new salt, cold and drought resistant genotypes [17]. Many studies on single or double biotic or abiotic stresses have been carried out [18-20] but not more than two biotic or abiotic stresses have not yet been fully illuminated. Because combined stresses involve numerous complex physiological, molecular and cellular factors and cause alterations in several plant processes [21].

Statistical analysis for complex characters like we had here require some detailed statistical methods involved. Analysis of variance (ANOVA) was followed by the least significant difference (LSD) comparison (25) in order to test wheat genotypes against salt, cold, and drought in a factorial restricted randomized block design which may serve as the first step in the stress testing procedures (24). Secondly, stress tolerance indices based on the cultivar rankings effectively differentiate tolerant and susceptible entries (24-27). Lastly, multivariate analysis of variance (MANOVA), Wilks' Lambda test, and Pillai's Trace tests, based on the most and the least deteriorated cultivars as well as the most degraded germination characters, comprehensively complete the stress testing through the sample means (26).

All of these abovementioned methods were, in this study, applied during the germination stage in order to investigate germination rate, germination power, coleoptile length, shoot length, root length, shoot to root length ratio, root fresh weight, root dry weight, and root fresh to dry weight ratio of 12 bread and 10 einkorn wheat entries under seven levels of salt, cold and drought stresses.

## 2. MATERIAL and METHODS

### 2.1. Plant material

The plant material was 12 bread (*Triticum aestivum* L.) and 10 einkorn wheats (*Triticum monococcum* ssp. *monococcum*) entries. Bread wheat cultivars grown in various wheat growing regions in Turkey were Gerek-79, İkizce-96, Kıraç-66, Kenanbey, Flamura-85, Momtchil, Bayraktar-2000, Tosunbey, Pandas, Pehlivan, Demir-2000 and Gün-91. Einkorn populations from different einkorn growing regions were 1, 2, 4, 5, 6, 9, 10, 11, 14, and 15 (Table 1). The bread wheat cultivar seeds were provided by various agricultural research institutes in Turkey while einkorn population seeds were collected from western Black Sea Region, Turkey (Table 1).

**Table 1.** Twelve bread and ten einkorn wheat entries tested against seven levels of salt, cold, and drought stresses.

| Numbers | Entry | Origin | Stress resistance/ tolerance |
|---|---|---|---|
| 1 | Gerek-79* | [2]TARI | D / S / C |
| 2 | İkizce-96 | [1]CRIFC | C |
| 3 | Kıraç-66 | [2] TARI | D / S / C |
| 4 | Kenanbey | [1] CRIFC | C |
| 5 | Flamura-85 | [3] Thrace ARI | S |
| 6 | Momtchil | [3] Thrace ARI | S |
| 7 | Bayraktar-2000 | [1] CRIFC | D / S / C |
| 8 | Tosunbey | [1]CRIFC | C |
| 9 | Pandas | [4] EMARI | S |
| 10 | Pehlivan | [3]Thrace ARI | S |
| 11 | Demir-2000 | [1]CRIFC | D / S / C |
| 12 | Gün-91 | [1]CRIFC | D / S / C |
| 13 | Population 1 | Bolu, Seben, Haccağız Village | |
| 14 | Population 2 | Bolu, Seben, Boğaz Region | |
| 15 | Population 4 | Bolu, Seben, Kavaklı Yazı Village, Field # 1 | |
| 16 | Population 5 | Bolu, Seben, Kavaklı Yazı Village, Field # 2 | |
| 17 | Population 6 | Bolu, Seben, Kavaklı Yazı Village, Field # 3 | |
| 18 | Population 9 | Kastamanonu, İhsangazi, Çatalyazı Village | |
| 19 | Population 10 | Kastamanonu, İhsangazi, Uzunoğlu District | |
| 20 | Population 11 | Kastamanonu, İhsangazi, Çay District | |
| 21 | Population 14 | Kastamanonu, İhsangazi, Center | |
| 22 | Population 15 | Kastamanonu, İhsangazi, Center | |

[1] CRIFC : Central Research Institute for Agricultural Research, Ankara,
[2] TARI : Transitional Zone Agricultural Research Institute, Eskişehir,
[3] Thrace ARI : Thrace Agricultural Research Institute, Edirne,
[4] EMARI : East Meditteranean Agricultural Research Institute, Adana.
* Growth type of bread wheat cultivars; W: Winter; W/F: Winter / Facultative; S: Spring. D: Drought; S: Salt; C: Cold.

## 2.2. Sterilization

Seeds in all salt, cold and drought tests were first surface-sterilized 30 seconds in 96% ethanol, 15 minutes in 10% NaClO, and rinsed twice in distilled $H_2O$ [22].

## 2.3. Stress tests

### 2.3.1. *Salt stress*

Ten seeds in each of three replicates for a given wheat entry were germinated on Whatman number 1 wet filter paper under seven salt (NaCl) levels. The levels were 5 ml doses of 0 (control), 0.05 M, 0.10 M, 0.15 M, 0.20 M 0.25 M and 0.30 M of salt. pH in each petri dish was kept at 5.9 ±1 [22].

### 2.3.2. *Cold stress*

Three replicates by ten seeds in each wheat entry were germinated on Whatman number 1 wet filter paper under seven cold stress levels of 2, 0, -2, -4, -6 and -8 °C, and the control (23±1 °C). pH was 5.9 ±1 during the tests [22].

### 2.3.3. *Drought stress*

Ten seeds in all three replicates of each wheat entry were germinated on Whatman number 1 under seven levels of drought stress induced by PEG 6000 of 0.09 M, 0.17 M, 0.25 M, 0.34 M, 0.43 M, 0.51 M and 0.00 M. The pH value was fixed at 5.9 ± 1 [22] during the experiment.

### 2.4. Germination Tests

The seeds were germinated at 23 ± 1 °C for 8 days (ISTA 2017) and tested *in vitro* under salt, cold, and drought. Each abovementioned stress consisted of seven stress levels. Germination rate, germination power, coleoptile length, shoot length, root length, shoot to root length ratio, root fresh weight, root dry weight, and root fresh to dry weight ratio were measured [23].

### 2.5. Statistical Analysis

The experiments were set up in a three-replicate (blocks) randomized complete block design (RCBD) with each stress in a factorial restriction [24]. Firstly, Fisher's protected test (F) and the mean separation by the least significant difference [LSD; 25] followed the analyses of variance (ANOVA), which were run in SPSS 21. Secondly, cultivar rank based stress tolerance indices, which were calculated in EXCEL differentiated six tolerant (Gerek-79, Gün-91, İkizce-96, Bayraktar-2000, Pehlivan, and Momtchil; approximately tolerant 25% of all entries) and six susceptible (Population 1, Population-2, Population 4, Population-5, Population 6, and Population 14; approximately susceptible 25% of all entries) wheat entries [24, 26-28] Finally, the sample mean of four the least (Gerek-79, İkizce-96, Bayraktar-2000, and Pehlivan) and the most deteriorated (Population 1, Population 4, Population 6, Population 14) wheat entries and germination characters (shoot length, root length, root fresh weight, and root dry weight) were utilized to end up the multivariate analysis of variance (MANOVA), Pillai's Trace, and Wilks' Lambda tests [26].

## 3. RESULTS and DISCUSSION

Abiotic stresses induced by environmental factors cause serious damages on crop plants including wheat. One or two-way stress studies have mostly been carried out in the literature up to now. In nature, however, stresses influence crop plants in somehow more than two-way combined manners as it we had tested here in this study. Germination is one of the most stress vulnerable growth stages of the crops. Biotic or abiotic factors during germination are so critical since they worsen crop establishment and reduce yield. Salt, drought and cold are the most devastating biotic factors, especially on salt sensitive plants during germination and early seedling stages. The higher the salt concentration in the soil occurs the lower the plants germinates (29). The decreased water intake by osmotic limitations under salt, cold and drought stresses prevents the germination (30).

Here, the most stress destructed germination characters shoot length, root length, root fresh weight, and root dry weight were presented but not the other least degraded ones i.e. germination rate, germination power, coleoptile length, shoot to root length ratio, and root fresh to dry weight ratio. Blocking was effective (Table 2) in this factorially restricted randomized complete block design except for root dry weight under salt and cold stresses. All salt, cold, and drought stress types highly differentiated ($p < 0.01$). Similarly, wheat entries did highly ($p < 0.01$) or just significantly ($p < 0.05$) except root fresh weight under cold and drought. Likewise, stress levels under all three stresses differentiated highly ($p < 0.01$) or just significantly ($p < 0.05$). No stress type by wheat entry interaction was determined.

**Table 2.** Fisher's protected F value for shoot length, root length, root fresh weight, and root dry weight.

| | | Shoot length (cm) | | | Root length (cm) | | | Root fresh weight (mg) | | | Root dry weight (mg) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sources of variation | D.F. | Salt | Cold | Drought | Salt | Cold | Drought | Salt | Cold | Drought | Salt | Cold | Drought |
| Blocks | 2 | 6.75 $^{**}$ | 12.88$^{*}$ | 8.64$^{*}$ | 8.87$^{**}$ | 4.19$^{**}$ | 3.34$^{*}$ | 4.08$^{*}$ | 4.27$^{**}$ | 9.02$^{**}$ | 0.42$^{ns}$ | 3.02$^{ns}$ | 2.97$^{ns}$ |
| Stress types | 153 | 27.50$^{**}$ | 92.16$^{**}$ | 56.25$^{**}$ | 17.26$^{**}$ | 24.69$^{**}$ | 44.18$^{**}$ | 22.88$^{**}$ | 20.86$^{**}$ | 56.16$^{**}$ | 18.15$^{**}$ | 17.34$^{**}$ | 39.53$^{**}$ |
| Entries | 21 | 2.21$^{*}$ | 28.89$^{*}$ | 1.98$^{*}$ | 4.00$^{**}$ | 7.16$^{**}$ | 5.91$^{**}$ | 6.91$^{**}$ | 50.72$^{ns}$ | 7.68$^{ns}$ | 5.22$^{**}$ | 5.47$^{**}$ | 7.11$^{*}$ |
| Stress Levels | 6 | 210.13$^{**}$ | 602.09$^{*}$ | 456.42$^{*}$ | 124.36$^{**}$ | 159.68$^{**}$ | 339.71$^{**}$ | 157.65$^{**}$ | 138.63$^{**}$ | 434.21$^{**}$ | 129.64$^{**}$ | 219.16$^{**}$ | 295.62$^{**}$ |
| Stress type by entry | 44 | 0.26$^{ns}$ | 1.15$^{ns}$ | 0.23$^{ns}$ | 0.16$^{ns}$ | 0.45$^{ns}$ | 0.25$^{ns}$ | 0.20$^{s}$ | 0.34$^{ns}$ | 0.27$^{ns}$ | 0.10$^{ns}$ | 0.65$^{ns}$ | 0.25$^{ns}$ |
| Error | 922 | | | | | | | | | | | | |

D.F.: Degrees of freedom; $^{*}$ statistically significant at p > 0.05; $^{**}$ statistically significant at p > 0.05; ns: non-significant.

### 3.1. Stress tolerance indices of genotypes under three stress

Stress rank indices differentiated bread and einkorn wheat entries under individual or multi-stresses. Approximately, 25% of all 22 wheat entries were determined tolerant and 25% susceptible under each stress alone or in two-way or three-way combinations [27, 28]. Bread wheat cultivars Bayraktar-2000, Gerek-79, İkizce-96, Demir-2000, Gün-91, Momtchil, and Flamura-85 and einkorn wheat populations 1, 6, 4, 2, 5, and 9 behaved tolerant under various stress combinations. Bread wheat cultivars Momtchil, Gerek-79, Bayraktar-2000 and einkorn populations 5, 6, 1 were the tolerant wheat entries where salt, cold and drought stresses evaluated together (Table 3).

Bread wheat and einkorn entries differed against salt, cold, and drought stresses alone or in combinations. These were einkorn populations 9, 10, 11, 14, 15 and bread wheat cultivars Demir-2000, Pehlivan, İkizce-96, Kıraç-66, Tosunbey, and Bayraktar-2000. Under three stresses, einkorn populations 10, 11, 14, 15 and bread wheat cultivars Tosunbey and Kıraç-66 were susceptible to salt-cold-drought. When two or three stresses were evaluated together Population 10 was the only genotype susceptible to salt-drought and salt-cold-drought. Kıraç-66 was susceptible under the combinations of salt-cold, salt-drought and cold-drought, with stress tolerance indices of 17.17, 14.06, and 14.48, respectively. Population 10 was the most susceptible to salt (20.56), drought (17.11), cold-drought (18.94), and salt-cold-drought (15.07), (Table 3). The most worsened characters were shoot length, root length, root fresh weight, and root dry weight under salt, cold, and drought.

**Table 3.** Six tolerant and six susceptible bread and einkorn wheat entries selected by their stress tolerance rank indices under one-, two-, and three-way salt, cold, and drought stresses.

| Tolerance / Susceptibility | Stress types | | | | | | |
|---|---|---|---|---|---|---|---|
| | Salt | Cold | Drought | Salt-Cold | Salt-Drought | Cold-Drought | Salt-Cold-Drought |
| Tolerant | Bayraktar-2000 | Population 6 | Kenanbey | Population 6 | Bayraktar-2000 | Gerek-79 | Momtchill |
| | 5.44 | 2.89 | 6.67 | 5.39 | 4.78 | 5.78 | 8.30 |
| | Gerek-79 | Population 1 | Bayraktar-2000 | Population 5 | İkizce-96 | Population 6 | Gerek-79 |
| | 5.44 | 5.44 | 7.33 | 6.33 | 6.17 | 6.06 | 8.48 |
| | İkizce-96 | Population 4 | Gün-91 | Population 11 | Gerek-79 | Population 1 | Population 5 |
| | 5.89 | 6.11 | 7.44 | 7.50 | 6.78 | 7.72 | 8.85 |
| | Gün-91 | Population 2 | Momtchil | Flamura-85 | Gün-91 | Flamura-85 | Population 6 |
| | 6.00 | 3.13 | 5.66 | 7.89 | 7.11 | 8.22 | 8.89 |
| | Demir-2000 | Population 5 | Population 9 | Population 9 | Demir-2000 | Population 2 | Bayraktar-2000 |
| | 6.78 | 6.56 | 9.00 | 8.00 | 8.00 | 8.44 | 9.44 |
| | Momtchil | Momtchil | İkizce-96 | Population 4 | Kenanbey | Population 5 | Population 1 |
| | 7.44 | 7.56 | 9.00 | 8.22 | 8.39 | 8.78 | 10.26 |
| Susceptible | Population 4 | Demir-2000 | Pehlivan | Bayraktar-2000 | Population 9 | Pandas | Population 11 |
| | 14.11 | 16.33 | 14.00 | 15.28 | 14.06 | 13.78 | 12.78 |
| | Population 14 | Pehlivan | Flamura-85 | Kenanbey | Kıraç-66 | İkizce-96 | Population 15 |
| | 14.56 | 16.78 | 14.00 | 15.39 | 14.06 | 14.00 | 14.26 |
| | Population 15 | İkizce-96 | Population 14 | Kıraç-66 | Population 15 | Population 14 | Tosunbey |
| | 17.11 | 17.78 | 14.22 | 17.17 | 15.67 | 14.17 | 14.26 |
| | Population 9 | Kıraç-66 | Kıraç-66 | Pehlivan | Population 14 | Bayraktar-2000 | Kıraç-66 |
| | 18.44 | 18.44 | 16.22 | 17.39 | 16.06 | 14.89 | 14.48 |
| | Population 11 | Tosunbey | Population 11 | Demir-2000 | Population 11 | Pehlivan | Population 14 |
| | 18.67 | 19.11 | 16.56 | 17.39 | 18.11 | 15.44 | 14.85 |
| | Population 10 | Bayraktar-2000 | Population 10 | İkizce-96 | Population 10 | Demir-2000 | Population 10 |
| | 20.56 | 19.22 | 17.11 | 18.22 | 18.94 | 15.67 | 15.07 |

The shoot length, root length, root fresh weight, and root dry weight were the worst decreased germination characters. The decrease gradually occurred under salt, cold, and drought stresses (Table 4). These four characters with the least worsened Gerek-79, İkizce-96, Bayraktar-2000, and Pehlivan bread wheat cultivars and the most worsened einkorn populations 1, 4, 6, 14 were further chosen for multivariate analysis.

**Table 4.** The most stress responsive shoot length, root length, root fresh weight, and root dry weight.

| Stress levels | Stress Types | Shoot length (cm) | Root length (cm) | Root fresh weight (mg) | Root dry weight (mg) |
|---|---|---|---|---|---|
| Control | Salt | 12.35A | 7.70A | 76.24A | 7.11A |
| | Cold | 14.94A | 8.36A | 77.46A | 9.23A |
| | Drought | 14.08A | 8.64AB | 87.26 A | 7.60A-C |
| Level 1 | Salt | 10.00B | 5.70AB | 65.88AB | 6.12AB |
| | Cold | 9.72B | 5.55AB | 52.72AB | 6.95AB |
| | Drought | 12.29B | 9.01A | 86.41AB | 9.90 AB |
| Level 2 | Salt | 5.47C | 4.00A-C | 48.34A-C | 4.81A-C |
| | Cold | 8.04BC | 4.86A-C | 45.31A-C | 4.49A-C |
| | Drought | 7.37BC | 7.48A-C | 68.04A-C | 9.96A |
| Level 3 | Salt | 2.16D | 2.39B-D | 32.30A-D | 3.52A-D |
| | Cold | 6.59CD | 4.45A-D | 40.67A-D | 3.88A-D |
| | Drought | 0.74 D | 4.49A-C | 36.87A-C | 6.73 A-C |
| Level 4 | Salt | 0.50E | 1.30B-E | 19.36B-E | 2.37B-E |
| | Cold | 3.03E | 2.38B-E | 22.03B-E | 2.11B-E |
| | Drought | 0.00DE | 1.85 C | 13.78 C | 2.94 A-C |
| Level 5 | Salt | 0.07E | 0.75C-F | 12.03C-F | 1.66C-F |
| | Cold | 1.31EF | 1.53B-F | 13.68B-F | 1.34C-E |
| | Drought | 0.00DE | 0.38 C | 3.53 C | 0.90 C |
| Level 6 | Salt | 0.00E | 0.00E | 5.92C-G | 1.00C-G |
| | Cold | 0.04FG | 0.46C-G | 4.05C-G | 0.41C-G |
| | Drought | 0.00DE | 0.08 C | 0.99 C | 0.24 C |
| LSD and CV% | Salt | 2.35; 12% | 2.19; 19% | 10.36; 12% | 0.99:13% |
| | Cold | 5.22; 20% | 0.36%8% | 24.74:20% | 2.28;24% |
| | Drought | 1.79; 11% | 3,31;22% | 1.05; 11% | 0.06; 12% |
| Decrease (%) | Salt | 100.00 | 100.00 | 92.24 | 85.94 |
| | Cold | 99.73 | 94.50 | 94.77 | 95.56 |
| | Drought | 100.00 | 99.07 | 98.87 | 97.60 |

The highest 100% decrease under salt stress was in the shoot length and root length while root dry weight (85.94%) and root fresh weight (92.24%) decreased the least, respectively. Decrease in shoot length under cold was 99.73%, in root dry weight was 95.56%, in root fresh

weight was 94.77%, and in root length was 94.50%. The highest decrease under cold was in shoot length (100%), followed by root length (99.07), root fresh weight (98.87%), and root dry weight (97.60%), respectively. A serious decrease was observed in shoot length at level 3 under salt and drought (i.e., 0.10 M salt and 0.17 M PEG 6000 drought). Level 6 stress (0.25 M, -6 °C, 0.43 M PEG 6000) dramatically decreased all four characters, at least up, to a percentage of 94.50% (Table 4). Further studies to evaluate the effect of stresses in combinations may help to comprehend the consequences of stresses on wheat genotypes.

### 3.2. Four the best bread wheat and four the worst einkorn wheat genotypes under three stress

Bread wheat cultivars Gerek-79, İkizce-96, Bayraktar-2000, and Pehlivan were destructed the least, tolerant in other words, under salt, cold and drought stresses. Einkorn populations 1, 4, 6 and 14 worsened the most. These eight entries were selected for multivariate analysis as mentioned below. Bread wheat cultivars had, in general, higher values for shoot length, root length, root fresh weight, and root dry weight than einkorn populations (Table 5; Figure 2).

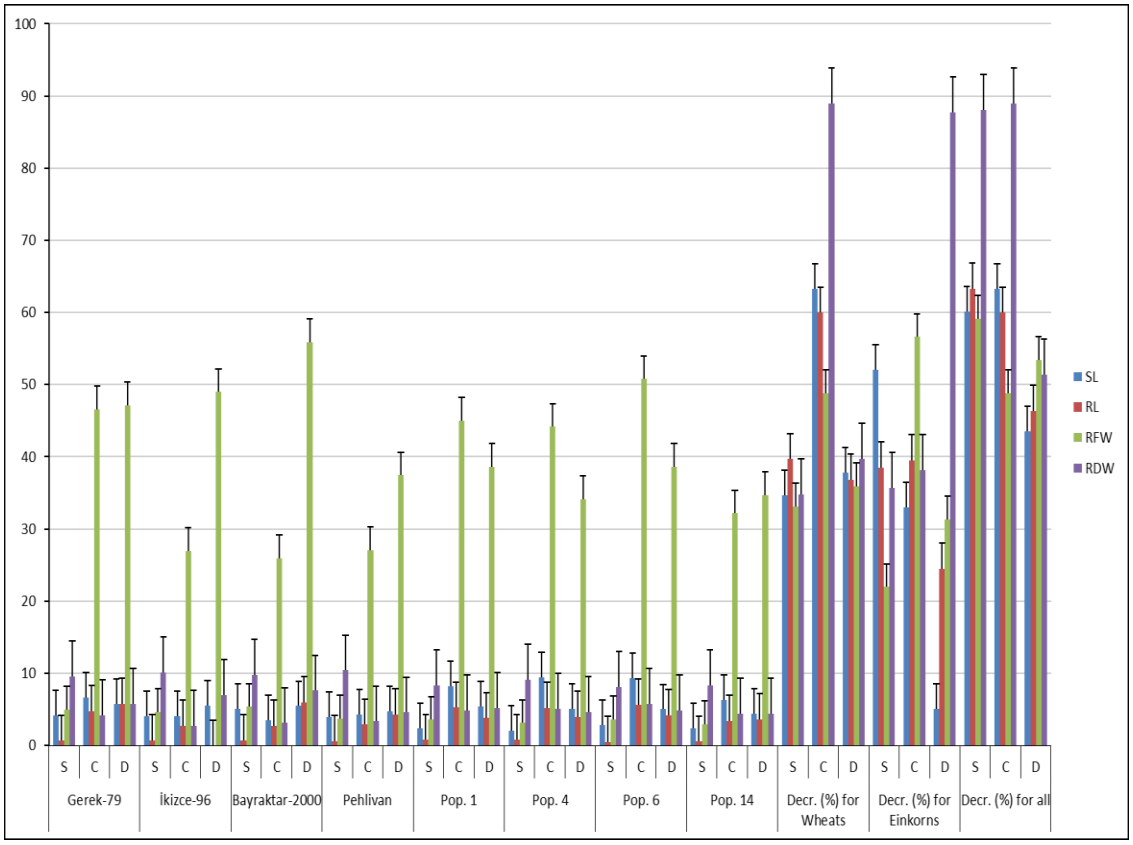


**Figure 1.** Four most worsened germination characters (shoot length, root length, root fresh weight, root dry weight), four einkorn populations (1, 4, 6 and 14), and four least worsened bread wheat cultivars (Gerek-79, İkizce-96, Bayraktar-2000, and Pehlivan) under salt, cold, and drought together.

Shoot length, root length, root fresh weight, and root dry weight in four bread wheat cultivars under salt stress decreased by 22.20%, 20.00%, 29.98% and 8.07%, respectively. The percentage decreases in the same characters were 40.80%, 43.09%, 42.15%, and 25.59%, under cold and 17.42%; 25.04%, 23.51% and 39.73%, under drought stress, respectively (Table 5). The same characters were also worsened in four einkorn populations, 27.30%, 32.91%, 16.24% and 8.56%, under salt stress; 32.94%, 39.85%, 36.68% and 24.19%, under cold stress; and 19.23%, 12.41%, 11.65% and 15.35%, under drought stress, respectively. The highest

decreases in entries were 59.72%, 32.91%, 44.32%, and 21.63% under salt; 63.25%, 51.77%, 49.11% and 42.14% under cold stress; and 23.17%, 34.69%, 38.88% and 41.97% under drought stress, respectively (Figure 1).

**Table 5.** Four most worsened germination characters (shoot length, root length, root fresh weight, root dry weight), four einkorn populations (1, 4, 6 and 14), and four least worsened bread wheat cultivars (Gerek-79, İkizce-96, Bayraktar-2000, and Pehlivan) under salt, cold, and drought together, which were used in multivariate analyses.

| Genotypes | Stress type | Shoot length (cm) | Root length (cm) | Root fresh weight (mg) | Root dry weight (mg) |
|---|---|---|---|---|---|
| Gerek-79 | Salt | 4.20ab | 0.66a-c | 5.00ab | 9.56b-l |
| | Cold | 6.69a-k | 4.78a-g | 4.65a-d | 4.18b-l |
| | Drought | 5.74a-c | 5.79ab | 4.71a-h | 5.79c-h |
| İkizce-96 | Salt | 4.11a-c | 0.75a-e | 4.65a-d | 10.10b-h |
| | Cold | 4.02g-s | 2.71j-t | 2.69i-t | 2.70m-u |
| | Drought | 5.56a-d | 5.26a-I | 4.89a-g | 6.99a-c |
| Bayraktar-2000 | Salt | 5.09a | 0.74a-e | 5.37a | 9.78b-I |
| | Cold | 3.48g-u | 2.72j-t | 2.58j-u | 3.11g-t |
| | Drought | 5.47a-f | 5.97 a | 5.58ab | 7.60 ab |
| Pehlivan | Salt | 3.96a-f | 0.60a-f | 3.76c-l | 10.40a-e |
| | Cold | 4.25g-r | 2.90j-r | 2.70i-s | 3.35e-o |
| | Drought | 4.74a-o | 4.34c-k | 3.74f-p | 4.58f-q |
| Population 1 | Salt | 2.40h-q | 0.78a-l | 3.57c-n | 8.30i-s |
| | Cold | 8.20a-e | 5.28a-b | 4.5.0a-e | 4.88a-d |
| | Drought | 5.46a-f | 3.82h-p | 3.85d-m | 5.21d-l |
| Population 4 | Salt | 2.05j-s | 0.79ao | 3.11g-p | 9.11b-m |
| | Cold | 9.47a | 5.17a-c | 4.41a-h | 5.07ab |
| | Drought | 5.12a-k | 3.96f-n | 3.41h-r | 4.66e-p |
| Population 6 | Salt | 2.82c-n | 0.53a-m | 3.61c-m | 8.15i-u |
| | Cold | 9.33a-b | 5.62a | 5.07a | 5.79a |
| | Drought | 5.02a-l | 4.19e-m | 3.86d-e | 4.88d-n |
| Population 14 | Salt | 2.36h-r | 0.54a-k | 2.99h-q | 8.33i-q |
| | Cold | 6.35a-l | 3.38g-p | 3.21e-n | 4.38b-g |
| | Drought | 4.41a-q | 3.67j-s | 3.46h-q | 4.41g-r |
| LSD and CV% | Salt | 0.85; 12% | 0.06:11% | 0.37; 11% | 0.84; 16% |
| | Cold | 0.14; 18% | 0.09%12% | 0.42;14% | 0.89%18% |
| | Drought | 0.27; 9% | 0.18;14% | 0.58: 15% | 0.61 |
| Decrease in wheat (%) | Salt | 22.20 | 20.00 | 29.98 | 8.07 |
| | Cold | 40.80 | 43.09 | 42.15 | 25.59 |
| | Drought | 17.42 | 25.04 | 23.51 | 39.73 |
| Decrease in einkorn (%) | Salt | 27.30 | 32.91 | 16.24 | 8.56 |
| | Cold | 32.94 | 39.85 | 36.68 | 24.35 |
| | Drought | 19.23 | 12.41 | 11.65 | 15.35 |
| Decrease in all (%) | Salt | 59.72 | 32.91 | 44.32 | 21.63 |
| | Cold | 63.25 | 51.77 | 49.11 | 42.14 |
| | Drought | 23.17 | 34.69 | 38.88 | 41.97 |

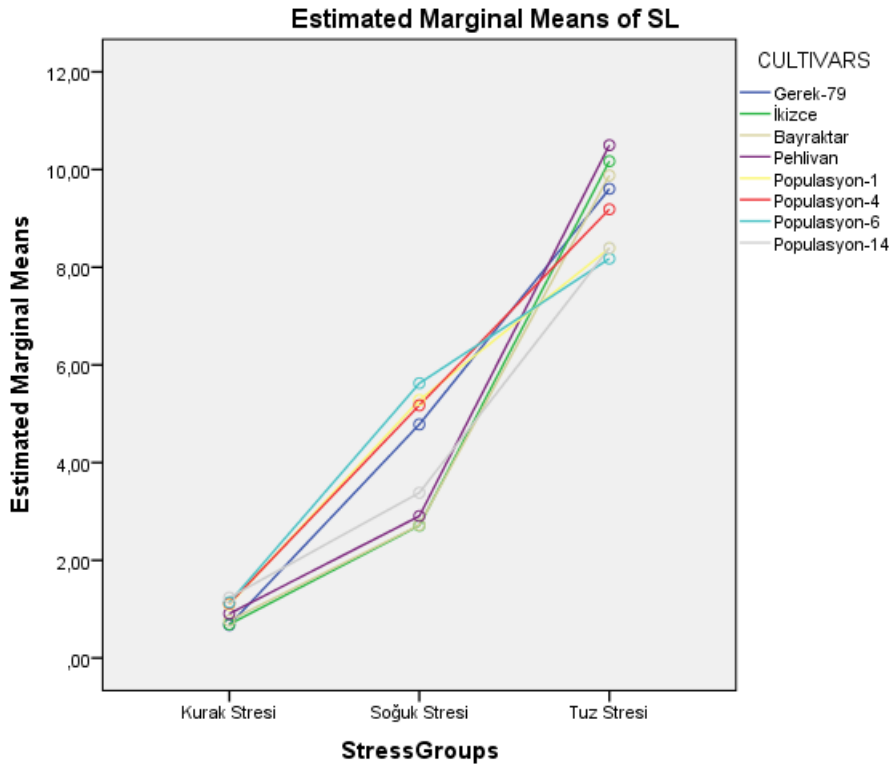


**Figure 2.** The shoot lengths of four most deteriorated einkorn populations (1, 4, 6 and 14), and four least worsened bread wheat cultivars (Gerek-79, İkizce-96, Bayraktar-2000 and Pehlivan) under salt, cold, and drought together, which were all three used in multivariate analyses.

### 3.3. Shoot length, root length, root fresh weight, and root dry weight of the least responsive four bread wheat and the most responsive four einkorn genotypes under salt, cold, and drought

A multivariate analysis of variance (MANOVA) was performed on germination characters, bread wheat cultivars, einkorn populations, and salt, cold, and drought stresses. Furthermore, Pillai's Trace and Wilks' Lambda tests were applied on data [26].

All salt, cold and drought stresses, wheat entries, and stress by wheat entry interaction(s) significantly ($P < 0.01$) affected the independent variables (Table 7). Partial Eta squared indicated a strong effect on all three stresses and a weak effect on wheat entries and stress by wheat entry interactions. Pillai's Trace and Wilks' Lambda were highly significant ($p < 0.01$) for stress types of salt, cold, drought, wheat entries, and stress type by entry interaction. The between-subject effects were also significant for all stresses and stress by wheat entry interactions ($P<0.05$), but insignificant for wheat entries. Partial Eta squared had the highest effect on the shoot length and the lowest on the root dry weight. Stress by wheat entry interactions affected shoot length the most (Table 6).

**Table 6.** MANOVA for four most worsened germination characters (shoot length, root length, root fresh weight, root dry weight), four einkorn populations (1, 4, 6 and 14), and four least worsened bread wheat cultivars (Gerek-79, İkizce-96, Bayraktar-2000, and Pehlivan) under salt, cold, and drought together.

| Source | Dependent Variable | df | F | Partial Eta Squared |
|---|---|---|---|---|
| Stress types | SL | 2 | 325.094** | 0.639 |
| | RL | 2 | 21.512** | 0.105 |
| | RDW | 2 | 10.890** | 0.056 |
| | RFW | 2 | 42.442** | 0.187 |
| Cultivars | SL | 7 | 0.571 | 0.011 |
| | RL | 7 | 0.816 | 0.015 |
| | RDW | 7 | 2.008 | 0.037 |
| | RFW | 7 | 0.919 | 0.017 |
| Stress types* Cultivars | SL | 14 | 3.633** | 0.121 |
| | RL | 14 | 2.892** | 0.099 |
| | RDW | 14 | 2.279** | 0.080 |
| | RFW | 14 | 2.784** | .096 |
| Error | SL | 368 | | |
| | RL | 368 | | |
| | RDW | 368 | | |
| | RFW | 368 | | |

**Table 7.** Pillai's trace and Wilks' lambda multivariate tests for intercept + stress types + cultivars + Stress types * cultivars.

| Effect | |
|---|---|
| Intercept | Pillai's Trace** |
| | Wilks' Lambda** |
| Stress types | Pillai's Trace** |
| | Wilks' Lambda** |
| Cultivars | Pillai's Trace** |
| | Wilks' Lambda** |
| Stress types * Cultivars | Pillai's Trace** |
| | Wilks' Lambda** |

**The tests are significant at the 0.01 level.
**The mean difference is significant at the 0.01 level.

All shoot length, root length, root dry weight, and root fresh weight differed between drought, cold, and salt pairs except root dry weight and root fresh weight between cold-salt stress pairs (Table 8). Pillai's Trace and Wilks' lambda were highly significant for shoot length, root length, root dry weight under slat, cold, and drought (Table 9).

**Table 8.** Multiple comparisons of shoot length, root length, root dry weight, and root fresh weight under salt, cold, and drought.

| Dependent Variable | Stress types (I) | Stress types(J) | Mean Difference (I-J) | Std. Error |
|---|---|---|---|---|
| SL | Drought | Cold | -3.1199** | 0.37594 |
| | | Salt | -8.3353** | 0.37594 |
| | Cold | Salt | -5.2155** | 0.26583 |
| RL | Drought | Cold | 2.2706** | 0.45295 |
| | | Salt | 2.9703** | 0.45295 |
| | Cold | Salt | 0.6997 | 0.32028 |
| RDW | Drought | Cold | 1.9190** | 0.41178 |
| | | Salt | 1.5070** | 0.41178 |
| | Cold | Salt | -0.4120 | 0.29117 |
| RFW | Drought | Cold | 32.7163** | 3.82036 |
| | | Salt | 33.1244** | 3.82036 |
| | Cold | Salt | -0.4081 | 2.70140 |

Fresh Weight. Based on observed means. The error term is Mean Square (Error) = 612,996.
*The mean difference is significant at 0.05 level and ** at the 0.01 level.

**Table 9.** Pillai's trace and Wilks' lambda multivariate tests for shoot length, root length, root dry weight, and root fresh weight under salt, cold, and drought.

| | Value | F |
|---|---|---|
| Pillai's trace | 1.451 | 241.710** |
| Wilks' lambda | 0.044 | 343.948** |

**The mean difference is significant at the 0.01 level.

Among four bread and einkorn multi-trait analyzed wheat entries, Gerek-79 and Population 14 significantly differed ($P < 0.05$) for root fresh weight. Gerek-79 - Population 14 and Pehlivan – Gerek-79 highly but not significantly differed (Table 10). Pillai's trace and Wilks' lambda tests also highly significant for these cultivar pairs in shoot length, root length, root dry weight, and root fresh weight (Table 11).

Many studies about the effect of stress on wheat are conducted individually; however, biotic and/or abiotic stresses attack wheat together. Recently, some studies have been undertaken by a two- or two-way biotic-biotic, biotic-abiotic stress study approaches. Thus, it seemed appropriate to engage in this current study on the individual effects of salt, cold, and drought stresses in a combined manner on wheat.

**Table 10.** Multiple comparisons of shoot length, root length, root dry weight, and root fresh weight observed with bread and einkorn wheats under salt, cold, and drought.

| Dependent Variable | (I) CULTIVARS | (J) CULTIVARS | Mean Difference (I-J) | Std. Error | Sig. |
|---|---|---|---|---|---|
| RL | Gerek-79 | Populasyon 14 | 1.5549 | 0.59305 | 0.151 |
| RDW | Pehlivan | Gerek-79 | -1.4550 | 0.53914 | 0.126 |
| RFW | Gerek-79 | Populasyon 14 | 15.9906* | 5.00202 | 0.032 |

Based on observed means.

**Table 11.** Pillai's trace and Wilks' lambda multivariate tests for shoot length, root length, root dry weight, and root fresh weight under salt, cold, and drought.

| | Value | F |
|---|---|---|
| Pillai's trace | 0.143 | 1.956** |
| Wilks' lambda | 0.861 | 1.995** |

**The mean difference is significant at the 0.01 level.

Plant germination decreases when salt concentration in the soil increases [29]. Reduced intake of water due to osmotic limitations and Na and Cl ion toxicity worsen germination characteristics [30]. Species and genotypes differ in their reactions to individual and most likely combined biotic stresses; thus, a detailed exploration is needed. Einkorn seemed more tolerant environmental stresses than bread wheats in the previous studies [19, 20, 31]. Mahmoodabad [32] reported the lowest germination rate at 2°C, with some differences among bread wheat cultivars of Gaspard, Sardari, Cascogen, Bezostaja-1, and MV-17. Similarly, the root length was the same in Gaspard, Bezostaya-1 and Cascogen as Bezostaya 1, which has been frequently reported to have the longest shoot and root lengths at the lower temperatures. The characteristics of cultivars under different stresses differed during the germination and early seedling stages in some previous studies. Chilling temperatures between 0-12°C significantly delayed the onset and reduced the germination rate in the cultivated plants [33, 34]. Cold also led to poor seed germination, uneven stand establishment, and poor crop performance [33]. Most commercial crop cultivars have been highly sensitive to cold during seed germination despite available genetic variation within and between related wild species [33, 34].

Among abiotic stresses, drought widely spreads around the world, upsetting seed germination and seedling growth, and resulting in a poor establishment and a decreased seedling growth. Poor establishment, in turn, decreases weed competitiveness, shades soil surface, restricts light interception, declines early season growth, and reduces yield. Similarly, shoot length, root length, root fresh weight, and root dry weight gradually decreased under salt, cold and drought stresses in this study (Table 4). This was parallel with the results of Mahmoodzadeh [35] who obtained significant shoot and root length differences in bread wheat cultivars. All four: shoot length, root length, root fresh weight and root dry weight characters similarly followed the same trend against salt, cold, and drought stresses in the present study.

Stress tolerance indices [24, 27, 28] successfully differentiated bread wheat cultivars and einkorn populations under individual or combined salt, cold, and drought stresses (Table 3). Bayraktar-2000, Gerek-79, İkizce-96, Demir-2000, Gün-91, Momtchil, and Flamura-85 bread wheat cultivars and 1, 6, 4, 2, 5, and 9 einkorn wheat populations were tolerant under various stress combinations. Bread wheat cultivars Momtchil, Gerek-79, and Bayraktar-2000 and einkorn populations 5, 6, and 1 were the tolerant wheat entries when salt, cold and drought stresses evaluated together (Table 3). The values by Ali and El-Sadak [36] who compared the stress susceptibility and stress tolerance indices, mean and geometric mean productivity were not parallel with the stress indices here. When multivariate analysis variance, partial Eta squared values, and Pillai's trace and Wilks' Lambda tests were considered partial Eta revealed a strong effect for three stresses and a weak effect for wheat entries and stress by wheat interaction (Table 7) similarly to the stress tolerance indices in this study.

## 4. CONCLUSION

Previous studies have mostly investigated individual stresses and their effects on plants because of an easy testing and a statistical analysis. However, plants are simultaneously exposed to more than one stress in their real-life cycles. Therefore, studying stresses in a combined approach by multivariate analysis methods is expected to provide a better

understanding of the stresses on wheat. This study, therefore, presented a novel approach and produced valuable results about the effects of three stresses on wheat and einkorn entries in a combined understanding.

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# *Scenedesmus obliquus*: A Potential Natural Source for Cosmetic Industry

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**Abstract**: Skin is the largest organ of our body and it protects interior organs against several environmental factors. Hyperpigmentation problem occurs as a result of abnormal melanin accumulation in the skin. A considerable amount of world's population uses skin whitening products. It is known that various algae-derived secondary metabolites play an important role in skin problems. Therefore, the tyrosinase inhibitory activities of *S. obliquus* ethanol and water extracts were evaluated in the present study. Tyrosinase activity was determined spectrophotometrically at 492 nm. The ethanol extract showed the higher inhibitory activity on tyrosinase enzyme ($IC_{50}$: 0.0270 g/mL) than water extract ($IC_{50}$: 0.2882 g/mL). This result may have stemmed from the vanillic, ferulic acid and rutin components that were identified by RP-HPLC only in the ethanol extract.

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## 1. INTRODUCTION

Hyperpigmentation and solar lentigines lead to the formation of dark spots on the skin and these spots constitute an important aesthetic problem for many people. These spots may also occur as a result of pregnancy, liver disease, Addison's disease and etc [1]. Although several methods such as chemical peeling, laser treatment, and so on can be used in order to reduce hyperpigmentation problem, the most common treatments are the topical treatments that target the inhibition of tyrosinase enzyme [2,3].

Tyrosinase enzyme (EC 1.14.18.1) plays a key role in melanin synthesis. Since melanin pigment is an important component of our body, both the abnormal loss and the accumulation of this pigment can cause serious disorders. Although hyperpigmentation problem is the most known problem caused by the accumulation of this pigment, there are more serious disorders such as neurodegeneration associated with Parkinson's disease and skin cancer related to this complication. Reducing melanin amount by inhibiting tyrosinase enzyme is an effective method to prevent above related illnesses [4].

Numerous tyrosinase inhibitors have been identifed up to date, but only a few of them are labelled as safe and effective ones. Although hydroquinone, arbutin and kojic acid are the well-

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known tyrosinase inhibitors, the use of all these inhibitors is limited due to their side effects. For example, hydroquinone is banned in the European Union and can only be used up to 2% in any formulation in USA. Arbutin is a pro-drug of hydroquinone and in the mode of action of arbutin, a hydroquinone release is formed. Therefore, The European Union Scientific Committee on Consumer Products-2008 [5] emphasized that the use of arbutin in cosmetic products is unsafe. Moreover, although The European Scientific Committee on Consumer Safety-2012 [6] specifies the use of kojic acid at the concentrations up to 1% in cosmetic products is safe, the use of this compound has been forbidden in many countries due to its serious side effects [3,7,8]. Therefore, effective natural tyrosinase inhibitors which are believed to be more reliable and safe attract more attention compared to synthetic counterparts.

Microalgae species with their high spectrum of biodiversity present an important source of biomolecules. Microalgae species are becoming an important part of daily life day by day as these biomolecules find applications in many sectors such as food, cosmetic, medicine, energy and etc [9,10]. In addition to the unnecessity of agricultural lands, the rich phytochemical content with various modifications based from cultivation medium makes these species unique sources for industrial applications [8]. Besides these general properties, *S. obliquus* species was chosen in the present study as a result of its specific features such as high growth rate, high tolerance to environmental change, having a rich content of amino acids, polyunsaturated fatty acids, vitamins and minerals. In this context, the present study was carried out to investigate the potential tyrosinase inhibitory effects of *S. obliquus* extracts.

## 2. MATERIAL AND METHODS

### 2.1. Chemicals

Tyrosinase, L-3,4-dihydroxyphenylalanine (L-DOPA), $KH_2PO_4$-$K_2HPO_4$, bovine serum albumin (BSA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, butylated hydroxytoluene (BHT) and all standard phenolic compounds used (Gallic acid (GA), p-OH benzoic acid, Vanillic acid, Caffeic acid, Syringic acid, p-Coumaric acid, Ferulic acid, Cinnamic acid, Protocatechuic acid, Hesperidin, Naringin, Rutin and Quercetin) were obtained from Sigma Chemical Co. Ethanol, methanol and acetic acid were purchased from Merck Chemical Company. All other reagents used were of analytical grade.

### 2.2. *S. obliquus* Cultivation Conditions

The *S. obliquus* species (from Ege University, Faculty of Aquaculture) was grown in Bold Basal (BB) medium at 30 ± 2 °C and 12 hours light / 12 hours dark photoperiod. Possible contaminations in *S. obliquus* culture were determined by microscopic analysis.

### 2.3. Preparation of Extracts from The Dried Biomass

Following the harvest, the obtained biomass was lyophilized at -52°C (Labconco 7670530, FreeZone). The lyophilized sample was extracted with ethanol or distilled water for 12 h in dark at 50°C in a temperature controlled shaker (Memmert, SV 1422). The extracts were dried by the removal of ethanol and water solvents by vacuum evaporation or lyophilisation, respectively and the resulting extracts were stored at -20°C until used.

### 2.4. Tyrosinase Enzyme Activity Assay and Inhibition Experiments

Tyrosinase activity was measured in phosphate buffer solutions (0.05 M, pH 6.8) by measuring the increase in absorbance related to DOPA quinone formation at 492 nm [11]. In inhibition experiments, firstly the *S. obliquus* extracts as an inhibitor were incubated with the enzyme solution at 30ºC for 10 min, then the tyrosinase activity was measured by using the enzyme-inhibitor mixture. The half maximal inhibitory concentration ($IC_{50}$) values were determined from the inhibition % – extract concentration curve. All experiments were performed in three replications and the results were expressed as mean ± S.D.

### 2.5. Determination of The Total Phenolic Contents of *S. obliquus* Extracts

The total phenolic contents of *S. obliquus* extracts were determined according to Folin–Ciocalteu total phenolics method at 760 nm [12]. The details of the method were given in my previous scientific report [8].

### 2.6. Phenolic Compound Identification by HPLC

Phenolic compounds were analyzed according to the modified method of Caponio et al. [13] using a reversed phase HPLC system (RP-HPLC, ThermoUltiMate 3000) equipped with a UV–vis photodiode-array detector. The mobile phase consists of a mixture of two solvents, one of them is the acetic acid solution 3% (A), and the other is methanol (B). The details of the mobile phase gradient conditions were also given in my previous scientific report [8]. The amount of phenolic compounds in the samples were determined according to the calibration curves formed in the same analysis conditions.

### 2.7. Determination of The DPPH Radical Scavenging Activities of *S. obliquus* Extracts

DPPH free radical scavenging activity of an extract was determined by the measurement of the decrease in absorbance at 515 nm [14]. BHT was used as a positive control in the present study. The required concentrations of the extract that cause a 50% decrease in the initial absorbance of DPPH ($IC_{50}$) were identified as the antioxidant activities of the *S. obliquus* extracts.

## 3. RESULTS and DISCUSSION

### 3.1. Tyrosinase Inhibitory Activity

Utilization of safe inhibitors from natural sources in order to control hyperpigmentation is gaining much importance in the last decade. Therefore, the present study focuses on to determine the inhibitory effect of *S. obliquus* extracts on tyrosinase enzyme. The results showed that both water and ethanol extracts of *S. obliquus* inhibited tyrosinase activity in a dose dependent manner (Figure 1,2).

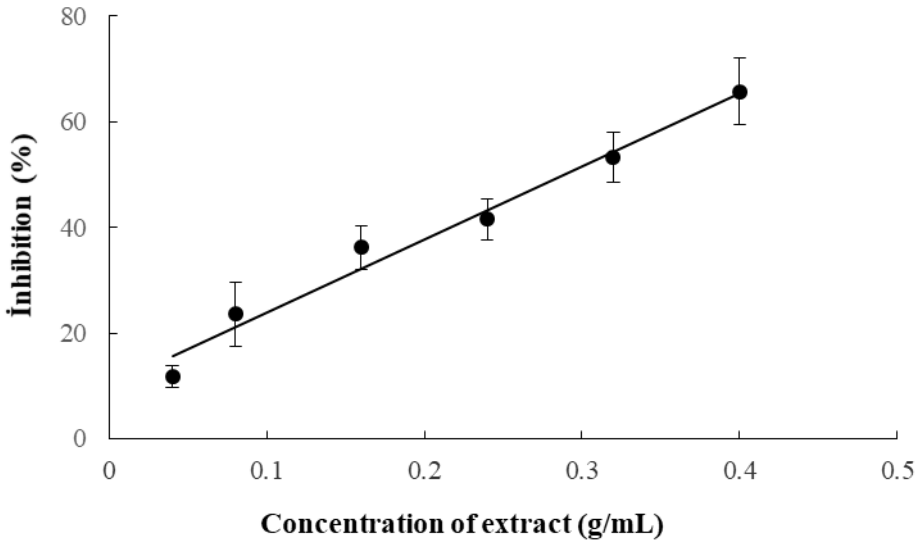


**Figure 1.** Concentration–inhibition (%) curve for tyrosinase inhibition by the water extract of *S. obliquus*. t= 30 °C, [L-DOPA]= 3 mM. The results are shown as mean ± SD of three independent experiment.

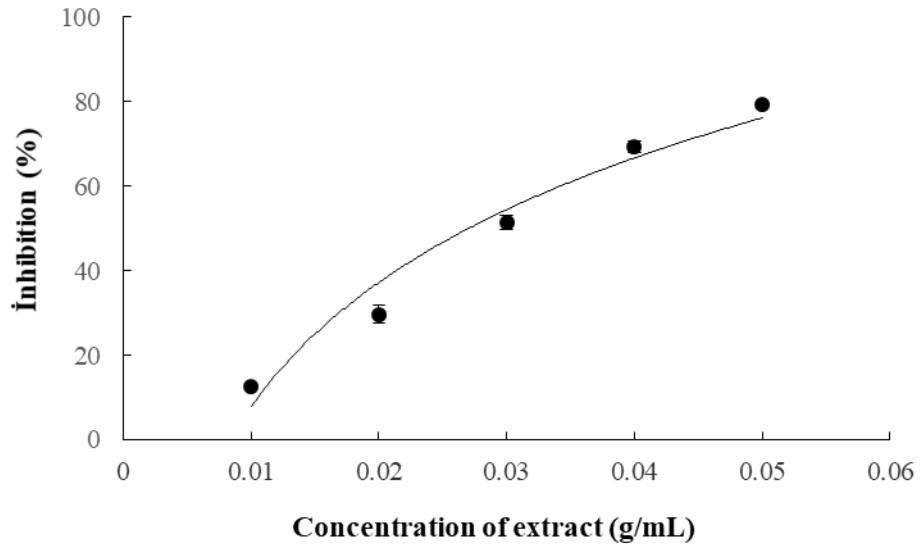


**Figure 2.** Concentration–inhibition (%) curve for tyrosinase inhibition by the ethanol extract of *S. obliquus*. t= 30 °C, [L-DOPA]= 3 mM. The results are shown as mean ± SD of three independent experiments.

The $IC_{50}$ values of tyrosinase inhibition with *S. obliquus* ethanol and water extracts were found as 0.0270 g/mL and 0.2882 g/mL, respectively. The comparison of $IC_{50}$ values found in the present study to previously reported ones seems not appropriate due to the variations in assay conditions such as the concentration of substrates, enzymes or the incubation times, etc [15]. Hence, a well-known and commercial tyrosinase inhibitor, kojic acid, was used to make our results more meaningful. The $IC_{50}$ value of kojic acid was found as 0.00028 g/mL. It is obvious that the inhibitory effect of kojic acid is higher than the *S. obliquus* extracts. But as indicated above, the use of kojic acid has been forbidden in many countries due to its undesirable side effects. Therefore, *S. obliquus* species used in the present study may serve an alternative material in obtaining safer tyrosinase inhibitors. There are only a few studies in the scientific literature which evaluates the inhibitory effect of microalgae species on tyrosinase enzyme. Fucoxanthine and phloroglucinol derivatives from brown algae and 7-phloroeckol and dieckol from *Ecklonia cava* have been reported as effective tyrosinase inhibitors [16-19]. The inhibitory effect of a cyanobacteria, *Artrosphira platensis*, extracts on tyrosinase enzyme were evaluated in a recent study of us. The $IC_{50}$ values of tyrosinase inhibition with *A. platensis* ethanol and water extracts were reported as 0.0014 g/mL and 0.0072 g/mL, respectively in the related study [8].

### 3.2. Total Phenolic Content of *S. obliquus* Extracts

Phenolic compounds of many types constitute an important kind of phytochemicals. Hence many researchers try to shed light to their wide range of biological effects in various mechanisms. In addition to having antioxidant, antiviral, antimicrobial, antiproliferative and so on effects**,** the structure similarities of these compounds to tyrosinase substrate make these compounds more important for this investigation. The positive correlation between the tyrosinase inhibitory activity and the abundance of hydroxyl groups were stated by various researchers [20-23].

The total phenolic content of *S. obliquus* water and ethanol extracts were determined as 48.0 mg GAE/g extract and 22.2 mg GAE/g extract, respectively. Stoica et al [24] used water, ethanol and their mixture in order to determine the total phenolic content of *Scenedesmus opoliensis*. They reported that the highest total phenolic content was found as 151.5 mg GAE/100 g of dry algal biomass for absolute water solvent, and the lowest total phenolic

content was found as 61.4 mg GAE/100 g of dry algal biomass for absolute ethanol solvent. Finally, the authors emphasized that the total phenolic content of any substances well correlated with the polarity of solvent. Bulut et al [25] determined the total phenolic content of thermo-tolerant *Scenedesmus* species and found that the total phenolic content of prepared extracts increased in order of hexane (1.13±0.11 mg GAE/g DW), water (1.97±0.03 mg GAE/g DW), ethyl acetate (3.73±0.65 mg GAE/g DW) and ethanol/water (5.40±0.28 mg GAE/g DW). In an another study of Jerez-Martel et al. [26], the total phenolic content of water and methanol extracts of several microalgae species were determined. The results of the related study presented that the extraction yield of water was higher than that of methanol. The total phenolic content of *S. obliquus* strain was found as 1.94 mg GAE/g DW in another study by Goiris et al. [27]. Actually, the content of phenolic compounds changes as a result of environmental differences due to being secondary metabolites of the defense system of any species [28,29]. Therefore, it is very difficult to compare the results of previous reports.

### 3.3. Phenolic Compounds Identification

Phenolic compounds of *S. obliquus* extracts were identified by RP-HPLC analysis and the results were shown in Table 1.

**Table 1.** The amount of phenolic compounds (ppm) in the studied extracts of *Scenedesmus obliquus*.

| Analysed phenolic compounds | The water extract of *S. obliquus* | The ethanol extract of *S. obliquus* |
|---|---|---|
| Gallic Acid | 12.771 | 1.487 |
| Caffeic Acid | 0.481 | - |
| Vanillic Acid | - | 0.678 |
| P-coumaric Acid | 1.184 | 0.426 |
| Hesperidin | 2.032 | - |
| Ferulic Acid | - | 0.315 |
| Rutin | - | 1.666 |
| Quercetin | 4.750 | 4.711 |
| Cinnamic Acid | 0.550 | 0.566 |

4 of studied 13 phenolic compounds were found in both extracts. On the contrary, caffeic acid and hesperidin were only found in water extract and vanillic acid, ferulic acid and rutin were only found in ethanol extract. While the main phenolic compound is gallic acid in water extract, it is quercetin in ethanol extract. Besides, very similar amount of quercetin was also found in water extract, too (Table 1).

In another investigation about *Scenedesmus* strain, two solvent systems were used in the extraction of phenolics. The author detected gallic acid, 4-hydroxy benzoic acid, vanillic acid, caffeic acid, chlorogenic acid and quercetin in ethanol/water extract and gallic acid, benzoic acid, 4-hydroxy benzoic acid, syringic acid, cinnamic acid, coumaric acid, caffeic acid, chlorogenic acid, rosmarinic acid, quercetin and rutin in ethyl acetate extract. It is obvious that the results are well in line with ours. Besides, the amount of gallic acid was significantly higher in polar extract than that of nonpolar extract as in our samples. The opposite was true for the rutin component as in ours, too. The authors determined that there were quite a high amount of quercetin in both extracts and emphasized its importance. This also applies to our results [25].

Vanillic acid, ferulic acid and rutin that were found in ethanol extract may be the main contributors responsible for the higher inhibition or the higher inhibition may be caused from the synergistic effect of all components. Chou et al [30] determined the tyrosinase inhibitory effect of vanillic acid from *Origanum vulgare* and found the inhibitory activity of this

compound was higher than the arbutin. Lee et al [31] presented that both ferulic acid ($IC_{50}$:0.559 mM) and caffeic acid ($IC_{50}$:0.037 mM) were efficacious tyrosinase inhibitors. The inhibitory effect of rutin on tyrosinase activity was evaluated in another report by Si et al [32]. They found that the $IC_{50}$ value of tyrosinase inhibition with rutin was found as 6.8±0.3 mM. Besides, the kinetic experiments revealed that rutin inhibited tyrosinase enzyme in a competitive manner by chelating with copper ion at active site.

### 3.4. DPPH Radical Scavenging Activity

DPPH radical scavenging activity method was used in order to determine the antioxidant potential of *S. obliquus* extracts. Since the present study focuses on the evaluation of the potential of *S. obliquus* extracts as a safer source for cosmetic industry, determination of the antioxidant capacity of these extracts are crucial. Undoubtedly, the existence of antioxidants in any product increases its value. The $IC_{50}$ values of water and ethanol extracts of *S. obliquus* were 2273.7 µg/mL and 938.7 µg/mL, respectively. Both of these results were lower than the value of 56.4 µg/mL that was found for the positive control, BHT. Since the phenolic compounds are effective antioxidants, a positive correlation is expected between the total phenolic content and antioxidant activity. Nevertheless, it is not the case for the present results like some previous results in the literature for various algae species. Sabeena Farvin and Jacobsen [33] found the antioxidant capacities of water extracts were higher than that of ethanol extracts of studied seaweeds, although the water extracts have lower total phenolic content. This unexpected result was explained by the possible presence of the other radical scavengers such as pigments, proteins or peptides. Bulut et al [25] highlighted that the high levels of polyunsaturated fatty acids may contribute to the antioxidant capacity of *Scenedesmus* sp. This observation confirms our result due to the expectation of higher amount of polyunsaturated fatty acids in ethanol extracts than that of water extracts.

## 4. CONCLUSION

Algae are a source of raw materials for many industries such as food, cosmetics, pharmaceuticals, and etc as a result of their biologically active metabolites waiting to be identified. The results of the present study revealed that the extracts of *S. obliquus* species can be used to combat hyperpigmentation problems. In addition to this, semi-synthetic analogues based on the natural ones may be designed in order to cope with the possible problems of natural inhibitors such as cytotoxicity, solubility, absorption, etc. However, more research is needed prior to use these inhibitors for practical applications.

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# Effect of Nitrogen, Phosphorus and Medium pH to Enhance Alkaloid Production from *Catharanthus roseus* Cell Suspension Culture

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**Abstract**: Several elevated levels of nitrogen and phosphate at varying pH of the medium which impart a major influence on callus and biomass development and subsequent production of alkaloids was investigated using suspension culture system of *Catharanthus roseus* in the present study. The B5 medium was buffered at pH 4.51, 5.82 and 7.32 by addition of different levels of (A) diammonium hydrogen phosphate $(NH_4)_2HPO_4$ and (B) ammonium dihydrogen orthophosphate $(NH_4H_2PO_4)$ representing the enhanced and varied supply of total nitrogen $(NH_4^+ + NO_3^-)$ and phosphate compared to MS medium (as control) for cell biomass production and alkaloid yield. The pH of the medium have shown significant effects with maximum biomass fresh wt., dry wt. and total alkaloid yield at 5.82 medium pH with elevated phosphate levels and total nitrogen concentration of 3710.10 mg/L compared to control MS medium with 2850 mg/L total nitrogen. At 3667.33 and 3752.48 mg/L of total nitrogen with enhanced phosphate supply showed reduced biomass fresh wt., dry wt. and total alkaloid yield at lower (4.51) and higher (7.32) medium pH respectively. Inclusion of 200 mg/L of tryptophan or phenylalanine as reduced nitrogen source in B5 medium buffered at 5.82 ± 0.2 pH showed enhanced biomass and alkaloid production. Hence, addition of nitrogen, phosphate, tryptophan, phenylalanine as nutrient in suspension culture stimulate their uptake to enhance cell biomass and total alkaloids production but as a function of pH of the medium.

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## 1. INTRODUCTION

The plant cell, tissue and organs cultured in vitro need to be supplied with complete nutrients in the medium [1]. Nitrogen and phosphate comprises the most important essential elements required for all plants growth and developmental process by feeding to the major metabolic pathways [2-6]. The nitrogen is available as nitrate and ammonia in the soil which are absorbed by plants through roots [7-9]. The nitrate gets transported through xylem reaches

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to the parenchymatous cells of root and shoot wherein it is stored in vacuoles and does not cause any toxicity to the plants but need to be converted to the active form ammonia for metabolisms leading to growth, development and stress tolerance [10]. The uptake of nitrate is dependent on various factors like plant species, root pH, temperature etc. [11]. The energy required for ammonium assimilation is low in comparison to nitrate. To avoid the toxicity in roots of the plants ammonia is immediately converted to organic biomolecules [12]. In suspension culture of *C. roseus* the effect of UV-B and nitrate was compared individually as well as in combinations for cell biomass growth and alkaloid accumulation [13]. The increased supply of nitrogen inhibited the repair mechanism of the cell for UV-B radiation damages as under decreased N supply, plant becomes sensitive to UV radiation [14].

Nitrate supplied as nutrients was found as an important factor in alkaloid production by *C. roseus* plants under salinity stress [15]. On the other hand, changes in many growth parameters such as reduction of biomass, leaf epidermis damages, change in leaf colour and leaf fall due to reduced resistance was implicated with excess N deposition in plants [16]. It has also been reported that secondary metabolite production is affected differently by nitrate and ammonia, where nitrate promote secondary metabolite synthesis but ammonium ions inhibit it [17-18].

The importance of phosphate in secondary metabolite production was demonstrated as depletion of its level showing decreased biomass and 20-hydroxyecdysone (20-HE) production in hairy root culture of *Ajuga* [19]. On the other hand, *C. roseus* batch culture added with different phosphate concentrations showed increased cell growth and alkaloids with higher intracellular phosphate level [20].

*Catharanthus roseus* a perennial medicinal herb of family Apocynaceae is the store house of more than 130 secondary metabolites. The most important are the alkaloids (vincristine and vinblastine) with anti-cancer activity, anti-hypertension (ajmalicine and serpentine) [21]. Majority of alkaloids possess nitrogen containing heterocyclic reaction centers for these activities. But the problem with these alkaloids is very low concentrations and gets stored in leaf vacuoles. As the genes involved in alkaloid synthesis are expressed in tissue specific manner might be the cause of its low yield [22-24].

Biosynthesis of alkaloids by *C. roseus* cell gets initiated by joining of amino acid and a monoterpenoid i.e. tryptophan and geraniol respectively. The whole process is expressed by the involvement of two regulatory genes controlling about 30 biosynthetic steps catalyzed by 30 enzymes to produce 35 intermediates compartmentalized among 7 intra- and inter- cellular components [25]. This emphasizes the importance of tryptophan in TIA biosynthesis. Addition of tryptophan, secologanin, phenylalanine etc as precursor have been applied in cell suspension and hairy root culture of *C.* roseus to enhance the alkaloid production [26-27]. The effect of tryptophan on metabolic flux of indole alkaloids in *Catharanthus* revealed tryptophan feeding at 17 days of culture mimicked auxin effect on the cultures and increased the flux density of the indole alkaloids [27].

Precursor feeding as elicitation is an important strategy for increasing secondary metabolite productions in plants [28]. Plants like *Crocus sativus* also showed enhanced ajmalicine and strictosidine production upon treatment with exogenous tryptamine and loganic acid [29]. The induced production of enzyme phenylalanine ammonia lyase (PAL) and tryptophan decarboxylase (TDC) in cell suspension culture of *C. roseus* are involved in the production of phenylalanine and tryptophan respectively [30]. The 2 amino acids are the branched point product of common intermediate chorismate of the pathway and serve as the precursors for the synthesis of phenolics and indole alkaloids. The shikimate and terpenoid pathway catalyzed the production of tryptamine and secologanin which are combined to produce strictosidine, the precursor for monomeric alkaloid ajamalicine, catharanthine and

vindoline. The coupling reaction between catharanthine and vindoline catalyzed by anhydrovinblastine synthase (AVLBS) to produce vinblastine from a-3',4'- anhydrovinblastine whose mechanism of conversion is still unclear [30]. Hence, the regulation of alkaloid production by *C. roseus* in response to nitrate and other cultural and environmental factors is not fully understood and need to be explored in detail.

There is pressing need for an alternative strategy for natural sources to produce enhanced level of alkaloids and other desirable substances. Therefore attempts are made in the present investigation to investigate the role of nitrogen along with phosphate for biomass growth leading to higher alkaloid production in suspension medium with varying pH. The experiments are designed to maintain different pH of the medium by buffering action with higher levels of nitrogen and phosphate as well as supplemented with reduced nitrogen (tryptophan and phenylalanine) sources to enhance total alkaloid synthe sis.

## 2. MATERIALS and METHODS

### 2.1. Selection of Plants and Preparation Explants

*Catharanthus roseus* plants grown as seedlings in the GITAM campus nursery were used for explant supply. Successful establishment of callus was induced only from leaf segments as other explants were not successful. Induction of callus was achieved by culturing 1 $cm^2$ as described earlier [31-33].

### 2.2. Culture Media and Treatments

The normal strength of Murashige and Skoog's (MS) [1] and Gamborgh's (B5) culture medium [34] containing 3% (w/v) sucrose and modified with 0.50 mg/L of 2,4-D, 1.0 mg/L of Kinetin and 2.0 mg/L NAA was used as the standard treatments for the induction of callus and cell proliferations in suspension cultures. Callus induction from leaf explants and multiplication was achieved by continuous incubation for four weeks in the presence of this standard treatment on agar medium. The cell suspension culture was established in normal strength B5 culture salts with above standard treatment for control and modified variously for different treatments. About 100 ml of suspension medium was modified for each treatment and dispensed 25 ml in 4 conical flasks of 150 ml capacity. Each 25 ml suspension medium was inoculated either with 100 -150 mg friable callus (4-weeks old) from agar medium or with 5 ml of 2-weeks old suspension cultures.

The pH of the MS medium was adjusted to 5.8 ± 0.2. To study the effect of pH the B5 medium was buffered at 4.51 ± 0.2, 5.82 ± 0.2 and 7.32 ± 0.2 by the addition of specific volumes of molar concentrations of i) ammonium di-hydrogen orthophosphate ($NH_4H_2PO_4$) and ii) di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) along with standard sucrose and PGR combination. The B5 control and medium buffered at pH 5.82 ± 0.2 was also supplemented with 0 – 200 mg/L of reduced nitrogen sources either as tryptophan or as phenylalanine as precursor feeding in alkaloid biosynthetic pathway.

All these media were sterilized by autoclaving (15 lbs, 121°C, 15 min). The suspension cultures were maintained under continuous shaking at 150rpm over an orbital shaker incubator at 25 ± 2 °C. The callus cultures were maintained in an environmentally controlled air conditioned room. Each culture shelves were fitted with fluorescent lamps providing 2,000-3,000 Lux photon flux density under 16/8 hrs photoperiodic cycle.

### 2.3. Growth Measurement

#### *2.3.1. Callus growth*

Induction and proliferation of callus from leaf explants was achieved as described by [31-33]. The callus grown on agar medium was harvested after 4-weeks of sub-culture. The cell biomass from suspension culture was harvested every 2- weeks of culture/sub-culture. The procedure for measurement of fresh and dry weight of callus and cell biomass was described earlier [31-33] and represented as:

Callus Fresh Wt = {Weight of filter paper and the callus – Initial weight of dried filter paper}

Callus Dry Wt = {Weight of dried callus along with filter paper – Initial weight of dried filter paper}

Biomass Fresh Wt = {Weight of moisture free filter paper and the cell biomass – Initial weight of moisture free filter paper}

Biomass Dry Wt = {Weight of the dried Biomass with Filter Paper – Weight of the dried Filter Paper}

### 2.4. Extraction and Quantifications of Total Alkaloids

Alkaloids were extracted using 20mg dried callus or cell biomass following the procedure detailed in by [31-33]. The total alkaloid was estimated following the modification of protocol [35; 36] along with Bismuth nitrate pentahydrate **(**$Bi(NO_3)_3.\ 5H_2O$**)** calibration curve. The alkaloid content was calculated as described earlier [31-33] and presented as:

Alkaloid content (mg/g dwt): {(Concentrations (µg) / 10mg Dry weight} x 1000

Alkaloid Production (mg/L) = Dry wt (g/L) x Alkaloid content (mg/g)

Alkaloid Productivity (mg/L/day) = {Alkaloid Production (mg/L) / no. of days the product is harvested}

Alkaloid Yield (% dwt) = {Alkaloid Content (mg/g) / 1000} x 100

### 2.5. Statistical Analysis

The mean of three replicates of experiments, standard deviation, analysis of variance (ANOVA) of results were performed by using SPSS 15 package for Window [37] in the present study.

## 3. RESULTS

### 3.1. Effect of Medium pH

The B5 medium is devoid of $NH_4NO_3$ and contain only $KNO_3$ as sources of nitrogen at a concentration lesser that present in MS medium. The results of the present study in B5 medium buffered at pH 4.51, 5.82 and 7.32 by addition of specific amount of molar solutions of **(A)** diammonium hydrogen phosphate **$(NH_4)_2HPO_4$** and **(B)** ammonium dihydrogen orthophosphate **($NH_4H_2PO_4$)** for the enhanced supply of total nitrogen ($NH_4^+$+$NO_3^-$) and phosphate compared to MS medium on alkaloid production is presented in Table 1.

**Table 1.** Effects of nitrogen and phosphate source (combination of diammonium hydrogen phosphate and ammonium dihydrogen orthophosphate) on modulation of medium pH for production of cellular biomass and yield of alkaloid in B5 suspension culture.

| Sl. No. | Volume of 1M (A) | Volume of 1M (B) | Total nitrogen conc. | pH | Biomass Fresh wt | Biomass Dry wt | Alkaloid Content | Alkaloid Production | Alkaloid Productivity | Alkaloid Yield |
|---|---|---|---|---|---|---|---|---|---|---|
| | (ml) | (ml) | (mg/L) | | g/L | g/L | mg/g dwt | mg/l | mg/L/d | % dwt |
| 1 | 0 | 0 | MS Medium (2850) | 5.80 ± 0.2 | 16.806 ± 1.076 | 1.668 ± 0.162 | 5.575 ± 0.270 | 9.276 ± 0.689 | 0.662 ± 0.049 | 0.558 ± 0.027 |
| 2 | 10 | 90 | 1167.33 (3667.33) | 4.51 ± 0.2 | 12.541 ± 0.098 | 1.463 ± 0.069 | 4.717 ± 0.044 | 6.900 ± 0.388 | 0.493 ± 0.028 | 0.472 ± 0.005 |
| 3 | 35 | 65 | 1210.10 (3710.10) | 5.82 ± 0.2 | 19.166 ± 0.145 | 2.096 ± 0.090 | 5.844 ± 0.154 | 12.239 ± 0.204 | 0.874 ± 0.015 | 0.584 ± 0.016 |
| 4 | 60 | 40 | 1252.48 (3752.48) | 7.32 ± 0.2 | 11.407 ± 0.093 | 1.317 ± 0.063 | 4.483 ± 0.087 | 5.905 ± 0.295 | 0.422 ± 0.021 | 0.448 ± 0.009 |
| | F(3, 11) | | | | 79.140 | 23.115 | 36.757 | 90.961 | 91.490 | 36.838 |
| | P<0.05 | | | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

The Gamborgh's (B5) suspension medium [34] was supplemented with 3% sucrose (w/v) and modified with 0.50 mg/L of 2,4-D, 1.0 mg/L of Kinetin and 2.0 mg/L NAA. For increased pH, total nitrogen and phosphorus, respective volume of 1M stock solution each of (A) diammonium hydrogen phosphate $(NH_4)_2HPO_4$ and (B) ammonium dihydrogen orthophosphate $(NH_4H_2PO_4)$ were mixed as shown in the table to get specific pH and the total amount of nitrogen and phosphorus in the medium. The suspension culture was established with 100-150 mg of callus (4-weeks) or by cell biomass (2- weeks) inoculated @ 20% per culture vessel with 25 ml medium. The growth of cell biomass in suspension culture was recorded after 2-week of subculture.

The B5 medium maintained at lower pH (4.51 ± 0.2) prevented the solidification of agar even at 2.5% concentration in the medium. While at higher pH (7.32 ± 0.2) precipitation of nutrient salts was common both in agar and suspension conditions of B5 medium. This led to reduced biomass and alkaloid production at lower and higher pH even in the presence of elevated levels of nitrogen and phosphate even in the suspension medium. The B5 medium maintained at pH of 5.82 ± 0.2 by supplementing with elevated levels of nitrogen and phosphate was almost indispensable for enhanced production of biomass (fresh and dry weight) and alkaloids (content, production, productivity and the yield) compared to control cultures in MS suspension medium. All the observable parameters such as fresh and dry weight of biomass as well as content, production, productivity and the yield of alkaloid was significantly reduced at both higher or lower pH even though media contained elevated levels of both nitrogen and phosphorus. The possible reason for this reduction in response to the media pH had been reported in various studies discussed in the following section.

### 3.2. Effect of Nitrogen and Phosphate

The increased supply of nitrogen and phosphate source in B5 medium showed a significant effect on biomass (fresh and dry wt.) and alkaloid (content, production, productivity and yield) as compared to the MS medium (Table 1). Maximum biomass production (fresh wt., dry wt.) and yield of total alkaloid was obtained with elevated phosphate levels and total nitrogen concentration of 3710.10 mg/L compared to control MS medium with 2850 mg/L of total nitrogen (Table 1) both maintained at 5.8 ± 0.2 pH. The B5 medium with 3667.33 mg/L of total nitrogen buffered at lower pH (4.51 ± 0.2) or with 3752.48 mg/L of total nitrogen adjusted at higher pH (7.32 ± 0.2) containing elevated levels of phosphate showed significant reduction in all the observable parameters compared to the control MS medium with normal levels of nitrogen and phosphate. These reductions might be attributed to the precipitation of nutrient salts as a function of medium pH. Hence, the elevated levels of nitrogen and phosphate showed significant effects on all observable parameter as a function of pH of the medium.

### 3.3. Effect of Nitrogen Phenylalanine and Tryptophan

The effects of tryptophan or phenylalanine added in the medium as reduced sources of nitrogen showed significant ($P<0.05$) response on all the observable parameters (Table 2 & 3). Both the reduced sources of nitrogen followed similar response trend while tryptophan showed slightly better response compared to phenylalanine. There was continuous increase in all the observable parameters with the addition of either the tryptophan or phenylalanine. Hence, addition of 200 mg/L of either tryptophan or phenylalanine in the B5 medium was the threshold for maximum alkaloid yield as well as other parameters.

**Table 2.** Effects of tryptophan and nitrogen source (combination of diammonium hydrogen phosphate and ammonium dihydrogen orthophosphate) on biomass production alkaloid yield.

| Sl. No. | Tryptophan Concentration | Biomass Fresh wt | Biomass Dry wt | Alkaloid Content | Alkaloid Production | Alkaloid Productivity | Alkaloid Yield |
|---|---|---|---|---|---|---|---|
| | mg/L | g/L | g/L | mg/g dwt | mg/L | mg/L/d | % dwt |
| 1 | 0.0 | 17.987 ± 0.122 | 2.089 ± 0.174 | 5.972 ± 0.160 | 12.492 ± 1.311 | 0.892 ± 0.093 | 0.597 ± 0.016 |
| 2 | 50 | 18.493 ± 0.101 | 2.293 ± 0.080 | 6.665 ± 0.160 | 15.287 ± 0.895 | 1.092 ± 0.064 | 0.666 ± 0.016 |
| 3 | 100 | 18.947 ± 0.122 | 2.450 ± 0.120 | 7.306 ± 0.203 | 17.913 ± 1.363 | 1.279 ± 0.097 | 0.731 ± 0.021 |
| 4 | 150 | 19.120 ± 0.120 | 2.672 ± 0.083 | 7.947 ± 0.118 | 21.240 ± 0.847 | 1.517 ± 0.061 | 0.794 ± 0.012 |
| 5 | 200 | 19.387 ± 0.061 | 2.766 ± 0.120 | 8.844 ± 0.231 | 24.478 ± 1.700 | 1.748 ± 0.121 | 0.884 ± 0.023 |
| F(4, 10) | | 78.876 | 15.726 | 116.539 | 42.169 | 42.266 | 116.374 |
| P<0.05 | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

The Gamborgh's (B5) suspension medium[34] was supplemented with 3% sucrose (w/v) and modified with 0.50 mg/L of 2,4-D, 1.0 mg/L of Kinetin and 2.0 mg/L NAA. Additionally, the media was fortified with 35 ml and 65 ml of 1M stock solution each of (A) diammonium hydrogen phosphate $(NH_4)_2HPO_4$ and (B) ammonium dihydrogen orthophosphate $(NH_4H_2PO_4)$ respectively to get enhanced amount of nitrogen, phosphorus and medium pH at 5.82. This medium was further supplemented with 0 – 200 mg/L tryptophan to study the combined effects of nitrogen source. The suspension culture was established with 100-150 mg of callus (4-weeks) or by cell biomass (2- weeks) inoculated @ 20% per culture vessel with 25 ml medium. The growth of cell biomass in suspension culture was recorded after 2-week of subculture.

**Table 3.** Effects of phenylalanine and nitrogen source (combination of diammonium hydrogen phosphate and ammonium dihydrogen orthophosphate) on biomass production alkaloid yield.

| Sl. No. | Phenylalanine Concentration | Biomass Fresh wt | Biomass Dry wt | Alkaloid Content | Alkaloid Production | Alkaloid Productivity | Alkaloid Yield |
|---|---|---|---|---|---|---|---|
| | mg/L | g/L | g/L | mg/g dwt | mg/L | mg/L/d | % dwt |
| 1 | 0.0 | 17.600 ± 0.080 | 1.942 ± 0.122 | 5.742 ± 0.247 | 11.174 ± 1.165 | 0.798 ± 0.083 | 0.574 ± 0.025 |
| 2 | 50 | 18.333 ± 0.122 | 2.106 ± 0.101 | 6.409 ± 0.160 | 13.502 ± 0.891 | 0.964 ± 0.064 | 0.641 ± 0.016 |
| 3 | 100 | 18.800 ± 0.120 | 2.317 ± 0.101 | 7.049 ± 0.248 | 16.346 ± 1.263 | 1.167 ± 0.090 | 0.705 ± 0.025 |
| 4 | 150 | 18.880 ± 0.120 | 2.499 ± 0.080 | 7.690 ± 0.204 | 19.229 ± 1.104 | 1.374 ± 0.079 | 0.769 ± 0.020 |
| 5 | 200 | 19.280 ± 0.160 | 2.593 ± 0.101 | 8.664 ± 0.248 | 22.478 ± 1.492 | 1.606 ± 0.107 | 0.866 ± 0.025 |
| F(4, 10) | | 44.971 | 21.064 | 76.585 | 42.022 | 41.911 | 77.094 |
| P<0.05 | | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

The Gamborgh's (B5) suspension medium [34] was supplemented with 3% sucrose (w/v) and modified with 0.50 mg/L of 2,4-D, 1.0 mg/L of Kinetin and 2.0 mg/L NAA. Additionally, the media was fortified with 35 ml and 65 ml of 1M stock solution each of (A) diammonium hydrogen phosphate $(NH_4)_2HPO_4$ and (B) ammonium dihydrogen orthophosphate $(NH_4H_2PO_4)$ respectively to get enhanced amount of nitrogen, phosphorurus and medium pH at 5.82. This medium was further supplemented with 0 – 200 mg/L phenylalanine to study the combined effects of nitrogen source. The suspension culture was established with 100-150 mg of callus (4-weeks) or by cell biomass (2-weeks) inoculated @ 20% per culture vessel with 25 ml medium. The growth of cell biomass in suspension culture was recorded after 2-week of subculture.

## 4. DISCUSSION

The growth of biomass and accumulation of products in cultured plant cells were dramatically altered by manipulating various media and environmental factors [38]. In the previous report enhanced production of biomass and alkaloid accumulation was optimized for the composition and strength of nutrient media, carbon source and plant growth regulators [31-33]. The normal or single strength B5 medium supplanted with 3% sucrose (w/v) was found as the best choice for the optimal biomass accumulation and production of secondary metabolite in cell suspension culture of *L. macranthoids* [39] and *C. roseus* [31-33]. Li et al [39] observed similar trends in biomass and chlorogenic acid production using 6-BA (2.0 mg/L) and NAA (0.5 mg/L) in B5 suspension culture of *Lonicera macranthoids*. In the present study production of biomass (fresh wt. and dry wt.) and alkaloid (content, production, productivity and yield) was investigated in response to increased supply of nitrogen (inorganic and organic) and phosphorus in suspension culture using B5 medium buffered at varying pH. The plant physiological process such as mineral nutrition, signaling, cell elongation, growth, development, environmental stress adaptation has been related to the pH of external media [40]. The pH of plant tissue culture media is mostly adjusted between 5 and 6 prior to the autoclaving which is dropped by 0.6 to 1.3 units after sterilization [41]. The cytosolic and vacuollar pH was increased by 3.0 and 1.3 units with a change in media pH from 4.5 to 6.3 in *Chenopodium rubrum* cell suspension culture [42] while the specific buffering potential was decreased continuously by 60% [40]. Production of biomass and withanolide A in *Withania somnifera* cell culture was not affected by media pH set at high or low. On the other hand *Daucus carota* cell culture excreted 90% of anthocyanin at 5.5 media pH than at 4.5 [43]. The release of secondary metabolites in to medium was due to cell membrane permeability associated with shift of media pH at low or high [44]. Reducing the media pH even for shorter time was found beneficial for up to 50% release of betalains from *Beta vulgaris* transformed root culture system [45]. The flavonolignan content in *Silybium marianum* hairy root culture was maximum at 5.0 medium pH and decreased with the increase of pH to 5.7, 6, and 7 [46]. The growth and ginsenoside production in hairy root culture of ginseng was maximum when the initial pH of the medium adjusted between 6.0 to 6.5 prior to autoclaving and significantly reduced at initial pH 4.0 or lower or above 7.0 [47]. Li et al [39] studied the effect of pH on the production biomass and chlorogenic acid in cell suspension cultures of *Lonicera macranthoids*. The highest biomass of 6.52 g/L and 6.76 g/L was achieved at 5.5 and 6.0 media pH respectively, showing maximum chlorogenic acid content. They found that production of biomass and secretion of chlorogenic acid was unaffected at higher or lower pH of the media, however, intracellular accumulation of acid was continued in medium at pH 7.0. Similar results were also observed in *E. ulmoides* cell suspension culture with maximum accumulation of chlorogenic acid at medium pH of 5.3 and continued production of chlorogenic acid up till the medium pH adjusted to 7.0 [48].

The inorganic and organic nutrients of culture media are easily manipulated for the enhanced and modified production of different chemical components of plant cells. In an earlier report Nowacki et al. [49] reported that the phosphate and nitrogen source provided by both ammonium di-hydrogen orthophosphate ($NH_4H_2PO_4$) and di-ammonium hydrogen phosphate ($(NH_4)_2HPO_4$) helps in increasing the alkaloid production by converting the excess amino acids produced to secondary metabolites. When different levels of fructose, galactose, glucose and sucrose carbon source were compared, the medium containing 3% sucrose showed maximum growth of cell biomass and the alkaloid yield [50]. In *L. aestivum* shoot culture, increased production of galanthamine was optimized applying different concentrations of sucrose, $NO_3^-$, $NH_4^+$, $PO_4$ ions [51]. Culture medium supplied with enhanced nitrogen levels and the ratio of $NH_4^+/NO_3^-$ (molar concentrations) showed to stimulate alkaloid synthesis. But the uptake of

nitrate from medium and assimilation of ammonia by plant cell decreased the medium pH [52]. The uptake of nutrients and buffering components ($NH_4^+$, $NO_3^-$, $PO_4^{3+}$) from the medium during initial growth phase or the acids (lactate, malate, succinate) secretion at the later stages in suspension culture caused change in hydrogen ion concentration affecting the secondary metabolite production [40; 53]. An optimum of 90 mM concentration of total nitrogen in 4:1 ratio of $NH_4^+/NO_3^-$ in hairy roots culture of *Anisodus acutangulus* showed highest yield of tropane alkaloid from the maximum biomass produced [50]. The hairy roots induced from leaf explants culture of *A. acutangulus* was significantly affected by variations in media pH. At pH 6.5 the hairy roots biomass increased two fold as compared to media with pH 4.5. Whereas, at pH 4.5 tropane alkaloid was produced with maximum yield [50]. Influence of external pH on alkaloid production and excretion by *C. roseus* resting cell suspensions reported [54]. In root cultures of *Brugmansia candida* production of scopolamine as well as hyoscyamine was increased at medium pH 5.5 adjusted with acetic acid inducing the release of these alkaloids [55]. While these alkaloids were reduced significantly at pH 3.5 and 4.5, but a medium pH of 4.5 and above showed significant release of scopolamine and hyoscyamine. A change in cell membrane permeability stimulated by acetic or citric acid might be the cause for the release of these two alkaloids [55].

Both tryptophan and phenylalanine at all the levels in B5 medium showed highly significant increase in all the parameters in comparison to the standard control conditions. The highest growth was observed at 200mg/l concentration of both the precursors across all parameters. A combination of the precursors along with the diammonium hydrogen phosphate and ammonium dihydrogen orthophosphate showed even higher response across all parameters. In conformity to the results of present study Whitmer et al. [56] reported that supplying tryptamine or tryptophan along with the iridoid precursors to transgenic cell lines S1 of *C. roseus* resulted in even further increase of alkaloid accumulation. Addition of a moderate concentration of reduced nitrogen source as L-glutamine followed by L-asparagine in medium also enhances the in vitro process of somatic embryo proliferation and maturation in *C. roseus* [57]. There was 2 fold increase in production of glucotropaeolin from *Tropaeolum majus* L. when phenylalanine and cystein fed as precursor amino acids in hairy root cultures [58]. Moreover, *Cistanche deserticola* cell culture fed with L- phenylalanine as precursor showed 75% higher phenylethanoid glycosides than the media without it [59]. On other hand, Namdeo et al. [60] reported that the key to successful protocol using precursor feeding to plant cell culture system lies in identification of cheapest by product of the other process which can be converted to desired secondary metabolites by selected plant cell line. Where as in *L. macranthoids* cell growth was inhibited by addition of phenylalanine at concentrations higher than 50 mg/L contrary to the content of chlorogenic acid which was gradually increased as compared to the control. The B5 medium added with 200 mg/L phenylalanine showed highest amount (18.0 mg/g DW) of chlorogenic acid production [39]. The total alkaloid of the *Fritillaria cirrhosa* cultures was significantly improved by adding different concentration of phenylalanine [61]. Similarly, soybean cell suspension cultures showed increased content of daidzein when medium was supplemented with phenylalanine for 48 h [62].

Nutrients and environment conditions such as potassium nutrition [63], nitrogen nutrition [12], and salinity [15] showed enhanced accumulation of alkaloids in *C. roseus*. Plants exposed to excess UV-B radiation and other physical or chemical factors [64-66] produce serious oxidative stress response to tolerate them. Similarly, *C. roseus* cell suspension cultures supplied with adequate nitrogen source showed enhanced alkaloid production under UV-B treatment [67]. The yield and accumulation of UV absorbing compounds in liverwort was found changing dynamically in response to UV-B and photosynthetic radiations to revert the inhibition due to UV-B radiation [68]. Malik et al. [69] also showed that an optimum pH of 5.5-6.5 enhanced *in vitro* shikonins production from various species like *Lithospermum, Arnebia, Alkanna,*

*Anchusa, Echium* and *Onosma*. Nitrogen is an important constituent of alkaloids and required for their synthesis while, phosphorus influence greatly the alkaloid synthesis in *C. roseus* L. [70]. Hassan et al. [71] showed enhanced alkaloid and other growth characteristics of *C. roseus* under the field application of nitrogen and potassium fertilizers. The highest yield of total alkaloid including the vincristine and vinblastine was obtained with maximum nitrogen but with lowest concentrations of potassium. Abdolzadeh et al. [72] found that feeding *C. roseus* plants with 11 mM of total nitrogen (nitrate+ammonia) significantly enhanced the vincristine, vinblastine and the total alkalods production with increased cellular pool of nitrogen, amino acids and proteins. Nitrogen deficiency showed reduction in total chlorophyll content [73; 74]. While highest plant biomass in *C. roseus* was reported at 200 mg N $dm^{-3}$ in the substrate [70] and the highest yield of alkaloids was obtained at 300 mg N $dm^{-3}$ [75].

Further, An et al. [76] and Zhang et al. [77] have pointed out that nitrate reductase-dependent nitric oxide signaling mediated the flavonoid accumulation in UV-B-induced plant leaves fed with nitrate. Adequate supply of nitrogen source showed significantly increased contents of $H_2O_2$ and MDA compared to control *C. roseus* plants indicating an enhanced tolerance to oxidative stress caused by UV-B radiation treatment [13]. The increased biosynthesis of different compounds with nitrogen, and alkaloids by extra nitrate supply might be involved in fighting the plants to various environmental and radiation stresses [13]. This hypothesis was further supported by the findings of unique catharanthine transporter as UV-B-induced signaling events under suspension cultures [67] and the involvement of ATP-binding cassette transporter with changing environmental conditions in *C. roseus* leaf surface cells [78].

Monnerat et al. [79] found that *C. roseus* plants supplied with nitrogen fertilizer in combinations with mycorrhizal fungi *C. etunicatum, G. margarita* and *R. intraradices,* showed increased production of ajmalicine. Hashemabadi et al. [80] reported enhanced level of leaf vindoline (1.94 mg/g DW) and root alkaloid (1.11 mg/g DW) and other plant characteristics by application of 40 mg $kg^{-1}$ soil nitrogen fertilizer along with *Azotobacter* and fungal compost. A positively significant effect of plant growth promoting rhizobacteria (PGPR) on the alkaloid content of *C. roseus* was reported recently [81-84]. Similarly, application of PGPR on the induction of secondary metabolite synthesis, particularly of alkaloids had been presented earlier [85].

Hence, all these studies demonstrated that both biotic and abiotic stresses have either inductive, stimulatory or enhancing effects on the production of alkaloids and other secondary metabolites under the field conditions as well as in cell culture system of *C. roseus* and many other plants by enhanced organic or inorganic nitrogen and phosphorus supply in a pH dependent manner similar to our present study.

## 5. CONCLUSION

The callus and cell suspension culture technique has proven as a feasible alternative for enhancing the production of alkaloids and other secondary metabolite. Since, *C. roseus* are categorized as high nitrogen demanding plants which tolerate wide range of soil nutrients, salinity and pH. Plants supplied with balanced level of essential nutrients have shown increased crop growth, biomass and yield of the various secondary metabolites. Applying similar strategies in cell suspension culture system would have positive effects on biomass and alkaloid accumulation. We found that B5 medium supplemented with enhanced levels of nitrogen and phosphorus as a combination of **(A)** diammonium hydrogen phosphate **$(NH_4)_2HPO_4$** and **(B)** ammonium dihydrogen orthophosphate (**$NH_4H_2PO_4$**) significantly affected all the observable parameters such as fresh and dry weight of cell biomass as well as alkaloid content, production, productivity and the yield in a pH dependent manner. The medium pH of 5.82 showed the maximum response of biomass and alkaloid yield while the lower and higher pH of the medium

were slightly inhibitory compared to the control cultures established in MS medium. The enhanced alkaloid biosynthesis in response to increased total nitrogen ($NH_4^+$+$NO_3^-$) and phosphate at an adequate pH of test culture medium might caused by uptake and transport of these nutrients increasing the intracellular pool of nitrogen containing compounds and the intermediates of the pathway as supported by various published reports reffered in the present study. Addition of tryptophan or phenylalanine as reduced nitrogen source in B5 medium buffered at 5.82 ± 0.2 pH further enhanced the biomass and alkaloid production. There might be involvement of more complex interactions mechanisms of pH, phosphate, organic and inorganic nitrogen as nutrient combined with other stresses to trigger the induction of alkaloid biosynthesis. There is need for further research to elucidate the mediation of molecular regulation and signaling mechanisms involved in the pathways leading to enhanced production of specific alkaloids.

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**Contribution of authors**

All the experiments were executed and performed by Mr. Malay Ranjan Mishra. The second author Dr. Rajesh K. Srivastava constantly reviewed the experiments and results of various experiments. The experiment design and planning for execution of entire study was conducted by the corresponding author Dr. Nasim Akhtar to achieve the objective of the major research project sanctioned to him by the funding agency University Grant Commission, New Delhi (F.: 42-207/2013 (SR) for the period 1.4.2013-31.3.2017).

**Conflicts of Interest**

All the authors declared that there is no conflict of interest with regards to any part of the manuscript.

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# Antioxidant Activities, Phenolic Contents and Electronic Nose Analysis of Black Garlic

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**Abstract**: Black garlic is a processed garlic product with a moisture-controlled high temperature heat treatment for a long time. In order to determine the secondary metabolites of black garlics treated in the study, firstly, in vitro antioxidant activities of black garlics purchased from Edovital company, Kastamonu, Turkey were determined, followed by qualitative and quantitative measurement of the phenolic compound content by HPLC and finally the electronic nose analysis of the content of nebulizer vapors in wood vinegar extract of black garlics were done successfully. Chlorogenic acid, vanillic acid, benzoic acid, gallic acid contents in detected 13 phenolic acids were quitely high. All quantitative results were expressed as mg gallic acid equivalent (GAE) per g dry matter of black garlic sample. ABTS and DPPH antioxidant activities were very low according to BHT standart and 2-Methylene-4-pentenal (18%) and Furfural (25%) were detected in high amount with electronic nose in nebulvapor contents of black garlic wood vinegar extract.

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## 1. INTRODUCTION

Black garlic is a processed state of fresh garlic under high temperature and humidity by the Maillard reaction. D,L-lactic acid, 5-hydroxymethyl-2-furfural, adenosine, uridine, (1S,3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, (1R,3S)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, and 2-acetylpyrrole were detected in black garlic ethyl acetate extracts with HPLC coupled with diode array detection analysis and NMR spectrometry [1]. Black garlic has many health-promoting properties: effective in the treatment of colon cancer, effective in the treatment of diabetes, having anti-allergic, anti-inflammatory, antioxidant, anticancer and cardiac protective effects [1-5]. Processed black garlic components are polysaccharides, reducing sugars, proteins, phenolic compounds, organic sulfur compounds and melanoidins [6]. The antioxidant capacity of black garlic is related to polyphenols [7]. Black garlic can prevent the development of atherosclerosis by clearing cholesterol [8]. In black garlic, the content of allicin, which gives a harsh aroma taste, is reduced and allisin in the browning process is transformed into antioxidant compounds such as bioactive alkaloid and

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flavonoid compounds [9]. Black garlic have been consumed in extracted form as well as being used widely as an food ingredient including beverages, candy due to sour taste of it [10]. Black garlic whose main volatiling components are organosulfur compounds such as thiosulfinates and sulfur [11]. High antioxidant activity of black garlic have been observed with S-allyl cystein (watersoluble compound) increases by fermentation [12]. In one study, as the concentration of black garlic increased, the DPPH radical clearing ability increased due to increased polyphenol content of treated black garlic [13]. In other study, ABTS scavenging activity was the highest in aged black garlic extract [14]. The aim of this study is finding out of phenolic and antioxidant profile of black garlic. In addition to this, with different method from literature, wood vinegar extract of black garlic converted into vapor phase by nebulizer and these vapors were analyzed to determine of content with electronic nose.

## 2. MATERIAL AND METHODS

### 2.1. Procurement of Plant Material and Extraction Process

Fermentable black Garlics (200 g, stock code: HBV000002R8CH, from Taskopru) were purchased Edovital Company, Kastamonu, Turkey in May, 2018. For extraction of dry black garlic sample powders (30 g, powdered via a blender (Model SHB 3062; Sinbo, Istanbul, Turkey)) were used methanol (300 mL) solvent with shaker for 24 h at room temperature. The methanolic extracts of the black garlic were filtered and collected into flasks, before they were dried with rotary evaporator stored at +4 °C in refrigerator (Bosch, Germany) until use [15].

### 2.2. DPPH (1,1-diphenyl-2-picrylhydrazyl) Radical Scavenging Activity

DPPH radical scavenging ability was determined with slight modifications of method of Zhang et al. (2011) at 517 nm by using a spectrophotometer [16]. 1 mL of 0.1mM DPPH radical (CAS Number 1898-66-4, 1 g, Sigma-Aldrich) solution prepared in methanol was mixed with 1 mL of the test sample (2 mg / mL, methanolic black garlic extract at 2 h 6000 rpm (Nuve laboratory technology)) dissolved in 100 mM acetate buffer (pH 7.2). The mixture was shaken and left to stand for 20 min in the dark until spectrophotometric measurement (with double beam UV-Vis spectrophotometer, Schimadzu (190 nm-1100 nm)) and butylated hydroxy toluene (BHT) (≥ 99%, Sigma-Aldrich) was used as a positive control.

The half inhibitory concentration ($IC_{50}$) (represents the concentration that caused a 50% inhibition of radical formation) value was used to express the results [16]. $IC_{50}$ value (Table 1) was obtained by using DPPH inhibition graph.

### 2.3. FRAP (Ferric Reducing Antioxidant Power) Activity

FRAP assay (is based on electron transfer) was performed by following the method described by Benzie and Straine (1996) [17], with soft modifications (FRAP reagent consist on mixing acetate buffer at pH 3.6, 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) (≥ 99.0%, HPLC grade, Merck) acidic solution and 20 mM $FeCl_3.6H_2O$ (97%, 5 g, Sigma-aldrich, CAS Number: 10025-77-1)). The reaction mixture (FRAP reagent and black garlic methanolic extract (2 mg / mL) (3:1)) was then incubated at 37 °C for 4 min (Nuve laboratory technology, BM 30 water bath). Absorbance was determined at 595 nm against a blank prepared using distilled water. The result was given as the average Trolox equivalent (TEAC) of 3 repeat measurements [17] (Table 1).

### 2.4. CUPRAC (Cupric Ion Reducing Antioxidant Capacity) Assay

The chromogenic redox reagent, bis (neocuproine) copper (II) chelate for the CUPRAC assay produced a stable and colorful Cu (I)- neocuproine chelate as a result of the redox reaction with the polyphenols at pH 7. The absorbance of the color was measured at 450 nm. Antioxidant activity was calculated as the average Trolox equivalent (TEAC) of 3 repeat measurements.

Black garlic methanolic extract (2 mg/mL) and CUPRAC reagent (1:1) were mixed and measured in 1 min [18] (Table 1).

### 2.5. ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) Radical Scavenging Activity

The determination of ABTS radicalic activity was based on the mechanism of Re et al. (1999) [19]. The stock solution of ABTS was mixed in dark bottle by dissolving 0.250 g of ABTS salt and 0.045 g $K_2S_2O_8$ in 100 mL of ultra pure water, then it was left in the fridge. The prepared ABTS solution was diluted with ultra pure water by using Milli-Q® IQ 7003/7005 Ultrapure Lab Water System (Merck) and before use it was kept at 25°C in darkness for 24 h. To measure the radical scavenging ability 0.05 mL of the black garlic methanolic extract (2 mg / mL) was added to 2.5 mL of a diluted ABTS solution. Absorbance was determined after 5 min incubation at 734 nm against water as blank. Butylated hydroxy toluene (BHT) (≥ 99%, Sigma-Aldrich) was used as a positive control. The rate of 50% inhibition ($IC_{50}$) was calculated [19] (Table 1).

**Table 1.** Antioxidant activity comparison of black garlic methanolic extract according to four different methods.

| DPPH ($IC_{50}$) (mg / mL) | ABTS ($IC_{50}$) (mg / mL) | FRAP (TEAC) (µM / methanolic extract (mg)) | CUPRAC (TEAC) (µM / methanolic extract (mg)) |
|---|---|---|---|
| 0.18±0.02 | 0.28±0.03 | 42.16±0.04 | 56.18±0.02 |

±SD: Average Standart Deviation, 95 % confidence interval, critical ratio: $p<0.05$

### 2.6. Phenolic Analysis with HPLC

RP-HPLC-DAD analysis system (Agilent 1100 Technologies, Waldbronn, Germany)of phenolic compounds was combined by using a purospher star reverse phase column (4.6 × 250 mm, 5 µm) (Merck, Germany), on a isocratic program with a solvent system (A: 2% formic acid in methanol:water [1:1]) at a constant solvent flow rate of 0.7 mL.min$^{-1}$ Injection volume was 50 µL and analysis time was 40 min. Signals were detected at 235, 240, 253, 265, 280, 295, 312, 345 nm by DAD and at 280 nm (For the phenolic compounds, maximum absorption) by UV detection. Column temperature has been set at room temperature, 25°C. 13 phenolic components were identified by comparing to standards in black garlic methanolic extract (2 mg / mL), qualitatively and quantitatively [20] (Figure 1) (Table 2).

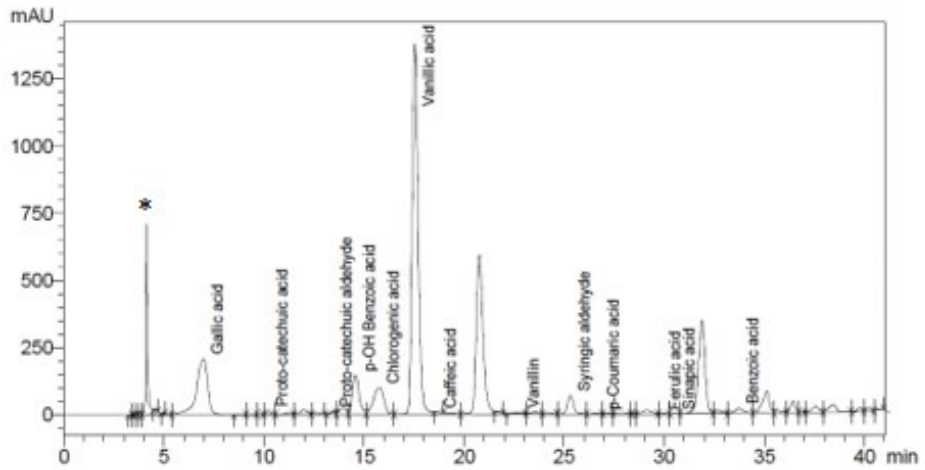


**Figure 1.** HPLC peak chromatogram of black garlic methanolic extract at 280 nm

**Table 2.** Detected qualitative and quantitative phenolic components of black garlic methanolic extract with HPLC at 280 nm (±SD: Average Standart Deviation, 95% confidence interval, critical ratio: $p<0.05$)

| Number | Phenolic Component Name | Retention Time | % Area | Concentration (mg / L) |
|---|---|---|---|---|
| * | Unknown | 4.15 | 0.01 | 0.1±0.01 |
| 1 | Gallic acid | 6.98 | 18.32 | 172.14±0.03 |
| 2 | Proto-catechuic acid | 10.22 | 0.49 | 4.89±0.02 |
| 3 | Proto-catechuic aldehyde | 13.41 | 0.20 | 1.11±0.04 |
| 4 | *p*-OH Benzoic acid | 14.57 | 7.48 | 94.62±0.02 |
| 5 | Chlorogenic acid | 15.77 | 7.69 | 183.08±0.03 |
| 6 | Vanillic acid | 17.54 | 60.97 | 750.95±0.01 |
| 7 | Caffeic acid | 18.63 | 0.07 | 0.92±0.03 |
| 8 | Vanillin | 22.77 | 0.36 | 2.88±0.01 |
| 9 | Syringic aldehyde | 25.31 | 3.10 | 95.27±0.03 |
| 10 | *p*-Coumaric acid | 26.76 | 0.16 | 1.50±0.01 |
| 11 | Ferulic acid | 29.84 | 0.06 | 1.24±0.04 |
| 12 | Sinapic acid | 30.53 | 0.01 | 0.09±0.01 |
| 13 | Benzoic acid | 33.73 | 1.10 | 223.72±0.03 |

## 2.7. Electronic Nose Analysis of Nebulizer Vapors of Black Garlic Wood Vinegar Extraction Oils

Wood vinegar (250 mL), which is a kind of dark brown liquid produced by slow pyrolysis of plant biomass, was purchased from Tu Hong Biotech Co., Ltd, Hebei, China (Mainland) and was used for clevenger distillated extraction (Sesim Kimya Laboratuvar, Ankara, Turkey) method of black garlic (30 g) powdered via a blender (Model SHB 3062; Sinbo, Istanbul, Turkey). Collected extraction oils of black garlic were placed in the chamber of nebulizer device (particle size <4 µm, Bayer company, Germany). Components analysis of obtained nebulous vapors were realized with electronic nose (PERES foodsniffer (Swiss Technology)) including volatile organic compound sensors combined smartphone (Samsung Galaxy S5 (Seoul, South Korea)) network library in a short time (120 second) [21, 22] (Figure 2).

## 2.8. Statistical analysis

All qualitative and quantitative statistical analysis were reported significantly ($p< 0.05$) with average standard deviation of 3 repeated measurements. Statistical analysis was carried out by using SPSS Version 21.0 software program and Microsoft Excel (Microsoft Office Corporation, 2010, Redmond, Washington).

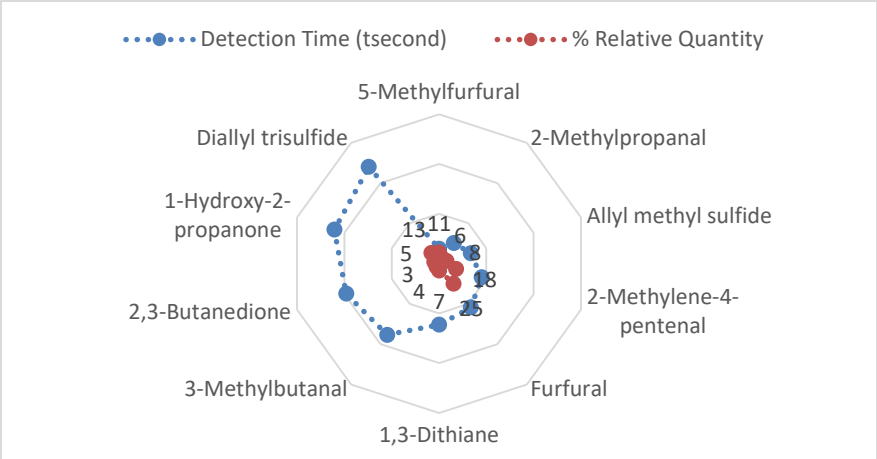


**Figure 2.** Volatile organic compounds detected with electronic nose

## 3. RESULTS and DISCUSSION

In one study, DPPH radical clearance of black garlic ethyl acetate extract was 30 % and was equivalent to 5 mg / mL gallic acid [1]. In another study, black garlic contained many organic acids occurring in nature. Lactic acid was the main organic acid in the black garlic detected in liquid analysis [23]. Therefore, lactic acid may be responsible for unique taste. Total phenolic content in black garlic was increased by about four to 10 times compared to white garlic. Hydroxycinnamic acid derivatives were found to be the major phenolic acids of black garlic at different processing stages [24].

In the other study, malondialdehyde content in black garlic groups at 20 and 40 mg / kg animal study doses significantly was reduced ($p$ <0.05). In addition to this, Serum superoxide dismutase activities which are showing the effectiveness of antioxidant system in black garlic groups at 20 and 40 mg / kg animal study doses were significantly higher ($p$ >0.05) [25]. Table 1 antioxidant activity data in this study support this. On the other hand, in volatile components analysis of black garlic, the main sulfur volatiles in the black garlic exhibited an inverse behavior throughout the heating. While the concentration on plant volatiles decreased throughout the warming, the volatile and roasted aroma volatile concentrations increased [26]. In the HPLC profile analysis of free, soluble esters and glycosylated phenolic acids, trans-hydroxycinnamic acids (caffeic, *p*-coumaric, ferulic and sinapic) in garlic were shown to be twice as high as onions [27]. In this fence, because of the fact that contents of phenolic acids and trans-hydroxycinnamic acids of black garlic were valuable for responsible about antioxidant activity, HPLC phenolic peak profiles were examined in this study.

Electronic nose method is a new trend for volatile content analysis as an alternative to GC-MS. For showing heat process effect on garlics, in a study, electronic nose was used [28]. The electronic nose's (have six sensors) spider radar graph analysis values showed that raw and heat-treated garlic's odour component characteristics were different [28].

In this study, ABTS and DPPH with CUPRAC and FRAP antioxidant activity results of black garlic methanolic extract supported each other in addition to this, DPPH activity was more effective to show the radical scavenging capacity of high antioxidative components than ABTS with increasing percentage of negative slope and CUPRAC activity was also more effective than FRAP about black garlic methanolic extract. Because copper ions were more effective linked to phenolic content from iron ions according to their reduction potentials about antioxidant capacity with positive slope in proportion to the increasing spectrophotometric color intensity (Table 1).

In a study the FRAP value in Taşköprü black garlic (640.76±86.98 $\mu mol.g^{-1}$) slightly decreased from FRAP value of black garlic in Chinese type (678.20±77.56 $\mu mol.g^{-1}$) [29] but in this study, FRAP value of black garlic (42.16±0.04 µM/mg) was found and this value was quitely high per gram (Table 1). In the other study, The $IC_{50}$ value of polyphenol extracted from black garlic for DPPH· radical inhibition was 140.79 µg/mL [25] but in this study, the $IC_{50}$ value of black garlic methanolic extract for DPPH· radical inhibition was found as 180 µg/mL (Table 1). The reason for this is that the total content of black garlic including flavonoid and sugar groups decreases the antioxidant activity. In an another study, n-hexane, dichloromethane, ethyl acetate, n-butanol and water extracts of black garlic in different polarities were used to isolate the active ingredients (total polyphenol content) [30]. In the DPPH·radical scavenging and iron reducing power antioxidant activity tests, the aqueous extract of black garlic was more effective [30]. Therefore, the methanolic extract form closest to the polarity of the water was preferred in this study. In the same study, DPPH· radical scavenging activity of the aquatical extract of black garlic was high at a concentration of 0.6 mg / mL and concentrations below 0.2 mg/mL were not studied [30]. The DPPH· radical scavenging activity of black garlic was measured as 0.18±0.02 in this study (Table 1), with the

idea that different solvent extracts (such as methanol) may exhibit better activity if these concentrations are studied. ABTS radical scavenging activity was higher in hexane, chloroform and ethyl acetate fractions of black garlic and reducing power was also significantly lower in butanol and water fraction (its polarity is close to methanol) [31] as opposite that ABTS radical scavenging activity was high in methanolic extract of black garlic in this study (Table 1).

While in a study, in phenolic analysis with HPLC, coumaric acid was the main ingredient in black garlic residue [32], in this study, in phenolic analysis with HPLC, from detected 13 components at 280 nm, vanillic acid (60,97 % peak area) was in the foreground as main flavor component of black garlic. Gallic acid (18,32 % peak area), chlorogenic acid (7,69 % peak area) and *p*-OH Benzoic acid (7,48 % peak area) with vanillic acid (60,97 % peak area) formed the major components of total phenolic content (Table 2) (Figure 1).

In the HPLC analysis of methanolic extract of black garlic, it is estimated by considering retention time and uv spectrum that the unknown component with asterisk is vitamin C (Figure 1) (Table 2). 2-Methylene-4-pentenal (18 %), furfural (25 %), diallyl trisulfide (13 %), 5-methylfurfural (11 %) were detected as volatile major organic compounds from 10 components with electronic nose (Figure 2). In other studies, for fermented black garlic according to purple garlic, there was no difference in the amount of ferulic acid, fernented black garlic had high amounts of coumaric acid and caffeic acid and chlorogenic acid decreased significantly [33]. In this study, as opposed to other studies [33], chlorogenic acid was quitely high (Table 2). In other studies, in black garlic, volatile compounds of allyl alcohol and S-alk(en)-yl-L-cysteine derivatives were strongly high and furfural rate was approximetly 4 % of total area of volatile compounds [33] but in this study, furfural compound and its derivatives were strongly high (Figure 2).

## 4. CONCLUSION

Black garlic can be a strong source of antioxidants and phenolic acids and can be used as an alternative fermented food product against oxidative stress. It can also be an aromatic food source as a component of flavor and odor. Most susceptible vitamins especially like ascorbic acid responsible for antioxidant activity can be lost during the heating process for fermented black garlic [33]. In the study, it was found that black garlic had high phenolic acid content especially like vanillic acid (60,97 % peak area) which have been responsible for sweet aroma from detected 13 components with HPLC-DAD-UV analysis and great antioxidant properties of black garlic were supported by 4 different methods (CUPRAC, FRAP, DPPH, ABTS). The results were statistically significant ($p < 0.05$) (Table 1 and Table 2). Electronic Nose Analysis of Nebulizer Vapors of Black Garlic Wood Vinegar Extraction Oils was applied firstly, in literature. Wood vinegar extraction increased the formation and involvement of furfural derivative compounds (furfural (25 %), 5-methylfurfural (11 %)) in oil phase (Figure 2). Thus, compounds which have low molecular weight and can easily fly were better detected with electronic nose combined nebulizer.

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# Adventitious roots formation for enhanced and sustainable production of antioxidants in *Brassica oleracea* var. acephala (Brassicaceae)

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**Abstract**: *Brassica oleracea* var. *acephala* is listed as the healthiest vegetable due to its high valued secondary metabolites content and antioxidant potential. This study was conducted to establish adventitious roots (ARs) culture as an alternative and feasible production of antioxidant secondary metabolites. ARs were induced from cotyledon explants in commercially available Murashige and Skoog (MS) plant nutrient media, gelled with 0.8% phyto-agar and supplemented with different concentration (0.1−1.5 mg·L-1) of auxins (α-Naphthalene acetic acid; NAA, or Indole acetic acid; IAA, or Indole-3-butyric acid; IBA). AR formation responses in MS media at varying concentrations (0− 50 g·L-1) of sucrose and initial media pH (4, 5.0, 5.8, 7 & 8) were also studied. The bioprocessing of ARs were studied in liquid MS media containing NAA (1.5 mg·L-1) as growth regulator. The growth curve, important antioxidants (phenols & flavonoids), and free radical scavenging potential of ARs were studied for a period of 9-weeks. The ARs at stationary phase (7-week) attained highest accumulation of phenols and flavonoids, which ultimately showed the highest reactive species scavenging potential. This study provides the base for production of *B. oleraceae* var. *acephala* secondary metabolites on large scale to strengthen the bio-based economy of developing world.

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## 1. INTRODUCTION

Roots are biosynthetic factories of nutritionally and pharmaceutically important metabolites such as alkaloids, phenols, polyacetylenes, sesquiterpenes and napthoquinones [1]. To render this potential adventious roots (ARs) culture is the promising alternative for large scale production. It is advantageous over cell, microbial and hairy roots cultures as it has fast multiplication rate, resistant to shear-stress, genetically stable, non-GMO, and easily scalable [2]. Unlike opines, toxic chemicals production in hairy roots culture makes it ideal and acceptable to the consumers [3,4].

*Brassica oleracea* var. *acephala* belonging to the family *Brassicaceae* (mustard family) is economically important due to its edibility, fodder and condiment usages and oil content [5, 6]. Traditionally, this plant has been utilized as a vegetable [7, 8], ornamental plant [9] and a

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medicinal plant. Locally in Kashmir, it is treated as an important foliage herbaceous plant, is used as vegetable and taxonomically it is the oldest form of cabbage [10]. In traditional medicine the intact plant has been reported for blister formation against inflammation and warts [11] and its juices were used to relieve bronchitis, chronic cough and asthma [12]. It has also been utilized for the treatment of cardiovascular diseases and carcinomas of colon, rectum and stomach [13, 14]. These qualities are linked to its metabolites profile which varies in quality and quantity due to specie type, plant part and age, and other agronomic factors (e.g. environmental and geographic location) [15, 11]. To overcome these constraints; in-vitro cultures have been selected as an attractive, rapid and reproducible method for production of specific metabolites in bulk scale [16].

The multiple utilization practices of this plant have made it ideal for in vitro cultures establishment. In this study, we established adventious root culture system of *Brassica oleracea* var. *acephala* and investigated the effects of different auxins, sucrose concentrations, and pH strength on ARs formation from cotyledon explants. Furthermore, the content of phenolic, flavonoids, and antioxidant potential in the bioprocessed roots were evaluated.

## 2. MATERIAL and METHODS

### 2.1. Explant source and adventitious root induction

The seeds of Brassica oleracea var. acephala were obtained from at Quaid-I-Azam University Islamabad, Pakistan. The surfaces of seeds were sterilized and then inoculated on Murashige and Skoog (1962) solid medium containing 30 $g \cdot L^{-1}$ sucrose and 8 $g \cdot L^{-1}$ agar [17].

For adventitious root induction, cotyledon and internode explants from 20 days old seed derived plantlet were inoculated on MS media supplemented with various concentrations (0.1, 0.5, 1.0 and 1.5 $mg \cdot L^{-1}$) of Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA) and α-naphthalene acetic acid (NAA) each. Additionally, the explants were cultured on MS media supplemented with varying sucrose concentrations (0, 20, 30, 40, 50, 60 and 70 $g \cdot L^{-1}$) and different pH levels (4, 5.0, 5.8, 7 and 8). The data were recorded in terms of percent root induction response, number of roots induced per explant, and roots fresh and dry weight (DW) after 4 weeks of culture.

All cultures were incubated in plant growth room, where room temperature (25 ± 1°C) and humidity (70%) were kept in control. The pH of culture media was maintained at 5.8 before autoclaving. The 16h photoperiod with light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was maintained in a growth room.

### 2.2. Submerged cultivation of adventitious roots

The 4-weeks old viable adventitious roots were aseptically excised from leaf explant and inoculated into MS medium, devoid of agar. For roots multiplication medium was supplemented with 1.0 $mg \cdot L^{-1}$ NAA and 30 $g \cdot L^{-1}$ sucrose. To ensure proper aeration, ~0.5 g ARs were cultured in 250 ml Erlenmeyer (conical) flask. The culture conditions in shaking incubator were set as 24h dark, 110 rpm and 25˚C. The adventitious roots from shake flask were sampled after each week of culturing for a total 9-weeks of period, and biomass and secondary metabolites accumulation were measured with the time course.

### 2.3. Phytochemical Analysis

The increase in fresh weight (FW) and dry weight (DW), and residual media electrical conductivity (EC) were measured according to Baque et al [18]. The antioxidants, phenols and flavonoids content in ARs were measured according to Ali & Abbasi [19], and Tariq et al [20] methods. For antioxidant activity 1-diphenyl-2-picrylhydrazyl (DPPH) was used as free radical producer and its scavenging potential in ARs were measured according to Abbasi et al [21].

### 2.4. Statistical analysis

All experiments were carried out in triplicate and were repeated two times. Mean values of the experimented data sets were analysed for variance and significance using Duncan's Multiple Range Test (DMRT) on Statistix (8.1) software. For graphical presentation OriginPro (8.5) was used and error bars represent standard error (SE).

### 3.1. Adventitious root induction

Adventitious root cultures of *Brassica oleracea* var. *acephala* were established in four steps i.e. seed germination, aseptic transfer of cotyledon explant to medium, adventitious root induction and submerged cultivation in shake flasks (Fig 1).

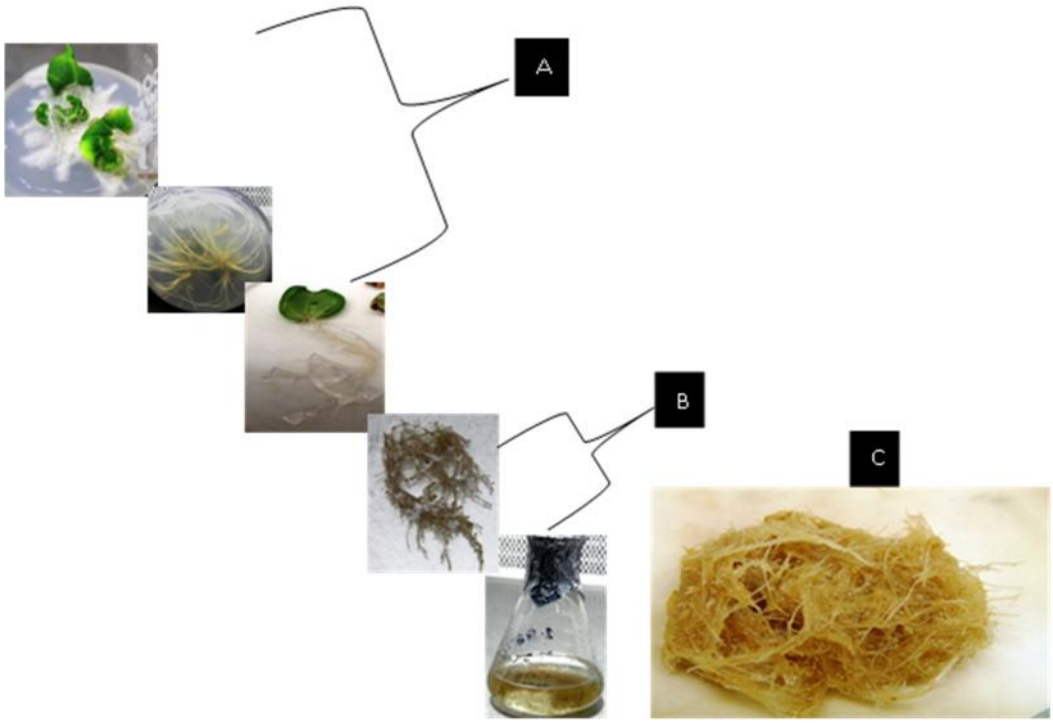


**Figure 1.** Adventitious root culture establishment **A)** Induction and elongation of adventitious roots **B)** Submerged cultivation, and **C)** Fresh Biomass.

Cotyledon explants cultured on to MS basal media supplemented with various amounts (0.1, 0.5, 1.0 and 1.5 mg·$L^{-1}$) of auxins; NAA, IBA or IAA failed to produce shoots and resulted in induction of the primary adventitious roots at the cut ends in varying frequency (Table 1). However, internode explants induced shorter adventitious roots with maximum frequency (34%) in response to 1.0 mg $L^{-1}$ NAA. The cotyledon explants inoculated on MS medium containing 0.5 mg·$L^{-1}$ NAA resulted into highest adventitious root induction frequency (87%) and maximum number of roots per explant (35), fresh weight (3.0 g) and dry weight (0.26 g). In response to 1.5 mg·$L^{-1}$ of IAA, maximum (70.7%) adventitious roots induction was observed. Different concentrations of IBA resulted into significantly lower biomass accumulation and rooting induction frequency.

**Table 1.** Auxin type and concentration effect adventitious root formation in cotyledon explant of *B. oleracea* var. *acephala*.

| Treatment (mg l-1) | Rooting frequency (%) | Number of roots | Fresh Weight (g/culture) | Dry Weight (g/culture) |
|---|---|---|---|---|
| $MS^0$ | ND× | ND | ND | ND |
| NAA 0.1 | 68.7 ± 2.96bc* | 14.3 ± 1.97c | 1.96 ± 0.034c | 0.150 ± 0.017abc |
| 0.5 | 87.3 ± 1.45a | 35.06 ± 2.31a | 2.96 ± 0.035a | 0.232 ± 0.088a |
| 1.0 | 79.0 ± 1.53ab | 24.33 ± 2.62b | 2.76 ± 0.038a | 0.239 ± 0.007a |
| 1.5 | 76.7 ± 2.60ab | 26.90 ± 1.07ab | 2.36 ± 0.037b | 0.199 ± 0.006ab |
| IAA 0.1 | ND | ND | ND | ND |
| 0.5 | 20.7 ± 2.33d | 2.00 ± 1.15d | 1.09 ± 0.042d | 0.088 ± 0.003abc |
| 1.0 | 32.7 ± 2.03d | 4.53 ± 1.47d | 1.15 ± 0.052d | 0.094 ± 0.004bc |
| 1.5 | 70.7 ± 1.76b | 7.33 ± 1.05d | 1.29 ± 0.058d | 0.136 ± 0.011abc |
| IBA 0.1 | ND | ND | ND | ND |
| 0.5 | 27.30 ± 2.34d | 1.60 ± 0.92d | 1.05 ± 0.028d | 0.063 ± 0.005bc |
| 1.0 | 31.23 ± 2.69d | 1.46 ± 0.41d | 1.053 ± 0.030d | 0.086 ± 0.008abc |
| 1.5 | 56.50 ± 2.18c | 3.59 ± 1.30d | 1.18 ± 0.056d | 0.010 ± 0.002bc |

Data values represent mean ± SE of three replicates.
*different alphabets in columns notes the significant difference at P<0.01
×ND: Not Detected

Significant variation in biomass accumulation was observed in response to different concentrations of sucrose while keeping the NAA concentration to 1.0 $mg \cdot L^{-1}$. MS medium supplemented with 40 $g \cdot L^{-1}$ sucrose was found to display maximum values for adventitious root induction frequency (86%), number of roots per explant (16.2) and fresh weight (2.3 g/culture) and dry weight (0.12 g/culture) (Table 2). Further increase in sucrose concentration beyond 40 $g \cdot L^{-1}$ significantly reduced adventitious root induction frequency.

**Table 2.** The effects of different sucrose concentrations in MS medium on induction of adventitious roots from cotyledon explant.

| Sucrose Concentration ($g\ L^{-1}$) | Rooting frequency (%) | Number of roots | Fresh weight (g/culture) | Dry weight (g/culture) |
|---|---|---|---|---|
| 0 | ND | ND | ND | ND |
| 20 | 40.64 ± 2.4c | 3.33 ± 1.3c | 0.81 ± 0.1b | 0.05 ± 0.012a |
| 30 | 59.90± 1.3b | 7.33 ± 1.9bc | 1.73 ± 0.4ab | 0.09 ± 0.023a |
| 40 | 86.67 ± 1.5a | 16.30 ± 1.5a | 2.29 ± 0.2a | 0.12 ± 0.034a |
| 50 | 77.80 ± 2.7a | 10.96±2.5ab | 1.42 ± 0.2ab | 0.06 ± 0.025a |
| 60 | 51.15±2.3bc | 4.59 ± 0.9c | 0.70 ± 0.3b | 0.04 ± 0.003a |
| 70 | 25.74 ± 1.9d | 2.67 ± 1.8c | 0.50 ± 0.1b | 0.03 ± 0.007a |

Data values represent mean ± SE of triplicates.
*ND; not detected
*values annotated with different alphabets are significant at P<0.01

Among different levels of pH tested, while keeping sucrose 30 $gL^{-1}$ and NAA 0.5 $mgL^{-1}$ constant, highest adventitious root induction frequency (84.6%) with maximum number of roots per explant (21.04) and fresh weight (2.5 g/culture) and 0.24 g dry weight (0.24 g/culture) were recorded in response to pH 5.8. Significant decrease in adventitious rooting was observed at all other pH levels compared to 5.8 (Table 3).

**Table 3.** The effects of hydrogen ion concentration (pH) on formation of adventitious roots from cotyledon explant.

| Hydrogen ion concentration (pH) | Rooting Frequency (%) | Number of Roots | Fresh Weight (g/culture) | Dry Weight (g/culture) |
|---|---|---|---|---|
| 4 | 52.21 ± 2.78c | 8.25 ± 2.03bc | 0.98 ± 0.13b | 0.07 ± 0.01a |
| 5 | 70.66 ± 1.503b | 10.05 ± 2.07b | 1.19 ± 0.11ab | 0.18 ± 0.08a |
| 5.8 | 84.63 ± 2.04a | 21.04 ± 2.77a | 2.50 ± 0.29a | 0.24 ± 0.07a |
| 7 | 47.49 ± 2.09c | 5.28 ± 1.79bc | 0.82 ± 0.35b | 0.06 ± 0.02a |
| 8 | 22.04 ± 2.65d | 1.81 ± 1.06c | 0.45 ± 0.26b | 0.02 ± 0.004a |

*values are means ± standard error of three replicates and different alphabets denotes significance at P<0.01

## 3.2. Adventitious root culture

Biomass formation of the adventitious root culture in suspension culture of *Brassica. olerace* var. *acephala* showed a swift inclined in growth curve. This inclined was characterized by an initial lag phase of 7 days for fresh and dry weight, followed by log phase of 35 days and a subsequent stationary phase during 63 days period of culture (Fig 2). Maximum fresh weight (FW) and dry weight (DW) of 3.19±0.044 and 0.338±0.006 g/culture, respectively, were observed on the 49th day of culture. A highest fresh weight (FW), ~6-times than the initial inoculum weight (0.5 g) of ARs were attained in shake flask bioreactor. This increase in roots FW was also characterized by steady decrease in culture volume with time, and was linked with the nutrients and water ingestion by roots for growth and biomass accumulation.

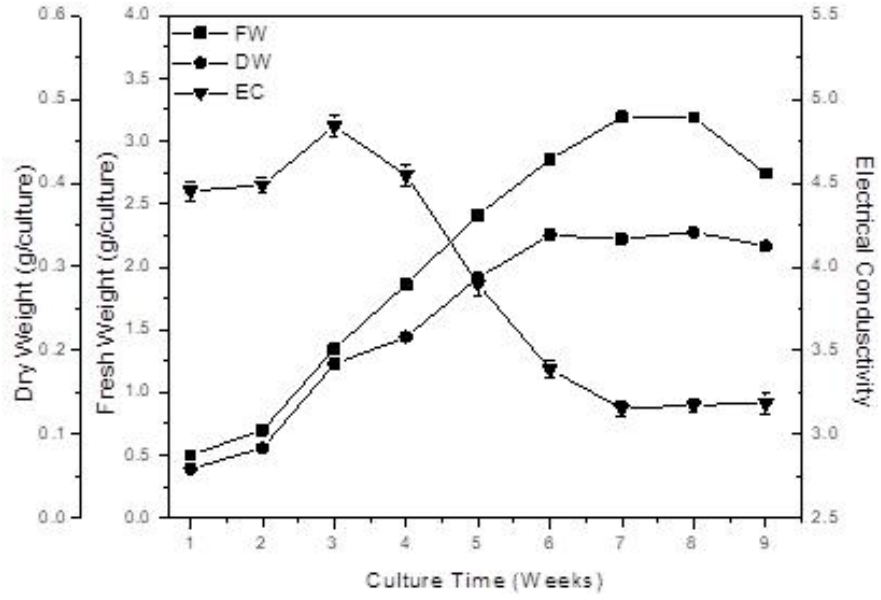


**Figure 3.** Total phenolic content (TPC) and total flavonoid content (TFC) with respect to dry weight (DW) accumulation.

The electrical conductivity (EC) of medium showed a gradual decline which can be linked with the nutrients consumption by the growing roots. This decrease was characterized by an initially increase and might be explained from an assumption that roots excrete metabolites for its adjustment to the new environment. The increase in biomass accumulation and decline in EC of exhausted MS media are associated with the nutrients uptake ($PO4^-$, $NH3^+$, $NO3^-$, etc.) from media by the ARs [24].

In the present study, the overall pattern of total phenolic content and total flavonoid content accumulation in adventitious root cultures displayed a growth-dependent pattern. TPC and TFC detected in 1 week old cultures were 3.39 $mg \cdot g^{-1}$ DW and 0.24 $mg \cdot g^{-1}$ DW, respectively, that reached to its respective maximum values of 27.4 mg·g-1 DW and 8.8 $mg \cdot g^{-1}$ DW in 7-week-old cultures (Fig 3). The phenolic and flavonoid content in ARs were termed as gallic acid and quercetin equivalent, respectively based on the used standers (gallic acid and quercetin).

### 3.3. Antioxidant activity

Antioxidant activity of adventitious root cultures was determined by three different methods; DPPH radical scavenging assay, reducing power assay and total antioxidant capacity (Fig 4). These activities were estimated as ascorbic acid equivalent (AAE). Highest levels of DPPH radical scavenging activity (73.1±5.9%), reducing power (1.51±0.17 mg AAE/g DW) and total antioxidant activity (3.66 ± 0.13 mg AAE/g DW) were displayed by 7-week old cultures (Fig 4).

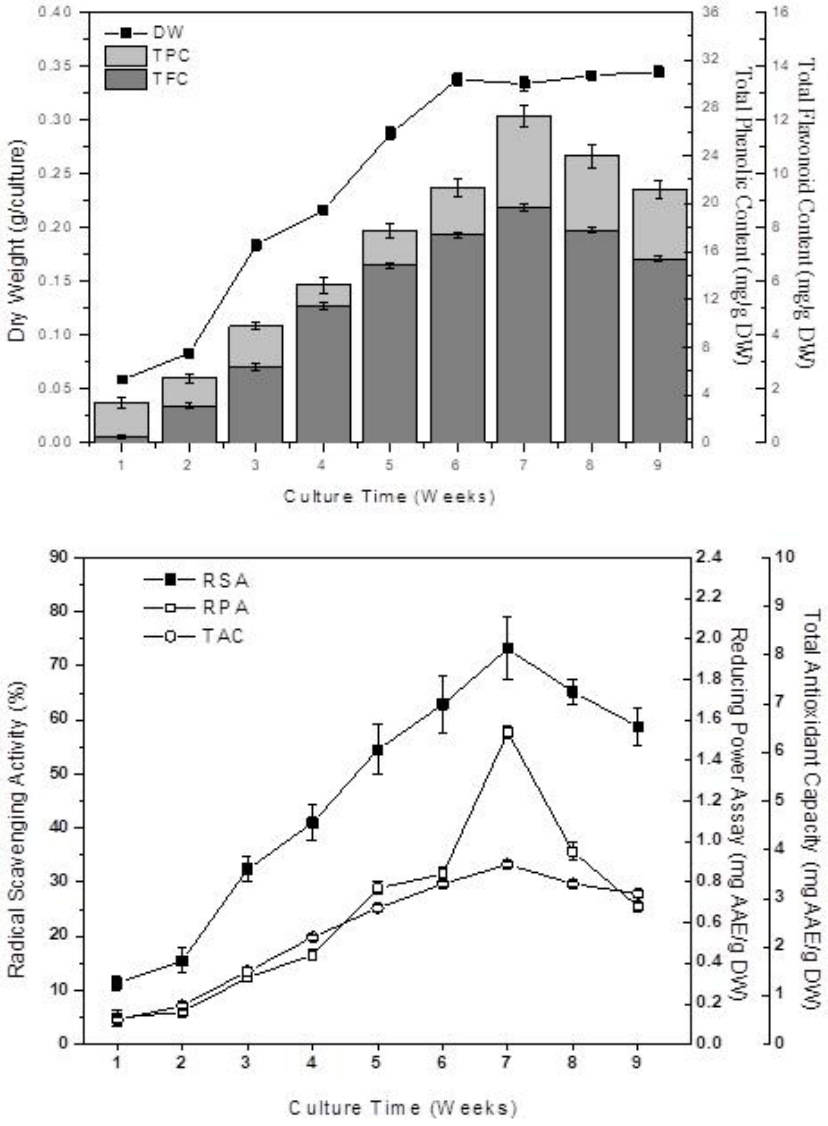


**Figure 4.** Antioxidant activities of adventitious root cultures as radical scavenging activity (RSA), reducing power assay (RPA) and total antioxidant capacity (TAC), with respect to time of harvest.

## 4. DISCUSSION

In the preliminary studies, internode and cotyledon explants were cultured for adventitious root induction. Among both the explants investigated, maximum 87% adventitious root formation was found in cotyledon explants; however internodes explants remained quiescent during the same culture conditions and minimum 34% rooting was observed. The morphological and physiological differences in these two explants explains the different rooting tendency at the same hormonal treatments [25]. By using cotyledon explants in the subsequent experiments, the adventitious root induction frequency was more pronounced with NAA compared to similar concentrations of IAA and IBA. These ARs in NAA containing MS medium were many in number, thick and white in appearance, and shorter in length without lateral branching. However, ARs in IAA supplemented media were few in number, profusely branched and slender in strength.

We found that sucrose concentration and pH level effected adventitious root induction significantly. MS medium augmented with 40 $g \cdot L^{-1}$ of sucrose was supportive for maximum percent root induction while sucrose level higher than 40 significantly impaired the roots formation in cotyledon explants. As has been reported, sucrose act as building block of living cell [26] and adjust the cellular osmotic potential [27], the decrease in biomass accumulation at elevated sucrose concentration might be attributed to higher osmotic pressure that is deleterious to root primordia growth [28]. Similar observations were also made by Baque et al. [4] and Wang & Weathers [28].

Medium pH is reported to effect the solubility of nutrient elements by changing their ionic forms [24]. The decrease in biomass accumulation at both extremes of pH (4.0 and 8.0) might be due to the availability of some nutrient elements, like trace elements at acidic pH are more available while calcium (Ca) and phosphorous (P) are less available [29].

Plants are the source of structurally diverse secondary metabolites which are grouped into several classes (phenols, alkaloids, saponins, etc) and among these polyphenols are considered as the largest class of organic antioxidants [30]. These phenolics antioxidants are safer then vitamin C and E supplements, and more potent to scavenge the reactive free radicals [31]. The present study reports the viable alternative for large scale production of antioxidants in ARs culture, which can be scaled up to bioreactor scale for commercial production. We found a positive correlation of phenolics and flavonoids with antioxidant activities in adventitious root cultures. Several studies attributed the antioxidant activity (DPPH, total antioxidant activity and reducing power) to phenolic content in plant samples [32, 33], which have been proved to be more potent than synthetic antioxidants. Earlier authors [34, 35] have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. Previously, adventitious root cultures for other medicinal plants have been reported but this study reports ARs culture of *Brassica oleracea* var. *acephala* for the first time to produce phenolic and flavonoids using shack flask bioreactor [4, 27, 36, 37]. The fast growth rate of ARs in this study also opens the gate to genetic engineers for recombinant therapeutic proteins production.

## 5. CONCLUSION

The present study showed growth-associated increase in total phenolic content and total flavonoid content, which were found in a linear correlation with antioxidant activities. Additionally, this protocol can be exploited for production of other medicinally valuable secondary metabolites including glucosinolates and brassinosteroids.

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Published at http://ijate.net/index.php/ijsm http://dergipark.org.tr/ijsm **Review Article**

# Biotechnological approaches for production of bioactive secondary metabolites in *Nigella sativa*: an up-to-date review

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**Abstract**: Medicinal and aromatic plants and their refined natural products have gained global attraction for their therapeutic potential against many human diseases. *Nigella sativa* is a medicinally important plant, commonly known as Black cumin or Black seed is a dicotyledon plant of the Ranunculaceae family. It is in common use for a longer time in history as preservative and spice and has also been extensively utilized by different communities around the globe. Black cumin has been an eminent component of traditional medicine systems like Unani and Tibb, Ayurveda and Siddha. Its biological activities include antidiarrheal, analgesic, antibacterial, liver tonic, diurectic, digestive agent and to treat several skin disorders. Furthermore, the therapeutic properties also include antidiabetic, anticancer, antihypertensive, anti-inflammatory, hepatoprotective, spasmolytic and bronchodialator. This is all because of its miraculous healing power that it has been ranked as top ranked, among evidence based herbal medicines. The literature supports that the pharmacological activities of *Nigella sativa* are mainly because of the essential oil and its constituents particularly thymoquinone. The current review is an attempt to present a detailed literature survey regarding chemical composition, phytochemistry, therapeutic potential and biotechnological approaches to enhance the medicinal potential of this valuable plant.

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*Nigella Sativa,*
Black cumin,
Phytochemistry,
Therapeutic potential,
Biotechnological approaches

## 1. INTRODUCTION

*N. sativa* Linn, a highly potent medicinal plant of Ranunculaceae family, is an annual flowering herb which usually grows 20-90 cm tall. *N. sativa* locally known as Black cumin or Black seed is natively found in the regions of Southern Europe, North Africa and Southwest Asia. Currently, it is cultivated in many countries across the globe [1,2]. *N. sativa* is a rabi crop and seeds

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of this crop are sown in the month of November and harvested in March or April. Sandy and loamy soil with pH 6.85, having 0.78% organic carbon is ideal for its cultivation [3,4]. The morphology of *N. sativa* flowers comprises 5 to 10 petals with color ranging from white, yellow, pink, pale blue to pale purple (Figure 1). The fruit appears like a big, inflated capsule having 3-7 united seeds containing follicles. Seeds are small, dicotyledonous and black in color with aromatic odor and bitter taste [5]. *N. sativa* is known with diverse names in different part of the world, such as in English: fennel flower, nutmeg flower, Roman coriander, blackseed or black caraway, black sesame; India: Assamese - kaljeera or kolajeera, Hindi/Urdu - kalaunji/ mangrail; Arabic: habbat al-barakah; French: nigelle de Crète, toute épice; Germany: Schwarzkümmel.

Due to availability of unique phytochemicals there are numerous therapeutic potentials of *N. sativa* such as anti-inflammatory, anti-analgesic, anti-stress, anticancer, antioxidant, antibacterial, antifungal, antiparasitic and antiasthmatic [1,5-8]. Plants have secondary metabolites for their defense mechanism whereas humans utilize these secondary metabolites for multiple purposes such as medicines, flavorings, and recreational drugs [9-11]. Due to phytochemicals and vast therapeutic potential, seeds of *N. sativa* got a great economic value in local and international market such as Rs. 275-500/kg in local market (Pakistan; Mingora, Dir, Peshawar, Pindi, Lahore, Gilgit). In Indian market Rs. 250-300/kg, whereas it is put up for sale in international market for Rs. 850-1000/gm [12]. Therefore it can be one of the ideal plants for farmers to cultivate and get a good income out of it [13,14]. Seeds of *N. sativa* can be stored for a year in airtight bags or jars to maintain its aroma. It should be kept away from other species (condiments) as it can affect the aroma and flavor of other species [13].

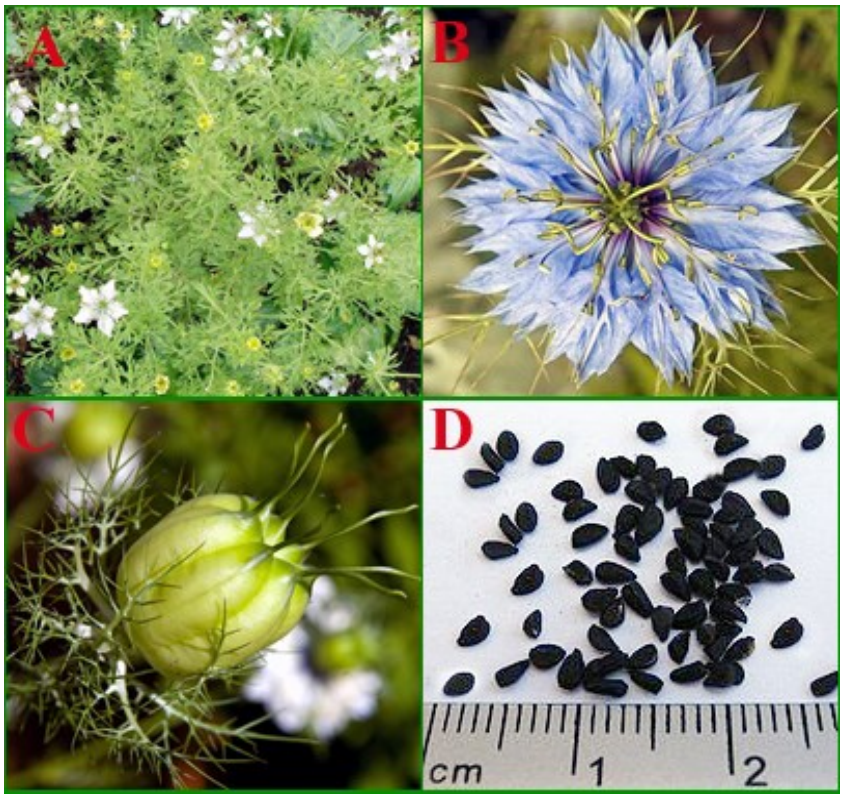


**Figure 1.** (A) Plant of *N. sativa*, (B) Flower, (C) Capsule or fruit, (D) Seeds

## 2. ACTIVE PHYTOCHEMICALS IN *N. SATIVA*

The plant has undergone an extensive phytochemical analysis owing to its various medicinal properties and a general composition was found to be volatile oils (1.6%), fixed oils (35.6-41.6%) and proteins (22.7%) [132]. The further composition based analysis of oils revealed that there are several important active constituents of oils like thymoquinone which comprises 30-48%, thymohydroquinone, dithymoquinone and p-cymene constitute 7%-15%, carvacrol (6%-12%), 4 terpineol (2%-7%), tanethol (1%-4%), sesquiterpene longifolene (1%-8%) α-pinene and thymol (Figure 2). There are found two different types of alkaloids in seeds of N. sativa i.e. isoquinoline alkaloids (nigellicimine and nigellicimine N-oxide) and pyrazol alkaloids/indazole ring containing alkaloids (nigellidine and nigellicine). Seeds of *N. sativa* also contain water soluble pentacyclic triterpene (alpha-hederin) along with saponins [7]. Other important constituents found in the seeds include protein, fat, carbohydrates, crude fibre, vitamins and minerals like Cu, P, Zn and Fe etc [8]. Additional chemical components are nigellone, avenasterol-5-ene, avenasterol- 7-ene, campesterol, cholesterol, citrostadienol, lophenol, obtusifoliol, stigmastanol, stigmasterol-7-ene, β-amyrin, butyro- spermol, cycloartenol, 24-methylene-cycloartanol, taraxerol, tirucallol, 3-O-[β-D xylopyranosyl (1→3)-α-L- rhamnopyranosyl (1→2)- α-L-arabino-pyranosyl]-28-O-[α- L-rhamnopyranosyl (1→4)- β-D-glucopyranosyl (1→6)-β-D- gluco-pyranosyl] hederagenin, volatile oil, fatty oil, oleic acid, esters of unsaturated fatty acids (Figure 3) and higher terpenoids, esters of dehydrostearic and linoleic acid, aliphatic alcohol, melanthin, melanthigenin, 3-O- [β-D-xylopyranosyl-(1→2)-α-L-rhamno- pyranosyl-(1→2)-β- D-glucopyranosyl]-11-methoxy- 16, 23-dihydroxy-28-methylolean-12-enoate,stigma-5, 22-dien- 3-β-D-gluco-pyranoside, cycloart-23-methyl-7, 20, 22- triene-3β, 25-diol, nigellidine-4-O-sulfite [10,15].

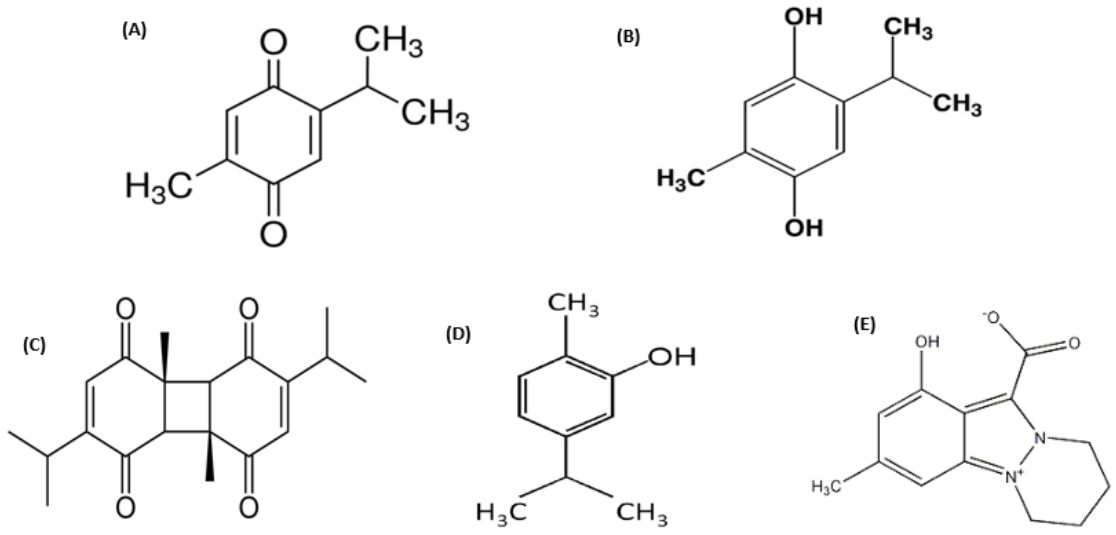


**Figure 2.** Structure of Thymoquinone (A), Thymohydroquinone (B), Dithymoquinone (C), Thymol (D), Nigellicine (E)

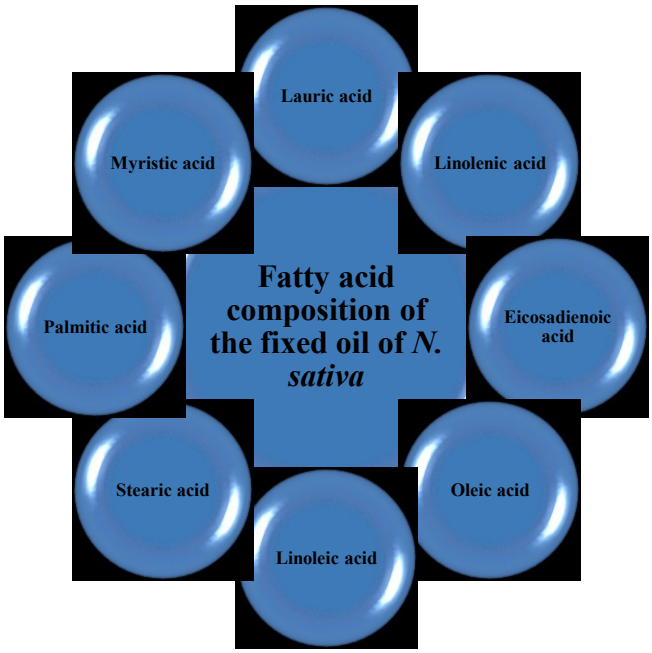


**Figure 3.** Fatty acid composition of the fixed oil of *N. sativa*

## 3. MEDICINAL and PHARMACOLOGICAL APPLICATIONS

To prevent and cure variety of diseases all over the world, seeds of *N. sativa* are used in herbal medicines. Prophet Mohammad (Peace Be Upon Him) said: "Use this Black Seed; it has a cure for every disease except death" (Sahih Bukhari). There are several ailments like skin disorders, respiratory disorders including asthma, bronchitis, disorders of joints like rheumatism and disorders of gastrointestinal track i.e. diarrhea and also hepatic one which are cured by the seeds of *N. sativa*. It gives strength to immune system and increase milk production in females [16]. Worm treatment is also reported by using the seeds and which are also helpful in the treatment of nausea. Oil of *N. sativa* has the ability to work as an antiseptic and a local anesthetic [17].

Different studies have proved that *N. sativa* and its active secondary metabolites can be effective in different pharmacological activities such as diuretic, antihypertensive, bronchodilator, gastroprotective, hepatoprotective, antidiabetic, anticancer and immunomodulatory, analgesic, antimicrobial, analgesics and anti-inflammatory, spasmolytic, renal protective and antioxidant properties, summarized in Table 1.

**Table 1.** Biological Activities of *N. sativa*

| Plant | Extract type/metabolite | Activities/ effect | Remarks | Experimental model | References |
|---|---|---|---|---|---|
| Nigella sativa | Thymoquinone | Antioxidant<br>Antiarthritic | | Wistar rat | [18, 19] |
| | | Anti hypertensive | averted the decrease of platelet numbers, prothrombotic events, systolic blood pressure, Leucocytosis and increased IL-6 concentration | Mice | [20] |
| | | Gastroprotective | | Animal model | [21] |
| | Seed oil | | | | |
| | Aqueous extract of seeds | Hepatoprotective activity | | Male Wistar rats | [22] |
| | Hexane extract of seeds | Prevented pregnancy | | Rats | [23] |
| | Ethanolic extract of seeds | Anti-fertility activity | | Male rats | [24] |
| | Seeds oil | Anti-oxytocic | inhibited uterine smooth muscle contraction | Rat and guinea pig | [25] |
| | Seed ethanol extract | Antihyperglycemic | amplified glucose-stimulated insulin secretion by more than 35%, accelerated β-cell proliferation, increased basal glucose uptake by 55% | *in vivo* | [26] |
| | Seed extracts | Anti-cancer | | *In vitro* and *in vivo* | [27] |
| | Essential oil and ethyl acetate extracts | | | | |
| | Melanin<br>Thymoquinone | Antimicrobial | | *In vitro* | [28, 29] |
| | Ethanolic extract | Antibacterial | | | [30] |
| | Ethyl ether extract | | inhibition of *Staphylococcus aureus Pseudomonas* | | [31] |

| | | | | |
|---|---|---|---|---|
| | | *aeruginosa*, *Escherichia coli* and *Candida albicans* | | |
| Crude extracts | | | | [17] |
| Seed oil | | | | [32] |
| Ethanolic extract of seeds | | Inhibitory of all tested strains of MRSA | | [30] |
| Thymoquinone<br>Thymohydroquinone | Anti-bacterial | | | [33] |
| Thymoquinone | | Effect against cocci (*Staph. aureus* ATCC 25923 and *Staphylococcus epidermidis* CIP 106510) | | [34] |
| | | Activity against *Streptococcus mitis, Streptococcus mutans, Strep. constellatus* and *Gemella haemolysans*<br>Activity against *Entero. faecalis, Entero. faecium* and *Streptococcus salivarius, Staph. Aureus* | | [35, 28] |
| Essential oil | | Activity against *oralis, Strep. mutans, Strep. constellatus* and *G. haemolysans* | | |
| Aqueous extract of seed<br>Methanol extract of seed | | Effective against *Strep. Pyogenes, Streptococcus. pneumoniae, Pseudo. aeruginosa and Proteus vulgaris* | | [36, 37] |
| Seed extract loaded in polymeric micelle | Antibacterial | | | |
| Methanol extract of seed<br>Chloroform extracts of seed total extract | Antibacterial activity | | animal study | [38] |

| | | | | |
|---|---|---|---|---|
| Essential oil of seeds | | | | |
| Thymoquinone | Antioxidant, Hepato-protectant, Anti bacterial | Prevents damage in an acute pyelonephritis (PYN) caused by *Esch. Coli* protective effect in kidney tissue | rat model | [19] |
| Methanol extract of seeds | Antibacterial | effective against bacteria cause mastitis | cows that have mastitis | [29] |
| Seeds | Anti *H. Pylori* activity | | patients with non-ulcer dyspepsia | [39] |
| Thymoquinone | Antidermatophyte effects | | *In vitro* | [40] |
| Ether extract of seed | | | | |
| Dithymoquinone | Anti yeast activity | | | [41] |
| Thymohydroquinone | | | | |
| Thymoquinone | | | | |
| Seed oil | Antidermatophyte effect | | | [42, 43] |
| Aqueous extract of seeds | Anti-fungal | inhibitory effect against candidiasis | *Candida albicans* infected mice | [44-46] |
| Methanolic extract of seeds | | | | |
| Chloroform extract of seeds | | | | |
| Thymoquinone | Anti-fungal | effective against vaginal candidiasis | prednisolone induced immune suppressed mice | [47] |
| Plant oil | Anti-schistosomal effects, antioxidant effects | improved hepatic function and the immunological system | mice infected with *Schistosoma mansoni* | [48-51] |
| Thymoquinone | | | | |
| Seeds | | | | |
| Oil | Antiviral effect | | murine cytomegalo virus (MCMV) model | [52] |
| | Antiviral, antioxidant activity | enhanced RBC and platelet counts | patient with hepatitis C virus (HCV) | [53] |

| | | | |
|---|---|---|---|
| Ethanolic extract | Anti-parasite activity | children infected with cestode worms | [54] |
| Methanolic extract of seeds | Antimalarial effect, antioxidant effect hepatoprotactent | Mice | [55] |
| Aqueous suspensions of seeds<br>Oil emulsions of seeds | Antiparasite effect, anticoccidial effects | coccidiosis in rabbits | [39] |

### 3.1. Patents

There are five different FDA (Food and Drug Administration) patents in the U.S.A. of *Nigella sativa* for the treatment of following diseases [56]:

1. Inhibition of cancer cell growth, Patent no - US 5,653,981, Inventor- R. D. Medenica.
2. Diabetes, No.-US 6,042,834, Inventor – Wasif Baraka.
3. Improvement of the Immune System, No.- US 5,482,711, Inventor – R. D. Medenica.
4. Viral Infections, No.- US 6,841,174, Inventor – S. I. A. Shalaby and E. M. A. H. Allah.
5. Psoriasis, No.- US 6,531,164, Inventor – H. H. R. Credé.

### 3.2. Cultivation Requirements and Challenges

*N. sativa* is a highly medicinal plant and its demand especially for its magical oil is increasing day by day. The *Nigella* crop takes around 140–160 days to reach to its harvesting period as it is a rabi (cool season) crop so it grows during the winter season in India. The requirements for sowing the seeds are warm weather with a temperature range of 20–25°C and cold weather is required for the early growth period. The seed formation also requires the warm sunny weather thus it is the main requirement for *N.sativa* from seed sowing to seed formation [57, 58]. Root rot is one of the common infections of *N. sativa* which is instigated by *Rhizoctonia* and *Fusarium*. In this disease, first the leaf color turn from green to yellow and early drying of plant occurs, which significantly decreases the crop yield. No unspoiled control procedures are available for this disease. Aphids (small sap-sucking insects), larvae of armyworm *Spodoptera litura* and *Cercospora nigellae* are also involved in damaging the crop [58]. All these issues with *Nigella* in wild grown conditions are dragging us towards an alternate method to meet the demands and requirements of this modern era.

Therefore*, in vitro* cultures are attractive technique that can play a vital role in fulfilling these demands by providing metabolites within reasonable time and quantity. Plant tissue culture is a technique through which one can grow and multiply cells, tissues and organs of plants on defined solid or liquid media under contamination free and ideal conditions. Different important metabolic compounds such as alkaloids, phenols, terpenoids, vitamins and other highly medicinal compounds can easily be isolated from this technology [59].

## 4. BIOTECHNOLOGICAL ASPECTS

### 4.1. Cell Culture Technology

Plant cell culture technology is an essential tool in basic and applied research for the production of seedlings and plants and it is very important on a commercial scale. To succeed in tissue culture, medium composition is very important [60]. Hormones such as auxins, cytokinins and gibberellic acid are used to control cell growth and division can be supplemented to the growth medium at the right time which poses an important role in the formation of callus, regeneration of other plant parts or organogenesis. These hormones can also lead to increase the amount of phenolics, flavonoids and terpenoids in different cultures such as, thidiazuron (TDZ)-induced stimulated production of phenolics and flavonoids in callus and cell suspension cultures of *A. absinthium* [61, 62]. Likewise, callus cultures of *A. absinthium* displayed the maximum levels of phenolic and flavonoid content in response to combining thidiazuron (TDZ) and naphthaleneacetic acid (NAA) [63]. Artemisinin concentration was found highest in *A. absinthium* callus cultures when treated with benzyl adenine (BA; 2.0 mg/L) [64]. Some parts of cultivated plants need auxin to produce callus while some require only cytokinin however most cultures need both. Optimal formula of medium varies depending on the species, type of genotype within the species, origin and the age of tissue culture. In most experiments in this field, culture has been conducted in base medium of Morashige and Skoog [65] at different ratios of plant hormones.

The most important feature of callus is that, this cellular mass has the necessary potential for organogenesis, embryogenesis and complete plant production. Beside PGRs callus formation also depends on the type of the explants used. A variety of explants are used for the callus and suspension cultures, organogenesis and embryogenesis which are summarized in Figure 4.

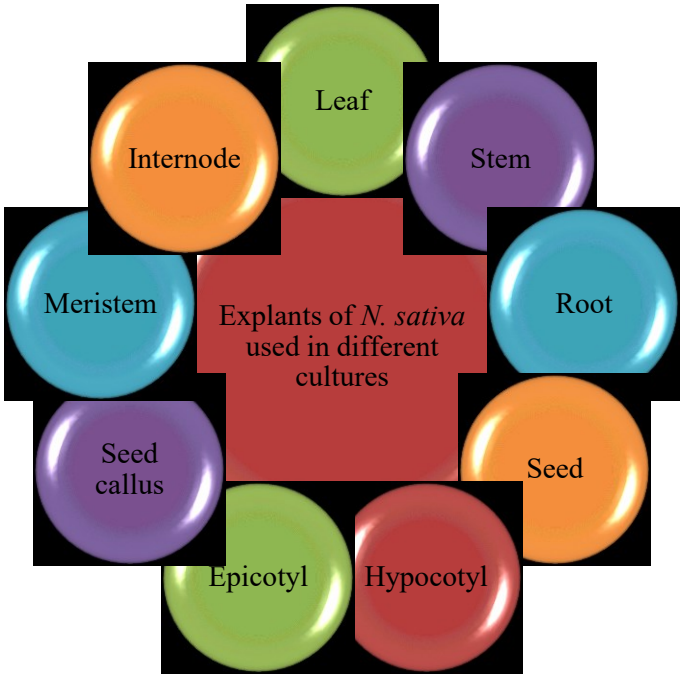


**Figure 4.** Explants of *N. sativa* used in different cultures

### 4.2. Significance of Plant in Vitro Cultures

There are numbers of drugs and medicines available in market for treatment of different diseases but we are still in search of novel chemical compounds which can help us in the decreasing the number of diseases and outbreaks. Therefore, we have to look towards natural resources where our synthetic drugs fail to cure us. Plants are rich with these novel chemical compounds known as phytochemicals or plant secondary metabolites, these phtochemicals are used by humans against different diseases [66]. Due to less number of availability of plant and high demand of phytochemicals, different approaches like in vitro cultures, are utilized to improve the quality and quantity of these metabolites [67]. One of the main reasons for utilization of in vitro cultures is the presence of trace amounts of these secondary plant compounds in the plants. The most likely reason for this is that different genes coordinate at different developmental stages indicating that production of useful important metabolites is growth dependent. There is also a general perception that during in vitro growth of the cells, the chemicals produced are mainly those which support the growth of the plant and production of secondary metabolites might be unnecessary or even toxic thus leading to deceased secondary metabolites production in vitro. Therefore, there is a need to optimize the in vitro growth conditions increased secondary metabolites production that would be a key to overcome this hurdle.

### 4.3. Strategies to Enhance Biomass and Secondary Plant Compounds

There are different strategies mentioned in Figure 5, which can enhance the phytochemicals in *in vitro* cultures. Studies have revealed that elicitation is the most effective

method for improving and increasing the production of secondary metabolites in *in vitro* cultures.


**Figure 5.** Strategies to enhance secondary metabolites

### 4.4. Effects of PGRs

Plant growth regulators are signaling compounds which help the plant growth and development and production of secondary metabolites [68]. Plant growth regulators (PGRs) have a significant effect on explant in plant tissue culture; they have a great impact of cell growth, differentiation, regeneration, and metabolite formation [69-71]. Different PGRs have been studied on different plants including *N. sativa* and each PGR has its own role in initiating in vitro growthand biomass formation. Datta et al. reported that 2,4-D and kinetin help in callus formation from hypocotyl segment [76]. In another study, MS media added with 2,4-D, NAA and IAA produced a significant amount of callus from leaf of *Nigella sativa* [75]. PGRs not only responsible for culture initiation and biomass formation, but also enhance the metabolites accumulation. Chaudhry et al. and Hoseinpanahi et al. concluded that combination of Kn + NAA and BAP + IAA enhanced terpenoid and thymol production in suspension culture of *N. sativa* [72, 73]. Al-Ani also reported that 2,4-D and Kn initiated callus formation from leaf explant of *Nigella sativa* with enhanced thymol concentration [74]. By enhancing metabolic content the antioxidant activity of culture is also increased, Further, TDZ + NAA enhanced biomass and antioxidant activity of callus of *Nigella sativa* [77].

### 4.5. Role of Elicitors

Elicitation is one of the most effective approachs for the enhancement and biotechnological production of secondary metabolites [134, 135]. An “elicitor” is a substance which initiates or stimulates the production of particular metabolites when applied at optimal concentrations. These compounds stimulate plant defense by promoting secondary metabolism for the protection of plant cell, to cope with the stress created by the them, as a result plant through a series of reactions such as activation of NADPH oxidase, production of reactive oxygen and nitrogen species, expression of defensive genes and secondary metabolites production [136-139]. Elicitors may be abiotic such as metal ions and inorganic compounds, or biotic from fungi, bacteria, viruses or herbivores, plant cell wall components [68]. Jasmonic acid (JA) is naturally synthesized inside plant and is responsible for different functions along with activation of production of secondary metabolites, therefore different mediators can be used to activate JA pathway [78, 79]. In several studies PGRs stimulated the production of

callus, organogenesis, phytochemicals which were further enhanced by the elicitors in many medicinal plants. GA3 enhanced artemisinin accumulation in hairy root cultures and shoot culture of *A. annua* [80, 81, 83-86] and *A. dubia* [82]. Similarly, Salicyclic acid (SA), AgNPs, MeJ, SPD and Chitosan enhanced stevioside biosynthesis in various cultures of Stevia [140-143]. A variety of elicitors have been used in different studies to initiate cultures of *N. sativa* with higher production of secondary metabolites. Casein hydrolysate promoted callus formation and embryogenesis with enhance biomass of *N. sativa* [87, 107]. In another study, SA enhanced the physiological parameters and also produced healthy biomass of Callus in *N. sativa* [88]. Increase in levels of monodesmosidic triterpene saponins α-hederin and kalopanaxsaponin I (KsI) in the leaves of *Nigella sativa* were observed when treated with methyl jasmonate (MeJA) [110], GA3 enhanced germination rate of *N. sativa* [89], $AgNO_3$ and SA increased secondary metabolites (fatty acids and essential oil) in callus culture [90], Nano-silver and yeast extract increased total phenol and flavonoids in *N. sativa* [91] (Table 2).

### 4.6. Light Effect

Light is a basic requirement for majority of plants for their growth and development and production of primary, secondary metabolites [92, 93]. Light in Plant tissue culture can play a very major part, it is a type of physical elicitation, fluctuation of intensity and color of light can produce some sort of stress in plant which may lead to initiate culture and also stimulate the production of phytochemicals. Light has stimulated the production of protopine in suspension cultures of Fumaria, [94] phenolics and flavonoids in callus cultures of *Stevia rebaudiana*, [95] caffeic acid derivatives in hairy root cultures of *Echinacea purpurea*, [93] phenolic acids in in vitro cultured *Ruta graveolens* and *Ruta graveolens divaricata* [96]. Several reports are available on light stimulated cultures of *N. sativa.* Complete dark helped in callus initiation from seeds [97], leaf explants [98], and stem of *N. sativa* [99], Somatic embryo formation was promoted when explants of *N. sativa* were kept in dark [98]. In another study, controlled dark conditions stimulated Melanin production in suspension culture of *N. sativa* [115] (Table 2).

### 4.7. Plant Cell Cultures Strategies for Phytochemical Production

For evaluation of biomass kinetics, optimize conditions for production of highly medicinal and important secondary metabolites, Cell suspension cultures play a very central role [100]. The factors making cell suspension cultures suitable for the analysis of complex physiological processes include the homogeneous nature of cell population, the availability of material in bulk, accelerated growth of cells and conditions reproducibility [101]. Some recent examples of medicinal secondary metabolites in suspension cultures include zerumbone production in *Zingiber zerumbet*, [102] ursolic acid production in *Eriobotrya japonica*, [103] lutein and tocopherol in carrot, [104] rosmarinic acid in *Satureja khuzistanica* [105] and taxane in *Taxus chinensis* [106], enhanced terpenoid and thymol production in *N. sativa* [72]. Al-Ani reported that from callus culture of *N. sativa* higher thymol concentration was extracted [74]. Enhanced biomass and antioxidant activity showed by the Cotyledon derived callus culture of *Nigella sativa* [77]. Enhanced Thymoquinone concentration was also extracted from callus culture of *Nigella sativa* [133].

**Table 2.** Strategies used to enhance Secondary metabolites (SMs) in various cultures of *Nigella sativa.*

| Specie | Explant | Culture | Medium/ PGRs | Elicitor treatments | Effect on sms | References |
|---|---|---|---|---|---|---|
| *Nigella sativa* | Epicotyls | Suspension culture | Kn (2 mg/L) + NAA (1 mg/L) and BAP (2 mg/L) + IAA (1 mg/L) | | Enhanced Terpenoid and Thymol production | [72] |
| | Leaf | Callus Culture | 2,4-D (1 mg/L) and kinetin (2.15 mg/L) | | Enhanced thymoquinone | [133] |
| | Leaf | Embryogenesis | IAA (0.5 mg/L) | casein hydrolysate | | [107] |
| | Hypocotyl | Callus culture | NAA (1 mg/L) | | | [108] |
| | Internode and hypocotyls | Micropropagation through Callus culture | BA, NAA | SA | | [88] |
| | Hypocotyledon, root | Callus Culture | BAP and 2,4-D | yeast extract, Silver nanoparticle | Higher production of phenolic and flavonoids | [91] |
| | Seed, cotyledon | Seed germination, Callus culture | Thidiazuron (TDZ) + (NAA) | Gibberellic acid (GA3) | Higher production of phenolics and flavanoids | [77] |
| | Leaf | Regeneration through Callus culture | NAA, BAP, IBA | | | [73] |
| | Meristem | Regeneration through Callus culture | BAP + NAA | | | [109] |
| | Callus | Suspension Culture | Kn + NAA | casein hydrolysate | | [87] |
| | | Hydroponic culture | Hoagland liquid medium | Methyl jasmonate (MeJA) | Higher levels of the monodesmosidic triterpene saponins α-hederin and kalopanaxsaponin I (KsI) | [110] |
| | Hypocotyl segment | Callus culture | 2,4-D (2 mg/L) and kinetin (1 mg/L) | | | [76] |
| | Leaf | Callus culture | 2,4-D (1 mg/L) and Kn (1.5 mg/L) | | Enhanced thymol | [74] |

| | | | | | |
|---|---|---|---|---|---|
| Leaf, stem, seed | Callus culture | IAA, NAA, Kinetin | Dark incubation of seeds | | [97] |
| Excised hypocotyls | callus culture | IAA, NAA, IBA, and 2,4-D | | | [111] |
| Leaf | callus culture | | AgNO3 and SA | Increased fatty acids and essential oil content | [90] |
| Root and sprout segments | Callus Culture | benzyl aminopurine and indole acetic acid. | MeJA | More feruloylquinic acid | [112] |
| Leaf, stem, root | somatic embryos | Kinetin. 2,4-D, NAA | Cultures incubation in complete dark | | [98] |
| Root, stem, leaf | Callus Culture | NAA, Coconut milk, IAA | | | [113] |
| Stem | Callus culture | Kinetin, NAA | Complete Dark | | [99] |
| Leaf | Callus culture | 2,4-D | Deltamethrine | Increase in protein content | [114] |
| Leaf, stem, root | Callus and suspension cultures | Kinetin, 2,4-D, NAA | controlled dark conditions | Melanin production | [115] |
| Seed | Callus culture | 2,4-D, kinetin coconut milk | yeast extract | Variation in chromosome number | [116] |
| Leaf | Organogenesis through Callus culture | 2,4-D + kinetin (6-furfuryl amino purine), coconut milk, IAA or NAA | casein hydrolysate | | [117] |
| Hypocotyl | Callus culture, Suspension culture | kinetin, 6-BA, 2,4-D, NAA, IBA, | | | [118] |
| Seed | Callus culture, Suspension culture | 2,4-D (2mg/L) and kinetin | | Protoplast isolation | [119] |
| Leaf | Callus Culture | kinetin, 2,4-D, NAA | | More Thymol production | [120] |
| Seed callus | Callus culture, suspension culture, Biotransformation | Kinetin + IAA | limonene dissolved in DMSO | Production of carveol, limonene-1,2-diol , p- | [121] |

| | | | | | |
|---|---|---|---|---|---|
| | | | | mentha-2,8-diene-1-ol-trans and carvone | |
| Stem | Callus culture | 2,4-D or PDA | | Activity of GDH was increased | [122] |
| Stem | Callus, suspension Culture | 2,4-D and Kin | Sulphanilamide | Enhanced thymol production | [123] |
| Seed, stem | Callus culture | 2,4-D | thymidine phosphorlase | Increase in the cellular contents of proteins, nucleic acids and folate extract | [124] |
| Root, hypocotylodon and leaf | Callus culture | 2,4-D, BAP | yeast extract and nano silver | More flavonoid content | [125] |

## 5. *NIGELLA SATIVA* IN NANOTECHNOLOGY

Plants have majority of phytochemicals such as phenols, acids, tannins, steroids, terpenes etc which can be utilize in synthesis of nanoparticles (Green synthesis). Plants derived nanoparticles are environment friendly with low cost and can be used in majority of therapeutic and pharmacological applications such as antibacterial, antitumor, and can also be used as biosensor. Seed extract of *N. sativa* and AgNO3 resulted in the formation of silver nanorods, which showed antidiabetic property, *in vitro* [126]. Silver nanorods were also prepared from the leaf extract of *N. sativa* [127]. Gold nanoparticles AuNPs have also been prepared from seed extract of *N. sativa* and aqueous chloroauric acid solution [128]. Plant extracts of *Nigella sativa*, *Dioscorea alata* was used to produce phytochemical capped Silver nanoparticles, thymoquinone, dioscorin and ferulic acid worked as capping agents [129]. Encapsulation of TQ into nanoparticles enhances its anti-proliferative, anti-inflammatory effects and can be used in variety of biomedical applications [130] Silver nanoparticles prepared from essential oil of *N. sativa*, showed inhibitory activity against pathogenic *Vibrio harveyi* and *V. parahaemolyticus* [131].

## 6. CONCLUSIONS

*Nigella sativa* has shown substantial therapeutic effects on several biological systems. The volatile oil as well as organic and aqueous fractions of the seeds has been proven to possess beneficial effects in terms of medicinal significance. The presence of active proteins and lipid soluble elements provide the clue to the several mechanisms of actions behind therapeutic potential. Although, the *Nigella sativa* has become the topic of research worldwide, still there is lot of room to be explored regarding this phytotherapeutic source and no doubt clinical trials need to be done to validate the therapeutic efficacy of the plant.

### Conflicts of Interest

All the authors declared that there is no conflict of interest with regards to any part of the manuscript.

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# The Effect of Nutrient-Allelochemicals Interaction on Food Consumption and Growth Performance of Alder Leaf Beetle, *Agelastica alni* L. (Coleoptera: Chrysomelidae)

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**Abstract**: In this study, the effects of secondary metabolites on the feeding preference and growth of generalist caterpillars, *Agelastica alni* L., were investigated. Feeding experiment has been applied with a total of 11 diet; 6 of which were prepared by adding different concentrations of gallic acid (1, 3, 5 %) and quinine (0.125, 0.25, 0.5 %) to the control diet, 3 diet of which prepared by adding different concentrations of gallic acid and quinine. According to the results, the amount of gallic acid consumed did not affect the food consumption and the amount of pupa lipids. However, the amount of gallic acid consumed positively affects the pupal mass and the pupal crude protein. In addition, the amount of quinine consumed negatively affected the developmental performance of larvae except for the food consumption. As the count of secondary metabolites in the diet increases, the pupal mass and the pupal crude protein decrease. Overall, during the co-evolution processs, *A. alni* larvae may be able to adapt to gallotannins. However, quinine, an alkaloid, is a feeding deterrence and growth suppressor for larvae.

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## 1. INTRODUCTION

Herbivorous insects consume a wide variety of plant parts for their growth and reproduction that require a balanced nutrient intake that is a challenge for all animals [1] and nutritional requirements of animal include a variety of macro- and micronutrients [2]. Nitrogen is a macronutrient and nitrogen content of plant foods is a critical important feature for herbivores. These compounds play important roles in development of organisms as structural components of membranes, nucleotides and nucleic acids [2, 3, 4]. Availability of nitrogenous compounds of plant foods to herbivores has influential effects on animals feeding on plants. However, it is known that the presence of some secondary metabolites in herbivores' food plant, especially the polyphenols' affects the nitrogen availability to herbivores [4].

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Tannins and alkaloids are two important classes of secondary metabolites that are distributed widely in dicotyledonous plants. These compounds may influence the target insects in many ways [5-6]. Tannins by binding to the natural polymers such as proteins and carbohydrates may reduce the enzyme actions and the digestion of carbohydrates and proteins in the digestive tracts of herbivores [7]. Also, the esters of gallic acid and its polymers with sugars known as gallotanins are one of the major groups of tannins [8]. [9] reported that complexes could be formed between gallotannins and many proteins, polysaccharides and alkaloids. These complexes may cause enzyme deactivation and the shortage of some microelements in the body [9]. Alkaloids with their bitter taste to most animals play an important role as feeding deterrent to many herbivores [10-11]. Alkaloids can be affect different regions of the nervous system and they may block acetylcholine receptors. In addition, they can affect the development and reproduction of insects, as well as mortality [12].

The larvae and mature individuals of *Agelastica alni* L. are oligophagous herbivores feeding on the leaves of *Alnus* spp. (especially *A. glutinosa*) and *Salix* spp. Their population growth in some years may reach to a level of population explosion on these plant species. If there is a shortage of their normal foods, *A. alni* individuals may feed on hazel nut, birch and hornbeam [13]. So, they may cause differentiation in the landscape in the forests; this may lead to soil erosion in the forested areas and eventually to decrease in the forested area size. *A. alni* has a significant distribution range on the coastline of Black Sea Region of Turkey.

Plant produce the mixtures of structurally different secondary metabolites against an array of different herbivore attacks and microbial invaders. However, some herbivores may overcome the adverse effects of secondary compounds on their survival by evolving detoxification and sequestrations as handling mechanisms. Some herbivores may be adapted and become dependent on certain secondary metabolites of those plants in order to find and feed on new food plants and to lay eggs [14]. So, in this study, we aimed that the synergistic effects of the nutrient and the plant secondary metabolites constituents of artificial agar based diets are investigated in the performance of *A. alni* larvae.

## 2. MATERIALS and METHODS

### 2.1. Insects and Experimental Chambers

*A. alni* adults were collected from *Alnus glutinosa* leaves in Yildizli location of the town of Arakli in Trabzon Province in Turkey in the late May in 2012 and allowed to mate and lay eggs in the laboratory. The eggs laid by the females were collected and used to maintain a colony in the laboratory. The caterpillars from the laboratory colony were fed on an agar based artificial diet originally designed by [15] until the final instar. Immediately the individuals were weighed in 0.0001 mg sensitive scale; then each one was placed singly into a plastic cup with a cover. Both the culture and the experimental chambers were kept at the constant temperature at 25 $^{0}$C with a 12h:12h light-dark regime.

### 2.2. Artificial Diets

The diet developed by Yamamoto has been modified. The diet developed by [15] has been modified. The protein and carbohydrate amounts of all diets are the same. Gallic acid, quinine or secondary metabolite mixture were added to the diets at different concentrations for feeding experiments. Totally eleven artificial diets were prepared. These artificial diets were labeled A, B, C, D, E, F, G, H, J, K and L. Diets and their contents are given in Table 1.

**Table 1.** The components of the artificial diets

| Artificial Diets | Secondary Metabolite |
|---|---|
| A (control diet) | No secondary metabolites |
| B | 1 % gallic acid (GA) |
| C | 3 % gallic acid |
| D | 5 % gallic acid |
| E | 0.125 % quinine |
| F | 0.25 % quinine |
| G | 0.5 % quinine |
| H | 1 % gallic acid + 0.5 % quinine |
| J | 1 % gallic acid + 0.125 % quinine |
| K | 5 % gallic acid + 0.125 % quinine |
| L | 5 % gallic acid + 0.5 % quinine |

### 2.3. Feeding Experiment

Each of the final instar larvae was weighed. Each food block prepared, as described above was pre-weighed before being presented to the larvae for each treatment. A total of 10 replicates were used for each diet treatment. Every other day, any food uneaten by the larvae remaining in the larval chamber was collected and replaced with fresh pre-weighed food block. The uneaten food left by the larva from each feeding chamber was collected separately and dried in an oven (50 $^0$C) and weighed after it reached a constant weight. Every other day, each larva was weighed. This procedure was repeated until all of the larvae entered the pupal stage [16].

### 2.4. Pupal Lipid and Crude Protein Analysis

The total amount of lipids stored in each pupa was determined with chloroform extraction by three times [17]. After each chloroform treatment the pupae were dried to the constant weight at a drying oven at 50 $^0$C. At the end of third chloroform extraction, the pupae were re-dried and re-weighed to calculate the lipid contents.

After the chloroform treatment, free lipid pupae were used for estimation of crude protein content. Crude protein content was determined by Dumas (Thermo Scientific Flash 2000 series-NCS analyzer) using a protein-to nitrogen conversion factor of 6.25 [18-19].

### 2.5. Statistical Analysis

The amount of food consumption by each larva fed on each artificial diet, the pupal dry weight, and the protein contents of the pupae, the lipid contents of the pupae were analyzed statistically using SPSS 17 version. The values were analysed with TUKEY test for identification of differences between groups. A Pearson correlation test was performed to determine whether there was an association between variables.

## 3. RESULTS

### 3.1. Food Intake

As expected, secondary metabolites at different concentrations affect the food consumption of larvae (Fig 1). The food consumption of larvae differs in different artificial diets (ANOVA, F=24355,96; $p<0.001$). The diet with the highest concentration of gallic asid was consumed by the larvae than other diets. The least consumed diet is that the diet containing the highest concentration quinine. (Fig 1). Interestingly, there is no difference in the food consumption between diet with the highest concentration of quinine and A diet. Diet A does not contain any secondary metabolite (Fig 1). The amount of quinine affected food consumption

(r=0.309, p<001). However, the amount of gallic acid consumed did not affect food consumption. It was determined that the concentration of gallic acid in the diets and the concentration of quinine affect the amount of consumption. While there was a positive correlation between gallic acid concentration and food consumption (r = 0.767, p <0.001), there was a negative correlation with quinine concentration and food consumption (r = -0.598, p> 0.001). Any relation was not found between the count of secondary metabolites in the diet and the amount of consumption (p>0.05).

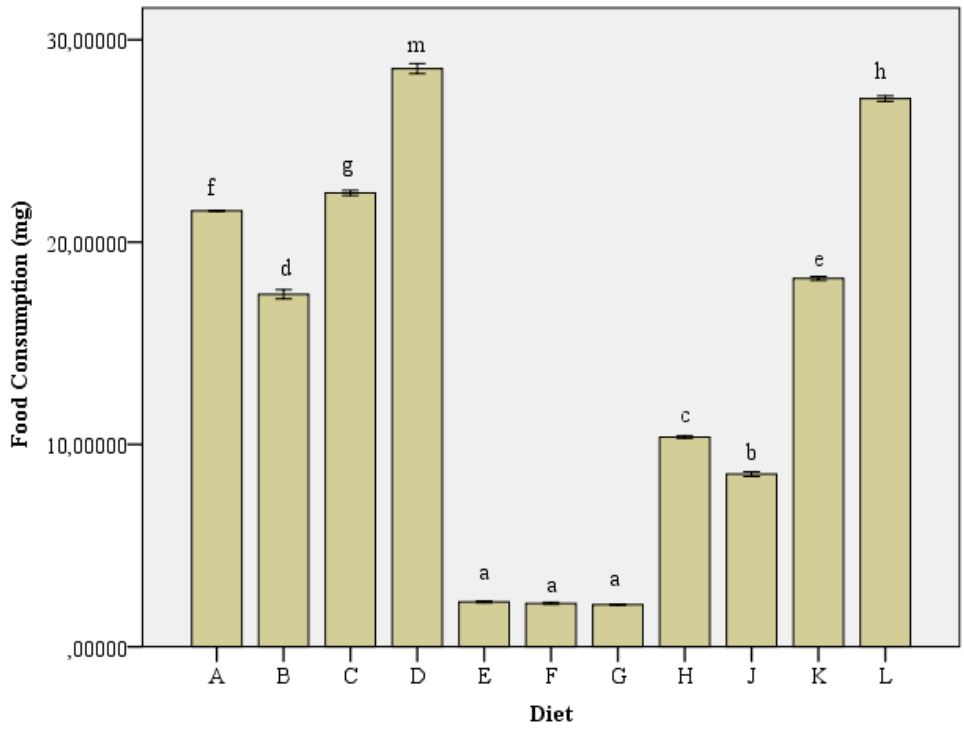


**Fig 1.** Food consumption on different artificial diet. *Diets with the same letter are not significantly different

### 3.2. Growth Performance

Pupal mass, amount of pupal crude protein and amount of pupa lipid were analysed to investigate the effect of diets on development performance. Differences were found between the pupal mass for different artificial diets (ANOVA F=169.662, p<0.001). The highest pupal mass was determined for A diet (no secondary metabolite). The minimum pupae mass was determined in the diet containing 0.25% quinine (Fig 2). The amount of gallic acid consumed and the amount of quinine consumed affects the pupal mass. The increase in the amount of gallic acid consumed causes the increase in pupa mass (r=0.22, p<0.05). The increase in the amount of quinine consumed resulted in a decrease in the pupa mass (r= -0.501, p<0.001). Gallic acid consumption affects the pupa mass, whereas gallic acid concentration of the diet does not have any effect on pupa mass (p>0.05) However, quinine concentration has a negative effect on the mass of the pupa mass just like the amount of quinine consumption (r= -0.695, p <0.001). Secondly, the number of secondary metabolite in the diet also negatively affected the pupal mass (r= -0.436, p<0.001).

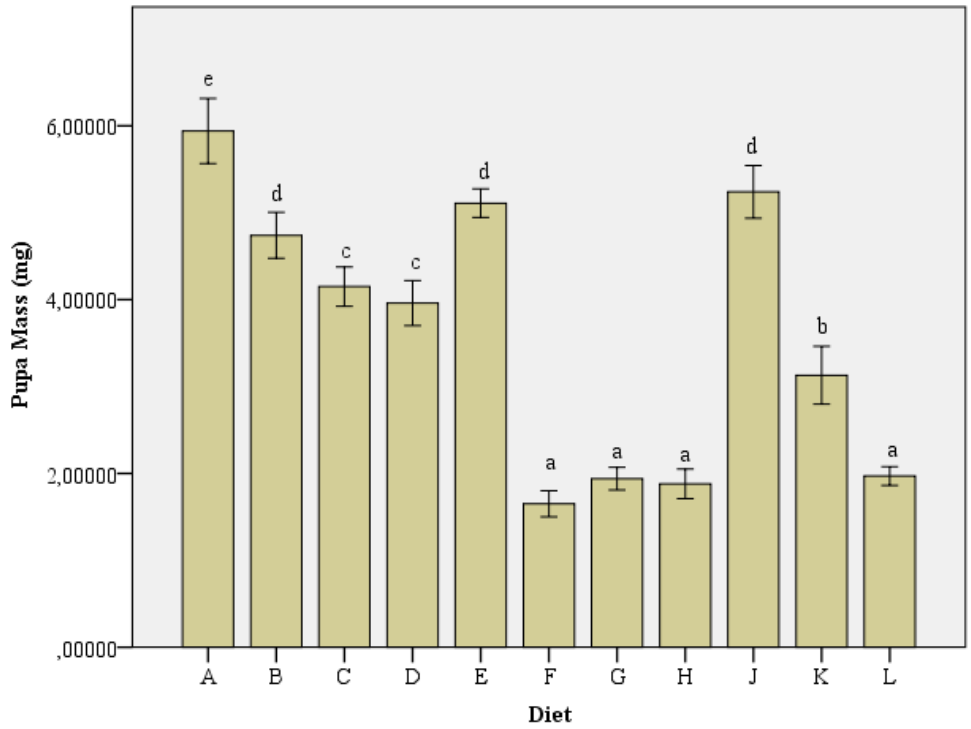


**Fig 2.** Pupa mass on different artificial diet (mg). *Diets with the same letter are not significantly different.

The amount of pupae protein and pupae lipid amount are very important for development. Feeding on different artificial diets influenced the pupae lipid amount (ANOVA, F=78.393, $p<0.001$). The maximum amount of pupa lipids was determined in the diet containing at least quinine. The lowest pupa lipid amount was determined for L diet (Fig 3). Concentrations of two secondary metabolites are the highest in L diet. Neither the amount of gallic acid consumed nor the gallic acid concentration influenced the amount of pupa lipid ($p>0.05$). In contrast, both the amount of quinine consumed and the concentration of quinine in the diet affected the amount of pupal lipid. Increased quinine consumption of the larvae as well as the increase in quinine concentration of the diet resulted in a decrease in the amount of pupal lipid (respectively, $r= -0.495$, $p<0.01$; $r= -0.534$, $p<0.01$). The count of secondary metabolite in the diet did not affect the amount of lipid ($p>0.05$).

Artificial diets affect the pupae crude protein ($F=50.086$, $p<0.01$). The maximum pupal crude protein was obtained with the control food A. A diet contains no secondary metabolite. The minimum pupal crude protein was obtained with the food H (Fig 4). The H diet is the diet with the minimum concentration of gallic acid and quinine. Both the concentration of secondary metabolite, the consumption amount of secondary metabolite and the count of secondary metabolite negatively affected the amount of pupae protein. Just ingested gallic acid amount affected the pupal crude protein positively ($r=0.327$, $p<0.01$) There is a negative relationship between gallic acid concentration of diet and pupa crude protein ($r= -0.191$, $p<0.01$). Also, there is a negative relationship between quinine concentration of diet, secondary metabolite counts and pupal crude protein (respectively, $r= -0.667$, $p<0.01$; $r= -0.577$, $p<0.01$). Ingested quinine negatively affected the pupal crude protein ($r= -0.429$, $p <0.001$).

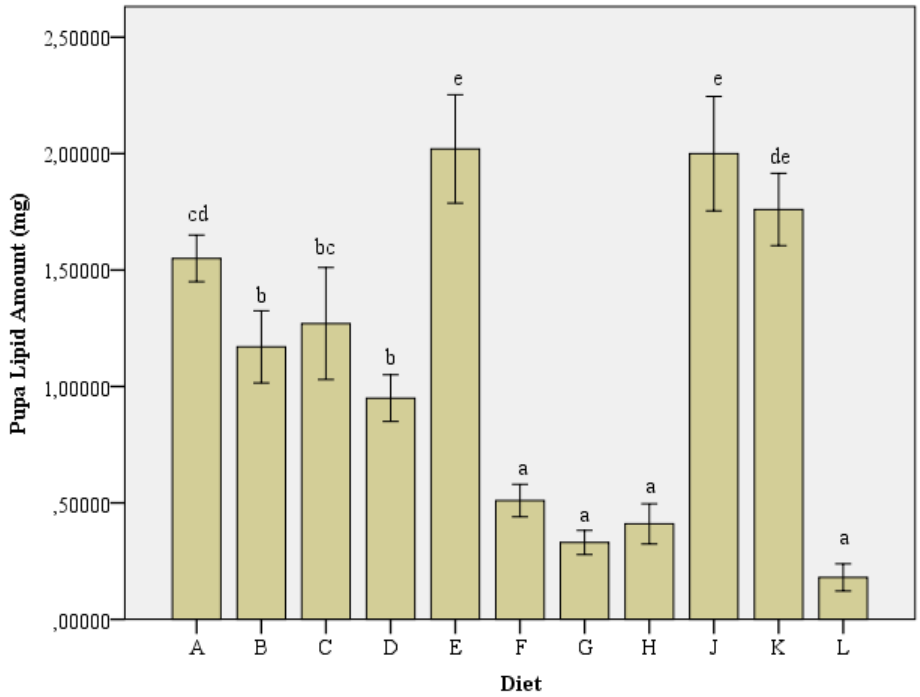


**Fig 3.** Pupa lipid amount on different artificial diet (mg). *Diets with the same letter are not significantly different.

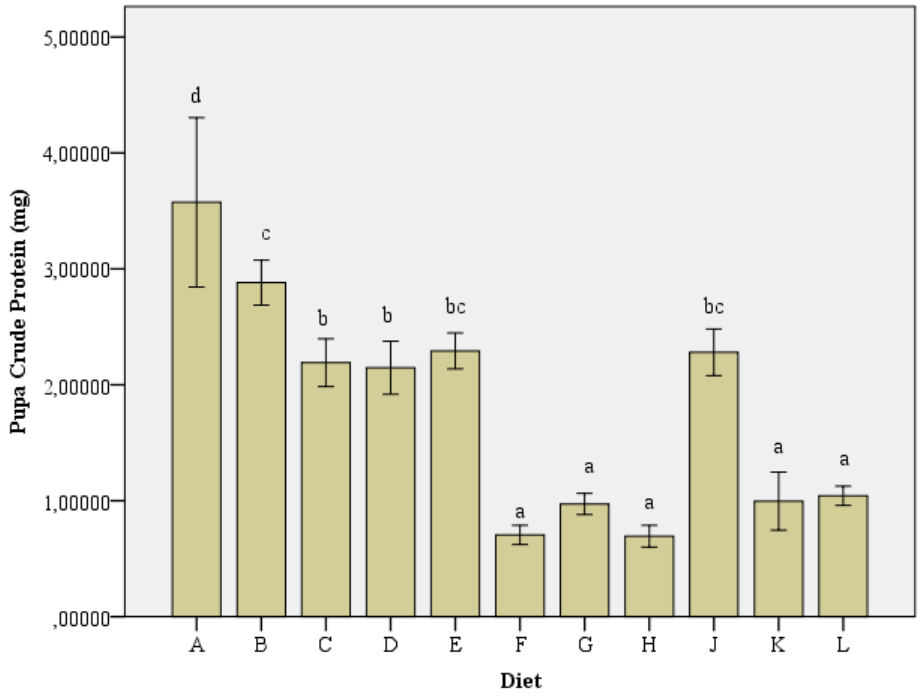


**Fig 4.** Pupa crude protein on different artificial diet. *Diets with the same letter are not significantly different

## 4. DISCUSSION

It is known that gallotanins with other secondary compounds and primary metabolites are effective protective agents against herbivores [20]. The results presented in the study show that the digestion, the post digestion regulation and the development of the larvae were affected by their consumed nutrients by the larvae and the nutrient-secondary metabolites interaction. [21] reported that morphology and plant nutritional content affect growth and development of

herbivores. Secondary metabolites and nutritional quality of food are main factors to regulate between plant and its environment [4, 22-23].

Tannins are divided into 2 groups as condensation tannins and hydrolysable tannins (such as gallotanens). The effects of tannins on herbivores are as feeding deterrents or to reduce the availability of nutrients [8, 24, 25]. The hydrolysable tannins may not affect the digestion of proteins. These tannins are reduced to smaller phenolic substances in the gut. These phenolics do not bind proteins. However, they have positive or negative effects in the digestive system according to hydrolysis products [8]. In our study, the amount of gallic acid consumed does not affect the consumption of larvae. Therefore, it supports the view in the literature that the hydrolysable tannins are not feeding deterrent. With increasing concentration of gallic acid, the pupae mass and pupa protein amount of *A. alni* individuals increased. According to the co-evolution theory, herbivores can adapt to secondary metabolites of plants and they can use nutrients [26]. *A. glutinosa* contains gallotannin as a secondary metabolite [27]. *A. alni* larvae have been adapted to the secondary metabolite of these plant for feeding and they have managed to be protected from the negative effects of gallotannins. Also, *A. alni* larvae by increasing the food consumption were able to obtain enough nutrients that were otherwise hardly obtainable because of the tannic acid in their foods [23].

Alkaloids are toxic compounds to herbivores. They have bitter, nitrogenous compounds. Alkaloids that have an impact on nervous system [28-29] and cell division inhibit to larval development and growth [23, 30-31]. According to the results of the study, quinine has a negative effect on the development of *A. alni*. Also, alkaloids have a negative effect on food consumption because of the bitter taste. The binding to chromosomes (especially to the chromosome Y) is well known characteristics of quinine [32]. The absorption of quinine through animals' guts into the blood stream may distribute it through out the body. When it reaches to the growth zones where mitotic division takes place, quinine may bind to the chromosomes and may cause the interruption of mitotic division; therefore, animals' bodies may not grow as their actual sizes [32]. The larvae were negatively affected by the toxic effects of quinine because they were not adapted to the quinine of their nutrients. Quinine may affect the DNA structure of *A. alni* larvae. Also this may cause possible decrease in the body size of the larvae.

Plants can provide an advantage to protect the herbivores by increasing the number of secondary substances. As the count of secondary metabolites in the diet increased, the mass of *A. alni* pupae and the amount of pupa protein decreased. Fecundity of individuals will be adversely affected as the count of secondary metabolite in the diet. Because the increase in the count of secondary metabolite caused a decrease for pupa mass. The development of larval stage is important for insects rather than the development of the adult stage [26]. Pupae proteins and pupa mass are also important parameters for reproduction.

Herbivores can adapt to secondary metabolites in the evolutionary process, so plants can provide protection against herbivores with more than one secondary metabolite [33]. In addition, this result indicates that the *A. alni* larvae were not able to adapt to alkaloids. As a result, *A. alni* larvae have been adapted to the gallotannins during the process of co-evolution. However, quinine is a deterrent for larvae and adversely affects the development of larvae. [34] submitted that new defense substances arose on the plants parts approximately distribution and speciation of insects at co-evolution process and new defenses evolved against insect herbivores.

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Published at http://ijate.net/index.php/ijsm http://dergipark.org.tr/ijsm **Review Article**

# Brief Review on *Lantana camera*

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**Abstract**: Medicinal plants are widely spread in nature with their unique habitats and effective medicinal properties. One of them plant is *Lantana Camera*. The *L. camera* is well known invasive weed. Used to cure several diseases in Ayurvedic preparations with different formulations. This plant has great ethnobotany and Pharmacology however it is lagging behind in the list of medicinal plants for their applications in drug preparations due to mere research study and awareness. Therefore present review may help to reveal different aspects of *L. camera* with their awareness in society.

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**KEYWORDS**
*Lantana camera*,
Ethnobotany,
Pharmacology

## 1. INTRODUCTION

The term "biodiversity hotspot" was coined by the British biologist Norman Myers in 1988. He described them as a biogeographic region by characterizing their exceptional levels of plant endemism and serious levels of habitat loss [1]. Further, in between 1989-1996, Conservation International (CI) adopted 'Myers hotspots criteria' and they made one organization for the reassessment of the hotspots concept and in 2005 they have published an revised title "Hotspots Revisited: Earth's Biologically Richest and Most Endangered Terrestrial Ecoregions" [2]. According to the CI report, there is the total of thirty-five biodiversity hotspots in the world, amongst them, four are in India. Mainly these three biodiversity hotspots are situated in the Himalaya, Indo-Burma, Sundaland, and the Western Ghats. Total 17000-18000 flowering plant species, 8000 medicinal plants, are documented in folk and Ayurveda, Unani, Siddha, and Homoeopathy [3]. Medicinal plants are a great resource base for the traditional medicine & herbal industry and also it provides livelihood and health security to a large segment of Indian inhabitants. India has the greater natural ecosystem from past two decades, there has

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been a marvelous augment in the use of herbal medicines; though there is still a noteworthy deficiency in the research of medicinal plants and it has the great resources of medicinal plants which are noteworthy to human beings in many ways [4].

According to the World Health Organization, medicinal plants are used as the best resource to obtain a variety of bioactive compounds in the development of different drugs, those are effective as an antimicrobial, anti-tuberculosis, antioxidant, anticancerous, anti-inflammatory, antidiabetic, anthelmintic, hepatoprotective activity, larvicidal activity [5,6]. One of them plant is *Lantana camera*, Linn. The word *Lantana camera* Linn, was derived from the Latin word 'lento' meaning is to bend. Linnaeus first time described and placed them in the binomial system under the family of Verbenaceae. They are present worldwide with the native place America and also obtained from Mexico, Trinidad, Jamaica, Brazil, Florida, Africa, and India. *L. camera* is regionally differing in their names such as in Marathi: Ghaneri, Hindi: Raimuniya, Tamil: Unnichedi, Kannada: Kakke, Manipuri: Samballei and Telugu: Pulikampa [7]. In India, *L. camera* is considered as poisonous plant and included in top ten as invasive weeds [8]. Invasive weeds are nothing but a species that is not native to the ecosystem under consideration whose introduction causes economic or environmental harm or to human harm. Due to invasive nature *L. camera* is lagging behind in the list of medicinal plants for their uses in the drug preparation [9,10].

Therefore the main objective of the present review is to create awareness about ethnobotany and pharmacological studies of *L. camera* in society.

## 2. HISTORY

*L. camera's* range extends from Bega Shire in southern NSW to Cape Melville in north Queensland. It is found on the Lord Howe and Norfolk Islands. The chief infestations are east of the huge in-between range in NSW and QLD [3,4].

Pink-edged red lantana grows on the:

1. North Coast around Kempsey, south-east of Dorrigo, Bellingen, in the Coffs Harbour and Grafton areas Central Coast [8].
2. Red varieties grow on the North Coast, around Kempsey, Bellingen and Coffs Harbour.
3. *L. camera* is improbable to invade new regions in NSW. It is increasing in density and invades new areas within its range [8].
4. *L. camera* comes from the tropical and subtropical regions of Central and South America. It was introduced to Australia in 1841 as a decorative plant. By the 1860s it was common in Sydney and Brisbane [16].

## 3. TAXONOMY [6,9,14]

Kingdom: Plantae
Class: Angiosperms
Order: Lamiales
Family: Verbenaceae
Genus: Lantana
Species: *L. camera*

## 4. ECOLOGY

### 4.1 Habit

*L. camera* belongs to the family Verbenaceae. It is also known as a red sage. It grows up to 1-3 meter and can spread to 3 meters in width. It is a thorny multistemmed, deciduous shrub. It contains leaves which are opposite, simple, and aromatic, with long petioles, with oval rough

and hairy blades at margins [2]. Leaves are 3-8 cm long by 3-6 cm wide with green color, the pungent smell will emit when it is in crushed condition. Flowers are small, stalked, with different colors such as orange-blue-red-yellow-and bright red flowers. They are dense in the flat-topped cluster with corolla having the narrow tube with the four short spreading lobes. Flowers always go under color changes with weather conditions. Flowering occurs in between March and August [6]. The stem is square in outline, covered with bristly hairs. Roots are dipped in soil up to 50-100cm. they are yellowish-whitish in color with strong nature. Berries are rounded, fresh, fleshy, poisonous, 2-seeded, initially green-purple in color and finally turns into blue-black color. Berries are attractive to insects and birds due to their attractive nature. It is 6 – 8 mm in diameter, round berries, in a group, green to start, shiny, dark purple-black when ripe, and with one seed per fruit [1,6,7,9].

### 4.2 Habitat

*L. camera* is a tropical origin plant [3]. It requires diverse and broad geographic distribution. It is perennial shrub therefore mainly grows in open, disturbed areas such as roadside, railway tracks, and canals. It establishes at altitudes from the sea level up to 2000 m and can flourish extremely well under rainfall ranging area from 750 to 5000 m per annum. It belongs to Central and Northern South America and the Caribbean by birth [6]. It is currently spread in 60 countries. In India, this plant is spread over 7-10 states includes Maharashtra, Himachal Pradesh, Karnataka, Kerala, Uttar Pradesh, Uttarakhand, Gujarat, Rajasthan, Goa, and Arunachal Pradesh [8,11]. They require high sunlight for their growth. They grow in mostly grow in sandy or loamy soil which has pH 4 -9. The lowest temperature requires for their growth [12].

**Table 1**. General Characteristics and habitat of *L. Camera*

| Sr. No | Parameters | Description |
|---|---|---|
| 1 | Native | Tropical region in Central and Northern south America |
| 2 | Distribution | Nearly spreaded in 60 countries between 35°N and 35°S latitude. |
| 3 | Conservation studies | Unknown |
| 4 | Plant colour | Dark green |
| 5 | Plant features | Aromatic, Evergreen, Poisonous |
| 6 | Flowers | Long lasting, small, stalked, with different colors |
| 7 | Tolerances | Wind, slope, pollution, drought, heat and humidity |
| 8 | Pollinators | Lepidopteron species and thrip |
| 9 | Light | High sunlight |
| 10 | pH | 4.5 to 8.5 |
| 11 | Temperature | Below 45°C |
| 12 | Soil | Sandy to clay |
| 13 | Water | Any water (semi-arid to normal) |
| 14 | Propagation methods | Herbaceous stem cuttings |

## 5. ETHNOBOTANY

*L. camera* is an important medicinal plant. It has several medicinal applications in the medicinal field. The explants such as leaves, stem, roots, flowers, and seeds are used to extract different bioactive compounds by using different solvents such as water, methanol, ethanol, n-Butanol [6,7]. Leaves are used to treat cuts, rheumatisms, ulcers, catarrhal infection, tetanus, rheumatism, malaria, cancer, chicken pox, asthma, ulcer, swelling, eczema, tumor, high blood pressure, and bilious fever, ataxy of abdominal viscera, sores, measles, fevers, cold and high

blood pressure. The whole plant is used to cure bronchitis and the powdered root in milk was given to children for stomach-ache and as a vermifuge. Lantana oil is used to treat a skin infection, itches, and as an antiseptic for wounds [4,8,11].

### 5.1. Phytochemistry

Phytochemistry of *L. camera* has been extensively studied from long back for different applications. The explants of *L. camera* such as leaves, stem, roots, flowers, and seeds revealed different phytochemicals such as tannin, alkaloids, catechin, steroids, saponins, phenol, anthraquinone, protein, terpenoids, flavonoids, glycosides, different reducing sugars and essential oils. The essential oil consists consist Sabiene, 1,8- Cineole, β-caryophyllene, α-humulene, two are sesquiterpenoids [2,8,11].

**Table 2.** Used explants for the analysis of Phytochemistry

| Plant | Explants | References |
|---|---|---|
| *Lantana camera* Linn. | Leaves | Sing et al., 1999 |
| | Flowers | Day et al., 2003 |
| | Stalks | Underwood et al., 2003 |
| | Roots | Prasad et al., 2001 |
| | Seeds | Day et al., 2003 |
| | Berries | Thakur et al., 1992 |

## 6. PHARMACOLOGICAL STUDIES

In India, the Lachhiwala village is situated near to Dehradun around 24 km. The scientists from NGO Himalayan Environmental Studies and Conservation Organization (HESCO) established one lakhs hectors of land with L. camera plants. The village people use *L. camera* and mud to make the walls of their houses and chicken coops. They are using bark, stems for furniture, trays and baskets purposes. The *L. camera* leaves have been used to make excellent mosquito repellents and incense sticks. Such innovative use of the weed brings in Rs 75,000 a year for each of the families there therefore they have named village name as a Lantana village. All lantana villagers are using this L. camara plant for economic purposes [6,9].

### 6.1. Antimicrobial Activity

Different extracts of *L. camara* explants are used against different microorganisms such as bacteria and fungi. The explants such as leaves, stem, roots, flowers, and seeds extracts revealed effective Phytochemistry and their useful applications against different microorganisms. Aqueous, ethanol and methanol extracts of leaves, roots stem, and flowers of *L. camara* showed effective antibacterial activity against *E. coli, Proteus vulgaris, Vibrio cholareae, Bacillus subtilis, P. aeruginosa* and *Staphylococcus aureus* by disc diffusion and micro dilution method [2,4,7,9]. Further antifungal activity of *L. camara* extract was screened against Alternaria species which causes dissimilar plant diseases in vegetable plants. The activity was performed by food poison plate method at three different concentrations of extract viz, 10 mg/ml, 15 mg/ml and 20 mg/ml. At 20mg/ml dose *L. camara* exhibited significant antifungal activity against Alternaria species. Both extracts exhibited well-organized antifungal activity against white and brown rot fungi, however ethanol extract was highly potential at very low concentration (0.01%) therefore it is concluded that *L. camara* has effective antimicrobial activity [9-11].

### 6.2. Antimotility activity

*L. camara* has great antimotility activity which was proved by the report of leaves methanol extract antimotility activity in mice. 1 g/kg body weight dose was used to check the intestinal motility by charcoal meal test in mice which resulted into completely inhibition and Intraperitoneal management of 125 and 250 mg/kg body heaviness the extracts considerably reduced the fecal production in castor oil induced diarrhoea in mice [1-3].

### 6.3. Antiulcerogenic activity

Antiulcerogenic activity of the methanol extract of leaves of *L. camara* was reported on aspirin, ethanol and cold resistant stress induced gastric lesions in rats. Pre-treatment of the effected rats with the extract (200 and 400 mg/kg body weight) showed noteworthy defensive effect in aspirin induced, ethanol induced and cold restraint stress induced ulcers in rats. The extract resulted in dose dependent antiulcerogenic activity in all models [4-6].

### 6.4. Hemolytic activity

The hemolytic activity of *L. camara* aqueous extract and their solvent fractions was performed by spectroscopic method with different concentrations such as 125, 250, 500, 1000 µg/ml which was resulted into very low hemolytic activity towards the human erythrocytes [7,8].

### 6.5. Antihyperglycemic activity

Different extracts of *L. camara* has been proved effective and digestive antihyperglycemic activity. Extracts includes methanol extract of leaves, aqueous extract of roots. These both extracts were reported in alloxan induced diabetic rats. Oral administration of the methanol extract of *L. camara* (400 mg/kg body weight) leaves revealed decreased blood glucose up to 121.94 mg/dl. Extract action also showed development in body weight, HbA1c profile as well as renewal of liver cells [6,8,16].

### 6.6. Wound healing activity

Wound healing activity of aqueous leaves extract of *L. camara* was reported in rats. 100 mg/kg/day of dose significantly enhanced the rate of wound contraction (98%), synthesis of collagen and decreased wound healing occasion. Then ethanol extract of leaves of *L. camara* was also reported for wound healing activity in adult male Wister rats. Histological analysis of healed wounds established the role of take out in healing [5,16].

### 6.7. Antiinflammatory activity

Anti inflammatory activity of aqueous extract of *L. camara* was reported in albino rats. The 500mg/kg body weight significantly decreased paw volume in carrageenan induced paw oedema rat tests [11,12].

### 6.7. Antiurolithiasis activity

Different extracts of the *L. camara* leaves was reported for antiurolithiasis activity against ethylene glycol and ammonium chloride induced calcium oxalate urolithiasis in male albino rats which revealed significant reduction of the deposition of calcium, oxalate and also reduced urinary excretion of calcium, oxalate and creatinine [12,13].

## 7. CONCLUSION

*Lantana camara* is the poisonous, invasive, a noxious weed, present worldwide however due to insufficient knowledge and myths weed is lagging behind in the list of medicinal plants. The Ethanopharmaceutical and pharmacological studies revealed different aspects of *Lantana camara* therefore present study may helpful to create awareness in society about the plant.

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Published at http://ijate.net/index.php/ijsm http://dergipark.org.tr/ijsm **Research Article**

# Antioxidant and anti-inflammatory activity of capitula, leaf and stem extracts of *Tanacetum cilicicum* (Boiss.) Grierson

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**Abstract**: In this study, various extracts obtained different parts of *Tanacetum cilicicum* were investigated for *in vitro* antioxidant and anti-inflammatory activity. Antioxidant activity was tested with three methods; namely 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, 2.2′-azinobis (3-ethylbenzothiazolin-6-sulphonic acid) (ABTS) radical cation scavenging capacity, and ferric-reducing antioxidant power (FRAP) assays. Total phenolic and flavonoid contents of extracts were determined by Folin-Ciocalteu and aluminum chloride methods, respectively. Also, anti-inflammatory activity of these extracts was evaluated by 5-lipoxygenase inhibition assay. Ethyl acetate extract of capitula of *T. cilicicum* (TCCEA) showed the highest antioxidant activity with $IC_{50}$ values of 22.44 and 30.86 µg/mL against DPPH and ABTS radicals, respectively. At the same time, the highest ferric reducing power was found in the TCCEA (42.2 mg TE/g extract). The highest total phenolic contents have been detected in TCCEA and ethyl acetate extract of leaves of *T. cilicicum* (TCLEA) with value of 174.1 and 175.6 mg GAE/g extract, respectively. Similarly, the highest total flavonoid contents have been detected in TCCEA and TCLEA with values of 26.94 and 30.48 mg QE/g extract, respectively. TCCEA exhibited strong anti-inflammatory activity with $IC_{50}$ value of 9.44 µg/mL when compared to standard indomethacine (22.39 µg/mL). These results demonstrate that TCCEA has a significant antioxidant and anti-inflammatory activity. Also, the results show that TCCEA is a good candidate for further bioactivity-guided fractionation in the search for new active anti-inflammatory and antioxidant compounds.

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## 1. INTRODUCTION

Inflammation is a physiopathological response of living tissues and occurs against injuries causing accumulation of local plasmatic fluid and blood cells [1]. Inflammation is a complex process involving many different factors such as prostaglandins, leukotrienes and platelet activating factor (PAF) [2]. Although it is a defense mechanism of the body, complex events and mediators involving the inflammatory reaction can induce, or even aggravate, many

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diseases. Thus, anti-inflammatory agents are known to be effective agents in the treatment of these pathologies [1].

Chronic inflammatory diseases, one of the most important health problems in the world, are increasingly developing worldwide. Today, both steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are used to heal inflammation. Although steroids have an important role in the treatment of inflammatory diseases, they can be used only for short periods due to their toxicity. Prolonged use of NSAIDs can cause serious side effects, especially gastrointestinal bleeding [3]. It is known that some natural products are used as a good anti-inflammatory agent without the risk of side effects [4]. Also, It is generally recognized that free radicals play an important role in the development of tissue damage and pathological events [5]. There are free radicals in the pathology of many diseases including cancer, atherosclerosis, malaria, rheumatoid arthritis and neurodegenerative diseases [6]. Endogenous and exogenous antioxidants act as free radical scavengers by preventing and repairing the damage caused by free radicals and can therefore increase immune defense and reduce the risk of cancer and degenerative disease [7]. Antioxidant compounds derived from plants can reduce the formation of free radicals and cure the diseases caused by oxidative stress. Medicinal plants are known to have an important antioxidant and anti-inflammatory activity. Especially, phenolic and flavonoid compounds derived from medicinal plants contribute to the antioxidant activity of plants and function as anti-inflammatory agents [8].

The genus *Tanacetum* L., the third largest genus of the Asteraceae family, grows in the temperate regions of Europe and West Asia and comprises about 200 species. In Turkey, the genus *Tanacetum* is represented by 47 species or 61 taxa including subspecies and varieties and 27 of those are endemic [9,10]. *Tanacetum cilicicum* is 60-70 cm tall. Cauline leaves is greyish, 5-15 cm tall and pinnatisect [11]. *Tanacetum* species have been used as antipyretic and to treat headache, tinnitus, dizziness in traditional medicine for centuries in Turkey [12]. For example, *T. parthenium* has been used for years for the therapy of fever, migraine, menstrual disases, stomachache, toothache due to its anti-inflammatory activity [13]. Also, *Tanacetum* species are used as tonic, appetizers, anthelmintics, diuretics, carminatives, stimulants, emmenagogues in Turkey [14]. The members of this genus are rich in phenolic compounds which are responsible for their biological activities such as anti-inflammatory, antimicrobial, antifeedant, cytotoxic, insecticidal etc. [15,16]. Also, it is reported that different *Tanacetum* species have anticancer activity [17]. It has been demonstrated in previous study that different parts of *T. cilicicum* contain *p*-coumaric acid, ferulic acid, gallic acid, gentisic acid, chlorogenic acid, quercetin, naringenin and catechin [18]. *Tanacetum* species also contain essential oil (*α*-pinene, sabinene, limonene, eucalyptol, camphor, linalool, *α*-terpineol, borneol) and sesquiterpene lactones as secondary metabolites [19-21].

In this study, various extracts obtained different parts of *Tanacetum cilicicum* were examined for *in vitro* antioxidant and anti-inflammatory activity. According to our detailed literature search, there is a limited number of the study on *T. cilicicum.* There are only two study in literature regarding antioxidant properties of *T. cilicicum* [9,18] but antioxidant activities of different extracts of *T. cilicicum* obtained by using different solvents such as hexane, chloroform, ethyl acetate, ethanol were examined for the first time in the present study. Also, there is no study regarding anti-inflammatory properties of *T. cilicicum.* Therefore, this is the first study that *T. cilicicum* extracts were evaluated according to *in vitro* anti-inflammatory method.

## 2. MATERIAL and METHODS

### 2.1. Plant material and chemicals

Plant samples were collected at their flowering period from the Pülümür district of Tunceli in Turkey and identified by Dr. Ahmet Doğan, a botanist of the Faculty of Pharmacy, University of Marmara. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Marmara University (MARE No: 17768).

Chemicals and solvents of 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, Folin–Ciocalteau reagent sodium nitrite, aluminum chloride hexahydrate, sodium hydroxide, iron (II) chloride, 2,4,6-tris (2-pyridyl)-s-triazine, type V soybean lipoxygenase, sodium acetate, boric acid, ferric chloride, gallic acid, quercetin, ascorbic acid, indomethacine, linoleic acid, ferrozine, methanol, ethanol, hexane, chloroform, ethyl acetate, asetic acid, hydrochloric acid were obtained from Merck (Darmstadt, Germany), Sigma–Aldrich (St. Louis, MO, USA) and were of analytical grade.

### 2.2. Extraction

About 15 g of each dried and ground the capitula (TCCE), leaves (TCLE), and stem (TCSE) of *Tanacetum cilicicum* were extracted with 8×200 mL EtOH, using an ultrasonic bath. After filtration and evaporation, the ethanol extracts were dissolved in 30 mL 60% aqueous ethanol, and subjected to solvent-solvent partition between *n*-hexane (5×50 mL), chloroform (3×50 mL), and ethyl acetate extract (2x50 mL). The *n*-hexane, chloroform, ethyl acetate extract and aqueous ethanol extracts of *T. cilicicum* capitula obtained by this method were coded as TCCH, TCCC, TCCEA and TCCAE, respectively. The *n*-hexane, chloroform, ethyl acetate extract and aqueous ethanol extracts of *T. cilicicum* leaves obtained by this method were coded as TCLH, TCLC, TCLEA and TCLAE, respectively. The *n*-hexane, chloroform, ethyl acetate extract and aqueous ethanol extracts of *T. cilicicum* stem obtained by this method were coded as TCSH, TCSC, TCSEA and TCSAE, respectively. Extraction yields have been summarized in Table 1. All extracts were stored under refrigeration for further analysis.

### 2.3. Antioxidant activity

#### 2.3.1. *DPPH radical scavenging activity*

DPPH radical scavenging capacity of each extract was determined by the method of Zou et al. [22]. Briefly, 10 µL of extracts in DMSO at different concentrations (250-0.048 µg/mL) were added to 190 µL methanol solution of DPPH (0.1 mM) in a well of 96-well plate. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. Absorbance readings were taken at 517 nm. The percent radical scavenging activity of extracts and standard against DPPH were calculated according to the following:

$$\text{DPPH radical-scavenging activity (\%)} = [(A_0 – A_1)/A_0]\times 100$$

where $A_0$ is the absorbance of the control (containing all reagents except the test compounds), and $A_1$ is the absorbance of the extracts/standard. Extract concentration providing 50% inhibition ($IC_{50}$) was calculated from the graph plotting inhibition percentage against extracts concentration. Tests were carried out in triplicate. Ascorbic acid was used as positive control.

#### 2.3.2. *ABTS radical-scavenging activity*

ABTS radical cation scavenging activity assay was carried out according to the method described by Zou et al. [22]. ABTS radical cations were prepared by mixing equal volume of ABTS (7 mM in $H_2O$) and potassium persulfate (4.9 mM in $H_2O$), allowing them to react for

12-16 h at room temperature in the dark. Then, ABTS radical solution was diluted with 96% ethanol to an absorbance of about 0.7 at 734 nm. 10 µL of extracts in DMSO at different concentrations (250-0.048 µg/mL) were added to 190 µL of ABTS radical solution in a well of 96-well plate. The mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 min. Absorbance readings were taken at 734 nm. The percent radical scavenging activity of extracts and standard against ABTS were calculated according to the following:

$$\text{ABTS radical-scavenging activity (\%)} = [(A_0 – A_1)/A_0] \times 100$$

where $A_0$ is the absorbance of the control (containing all reagents except the test compounds), and $A_1$ is the absorbance of the extracts/standard. Extract concentration providing 50% inhibition ($IC_{50}$) was calculated from the graph plotting inhibition percentage against extracts concentration. Tests were carried out in triplicate. Trolox was used as positive control.

### 2.4. Determination of total phenolic contents (TPC)

Total phenolic contents of *Tanacetum cilicicum* extracts were measured using Folin–Ciocalteau reagent [23]. The assay was adapted to the 96 well microplate format. 10 µL of extracts in various concentrations (151.52-18.94 µg/mL) were mixed with 20 µL Folin-Ciocalteu reagent (Sigma), 200 µL of $H_2O$, and 100 µL of 15% $Na_2CO_3$, and the absorbance was measured at 765 nm after 2 h incubation at room temperature. Gallic acid was used as a standard and the total phenolics were expressed as mg GAE/g extract.

### 2.5. Determination of total flavonoid contents (TFC)

Total flavonoid content was determined following a method by Zhang at al. [24]. The assay was adapted to the 96 well microplate format. 25 µL extract of in various concentrations were mixed 125 µL of ultra pure water and 7.5 µL of 5 % $NaNO_2$. After 6 min, 15 µL of 10% $AlCl_3.6H_2O$ was added. After 5 min, 50 µL NaOH (1 M) was added and this solution completed with 250 µL of ultra pure water. The absorbance was measured against the reagent blank at 510 nm. The standard curve for total flavonoids was made using quercetin standard solution (0.488 to 250 µg/mL) under the same procedure as earlier described. The total flavonoids were expressed as milligrams of quercetin equivalents per g of dried fraction.

### 2.6. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power assay was carried out according to the method described by Zou et al. [22]. This method was developed to measure the ferric reduction ability at a low pH. When the ferric 2,4,6-tripyridyl-s-triazine complex (Fe3+-TPTZ) is reduced to the ferrous form (Fe2+-TPTZ), an intense blue color is developed. Briefly, the FRAP reagent was prepared by mixing 10 volumes of 250 mM acetate buffer (pH 3.6), with 1 volume of 10 mM TPTZ in 40 mM HCl and with 1 volume of 20 mM $FeCl_3.6\ H_2O$. A total of 10 µL of properly diluted samples and 30 µL of distilled water was added to 260 µL of freshly prepared FRAP reagent in a well of a 96-well plate. The mixture was incubated at 37 °C throughout the reaction. After 8 min, the absorbance was read at 593 nm against reagent blank. Tests were carried out in triplicate. The FRAP value of extracts was expressed as mg trolox/g extract.

### 2.7. *In vitro* anti-inflammatory activity

The anti-inflammatory activity was evaluated with slight modifications according to the method described by Phosrithong et al. [25]. The method was adapted to the 96 well transparent microplate with some modification. 10 µL at different concentrations of *Tanacetum cilicicum* extracts (5000-9.77 µg/mL) or standard indomethacine (250-0.49 µg/mL) were added to 20 µL ethanol, 20 µL pure water, 25 µL of sodium borate buffer solution (0.1 M, pH 9) followed by addition of 25 µL of type V soybean lipoxygenase solution in buffer (pH 9, 20.000 U/mL).

After the mixture was incubated at 25 °C for 5 min, 100 µL of 0.6 mM linoleic acid solution was added, mixed well and the change in absorbance at 234 nm was recorded for 6 min. Indomethacine was used as a reference standard. The percent inhibition was calculated from the following equation:

$$\% \text{ inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

A dose-response curve was plotted to determine the $IC_{50}$ values. $IC_{50}$ is defined as the concentration sufficient to obtain 50% of a maximum anti-inflammatory activity. All tests and analyses were performed in triplicates.

### 2.8. Statistical analysis

The data were given as means±standard deviations and analysed by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison tests using GraphPad Prism 5. Differences between means at $p<0.05$ levels were considered significant.

## 3. RESULTS

As shown in Table 1, all plant extracts were found to possess concentration-dependent inhibitory activity against DPPH radical except for hexane extracts. A low $IC_{50}$ value shows the high antioxidant activity. Therefore, TCCEA with $IC_{50}$ value of 22.44 µg/mL was superior than other extracts of *T. cilicicum* for DPPH assay. DPPH radical scavenging activity of *T. cilicicum* extracts decreased in the following order: TCCEA > TCLEA > TCCE > TCCC > TCLE > TCSEA > TCLAE > TCCAE > TCLC > TCSE > TCSC > TCCH > TCLH > TCSH (Table 1). In ABTS assay, TCCEA with $IC_{50}$ value of 30.86 µg/mL was better than other extracts of *T. cilicicum* for ABTS assay. ABTS radical scavenging activity of *T. cilicicum* extracts decreased in the following order: TCCEA > TCLEA > TCCE > TCCC > TCSEA > TCLAE > TCLC > TCLE > TCSC > TCCAE > TCSE > TCLH > TCLAE > TCSH > TCCH (Table 1). FRAP activity of the plant extracts were determined according to the equation ($y=0.0021x+0.0118$, $R^2=0.9922$) and expressed in terms of trolox equivalent (mg trolox/g extract). It was found that TCCEA (42.02 mg trolox/g extract) possessed the highest antioxidant activity in FRAP assay. Ferric reducing antioxidant power for extracts were reduced in the following order: TCCEA > TCCE > TCCC > TCSEA > TCLE > TCSC > TCLEA > TCLC > TCLAE > TCSE > TCCAE > TCSAE > TCLH > TCSH > TCCH.

Total phenolic contents were calculated according to the equation ($y=0.0056x+0.0466$, $R^2=0.9978$) obtained from calibration curve as gallic acid equivalent (mg/g extract), while total flavonoid contents were calculated according to the equation ($y=0.004x+0.0514$, $R^2=0.9967$) obtained from calibration curve as quercetin equivalent (mg/g extract). As shown in Table 2, the amount of total phenolics in extracts varied from 6.106 to 175.6 mg of gallic acid equivalent per gram dried extract and the total flavonoid contents varied from 6.136 to 30.48 mg quercetin equivalent per gram dried extract. The highest total phenolic and flavonoid levels have been detected in TCLEA and TCCEA which showed highest activity in DPPH, ABTS tests.

TCCEA inhibited 5-lipoxygenase activity by 99.21% at a concentration of 250 µg/mL. TCCEA exhibited very strong anti-lipoxygenase activity with $IC_{50}$ values of 9.44 µg/mL when compared to standard ($IC_{50}$ for indomethacin: 22.39 µg/mL) (Table 1). Also, TCSEA extract at a concentration of 250 µg/mL showed lowest anti-lipoxygenase activity with inhibition rate of 34.41% (Figure 1).

**Table 1.** Antioxidant and anti-inflammatory activities of various extracts obtained from different parts of *Tanacetum cilicicum*

| Extracts*/ Standards | Yield (%) | DPPH activity $IC_{50}$ (µg/mL) | ABTS activity (mg trolox/g extract)** | ABTS activity $IC_{50}$ (µg/mL) | FRAP activity (mg trolox/g extract)** | Anti-inflammatory activity $IC_{50}$ (µg/mL) |
|---|---|---|---|---|---|---|
| TCCE*** | 18.35 | 117.5±0.42$^{c}$ | 326.1 ± 1.89$^{c}$ | 42.32±0.13$^{d}$ | 41.89±0.90$^{a}$ | 156.0 ± 1.20$^{c,d}$ |
| TCCH | 25.87 | 981.00 ± 1.49$^{h}$ | 47.47 ± 0.00$^{j}$ | 238.4 ± 0.0$^{k}$ | 7.18 ± 0.05$^{i}$ | 167.5 ± 0.21$^{c,d,e}$ |
| TCCC | 24.40 | 126.5 ± 0.28$^{c}$ | 306.1 ± 7.55$^{d}$ | 45.62 ± 0.59$^{d,e}$ | 40.94 ±0.76$^{a,b}$ | 127.0 ± 2.05$^{b,c}$ |
| TCCEA | 12.07 | 22.44 ± 0.057$^{a}$ | 423.5 ± 0.00$^{a}$ | 30.86 ± 0.0$^{b}$ | 42.02 ± 1.31$^{a}$ | 9.44 ± 3.60$^{a}$ |
| TCCAE | 31.33 | 137.8 ± 0.35$^{c,d}$ | 214.1 ± 1.89$^{g}$ | 61.66 ± 0.17$^{h}$ | 16.82 ± 0.69$^{g}$ | 240.9 ± 24.25$^{f,g}$ |
| TCSE*** | 6.69 | 183.0 ± 1.27$^{e}$ | 212.8 ± 7.54$^{g}$ | 63.97 ± 0.84$^{h}$ | 30.39 ± 0.32$^{f}$ | 255.7 ± 14.99$^{g}$ |
| TCSH | 6.46 | 2052.00 ± 19.80$^{i}$ | 54.14 ± 9.43$^{i,j}$ | 210.4 ± 4.46$^{j}$ | 7.28 ± 0.15$^{i}$ | 202.9 ± 12.02$^{d,e,f,g}$ |
| TCSC | 10.53 | 191.0 ± 0.64$^{e}$ | 272.8 ± 1.88$^{f}$ | 51.73 ± 0.20$^{g}$ | 38.80 ± 0.10$^{b,c,d}$ | 112.4± 6.08$^{b,c}$ |
| TCSEA | 4.62 | 132.2 ± 0.35$^{c,d}$ | 291.5 ± 1.89$^{d,e}$ | 46.75 ± 0.18$^{d,e,f}$ | 40.32 ± 0.12$^{a,b}$ | 395.1± 2.76$^{i}$ |
| TCSSE | 69.65 | 557.5 ± 4.60$^{g}$ | 78.14 ± 1.89$^{h}$ | 179.7 ± 0.78$^{i}$ | 11.11 ± 0.72$^{h}$ | 360.3 ± 50.63$^{h}$ |
| TCLE*** | 22.55 | 130.3 ± 0.21$^{c}$ | 267.5 ± 1.89$^{f}$ | 51.48 ± 0.13$^{f,g}$ | 39.77 ± 0.17$^{a,b,c}$ | 187.4 ± 2.83$^{d,e,f}$ |
| TCLH | 17.62 | 1455.00 ± 9.19$^{i}$ | 64.80 ± 1.88$^{h,i}$ | 175.3 ± 0.71$^{i}$ | 10.12 ± 0.17$^{h}$ | 149.8 ± 3.75$^{c,d}$ |
| TCLC | 17.94 | 153.9 ± 0.28$^{d}$ | 279.5 ± 0.00$^{e,f}$ | 50.45 ± 0.46$^{f,g}$ | 36.57 ± 0.1$^{d,e}$ | 148.1± 1.06$^{c,d}$ |
| TCLEA | 7.41 | 61.49 ± 0.16$^{b}$ | 387.5 ± 1.89$^{b}$ | 35.43 ± 0.33$^{c}$ | 37.55 ± 1.62$^{c,d}$ | 85.18 ± 3.50$^{b}$ |
| TCLSE | 57.93 | 136.8 ± 0.14$^{c,d}$ | 278.1 ± 5.66$^{e,f}$ | 49.69 ± 0.59$^{e,f,g}$ | 34.44 ± 0.03$^{e}$ | 220.3 ± 4.31$^{e,f,g}$ |
| AA**** | | 17.6 ± 0.37$^{a}$ | | 14.5 ± 0.32$^{a}$ | | |
| Trolox**** | | 14.54 ± 0.18$^{a}$ | | 13.00 ± 0.21$^{a}$ | | |
| BHA**** | | 57.15 ± 0.09$^{b}$ | | 17.06 ± 0.58$^{a}$ | | |
| BHT**** | | 213.6 ± 15.20$^{f}$ | | 26.82 ± 1.12$^{b}$ | | |
| INDO | | | | | | 22.39 ± 0.2546$^{a}$ |

* Abbreviations: TCCE, TCCH, TCCC, TCCEA, TCCAE show the ethanol extracts and its *n*-hexane, chloroform, ethyl acetate, and aqueous ethanol fractions of the capitula of *Tanacetum cilicicum*, respectively. TCSE, TCSH, TCSC, TCSEA, TCSAE show the ethanol extracts and its *n*-hexane, chloroform, ethyl acetate, and aqueous ethanol fractions of the stems of *Tanacetum cilicicum* respectively. TCLE, TCLH, TCLC, TCLEA, TCLAE show the ethanol extracts and its *n*-hexane, chloroform, ethyl acetate, and aqueous ethanol fractions of the leaves of *Tanacetum cilicicum*, respectively.

** Results were expressed as trolox equivalent for ABTS and FRAP.

*** The yield of these extracts was calculated from the powdered dry plant. The yield of the remaining extracts was calculated from dry ethanol extracts.

**** AA: Ascorbic acid; BHA: Butylhydroxyanisole; BHT: Butylhydroxytoluene; INDO: Indomethacine

***** Each value in the table is represented as mean ± standard deviation (n=3). Different letter superscripts in the same column indicate significant differences ($P<0.05$).

**Table 2.** Total phenol and total flavonoid contents of various extracts obtained from different parts of *T. cilicicum*

| Extracts*/ Standards | TPC (mg GAE/g extract)** | TFC (mg QE/g extract)** |
|---|---|---|
| TCCE | $87.01 \pm 0.64^{b}$ | $14.25 \pm 0.64^{c,d}$ |
| TCCH | $10.28 \pm 0.20^{i,j}$ | $7.82 \pm 0.15^{d,e,f}$ |
| TCCC | $72.37 \pm 4.57^{c,d}$ | $13.68 \pm 1.55^{c,d,e}$ |
| TCCEA | $174.1 \pm 2.72^{a}$ | $26.94 \pm 0.56^{a,b}$ |
| TCCAE | $42.45 \pm 0.48^{g}$ | $8.57 \pm 1.94^{d,e,f}$ |
| TCSE | $28.44 \pm 0.16^{h}$ | $9.55 \pm 0.21^{d,e,f}$ |
| TCSH | $6.11 \pm 0.13^{j}$ | $5.84 \pm 3.58^{f}$ |
| TCSC | $68.85 \pm 0.48^{d,e}$ | $12.40 \pm 0.57^{c,d,e,f}$ |
| TCSEA | $77.36 \pm 0.70^{c}$ | $19.25 \pm 2.17^{b,c}$ |
| TCSAE | $12.08 \pm 0.0^{i,j}$ | $6.14 \pm 1.31^{e,f}$ |
| TCLE | $57.58 \pm 1.62^{f}$ | $11.43 \pm 0.39^{c,d,e,f}$ |
| TCLH | $13.63 \pm 0.22^{i}$ | $9.03 \pm 2.94^{d,e,f}$ |
| TCLC | $63.28 \pm 0.35^{e,f}$ | $15.29 \pm 2.72^{c,d}$ |
| TCLEA | $175.6 \pm 0.0^{a}$ | $30.48 \pm 0.48^{a}$ |
| TCLAE | $40.59 \pm 0.19^{g}$ | $11.16 \pm 3.80^{d,e,f}$ |

* Abbreviations: TCCE, TCCH, TCCC, TCCEA, TCCAE show the ethanol extracts and its *n*-hexane, chloroform, ethyl acetate, and aqueous ethanol fractions of the capitula of *Tanacetum cilicicum*, respectively. TCSE, TCSH, TCSC, TCSEA, TCSAE show the ethanol extracts and its *n*-hexane, chloroform, ethyl acetate, and aqueous ethanol fractions of the stems of *Tanacetum cilicicum* respectively. TCLE, TCLH, TCLC, TCLEA, TCLAE show the ethanol extracts and its *n*-hexane, chloroform, ethyl acetate, and aqueous ethanol fractions of the leaves of *Tanacetum cilicicum*, respectively.

** Results were expressed as gallic acid equivalent (GAE) for TPC, as quercetin equivalent for TFC.

*** Each value in the table is represented as mean ± standard deviation (n=3). Different letter superscripts in the same column indicate significant differences ($P<0.05$).

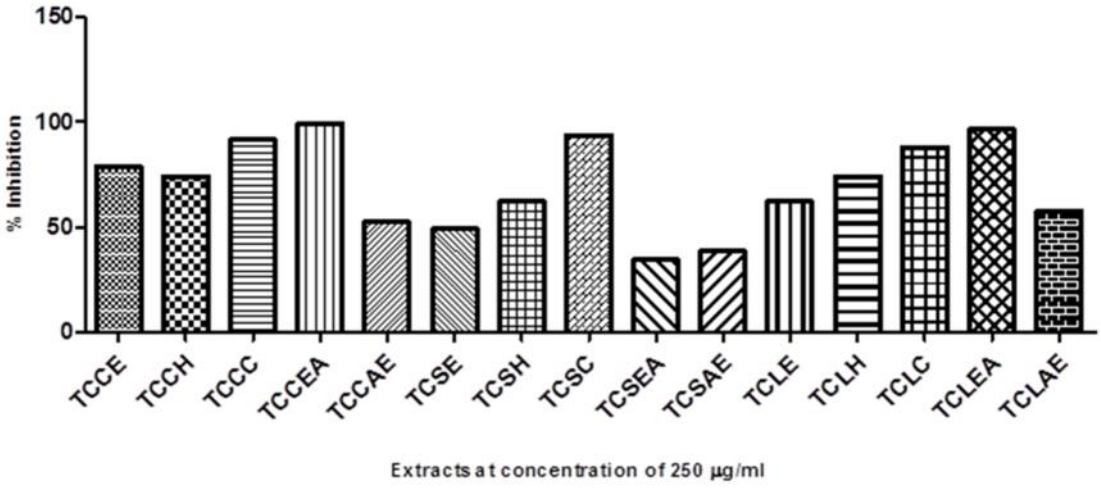


**Figure 1.** Anti-inflammatory activity of various extracts obtained from different parts of *T. cilicicum*

## 4. DISCUSSION

In the present study, the antioxidant and anti-inflammatory activities of ethanol extracts of the capitula, leaves, and stem of *Tanacetum cilicicum* and their *n*-hexane, chloroform, ethyl acetate and aqueous ethanol fractions were evaluated. TCCEA against DPPH radical, TCCEA, TCLEA against ABTS radical, and TCCEA, TCCE, TCCC, TCSEA against FRAP exhibited strong antioxidant activity.

The antioxidant activity of the extracts was investigated by three methods; DPPH radical-scavenging activity, ABTS radical-scavenging activity, and Ferric reducing antioxidant power (FRAP) assay. DPPH is a purple colored radical that transforms into a yellow non-radical DPPH in the presence of a strong antioxidant molecule. This color change occurs when the DPPH radical receives a hydrogen from the antioxidant molecule [26]. The ABTS radical cation decolorization assay is a method for screening antioxidant activities of molecules and is applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxysinamates, carotenoids and plasma antioxidants. Pre-formed radical monocation of 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) is produced by oxidation of ABTS with potassium persulfate and decreases in the presence of hydrogen-donating antioxidants [27]. The FRAP test measures the reduction capacity of the antioxidant agents by reducing $Fe^{3+}$ to $Fe^{2+}$. In this reaction, the bright red salt of potassium ferricyanide solution is reduced to the potassium ferrocyanide complex by taking an electron from the antioxidant molecule. The color of reaction mixture changes from green-yellow to Perl's Prussian blue color [26].

In one study, antioxidant activity of methanol extracts of flower, leaf and stem of *T. cilicicum* were investigated by various antioxidant experiments. It was reported that leaf, flower and stem of this species showed DPPH scavenging activity with $IC_{50}$ values of 25.95 µg/mL, 30.26 µg/mL and 117.48 µg/mL, respectively [18]. In another study, it was found that methanol extract prepared with aerial parts of *T. cilicicum* had an $IC_{50}$ value of 249.17 µg/mL against DPPH radical [9]. When these studies compare with activities of ethanol extracts (because they have approximately the same polarity) prepared from capitula (117.5 µg/mL), stem (183.0 µg/mL), and leaves (130.3 µg/mL) of *T. cilicicum* in our current study, we found that they had lower activity than the results of the first study but higher than the results of the second study. Also, ethyl acetate extract prepared from capitula of *T. cilicicum* (TCCEA) in our present study showed a strong activity with $IC_{50}$ value of 22.44 µg/mL against DPPH radical. TCCEA was found to have higher activity than BHT (213.6 µg/mL) and BHA (57.15 µg/mL) and close to ascorbic acid (17.6 µg/mL) and trolox (14.54 µg/mL) compared to the standards.

Arıtuluk et al. (2016) revealed that trolox equivalent antioxidant capacity (TEAC) of methanol extract of *T. cilicicum* aerial parts in ABTS test was 66.98 mg trolox equivalent per g extract (TE)[9]. When this study compare with activities of ethanol extracts (because they have approximately the same polarity) prepared from capitula (326.1 mg TE/g), stem (212.8 mg TE/g), and leaves (267.5 mg TE/g) of *T. cilicicum* in our current study, we found that they had the higher trolox equivalent antioxidant capacity. Also, ABTS activity of TCCEA in our present study, it was found to be quite high compared to the activities of all extracts (423.5 mg TE/g or $IC_{50}$: 30.86 µg/mL). Also, ethyl acetate extract prepared from capitula of *T. cilicicum* (TCCEA) in our present study showed a significant activity with $IC_{50}$ value of 30.86 µg/mL against ABTS radical. TCCEA was found to have lower activity than BHA (17.06 µg/mL), ascorbic acid (14.5 µg/mL) and trolox (13.00 µg/mL) and close to BHT (26.82 µg/mL) compared to the standards.

Arıtuluk et al. (2016) showed that methanol extract of aerial parts of *T. cilicicum* has ferric reducing antioxidant power of 198.19 mg QE/g extract [9]. In contrast to this study, ethanol extracts were obtained from the plant in our current study and the results were expressed as trolox equivalent, not as quercetin. When this study compare with activities of ethanol

extracts (because they have approximately the same polarity) prepared from capitula (41.89 mg TE/g), stem (30.39 mg TE/g), and leaves (39.77 mg TE/g) of *T. cilicicum* in our current study, we found that they had the lower ferric reducing antioxidant power. Also, TCCEA possessed the highest FRAP activity with 42.02 mg trolox/g extract while TCCH had the lowest FRAP activity with 7.175 mg trolox/g extract.

Gecibesler et al. (2016) reported that total phenolic amount of methanol extracts obtained from different parts of *T. cilicicum* were found ranging from 99.53±2.39 to 268.02±0.97 mg GAE/g extract. They found that phenolic contents for the methanol extract of flower of *T. cilicicum* were higher than leaf and stem of *T. cilicicum* [16]. In other study, Arıtuluk et al. (2016) indicated that amount of total phenol of methanol extract of *T. cilicicum* aerial parts was 33.14 mg gallic acid equivalent per g extract (GAE) [9]. When compared to our present study, total phenolic amounts for ethanol extracts of capitula (87.01 mg/g), leaves (57.58 mg/g) and stem (28.44 mg/g) of *T. cilicicum* were found to be lower than the results of the first study but higher than the results of the second study except for TCSE. Also, total phenol contents of TCLEA and TCCEA were found to be considerably high with values of 175.6 mg GAE/g extract and 174.1 mg GAE/g extract, respectively. The lowest phenolic amount was found in hexane extract of flower of *T. cilicicum* (6.106 mg GAE/g).

Gecibesler et al. (2016) reported that total flavonoid amounts of methanol extracts obtained from different parts of *T. cilicicum* varied from 26.76 ± 3.53 to 86.41 ± 1.62 mg QE/g of the extract. It was found that the flavonoid content of the flower parts (26.76 ± 3.53 mg QE/g extract) was the lowest followed by stem (29.59 ± 2.58 mg QE/g extract). However, the leaves had the highest total flavonoid content. (86.41± 1.62 mg QE/g extract). [16]. In other study, Arıtuluk et al. (2016) indicated that amount of total flavonoid of methanol extract of *T. cilicicum* aerial parts was 18.73 mg quercetin equivalent per g extract (QE) [9]. When compared to our present study, total flavonoid contents for ethanol extracts of capitula (14.25 mg/g), leaves (11.43 mg/g) and stem (9.55 mg/g) of *T. cilicicum*, were found to be lower than the results of the previous studies. However, total flavonoid content of TCLEA and TCCEA were found to be high with values of 30.48 and 26.94 mg QE/g extract when compared to the study of Arıtuluk et al. (2016), respectively. The lowest flavonoid content was observed in TCSH with 5.837 mg QE/g extract.

In addition, the total phenol and flavonoid contents of TCCEA and TCLEA, which had a high antioxidant activity compared to other extracts, were found to be high. Therefore, these compounds can be considered responsible for the antioxidant activity of the extract. Although the antioxidant activity of TCCEA was higher than TCLEA, the total amount of phenolic and flavonoid compounds was lower than TCLEA (although there was no statistically significant difference). This indicates that the activity of TCCEA may be due to not only phenolic compounds found in the extract but also non-phenolic compounds such as terpenoid compounds. It supports this view in a previous study conducted by Ali et al. In this study, the sesquiterpene and triterpene compounds were isolated from the ethyl acetate extract of a different *Tanacetum* species, *Tanacetum abrotonifolium* [28].

Polar solvents are more effective in obtaining phenolic compounds from plants compared to less polar solvents [26]. Less polar solvents such as hexane and $CHCl_3$, carry non-polar and non-phenolic compounds such as terpenoids and methoxylated flavonoids [26,29-30].For example, in our current study, polar extracts as EtOAc and MeOH extracts had higher total phenolic and flavonoid content than hexane and $CHCl_3$ extracts. The results of present study are similar to those of See et al. (2017).

No study has been found about anti-inflammatory activity of the plant in literature. However, some parts of *Tanacetum* species traditionally have been used by the people for anti-inflammatory purposes [31] and there are some studies regarding anti-inflammatory properties

of other *Tanacetum* species [13]. In our current study, it was found that especially the TCCEA had significant anti-inflammatory activity with $IC_{50}$ value of 9.44 µg/mL. In addition, it has been found that it has a higher anti-lipoxygenase activity than indomethacin (22.39 µg/mL) which is used as standard. These results confirm ethnobotanical use of the *Tanacetum* species. Also, flavonoids and sesquiterpene lactones are known as important compounds with anti-inflammatory activity [32]. Therefore, the reason why TCCEA exhibits good anti-inflammatory activity may be due to the presence of high phenolic compounds.

## 5. CONCLUSION

These results show that ethyl acetate extract from capitula of *Tanacetum cilicicum* has a strong anti-inflammatory and antioxidant potency. In addition to the *in vivo* experiments that will be carried out on TCCEA, it is clear that more scientific studies are needed to elicit compounds that are responsible for antioxidant and anti-inflammatory effect.

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