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RESEARCH ARTICLE

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Phytochemical analysis of *Robinia pseudoacacia* flowers and leaf: quantitative analysis of natural compounds and molecular docking application

Robinia pseudoacacia çiçekleri ve yaprağının fitokimyasal analizi: doğal bileşiklerin kantitatif analizi ve moleküler doking uygulaması

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Anahtar Kelimeler: BChE, DD peptidaz Kantitatif analiz, LC-MS/MS, Moleküler yerleştirme, *Robinia pseudoacacia*

ABSTRACT

Phenolic compounds are widely found and well-known secondary metabolites in plants. Identification, and quantification of phenolic compounds, and determination of their biological activities reveal the unknown secrets of plants. *Robinia pseudoacacia* (RP) is known as the white-flowered false acacia and is distributed in Northern Anatolia in Turkey. Spectrophotometric and chromatographic techniques are used to identify the presence and amount of phenolics. In this study, RP flowers and leaves were extracted with methanol and analyzed by LC-MS/MS to determine their phytochemical content. Salicylic acid and syringic acid were found as major products in leaves and flowers. RP extracts have been reported to have antibacterial activity and BChE inhibitory properties. Therefore, the BChE and DD peptidase enzyme inhibitory properties of the main components salicylic acid and syringic acid were investigated by molecule docking (MolDock). According to MolDock results, syringic acid interacted with BChE and DD peptidase and was calculated as a MolDock score of -79.38, and -71.25, with binding energies -5.90, and -5.40 kcal/mol respectively. Salicylic acid interacted with BChE and DD peptidase and was calculated as a MolDock score of -63.54, and -66.18, with binding energies of -6.10, and -5.70 kcal/mol respectively. As a result, salicylic acid had higher binding energy in its interactions with BChE and DD peptidase enzymes. In theory, salicylic acid can be used as a good BChE and DD peptidase inhibitor.

Öz

Fenolik bileşikler bitkilerde yaygın olarak bulunan ve iyi bilinen sekonder metabolitlerdir. Fenolik bileşiklerin tanımlanması, miktarlarının belirlenmesi ve biyolojik aktivitelerinin belirlenmesi, bitkilerin bilinmeyen sırlarını ortaya çıkarmaktadır. *Robinia pseudoacacia* (RP), beyaz çiçekli sahte akasya olarak bilinir ve Türkiye'de Kuzey Anadolu'da yayılış gösterir. Fenoliklerin varlığını ve miktarını belirlemek için spektrofotometrik ve kromatografik teknikler kullanılır. Bu çalışmada, RP çiçekleri ve yaprağı metanol ile ekstrakte edilmiş ve fitokimyasal içeriğini belirlemek için LC-MS/MS ile analiz edilmiştir. Yaprak ve çiçeklerde salisilik asit ve siringik asit ana ürünler olarak bulunmuştur. RP ekstraktlarının antibakteriyel aktiviteye ve BChE inhibitör özelliklerine sahip olduğu rapor edilmiştir. Bu nedenle, ana bileşenler olan salisilik asit ve siringik asidin BChE ve DD peptidaz enzimini inhibe edici özellikleri, molekül yerleştirme (MolDock) yöntemiyle araştırıldı. MolDock sonuçlarına göre, siringik asit, BChE ve DD peptidaz etkileşimi sonucu MolDock skoru -79.38, -71.25 ve bağlanma enerjileri -5.90, -5.40 kcal/mol olarak sırasıyla hesaplandı. BChE ve DD peptidaz ile etkileşimi sonucu salisilik asidin, MolDock skoru -63.54, -66.18 ve bağlanma enerjileri -6.10, -5.70 kcal/mol olarak sırasıyla hesaplandı. Sonuç olarak salisilik asidin BChE ve DD peptidaz enzimleri ile etkileşimlerinde daha yüksek bağlanma enerjisine sahip olduğu görüldü. Teorik olarak salisilik asit iyi bir BChE ve DD peptidaz inhibitörü olarak kullanılabilir.

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1. INTRODUCTION

Herbal medicines, one of the most important components of traditional medicine, have unique variations in chemical components (Topçu et al., 1999; Elmastas et al., 2004; Demirtas et al., 2013; Sahin Yaglioglu et al., 2013). For this reason, compounds effective in the treatment of many diseases have been isolated from plants with a wide range of biological activities and introduced to the pharmaceutical industry (Aksit et al., 2014; Bayir et al., 2014; Erenler et al., 2014; Türkmen et al., 2014). In recent studies, it has been shown that phytochemical natural compounds (steroids, carotenoids, phenolic compounds, alkaloids, and vitamins) treat diseases and prevent the formation of diseases in humans and animals. Treatments of existing diseases in the world are evaluated in many ways with the developing technology and solutions are sought (Erenler et al., 2015; Aydin et al., 2016; Elmastas et al., 2016; Erenler, et al., 2016a; Erenler, et al., 2016b). Interest in medicinal plants, which are widely used among the public in Turkey, is increasing day by day. For this reason, with the developing technology, the research of plants used for medicinal purposes in our country and the detection of active ingredients contribute greatly to the country's economy (Erenler, 2016c; Erenler et al., 2016d; Karan et al., 2016; Erenler et al., 2017a; Erenler et al., 2017b). Natural products have been used commonly in nanotechnology, basically green synthesis of nanoparticles (Erenler et al., 2023a; Erenler et al., 2023b; Erenler et al., 2023c; Erenler & Hosaflioglu 2023; Karan et al., 2024a; Karan et al., 2024b).

Robinia species is a perennial plant belonging to the Fabaceae family, popularly called acacia in our country. *Robinia pseudoacacia* (RP) is known as the white-flowered false acacia and is distributed in Northern Anatolia in Turkey. RP is rich in compounds such as polyphenolics, tannins, and monoterpenes. Additionally, different extracts have been reported to have antibacterial activity and AChE and BChE enzyme inhibitory properties. It has a strong backup potential thanks to the polyphenols contained in the flowers. It is used among the public as a stomach acid reducer, sedative, and antispasmodic (Vítková et al., 2017).

Enzyme Inhibitors are a control mechanism in biological systems. It is known that enzyme inhibitors are effective in the treatment of many diseases. There are some herbs commonly used in traditional medicine to reduce the symptoms of Alzheimer's disease. Acetylcholinesterase (AChE) inhibitors inhibit the breakdown of acetylcholine, thus strengthening central and peripheral cholinergic function. One of the most commonly used approaches in the treatment of Alzheimer's, senile dementia, ataxia, myasthenia gravis, and Parkinson's diseases is AChE inhibition (Mukherjee et al., 2007; Atalar et al., 2023). Butyrylcholinesterase (BChE); is found in the pancreas, central nervous system, liver, and serum. The esterase activity of BChE is important in removing organophosphate and carbamate inhibitors from the circulation before they reach AChE, and in controlling cholinergic neurotransmission in AChE deficiency (Çokuğraş et al., 2003; Rao et al., 2007). Bacterial diseases occur when bacteria enter the body, destroying healthy bacteria, or beginning to grow in sterile tissues (Aissous et al., 2023). DD-peptidase enzyme (penicillin-binding) catalyzes the final reaction in the biosynthesis of the cell wall of the bacterium. The main approach to the treatment of bacterial infections is the use of antibiotics. The importance of discovering new drugs containing natural molecules without side effects is increasing. Therefore, natural compounds can be medicines; Their interactions with enzymes are observed with programs such as molecular docking. Molecular docking is a web-based method that predicts binding or non-binding orientation in the ligand-protein complex. It is an in silico approach used to theoretically predict the binding affinity and activity of the molecule to protein targets (Boussaha et al., 2024).

RP flowers and leaf methanol extracted were analyzed by LC-MS/MS to determine their phytochemical content. Biological activity applications are a costly and time-consuming process. Enzyme inhibitory properties of molecules are calculated theoretically through molecular docking applications. Therefore, Syringic acid and salicylic acid, which are determined as the common and main components in the flower and leaf; BChE and DD peptidase inhibitory properties, were investigated. Thus, the accuracy of this plant's antibacterial properties as well as its use in the treatment of memory loss and central nervous system disorders was determined.

2. MATERIALS AND METHODS

2.1. Plants

RP was harvested in the city of Van and identified by Dr. Murat Ünal, Van Yüzüncü Yıl University, a voucher specimen was deposited in the Herbarium of the same university (VANF20432).

2.2. Extraction

Sex- Each leaf and flower of a plant (30 g) were extracted with methanol (500 mL) in a flask separately. The solvent

extract mixture was filtered and the solvent was evaporated using a rotary evaporator to yield the crude extract (Gecer & Erenler, 2023).

2.3. Quantitative analysis

The phenolic contents and amounts of RP leaf and flower of methanol extract were determined by LC-ESI-MS/MS analysis (Erenler et al., 2023c; İpek et al., 2024). Forty-one phenolic standards were employed for LC-ESI-MS/MS analysis (Figure 1).

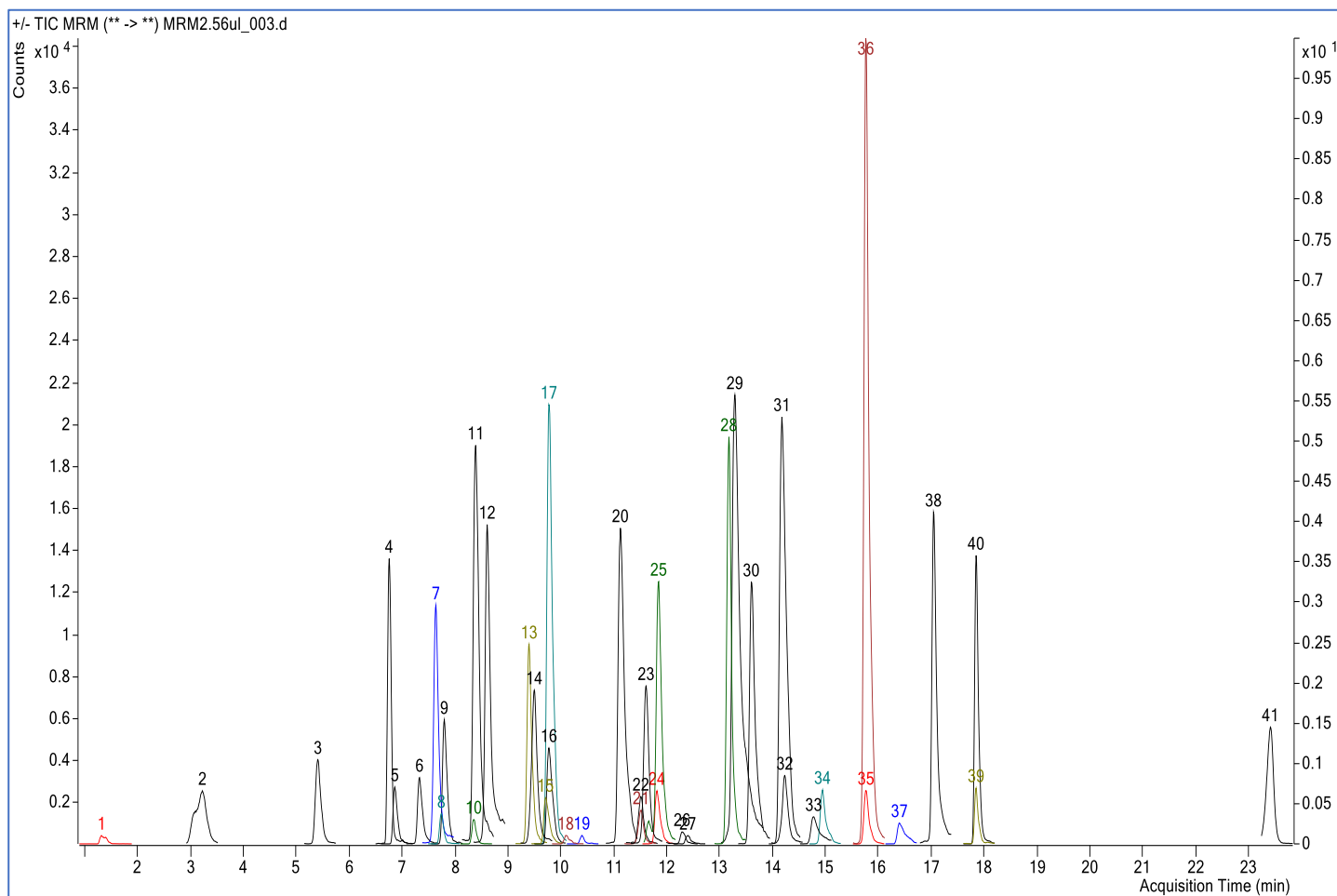


Figure 1. LC-MS/MS standard chromatogram: Shikimic acid (1), Gallic acid (2), Protocatechuic acid (3), Epigallocatechin (4), Catechin (5), Chlorogenic acid (6), Hydroxybenzaldehyde (7), Vanillic acid (8), Caffeic Acid (9), Syringic acid (10), Caffein (11), Vanillin (12), o-Coumaric acid (13), Salicylic acid (14), Taxifolin (15), Resveratrol (16), Polydatine (17), *Trans*-ferulic acid (18), Sinapic acid (19), Scutellarin (20), p-Coumaric acid (21), Coumarin (22), Protocatechuic ethyl ester (23), Hesperidin (24), Isoquercitrin (25), Rutin (26), Quercetin-3-xyloside (27), Kaempferol-3-glucoside (28), Fisetin (29), Baicalin (30), Chrysin (31), *Trans*-cinnamic acid (32), Quercetin (33), Naringenin (34), Hesperetin (35), Morin (36), Kaempferol (37), Baicalein (38), Luteolin (39), Biochanin A (40), Diosgenin (41)

2.4. Molecular docking application

The drawing, 3D structure, and minimum energy of the syringic acid and salicylic acid were calculated in the ChemDraw software. The enzymes chosen for this docking study were BChE [PDB ID: 1POI] and DD peptidase [PDB ID:3PTE]. Syringic acid and salicylic acid molecules, DD peptidase, and BChE interactions were determined using the Molegro Virtual Docker (MVD) program (Başar et al., 2024). 2D and 3D images of the interactions were taken with the BIOVIA Discovery Studio Visualizer program. Also, The AutoDock Vina program was used to calculate the binding affinities (Başar et al., 2023; Yenigün et al., 2023).

3. RESULTS AND DISCUSSIONS

3.1. Quantitative analysis of bioactive compounds

When using the plant for medicinal purposes, its phytochemical content must be known (Erenler et al., 2023d). For this purpose, the quantitative analysis of bioactive compounds (phenolics, flavonoids, etc.) in plants is determined by devices such as HPLC, LC-MS, and

LC-MS/MS (Erenler et al., 2018). In this study, a quantitative analysis of phenolic compounds in the methanol extract of the leaf and flower parts of the RP plant was performed (Table 1). According to the analysis results, on the leaf; syringic acid (24.78 µg/g extract), salicylic acid (14.86 µg/g extract), and kaempferol (10.30 µg/g extract), in flower; rutin (199.74 µg/g extract), salicylic acid (26.41 µg/g extract), and syringic acid (19.23 µg/g extract) were detected in the highest amount (Table 1). Syringic acid and salicylic acid are the main components in the flower and leaf. Syringic acid is a naturally occurring phenolic compound found in a wide variety of herbal products (Erenler et al., 2023e). It has biological activities such as antioxidant, antimicrobial, anticancer, antidiabetic, and anti-inflammation. Moreover, it is known to be friendly to the central nervous system, brain, heart, and liver. Salicylic acid is a β-hydroxy acid derived from salicin. Salicylic acid has been reported to have biological activities such as anti-inflammatory and anti-tumor, anti-cancer, anti-bacterial, and anti-oxidant (Randjelović et al., 2015).

Table 1. Quantitative analysis of natural compounds of RP leaf and flowers (µg/g extract)

No	Compound	RT	RPL	RPF
1	Gallic acid	3.23	3.39	4.37
2	Protocatechuic acid	6.08	-	2.46
3	Chlorogenic acid	7.11	-	1.10
4	Hydroxybenzaldehyde	7.60	1.46	1.96
5	Caffeic Acid	7.77	-	1.11
6	Syringic acid	9.11	24.78	19.23
7	Vanillin	9.26	2.12	2.49
8	o-coumaric acid	9.39	1.26	1.32
9	Salicylic Acid	9.54	14.86	26.41
10	<i>Trans</i> -ferulic acid	10.12	5.13	4.019
11	Sinapic acid	10.77	2.79	2.56
12	p-coumaric acid	11.54	1.35	1.30
13	Hesperidin	11.84	-	67.29
14	Isoquercitrin	11.81	-	4.30
15	Rutin	13.09	-	199.74
16	Kaempferol-3-glucoside	13.29	-	11.05
17	Fisetin	13.44	1.95	2.19
18	Naringenin	15.07	3.47	3.58
19	Hesperetin	15.87	3.08	3.01
20	Kaempferol	16.12	10.30	10.69
21	Luteolin	17.88	10.14	-
22	Diosgenin	23.56	1.48	-

3.2. Molecular Docking Result

According to the LC-MS/MS analysis results; syringic acid and salicylic acid are the common and most abundant compounds in leaves and flowers. In the literature, RP plant extracts have been reported to have anti-alzheimer and antibacterial activity (Vítková et al., 2017). Therefore, the BChE (anti-Alzheimer) and DD peptidase (antibacterial) inhibitory properties of syringic acid and salicylic acid molecules, which are the main components of the RP plant extract, were investigated (Figure 2- Figure 3).

Syringic acid molecule interacted with BChE; Five conventional hydrogen bonds with amino acid THR120, TYR128, GLU197, TRP82, one carbon-hydrogen bond with amino acid TRP82, one pi-sigma with amino acid TRP82, pi-pi-stacked with amino acid TRP82, alkyl with amino acid with LEU125 and pi-alkyl amino acid with TRP82 (Figure 2-Table2). Syringic acid with BChE was calculated as a MolDock score of -79.38, with binding energies of -5.90 kcal/mol.

Table 2. Interaction categories, types, and distances of molecular insertion of the syringic acid molecule with BChE

No	Name	Distance	Category	Type	Transmitter	From Chemistry	Receiver	To Chemistry
1	A:THR120:OG1 - :[001:O4	3.02952	Hydrogen Bond	Conventional Hydrogen Bond	A:THR120:OG1	H-Donor	:[001:O4	H-Acceptor
2	A:THR120:OG1 - :[001:O5	3.14928	Hydrogen Bond	Conventional Hydrogen Bond	A:THR120:OG1	H-Donor	:[001:O5	H-Acceptor
3	A:TYR128:OH - :[001:O3	3.09903	Hydrogen Bond	Conventional Hydrogen Bond	A:TYR128:OH	H-Donor	:[001:O3	H-Acceptor
4	:[001:H3 - A:GLU197:OE1	2.09025	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H3	H-Donor	A:GLU197:OE1	H-Acceptor
5	:[001:H7 - A:TRP82:O	2.06705	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H7	H-Donor	A:TRP82:O	H-Acceptor
6	:[001:H5 - A:TRP82:O	2.62529	Hydrogen Bond	Carbon Hydrogen Bond	:[001:H5	H-Donor	A:TRP82:O	H-Acceptor
7	A:TRP82:CB - :[001	3.88238	Hydrophobic	Pi-Sigma	A:TRP82:CB	C-H	:[001	Pi-Orbitals
8	A:TRP82 - :[001	4.99669	Hydrophobic	Pi-Pi Stacked	A:TRP82	Pi-Orbitals	:[001	Pi-Orbitals
9	A:TRP82 - :[001	4.97275	Hydrophobic	Pi-Pi Stacked	A:TRP82	Pi-Orbitals	:[001	Pi-Orbitals
10	:[001:C8 - A:LEU125	3.44254	Hydrophobic	Alkyl	:[001:C8	Alkyl	A:LEU125	Alkyl
11	A:TRP82 - :[001:C9	5.26866	Hydrophobic	Pi-Alkyl	A:TRP82	Pi-Orbitals	:[001:C9	Alkyl
12	A:TYR128 - :[001:C8	5.01949	Hydrophobic	Pi-Alkyl	A:TYR128	Pi-Orbitals	:[001:C8	Alkyl

Syringic acid molecule interacted with DD peptidase; Three carbon-hydrogen bonds with amino acid VAL302, SER326, THR123, one pi sigma with amino acid PHE120, one amide-pi stacked with amino acid VAL302, and one

pi-alkyl with amino acid TYR306 (Figure 2-Table 3). Syringic acid with DD peptidase was calculated as a MolDock score of -71.25, with binding energies of -5.40 kcal/mol.

Table 3. Interaction categories, types, and distances of molecular insertion of the syringic acid molecule with DD peptidase

No	Name	Distance	Category	Type	Transmitter	From Chemistry	Receiver	To Chemistry
1	A:VAL302:HA - :[001:O2	2.73206	Hydrogen Bond	Carbon Hydrogen Bond	A:VAL302:HA	H-Donor	:[001:O2	H-Acceptor
2	A:SER326:HB2 - :[001:O1	2.43792	Hydrogen Bond	Carbon Hydrogen Bond	A:SER326:HB2	H-Donor	:[001:O1	H-Acceptor
3	:[001:H9 - A:THR123:O	2.35551	Hydrogen Bond	Carbon Hydrogen Bond	:[001:H9	H-Donor	A:THR123:O	H-Acceptor
4	:[001:H7 - A:PHE120	2.82865	Hydrophobic	Pi-Sigma	:[001:H7	C-H	A:PHE120	Pi-Orbitals
5	A:VAL302:C,O;GLN303:N-:[001	4.56129	Hydrophobic	Amide-Pi Stacked	A:VAL302:C,O;GLN303:N	Amide	:[001	Pi-Orbitals
6	A:TYR306 - :[001:C1	5.1516	Hydrophobic	Pi-Alkyl	A:TYR306	Pi-Orbitals	:[001:C1	Alkyl

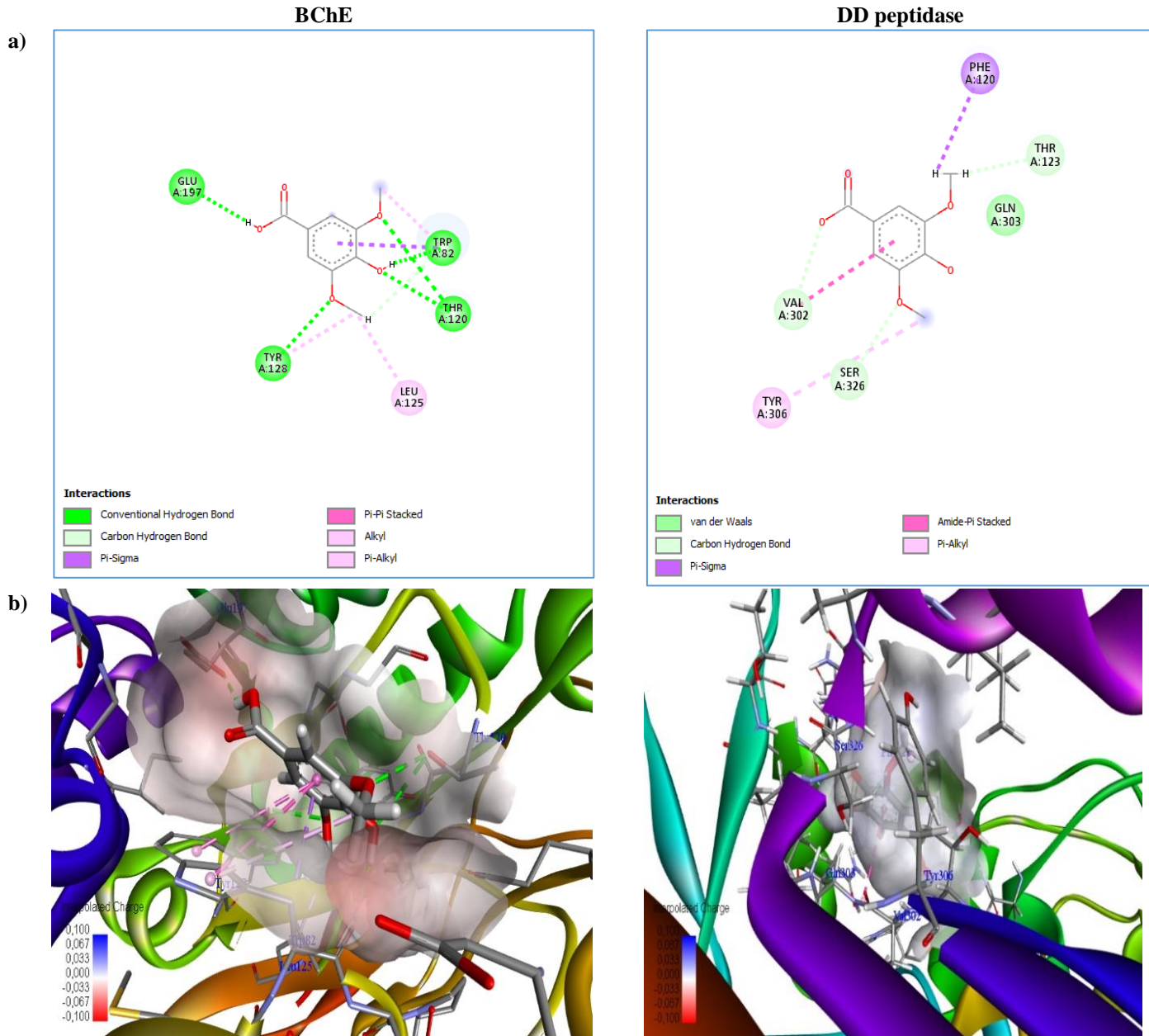


Figure 2. Syringic acid interaction with BChE and DD peptidase, a) 2D images b) interpolated load view

Salicylic acid molecule interacted with BChE; Five conventional hydrogen bonds with amino acid GLY116, GLY117, SER198, HIS438, three pi-pi-t shaped with amino acid TRP231, PHE329, and pi-alkyl with amino acid

LEU286 (Figure 3-Table 4). Salicylic acid with BChE was calculated as a MolDock score of -63.54, with binding energies of -6.10 kcal/mol.

Table 4. Interaction categories, types, and distances of molecular insertion of the salicylic acid molecule with BChE

No	Name	Distance	Category	Type	Transmitter	From Chemistry	Receiver	To Chemistry
1	A:GLY116:N - :[001:O1	3,03493	Hydrogen Bond	Conventional Hydrogen Bond	A:GLY116:N	H-Donor	:[001:O1	H-Acceptor
2	A:GLY117:N - :[001:O1	2,87395	Hydrogen Bond	Conventional Hydrogen Bond	A:GLY117:N	H-Donor	:[001:O1	H-Acceptor
3	:[001:H5 - A:SER198:OG	2,41903	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H5	H-Donor	A:SER198:OG	H-Acceptor
4	:[001:H5 - A:HIS438:NE2	1,85719	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H5	H-Donor	A:HIS438:NE2	H-Acceptor
5	:[001:H6 - A:SER198:OG	1,7192	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H6	H-Donor	A:SER198:OG	H-Acceptor
6	A:TRP231 - :[001	4,97577	Hydrophobic	Pi-Pi T-shaped	A:TRP231	Pi-Orbitals	:[001	Pi-Orbitals
7	A:TRP231 - :[001	4,92386	Hydrophobic	Pi-Pi T-shaped	A:TRP231	Pi-Orbitals	:[001	Pi-Orbitals
8	A:PHE329 - :[001	5,68254	Hydrophobic	Pi-Pi T-shaped	A:PHE329	Pi-Orbitals	:[001	Pi-Orbitals
9	:[001 - A:LEU286	5,07008	Hydrophobic	Pi-Alkyl	:[001	Pi-Orbitals	A:LEU286	Alkyl

Salicylic acid molecule interacted with DD peptidase; two conventional hydrogen bonds with amino acid GLN303, THR123, one carbon-hydrogen bond with amino acid THR123, and one pi-pi-t shaped with amino acid THR123 (Figure 3- Table 5). Salicylic acid with DD peptidase was calculated as a MolDock score of -66.18, with binding energies of -5.70 kcal/mol.

4. CONCLUSION

RP, also known as white-flowered false acacia; is distributed in Northern Anatolia in Turkey. RP's different extracts have been reported to have antibacterial activity and BChE enzyme inhibitory properties. RP flowers and leaf was extracted with methanol and analyzed by LC-MS/MS to determine their phytochemical content. According to the LC-MS/MS

result, salicylic acid and syringic acid were the main components. The interactions of syringic acid and salicylic acid molecules with BChE and DD-phosphate enzymes were calculated theoretically by molecular docking application. The binding affinity of the molecular interaction of the salicylic acid molecule with BChE was determined to be higher than that of syringic acid. However, the mold lock score of the molecular interaction of the salicylic acid molecule with BChE was observed to be lower than that of the syringic acid molecule. Moreover, the binding affinity of the molecular interaction of the salicylic acid molecule with DD peptidase was observed to be higher than that of the syringic acid molecule, and the MolDock score was lower. As a result, salicylic acid has higher BChE and DD peptidase enzyme binding affinity. Therefore, the BChE and DD peptidase inhibitory properties of these molecules can be investigated *in vitro*.

Table 5. Interaction categories, types, and distances of molecular insertion of the salicylic acid molecule with DD peptidase

No	Name	Distance	Category	Type	Transmitter	From Chemistry	Receiver	To Chemistry
1	A:GLN303:HE22-:[001:O2	2,25256	Hydrogen Bond	Conventional Hydrogen Bond	A:GLN303:HE22	H-Donor	:[001:O2	H-Acceptor
2	:[001:H5 - A:THR123:O	2,23203	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H5	H-Donor	A:THR123:O	H-Acceptor
3	A:THR123:HA - :[001:O1	2,73968	Hydrogen Bond	Carbon Hydrogen Bond	A:THR123:HA	H-Donor	:[001:O1	H-Acceptor
4	A:PHE120 - :[001	5,19155	Hydrophobic	Pi-Pi T-shaped	A:PHE120	Pi-Orbitals	:[001	Pi-Orbitals

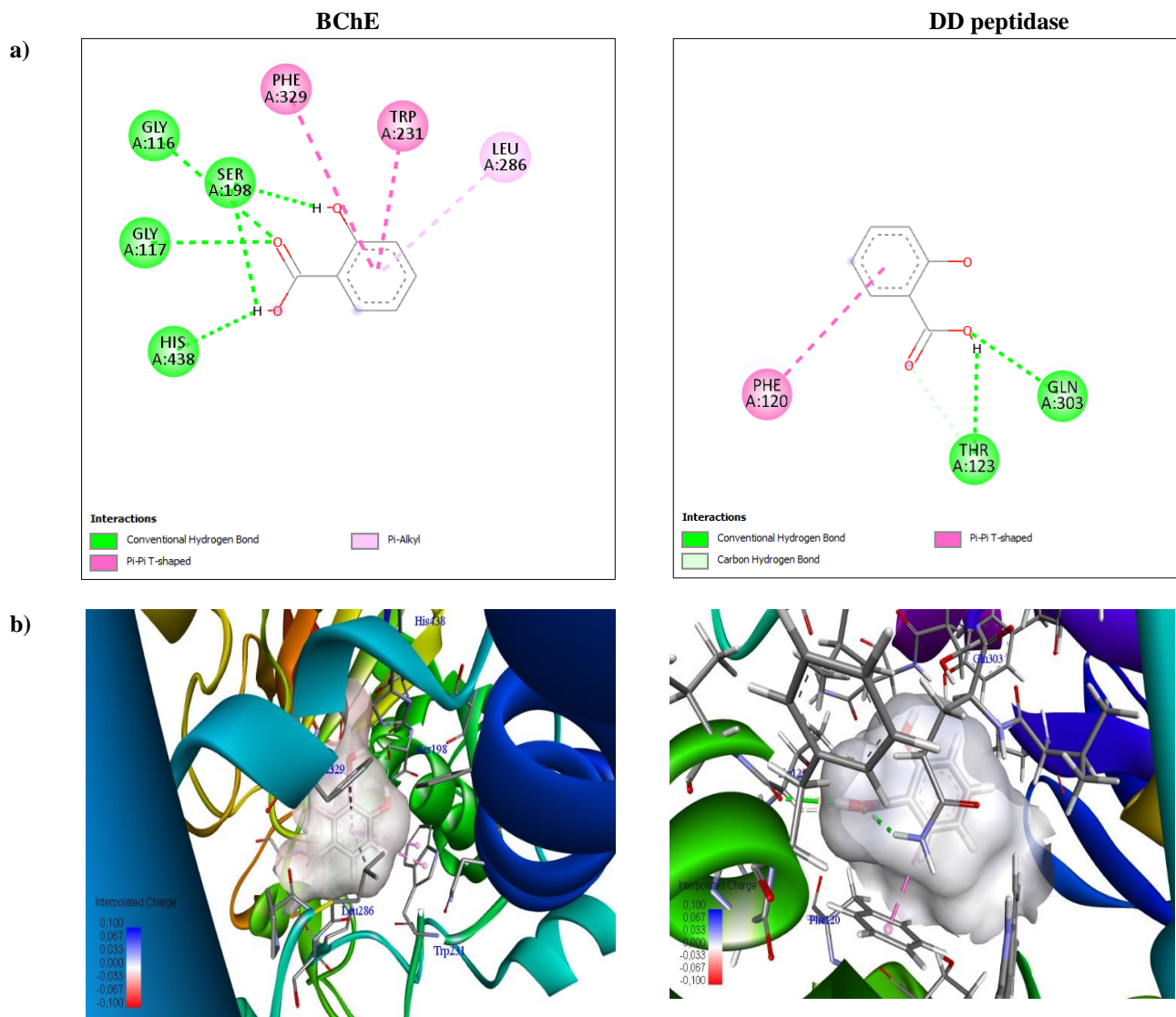


Figure 3. Salicylic acid interaction with BChE and DD peptidase a) 2D images b) interpolated load view

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RESEARCH ARTICLE

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Yüksek oranda fenolik bileşen içeren *Thymus* (Lamiaceae) türlerinin modifiye edilmiş CTAB yöntemiyle genomik DNA izolasyonu

Genomic DNA isolation of *Thymus* (Lamiaceae) species containing a high rate of phenolic components using the modified CTAB method

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Anahtar Kelimeler: *Thymus*, DNA izolasyonu, CTAB, PVP 40, STE

Keywords: *Thymus*, DNA isolation, CTAB, PVP 40, STE

Öz

Sistematik botanik çalışmalarında morfolojik karakterlerin yetersiz kaldığı durumlarda moleküler çalışmalar yapılmaktadır. Çalışmanın güvenilir olabilmesi için kaliteli ve yeterli miktarda DNA elde edilmelidir. Bu çalışmada *Thymus* L. cinsine ait bazı taksonlar kullanılarak 3 farklı izolasyon yöntemi denenmiştir. Araştırmalarımız ile klasik CTAB yöntemi modifiye edilmiştir. Yapılan yeni protokolda CTAB protokolüne ek olarak STE, PVP 40 gibi kimyasallar eklenmiş ve bazı inkübasyon süreleri değiştirilmiştir. İzolasyon sonucunda elde edilen DNA'da klasik yöntemlere ve hazır kit kullanımına oranla daha saf genomik DNA'nın elde edildiği görülmüştür.

ABSTRACT

In cases where morphological characters are insufficient in systematic botanical studies, molecular studies are carried out. For the study to be reliable, high quality and sufficient amount of DNA must be obtained. In this study, 3 different isolation methods were tried using some taxa belonging to the *Thymus* L. genus. With our research, the classical CTAB method has been modified. In the new protocol, in addition to the CTAB protocol, chemicals such as STE and PVP 40 were added and some incubation times were changed. It has been observed that purer genomic DNA was obtained from the DNA obtained as a result of isolation compared to classical methods and the use of ready-made kits.

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1. GİRİŞ

Aromatik bitkiler, doğal olarak oluşan polifenolikler, fenolikler ve flavonoid (Maltas vd., 2011) içerdiklerinden dolayı bitki araştırmacıları tarafından sistematik ve moleküler çalışmalarda daha fazla ilgi çekmektedir. Bu bileşikler, proteinleri parçalayabildikleri veya enzimlere müdahale edebildikleri için bu tür dokulardan nükleik asit veya protein ekstraksiyonunda zorluklar yaşanmaktadır (Hills & Staden, 2002). Fenolik bileşikler DNA ekstraksiyonu sırasında nükleik asitlere bağlanarak DNA'nın parçalanmasına sebep olur. Bu nedenle bitkilerden

yüksek saflıkta ve miktarda DNA elde edebilmek için klasik CTAB yöntemine (Doyle & Doyle, 1990) bazı modifikasyonlar (Drábková, 2014) uygulanmalıdır.

Lamiaceae familyasına ait *Thymus* L. cinsi bu gruba en iyi örneklerden biridir (Aneva vd., 2022). Uzun yıllardır halk arasında tedavi edici özellikleri nedeniyle sıklıkla kullanılan bu bitkinin yoğun uçucu yağ içeriğiyle ilgili birçok araştırma yapılmıştır. Henüz evrimini tamamlamamış olan *Thymus* L. kimyasal polimorfizm ve kolay melez oluşturma özelliklerinden dolayı çiçekli bitkilerin taksonomik olarak sorunlu türlerindedir (Yıldız vd., 2004).

Sistemik çalışmalar öncelikle morfolojik veya anatomik özellikler (Deniz, 2007) dikkate alınarak yapılmaktadır. Fakat *Thymus* L. gibi cinslerde bu veriler yetersiz kaldığı için güvenilir bir sonuç elde edilememektedir. Bu gibi durumlarda moleküler çalışmaların önemi daha da artmaktadır (Demirpolat, 2017).

Bitki moleküler biyolojisi çalışmalarının en önemli adımı yüksek kalitede genomik DNA elde etmektir (Şimşek vd., 2008). Bitkilerden genomik DNA izole edebilmek için hücre zarının eritilmesi (Sancar, 2021), proteinlerden arındırılması, fenolik bileşenlerin uzaklaştırılması ve sekonder metabolitlerin yıkanması gerekmektedir. Bu amaçla yapılan birçok izolasyon protokolü vardır. Fakat her bitkinin biyokimyasal yapısının farklı olmasından dolayı her protokol her takson için başarılı sonuç vermeyebilir.

Bu çalışmamızda da klasik CTAB yönteminin veya hazır kitlerin sonuç vermediği *Thymus* L. cinsine ait bazı

taksonlar kullanılarak yeni bir protokol denenmiştir. *Thymus* L. cinsi içerdiği bileşenlerden dolayı tıp, eczacılık, parfümeri, kozmetik gibi birçok alanda kullanımı yaygın olan önemli bir bitki grubudur. Bu yüzden bu türlerin taksonomik olarak yerlerinin belirlenmesi de sistematikçiler tarafından oldukça önemlidir. Bu araştırma ile ilerde bu cinse dahil türler üzerinde yapılacak olan moleküler filogenetik çalışmalarına katkı sağlaması amaçlanmıştır.

2. MATERYAL VE METOD

2.1. Bitki Örnekleri

Bu çalışmada kullanılan bitki örnekleri Prof. Dr. H. Aşkın Akpulat ve Prof. Dr. Bayram Yıldız tarafından Sivas ilinde toplanmıştır. Toplanan türler preslenerek kurutulup herbaryum materyali haline getirilmiştir.

Tablo 1. DNA izolasyonu yapılan türler ve lokaliteleri

Takson	Lokalite
<i>Thymus cappadocicus</i> var. <i>cappadocicus</i>	B6 Sivas: Gürün-Kangal arası, Böğrüdelik köyü batısı, kalker kayalıkları, 2000-2100m, 04.07.1997, B. YILDIZ, 14250!
<i>Thymus pectinatus</i> var. <i>pectinatus</i>	B6 Sivas: Hafik, Lota Gölleri doğu kısmı, jipsli alanlar, 1295-1300 m., 20.07.2017, Akpulat, 6354!
<i>Thymus pectinatus</i> var. <i>pallasicus</i>	B6 Sivas: Şarkışla, Kayadibi-Yeniçubuk arası, 1300-1400m, 12.06.2003, Akpulat, 3305!
<i>Thymus sipyleus</i> subsp. <i>sipyleus</i>	B6 Sivas: Hafik, Lota Gölleri doğu kısmı, jipsli alanlar, 1295-1300 m., 27.06.2017, Akpulat, 6396!
<i>Thymus sipyleus</i> subsp. <i>rosulans</i>	B6 Sivas: Hafik, Lota Gölleri batı kısmı, jipsli alanlar, 1290-1295 m., 27.06.2017, Akpulat, 6390!
<i>Thymus leuchotrichus</i> var. <i>leuchotrichus</i>	Sivas: Suşehri-Zara arası, Arabca k. Kuzeyi, Quercus çalılığı, 1300m, 30.06.1985, B. YILDIZ, 6112!
<i>Thymus convolutus</i>	Sivas: Divriği, Gedikbaşı köyü, kaya üzeri, 1100m, 11.07.1999, B. YILDIZ, 14471!
<i>Thymus fallax</i>	B7 Sivas: İmranlı, Kızıl Da, bazalt, 2200m, 22.07.2000, B. YILDIZ, 14901!

2.2. DNA izolasyon metodları

2.2.1. EZ-10 Spin Column Genomic DNA isolation kit protokolü

Kullanılan Kimyasallar:

1. Buffer PCB
2. Buffer BD

3. PW Solüsyonu
4. Yıkama Solüsyonu
5. TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8,0)
6. EZ-10 Column & Collection Tube
7. İzopropanol
8. β -merkaptöetanol
9. Etil alkol (%96)
10. RNaz
11. Kloroform

Kit ilk defa kullanılıyorsa PW solüsyonuna 24 ml izopropanol, yıkama solüsyonuna ise 45 ml etanol eklenip 65°C'lik su banyosunda 5 dakika bekletilmelidir. Daha sonra kimyasallar kullanıma hazır gelecektir.

Bu yöntemin uygulama şekli şöyledir; 100 mg taze veya 20 mg kurutulmuş bitki dokusu sıvı azot ile öğütülür. Örnekler 1,5 ml'lik ependorf tüplere aktarılır. 600µl Buffer PCB ve 12 µl β -merkaptotanol eklenerek vortekslenir. 25 dakika boyunca 65°C inkübasyona bırakılır. Eğer RNA içermeyen bir izolat elde edilmek isteniyorsa tüplere 20 µl RNaz eklenip vortekslenir. Daha sonra tüplere 0,6 ml kloroform eklenir. İyice karıştırılmak suretiyle en az on defa alt üst edilmelidir. 12000 g'de 2 dakika santrifüj edilir ve süpernatant yeni tüplere aktarılır. Yeni tüplerin üzerine 200 µl Buffer BD eklenip vorteksle karıştırılır. Eğer bu aşamada jelatinimsi bir yapı oluşursa 70°C'de 10 dakika inkübe edilmelidir. 200 µl, %96'lık etanolde eklendikten sonra elde edilen karışım EZ-10 kolonlarına aktarılır. 9000 g'de 1 dakika santrifüj edilir ve kolonun altında kalan kısım dökülür. 500 µl PW solüsyonu eklenip 9000 g'de 1 dakika santrifüj edilir ve kolonun dibindeki çökelti atılır. 500 µl yıkama solüsyonu eklenip tekrar 1 dakika süresince 9000 g'de santrifüj edilir. Yine kolonun altındaki çökelti uzaklaştırılır. Kolonun üst kısmında elde edilmek istenen DNA bulunur. Daha temiz bir DNA için 2 dakika boyunca 9000 g'de santrifüj edilip kimyasalların uzaklaşması sağlanır. 50 veya 100 µl TE Buffer eklenip 1 dakika oda sıcaklığında inkübe edilip 1 dakika boyunca da 9000 g'de santrifüj edilir.

2.2.2. CTAB protokolü

Kullanılan Kimyasallar:

1. CTAB tamponu (2 gr %2'lik CTAB, 28 ml 5M NaCl, 40 ml 0,5M EDTA, 10 ml 0,1M Tris-HCl, 20 ml dH₂O)
2. β – merkaptotanol
3. Kloroform:izoamil alkol (24:1)
4. İzopropanol
5. Etil alkol (%70)
6. TE Tamponu (1 ml 1M Tris-HCl ph: 8,0, 0,2 ml 0,5M EDTA ph: 8,0 ve dH₂O)

Bu yöntemin uygulama şekli şöyledir; yaklaşık 1 g taze ya da 100 g kuru bitki dokusu sıvı azot ile bir havan içerisinde parçalanır. Parçalanmış doku 2 ml'lik tüplere aktarılır ve üzerine 500 µl CTAB eklenir. Daha sonra 5 µl β -merkaptotanol eklenir ve vortekslenir. Ardından bir saat süreyle 65°C'de inkübasyona bırakılır. İnkübasyon sonrasında tüpler 14000 rpm'de 20°C'de 10 dakika

boyunca santrifüj edilir. Santrifüj sonrası oluşan süpernatant yeni tüplere aktarılır ve pelet atılır. Yeni tüplerin üzerine 750 µl 24:1 oranında kloroform:izoamil alkol eklenerek alt üst edilir. Tekrar 14000 rpm'de 10 dakika boyunca santrifüj edilir. Santrifüjden sonra tüplerde 3 faz görünür. En altta fenolik bileşenler, orta fazda bitki partikülleri ve en üstteki fazda ise DNA'yı içeren renksiz sulu faz bulunur. En üstteki sulu faz yeni tüplere aktarılarak üzerine 450 µl izopropanol eklenip 24 saat boyunca -20°C'de bekletilir. 24 saatin sonunda tüpler çıkarılıp 14000 rpm'de +4°C'de 10 dakika süresince santrifüj edilir. Santrifüjden sonra tüpün en dip kısmında pelet halinde DNA'nın görünmesi gerekir. Tüpte kalan izopropanol dökülüp tamamen uzaklaştırılır. Üzerine 500 µl %70'lik etil alkol eklenir ve DNA'nın yıkanması sağlanır. Yıkama işleminden sonra yeniden 14000 rpm'de +4°C'de 5 dakika santrifüj edilir. Gerek görülürse bu işlem birkaç defa tekrarlanabilir. Son olarak tüpler kurutma kağıdı üzerinde yan yatırılarak etil alkolün uçup uzaklaşması beklenir. Kuruyan pelet üzerine de 50 µl TE tamponu eklenip DNA'nın çözünmesi sağlanır.

2.2.3. Modifiye edilmiş CTAB yöntemi

Kullanılan Kimyasallar:

1. STE (0,25 M sükröz, 0,03 M Tris, 0,05 M EDTA)
2. CTAB (1,4 M NaCl, 0,2 M EDTA, 100 mM Tris/HCl pH: 8,03)
3. PVP (%10)
4. β – merkaptotanol (%1)
5. Kloroform:izoamil alkol (24:1)
6. İzopropanol
7. Etil alkol
8. Molecular Grade Water

Bu yöntemin uygulama şekli şöyledir; 1 g kuru yaprak havanda sıvı azot ile toz haline getirilene kadar ezilir. 2000 µl'lik tüplere aktarılır. Toz haline gelen örnek üzerine 1 ml STE tamponu eklenir ve vorteks ile homojen şekilde karıştırılır. Daha sonra 10 dakika boyunca 10.000 rpm'de santrifüj edilir. Santrifüj sonrasında süpernatant uzaklaştırılır. Ardından 600 µl CTAB tamponu, %10'luk PVP'den 200 µl ve %1'lik β – merkaptotanol'den ise 12 µl eklenir. Tüpler vorteks yardımıyla homojen hale getirilir ve bir saat süreyle 65° C'de inkübe edilir. İnkübasyon işlemi bitince örnekler 10.000 rpm, + 4° C'de 10 dakika santrifüj edilir. Santrifüj sonrası üst faz yeni

tüpe aktarılır ve pelet atılır. Üst fazla eşit hacimde olacak şekilde kloroform:izoamil alkol eklenir ve vortekslenir. 10.000 rpm, + 4°C'de on dakika santrifüj edildikten sonra üst faz tekrar yeni bir tüpe aktarılır. Üst fazın hacmi kadar soğuk izopropanol eklenir. 24 saat boyunca örnekler -20°C'de bekletilir. İzopropanol DNA molekülünün çökmesini sağlar. 24 saatin sonunda çıkarılan örnekler 14.000 rpm, + 4°C'de on dakika boyunca santrifüj edilir. Santrifüj işleminden sonra DNA pelet olarak tüpün dibinde görünür hale gelecektir. İzopropanol tamamen uzaklaştırılarak %70'lik EtOH ile DNA yıkanır. Etil alkolü uzaklaştırmak için 14.000 rpm, + 4°C'de 5 dakika santrifüj yapılır. Tüpler kurutma kağıdı üzerinde yan yatırılarak etil alkolün tamamen uçması sağlanır. Son olarak pelet üzerine molecular grade water eklenerek çözündürülür.

2.3. Elektroforez ve saflık tayini

İzole edilen DNA'ların elektrik akımıyla yürütülmesi sağlanır. Bunun için %1'lik agaroz jel hazırlanır. Hazırlanışı şu şekildedir; 1 g agaroz terazide tartılır ve behere konulur. Üzerine 100X Tris/EDTA tamponu eklenir ve mikrodalgada kaynatılır. Kaynatma işlemi

bitince 1 µl etidyum bromür eklenir. Etidyum bromür UV translimunator üzerinde DNA'nın görünür olmasını sağlar. 80 W'da 45 dakika boyunca elektroforezde koşturulur.

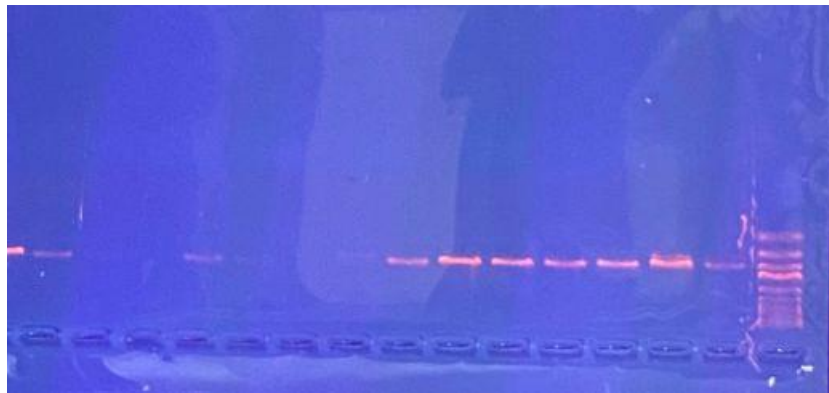
Elde edilen genomik DNA'nın saflık derecesini belirlemek için nanodrop cihazı ile 260 ve 280 nm'de absorban değerleri alınır. Bu değerlerin A_{260} / A_{280} nm oranları hesaplanır.

3. SONUÇ VE TARTIŞMA

Lamiaceae familyasına ait bitkilerin fenolik bileşen ve sekonder metabolit içeriklerinden dolayı araştırmacıların oldukça dikkatini çekmektedir. Ancak bu içeriklerinden dolayı moleküler çalışmaların yapılmasında da bazı zorluklar yaşanmaktadır. Moleküler çalışmaların başarılı olabilmesi için genomik DNA'nın izole edilebilmesi gerekmektedir. Lamiaceae familyasındaki fenolik bileşenler izolasyon aşamasında DNA'nın kırılmasına veya saf bir şekilde izole edilmesinde engel oluşturmaktadır.



Şekil 1. EZ-10 DNA izolasyon kiti kullanılarak izole edilen gDNA'nın jel görüntüsü



Şekil 2. Klasik CTAB yöntemi ile izole edilen gDNA'nın jel görüntüsü



Şekil 3. Modifiye edilen CTAB yöntemi ile izole edilen gDNA'nın jel görüntüsü

Tablo 2. Farklı yöntemler kullanılarak elde edilen gDNA'ların A_{260} / A_{280} oranları

	<i>Thymus cappadocicus</i>	<i>Thymus pectinatus</i> var. <i>pectinatus</i>	<i>Thymus pectinatus</i> var. <i>pallasicus</i>	<i>Thymus sipyleus</i> subsp. <i>sipyleus</i>	<i>Thymus sipyleus</i> subsp. <i>rosulans</i>	<i>Thymus leuchotrichus</i>	<i>Thymus convolutus</i>	<i>Thymus fallax</i>
EZ-10 Kit	0.872	0.564	0.652	0.924	0.854	0.451	0.554	0.745
CTAB	1.258	1.302	1.045	1.345	0.974	1.245	1.157	1.008
Mod. CTAB	1.924	1.851	1.801	1.840	1.785	1.914	1.648	1.715

Bu çalışmada Sivas ilinde yetişen Lamiaceae familyasına ait *Thymus* L. cinsine ait 8 takson kullanılmıştır. Bu taksonlar için 3 farklı izolasyon yöntemi denenmiştir. İlk olarak hazır izolasyon kiti kullanılmıştır ve izole edilen DNA'lar %1'lik agaroz jelde koşturulmuştur. Çalışmanın güvenilirliğini anlayabilmek için pozitif ve negatif kontrollerde dahil edilmiştir. Jel görüntüleri Şekil 1' de gösterilmiştir. Jelde pozitif kontrol dışında bir bant oluşumu gözlenmemiştir. Nanodrop ölçümleri de ortamda bulunan protein fazlalığına dikkat çekmektedir. Bu yöntemde DNA'nın saflık değeri düşük olduğu için farklı bir yöntem denenmiştir.

Klasik CTAB yöntemi hazır kit kullanımına oranla daha fazla zaman ve uğraş gerektiren bir yöntemdir. Fakat kite göre daha iyi sonuçlar vermiştir. Şekil 2' de zayıf ve silik bantlar gözlemlenmiştir. Bu bantlar istenilen kalite ve miktarda DNA olmadığını gösterse de nanodrop ölçümleriyle de gözlemin doğruluğu kanıtlanmıştır. Bu yöntemde de protein kirliliği oldukça fazla olmasına rağmen kit kullanımına göre daha az olduğu

görülmektedir. Saf DNA eldesi için bu yöntemin de uygun olmadığı anlaşılmıştır.

Modifiye ettiğimiz CTAB yöntemi de daha uzun ve ağır kimyasallar içeren bir yöntemdir. Bu yöntemle izole edilen DNA'lar da %1'lik agaroz jelde koşturulup UV transilluminator ile görüntülenmiştir. Şekil 3' de görüldüğü gibi bütün türlerde başarılı bir izolasyon yapılmış olup herhangi bir kirliliğe rastlanmamıştır. Yapılan deneylerde STE işleminin iki defa yapılmasının fenolik bileşikler uzaklaştırmada daha etkili olduğu görülmüştür. Ayrıca STE ile bitkilerde selülozun birikmesiyle oluşan ikincil hücre zarının parçalanması sağlanır. Her cins veya tür için izolasyon yöntemlerinde bazı değişiklikler yapılması gerekebilir. Çünkü her bitki içeriği birbirinden farklı bileşenler içermektedir. Bu yöntem zararlı kimyasal maddelerin kullanılmasını gerektiren ve uzun süren bir yöntem olmasına rağmen sonuçlar başarılı olmuştur.

Moleküler biyoloji çalışmalarında izolasyon en önemli aşamadır ve çalışmanın geri kalanını önemli ölçüde

etkilemektedir. İzolasyon sonrası yapılan PCR reaksiyonlarında DNA'nın miktarı ve kalitesi çalışmayı önemli oranda etkilemektedir. Şekil 3'de gösterildiği gibi agaroz jel görüntülerinde RNA veya sekonder metabolit kirlenmesi olmadığı görülmektedir. Bu da izolasyonu yapılan türlerin PCR çalışmaları için uygun olduğunu kanıtlamaktadır.

Sonuç olarak uyguladığımız bu yöntemin *Thymus* L. türleri için iyi bir yöntem olduğu görülmüştür.

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RESEARCH ARTICLE

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The phytochemical study of the *Pastinaca pimpinellifolia*

Pastinaca pimpinellifolia'nın fitokimyasal çalışması

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Anahtar Kelimeler: *Pastinaca pimpinellifolia*, doğal ürünler, peucenidin, oroselon, tomazin

ABSTRACT

The *Pastinaca* genus belongs to *Umbelliferae* family. *Pastinaca pimpinellifolia* M.Bieb grows in the north-west (Gadabay region) of Azerbaijan. In the present study, phytochemicals of the plants have been isolated and their structures have been elucidated. The mix of extractive substances was obtained by finely cutting the body of *Pastinaca pimpinellifolia*, then drying in the room conditions (100 g) and extracting by 95% alcohol 3 times (3 days for each time). The alcohol was filtered and evaporated on a water bath. The residue was a brown resin (6 g, 3% yield). The resin (6 g) was dissolved in CHCl_3 (50 mL) and chromatographed over a column of neutral Al_2O_3 with elution by hexane, benzene, chloroform and alcohol, in different ratios. The volume of each fraction was 100 mL. The separation and isolation process was carried out using column (silica gel) and thin layer chromatographic (TLC) methods. Current research is devoted to studying the chemical composition of the wild plants *Pastinaca pimpinellifolia*. The alcohol extraction resulted in the procurement of three individual substances *peucenidin* 1,9% *oroselon* 2.3% and *tomazin* 2.7%.

Öz

Pastinaca cinsi, *Umbelliferae* familyasına aittir. *Pastinaca pimpinellifolia* M.Bieb, Azerbaycan'ın kuzeybatısında (Gadabay bölgesi) yetişmektedir. Bu çalışmada, bitkilerin fitokimyasalları izole edilmiş ve yapıları belirlenmiştir. *Pastinaca pimpinellifolia* gövdesi ince doğranarak, oda koşullarında kurutulmuş (100 g) ve %95 alkolle üç kez (her seferinde 3 gün) ekstrakte edilmiştir. Alkol filtrelenmiş ve su banyosunda buharlaştırılmıştır. Elde edilen kalıntı, kahverengi bir reçinedir (6 g, %3 verim). Reçine (6 g) CHCl_3 (50 ml) içinde çözülmüş ve nötr Al_2O_3 sütunu üzerinde heksan, benzen, kloroform ve alkol ile farklı oranlarda elüsyon yapılarak kromatografik olarak ayrılmıştır. Her bir fraksiyonun hacmi 100 mL idi. Ayırma ve izolasyon süreci, kolon (silika jel) ve ince tabaka kromatografisi (TLC) yöntemleri kullanılarak gerçekleştirilmiştir. Bu araştırma, yabani bitki *Pastinaca pimpinellifolia*'nın kimyasal bileşiminin incelenmesine yönelik olup, alkol ekstraksiyonu sonucunda üç bireysel madde, peucenidin %1,9, oroselon %2,3 ve tomazin %2,7 oranlarında elde edilmiştir.

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1. INTRODUCTION

Of the 15 species of the genus *Pastinaca* L. (Chimi) belonging to the family *Apiaceae* Lindl, distributed in Eurasia and the Caucasus, 4 species are known in Azerbaijan: *P. umbrosa*, *P. armena*, *P. pimpinellifolia*, *P. glandulosa*. In the flora of Azerbaijan, these species are found in the middle mountain range to the alpine zone, in forests and shrubs, meadows, arid slopes (Askerov, 2006; Ibadullayeva, 2004; Flora of Azerbaijan 1955; Mammadova et al., 2022).

According to the latest literature data, *P. pimpinellifolia* M.Bieb.; Fl. Taur.-Caucasus 1: 237 (1808) is distributed in Turkey, Azerbaijan, Georgia, and other regions of the Caucasus (Ahmet et al., 2014). In the flora of Azerbaijan, the species *P. pimpinellifolia* is found in meadow formations around the village of Keleman in the Gadabay region. This formation is very rich in species composition.

103 species of the genus *Pastinaca* registered in the Plant List, one of which is *P. pimpinellifolia* M.Bieb. is a

plant. *Apiaceae Lindl* species have an interesting coumarin composition (Adriana et al., 2009). According to the literature coumarin derivatives have been identified in more than 1626 plant species belonging to 134 genera and 568 genera in the world flora (Serkerov et al., 2017; Gebhardt et al., 2005). Current research is devoted to study the chemical composition of *P. umbrosa*.

2. MATERIALS AND METHODS

Surface parts of *P. pimpinellifolia* species collected in the flowering phase from the territory of Gadabay region were used as the object of research.

Column chromatography method was used to get the individual items. Thus, the sum of 6 g ingredients was chromatographed in the glass column (h=80; d=2.5 cm) filled with Al₂O₃ (neutral, with III-IV degrees of activity). The volume of each fraction was taken 100 ml. The column chromatography was eluated by Hexane (10 fractions), hexane+benzen (20 fractions), benzene (30 fractions), benzene+chloroform (25 fractions), chloroform (7 fractions) and chloroform+etanol (3 fractions).

The obtained substances were compared based on the ¹H and ¹³C NMR information provided in literature on the structure of the individual items. The individuality of the substances was defined using thin layer chromatography method (Silufol UV 254, solvent-benzol+chloroform 1:1), IR-spectrums were drawn in the Cary 630 FTIR Spectrometer. ¹H, ¹³C, ¹³C Dept 135, Dept 90 spectrums

Substances 1. *Peucenidin* (C₂₁H₂₂O₇)

Table 1. *Peucenidin* infrared spectroscopy (IR), ultraviolet (UV) and melting temperature (Serkerov et al., 2006)

IR-spectr, v, cm ⁻¹	UV-specter, nm (lgε)	Melting point, °C
(v, cm ⁻¹): 806, 847, 862, 937, 980, 1005, 1024, 1053, 1076, 1115, 1131, 1320, 1353, 1490, 1575, 1621, 1646, 1727, 1751, 3092.	λ _{max} 207 (lgε 5.12), 226 (lgε 4.12), 298 (lgε 3.77), 322 (lgε 4.68)	122,0-225,0°C

Mass-spectra, m/z (Icomp, %): 386 (15), 326 (12), 311 (13), 303 (8), 286 (22), 261 (9), 244 (28), 243 (15), 229 (40), 227 (32), 201 (24), 187 (22), 83 (100), 43 (25). ¹H NMR spectra (CDCl₃, δ, ppm, J/Hz): 1.61, 1.70 (both s, 3H, CH₃ at C-4'), 1.86, 2.11 (both s, 3H, CH₃ at C-5''), 2.02 (3H, s, CH₃C=O), 5.15 (1H, d, J=7.0, H-2'), 5.58 (1H, broad s, H-4''), 6.21 (1H, d, J=9.6, H-3), 6.82 (1H, d, J=8.5, H-6), 6.97 (1H, d, J=7.0, H-3'), 7.40 (1H, d, J=8.5, H-5), 7.61 (1H, d, J=9.6, H-4).

Substances 2. *Oroson* C₁₄H₁₀O₃

were registered in the Bruker 300 MHz spectrometer for the isotope ¹H with frequency 300 MHz and for the ¹³C isotope with 75 MHz frequency using DMSO-d₆ as a solvent.

Three crystalline substances (*peucenidin*, *oroselon*, *tomazin*) were obtained individually by chromatography in a glass column filled with neutral, grade III-IV activity Al₂O₃ from the total amount of extractives extracted from the surface part of the *P. pimpinellifolia* plant.

3. RESULTS AND DISCUSSIONS

Natural products have begun to gain popularity worldwide for designing new and safe drugs or food additives. In this sense, novel plant products or plant metabolites are today one of the wild plant's most interesting subjects in scientific explorations. Especially uninvestigated wild plants are considered as an important pool for new pharmaceuticals and bioactive food additives, often termed nutraceuticals.

Current research is devoted to studying the chemical composition of the wild plants *P. pimpinellifolia*.

The current study aimed to conduct a chemical passporting of plants of the *Apiaceae* family, focusing on the coumarin content of the above-ground part of the *Pastinaca pimpinellifolia*. The alcohol extraction resulted in the procurement of three individual substances (*peucenidin* 1.9% *oroselon* 2.3% and *tomazin* 2.7 %). The elution of the obtained substances was executed using column chromatography with Al₂O₃.

Table 2. *Oroselon* infrared spectroscopy (IR), ultraviolet (UV) and melting temperature (Serkerov, 2005)

IR-spectr, ν , cm^{-1}	UV-spectr, nm ($I_{g\epsilon}$)	Melting point, $^{\circ}\text{C}$
ν_{max} 761, 813, 832, 907, 1021, 1124, 1153, 1373, 1554, 1617, 1680	λ_{max} 230 ($I_{g\epsilon}$ 4,11), 259 ($I_{g\epsilon}$ 3,67), 300 ($I_{g\epsilon}$ 3,74), 363 nm ($I_{g\epsilon}$ 4,08)	268,0-272,0 $^{\circ}\text{C}$

Mass-spectra, m/z (Icomp, %): 226 (100), 198 (82), 183 (48), 171 (24), 155 (53), 141 (28), 115 (43), 101 (12), 75 (26), 63 (78). IR spectra (ν , cm^{-1}): 760, 812, 831, 907, 1020, 1123, 1154, 1374, 1553, 1617, 1680. ^1H NMR spectra (CDCl_3 , δ , ppm, J/Hz): 2.13 (3H, s, CH_3), 5.24 (1H, d, s, H-5'), 5.82 (1H, s, H-5'), 6.36 (1H, d, J=9.6, H-3), 6.96 (1H, s, H-3'), 7.30, 7.34 (both d, 1H, J=8.5, H-5,6), 7.77 (1H, d, J=9.6, H-4).

Substances 3. Tomazin (Edultin) $\text{C}_{21}\text{H}_{22}\text{O}_7$

Table 3. Tomazin infrared spectroscopy (IR), ultraviolet (UV) and melting temperature (Serkerov et al., 2006)

IR-spectr, ν , cm^{-1}	UV-spectr, nm ($I_{g\epsilon}$)	Melting point, $^{\circ}\text{C}$
ν_{max} 3484 (OH), 3137 (CH), 1716 (CO- δ -lactones), 1650, 1614, 1589	λ_{max} 207 ($I_{g\epsilon}$ 5.12), 232 ($I_{g\epsilon}$ 4.21), 300 ($I_{g\epsilon}$ 3,36), 318 nm ($I_{g\epsilon}$ 4.21)	154-156 $^{\circ}\text{C}$

Mass-spectra, m/z (Icomp, %): 386 (18), 311 (12), 287 (12), 261 (8), 243 (32), 227 (18), 187 (73), 83 (100), 43 (54). ^1H NMR spectra (CDCl_3 , δ , ppm, J/Hz): 1.59, 1.71 (both s, 3H, CH_3 at C-4'), 1.81, 1.92 (both s, 3H, CH_3 at C-2''' and C-3'''), 2.01 (3H, s, $\text{CH}_3\text{C}=\text{O}$), 5.28 (1H, d, J = 7.0, H-2'), 6.08 (1H, m, H-3'''), 6.23 (1H, d, J=9.6, H-3), 6.85 (1H, d, J=8.5, H-6), 7.02 (1H, d, J=7.0, H-3'), 7.41 (1H, d, J=8.5, H-5), 7.62 (1H, d, J=9.6, H-4).

Obtained 3 substances were peucenidin, oroselon, tomazin. The ^1H -NMR spectra were recorded at 300 MHz on a Bruker AM300 NMR spectrometer. The ^{13}C -NMR spectra were recorded at 75 MHz on the same instrument. Further investigation needed for the isolation of other phytochemicals and also an investigation of biological properties.

4. CONCLUSION

Three compounds were isolated for the first time from the extracts obtained from the aerial parts of *P. pimpinellifolia*. The structures of the isolated compounds were identified as *peucenidin*, *oroselon* and *tomazin* on the basis of spectroscopic and by comparing their physical properties reported in the literature.

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RESEARCH ARTICLE

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Phytochemical analysis of *Silybum marianum* flowers: Quantitative analysis of natural compounds and molecular docking application

Silybum marianum çiçeklerinin fitokimyasal analizi: Doğal bileşiklerin kantitatif analizi ve moleküler yerleştirme uygulaması

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Anahtar Kelimeler: Deve dikeneni, GC-MS/MS, MolDock, *Silybum marianum*, LC-MS/MS, üreaz.

ABSTRACT

Secondary metabolites in plants, identifying, quantifying, and determining the biological activity of plants enables the use of plants in different fields such as pharmacology, food, and cosmetics. Different chromatographic methods such as GC-MS/MS (volatile compounds, fatty acid) and LC-MS/MS (phenolic compounds) are used to identify and quantify these secondary metabolites. *Silybum marianum* is a member of the Asteraceae family and grows naturally. It is known among the public by names such as Thistle, Virgin Mary Thorn, and Milky Kengel. In this study, *S. marianum* hexane extract was analyzed by GC-MS/MS, and the methanol-chloroform (1:1 v/v) extract was analyzed by LC-MS/MS. Palmitic acid methyl ester (17.96%), linoleic acid methyl ester (14.20%), and sesquiceneole (10.22%) were determined by GC-MS/MS. Moreover, LC-MS/MS analysis resulted in the quantification of chlorogenic acid (250.171 µg/g extract), salicylic acid (234.95 µg/g extract), isoquercitrin (210.65 µg/g extract), and rutin (102.05 µg/g extract). According to the analysis results, palmitic acid and chlorogenic acid were detected as the main components of fatty acid and phenolic compound respectively. Molecular docking was applied to determine their interaction with the urease enzyme. Palmitic acid and chlorogenic acid interaction with urease were calculated as a MolDock score of -104.63, and -113.21, with binding energies of -3.70, and -6.50 kcal/mol respectively. According to the results, chlorogenic acid may be a urease enzyme inhibitor.

ÖZ

Bitkilerdeki sekonder metabolitler, Bitkilerin biyolojik aktivitesinin tanımlanması, ölçülmesi ve belirlenmesi, bitkilerin farmakoloji, gıda ve kozmetik gibi farklı alanlarda kullanılmasına olanak sağlamaktadır. Bu ikincil metabolitleri tanımlamak ve ölçmek için GC-MS/MS (esansiyel yağ, yağ asidi) ve LC-MS/MS (fenolik bileşikler) gibi farklı kromatografik yöntemler kullanılır. *Silybum marianum* Asteraceae familyasının bir üyesidir ve doğal olarak yetişir. Halk arasında Devedikeneni, Meryem Dikeneni, Sütlü Kengel gibi isimlerle tanınır. Bu çalışmada, *S. marianum* heksan ekstraktı GC-MS/MS ile, metanol-kloroform (1:1 v/v) ekstraktı ise LC-MS/MS ile analiz edildi. Palmitik asit metil ester (%17.96), linoleik asit metil ester (%14.20), seskisinol (%10.22) bileşikleri GC-MS/MS ile belirlendi. Ayrıca LC-MS/MS analizi, klorojenik asit (250.171 µg/g ekstrakt), salisilik asit (234.95 µg/g ekstrakt), izokersitrin (210.65 µg/g ekstrakt) ve rutin (102.05 µg/g ekstrakt) miktarlarının belirlenmesiyle sonuçlandı. Analiz sonuçlarına göre ana bileşenler olarak sırasıyla yağ asidi olarak palmitik asit ve fenolik bileşik olarak klorojenik asit belirlendi. Üreaz enzimi ile etkileşimlerini belirlemek için moleküler doking uygulandı. Palmitik asit ve klorojenik asitin üreaz ile etkileşimi, sırasıyla -103,16 ve -113,21 olarak belirlendi. Bağlanma enerjisi ise sırasıyla -3.70 ve -6.50 kcal/mol olarak hesaplandı. Sonuçlara göre klorojenik asit bir üreaz enzim inhibitörü olabilir.

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1. INTRODUCTION

Throughout human history, plants have been used for food, fuel, and pharmaceutical purposes (Elmastas et al., 2004; Topçu et al., 1999). Medicinal plants used in traditional treatments have become known as alternative medicine with the development of modern medicine (Erenler et al., 2016). Due to the side effects of drugs derived from synthetic substances, the tendency towards drugs with natural bioactive components is increasing steadily (Demirtas et al., 2013). Research on plants to identify natural bioactive components and to find new natural drug raw materials is increasing currently (Aksit et al., 2014; Bayir et al., 2014; Erenler et al., 2014; Sahin Yaglioglu et al., 2013). After the improvement of spectroscopy, bioactive compounds began to be isolated from natural sources (Erenler et al., 2017). It has been reported that many medicinal plants show biological activity due to secondary metabolites and studies on the flora of Turkey have gained great interest in identifying new plant species that can be used in medicines (Beğen Akyıldırım & Eminağaoğlu, 2022; Önal & Eminağaoğlu, 2022; Palaşoğlu & Eminağaoğlu, 2022).

Milk thistle (*S. marianum*) is a member of the Asteraceae family and grows naturally in North Africa, Anatolia, Southern Europe, and Northern Russia (Demirezer et al., 2007). Thistle grows uncontrolled on roadsides, in meadows, and in all kinds of terrain. It consists of a head part, spiny and light green leaves, and small purple flowers. There are dark-colored seeds in the head. It ripens in July-August. It is known among the public by names such as Camel Obstacle, Virgin Mary Thorn, and Milky Kengel (Corchete, 2008). Its structure contains compounds such as flavonolignan (silymarin etc.), flavonoids (apigenin, kaemferol, naringerin, quercetin), fatty acids (linoleic acid, linolenic acid, oleic acid, palmitic acid), protein, tocopherol and sterol (AbouZid, 2012). It has been reported that milk thistle seeds protect the liver from the harmful effects of chemical drugs, poisonous mushrooms, and alcohol. In addition, milk thistle seed is used especially in the treatment of such as stomach, liver, and gall bladder disorders (Agarwal et al., 2006). Among the public, the aboveground part is used as a diuretic, antipyretic, sedative, appetite stimulant, and for rheumatism pain (Baytop, 1999).

The molecular docking method is a method that examines the binding of two molecules to each other in stable conformations. The key-lock model is used to describe compounds that bind to proteins. Here, the protein can be defined as the lock, and the ligand to be bound can be defined as the key. Similarly, docking examines the binding possibilities of the inhibitor or substrate to suitable spaces in the enzyme (Yenigün et al., 2023). Studies on urease inhibitors are very important to develop drugs to be used in the treatment of diseases caused by urease-containing pathogens in humans and animals and to repair these negative effects on the environment (Amtul et al., 2004).

In this study, the fatty acid content of the hexane extract of the flower was analyzed by GC-MS/MS, and the phenolic content of the methanol-chloroform extract was analyzed by LC-MS/MS. A molecular docking study was conducted for the major compounds of *S. marianum* to detect their interaction with the urease enzyme. Thus, the interactions of the palmitic acid and chlorogenic acid molecules with the urease enzyme were determined.

2. MATERIALS AND METHODS

2.1. Plants

S. marianum plant was collected on 11 August at Iğdır University Şehit Bülent Yurtseven campus. Identification of plant was carried out by Prof. Dr. Ahmet Zafer Tel at Iğdır University Faculty of Agriculture, Department of Agricultural Biotechnology.

2.2. Extraction

The flowers of the dried *S. marianum* plant were ground with a blender. Then, 300 grams of sample was weighed and transferred to the volumetric flask. Hexane solvent was added to the flask and extracted for 2 days. Afterward, the hexane was removed by reduced pressure to yield the crude extract to be used for fatty acid analysis. The flowers that were extracted with hexane were extracted with the methanol-chloroform (1:1, v/v) for 2 days. After filtration and evaporation of the solvent, crude extract was acquired for phenolic compounds analysis (Elmastas et al., 2016).

2.3. GC-MS/MS Analysis

GC-MS/MS analysis of hexane extract of *S. marianum* was determined using an Agilent (7890A GC System, 5975C by Triple-Axis Detector MS), a built-in-Autosampler, HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm). Helium was the transporter gas at a flow rate of 1.0 mL min⁻¹. The injector temperature was set at 250°C. The column temperature was 50°C and raised to 270°C at a rate of 4°C/min. The material was diluted with hexane to 1.0 L split/splitless (10:1) and transferred to the column. Molecules were identified through comparison with the Willey and NIST library (Başar et al., 2024a).

2.4. LC- ESI-MS/MS Analysis

We determined the phenolic contents and amounts of *S. marianum* flower of methanol-chloroform extract by LC-ESI-MS/MS analysis. An Agilent 6460 Triple Quad device combined with liquid chromatography (HPLC) and mass spectrometry (MS) was used to analyze the chemical as explained in our previously published article (Erenler et al., 2023; İpek et al., 2024). It was analyzed with 34 phenolic standards on the LC- ESI-MS/MS device.

2.5. Molecular Docking Application

The drawing, 3D structure, and minimum energy of the palmitic acid and chlorogenic acid were conducted by the ChemDraw program. The enzymes chosen for this docking investigation were urease, and urease interactions with palmitic acid and chlorogenic acid molecules were determined using the Molegro Virtual Docker (MVD) program (Başar et al., 2024b). 2D and 3D images of the interactions were taken with the BIOVIA Discovery Studio Visualizer program. Also, The AutoDock Vina program was used to calculate the binding affinities (Başar et al., 2023).

3. RESULTS AND DISCUSSIONS

The flower part of the *S. marianum* was ground with its seeds, and extraction was performed with hexane and methanol-chloroform sequentially. Then, the hexane extract was determined for fatty acids by GC-MS/MS, and the phenolic content of the methanol-chloroform extract was determined by LC-ESI-MS/MS. Phytochemical analysis is very important for food, pharmacy, and biotechnology. The isolation, identification, and evaluation of bioactive compounds contribute to the new drug invention. In addition, synthetic chemists continue to work intensively to synthesize compounds with the frame of natural products (Cakmak et al., 2006; Çelik et al., 2003; Erenler, 2011; Erenler & Biellmann, 2005; Erenler & Biellmann, 2007; Erenler & Cakmak, 2004; Erenler et al., 2009; Lu et al., 2014).

3.1. GC-MS/MS Analysis Results

Phytochemicals are naturally occurring chemical compounds found in plants that have biological activity. It is found in different parts of plants such as roots, stems, leaves, flowers, fruits, and seeds (Costa et al., 1999; Erenler et al., 2015). In this study, natural compounds analysis of the flowers of *S. marianum* hexane extract was performed (Figure 1).

According to the analysis results, a total of 68 volatile compounds and fatty acid compositions were detected. Palmitic acid methyl ester (17.96%), linoleic acid methyl ester (14.20%), sesquicineole (10.22%), oleic acid methyl ester (6.74%), stearic acid methyl ester (5.59%), nonacosane (4.20%) and 2-ethylhexanol (3.28%) were determined in the highest amount (Figure 3-Table 1). Therefore, fatty acid components were found to be in higher amounts.

In a similar study on *S. marianum* seeds, palmitic acid (8.25%), stearic acid (6.67%), oleic acid (31.58%), linoleic acid (45.36%), arachidic acid (4.11%) were determined to be the main components (Hasanloo et al., 2008).

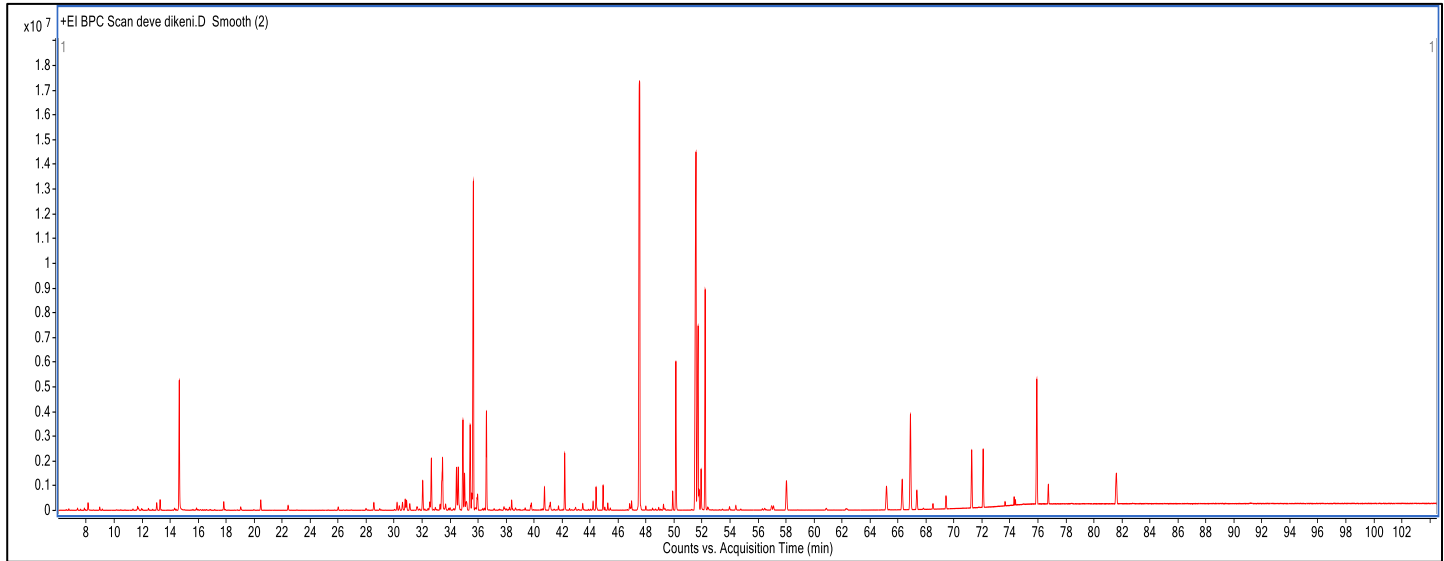


Figure 1. GC-MS/MS analysis chromatogram of hexane extract from *S. marianum* flowers

In another study, the main components in the seed part were linoleic acid (53.3%), oleic acid (21.3%) palmitic acid (9.4%), and stearic acid (6.6%) (El-Mallah et al., 2003). The results in our study are generally similar to the literature. But changes in rates in content analysis;

The climate where the plant grows, soil structure, altitude, and different parts of the plant affect it. However, the main components identified in the literature and our study are almost common.

Table 1. GC-MS/MS content analysis results of hexane extract from *S. marianum* flower

Peak	Compound	RT	RI	%Content
1	Ethylbenzene	8.13	855	0.19
2	p-Ethyltoluene	13.04	954	0.20
3	Decane	13.28	1000	0.25
4	2-Ethylhexanol	14.65	1030	3.38
5	Undecane	17.83	1100	0.23
6	Caprylic acid methyl ester	19.04	1126	0.10
7	Dodecane	22.43	1200	0.14
8	Elemene isomer	28.55	1344	0.23
9	Copaene	30.21	1376	0.23
10	Daucene	30.37	1381	0.14
11	β -Bourbonene	30.60	1384	0.24
12	7-epi-Sesquithujene	30.79	1391	0.31
13	β -Elemene	30.89	1391	0.29
14	Tetradecane	31.12	1400	0.19
15	α -Gurjunene	31.64	1409	0.14
16	Caryophyllene	32.05	1419	0.88
17	β -Gurjunene	32.55	1432	0.23
18	trans- α -Bergamotene	32.66	1435	1.48
19	Guaia-6,9-diene	33.28	1443	0.16
20	Humulene+ β -Famesene	33.46	1457	2.21
21	Alloaromadendrene	33.68	1461	0.22
22	Germacrene D	34.46	1481	1.18
23	Aristolochene	34.58	1487	1.25
24	Valencene	34.92	1492	2.52
25	β -Cyclogermacrene	35.03	1495	0.87
26	β -Bisabolene	35.43	1509	2.52

27	Sesquicineole	35.66	1516	10.22
28	Lauric acid, methyl ester	35.93	1526	0.24
29	β -Cadinene	35.97	1518	0.35
30	cis-Sesquisabinene hydrate	36.60	1543	2.61
31	2.6.10-Trimethyltetradecane	38.40	1539	0.25
32	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-trans-	39.76	1544	0.10
33	Guaia-3,9-diene	39.80	1556	0.17
34	trans-Sesquisabinene hydrate	40.74	1581	0.63
35	Viridiflorol	41.16	1591	0.25
36	Norphytane	41.75	1687	0.10
37	Myristic acid, methyl ester	42.19	1725	1.42
38	1(2H)-Naphthalenone, 3,4,4a,5,6,7-hexahydro-4a,5-dimethyl-3-(1-methyl ethenyl)-	43.48	1756	0.18
39	Octadecane	44.22	1800	0.22
40	Dehydrosaussurea lactone	44.43	1838	0.61
41	Pentadecanoic acid methyl ester	44.93	1820	0.61
42	Neophytadiene	45.27	1837	0.16
43	Methyl 6,9,12-hexadecatrienoate	46.83	1871	0.17
44	Palmitoleic acid, methyl ester	46.97	1899	0.23
45	Palmitic acid, methyl ester	47.53	1926	17.96
46	Eicosane	49.25	2000	0.14
47	Margaric acid methyl ester	49.91	2028	0.44
48	Linoleic acid, methyl ester	51.57	2092	14.20
49	Oleic acid, methyl ester	51.72	2091	6.74
50	Oleic acid, methyl ester-isomer	51.78		0.38
51	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	51.93	2116	0.95
52	Stearic acid, methyl ester	52.22	2128	5.59
53	cis-10-Nonadecenoic acid, methyl ester	54.41	2209	0.15
54	Tricosane	56.97	2300	0.16
55	cis-Methyl 11-eicosenoate	57.10	2306	0.15
56	Arachidic acid methyl ester	58.03	2329	1.18
57	Pentacosane	65.18	2600	1.01
58	Behenic acid, methyl ester	66.30	2528	1.18
59	2-Methylpentacosane	67.35	2561	0.72
60	Hexacosane	68.50	2600	0.16
61	Tricosanoic acid, methyl ester	69.43	2628	0.39
62	Heptacosane	71.27	2700	1.84
63	Lignoceric acid methyl ester	72.08	2728	1.81
64	Squalene	74.30	2832	0.16
65	Pentacosanoic acid, methyl ester	74.39	2823	0.10
66	Nonacosane	75.92	2900	4.20
67	Cerotic acid methyl ester	76.74	2935	0.53
68	triacontane	81.60	3000	1.32

RT: Retention time, RI: Retention index

3.2. LC-ESI-MS/MS Analysis of Bioactive Compounds

In LC-MS/MS analysis, 22 compounds were detected (Figure 2). Chlorogenic acid (250.171 $\mu\text{g/g}$ extract), salicylic acid (234.95 $\mu\text{g/g}$ extract), isoquercitrin (210.65 $\mu\text{g/g}$ extract), and rutin (102.05 $\mu\text{g/g}$ extract) were determined in the highest amount (Figure 3-Table 2).

Chlorogenic acid is a polyphenol that shows important therapeutic and biological activities such as antibacterial, hepatoprotective, radical scavenger, central nervous system stimulant cardioprotective, anti-inflammatory, antipyretic, neuroprotective, anti-obesity, anti-viral, anti-microbial, anti-hypertension (Naveed et al., 2018). Salicylic acid is a phenolic compound and has beneficial

effects on human health. Salicylic acid has anti-inflammatory and antioxidant activity. Also, it is effective in treating cardiovascular disease (Randjelovic et al., 2015). Isoquercitrin is the monoglucoside of the quercetin. It is widely found in the plant kingdom, but its quantity is small. Isoquercitrin has biological activities such as oxidative stress, antioxidants, cancer,

cardiovascular disorders, diabetes, and chemoprotective (Valentová et al., 2014). Rutin is a flavonoid with pharmacological properties such as antimicrobial, anti-inflammatory, anticancer, and antidiabetic (Gullón et al., 2017). Therefore, *S. marianum* is a plant species that contains phenolic and flavonoid compounds with biological and pharmacological activities.

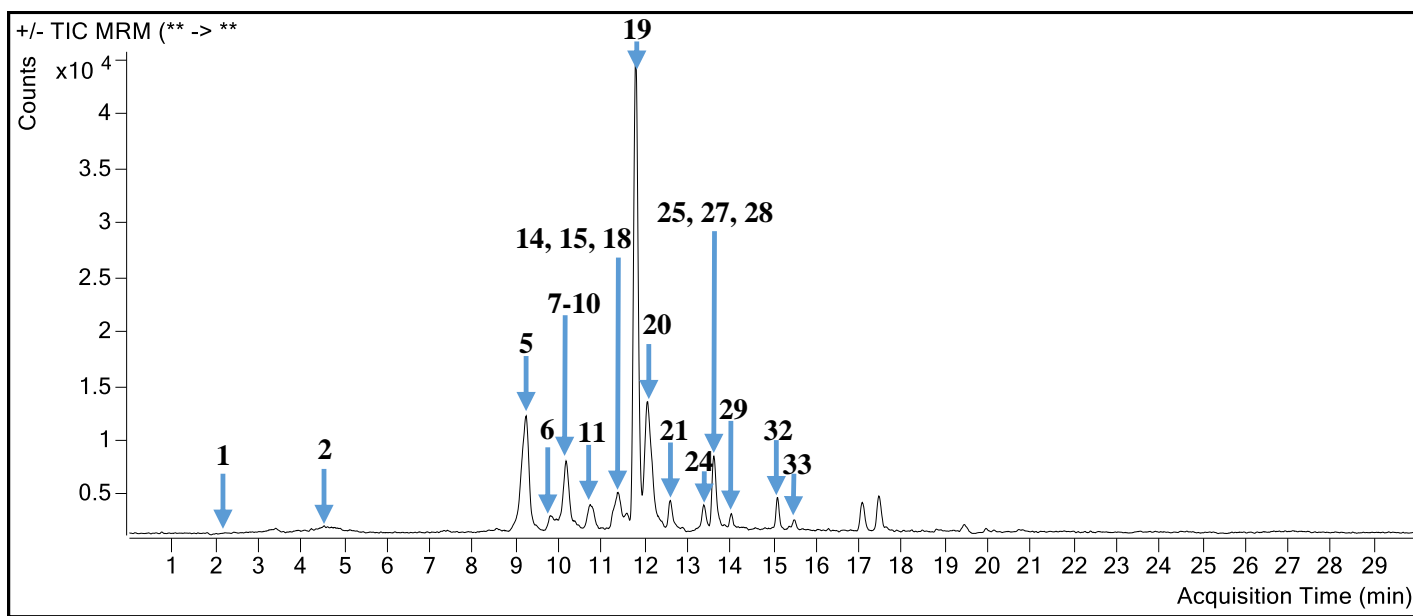


Figure 2. LC- ESI-MS/MS analysis chromatogram of methanol-chloroform extract from *S. marianum* flowers. Shikimic acid (1), Gallic acid (2), Epigallocatechin (3), Catechin (4), Chlorogenic acid (5), Caffeine (6), Vanillic acid (7), Caffeic Acid (8), Hydroxybenzaldehyde (9), Syringic acid (10), Vanillin (11), Polydatine (12), Resveratrol (13), o-Coumaric acid (14), Trans-ferulic acid (15), Taxifolin (16), Sinapic acid (17), Salicylic acid (18), Rutin (19), Isoquercitrin (20), Hesperidin (21), Quercetin-3-xyloside (22), Protocatechuic ethyl ester (23), Quercetin (24), Naringenin (25), Hesperetin (26), Morin (27), Trans-cinnamic acid (28), Kaempferol (29), Baicalein (30), Biochanin A (31), Luteolin (32), Chrysin (33), Diosgenin (34)

Table 2. LC-ESI-MS/MS analysis result of methanol-chloroform extract ($\mu\text{g/g}$ extract) from *S. marianum* flowers

No	Compound	RT	Quantity
1	Shikimic acid	2.16	24.48
2	Gallic acid	4.69	2.27
3	Chlorogenic acid	9.13	250.71
4	Caffeine	9.82	31.26
5	Vanillic acid	10.03	63.51
6	Caffeic acid	10.12	22.43
7	Hydroxybenzaldehyde	10.19	28.08
8	Syringic acid	10.35	79.10
9	Vanillin	10.80	8.78
10	o-coumaric acid	11.39	13.10
11	trans-ferulic acid	11.58	31.94
12	Salicylic acid	11.80	234.95
13	Rutin	12.02	102.05
14	Isoquercitrin	12.10	210.65
15	Hesperidin	12.20	57.05

16	Quercetin	13.38	86.99
17	Naringenin	13.62	24.63
18	Morin	13.70	29.70
19	trans-cinnamic acid	13.73	15.30
20	Kaempferol	14.03	76.93
21	Luteolin	14.96	0.99
22	Chrysin	15.09	12.57

RT: Retention time

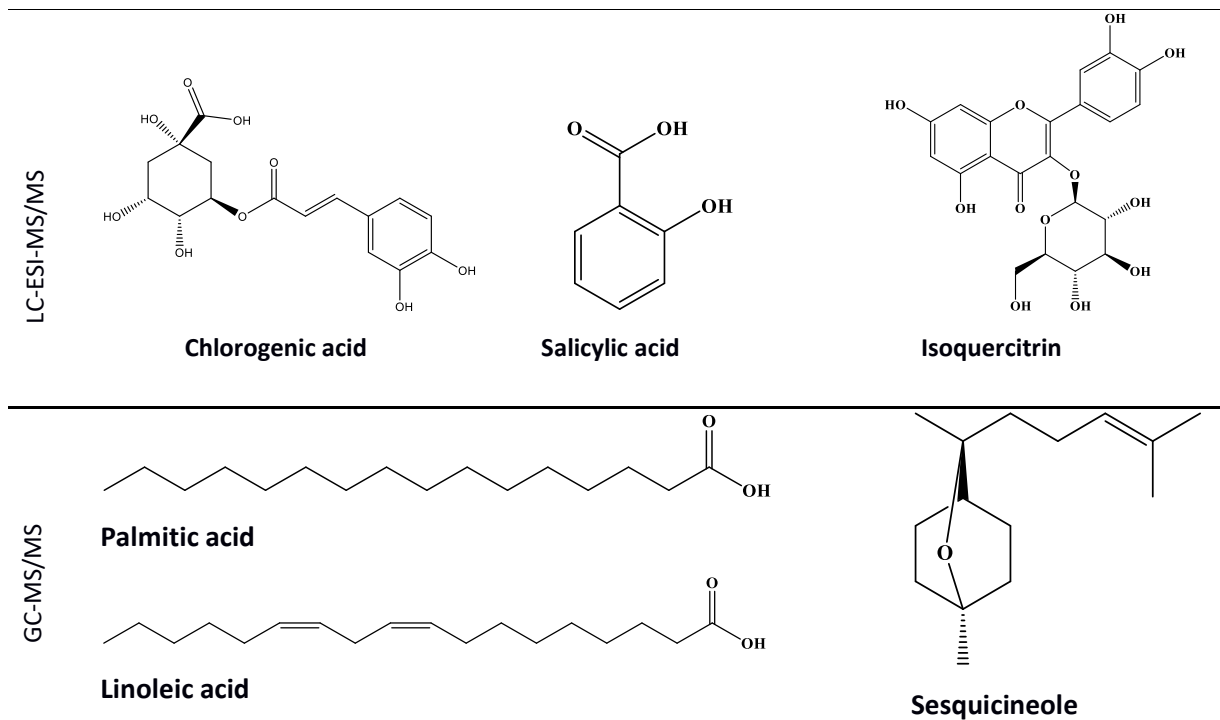


Figure 3. The molecular structures of the main components determined by LC-MS/MS and GC-MS/MS analyses

3.3. Molecular Docking Results

S. marianum plant is used among the public in stomach treatment. The urease enzyme produced by *Helicobacter pylori* catalyzes the hydrolysis reaction of urea into ammonia and carbon dioxide. Ammonia formed as a result of catalysis neutralizes stomach acid and causes bacteria to form colonies (Demiray & Yilmaz, 2007).

Therefore, inhibitors are needed, which inhibit the action of the urease enzyme. According to GC-MS and LC-MS/MS results, Palmitic acid and chlorogenic acid molecules were determined as the main components; Their interactions with the urease enzyme were observed. Moreover, the urease inhibitor properties (Moldock score, binding energy) of the molecules were calculated theoretically.

Palmitic acid molecules interacted with urease by two conventional hydrogen bonds with amino acid LYS709, ASP730, six alkyls with amino acid VAL36, ALA37, LYS716, PRO743, and three pi-alkyl with amino acid TYR32, PHE712 (Figure 4-Table 3).

Palmitic acid with urease was calculated as a MolDock score of -104.63, with binding energies of -3.70 kcal/mol.

Chlorogenic acid molecules interacted with urease by seven conventional hydrogen bonds with amino acid LYS716, MET746, THR33, TYR32, ASP730, THR715, two carbon-hydrogen bond with amino acid LYS745, one alkyl with amino acid VAL744, five pi-alkyl with amino acid TYR32, PHE712, LYS716, PRO717, MET746 (Figure 5-Table 4).

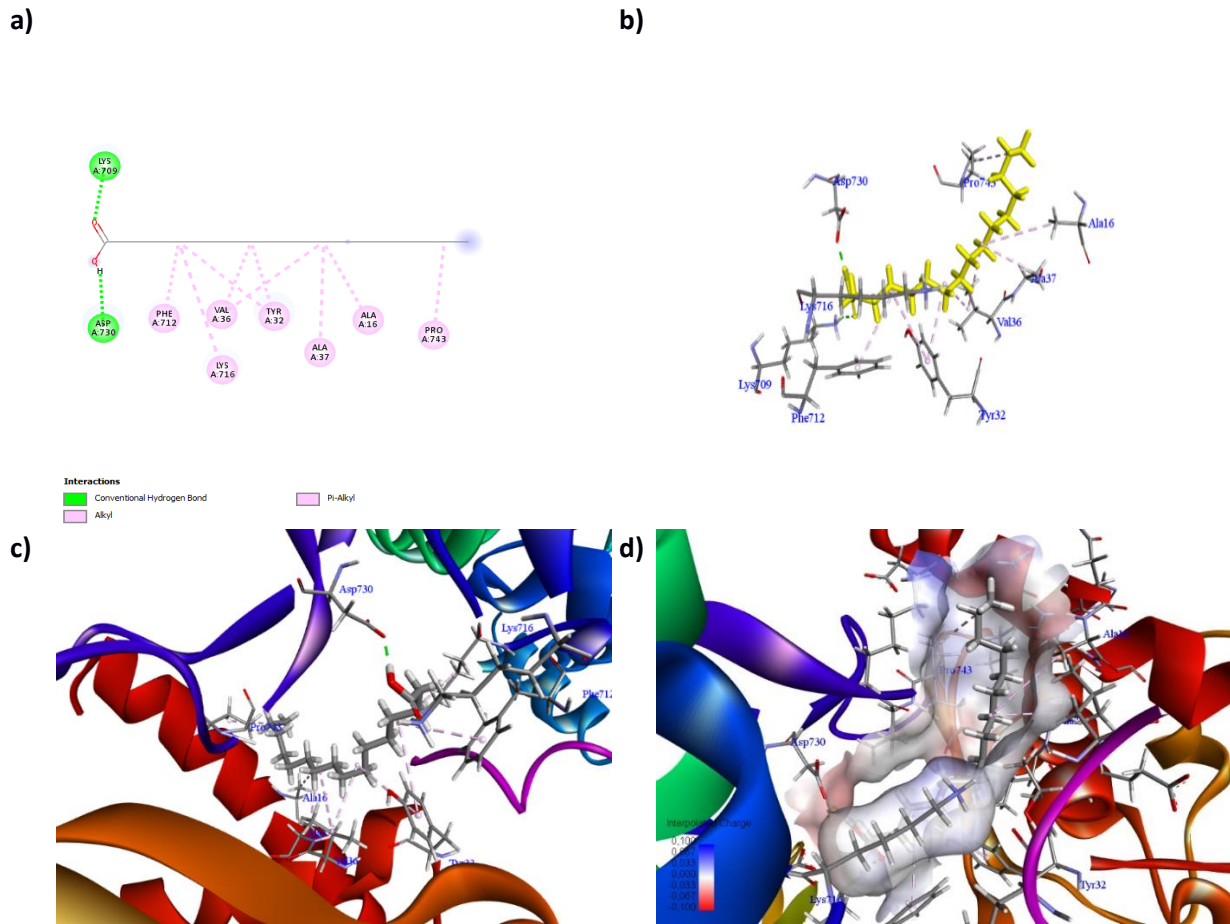


Figure 4. Palmitic acid interaction with urease, a) 2D images, b) interaction images c) 3D images, d) interpolated load view

Table 3. Interaction categories, types, and distances of molecular insertion of the palmitic acid molecule with urease

No	Name	Distance	Category	Type	Transmitter	From Chemistry	Receiver	To Chemistry
1	A:LYS709:HZ1- :[001:O2	2.28934	Hydrogen Bond	Conventional Hydrogen Bond	A:LYS709:HZ1	H-Donor	:[001:O2	H-Acceptor
2	:[001:H13-A:ASP730:OD2	1.98395	Hydrogen Bond	Conventional Hydrogen Bond	:[001:H13	H-Donor	A:ASP730:OD2	H-Acceptor
3	A:ALA16 - :[001	5.34477	Hydrophobic	Alkyl	A:ALA16	Alkyl	:[001	Alkyl
4	A:VAL36 - :[001	4.09893	Hydrophobic	Alkyl	A:VAL36	Alkyl	:[001	Alkyl
5	A:VAL36 - :[001	5.20353	Hydrophobic	Alkyl	A:VAL36	Alkyl	:[001	Alkyl
6	A:ALA37 - :[001	4.58918	Hydrophobic	Alkyl	A:ALA37	Alkyl	:[001	Alkyl
7	A:LYS716 - :[001	5.1705	Hydrophobic	Alkyl	A:LYS716	Alkyl	:[001	Alkyl
8	:[001:C15 - A:PRO743	4.69701	Hydrophobic	Alkyl	:[001:C15	Alkyl	A:PRO743	Alkyl
9	A:TYR32 - :[001	4.79773	Hydrophobic	Pi-Alkyl	A:TYR32	Pi-Orbitals	:[001	Alkyl
10	A:TYR32 - :[001	5.02732	Hydrophobic	Pi-Alkyl	A:TYR32	Pi-Orbitals	:[001	Alkyl
11	A:PHE712 - :[001	4.66364	Hydrophobic	Pi-Alkyl	A:PHE712	Pi-Orbitals	:[001	Alkyl

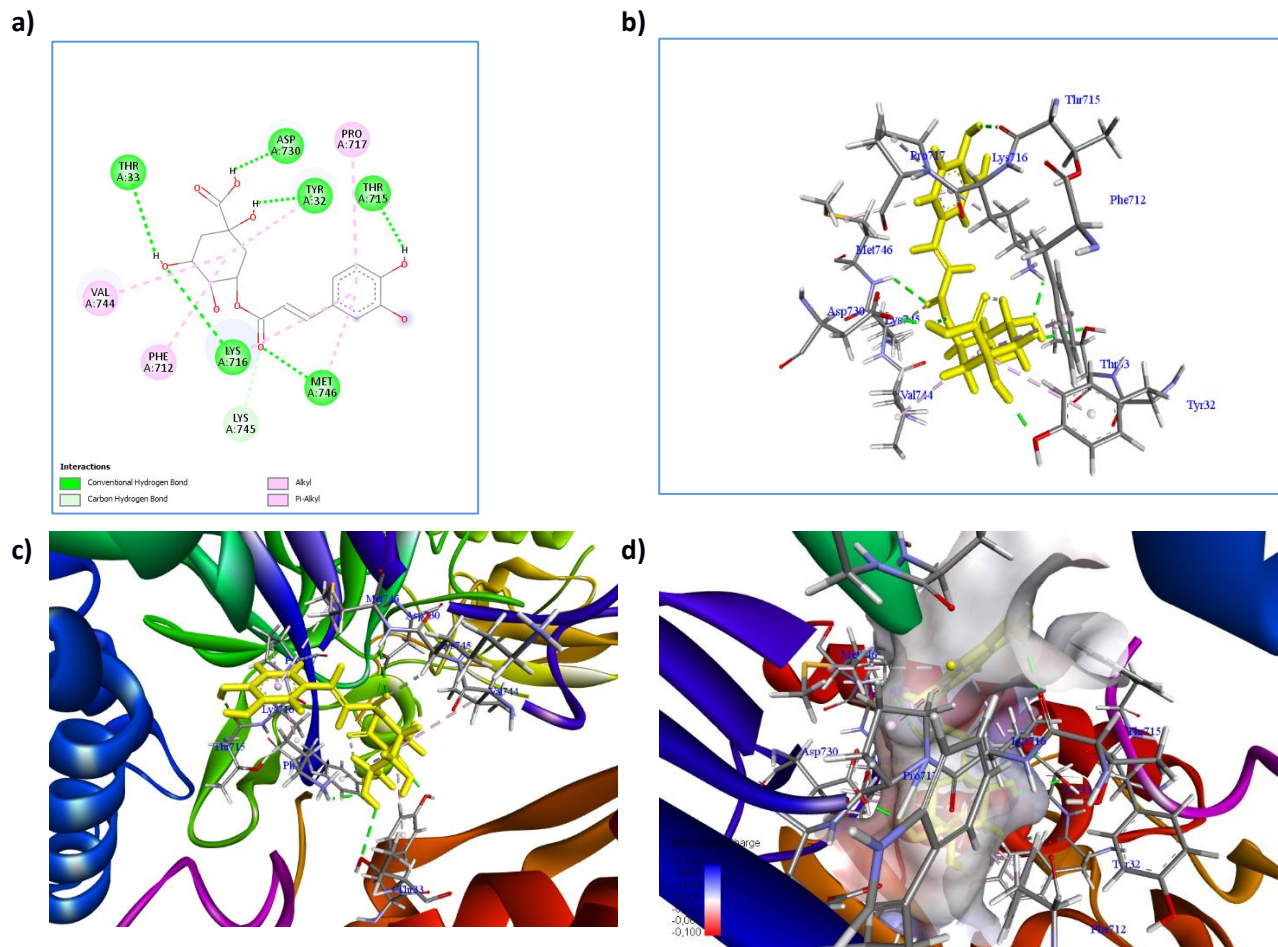


Figure 5. Chlorogenic acid interaction with urease, a) 2D images, b) interaction images c) 3D images, d) interpolated load view

Table 4. Interaction categories, types, and distances of molecular insertion of the chlorogenic acid molecule with urease

No	Name	Distance	Category	Type	Transmitter	From Chemistr	Receiver	To Chemistry
1	A:LYS716:HZ1	-	Hydrogen	Conventional	Hydrogen			
	: [001:O1	2.08972	Bond	Bond	A: LYS716:HZ1	H-Donor	: [001:O1	H-Acceptor
2	A:MET746:HN	-	Hydrogen	Conventional	Hydrogen	A: MET746:HN	H-Donor	: [001:O7
	: [001:O7	2.73541	Bond	Bond				H-Acceptor
3	: [001:H8	-	Hydrogen	Conventional	Hydrogen			
	A:THR33:OG1	2.86661	Bond	Bond	: [001:H8	H-Donor	A: THR33:OG1	H-Acceptor
4	: [001:H10	-	Hydrogen	Conventional	Hydrogen			
	A:TYR32:OH	1.98119	Bond	Bond	: [001:H10	H-Donor	A: TYR32:OH	H-Acceptor
5	: [001:H11	-	Hydrogen	Conventional	Hydrogen			
	A:ASP730:OD2	1.68883	Bond	Bond	: [001:H11	H-Donor	ASP730:OD2	H-Acceptor
6	: [001:H11 - : [001:O4	2.85831	Hydrogen	Conventional	Hydrogen			
	Bond		Bond		: [001:H11	H-Donor	: [001:O4	H-Acceptor
7	: [001:H18	-	Hydrogen	Conventional	Hydrogen			
	A:THR715:O	2.19567	Bond	Bond	: [001:H18	H-Donor	A: THR715:O	H-Acceptor
8	Hydrogen							
9	A:LYS745:HA - : [001:O7	2.41686	Hydrogen	Carbon Hydrogen Bond	A: LYS745:HA	H-Donor	: [001:O7	H-Acceptor
	Bond							
10	: [001:H3 - : [001:O6	2.53425	Hydrogen	Carbon Hydrogen Bond	: [001:H3	H-Donor	: [001:O6	H-Acceptor
	Bond							
11	A:VAL744 - : [001	5.15651	Hydrophobic	Alkyl	A:VAL744	Alkyl	: [001	Alkyl
12	A:TYR32 - : [001	5.48771	Hydrophobic	Pi-Alkyl	A: TYR32	Pi-Orbitals	: [001	Alkyl
13	A:PHE712 - : [001	5.44746	Hydrophobic	Pi-Alkyl	A: PHE712	Pi-Orbitals	: [001	Alkyl
14	: [001 - A:LYS716	3.55539	Hydrophobic	Pi-Alkyl	: [001	Pi-Orbitals	A: LYS716	Alkyl
15	: [001 - A:PRO717	5.44682	Hydrophobic	Pi-Alkyl	: [001	Pi-Orbitals	A: PRO717	Alkyl
16	: [001 - A:MET746	5.18975	Hydrophobic	Pi-Alkyl	: [001	Pi-Orbitals	A: MET746	Alkyl

Chlorogenic acid with urease was calculated as a MolDock score of -113.21, with binding energies of -6.50 kcal/mol.

4. CONCLUSION

S. marianum is a member of the Asteraceae family and grows naturally. In our study, *S. marianum* hexane extract was analyzed by GC-MS/MS, and the methanol-chloroform (1:1 v/v) fraction was analyzed by LC-MS/MS. GC-MS/MS analysis resulted in the determination of palmitic acid methyl ester (17.96%) as a chief compound. Chlorogenic acid (250.171 µg/g extract) was detected as a major compound in LC-MS/MS analysis. Molecular docking (MolDock) was applied theoretically to determine the interactions of the main components, palmitic acid, and chlorogenic acid molecules with the urease enzyme. The interactions, binding energies, and MolDock score of palmitic acid and chlorogenic acid with urease were calculated. The analysis showed that the chlorogenic acid molecule could be a urease enzyme inhibitor. Hence, *S. marianum*, with its high secondary metabolite content, may further increase its use in sectors such as pharmacology and food.

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RESEARCH ARTICLE

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Bingöl Tuzlası Gölü (Sivas, Türkiye) ve çevresinin tuzcul florası

Saltwater flora of Bingöl Salt Lake (Sivas, Turkey) and Its surroundings

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Keywords: Flora, saline plant, salty lake, Sivas

ÖZ

Bu çalışmada Bingöl Tuzlası Gölü ve çevresinin tuzcul florası araştırılmıştır. Araştırma alanından, 2023-2024 yılları arasında toplanan 1127 örnekten, 54'ü tuzcul bitkidir. Araştırma sonucunda teşhis edilen tuzcul bitkilerden, 8 familya, 18 cinsle ilişkin 24 takson kaydedilmiştir. Tanımlanan tuzcul bitki taksonlarının hepsi Spermatophyta bölümüne aittir. Angiospermae alt bölümüne ait taksonların 24'ü de Dicotyledoneae sınıfında yer almaktadır. İçerdikleri takson sayısına göre en zengin ilk beş familya: Amaranthaceae 8, Asteraceae 6, Plantaginaceae 3, Plumbaginaceae 3, Fabaceae 1, Brassicaceae 1, Liliaceae 1, ve Tamaricaceae 1 takson. İçerdikleri takson sayısına göre en zengin ilk 5 cins: Amaranthaceae 6, Asteraceae 5, Plumbaginaceae 2, Plantaginaceae 1, Fabaceae 1, Brassicaceae 1, Asparagaceae 1, ve Tamaricaceae 1 takson. Tespit edilen taksonların fitocoğrafik bölgelere göre dağılımı şöyledir: İran-Turan elementleri 4 (%16,66), Avrupa-Sibirya elementleri 3 (%12,50), Akdeniz elementi 1 (%4,17), coğrafi bölgesi bilinmeyenler ve birden fazla bölgeyi tercih edenler ise 16 (%66,67)'dir. Taksonların 11'i B6 karesi için yeni kayıttır.

ABSTRACT

In this study, the saline flora of Bingol Salt Lake and its surroundings was investigated. Of the 1127 samples collected from the research area between 2023-2024, 54 are saline plants. As a result of the research, 24 taxa belonging to 8 families and 18 genera were recorded. All identified taxa belong to the Spermatophyta division. 24 of the taxa belonging to the Angiospermae subsection are in the Dicotyledoneae class. The first five richest families according to the number of taxa they contain are: Amaranthaceae 8, Asteraceae 6, Plantaginaceae 3, Plumbaginaceae 3, Fabaceae 1, Brassicaceae 1, Liliaceae 1 and Tamaricaceae 1 taxon. The top 5 richest genera according to the number of taxa they contain Amaranthaceae 6, Asteraceae 5, Plumbaginaceae 2, Plantaginaceae 1, Fabaceae 1, Brassicaceae 1, Asparagaceae 1 and Tamaricaceae 1 taxon. The distribution of the detected taxa according to phytogeographic regions is as follows: Irano-Turanian elements 4 (16.66%), Euro-Siberian elements 3 (12.50%), Mediterranean element 1 (4.17%), those whose geographical region is unknown and those that prefer more than one region is 16(66.67%). 11 of the taxa are new records for square B6.

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1. GİRİŞ

Tuza toleranslı bitkiler, yaşam döngülerini tuzlu yüzeylerde tamamlamak için iyi adapte olmuş belirli bir organizma grubudur (Koyro vd., 2009). Tuzlu habitatlar,

özellikle tuzlu sudan etkilenen kıyı bölgelerinde olmak üzere dünya genelinde doğal olarak bulunur. İç bölgelerde, özellikle kurak ve yarı kurak bölgelerde düzensiz olarak dağılmışlardır (Chapman, 1960; Dítě vd., 2023; Szabolcs, 1974).

Türkiye’de 31 tane tatlı su gölü ve 10 tane de tuzlu su gölü bulunmaktadır. Tuzlu su içeren büyük göllerimiz; Tuz Gölü (Ankara-Konya-Aksaray), Palas Tuzla Gölü (Kayseri), Marmara Gölü (Manisa), Sarıkum Gölü (Sinop), Bolluk Gölü (Konya), Yayla Gölü (Denizli), Tuzla Gölü, Seyfe Gölü (Kırşehir), Çöl Gölü (Ankara) ve Paradeniz Gölü (Mersin)’dür. Bunların yanında Bingöl Gölü de tuzlu su içeren küçük bir göldür (Şekil 1).



Şekil 1. Bingöl Gölü ve Tuzla havuzları

Türkiye’deki flora çalışmaları 18. yüzyıldan günümüze kadar devam etmektedir. Bu çalışmalar incelendiğinde Türkiye ve farklı ülkelerden çok sayıda botanikçi tarafından yapıldığı görülmektedir. Bu çalışmaların sonuçları incelendiğinde, Türkiye’nin bitki tür çeşitliliği yönünden zengin olduğunu göstermektedir. Örneğin Avrupa kıtasında 11557 bitki türü; Britanya adalarında 2000 bitki türü bulunmaktadır (Heywood & Tutin, 1964-1981). Türkiye’de ise yaklaşık 9222 bitki türüne ev sahipliği yapmaktadır (Davis vd., 1988; Davis, 1965-1985; Güner vd., 2012).

Ülkemizde bu kadar çok tür çeşitliliğinin olmasının nedenleri; Türkiye’nin İran-Turan, Akdeniz ve Avrupa-Sibirya bitki coğrafyalarının kesiştiği alanda yer alması, Türkiye’nin jeopolitik konumu yani Asya ile Avrupa’yı birbirine bağlayan bir köprü konumunda olması, Türkiye’de iklimsel farklılıkların olması, Türkiye’de topoğrafik yapının çeşitliliği ve jeolojik farklılıklarının olması, Türkiye’de 0-5000 metre arasında yükseklik farklılıklarının olması, Türkiye’de deniz, nehir, bataklık ve göl gibi değişebilen sulak alanlara ve ekosistemlere sahip olmasıdır (Davis, 1965-1985; Ekim, 1997).

İç Anadolu Bölgesi’nde yer alan Tuz Gölü ve çevresinde tuz oranının fazla olduğu araziler üzerinde halofit bitkiler

yayılsı göstermektedir. Birçoğu endemik olan bu halofit bitkiler önemli birer gen kaynağını teşkil etmektedir. Ayrıca bölgedeki çevre baskısının olması nedeniyle bu gen kaynakları tehdit altındadır (Adıgüzel vd., 2005).

Araştırma alanı olarak Bingöl Köyü Tuzla Gölü’nün seçilmesinin nedeni, göl ve çevresine ait daha önce yapılmış herhangi bir floristik çalışmanın bulunmamasıdır. Bingöl Köyü Tuzla Gölü ve çevresinde yapılacak bu floristik çalışma ile Türkiye Florası’na yeni taksonların eklenmesi, ekonomik öneme sahip bazı zirai, tıbbi bitkilerin saptanması ve böylece daha sonra yapılacak bilimsel çalışmalara katkı sağlaması amaçlanmıştır.

2. MATERYAL VE METOD

Bingöl Köyü Tuzla Gölü “Flora of Turkey and the East Aegean Islands” adlı 11 ciltlik eserde, Davis’in kareleme sistemine göre B6 karesinde bulunmaktadır (Davis vd., 1988; Davis, 1965-1985; Ekim, 1997). Bingöl Köyü, Sivas il merkezine yaklaşık olarak 12 km uzaklıktadır. Köyde bulunan tuzlu su içeren göl, her yıl eriyen kar suları, yağmur suları ve yer altından çıkan tuzlu kaynak sularının da eklenmesiyle çekilen suların geri gelmeye başladığı izlenmiştir (Anonim, 2024a). Suların birikmesi ve havaların Mart ayının sonu Nisan ayının başlarında ısınmasına bağlı olarak Bingöl gölüne turna, leylek, angut gibi çeşitli göçmen kuş türleri gelmekte, göl bu kuşlara ev sahipliği yapmaktadır (Anonim, 2024b). Temmuz, Ağustos aylarında gölün suları hava sıcaklıklarının etkisiyle buharlaşmaktadır. Bingöl gölünün çekilen kısımlarında tuz birikintileri (Şekil 2) ve çeşitli tuzcul bitkiler görülmektedir (Şekil 3).



Şekil 2. Sivas Merkez-Bingöl Tuzlası Gölü suyunun çekilmesiyle ortaya çıkan tuz birikintileri

3.2. Sistematik liste

Angiospermae

Dicotyledoneae (Magnoliopsida)

Amaranthaceae (Chenopodiaceae)

Atriplex micrantha Ledeb. 8; 30.09.2023, Erdoğan 2070, İran Turan elementi, A, Hal

Chenopodium berlandieri Moq. 6; 30.08.2023, Erdoğan 2013, A, Th, NE

Chenopodium album L. subsp. *iranicum* 7; 30.08.2023, Erdoğan 2004, A, Th, NE

Chenopodium vulgare Gueldenst. ex. Ledeb. 8; 30.9.2023, Erdoğan 2071, A, Th, NE

Noaea mucronata (Forssk.) Asch. & Schweinf. 6; 30.08.2023, Erdoğan 2006, P, Ch,

Salicornia europaea (Mass.) Lambinon & Vanderp 2; 15.9.2023, Erdoğan 2030; 13.10.2023, Erdoğan 2116; 11.10.2023, Erdoğan 2131, A, Hal,

Suaeda vera Forssk. Ex J.F. Gmel 7; 05.08.2023, Erdoğan 1888, A, Hal, İran-Turan elementi.

Salsola kali L. 8; 30.09.2023, Erdoğan 2065, A, Th,

Asparagaceae

Prospero autumnale (L.) Speta 8; 05.08.2023, Erdoğan 1914, P, Geo, Hal, geniş yayılışlı

Asteraceae

Artemisia araratica Krasch 6; 30.08.2023, Erdoğan 2005, P, İran-Turan elementi.

Artemisia campestris L.7; 05.08.2023, Erdoğan 1898, P,

Inula montana L.1; 11.07.2023, Erdoğan 1667; 11.07.2023, Erdoğan 1687, P, Hal, Avrupa-Sibirya elementi

Pulicaria vulgaris Gaertn. 1; 15.08.2023, Erdoğan 1966; 15.09.2023, Erdoğan 2039; 2; 15.09.2023, Erdoğan 2033; 4; 15.08.2023, Erdoğan 1932; 15.09.2023, Erdoğan 2017; 7; 05.08.2023, Erdoğan 1892; 8; 30.08.2023, Erdoğan 1994, A, Th, Avrupa-Sibirya elementi

Scorzonera cana (C.A. Mey.) Hoffm. var. *jacquiniana* (W. Koch.) Cham.

1; 23.06.2023, Erdoğan 1503; 23.06.2023, Erdoğan 1523; 15.09.2023, Erdoğan 2053; 2; 23.06.2023,

Erdoğan 1545; 7; 04.06.2023, Erdoğan 1447, P, Hal, geniş yayılışlı

Taraxacum bessarabicum (Horn.) Hand.-Mazz. subsp. *bessarabicum* 2; 15.09.2023, Erdoğan 2028; 13.10.2023, Erdoğan 2114; 3; 15.08.2023, Erdoğan 1938; 13.10.2023, Erdoğan 2109; 7; 30.09.2023, Erdoğan 2077, P, Hal, geniş yayılışlı

Brassicaceae

Lepidium perfoliatum L. 3; 23.04.2023, Erdoğan 1076; 7; 21.05.2023, Erdoğan 1273, A, B, Hal, Th, geniş yayılışlı, VU

Fabaceae

Lotus maritimus L. 7; 04.04.2023, Erdoğan 1444, P, Hal, geniş yayılışlı

Plantaginaceae

Plantago lagopus L. 1; 11.07.2023, Erdoğan 1697; 4; 19.07.2023, Erdoğan 1764; 7; 21.05.2023, Erdoğan 1272, P, Hem, geniş yayılışlı

Plantago major L. subsp. *intermedia* 2; 15.09.2023, Erdoğan 2147; 7; 30.08.2023, Erdoğan 2003, P, Hem, geniş yayılışlı

Plantago lanceolata L. 5; 04.06.2023, Erdoğan 1350; 7; 21.05.2023, Erdoğan 2132, P, Hem, geniş yayılışlı

Plumbaginaceae

Limonum bellidifolium (Gouan.) Dumort. 8; 30.08.2023, Erdoğan 1992, P, Hal, Avrupa-Sibirya elementi.

Limonum narbanense Mill. 8; 30.08.2023, Erdoğan 1991; 2; 15.09.2023, Erdoğan 2148; 3; 15.08.2023, Erdoğan 1942; 2; 11.07.2023, Erdoğan 1745; 3; 19.07.2023, Erdoğan 1788, P, Hal, Akdeniz elementi

Acantholimonum venustum Boiss. subsp. *venustum* 1; 26.07.2023, Erdoğan 1860; 5; 30.09.2023, Erdoğan 2088; 8; 05.08.2023, Erdoğan 1909, P, İran-Turan elementi.

Tamaricaceae

Tamarix gracilis Willd. 2; 13.10.2023, Erdoğan 2113; 3; 20.05.2023, Erdoğan 1210; 7; 30.09.2023, Erdoğan 2072, P, Pha, Hal, geniş yayılışlı

4. TARTIŞMA, SONUÇ VE ÖNERİLER

Araştırma alanında Nisan, Mayıs, Haziran, Temmuz, Ağustos, Eylül, Ekim, Kasım 2023 ve Mart, Nisan, Mayıs 2024 (11 ay) boyunca toplanan örneklerden 24 tuzcul bitki taksonu tespit edilmiştir. 24 taksondan yurdumuz için endemik olan tür yoktur. Araştırma bölgesinin florası

8 familya ve 18 cinse ait 24 takson'dan meydana gelmektedir. Toplam taksonlardan 24'ü Spermatophyta bölümlerine aittir. Angiospermae alt bölümüne ait 24 taksona sahiptir. Angiospermae alt bölümüne ait taksonların 24'ü de Dicotyledonea sınıfında yer almaktadır. B6 karesinden toplanan 11 takson yeni kayıt durumundadır (Tablo 1).

Tablo 1. B6 Karesi İçin Yeni Kayıt Bitkiler

Tür Adı
<i>Atriplex micrantha</i> Ledeb.
<i>Chenopodium album</i> L. subsp. <i>iranicum</i>
<i>Salicornia europaea</i> (Mass.) Lambinon & Vanderp
<i>Suaeda vera</i> Forssk. Ex J.F. Gmel
<i>Artemisia araratica</i> Krasch
<i>Scorzonera cana</i> (C.A. Mey.) Hoffm. var. <i>jacquiniana</i> (W. Koch.) Cham.
<i>Lotus maritimus</i> L.
<i>Plantago lagopus</i> L.
<i>Plantago major</i> L. subsp. <i>intermedia</i>
<i>Limonum bellidifolium</i> (Gouan.) Dumort.
<i>Tamarix gracilis</i> Willd.

Taksonların bitki coğrafyası bölgelerine dağılım oranları şöyledir: İran-Turan elementleri 4 (%16,66), Avrupa-Sibirya elementleri 3 (%12,50), Akdeniz elementi 1 (%4,17), Geniş yayılışlı ve yayılışlı bilinmeyenler 16 (%66,67).

Tür sayısı bakımından familyaların sıralaması şu şekildedir:

Amaranthaceae (Chenopodiaceae) (8), Asteraceae (Compositae) (6), Plantaginaceae (3), Plumbaginaceae (3), Fabaceae (Leguminosae) (1), Brassicaceae (1), Asparagaceae (1), ve Tamaricaceae (1).

Araştırma alanındaki en bol cinsler ve takson sayıları şöyledir:

Amaranthaceae (6), Asteraceae (5), Plumbaginaceae (2), Plantaginaceae (1), Fabaceae (1), Brassicaceae (1), Liliaceae (1), ve Tamaricaceae (1).

Baysal'ın 2010 yılında Tuz Gölü ve çevresinde toplanan familya ve takson sayıları:

Toplanan taksonlar 14 farklı familyadan olup, dikotiledonlara ait 11 familyadan 26 takson [Brassicaceae (1), Caryophyllaceae (2), Chenopodiaceae (Amaranthaceae) (3), Hypericaceae (1), Leguminosae (Fabaceae) (2), Umbelliferae (Apiaceae) (1), Compositae (Asteraceae) (10), Boraginaceae (1), Scrophulariaceae (1), Labiatae (Lamiaceae) (1), Plumbaginaceae (3)], monokotiledonlara ait 3 familyadan 4 takson [Liliaceae (2), Iridaceae (1), Gramineae (Poaceae) (1)] toplanmıştır (Baysal Furtana, 2010).

Çalışma alanımız Bingöl Gölü ve Çevre florası ile Tuz Gölü ve çevre florası karşılaştırıldığında;

Amaranthaceae (Chenopodiaceae), Brassicaceae, Asteraceae (Compositae), Fabaceae (Leguminosae), Plumbaginaceae, familyalarının olması benzerlik göstermektedir. Aynı zamanda araştırma alanımız arasında ilk üç familya sıralaması bakımından sadece ilk iki familyanın uyumluluğu bulunmaktadır (Tablo 2).

Tablo 2. Yakın yerlerde yapılan çalışmalardaki ilk üç familya sıralamasının araştırma alanıyla karşılaştırılması.

Araştırma alanı	İlk üç familya sıralaması
Tuz Gölü ve Çevre Florası (Konya)	Asteraceae-Amaranthaceae-Fabaceae
Bingöl Gölü ve Çevre Florası (Sivas)	Amaranthaceae-Asteraceae- Plumbaginaceae/Plantaginacea

Sonuç olarak yapılan bu çalışma ile tuzcul bitki florası orta derecede bilinen bir yer olan araştırma alanının (Davis, 1974), daha iyi derecede bilinir hale gelmesi sağlanmıştır. Dolayısı ile Türkiye Florası'na az da olsa bir katkı sağlanmaya çalışılmıştır. Bazı taksonların yayılış alanları genişletilmiş, ait olduğu bitki coğrafyası bölgesi bilinmeyen bazı taksonların yayılışları gözden geçirilmiş ve B6 karesi için yeni kayıt durumunda olan bazı bitkiler saptanmıştır (Tablo 1). Tuzcul alanlar özel habitatlar olup korunması gereken özel bölgelerdir. Tuzcul alanlara ve türlere dikkat çekilmeye çaba gösterilmiştir. Araştırma alanında toplanan tuzcul bitkilerin fotoğrafları Şekil 6-23'de verilmiştir.



Şekil 7. *Tamarix gracilis* Willd.



Şekil 6. *Salicornia europaea* (Mass.) Lambion & Vanderp



Şekil 8. *Suaeda vera* Forssk. ex J.F. Gmel



Şekil 9. *Plantago lanceolata* L.



Şekil 12. *Limonum narbanense* Mill.



Şekil 10. *Plantago major* L. subsp. *intermedia*



Şekil 13. *Atriplex micrantha* Ledeb.



Şekil 11. *Plantago lagopus* L.



Şekil 14. *Artemisia campestris* L.



Şekil 15. *Noaea mucronata* (Forsk.) Asch. & Schweinf.



Şekil 18. *Artemisia araratica* Krasch



Şekil 16. *Chenopodium berlandieri* Moq.



Şekil 19. *Chenopodium album* L. subsp. *iranicum*



Şekil 17. *Limonium bellidifolium* (Gouan.) Dumort.



Şekil 20. *Chenopodium vulgare* Gueldenst. ex Ledeb.



Şekil 21. *Acantholimon venustum* Boiss. subsp. *venustum*



Şekil 22. *Salsola kali* L.



Şekil 23. *Inula montana* L.

Çalışma alanındaki toprak tuzluluğu arazi kayıplarına neden olmakla birlikte, tarımsal faaliyetlerin yapılmaması, birçok endemik bitki ve göçmen su kuşlarına barınak olması nedeniyle korunması gerekli çok önemli alanlardan biridir.

Teşekkür

Bu makale birinci yazarın “Bingöl Tuzlası Gölü (Sivas) Çevresi Florası” adlı yüksek lisans tezinden türetilmiştir.

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RESEARCH ARTICLE

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In vitro production of *Phalaenopsis* orchids

Phalaenopsis orkidelerinin *in vitro* üretimi

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Anahtar Kelimeler: Besin ortamı, bitki büyüme düzenleyicileri (PGR), doğrudan organogenez, gibberellik asit (GA3), sitokinin.

ABSTRACT

Phalaenopsis – known as moth orchids – are the most popular orchids cultivated indoors as decorative house plants. This makes propagation and cultivation of *Phalaenopsis* important for commercial growers. Enhancements to the micropropagation of *Phalaenopsis* would have pronounced economic benefits through reduced losses and wastage. We examined the effects of several nutrient media and specific plant growth regulators (PGRs) belonging to the gibberellic acid and cytokinin groups on the *in vitro* germination of *Phalaenopsis* seeds, utilizing a single group pretest-posttest model. The effects of several nutrient media such as: Knudson C (KCM), Lindemann (LM), Orchimax (-OM), Orchimax + activated charcoal (+OM), Murashige & Skoog (MS), as well as various PGRs such as 6-Benzylaminopurine (6BA), 6-Furfurylaminopurine (KIN), Adenin hemisulfate (AHS), Thidiazuron (TDZ), 2-Isopentenyl adenine (2iP), and Gibberellic acid (GA3), on the process of germination were also investigated. The explants obtained from the germinating seedlings were subjected to direct organogenesis, and the optimal PGR and tissue fragments were determined. The +OM medium facilitated the shortest germination period (in days). An inverse relationship between the concentration of TDZ and the percentage of germination in the context of the employed PGRs was observed. Apart from TDZ, the remaining PGRs exhibited a positive correlation with concentration. However, no significant difference in germination was observed in comparison to the control. The findings of direct organogenesis investigations revealed that the medium that exhibited the highest productivity was enriched with 5.0 ppm of 6BA. The media containing TDZ exhibited a reduced level of efficiency. Particularly, the group treated with 1.0 ppm of TDZ exhibited reduced efficacy compared to the control group. All concentrations of cytokinin in root elongation stage exhibited a favorable impact in comparison to the control. The variance between these PGRs was not statistically significant.

ÖZ

Phalaenopsis, ev dekorasyonu için iç mekanlarda yetiştirilen en popüler orkidelerdir. Bu nedenle, *Phalaenopsis*'in çoğaltılması ve yetiştirilmesi ticari yetiştiriciler için büyük önem taşımaktadır. *Phalaenopsis*'in mikro çoğaltılmasındaki iyileştirmeler, kayıpların ve israfın azaltılmasını sağlayarak ekonomik faydalar sunabilir. Bu çalışmanın amacı, bir grup ön test-son test modeli kullanarak *Phalaenopsis* tohumlarının *in vitro* çimlenmesi üzerinde çeşitli besin ortamlarının ve bazı bitki büyüme düzenleyicilerinin (PGR'ler) etkilerini incelemektir. Araştırmada kullanılan besin ortamları arasında Knudson C (KCM), Lin-demann (LM), Orchimax (-OM), Orchimax + aktif kömür (+OM) ve Murashige & Skoog (MS) yer alırken, PGR'ler olarak 6-Benzilaminopurin (6BA), 6-Furfurylaminopurin (KIN), Adenin hemisülfat (AHS), Thidiazuron (TDZ), 2-Isopentenil adenin (2iP) ve Gibberellik asit (GA3) incelenmiştir. Çimlenen fidelerden elde edilen eksplantlar, doğrudan organogenez işlemine tabi tutularak optimal PGR ve doku fragmanları belirlenmiştir. Sonuçlar, +OM ortamının en kısa çimlenme süresini (gün cinsinden) sağladığını göstermiştir. TDZ konsantrasyonu ile çimlenme yüzdesi arasında ters bir ilişki gözlenmiş, diğer PGR'ler ise konsantrasyon ile pozitif bir korelasyon göstermiştir. Ancak, kontrol ile karşılaştırıldığında çimlenme açısından önemli bir fark bulunmamıştır. Doğrudan organogenez araştırmalarının bulguları, en yüksek üretkenliğe sahip olan ortamın 5.0 ppm 6BA ile zenginleştirilmiş olduğunu ortaya koymuştur. TDZ içeren ortamlar düşük düzeyde bir verimlilik sergilemiş, özellikle 1.0 ppm TDZ grubu, kontrol grubuna kıyasla azalmış etkinlik göstermiştir. Kök uzatma aşamasındaki tüm sitokinin konsantrasyonları, kontrole kıyasla olumlu bir etki göstermiş, ancak bu PGR'ler arasındaki fark istatistiksel olarak anlamlı bulunmamıştır.

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1. INTRODUCTION

Ornamental plants, especially *Phalaenopsis* orchids, constitute a significant sector in the realm of plant life, as they exert a considerable influence on human health

and psychology, thereby enhancing the overall quality of life. The use of plants in interior spaces enhances the aesthetic appeal and olfactory experience, while also promoting a sense of connection between nature and the built environment (Akça, 2021). Moreover, the longevity of these plants further contributes to the liveliness of the designated areas. According to estimates, orchids comprise 10% of the global floriculture industry, serving as both potted plants and cut flowers. The plant *Phalaenopsis* holds a huge portion of over 500 million USD in the worldwide production and consumption markets (Yuan et al., 2021). The popularity of orchids stems from the diverse range of colors, sizes, shapes, and scents exhibited by their flowers. Furthermore, there has been a notable rise in commercial demand for orchids in recent years. The Orchidaceae family comprises approximately 800 genera and nearly 30,000 species, exhibiting a broad geographical distribution across the globe (Arditti & Ghani, 2000). The Orchidaceae family is classified under the order Asparagales and is considered the second most extensive group of angiosperms. Hinsley et al. (2017) estimated 31,000 species of this plant, based on 880 genera and 29,199 species. Despite its ornamental nature, *Phalaenopsis* exhibits a high concentration of chemical metabolites, including polysaccharides and alkaloids. Consequently, they are employed in diverse regions of the globe by the food and pharmaceutical industries as well (Aytar & Kömpe, 2021). The *in vivo* propagation of *Phalaenopsis* orchids is limited by the capacity of their seeds to undergo germination. Consequently, the horticultural industry necessitates the utilization of *in vitro* germination (Park et al., 2018). Therefore, the use of *in vitro* techniques for the propagation of *Phalaenopsis* orchids has shown significant reliability when compared to conventional methods, especially in the successful cultivation of a wide range of cultivars (Paek & Murthy, 1977). The utilization of *in vitro* propagation with the involvement of various PGRs and nutrient media enables the possibility of largescale production of valuable commercial hybrids and the conservation of endangered species through clonal reproduction.

The aims of the study were:

i) to initially examine the impact of basic nutrient media and PGRs, specifically gibberellic acid and cytokinin, on the *in vitro* germination of *Phalaenopsis* sp. plant seeds. ii) to determine the optimal concentrations of PGRs and nutrient media for the *in vitro* root proliferation and shoot elongation of *Phalaenopsis* orchids. iii) to investigate the potential for direct organogenesis from shoot tip explants of *Phalaenopsis* orchids.

2. MATERIALS AND METHODS

The research was conducted at the Tissue Culture Laboratory of the Department of Plant Production and Technologies at Konya Food and Agriculture University. A plant growth facility was utilized for the purposes of seed germination, tissue culture, and the micropropagation process. The ambient temperature within the room was maintained at 25°C ± 2°C, and ventilation was provided by a 24000 BTU air conditioning system. Experimental conditions included setting the illumination to 2500 Lux and implementing an 8/16 photoperiod alternating between periods of darkness and light.

2.1. Plant materials

The plant materials, from which the seeds for this investigation were assessed, were procured from Koçtaş Store. Botanical specimens, whose floral structures remained in the prebloom developmental stage, were obtained and subsequently transported to a controlled laboratory setting, where they were maintained at a constant temperature of 25°C until the flowers reached full bloom. Fertilization was conducted after the complete expansion of the flowers. The stamen was extracted from its filaments and subsequently inserted into the ovary after incising the stylus. This procedure was executed on identical floral specimens. Upon the completion of the manual fertilization procedure, the plants were transferred to a growth medium and subjected to regulated environmental conditions to facilitate their growth. This procedure was sustained until viable capsules were produced through the process of fertilization (Figure A4). Upon the capsules' transition from a green hue to yellow, the process of seed extraction was initiated by separating them from the maternal plant.

2.2. Basic nutrient media

In the initial phase of the study, the germination experiments involved the evaluation of four distinct nutrient media that varied in their inorganic and organic compositions as given elsewhere (Bektaş, 2014).

2.3. Method

2.3.1. Determination of plant growth regulators

The germination and micropropagation investigations of *Phalaenopsis* sp. seeds exclusively employed Thidiazuron as source of cytokinin-like growth regulator. Gibberellic acid (GA3) was also employed in the germination experiments.

2.3.2. Preparation of nutrition media

All the basic media were purchased from Duchefa Biochemie, The Netherlands. In addition to Orchimax, a 2% sucrose solution was incorporated into the medium. The media were solidified using 0.6% agar (Phytoagar, Duchefa Biochemie, Netherlands) and enriched with 20 g/L sucrose as the primary carbon source. Following the addition of PGRs, the solution's volume was augmented to 1000 mL and the pH was subsequently modified to a range of 5.7-5.8. The sterilization process for the media involved placing them in capped bottles that were capable of withstanding autoclaving. The autoclaving process was conducted at a pressure of 1.5 atm and a temperature of 121 °C for a duration of 15 min. The agar containing media retrieved from the autoclave was subjected to gentle agitation to promote uniform distribution, after which it was allowed to cool to room temperature. The efficacy of PGRs was found to diminish under high temperature conditions. To mitigate this issue, sterilization of the regulators was conducted using filters with a diameter of 0.22 µm. Following sterilization, the regulators were added to the media after autoclaving and prior to cooling. The media, which underwent a cooling process to approximately 40°C, were subsequently transferred to culture dishes within a sterilized cabinet. In the subsequent micropropagation and rooting stages, either Orchimax activated charcoal or charcoal-free media were employed as the fundamental media.

2.3.3. Determination of viability percentages of seeds

The viability of seeds was assessed both pre- and post-sterilization using a methodology outlined in Lauzer et al.

(1994)'s study. The seeds from each experimental group were immersed in a 1% solution of TTC (2,3,5-Triphenyltetrazoliumchloride, Sigma-Aldrich, Austria) and incubated in the dark at a temperature of 30 °C for a period of 48 hours. After this time frame, the seeds were subjected to scrutiny using a binocular microscope, whereby seeds exhibiting an orange-red hue were deemed to possess the capacity to germinate. Our study determined the impact of various concentrations of surface sterilization chemicals on seed viability, based on the data obtained.

2.3.4. Activated charcoal powder

The utilization of activated charcoal powder is employed for the purpose of eliminating phenolic compounds that could potentially arise within the culture media. Only 1% of activated charcoal was employed in the pertinent segments of this investigation.

2.3.5. Sterilization of capsules

Upon completion of a six-month duration, the capsules, which exhibited a chromatic alteration, were extracted from the rootstock, and subjected to a one-minute rinse in tap water. Subsequently, the capsules were subjected to immersion in a solution of 70% ethanol for a duration of 60 seconds. Subsequently, the capsules' seeds were longitudinally dissected using a sterile scalpel and then transferred onto the pre-prepared media in the most diluted form feasible. A meticulous approach was employed to ensure that approximately 200 seeds were present in each culture dish (magenta) during microscopic observation.

2.3.6. Initiation of *in vitro* cultures

The objective of our study was to examine the effects of nutrient media and PGRs on seed germination throughout the germination process (Figure A5). Accordingly, the task at hand can be outlined in the following two distinct stages:

2.3.6.1. Determination of the effects of various media on germination of seeds

During the preliminary phase of the study, the seeds extracted from the capsules were meticulously sown in the nutrient media. The magentas, which contained seeds, underwent a three-month cultivation period within a plant growth chamber utilizing the physical medium outlined in the initial section of the Materials

and Methods. Following the process of seed sowing, a quantitative analysis was conducted to determine the number of seeds present in the growth medium. Subsequently, the cultures were subjected to germination and monitored for a period of one week. After two months, the quantity of germinated seeds within the culture pots was assessed, and subsequently, the germination percentages were calculated based on the number of viable seeds. Based on the findings, the optimal conditions for seed germination were identified. The rates of germination and protocorm formation are expressed as percentages (%).

2.3.6.2. Determination of the effects of various PGRs on the germination of seeds

The second stage of the study utilized Orchimax activated charcoal medium as the foundational medium, based on an assessment of the outcomes of the prior investigation. This medium was assessed using solely three distinct concentrations of cytokinins, TDZ, and gibberellic acid (GA3). The concentrations of the growth regulators were selected based on the recommended, low, medium, and highest effective doses for each respective regulator. The growth regulators selected for this study and their respective concentrations are as follows: 6BA at 1.0, 2.5, and 5 ppm, 2iP at 1.0, 2.5, and 5 ppm, KIN at 1.0 2.5, and 5 ppm, AHS at 10, 50, and 100 ppm, TDZ at 0.1, 0.5, and 1.0 ppm, and GA3 at 0.5, 2.0, and 5.0 ppm. The control group involved in the study was orchimax activated charcoal medium that lacked a growth regulator.

2.4. Statistical Analysis

The statistical analysis of the data was conducted utilizing Microsoft Excel (Office 2007, Tool Pack Analyzer) and SPSS, Version 17.0 (SPSS Inc., Chicago, IL, USA). The statistical disparities among the means and the consequentiality of the disparities were computed at $P < 0.05$ through the utilization of the Duncan test for one way ANOVA variable analysis. The Spearman two tail test was employed to establish the correlation between the fluctuations in PGR concentrations and the examined parameters.

3. RESULTS

3.1. Vitality percentages of seeds

The study determined that the seeds' viability percentage and contamination rate acquired through direct capsule opening were $72\% \pm 4.0\%$ and $6 \pm 0.4\%$, respectively. Prior to conducting each experiment, assessments of seed viability were conducted. The viability percentages obtained were then utilized to determine the germination rates of the seeds.

3.2. Effect of basic nutrient media on *in vitro* seed germination

The study assessed the growth of mature *Phalaenopsis* seeds that were cultivated in five distinct nutrient media, each with its own specific composition. The seeds were observed after a period of approximately four months in these respective media. The germination rates and durations of the seeds were assessed utilizing a stereomicroscope (Table 1).

Table 1. Germination percentages of *Phalaenopsis* sp. and durations of seeds in different media

Germination	Nutrient media				
	KCM	LM	OM	OM+	MS
Percentage	22.40±1.8 ^d	29.20±2.4 ^c	49.90±3.9 ^b	57.90±3.10 ^a	36.20±3.0 ^b
Duration (days)	62	57	32	28	42

*KCM: Knudson Culture Medium; LM: Lindemann Medium; OM: Orchimax Medium; OM+: Orchimax Medium with active charcoal; MS: Murashige and Skoog Basal Medium. Similar letters in the same line are not different according to Duncan's multiple comparison test ($P < 0.05$).

It is evident that the Orchimax basic nutrient medium (OM+) supplemented with activated charcoal results in the shortest germination time (in days). Subsequently, a

period of 32 days was observed during which Orchimax (OM) was administered in the absence of activated charcoal. The onset of germination was noted to occur

on the 62nd and 57th days in Knudson C (KCM) and Lindemann media, respectively. Germination was observed on the 42nd day in the commonly utilized MS media. The emergence of root rhizoids in all experimental trials indicates the initiation of germination. Upon comparison of the germination percentages at the fourth month, it was observed that the Orchimax activated charcoal media exhibited the highest germination percentage (57.90%). The medium exhibiting the highest germination percentage (49.90%) was observed to be the one devoid of activated charcoal, namely Orchimax medium. The present study revealed that the MS medium exhibited a moderate level of germination percentage in comparison to the other tested media. Conversely, Lindemann medium exhibited the least germination percentage (22.40%). The germination rate of Knudson C media was observed to be low, measuring at 29.40%. As a result of dividing the highest germination value with the percentage of viability, the germination rate was calculated as 80.04% on OM+ medium that does not contain growth regulators.

3.3. Effects of some PGRs on *in vitro* seed germination

In this phase of the research, the seeds extracted from the capsules of the second group were evaluated for

Table 2. Effects of some PGRs on germination time and rate of *Phalaenopsis* seeds

Treatments	Concentration (ppm)	Germination (day)	Germination (%)
TDZ	0.1	30	58.75±1.5 ^a
	0.5	32	54.60±2.7 ^b
	1.0	32	50.55±1.8 ^b
	1.0	30	58.9±2.9 ^a
KIN	2.5	29	59.8±3.4 ^a
	5.0	30	60.50±3.6 ^a
	1.0	32	58.8±3.1 ^a
6BA	2.5	33	59.5±4.7 ^a
	5.0	30	60.6±4.9 ^a
	1.0	32	57.8±4.2 ^a
2iP	2.5	30	58.9±2.8 ^a
	5.0	30	58.7±1.5 ^a
	10.0	30	57.8±2.4 ^a
AHS	50.0	30	59.4±3.6 ^a
	100.0	30	59.5±4.0 ^a
	0.5	29	58.9±3.6 ^a
GA ₃	2.0	29	60.00±2.7 ^a
	5.0	28	62.55±3.5 ^a
	N/A*	29	59.10±2.35 ^a

*Similar letters in the same column are not different according to Duncan's multiple comparison test (P<0.05). Not applicable.

their viability and contamination rate. The results indicate that the percentage of viability was 70% ± 3.6, while the contamination rate was 6 ± 0.6%. The control group in this study was treated with Orchimax (OM+) containing activated charcoal but without the use of any growth regulator. Table 2 presents the impact of specific concentrations of certain cytokinins, cytokinin-like TDZ, and gibberellic acid on the duration and timing of germination. The germination percentages are recorded at the onset of the fourth month. The results indicate that the highest percentage of germination was observed at a concentration of 0.1 ppm, with a value of 58.75%, while the lowest percentage was recorded at a concentration of 1.0 ppm, with a value of 50.55%. The impact of KIN on seed germination suggests that the maximum concentration has a favorable influence, albeit lacking statistical significance when compared to the control cohort. The germination percentages were determined to range from 59.8% to 60.50%. The application of gibberellic acid (GA₃) had a positive impact on germination, with no significant difference in the statistical analysis. The results indicate that using 5.0 ppm of GA₃ resulted in a germination rate of approximately 90% compared to the proportion of viable seeds.

3.4. Shoot formation studies

To facilitate the cultivation of seedlings intended for direct organogenesis as explant sources, the final phase of our investigation involved the cultivation of robust plants that had germinated in various growth media. These plants were individually cultivated in media supplemented with 5.0 ppm of 6BA and 5.0 ppm of KIN. The plants were subcultured at 28 days intervals. At the conclusion of the fifth month, explants measuring approximately 10 mm were obtained from the shoot tip, root, and leaves of the juvenile seedlings that had developed shoots, leaves, as well as roots (Figure 1 & Figure A1).

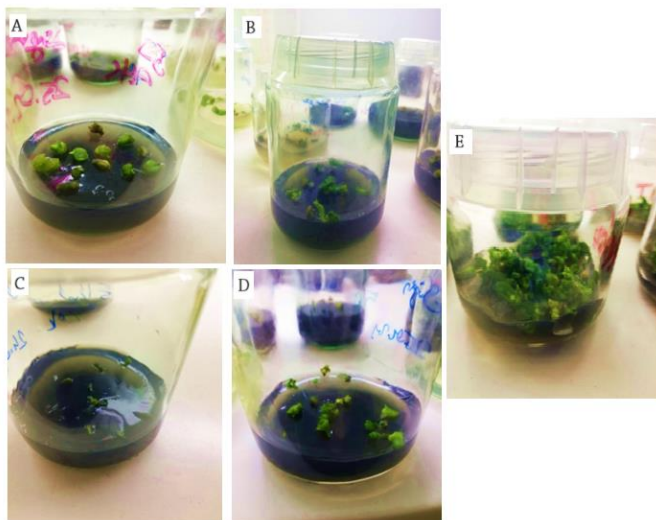


Figure 1. Transfer of shoot explants (A); Development of explants in culture media (B); Leaf explants status (C); Development of shoot explants (D); Elongation of shoot explants (E).

3.5. Direct organogenesis from shoot tip explant

The shoot tips yielded a positive response exclusively. The root and leaf explants exhibited complete blackening, with a 100% response rate. Hence, Table 3 denotes data exclusively derived from shoot tip explants. The initial parameter being assessed in this study pertains to the response observed in shoot formation. In alternative terms, it refers to the ability of each explant to engage in a shoot out. The percentage yield values obtained from a sample of 10 explants, which were selected from a larger population of 100 explants. These explants were transferred to nutrient medium. Based on the observations, it was found that the media supplemented with a concentration of 5.0 ppm of 6BA

exhibited the highest level of productivity. Conversely, media containing concentrations of 0.5 and 1.0 ppm of TDZ resulted in lower levels of efficiency. Specifically, the group treated with a concentration of 1.0 ppm of TDZ exhibited decreased efficiency in comparison to the control group, with a recorded efficiency of 41%. The effectiveness of KIN and 2iP in the obtained shoot yield from the explant has also been observed. While the efficacy of the alternative PGR, the AHS, was found to be lower compared to other treatments, it still exhibited a statistically significant improvement when compared to the control group. TDZ treatment resulted in a significant increase in shoot elongation, while the impact on explant yield was comparatively limited. Particularly at a concentration of 0.5 ppm, TDZ exhibited superior efficacy compared to the other experimental groups. Increased TDZ concentration of 1.0 ppm had a detrimental impact on shoot elongation, resulting in a decrease of 62.40 mm. A positive correlation was observed between the concentration of the substance and the elongation of the shoot. Application of 200 ppm of AHS resulted in a significant increase in shoot elongation, with a recorded measurement of 53.55 mm. The observation indicates that lower concentrations of KIN have a favorable impact on shoot elongation, whereas higher concentrations have an adverse effect. The optimal concentration of KIN for promoting shoot elongation was determined to be 5.0 ppm. The experimental groups with varying concentrations of 6BA exhibited significantly more favorable outcomes compared to the control group. While the concentration of 5.0 ppm exhibited the highest yield, there was no statistically significant difference observed when compared to the other concentrations of 6BA.

The study revealed that the growth regulator 2iP, which belonged to the cytokinin group evaluated in this investigation, induced shoot elongation at a comparatively lower rate compared to the remaining groups. The shoot elongation values range from 36.96 to 39.40 mm. The value fluctuates within the range. The values observed in this study corresponded to the lowest yields among all growth regulators examined. There was no observed positive correlation between the presence of 2iP and the elongation of shoots.

Regarding root elongation, all cytokinins and their respective concentrations exhibited a favorable impact compared to the control group. However, no statistically significant difference was observed between these

growth regulators. Regarding the formation of roots, all cytokinins and their respective concentrations tested exhibited favorable outcomes. Statistical analyses revealed that the number of root formations decreased with increasing concentrations, except for the increased 2-ropes. All *in vitro*-grown seedlings were prepared to be transferred to the external environment. (Figure 2; Figure A2; Figure A3).

revealed that the number of root formations decreased with increasing concentrations, except for the increased

Table 3. Effects of PGRs on shoot and root formation of *Phalaenopsis* shoot tip explants

Treatments	Concentration (ppm)	PS ¹ (%)	AE ² (mm)	RL ³ (mm)	NR ⁴	NL ⁵
TDZ	0.1	54 ^c	52.50±4.60 ^b	12.28±1.10 ^a	3.8 ^a	4.8 ^b
	0.5	47 ^d	62.40±3.70 ^a	12.95±0.90 ^a	3.1 ^b	5.7 ^{a,b}
	1.0	41 ^d	50.00±3.60 ^b	12.75±0.80 ^a	3.0 ^b	5.2 ^b
AHS	50.0	58 ^c	44.60±2.68 ^c	12.86±0.80 ^a	3.7 ^a	4.2 ^c
	100.0	64 ^b	49.50±3.60 ^{b,c}	13.65±0.70 ^a	3.3 ^b	3.9 ^c
	200.0	70 ^{a,b}	53.55±3.00 ^b	12.29±0.40 ^a	3.2 ^b	4.0 ^c
KIN	1.0	72 ^{a,b}	50.83± 3.40 ^b	10.05±0.9 ^{a,b}	3.5 ^{a,b}	6.6 ^a
	5.0	77 ^a	52.24± 3.0 ^b	11.55±0.90 ^a	3.5 ^{a,b}	6.8 ^a
	10.0	69 ^{a,b}	42.31± 2.80 ^c	10.20±0.7 ^{a,b}	3.0 ^b	6.6 ^a
6-BA	1.0	78 ^a	46.60±2.75 ^{b,c}	12.63±0.80 ^a	3.0 ^b	5.9 ^{a,b}
	5.0	80 ^a	49.15±2.80 ^{b,c}	13.41±0.95 ^a	2.8 ^{b,c}	6.8 ^a
	10.0	72 ^{a,b}	46.74±2.50 ^{b,c}	13.86±0.90 ^a	2.7 ^{b,c}	6.0 ^a
2-IP	5.0	68 ^{a,b}	39.40±3.40 ^c	9.56±0.70 ^b	3.2 ^{b,c}	4.2 ^c
	10.0	74 ^{a,b}	36.96±3.10 ^c	9.95±0.50 ^b	3.7 ^a	4.3 ^c
	20.0	70 ^{a,b}	38.00±2.80 ^c	10.83±0.60 ^{a,b}	2.8 ^c	4.0 ^c
Control	N/A*	45 ^d	19.80±1.72 ^d	8.31±0.5 ^c	2.0 ^d	2.7 ^d

*PS: Percentage of Shooting, AE: The amount of elongation. The differences between the final height and the initial height of the 4-month-old seedlings were taken, and the averages of these differences were given with ± standard deviations. Measurements were taken from 10 samples for each media., RL: Root length. Root lengths were given with ± standard deviations by taking the averages of the roots of the 4-month-old seedlings with the longest length. Measurements were taken from 20 samples for each media, NR: Number of roots. The average root numbers of 4-month-old seedlings were calculated with ± standard deviations. Measurements were taken from 20 samples for each media, NL: Number of leaves. The average of the leaf numbers of the 4-month-old seedlings was given with ± standard deviations. Measurements were taken from 25 samples for each media.*Not applicable. Similar letters in the same column are not different according to the Duncan multiple comparison test (P<0.05).



Figure 2. *In vitro* propagated seedlings are ready to be transferred to *in vivo*

4. DISCUSSION

The elimination of mycorrhizal fungi is achieved by supplementing the *in vitro* medium with the essential nutrients needed for seed germination. In our investigations, the germination of mature seeds from *Phalaenopsis* sp. was examined under varying nutrient compositions in the growth media. Notably, it was observed that the seeds exhibited a higher germination rate (57.90%) when subjected to the OM+ media. Orchimax, lacking activated charcoal at a concentration of 49.90%, exhibits the second highest germination percentages among the various nutrient media investigated in this study. The MS media exhibited a moderate germination percentage in comparison to the other tested mediums. The germination rate in the OM+ medium without growth regulator was determined to be 80.04% by proportioning the highest germination value with the percentage of viability. Merely a fraction of 5% of orchid seeds exhibit the ability to undergo germination within their native habitat, as they patiently await the arrival of optimal climatic conditions conducive to this process. Based on these data, the values associated with OM+ media exhibit a considerable

degree of significance, particularly with regards to the duration and speed of germination. The orchid species *Orchis sancta* L. and *Serapias vomeracea* exhibit similarities to the subject of our study. The highest rate of germination was observed in the OM+ media, as reported by [Bektas \(2014\)](#).

Numerous studies have documented that orchid seed germination rates are significantly higher when cultivated in a growth medium containing both organic and inorganic nitrogen sources ([Stewart & Kane, 2006](#)). In our study, the OM+ and OM- nutrient media, in addition to inorganic nitrogen, also contained tryptone as an organic nitrogen source. This suggests that the higher germination rates observed in these two specific environments, compared to others, can be attributed to the presence of an organic nitrogen source. Notably, previous research by [Raghavan and Torrey \(1964\)](#), and [Van and Debergh \(1986\)](#) found that the inclusion of an inorganic nitrogen source during orchid seed germination can impede the process, which aligns with our study's findings.

There is a discernible disparity in the rate of germination between the OM+ and OM-media. The inclusion of an additional 1% of activated charcoal in OM+ media is believed to create a light-restricted environment for the seeds within the medium, thereby enhancing the germination process. According to the study of Paek and Murthy (1977), it has been observed that the presence of activated carbon in the growth medium can enhance rooting by impeding the penetration of light. Nevertheless, it has been indicated that the utilization of activated carbon has the potential to elevate the pH level, thereby facilitating nitrogen absorption. Consequently, there was an observed increase in growth (Eymar et al., 2000). In contrast to findings in other studies, Waes (1987) reported a reduction in germination due to the presence of activated carbon in the environment. The pH levels of all the media used in our investigation were adjusted to a range of 5.7 to 5.8. Additionally, the cultured samples were incubated at a temperature of 23 ± 2 °C with a photoperiod of 16/8 hours. These pH values and incubation temperatures align with those reported in previous research. Van Waes and Deberg (1986) reported the optimal pH level for orchid germination in temperate zones as 5.8. In contrast, research by Arditti (1967) and Arditti (1979) indicated that the most favorable temperature range for germination and subsequent seedling growth falls within 20–25 °C. Contrary to our findings, Mead and Bulard (1975) reported that orchid germination is optimal in a dimly lit environment. Valletta et al. (2008) found that *Orchis mascula* seeds have the most favorable germination process under a photoperiod consisting of 16 hours of light followed by 8 hours of darkness. In contrast, seeds incubated in continuous darkness did not germinate. The 16-hour light/8-hour darkness photoperiod, which we implemented in our study, proved highly effective in promoting germination.

In our investigation, viability assessments conducted on the seeds of *Phalaenopsis* sp. revealed that a portion of the seeds did not exhibit signs of viability. The study involved determining the viability percentages and contamination rates of seeds obtained through the direct opening of the capsule. The viability percentages were found to be $72 \pm 4.0\%$, while the contamination rate was determined to be $6 \pm 0.4\%$. The germination rates of the seeds were subsequently determined based on the viability percentages obtained. According to Crafts and Miller (1974), it has been asserted that the

fungi generate cytokinins within this symbiotic association, consequently leading to the germination of the seeds.

Considering the observed elongation in seedlings at the age of four months of our study, it was observed that the application of TDZ resulted in a significant increase in shoot elongation, while not significantly affecting explant yield. Particularly at a concentration of 0.5 ppm, TDZ exhibited superior efficacy compared to the other experimental groups. Nevertheless, it was noted that the increase in shoot elongation was adversely impacted by higher concentrations of TDZ (1.0 ppm). The alternative group of individuals from the AHS also exhibited shoot elongation in comparison to the control group during the shoot elongation experiment. A significant correlation was observed between the concentration levels and the extent of shoot elongation.

In a study conducted by Chen and Chang (2000), it was observed that lower concentrations of TDZ exhibited favorable outcomes in terms of shoot formation. However, in our study higher concentrations of TDZ were found to have an impact on the formation of callus and adventitious shoots. The utilization of TDZ has demonstrated greater efficacy compared to 6BA, as indicated by Ernst (1994) and Chang and Chang (1998). Previous studies in the literature have documented the positive impact of TDZ on the promotion of shoot formation and reproductive processes in orchids (Chen & Piluek, 1995; Nayak et al., 1997; Chen et al., 2004; Ferreira et al., 2006). In our study, regarding the elongation of roots, it was observed that all cytokinins, regardless of their concentrations, exhibited a favorable impact when compared to the control group. Nevertheless, there was no statistically significant difference observed between these growth regulators. Regarding the quantity of roots generated, it was observed that all cytokinins tested, along with their respective concentrations, exhibited favorable outcomes when compared to the control group.

The statistical analyses of our study revealed that higher concentrations, apart from the increased 2-rope concentration, were associated with a decrease in the number of root formations. Previous studies have documented that the presence of auxins in the environment is essential for the promotion of root formation (Bhojwani & Razdan, 1986; Mansuroğlu & Gürel, 2001; Díaz & Álvarez, 2009).

The statistical analysis results also indicated that, overall, the use of 2-ropo as a PGR had a smaller impact on leaf formation when compared to other regulators. Previous research has documented that cytokinin group growth regulators exhibit positive effects on shoot formation as well as inhibit leaf formation (Gaspar et al., 1996; Haberer & Kieber, 2002). Nevertheless, in a study conducted by Bektaş et al. (2013), it was observed that the concurrent application of 2.0 mg/L of Zeatin (ZEA) and 0.5 mg/L of Indole-3-butyric acid (IBA) resulted in a greater stimulation of leaf development. In our investigation, exclusively cytokinins and their varying concentrations were examined. In prospective investigations, it is hypothesized that the concurrent application of auxin and cytokinins will yield a more favorable outcome in terms of leaf development.

5. CONCLUSION

Since our study solely aimed to investigate the impact of basic nutrient media and specific growth regulators, namely gibberellic acid and cytokinins, on the *in vitro* germination of plant seeds belonging to the orchid species *Phalaenopsis* sp. therefore, as a continuation of the work we suggest the following stages for further investigations:

- Assessment of auxins along with cytokinins on leaf and root formation in determining the effects of plant growth regulators.
- Examination of callus formation, where cytokines and auxins are used equally for indirect organogenesis.
- Protocorm-like structures are structures that are composed of many meristematic cell centers capable of forming shoots and roots (Da Silva et al., 2000). *Phalaenopsis* sp. creates synthetic seeds by coating protocorm-like structures (PBY) with alginate. Therefore, more investigation of the germination capabilities of the synthetic seeds in the culture media and soil conditions should be conducted.
- It can be presented under new research titles such as adaptation studies of seedlings grown in culture medium to an external environment.

Appendix



Figure A1. *In vitro* completed development of seedlings



Figure A2. *In vitro* propagated plantlets transferred to the external environment

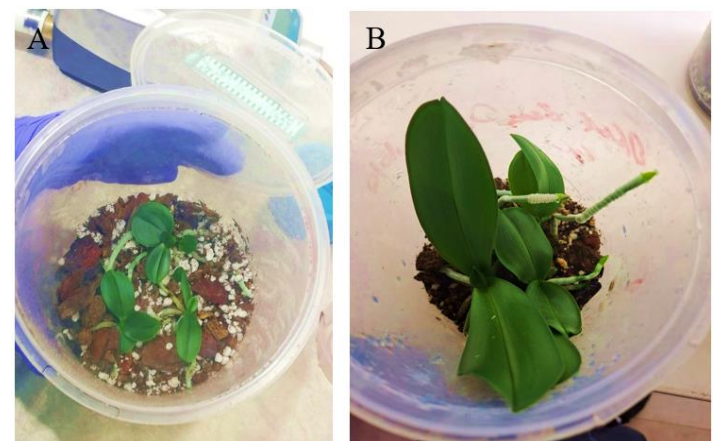


Figure A3. Seedlings transferred to *in vivo* (A); Orchids growing outdoors (B)

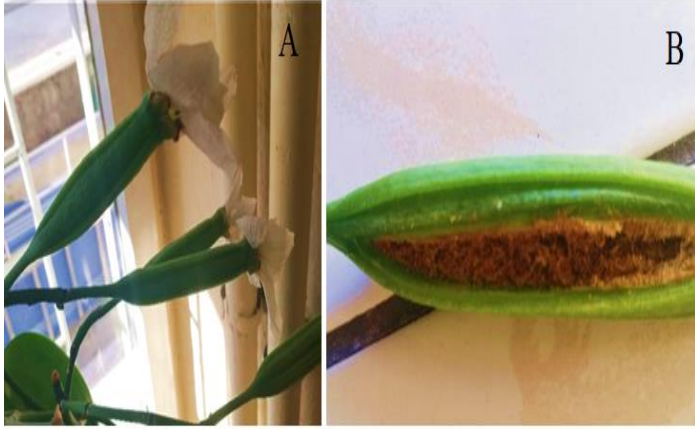


Figure A4. (A) Seed stubs formed after fertilization; (B) Seeds inside the seed pod

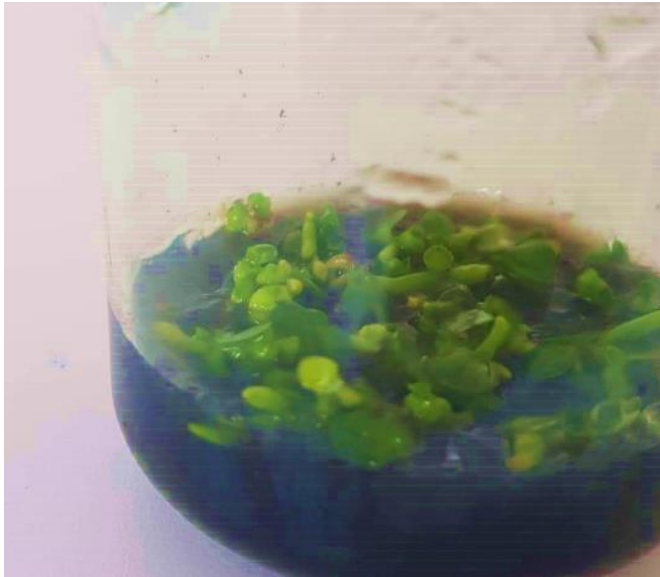


Figure A5. Germination of orchid seeds



Figure A6. Explant preparation from developing seedlings

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