

**T.C.
YEDİTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF PHARMACOGNOSY**

**Pharmacognostic researches on *Pinus* species growing
in Turkey**

DOCTOR OF PHILOSOPHY THESIS

Gülşah Selin AKYÜZ, Pharm.

İSTANBUL-2022

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Prof. Dr. Erdem Yeşilada**

İSTANBUL-2022

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APPROVAL

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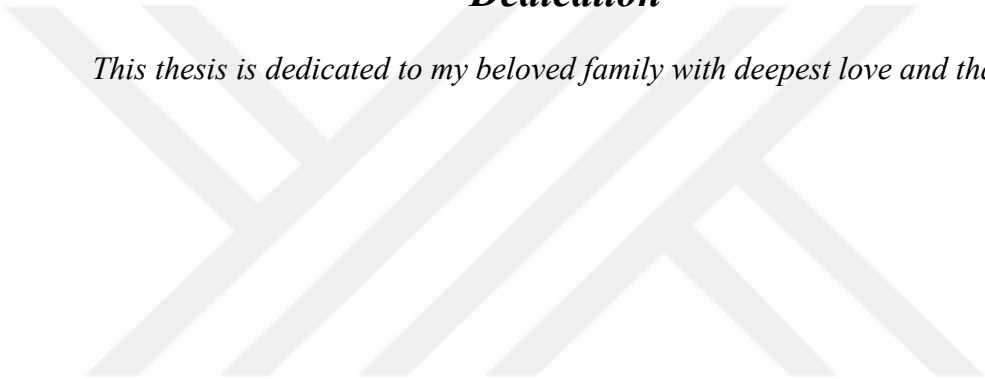
DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

11.02.2022

Gülşah Selin AKYÜZ





Dedication

This thesis is dedicated to my beloved family with deepest love and thanks

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ABBREVIATIONS

AGEs: Advanced Glycation End Products
AAE: Ascorbic Acid Equivalent
ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ADC2: Automatic Development Chamber 2
AE: Aescin Equivalent
ANOVA: Analysis of Variance
BaVI: Bioavailability Index
BD: Before Digestion
BHT: Butylated Hydroxytoluene
BSA: Bovine Serum Albumine
BP: Bioavailable Phase
CaCl₂: Calcium chloride
CAE: Caffeic Acid Equivalent
CE: Catechin Equivalent
CG: Catechin Gallate
CHCl₃: Chloroform
CUPRAC: Cupric Reducing Antioxidant Capacity
CuSO₄: Copper(II) sulfate
DM: Diabetes Mellitus
DMPD: (N,N-dimethyl-p-phenyldiamine)
DNA: Deoxyribonucleic Acid
DNS: 3,5-dinitrosalicylic acid reagent
DPPH: 2,2-Diphenyl-1-picrylhydrazyl
EGC: Epigallocatechin
EGCG: Epigallocatechin Gallate
EtOAc: Ethyl Acetate
Fe³⁺: Ferric Ion
FeCl₃.6H₂O: Iron(III) chloride hexahydrate
FCR: Folin-Ciocalteu Reagent
FRAP: Ferric Reducing Antioxidant Power
GAE: Gallic Acid Equivalent
GI: Gastro-Intestinal

GF: Gastric Phase
HCl: Hydrochloric Acid
HPTLC: High Performance Thin Layer Chromatography
IC₅₀: The half aximal inhibitory concentration
IL-1 α : Interleukin 1 alpha
IL-1 β : Interleukin 1 beta
KCl: Potassium Chloride
MeOH: Methanol
n-BuOH: *n*-Butanol
NaCl: Sodium Chloride
QE: Quercetin Equivalent
PaA1: Proanthocyanidin A1
PaB1B2: Proanthocyanidin B1/B2 mixture
PaC1: Proanthocyanidin C1
PYC: Pycnogenol[®]
ROS: Reactive Oxygen Species
TFC: Total Flavonoid Content
TLC: Thin Layer Chromatography
TOAC: Total Antioxidant Capacity
TPC: Total Phenolic Content
TPaC: Total Phenolic Acid Content
TPrC: Total Proanthocyanidin Content

ABSTRACT

Akyüz, G.S. (2022). Pharmacognostic researches on *Pinus* species growing in Turkey . Yeditepe University, Institute of Health Sciences, Department of Pharmacognosy, Ph.D. Thesis, İstanbul.

Current environmental factors and lifestyles deteriorate the balance of oxidants and antioxidants, and hence, oxidative stress and various diseases trigger in the human body. Substantial evidence suggests that oxidative stress is associated directly with diabetes and its microvascular and cardiovascular complications. Numerous scientific research has indicated that natural products could prevent the body from oxidative stress and alleviate many oxidative stress-related diseases. Pycnogenol[®], a patented-standardized extract from *P. pinaster* barks, is one of the pharmacologically active natural products. This thesis was planned to evaluate the phytochemical profile, antioxidant and anti-diabetic activities of hot water extracts from the barks of two naturally growing *Pinus* species in Turkey; *P. brutia* Ten., *P. pinea* L., and one cultivated species in Turkey; *P. pinaster* Ait. Their chemical and activity profiles were compared to Pycnogenol[®] before and after submitting to an *in vitro* gastrointestinal human digestion simulation system. *In vitro* antioxidant assays indicated that hot water bark extracts of all studied plants possess high antioxidant activity in terms of free radical scavenging, metal-reducing activity, and total antioxidant activity. In addition, the results showed that all studied extracts had potent inhibitory activities on α -glucosidase, α -amylase enzymes and advanced-glycation end products (AGEs). The total phenolic, phenolic acid, proanthocyanidin, and flavonoid contents and also the amounts of some proanthocyanidins (proanthocyanidin A1, a proanthocyanidin B1/B2 mixture, proanthocyanidin C1), and flavan-3-ol derivatives (catechin/epicatechin mixture, epigallocatechin, epigallocatechin gallate, and catechin gallate), and several other phenolic components (taxifolin, caffeic acid, and ferulic acid) were measured to reveal the phytochemical contents of all studied extracts. High Performance Thin Layer Chromatography (HPTLC) system was used to identify and quantify of these metabolites. The results indicated that even though *in vitro* human gastrointestinal system negatively influenced the phytochemical profile, antioxidant and anti-diabetic potentials, all extracts possess high phenolic content and potent biological activities.

Key words: *Pinus* species, Phenolic compounds, Antioxidant activity, *In vitro* Gastrointestinal Digestion Simulation system, HPTLC, Antidiabetic Activity

ÖZET

Akyüz, G.S (2022). Türkiye’de Yetişen *Pinus* türleriyle ilgili Farmakognostik çalışmalar. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Farmakognozi ABD, Doktora Tezi, İstanbul.

Mevcut çevresel faktörler ve yaşam biçimleri oksidan ve antioksidan dengesini bozmakta ve insan vücudunda oksidatif stres ve çeşitli hastalıklar tetiklenmektedir. Önemli kanıtlar, oksidatif stresin doğrudan diyabet ve onun mikrovasküler ve kardiyovasküler komplikasyonları ile ilişkili olduğunu göstermektedir. Bilimsel araştırmalar doğal ürünlerin vücudu oksidatif stresten ve buna bağlı birçok hastalığı hafifletme yeteneğine sahip olduğunu göstermiştir. *P. pinaster* kabuklarından elde edilen patentli standardize bir ekstrakt olan Pycnogenol®, farmakolojik olarak aktif doğal ürünlerden biridir. Bu tez Türkiye’de doğal olarak yetişen iki *Pinus* türünden; *P. brutia* Ten., *P. pinea* L., ve Türkiye’de yetiştirilen bir *Pinus* türünden; *P. pinaster* Ait., elde edilen kabuklarının sıcak su ekstraktlarının fitokimyasal profilini, antioksidan ve antidiyabetik aktivitelerini değerlendirmek için planlanmıştır. Ekstraktların kimyasal ve aktivite profilleri Pycnogenol® ile *in vitro* gastrointestinal insan sindirim simülasyon sistemine uygulanmadan önce ve sonra karşılaştırılmıştır. İncelenen tüm bitkilerin sıcak su kabuğu ekstraktlarının, serbest radikal temizleme, metal indirgeme aktivitesi ve toplam antioksidan aktivite açısından yüksek antioksidan aktiviteye sahip olduğu bulunmuştur. Ayrıca sonuçlar, çalışılan tüm ekstraktların α -glukosidaz, α -amilaz enzimleri ve ileri glikasyon son ürünleri (AGE’ler) üzerinde güçlü inhibitör aktivitelere sahip olduğunu göstermiştir. Toplam fenolik, fenolik asit, proantosiyanidin ve flavonoid içerikleri ve ayrıca bazı proantosiyanidinlerin (proantosiyanidin A1, proantosiyanidin B1/B2 karışımı, proantosiyanidin C1), bazı flavan-3-ol türevlerinin (kateşin/epikateşin karışımı, epigallokateşin, epigallokateşin gallat, kateşin gallat), ve diğer bazı fenolik bileşiklerin (taksifolin, kafeik asit ve ferulik asit) miktarları çalışılan tüm ekstraktların fitokimyasal içeriğini aydınlatmak için ölçülmüştür. Bu bileşenlerin tespiti ve miktar tayini amacıyla Yüksek Performanslı İnce Tabaka Kromatografisi (YPİTK) sistemi kullanılmıştır. Sonuçlar, *in vitro* insan gastrointestinal sisteminin fitokimyasal profili, antioksidan ve anti-diyabetik potansiyelleri olumsuz etkilemesine rağmen, tüm ekstraktların yüksek fenolik içeriğe ve güçlü biyolojik aktivitelere sahip olduğunu göstermiştir.

Anahtar kelimeler: *Pinus* türleri, Fenolik bileşikler, Antioksidan aktivite, *In vitro* Gastrointestinal Sindirim Simülasyonu, YPİTK, Antidiyabetik aktivite

1. INTRODUCTION AND AIMS OF THE STUDY

There is much substantial evidence in the scientific literature that suggests the natural products are the lynchpin for ameliorating human health by preventing and treating diseases and metabolic disorders. In particular, increasingly growing interest in the influence of natural products on human health is due to transpiring the inevitable side effects of synthetic drugs in recent years. Hence, the investigation on the health benefits of natural products, the description of their active components, and their mechanisms of action have gained importance for maintaining human health.

Oxidative stress, which is directly associated with the augmentation of reactive oxygen species, is the principal reason in the pathogenesis of various diseases and metabolic disorders, including diabetes mellitus, Alzheimer's disease, cardiovascular and neurodegenerative diseases, and cancer. There is a growing belief that the first step for preventing and healing these abnormalities is to balance this augmentation of reactive oxygen species in the human body. Indeed, many scientific studies are in good agreement with this phenomenon. Fortunately, natural antioxidants expedite the counterbalance of this imbalance with the natural antioxidant defence system in the body (1).

Diabetes Mellitus is characterized by the augmentation of glucose in the blood and an imbalance of insulin secretion in the body. In particular, as parallel to the modernization of society in the recent decades, the incidence of diabetes and insulin resistance has grown due to changing dietary habits and shifting to a sedentary lifestyle. The considerable evidence suggests that oxidative stress is associated closely with diabetes and its microvascular and cardiovascular complications. Advanced glycation end products (AGEs), which occur after the glycation reaction, play a crucial role in the pathogenesis of diabetes and its complications. The formation of these metabolites is triggered by raising blood sugar, and also the production of reactive oxygen radicals is induced by increasing AGEs. This circle contributes to the development of various diseases and metabolic syndrome. Therefore, it is important to investigate the natural products effective against oxidative stress and diabetes. Many studies have established that many natural compounds exert potent antioxidant and antidiabetic activities (2,3).

On the other hand, it is well known that natural products strive for health-markedly promoting effects; their bioactive forms must be absorbed from the gastrointestinal system into the bloodstream to effectuate these health-promoting effects. The bioavailability of natural products is influenced by several factors, including; pH changes, enzymes in the gastrointestinal tract, peristaltic movements and microbiota. Moreover, the physicochemical characteristics of natural products, such as crystalline, also affect their bioavailability in the body. Therefore, *in vitro* gastrointestinal digestion simulation models have been suggested to obtain more reliable pharmacological results (4).

The genus *Pinus*, composed of trees, is the largest family member of Pinaceae, which is widely distributed naturally in the northern hemisphere and cultivated in the southern hemisphere. Five species of this genus, *P. brutia*, *P. pinea*, *P. nigra*, *P. sylvestris*, and *P. halepensis*, grow naturally in Turkey. In addition, several *Pinus* species, including *P. pinaster* are cultivated in Turkey (5,6). The different parts of *Pinus* species have been used in Turkish traditional medicine to heal various disorders. On the other hand, Pycnogenol[®], a patented-standardized extract from *P. pinaster* barks, is recognized as a potent natural antioxidant component. This component has been shown to possess widely diverse pharmacological activities such as antidiabetic, anti-inflammatory, cardioprotective, etc. by experimental and clinical investigations (7).

In order to evaluate the possible antioxidant and antidiabetic potentials of the natural *Pinus* species of Turkey, a comparative study has been planned. For this purpose, the antioxidant and anti-diabetic activities of the extracts obtained from *P. brutia* and *P. pinea* barks (indigenous species) were compared with *P. pinaster* (cultivar) and Pycnogenol[®] by using *in vitro* methods. For antioxidant activity, two free radical scavenging activity assays; DPPH and DMPD, two metal-reducing activity assays; CUPRAC and FRAP, and a total antioxidant capacity assay, were used. For the anti-diabetic activity *in vitro*, α -amylase and α -glucosidase enzyme inhibitory activities and effect on AGEs inhibition of these extracts were evaluated. For evaluation of the true antioxidant and antidiabetic activity potentials, all extracts were subjected to *in vitro* gastrointestinal digestion simulation procedures. In addition, marker metabolites of the extracts were characterized using the HPTLC system.

2. THEORETICAL INFORMATION

2.1. Botanical Information

2.1.1. Pinaceae family

Pinaceae family, composed of trees, is one of the main members of Gymnospermae. Leaves are linear or linear-oblong, and always have resin canals. They are borne in fascicles or whorls on the short shoots or singly on long shoots. Fruit is a woody cone. According to ‘*The Flora of Turkey and the East Aegean Islands*’, there are four genera of Pinaceae family in Turkish flora (6). The dichotomous key for the identification of these genera is as follows:

1. Mature leaves borne on short shoots, in whorls or fascicles of two
 2. Leaves in fascicles of two, each fascicle surrounded by a sheath at the base.....*Pinus*
 2. Leaves in whorls, without sheaths at the base.....*Cedrus*
1. Mature leaves borne spirally on long shoots; short shoots absent
 3. Branchlets with numerous peg-like projections persisting after leaf-fall; cones pendulous, falling as a whole.....*Picea*
 3. Branchlets without such projections; cones erect, the scales falling from the persistent axis..... *Abies*

2.1.1.1. *Pinus* genus

The genus of *Pinus* is composed of evergreen trees whose branches are whorled. Leaves in pairs (in Turkish species) are borne on short shoots. Cones fall in their entirety, cylindrical to ovoid. The exposed scale (apophysis) is with a prominent umbo, often mucronate. Seeds are winged or unwinged. The genus *Pinus* is represented by five species in “*Flora of Turkey and The East Aegean Islands*”. Dichotomous key for the determination of these species is as follows (Flora of Turkey):

1. Buds not resinous; leaves flaccid, light green
 2. Crown umbrella-shaped; seeds unwinged.....*pinia*
 2. Crown irregular; seeds winged
 3. Cones clearly stalked, pendulous.....*halepensis*
 3. Cones ± sessile, spreading.....*brutia*

1. Buds not resinous; leaves flaccid, light green

4. Twigs reddish; leaves glaucous, twisted: cone pendulous.....*sylvestris*

4. Twigs blackish; leaves dark green, not twisted: cone erect or ascending
.....*nigra* subsp. *pallasiana*

2.1.1.1.1. *Pinus brutia* Ten.

P. brutia is an evergreen, with a straight trunk and branches, up to 25 m. Twigs are glabrous, usually reddish in the first year, becoming greyish brown, rarely all greyish. Buds are non-resinous, the scales recurved and fimbriated. Leaves are (5–)10–18(–29) cm long, 1–1.5 mm wide, flaccid, light green. Barks are red-brown and deeply fissured. Cones are elongate, brown, usually more than two borne together. Peduncles are very short to almost absent, and the cones are erect or ascending, never deflexed. Seeds are winged, obovoid-oblique (6).

Habitat: Dominant forest tree

Altitude: 1–1200 m.

Turkish names: Akçam,çam (8), kızılçam (9)

English names: Calabrian pine, Brutia pine(9)

Distribution in Turkey: Outer Anatolia, Turkey-in-Europe & Islands.

Worldwide distribution: South Italy, South Aegean, Crimea, Cyprus, North Iraq, West Syria, West Caucasus.



Figure 1. *Pinus brutia* Ten.

2.1.1.1.2. *Pinus pinea* L.

P. pinea is an evergreen tree, up to 25 m, with a large, umbrella-shaped crown. Twigs are glabrous, very rough. The young twigs are yellowish, while the older grey-brown. Buds are non-resinous, scales fimbriated. Leaves are 8–12(–15) cm long, 1.2–1.8mm wide, and dark green. Barks are orange-brown to reddish-brown with grey patches. Cones are very large, 8-14 x c. 10 cm, brown, sessile, and pendulous. Seeds are unwinged or with a very short wing (6).

Habitat: Dominant forest tree

Altitude: 1-350 m.

Turkish names: Fıstık çamı, Kürnar (8,10)

English names: Stone pine, Umbrella pine (5)

Distribution in Turkey: Scattered in Outer Anatolia

Worldwide distribution: Mediterranean area, Portugal





Figure 2. *Pinus pinea* L.

2.1.1.1.3. *Pinus pinaster* Ait.

P. pinaster Ait. is an evergreen tree, up to 40 m, with a regular, ovoid-conic shaped crown. Buds are non-resinous and reflexed. Leaves are 10-25 cm long and 1.5-2 mm wide, and dark green. Barks are reddish-brown or grey-brown and deeply fissured. Cones are light brown, pedunculate, reflexed 10–20(–22) cm long and 5–8 cm wide. Seeds are winged, grey or blackish-grey and obovoid-oblong (5).

Habitat: Dominant forest tree

Altitude: 1-2000 m.

Turkish names: Sahil çamı

English names: Maritime pine⁵

Distribution in Turkey: Cultivation

Worldwide distribution: South Europe; North Africa, Morocco, border between Algeria and Tunisia



Figure 3. *Pinus pinaster* Ait.

2.2. General Information on Diabetes Mellitus

In general, sufficient insulin is secreted from β -cells of the islets of Langerhans in the pancreas, and insulin diffuses towards peripheral tissues to uptake the glucose from the blood to these tissues. Moreover, the elevated glucose level in the blood is converted to glycogen, a multibranched polysaccharide of glucose, by insulin in the liver. Glucose transport throughout the cell membranes is ensured with glucose transporter (GLUT) proteins, and sodium-dependent glucose facilitates transporters (SGLTs). In addition to these activities, insulin also indirectly liberates the lipid triglycerides into glycerol and fatty acids by stimulation of lipoprotein lipase enzyme. Glucagon, secreted from α -cells in the pancreas, is a counterbalance of insulin, and Glucagon triggers the breakdown of glycogen back to glucose. This situation is known as “Glycogenolysis”. The detriment of glucose homeostasis leads to many diseases and metabolic syndromes, especially diabetes mellitus (DM) (11,12).

Diabetes mellitus, a chronic endocrine disorder, is caused by inadequacy of insulin production and resistance to insulin and elevated blood sugar levels. Type I-DM, is called insulin-dependent diabetes, characterized by a lack of insulin production in pancreatic β -cells. The first causation of Type II DM is a decrease in interaction between insulin and peripheral tissues, and this situation is known as “Insulin resistance”. Secondly, the pancreas does not produce adequate insulin due to pancreatic β -cells dysfunction. In addition to these two factors, increased gluconeogenesis in the liver may lead to Type II DM (13,14).

High glucose levels spark microvascular and macrovascular abnormalities due to damage to the endothelial cells. The destructions of small blood vessels promote microvascular abnormalities, including diabetic retinopathy, nephropathy, and neuropathy. Macrovascular abnormalities as atherosclerosis, hypertension, cerebrovascular disease are examples of the highest prevalence in DM patients (15).

The deterioration of endothelial cells is carried on five main mechanisms (3).

- I. The polyol pathway in which transubstantiates glucose into polyols is induced with hyperglycemia.
- II. The elevated blood sugar levels culminate in excessive hexosamine production.

- III. Overactivity of protein kinase C is begotten by hyperglycemia.
- IV. Hyperglycemia generates the formation of advanced glycation end products (AGE).
- V. The expression of the receptor for AGE (RAGE) is sparked by hyperglycemia.

Oxygen is one of the vital molecules for aerobic organisms and its reduced forms, known as reactive oxygen species (ROS), occur in routine cell activities in the body. ROS, which forms at high concentrations, attacks endothelial cells with endogenous and exogenous sources, including mitochondrial electron transport chain, haem proteins, sun exposure, or drugs. Consequently, the overproduction of ROS contributes to oxidative stress¹. Oxidative stress is the utmost causation in both microvascular and macrovascular abnormalities. The increased mitochondrial superoxide formation in endothelial cells is the mutual point of these five mechanisms (3).

2.2.1. Advanced-Glycation End Products

Glycation is a natural chemical reaction occurring both inside and outside the cells. This reaction may ensue enzymatically (glycosylation) or non-enzymatically. Non-enzymatic glycation is simply defined as reducing sugar to a molecule such as proteins and DNA. In the first step of the glycation, the carbonyl group of reducing sugars such as glucose react with a free amino group of proteins. As a result of this reaction, it yields unstable and reversible molecules known as Schiff base. After that, more stable intermediates, Amadori adducts, form from Schiff bases. Eventually, some chemical reactions of Amadori adducts, such as oxidation, generate heterogeneous, stable, and irreversible molecules. These molecules are called advanced glycation end products (2). Furthermore, the oxidation of lipid molecules and glycolysis lead to the production of dicarbonyl intermediates such as methylglyoxal (MGO) and glycolaldehyde. AGEs are formed in which the generated intermediates attack the free amino group of proteins. Besides, exogenous sources, including smoking and diet with high AGEs products, are also accumulated AGEs tremendously in the body (16).

On the cell surface, there are specific receptors for AGEs (RAGE). These receptors, which are a member of the immunoglobulin superfamily, play a significant role in the many ailments by AGEs. Apart from RAGE, there are also some receptors for

AGEs such as galectin or 80 K-H phosphoprotein (2). Stimulation of these receptors by ROS (reactive oxygen species) or an excessive amount of AGEs trigger the production of some proinflammatory mediators such as tumor necrosis factor- α (TNF- α) and matrix metalloproteinases (MMPs). These mediators induce cell inflammation signalling pathways linked to metabolic and autoimmune diseases (3).

The exorbitant formation of AGEs is one of the major agents in the aging process, inflammation as well as diabetic complications. For instance, in the case of hyperglycemia, AGEs form a complex with RAGE on peripheral nerves, increasing the free radicals that provoke AGEs production by nitric oxide. Diabetic neuropathy is one of the diabetic complications that developed in this way (2). In addition to these reasons, many other different mechanisms boost hyperglycemia and cause diabetes. In diabetic retinopathy, overactivity of AGEs, especially carboxymethyl lysine (CML), attacks the retinal vascular endothelial cells via inducing RAGE expression on perivascular cells. Hereby, apoptosis of perivascular cells is induced with the thickening capillary basement membrane, exudation of the inner blood-retinal barrier (16). Diabetic nephropathy is a chronic syndrome that emerges by promoting urine albumin excretion, diminishing glomerular filtration, and enhancing glomerular lesions in the kidney (17). The kidney is the central organ of the clearance of metabolites such as AGEs. AGEs-RAGE complex induces thickening glomerular basement membrane, loss of podocyte, and abridgement of the glomerular filtration rate (18).

Apart from microvascular abnormalities, AGEs are related to impaired wound healing and macrovascular abnormalities, including atherosclerosis. TNF- α and MMPs that hobble the wound healing process are elevated by AGEs-RAGE complex (2). In atherosclerosis, AGEs enhance many functions, including the glycation of low-density lipoprotein (LDL), plaque formation, and the transformation of monocytes to macrophages. Furthermore, AGEs-RAGE complex triggers atherosclerosis via the improvement of the expression of vascular cell adhesion molecule-1 (19).

2.2.2. Diabetes-Related Enzymes

α -Amylase (1,4-D-glucan-glucanohydrolase), located in the saliva and pancreas, activates the hydrolysis of α -(1,4)-type glucan of polysaccharides. The digestion of carbohydrates starts partially by α -amylase in the salivary. These molecules that pass on

the intestinal system are hydrolyzed to smaller types by pancreatic α -amylase. In the brush border of the intestine's enterocytes, there is another digestive enzyme known as α -glucosidase enzyme. This enzyme breaks down oligosaccharides to glucose, which is absorbed by the blood circulation, thus elevating the blood glucose level (20).

2.3. Chromatography

Therapeutic activities of natural products such as plants and marine organisms are related to their chemical components and pharmacological effects. Natural products used for therapeutic activities must be accurately standardised to expose their pharmacological activities exactly. Several techniques have been used for qualitative and quantitative analysis of natural products for the standardization process (21).

Chromatography is one of the utmost essential techniques for qualitative and quantitative analysis. Chromatography, known as written in color, is a separation technique in which molecules are distributed between two phases according to their affinities. There are three main categories according to their physical state of mobile phase, mechanism of separation and development technique (22).

2.3.1. High-Performance Thin-Layer Chromatography (HPTLC)

Thin-layer chromatography (TLC), a basic chromatographic method, is used to identify and quantify molecules by using solid and liquid phases. The separation of molecules is related to retention times on the solid phase. TLC is improved with increasing new technological development by scientists. HPTLC is one of the superior techniques of TLC. The automated sample application, automated developing chamber, densitometric measurements, minimum sample size, and combination to mass spectrometry (MS) are some advantages of the HPTLC technique (23).

Moreover, all steps of HPTLC can be controlled with software and provide more sensitivity, reproducible analysis, and spending less time. The particle size of stationary phase is smaller than TLC plates and provides more accurate results using less mobile phase. The reliable qualitative analysis of HPTLC depends on chromatographic fingerprints of molecules that can be visualized and deposited as images. The quantitative data is obtained by measuring peak area with densitometry (22).

HPTLC is widely used in pharmacognosy, forensic chemistry, environmental analysis, cosmetology, and many other fields. Apart from qualitative and quantitative analysis, it is used to detect adulteration, stability control and also to perform some activity analysis such as DPPH radical scavenging activity (22,23). After the development process, HPTLC plates are sprayed with DPPH solution. It is well-known that the characteristic purple color of DPPH solution transforms to yellow color in the presence of the antioxidant molecules due to DPPH radical reduced to diphenyl picryl hydrazine. Hence, qualitative and quantitative DPPH analysis can be carried out using the HPTLC system (22).



2.4. General Information on *Pinus* species

2.4.1 Traditional Medicinal Uses of *Pinus* species in Turkey

Leaves, cones, resin, tar and stems of *Pinus* species have been used for various complaints in Turkish traditional medicine. Tables 1-4 summarize the use of *P. brutia* Ten., *P. pinea* L., *P. nigra* J.F. Arnold, and *P. sylvestris* L. species in traditional medicine. However, there is no record of the traditional uses of *P. halepensis* Mill.

Table 1. Traditional Medicinal Uses of *P. brutia* from Turkey

Part(s) used	Administration	Usage
Tar	Externally	Fissure, skin lesions, as antiseptic, and to heal wounds (8,9,24,25)
	Externally; mixed with olive oil and salt	Abdominal pain and rheumatism (8)
	Externally; with onion and olive oil	Diarrhea and bloating (26)
Resin	Externally	Hemorrhoids, abscess and abdominal pain (27,28)
	Internally; with honey	Common cold and cough (9,25)
	Internally; eaten with hot water	Peptic ulcer (29)
Cones	Internally; boiled with sugar	Stomachache, bronchitis, and cough)30)
	Internally; decoction	Shortness of breath (8), Anti-diarrhoea (26), antitussive, asthma, hoarseness (31), cough (25)
	Internally; infusion	Bronchitis (24,31)
Barks	Internally; decoction	Pneumonia (26)
Leaves	Internally; decoction	Bronchitis (30)
Stems	Externally; infusion; as a gargle	Dental hygiene (31)

Table 2. Traditional Medicinal Uses of *P. pinea* from Turkey

Part(s) used	Administration	Usage
Branches	Internally; infusion	Bronchitis, cardiovascular diseases (29)
Barks	Internally; decoction	Stomach ailments (32)
Cones	Internally; eaten raw	Aphrodisiac (24,33)
Gum	Internally; decoction	Wounds (34,35)
Seeds	Internally; eaten in honey	Bronchitis (10)

Table 3. Traditional Medicinal Uses of *P. sylvestris* from Turkey

Part(s) used	Administration	Usage
Cones	Internally; decoction	Bronchitis, common cold, cough, and as hypoglycaemic (25,36-38)
	Internally; eaten while fresh	Common cold, as expectorant (37)
Shoots	Internally; eaten	Bronchitis and common cold (25,36)
	Internally; decoction	Cough (25,38) and tuberculosis (37)
Branches	Externally; decoction	Rheumatic pain (36)
Resin	Externally; chewed	Cough (25)
	Externally; with beeswax and butter	Wounds (37)
	Internally; boiled in milk	Common cold, as an expectorant and as anthelmintic (37)
	Internally; with honey	Tuberculosis and ulcers (38)
Tar	Externally; with butter	Wounds (39)
	Externally; with egg yolk	For snake bite (39)

	Externally; with the tar of <i>Juniperus sp.</i> and leaves of <i>Sambucus ebulus</i>	Rheumatic pain (37)
Stem barks (floem)	Internally; with milk	Tuberculosis ^{36,39}
	Internally	Peptic ulcer ³⁶
	Internally; eaten as raw	Tuberculosis ³⁸

Table 4. Traditional Medicinal Uses of *P. nigra* from Turkey

Part(s) used	Administration	Usage
Cones	Internally; decoction	Shortness of breath (8), hemorrhoids, and as anthelmintic (37)
	Internally; decoction	Diabetes and fungal infections (27)
Barks	Internally; eaten	Antidiarrheal (9)
Resin	Internally; swallowed	Gastric ulcer (9)
	Externally	Fractured bones, burns (9), respiratory and urinary diseases (24), abcess (9,37), wound (27), Itch (8)
	Externally; heated	Itch (8)
	Internally; with honey	Bronchitis, cough (37), and stomachache (27)
	Internally; with styrax	Bronchitis (27)
	Internally; as pills	Common cold and cough (27)
	Internally; with roots of <i>Astragalus sp.</i> in milk	Abdominal ptosis (28)
	Internally; eaten raw	Tuberculosis (40)
Tar	Externally; on the abdomen	Abdominal pain and diarrhea (9)
	Internally; with water	Abdominal pain and diarrhea (9)
	Externally	Snake bite, wound, common cold, fissure (9)
	Externally; with pounded wheat	Abcess (9)
	Externally; with salt	Abcess (37)

Stem barks (floem)	Internally; eaten	Tuberculosis, bronchitis, stomachache, and as anthelmintic (37)
	Internally; eaten as raw	As panacea (27)
Shoots	Internally; decoction	Rheumatism (37)
	Internally	Piles (28)

2.4.2. Traditional Medicinal Uses of *Pinus* species in other countries

The genus *Pinus* consists of approximately 113 species throughout the world. The different parts of *Pinus* species have been used in traditional medicine in other world regions.

Different parts of *P. halepensis* Mill. have been used against many ailments such as diarrhea, wounds, skin disorders, sexual disorders, hemorrhoids, tuberculosis, rheumatism, urinary problems, asthma, and hypertension in various Mediterranean countries (41-43). The resin was obtained from the stem of *P. chiapensis* Mart. is applied on the skin for wounds and fractured bones in southern Mexico and northern Guatemala (44). The resin of *Pinus roxburghii* Sarg. is used against many disorders such as cough, injuries, cracks, gastric ailments, tuberculosis, hair loss and snake bites in Nepal (45). Cones of *P. cembra* L. is used as a digestive in North-Western Italy (46). Both cones and shoots of *P. nigra* and *P. sylvestris* are utilized against cough and sore throat in the North-Western Ligurian Alps (47). In Kopaonik Mountain (Central Serbia), a mixture prepared from buds of *P. sylvestris* and honey is eaten for chronically bronchitis (48). In Bosnia and Herzegovina, traditional balms prepared from resins of *P. sylvestris*, *P. mugo* Turra, *P. heldreichii* Christ, *P. nigra* are used for wound healing (49). In the Himalayan region, the resin of *P. wallichiana* A.B Jacks. is used to treat wounds, fractures, influenza and as antiphlogistic (50). In Southern Spain, cones of *P. pinea* are used as antiscorbutic and astringent, while cones of *P. pinaster* are used for acute renal colic and depurative properties (51). Different parts of *P. kochiana* Klotzsch ex K. Koch are employed against asthma, bronchitis, cough, and renal problems in Caucaus (52). In Portugal, decoctions prepared from young male strobilus of *P. pinaster* are drunk against cold and cough (53). Alcoholic extracts prepared from young leaves and cones of *P. yunnanensis* Franch. are taken orally against rheumatic diseases in Yunnan province, China (54). According to

Ayurvedic Pharmacopoeia, *P. gerardiana* Wallich ex. D. Don. is used to treat many diseases such as epilepsy, cough, wound, anaemia, gout, asthma (55). In the USA, different parts of *Pinus edulis* Engelm. and *Pinus monophylla* Torr. & Fr. are used traditionally to relieve cold symptoms by inhalation (56).

Table 5. Traditional Medicinal Uses of *Pinus* species in other countries

Plant name	Part(s) used	Administration	Locality	Usage
<i>P. halepensis</i> Mill.	Leaves and seeds	Internally; Decoction	Eastern Region of Mediterranean	Sexual weakness (42)
	Leaves and resin	Internally; decoction	Northeast Algeria	Bronchitis, pneumonia, colds, urinary problems, parasitosis, wounds (43)
	Inflorescence	Internally; decoction	Southern Spain	Asthma (41)
	Leaves and barks	Internally; decoction	Morocco	Skin disorders, wounds, eczema, astringent, tuberculosis (41)
	Fruits	Internally; infusion	Algeria	Hemorrhoids (41)
	Needles	-	Middle-Atlas of Morocco	Rheumatism (41)
<i>P. chiapensis</i> Mart.	Resin	Externally; ointment	Southern Mexico and northern Guatemala	Wounds and fractured bones (44)
<i>P. roxburghii</i> Sarg.	Resin	Externally; ointment	Nepal	Cough, wounds, cracks, gastric ailments, tuberculosis,

				hair loss and snake bites (45)
<i>P. cembra</i> L.	Cones	Internally; macerated in alcohol	North-Western Italy	As digestive (46)
<i>P. nigra</i> J.F. Arnold	Cones and shoots Resin	Internally; decoction Externally; balms	North-Western Ligurian Alps Bosnia and Herzegovina	Cough and sore throat (47) Wounds (49)
<i>P. sylvestris</i> L.	Cones and shoots Buds Resin	Internally; decoction Internally; with honey Externally; balms	North-Western Ligurian Alps Central Serbia Bosnia and Herzegovina	Cough and sore throat (47) Bronchitis (48) Wounds (49)
<i>P. mugo</i> Turra	Resin	Externally; balms	Bosnia and Herzegovina	Wounds (49)
<i>P. heldreichii</i> Christ	Resin	Externally; balms	Bosnia and Herzegovina	Wounds (49)
<i>P. wallichiana</i> A.B Jacks.	Resin Resin	Externally Internally; with milk	Himalayan province Himalayan province	Wounds and fractures (50) Influenza and as antiphlogistic (50)
<i>P. pinea</i> L.	Cones	Internally	Southern Spain	As antiscorbutic and astringent (51)
<i>P. pinaster</i> Ait.	Cones	Internally	Southern Spain	Acute renal colic and depurative properties (51)

	Cones	Internally; decoction	Portugal	Cold and cough (53)
<i>P. kochiana</i> Klotzsch ex K. Koch	Cones, leaves, and stems	-	Caucasus	Asthma, bronchitis, cough, and renal problems (52)
<i>P. yunnanensis</i> Franch.	Leaves and cones	Internally; alcoholic extracts	Yunnan province	Rheumatic diseases (54)
<i>P. gerardiana</i> Wallich ex. D. Don.	-	-	Himalayan region (Ayurvedic Pharmacopoeia)	Epilepsy, Cough, Wound, Anaemia, Gout, Asthma (55)
<i>P. edulis</i> Engelm.	Leaves and gum	Inhalation	USA	Cold symptoms by inhalation (56)
<i>P. monophylla</i> Torr. & Fr.	Leaves and gum	Inhalation	USA	Cold symptoms by inhalation (56)

2.5. Bioactivity Studies on *Pinus* species

2.5.1. Studies on the Antioxidant Activity

Guri et al. (57) studied antioxidant activities of fresh needles, dry needles and barks of *P. pinea*, *P. brutia*, *P. radiata*, *P. halepensis*, *P. attenuata*, and *P. nigra* with Co(II)/EDTA induced luminol plateau chemiluminescence assay. The highest antioxidant activities were observed in barks of *P. pinea* and dry needles of *P. brutia* (IC_{50} ; $10.69 \pm 0.1 \times 10^{-2}$ and $18.38 \pm 0.6 \times 10^{-4}$ $\mu\text{g/mL}$).

In another comparative study, essential oils and various solvent extraction of twigs and needles of *P. pinea*, *P. brutia*, *P. sylvestris*, *P. halepensis*, *P. nigra* were compared with Pycnogenol® via ferric reducing and ferrous ion-chelating antioxidant potential, DPPH and DMPD radical scavenging activities (58). In comparison to *Pinus* extracts, Pycnogenol® showed the most potent DPPH and DMPD radical scavenging and ferric reducing antioxidant activities, whereas it had the lowest ferrous ion-chelating activity. Results also indicated that essential oils had no or low activity in DPPH and DMPD radical scavenging assays. On the other hand, essential oil obtained from twigs of *P. sylvestris* had the best ferrous ion-chelating activity.

Cretu et al. (59) performed some *in vitro* assays to evaluate the antioxidant activity of barks of *P. brutia* methanolic extracts and its ethyl acetate, diethyl ether, n-butanol, and aqueous fractions. Accordingly, there was no significant change among the fractions in the reducing power and Trolox equivalent antioxidant capacity assays, whereas results of the superoxide anion radical scavenging assay demonstrated important differences by far (EC_{50} value of aqueous fraction; 33.5 ± 1.1 $\mu\text{g/mL}$ and EC_{50} value of diethyl ether fraction; 70.30 ± 2.65 $\mu\text{g/mL}$). In the hydroxyl radical scavenging assay evaluation, the aqueous fraction showed the most potent activity ($EC_{50} = 0.47 \pm 0.06$ mg/mL), whereas the lowest activity was seen in the diethyl ether fraction ($EC_{50} = 1.07 \pm 0.06$ mg/mL).

In a comparative antioxidant activity study among bark extracts of *P. thunbergia* and *P. densiflora* from Korea with Pycnogenol®, ABTS free radical scavenging assay as well as total phenolic and total proanthocyanidin content were determined by Kim et al. (60). The main hot water extraction and ethyl acetate and proanthocyanidin fractions were

studied in this research. Results showed that all fractions of *P. densiflora* had the richest phenolic and proanthocyanidin content. In addition, the most potent antioxidant activities were also seen in the same plant. For all tests, fractions and Pycnogenol® can be ranked in the following order; *P. densiflora* > Pycnogenol® > *P. thunbergia*.

Zulfqar et al. (60) demonstrated the antioxidant activity of pine nut of *P. gerardiana* using different techniques. Their results indicated that methanolic extract showed higher antioxidant capacity than ethyl acetate extract in DPPH and FRAP assays. However, both extracts had lower potential than reference compounds. In addition to *in vitro* assays, the methanolic extract at 750 mg/kg exhibited a significant activity by triggering catalase, superoxide dismutase and peroxidase activities in the liver of diabetic mice.

In another *in vitro* study, the antioxidant activity of ethanolic-water extract of shoots of *P. sylvestris*, using different drying methods including freeze, vacuum, and natural drying, was evaluated by FRAP and DPPH assays ⁶². The freeze-drying extract showed the best DPPH activity, with the lowest FRAP activity. Similarly, the vacuum-drying extract showed greater FRAP activity, displaying the lowest DPPH activity.

Tsvetkov et al. (63) evaluated the antioxidant activity of extracts of the knot wood of *P. roxburghii* by the amperometric method. The results indicated that the most potent antioxidant capacity was observed in crude aqueous acetone extract. In addition, a lignan molecule (2,3-bis(4-hydroxy-3-methoxybenzyl)- butane-1,4-diol) isolated from crude aqueous acetone extract also showed significant antioxidant activity.

Another study on antioxidant properties of *P. densiflora* needle and bark using different solvents and extraction time was performed by Venkatesan et al. (64). Both needles and barks were extracted with 0%, 20%, 40%, and 60% ethanol with water (v/v) at 6, 9, and 15 hours. The antioxidant capacity of extracts was measured with ORAC, DPPH, and ABTS assays. According to the results, 40% of ethanolic extracts showed the most potent antioxidant capacity in both needles and barks at all extraction span. Bark extracts were significantly more active than needle extracts, and water extracts showed the lowest activities in all assays. In addition, 6 hours of extraction time decreased the antioxidant capacity in all extracts.

Gülçin et al. (65) demonstrated the antioxidant activity of ethanolic extract of *P. nigra* Arn. subsp. *pallasiana* resin at different concentrations using different assays, including thiocyanate method, reducing power, superoxide anion radical activity, DPPH, metal chelating, and H₂O₂ scavenging activity assays. The results indicated that the antioxidant potentials of extract escalated with increasing concentration.

In vitro antioxidant capacity of ethanolic extract of *P. densiflora* needles was measured using DPPH and lipid peroxidation assays by Kwak et al. (66). According to results, extracts had a significant scavenging effect similar to α -tocopherol as reference (EC₅₀: 95.12 μ g/mL and 95.54 μ g/mL, respectively). In lipid peroxidation assay, the extract showed an inhibitory effect but was lower than α -tocopherol as a reference (IC₅₀: 53.48 μ g/mL and 18.15 μ g/mL, respectively).

2.5.2. Studies on Anti-inflammatory Activity

Tümen et al. (67) demonstrated the anti-inflammatory activity of essential oil, *n*-hexane, and acetone extracts obtained from the needles, wood and cones of *P. pinaster* using three different models, including carrageenan-induced hind paw edema, Whittle, and TPA-induced ear edema models. Anti-inflammatory activity in the Whittle model was seen in essential oil obtained from the cones (inhibition value; 30.3% at 100 mg/kg dose). Other extracts showed no activity in all assays.

Ince et al. (68) investigated the anti-inflammatory activity of *P. brutia* bark extract and Pycnogenol® by using carrageenan-induced inflammation models in rats. *P. brutia* bark extract showed the highest anti-inflammatory activity at 75 mg/kg compared to Pycnogenol® and indomethacin as a reference, whereas Pycnogenol® had better activity at 100 mg/kg.

In another study, polysaccharides extract obtained from *P. halepensis* seeds using acetone, ethanol, and propanol solvents were shown to possess anti-inflammatory activity using methods based on inhibition of protein denaturation; antiproteinase, and anti-hemolytic assays (69). Acetone extract exerted the highest inhibition on protein denaturation and anti-hemolytic activities, while the best antiproteinase activity was seen in the ethanol extract.

Karoneen et al. (70) investigated *in vitro* anti-inflammatory activity of *P. sylvestris* bark extracts by measuring the inhibitory effects on inflammatory mediators, including nitric oxide (NO), prostaglandin E₂ (PGE₂). In addition, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expressions were studied by western blot analysis. Besides crude aqueous acetone extract, chloroform and semipreparative fractions from the crude extract were tested at 20 µg/mL and 50 µg/mL concentrations. The results showed that chloroform and semipreparative fractions had significant inhibitory activity on NO and PGE₂ productions. However, only the semipreparative fraction had an inhibitor activity on iNOS protein expression. In addition, none of the extracts showed inhibitory activity on COX-2 protein expression.

Sharma et al. (71) demonstrated *in vitro* anti-inflammatory activity of *P. roxburghii*, *P. wallichiana* and *P. gererdiana* hydro-alcoholic bark extracts using albumin denaturation and human red blood cells membrane stabilization tests. In both assays, anti-inflammatory potentials were ranked in the following order; *P. roxburghii* > *P. gererdiana* > *P. wallichiana*.

In another study, the essential oil obtained from different parts of *P. heldreichii*, *P. peuce*, and *P. mugo* were studied *in vitro* anti-inflammatory activity using murine macrophages (72). Essential oil from the twigs of all plants had the best reduction activity on cytokine IL-6 compared to others.

Süntar et al. (73) investigated the anti-inflammatory activity of the essential oils obtained from *P. pinea*, *P. brutia*, *P. sylvestris*, *P. halepensis*, *P. nigra* cones, and needles using the acetic acid-induced increase in capillary permeability assay. The results suggested that the essential oils obtained from *P. pinea* and *P. nigra* had the best anti-inflammatory activity among all essential oils.

The extract from *P. densiflora* needles was analysed by Kim et al. on nitric oxide (NO) production in the murine macrophage cell line (RAW 264.7) in different concentrations, and a decrease of NO was seen in all concentrations (74).

2.5.3. Studies on Wound-healing Activity

The essential oil, *n*-hexane, and acetone extracts obtained from the needles, wood, and cones of *P. pinaster* were studied using both *in vivo* and *in vitro* wound healing models (67). The wound healing activity of the essential oil obtained from the cones showed a significant increase similar to the reference drug Madecassol® in both circular excision and linear incision wound models. The same extract also had the best activity in *in vitro* assays.

The essential oils obtained from *P. pinea*, *P. brutia*, *P. sylvestris*, *P. halepensis*, *P. nigra* cones, and needles were tested for their *in vivo* wound healing activity using linear incision and circular excision wound models (73). According to results, essential oils obtained from *P. pinea* and *P. halepensis* cones showed the highest wound healing activity, whereas the activity of other essential oils was found weak.

2.5.4. Studies on Cytotoxic and Anticancer Activities

Venkatesan et al. (64) investigated *in vitro* cytotoxic and cytoprotective activity of *P. densiflora* needle and bark extracts prepared with different solvents and extraction times by methylthiazolyldiphenyl-tetrazolium bromide (MTT) colorimetric assay in HepG2 cells. None of the extracts showed cytotoxicity up to 15 µg/mL. The bark extracts exerted significant cytoprotective activity up to 7.5 µg/mL, whereas needle extracts had no cytoprotective effect.

In vivo antitumor effect and *in vitro* antimutagenicity and cell viability tests of the ethanolic extract of *P. densiflora* needles was studied by Kwak et al. (66). Antimutagenicity test on *Salmonella typhimurium* TA98 and TA100 strains demonstrated the inhibitory effect of extracts on both strains. Human breast adenocarcinoma, human gastric carcinoma, and human leukemia cells as cancer cell lines and human diploid fibroblast as normal cell lines were used to measure the cell viability potential. The extract was found markedly cytotoxic to cancer cells compared with normal cells. S-180 inoculated mice and DMBA-induced mammary cancer models in rats were used to study the antitumor activity and the extract exhibited a significant antitumor effect in both models.

The crude aqueous acetone extract, chloroform, and semipreparative fractions from the crude extracts of *P. sylvestris* bark were studied on cytotoxic effects using the XTT test (70). The cytotoxic activity was not seen in any extracts.

The cytotoxic activity of essential oils obtained from different parts of *P. heldreichii*, *P. peuce*, and *P. mugo* was studied by MTT-assay (72). HeLa, CaCo-2, and MCF-7 cancer cell lines were used in this research. The results showed that all essential oils exerted a significant cytotoxic activity on all cancer lines. The highest cytotoxic effects on HeLa and CaCo-2 cell lines were seen in the essential oils of needle and twigs from *P. peuce*, and *P. mugo*, whereas the essential oil of *P. heldreichii* needle had the most cytotoxic on MCF-7 cell lines.

Hong et al. (75) demonstrated the cytotoxic activity of the ethanolic extract of *P. koraisensis* leaves in hepatocellular carcinoma cells (HepG2 cells) with MTT assay. Results indicated that the extract did not show significant cytotoxic activity. Additionally, the anticarcinogenic effects of the extracts and their kinetics were studied in four cell lines, including the human prostate (PC-3, DU 145, LNCaP) and breast adenocarcinoma (MCF7) by the MTT assay. Cell viability was reduced to 40% by extracts of *P. pinea*, and *P. sylvestris* in PC-3 cells showing a similar effect like the positive control, CPT-11.

In vitro cytotoxic activity of *P. brutia*, *P. pinea*, *P. sylvestris*, and *P. nigra* bark extracts on human prostate adenocarcinoma (PC-3), human breast adenocarcinoma (MCF7), human prostate carcinoma (DU 145), and human prostate carcinoma (LNCaP) was studied by Yeşil-Çeliktaş et al. (76). Accordingly, *P. pinea*, and *P. sylvestris* extracts at doses of 10 µg/ml markedly inhibited the proliferation of PC-3 cells. However, none of the extracts effectively inhibited MCF7, DU 145, and LNCaP cells.

Milic et al. (77) investigated the cytotoxic activity of dimethyl sulfoxide (DMSO) extract of *P. nigra* bark on human cervix epithelioid carcinoma (HeLa), breast adenocarcinoma (MCF7), colon adenocarcinoma (HT-29), and human fetal lung (MRC-5) cell lines by Sulforhodamine B (SRB) assay. Results revealed that extract markedly inhibited all cell lines, and the best inhibitory activity was seen in MCF7 and MRC-5, respectively.

Mao et al. (78) evaluated the cytotoxic activity of the extract of *P. massoniana* barks on the human lung cancer A549 cell line using the MTT assay. Results indicated that the extract markedly inhibited the growth and the matrix metalloproteinase-9 (MMP-9) activity in lung cancer cells.

Zhang et al. (79) investigated the cytotoxic activity of procyanidins isolated from aqueous and alcoholic extracts of *P. caribaea* barks on promyelocytic cells (HL-60), human stomach adenocarcinoma cells (BGC-823), human hepatocellular carcinoma cells (BEL-7402), and human lung carcinoma cells (A549) using MTT assay. Both extracts had a significant inhibitory activity on HL-60 and BGC-823 cell lines. BEL-7402 cell lines were only inhibited by procyanidins from aqueous fractions, while none of the extracts inhibited the A549 cell lines.

2.5.5. Studies on Antimicrobial and Antifungal Activities

The antimicrobial activity of *P. roxburghii*, *P. wallichiana*, and *P. gererdiana* hydro-alcoholic bark extracts against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Klebsiella pneumoniae* (*K. pneumoniae*) using the agar well diffusion plate method (71). According to the results, none of the extracts was effective on *E. coli* strains. *P. wallichiana* had marked inhibition activity on *S. aureus*, *P. aeruginosa*, and *K. pneumoniae*, while *P. roxburghii* was effective on *S. aureus* and *K. pneumoniae*. In the case *P. gererdiana* showed only inhibition on *P. aeruginosa*. This research also studied the antifungal activities of the extracts against *Candida albicans* and *Saccharomyces cerevisiae*. All extracts significantly inhibited *Candida albicans*, but were not active on *Saccharomyces cerevisiae*.

Apetri et al. (80) studied the antimicrobial and antifungal activities of 80% methanolic extracts from the barks and needles of *P. cembra* using the agar diffusion assay. *Staphylococcus aureus*, *Sarcina lutea*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* strains for antimicrobial activity, and *Candida albicans* strains for antifungal activity were used in this study. According to the results, the inhibition activities on all strains were observed in both extracts at 4 mg/well, whereas bark extract showed higher activity than needles.

Ulukanlı et al. (81) investigated the antimicrobial activity of the essential oils obtained from *P. brutia* and *P. pinea* resins on four Gram-negative and seven Gram-positive bacteria strains. Both essential oils had a significant inhibitory effect on all strains except *P. aeruginosa* and *K. pneumoniae*. Moreover, the antimicrobial potential of *P. pinea* essential oil on *Micrococcus luteus*, *Bacillus cereus*, *Bacillus subtilis*,

Staphylococcus aureus, and *Enterobacter hormaechei* was greater than that of *P. brutia*. Besides, both essential oils exerted insecticidal activity on *Ephestia kuehniella* eggs.

The antimicrobial and antifungal activities of the essential oils obtained from needles of two different *P. pinaster* varieties were studied by Soudes et al. (82). The antibacterial activity was tested against *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, *Listeria monocytogenes*, and *Bacillus cereus*, while the antifungal activity was tested on *Aspergillus flavus*, *Aspergillus niger* and *Candida albicans* by disk diffusion assay. Accordingly, both essential oils significantly inhibited all strains of bacteria, whereas no antifungal activity was observed.

2.5.6. Studies on Antidiabetic Activity

Rohdewald et al. (83) investigated the antidiabetic activity of Pycnogenol® in type 2 diabetic patients. Patients received different doses of Pycnogenol® for 12 weeks in this open-controlled and dose-depending study. The highest blood-glucose-lowering effect of Pycnogenol® was observed at 200 mg, whereas there was no significant change at 300 mg. HbA1c levels were markedly ameliorated at 200 mg at the end of the study. Nonetheless, there was no effect on insulin level.

In another study, *in vitro* α -amylase and *in vivo* antidiabetic activities of the methanolic and ethyl acetate extracts from *P. gerardiana* pine seed were investigated to evaluate their antidiabetic activity (61). The results suggested that the methanolic extract inhibited α -amylase more than the ethyl acetate extract ($88.67 \pm 5.13\%$ and $82.3 \pm 5.51\%$, respectively). *In vivo* antidiabetic activity was tested on alloxan monohydrate-induced diabetic mice. The incidence of alloxan-induced organ damage was reduced in mice's liver, pancreas, and kidney.

Salhi et al. (84) investigated *in vitro* and *in vivo* antidiabetic activities of aqueous and ethanolic extracts of *P. halepensis* barks. The results suggested that ethanolic extract significantly inhibited α -amylase and α -glucosidase enzymes compared to aqueous extract. Oral glucose tolerance and oral starch tolerance assays were conducted on non-diabetic rats fed with 250 and 500 mg/kg extracts. The aqueous extract at both doses and the ethanolic extract at 250 mg/kg was ineffective in the oral glucose tolerance test. In

contrast, the ethanolic extract at 500 mg/kg showed significant activity. Both extracts had a significant antihyperglycemic activity in the oral starch tolerance test.

Kaushik et al. (45) evaluated the α -amylase inhibition activity of petroleum ether, chloroform, ethyl acetate and ethanol extracts of *P. roxburghii* barks and quercetin, isolated from the ethanolic extract. Results indicated that the ethanolic extract and quercetin provided the best inhibition (43.4% and 49.6% inhibition, respectively).

Another study focused on the antidiabetic effect of hydroalcoholic extract of *P. elderica* seeds (85). The fasting blood glucose levels of hypercholesterolemic diabetic rats were fed with different doses of the extracts. After four weeks, extracts at 200 and 400 mg/kg doses plummeted the fasting blood glucose levels. Nevertheless, extracts did not ameliorate the hypercholesterolemic parameters.

Hu et al. (86) investigated the inhibitory activity of human protein tyrosine phosphates 1 B (PTP1B) of isolated compounds from the methanolic extracts of *P. kwangtungensis* needles and twigs. Fifteen diterpene molecules, including new types, were isolated and lambertianic acid (labdane type) and cassipourol (retinane type) significantly inhibited PTP1B.

2.5.7. Studies on Anti-*Helicobacter pylori* Activity

Rohdewald and Beil (87) studied the anti-*Helicobacter pylori* activity of Pycnogenol®. According to results, Pycnogenol® inhibited *H. pylori* adherence and growth adherent to gastric epithelial cells.

Kim et al. (88) investigated the *in vitro* and *in vivo* anti-*Helicobacter pylori* activity of phytoncide obtained from *P. koraiensis* cone waste. Results indicated that phytoncide decreased the gastric complaint in *H. pylori*-infected mice by inhibiting pro-inflammatory cytokines in the gastric mucosa. *H. pylori* IgG antibodies in mice were measured after two weeks. Results indicated that phytoncide significantly inhibited *H. pylori* in the gastrointestinal system of mice.

2.5.8. Studies on Cardiovascular Activity

A randomized, double-blind, placebo-controlled crossover study was conducted in patients with coronary artery diseases with Pycnogenol® for eight weeks (89). According to the results, flow-mediated dilatation ameliorated in patients who received Pycnogenol® at a dose of 200 mg at the end of the study. Nevertheless, no significant change was observed in blood pressure and platelet adhesion.

Kim et al. (90) investigated the antihypertensive activity of *P. densiflora* bark extract in spontaneously hypertensive rats. The angiotensin-converting enzyme (ACE) activity, angiotensin II content, and malondialdehyde (MDA) levels of rats who received the extract at a dose of 50 mg/kg/day extract were measured after seven weeks. Accordingly, the ACE activity, angiotensin II content, and malondialdehyde (MDA) levels, as well as blood pressure, markedly plummeted in comparison to the control group.

2.6.9. Other Biological Activities

Üstün et al. (58) investigated *in vitro* acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition activity of the essential oils and acetone, ethyl acetate and ethanol extracts of *P. pinea*, *P. brutia*, *P. sylvestris*, *P. halepensis*, *P. nigra* needles, and twigs comparing with Pycnogenol®. The best inhibition activities for both assays were observed in the needle ethanol extract and essential oil obtained from *P. halepensis* twigs.

Hong et al. (75) demonstrated the anti-alcoholic fatty liver activity (anti-AFL) of the ethanolic extract of *P. koraisensis* leaves. Results suggested that the extract increased phosphorylation of liver kinase B1 (LKB1)-AMP-activated protein kinase signalling, while fatty acid biosynthesis-related proteins have plummeted in HepG2 cells. Moreover, AST (aspartate aminotransferase) and triglyceride levels were significantly ameliorated in Sprague Dawley (SD) rats.

The antiviral activity of the compounds isolated from methanol extract and its subfractions from *P. densiflora* against the influenza A virus was studied by Ha et al. (91). The cytopathic effect (CPE) inhibition and neuraminidase (NA) inhibition assays were tested in this study. The isolated compounds including; diterpenoids; 7 α -methoxydehydroabietic acid, ampelopsin, 15-hydroxydehydroabietic acid, abieta-

8,11,13,15-tetraen-18-oic acid, karamatsuic acid, 7-oxo-13 β -hydroxyabiet-8(14)-en-18-oic acid, 8(14)-podocarpin-13-on-18-oic acid, 8(14)-podocarpin-7,13-dione-18-oic acid, 4-epi-trans-communic acid, (13*S*)-15-hydroxyabund-8(17)-en-18-oic acid, and a flavonoid; ampelopsin, markedly inhibited the CPE. The NA inhibition activity was seen only in ampelopsin.



2.6. Phytochemical Studies on *Pinus* species

2.6.1. Phenolic acids from *Pinus* species

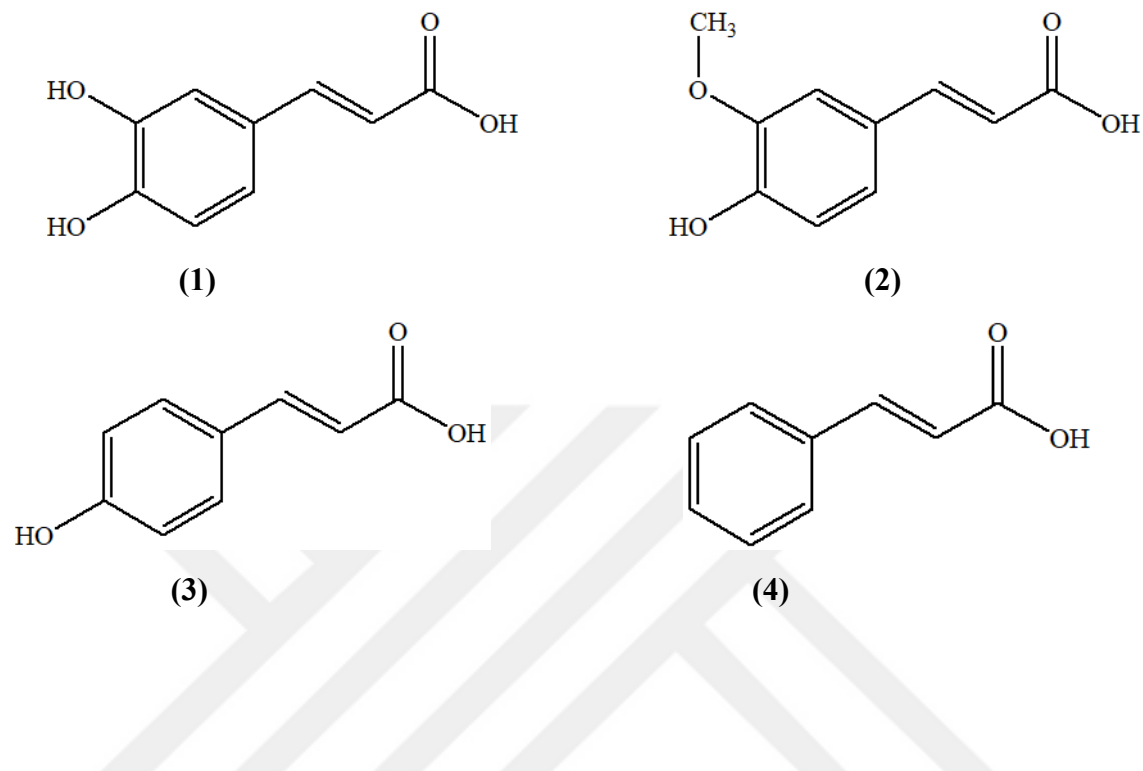


Table 6. Phenolic acids from *Pinus* genus

Compounds	Species	Reference
Caffeic acid (1)	<i>brutia</i> (barks)	92
	<i>pinaster</i> (knots)	93
	<i>elderica</i> (barks)	94
	<i>halepensis</i> (roots/needles)	95
Ferulic acid (2)	<i>brutia</i> (barks)	92
	<i>sylvestris</i> (barks)	70
	<i>elderica</i> (barks)	94
	<i>pinaster</i> (knots)	93
<i>p</i> -coumaric acid (3)	<i>halepensis</i> (roots/needles)	95
Cinnamic acid (4)	<i>halepensis</i> (bark)	96

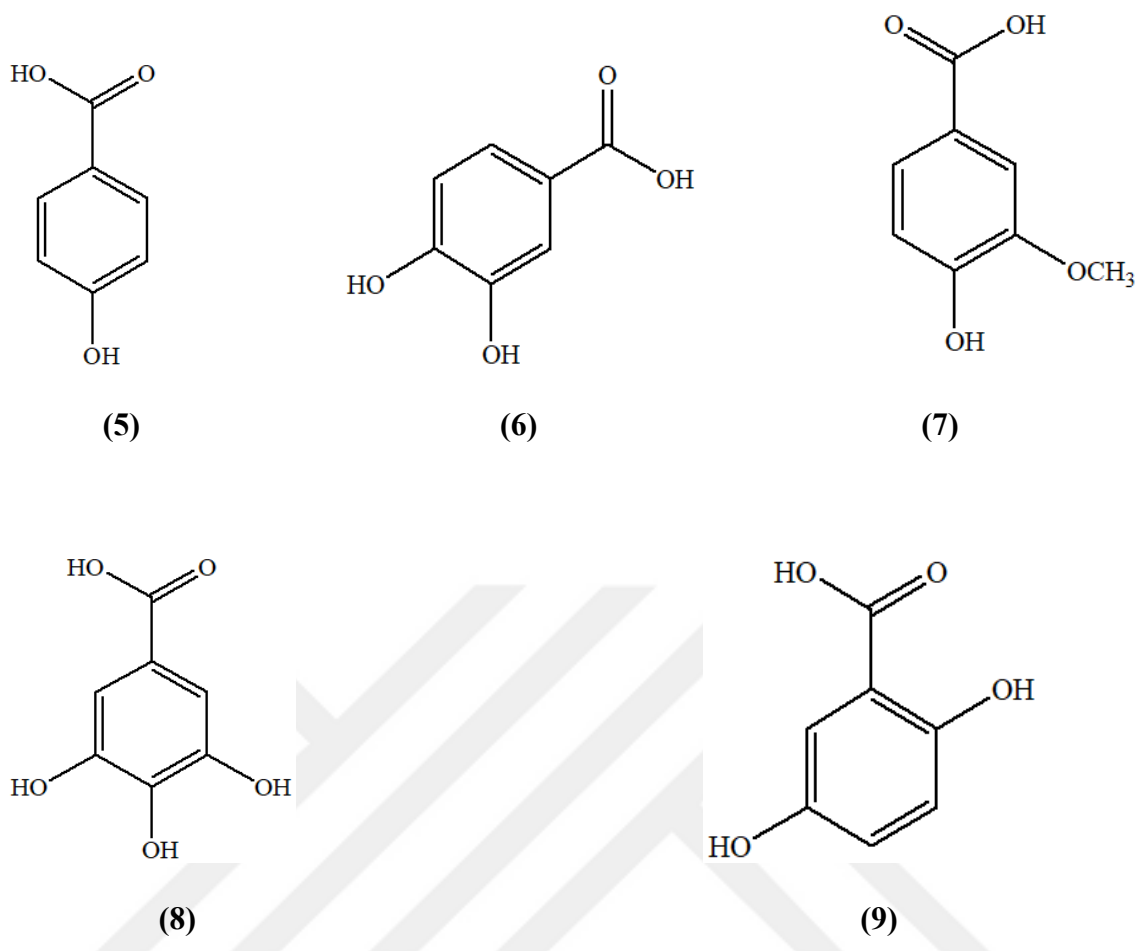


Table 7. Phenolic acids from *Pinus* genus

Compounds	Species	Reference
4-hydroxy benzoic acid (5)	<i>brutia</i> (barks)	92
	<i>pinaster</i> (barks)	97
	<i>halepensis</i> (roots/needles)	95
Protocatechuic acid (6)	<i>brutia</i> (barks)	92
	<i>halepensis</i> (roots/needles)	95
Vanillic acid (7)	<i>brutia</i> (barks)	92
	<i>pinaster</i> (knots)	93
Gallic acid (8)	<i>brutia</i> (barks)	92
	<i>pinaster</i> (barks)	98
	<i>patula</i> (barks)	53
	<i>halepensis</i> (roots/needles)	99
	<i>halepensis</i> (bark)	96
Gentisic acid (9)	<i>brutia</i> (barks)	92

2.6.2. Flavonoids from *Pinus* species

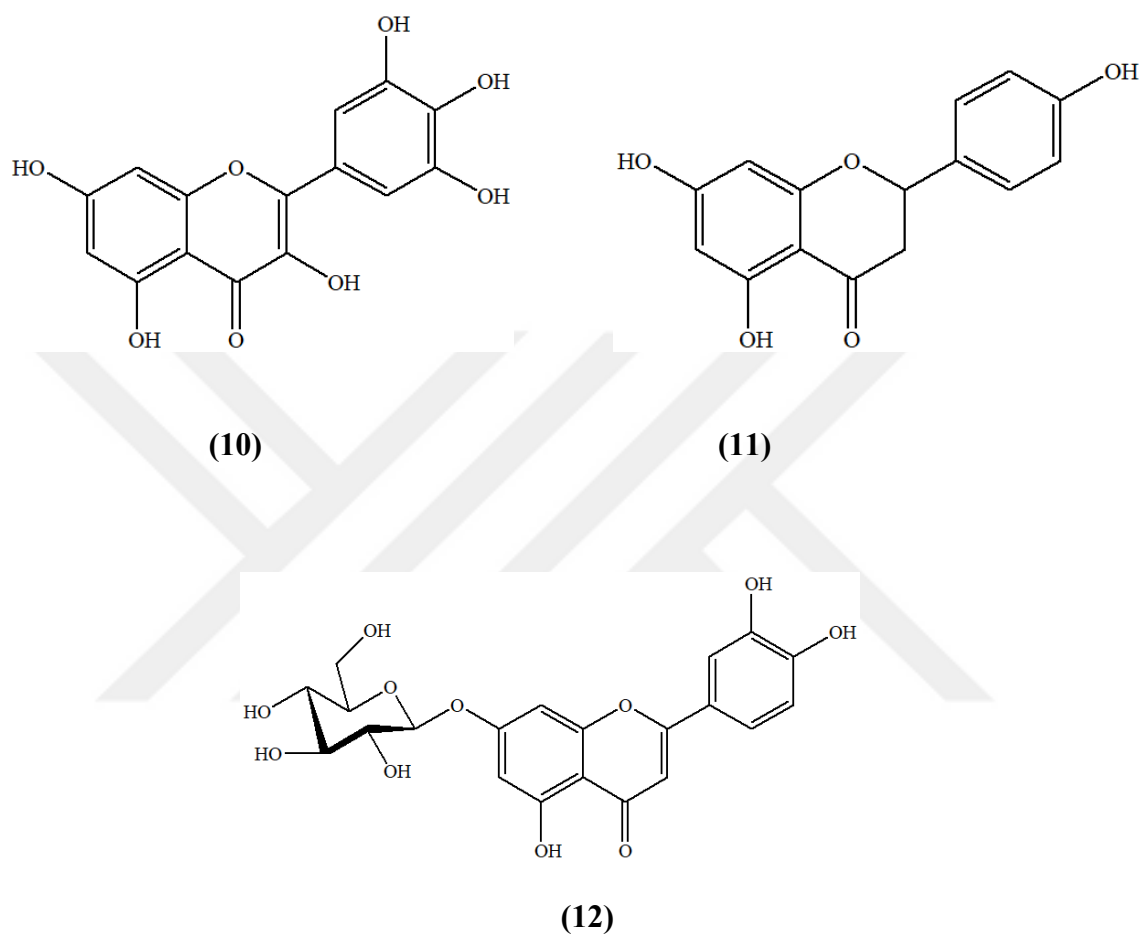


Table 8. Flavonoids from *Pinus* genus

Compounds	Species	Reference
Myricetin (10)	<i>brutia</i> (barks)	92
Naringenin (11)	<i>brutia</i> (barks)	92
Apigenin-7-glucoside (12)	<i>sylvestris</i> (barks) <i>halepensis</i> (barks)	70 96

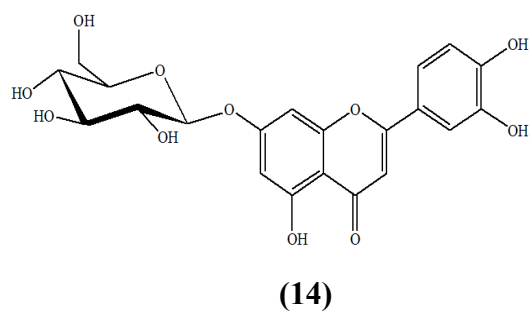
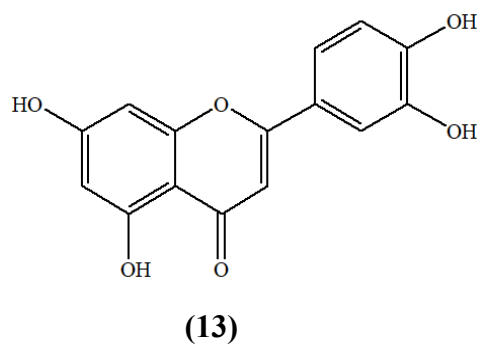
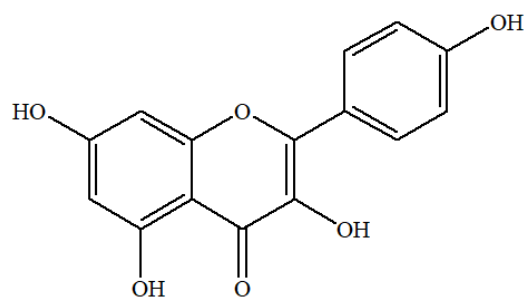
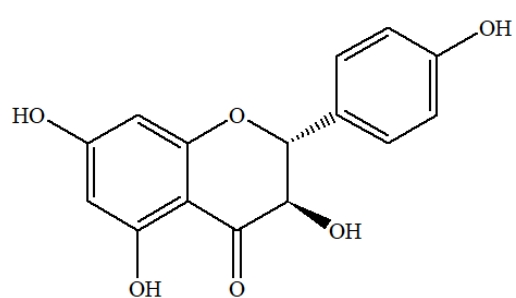


Table 9. Flavonoids from *Pinus* species

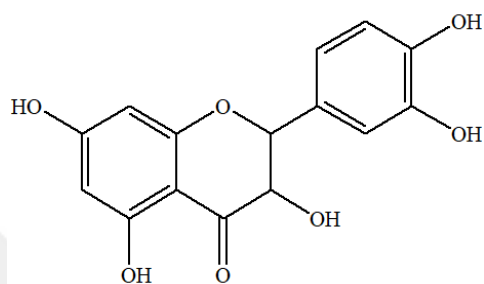
Compounds	Species	Reference
Luteolin (13)	<i>brutia</i> (barks)	92
Luteolin-7-glucoside (14)	<i>halepensis</i> (barks)	96



(15)



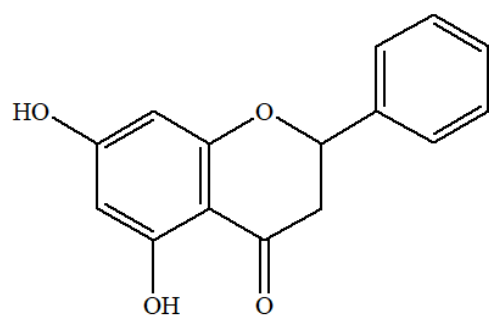
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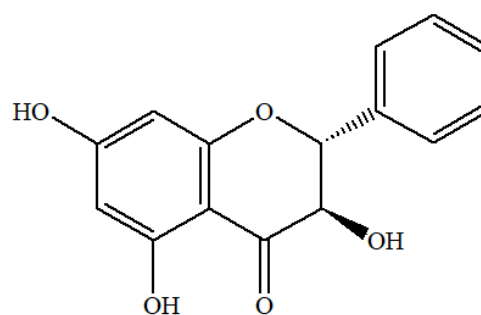
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Table 10. Flavonols from *Pinus* species

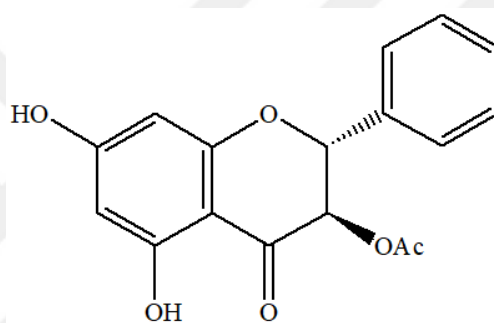
Compounds	Species	Reference
Kaempferol (15)	<i>brutia</i> (barks)	92
Dihydrokaempferol (16)	<i>pinaster</i> (knots)	93
Taxifolin (17)	<i>brutia</i> (barks)	92
	<i>pinea</i> (barks)	100
	<i>nigra</i> (barks)	100
	<i>pinaster</i> (barks)	97
	<i>pinaster</i> (knots)	93
	<i>sylvestris</i> (barks)	70
	<i>elderica</i> (barks)	93



(18)



(19)



(20)

Table 11. Flavan-3-ols from *Pinus* species

Compounds	Species	Reference
Pinocembrin (18)	<i>pinaster</i> (knots)	93
Pinobanksin (19)	<i>pinaster</i> (knots)	93
pinobanksin 3-acetate (20)	<i>pinaster</i> (knots)	93

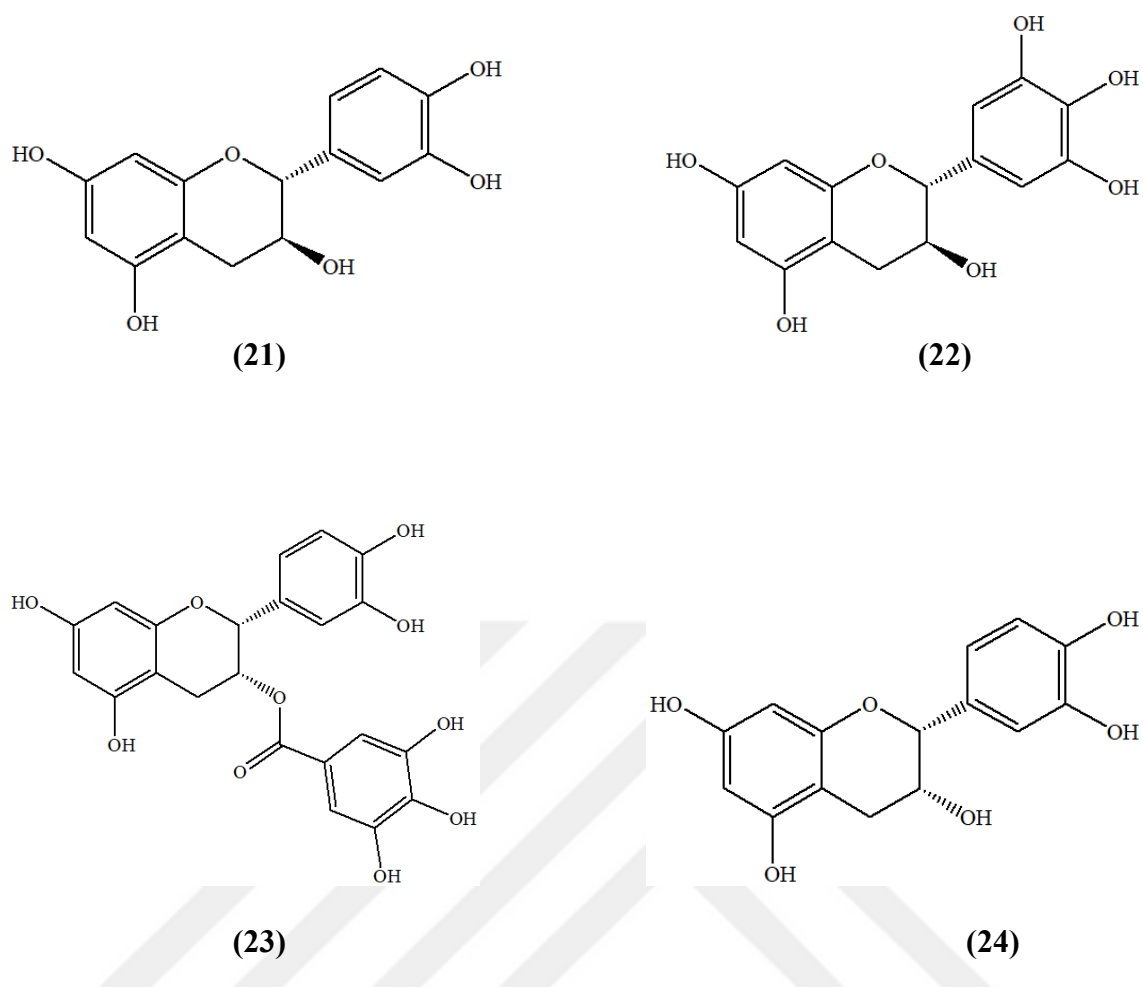
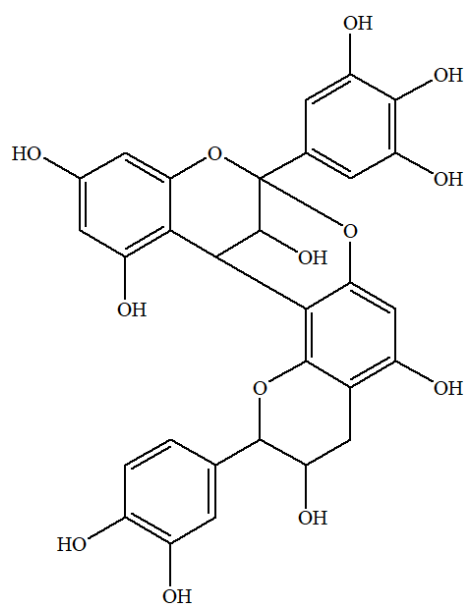


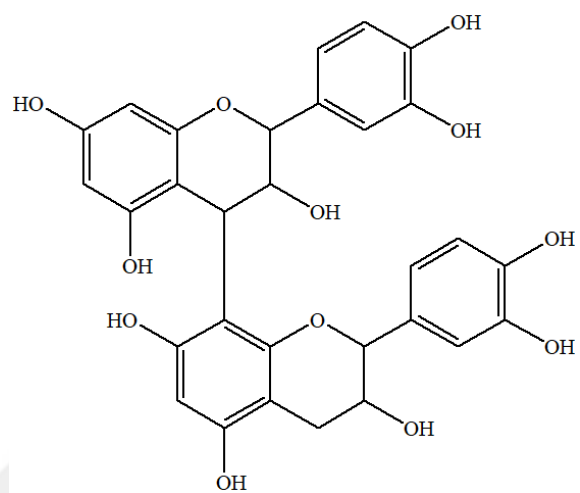
Table 12. Flavan-3-ol from *Pinus* species

Compounds	Species	Reference
Catechin (21)	<i>brutia</i> (barks)	92
	<i>pinaster</i> (barks)	98
	<i>patula</i> (barks)	53
	<i>elderica</i> (barks)	94
Gallocatechin (22)	<i>pinaster</i> (barks)	98
Epicatechin gallate (23)	<i>pinaster</i> (barks)	98
Epicatechin (24)	<i>pinaster</i> (barks)	92
	<i>patula</i> (barks)	53

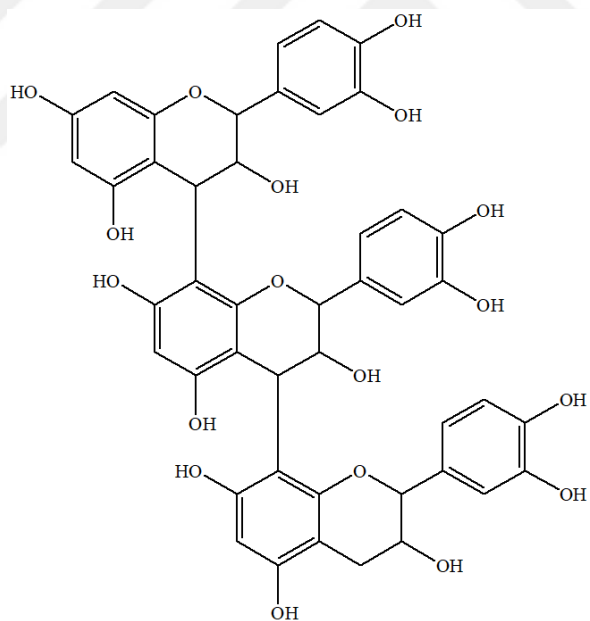
2.6.3. Proanthocyanidins from *Pinus* species



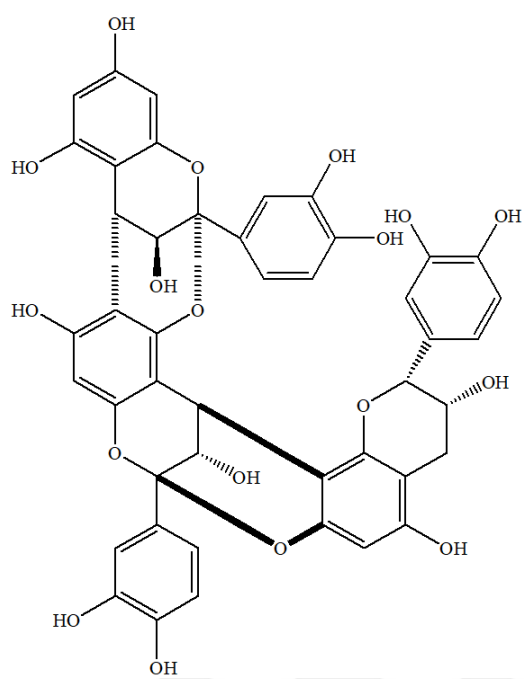
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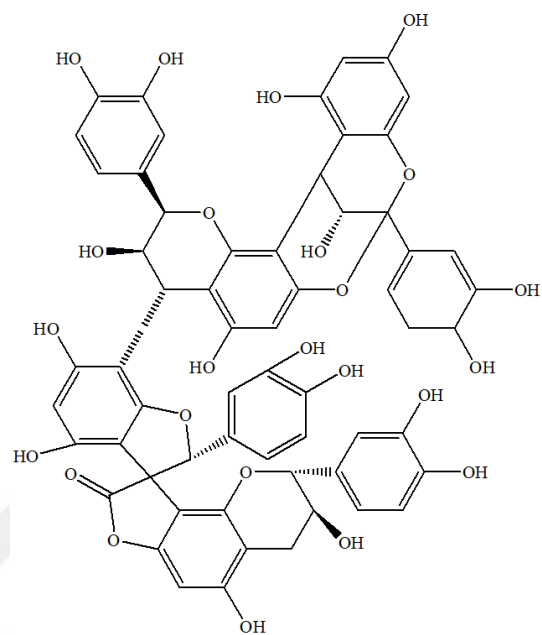
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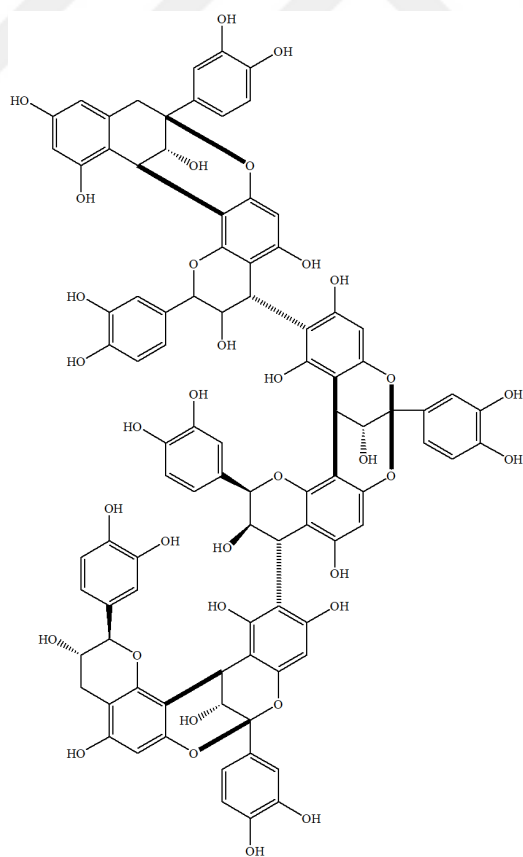
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(28)



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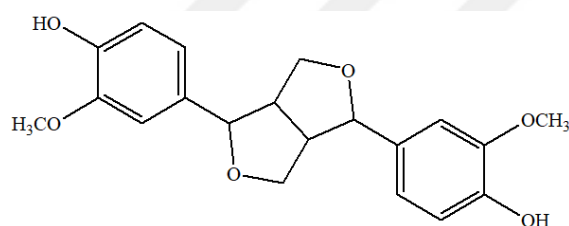


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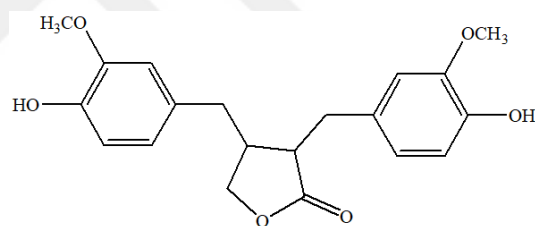
Table 13. Proanthocyanidins from *Pinus* species

Compounds	Species	Reference
A-type Proanthocyanidin (25)	<i>pinaster</i> (barks) <i>pinea</i> (barks)	101,102 101
B- type dimer Proanthocyanidin (26)	<i>pinea</i> (barks) <i>pinaster</i> (barks) <i>wallichiana</i> (barks) <i>roxburghii</i> (barks) <i>gerardiana</i> (barks)	101 101,102 103 103 103
B-type trimer Proanthocyanidin(27)	<i>pinaster</i> (barks) <i>pinea</i> (barks)	101 101
Pinutwindoublin (28)	<i>massoniana</i> (barks)	104
Pinuspirotetrin (29)	<i>massoniana</i> (barks)	104
Pinumassohexin (30)	<i>massoniana</i> (barks)	104

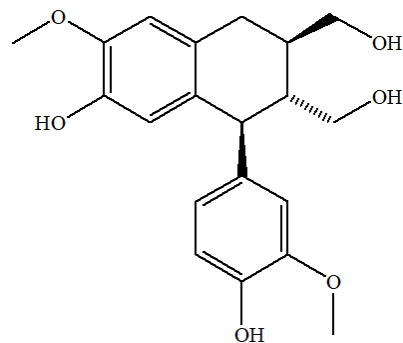
2.6.4. Lignans from *Pinus* species



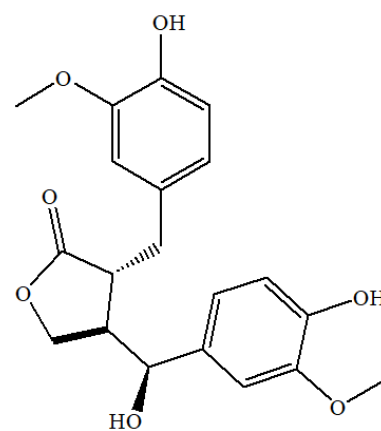
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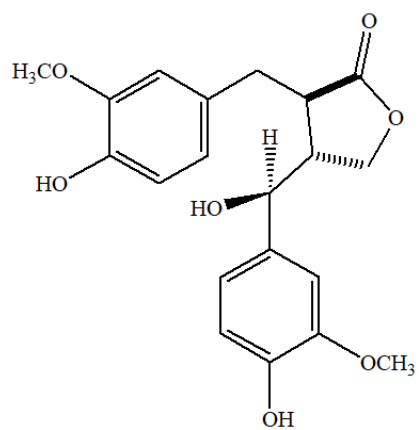
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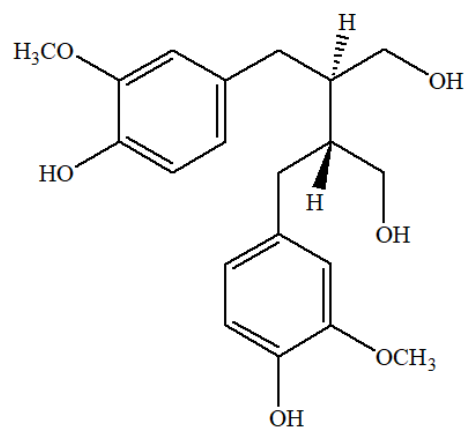
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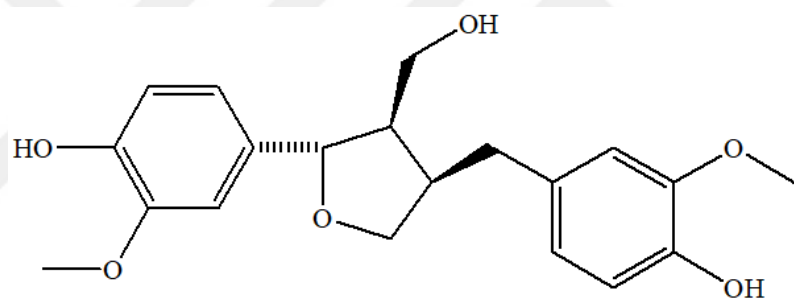
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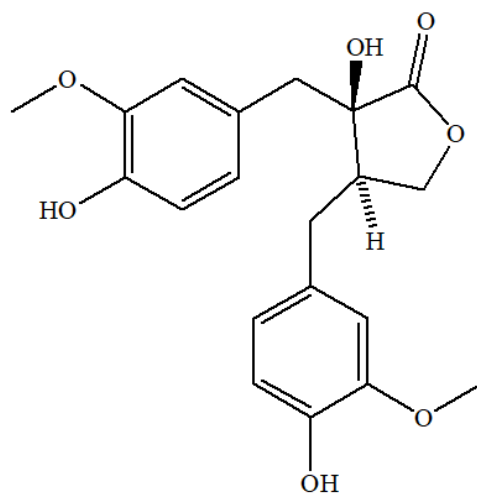
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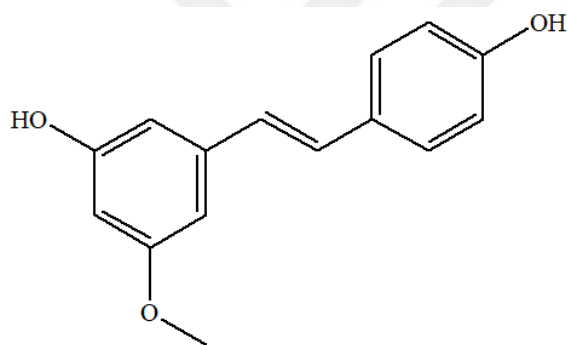


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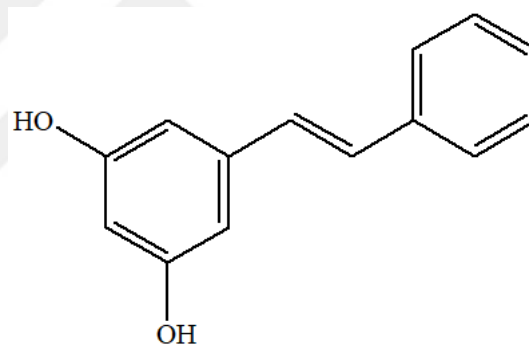
Table 14. Lignans from *Pinus* species

Compounds	Species	Reference
Pinoresinol (31)	<i>sylvestris</i> (barks) <i>pinaster</i> (knots)	70 93
Matairesinol (32)	<i>sylvestris</i> (barks) <i>pinaster</i> (knots)	70 93
Isolariciresinol (33)	<i>pinaster</i> (knots)	93
Hydroxymatairesinol (34)	<i>pinaster</i> (knots)	93
Allohydroxymatairesinol (35)	<i>pinaster</i> (knots)	93
Secoisolariciresinol (36)	<i>pinaster</i> (knots)	93
Lariciresinol (37)	<i>pinaster</i> (knots)	93
Nortrachelogenin (38)	<i>pinaster</i> (knots)	93

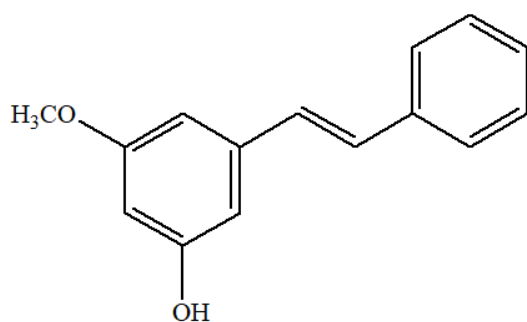
2.6.5. Stilbenes from *Pinus* species



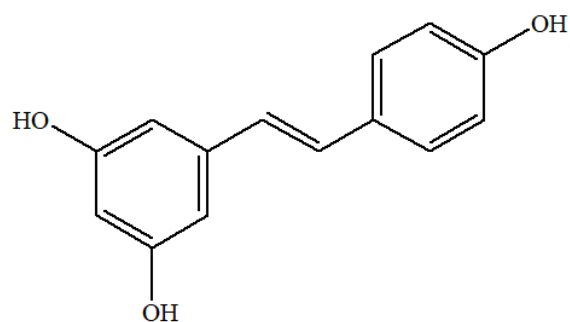
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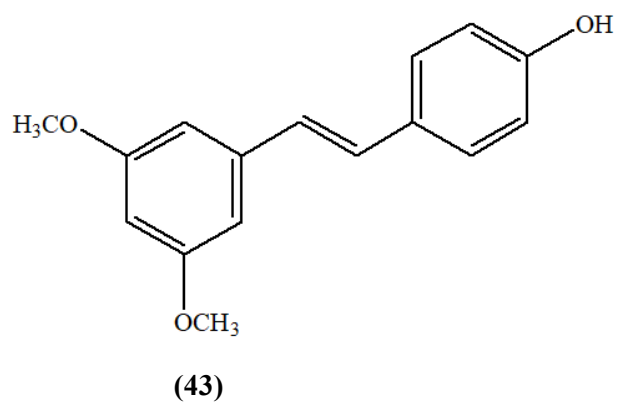
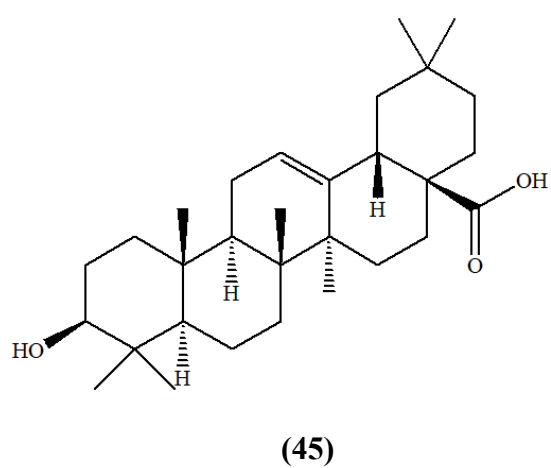
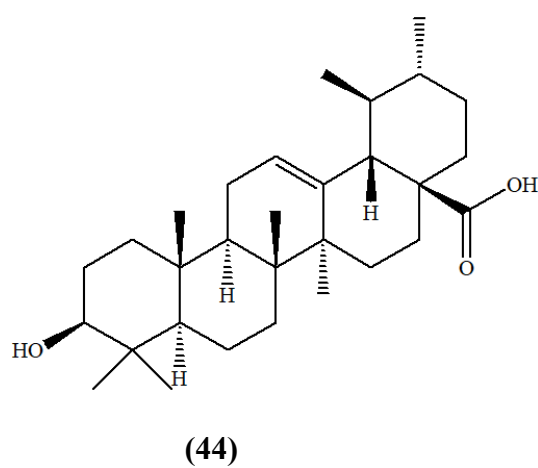


Table 15. Stilbenes from *Pinus* species

Compound	Species	Reference
pinostilbene (39)	<i>pinaster</i> (knots)	93
Pinosylvin (40)	<i>pinaster</i> (knots)	93
pinosylvin monomethyl ether (41)	<i>pinaster</i> (knots)	93
Resveratrol (42)	<i>pinaster</i> (knots)	93
Pterostilbene (43)	<i>pinaster</i> (knots)	93

2.6.6. Triterpenoids from *Pinus* species



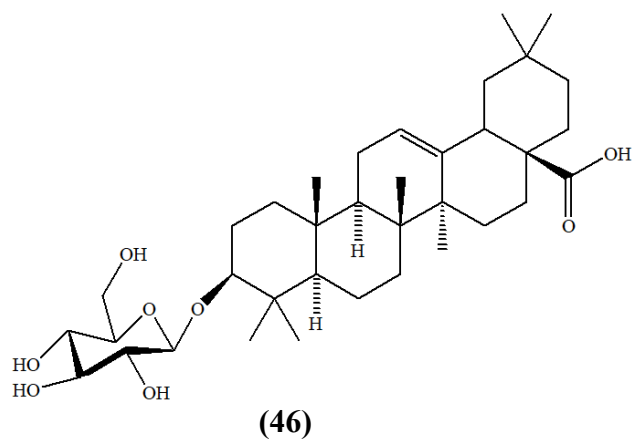


Table 16. Triterpenoids from *Pinus* species

Compound	Species	Reference
Ursolic acid (44)	<i>massoniana</i> (barks)	105
Oleanolic acid (45)	<i>massoniana</i> (barks)	105
Oleanolic acid 3-O- β -D-glucopyranoside (46)	<i>massoniana</i> (barks)	105

2.6.7. Other compounds from *Pinus* species

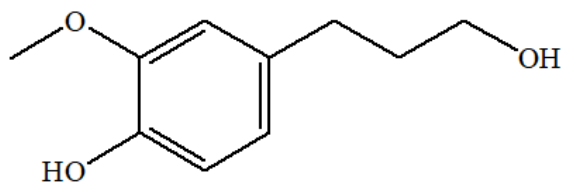
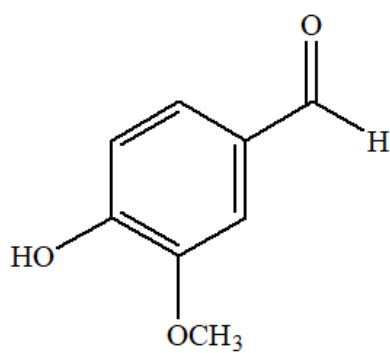


Table 17. Other compounds from *Pinus* species

Compound	Species	Reference
Vanillin (47)	<i>brutia</i> (bark)	92
	<i>sylvestris</i> (bark)	70
Dihydroconiferyl alcohol (48)	<i>sylvestris</i> (bark)	70

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Plant Materials and Pycnogenol®

Barks of *Pinus brutia* and *Pinus nigra* were collected from Üzümlü-Evciler villages, Ida mounts, Çanakkale, Turkey in June 2018. Barks of *Pinus pinea* were gathered from Didim, Aydın, Turkey in July 2018. Barks of *P. halepensis* were collected from Yumurtalık, Adana, Turkey in July 2018. Barks of *Pinus sylvestris* were gathered from Bolu mountain, Bolu, Turkey in August 2018. Barks of *Pinus pinaster* were collected from Yeditepe University Campus, Kayışdağı, İstanbul in August 2018. The specimens were authenticated before any process and dried. The voucher specimens for *P. pinaster* (YEF18032), *P. pinea* (YEF18033) and *P. brutia* (YEF18034) are stored at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, İstanbul, Turkey. Pycnogenol® was purchased from a pharmacy.

3.1.2. Chemicals and Solvents

2-aminoethyl diphenylborinate	Sigma Aldrich; 42810
2,2-diphenyl picryl hydrazyl	Sigma Aldrich; 056K1147
4-dimethylaminocinnamaldehyde	Sigma Aldrich; C6293
3,5- dinitrosalicylic acid	Sigma Aldrich; 128848
α -amylase	Sigma Aldrich; A3176
α -glucosidase	Merck; G5003
α -PNG (4-Nitrophenyl α -D-glucopyranoside)	Sigma Aldrich; N1377
Aescin	Sigma Aldrich; E1378
Acarbose	Sigma Aldrich; A8980
Acetic acid	Riedel de Haen; 30990
Aluminum chloride	Merck; 8.01081.1000
Ammonium acetate	Carlo Erba; 313507
Ammonium molybdate	Riedel de Haen; 31402
Ascorbic acid	Sigma Aldrich; 065K0003
Bile acid	Sigma Aldrich; B8631
Bovine serum albumin	Sigma Aldrich; A7906
Butylated hydroxytoluene	Sigma Aldrich; MKBD8339
Caffeic acid	Sigma Aldrich; C0625

Copper (II) sulfate	Sigma Aldrich; 209201
Catechin gallate	Sigma Aldrich; C0692
(+)-Catechin hydrate	Sigma Aldrich; C1251
Copper (II) sulphate	Carlo Erba; 364757
Dialysis tubing cellulose membrane	Sigma Aldrich; D9527
Dichloromethane	Merck; 1.06044.2500
Ethyl acetate	Sigma Aldrich; SZBA113S
Epicatechin	Sigma Aldrich; 490460
Epigallocatechin	Sigma Aldrich; 970741
Epigallocatechin gallate	Sigma Aldrich; 989515
Ferric chloride hexahydrate	Sigma Aldrich; 236489
Ferrozine	Sigma Aldrich; MKBD0707
Ferulic acid	Sigma Aldrich; 1135246
Folin-Ciocalteu Reagent	Sigma Aldrich; F9252
Formic acid	Sigma Aldrich; 27001
Gallic acid	Sigma Aldrich; 67384
D-Glucose	Merck; 47829
Hydrochloric acid (36.5%)	Sigma Aldrich; SZBA2250
Hyperoside	HWI Pharma; 0018-05-85
Iron (II) chloride	JT Baker; 0125
Iron (II) sulfate heptahydrate	JT Baker; 0126
Methanol	Sigma Aldrich; SZE9365S
<i>n</i> -Hexane	Sigma Aldrich; SZBA0655
Neocuproine	Sigma Aldrich; N1501
N,N-dimethyl-phenylendiamine	Sigma Aldrich; D4139
Quercetin dihydrate	Sigma Aldrich; Q4951
Pancreatin	Sigma Aldrich; P7545
Pancreatin	Sigma Aldrich; P7545
Pepsin	Sigma Aldrich; P7000
Pronthocyanidin A1	Sigma Aldrich; 103883
Pronthocyanidin B1	Sigma Aldrich; 20315
Pronthocyanidin B2	Sigma Aldrich; 29106
Pronthocyanidin C1	Sigma Aldrich; 37064
Sodium carbonate	Sigma Aldrich; 223484
Sodium chloride	Sigma Aldrich; 31434
Sodium dodecyl sulphate	Merck; 8.2205.1000

Sodium hydroxide	Sigma Aldrich; 06203
Sodium molybdate	Sigma Aldrich; 243655
Sodium nitrite	Fluka; 71760
Sodium phosphate monobasic	Sigma Aldrich; 04269
Sodium potassium tartarate	Sigma Aldrich; 217255
Starch	Sigma Aldrich; 33615
Sulfuric acid (98%)	Riedel de Haen; 62260
Taxifolin	Sigma Aldrich; 480182
Trichloroacetic acid	Riedel de Haen; 23100
Tripyridyltriazine	Sigma Aldrich; 93285
Trolox®	Sigma Aldrich; BCBF4547V
Vanillin	Fluka; 94752

3.1.3. Equipments and Instruments

96-well plate	TPP; 92096
Balance	Ohaus Explorer
Beaker (50, 100, 250, 1000 mL)	
Centrifuge	Sigma 3-16 PK
Eppendorf tubes (1,5,15 mL)	
Erlenmayer flask (50, 100, 250, 1000 mL)	
Hairdryer	Arçelik
HPTLC system	CAMAG
Lyophilizator	Christ Alpha 2-4 LD
Micropipette (100-1000 microlt)	Gilson
Micropipette (500-5000 microlt)	Rainin
Micropipette (20-200 microlt)	Eppendorf
Microplate reader	Thermo Multiskan Ascent
Microplate reader	Varioskan Lux
Milli Q water device	Millipore
Oven	Binder
pH meter	Mettler-Toledo MP220
Refrigerator	Arcelik
Rotatory evaporator	Heidolph
Spectrophotometer	Spekol 1300
HPTLC Developing Chambers	CAMAG
HPTLC Plates, Silica gel 60 F ₂₅₄	CAMAG

Ultrasonic bath	Sonorex RK156BH
Volumetric flasks (25, 100, 500, 1000 mL)	
Vortex	Heidolph Reax
Waterbath	GFL

3.2. Extraction Procedures

3.2.1. Proanthocyanidin Extraction

The proanthocyanidin extraction from *Pinus* bark samples was carried out by the standardized method developed by Masquelier with some modifications (106). The barks were dried in the shade for about one week and grounded to fine powders in a waring stainless steel blender. Firstly, each powdered bark sample (100 g) was extracted with 600 mL of boiling distilled water (dH₂O) and then macerated in an orbital shaker at 100 revolutions per minute (rpm) for 30 minutes in an amber glass beaker. In proanthocyanidin extractions, hot water is one of the most preferred solvents due to breaking hydrogen bonds. Then, the extract was filtered with fluted filter papers while it was hot. The collected liquid phase was left to cool down to room temperature following the filtration process. After that, sodium chloride (NaCl) was added slowly to the liquid phase until a precipitate formed. The precipitate formed was eliminated by filtration with fluted filter papers. In this way, the water phase becomes saturated for the desired molecules to pass into the ethyl acetate phase.

The saturated water phase was placed into a separating funnel and extracted with certain volumes of ethyl acetate. This process was repeated three times to enhance the yield. Even though an intermediate phase appeared between two phases, following ethyl acetate addition to the water phase, this intermediate phase disappeared in time. As mentioned previously, the desired molecules such as proanthocyanidins, flavonoids, and phenolic acids in *Pinus* barks remained in the ethyl acetate phase because the water phase was saturated. The ethyl acetate phase was collected, and anhydrous sodium sulfate was added to discard the remaining water from the ethyl acetate phase. Then, the volume of the ethyl acetate phase was reduced to 1/5 in a rotary vacuum evaporator, and thereby, the remaining ethyl acetate phase was placed into a beaker. After that, three volumes of chloroform was added drop-by-drop to one volume of ethyl acetate phase while stirring mechanically to precipitate proanthocyanidins. The ethyl acetate-chloroform mixture, including the precipitated proanthocyanidin, was placed into centrifuge tubes and

separated by centrifuge at 4500 rpm for 20 min. After removing the ethyl acetate-chloroform supernatant phase, precipitates are put into the vacuum drying chambers overnight to remove residual solvents. The light beige colored powder obtained was stored at -20°C . The proanthocyanidins from *P. brutia*, *P. pinea*, and *P. pinaster* barks were dissolved in distilled water and methanol before the tests. The proanthocyanidins from *P. sylvestris*, *P. nigra*, and *P. halepensis* barks could not be studied for phytochemical and biological activities due to low extraction yields of proanthocyanidins.

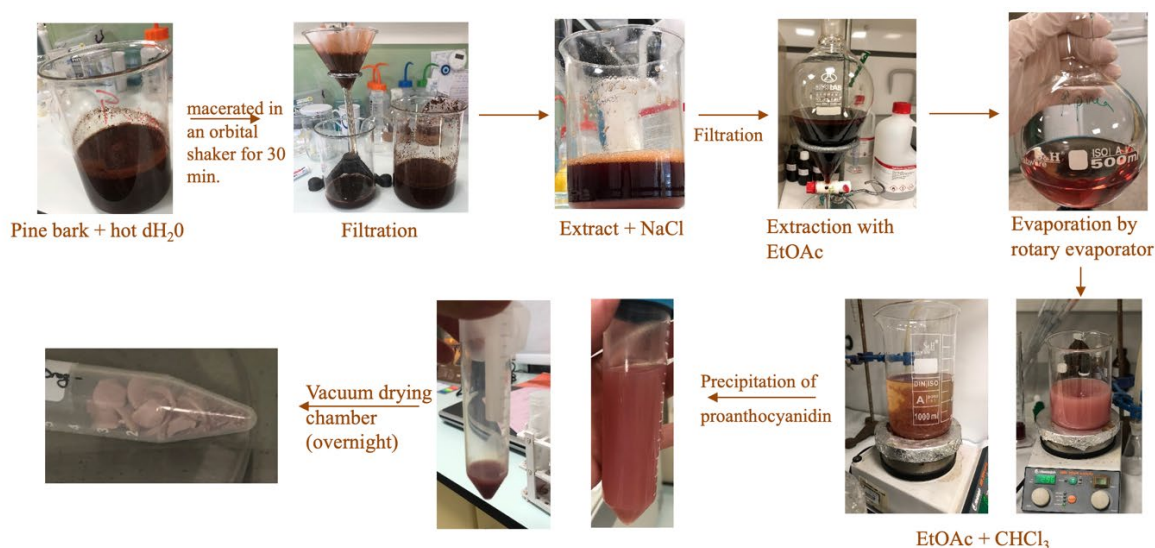


Figure 4. Schematic representation of proanthocyanidin extraction procedure.

Table 18. Proanthocyanidin Extraction yields of *Pinus* species.

Plant Name	Part Used	Yield (%)
<i>P. brutia</i>	barks	0.105 %
<i>P. pinea</i>	barks	0.22 %
<i>P. pinaster</i>	barks	0.1 %
<i>P. sylvestris</i>	barks	0.023%
<i>P. nigra</i>	barks	0.025%
<i>P. halepensis</i>	barks	0.016%

3.2.2. *In vitro* Gastrointestinal Digestion Simulation Procedure

The simulation model of gastrointestinal digestion was performed according to an *in vitro* model, as explained elsewhere (107). This model consists of two steps; gastric digestion and intestinal digestion. To simulate the gastric fluid, 1.6 g pepsin as a gastric enzyme, 1 g NaCl as an electrolyte was dissolved in 500 mL of distilled water, and 5 M of HCl for pH adjustment (pH:2) was added to this mixture. This mixture defines as simulated gastric fluid (SGF). Following 2.5 mL of *Pinus* extracts mixed with 17.5 mL of SGF, the mixture was put to the shaking water bath at proper body temperature for 2 hours to beget the peristaltic force. After that, the mixture was put on an ice bath to cap off the gastric simulation. 2 mL of sample was received to evaluate the gastric fraction (GF). In order to mimic the small intestinal conditions, pancreatin with bile acids was added, and cellulosic dialysis membrane (CDM) was used to imitate intestinal absorption. CDM in which a proper amount of NaHCO_3 (1 M) was added to adjust until pH of the intestinal medium (pH:7) was placed to the remaining mixture in a tube. After simulating the pH of intestinal digestion, pancreatin with bile acid mixture was added to the tube. It was left in the shaking water bath at proper body temperature for 2 hours. After 2 h, the mixture in the CDM was set aside as the “bioavailable” phase (BP). All samples were stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

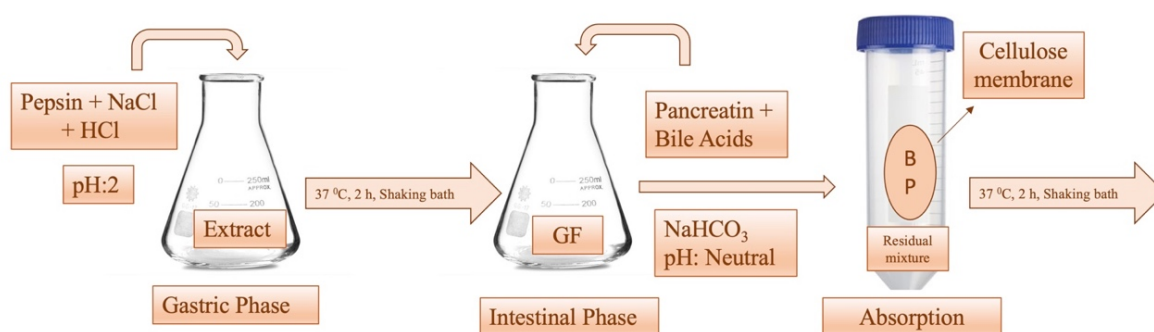


Figure 5. Schematic representation of *in vitro* digestion simulation procedure.

3.3. *In vitro* studies

3.3.1. *In vitro* evaluation of phenolic profile

3.3.1.1. Total Phenolic Content

The evaluation of the total phenolic content of the samples was measured spectrophotometrically by using Folin-Ciocalteu reagent (FCR) (108). Folin-Ciocalteu reagent, composed of phosphomolybdate and phosphotungstate, is a specific redox solution. This method relies on generating a chromophore group in the phenolic metabolites by using the phosphotungstic-phosphomolybdenum complex in an alkaline medium. 75 μL of Na_2CO_3 was mixed thoroughly with 20 μL of extracts and reference solution to create an alkaline medium. After adding 100 μL of FCR, the mixture was left for incubation at room temperature in the dark. After 30 minutes, the absorbance was measured at 690 nm. The calibration curve was obtained to use the different concentrations of gallic acid as the standard solution (31.25-1000 $\mu\text{g/mL}$). The results were expressed as gallic acid equivalents (GAE) in 1 g dry extract.

3.3.1.2. Total Proanthocyanidin Content

The evaluation of the total proanthocyanidin content of the samples was carried out spectrophotometrically in a 96-well plate template, as explained earlier in detail (109). This assay, known as vanillin-HCl assay, depends on the production of the colored complex due to a reaction between proanthocyanidin and vanillin in an acidic medium. Concisely, the mixture in which 150 μL of 4% vanillin and 75 μL of 32% HCl were added to 25 μL of extracts incubated at room temperature in the dark for 15 min. The same procedure was performed with methanol as a blank group. Following the incubation period, the absorbance was measured at 492 nm. A calibration curve was created by different concentrations of catechin hydrate as a reference (31.25-1000 $\mu\text{g/mL}$). The total proanthocyanidin content of the samples was disclosed as catechin equivalents (CE) in 1 g dry extract.

3.3.1.3. Total Flavonoid Content

The method explained in our previous study was performed spectrophotometrically for the evaluation of total flavonoid content (1). The solution comprising 10 μL of aluminium chloride, 10 μL of 1M sodium acetate with 150 μL of 75% ethanol were combined with 50 μL of extracts or reference solutions before the

incubation process at room temperature. Different concentrations of quercetin as a reference solution (31.25-1000 µg/mL) were used to obtain a calibration curve. After 30 min, the absorbance was measured at 405 nm. The results were defined as mg quercetin equivalents (QE) in 1 g dry extract.

3.3.1.4. Total Phenolic Acid Content

The spectrophotometric evaluation of the total phenolic acid content of the samples was determined according to a published method (110). Arnou reagent was attained with 10% sodium nitrite and 10% sodium molybdate in distilled water. After 1 mL of this reagent mixed with 1 mL of the extracts, equal volumes of 1 M NaOH and 0.1 M HCl solutions were added quickly to the mixture. The same procedure was also conducted to different concentrations of caffeic acid as a reference solution (31.25-1000 µg/mL). The absorbance was measured at 490 nm. The total phenolic acid content of the samples was indicated as caffeic acid equivalents (CAE) in 1 g dry extract.

3.3.2. *In vitro* Evaluation of Antioxidant Potential

3.3.2.1. Free Radical Scavenging Activity Assays

3.3.2.1.1. DPPH Radical Scavenging Activity Assay

2,2- diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay was evaluated by slightly modifying assay used by Celep et al. (111). Firstly, 11.83 mg DPPH was dissolved in 200 mL of methanol to obtain 150 µM DPPH solution. 200 µL of this solution was mixed with 25 µL of extracts in a 96-well plate. The mixture was incubated at room temperature in the dark for 50 min. The same procedure was also performed with methanol as a control solution and butylated hydroxytoluene (BHT) as a reference solution. After the incubation period, the absorbance was read at 517 nm.

$$\text{DPPH radical scavenging activity \%} = [(A_{\text{control}} - A_{\text{sample}}) / (A_{\text{control}})] \times 100$$

Where A_{control} = absorbance of the control group, A_{sample} =absorbance of the extracts or reference.

3.3.2.1.2. DMPD Radical Scavenging Activity Assay

DMPD (N, N-dimethyl-p-phenylenediamine) radical scavenging assay was accomplished spectrophotometrically according to a previously described method (112). Briefly, DMPD⁺ radical mixture was obtained by mixing 1 mL of 100 mM DMPD, 0.2 mL of 0.05 M FeCl₃, and 100 mL of 0.1 M sodium acetate buffer (pH: 5.25). A 210 µL aliquot was combined with 15 µL of the extracts and allowed to stand at room temperature in the dark for 50 min. The same protocol was applied to a blank solution (sodium acetate buffer) and different concentrations of reference solution (Trolox). The absorbance was read at 492 nm. After preparing the calibration curve with the absorbance of Trolox, results were expressed as Trolox equivalents (TE) in 1 g dry extract.

3.3.2.2. Metal Reducing Activity Assays

3.3.2.2.1. Ferric Reducing Antioxidant Power Assay

The method described previously was performed to evaluate the reduction potential of antioxidant molecules on ferric tripyridyl triazine complex (Fe⁺³-TPTZ) to ferrous form (Fe⁺²) (113). Concisely, 10 µL of extracts and 30 µL of dH₂O were added to 260 µL of FRAP reagent comprising 2.5 mL of ferric-tripyridyl triazine, 25 mL of 300 mM acetate buffer (pH:3.6), and 2.5 mL of 2 mM FeCl₃.6H₂O. After the mixture was allowed to stand at 37 °C for 30 min, the absorbance was measured at 593 nm. The calibration curve was created by different concentrations of ferrous sulfate (0.25–2 mM), and different concentrations of butylated hydroxyl toluene (31.25-1000 µg/mL) were used as a control solution. The results were disclosed as mM FeSO₄ in 1 g dry extract.

3.3.2.2.2. Cupric Reducing Antioxidant Power Assay

CUPRAC assay is based on the formation of chelation between CuSO₄ and neocuproine by a redox reaction (114). Firstly, 85 of µL 10 mM CuSO₄ and 85 µL of neocuproine were combined in an equal volume of ammonium acetate buffer. After that, 43 µL of extracts or reference solution and 51 µL of dH₂O were added to this mixture. The mixture was incubated for 20 min at the proper temperature, and the absorbance was read at 450 nm. To obtain a calibration curve, cupric reducing activities of different concentrations (31.25-1000 µg/mL) of ascorbic acid were measured. The results indicated mg ascorbic acid equivalent (AAE) per g extract.

3.3.2.3. Total Antioxidant Capacity Assay

The total antioxidant activity of the samples was determined spectrophotometrically according to a method described previously by Celep et al. (107). Firstly, TOAC reagent was attained freshly by 247.2 mg of ammonium molybdate tetrahydrate, 164.6 μ L of sulfuric acid, and 280 mg of sodium phosphate monobasic dissolving in 50 mL of distilled water. 300 μ L of this reagent was added to 30 μ L of extracts and incubated for 90 min at 95°C in a water bath. The absorbance was measured at 690 nm. The calibration curve was obtained using different ascorbic acid concentrations as a reference solution (31.25-1000 μ g/mL). The results were stated as ascorbic acid equivalents (AAE) in 1 g dry extract.

3.3.3. *In vitro* Anti-diabetic Activity Assays

3.3.3.1. Inhibition of α -amylase Activity Assay

This spectrophotometric method was performed to investigate the inhibition potential of α -amylase enzyme of the extracts using a published method with minor modifications (110). In this assay, 3,5-dinitrosalicylic acid (DNS) is used as a coloring reagent, and thereby, the intensity of the color was measured spectrophotometrically at 540 nm. Maltose, which is liberated from starch by α -amylase enzyme, transforms the yellow color of DNS to the orange color. First of all, to obtain 96 mM of DNS, 347.99 g of DNS was dissolved in 20 mL of distilled water, and 12 g sodium potassium tartrate was dissolved in 8 mL of 2 M NaOH. Then, two solutions were mixed while stirring mechanically slowly. Secondly, sodium phosphate buffer with 6.7 mM sodium chloride (pH:6.9) was prepared at 20 °C. 15.625 mg of the α -amylase enzyme was dissolved in 250 mL of buffer solution (1U/mL). 0.5 g starch as the substrate was also dissolved in 50 mL of buffer solution. 500 μ L of the extracts and 500 μ L of α -amylase solution were combined in tubes and preincubated for 30 min at 37 °C. End of the preincubation period, 500 μ L of 1% starch solution was added to the mixture, and the incubation period was repeated once more. Finally, 1 mL of DNS reagent was mixed and incubated in a boiling water bath for 15 min. The absorbance was measured at 540 nm following the mixtures reached room temperature. The background samples prepared without α -amylase enzyme by the same procedure were tested to eliminate the absorbance of the color of the sample. The control group prepared without samples by the same procedure was analyzed to check the inhibitory activity. Different concentrations of acarbose solutions (31.25-1000

µg/mL) were used as a positive control. Two concentrations of extracts (0.25 mg/mL and 0.125 mg/mL) were used for evaluation of α-amylase enzyme inhibitory activity. The results were indicated as percentage of α- amylase inhibition estimated by the following equation:

$$\text{Inhibition of } \alpha\text{- amylase activity \%} = [(A_{\text{control}} - A_{\text{sample}} / (A_{\text{control}})] \times 100$$

Where A_{control} = absorbance of the control group, A_{sample} =absorbance of the extracts or reference.

3.3.3.2. Inhibition of α-glucosidase Activity Assay

The inhibition potential of α-glucosidase of samples was performed spectrophotometrically using a published method with a slight modification (110). The mixture, which was procured by combined 20 µl of the sample and 20 µl of α-glucosidase (0.2 U/mL) solution in 170 µl of 100 mM phosphate buffer (pH 7.0), was placed into a 96-well plate and incubated for 15 min at 37 °C. 20 µl of 2.5 mM of 4- nitrophenyl-α-D- glucopyranoside (p-NPG), as the substrate, was added to the mixture, and the incubation period was repeated once more. 80 µl of 0.2 M sodium carbonate was put into the wells to stop the reaction, and the absorbance was measured at 405 nm. The same procedure set up the control group prepared without samples to check the inhibitory activity. Different concentrations of quercetin solutions (31.25-1000 µg/mL) were used as a positive control. The results were given as per cent of α-glucosidase inhibition estimated by the following equation:

$$\text{Inhibition of } \alpha\text{-glucosidase activity \%} = [(A_{\text{control}} - A_{\text{sample}} / (A_{\text{control}})] \times 100$$

Where A_{control} = absorbance of the control group, A_{sample} =absorbance of the samples or positive control.

3.3.3.3. Inhibition of advanced glycation end products

Bovine serum albumin/glucose model was performed to evaluate the inhibitory activity of advanced glycation end-products of samples according to the method published previously (115). Bovine serum albumin (BSA) (10 mg/ml) and 0.5 M glucose were prepared with 0.1 M phosphate buffer saline (pH 7.4) separately. First of all, 1 mL of extracts were mixed equally with BSA. After that, to trigger the glycation reaction, an

equal volume of glucose was combined with the mixture. The proper amount of sodium azide was added to the mixture to prevent microbial growth. The mixture was allowed to stand for incubation at 55 °C for 40 h in a shaking water bath. Different concentrations of quercetin solutions (31.25-1000 µg/mL) were used as a positive control. The background samples were attained without glucose and were analyzed parallelly. The control group was tested by replacing the sample with phosphate buffer to form AGEs. The fluorescence intensity of the mixture was estimated at the 370 nm excitation and emission 440 nm. The following equation was used estimation the inhibitory activity:

$$\text{Inhibitory activity (\%)} = \{1 - [(IF_{E/PC} - IF_{blank}) / (IF_{control})]\} \times 100$$

where $IF_{E/Q}$ = the fluorescence intensity of extracts or quercetin solution (positive control), IF_{blank} = the fluorescence intensity in the absence of glucose, and $IF_{control}$ = the fluorescence intensity in the absence of extracts.

3.4. Chromatographic Analysis

3.4.1. Qualitative and Quantitative Analysis with HPTLC

The chromatographic analysis of samples was carried out on the HPTLC system from CAMAG to perform qualitative and quantitative tests following the validated method with some modifications (116). This system, controlled by WinCATS software, consisted of Plate Heater III, Automatic Developing Chamber 2 (ADC2), Linomat 5 sample applicator with a 100 µL syringe, TLC Visualizer, Immersion Device, and Automatic Developing Chamber 2 (ADC2). HPTLC plates of silica 60F₂₅₄ were used as stationary phase. The reference compounds given in Table 20 were dissolved in MeOH (100 µg/mL). The samples were adjusted to proper concentrations and filtered by a syringe. The conditions in operation were: syringe delivery speed; 100 nL/s, band width of analytes; 8 mm, distance from bottom; 15 mm, injection volume; 1-6 µL reference solutions and 1-3 µL sample solutions were, length of chromatogram; 70 mm from the application point. The relative humidity was adjusted to 33% by saturated magnesium chloride hexahydrate solution.

Table 19. Reference compounds used for HPTLC analysis

Reference Compounds	
Proanthocyanidins	Proanthocyanidin A1
	Proanthocyanidin B1/B2 mixture
	Proanthocyanidin C1
Flavonoids	Taxifolin
	Catechin/epicatechin mixture
	Epigallocatechin
	Epigallocatechin gallate
	Catechin gallate
Phenolic acids	Caffeic acid
	Ferulic acid

Table 20. Mobile phases used for HPTLC analysis

Mobile phase	Reference Compounds
Toluene:Acetone:Formic acid (3:6:1, v/v/v)	Proanthocyanidin A1
	Proanthocyanidin B1/B2 mixture
	Proanthocyanidin C1
	Catechin/epicatechin mixture
	Epigallocatechin
	Epigallocatechin gallate
	Catechin gallate
Toluene:Ethylacetate:Formic acid (5:4:1, v/v/v)	Taxifolin
	Caffeic acid
	Ferulic acid

Table 21. Derivatization reagents used for HPTLC analysis

Derivatization reagent	Reference Compounds
4-dimethylaminocinnamaldehyde (DMACA)	Proanthocyanidin A1
	Proanthocyanidin B1/B2 mixture
	Proanthocyanidin C1
	Catechin/epicatechin mixture
	Epigallocatechin
	Epigallocatechin gallate
	Catechin gallate
Natural Product Reagent (NPR)	Taxifolin
	Caffeic acid
	Ferulic acid

The reference compounds seen in Table 19 were prepared at different concentrations (100 µg/mL to 1 mg/mL). Both sample and reference solutions were filtered through a 0.45 mm syringe filter, before the application process. The mobile phases used for the development process are given in Table 20. The mobile phases were toluene:acetone:formic acid (3:6:1 by volume) and toluene:ethylacetate:formic acid (5:4:1 by volume) for the development process which are conducted by Automated Development Chamber (ADC 2). After The plates were dried properly using Plate Heater III following the development process. The derivatization reagents seen in Table 21 were used to obtain the visual documentation by using TLC Visualizer. 4-dimethylaminocinnamaldehyde (DMACA) dipping detection reagent was prepared by dissolving 60 mg of DMACA in 13 mL of 37% hydrochloric acid, which was made up to 200 mL with ethanol. Natural Product Reagent (NPR) dipping detection reagent was prepared by dissolving 1g of 2-aminoethyl-diphenylborinate in 200 mL of ethylacetate. In quantitative analysis, the densitometry scan (280 nm for proanthocyanidin A1, proanthocyanidin B1/B2 mixture, proanthocyanidin C1, catechin/epicatechin mixture,

epigallocatechin, epigallocatechin gallate, and catechin gallate, 330 nm for taxifolin, ferulic acid, and caffeic acid) was used to determine the calibration curve of the samples by measuring the peak areas of reference compounds. The correlation coefficients (r^2) were found to be >0.99 for the analysis of all samples.

3.4.2. HPTLC-DPPH Method

HPTLC-DPPH assay was performed to determine the DPPH scavenging activities of extracts qualitatively on plates following the validated method explained earlier by Güzelmeriç et al. (22). 10 mg/mL *Pinus* extracts and 0.25 mg/mL reference compounds as given in Table 19 were applied on the silica gel 60 F₂₅₄ HPTLC glass plate. After saturating the chamber for 20 min, the plate was developed with a proper mobile phase according to applying reference compounds up to seven cm. Before the derivatization process, 0.05% DPPH, used as the derivatized reagent, was freshly obtained in methanol. After the developing process, the plate quickly dried with cold air was derivatized with DPPH solution. The derivatized plates were executed under white light using TLC Visualizer at 0., 15., and 30. min.

3.5. Determination of Bioavailability Index

The theoretical calculation of the bioavailability index (BAvI) was performed by using the formula described by Swieca et al. (117). According to this formula:

$$\text{BAvI} = C_{\text{BP}}/C_{\text{BD}}$$

The bioavailability index (BAvI) is the mathematical ratio of phenolic molecules in the bioavailable phase compared to before digestion (BD).

3.6. Statistical Analysis

All *in vitro* assays and chromatographic analyses were performed in triplicate and the results were expressed as mean $\pm$ standard deviation (SD). Statistical analysis of results was done by using GraphPad Prism 6.1 software program. The statistical analysis of *in vitro* assays except for α -amylase, α -glucosidase and AGEs inhibitory activities were conducted by 1-way ANOVA Tukey's Multiple programs, whereas the results of other assay were estimated statistically by 2-way ANOVA Sidak's Multiple programs. Statistically significant results were defined as $p < 0.05$.

4. RESULTS

4.1. Results of Phenolic Content Determination

4.1.1. Total Phenolic Contents of Extracts

A striking downtrend of total phenolic content was observed from before-digestion to bioavailable phase for all *Pinus* species. The highest amount of TPC was observed in non-digested extracts of all plants. Before-digestion, higher total phenolic contents were observed for the extracts than the gastric fraction and bioavailable phase; for PB 1.14 and 1.66, PP 1.08 and 1.97, PPR 1.21 and 2.18 and PYC 1.22 and 2.55 folds higher, respectively. Even though before-digestion and gastric fraction PP contained a higher amount of TPC among all pine samples (408.94 ± 1.09 and 375.68 ± 3.96 mg GAE/g respectively), the bioavailable fraction PB had higher TPC than the bioavailable fraction PP (230.98 ± 2.82 and 207.42 ± 3.17 mg GAE/g, respectively). PYC had the lowest amount of TPC in all fractions. In addition, the most potent descent from before-digestion to bioavailable fraction was observed in PYC (351.88 ± 2.11 and 137.86 ± 1.90 mg GAE /g, respectively).

Table 22. Spectrophotometric determination of total phenolic profile of extracts

<i>Proanthocyanidins of Pinus sp. (1mg/ml)</i>	<i>TPC (mg GAE/g)*</i>		
	<i>BD⁺</i>	<i>GF</i>	<i>BP</i>
<i>P. brutia</i>	383.58 ± 2.82^a	336.42 ± 2.48^b	230.98 ± 2.82^c
<i>P. pinea</i>	408.94 ± 1.09^a	375.68 ± 3.96^b	207.42 ± 3.17^c
<i>P. pinaster</i>	396.93 ± 2.92^a	327.17 ± 1.25^b	182.17 ± 1.45^c
Pycnogenol®	351.88 ± 2.11^a	287.64 ± 3.62^b	137.86 ± 1.90^c

⁺ The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase.

* Results were expressed as the mean of triplicates $\pm$ standard deviation (S.D.) and as mg gallic acid equivalents (GAE) in 1 g sample.

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species were compared among themselves with GraphPad software version 8.1 programs and the statistical differences were demonstrated by a, b and c. The same statistical analysis was applied to different fractions of Pycnogenol® ($p \leq 0.0001$).

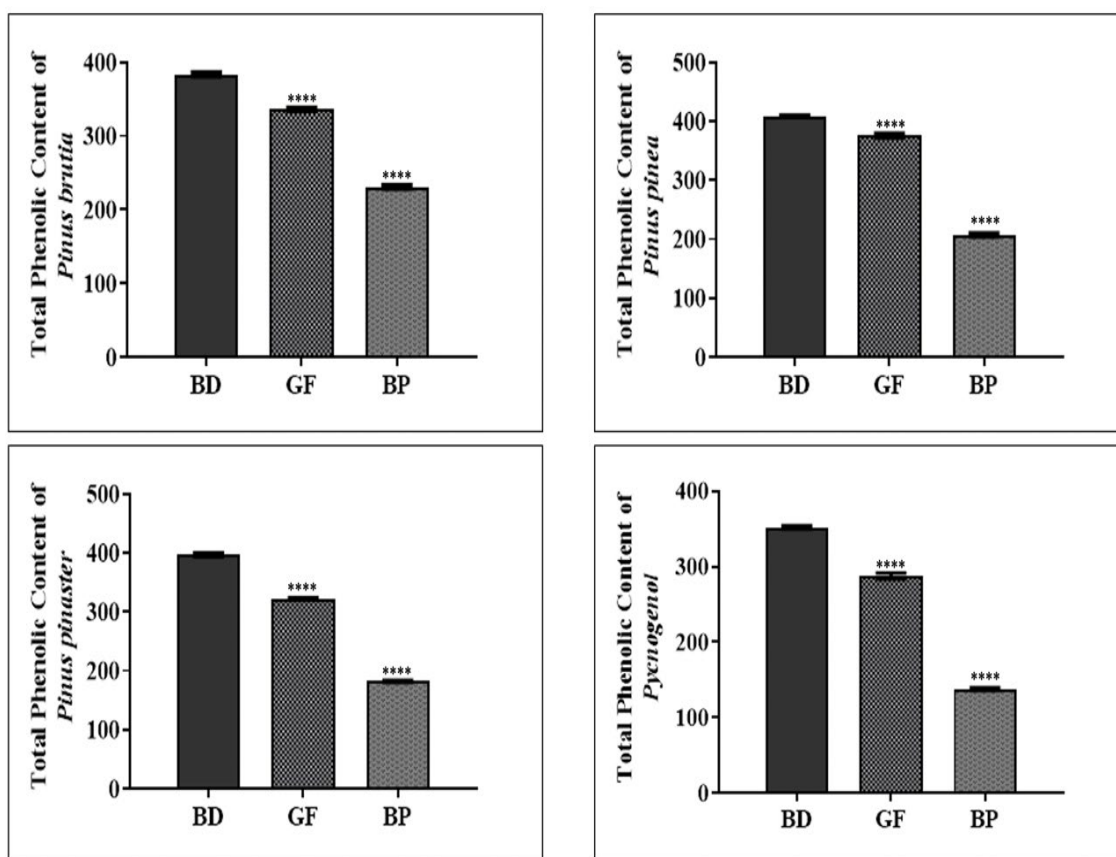


Figure 6. Comparison of the total phenolic profile of each different *Pinus* species and Pycnogenol®. The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase. The BD, GF, and BP fractions of each *Pinus* species were compared among themselves by GraphPad software version 8.1 programs with 1way ANOVA multiple comparisons and the statistical differences were observed by **** ($p \leq 0.0001$). The same statistical analysis was applied to different fractions of Pycnogenol®.

4.1.2. Total Phenolic Acid Contents of Extracts

As seen in Table 23, before-digestion fractions in all extracts contained the highest total phenolic acid content. Before-digestion PB had 1.49 times higher total phenolic acid content in comparing gastric fraction, while a striking change as 8.02 folds was observed between before-digestion and bioavailable fractions. The same differentiation was seen in PYC. Before-digestion PYC contained higher total phenolic acid content than the gastric fraction and bioavailable phase by 1.50 and 6.47 folds. Before-digestion PP and PPR had higher total phenolic acid content compared to gastric fractions and bioavailable phases (PP; 1.34, 3.11 and PPR 1.80, 3.68, respectively). This alteration was slightly lower than the change of PB and PYC. The utmost amount of TPaC was observed in

before-digestion PP, while PYC had the lowest amount of TPaC (193.93 ± 3.36 and 112.64 ± 3.27 mg CAE/g, respectively). Likewise, GF exerted the same trend. Among the samples, the amount of TPaC in PP was 144.55 ± 4.06 , followed by PB, PPR, and PYC (125.74 ± 2.89 , 90.74 ± 3.60 , and 75.26 ± 2.06 mg CAE/g, respectively). The highest amount of TPaC was seen in PP and then PPR, PB, and PYC, respectively.

Table 23. Spectrophotometric determination of total phenolic acid profile of samples

<i>Proanthocyanidins of Pinus</i> <i>sp. (1mg/ml)</i>	<i>TPaC (mg CAE/g)*</i>		
	<i>BD</i> ⁺	<i>GF</i>	<i>BP</i>
<i>P. brutia</i>	187.21 ± 2.38^a	125.74 ± 2.89^b	23.34 ± 2.14^c
<i>P. pinea</i>	193.93 ± 3.36^a	144.55 ± 4.06^b	62.40 ± 4.06^c
<i>P. pinaster</i>	163.12 ± 3.21^a	90.74 ± 3.60^b	44.31 ± 3.38^c
Pycnogenol [®]	112.64 ± 3.27^a	75.26 ± 2.06^b	17.40 ± 1.09^c

⁺ The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase

* Results were expressed as the mean of triplicates $\pm$ standard deviation (S.D.) and as mg caffeic acid equivalents (CAE) in 1 g sample.

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species were compared with GraphPad software version 8.1 programs and statistical differences were demonstrated by a, b and c. The same statistical analysis was applied to different fractions of Pycnogenol[®] ($p \leq 0.0001$).

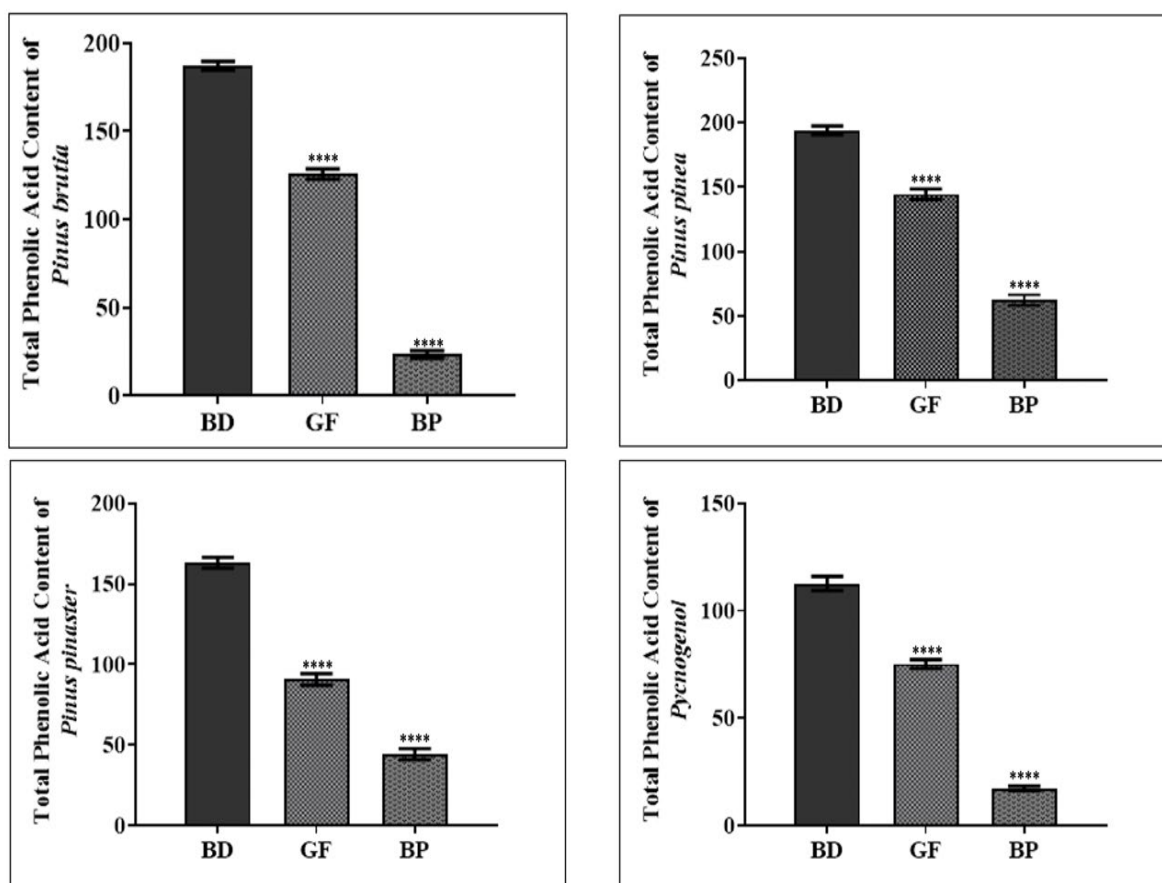


Figure 7. Comparison of the total phenolic acid profile of each *Pinus* species and Pycnogenol[®]. The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase. The BD, GF, and BP fractions of each *Pinus* species were compared among themselves by GraphPad software version 8.1 programs with 1way ANOVA multiple comparisons and statistical differences were demonstrated by **** ($p \leq 0.0001$). The same statistical analysis was applied to different fractions of Pycnogenol[®].

4.1.3. Total Proanthocyanidin Content of Extracts

The total proanthocyanidin content of samples ranged between 124.52 ± 2.63 and 623.61 ± 2.78 mg, expressed as catechin hydrate equivalents (CAE)/g samples. A substantial decrease from BD to bioavailable phase was revealed in all plants, as shown in Table 23. Before-digestion PB had 1.37 and 2.15 times higher total proanthocyanidin content compared to gastric-fraction and bioavailable phase, respectively. The total proanthocyanidin content of before-digestion PP was higher than the gastric-fraction and bioavailable phase by 1.24 and 2.42 folds, respectively. Before-digestion PPR contained 1.50 and 3.16 times higher total proanthocyanidin content compared to gastric-fraction and bioavailable phase, respectively. The total proanthocyanidin content in before-digestion PYC was higher than gastric-fraction and bioavailable phase by 1.42 and 3.05 folds, respectively. The most abundant TPrC was seen in BD PB, followed by PP, PPR, and PYC (623.61 ± 2.78 , 528.68 ± 1.80 , 471.33 ± 2.85 , and 379.29 ± 4.55 mg CHE/g, respectively). This trend was explicitly seen in both GF and BP. The gastric-fraction and bioavailable phase obtained from PYC were found to have the least TPrC (267.09 ± 2.08 and 124.52 ± 2.63 mg CHE/g, respectively).

Table 24. Spectrophotometric determination of total proanthocyanidin profile of samples

<i>Proanthocyanidins of Pinus</i> <i>sp. (1mg/ml)</i>	<i>TPrC*</i> (mg CHE/g)		
	<i>BD</i> ⁺	<i>GF</i>	<i>BP</i>
<i>P. brutia</i>	623.61 ± 2.78^a	454.97 ± 2.84^b	290.58 ± 0.69^c
<i>P. pinea</i>	528.68 ± 1.80^a	427.09 ± 3.80^b	218.91 ± 3.18^c
<i>P. pinaster</i>	471.33 ± 2.85^a	313.23 ± 2.39^b	149.14 ± 3.98^c
Pycnogenol®	379.29 ± 4.55^a	267.09 ± 2.08^b	124.52 ± 2.63^c

⁺ The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase

* Results were expressed as the mean of triplicates $\pm$ standard deviation (S.D.) and as mg (+)- catechin hydrate equivalents (CHE) in 1 g sample.

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species were compared with GraphPad software version 8.1 programs and statistical differences were demonstrated by a, b and c. The same statistical analysis was applied to different fractions of Pycnogenol® ($p \leq 0.0001$).

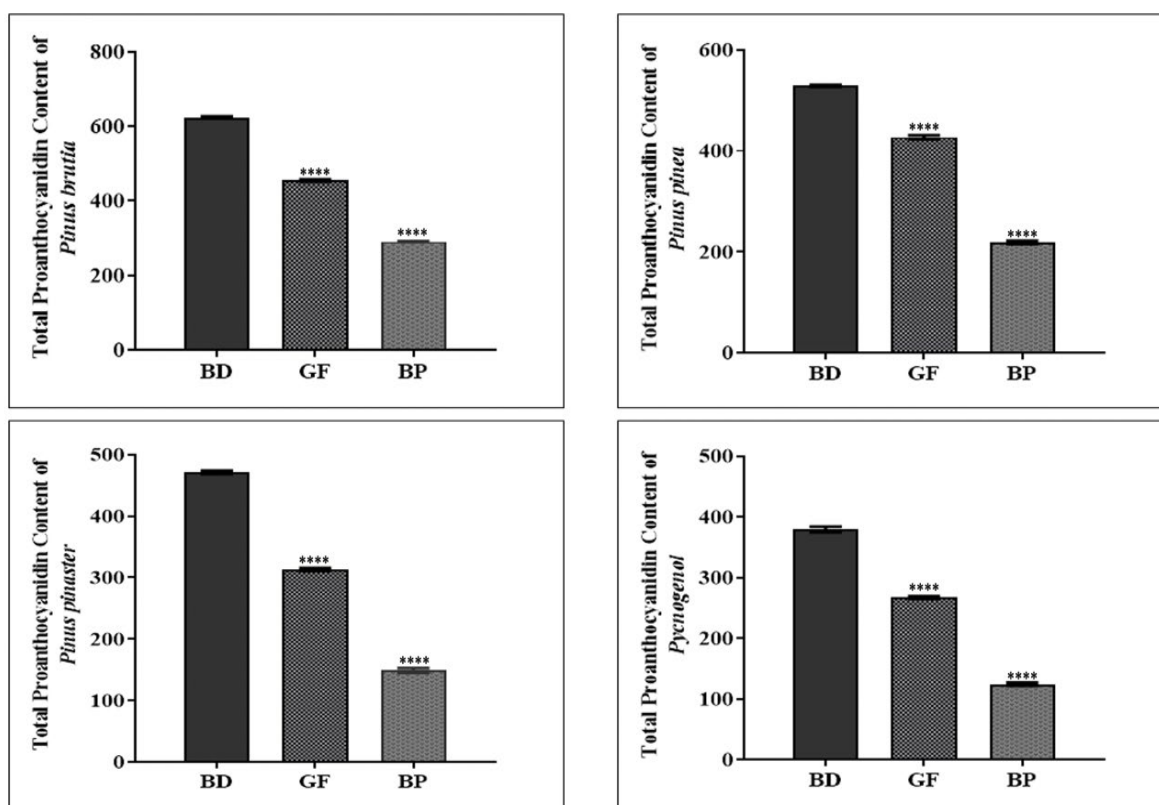


Figure 8. Comparison of total proanthocyanidin profile of each different *Pinus* species and Pycnogenol®. The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase. The BD, GF, and BP fractions of each *Pinus* species were compared by GraphPad software version 8.1 programs with one-way ANOVA multiple comparisons. As a result of the analysis, statistical differences were exhibited by **** ($p \leq 0.0001$). The same statistical analysis was studied in the different fractions of Pycnogenol®.

4.1.4. Total Flavonoid Contents of Extracts

Total flavonoid content assay was performed on all fractions. However, none of the fractions was contained a quantifiable TFC. This result showed that the amount of TFC in samples was lower than the limit of quantification.

4.2. Results of Free Radical Scavenging Activity Assays

4.2.1. DPPH Radical Scavenging Potential of Extracts

DPPH radical scavenging potential of samples is presented in Table 25. A minor alteration ranging between 90.87 ± 0.09 and 89.96 ± 0.52 was found among BD fractions in DPPH radical scavenging assay. Likewise, PB, PP, and PYC established no significant change from BD to GF fractions, but DPPH radical scavenging potent of gastric-fraction PB was slightly more than BD. However, PPR had a downtrend of DPPH radical scavenging potential from BD to GF fraction (90.67 ± 0.09 and 73.36 ± 2.79 , respectively). DPPH radical scavenging potential of the bioavailable phases indicated marked descent in comparing BD and GF for all samples. Before-digestion PB exerted 1.73 times DPPH radical scavenging potential than PB bioavailable phase. DPPH radical scavenging potential of before-digestion PP was higher 2.01 folds than PP bioavailable phase. Before-digestion PP and PPR indicated the biggest change among samples (2.48 and 3.17 folds, respectively). Among the bioavailable phases, PB showed the highest DPPH radical scavenging potential, whereas the lowest was seen in PYC.

Table 25. DPPH radical-scavenging activity of samples

<i>Proanthocyanidins of Pinus sp. (0.25 mg/ml)</i>	<i>DPPH^{##} (%)</i>		
	<i>BD⁺</i>	<i>GF</i>	<i>BP</i>
<i>Pinus brutia</i>	90.87 ± 0.09^a	91.12 ± 0.37^a	57.79 ± 1.49^b
<i>Pinus pinea</i>	90.43 ± 0.09^a	90.77 ± 0.30^a	45.09 ± 2.08^b
<i>Pinus pinaster</i>	90.67 ± 0.09^a	73.36 ± 2.79^b	36.51 ± 2.90^c
Pycnogenol [®]	89.96 ± 0.52^a	85.52 ± 1.27^b	28.37 ± 2.37^c

⁺The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase

* Results were expressed as the mean of triplicates $\pm$ standard deviation (S.D.) and DPPH radical-scavenging activity of the reference compound “butylated hydroxytoluene (BHT)” is found to be $\% 91.67 \pm 0.15$ in 1 g sample.

[#] 2,2-diphenyl-1-picrylhydrazyl.

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c). The same statistical analysis was applied to different fractions of Pycnogenol[®].

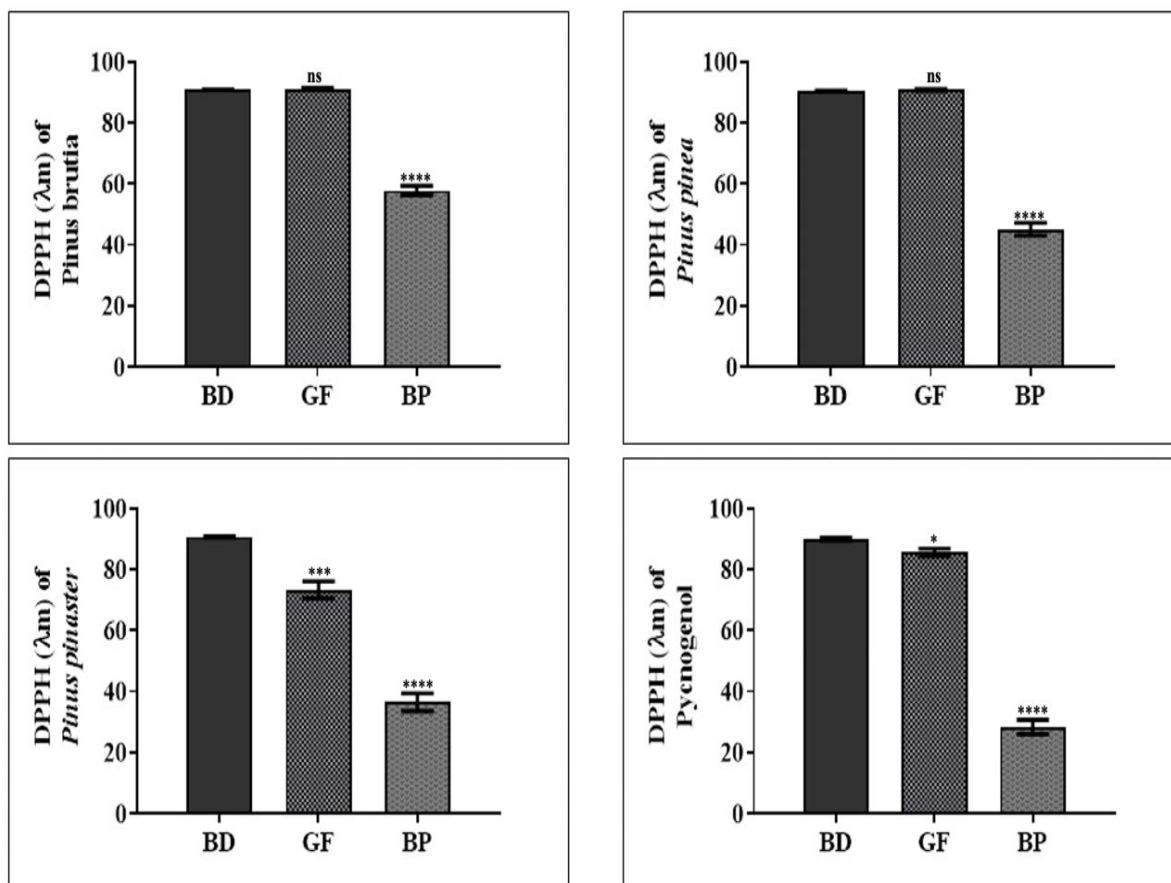


Figure 9. Comparison of DPPH radical scavenging potential of each different *Pinus* species and Pycnogenol®. The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase. The BD, GF, and BP fractions of each *Pinus* species were compared by GraphPad software version 8.1 programs with one-way ANOVA multiple comparisons. As a result of the analysis, non-significant results were symbolized with ns, * was used for $p \leq 0.05$, *** was used for $p \leq 0.001$ and **** was $p \leq 0.0001$.

4.3.2. DMPD Radical Scavenging Potential of Extracts

DMPD radical scavenging potential of samples ranged between 127.80 ± 2.65 and 51.13 ± 2.08 . The before-digestion fractions had the most potent DMPD activity in all plants, as shown in Table 26. The highest activity was seen in PP, whereas PYC had the least (127.80 ± 2.65 and 102.47 ± 2.52 , respectively) among before-digestion fractions. Results of the DMPD assay had a similar downtrend with the DPPH assay in every step of the digestion model. Gastric-fraction PB exhibited a minor increment against before-digestion PB in DMPD assay (109.47 ± 0.58 and 104.13 ± 1.53 , respectively). However, before-digestion PP, PPR, and PYC showed higher DMPD radical scavenging potential than their gastric fractions as 1.38, 2.04, and 2.00 times, respectively. As seen in Table X, PP showed the most increased activity among bioavailable phases as 71.47 ± 2.89 , followed by PB, PPR, and PYC. Before-digestion PB, PP, PYC, and PYC had higher DMPD potential than their bioavailable phases by 1.38, 1.58, 2.04, and 2.00, respectively.

Table 26. DMPD radical-scavenging activity of samples

<i>Proanthocyanidins of Pinus</i> <i>sp.</i> (0.25 mg/ml)	DMPD* [#]		
	<i>BD</i> ⁺	<i>GF</i>	<i>BP</i>
<i>P. brutia</i>	104.13 ± 1.53^a	109.47 ± 0.58^b	66.80 ± 2.00^c
<i>P. pinea</i>	127.80 ± 2.65^a	92.80 ± 2.73^b	71.47 ± 2.89^c
<i>P. pinaster</i>	118.47 ± 1.53^a	74.80 ± 2.00^b	58.13 ± 1.53^c
Pycnogenol [®]	102.47 ± 2.52^a	93.13 ± 3.06^b	51.13 ± 2.08^c

⁺The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase.

* Results were expressed as the mean of triplicates $\pm$ standard deviation (S.D.) and as mg Trolox equivalents (TE) in 1 g sample

[#] N,N-dimethyl-p-phenyldiamine.

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species were compared with GraphPad software version 8.1 programs and the statistical differences were demonstrated by a, b and c. The same statistical analysis was applied to different fractions of Pycnogenol[®] ($p \leq 0.05$).

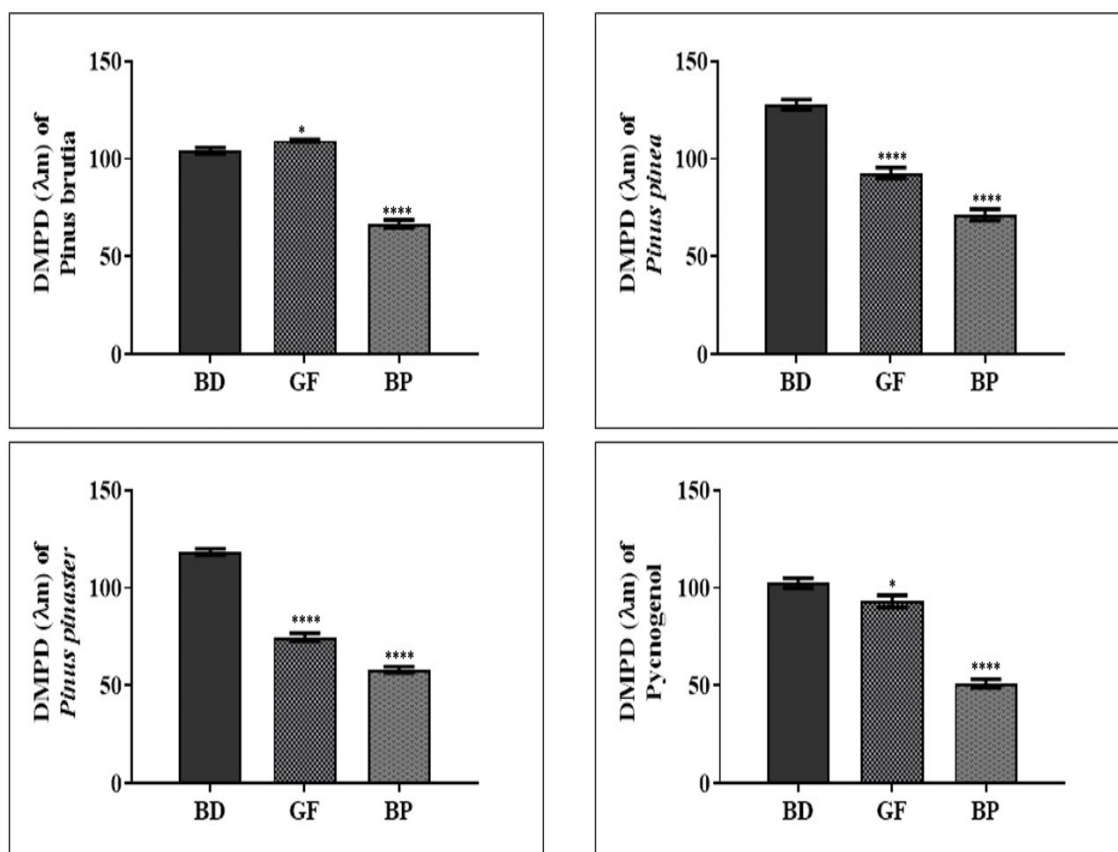


Figure 1. Comparison of DMPD radical scavenging potential of each different *Pinus* species and Pycnogenol®. The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase. The BD, GF, and BP fractions of each *Pinus* species were compared by GraphPad software version 8.1 programs with one-way ANOVA multiple comparisons. As a result of the analysis, statistical differences were exhibited by * ($p \leq 0.05$) and **** ($p \leq 0.0001$). The same statistical analysis was studied in the different fractions of Pycnogenol®.

4.3. Total Antioxidant Potential of Extracts

The total antioxidant potential of samples, presented in Table 27, ranged between 111.29 ± 2.60 and 51.29 ± 4.16 . Results indicated that the total antioxidant potential of samples was decreased after the digestion model. PB showed the highest activity in all fractions (BD: 111.29 ± 2.60 , GF: 106.29 ± 1.44 , and BP: 71.50 ± 3.80). Before-ion B, PP, and PYC slightly abated throughout gastric fractions (1.09, 1.08, and 1.10 folds, respectively). However, before-digestion PPR indicated a significant total antioxidant potential compared to the gastric fraction by 1.46 folds. The total antioxidant potential of before-digestion PB, PP, PPR, and PYC was higher than the bioavailable phases by 1.39, 1.56, 1.64, and 1.69 times. PYC had the lowest total antioxidant potential in both the before-digestion and bioavailable phase, whereas PPR exhibited the least activity in gastric fraction.

Table 27. Total antioxidant capacity of samples

<i>Proanthocyanidins of Pinus</i> <i>sp.</i> (0.25 mg/ml)	TOAC*		
	<i>BD</i> ⁺	<i>GF</i>	<i>BP</i>
<i>P. brutia</i>	97.62 ± 2.04^a	89.83 ± 1.69^b	70.25 ± 2.25^c
<i>P. pinea</i>	111.29 ± 2.60^a	106.29 ± 1.44^a	71.50 ± 3.80^b
<i>P. pinaster</i>	103.17 ± 3.12^a	70.88 ± 3.80^b	62.75 ± 3.31^b
Pycnogenol®	86.50 ± 3.75^a	78.38 ± 2.72^a	51.29 ± 4.16^b

⁺The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase

* Results were expressed as the mean of triplicates $\pm$ standard deviation (S.D.) and as mg ascorbic acid equivalents (AAE) in 1 g sample.

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c). The same statistical analysis was applied to different fractions of Pycnogenol®.

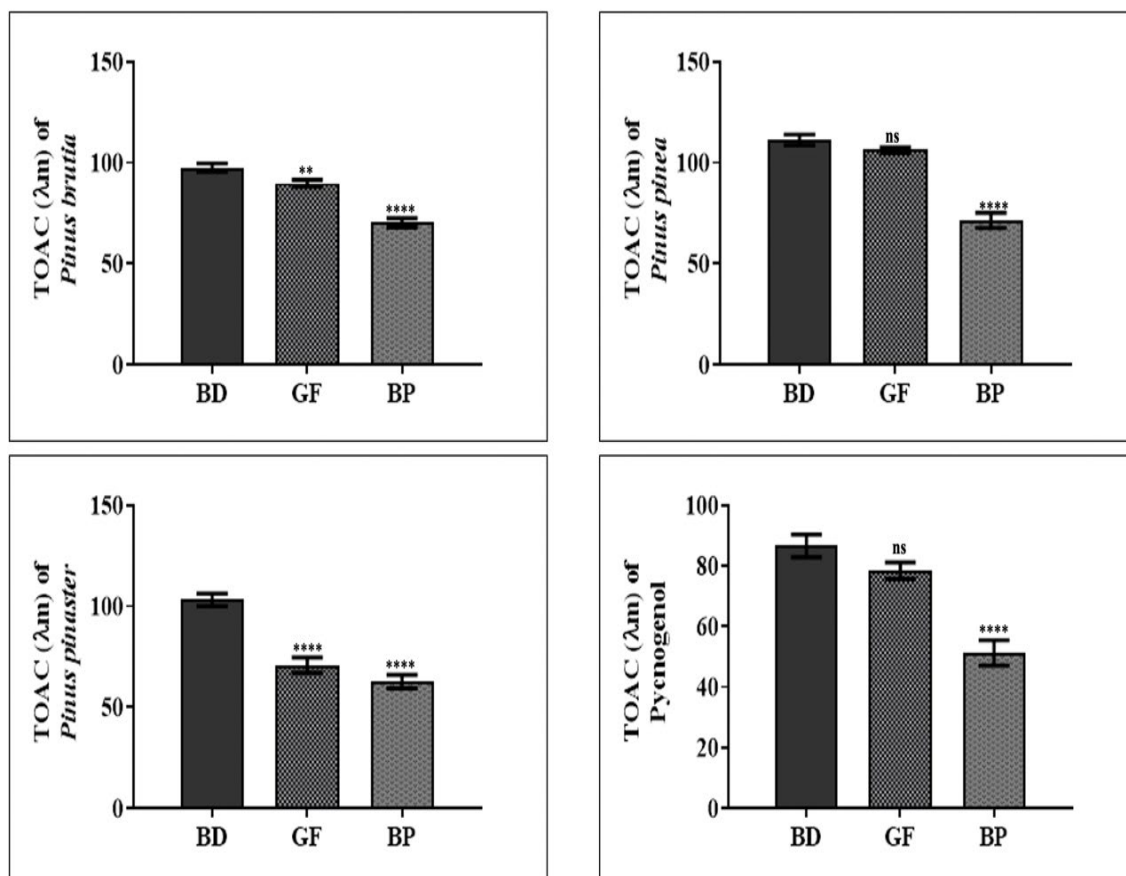


Figure 2. Comparison of TOAC potential of each different *Pinus* species and Pycnogenol®. The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase. The BD, GF, and BP fractions of each *Pinus* species were compared by GraphPad software version 8.1 programs with one-way ANOVA multiple comparisons. As a result of the analysis, ns was used for non-significant data, and statistical differences were exhibited by ** ($p \leq 0.01$) and **** ($p \leq 0.0001$). The same statistical analysis was studied in the different fractions of Pycnogenol®.

4.4. Results of Metal Reducing Activity Assays

4.4.1. Cupric Reducing Antioxidant Potential of Extracts

As in other assays, the utmost activity was seen in before-digestion fractions of all samples in cupric reducing antioxidant assay. In these assays, before-digestion PB, PP, and PYC had a slight alteration compared to their gastric fractions by 1.15, 1.24, and 1.21 times, whereas PPR showed 1.77 times higher CUPRAC activity than its gastric fractions. However, bioavailable phases of all samples presented a striking descent in CUPRAC activity. Before-digestion PB, PP, PPR, and PYC showed higher CUPRAC activity by 2.15, 2.41, 3.16, and 3.05 folds, respectively. PP demonstrated the highest CUPRAC activity in all fractions (BD; 528.68 ± 1.80 , GF; 427.09 ± 3.80 , BP; 218.91 ± 3.18). Even though before-digestion PPR had the higher CUPRAC activity than before-digestion PYC and PB, gastric fraction PPR showed the lowest CUPRAC activity. Among bioavailable phases, the highest CUPRAC activity was seen in PP, followed by PB, PPR, and PYC.

Table 28. Cupric Reducing Antioxidant Potential of Extracts

<i>Proanthocyanidins of Pinus</i> <i>sp.</i> (0.25 mg/ml)	CUPRAC* ^{&}		
	<i>BD</i> ⁺	<i>GF</i>	<i>BP</i>
<i>P. brutia</i>	409.14 ± 1.38^a	354.83 ± 2.84^b	190.58 ± 0.69^c
<i>P. pinea</i>	528.68 ± 1.80^a	427.09 ± 3.80^b	218.91 ± 3.18^c
<i>P. pinaster</i>	471.33 ± 2.85^a	267.09 ± 2.08^b	149.14 ± 3.98^c
Pycnogenol [®]	379.29 ± 4.55^a	313.23 ± 2.39^b	124.52 ± 2.63^c

⁺ The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase

* Results were expressed as the mean of triplicates $\pm$ standard deviation (S.D.) and as mg ascorbic acid equivalents (AAE) in 1 g sample.

[&] Cupric reducing antioxidant capacity

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species were compared by GraphPad software version 8.1 programs and statistical differences were demonstrated by a, b and c. The same statistical analysis was applied to different fractions of Pycnogenol[®] ($p \leq 0.0001$).

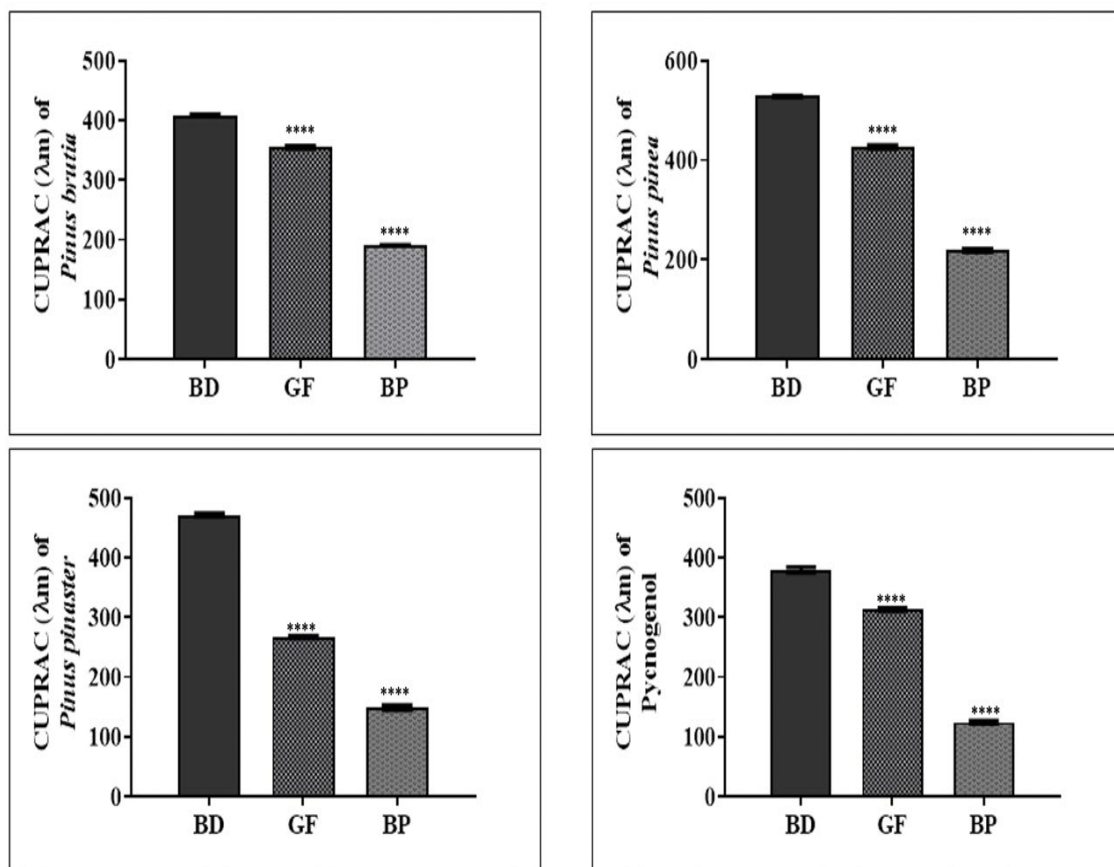


Figure 3. Comparison of CUPRAC potential profile of each different *Pinus* species and Pycnogenol®. The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase. The BD, GF, and BP fractions of each *Pinus* species were compared by GraphPad software version 8.1 programs with one-way ANOVA multiple comparisons. As a result of the analysis, statistical differences were exhibited by **** ($p \leq 0.0001$). The same statistical analysis was studied in the different fractions of Pycnogenol®.

4.4.2. Ferric Reducing Antioxidant Potential of Extracts

Ferric reducing antioxidant potential of samples were given in Table 29. All studied fractions showed a significant FRAP activity compared with BHT as a reference molecule. In all assays, before-digestion fractions of samples showed the highest activity. Furthermore, a substantial reduction from before-digestion fractions to bioavailable phases was observed in the FRAP assay. Before-digestion PB, PP, PPR, and PYC showed higher FRAP activity than their bioavailable phases by 2.09, 2.41, 3.19, and 3.50 times, respectively.

Nonetheless, there is a slighter decrease in FRAP activity from before-digestion to gastric fractions. Among the before-digestion fractions, the highest activity was seen in PP, followed by PPR, PB, and PYC. PP showed the highest FRAP activity in the gastric fraction, followed by PB, PYC, and PPR this time. Among the bioavailable phases, PP indicated the utmost FRAP activity, whereas the lowest was seen in PYC.

Table 29. Ferric Reducing Antioxidant Potential of Extracts

<i>Proanthocyanidins of Pinus sp.</i> (0.25 mg/ml)	FRAP* ^{&}		
	<i>BD</i> ⁺	<i>GF</i>	<i>BP</i>
<i>P. brutia</i>	1.38 ± 0.04 ^a	1.26 ± 0.06 ^a	0.66 ± 0.03 ^b
<i>P. pinea</i>	1.81 ± 0.12 ^a	1.49 ± 0.11 ^b	0.75 ± 0.02 ^c
<i>P. pinaster</i>	1.66 ± 0.18 ^a	0.95 ± 0.07 ^b	0.52 ± 0.03 ^c
Pycnogenol [®]	1.33 ± 0.04 ^a	1.05 ± 0.05 ^b	0.38 ± 0.02 ^c

⁺ The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase

* Results were expressed as the mean of triplicates ± standard deviation (S.D.) and as Mm FeSO₄ equivalents in 1 g sample.

[&]Ferric reducing antioxidant power

P.S. FRAP activity of the reference compound “butylated hydroxytoluene (BHT)” is found to be 2.10 ± 0.14.

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c). The same statistical analysis was applied to different fractions of Pycnogenol[®].

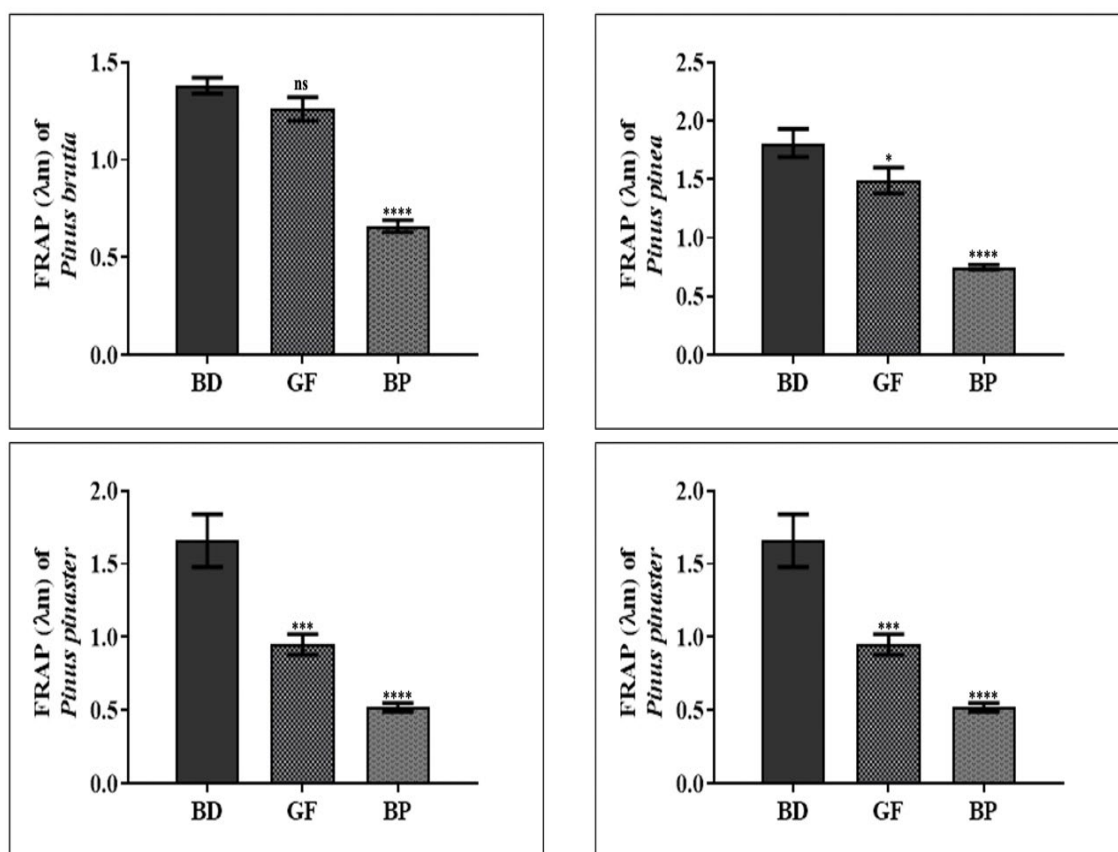


Figure 4. Comparison of FRAP potential of each different *Pinus* species and Pycnogenol®. The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase. The BD, GF, and BP fractions of each *Pinus* species were compared by GraphPad software version 8.1 programs with one-way ANOVA multiple comparisons. As a result of the analysis, ns was used for non-significant data and statistical differences were exhibited by * ($p \leq 0.05$), *** ($p \leq 0.001$) and **** ($p \leq 0.0001$). The same statistical analysis was studied in the different fractions of Pycnogenol®.

4.5. Results of *in vitro* Anti-diabetic Activity Assays

4.5.1. Inhibition potential of Advanced Glycation End Products of Extracts

The antiglycation activity of the samples was studied at two different concentrations (0.250 and 0.125 mg/ml), and the results were given as % inhibition in Table 30 and Figure 14, ranging from 80.37 ± 1.74 to 33.00 ± 2.16 . Quercetin was used as the reference compound. It was observed that the antiglycation activity was increased depending on the concentration in all samples. The antiglycation activities of PP showed higher activity in all fractions and at both concentrations. The highest activity was seen in the before-digestion PP, followed by PPR, PB, and PYC at 0.250 mg/ml concentrations (80.37 ± 1.74 , 75.55 ± 1.74 , 66.14 ± 1.93 , 54.94 ± 2.32). In both concentrations and all fractions, PP stated higher inhibition activity compared to other species and was followed by PPR, PB, and PYC. Moreover, the inhibition activities of the before-digestion fraction of PP at both concentrations were higher than the quercetin reference compound at the same concentration. The highest antiglycation activity among the samples was seen in all concentrations of before-digestion fractions. In addition, in all assays, a significant reduction in antiglycation activity was seen from the before-digestion to gastric fractions and bioavailable phases, respectively. Before-digestion PP, PPR, PB, and PYC showed higher antiglycation activity compared to their gastric fractions and bioavailable phases at 0.250 mg/ml (PP; 1.10, 1.32, PPR 1.20, 1.43, PB; 1.14, 1.32 and PYC; 1.17, 1.38 folds, respectively). The same alteration was seen at 0.125 mg/ml. PYC indicated the lowest antiglycation activity in all fractions and at both concentrations.

Table 30. Inhibition potential of Advanced Glycation End Products (%) of Extracts

<i>Proanthocyanidins of Pinus sp.</i>	<i>Concentrations (mg/ml)</i>	<i>Antiglycation ^{*&}</i>		
		<i>BD ^A</i>	<i>GF</i>	<i>BP</i>
<i>P. brutia</i>	0.25	66.14 ± 1.93 ^a	57.99 ± 0.97 ^b	49.65 ± 0.62 ^c
<i>P. brutia</i>	0.125	57.63 ± 1.68 ^a	43.26 ± 0.95 ^b	37.90 ± 0.58 ^c
<i>P. pinea</i>	0.25	80.37 ± 1.74 ^a	72.79 ± 1.09 ^b	60.81 ± 0.81 ^c
<i>P. pinea</i>	0.125	66.82 ± 3.24 ^a	51.16 ± 1.13 ^b	48.10 ± 1.76 ^b
<i>P. pinaster</i>	0.25	75.55 ± 1.74 ^a	63.04 ± 0.96 ^b	52.93 ± 0.93 ^c
<i>P. pinaster</i>	0.125	61.03 ± 0.67 ^a	49.44 ± 3.10 ^b	40.82 ± 0.44 ^c
Pycnogenol [®]	0.25	54.94 ± 2.32 ^a	47.02 ± 1.23 ^b	39.80 ± 0.19 ^c
Pycnogenol [®]	0.125	48.00 ± 1.42 ^a	38.99 ± 1.35 ^b	33.00 ± 2.16 ^c

^A The abbreviations for samples are *BD*: before-digestion, *GF*: gastric fraction, *BP*: bioavailable phase.

* Percent of antiglycation activity of quercetin as a positive control was found to be 73.51 ± 2.37 at 0.250 mg/mL and 64.31 ± 4.00 at 0.125 mg/mL.

& Different letters in the same row indicate significance (p < 0.05).

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a, b, and c). The same statistical analysis was applied to different fractions of Pycnogenol[®].

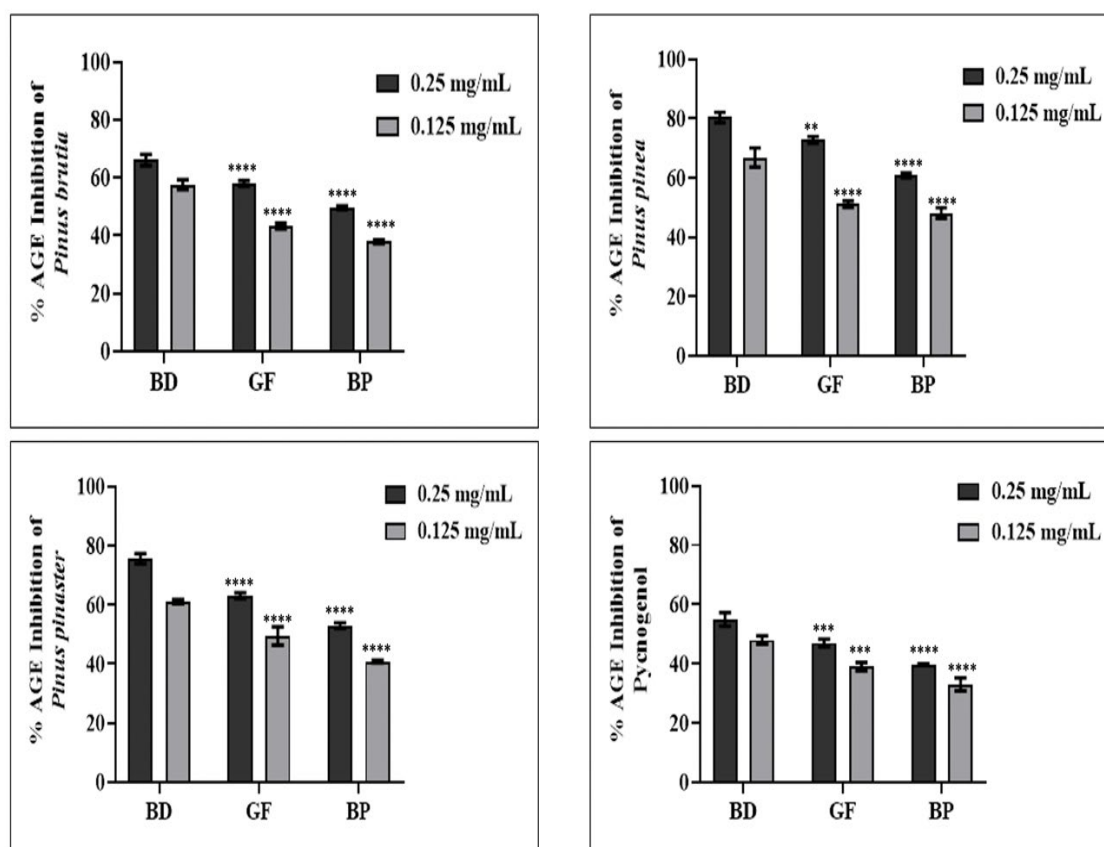


Figure 5. Comparison of Inhibition potential of Advanced Glycation End Products of each *Pinus* species and Pycnogenol®. The abbreviations for samples are BD: *before-digestion*, GF: *gastric-fraction*, BP: *bioavailable phase*. The BD, GF, and BP fractions of each *Pinus* species at the same concentrations were compared by GraphPad software version 8.1 programs with 2-way ANOVA multiple comparisons. As a results of analysis, statistical differences were exhibited by ** ($p \leq 0.01$), *** ($p \leq 0.001$) and **** ($p \leq 0.0001$). The same statistical analysis was studied in the different fractions of Pycnogenol®.

4.5.2. Inhibition Potential of α -amylase Activity of the Extracts

α -amylase inhibitory activities of samples were given in Table 31. Acarbose was used to compare the enzyme activities of samples as a reference compound with a proven α -amylase inhibitor. The α -amylase inhibitory activities of all samples upsurged with increased concentration of samples. The α -amylase inhibitory activities of all samples at a concentration of 0.25 mg/mL were greater than that of 0.125 mg/mL. According to results, the reference compound, acarbose, showed the lowest inhibitory activity at both concentrations among all fractions except the bioavailable phase of PYC. At 0.125 mg/mL concentrations, the bioavailable phase of PYC BP showed inhibitory activity as 31.16 ± 0.92 ; in the meantime, inhibition activity of acarbose was 39.68 ± 0.86 . Before-digestion PP demonstrated the highest inhibitory activity, followed by PPR, PB, and PYC at 250 mg/mL (91.10 ± 2.76 , 85.11 ± 1.14 , 82.58 ± 1.12 , 77.17 ± 0.63). A slight decreasing trend was observed in all samples and concentrations after *in vitro* digestion simulation. According to results, before-digestion PP, PPR, PB, and PYC inhibited α -amylase highly compared to their gastric fractions by 1.06, 1.09, 1.06, and 1.20 times. In addition, bioavailable phases PP, PPR, PB, and PYC showed 1.13, 1.19, 1.19, and 1.26 times lower inhibition activity than the before-digestion fractions.

Table 31. Inhibition Potential of α -amylase Activity (%) of Extracts

<i>Proanthocyanidins of Pinus sp.</i>	<i>Concentrations (mg/ml)</i>	<i>α-amylase inhibition^{*&}</i>		
		<i>BD⁺</i>	<i>GF</i>	<i>BP</i>
<i>P. brutia</i>	0.25	82.58 $\pm$ 1.12 ^a	77.55 $\pm$ 1.49 ^b	69.15 $\pm$ 1.03 ^c
<i>P. brutia</i>	0.125	67.60 $\pm$ 2.02 ^a	53.74 $\pm$ 1.97 ^b	48.92 $\pm$ 0.64 ^c
<i>P. pinea</i>	0.25	91.10 $\pm$ 2.76 ^a	85.87 $\pm$ 1.56 ^{ab}	80.60 $\pm$ 1.03 ^b
<i>P. pinea</i>	0.125	80.14 $\pm$ 0.29 ^a	66.30 $\pm$ 1.43 ^b	56.88 $\pm$ 2.92 ^c
<i>P. pinaster</i>	0.25	85.11 $\pm$ 1.14 ^a	77.91 $\pm$ 1.48 ^b	71.50 $\pm$ 0.49 ^c
<i>P. pinaster</i>	0.125	68.58 $\pm$ 1.22 ^a	64.59 $\pm$ 1.72 ^b	61.00 $\pm$ 0.78 ^{bc}
Pycnogenol [®]	0.25	77.17 $\pm$ 0.63 ^a	64.49 $\pm$ 1.08 ^b	61.31 $\pm$ 1.24 ^c
Pycnogenol [®]	0.125	52.59 $\pm$ 0.14 ^a	49.02 $\pm$ 1.63 ^b	31.16 $\pm$ 0.92 ^c

⁺The abbreviations for samples are *BD*: before-digestion, *GF*: gastric fraction, *BP*: bioavailable phase.

^{*} Percent of α -amylase inhibition activity of acarbose as positive control was found to be 55.92 $\pm$ 0.61 at 0.250 mg/mL and 39.68 $\pm$ 0.86 at 0.125 mg/mL.

[&] Different letters in the same row indicate significance ($p < 0.05$).

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a, b, and c). The same statistical analysis was applied to different fractions of Pycnogenol[®].

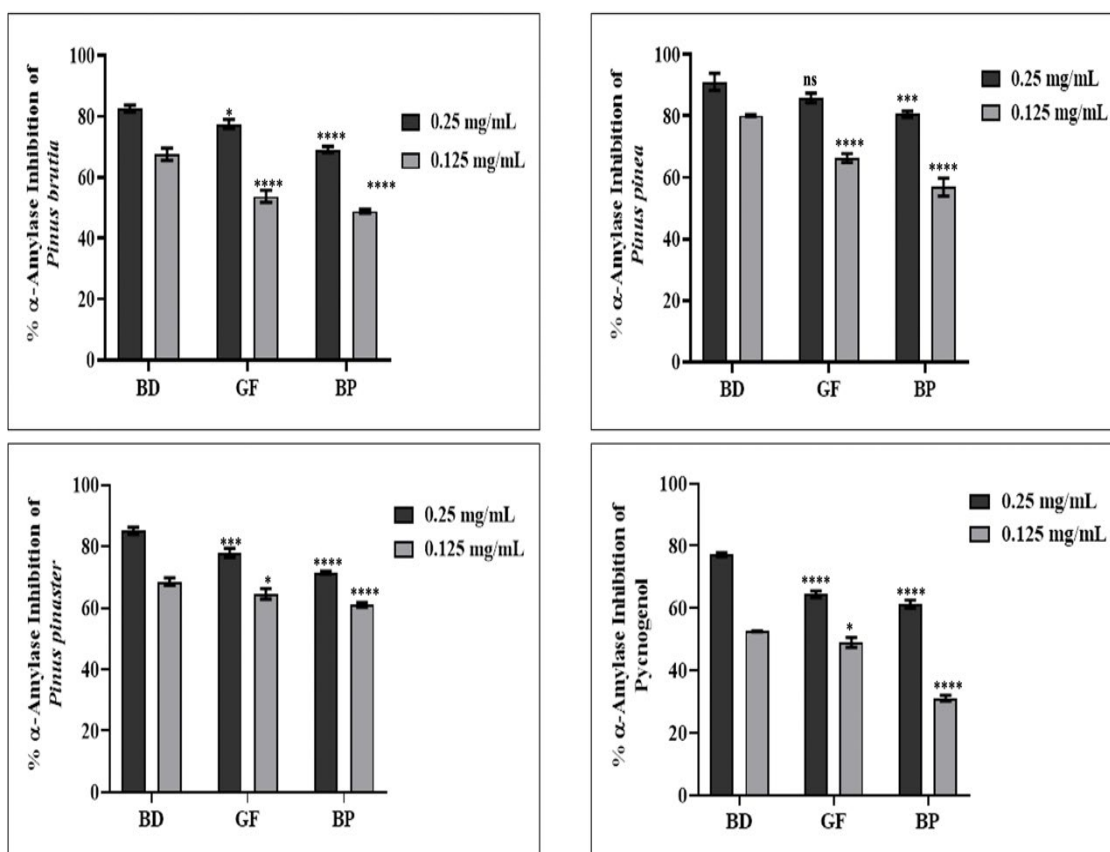


Figure 6. Comparison of Inhibition Potential of α -amylase Activity of each *Pinus* species and Pycnogenol®. The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase. The BD, GF, and BP fractions of each *Pinus* species at the same concentrations were compared by GraphPad software version 8.1 programs with 2-way ANOVA multiple comparisons. As a result of the analysis, ns was used for non-significant data and statistical differences were exhibited by * ($p \leq 0.05$), *** ($p \leq 0.001$) and **** ($p \leq 0.0001$). The same statistical analysis was applied in the different fractions of Pycnogenol®.

4.5.3. Inhibition Potential of α -glucosidase Activity of Extracts

The α -glucosidase inhibitory activity of the samples is shown in Table 32. This assay was repeated three times and at two different concentrations (0.250 and 0.125 mg/mL), and a concentration-dependent increase in the activity was observed. The highest inhibitory activity was seen in before-digestion PP compared with other samples and quercetin as a reference compound (71.67 ± 1.68). Furthermore, similar to results of other antidiabetic activity, before-digestion PP was pursued by PPR, PB, and PYC, respectively. The inhibitory activities of samples were decreased throughout the *in vitro* digestion system. Before-digestion fractions of PP, PPR, PB, and PYC possessed higher inhibitor activity than gastric fractions by 1.09, 1.11, 1.30, and 1.29, respectively. In addition, the inhibitory activities of the before-digestion fractions of PB, PP, PPR, and PYC were higher than bioavailable phases by 1.27, 1.32, 1.58, and 1.46 times, respectively.

Table 32. Inhibition Potential of α -glucosidase Activity (%) of Extracts

<i>Proanthocyanidins of Pinus sp.</i>	<i>Concentrations (mg/ml)</i>	<i>α- glucosidase inhibition^{*&}</i>		
		<i>BD⁺</i>	<i>GF</i>	<i>BP</i>
<i>P. brutia</i>	0.25	62.50 ± 0.90^a	47.89 ± 1.90^b	39.58 ± 0.61^c
<i>P. brutia</i>	0.125	47.45 ± 1.74^a	41.78 ± 1.13^b	30.16 ± 1.20^c
<i>P. pinea</i>	0.25	89.90 ± 2.02^a	82.51 ± 1.39^b	70.55 ± 1.06^c
<i>P. pinea</i>	0.125	69.94 ± 0.68^a	60.00 ± 2.18^b	51.05 ± 2.40^c
<i>P. pinaster</i>	0.25	80.26 ± 0.17^a	72.27 ± 0.97^b	60.72 ± 0.51^c
<i>P. pinaster</i>	0.125	62.06 ± 0.97^a	54.46 ± 1.62^b	40.82 ± 0.44^c
Pycnogenol [®]	0.25	57.03 ± 0.27^a	44.36 ± 1.01^b	39.04 ± 4.39^{bc}
Pycnogenol [®]	0.125	45.31 ± 1.06^a	39.71 ± 0.99^a	28.42 ± 1.63^b

⁺ The abbreviations for samples are *BD*: before-digestion, *GF*: gastric fraction, *BP*: bioavailable phase.

^{*} Percent of α -glucosidase inhibition of quercetin as a positive control was found to be 71.67 ± 1.68 at 0.250 mg/mL and 62.43 ± 1.82 at 0.125 mg/mL.

[&] Different letters in the same row indicate significance ($p < 0.05$).

^{a,b,c} The BD, GF, and BP fractions of each *Pinus* species at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c). The same statistical analysis was applied to different fractions of Pycnogenol[®].

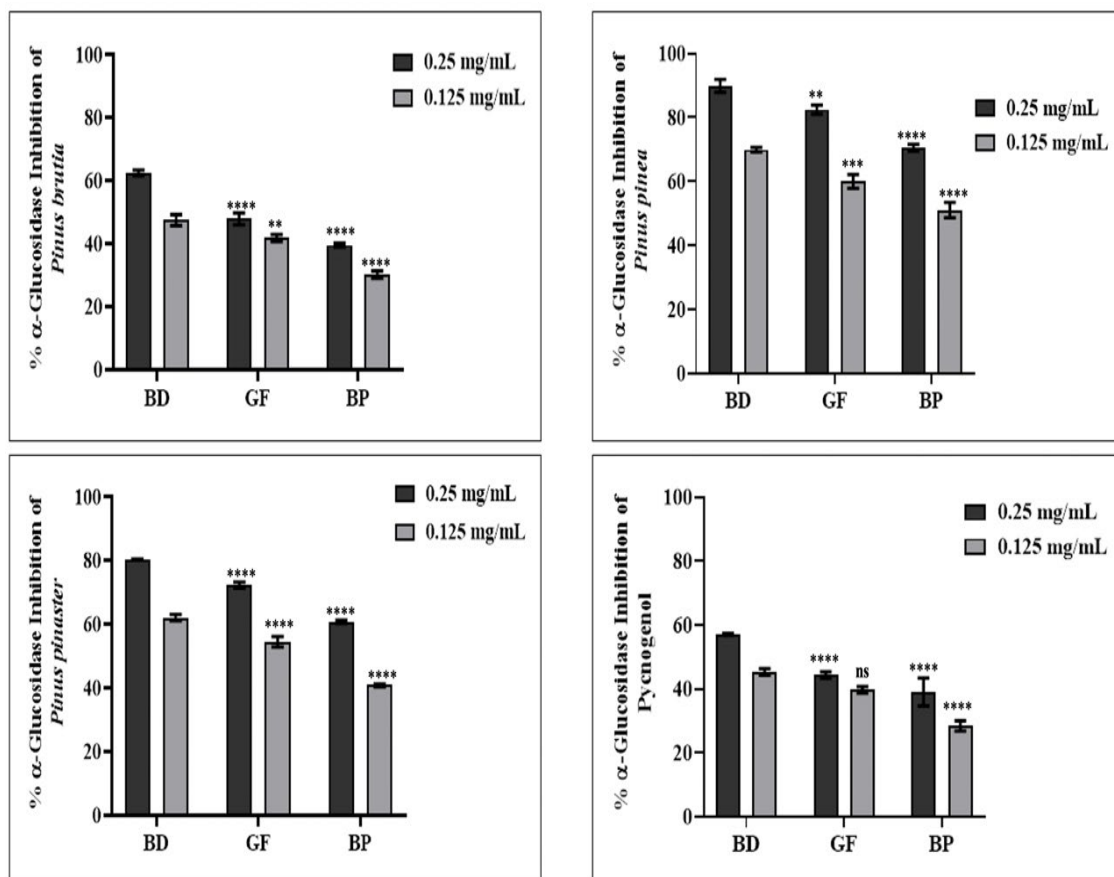


Figure 7. Comparison of Inhibition Potential of α -glucosidase Activity of each *Pinus* species and Pycnogenol®. The abbreviations for samples are *BD*: before-digestion, *GF*: gastric-fraction, *BP*: bioavailable phase. The BD, GF, and BP fractions of each *Pinus* species at the same concentrations were compared by GraphPad software version 8.1 programs with 2-way ANOVA multiple comparisons. As a result of the analysis, ns was used for non-significant data, and statistical differences were exhibited by ** ($p \leq 0.01$), *** ($p \leq 0.001$) and **** ($p \leq 0.0001$). The same statistical analysis was applied in the different fractions of Pycnogenol®.

4.6. Results of Chromatographic Analysis

4.6.1. Quantitative and Qualitative Analysis of Marker Metabolites

4.6.1.1. HPTLC Analysis of Proanthocyanidins from *Pinus* species

4.6.1.1.1. HPTLC Analysis of Proanthocyanidins from *Pinus brutia*

Quantitative and qualitative analyses of proanthocyanidins in *P. brutia* were given in Table 33 and Figure 17. HPTLC results showed that proanthocyanidin A1 could not be determined in any of the extracts, and thereby, the bioavailability index of proanthocyanidin A1 in extracts was not detectable. All fractions of *P. brutia* had the higher amount of proanthocyanidins compared to the extracts from other *Pinus* species. Moreover, bioavailability indexes of all fractions of *P. brutia* were strikingly higher than the other extracts (proanthocyanidin B1/B2: 76%, proanthocyanidin C1: 60%). Proanthocyanidin B1/B2 mixture was found higher than proanthocyanidin C1 in all fractions of *P. brutia*. The concentration of these proanthocyanidins had tended to slightly descent from before-digestion to gastric fractions and bioavailable phase, successively.

Table 33. Quantitative determination of proanthocyanidins from *Pinus brutia*

Samples [*]	BD ^A	GF	BP	BAvI (%)
Proanthocyanidin A1 ^B	nd	nd	nd	-
Proanthocyanidin B1/B2	16.47 ± 0.41 ^a	17.6 ± 0.33 ^b	12.66 ± 0.48 ^c	76%
Proanthocyanidin C1	10.48 ± 0.41 ^a	9.28 ± 0.34 ^b	6.30 ± 0.36 ^c	60%

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected

^B Results were expressed as mg/g dry extract, and analyses were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was symbolized with $\pm$.

a,b,c The BD, GF, and BP fractions of *P. brutia* at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

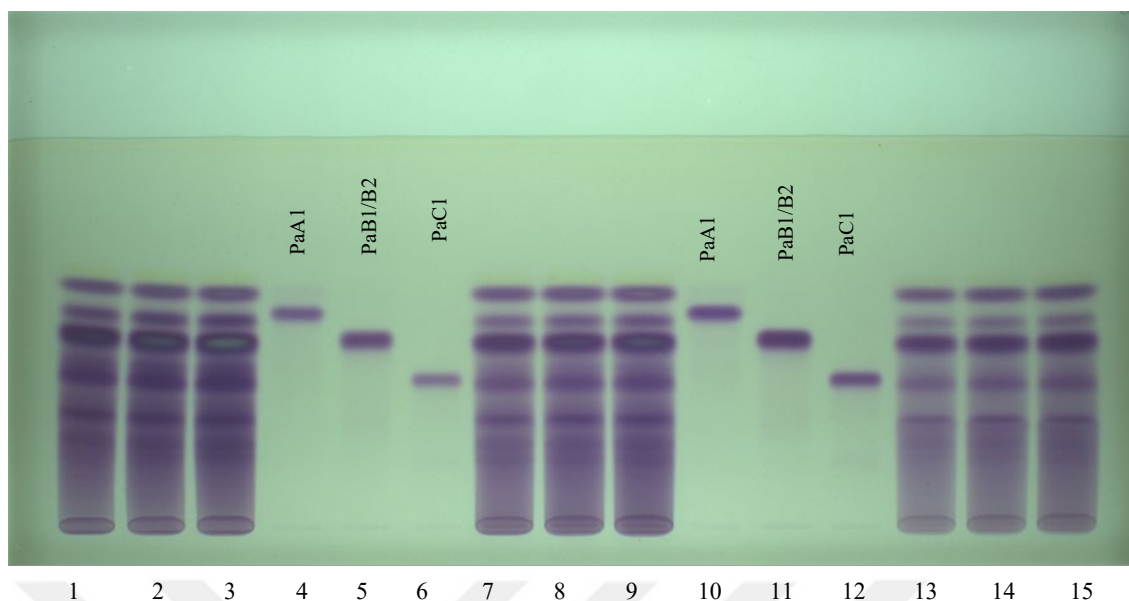


Figure 8. HPTLC chromatograms of BD, GF, BP samples from *P. brutia* under the white light. Tracks. 1-3: *P. brutia* BD, 4,10: Proanthocyanidin A1 (PaA1), 5,11: Proanthocyanidin B1/B2 (PaB1/B2), 6,12: Proanthocyanidin C1 (PaC1) , 7-9: *P. brutia* GF, 13-15: *P. brutia* BP.

4.6.1.1.2. HPTLC Analysis of Proanthocyanidins from *Pinus pinea*

Quantitative and qualitative analyses of proanthocyanidins in *P. pinea* are exhibited in Table 34 and Figure 18. Proanthocyanidin B1/B2 amount of all fractions of *P. pinea* was notably higher than proanthocyanidin C1. Proanthocyanidin B1/B2 amount of BD was 2.17 times higher than proanthocyanidin C1 amount of BD. Even though the same enhancement was seen in ratios of bioavailability indexes (proanthocyanidin B1/B2: 34%, proanthocyanidin C1: 26%), the amount of Proanthocyanidin B1/B2 mixture and C1 decreased notably in all steps of the digestion.

Table 34. Quantitative determination of proanthocyanidins from *Pinus pinea*

Samples [*]	BD ^A	GF	BP	BAvI (%)
Proanthocyanidin A1 ^B	nd	nd	nd	-
Proanthocyanidin B1/B2	15.34 ± 0.44 ^a	12.04 ± 0.24 ^b	5.15 ± 0.21 ^c	34%
Proanthocyanidin C1	7.08 ± 0.39 ^a	4.68 ± 0.29 ^b	1.74 ± 0.15 ^c	26%

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected

^B Results were expressed as mg/g dry extract and analyses were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was symbolized with $\pm$.

a,b,c The BD, GF, and BP fractions of *P. pinea* at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

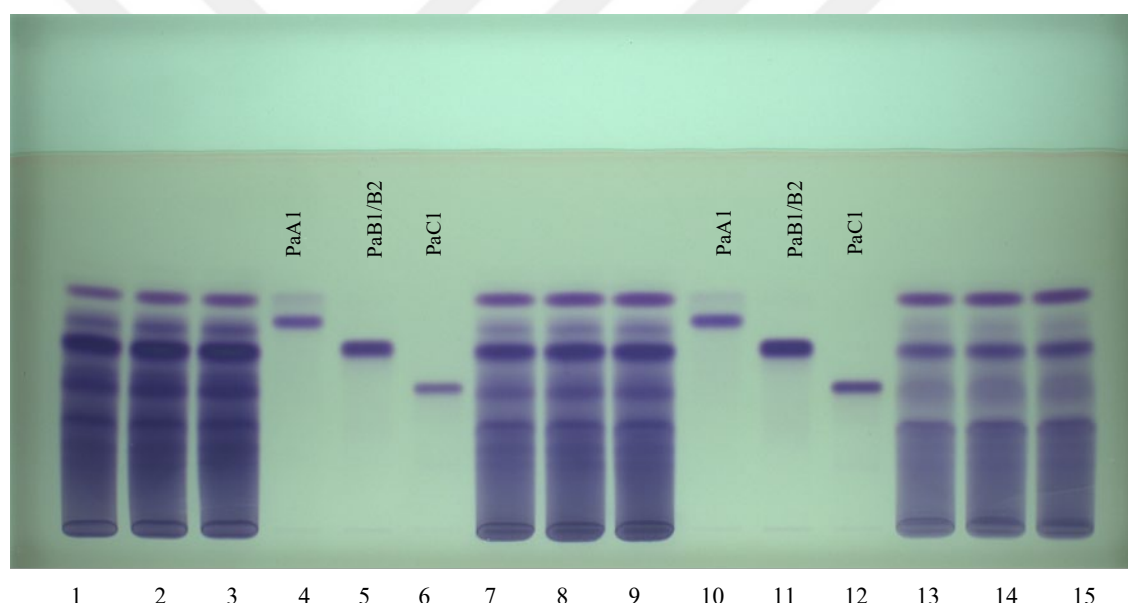


Figure 18. HPTLC chromatograms of BD, GF, BP samples from *P. pinea* under the white light Tracks. 1-3: *P. pinea* BD, 4,10: Proanthocyanidin A1 (PaA1), 5,11: Proanthocyanidin B1/B2 (PaB1/B2), 6,12: Proanthocyanidin C1 (PaC1), 7-9: *P. pinea* GF, 13-15: *P. pinea* BP.

4.6.1.1.3. HPTLC Analysis of Proanthocyanidins from *Pinus pinaster*

Quantitative and qualitative analyses of proanthocyanidins in *P. pinea* were demonstrated in Table 35 and Figure 19. According to data, both proanthocyanidins amounts expressively declined in BP fraction. Like other extracts, proanthocyanidin C1 amounts were significantly lower than proanthocyanidin B1/B2 amounts. The

bioavailability index of the proanthocyanidin B1/B2 was measured as 26%, and in the meantime, bioavailability index of proanthocyanidin C1 was measured 23%.

Table 35. Quantitative determination of proanthocyanidins from *Pinus pinaster*

Samples [*]	BD ^A	GF	BP	BAvI (%)
Proanthocyanidin A1 ^B	nd	nd	nd	-
Proanthocyanidin B1/B2	13.82 ± 0.38 ^a	9.93 ± 0.64 ^b	3.54 ± 0.27 ^c	26%
Proanthocyanidin C1	8.82 ± 0.46 ^a	5.69 ± 0.44 ^b	1.99 ± 0.13 ^c	23%

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected

^BResults were expressed as mg/g dry extract and analysis were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was symbolized with $\pm$.

a,b,c The BD, GF, and BP fractions of *P. pinaster* at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

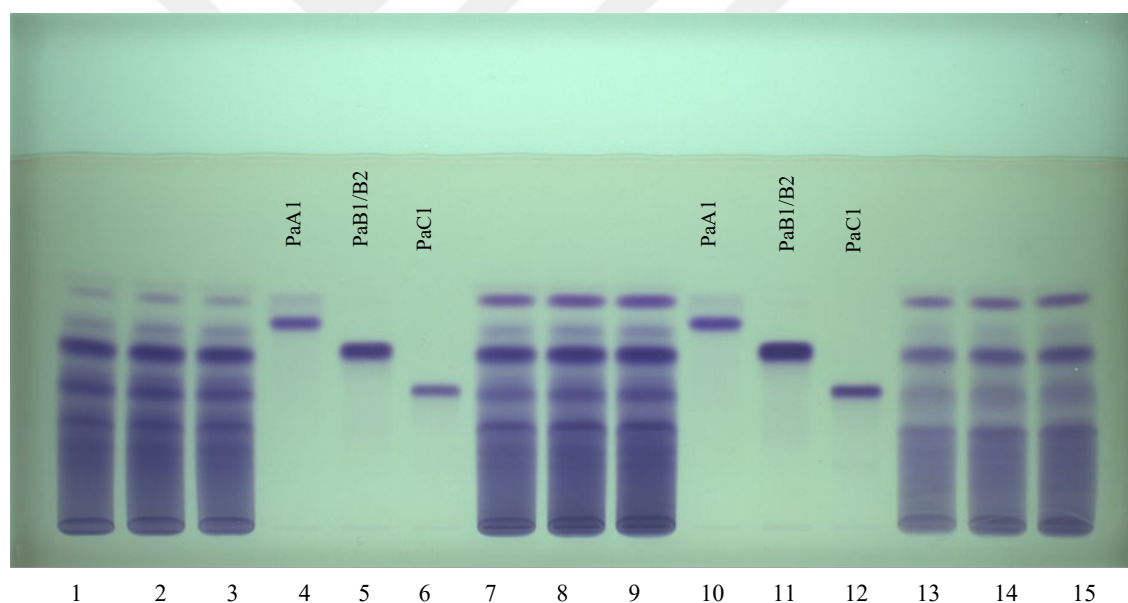


Figure 19. HPTLC chromatograms of BD, GF, BP samples from *P. pinaster* under the white light. Tracks 1-3: *P. pinaster* GF, 4,10: Proanthocyanidin A1 (PaA1), 5,11: Proanthocyanidin B1/B2 (PaB1/B2), 6,12: Proanthocyanidin C1 (PaC1), 7-9: *P. pinaster* BD, 13-15: *P. pinaster* BP.

4.6.1.1.4. HPTLC Analysis of Proanthocyanidins from Pycnogenol®

Quantitative and qualitative analyses of proanthocyanidin content of Pycnogenol® are presented in Table 36 and Figure 20. Accordingly, proanthocyanidin C1 was not found in any fraction, contrary to the extracts from other *Pinus* species. The gastrointestinal

system adversely affected the Proanthocyanidin B1/B2 quantity, as seen in all pine extracts. A striking decline was seen from BD to BP (9.19 ± 0.42 and 1.62 ± 0.14 , respectively). Thus, the bioavailability index of proanthocyanidin B1/B2 was determined as 26%.

Table 36. Quantitative determination of proanthocyanidins from Pycnogenol®

Samples*	<i>BD</i> ^A	<i>GF</i>	<i>BP</i>	BAvI (%)
Proanthocyanidin A1 ^B	nd	nd	nd	-
Proanthocyanidin B1/B2	9.19 ± 0.42^a	8.01 ± 0.14^b	1.62 ± 0.14^c	26%
Proanthocyanidin C1	nd	nd	nd	-

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected

^B Results were expressed as mg/g dry extract and analyses were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was symbolized with $\pm$.

a,b,c The BD, GF, and BP fractions of Pycnogenol® at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

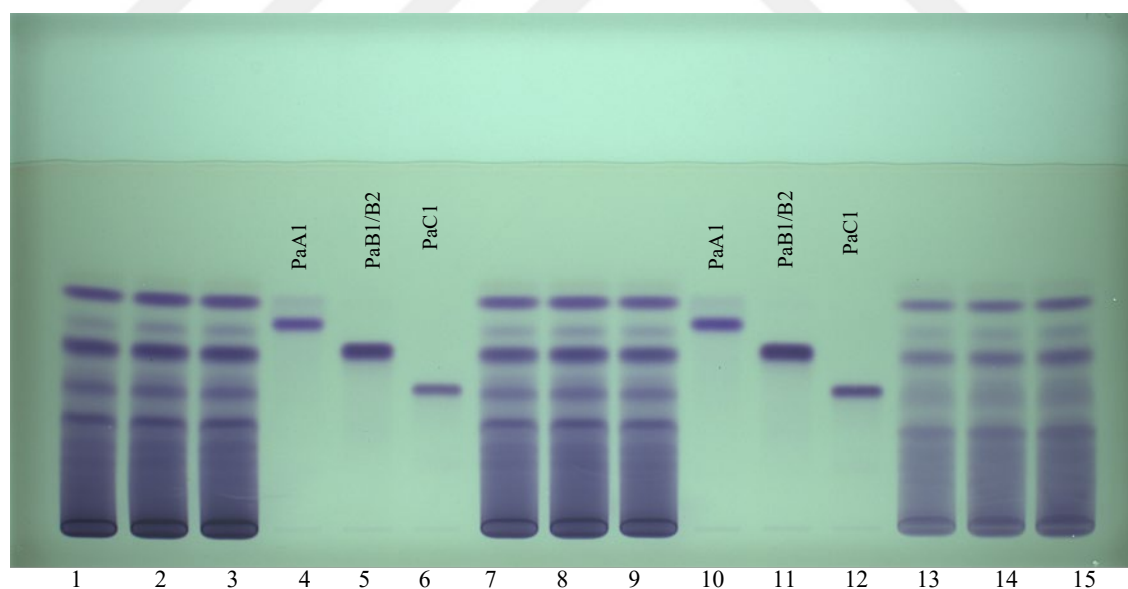


Figure 9. HPTLC chromatograms of BD, GF, BP samples from Pycnogenol® under the white light. Tracks 1-3 Pycnogenol® BD, 4,10: Proanthocyanidin A1 (PaA1), 5,11: Proanthocyanidin B1/B2 (PaB1/B2), 6,12: Proanthocyanidin C1 (PaC1), 7-9: Pycnogenol® GF, 13-15: Pycnogenol® BP.

4.6.1.2. HPTLC Analysis of Flavan-3-ols from *Pinus* species

4.6.1.2.1. HPTLC Analysis of Flavan-3-ols from *Pinus brutia*

The results of quantitative and qualitative analyses of flavan-3-ols in *P. brutia* are presented in Table 37 and Figure 21. HPTLC results indicated that epigallocatechin, epigallocatechin, and epigallocatechin gallate were not found in any of the pine extracts;. In contrast to proanthocyanidin B1/B2 and C1, the highest quantity of catechin/epicatechin was seen in gastric fraction (7.33 ± 0.24). The lowest quantity of catechin/epicatechin was seen before digestion (5.50 ± 0.24). The lowest quantity of catechin/epicatechin was seen before digestion. Nevertheless, no significant alteration was found between the before digestion and the bioavailable phases (5.50 ± 0.41 and 5.96 ± 0.36 , respectively). The bioavailability index of the catechin/epicatechin mixture was measured as 108%. Furthermore, the catechin/epicatechin amount of *P. brutia* showed the highest level among the other extracts in all fractions.

Table 37. Quantitative determination of flavan-3-ols from *Pinus brutia*

Samples	<i>BD</i> ^A	<i>GF</i>	<i>BP</i>	BAvI (%)
Catechin/epicatechin	5.50 ± 0.41^a	7.33 ± 0.24^b	5.96 ± 0.36^c	108%
Epigallocatechin	nd	nd	nd	-
Epigallocatechin gallate	nd	nd	nd	-
Catechin gallate	nd	nd	nd	-

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected

^B Results were expressed as mg/g dry extract and analyses were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was presented with $\pm$.

a,b,c The BD, GF, and BP fractions of *P. brutia* at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

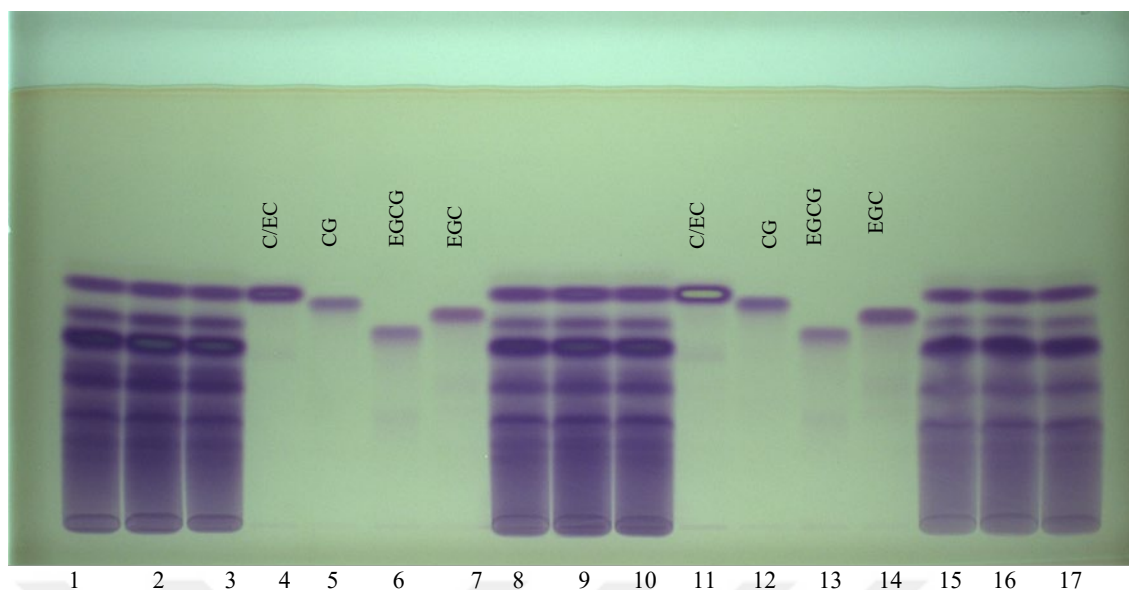


Figure 10. HPTLC chromatograms of BD, GF, BP samples from *P. brutia* under the white light. Tracks 1-3: *P. brutia* BD, 4,11: Catechin/Epicatechin (C/CE), 5,12: Catechin gallate (CG), 6,13: Epigallocatechin gallate (EGCG), 7,14: Epigallocatechin (EGC), 8-10: *P. brutia* GF, 15-17: *P. brutia* BP.

4.6.1.2.2. HPTLC Analysis of Flavan-3-ols from *Pinus pinea*

The quantitative and qualitative analyses of flavan-3-ols in *P. pinea* are shown in Table 38 and Figure 22. HPTLC results presented that only catechin/epicatechin was measured among the other flavan-3-ols investigated in this study. The concentration of proanthocyanidin B1/B2 and C1, during *in vitro* gastrointestinal simulation, was a descent trend from before digestion to gastric fraction and bioavailable phase, respectively (BD: 4.52 ± 0.43 , GF: 3.53 ± 0.22 , BP: 3.14 ± 0.13). The bioavailability index of the catechin/epicatechin was measured as 69%.

Table 38. Quantitative determination of flavan-3-ols in *Pinus pinea*

Samples *	<i>BD</i> ^A	<i>GF</i>	<i>BP</i>	BAvI (%)
Catechin/epicatechin	4.52 ± 0.43 ^a	3.53 ± 0.22 ^b	3.14 ± 0.13 ^c	69%
Epigallocatechin	nd	nd	nd	-
Epigallocatechin gallate	nd	nd	nd	-
Catechin gallate	nd	nd	nd	-

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected

^B Results were expressed as mg/g dry extract and analyses were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was presented with $\pm$.

a,b,c The BD, GF, and BP fractions of *P. pinea* at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

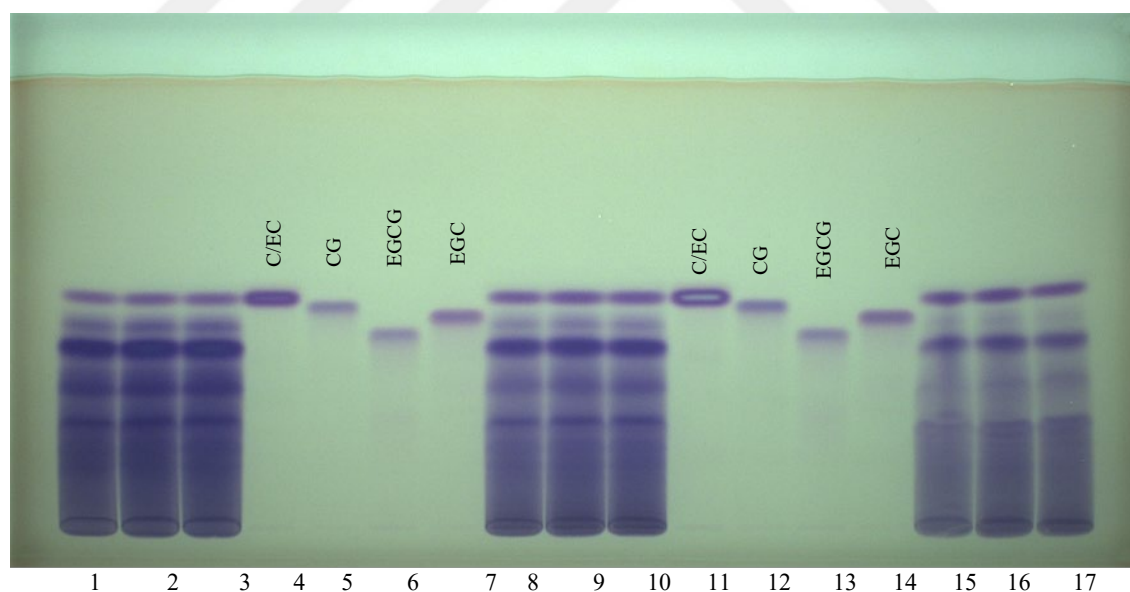


Figure 11. HPTLC chromatograms of BD, GF, BP samples from *P. pinea* under the white light. Tracks 1-3: *P. pinea* BD, 4,11: Catechin/Epicatechin (C/CE), 5,12: Catechin gallate (CG), 6,13: Epigallocatechin gallate (EGCG), 7,14: Epigallocatechin (EGC): *P. pinea* GF, 15-17: *P. pinea* BP.

4.6.1.2.3. HPTLC Analysis of Flavan-3-ols from *Pinus pinaster*

The results of quantitative and qualitative analyses of flavan-3-ols in *P. pinaster* are given in Table 39 and Figure 23. Like other extracts, catechin/epicatechin, among other flavan-3-ols investigated, was only measurable flavan-3-ol molecules in this study. A substantial reduction was observed in the catechin/epicatechin quantity in *P. pinea* after *in vitro* gastrointestinal simulation. The bioavailability index of the catechin/epicatechin was determined as 62%.

Table 39. Quantitative determination of flavan-3-ols from *Pinus pinaster*

Samples *	BD ^A	GF	BP	BAvI (%)
Catechin/epicatechin	3.41 ± 0.15 ^a	3.01 ± 0.12 ^b	2.12 ± 0.07 ^c	62%
Epigallocatechin	nd	nd	nd	-
Epigallocatechin gallate	nd	nd	nd	-
Catechin gallate	nd	nd	nd	-

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected

^B Results were expressed as mg/g dry extract with and analysis were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was symbolized with $\pm$.

a,b,c The BD, GF, and BP fractions of *P. pinaster* at same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

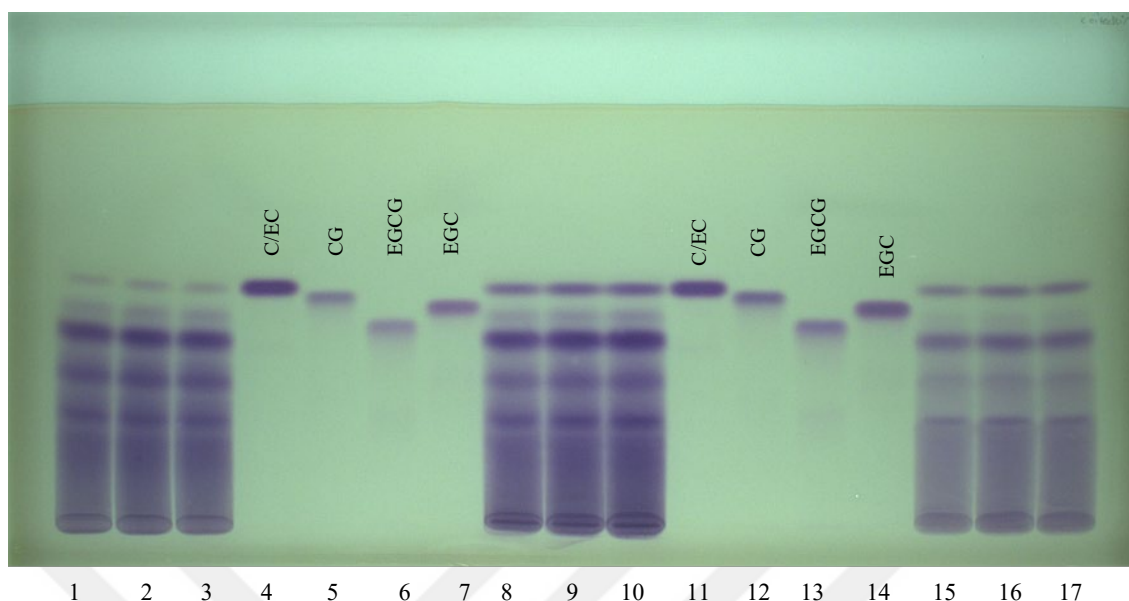


Figure 23. HPTLC chromatograms of BD, GF, BP samples from *P. pinaster* under the white light. Tracks 1-3: *P. pinaster* GF, 4,11: Catechin/Epicatechin (C/CE), 5,12: Catechin gallate (CG), 6,13: Epigallocatechin gallate (EGCG), 7,14: Epigallocatechin (EGC): *P. pinea* BD, 15-17: *P. pinaster* BP.

4.6.1.2.4. HPTLC Analysis of Flavan-3-ols from Pycnogenol®

The quantitative and qualitative analyses of flavan-3-ols in Pycnogenol® are presented in Table 40 and Figure 24. The lowest catechin/epicatechin concentrations were found in the fractions of Pycnogenol® among all pine extracts. The lowest bioavailability index of the catechin/epicatechin, 36%, was found among all extracts. The catechin/epicatechin quantity decreased significantly after *in vitro* gastrointestinal simulation.

Table 40. Quantitative determination of flavan-3-ols from Pycnogenol®

Samples *	BD ^A	GF	BP	BAvI (%)
Catechin/epicatechin	3.26 ± 0.3 ^a	2.75 ± 0.22 ^b	1.18 ± 0.10 ^c	36%
Epigallocatechin	nd	nd	nd	-
Epigallocatechin gallate	nd	nd	nd	-
Catechin gallate	nd	nd	nd	-

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non detected

^B Results were expressed as mg/g dry extract with and analysis were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was symbolized with $\pm$.

a,b,c The BD, GF, and BP fractions of Pycnogenol® at the same concentrations were compared among themselves with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

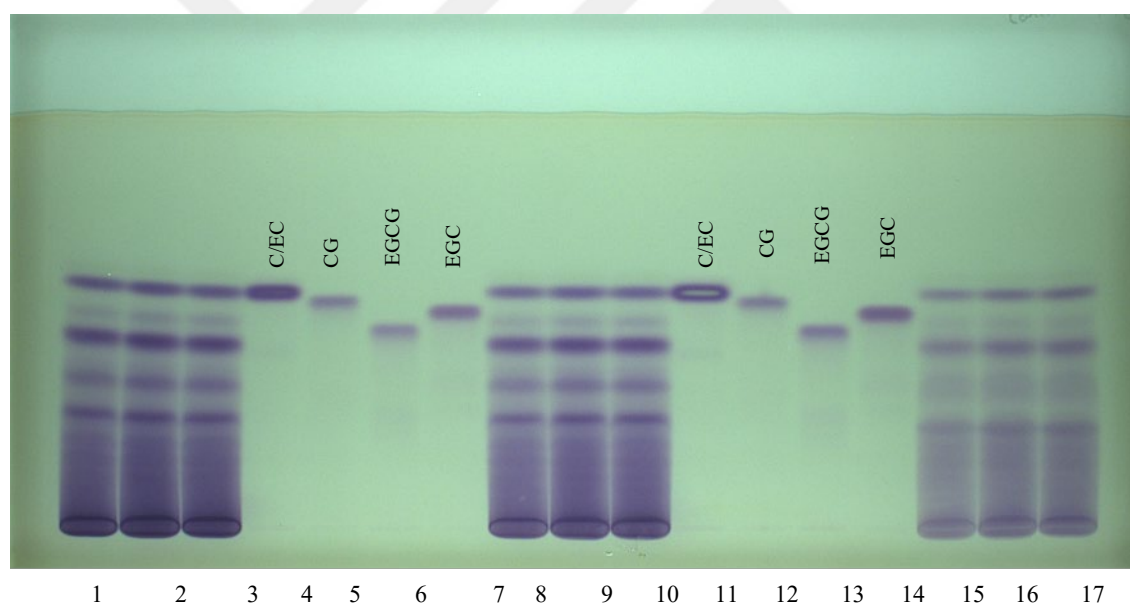


Figure 12. HPTLC chromatograms of BD, GF, BP samples from Pycnogenol® under the white light. Tracks 1-3: Pycnogenol® BD, 4,11: Catechin/Epicatechin (C/CE), 5,12: Catechin gallate (CG), 6,13: Epigallocatechin gallate (EGCG), 7,14: Epigallocatechin (EGC): Pycnogenol® GF, 15-17: Pycnogenol® BP.

4.6.1.3. HPTLC Analysis of Other Phenolic Compounds in *Pinus* species

4.6.1.3.1. HPTLC Analysis of Other Phenolic Compounds from *Pinus brutia*

Quantitative and qualitative analyses of taxifolin, caffeic acid, and ferulic acid in *P. brutia* are presented in Table 41 and Figure 25. HPTLC results indicated that caffeic acid and ferulic acid were not found in any fractions. The amount of taxifolin was observed at the highest level in the gastric phase (15.51 ± 0.58). However, no significant alteration was observed during the *in vitro* gastrointestinal simulation (BD: 14.25 ± 0.26 and BP: 12.38 ± 0.44). The highest bioavailability index of taxifolin was observed in *P. brutia* among the other pine extracts (86%).

Table 41. Quantitative determination of other phenolic compounds from *Pinus brutia*

Samples [*]	BD ^A	GF	BP	BAvI (%)
Taxifolin ^B	14.25 ± 0.26^a	15.51 ± 0.58^b	12.38 ± 0.44^c	86%
Caffeic Acid	nd	nd	nd	-
Ferulic Acid	nd	nd	nd	-

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected

^B Results were expressed as mg/g dry extract and analyses were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was symbolized with $\pm$.

a,b,c The BD, GF, and BP fractions of *P. brutia* at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

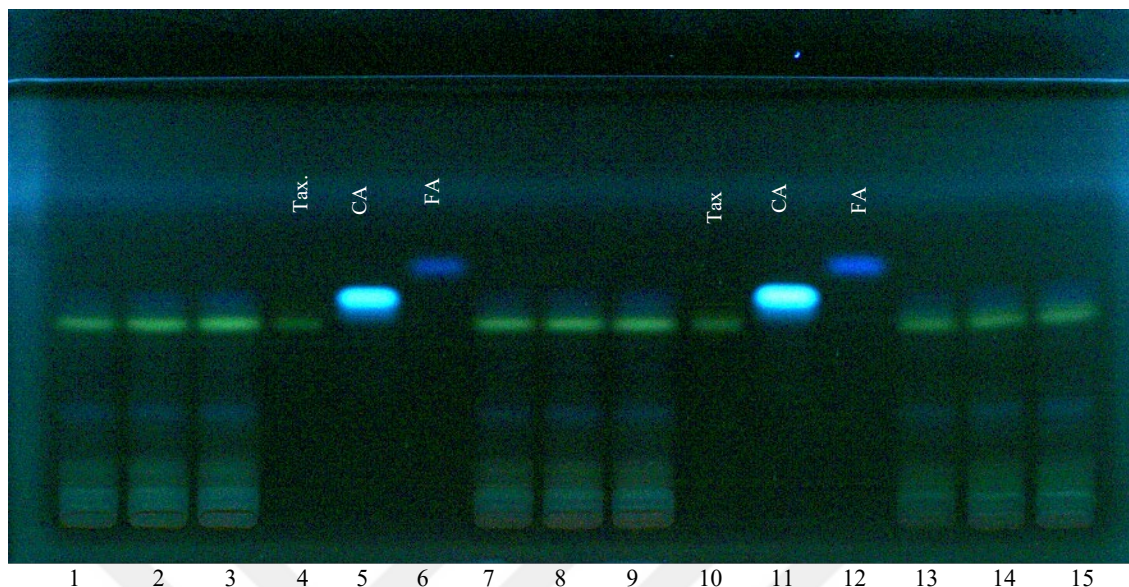


Figure 25. HPTLC chromatograms of BD, GF, BP samples from *P. brutia* at 366 nm. Tracks 1-3: *P. brutia* BD, 4,10: Taxifolin (Tax), 5,11: Caffeic acid (CA), 6,12: Ferulic acid (FA), 7-9: *P. brutia* GF, 13-15: *P. brutia* BP.

4.6.1.3.2. HPTLC Analysis of Other Phenolic Compounds from *Pinus pinea*

The quantitative and qualitative analyses of taxifolin, caffeic acid, and ferulic acid in *P. pinea* are presented in Table 42 and Figure 26. HPTLC results displayed amounts of caffeic acid and ferulic acid, and so their bioavailability indexes could not be determined in any of the fractions. The amount of taxifolin in the fraction before digestion was 9.15 ± 0.31 mg/g dry extract was the highest, and a significant decline was observed after *in vitro* gastrointestinal simulation (GF: 7.36 ± 0.26 and BP: 4.33 ± 0.49). The bioavailability index of the taxifolin was found as 47%.

Table 42. Quantitative determination of other phenolic compounds from *Pinus pinea*

Samples [*]	<i>BD</i> ^A	<i>GF</i>	<i>BP</i>	BAvI (%)
Taxifolin ^B	9.15 ± 0.31 ^a	7.36 ± 0.26 ^b	4.33 ± 0.49 ^c	47%
Caffeic Acid	nd	nd	nd	-
Ferulic Acid	nd	nd	nd	-

^A*BD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected*

^BResults were expressed as mg/g dry extract and analyses were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was presented with $\pm$.

a,b,c The BD, GF, and BP fractions of *P. pinea* at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

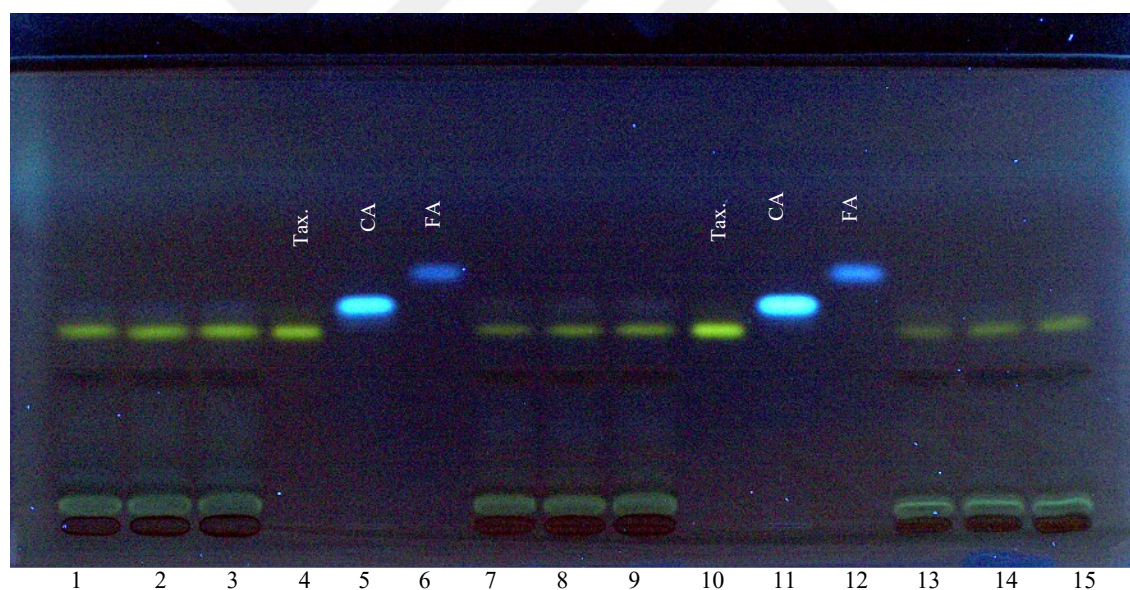


Figure 13. HPTLC chromatograms of BD, GF, BP samples from *P. pinea* at 366 nm. Tracks 1-3: *P. pinea* BD, 4,10: Taxifolin (Tax), 5,11: Caffeic acid (CA), 6,12: Ferulic acid (FA), 7-9: *P. pinea* GF, 13-15: *P. pinea* BP.

4.6.1.3.3. HPTLC Analysis of Other Phenolic Compounds from *Pinus pinaster*

Quantitative and qualitative analyses of taxifolin, caffeic acid, and ferulic acid in *P. pinaster* are exhibited in Table 43 and Figure 27. According to the results, the amount of caffeic acid and ferulic acid that were the investigated phenolic acids in this study could not be found in any fractions. The lowest amount of taxifolin was observed in the fractions of *P. pinaster*, among the other pine extracts. Moreover, this extract showed the lowest bioavailability ratio (BAvI: 33%). A comparison between the fractions showed a notable decrease after *in vitro* digestion (BD: 5.19 ± 0.23 , GF: 3.63 ± 0.20 , and BP: 1.72 ± 0.15).

Table 43. Quantitative determination of other phenolic compounds from *Pinus pinaster*

Samples	BD ^A	GF	BP	BAvI (%)
Taxifolin ^B	5.19 ± 0.23	3.63 ± 0.20	1.72 ± 0.15	33%
Caffeic Acid	nd	nd	nd	-
Ferulic Acid	nd	nd	nd	-

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected

^B Results were expressed as mg/g dry extract and analyses were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was presented with $\pm$.

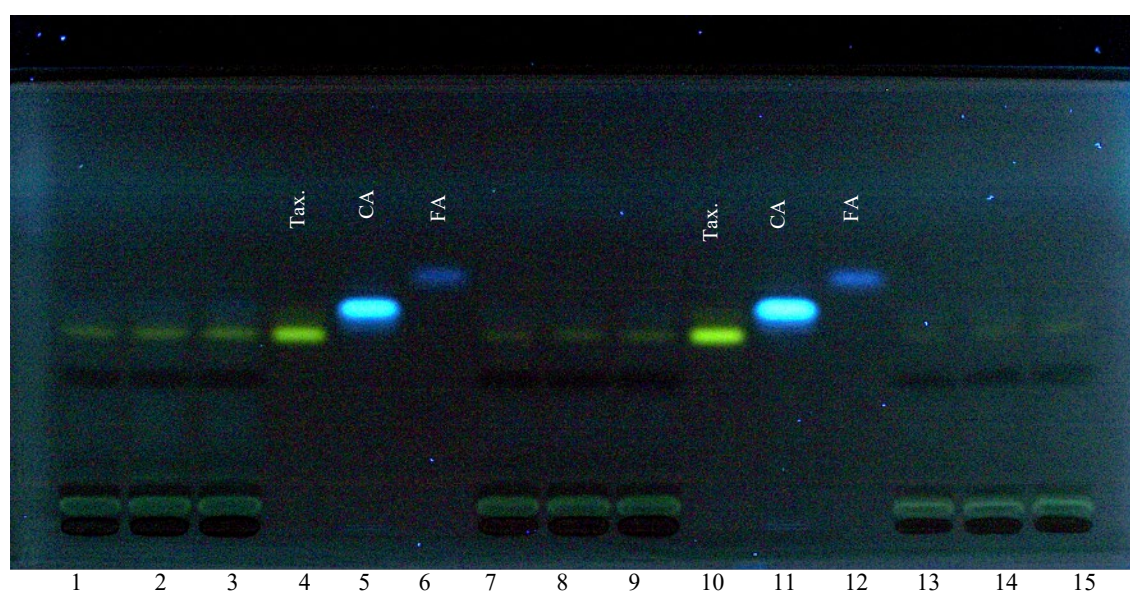


Figure 14. HPTLC chromatograms of BD, GF, BP samples from *P. pinaster* at 366 nm. Tracks 1-3: *P. pinaster* BD, 4,10: Taxifolin (Tax), 5,11: Caffeic acid (CA), 6,12: Ferulic acid (FA), 7-9: *P. pinaster* GF, 13-15: *P. pinaster* BP.

4.6.1.3.4. HPTLC Analysis of Other Phenolic Compounds from Pycnogenol®

Quantitative and qualitative analyses of taxifolin, caffeic acid, and ferulic acid in Pycnogenol® are presented in Table 44 and Figure 28. Similar to other pine extracts, taxifolin was found in all fractions of Pycnogenol®. However, in contrast to others, all fractions of Pycnogenol® also possess caffeic acid and ferulic acid. According to the results, a slight change in taxifolin amount was observed from before digestion to the gastric phase, whereas a striking decline was found at the end of the intestinal digestion (BD: 7.48 ± 0.32 , GF: 7.07 ± 0.08 , BP: 3.14 ± 0.18). The bioavailability index of taxifolin was found as 42%. Similar to taxifolin, the same notable decline trend was observed in the analysis of phenolic acids. Caffeic acid quantity of the fraction before digestion was determined to be 3.36 ± 0.13 , which was higher than the ferulic acid amount, 2.1 ± 0.12 . Moreover, as seen in Table 38, the quantity of caffeic acid was higher than ferulic acid in both gastric fraction and bioavailable phase. The bioavailability index of caffeic acid was measured as 50%; meanwhile, the bioavailability index of ferulic acid was 59%.

Table 44. Quantitative determination of other phenolic compounds from Pycnogenol®

Samples*	BD ^A	GF	BP	BAvI (%)
Taxifolin ^B	7.48 ± 0.32^a	7.07 ± 0.08^b	3.14 ± 0.18^c	42%
Caffeic Acid	3.36 ± 0.13^a	2.98 ± 0.13^b	1.69 ± 0.06^c	50%
Ferulic Acid	2.1 ± 0.12^a	1.66 ± 0.13^b	1.24 ± 0.06^c	59%

^ABD: Before-digestion, GF: Gastric-fraction, BP: Bioavailable phase, BAvI: Bioavailability index, nd: Non-detected

^B Results were expressed as mg/g dry extract and analyses were conducted independently three different times.

* Significant results were shown with $p < 0.05$ and standard deviation was symbolized with $\pm$.

a,b,c The BD, GF, and BP fractions of Pycnogenol® at the same concentrations were compared with GraphPad software version 8.1 programs. In the statistical analysis, no statistical difference was detected between the results indicated with the same letter and statistical changing was shown by different letters (a,b, and c).

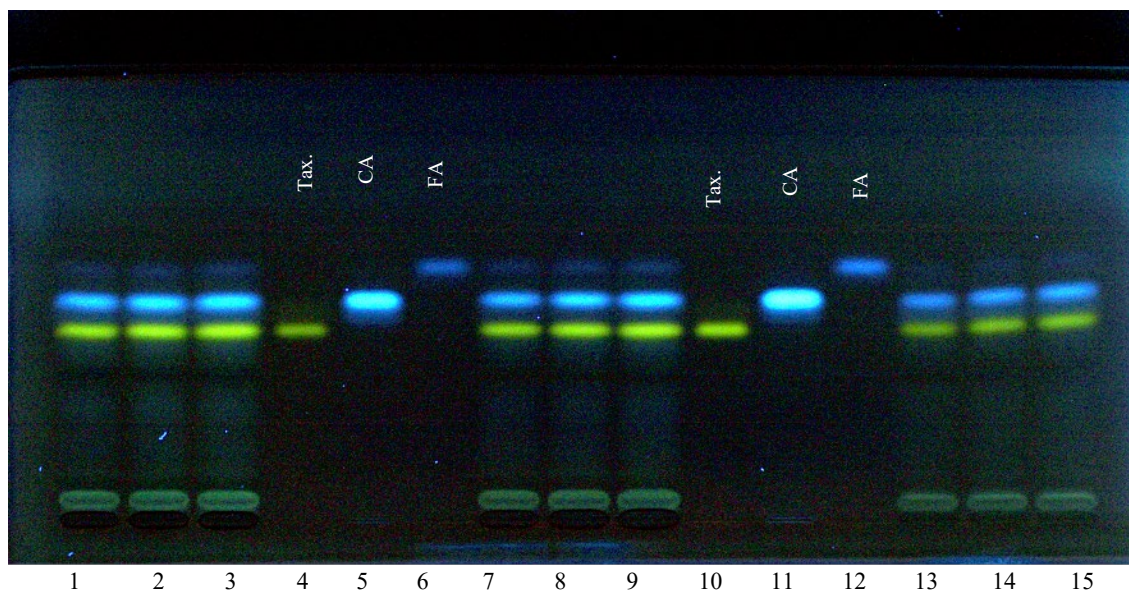


Figure 28. HPTLC chromatograms of BD, GF, BP samples from Pycnogenol® at 366 nm. Tracks 1-3: Pycnogenol® BD, 4,10: Taxifolin (Tax), 5,11: Caffeic acid (CA), 6,12: Ferulic acid (FA), 7-9: Pycnogenol® GF, 13-15: Pycnogenol® BP.

4.6.2. HPTLC-DPPH Analysis

4.6.2.1. HPTLC-DPPH Analysis of Proanthocyanidins from *Pinus* species

The qualitative HPTLC-DPPH analyses of proanthocyanidins in *Pinus* species are presented in Figures 29, 30, 31, and 32. This method allowed the preliminary screening of antioxidant activities of the individual components in proanthocyanidins. According to the results, proanthocyanidins have contributed significantly to the total antioxidant activity potentials of the fractions.

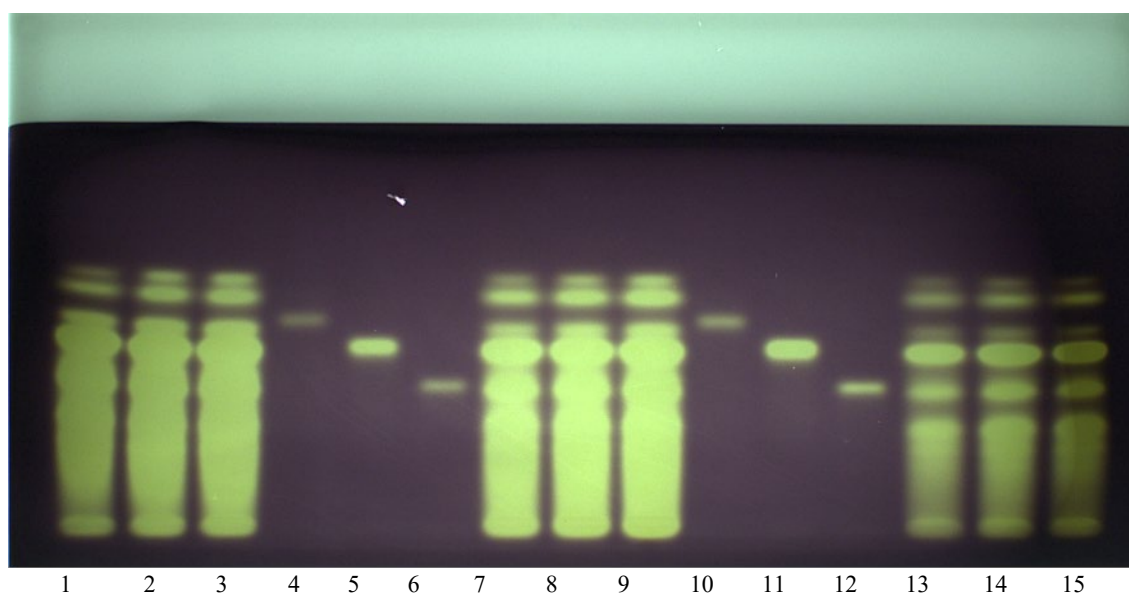


Figure 15. HPTLC-DPPH chromatograms of BD, GF, BP samples from *P. brutia* under the white light Tracks. 1-3: *P. brutia* BD, 4,10: Proanthocyanidin A1, 5,11: Proanthocyanidin B1/B2, 6,12: Proanthocyanidin C1, 7-9: *P. brutia* GF, 13-15: *P. brutia* BP.

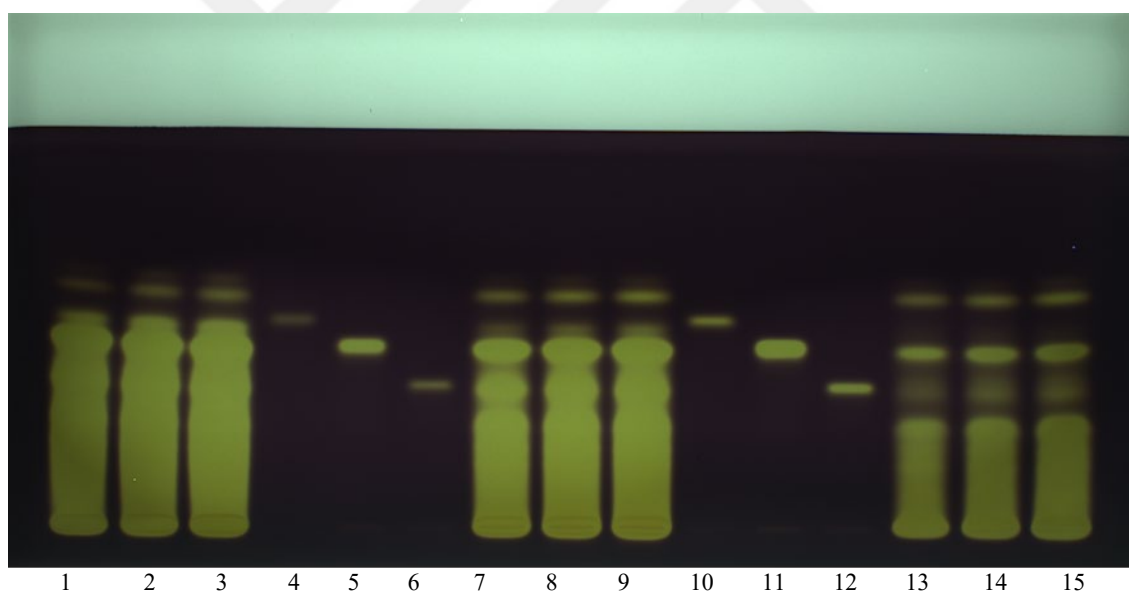


Figure 30. HPTLC-DPPH chromatograms of BD, GF, BP samples from *P. pinea* under the white light Tracks. 1-3: *P. pinea* BD, 4,10: Proanthocyanidin A1, 5,11: Proanthocyanidin B1/B2, 6,12: Proanthocyanidin C1, 7-9: *P. pinea* GF, 13-15: *P. pinea* BP.

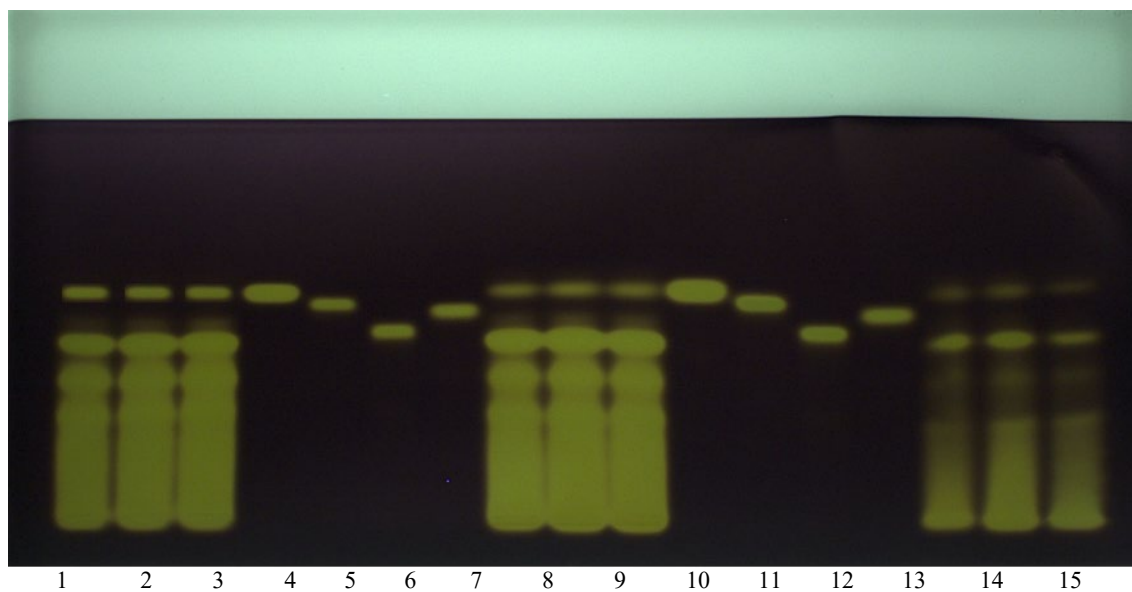


Figure 31. HPTLC-DPPH chromatograms of BD, GF, BP samples from *P. pinaster* under the white light. Tracks 1-3: *P. pinaster* BD, 4,10: Proanthocyanidin A1, 5,11: Proanthocyanidin B1/B2, 6,12: Proanthocyanidin C1, 7-9: *P. pinaster* GF, 13-15: *P. pinaster* BP.

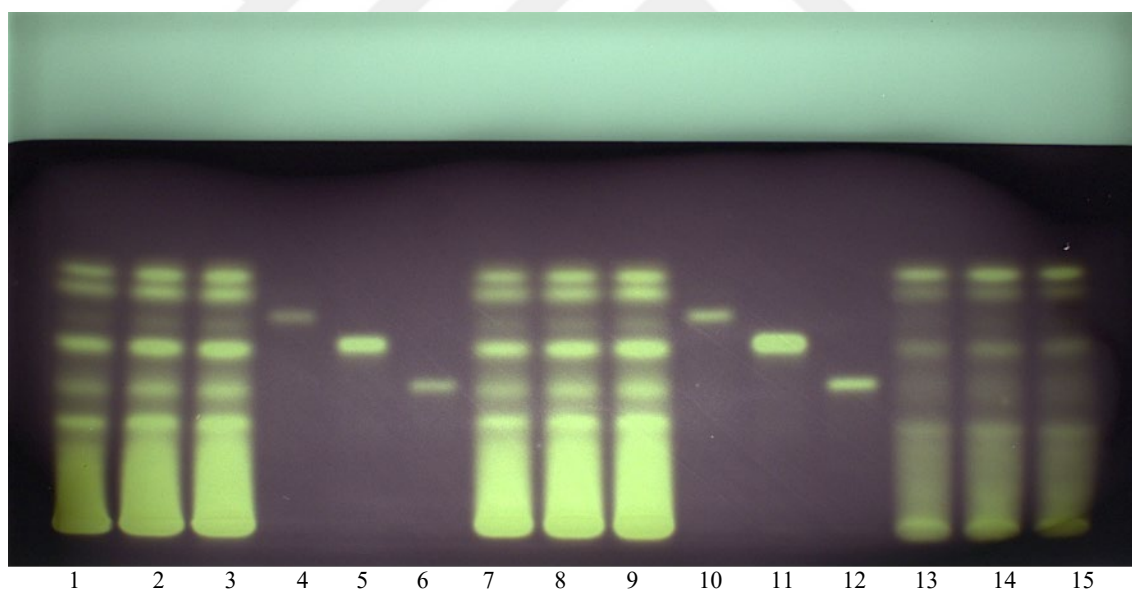


Figure 32. HPTLC-DPPH chromatograms of BD, GF, BP samples from Pycnogenol[®] under the white light. Tracks 1-3 Pycnogenol[®] BD, 4,10: Proanthocyanidin A1, 5,11: Proanthocyanidin B1/B2, 6,12: Proanthocyanidin C1, 7-9: Pycnogenol[®] GF, 13-15: Pycnogenol[®] BP.

4.6.2.2. HPTLC-DPPH Analysis of Flavan-3-ols from *Pinus* species

Qualitative HPTLC-DPPH analyses of flavan-3-ols in *Pinus* species are demonstrated in Figures 33, 34, 35, and 36. The screening of antioxidant activities of flavan-3-ols were detected qualitatively on HPTLC plates after derivatized with DPPH solution. According to the results, all flavan-3-ols had noteworthy DPPH radical scavenging activity.

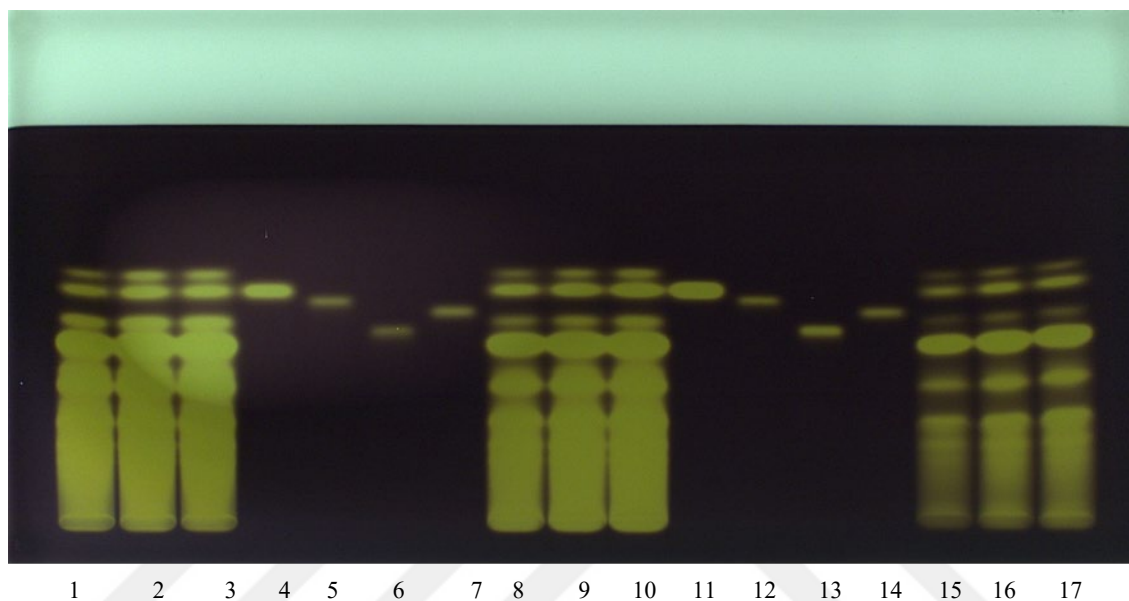


Figure 33. HPTLC-DPPH chromatograms of BD, GF, BP samples from *P. brutia* under the white light. Tracks 1-3: *P. brutia* BD, 4,11: Catechin/Epicatechin, 5,12: Catechin gallate, 6,13: Epigallocatechin gallate, 7,14: Epigallocatechin, 8-10: *P. brutia* GF, 15-17: *P. brutia* BP.

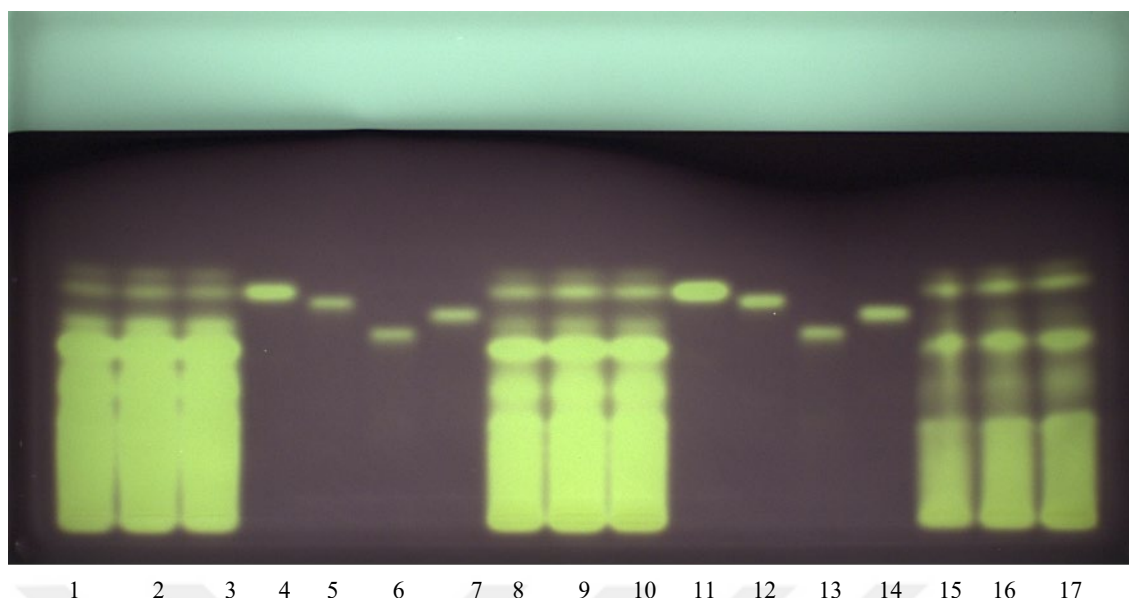


Figure 34. HPTLC-DPPH chromatograms of BD, GF, BP samples from *P. pinea* under the white light. Tracks 1-3: *P. pinea* BD, 4,11: Catechin/Epicatechin, 5,12: Catechin gallate, 6,13: Epigallocatechin gallate, 7,14: Epigallocatechin, 8-10: *P. pinea* GF, 15-17: *P. pinea* BP.

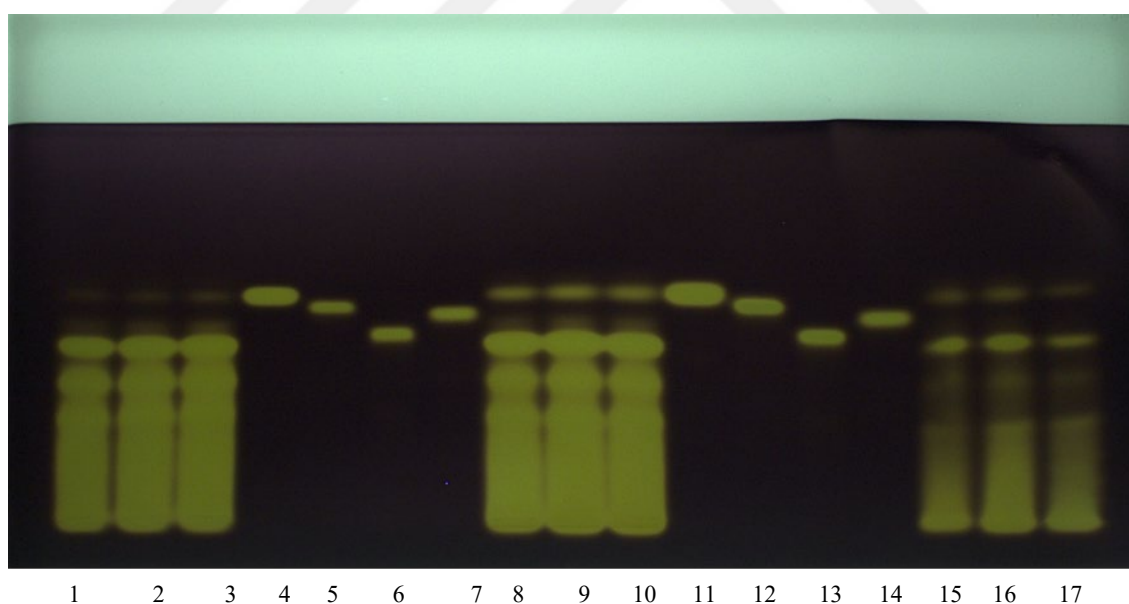


Figure 16. HPTLC-DPPH chromatograms of BD, GF, BP samples from *P. pinaster* under the white light. Tracks 1-3: *P. pinaster* BD, 4,11: Catechin/Epicatechin, 5,12: Catechin gallate, 6,13: Epigallocatechin gallate, 7,14: Epigallocatechin, 8-10: *P. pinea* GF, 15-17: *P. pinaster* BP.

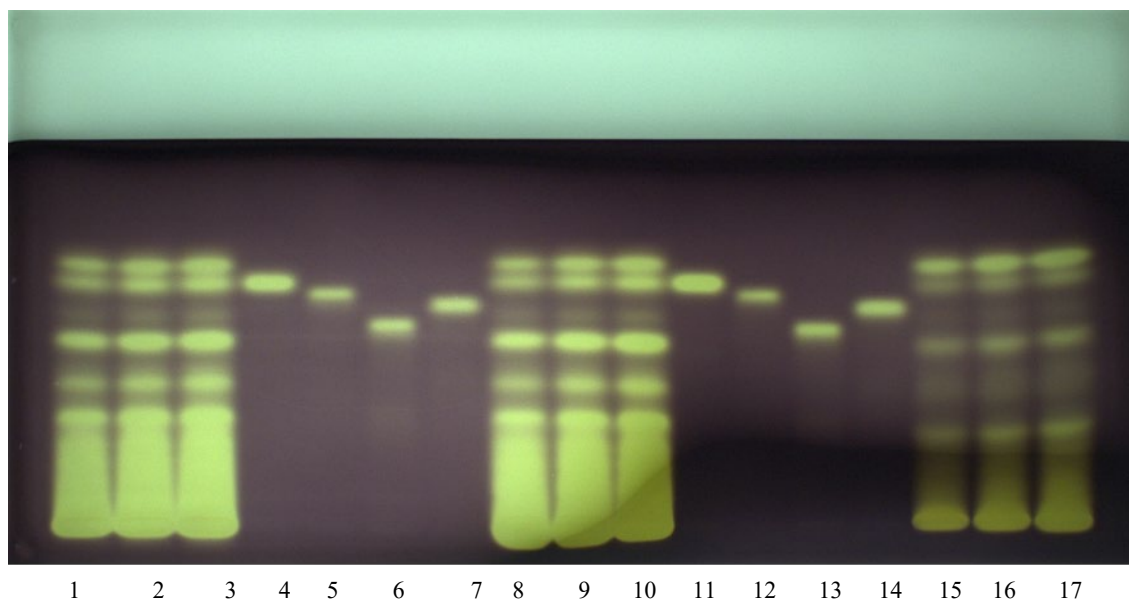


Figure 17. HPTLC-DPPH chromatograms of BD, GF, BP samples from Pycnogenol[®] under the white light. Tracks 1-3: Pycnogenol[®] BD, 4,11: Catechin/Epicatechin, 5,12: Catechin gallate, 6,13: Epigallocatechin gallate, 7,14: Epigallocatechin, 8-10: Pycnogenol[®] GF, 15-17: Pycnogenol[®] BP.

4.6.2.3. HPTLC-DPPH Analysis of Other Phenolic Compounds from *Pinus* species

Qualitative HPTLC-DPPH analyses of taxifolin, caffeic acid, and ferulic acid in *Pinus* species are presented in Figures 37, 38, 39, and 40. The yellow zone appeared on the plates after derivatized by DPPH solution due to antioxidant molecules in all fractions. These plates demonstrated that taxifolin, caffeic acid, and ferulic acid showed significant DPPH radical scavenging activity.

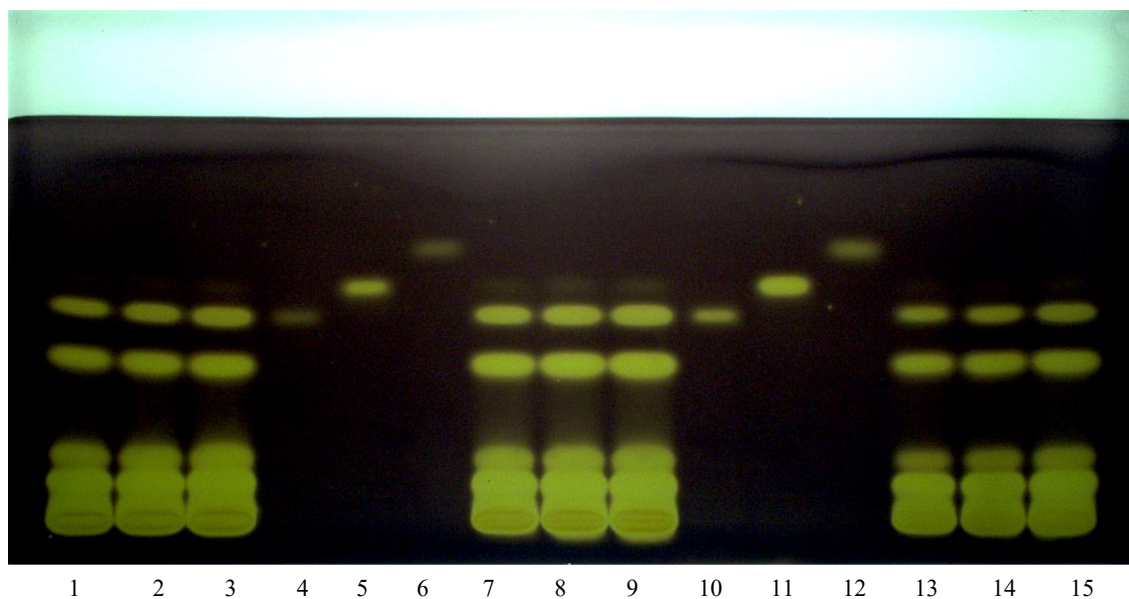


Figure 18. HPTLC-DPPH chromatograms of BD, GF, BP samples from *P. brutia* at 366 nm. Tracks 1-3: *P. brutia* BD, 4,10: Taxifolin, 5,11: Caffeic acid, 6,12: Ferulic acid, 7-9: *P. brutia* GF, 13-15: *P. brutia* BP.

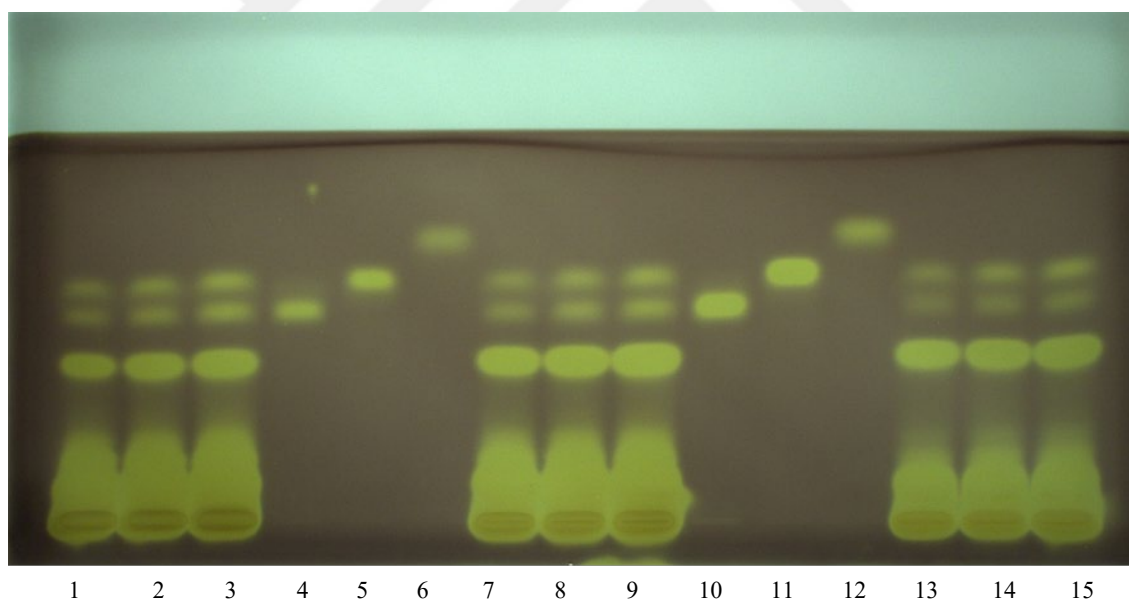


Figure 19. HPTLC-DPPH chromatograms of BD, GF, BP samples from *P. pinea* at 366 nm. Tracks 1-3: *P. pinea* BD, 4,10: Taxifolin, 5,11: Caffeic acid, 6,12: Ferulic acid, 7-9: *P. pinea* GF, 13-15: *P. pinea* BP.

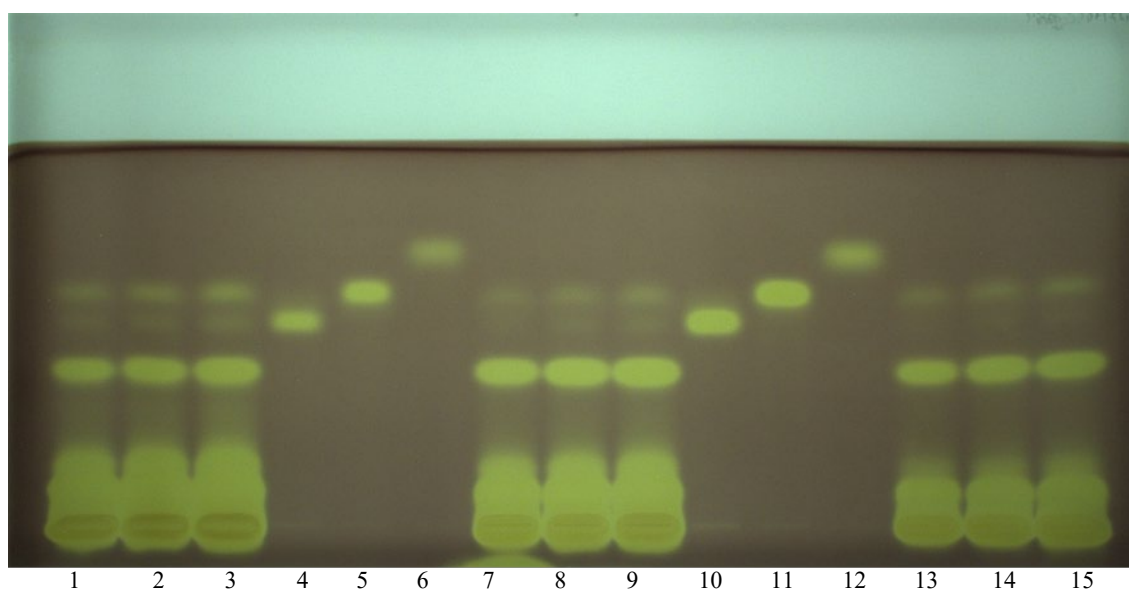


Figure 39. HPTLC-DPPH chromatograms of BD, GF, BP samples from *P. pinaster* at 366 nm. Tracks 1-3: *P. pinaster* BD, 4,10: Taxifolin, 5,11: Caffeic acid, 6,12: Ferulic acid, 7-9: *P. pinaster* GF, 13-15: *P. pinaster* BP.

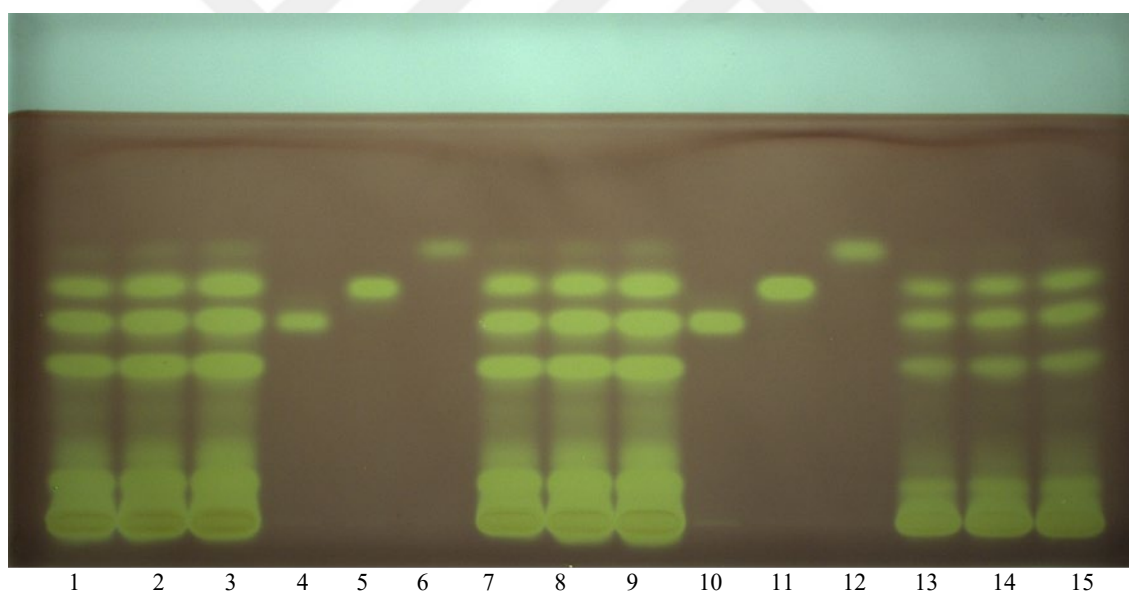


Figure 20. HPTLC-DPPH chromatograms of BD, GF, BP samples from Pycnogenol® at 366 nm. Tracks 1-3: Pycnogenol® BD, 4,10: Taxifolin, 5,11: Caffeic acid, 6,12: Ferulic acid, 7-9: Pycnogenol® GF, 13-15: Pycnogenol® BP.

4.6.3. UV spectral analysis of the marked metabolites from *Pinus* species

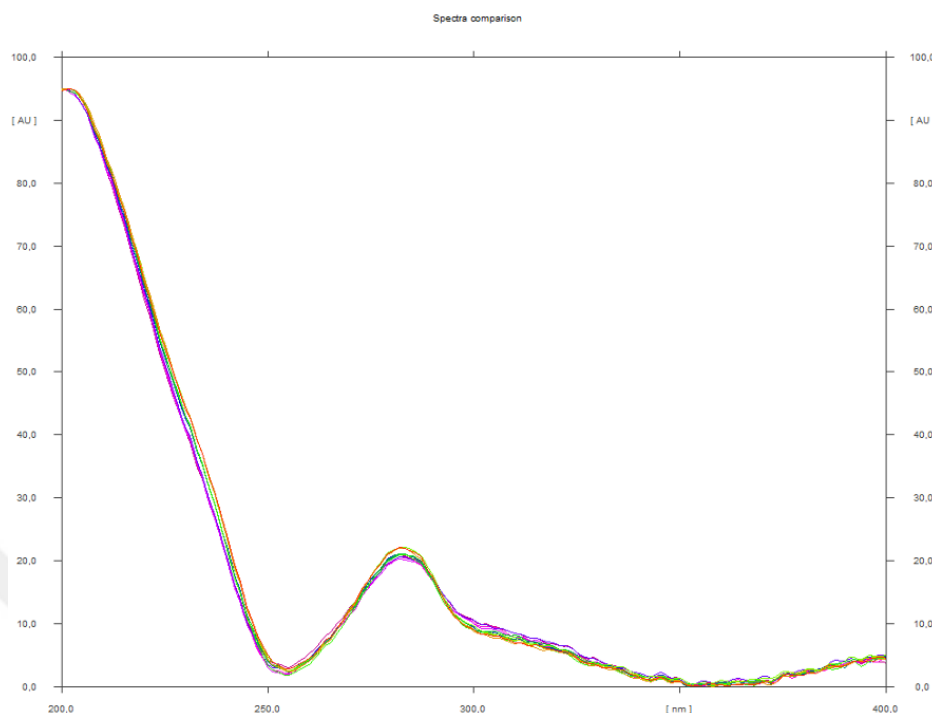
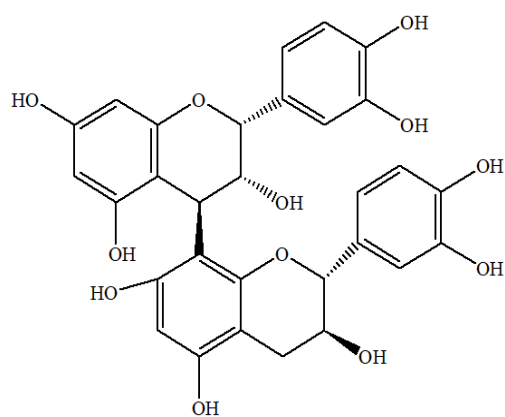
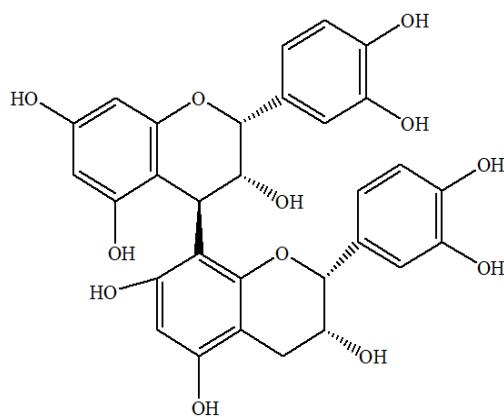


Figure 21. Overlay UV spectra of proanthocyanidins B1/B2 and the corresponding spots in the tracks of all extracts.



Proanthocyanidin B1



Proanthocyanidin B2

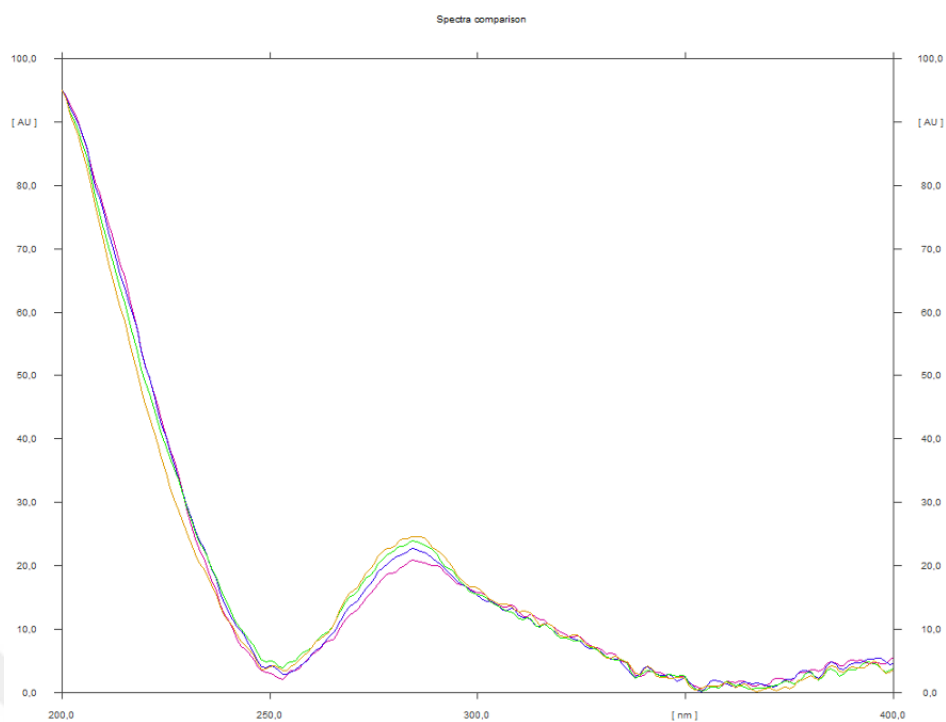
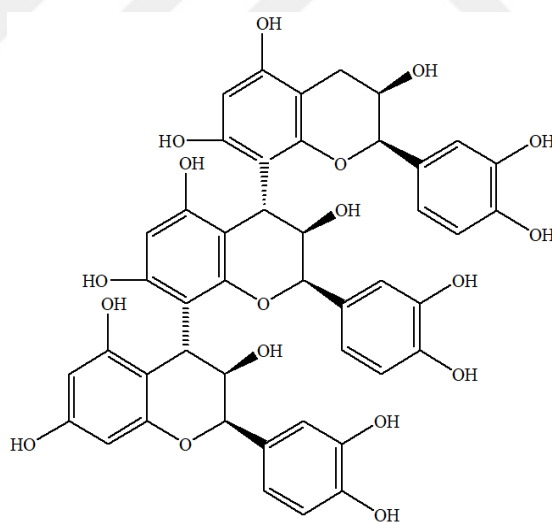


Figure 22. Overlay UV spectra of proanthocyanidins C1 and the corresponding spots in the tracks of all extracts.



Proanthocyanidin C1

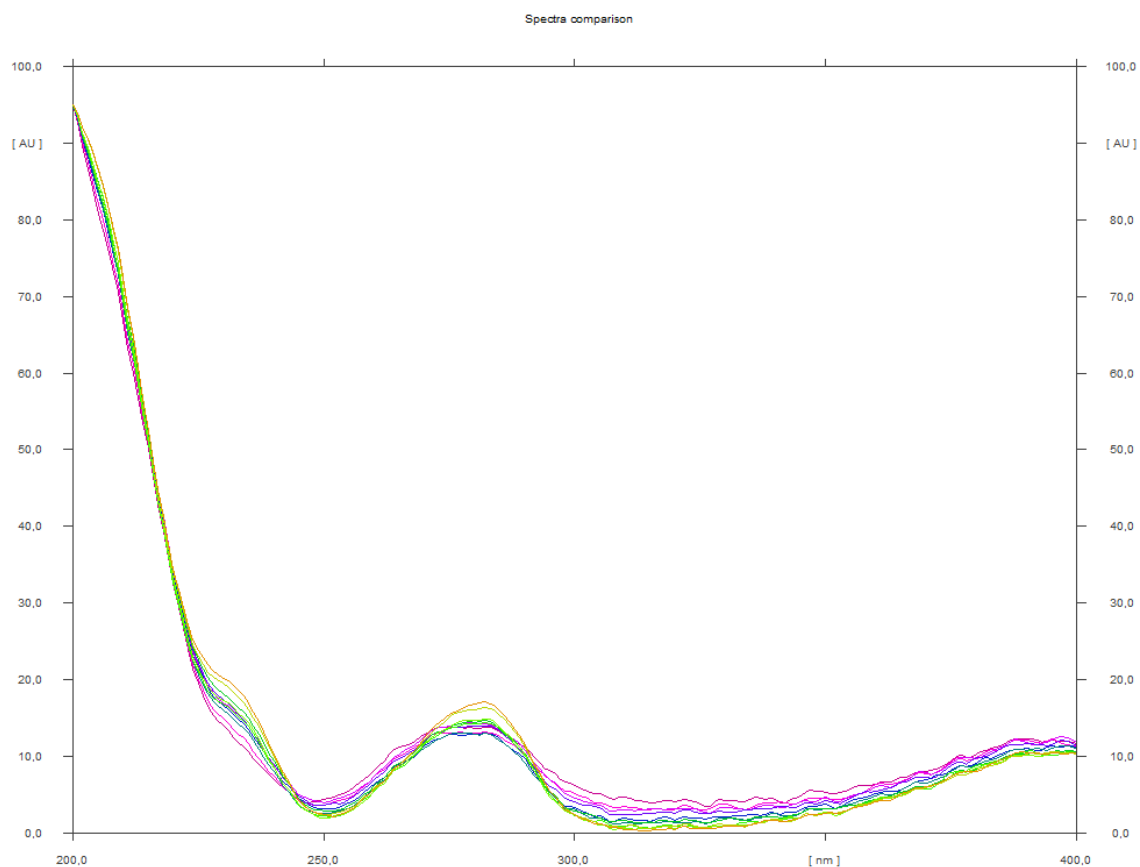
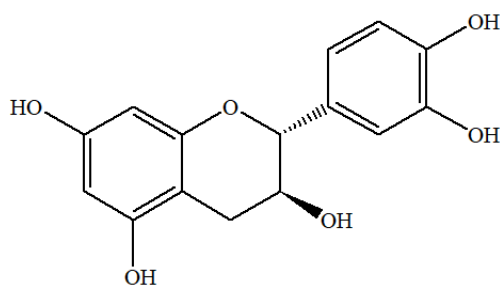
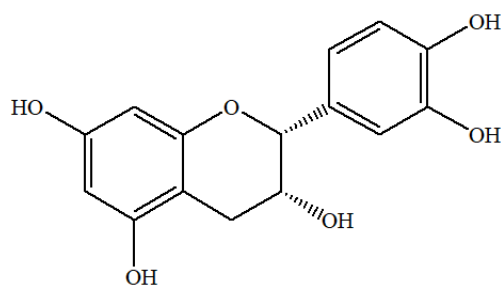


Figure 23. Overlay UV spectra of catechin/epicatechin and the corresponding spots in the tracks of all extracts.



Catechin



Epicatechin

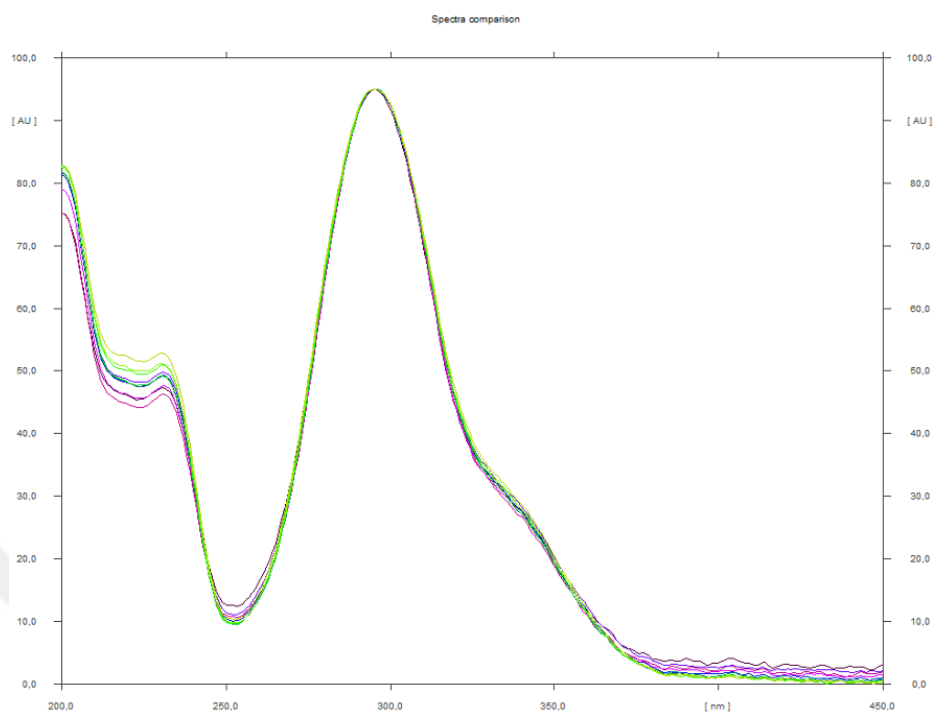
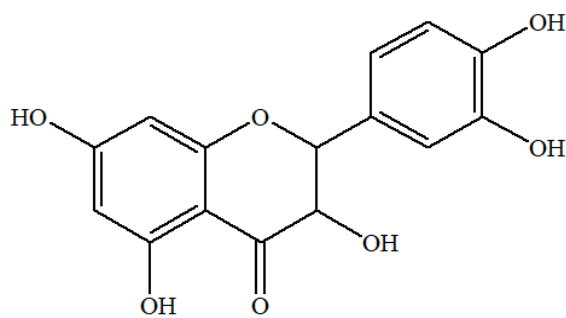


Figure 24. Overlay UV spectra of taxifolin and the corresponding spots in the tracks of all extracts.



Taxifolin

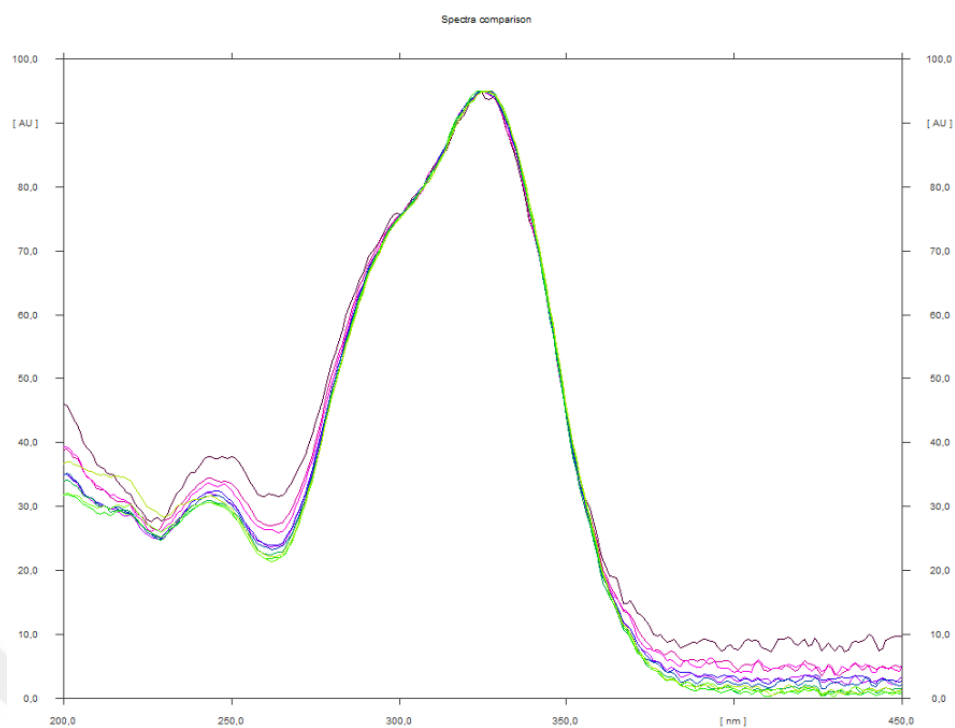
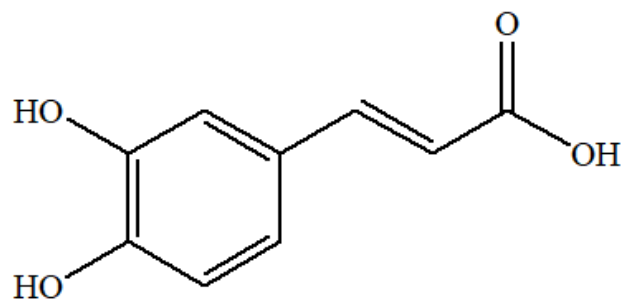


Figure 25. Overlay UV spectra of caffeic acid and the corresponding spots in the tracks of all extracts.



Caffeic Acid

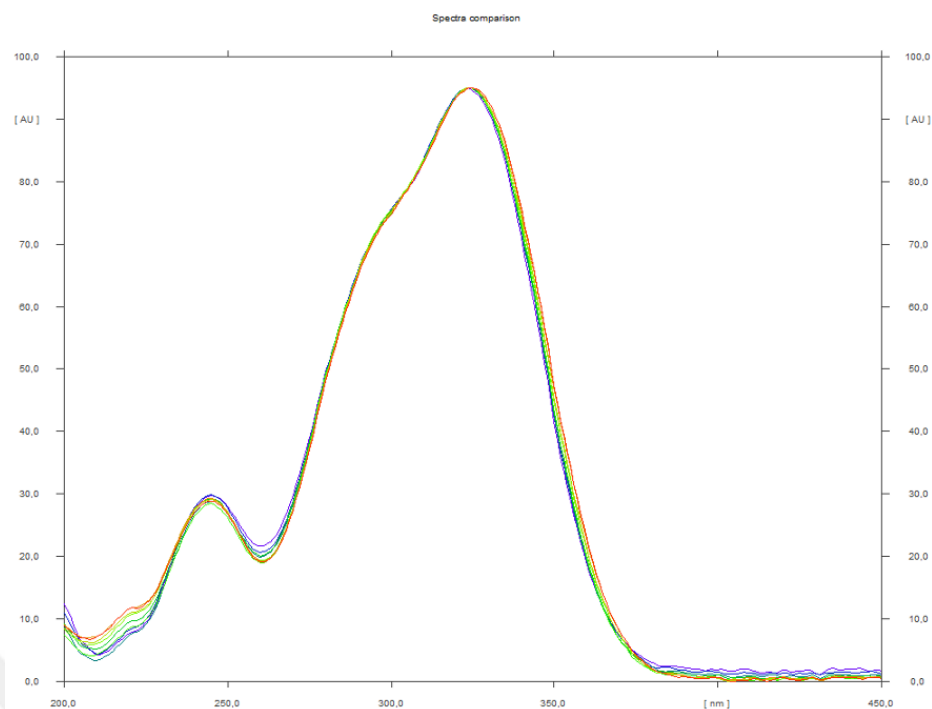
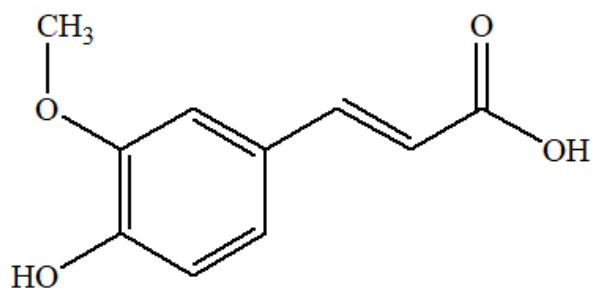


Figure 26. Overlay UV spectra of ferulic acid and the corresponding spots in the tracks of all extracts.



Ferulic Acid

5. DISCUSSION

Current environmental factors and lifestyles, including UV radiation, smoking, alcohol, stress, and chemicals, are the preliminary driving forces to enhance the risk for oxidative stress in the human body. It is well-known that human cells use molecular oxygen and thereby produce energy via mitochondrial oxidative metabolism to satisfy their functions. However, reactive oxygen species, including free radicals, are produced as a consequence of this metabolism inducing oxidative damage (1). Various diseases such as neurodegenerative diseases, inflammatory disorders, and metabolic syndromes such as diabetes stem from the surplusage of oxidative stress in the body (118). Although endogenous antioxidant mechanisms in the body withstand the free radicals, oxidative stress might outweigh this mechanism due to the factors above. Consequently, free radicals attacking proteins, lipids, and nucleic acids generate various unstable molecules (119).

Advanced glycation end products (AGEs) are numerous, heterogeneous, and vary according to fluorescent and cross-linking. There are considered important examples of these molecules, such as pentosidine (fluorescent cross-linking), carboxymethyl lysine (non-fluorescent cross-linking), and methylglyoxal-lysine dimers (non-fluorescent cross-linking). The generation of AGEs occurs inside and outside of the cells and relies on slow, progressive, enzyme-induced or not, and mostly irreversible chemical reactions. Glycation is commonly used for non-enzymatic reactions, whereas glycosylation defines enzymatic reactions (2,120). Besides, the combined effect of glycation and oxidation, named glycooxidation, generates AGEs (121). Glycation begins with an amino group of molecules, including proteins and lipids, reacting with the carbonyl group of reducing sugars. This reaction generates a reversible and unstable Schiff base. Amadori products occur due to dehydration and rearrangement of Schiff bases into more stable intermediates. In conclusion, these intermediates transform irreversibly to AGEs through certain reactions such as oxidation or dehydration. Furthermore, the oxidation of lipid molecules and glycolysis lead to the production of dicarbonyl intermediates such as methylglyoxal. These generated intermediates attack the free amino group of proteins (2,121). On the cell surface, there are specific receptors for AGEs (RAGE). Stimulation of these receptors by ROS (reactive oxygen species) or an excessive amount of AGEs, trigger the production of some proinflammatory mediators such as tumor necrosis factor- α (TNF- α), and matrix metalloproteinases (MMPs) (122). These mediators induce cell

inflammation signalling pathways linked to metabolic and autoimmune diseases. Although the accumulation of AGEs rises naturally with ageing, hyperglycemia also escalates this accumulation through some mechanisms (3). For instance, in the case of hyperglycemia, AGEs form a complex with RAGE on peripheral nerves. This complex promotes free radical formation, and as a result, AGEs production is provoked. Consequently, excessive accumulation of AGEs contributes to diabetic complications as well as secondary complications. Diabetic neuropathy is one of the diabetic complications developed in this way (2). To sum up, it is well known that an irrefutable relevance is found among oxidative stress, production of AGEs, and many pathological abnormalities, including diabetes and its microvascular and cardiovascular complications (3,118,119). In recent years, natural products have been preferred for the prophylaxis and treatment of these abnormalities due to the common risks of chemical drugs (2).

Humankind has widely utilized plants for nutrition, shelter, clothing, and prevention or treatment of many diseases since ancient times. Plants synthesize primary metabolites such as; carbohydrates, lipids, and secondary metabolites such as phenolics, alkaloids, etc.. Primary metabolites that are identical among higher plants are indispensable molecules for growth and survival throughout some pathways, such as hexose monophosphate (HMP) pathway etc. (123). Moreover, numerous types of secondary metabolites that vary according to the species, altitude, climatic zone, genotype etc., are produced widely via many different pathways (124). Plants generate secondary metabolites as a response to various conditions such as; increasing the attractiveness for animals to disperse their pollens or seeds, self-protection against insects, herbivores, or environmental changes, alleviating the scars caused by pathogen attacks or animals (125). Fortunately, these secondary metabolites also have unique and beneficial effects on human health (126).

Phenolic compounds that constitute the second largest class of secondary metabolites after terpenoids have unique activities on human health. Phenolic acids, flavonoids, and procyanidins are examples of the subclass of phenolic metabolites (126). Procyanidins, oligomeric or polymeric phenolic metabolites, are divided into two sub-group; hydrolysable and condensed tannins. Hydrolysable tannins are presented in limited species, whereas condensed tannins are highly distributed. Hydrolysable tannins, classified as gallotannins and ellagitannins, are derived from gallic and ellagic acid,

respectively. Condensed tannins are generated as final metabolites of the phenylpropanoid biosynthetic pathway (127). Proanthocyanidins, also known as condensed tannins, are oligomeric or polymeric metabolites of flavan-3-ols metabolites such as catechin, epicatechin. Proanthocyanidins are categorized according to the types of monomeric units, which are mainly bonded through C₄-C₈ or C₄-C₆ interflavan bonds. For example, procyanidins, a prominent class of proanthocyanidins, are formed transformation of catechin and epicatechin units, whereas prodelphinidins arise from the condensation of epigallocatechin metabolites (128-130).

Numerous studies have shown that proanthocyanidins affect human health through many different mechanisms. These metabolites, which exert antioxidant, cardioprotective, antidiabetic, neuroprotective activity, cardioprotective activity, etc., are found in many plant species such as pine bark and peanut skin (127,129). Pycnogenol[®], a standardized commercial product isolated from *Pinus pinaster* (French maritime), consists of oligomeric proanthocyanidins, flavonoids, and phenolic acid metabolites (128). A tea mixture prepared from barks of a tree was used by Native Americans to heal scurvy in 1535 by Jacques Cartier and crew members in North America. In 1951, A French researcher, Jacques Masquelier reconnoitred the writings of Cartier and began to scrutinize this tea mixture. At the end of the 1970s, the term ‘Pycnogenol[®]’, a mixture of oligomeric proanthocyanidin compounds, was introduced to the literature by Masquelier (7).

Pycnogenol[®] is obtained via a patented-standardized hot water extraction method, first described by Masquelier. It contains mainly procyanidins but also phenolic acids and flavonoids such as taxifolin (7). Moreover, many researchers reported that Pycnogenol[®] has diverse pharmacological activities such as antioxidant, cardioprotective, antidiabetic etc. (7,131). As seen in section 2.4.1, in Turkey, ethnobotanical records have revealed that tea prepared from the bark of *Pinus* species has been used against many diseases in traditional medicine. However, few studies on the proanthocyanidin contents from barks of *Pinus* species are available in Turkey. Furthermore, none of them has provided the data on the possible structural transformations that proanthocyanidins might be exposed to in the gastrointestinal system.

It is a well-known fact that secondary metabolites exert promoting effects on health. However, secondary metabolites and their bioactive forms must be absorbed from the gastrointestinal system into the bloodstream to effectuate their activities. Polyphenolics, including proanthocyanidins and phenolic acids, are subjected to chemical

or mechanical reactions during the digestion process in the gastrointestinal system (4). One of the critical reactions is that enzymes found in the stomach convert the macromolecules to aglycone or glycone forms of phenolics. The bioavailability of secondary metabolites depends on some preeminent gastrointestinal parameters such as food matrix, pH, enzyme profiles, and also the physicochemical characteristics of the secondary metabolites (107). It is quite well known that clinical and *in vivo* studies are the best options to determine the bioavailability of these metabolites. Nonetheless, such studies have certain ethical considerations and require highly costly procedures. Conversely, *in vitro* gastrointestinal digestion simulation system is a feasible method, and it provides valuable information for evaluating the bioavailability of secondary metabolites as the first approach (132). As a whole, this study was designed to investigate the phenolic profile, antioxidant and anti-diabetic activities of Pycnogenol® and proanthocyanidin fraction obtained from the barks of *P. brutia*, *P. pinea*, and *P. pinaster* using *in vitro* gastrointestinal simulation system.

Numerous studies focused on the phenolic profiles of *Pinus* species using different assays such as chromatographic approach or total content assays. Guri et al. (57) investigated the total phenolic content of the bark extracts of different *Pinus* species obtained by various organic solvents. Their findings were much lower than our results (from 1.1 to 3.2 mg GAE/g), which might be due to the extraction method and solvents used. However, the total phenolic content of *P. pinea* was found higher than *P. brutia* in all fractions, and this ranking seems consistent with our findings. Yesil-Celiktas et al. (76) evaluated total proanthocyanidin contents and the quantity of the two marker molecules; catechin and taxifolin by HPLC, and antioxidant activities of the barks of different *Pinus* species and Pycnogenol®. Accordingly, *P. brutia* had the highest taxifolin and catechin content, followed by *P. pinea* and Pycnogenol®, respectively. Total proanthocyanidin content of the extracts was reported as 367.7 mg/g extract for *P. brutia*, 280.0 mg/g extract for *P. pinea*, and 12.8 mg/g extract Pycnogenol®, respectively. As seen in Table 28, 29, and 30, the current study far exceeds these results. This inconsistency was probably due to the methods used to quantify total proanthocyanidin content. Moreover, DPPH radical scavenging activity assay was conducted to determine the antioxidant activities of extracts. Their results showed that *P. brutia* and *P. pinea* had almost the same scavenging activity, while the lowest scavenging activity was found in Pycnogenol®. These results are consistent with our findings.

In the present study, the utmost total phenolic and phenolic acid contents were found in the fractions of *P. pinea*; meanwhile, the highest proanthocyanidin content was observed in the digestion fractions of *P. brutia*. Pycnogenol® had the lowest total phenolic, phenolic acid, and proanthocyanidin contents. It is quite well known that the antioxidant properties of plants depend mainly on their phenolic contents. For this reason, it is not surprising that Pycnogenol® exerts the lowest antioxidant properties in all assays. In the case of phenolic acids, antioxidant activity depends largely on the number and position of hydroxyl groups, electron donation moiety, esterification of the molecules¹³³. Bendary et al. (134) reported that catechol was the most potent antioxidant molecule due to the existence of *o*-dihydroxyl groups among the phenolic molecules. The antioxidant activity of flavonoids is influenced by the flavan nucleus, the types of moieties and their numbers and positions. The ortho-dihydroxy group in the B ring also evokes the enhancement of the antioxidant activity of flavonoids (135). The antioxidant capacity of proanthocyanidins augments with the increasing number of catechin molecules binding with C₄→C₈ bonds, and the hydroxy group (136). Besides, dimeric and trimeric proanthocyanidins possess higher potency than monomeric flavonoids against oxidant molecules (135). Kivrak et al. (92) compared the phenolic compositions of the ethanolic extract of *P. brutia* bark and Pycnogenol® by using UPLC-ESI-MS/MS. Unlike the current study, caffeic acid and ferulic acid were quantified in *P. brutia* and Pycnogenol®, together with other phenolic molecules. Results in Table 44 revealed that caffeic acid and ferulic acid were found only in Pycnogenol® by using HPTLC. It is well-known that UPLC-ESI-MS/MS system measure lower amounts of molecules than HPTLC and HPLC systems (137). However, their results indicated that the caffeic acid and ferulic acid amounts were close in both *P. brutia* and Pycnogenol® extracts. The discrepancy in the results was most presumably due to different extraction methods. The extraction method developed by Masquelier provides a pure proanthocyanidin mixture with a low quantity of phenolic acids, whereas ethanolic extraction yields a mixture composed of various types of phenolic compounds such as phenolic acids and flavonoids, and proanthocyanidins (106,138). In another report, antioxidant activity and phenolic contents of 80% aqueous methanolic extract and its fractions (diethyl ether, ethyl acetate, *n*-BuOH, and remaining aqueous phase fractions) from the barks of *P. brutia* were investigated (59). According to their results, the highest total phenolic content was found in the EtOAc fraction (448.90 mg GAE/g). In the meantime, the highest proanthocyanidin content was seen in the aqueous phase (250.40 mg CHE/g). Additionally, the results showed that the

EtOAc fraction and aqueous phase exerted significant antioxidant activity compared to other fractions. In the current study, aqueous bark extract was also fractionated with EtOAc to purify proanthocyanidin. Total phenolic content was lowest in the EtOAc phase (383.58 mg/g GAE), while total proanthocyanidin content was highest in the aqueous phase (623.61 mg/g CHE). The importance of the extraction method is conspicuous once again. Ince et al. (68) evaluated the anti-inflammatory activity and the phytochemical composition of the *P. brutia* bark extracts obtained by the same extraction method in the current study and Pycnogenol®. They reported three flavan-3-ol derivatives; catechin, epicatechin, catechin gallate, and one flavanone molecule; taxifolin, by using HPLC system in both extracts. Unlike the present report, the authors detected catechin gallate in the extracts might be due to the higher sensitivity of HPLC method (139).

As mentioned previously, even though phytochemicals exert considerable activities on human health, their bioavailability in the body has supreme importance to reveal these activities. There are a huge number of studies on Pycnogenol® and its effects on human health and chemical composition (7,140-142). However, the researches of Pycnogenol® up to the present are not enough yet to enlighten its bioavailability (141). Moreover, there are no studies on the bioavailability of the other pine extracts studied in the current study. The bioavailability of molecules depends on the plant matrix, the interaction between the components in the extract, and gastrointestinal physiology and functions of the individual (4). Moreover, the properties of molecular structure such as molecular weight, hydrophobicity, aglycone, or glycoside form have significantly affected the bioavailability of the phytochemicals. Scalbert et al. (143) reported that the aglycone form of a glycoside demonstrates higher bioavailability by passive diffusion through their hydrophobic properties. Day et al. (144) proved that quercetin glycosides should be hydrolyzed in the gut to release the aglycone to be absorbed into the body. Piskula et al. (145) investigated the bioavailability of isoflavones; daidzein and genistein and their glycosidic forms in the rats. Their results showed that the aglycone form was absorbed in rat's stomach. Catechin and epicatechin are two of the major flavan derivatives of Pycnogenol®. Many reports indicated that they were readily bioavailable in the human body (146-148). In the current study, catechin/epicatechin mixture was found in different amounts in each extract. However, the bioavailability of the catechin/epicatechin mixture varied depending on the extracts. The highest bioavailability of catechin/epicatechin mixture was seen in *P. brutia* (108%), followed by *P. pinea* (69%), *P. pinaster* (62%), and Pycnogenol® (36%), respectively. Similarly, the

bioavailability of the proanthocyanidin B1/B2 mixture and proanthocyanidin C1 was also different in the extracts. The bioavailability of the proanthocyanidin B1/B2 mixture (76%) and proanthocyanidin C1 (60%) of *P. brutia* was found almost two times higher than the other pine extracts. These differences probably lean on the synergistic or antagonistic interaction between the other components in the extracts. Proanthocyanidin B1 and B2 are the dimeric procyanidins consisting of epicatechin-catechin and epicatechin- epicatechin subunits linked through $4\beta\rightarrow 8$ bonds, respectively, whereas proanthocyanidin C1 is the proanthocyanidin trimer composed of three epicatechin subunits linked through $4\beta\rightarrow 8$ bonds (128). As seen in Table 28, 29, 30, and 31, the utmost level of proanthocyanidin B1/B2 mixture was observed in the before-digestion fractions of *P. brutia*, followed by *P. pinea*, *P. pinaster*, and Pycnogenol[®], respectively. This ranking was also observed after gastric and intestinal digestion. As shown in Figure 17, 18, 19, and 20, the amounts of proanthocyanidin B1/B2 mixture and proanthocyanidin C1 were markedly decreased in all extracts after the gastric and intestinal digestion. Similarly, the highest level of proanthocyanidin C1 was found in *P. brutia*. Nonetheless, conversely, proanthocyanidin C1 level of *P. pinaster* was more pronounced than *P. pinea*. Even though proanthocyanidin C1 could be observed in qualitative analysis of proanthocyanidin C1 in Pycnogenol[®], it was not measurable in quantitative analysis by HPTLC. Moreover, proanthocyanidin A1, (+)-epicatechin-($4\beta\rightarrow 8$, $2\beta\rightarrow O-7$)-catechin were not detected in all extracts. The bioavailability of the catechin/epicatechin mixture was higher than the bioavailability of proanthocyanidin B1/B2 and proanthocyanidin C1 in all extracts. It is also well-known that catechin is one of the major degradation products of proanthocyanidin (135). In this regard, it is not surprising that the highest level of catechin/epicatechin mixture was observed in the gastric phase of *P. brutia*, followed by bioavailable and before-digestion fractions, respectively. Conceivably, proanthocyanidins found in *P. brutia* before-digestion would be converted to catechin or epicatechin after gastric and intestinal digestions. In fact, a catechin/epicatechin mixture was found in *P. brutia* before-digestion fraction; it was degraded under the gastrointestinal system conditions, and eventually, the quantity of catechin/epicatechin increased. In contrast to *P. brutia*, the catechin/epicatechin mixture in the other pine extracts were slightly reduced during gastrointestinal digestion. This situation might be due to the chemical type of proanthocyanidin structure or their amounts in the extracts.

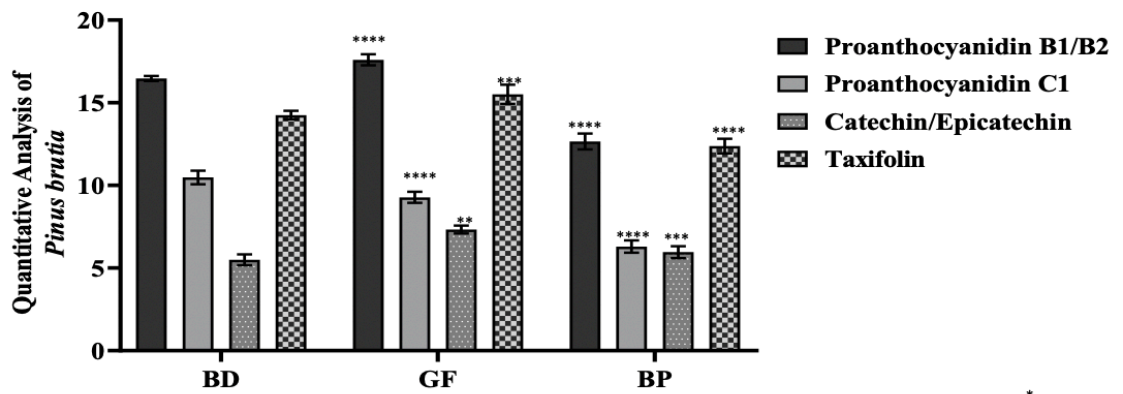


Figure 47. Comparison of marker compounds in BD, GF, BP samples from *P. brutia*.

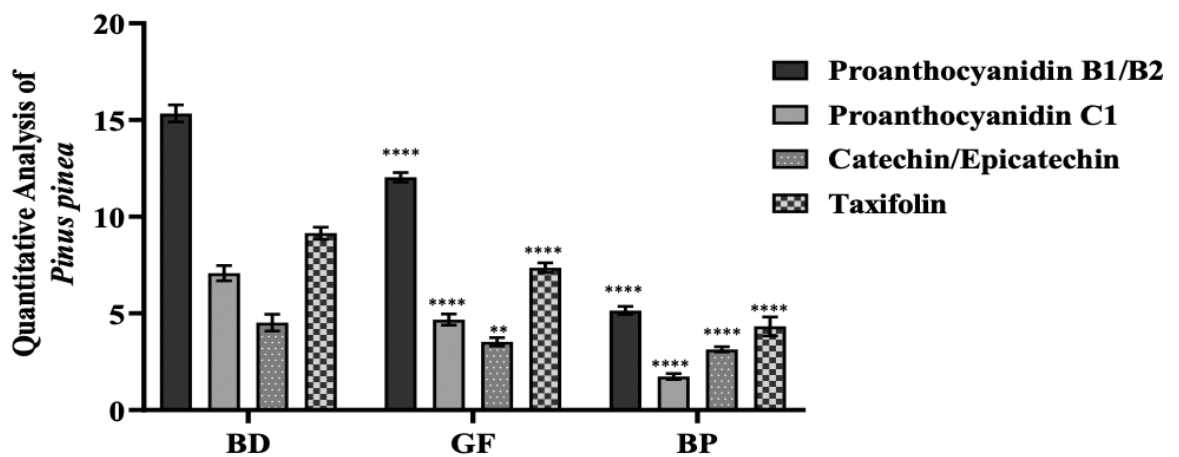


Figure 48. Comparison of marker compounds in BD, GF, BP samples from *P. pinea*.

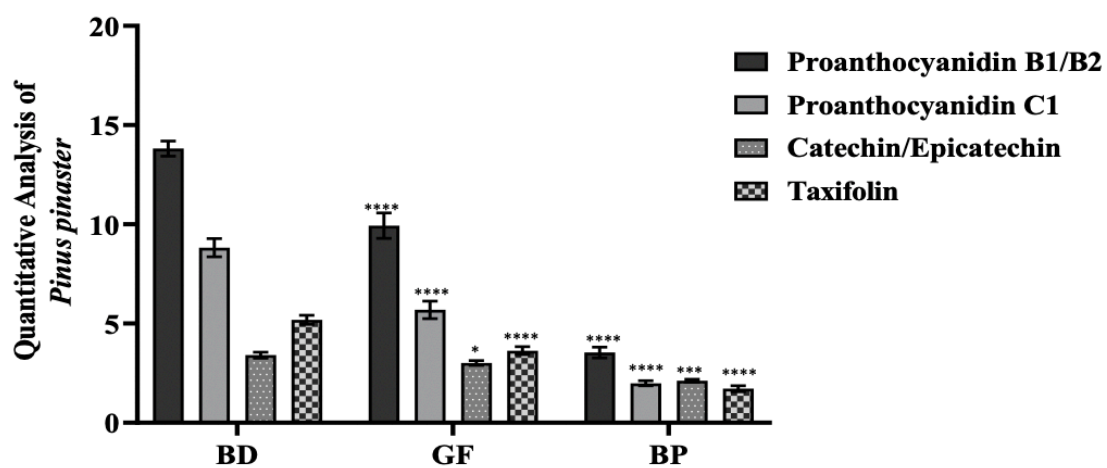


Figure 49. Comparison of marker compounds in BD, GF, BP samples from *P. pinaster*.

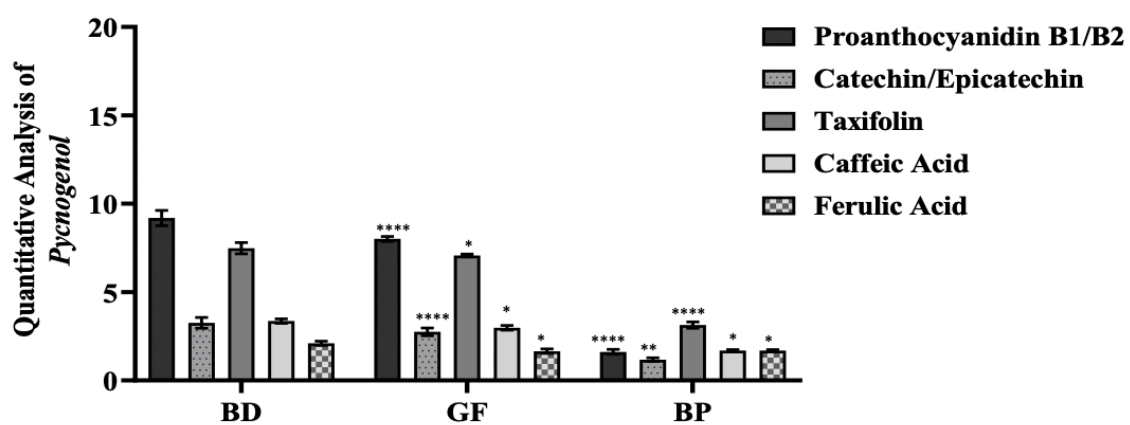
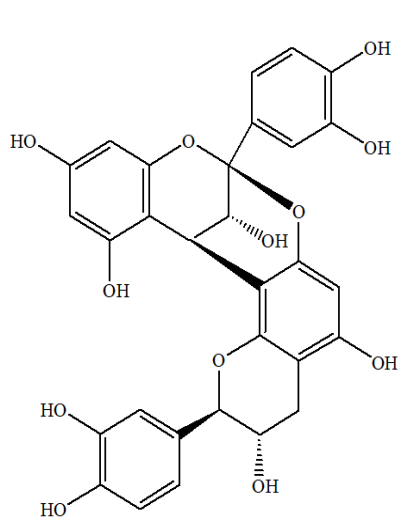
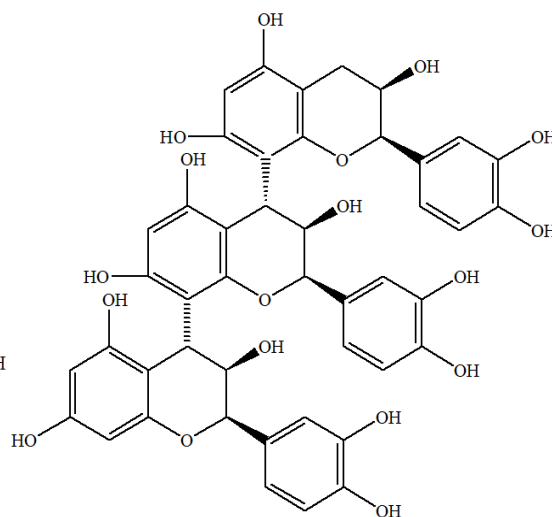


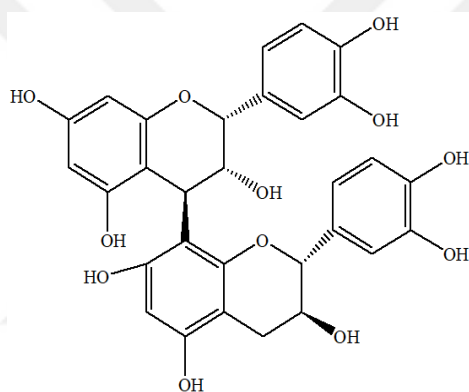
Figure 50. Comparison of marker compounds in BD, GF, BP samples from Pycnogenol®.



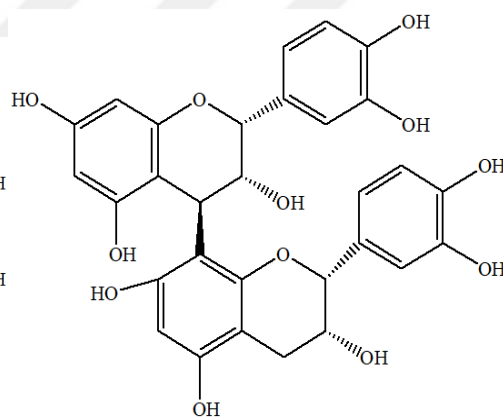
Proanthocyanidin A1



Proanthocyanidin C1



Proanthocyanidin B1



Proanthocyanidin B2

Tarko et al. (149) studied the phenolic components and their antioxidant activities in several fruits after submitting *in vitro* gastrointestinal simulation system. The amounts of proanthocyanidins B1, B2, and C1 in the extract from Malinowka apple were decreased markedly after the digestion system. Moreover, flavonoid glycosides and phenolic acids concentrations were also reduced during digestion. Similar to our results, a minor decline was observed in the catechins quantity of the extract. Flavonoids in plants are known to be more prevalently occur in glycosidic forms. However, aglycone forms have higher stability than glycosidic forms in the digestion system. Taxifolin (dihydroquercetin), a flavanonol aglycone, is one of the major phenolic components in pine bark extracts (59,76,150). Şeker et al. (100) determined the quantities of catechin, epicatechin, epicatechin gallate, taxifolin, and ferulic acid in five *Pinus* species. Like our results, *P.*

brutia had higher amounts of catechin, epicatechin, and taxifolin than *P. pinea*, while epicatechin gallate and ferulic acid were not detected in these extracts. According to our results, before-digestion fraction of *P. brutia* had the highest level of taxifolin, followed by *P. pinea*, *P. pinaster*, and Pycnogenol[®], respectively. A decrease was seen in all extracts, except *P. brutia*, after gastric and intestinal digestions. In fact, a slight increase was observed in *P. brutia*, from the before-digestion to gastric phase (14.25 ± 0.26 , 15.51 ± 0.58 mg/g, respectively), and then a minor reduction was seen in the bioavailable fraction (12.38 ± 0.44 mg/g). The highest bioavailability was seen in *P. brutia* as 86%, followed by *P. pinea* as 47%, Pycnogenol[®] as 42%, and *P. pinaster* as 33%, respectively. Various studies also reported the negative influence of gastrointestinal digestive system on taxifolin level in pine bark extracts (151,152). Our results also confirmed the fact, as previously stated in many studies, that caffeic acid and ferulic acid are the preeminent phenolic acids in Pycnogenol[®] (140,150). Like other marker components, caffeic acid and ferulic acid contents also decreased after gastrointestinal digestion. This decrease might be due to the unstable structure of phenolic acids in alkaline medium (153). Moreover, the amount of caffeic acid (3.36 ± 0.13 mg/g dry ext.) was higher than the amount of ferulic acid (2.1 ± 0.12 mg/g dry ext.), in the meantime, the bioavailability of caffeic acid (as 50%) was lower than that of ferulic acid as 59%. The decrease in phenolic content might be due to their sensitivity to the alkaline medium during digestion, and many studies are in good agreement with this approach)154-156).

The bioavailability of phytochemicals may be evaluated in various ways. *In vitro* gastrointestinal digestion simulation technique has some drawbacks compared to *in vivo* and clinical studies. It is rather designed to obtain the preliminary data on the bioavailability of phytochemicals by reducing the ethical and economic considerations (4). The effects of digestion on the phytochemicals fluctuate depending on the many situations. Celep et al. (111) investigated *in vitro* antioxidant activity, the amounts of marker compounds and phenolics in the methanolic extract of *Lavandula stoechas* L. after *in vitro* gastrointestinal digestion simulation system. This study observed a descending trend in the amounts of marker compounds and antioxidant activities. İnan et al. (114) investigated total phenolic contents, antioxidant and anti-diabetic activity assays, and marker compounds in the methanolic extract from *Salvia virgata* Jacq. using a gastrointestinal digestion simulation system. A declined trend was reported in all assays in the quantities of marker compounds. Green et al. (157) evaluated the amounts of epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate in the

different green tea formulations after the *in vitro* gastrointestinal digestion system. Their results indicated that catechin degradation occurred mainly in the small intestine due to its instability under neutral and basic pH. Laurent et al. (158) determined the quantities of catechin, epicatechin, proanthocyanidin B1, and proanthocyanidin B2 in the grape seed extract throughout the *in vitro* digestion simulation model. The amount of all compounds was declined after intestinal digestion. Lopez-Nicolas et al. (152) investigated the effects of *in vitro* digestion system on gallic acid, chlorogenic acid, caffeic acid, ferulic acid, and taxifolin as the phenolic compounds found in pineapple and red fruit juices enriched with pine bark extract. As expected, after the addition of pine bark extract into fruit juices, the quantity of marker compounds increased significantly. After being submitted to the gastrointestinal digestion system, the quantities of all phenolic compounds diminished in pineapple and red fruit juices. Ferreira-Santos et al. (151) compared the phenolic content, levels of some marker phenolic compounds, antioxidant, and antimicrobial activities of the aqueous ethanolic extract from *P. pinaster* barks and its encapsulated form after being submitted to *in vitro* gastrointestinal digestion system. Results showed that the phenolic composition, antioxidant, and antimicrobial activities of both non-encapsulated and encapsulated forms, were adversely affected by this process; however, the reduction was lower in encapsulated form. As expected encapsulation protected the phenolic compounds against degradation due to gastrointestinal conditions. They determined the total phenolic content of non-encapsulated form before-digestion as 222 mg GAE/g dry extract, while in the present study, total phenolic content of *P. pinaster* was 396.93 ± 2.92 . This incongruity might probably due to the extraction method. However, total phenolic contents in both studies reduced nearly two folds following the digestion system.

In contrast to these studies, many scientists also reported contrary results. For instance, on methanolic extract of *Hypericum perforatum* L., phenolic contents were increased after the gastrointestinal digestion system (112). As expected, the same trend was observed in antioxidant activity assays due to the association between phenolic compounds and antioxidant activity. In our previous study, we reported the effect of the gastrointestinal digestive system on antioxidant activity, the results of enzyme assays related to diabetic activity, the concentrations of marker compounds and phenolics of the methanolic extract from *Hypericum olympicum* L. (110). The highest levels of marker metabolites were observed in the bioavailable phase, and the lowest levels were seen in before digestion fraction. These results have shown that the *in vitro* gastrointestinal

digestion system may affect the quantity of secondary metabolites differently and their biological activities.

Obviously, the *in vitro* gastrointestinal digestion system affects the phenolic contents negatively or positively and eventually the biological activities. In the current study, total phenolic, proanthocyanidin, and phenolic acid contents were reduced in all pine extracts during the steps of gastrointestinal simulation. The instability of phenolic compounds may be due to pH changes, gastrointestinal enzymes, peristaltic movement and/or the plant matrix. For instance, phenolic compounds bound to macromolecules such as fibers or lipids must be liberated from the plant matrix to become bioavailable (4). Proanthocyanidins were the marker molecules in our extracts with higher molecular weight than other phenolic compounds due to their complex oligomeric structures. Oligomeric proanthocyanidins transform to dimeric or monomeric, and also other oligomeric forms in mild acidic medium (159). In this study, total proanthocyanidin contents decreased in all extracts after submitting to gastric simulation, confirming the aforementioned findings. In the intestinal medium, catechol moiety, found in most proanthocyanidins structures, degrades rapidly via oxidation reaction due to the alkaline conditions. Yoshino et al. (160) indicated that the level of epigallocatechin gallate decreased 81.6% after being submitted to an intestinal medium. The results revealed that the bioavailability of marker molecules might vary depending on the plant material. This situation was possibly due to the synergistic or antagonistic effects of the components in the extracts.

Many studies indicated that the bioavailability of phytochemicals depends on the plant matrix, interaction with other macromolecules, enzymes found in the digestive system, etc (161-164). Results of the present study have shown that total phenol, phenolic acid, and proanthocyanidin contents of the extracts decreased throughout the gastrointestinal digestion simulation. Accordingly, antioxidant activity assays using different mechanistic approaches on the extracts revealed a reduction trend from before-digestion to gastric phase and bioavailable fraction, respectively. Many researchers have reported a linear correlation between the total phenolic content and antioxidant activity so far (165,166).

As mentioned before, various studies showed the anti-hyperglycemic activity of Pycnogenol® and *Pinus* species (131,140,167). In the current study, α -glucosidase, α -amylase inhibitory and anti-glycation activities of the pine extracts were studied to evaluate the variations in their anti-diabetic potentials after subjecting to *in vitro*

gastrointestinal digestion. As mentioned before, hyperglycemia triggers diabetic complications by many mechanisms such as activating the protein glycation or protein kinase C, etc. (2). In addition, increased oxidative stress in the body contributes to augmenting these factors markedly. Numerous studies focused on the prevailing potentials of the phytochemicals to chemical drugs, yielding lower risks than conventional drugs, for prevention and treatment of diabetes and its complications (12,168,169). Various studies indicated that phenolic compounds such as proanthocyanidins and flavonoids have an unrivalled protective role against diabetes mellitus by different mechanisms such as the inhibition of advanced glycation end-products, inhibition of α -glucosidase or α -amylase enzymes etc. (2,169,170). Several studies showed the inhibitory effect of epigallocatechin-3-gallate (EGCG), one of the major components in green tea, on glucose production via hepatic gluconeogenesis. and the anti-diabetic activity (171,172). According to their results, EGCG reduced body, liver, and kidney weights and insulin and glucose levels. Moreover, a significant decrease was observed in the amounts of AGEs in the liver and plasma. In another study, the anti-hyperglycemic activity of chlorogenic acid was evaluated using different assays. It has been reported that chlorogenic acid inhibited α -glucosidase and α -amylase enzymes and reduced glucose 6-phosphate mediated glucose production in the liver by glycogenolysis and gluconeogenesis (173). Toh et al. (174) compared the α -amylase inhibitory activity of dracoflavan B and proanthocyanidin A2 isolated from *Daemonorops draco* resin. Even though proanthocyanidin A2 and dracoflavan B have the same skeleton, dracoflavan B exerted a higher inhibition than proanthocyanidin A2 due to its hydrophobic nature. Zhang et al. (175) evaluated the α -glucosidase inhibitory, anti-glycation, and antioxidant activities of ethanolic extract from *Liquidambar formosana* Hance leaves and its dichloromethane, ethyl acetate, water, and *n*-butanol fractions. The phenolic profile of these fractions was studied by HPLC-QTOF-MS/MS. They reported that phenolics, condensed, and hydrolysable tannins contents of ethyl acetate subfraction were strikingly higher than the other fractions, while the highest total flavonoid content was in the *n*-butanol subfraction. Therefore highest antioxidant activity was observed in the ethyl acetate fraction; meanwhile, the water fraction showed the lowest as the phytochemical content was the lowest. The ethyl acetate fraction also significantly inhibited the α -glucosidase and AGEs production than the other fractions. As it is well known, high phenolic and tannins contents are correlated with the biological properties of extracts, this outcome is correlated with the present study.

Similar results were reported by Salhi et al (84). They investigated the total phenolic content, antioxidant, and antidiabetic activities of the aqueous and ethanolic extracts from *P. halepensis* barks, comparatively. The ethanolic extract exerted higher antioxidant and antidiabetic activities due to its higher phytochemicals content. Lee et al.¹⁷⁶ investigated the antiglycation, α -glucosidase, and α -amylase inhibitory activities of polymeric and oligomeric proanthocyanidins isolated from persimmon peel. Their results showed that polymeric proanthocyanidins more strongly inhibited the α -amylase enzyme than oligomeric forms, possibly due to the degree of polymerization. However, proanthocyanidin oligomers provided the most potent inhibition in α -glucosidase enzyme and AGEs. This situation might be due to hydroxyl and gallol moieties of proanthocyanidins. In our study, although *P. pinea* exerted the highest inhibition activity on AGEs, α -glucosidase, and α -amylase enzymes, the highest quantity of proanthocyanidin oligomers, proanthocyanidin B1, B2, and C1, were found in *P. brutia*. This might be due to the polymeric proanthocyanidins in the extracts. Ho et al. (177) evaluated the α -glucosidase, and α -amylase inhibitory activities of proanthocyanidin B2, B5, and C1 isolated from elderberry. Proanthocyanidin C1 showed the highest inhibitory activity on both enzymes, possibly due to its higher molecular weight. Another study also confirmed that the α -glucosidase inhibitory potential of proanthocyanidins was augmented with the increasing molecular weight of proanthocyanidin (178). In another study, the antioxidant and α -glucosidase inhibitory activities of some phytochemicals isolated from *Ipomoea batatas* (L.) Lam. leaves were evaluated by establishing the structure-activity relationship (179). The increasing caffeoyl moiety in quinic acid strengthened the antioxidant activity of molecules according to this result. In addition, 3,4,5-tricaffeoylquinic acid and *trans*-N-(*p*-coumaroyl) tyramine had the strongest inhibitory activity on the α -glucosidase enzyme. Accordingly, adding a methyl moiety to the hydroxyl group in phenethyl cinnamides and *cis* form would weaken the α -glucosidase inhibitory potential. Consequently, the chemical composition of extracts should be comprehensively elucidated to reveal their exact mechanism of action.

Kawakami et al. (180) studied the effect of seasonal changes in the water-soluble proanthocyanidins content of aqueous acetone extract from *Diospyros kaki* leaves and their α -amylase inhibitory potential. They found that the highest proanthocyanidins concentration was attained in June as parallel to the highest α -amylase inhibitory activity of the extract. The results indicated a direct correlation between the proanthocyanidins content and the α -amylase inhibitory activity. In the current study, *P. brutia* had the

richest level of total proanthocyanidin content, whereas *P. pinea* possessed the highest α -amylase inhibitory activity. In contrast, the total phenolic and phenolic acid contents were found in the highest levels in *P. pinea*. Ferreira-Santos et al. (181) reported the anti-hyperglycemic activity of ethanolic and aqueous extracts from *P. pinaster* barks by α -glucosidase and α -amylase inhibition assay, while the reference compound, acarbose, had the lowest inhibitor activity in α -glucosidase inhibition assay. El-zein et al. (182) investigated the anti-hyperglycemic activity of the ethanolic extract from *P. pinea* bark. Their results showed the extract declined glucose transport strikingly by triggering the p38 mitogen-activated kinase. In another study, the α -glucosidase inhibitory activity of various fractions of Pycnogenol[®], and some marker compounds were compared to acarbose and green tea extract (142). Accordingly, Pycnogenol[®] exerted the highest α -glucosidase inhibitory activity, whereas catechin was ten folds lower. The highest α -glucosidase inhibitory activity among the fractions of Pycnogenol[®] was seen in oligomeric fractions, followed by tetrameric up to hexameric, dimeric, and phenolic acid fractions, successively. Consequently, the α -glucosidase inhibitory activity of Pycnogenol[®] was predicated mainly on its proanthocyanidin content. It is worth noting that phenolic compounds, to some extent, may influence the enzyme inhibition synergistically or antagonistically (183). Su et al. (184) demonstrated a dose-dependent α -glucosidase and α -amylase inhibitory activity of by *in vivo* and *in vitro* assays. They reported that increasing doses augment the inhibitory activity of taxifolin. Taxifolin had potent hypoglycemic activity in rats. In *in vitro* assays, taxifolin inhibited the α -glucosidase and α -amylase enzymes stronger than acarbose. Moreover, in a molecular docking study, many interaction types, including hydrogen bond and π - π stack, were demonstrated to interact taxifolin with both enzymes. These results seem correlated with the present study. According to the current study, *P. pinea* had the highest α -glucosidase inhibitory activity, followed by *P. pinaster*, *P. brutia*, and Pycnogenol[®], respectively. This order was observed in all fractions. However, as stated previously, *P. brutia* had the richest proanthocyanidin content, while the highest amounts of total phenolic and phenolic acid contents were found in *P. pinea*. In addition, total phenolic, phenolic acids, proanthocyanidin contents, and the quantities of proanthocyanidin B1/B2, proanthocyanidin C1, and catechin/epicatechin were observed significantly decreased throughout the *in vitro* digestion system. These data might divulge the descent in the α -glucosidase inhibitory activity of the extracts from the before-digestion fraction to the bioavailable phase of *in vitro* digestion system.

In the present study, antiglycation, α -glucosidase, and α -amylase inhibitory assays were conducted at two concentration levels (0.125 mg/mL and 0.25 mg/mL). The result showed that all extracts had a concentration-dependent α -glucosidase, α -amylase inhibitory, and antiglycation activities. Among the before-digestion fractions, the utmost inhibitory activities in these three assays were observed in *P. pinea*, followed by *P. pinaster*, *P. brutia* and Pycnogenol® at the same concentrations. The same ranking was observed after being subjected to the digestion system in all assays.

Phenolic compounds are well-known to be positively correlated with many biological activities, including antioxidant and anti-diabetic properties. As discussed above, the amounts of total phenolic, phenolic acid, proanthocyanidin, and marker compounds were decreased after gastric and intestinal digestion. Thus, our results promoted this approach. Ho et al. (185) investigated the antiglycation activities, total phenolic and flavonoid contents of some herbal teas, including green, rosemary, mint, and sage teas. According to their results, phenolic contents were positively correlated with antiglycation activity. Spinola et al. (186) evaluated the phytochemical compositions, antioxidant and antidiabetic activities of methanolic extracts from *Elaeagnus umbellata* and *Sambucus lanceolata* leaves and berries by *in vitro* digestion simulation system. Their results showed that antioxidant activity, antiglycation, α -glucosidase and α -amylase inhibitory activities of the extracts had reduced gradually during the gastrointestinal digestion as parallel to the reduced rate of phytochemicals in the extracts. These results are in good agreement with the findings of the current study. Peng et al. (187) studied the antiglycation activity of ethyl acetate, water, and *n*-butanol extracts and their subfractions from cinnamon barks and the isolated components, catechin, epicatechin, and proanthocyanidin B2. Accordingly, ethyl acetate and *n*-butanol extracts had the highest inhibitory activity due to their rich phenolic content, as expected. In addition, proanthocyanidin B2, epicatechin, and catechin exerted potent antiglycation activity. Lavelli et al. (188) compared the methanolic extracts from apple puree (*Malus domestica* Borkh.) and apple puree fortified with green tea in terms of their antiglycation activity and amounts of certain marker molecules. The catechin, epicatechin, proanthocyanidin B2 and total proanthocyanidin contents were markedly increased in the fortified extract, and thus a higher antiglycation activity was observed than apple puree. They suggested gallate moiety at the C-3 position in the flavan-3-ol structure augments the antiglycation activity.

The biological activities of phytochemicals depend on their molecular structures. Structure-activity relationship (SAR) studies on phytochemicals have provided notable data, especially for evaluating their bioavailability. A double bond between C2-C3 in polyphenolic skeleton improves the antioxidant and antiglycation activity potential due to stabilization of the hydrogen bond and modification of the electron delocalization (122). The number and position of subunits, including hydroxyl, methoxyl, or galloyl moieties affect their activities. For instance, increasing the hydroxyl and galloyl moieties of flavan-3-ol improve the α -glucosidase inhibitory activity. However, antiglycation activity is fortified by increasing the hydroxyl moiety on A and B rings, while the activity weakens by the hydroxylation of the C ring (189). Oligomeric proanthocyanidins demonstrate a stronger inhibitory activity on α -glucosidase, α -amylase, and AGEs (189,190). Sun et al. (191) showed that proanthocyanidin B1 and B2 had higher antiglycation activity than the catechin and epicatechin monomers. Despite a loss in antioxidant activity in the current study, a decrease in the inhibitory potentials of α -glucosidase and α -amylase enzymes, and AGEs through the gastrointestinal digestion steps were observed in all extracts or fractions, which was correlated with their phenolic contents.

6. CONCLUSION

In this current study, proanthocyanidin mixtures were obtained from *P. brutia* and *P. pinea* barks, grow naturally in Turkey, as well as *P. pinaster* barks, a cultivated tree, by a patented method. These proanthocyanidin mixtures and Pycnogenol[®], a standardized *P. pinaster* bark extract, were compared in their phenolic profiles, total antioxidant, and anti-diabetic potentials. Even though the earlier studies have focused on the phytochemical profiles, total antioxidant, and anti-diabetic potentials of these extracts, for the first time, this study was designed to investigate the influence of *in vitro* human gastrointestinal digestion on these activities and their phytochemical profiles.

Total phenolic, phenolic acid, flavonoid, and proanthocyanidin contents of the extracts before digestion (BD), in the gastric fractions (GF), and bioavailable phases (BP) were investigated by *in vitro* assays. In addition, Proanthocyanidin A1 (PaA1), proanthocyanidin B1/B2 mixture (PaB1B2), proanthocyanidin C1 (PaC1), catechin/epicatechin mixture, epigallocatechin (EGC), epigallocatechin gallate (EGCG), catechin gallate (CG), taxifolin, ferulic acid, and caffeic acid metabolites were used to elucidate their phytochemical profiles by HPTLC.

Two free radical scavenging activity assays; DPPH and DMPD, two metal-reducing activity assays; CUPRAC and FRAP, and total antioxidant capacity assay, were used to reveal the *in vitro* antioxidant capacity of extracts. In addition, *in vitro* inhibitory α -amylase and α -glucosidase enzyme activities and AGEs inhibition activity of extracts were evaluated for investigation of the anti-diabetic activities of extracts.

The results showed that *in vitro* human gastrointestinal digestion affected the phytochemical profile and the antioxidant and anti-diabetic potentials of the extracts. PaA1, EGC, EGCG, and CG were not in detectable quantity in all pine extracts. PaB1B2 mixture, catechin/epicatechin mixture, and taxifolin were found in all samples, whereas PaC1 was not detected in Pycnogenol[®]. In addition, ferulic acid and caffeic acid were only found in Pycnogenol[®]. BD fractions had the highest metabolites concentrations and thus the highest TPC, TPaC, TPrC contents in all pine extracts. Furthermore, a decreasing trend was observed in antioxidant and antidiabetic assays after the gastrointestinal digestion for all metabolites. In addition, even though a descending trend was observed

from BD to BP, all extracts exerted potent antioxidant and antidiabetic activities. *P. pinea* had the highest inhibitory activity on α -amylase and α -glucosidase enzymes and AGEs activity among the pine extracts, whereas the lowest inhibitory activities were seen in Pycnogenol®. The same trend was also seen in the antioxidant assays.



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