

T.R.
KASTAMONU UNIVERSITY
INSTITUTE OF SCIENCE
FOOD ENGINEERING DEPARTMENT



**INVESTIGATION OF PINE (*Pinus nigra*) BARK EXTRACTS
CONTAINING PHENOLIC COMPOUNDS**

MERILYN AMLANI

MASTER THESIS

Assist. Prof. Dr. Senem YETGİN

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THESIS APPROVAL

The thesis study prepared by **MERILYN AMLANI** entitled “**INVESTIGATION OF PINE (*Pinus nigra*) BARK EXTRACTS CONTAINING PHENOLIC COMPOUNDS**” was held in **December 08, 2022** and it was accepted as Master thesis of the Food Engineering Department, Institute of Science, Kastamonu University by unanimously/by majority of votes of the following jury

Supervisor	Assist. Prof. Dr. Senem YETGİN	
	Kastamonu University	
Jury Member	Assoc. Prof. Dr. İdris YAZGAN	
	Kastamonu University	
Jury Member	Assist. Prof. Dr. Duygu ALTIOK	
	Giresun University	

This thesis, which was accepted by the jury members, was approved by the Kastamonu University.

Institute Director	Assoc. Prof. Dr. Osman ÇİÇEK	
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COMMITMENT

In the design, preparation, conduct, research and analysis of the findings of this thesis, all information obtained and presented within the framework of ethical behavior and academic rules; I also declare and undertake that in this study, which is prepared in accordance with the thesis writing rules, all kinds of statements and information that do not belong to me have been fully cited, and that the source has been cited in accordance with scientific ethics.



Merilyn AMLANI

Signed

ABSTRACT

MSC THESIS

INVESTIGATION OF PINE (*Pinus nigra*) BARK EXTRACTS CONTAINING PHENOLIC COMPOUNDS

MERILYN AMLANI

**KASTAMONU UNIVERSITY INSTITUTE OF SCIENCE
DEPARTMENT OF FOOD ENGINEERING**

SUPERVISOR: Assist. Prof. Dr. Senem YETGİN

Chemical pollution, which occurs in proportion to the increase in the rate of industrialization seen today, and the increase in the duration and amount of exposure, seriously affect human health. The public's awareness of the relevance of the issue is caused by the direct or indirect interaction of chemicals like poisonous, carcinogenic, and pesticides with nature. It is not only for the elimination of these reservations and the protection of health; At the same time, it is the most basic approach to use plants in studies made as a result of the body's vitality and effort to live longer, and this approach is as old as human history.

As a result, today's interest in food has shifted from providing the nutrients needed to sustain life and growth to preventing or actually curing various types of disease. Moreover, recent technological advances, lifestyle changes in the population and socio-economic trends worldwide indicate the growing need for foods with health benefits. Even if for people with unhealthy eating habits beyond a healthy diet, supplemental products are recommended to eliminate the harmful effects of metabolic reactive oxygen species. In short, it is desired that nutrition reduces oxidative stress in a straightforward way. Within the subject, the interest in the so-called “*natural*” products that are potentially under-processed has increased tremendously. As a result, herbal supplements have gained acceptance as an alternative to synthetic medications and have established a market for themselves, even if research on their therapeutic effects is still ongoing.

The bioactive compounds found in plants have been used by humans from the earliest times to treat a variety of diseases naturally in addition to providing food and shelter. These bioactive substances, which are important in various fields, are formed as a result of crucial metabolic processes in plants. As a result of different chemical reactions, they have a wide variety and are classified as primary and secondary metabolites. Plants; through primary metabolism, they produce primary metabolites, including carbohydrates, proteins, lipids, and other organic acids that are essential for both human nutrition and the growth and development of plants. On the other hand, plants' secondary metabolism also helps to produce secondary metabolites, including terpenes, compounds that contain nitrogen, and phenolic compounds (PCs). These compounds are not directly important for plant growth and development. However, they are important for their ecological function as an attractant, pollinator, and even protecting plants from anything that could harm them. In addition, these compounds, especially PCs, are widely used in different fields such as pharmacology, cosmetics and the food industry. PCs are widely used as an active ingredient in the pharmacological field due to their various therapeutic functions such as anti-inflammatory,

anticancer, anti-proliferative, antidiabetic and, most importantly, their antioxidant capacity. The use of PCs in the food industry has also become widespread due to their antioxidant capacity, which can be used as an alternative to synthetic antioxidants in the protection of food products containing high unsaturated fatty acids prone to oxidation. Therefore, an increasing number of studies are carried out on these substances in terms of their biological effects and natural resources.

PCs are found in a variety of plant species, including herbs, shrubs, trees, and others. These include species such as pine trees that are often used as potential sources of PCs. *Pinus pinaster* and *Pinus maritima* are pine tree species that attract attention for the abundance of PCs. The bark of this tree is a great source of proanthocyanidin compounds and is sold commercially as dietary supplements under the trade name *Pycnogenol* (PYC). With the demand and popularity of this pine tree, other pine tree species have also been discovered as potential sources of PCs. One of them is *Pinus nigra*. *P. nigra*, the second most abundant pine species in Türkiye, is especially common in Küre and Ilgaz Kastamonu National Parks. It has a total forest area of 4.7 million hectares and is one of the important pine tree species in the wood industry. *P. nigra* is already used in traditional Turkish medicine to treat a variety of conditions, including respiratory, gastrointestinal, and back problems.

PCs can be found in various plant parts. According to various studies, pine tree components such as needles, cones, roots and especially bark can be used as possible sources of PCs. Pine bark, which constitutes 9-15% of the waste by-product in the wood sector, is an ideal source to be used to reduce waste and obtain products rich in PCs with high added value. Different methods, such as conventional and non-traditional extraction methods, are used for the isolation of PCs. While unconventional methods are advantageous in terms of shorter extraction time, less solvent usage and other aspects, traditional methods such as maceration are still widely used today due to their simplicity and accessibility.

In this work, *P. nigra* bark extracts (PNBE) abundant in the province of Kastamonu, Türkiye, particularly in Ilgaz and Küre national parks, were used as a potential plant source of PCs and aimed to utilize it to establish a cost-effective and efficient way to extract. The PNBE was extracted using maceration (MAC), where the effects of time, temperature, and solvent concentrations on the total phenolic contents (TPC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant capacity was optimized using response surface methodology (RSM). The extraction yield (EY) ($6.40 \pm 0.66\%$ d.w), diffusion coefficients (D_{eff}) (1.01×10^{-12} m²/s), TPC (42.56 ± 1.13 mgGAE/100g d.w), DPPH (IC₅₀ 6.24 ± 0.04 µgAAE/ml), and FRAP (18.42 ± 0.56 mgAAE/100g d.w) antioxidant capacity were determined using the optimized parameters at 60% EtOH, 240 mins, and 60°C, with constant solid/liquid ratio (100 g/L) and particle size (0.250-0.500 mm). Analysis in phenolic profiling using reverse phase high-performance liquid chromatography (RP-HPLC-DAD) revealed that 2,5 dihydroxybenzoic acid, myricetin, catechin, naringin, and ferulic acid are the major components in PNBE, accounting for 19.42, 19.33, 16.88, 11.04, and 10.15% of the total amount measured, respectively, while others are less than 10%. For SPME-GC-MS, there were four main peaks detected: decane, dodecane, tetradecane, and hexadecane, with 8.30, 15.71, 19.00, and 7.92% total area detected, respectively. Compounds such as phenol and terpenes, including the cadinene family of sesquiterpenes, which made up 0.98 and 0.24% of the total area, were also observed. Antibacterial properties of PNBE were also assessed, and it was found that their ability to stop bacterial development is less effective than that of other pine species. Furthermore, heavy metals such as As, Cr, Cd, Cu, and Pb were found in PNBE with concentration levels of 192 ± 3.49 , 94.85 ± 0.61 , 16.65 ± 0.05 , 177.62 ± 1.22 , and 215.82 ± 0.53 ppb, respectively. These concentrations are less than the acceptable levels established by the World Health Organization (WHO), European Union (EU), and Codex Stan 193-1995.

Based on the findings of this study, it can be concluded that PNBE is an excellent source of PCs that are very important for pharmacological uses, especially the finding of the antioxidant ability of *in-vitro* analysis. The impacts of various variables and amounts are also demonstrated for the various PCs in PNBE. According to the completed study, ideal conditions were obtained for the extraction of PNBE using the RSM technique. This optimization technique has been identified as an effective approach to intensify different extraction factors. The presence of heavy metals in PNBE may pose a risk to human health because pine species extracts are essential for many medicinal applications. Additionally, PNBE has been found to have the antibacterial capacity, but at some points their ability to inhibit bacterial growth is less effective than other strains.

In conclusion, this study is significant because it demonstrates that high value-added products can be produced using a simple extraction method, such as the maceration of tree bark which is considered a waste product of the timber industry. Only in this way, it should be tested with further studies supporting its applicability in nutraceutical and cosmetic products in order to prevent damage caused by oxidative stress in the human organism and more. Finally, another important result of this study is that the commercially imported product substitution was made in Kastamonu, which includes the Ilgaz and Küre national parks.

KEYWORDS: Pine Bark Extracts, *Pinus nigra*, Vitamin P, Phenolic compounds, Antioxidant, Antibacterial, Heavy Metals.

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GENİŞLETİLMİŞ ÖZET

YÜKSEK LİSANS TEZİ

FENOLİK BİLEŞİKLER İÇEREN KARAÇAM (*Pinus nigra*) KABUĞU EKSTRAKTLARININ İNCELENMESİ

MERILYN AMLANI

KASTAMONU ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ
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DANIŞMAN: Dr. Öğr. Üyesi Senem YETGİN

Günümüzde görülen sanayileşme hızındaki artış ile orantılı şekilde oluşan kimyasal kirlenme ve buna bağlı kirlenmeye maruz kalma süresi ve miktarındaki yükseliş, insan sağlığını ciddi anlamda etkilemektedir. Toksik, kanserojen, pestisit gibi kimyasalların doğaya direkt ya da dolaylı olarak karışması, konuya duyulan önemin kamuoyunda hassasiyetine neden olmaktadır. Bu çekincelerin giderilmesi ve sadece sağlığın korunması için değil; aynı zamanda vücudun zindeliği ve daha uzun yaşama çabası amacıyla yapılan çalışmalarda bitkilerden yararlanmak bilinen en temel yaklaşımdır ve bu yaklaşım insanlık tarihi kadar da eskidir.

Bunun neticesinde, günümüzde gıdalara yönelik ilgi, yaşamı ve büyümeyi sürdürmek için gerekli besinleri sağlamaktan, çeşitli hastalık türlerini önlemeye veya gerçekten iyileştirmeye doğru kaymıştır. Dahası, son teknolojik gelişmeler, nüfusun yaşam tarzı değişiklikleri ve dünya çapındaki sosyo-ekonomik eğilimleri, artan sağlık yararları olan gıdalara olan ihtiyacı göstermektedir. Sağlıklı beslenmenin ötesinde sağlıksız beslenme alışkanlıkları olan insanlar için bile metabolik reaktif oksijen türlerinin zararlı etkilerini bertaraf etmek için destekleyici ürünler önerilmektedir. Kısacası, beslenmenin kısa yoldan oksidatif stresi azaltması istenmektedir. Konu dâhilinde potansiyel olarak az işlenmiş “doğal” olarak tabir edilen mamullere olan ilgi fazlası ile artmıştır. Sonuç olarak; bitkisel kaynaklı katkılar, terapatik etkileri üzerine halen çalışmalar devam etmesine rağmen, alternatif tıp olarak kabul edilip, sentetik ilaçlara kıyasla günümüzde takviye amaçlı kullanım alanı bulmuş ve bir sektör haline gelmiştir.

Zamanın başlangıcından beri insanlar bitkileri sadece yiyecek ve barınak yapmak amaçlı değil, aynı zamanda içeriğindeki biyoaktif maddeler sayesinde çeşitli hastalıkları tedavi etmek amacı ile doğal tedavi yöntemi olarak da kullandılar. Çeşitli alanlarda önemli olan bu biyoaktif maddeler bitkilerde önemli metabolik süreçler sonucunda, farklı kimyasal reaksiyonlar sonucunda oluştuklarından; geniş çeşitliliğe sahiptirler ve birincil (*primer*) ve ikincil (*seconder*) metabolitler olarak sınıflandırılırlar. Bitkiler; birincil metabolizmalarda insan beslenmesinin yanı sıra bitkilerin büyümesi ve gelişmesi için gerekli olan karbonhidratlar, proteinler, lipitler ve diğer organik asitler gibi birincil metabolitleri oluştururlar. Öte yandan, bitkilerin ikincil metabolizmaları, aynı zamanda terpenler, nitrojen içeren kimyasallar ve fenolik bileşikler gibi ikincil metabolitlerin üretilmesine de katkı sağlar. Bu bileşikler, bitkinin büyümesi ve gelişmesi için doğrudan önemli değildir, ancak cezbedici, tozlayıcı ve hatta bitkileri kendilerine zarar verebilecek her şeyden koruyan ekolojik işlevleri açısından önemlidir. Ayrıca bu bileşikler özellikle fenolik bileşikler farmakolojik, kozmetik ve gıda sanayi gibi farklı alanlarda da yaygın olarak

kullanılmaktadır. Fenolik bileşikler, anti-enflamatuar, antikanser, anti-proleferatif, antidiyabetik gibi çeşitli terapötik fonksiyonları ve en önemlisi antioksidan kapasiteleri nedeniyle farmakolojik alanda aktif bir bileşen olarak yaygın olarak kullanılmaktadır. Fenolik bileşiklerin oksidasyona yatkın yüksek doymamış yağ asidi içeren gıda ürünlerinin korunmasında sentetik antioksidanlara alternatif olarak kullanılabilecek antioksidan kapasiteleri nedeniyle gıda endüstrisinde de kullanımı yaygınlaşmıştır. Bu nedenle, biyolojik etkileri ve doğal kaynakları açısından, bu maddeler üzerinde giderek artan sayıda çalışma yapılmaktadır.

Fenolik bileşikler, otlar, çalılar, ağaçlar ve diğerleri dahil olmak üzere çeşitli bitki türlerinde bulunur. Bunlar, sıklıkla olası fenolik bileşik kaynakları olarak kullanılan çam ağaçları gibi türleri de içerir. Fenolik bileşiklerin bolluğu açısından çok dikkat çeken bir çam ağacı türü *Pinus pinaster* ve *Pinus maritima*' dır. Bu ağacın kabuğu, büyük bir proantosiyanidin bileşikleri kaynağıdır ve ticari olarak *Pycnogenol* (PYC) ticari adı altında diyet takviyeleri olarak satılmaktadır. Bu çam ağacının talep görmesi ve popüleritesi ile diğer çam ağacı türleri de potansiyel bir fenolik bileşik kaynağı olarak keşfedilmiştir. Bunlardan biri *Pinus nigra*' dır. Türkiye' de en çok bulunan ikinci çam türü olan *P. nigra*, özellikle Kastamonu Küre ve Ilgaz Milli Parklarında yaygındır. Kastamonu, 4.7 milyon hektarlık bir orman alanına ve ahşap endüstrisindeki önemli çam ağacı türlerinden birine sahiptir. *P. nigra*, Türk geleneksel ilaçlarında solunum, mide ağrısı, sırt ağrısı ve daha pek çok rahatsızlığı tedavi etmek için hali hazırda kullanılmaktadır.

Fenolik bileşikler, çeşitli bitki kısımlarında bulunabilir. Çeşitli araştırmalara göre, iğneler, kozalaklar, kökler ve özellikle kabuk gibi çam ağacı bileşenleri olası fenolik bileşik kaynakları olarak kullanılabilir. Ahşap sektöründeki atık yan ürünün % 9-15' ini oluşturan çam ağacı kabuğu, atıkların azaltılarak katma değeri yüksek fenolik bileşiklerce zengin ürün eldesi için kullanılmak adına ideal bir kaynaktır. Fenolik bileşiklerin izolasyonu için geleneksel ve geleneksel olmayan ekstraksiyon yöntemleri gibi farklı yöntemler kullanılmaktadır. Geleneksel olmayan yöntemler, daha kısa ekstraksiyon süresi, daha az solvent kullanımı ve diğer yönler açısından avantajlı olsa da, maserasyon gibi geleneksel yöntemler, basitliği ve erişilebilirliği nedeniyle günümüzde hala yaygın olarak kullanılmaktadır.

Bu çalışmada, *P. nigra* kabuğu özütleri (PNBE) fenolik bileşikleri, antioksidan aktivitesi, antibakteriyel aktivitesi ve ağır metal kompozisyonu araştırılmış ve karakterize edilmiştir. PNBE, MAC ekstraksiyonu kullanılarak ekstre edilmiştir. Ekstraksiyon prosedüründe, Toplam Fenolik İçerik (TPC) ve 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioksidan kapasitesi üzerindeki zaman, sıcaklık ve çözücü konsantrasyonlarının etkileri, RSM kullanılarak optimize edilmiştir. EY (%6.4 ± 0.66 d.w), difüzyon katsayısı (D_{eff}) (1.01×10^{-12} m²/s), TPC (42.56 ± 1.13 mgGAE/100g d.w), DPPH (IC₅₀ 6.24 ± 0.04 µgAAE/ml) ve FRAP (18.42 ± 0.56 mgAAE/100g d.w) antioksidan deneyleri, sabit katı/sıvı oranı (100 g/L) ve partikül boyutu (0.250-0.500 mm) ile %60 etanol, 240 dakika ve 60°C' de optimize edilmiş parametreler. RP-HPLC-DAD analizi, 2,5 dihidroksibenzoik asit, mirsetin, kateşin, naringin ve ferulic asid' in toplamın %19.42' sini, %19.33' ünü, %16.88' ini, %11.04' ünü ve %10.15' ini temsil eden fenolik bileşime ana katkıda bulunduğunu ortaya koymuştur. Fenolik bileşiklerin nicel miktarı, diğerleri ise %10' dan azını temsil etmektedir. Uçucu organik bileşiklerin analizi için SPME-GC-MS için sırasıyla %8.30, 15.71, 19.00 ve 7.92 toplam alanla dekan, dodekan, tetradekan ve heksadekan gibi 4 temel kimyasal madde tespit edilmiştir. Eser miktarda da olsa; kadinen sekiterpen dahil olmak üzere fenol ve terpenler varlığı da belirlenmiştir. PNBE' nin antibakteriyel özellikleri de değerlendirilmiş ve antibakteriyel kapasiteye sahip olduğu, ancak bazı noktalarda bakteri gelişimini durdurma yeteneklerinin diğer türlere göre daha az etkili olduğu görülmüştür. Ayrıca, PNBE' nin genel kalitesini sağlamak için ağır metal bileşimleri de değerlendirilmiştir. As, Cd, Cr, Cu ve Pb gibi ağır metaller PNBE' de sırasıyla 192 ± 3.49, 16.65 ± 0.05, 94.85 ± 0.61, 177.62 ± 1.22

ve 215.82 ± 0.53 ppb konsantrasyonlarında belirlenmiştir. Bu konsantrasyon seviyeleri Dünya Sağlık Örgütü (WHO), Avrupa Birliği (EU) ve Codex Stan 193-1995 tarafından belirlenen limit değerlerinin çok altındadır. Bu nedenle PNBE, insan sağlığını tehlikeye atabilecek ağır metal toksikolojisi açısından güvenlidir.

Bu çalışmanın bulgularına dayanarak, PNBE'nin farmakolojik kullanımlar için çok önemli olan üstün bir fenolik bileşik kaynağı olduğu sonucuna varılabilir, özellikle *in-vitro* analizin antioksidan yeteneği hakkındaki bulgusu. Farklı miktarlarda ve farklı faktörlere bağlı olarak farklı fenolik bileşikler de PNBE üzerinde etkilidir. Tamamlanan çalışmaya göre, RSM tekniği kullanılarak PNBE' nin ekstraksiyonu için ideal koşullar elde edilmiştir. Bu optimizasyon tekniği, farklı ekstraksiyon faktörlerinin yoğunlaştırılması için etkili bir yaklaşım olarak belirlenmiştir. PNBE' de ağır metallerin varlığı, insan sağlığı için risk oluşturabilir çünkü çam türleri özleri birçok tıbbi uygulama için gereklidir. Ek olarak, PNBE'nin antibakteriyel kapasiteye sahip olduğu, ancak bazı noktalarda bakteri gelişimini durdurma yeteneklerinin diğer türlere göre daha az etkili olduğu bulunmuştur.

Sonuç olarak bu çalışma; kereste sanayi atığı olarak adlandırılan ağaç kabuklarının sadece maserasyon gibi basit ekstraksiyon prosesi ile; katma değer açısından yüksek mamul imalini mümkün hale getirebileceğini göstermesi açısından önemlidir. Sadece bu sayede insan organizmasında oksidatif stresin neden olduğu hasarları ve dahasını önlemek için nutrasötik ve kozmetik ürünlerde kullanılabilirliğini destekleyici ileri çalışmalar ile test edilmelidir. Son olarak bu çalışma ile ticari olarak ithal edilen ürün ikamesinin Ilgaz ve Küre milli parklarını sınırları içerisinde bulunduran Kastamonu özelinde yapılmış olması diğer bir önemli sonuçtur.

ANAHTAR KELİMELEER: Çam Kabuğu Özütleri, *Pinus nigra*, vitamin P, Fenolik Bileşik, Antioksidan, Antibakteriyel, Ağır Metaller

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols and Units

mgAAE/100g or $\mu\text{gAAE/ml}$	: Ascorbic Acid Equivalent
mgCE/g	: Catechin Equivalent
CV%	: Coefficients Variation
CFU/g	: Colony Forming Unit
C_A	: Concentration of Solute
β_0	: Constant in RSM
mgCyaE/g	: Cyanidin Equivalent
D_{AB}	: Diffusion Coeffecients
r	: Diffusion Radius (Distance)
D_{eff}	: Effective Diffusion Coefficients
m^2/s	: Effective Diffusion Coefficients (Units)
eV	: Electronvolts
mgGAE/100g	: Gallic Acid Equivalent
mgGE/g	: Glucose Equivalen
GHz	: Gigahertz
IC ₅₀	: Half-maximal Inhibitory Concentration
B	: Intercept
kPa	: Kilopascal (Pressure)
MPa	: Megapascal (Pressure)
Ln (Y)	: Natural Nmuber of Experimental Data
∂	: Partial Derivative
bar	: Pressure
R ²	: Regression Coefficients
$\beta_i, \beta_{ii}, \text{ and } \beta_{ij}$	: Regression Coefficients for the Response Model
ε	: Residual associated to the experiments in RSM
TAX/g	: Taxifolin Equivalent
T	: Time
W	: Watts

Abbreviations

ABTS	: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	: Analysis of Variance
Ace	: Aceton
MeCN	: Acetonitrile
BHA	: Butylated Hydroxyanisol
CCRD	: Central Composite Rotatable Designs
DAD	: Diode Array Detector
DES	: Deep Eutectic Solvent
DPPH	: 2,2-diphenyl-1-picrylhydrazyl Assay

DP	: Degree of Polymeration
DOE	: Design of Experiments
EtOH	: Ethanol
EU	: European Union
EY	: Extraction Yield
FRAP	: Ferric Reducing Antioxidant Power Assay
FC	: Folin-Ciocalteu
GC-FID	: Gas Chromatography-Flame Ionization Detector
GC-MS	: Gas Chromatography-Mass Spectrometry
HIV	: Human Immunodeficiency Virus
ICP-OES	: Inductively Coupled Plasma Optical Emission Spectrometry
IUPAC	: International Union of Pure and Applied Chemistry
LLE	: Liquid-Liquid Extraction or Solvent Extraction
MAC	: Maceration
MeOH	: Methanol
MAE	: Microwave-Assisted Extraction
MAC-MD	: Modified Maceration (Optimized)
MAC-NM	: Normal Maceration
NP-HPLC	: Normal-Phase High Performance Liquid Chromatography
OB	: Old Bark
ORAC	: Oxygen Radical Adsorbance Capacity
ROS	: Reactive Oxygen Species
RSV	: Respiratory Syncytial Virus
RSM	: Response Surface Methodology
RP-HPLC	: Reverse Phase High Performance Liquid Chromatography
PCs	: Phenolic Compounds
PBS	: Phosphate Buffer Solution
PBE	: Pine Bark Extracts
PNBE	: <i>Pinus nigra</i> Bark Extracts
PWC	: Platinum Wilderness Certificates
PLE	: Pressurized Liquid Extraction
PHWE	: Pressurized hot water extraction
PA	: Proanthocyanidin
PAN	: Protected Areas Network
PYC	: Pycnogenol
SEM	: Scanning Electron Microscopy
S/L	: Solid-Liquid Ratio
SLE	: Solid-Liquid Extraction
SPME	: Solid Phase Micro-Extraction
SOX	: Soxhlet
SFE	: Supercritical Fluid Extraction
TBHQ	: Tert-butylhydroxyquinone
TPC	: Total Phenolic Contents
TEAC	: Trolox Equivalent Antioxidant Capacity
UAE	: Ultrasonic Assisted Extraction
VOCs	: Volatile Organic Compounds
YB	: Young Bark
WHO	: World Health Organization

1. INTRODUCTION

Humans historically used plants not only for food and shelter but also as natural medicines to treat various diseases. They are considered safe and healthy due to their natural form (Amlani and Yetgin, 2022; Dorta et al., 2014; Shaheen et al., 2022). Despite the recent development in modern medicine, the usage of medicinal plants has increased dramatically throughout the last three decades, with at least 80% of the people still utilizing them as their primary source of healthcare globally (Akther et al., 2022; Ekor, 2014; Sadiku et al., 2022). Today, medicinal plants and herbal supplements are acknowledged as a viable alternative to modern drugs, even though study on their various therapeutic properties is still ongoing. Thus, most medicines today are made almost from natural plant materials (Najmi et al., 2022; Liu et al., 2021).

Plants such as vegetables, fruits, green tea, oils, jams, fruit purée, juices, sugars, spices, herbs and other medicinal plants are natural sources of essential bioactive components known as metabolites. Metabolites in plants are represented by primary and secondary. All species of plants produce primary metabolites as part of their normal metabolic processes, which may help their growth and development, respiration, and protein synthesis. Carbohydrates, proteins, lipids, and certain organic acids are some of the primary metabolites which are also required for human body development in the form of nutrition (Chiocchio et al., 2021; Hashim et al., 2021). In response to environmental stressors and other conditions, plants may develop additional chemical substances known as secondary metabolites, such as terpenes, nitrogen-containing compounds and phenolic compounds (PCs) (the three largest classifications of secondary metabolites) (Korfii et al., 2022; Shakya, 2016; Zhang et al., 2020). Even though they do not play a part in plants' physiological or morphological development, secondary metabolites are crucial for their ecological activities since they help pollinate and protect the plants from potential threats due to their distinct aroma and astringent taste. In addition to their importance, secondary metabolites are distinctive in that they can be employed for other industrial purposes, such as flavoring, additives, and active ingredients in pharmaceutical industries

(Akula and Ravishankar, 2011). Furthermore, secondary metabolites can sometimes be used as taxonomical markers for plant classification because each plant includes a unique combination of secondary metabolites (Chen et al., 2022a). Given the importance of secondary metabolites in pharmaceutical applications, it is necessary to understand and be familiar with the fundamentals of these unique molecules found in plants.

Modern-day human diets contain significant amounts of PCs isolated from plants (Rana et al., 2022). Due to their possible uses as natural flavoring, colorants, and antioxidants, as well as their numerous health benefits, they are of significant interest. However, their antioxidant defense mechanisms have drawn more and more attention over time amid all their potential applications (Stagos, 2019). PCs are found in different vegetables, fruits, and dietary supplements (Cádiz-Gurrea et al., 2014). They have several biological actions, including antibacterial, anticarcinogenic, anti-inflammatory, and antiviral effects and are potent free radical scavengers. These biological properties are primarily attributed to the PC's capacity to fight free radicals and act as antioxidants (Parham et al., 2020).

Plants are the primary source of essential PCs, and trees are among them (Elansary et al., 2019). Trees are a type of plant with a complex, woody stem covered with bark, a longer lifespan, and a comparatively robust structure compared to other types of plants. The trees, which are anatomically most susceptible to the activity of infections, have abundant quantities of bioactive components. These bioactive components are predominantly present in the trees' sprouts, fruits, leaves, needles, and bark (Szwajkowska-Michalek et al., 2020). Many woody plants, including green tea, apples, berries, cacao beans, and many others, contain well-known PCs such as proanthocyanidin or condensed tannins (Hollands et al., 2018; Ma et al., 2022a). However, they are frequently found in two plant sources: white pine (*Pinus maritime*, *Pinus pinaster*) and grape seed (*Vitis vinifera*) (Adam and Newair, 2022; Cádiz-Gurrea et al., 2014; Habib et al., 2022; Hussein et al., 2022; Rauf et al., 2019; Thornfeldt, 2022; Ugoeze and Odeku, 2022; Wang et al., 2022b).

In the past, the bark of pine trees was traditionally removed during processing. It is viewed as undesired waste material in the wood industry, and around 9-15% of the total wood employed is treated as waste (Kumar et al., 2021a; Lee et al., 2020). Recently, pine bark has been widely used in obtaining PCs essential for nutrition, health, medicine (Li et al., 2015), and even cosmetics (Trehan et al., 2019). PCs are isolated from different parts of pine trees, including needles, seeds, cone, and barks, by using a variety of organic solvents (Dziedziński et al., 2021). A commercially available food supplement from pine bark is already known as *Pycnogenol* (PYC) (Saonere et al., 2021).

PYC is a proanthocyanidin-rich extract manufactured from pine bark trees, a PC-rich source (Pals et al., 2022). It comprises flavonoids such as catechin and epicatechin, procyanidins ranging between 60-75% oligomeric taxifolin, and some phenolic acids such as gallic acid, caffeic acid, and ferulic acid as the minor components (Alonso-Esteban et al., 2021; Csikós et al., 2021; Giglio et al., 2018; Rohdewald, 2018). The term PYC is from Horphag Research Ltd., UK, Geneva, Switzerland is a patented name for Pine Bark Extract (PBE) derived from the bark of the French maritime pine *Pinus pinaster* (originally as *Pinus maritima* Aiton, subspecies *Atlantica* des Villar) (Olaifa et al., 2022; Rašković et al., 2019; Simpson et al., 2019). *P. pinaster* is a pine tree with more than 100 different species and one of the most studied phenolic tree extracts (Charpin et al., 2022; Dziedziński et al., 2021). It is grown in coastal areas such as France and a few nations in northwest Africa (Chupin et al., 2013). It can also be found in Portugal, Spain, Italy, and Morocco (Mármol et al., 2019). *Pinus* (*Pinaceae*) is regarded as the most prominent genus of conifers (Jin et al., 2021) where Linnaeus initially used the term *Pinus* to refer to a collection of 10 plants in his book "*Species Plantarum* ."This tree has a variety of nutrients, minerals, PCs, and essential oils found in its seeds, needles, bark, sprout, roots, and cones (Dziedziński et al., 2021). In the United States, this commercially available herbal remedy was ranked 117th among all-selling herbal dietary supplements in mainstream retail outlets such as food, drug, and mass-market stores and top 63rd in the natural channel such as health food stores in 2017 (American Botanical Council, 2019). In addition, there are many pharmaceutical functions of PYC that have been reported: this includes it is antioxidant (Rašković et al., 2019), anti-diabetic and antiviral properties

(Ezzikouri, 2016), pro-cardiovascular (Luo et al., 2015), anti-inflammatory activity (Gulati, 2015), and antibacterial (Mármol et al., 2022) as well as in skin (Grether-Beck et al., 2016), and cognitive disorders (Schoenlau, 2021). However, PYC's potential to efficiently scavenge the reactive oxygen species such as hydroxyl radicals, superoxide radical anion, lipid peroxy radicals, and reactive nitrogen species is its most prevalent activity (D'Andrea, 2010). These are only a few of the countless free radicals that PCs have shown to neutralize both *in-vitro* and *in-vivo* by resonance (Engwa et al., 2022; Sang Min Kim, 2012). Pine trees are an important tree species in the wood industry worldwide due to their valuable wood pulp, turpentine, timber, seeds, and cambium. Cambium, the inside of the bark, is regarded as food in various nations, including Finland. They used this cambium with rye flour to make pine bark bread (locally known as *Pettuleipä*) (Cisar-Erlach, 2019; Huuhka, 2018; Nisula, 2018).

In Türkiye, there are many pine trees present, especially in Kastamonu province's Küre and Ilgaz mountains, which are designated as national parks and have Protected Areas Network (PAN) and Platinum Wilderness Certificates (PWC) (Şen and Güngör, 2018). Kastamonu province is mountainous and covered with forest to 65%, of which 79.8% is regarded as productive forest and 20.1% as degraded forest (Şen et al., 2022). The pine tree species can be found in this area are represented by five species: such as *Pinus nigra* (European Black Pine), *Pinus pinea* (Stone pine), *Pinus sylvestris* (Scots pine), *Pinus brutia* (Turkish pine), and *Pinus halepensis* (Aleppo pine) with a recorded area of 4,200.000, $\leq 100,000$, 1,200.00, 5,400.000, $\leq 10,000$ hectares, respectively as shown in Figure 1.1 from Akkemik, (2018) and Dutkuner, (2012). However, only three species are being utilized to produce timber, as The Turkish Ministry of Forestry reported. These are *P. sylvestris*, *P. brutia*, and *P. nigra* (Çakır, 2017; Dıġrak et al., 1999; Şen et al., 2022; Ustun et al., 2012). Among all the species of pine trees mentioned, *P. nigra* is one of the least studied pine tree species in terms of its biological components and properties (Milić et al., 2021; Nisca et al., 2021). Therefore, this particular study concentrated only on this pine tree species.

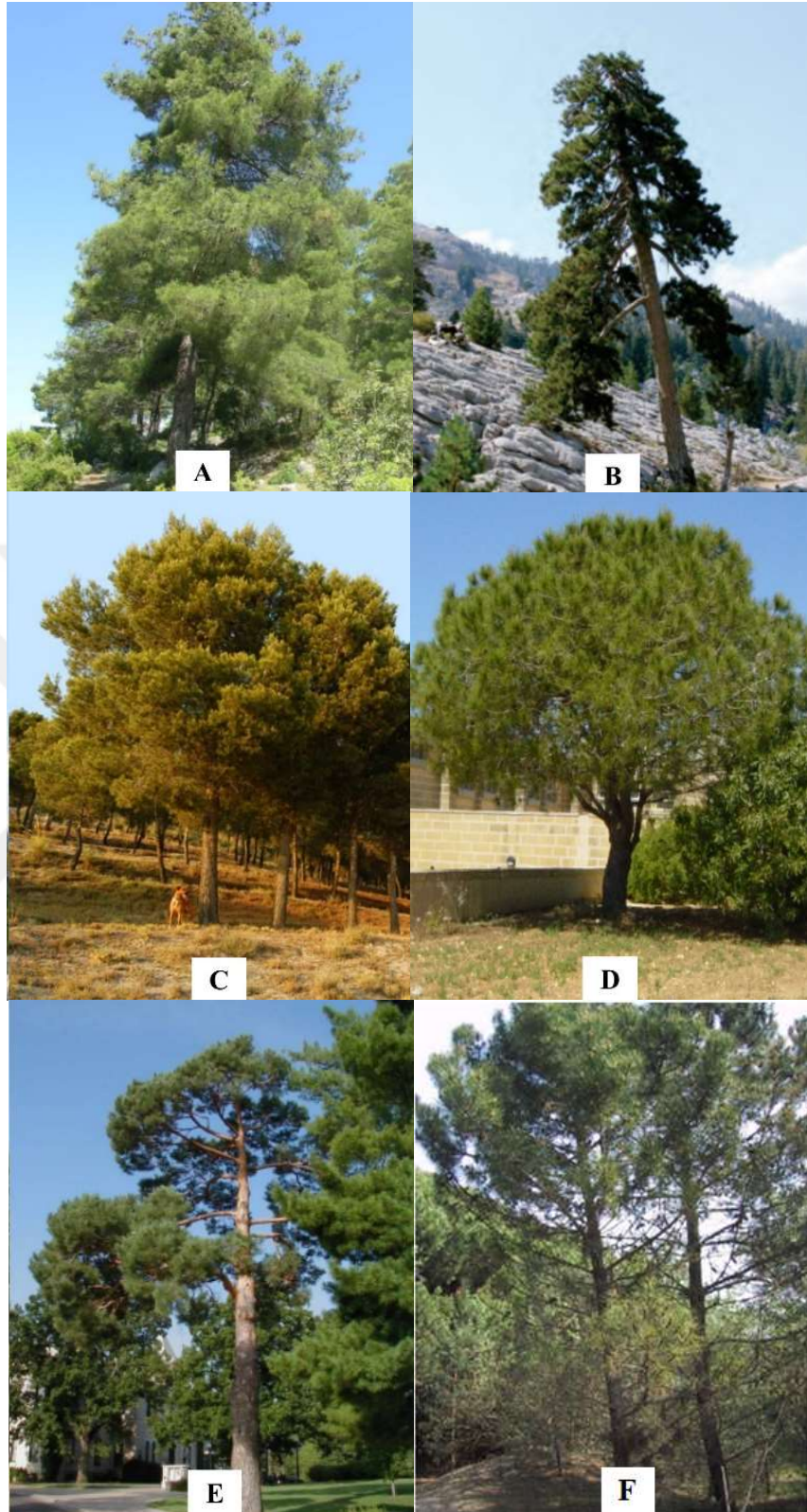


Figure 1.1. Examples of Pine Species (A: *P. brutia*, B: *P. nigra*, C: *P. halepensis*, D: *P. pinea*, E: *P. sylvestres*, and F: *P. pinaster*)

P. nigra, or European black pine, is abundant in Türkiye, especially in Kastamonu province, as shown in Figure 1.2 from the study of Atalay and Efe, (2012), which has a 4.2-4.7 million ha, and it is an essential species in the wood industry due to its good wood properties (Arslan et al., 2021; Seki and Sakici, 2017).

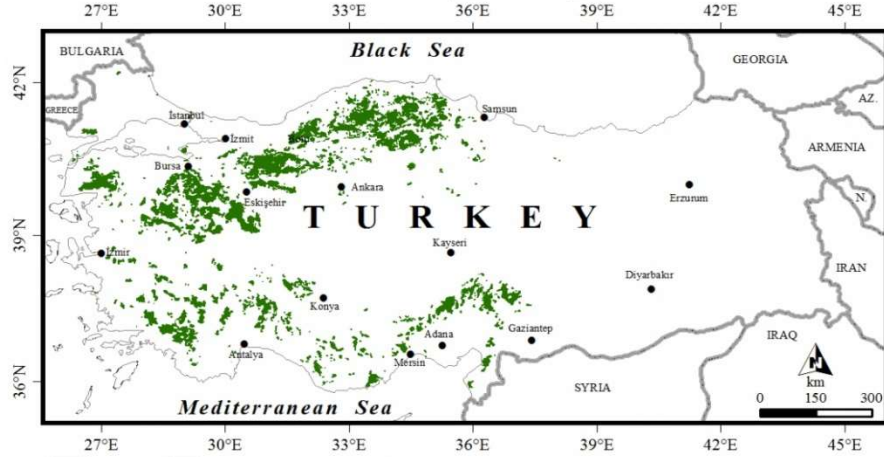


Figure 1.2. Abundance of *P. nigra* in Türkiye

P. nigra is a vast, evergreen conifer species (gymnosperm) that can reach heights of up to 20-30 meters and occasionally even up to 40 or 50 to 55 meters (Ghoreishi et al., 2016). The bark is typically found to be a light to dark greyish-brown color (Dubach et al., 2022; Jurišić et al., 2022). The tree thrives in rough terrain and sluggish soil (Puri et al., 2020). For younger trees, the cones are distinctly conical. However, as the tree grew older, the longitudinal furrows deepened, and the cones took on an umbrella-like form. The tree's needles come in pairs 8–16 cm long and 1-2 mm diameter and can be finely serrated, straight or curved. The black pine is a monoecious plant. The seed matures in the second year, opens in the third, and is propelled through the air by the wind for pollination. The cones hold between 30 and 40 gray, 5-7 mm long seeds with 19–26 mm long wings. The lifespan of the black pine can reach 400 years. According to reports, one instance in Germany, known as the "*Vier-Brüder-Baum*" from its four significant trunks, is over 1000 years old and has a girth of nearly 7 m (Enescu et al., 2016; Isajev et al., 2004). In the Irano-Turanian and Mediterranean regions, *P. nigra* is among the most widely used medicinal herbs. This pine species, which belongs to the *Pinaceae* family, is grown in Anatolia's south and west, particularly in the Toros Mountains. Due to its antiseptic properties, the turpentine found in this type of pine has been utilized in

Turkish traditional herbal medicine for years to cure conditions like respiratory and urinary illnesses. In addition, it is used in dermatology as an analgesic remedy and as back pain treatment, especially for the natives of Kastamonu province (Horozic et al., 2019; Şen et al., 2022). The essential bioactive components that are found in the bark of the extracts are known to have these medicinal properties, and the only way to obtain them is by extracting the molecules from the bark itself using a variety of extraction techniques and solvents.

PCs are among the most significant bioactive substances that receive much attention due to their numerous important applications in various sectors, most notably in the pharmaceutical, cosmeceutical and food industries (Pateiro et al., 2021). PCs are secondary metabolites created in secondary metabolisms at various plant metabolic pathways (Giri and Giri et al., 2022). They are ecologically significant because they function as attractants and pollinators, shield plants from environmental challenges, and aid in plant adaptation to their surroundings (Divekar et al., 2022). PCs are known for their ability to act as antioxidants (Alfieri et al., 2022). These compounds are not only used as active ingredients in some medications. However, they are also being used in the food industry as an alternative to synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Darmawan and Öztürk., 2022). Many foods contain many unsaturated fatty acids, prone to oxidation-induced degradation. Antioxidants must be added to food to slow down this deterioration and increase the shelf life (Mueed et al., 2022). Furthermore, consumers today prefer natural products over synthetic ones; hence, making PCs from natural plant materials is highly significant (Siddiqui et al., 2022). These essential PCs can be isolated using different methods of extraction, which are being developed, enhanced, and employed. Extraction is a standard method that has been developed to obtain PCs from both plants and pine bark. Nowadays, there are different types of extraction methods have been employed and developed, such as Ultrasound-Assisted Extraction (UAE), Pressurized Liquid Extraction (PLE), Microwave-Assisted Extraction (MAE), and Supercritical Fluid Extraction (SFE). However, traditional methods like Maceration (MAC) extraction and Soxhlet (SOX) extraction are still in use. MAC is still in use today due to its simplicity and continued ability to extract the essential bioactive components found in plants

(Oreopoulou et al., 2019), while SOX, commonly known as the hot continuous extraction technique, has the benefit of achieving complete extraction while using the least quantity of solvent (Gopalasatheeskumar, 2019). In the extraction process, the mass transfer laws of the molecules are described as the driving force, and the diffusion of the PCs from the solid material to the solvents triggers it (More et al., 2022). Furthermore, the final content of PCs mainly depends on several factors, including time, temperature, solvent type, and concentrations, affecting how well PCs are extracted from plants (Dulyanska et al., 2022). Therefore, it is essential to define and enhance the extraction method depending on samples to guarantee the best results. After the extracts have been successfully obtained from the plant materials, it is crucial to assess the extraction efficiency to measure the quantities that have been extracted and the characterization of the individual compounds to determine their full potential. Thus, numerous analysis methods are used to analyze the plant extracts.

Several techniques, including spectrophotometric and chromatographic methods, have been used for the analysis of the PCs from the extracts. The Folin-Ciocalteu method (FC) is the most widely used technique for calculating plant extracts' total amount of phenolic contents (TPC) using spectrophotometric analysis. FC is the most widely used methodology for measuring PCs in virtually all foods and plant materials globally (Munteanu and Apetrei, 2021). However, this assay lacks sufficient specificity because other reducing compounds, such as amino acids, proteins, and others, could substantially impact the ability of FC protocol to measure the TPC (Ciulu et al., 2018). Hence, characterizing the extracts by high-performance liquid chromatography (HPLC) is crucial in determining the composition of phenolic contents (Cádiz-Gurrea et al., 2014). HPLC is one of the popular chromatographic methods used to characterize the extracts fully. In addition, gas chromatography-mass spectrometry (GC-MS) is also used to characterize the PCs from plant materials (Singh and Sharma, 2022; Abdulkader et al., 2021). Numerous bioactive compounds exhibit antioxidant and antibacterial activity, and to assess their potentiality, *in-vitro* antioxidant and antibacterial analysis are used. *In-vitro* antioxidant analysis such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and oxygen radical

adsorbance capacity (ORAC) are frequently employed to evaluate antioxidant capacity (Cádiz-Gurrea et al., 2014), whereas, for *in-vitro* antibacterial analysis screening process such as disc diffusion method are used due to its easy process (Tan and Lim, 2015). In addition, it is also essential to identify any heavy metal elements that may be present in the extracts to ensure that they are free of toxicity because it is well-known that plants grow in nature and that heavy metals are everywhere (Kumari and Mishra, 2020). Inductively coupled plasma-optical emission spectrometry (ICP-OES), frequently used to assess heavy metals, is one of the most powerful methods for quickly analyzing many elements because of its excellent sensitivity (Sharma, 2020). Furthermore, just as important as the investigation of the extracts themselves is the examination of the structural and morphological changes in the surface of the extracted materials before and after the extraction. This can be analyzed using scanning electron microscopy (SEM). SEM is one of the most powerful technologies for studying surface morphology because it provides high-resolution images of the surface's components. (Sobhani et al., 2020).

Hence, the main objectives of this study were to use a by-product of the forest industry in Kastamonu, Türkiye, such as the *P. nigra* bark, as a potential source of PCs and to establish an effective and affordable method for extracting PCs by optimizing the MAC extraction for *P. nigra* Bark Extracts (PNBE). Using the optimized parameters, it aimed to determine the diffusion coefficients, EY, characterized bioactive components by HPLC and SPME GC-MS, measure the antioxidant potential and antibacterial activity, and evaluate the heavy metals composition of PNBE. Furthermore, this study aimed to determine the surface structural changes brought about by the extraction methods on the bark sample. Finally, the product to be produced in this study was aimed to be a potential source of income in the province of Kastamonu, Türkiye, most especially for the people living in the area where *P. nigra* bark is available and where only considered as waste in the wood industry.

2. LITERATURE REVIEW

Natural resources such as plants and trees have since been used by humans for a variety of reasons, including food, shelter, and even for the production of medications that are essential for survival (Jain et al., 2019). Natural products have inspired interest not just for academic purposes, but also for practical industrial applications such as in pharmaceuticals (Laird and ten Kate, 2019), glues (Mukherjee et al., 2019), oils (Martakos et al., 2019), plastics (Muneer et al., 2021), dyes for fabrics (Salsabillah and Arumsari, 2021), waxes (Aguieiras et al., 2019), fragrance (Sharmeen et al., 2021), flavouring (Lugo-Flores et al., 2021), and food additives or as functional food ingredients (Chamorro et al. 2022; Wedamulla et al. 2022) due to the presence of their essential bioactive compounds. These reasons explain why, since then, researchers have been fascinated by these essential bioactive compounds and have actively researched their chemical properties for different purposes.

2.1 Plants as a Natural Sources of Essential Bioactive Compounds

Plants and trees are essential primary producers worldwide. They produce several essential bioactive components with numerous pharmacological effects that are essential for maintaining human's health (Süntar, 2020; Wilkinson, 2019). The two metabolic processes in plants are primary and secondary metabolisms (Hussein and El-Anssary, 2019) as shown in Figure 2.1 which is adapted from Sinha and co-workers (2019). All metabolic processes required for plant cell viability are referred to as primary or basic metabolisms and this is where primary metabolites are produced. On the other hand, secondary metabolism is where the secondary metabolites are produced as a result of the breakdown of different substances in primary metabolisms through different pathways that are essential to the overall plant organism's survival. Unlike primary metabolites, secondary metabolites have no impact on a plant's fundamental metabolism, such as growth, reproduction, photosynthesis and/or any other primary function of the plant's cells (Twaij and Hasan, 2022). Even so, secondary metabolites play crucial ecological roles as they may promote and act as pollinator attractants, helps the plants to adjust to their

environments, and most importantly, protect plants from anything that could harm them (Jan et al., 2021). Over time humans and animals have learned to harness many of these plant compounds to serve their own needs.

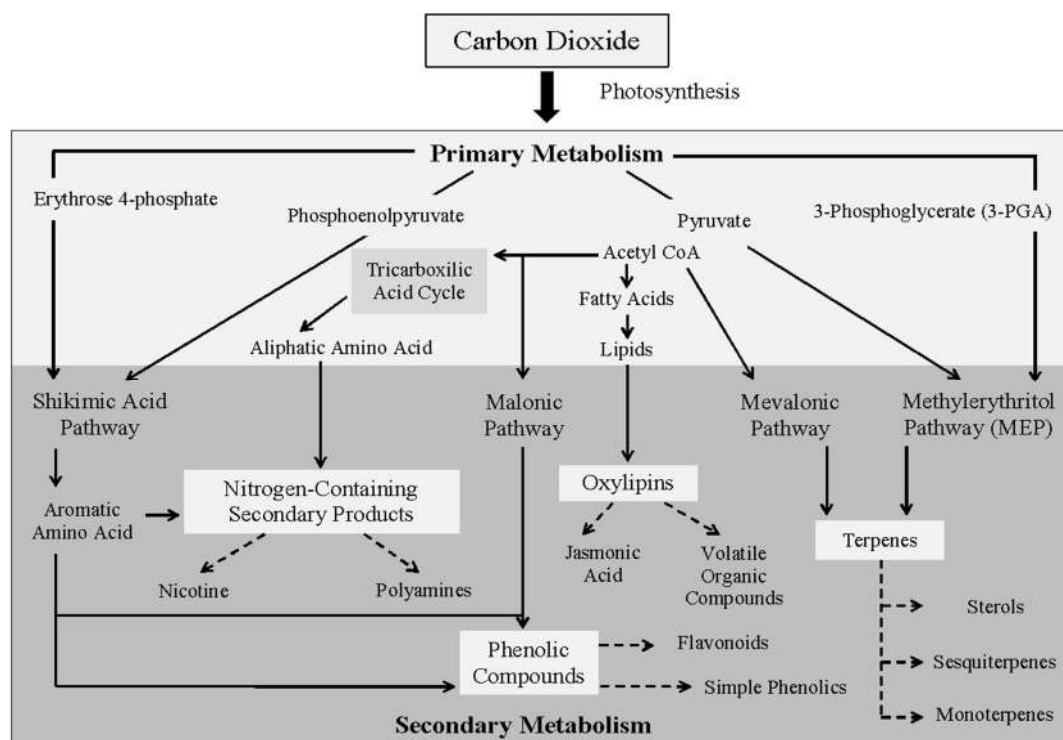
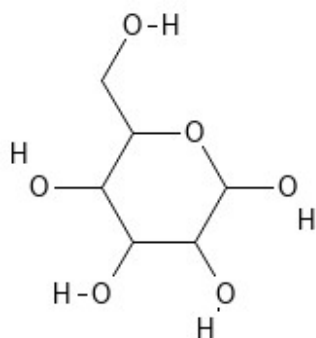


Figure 2.1. Primary and Secondary Metabolism

2.1.1 Plant Primary Metabolites

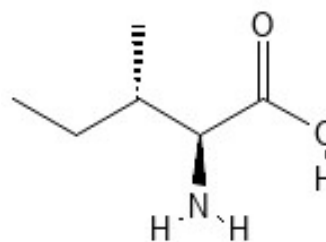
Plants produce primary metabolites as part of their regular metabolic activities, which begin when the required nutrients are present in a growth medium and occur during the active developmental phase. These are essential for the basic primary metabolisms of the cells, that are directly related to growth, reproduction, and development as well as sustaining the body's regular physiological activities. Usually, primary metabolites are produced, including carbohydrates, lipids, proteins, and organic acids as shown in Figure 2.2 (Zaynab et al., 2019). In addition to the molecules already involved in primary metabolism, secondary metabolism uses modified biosynthetic pathways from primary metabolite to create secondary metabolites in response to environmental stressors that help plants to adapt to their surroundings.

Carbohydrate



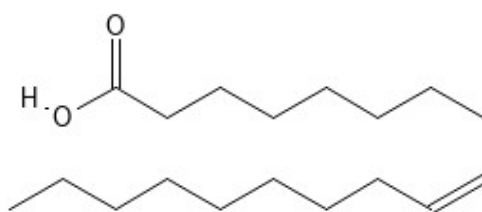
(Glucose)

Amino Acid



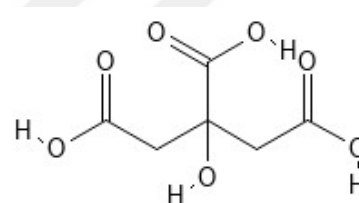
(Isoleucine)

Fatty Acid



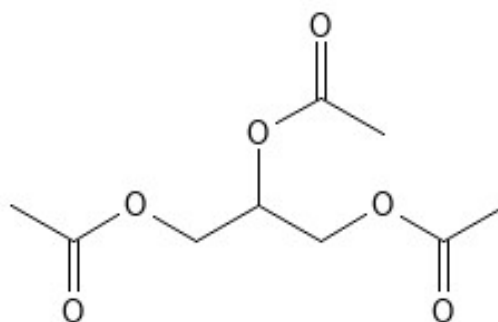
(Oleic Acid)

Organic Acid



(Citric Acid)

Lipids (Tryglycerides)



(Triacetin)

Figure 2.2. Examples of Primary Metabolites Present in Plants

2.1.2 Plant Secondary Metabolites

Part of plant's metabolic process involves the synthesis and breakdown of primary metabolites into various complex compounds along various pathways to produce secondary metabolites in reaction to various stressors, to fulfill crucial physiological roles in the establishment of symbioses, pollination, and the provision of structural elements for the lignified cell walls of vascular tissues. These metabolites are also vital to the pharmaceutical industry (Bondam et al. 2022), cosmetics (Soleimani et al., 2022), and the manufacturing of dyes (Khataee et al., 2022), scents (Li et al. 2022), food additives (Chamorro et al., 2022), medicines (Ghorbani et al., 2022), and dietary supplements (Guerriero et al., 2018). The lack of these metabolites in plants does not result in the death of the plant, in contrast to primary metabolites. However, they could have a long-term negative impact on the plant's ability to reproduce, survive, and even the appearance. These molecules are exclusive to specific plant species, and for each species growing in a distinct environment, they differ in terms of quality and quantity. Consequently, they may occasionally function as taxonomical markers. Furthermore, they also protect the plants from different species of animals (Jain et al., 2019). Over 2,140,000 secondary metabolites (Lakshmaiah et al., 2022) are categorized based on their wide range of chemical structures, components, functions, and biosynthetic pathways. They are represented by terpenes, nitrogen/sulphur containing (alkaloids), and PCs are secondary metabolites as shown in Figure 2.3 together with some of their examples (Chiocchio et al., 2021; Thirumurungan et al., 2018; Twajj et al., 2022).

Secondary Metabolites		
Terpenes	Nitrogen Containing Compound	Phenolic Compounds
• Monoterpenes • Sesquiterpenes • Diterpenes • Sesterterpenes • Triterpenes • Sesquaterpenes • Tetraterpenes • Polyterpenes	• Alkaloids • Cyanogenic Glucoside • Non-Protein Amino Acid	• Coumarin • Furano-Coumarin • Lignin • Flavonoids • Isoflavonoids • Tannins

Figure 2.3. Major Classification of Secondary Metabolites

2.1.2.1 Terpenes

Terpenes are the primary division and largest class of secondary metabolites in plants that are entirely made of carbon and hydrogen. (Chen et al., 2018). They are produced by two of the plant's biosynthesis processes, the methylerythritol and mevalonate pathways. Terpenes and terpenoids are distinct from one another, despite the fact that some authors used these two terms interchangeably. Terpenes are hydrocarbon compounds with usually between 10 and 15 carbon atoms, when modified they are called terpenoids in which the methyl groups are either shifted or deleted when hydrocarbon molecules are added with oxygen (Reyes et al., 2018). Terpenoids are composed of five isoprene carbons and are categorized by its fundamental structure and functional divisions, such as mono with two units, sesqui with three, di with four, tri with six, and so on. These molecules are ubiquitous since they are present in all living things (Nagegowda and Gupta, 2020). They assist plants in producing flavorful, fragrant, and colorful flowers, fruits, and leaves. Terpenoids are also crucial for the growth development of plants, survival against pests and other predators, and defense against fungal infections and illnesses. In animals, they act as precursors to steroid and sterol synthesis (Cox-Georgian et al., 2019). Artemisinin as shown in Figure 2.4 is used to treat malaria (Pinheiro et al., 2018), vincristine, and taxol, which treats cancer (Gezici and Şekeroğlu, 2019) are some examples of terpenoids in pharmaceuticals areas. Plants like *Elephantopus scaber* known as elephant foot plant have been shown to have antidiabetic properties since they contain Triterpenoids (Rusdi and Efendi, 2021).

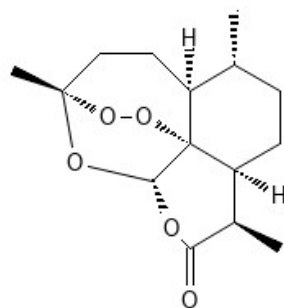


Figure 2.4. Structure of Terpene: (Artemisinin)

2.1.2.2 Nitrogen containing compounds

Another classification of secondary metabolites is known as '*Nitrogen Containing Compounds*'. These compounds are represented mainly by Alkaloids (they have a heterocyclic ring with at least one nitrogen atom), as the major groups of nitrogen containing compounds (Bribi, 2018). Alkaloids are incredibly important in terms of their pharmacological or therapeutic effects on individuals since they affect the central nervous system (Lin et al., 2020) and have some sort of psychological impact on people (Kurgat et al., 2016). The compounds that plants produce and synthesize usually serve to deter animals from eating them, but occasionally they don't, as in the case of a number of alkaloids that are widely abused by people, including mescaline and other indole alkaloids that cause hallucination effects like psilocybin, ibogaine, and dimethyltryptamine (Gonçalves et al., 2021; Mallarino, 2022). Therapeutic effects of alkaloids serve as anesthetics, cardioprotective, and anti-inflammatory agents, these therapeutic effects are just some of the essential uses of alkaloids worldwide. Alkaloids are common in market and they are popularly known as aconitine, boldine, caffeine, ephedrine, harmine, morphine, nicotine (Figure 2.5), pilocarpine, quinine, strychnine (Adejoke et al., 2019; Heinrich et al., 2021). Plant families such as *Fabaceae*, *Rutaceae*, *Solanaceae*, *Papaveaceae*, *Menispermaceae*, *Asteraceae*, *Loganiaceae*, *Rubiaceae*, *Apocynaceae*, *Berberidaceae*, *Boraginaceae*, *Chenopodiaceae*, *Euphorbiaceae*, and *Lamiaceae* contains the majority of alkaloids. However, because they contain lots of alkaloids, they could cause dermatitis (Janjić, 2021). Alkaloids are mostly concentrated on certain plant components, such as leaves, bark, or roots (Awuchi, 2019). Among all known alkaloids present in trees, cinchona is the most well-known and they are mostly found in the *Rubiaceae* family's Andean genera *Cinchona* and *Remjia* (Raza et al., 2021).

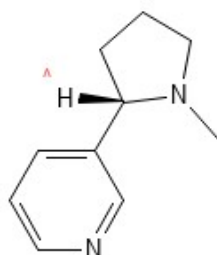


Figure 2.5. Alkaloid: (Nicotine)

In addition to this bioactive compounds, plants also synthesized PCs for their overall survival in their settings. These compounds are made up of different important substances that are employed for numerous applications, including cosmetic and medicinal ones. Due to its widespread occurrence in plants and significant applications, it has emerged as a crucial area of study. Hence, this compounds are also the focused of this study.

2.2 Phenolic Compounds in Plants

PCs are ubiquitous in nature meaning they can be found everywhere PCs comprise the bulk of phytochemicals (Shahidi et al., 2019). They synthesize in plants through the shikimic acid and phenylpropanoid pathways (Laura et al., 2019). Only a few of the numerous phenolic and polyphenolic natural substances include quinones, flavonoids, tannins, and coumarins. These secondary metabolites, and among other things, play an important part in plants' chemical ecology, assisting in tissue pigmentation, defending against microbe infections, and more (Bhatti et al., 2022). Many of these substances are used in human medicine because of their numerous biological actions, including antioxidant, anti-agent, anti-inflammatory, anti-proliferative, and other cardiovascular disorders (Lin et al., 2016).

2.2.1 Types and Structures of Phenolic Compounds

Simple and polyphenolic compounds are two categories into which PCs are frequently divided (Vuolo et al., 2019).

2.2.1.1 Simple phenolic compounds

Simple PCs contain one phenol unit or one of its derivatives. The C₆ general skeleton is the sole depiction of simple PCs (Gan et al., 2019). A subclass of PCs is phenols. They are made up of substances with an aromatic hydrocarbon group and either a hydroxyl group or an OH bond attached directly to them (Swallah et al., 2020).

Carbolic acid as shown in Figure 2.6 A, often known as phenol, is the most basic substance in this family and has only one hydroxyl group connected to a benzene ring (Białecka-Florjańczyk et al., 2018). Sir Joseph Lister, a British surgeon, first used this simple substance, which was first extracted from coal tar in 1834, in his ground-breaking study on the utilization of antibiotics to lower hospital infections (Andriani and Kaminska, 2021). It is still an active ingredient in several oral analgesics even if it is no longer utilized in surgical operations (Ayres, 2021).

Pyrocatechol as shown in Figure 2.6 B is dihydroxybenzenes representative of simple PCs that is commonly known as catechol. It is a common building block in the synthesis of organic compounds. H. Reinsch discovered catechol in 1839 by extracting catechin from *Mimosa catechu* (*Acacia catechu* L.f) juice (Özgen, 2011). Fruits and vegetables naturally contain small levels of catechol as well as the enzyme polyphenol oxidase. The colorless catechol reacts with air to produce reddish-brown benzoquinone derivatives just like what happens when a potato or apple is chopped (Chen, 2021). According to claims, benzoquinone has antibacterial properties that delay rotting in fruit and other plant tissues (Pongprasert and Srilaong, 2017). In its 1993 recommendations for the nomenclature of organic chemistry, the International Union of Pure and Applied Chemistry (IUPAC) suggested using the name pyrocatechol. Pyrocatechol is found in small amounts (>0.01-0.06 mg/g extracts dried weight) in the leaves, flowers, and stems of *Crithmum maritimum* L. (Pereira et al., 2017). In addition, when phenol is joined by a carboxyl group, a phenolic acid is produced.



Figure 2.6. Examples of Simple Phenolics

2.2.1.2 Phenolic acids

Phenolic acids can be distinguished from other types of acids when a carboxyl group is attached to a phenol (Marchiosi et al., 2020). Despite having a relatively simple structure and getting less attention unlike flavonoids, still these compounds have proven to be very useful in medicinal applications (Kumar and Goel, 2019). For example, salicylic acid is a member of this class. It regularly appears in over-the-counter topical remedies for acne and, by chance, was the original aspirin precursor (Mahmud and Rosen, 2019). The two types of phenolic acids are hydroxybenzoic acids and hydroxycinnamic acids (Skroza et al., 2022).

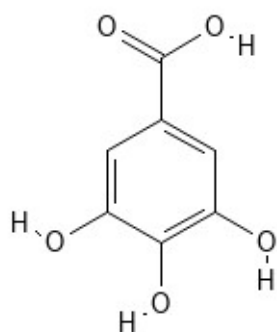
2.2.1.2.1 Hydroxybenzoic acids

The hydroxyl group is substituted for the benzoic acid in hydroxybenzoic acids. They are phenols that have had a carboxylic acid functional group substituted in a direct bond with the phenol group (Godlewska-Żyłkiewicz et al., 2020).

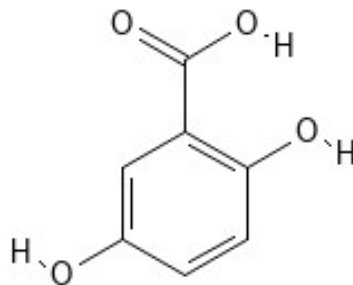
Gallic acid (3,4,5-trihydroxybenzoic acid) as shown in Figure 2.7 A became the most widely used type of hydroxybenzoic acid (Bai et al., 2021). It has several pharmacological actions that have been demonstrated, including those that are antibacterial, anti-inflammatory, antioxidant, anti-obesity, and many more (Lee and Yoon, 2022). However, its antioxidant effects are the best known biological functions (Mehraban et al., 2019). Additionally, its effective anti-inflammatory properties are receiving more and more attention recently (Shabani et al., 2020). Gallic acid can be isolated from many plants, such as in fruits, nuts, and other crops (Akanbi et al., 2019).

Another example of hydroxybenzoic acid is 2-5 hydroxybenzoic acid (Gentisic acid) as shown in Figure 2.7 B, which is a small result of the metabolism of aspirin and a benzoic acid derivative. In addition to being widely present in plants like *Citrus* spp., *Gentiana* spp., *Vitis vinifera*, and others. 2,5-dihydroxybenzoic acid is also present in many traditional alcoholic beverages and herbal remedies. In addition, it is also well known for being a powerful inhibitor of fibroblast growth factors (Abotaleb et al., 2020). Gentisic acid has been linked to positive impacts on the health of human and

others including antioxidant activity, antigenotoxic, anti-inflammatory, antibacterial, hepatoprotective, and neuroprotective (Abedi et al., 2020)



A: (Gallic Acid)



B: (Gentisic Acid/2-5 hydroxybenzoic Acid)

Figure 2.7. Examples of Hydroxybenzoic Acids

2.2.1.2.2 Hydroxycinnamic acids

Hydroxycinnamic acids, produced when a double bonded carbon separates the phenol ring from the carboxylic acid functional group. Examples are caffeic acid, ferulic acid, p-Coumaric acid, as shown in Figure 2.8 and many more (Godlewska-Żyłkiewicz et al., 2020).

Caffeic acid is the primary hydroxycinnamic acid because it is crucial in lignin production (Espinoza-Acosta et al., 2022). They can be found in a variety of plants, not just those that make up 75% of the total hydroxycinnamic acids, such blueberries, tomatoes, apples, and apricots (Rudra et al., 2021). Caffeic acid is a by-product of the biosynthetic process of hydroxylation of the coumaroyl ester of quinic ester (Park et al., 2021). This substance can lessen aflatoxin production (Owumi et al., 2022) and has shown promise as an anti-inflammatory, carcinogen, immunomodulator, antioxidant (Torres et al., 2020) and antibacterial (Khan et al., 2021).

Alike caffeic acid, ferulic acid also shows promising anti-inflammatory, carcinogen, immunomodulator, and antioxidant (Torres et al., 2020). They are abundant in oats, tomatoes, and sweet corn, all of which help lower blood pressure and protect the skin

(Paulo et al., 2017). Like vitamin E, ferulic acid also prevents phospholipid membranes' oxidative chain reactions caused by UV exposure (Burke, 2009).

p-Coumaric acid a hydroxycinnamic acid that is synthesized from phenylalanine and tyrosine, which serve as precursors. Many edible plants naturally contain *p*-coumaric acid, which has been shown in numerous animal models to have antioxidant properties that reduce oxidative stress, inflammatory responses and antimicrobial activity (Ferreira et al., 2019). The solubility of *p*-Coumaric acid affected by the solvent types, they are more soluble in organic solvents rather than water (H₂O) or aqueous buffer (Ferreira et al., 2019). The best structural fit for *p*-coumaric acid is as a competitive tyrosinase inhibitor, which catalyzes important events in the melanin biosynthesis pathway (Boo, 2019).

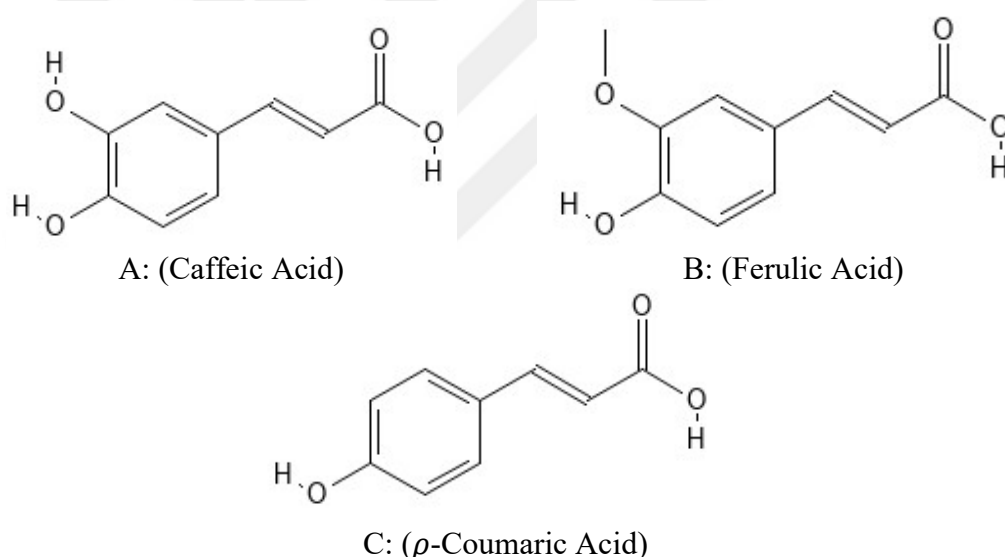


Figure 2.8. Examples of Hydroxycinnamic Acids

2.2.1.3 Coumarins

Similarly, coumarins like cinnamic acids contain a C₆ and C₃ skeleton, but in addition, they have an oxygen heterocycle as part of the C₃ region of their structure (Corso et al., 2020). Tonka bean (*Dipteryx odorata*) also known as '*kumaru*' in French, in 1820 was the first source where the chemical coumarin was first identified (Lončarić et al., 2020). In addition, commonly coumarin contributes to the aroma of

newly cut grass through its scent (Xiao et al., 2022). The development of phytoalexins, which have broad-spectrum antibacterial and antioxidant effects, is enhanced during plant infections and coumarins perform a critical protective function for plants (Yadav et al., 2020). Such biologically active coumarins include esculetin (Garg et al., 2022), scopoletine (Sakthivel et al., 2022), and umbelliferon (Amin et al., 2020). However, coumarins are not extensively dispersed among botanical families but are most frequently found in the bean, carrot, citrus, and daisy families (Vázquez-Fresno et al., 2019).

As shown in Figure 2.9, warfarin and dicoumarol are two examples of 4-hydroxycoumarins that have potent biological anticoagulant effects (Pereira et al., 2018). Warfarin with a brand name of coumadin continues to be employed as a rodent insecticide, as it was intended to be (Bell, 2022; Manao et al., 2022). It is also used in medicine to stop blood clots from forming and moving around the body (Lateef et al., 2018). The distinction between poison and medicine is frequently blurred when it comes to warfarin, as it is with many other drugs (Sharifi-Rad et al., 2021). As for dicoumarol, it was first discovered after an investigation of silage, which is moldy or fermented hay containing sweet clover and was linked to hemorrhagic bleeding disorders in the cattle that fed on it (Nisar et al., 2020). Silage is sweet clover that has undergone the chemical transformation of coumarin into a 4-hydroxycoumarin by a number of fungal species (Ruiz et al., 2022).

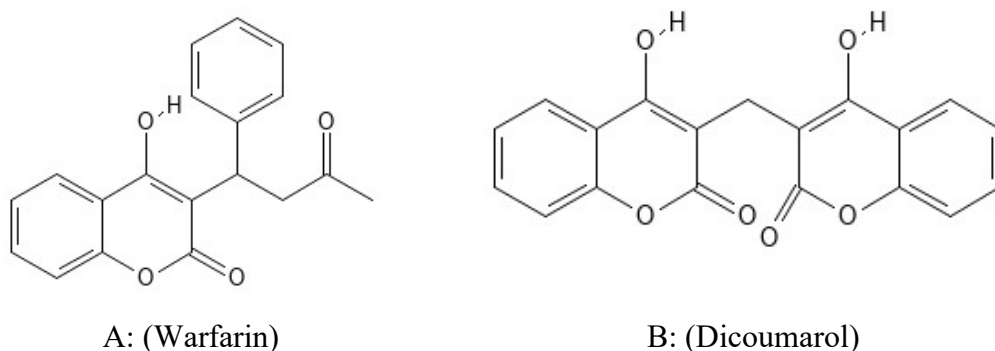


Figure 2.9. Examples of Coumarins

2.2.1.4 Polyphenols

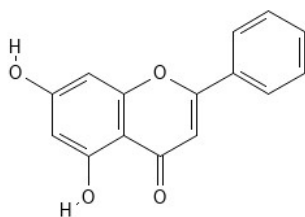
Polyphenols lack nitrogen compounds and are made up of several phenolic units. They have a C15 general skeleton representation. These compounds are typically categorized as phenolic cycle numbers and the presence of carbon atoms in the basic skeleton. (Hano and Tungmunthum, 2020). Both phenolic acids, like cinnamic and benzoic acid, and their derivatives, were produced by plants. (Sarker and Oba, 2020).

2.2.1.4.1 Flavonoids

Flavonoid is another group belonging to PCs which attained greater attention among all types of PCs including catechin and epicatechin (Kumar and Goel, 2019). Plants produce flavonoids in response to infection, and several of them are effective against bacteria and viruses, including the Human Immunodeficiency Virus (HIV) and Respiratory Syncytial Virus (RSV) (Wang et al., 2020). Plant flavonoids frequently appear as plant pigments that are frequently yellow or orange, and “*flavis*” is the Latin word for yellow. Because they are found across the whole plant species, most likely, one of their primary responsibilities in nature is to change the flavor of plants or draw insects and birds to their color (Wang et al., 2022a). Flavonoids have significant dietary importance since they are PCs, which are also high in antioxidant. Some flavonoids are exceedingly bitter and astringent, while others are intensely sweet (Mlcek et al., 2021). The six classes of flavonoids are flavones, flavonols, anthocyanins, isoflavones, flavanones, and flavanols (Ku et al., 2020). Flavones, flavonols, and all flavonoids have a few structural similarities with slight variations with each group (Liu et al., 2022b; Shen et al., 2022). In Figure 2.10, a ring C that is connected to the carbon atom C2 of a ring B is known as a flavone, flavonol, flavanone, or anthocyanin. The double bond between the carbons at locations C2 and C3 and at the fourth position of the C ring where ketone group is present is make up the fundamental structure of flavone. A flavone and a flavanone are only distinguished by the lack of C2=C3. This resulted in a saturated C ring in the flavanones. Flavonols and flavones share the same structure only that flavonols include an extra hydroxyl group at the C3 position. Regarding anthocyanin, the C ring's initial oxygen atom bears a positive charge and lacks a ketone group at the

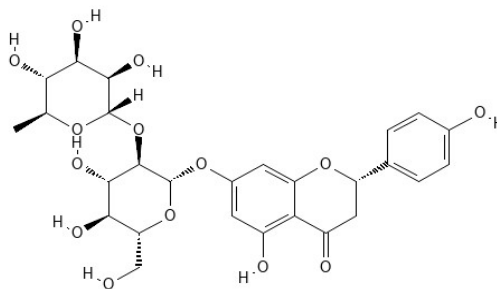
fourth position. Anthocyanins is an oxidized flavonols that give fruits like blueberries and fruit-based beverages like red wine their vivid blues, deep reds, and purple hues (Kalt et al., 2020; Nurtiana, 2019). Anthocyanins have been demonstrated to be particularly effective in scavenging oxygen radicals by *in-vitro* and *in-vivo* antioxidant analyses (Chensom et al., 2020; Yang et al., 2019). Then, if the C is linked to ring B's C3, the chemical is then classified as an isoflavone. On the other hand, Chalcone, a unique class of flavonoids, has a 3-carbon linear chain instead of a ring connecting its rings A and C. Furthermore, flavanol and flavanone also belong to flavonoids. The fourth position of the C rings' ketone group and the lack of C2=C3 are what set flavanol apart from other compounds. Lastly, Flavanone is the 3-hydroxy derivatives of flavanones (Ku et al., 2020).

Flavone



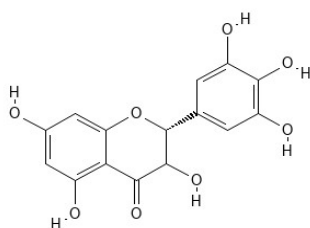
A: (Chrysin)

Flavonone

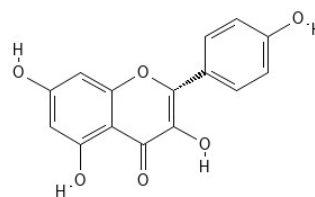


B: (Naringin)

Flavonol

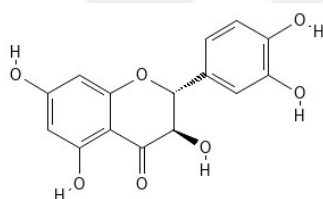


C: (Myricetin)



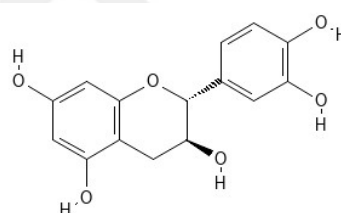
D: (Kaempferol)

Flavanonol



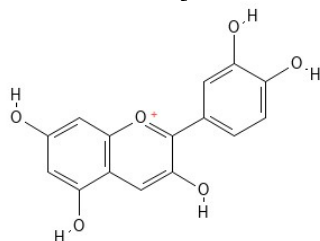
E (Taxifolin)

Flavanol



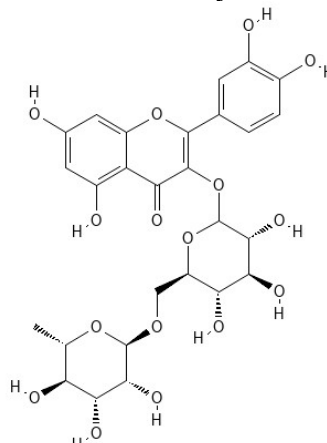
F: (Catechin)

Anthocyanin



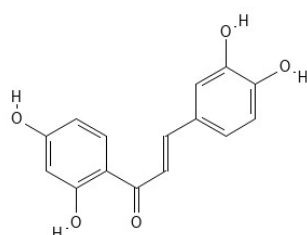
G: (Cyanidin)

Flavonoid Glycoside



I: (Rutin)

Chalcone



H: (Butein)

Figure 2.10. Examples of Flavonoids

2.2.1.5 Tannins

Many foods include tannins because of their well-known astringency and bitterness. The capacity of tannins to attach to proteins is one of their important characteristics, and it is for this reason that tannins have been used for tanning leather (Molino et al., 2019; Watrelot and Norton, 2020). As shown in Figure 2.11, tannins are H₂O-soluble and are split into two main categories: condensed or non-hydrolyzable tannins, and hydrolyzable tannins (Faye et al., 2021). Simple PCs are the source of tannins, which are then esterified with a sugar to form a bond. Additionally, non-hydrolyzable tannins or condensed tannins are generated by the hydrolysis of flavonoids. The base can hydrolyze them to produce simple acids and sugars (Bodoira and Maestri, 2020; Dwivedi, 2022). These tannins are mainly utilized for their astringent qualities. They can be helpful as anti-diarrheal medicines (Wagarachchi et al., 2020), for example, the dog rose produces a gall as a defense mechanism against pests; this gall is high in ellagitannin concentration (Olennikov et al., 2021) and is used in a medicinal tea in Anatolia to cure diarrhea (Ayati et al., 2018). However, protein and other nutrients may be absorbed less effectively after tannin excessive consumption. Additionally, they may trigger the precipitation of additional substances and herbal medicines (Ojo, 2022; Sarkar et al., 2022).

One of the key ingredients in the ellagitannin family that gives pomegranates their antioxidant benefits is ellagic acid (Figure 2.11 A). Ellagic acid is a hydrolyzable tannin and dimeric derivative of gallic acid. It can be found in certain berries, pomegranates, grapes, walnuts, and a particular species of edible mushrooms in their natural form or as a complex inside ellagitannin. Ellagic acid can potentially have antiproliferative and apoptotic effects in cancer treatment due to its capacity to prevent malignant cells (Evtyugin et al., 2020; Ríos et al., 2018).

Another typical tannin type is proanthocyanidin, which is abundant in pine bark extracts and commercially available in the name of PYC (Bhardwaj et al., 2021). Proanthocyanidins (PA) are condensed tannins found in various plants such as flowers, leaves, seeds, nuts, fruits, bark, and roots (Maleš et al., 2022; Sharma and Beniwal, 2022). Due to the astringent properties of proanthocyanidins, they are used

by plants to protect themselves from various pathogens and predators (Przybylska et al., 2022). They are classified as polymeric PAs ($DP > 10$) and oligomeric PAs ($DP \leq 10$) based on their polymerization degree (DP) (Ge et al., 2016). The most common subclass of PA is procyanidin, composed of flavan-3-ols such as (+) catechin and (-) epicatechin (Mustafa et al., 2022). Two types of Procyanidins are based on monomers' linkages: A-type and B-type procyanidins; they are both classified as having a single interflavan bond. However, A-type procyanidins have second ether that links the A-ring hydroxyl group and C2 of the A-ring with A1 and A2 as the typical A-type procyanidin (Figure 2.11 B). B-type procyanidins have one interflavan bond between the C4 of the B-ring and C8 or C6 of the C-ring. B-type is the sufficient procyanidins in which the B1, B2, B3, and B4 are the procyanidins that mainly occurred (Figure 2.11C) (Blanck et al., 2022; Rue et al., 2018).

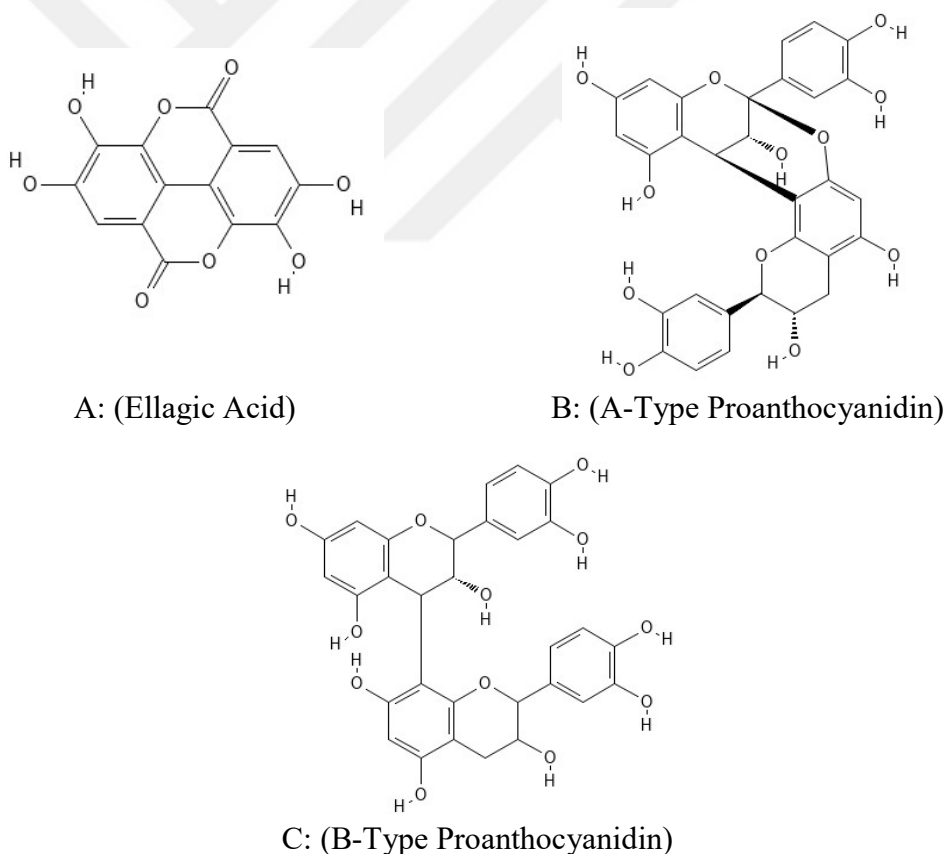


Figure 2.11. Examples of Tannins

2.2.1.6 Other phenolic compounds

Some PCs, in addition to the major categories of PCs mentioned above, are also found in plant-based products. They are stilbenes, lignans, and lignins. Stilbenes are PCs in which two double-bonded carbons connect the phenol units. Similarly, lignans, another phenolic substance, consist of two phenol units joined by four carbons. On the other hand, the lignins are composed of PCs linked to one another by carbon chains and polymers with high molecular weight (Al Mamari, 2021).

2.2.2 Sources and Uses of Phenolic Compounds

Several uses for different PCs are listed in the literature, including uses in cosmetics, food, dyes, and many other areas (Albuquerque et al., 2021). However, PCs are of great interest because of their various pharmacological applications (Tungmunthum et al., 2018). Table 2.1 demonstrates the varying significance of several of the mentioned PCs for pharmacological applications. Table 2.1 demonstrates that PCs are well known for their numerous biological functions, including those that are stated there. However, the most potential properties of these PCs are their ability to scavenge radical species hence their strong antioxidant capacity.

The crucial elements mentioned above are just a few examples of PCs, which are abundant in plants because many plant species contain different PCs with essential medicinal properties (Azmir et al., 2013). Therefore, it is essential to isolate them to evaluate their potentiality. Several extraction procedures have been developed recently to isolate PCs from their sources and identify their active components, which may be necessary for therapeutic development.

Table 2.1. Some Phenolic Compounds and their Bioactive Properties in Plants

Phenolic Compounds	Sources	Bioactive Properties	References
• Hydroxybenzoic Acid • Gallic Acid • 2-5 Dihydroxybenzoic Acid	• Grapes, tea and wine • Apples, avocados, pears, bitter melon, batoko plums, kiwis, blackberries, and some mushrooms	• Neuroprotective, anti-inflammatory, antibacterial, antioxidant, and anticancer properties. • Hepatoprotective, neuroprotective, antigenotoxic, antibacterial, antioxidant, and anti-inflammatory actions	• Heleno et al., (2015); Wang et al., (2019) • Abedi et al., (2020)
• Hydroxycinnamic Acid • Ferulic Acid • <i>p</i> -Coumaric Acid • Caffeic Acid	• Rice, fruits, mushrooms, and cereal grains • Tomatoes, carrots, coffee, garlic, grapes, and spinach • Propolis, coffee, and mushrooms	• Activities include antimicrobial, antioxidant, antitumor, and antidiabetic, anti-inflammatory, antimicrobial, antioxidant, antiviral, antitumoral, and neuroprotective activities. • Anti-viral, antioxidant, anti-microbial, anti-inflammatory, and antitumor properties.	• Heleno et al., (2015) • Heleno et al., (2015) • Heleno et al., (2015); Abdel-Moneim et al., (2019)
• Flavonol • Myricetin • Kaempferol • Quercetin	• Berries, herbs and vegetables • Cabbage, cauliflower, propolis and spinach • Pears, apples, berries, and onions	• Anti-inflammatory, antitumoral, and antioxidant properties. • Activities that are anti-inflammatory, antioxidant, antitumor, and cardioprotective. • Activities that are anti-inflammatory, antioxidant, antitumor, and cardioprotective.	• Zhang et al., (2015) • Durazzo et al., (2019) • Durazzo et al., (2019)
• Flavanone • Naringin	• Grapefruit and citrus fruits	• Activities that are anti-inflammatory, antibacterial, antioxidant, anticancer, and anti-lipidemic.	• Durazzo et al., (2019); Tajaldini, et al., (2020)

Cont. Table 2.1

Phenolic Compounds	Sources	Bioactive Properties	References
• Flavone • Chrysin	• A wide variety of herbs, honey, and propolis	• Antioxidant characteristics. anticancer, pro-apoptotic, inhibits angiogenesis, inhibits metastasis, and modulates immunity	• Mani and Natesan, (2018)
• Flavononol • Taxifolin	• Seeds and bark (<i>pinus or larix</i>)	• Activities that are anti-inflammatory, antibacterial, antioxidant, antiviral, anti-tumor, and neuroprotective.	• Zu et al., (2014)
• Chalcone • Butein	• Lacquer tree, camel's tail, aromatic rosewood heartwood, and cashew tree stem bark	• Properties that fight cancer and tumors.	• Tuli et al., (2021)
• Anthocyanin	• Purple carrots, grapes, and berries	• Cardioprotective, anti-proliferative, anti-microbial, antioxidant, and anti-inflammatory properties.	• Durazzo et al., (2019)
• Tannin • Ellagic Acid	• Berries	• Anti-inflammatory, antioxidant, and antitumoral activities.	• Durazzo et al., (2019)
• Simple Phenolics • Pyrocatechol	• Lichen <i>Cetraria islandica</i>	• Antimicrobial, Antioxidant, genotoxic and anticancer activities	• Grujičić et al., (2014)
• Triglyceride • Triacetin	• Wine grape	• Antifungal agents and plasticizers	• Darabian et al., (2020) • NCBI, (2022)
• Flavonoid Glycoside • Rutin	• Fruits and vegetables, including citrus fruits and berries, as well as medicinal herbs like buckwheat and asparagus	• Vasoactive, hypolipidemic, antiplatelet, antispasmodic, antibacterial, antiprotozoal, anticancer, antiallergenic, antiviral, and antihypertensive.	• Patel and Patel, (2019) • Chua, 2013)

2.3 Extraction Process

Extraction is a crucial primary step in the study of PCs from plant materials in order to make sure that the target compounds in the plants are not vanish or damaged during the processing of the plant material (Yahya et al., 2018). The basic definition of extraction is the process of removing components from a liquid or solid combination while employing a liquid solvent. The solubility behavior of each component in the corresponding phase or the rate of solute diffusion through the liquid boundary layer at the interface regulates the mass transfer of molecules from phase 1 to phase 2 (Sapkale et al., 2010). The extract phase and the raffinate phase are the two phases that result from the extraction step. The active molecules are concentrated in the extract phase, while the remains of the extraction are in the raffinate phase or the residuals of the solute. After the extraction process, the solvent is obtained using a different separation technique to regenerate it (Seader et al., 2016). The basic extraction process is shown in Figure 2.12 where component B is extracted using solvent C from the combination of components A and B. The components of the feed are A+B, C is the solvent, A(+B) is the raffinate, and C+B are the extracts. Here the phenomenon arises when a solvent penetrates a solid and dissolves the required molecules; this is where internal diffusion takes place. Then, external diffusion occurs as it moves from the solid material's interior to the liquid solvent. This process is what the mass transfer principle which mostly controls the extraction process (Mosca et al., 2018).

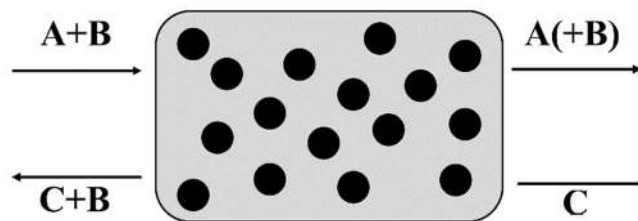


Figure 2.12. Typical Step of Parallel Extraction Process

There are two types of generally used extraction method such as Liquid-Liquid Extraction or Solvent Extraction (LLE) and Solid-Liquid Extraction (SLE) (Hidayah and Abidin, 2017).

In LLE, the extraction process comprises employing the liquid solvent to separate constituents from a liquid mixture. The solvents must dissolve the desired components (Hanson, 2013). For ease of comprehension, this extraction type consists of three parts: transition component A, solvent B, and carrier C. Additionally, LLE might be a viable option in order to separate and purify PCs since it performs well with diluted feed streams and provides a high degree of flexibility when the intended PCs are heat sensitive (Silva et al., 2018). Figure 2.13 illustrates the fundamental idea behind this approach. Component A and carrier C are mixed to create the first mixture (Feed). The feed is then combined with solvent B. It is intended that component A's solubility in solvent B be greater than its solubility in carrier C.

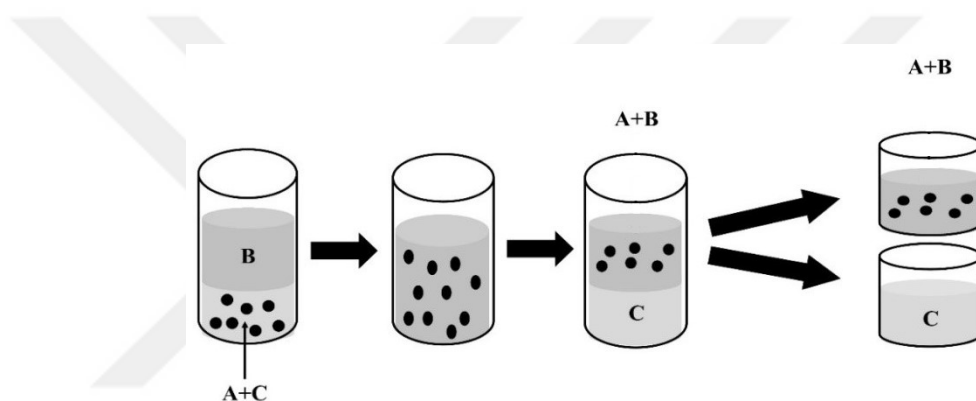


Figure 2.13. Ideal LLE Process

For SLE, the concept behind this approach is the same as it is for LLE, except the extraction happens from a solid solute which enables the separation of the solid substance from the solvent-soluble components. In this approach, the solute must be transfer to the solvent phase by desorption or by dissolution under the influence of physical or chemical forces. Then, in the liquid phase in bulk, the solvent and solute mixture diffuses to the surface of the solids and spreads over the particles interface which guarantee the effective extraction process. Thus, this technique has been applied frequently in plant extraction to isolate bioactive compounds from their sources. However, SLE is known for having low quantitative efficiency, hence several measures have been taken to improve it. One such measure is the use of high temperature or high pressure during the extraction process (Priego-Capote, 2021). In addition to this, a number of additional factors, such as but not limited to time, temperature, type of the solvent, concentration, solid/liquid (S/L) ratio, particle size

and extraction techniques, as well as various associated phenomena like the diffusion, convection, and solubility, have an immediate impact on the effectiveness of the extraction process and the quality of the extracts (Azmir et al., 2013; Gerke et al., 2018). Similar to LLE, other separation processes like evaporation and distillation can also be used to recover the solvent following extraction. Figure 2.14 represent the schematic diagram for the basic principle of SLE.

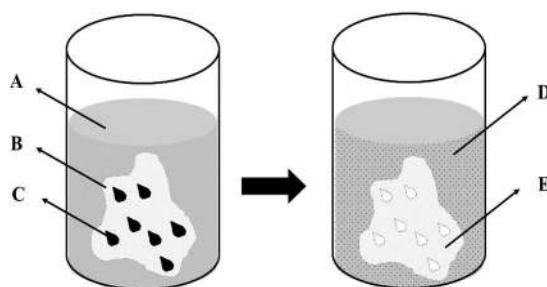


Figure 2.14. Schematic Extraction of SLE. Solvent (A), Extraction Material- Solid Carrier Phase (B), Transition Component (C), Solvent with Transition Component (D), and Extraction Remains (E).

2.3.1 Mass Transfer: Diffusion Coefficients

Mass transfer is a vital concept to understand in many scientific and technical domains. It is brought on by the polarity and chemical potential of the components' solubility (Sun et al., 2022). This phenomena occurs when a component in a mixture migrates from one phase to another due to a change in concentrations (Dawe, 2022). During the SLE, mass transfer laws rules the extraction process and takes place in two steps. First, the solvents dissolve the wanted or extractable compounds by penetrating the solid materials. Next, the desired compounds diffuse out of the solid materials and into the primary solvent (liquid). Diffusion controls this mass transfer rate throughout this process (Yiğitarıslan, 2017). The diffusion rate can be defined and best described using Fick's second law, as seen in equation (3.1) (Xu et al. 2022) and assuming that the particles have a spherical shape, then the same equation can be written according to spherical coordinates as seen in equation (3.2) (Mosca et al. 2018).

$$\frac{DC_A}{Dt} = D_{AB} \nabla^2 C_A \quad (3.1)$$

$$\frac{\partial C}{\partial t} = D_{eff} \left(\frac{1}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial C}{\partial r} \right) \right) \quad (3.2)$$

Where C_A = concentration of the compound A, D_{AB} = diffusion coefficients of the mixture of compound A (solute) and B (solvent), r = diffusion radius, and D_{eff} = effective diffusion coefficients that is mostly calculated based on the experimental determination of kinetics. Furthermore using the equation applied from the study of Mosca and co-workers (2018) and Yiğitarlan, (2017). The D_{eff} can be determine using the equation (3.3). The effective diffusivities are related to the thermal motion of molecules at temperatures above absolute zero known as the molecular diffusion coefficients or referred to as diffusivity, defined as the amount of a material that, when diffusing from one area to another with a volume-concentration gradient of unity, passes through each unit of cross section in a unit of time (Chen et al., 2022b).

$$\ln(Y) = B - \frac{\pi^2 D_{eff}}{r^2} t \quad (3.3)$$

The slopes in the experimental data (Y) may be used to calculate the D_{eff} using linear regression, t = time, and B = intercept

$$\text{Slope} = - \frac{\pi^2 D_{eff}}{r^2} t \quad (3.4)$$

$$Y = \frac{mgGAE_0 - mgGAE(t)}{mgGAE_0} \quad (3.5)$$

Where $mgGAE_0$ = equal to the maximum extractable amount of plant material using the extraction method with the highest obtained EY and $mgGAE_t$ = measure TPC at specific time.

Depending on the selected unit system, the units for the diffusion coefficient (D_{AB}) and D_{eff} in the international system are $[m^2/s]$ and $[cm^2/s]$ respectively. When the various states of matter aggregation are taken into account, this variance becomes much more significant because the diffusivities of gases often fluctuate between 10^{-5} and $10^{-4} [m^2/s]$ in consideration of the mobility of the molecules, whereas the variations for liquids are observed between 10^{-10} and $10^{-9} [m^2/s]$. In addition, in terms of the diffusivity in the solids is much smaller than the two states and approximately from 10^{-10} to $10^{-14} [m^2/s]$ (Benitez, 2022). The D_{AB} are calculated during the extraction of PCs from plant material to determine the various effects of the extraction process as well as the movements and separation of the molecules or PCs from the plant materials. Table 2.2 contains a list of some plant materials for which the diffusion coefficients have been calculated.

Table 2.2. Diffusion Coefficients of Some Plant Materials

Phenolic Compounds (PCs)	Plant Species	Diffusion Coefficients (m ² /s)	References
PCs	<i>Syzygium cumini</i> L. (fruit)	Deep eutectic solvent (DES, 1:1): choline chloride: citric acid monohydrate (40-70 °C): 5.70×10^{-12} – 10.52×10^{-12}	Sharma and Dash, (2022)
Anthocyanin		DES, 1:1: choline chloride: citric acid monohydrate (40-70 °C): 2.485×10^{-12} – 8.507×10^{-12}	
PCs	<i>Phyllanthus niruri</i> (leaves)	H ₂ O (40-70°C): 2.62 – 2.64×10^{-12}	Alara et al. (2022)
PCs	<i>Moringa oleifera</i> (leaves)	60% EtOH (30.85, 31.85, 34.85, and 35.85°C): 3.058×10^{-10} , 3.114×10^{-10} , 5.912×10^{-10} , and 10.38×10^{-10}	Albarri et al. (2021)
PCs	<i>Azadirachta indica</i> (leaves)	MeOH (65 °C): 1.14×10^{-12} to 6.85×10^{-12}	Patil et al. (2021)
PCs	<i>Hibiscus sabdariffa</i> calyces	H ₂ O (40-80°C): 2.042×10^{-12} to 2.130×10^{-12}	Alara and Abdurahman, (2019)
PCs	<i>Sterculia apetala</i> (seed)	H ₂ O (25 and 60°C): 1.15 and 2.10×10^{-11} 50% EtOH (25 and 60°C): 3.71 and 1.38×10^{-11}	Mosca et al. (2018)
PCs	<i>Capsicum annuum</i> L.	EtOH (70°C): $2.67 \cdot 10^{-10}$	Yiğitarıslan, (2017)
PCs	<i>Vitis vinifera</i> L (pomace)	50% EtOH (40°C and 6.8–47.4 W/L): 1.47 – 5.82×10^{-11} 50% EtOH (40°C and 6.8–47.4 W/L): 3.82 – 4.98×10^{-12}	Tao et al. (2014)
PCs	Milled <i>Vitis vinifera</i> L (seed)	50% EtOH (25 and 50°C): 7.2 and 9.2×10^{-13}	Bucić-Kojić et al. (2013)
Vanillin	<i>Vanilla planifolia</i> (pod)	60% EtOH (50°C): 1.91×10^{-11}	Rodríguez-Jimenes et al (2013)
p-Hydroxybenzaldehyde		60% EtOH (50°C): 1.30×10^{-11}	
p-Hydroxybenzoic acid		60% EtOH (50°C): 1.55×10^{-11}	

2.3.2 Factors Affecting the Extraction Process

The extraction process of phytochemicals, most notably the PCs, from any plant materials is significantly influenced by a number of variables that directly affect mass transfer and diffusion from solid materials. These factors—which include but are not limited to temperature, time, type of the solvent, solvent concentration, S/L ratio, particle sizes and extraction techniques—are the crucial ones that significantly influenced the extraction process (Kumar et al., 2021b; Ma et al., 2022b; Munir et al., 2018; Riciputi et al., 2018). These factors are some of the critical variables to consider during the extraction of some plant materials (Aydar, 2018).

Temperature is an important factor that greatly influence the extraction process which has the direct effects on the PCs of the extracts (Haida et al., 2022). Higher extraction temperatures change the solvent's polarity and make less polar molecules more soluble by changing the solvent's physical characteristics (Quitério et al., 2022). A temperature rise often makes compounds more soluble and weakens the interaction between the matrix and analyte, resulting in increased diffusion rates (Raspe et al., 2022). Additionally, when the temperature rises, the solvent's viscosity and surface tension decrease, allowing it to permeate the solid more effectively and speeding up molecular mass transfer rates (Doan-Nguyen et al., 2022). This cause an increase in the PCs solubility and diffusion coefficient which increased the EY (Bimakr et al., 2011; Sheng et al., 2011; Thiruvankadam et al., 2015). However, increasing temperatures too high can also affects the phenolic contents in plants due to the competition on processes such as decomposition and epimerization (Ananingsih et al., 2013; Elboughdiri, 2018; Gaona et al., 2022). In some cases, the PCs can sometimes decrease as temperature rises higher because most PCs are temperature sensitive and many are volatiles (Xu et al., 2022). In addition, temperature also promotes oxidation of the PCs in the plant materials similar to time (Hu et al., 2019).

Time is another important factor influencing the extraction process (Ahangari et al., 2021). Generally, the extraction efficiency encreases as the extraction period is prolonged by enabling the solvent to penetrate the plant tissue for a sufficient amount

of time to dissolve the solute and then move the solution to the extraction media (Míguez et al., 2022). However, a longer extraction period with the combined effects of temperature, light and oxygen causes the decrease of the PCs isolated due to its risks in phenolic oxidation resulted from the extended exposure. In addition, since PCs is sensitive that is why determining the combined effects of different factors is essential (Che Sulaiman et al., 2017). Furthermore, an increase in extraction time is also uneconomical from an industrial standpoint (Tan et al., 2013).

Types of solvents also have a significant impact in the extraction process, in addition to temperature and time. Solvents are widely recognized as the most important parameters influencing the yield and phenolic content of a specific plant material (Zannou et al., 2022). Polar solvents are usually utilized in isolating PCs from plant matrices such as methanol (MeOH), ethanol (EtOH), and acetone (Ace). These solvents are commonly utilized because they are the most effective in extracting polar chemicals such as PCs (Do et al., 2014; Ezez, 2022). Among all the polar solvents employed for PCs extraction, EtOH is commonly utilized to extract phytochemicals from plant material since it is known as being generally safe for human ingestion (Do et al., 2014; Lohvina et al., 2021). However, researches have showed that mixtures of H₂O and EtOH or other organic solvents may facilitate more than the pure EtOH or other organic solvents for better extraction of PCs (Hashemi et al., 2022; Hikmawanti et al., 2021; Premanath et al., 2011; Sánchez-Arévalo et al., 2022; Vieito et al., 2018). This may be true because the amount of EtOH in a solvent affect all of its physical properties, such as density, boiling point, dynamic viscosity, and dielectric constant. Additionally, it would also have an impact on chemical solubility, which would affect phenolic extraction (Elboughdiri et al., 2018). Additionally, because PCs include hydroxyl groups, they are classified as having medium to high polarity (Zhang et al., 2023). Hence, the extraction of PCs is preferred using aqueous EtOH (mixture of two most polar solvents). In the study of Irakli and co-workers (2018), they were successfully obtained high PCs from olive leaf using 50% concentrations of aqueous organic solvents such as EtOH and among others.

In addition to this, the S/L ratio is another factor to consider while extracting PCs. Effect of S/L ratio is yet another crucial issue to take into account. Low S/L ratio are advantageous because they allow for the use of more concentrated extracts, which is frequently the case when no saturation effects exist. This seems obvious given that, in each case, using less solvent or using more solids increased the concentration of the extracted material. These are consistent with mass transfer principles, according to which chemicals diffusion from the extracted solid material into the solvent occurs more quickly at larger S/L ratio due to a bigger concentration gradient driving mass transfer (Predescu et al., 2016). However, increasing the S/L ratio further could make mass transfer less effective (Caldas et al., 2018).

As for the particle size, it is an important consideration because it is the first step in getting the plant materials ready for extraction. The samples used for the extraction might be whole, powdered, dried, or fresh. Usually dried powdered samples are often preferred over whole fresh ones because of their higher surface area which facilitates a better contact between the samples and the solvents made possible by the decreased in particle size (Da Silva et al., 2016). That is why, in the preparation for the extraction process, the samples must be provided in small sizes by being crushed into smaller particles to give the solvent in the extraction process a large exchange surfaces and short diffusion routes to maximize the speed and potential (Rajha et al., 2014). This is because smaller particle sizes have a higher surface area, allowing for greater penetration and increased solvent-particle interaction, thus increased in the mass transfer rate. Contrarily, using a larger particle size results in a reduced relative contact area between the sample and the solvent, reducing the mass transfer rate (Qu et al., 2010). The use of samples with small particle sizes led to increase in the extraction yield (EY), but it is crucial to emphasized that these samples can also induce agglutination, which makes it harder for the solvent to permeate and leads to extraction process clogging issues in the case for unconventional extraction method (Sulaiman et al., 2019; Sumere et al., 2018). Thus type of extraction method is also as important like other factor that needs to be focus on. Different extraction techniques have different effects to the extraction process of PCs. Thus it is important to know and understand the difference between each factor.

2.3.3 Classification of Extraction Method

In plant extraction there are two general classifications such as conventional (traditional) and non-conventional (non-traditional, or green methods) methods (Alara et al., 2021). Conventional or traditional extraction techniques have been used due to their simplicity and ease of execution. Several conventional techniques such as maceration (MAC), soxhlet extraction (SOX), decoction (DEC), digestion (DIG), infusion (INF), percolation (PER), and serial exhaustive extraction (SEE) (Alara et al., 2021) are used in many laboratories as well as used on a daily basis. However, as for the purpose of this study MAC and SOX extraction were only focused. Currently MAC and SOX extraction are still widely used due to their simple, inexpensive, and accessibility (Đurović et al., 2022; Tambun et al., 2021). MAC is a simple, popular, and low-cost process where it just requires immersing a pulverized sample in the proper solvent for a prolonged length of time in a closed system at room temperature (Farooq et al., 2022; Toumi et al., 2022). Afterward, the solvent is separated from the solute material either by filtration and decantation (Ćujić et al., 2016). A further modification to MAC is called as DIG, which incorporates the use of moderate heating as long as the temperature does not impact the bioactive compounds in the plants (Alara et al., 2021). In contrast, the SOX method placed the crushed materials in a thimble in the device extraction chamber directly above the collecting flask and below the reflux condenser. The solvent is heated further to produce vapour, which circulates before passing through the condenser and eventually condensing back into contact with the samples in the extraction chamber. When the collecting chamber is full, the solvent will then spill over into the heating flask once more. This method does not require filtration because the solvent and solid materials are initially separated where the samples are placed in a thimble, a cellulose-based substance (López-Bascón et al., 2020). Due to the shorter extraction time required by SOX, it offers an advantage over MAC. On the other hand, this method might affect the other bioactive compounds because it uses a higher temperature (Alara et al., 2018). Furthermore, because these methods call for using a larger amount of the solvent to be utilized and also require labor-intensive manual processes, non-conventional methods were developed to overcome these issues.

Green methods are another name for unconventional (non-traditional) methods. They are considered alternative methods to conventional methods that operate at significantly lower temperatures and with shorter extraction times than conventional processes, and the solvents are widely regarded as safe because of the use of deep eutectic solvents (DESs). DES has earned the title of "*green solvents*", displaying exceptional qualities such as minimal or no toxicity to human health and the environment, utilization of renewable resources, lowering dangers, using less energy, sustainability, etc (Chemat et al. 2019). Whereas, conventional methods are still using conventional organic solvents such as EtOH, MeOH, Ace, and others (Kupnik et al., 2022). Despite the fact that conventional organic solvents can effectively extract both hydrophilic and hydrophobic compounds, depending on the solvent's polarity which shows highly effective extraction, there are still some environmental issues that need to be addressed (De Jesus et al., 2020). Additionally, because the majority of these solvents are volatile organic compounds (VOCs) that are derived from non-renewable resources such as fossil fuels, they have a number of disadvantages including high toxicity, high flammability, and non-biodegradability (Fuad et al., 2021). Thus, a variety of unconventional methods have been developed, enhanced, and utilized for extracting PCs over the years with the addition of green solvents and some still use organic solvents. Table 2.3 lists the distinctions between these green methods, which include pressurized liquid extraction (PLE), microwave aided extraction (MAE), supercritical fluid extraction (SFE), and ultrasound assisted extraction (UAE). Additionally, because PCs are unstable in nature, the variations between these methods have a significant impact on the extraction process. As a result, optimization of the extraction process is crucial to achieving a greater yield and higher quality of PCs.

Table 2.3. Differences between the Unconventional Methods

Non-Conventional Methods	Extraction Procedure (Conditions)	Advantage
Microwave-Assisted Extraction (MAE)	- Solvent: H₂O, EtOH: H₂O, Ace: H₂O, MeOH: H₂O - Temperature: 30-150 °C - Microwave power: 2.5 GHz, 100-1500 W - Irradiation time: 0.5-10 min - S/L ratio: 22-250	- The capability of heating a matrix both internally and externally without producing a thermal gradient. - Molecules like phenolic compounds and ionic solutions that have a persistent dipole moment are very good at absorbing microwave radiation. - Microwaves cause a sample's H₂O molecules to become internally superheated, which encourages cell disintegration and improves the isolation.
Pressurized Liquid Extraction (PLE)	- Solvent: H₂O, ethyl acetate, hexane, EtOH: H₂O, acidified H₂O - Temperature: 25-275 °C - Pressure: 20-200 bar - Time: 5-480 min - S/L ratio: 5-480	The kinetics of extraction can be increased at temperatures over the solvent boiling point but below the critical point by using high pressures to keep the solvents in a liquid condition.
Supercritical Fluid Extraction (SFE)	- Solvent: scCO₂, scCO₂:EtOH, RS-L/CO₂(%),SMR (g EtOH/g CO₂) - Temperature: 30-70 °C - Pressure: 100-400 bar - Time: 13-210 min - S/L ratio: 20-180	It uses fluids at pressures and temperatures exceeding critical levels. Because of their comparatively large density, supercritical fluids have a significant solvation power in the zone above the critical point.
Ultrasonic-Assisted Extraction (UAE)	Solvent: H₂O, EtOH: H₂O, Ace: H₂O, MeOH: H₂O, MeOH:10%HCl, EtOH:10%HCl, Ace:10%HCl, 70% MeOH:70% Ace, DES, MeOH: H₂O:HCl	- The term "ultrasound" describes frequencies between 20 kHz and 10 MHz. Diagnostic ultrasonography (2–10 MHz) and traditional power ultrasound (>10 MHz) are both used in the food sector (20–100 kHz) - In the liquid media, cavitation creates a series of compressions and rarefactions that vary the pressure and cause bubbles to form and burst. - Cavitation results in the breakdown of the matrix's cell walls, enabling the extraction solvent to penetrate deeply and enhancing mass transfer. - Correlated with improved EY, faster extraction rates, lower extraction temperatures, and less solvent usage

- scCO₂, Supercritical CO₂; RS-L/CO₂(%), volume of compressed carbon dioxide, solids, and liquids. Source: (Osorio-Tobón, 2020)

2.3.4 Optimization of the Extraction Process

Optimization is one of the first and critical process in the extraction of any plant material, which aims to evaluate the impacts of the chosen process parameters (Belwal et al., 2018). However, the optimization strategy calls for performing numerous tests with all possible parameter combinations, which is seen to be both costly and unfeasible. Therefore, statistical approach and experimental planning helps in the understanding more about the optimum conditions (Aydar, 2018). Traditional optimization methods, such as single-factor analysis, involve modifying one condition at a time while maintaining the values of all other conditions, allowing for the study of the effects of those specific parameters on the process performance (Liyana-Pathirana and Shahidi, 2005; Simić et al., 2016). However, this procedure is lengthy, arduous, require additional experimental data sets, and unable to show how the variables interact with one another (Nde and Foncha, 2020; Prasad et al., 2005; Şahin and Şamlı, 2013). All of these issues can be solved using the response surface methodology (RSM), a practical and popular method for optimization that enables the accounting of potential interaction effects between variables. This statistical tool may provide the optimum conditions that would improve a process (Khuri, and Mukhopadhyay, 2010; Majeed et al., 2016; Patel et al., 2020; Silva et al., 2007). The RSM frequently used to design experiments by minimizing the number of experiments for particular numbers of factors and levels at random; which made more advantageous than any other traditional optimization method (Dean et al., 2017; Chelladurai et al, 2021). The fundamental and essential component of RSM is the design of experiments (DOE). This seeks to choose the appropriate areas where the response should be thoroughly analyzed. Box-Behnken Design (BBD) and Central Composite Designs (CCD) are the two primary experimental designs in RSM, and in recent years, Central Composite Designs Rotatable (CCRD) and Face Central Composite Designs (FCCD) have seen widespread use in the extraction of plant materials (Aydar, 2018). Furthermore, the best fitted model of response can be obtained by emphasizing the following statistical parameters: regression p-value summarized at the 95% confidence level, regression coefficients (R^2), adjusted regression coefficients (adjusted R^2), lack of fit, coefficients variation (CV%), regression F-value (Derkyi, et al., 2011). Multiple regression and the simplified

model ($p < 0.05$) were used to analyze the experimental data (Che Sulaiman et al., 2017). Most topics pertaining to the best way to design experiments involve different mathematical models of the process, which are typically represented by polynomials with an unknown structure. The equation used to describe how different factors interact and identify crucial points, such as the lowest, highest, and saddle points, which are not given by the linear formula, is usually expressed in the second order of the quadratic formula, as illustrated in equation 3.6. Furthermore, this equation is frequently used in the designing DOE for RSM optimizations (Breig and Luti, 2021).

$$\left[Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i=1}^k \sum_{j=i+1}^k \beta_{ij} x_i x_j + \varepsilon \right] \quad (3.6)$$

Where Y = predicted response, β_0 = constant, β_i , β_{ii} , and β_{ij} = regression coefficients for the response model, x_i and x_j = independent variables and ε = residual associated to the experiments.

2.4 Characterization of Bioactive Compounds

Following the successful extraction of PCs from their sources, it is important to characterize them. This includes determining their quantitative and qualitative characteristics in order to understand their full potential. Since PCs come in such a wide diversity, it is impossible to determine their antioxidative power and content in plants using a single method. The chemical make-up of PCs, the method of extraction used, interfering chemicals, and analytical technique all have a significant impact on their measurement in plant extracts. The quantitative and qualitative examination of plant-derived PCs are represented by various experimental assays that are widely used in the research areas such as spectrophotometric assay, chromatographic analysis and many more.

2.4.1 Spectrophotometric Assay and Chromatographic Analysis for Phenolic Compounds

Traditionally, in quantifying the PCs from plants it is employed by spectrophotometric assays such as Folin-Ciocalteu (FC) assay, a method for calculating total phenolic contents (TPC). This method is popular, simple and reproducible and also serves as the most generally adopted methodology for the worldwide measurement of phenolics in practically all food and plant materials in the research areas (Munteanu and Apetrei, 2021). The FC reagent is used as a reference standard in this assay, which measures all phenolic hydroxyl groups contained in the plant extracts. The phosphotungstic-phosphomolybdenum complex, which is formed when the reference standard is combined with the plant extract, gives off a blue color (Shi et al., 2022). Using spectrophotometry, it can measure the complex's absorbance in visible light and estimate the number of PCs present in the plant extract (Widyawati et al., 2022). However, this method lacks sufficient specificity because the presence of other reducing chemicals, such as proteins, amino acids, and others, could have a substantial impact on the FC protocol's ability to determine the TPC (Ciulu et al., 2018).

Therefore, characterizing the extracts by high performance liquid chromatography (HPLC) is an important analysis to determine the composition of the PCs (Cádiz-Gurrea et al., 2014). HPLC is a type of chromatography based on the principle of dispersion of substances between the stationary phase and the liquid-containing mobile phase at high pressure (Coskun, 2016). Common reason for its use includes sensitivity, adaptability to quantitative measurements, and suitability for separating non-volatile compounds that are quickly destroyed by heat. Most significantly, it is broadly applicable to compounds that are of the great importance to business, several scientific fields, and society. Examples of these materials include amino acids, proteins, nucleic acids, carbohydrates, medicines, and insecticides (Dong et al., 2019). Both normal-phase (NP) and reverse-phase (RP) techniques are applicable in HPLC and operate under high pressure. They often have a better resolution power than standard liquid chromatography (Hamacher and Schrader, 2022). The major distinction of the NP-HPLC and RP-HPLC is related to their polarity differences in

their respective phases. For NP-HPLC it has a polar stationary phase and a non-polar mobile phase, whereas RP-HPLC is simply as the opposite of the NP-HPLC. In addition, the stationary phase of RP-HPLC is a modified silica substrate with long hydrophobic long chains, and the mobile phase is primarily H₂O, MeOH, or acetonitrile (MeCN). In contrast, the stationary phase of NP-HPLC is primarily pure silica, and the mobile phase is a non-aqueous solvent such as chloroform (Keller et al., 2022). However, the high reproducibility of the retention time, which is primarily attained by making the stationary phase non-polar, provides RP-HPLC a greater advantage than NP-HPLC (Nahar and Sarker, 2022). Furthermore, the eluents may be acidified to lessen peak asymmetry the dispersion of the analyte concentration zone. In this method, an organic acid can be added to the mobile phase, which is commonly a mixture of solvents such H₂O, EtOH, MeOH, and MeCN, to prevent the ionization of the phenolic acids (Liaudanskas et al., 2017). To prevent phenolic acids from ionizing in the mobile phase, the most often employed additions are sulfuric, phosphoric, formic, acetic, and trifluoroacetic acids. According to related studies, the C18 column is the most popular column in RP-HPLC separations of PCs (Şeker et al., 2021; Žuvela et al., 2019). Other alkyl chains, silicon dioxide or polyamide chains are also used in RP-HPLC with normal column length is between 100 and 300 mm (Liaudanskas et al., 2017).

2.4.2 Chromatographic Analysis for Volatile Organic Compounds

Another chromatographic analysis used for the characterization of bioactive compounds is gas chromatography coupled with mass spectroscopy (GC-MS). It is also a versatile method for defining and estimating the quantity of bioactive chemicals in plant materials (Varshney et al., 2023). It is a chromatographic methods where the chemical mixtures are separated in the GC components and identifies the components in its MS components at molecular levels (Qian, 2021). It is also one of the most accurate methods for analyzing environmental materials, such as identifying the presence of pesticides (Srinivasan et al., 2021) and separating VOCs in plant extracts (Gonzalez et al., 2022). The GC is based on the idea that heating a mixture causes it to split into its constituent parts. Then an inert gas such as helium is used to carry the heated gases through a column. Then, the separated compounds enter the

MS as they exit the column aperture. By measuring the mass of the analyte molecule, MS which is regarded as the sole reliable analytical detector can identify substances (Charbonnet et al., 2022; Soni et al., 2022). Furthermore, GC-MS can be also used coupled with solid phase microextraction (SPME) method for further characterization of the extracts specially for VOCs. This method is practical substitute for more traditional extraction techniques for organic and semi-organic VOCs from various sample sources. It merges extraction, pre-concentration, and sample insertion into a single phase, removes the need for organic solvents, and is incredibly quick, inexpensive, and easy (Özgenç et al., 2017). Furthermore, it concentrates organic molecules from liquid or gaseous onto a thin, fused-silica fiber that has been coated with a stationary-phase material by passively extracting organic compounds (Kyle, 2017). In SPME, there are three different extraction modes including direct, headspace, and membrane (Ji, 2022). When employing the direct method, which includes immersing the fiber in a H₂O or air sample, the analytes are instantly adsorbed onto or absorbed into the fiber coating from the sample. In the headspace mode (HS), samples are placed in vials, followed by the placement of a fiber in the air directly above the sample. Analytes are subsequently distributed from the sample via the air to the fiber coating. To preserve the fiber and to stop fouling brought on by high molecular weight chemicals and other non-volatile interferences in the sample medium, the air in the vial acts as a barrier between the fiber and the sample. Finally, the third mode employs a membrane to shield the fiber from severely polluted samples that might harm it. Furthermore, HS-SPME mode is a simple and sensitive extraction method that is widely used for the investigation of VOCs in plant extracts and food substrates without any side effects and with the combination of GS-MS is often used in flavor analysis in the food industry (Ji, 2022; Lancioni et al., 2022; Liu et al., 2022a). When the extraction is finished, SPME enables quick transmission to the desired analytical equipment such as GC-MS, where the analyte is typically thermally desorbed (He et al., 2019).

2.4.3 Antioxidant Capacity of the Phenolic Compounds

Lipid oxidation is common in food and can be avoided by adding synthetic antioxidants such as butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) (Ousji and Sleno, 2020; Zhang et al., 2015). However, their use is limited because of their potential to cause cancer (Khezerlou et al., 2022). Thus, research into natural antioxidants is growing (Vieira et al., 2022). The literature uses a variety of antioxidant assays based on methodological differences to check the antioxidant potential of plant extracts (Apak et al., 2016). The antioxidant capacities of PCs from plants were mostly evaluated using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities and many more (Ferreira-Santos et al., 2020).

The DPPH assay, is one of the most popular and widely used colorimetric assays for measuring antioxidant potential by estimating the radical scavenging activity of plant extracts (Akar et al., 2017). This method is simple, accurate, inexpensive, rapid and easy to perform. It also provides a broad screening of antioxidant activity and is based on DPPH, a stable and synthetic radical (Sirivibulkovit et al., 2018). When samples with hydrogen-donating antioxidants were present (Adjaoud et al., 2022), the DPPH solution's color changed from violet to yellow when it was exposed to extracts containing antioxidant chemicals, losing its free radical property (Gonçalves et al., 2018; Sanna et al., 2012). The results can be shown by the percent reduction and by the value of half-maximal inhibitory concentration (IC_{50}). Lower IC_{50} or SC_{50} value means having high antioxidant capacity (Jadid et al., 2017).

Another widely used antioxidant analysis is FRAP assay. It is based on the idea that antioxidants can decrease a colorless ferric complex into a blue ferrous complex at low pH by serving as electron donors (Benzie and Devaki, 2018). It is widely used in many laboratories because it requires only few reagents, is simple, and is easy to perform, yielding highly reproducible results (Agarwal et al., 2021). However, since any extractives with a redox potential of less than 0.77 V can decrease iron (III) ions, the test also measures additional chemicals in addition to antioxidants (Hofmann et

al., 2015). Furthermore, few studies also reported the combined antioxidant and antibacterial action of PCs (Değirmenci et al., 2020; Öztürk et al., 2021; Petropoulos et al., 2018).

2.4.4 Antibacterial Activity of the Phenolic Compounds

Food-borne illnesses are extremely prevalent around the world and can occasionally pose a threat to people's lives (Kocabaş et al., 2008; Todd, 2020). Both gram-positive and gram-negative bacteria exhibit antimicrobial resistance, which raises morbidity, death, and healthcare costs. It is also widely acknowledged that many of the antifungal and antibacterial drugs available today have negative side effects. Thus, pharmaceutical companies have recently spent a lot of time developing new and more potent antibiotics (Sadeghi et al., 2016). Recently, there has been an increase interest in the discovery of novel antimicrobial agents based on natural ingredients. Many naturally occurring substances found in plants, trees, spices, and herbs have been shown to have significant potential as antibacterial agents in addition to their well-known antioxidant properties (Mayekar et al., 2021; O'Connell et al., 2013; Saeed et al., 2019). Many plants including pine tree exhibit a wide range of antibacterial actions against gram-negative and gram-positive food-borne diseases based on the studies for the antimicrobial activity of various plants (Ghasemi et al., 2010).

2.4.5 Heavy Metals Composition Analysis

Heavy metals are considered toxic metals. They are well-known to be harmful to the health of humans, animals, and plants (Mahurpawar, 2015). Thus, their release into the environment must be limited, and their concentrations in various environments must be regularly monitored (Ali et al., 2019). However, due to a variety of anthropogenic activities, toxic heavy metals such as chromium (Cr), lead (Pb), nickel (Ni), cadmium (Cd), mercury (Hg), and arsenic (As) are constantly discharged into the environment (Mahar et al., 2016). Furthermore, due to the vast range of uses for plants and the fact that they grow outside where they are very susceptible to pollution, plants and trees may become polluted or absorb this type of pollution.

Thus, the World Health Organization (WHO) highly suggests checking the levels of heavy metals in the plants that will be utilized to create the other components of the finished products. Table 2.4 shows some of the permissible concentrations (ppm) levels of heavy metals in foods and plant extracts established by WHO, European Union (EU) and Codex Standard (193-1995).

Table 2.4. Permissible Concentrations of Some Heavy Metals in Foods and Plant Extracts.

Heavy Metals	Permissible Concentrations (ppm)	References
As	2 to 5	WHO (2007); Osmani et al., (2015); Ullah et al., (2022)
Cd	0.02 to 1	
Cr	0.02 to 2	
Cu	3 to 150	
Pb	2 to 20	
Zn	0.60 to 27.4	Osmani et al., (2015)
Ni	10	
Mn	2	Ullah et al., (2022)
As	0.1 to 0.2	European Union, (2015)
Cd	0.05 to 3	
Hg	0.1 to 1	
As	0.01 to 0.5	Codex Stan (193-1995)
Cd	0.003-2	
Hg	0.0001-0.1	
Pb	0.05-2	
Tn	50-250	

Numerous research on the presence of heavy metal pollution in herbal medicines have been conducted in order to investigate and ensure the safety of plants, particularly those are used to make herbal remedies (Lajayer et al., 2017; Li et al., 2019; Mulaudzi et al., 2017, Kim et al., 2016; Singh et al., 2016; Pradhan et al., 2015). Though several researches on heavy metal contamination in herbal plants have been done, the majority used small sample sizes and narrow classifications (Luo et al., 2021). Heavy metals in the samples are widely investigated using inductively coupled plasma optical emission spectroscopy (ICP-OES) (Tunali et al., 2021). ICP-OES use the capacity of atoms and ions to absorb energy in order to move electrons from the ground state to an excited state. The energy source in ICP-OES is heat from an argon plasma running at 10,000 kelvin (°K). In order for the ICP-OES principle to

work, excited atoms must release light at certain wavelengths when they decelerate to a lower energy level (Khan et al., 2022).

2.4.6 Surface Morphological Analysis

The utilization of scanning electron microscope (SEM) is prominent in business, scientific, and industrial settings (Shakya, 2021). There are several beneficial uses for the current SEM, ranging from trimming fabrication methods to forensic ones (Kathirvel et al., 2022). It produces high-resolution, three-dimensional pictures using focused electron beams and accurately measures extremely minute features and objects (Mohammed and Abdullah, 2018). The images provided by the SEM analysis gave information on the topography, morphology, and composition of the samples (Malenica et al., 2021; Sharma and Bhardwaj, 2019). Thus, this equipment is far superior than an optical microscope (Zuo and Ding, 2021) .

2.5 Extraction of Phenolic Compounds from Pine Bark Species.

The pine tree species is one of the best sources of bioactive substances, particularly for its extensive array of PCs, which are listed in Table 2.5. These PCs isolated from certain pine tree species, are also employed for a variety of commercial applications, most notably in pharmacology, where it has been demonstrated that they improve people's health.

Table 2.5. Some Phenolic Compounds Present in Pine Bark Species

Phenolic Compounds	Sources	Amount	References
Phenolic Acids	<i>P. sylvestris</i>	0.77-15.1*	Drózdź and Pyrzynska, (2021)
• Chlorogenic Acid			
Hydroxybenzoic Acid	<i>P. sylvestris</i>	<LOD-6.10*	Drózdź and Pyrzynska, (2021)
• Gallic Acid	<i>P. brutia</i>	2.2**	Demirtaş, (2020)
	<i>P. brutia</i>	0.177***	Kivrak et al., (2013)
	PYC	0.593***	
• 2-5 Dihydroxybenzoic Acid	<i>P. brutia</i>	2.229***	Kivrak et al., (2013)
	PYC	0.897 ***	
• Protocatechuic acid	<i>P. sylvestris</i>	6.15-9.24*	Drózdź and Pyrzynska, (2021)
	<i>P. densiflora</i>	6.1*	Kim et al., (2018)
	<i>P. pinea</i>	0.041*	Karaçelik et al., (2022)
	<i>P. sylvestris</i>	0.271*	
	<i>P. pinaster</i>	0.138*	
	<i>P. brutia</i>	1.4**	Demirtaş, (2020)
• Vanilic acid	<i>P. sylvestris</i>	0.018*	Karaçelik et al., (2022)
	<i>P. pinaster</i>	0.010*	
• P-hydroxy benzoic acid	<i>Pinus brutia</i>	0.9**	Demirtaş, (2020)
	Ten	0.0332 ***	Kivrak et al., (2013)
	<i>P. brutia</i>	0.383*	
	PYC		
Hydroxycinnamic Acid	<i>P. sylvestris</i>	15.9-22.4*	Drózdź and Pyrzynska, (2021)
• Ferulic Acid	<i>P. brutia</i>	0.2**	Demirtaş, (2020)
• ρ -Coumaric Acid	<i>P. brutia</i>	0.2 **	Demirtaş, (2020)
	<i>P. brutia</i>	0.057 ***	Kivrak et al., (2013)
	PYC	0.112 ***	
• Caffeic Acid	<i>P. pinea</i>	0.104*	Karaçelik et al., (2022)
	<i>P. brutia</i>	1.2**	Demirtaş, (2020)
Flavonol	<i>P. sylvestris</i>	20.801 ****	Hamad et al., (2019)
• Myricetin	<i>P. brutia</i>	0.227 ***	Kivrak et al., (2013)
	PYC	0.065 ***	
• Kaempferol	<i>P. sylvestris</i>	0.805 ****	Hamad et al., (2019)
	<i>P. brutia</i>	0.2 **	Demirtaş, (2020)
	<i>P. brutia</i>	0.036 ***	Kivrak et al., (2013)
	PYC	0.011 ***	

*mg/g, **µg/g, ***mg/100g, ****mg/ml

Cont. Table 2.5.

Phenolic Compounds	Sources	Amount	References
• Quercetin	<i>P. sylvestris</i> <i>P. brutia</i> Ten	3.982 **** 17.7**	Hamad et al., (2019) Demirtaş, (2020)
Flavanol	<i>P. sylvestris</i>	242-378*	Drózdź and Pyrzynska, (2021)
• Catechin	<i>P. densiflora</i> <i>P. pinea</i> <i>P. sylvestris</i> <i>P. pinaster</i> <i>P. brutia</i> <i>P. brutia</i> <i>P. pinea</i> <i>P. sylvestris</i> <i>P. nigra</i> PYC	24.5* 3.586* 0.681* 1.231* 6.4** 70.4* 35.8* 1.78* 3.77* 1.5*	Kim et al., (2018) Karaçelik et al., (2022) Demirtaş, (2020) Yeşil-Çeliktas et al., (2009)
• Epicatechin	<i>P. sylvestris</i> <i>P. pinea</i> <i>P. pinaster</i> <i>P. brutia</i>	73.0-191* 0.022* 0.011* 5.8**	Drózdź and Pyrzynska, (2021) Karaçelik et al., (2022) Demirtaş, (2020)
Flavanone			
• Naringin	<i>P. sylvestris</i>	0.38 ****	Hamad et al., (2019)
• Naringenin	<i>P. brutia</i> PYC	0.135 *** 0.017 ***	Kivrak et al., (2013)
Flavone	<i>P. brutia</i> PYC	ND ND	Kivrak et al., (2013)
• Chrysin			
Flavone	<i>P. sylvestris</i> <i>P. brutia</i> <i>P. brutia</i> PYC	0.099 **** 0.2** 0.146 *** 0.13	Hamad et al., (2019) Demirtaş, (2020) Kivrak et al., (2013)
• Luteolin			
• Apigenin	<i>P. brutia</i>	0.3**	Demirtaş, (2020)
Flavononol	<i>P. densiflora</i> <i>P. pinea</i> <i>P. sylvestris</i> <i>P. pinaster</i> <i>P. brutia</i> <i>P. sylvestris</i> <i>P. brutia</i> <i>P. pinea</i> <i>P. sylvestris</i> <i>P. nigra</i> PYC	7.7* 1.866* 0.500* 0.360* 1.869** 1.869 **** 185.6* 2.87* 6.6* 3.0* 1.7*	Kim et al., (2018) Karaçelik et al., (2022) Demirtaş, (2020) Hamad et al., (2019) Yeşil-Çeliktas et al., (2009)
Chalcone	<i>P. sylvestris</i>	1.073 ****	Hamad et al., (2019)
• Butein			
Phenolic Aldehyde	<i>P. densiflora</i> <i>P. sylvestris</i> <i>P. brutia</i>	2.3* 0.024* 0.4**	Kim et al., (2018) Karaçelik et al., (2022) Demirtaş, (2020)
• Vanilin			
• Protocatechuic aldehyde	<i>P. sylvestris</i>	0.030*	Karaçelik et al., (2022)

*mg/g, **µg/g, ***mg/100g, ****mg/ml

PCs from pine trees can be isolated from its different parts such as the aerial and ground parts as shown in Figure 2.15 adapted from the book of Akkemik, (2018). However, the isolation of PCs from pine trees is also as challenging as other extraction process from other plants. Inlined with the purposed of this study, PCs are mostly isolated from the bark of the pine trees. One example is the PYC, which is a commercial dietary supplements rich in PCs. This supplement is from the bark of *P. pinaster*, a one famous species of pine trees.



Figure 2.15. Different Parts of Pine Tree as Potential Source of Phenolic Compounds

Like other plants PCs from pines can also be extracted using different extraction methods whether conventional or non-conventional, as seen in Tables 2.6 and 2.7, respectively. It is clearly demonstrated that traditional methods for the extraction of pine bark from various species, such as the MAC and SOX extraction procedures, are still commonly employed today due to their simplicity and accessibility and their effectiveness in extracting PCs from the pine bark. Additionally, as various extraction techniques have been improved and developed, including green techniques, they are also employed for the extraction of pine bark, particularly when it comes to higher-level and more technical researches.

Table 2.6. Conventional Methods for the Extraction of Phenolic Compounds from Pine Bark Species

Method	Pine Species	Results	Extraction Process	Reference
Maceration (MAC)	<i>P. eldarica</i>	• TPC (mgGAE/100g extract): 362.8±5.4 • PC (mg Cya/g): 174.386±2.5	• Weight of Sample: 1kg • Solvent volume: 5L • Solvent: 70% EtOH • S/L: 1:10 • Temperature: Room temperature • Time: 72 hr	Esmacili et al., (2021)
	<i>P. sylvestris</i>	• EY (%): 2.20±0.16 • TPC (mg GAE/100g): 488±15 • CT (mg CE/100 g): 238±19 • DPPH (mg AAE/100g): 296±3	• Weight of Sample: 100 g • Solvent volume: 1000 ml • Solvent: H₂O • S/L: 1:10 • Temperature: 50 °C • Time: 60 min	Pap et al., (2021)
	<i>P. taeda</i>	• EY (%)-5.87-11.22 • TPC (g GAE/100g bark)- 2.53-7.01 • CT (g CE/100g bark)- 2.82-4.95 • FRAP (mmol AAE/100g)-8.60-25.42	• Sample weight: 100 g • Solvent volume: 250 ml • Solvent: EtOH: H₂O 20-80% • Temperature: 20-60 °C • Time: 90 min • S:L ratio: 1/5-1/15	Xavier et al., (2021)
	<i>P. halepensis</i>	• EY (%): EtOH-7.0, MeOH-22.1, Hexanoic-3.0 • TPC (mgGAE/100g extract): EtOH-397.79, MeOH-369.00, Hexanoic-17.62 • TFC: (mg CE/g extract): EtOH-71.02, MeOH-126.58, Hexanoic-12.69 • DPPH (EC₅₀): EtOH-0.0029, MeOH-0.0031, Hexanoic-0.0960	• Weight of Sample: 50 g • Solvent volume: 250 ml • Solvent: 80 % MeOH, EtOH and hexane • Temperature: Room temperature • Time: 3 h	Salim et al., (2019)

EY- extraction yield, TPC- total phenolic contents, TFC-total flavonoid contents, CT- condensed tannins, PT-proanthocyanidin contents, GAE/g- gallic acid equivalents, CE- catechin equivalent, CyaE-cyanidin equivalent, TAX-taxifolin

Cont. Table 2.6.

Method	Pine Species	Results	Extraction Process	Reference
	<i>P. pinaster</i>	- EY (%): 31.30±10.42 - TPC (mgGAE/100g bark): 22.01±4.91 - TFC (mg CE/g bark): 2.18±0.57 - BuOH-HCl (mg CyaE/g bark): 1.20±0.44	- Solvent: H₂O:5% NaOH:0.25% Na₂SO₃:0.25% NaHSO₃ - Temperature: 80°C - S:L ratio: 1/9	Chupin et al., (2013)
	<i>P. radiata</i>	- EY (%)-15.3±0.9 - TPC (mg CE/g bark)- 645±18 - Tannins (mg CE/g bark)- 68.3±3.1	- Solvent: Ace: H₂O (7:3, v/v) - Temperature: 40 °C - Time: 2.5-360 min - S/L ratio: 1:10, w/v - Shaking: 200 rpm - Stage of extraction:3	Aspé and Fernández, (2011)
Soxhlet Extraction	<i>P. halepensis</i>	- EY (%): EtOH- 13.3, MeOH- 23.8, Hexanoic- 6.4 - TPC (mgGAE/100g extract): EtOH- 253.65, MeOH- 314.34, Hexanoic- 12.10 - TFC: (mgCE/g extract): EtOH- 64.92, MeOH- 87.97, Hexanoic- 12.14 - DPPH (EC₅₀): EtOH- 0.0062, MeOH- 0.0047, Hexanoic- 0.1640	- Weight of Sample: 50 g - Solvent volume: 250 ml - Solvent: 80 % MeOH, EtOH and hexane - Time: 6 h	Salim et al., (2019)
	<i>P. nigra</i>	Taxifolin (mg TAX/g): 0.94	- Weight of Sample: 4 g - Solvent volume: 250 ml - Solvent: EtOH - Time: 8 h	Ghoreishi et al., (2016)
	<i>P. radiata</i>	- EY (%)-12.3±0.1 - TPC (mg CE/g bark)- 580±23 - Tannins (mg CE/g bark)- 70.6±0.1	- Solvent: Ace: H₂O (7:3, v/v) - Solvent volume: 200 ml - Temperature: 82 °C - Time: 60-360 min - S/L ratio: 1:10	Aspé and Fernández, (2011)

EY- extraction yield, TPC- total phenolic contents, TFC-total flavonoid contents, CT- condensed tannins, PT-proanthocyanidin contents, GAE/g- gallic acid equivalents, CE- catechin equivalent, CyaE-cyanidin equivalent, TAX-taxifolin

Table 2.7. Non-Conventional Methods for the Extraction of Phenolic Compounds from Pine Bark Species

Non-Conventional Methods	Pine Species	Results	Extraction Process	Reference
Microwave-Assisted Extraction (MAE)	<i>P. pinaster</i>	- EY (%) - 9.24±0.117 - TPC (mgGAE/100g bark) - 28.30±2.937 - Vanillin test (mg CE/g bark) - 37.14±3.447 - Butanol-HCl (mg CyaE/g bark) - 7.66±1.335 - Total sugars (mg GE/g bark) - 3.51±1.117	- Solvent: EtOH: H₂O (80–20, v–v) - S/L ratio: 1–10 (w–v) - Time: 3 min - Microwave Power: 100 W - Particle size: below 1mm	Chupin et al., (2015)
Supercritical Fluid Extraction (SFE)	<i>P. nigra</i>	Taxifolin: 34.13%.	- Solvent: scCO₂ with EtOH - Pressure: 19.3 MPa - Temperature of 42.8°C - CO₂ flow rate: 1.9 ml/min - Dynamic extraction time: 137.9 min - Weight of sample: 2g	Ghoreishi et al., (2016)
Ultrasonic-Assisted Extraction (UAE)	<i>P. brutia</i>	- CT (mg CE/g) - 56.01 - Reducing sugar (mg/g) - 15.08	- Solvent: EtOH: H₂O (49.48:50.52 v/v to 82.73:17.22) - S/L ratio: 1:8 (w–v) - Time: 5min - Temperature: 40–53.20 °C	Gürgen et al., (2022)
mgGAE/100g bark: Gallic acid equivalents, mg CE/g bark: catechin equivalent, mg CyaE/g bark, cyanidin equivalent, mg GE/g bark: glucose equivalent				

Several factors also affect the extraction of PCs from bark of pine trees, for instance, the effects of time. In the study of Aspé and Fernández, (2011), at 180 minutes, there was a greater effect of time on the EY of the MAC and SOX extractions of *P. radiata* bark. Nevertheless, SOX outperformed MAC at the same extraction time. Adding increased temperature to the extraction process typically reduces the amount of time needed for the extraction (Elboughdiri, 2018). As for the study by Vieito and co-workers (2018), they studied phenolic extraction from *P. pinaster* subsp. *Atlantica*, their observations were made utilizing a variety of solvents, and was found that 50% aqueous EtOH had the highest EY% and TPC. Furthermore, Milić and co-workers (2021) study the polyphenolic profiles, antioxidative, and antiproliferative activity of the *P. nigra* Arnold bark extracts from Mokra Gora (MG) and Tara mountain. The solvents employed for the comparison were EtOH, MeOH, and Ace. When compared to the Ace and MeOH extracts from both sites, the results showed that EtOH extract from MG had the highest TPC (35.68 ± 1.74 mgGAE/100g), flavonoid content (1.22 ± 0.13 mg QE/g), tannin content (21.64 ± 0.3 mgGAE/100g), and proanthocyanidin content (3.76 ± 0.04 mg CE/g). According to these results, polar solvents like EtOH are more effective at extracting PCs (Milić et al., 2021). Additionally, Venkatesan and co-workers (2019)'s study on the effects of various extraction solvents on the TPC and antioxidant capacity of *P. densiflora* bark extracts revealed a significant phenolic content in the extracts made from an aqueous EtOH mixture. Thus, aqueous EtOH or other aqueous-organic solvent extracts make the optimal solvents for recovering PCs from plants since it may make it simpler to extract chemicals that are soluble in both H₂O and organic solvents. For S/L ratio, in line by of Jerez and co-workers (2006) on the degree of proanthocyanidin polymerization by thiolysis of *P. pinaster* extracts. They found out that a certain ratio of solid to liquid is favorable to the extraction of PCs. However, when increasing the S/L ratio further, the TPC were also affected as resulted in the ineffective of mass transfer (Caldas et al., 2018). Chupin and co-workers (2015) investigated in their study the effects of particle size on the extraction of PCs from the *P. pinaster* bark by MAE and MAC. They found based on their research that a particle size of 400 μ m produced the highest TPC. They also discovered that when the size of the particles decreased below 400 μ m, the extraction gets better. Additionally, they noted that MAC bark extraction produces higher EY than MAE. As for the effects of

temperature, this factor is related to the effects of extraction techniques. Aspé and Fernández (2011) study the impact of various extraction methods on EY, TPC, and antioxidant capacity of extracts from *P. radiata* bark. The study examined four extraction methods with different extraction stages, including traditional MAC and SOX extraction as well as non-conventional extraction methods, including MAE and UAE. $SOX > MAE > UAE > MAC$ ($p < 0.05$) was the sequence in which the extracted mass decreased at one point. In this study, SOX obtained the highest EY, TPC, and tannin levels, whereas MAC obtained the lowest. This result is caused by the different temperatures utilized during the extraction process. SOX extraction, for example, operates at a high temperature of about 82°C or higher since the solvent must boil in order to evaporate and flow inside the soxhlet apparatus. The saturated equilibrium constant is moved by an increase in temperature, favoring extraction and resulting in an increase in process efficiency (Rodriguez-Rojo et al., 2012). In some cases, the TPC can sometimes decrease as temperature rises higher because most phenolic contents are temperature sensitive and many are volatile (Xu et al., 2022). In these situations, phenolic oxidative degradation is most likely to blame for the low TPC obtained (Tomás-Barberán and Espín 2001; Vuong, et al., 2013). Thus, optimization is important. Table 2.8 represents some studies related to the used of RSM as a statistical method for the extraction of PCs from the bark of pine species.

Table 2.8. Previous Studies using RSM to Optimize the Extraction Process for Pine Bark

Pine Species	Extraction Method	Operating Variables	Optimized Parameters	Results (Actual)	References
<i>P. taeda</i>	Maceration	- Solvent: EtOH: H₂O 20-80% - Temperature: 20-60 °C - S:L ratio: 1/5-1/15	- Solvent: EtOH:H₂O 50% - Tempearture: 50 °C - S/L ratio: 1/10	- EY (%) -9.83±0.03 - TPC (g GAE/100g bark)- 5.00±0.67 - CT (g CE/100g bark)- 4.01±0.08 - FRAP (mmol AAE/100g)- 20.89±1.43	Xavier et al., (2021)
<i>P. nigra</i>	Supercritical CO ₂ with ethanol as modifier	- Temperature: (40–60 °C) - Pressure: (10–30 MPa) - Dynamic extraction time: (30–150 min) - CO₂ flow rate: (0.4–2 ml/min)	- Pressure: 19.3 MPa - Temperature: 42.8°C - CO₂ flow rate: 1.9 ml/min - Dynamic extraction time: 137.9 min.	Taxifolin: TAX/g: 34±2%	Ghoreishi et al., (2016)
<i>P. caribaea</i>	Maceration	- Temperature: Ace: H₂O -35-60°C, EtOH: H₂O - 35-80°C - Time: (30-180 min) - Solvent: 10-100% (Ace: H₂O, EtOH: H₂O) - Stage of Extraction: 1-6 - S/L ratio: 10-50	- Temperature: 71.46°C - Time: 79.2 min - Solvent: 21.9 - Stage of Extraction: 1-6 - S/L ratio: 26.4:1	Tannin yield (%): 20.68	Derkyi et al., (2011)

Previous studies related to the characterization of PCs from pine bark species are presented and summarized in Tables 2.9, 2.10 and 2.11. The total amount of PCs and the PBE's antioxidant ability are shown in Table 2.9. It is clear from the papers described that in addition to measurements of total phenolic levels, DPPH antioxidant capacity were also reported, but was different for FRAP assay, because some studies just only used the DPPH assay. In the study of Karaçelik and co-workers (2022), they evaluate the TPC and antioxidant capacity of aqueous *P. pinea*, *P. sylvestres*, and *P. pinaster* extracts. For TPC they reported that aqueous extracts have 984.46 ± 4.08 , 361.53 ± 3.52 , and 816.92 ± 2.31 GAE $\mu\text{g/ml}$ with an antioxidant capacity of 1.64 ± 0.00003 , 6.05 ± 0.09 , 1.83 ± 0.02 SC₅₀ $\mu\text{g/ml}$, in terms of DPPH respectively. They also reported the total antioxidant capacity of the three pine species based on FRAP assay as expressed as trolox equivalent (TEAC) μM , the total antioxidant capacity were 1428.75 ± 5.62 , 549.37 ± 8.59 , and 1279.68 ± 3.97 respectively. However, in the study of Ramos and co-workers (2022), they didn't performed the FRAP assay only the DPPH and TPC of *P. pinea* and *P. pinaster*, where the IC₅₀ reported were 6.79 ± 0.48 and 6.46 ± 0.36 IC₅₀ $\mu\text{g/ml}$. For TPC they reported 539 ± 26 and 568 ± 12 mgGAE/100g of Extract. Similar to the study of Pap and co-workers (2021), they didn't also performed the FRAP assay but in their study, they only focused on the extracts of *P. sylvestres* by MAC and hot H₂O extraction (PHWE). They reported that the TPC of MAC (488 ± 15 mg GAE/100g) is higher than the PHWE (194 ± 6 mg GAE/100g) similar to the antioxidant capacity MAC (296 ± 3 mgAAE/100g) is also higher than PHWE (162 ± 3 mgAAE/100g). As for *P. nigra* in the study of Milić and co-workers (2021). They used Ace, EtOH, and MeOH as the organic solvent for the extraction of *P. nigra*. The results they reported that EtOH extracts obtained that highest TPC (5.68 ± 1.74 mgGAE/100g) and antioxidant capacity in terms of FRAP assay (57.44 ± 1.41 mg TEAC/g). In study of Vieito and co-workers (2018) highlighted the advantages of using aqueous EtOH (50%) as good organic solvent for the extraction of *P. pinaster* for it obtained the highest TPC and antioxidant capacity in terms of DPPH assay when compared to pure H₂O and EtOH.

Table 2.9. Total Phenolic Contents and Antioxidant Capacity of Pine Bark based on FC, DPPH, and FRAP Assay

Pine Species	TPC	DPPH Assay	FRAP Assay	References
<i>P. pinea</i>	• H ₂ O: 984.46 ± 4.08 GAE, µg/ml	• H ₂ O: 1.64310 ± 0.00003 SC ₅₀ , µg/ml	• H ₂ O: 1428.75 ± 5.62 TEAC, µM	Karaçelik et al., (2022)
<i>P. sylvestris</i>	• H ₂ O: 361.53 ± 3.52 GAE, µg/ml	• H ₂ O: 6.04765 ± 0.09043 SC ₅₀ , µg/ml	• H ₂ O: 549.37 ± 8.59 TEAC, µM	Karaçelik et al., (2022)
<i>P. pinaster</i>	• H ₂ O: 816.92 ± 2.31 GAE, µg/ml	• H ₂ O: 1.83300 ± 0.01108 SC ₅₀ , µg/ml	• H ₂ O: 1279.68 ± 3.97 TEAC, µM	Karaçelik et al., (2022)
<i>P. pinaster Ait</i>	• 539 ± 26 mgGAE/100g of Extract	• 6.79 ± 0.48 IC ₅₀ (µg/ml)	•	Ramos et al., (2022)
<i>P. pinea</i>	• 568 ± 12 mgGAE/100g of Extract	• 6.46 ± 0.36 IC ₅₀ (µg/ml)	•	Ramos et al., (2022)
<i>P. sylvestris</i> (MAC)	• 488 ± 15 mg GAE/100g	• 296±3 (mgAAE/100g)	•	Pap et al., (2021)
<i>P. sylvestris</i> (PHWE)	• 194±6 mg GAE/100g	• 162±3 (mgAAE/100g)	•	Pap et al., (2021)
<i>P. nigra</i>	• Ace: 4.62 ± 1.74 (mgGAE/100g) • EtOH: 5.68 ± 1.74 (mgGAE/100g) • MeOH: 2.5 ± 3.17 (mgGAE/100g)	• Ace: 5.55 ± 0.78 mg TEAC/g • EtOH: 9.45 ± 0.82 mg TEAC/g • MeOH: 10.6 ± 0.53 mg TEAC/g	• Ace: 28.4 ± 0.77 mg TEAC/g • EtOH: 57.44 ± 1.41 mg TEAC/g • MeOH: 52.11 ± 1.41 mg TEAC/g	Milić et al., (2021)
<i>P. pinaster</i>	• H ₂ O: 50.09 ± 4.70 mgGAE/100g • EtOH: 63.38 ± 1.26 mgGAE/100g • EtOH:H ₂ O-73.48 ± 1.84 mgGAE/100g	• H ₂ O: 82.24 ± 4.65 mg AAE/g • EtOH: 95.58 ± 0.55 mg AAE/g • EtOH: H ₂ O -108.74 ± 2.02 mg AAE/g	•	Vieito et al., (2018)
<i>P. pinaster</i>	• 847.62 ± 39.74 mgGAE/100g	•	• 4.83 ± 0.15mmol TEAC equivalents g	Cádiz-Gurrea et al., (2014)

- PHWE: pressurized hot H₂O extraction

As seen in the Table 2.10, catechin, taxifolin, gallic acid are mostly found in the bark of pine trees according to all of the studies listed. However, some PCs are not detected. In the list, some studies detected the same PCs in same the pine species but other study didn't manage to find similar PCs. For instance, in the study of Karaçelik and co-workers (2022), he didn't manage to detect some PCs such as caffeic acids and ferulic acid in *P. pinaster* but Miliç and co-workers (2021) found these compounds in the species of *P. nigra*. This means that not all PCs found in one pine species are can be found in the other species. Hence, it is important to characterize each plant species in addition to the determination of the overall contents in the pine bark species. In addition, Pap and co-workers (2021) and Amalinei and co-workers (2014) reported the presence of proanthocyanidin in *P. sylvestres*, which is also abundant in the bark of the *P. pinaster* and is sold commercially as dietary supplements under the trade name PYC. This compound is in addition to the typical PCs present in pine bark as listed in the same Table 2.10. To properly understand the potential of plant extracts, it is crucial to know all of the PCs present and how they function biologically. Since then, plant extracts have been analyzed for their biological activity or bioactive characteristics; one usually used for PCs is the antioxidant and antibacterial capability. Other biological functions such as anti-inflammatory, anticancer, antidiabetic, anti proliferative and many more are also the potential functions of PCs from various pine bark species.

Table 2.10. HPLC Analysis of Phenolic Compounds from Pine Bark Species

HPLC Method	Pine Species	HPLC Conditions	Phenolic Compounds	Reference
Reversed-Phase High Performace- Liquid Chromatography (RP-HPLC)-DAD	<i>P. pinea</i>	• Detector: Diodearray • Column: C18 column (150 mm x4.6 mm, 5µ; Fortis) • Mobile Phase: A: 2% acetic acid-ultrapure H₂O, C: 50-50% MeCN ultra pure H₂O solution in 0.5% acetic acid, and D: MeCN • Flow Rate: 0.7 ml/min • Sample: 20µL • Temperature: 25 °C • Detection Wavelength: 260, 280, 308, 324 nm • Time: 30 min	• Protocatechuic acid • Catechin • Caffeic acid • Epicatechin • Taxifolin • Not detected: • Protocatechuic aldehyde • Vanilic Acid • Vanillin • Ferulic Acid • Gallic Acid • Ellagic Acid	Karaçelik et al., (2022)
	<i>P. sylvestris</i>	• Detector: Diodearray • Column: C18 column (150 mm x4.6 mm, 5µ; Fortis) • Mobile Phase: A: 2% acetic acid-ultrapure H₂O, C: 50-50% MeCN ultra pure H₂O solution in 0.5% acetic acid, and D: MeCN • Flow Rate: 0.7 ml/min • Sample: 20µL • Temperature: 25 °C • Detection Wavelength: 260, 280, 308, 324 nm • Time: 30 min	• Protocatechuic acid • Protocatechuic aldehyde • Catechin • Vanillic acid • Vanilin • Taxifolin • Not detected: • Epicatechin • Ferulic Acid • Caffeic Acid • Gallic Acid • Ellagic Acid	

Cont. Table 2.10.

HPLC Method	Pine Species	HPLC Conditions	Phenolic Compounds	Reference
Reversed-Phase High Performace- Liquid Chromatography (RP-HPLC)-DAD	<i>P. pinaster</i>	• Detector: Diodearray • Column: C18 column (150 mm x4.6 mm, 5μ; Fortis) • Mobile Phase: A: 2% acetic acid-ultrapure H₂O, C: 50-50% MeCN ultra pure H₂O solution in 0.5% acetic acid, and D: MeCN • Flow Rate: 0.7 ml/min • Sample: 20μL • Temperature: 25 °C • Detection Wavelength: 260, 280, 308, 324 nm • Time: 30 min	• Protocatechuic acid • Catechin • Vanillic acid • Epicatechin • Taxifolin • Not detected: • Protocatechuic aldehyde • Ferulic Acid • Caffeic Acid • Gallic Acid • Vanilin • Ellagic Acid	Karaçelik et al., (2022)
Reversed-Phase High Performace- Liquid Chromatography (RP-HPLC)-DAD	<i>P. sylvestris</i>	• Detector: Diodearray • Column: Gemini C18 column (150 × 4.6 mm, 5 μm) • Mobile Phase: MeCN into 5% formic acid (aq).	• (+)-Catechin • Taxifolin • Proanthocyanidins • Procyanidins	Pap et al., (2021)
Reversed-Phase High Performace- Liquid Chromatography (RP-HPLC)-DAD	<i>P. nigra</i>	• Detector: diodearray • Column: XDB-C18(4.6 × 50 mm, 1.8μm) • Mobile Phase: 1% formic acid • Flow Rate: 1 ml/min • Sample: 5μl • Temperature: 30°C • Detection Wavelength: 280, 330, and 350 nm • Time: 30 min	• Protocatechuic acid • Catechin and derivatives • Caffeic acid and derivatives • Syringic acid and derivatives • Epicatechin and derivatives • p-Coumaric acid • Ferulic acid and derivatives • Taxifolin	Milić et al., (2021)

Cont. Table 2.10.

HPLC Method	Pine Species	HPLC Conditions	Phenolic Compounds	Reference
Reversed-Phase High Performace- Liquid Chromatography (RP-HPLC)	<i>P. sylvestris</i>	• Detector: electrospray ionization-mass spectrometric detection • Column: 100 RP-18 column (75 mm×4mm, i.d. 4 µm) • Mobile Phase: (A) MeCN and (B) H₂O and formic acid (99:1, v/v) • Flow Rate: 1 ml/min • Detection Wavelength: 280 nm	• Taxifolin-hexoside, taxifolin • Procyanidins (two monomers, three dimers and three trimers)	Amalinei et al., (2014)
Reversed-Phase High Performace- Liquid Chromatography (RP-HPLC)-DAD	<i>P. pinaster</i>	• Detector: Diodearray • Column: acclaim 120 C18, 250 × 4.6 mm, 5 m column. • Mobile Phase: [A] 0.1% (v/v) aqueous TFA, [B] MeCN. • Temperature: 25 °C • Detection Wavelength: 220, 254, 272 and 280 nm	• Catechin • Epicatechin • Epicatechin gallate • Gallic acid	Chupin et al., (2013)
Reversed-Phase High Performace- Liquid Chromatography (RP-HPLC)-DAD	<i>P. sylvestris</i> (H ₂ O and EtOH)	• Detector: Diodearray • Column: C18 column (250 mm × 4.6 mm, 5 µm) • Mobile Phase: H₂O: MeCN • Flow Rate: 1.0 ml/min • Temperature: 33 °C	• 3,4-dihydroxybenzoic • 4-hydroxybenzoic • Anisic • Caffeic • Cinnamic • Ferulic • Gallic • Syringic • Vanillic • Catechin • Kaempferol • Quercetin • Maltol	

Compounds such as VOCs in PBE have been analyzed with different detectors combined with headspace approach like GC-MS, SPME GC-MS or SPME GC-flame ionization detection (FID) represents in Table 2.11 the list of related studies. According to Maimoona and co-workers (2011), GC-MS mostly used for the characterization of oils from *P. pinaster*. On the other hand, other studies used also GC-MS to characterized PCs. Furthermore, in the results based on the studies, the compounds mostly identified where VOCs such as terpenes. These are the compounds present in solid and/or liquid material that have a high vapor pressure and low H₂O solubility. In the study of Ucar and co-workers (2013), they used GC-MS to identify PCs in the ethyl acetate and Ace extracts of *P. brutia* where they determined some PCs such as taxifolin with minor amount of taxifolin isomer, catechin, quercetin, ferulic acid, glycerol, p-hydroxy benzoic-, and 3,4-dihydroxy benzoic acid as well as glucopyranose (16.5, 20.1% of the total peak area α - and β -D-glucose), arabinose, and some other monosaccharides and inositol. In addition, Sharma and co-workers (2020) also used GC-MS to evaluate VOCs in *P. roxburghii* where they mostly determined monoterpene and sesquiterpene compounds. As for GC with SPME, Nisca and co-workers (2021) used SPME GC-FID to determined VOCs in *P. nigra* such as α -pinene, β -pinene, camphene, 3-carene, α -phellandrene, limonene, sabinene, myrcene, tricyclene. Whereas, in study of Özgenç et al., (2017) similarly they used SPME but with GC-MS and they determined a total of 96 VOCs such as monoterpene hydrocarbon and limonene as the major compound determined in different *coniferous* species. Furthermore, Szmigielski and co-workers (2012) also used SPME GC-MS to determined VOCs such as terpenes, oxygenated terpenes, sesquiterpenes and diterpenes in *P. sylvestres*. Based on the studies mentioned it can be said that SPME GC-MS and/or SPME GC-FID is the best method for the analysis of VOCs in the plant materials particularly the bark where monoterpene are found to be major VOCs in pine bark. These VOCs are the compounds that also gave fragrance to the plant materials and are easily be evaporated because they have a vapor pressure at room temperature. As well as having a low boiling point and solubility.

Table 2.11. GS-MS Analysis of Compounds from Pine Bark Species

GC-MS Method	Pine Species	GC-MS Conditions	Bioactive Compounds	Reference
GC with a FID detector (GC-FID)	<i>P. nigra</i>	• Column: (60 m × 0.32 mm ID × 0.25 µm film thickness) • Volume: 1 µL • Split ratio of 1:10 • Carrier gas: helium • Flow rate: 1.2 ml/min • Column temperature program: 40 (2 min) to 90 °C with a 5 °C/min rate, 120 °C (2 min) with a 10 °C/min rate and 180 °C (4 min). • Injector and detector temperatures: 240 °C and 270 °C	• α-pinene, β-pinene, camphene, 3-carene, α-phellandrene, limonene, sabinene, myrcene, tricyclene	Nisca et al., (2021)
Gas Chromatography-Mass Spectrophotometry (GC-MS)	<i>P. roxburghii</i>	• Column : (30 m × 0.25mm;0.25 µm) • Oven temperature: 60°Cfor 2.0 min • Increase: 3°C/min and 250°C for 5.0 min • Sample: 1.0 µl • Carrier gas: Helium gas (99.99 % pure) • Flow rate: 1 ml/min • Injector temperature: 250°C • MS transfer line temperature: 260°C • Ion sourcetemperature: 230°C • Run time: 5-67.44 min	• Monoterpene (3-Carene, camphene, α-Pinene, α-Myrcene, D-Limonene, cadinol, and 4-Carene) • Sesquiterpene (Thujopsene, longifolene, Humulene, and Bornyl acetate)	Sharma et al., (2020)

Cont. Table 2.11.

GC-MS Method	Pine Species	GC-MS Conditions	Bioactive Compounds	Reference
Solid Phase Microextraction (SPME-GC-MS)	<i>A. nordmanniana</i> , <i>P. brutia</i> , <i>P. orientalis</i> , <i>P. pinaster</i> , <i>P. sylvestris</i> , <i>C. liban</i>	• SPME analyses • Temperature: 50 °C • Incubation time: 5 min • Extraction time: 10 min • GC-MS injector (split mode) • The initial temperature of 60 °C lasted 2 min, 240 °C lasting 3 min, 250 °C held for 4 min • Carrier gas: Helium (99.999%) • Flow-rate: 1 ml/min • Ionization voltage: 70 eV • Scan mode: 40–450 m/z	• 96 VOCs • Monoterpene hydrocarbon (14.8%, 31.4%, 39.3%, 40.4%, 26.6% and 51.3%, respectively) • Limonene (1.2%, 6.0%, 8.2%, 28.8%, 4.9% and 4.7% respectively)	Özgenç et al., (2017)
Gas Chromatography-Mass Spectrophotometry (GC-MS)	<i>P. brutia</i>	• Saponified: 0.5 M alcoholic potassium hydroxide • Reflux temperature: 70 °C • Ethyl Acetate	• Taxifolin (≥80% of total peak area) with minor amount of a taxifolin isomer (2%). • Catechin (2%) • Quercetin (0.4%), • Ferulic acid (<0.1%), • Glycerol (2.5%), • p-hydroxy benzoic- (0.5%) • 3,4-dihydroxy benzoic acid (2.5%)	Ucar et al., (2013)
Gas Chromatography-Mass Spectrophotometry (GC-MS)	<i>P. brutia</i>	• Saponified: 0.5 M alcoholic potassium hydroxide • Reflux temperature: 70 °C • 98% Ace	• Glucopyranose (16.5, 20.1% of the total peak area α- and β-D-glucose), • 5–7% arabinose, • Some other monosaccharides and inositol (7–8%), • Glycerol (4%), • Taxifolin (25–30%) • Catechin (1%)	Ucar et al., (2013)

Cont. Table 2.11.

GC-MS Method	Pine Species	GC-MS Conditions	Bioactive Compounds	Reference
Solid Phase Microextraction (SPME-GC-MS)	<i>P. sylvestris</i>	- Column: HP-5MS fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm; crosslinked 5% Phenyl-Methylpolysiloxane) - Desorption: plitless mode at 250°C for 30s - MS system - Scan mode: 30–550 u - Ionisation potential: 70 eV - Source temperature: 230°C - MS quadrupole temperature: 150°C. - Carrier gas: helium (1.0 ml/min) - Temperature programme: - Isothermal hold: 50°C for 5 min - Temperature ramp of 10°C/min up to 270°C - Isothermal hold at 270°C for 5 min.	- Terpenes (α-pinene, Δ-3-carene, and para-cymenene), - Oxygenated terpenes (α-terpineol and verbenone) - Sesquiterpenes (α-longipinene, longifolene, E-β-farnesene, γ-cadinene and pentadecane), - Diterpenes (manoyl oxide and (+)-pimaral).	Szmigielski et al., (2012)

As mentioned in the literature that some of the studies for the antibacterial activity of the phenolic extracts were also reported along side with antioxidant capacity. For pine species the majority of are inactive against gram negative bacteria especially *P. aeruginosa*, whereas PBE usually have an antibacterial impact on gram positive bacteria. In the literature, the antibacterial properties of pine species are mostly associated with their aerial portions, specially their essential oils from needles, some examples are *P. halepensis* (Ramos et al., 2022), *P. nigra* (Ameur et al., 2022), and *P. sylvestris* (Oyewole et al., 2021). However, research on the PBE's antibacterial properties is currently limited (Wahid et al., 2019). As previously indicated, PBE are abundant in PCs with a wide range of medicinal characteristics, including catechin, epicatechin, taxifolin, and phenolic acid (Dziedziński et al., 2021). It's possible that PCs like catechin, proanthocyanidin, and quinic acid are what give plant extracts and pine trees their antibacterial properties (Ramos et al., 2022). An initial investigation on *P. nigra* bark extracts in chloroformic, Ace, and MeOH examined the extracts' antibacterial activity against several bacteria, including strains of *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *S. aureus*. With the exception of *E. coli*, the extracts prevented all bacterial strains from growing and developing (Dığrak and Hakkı Alma, 1999). In Table 2.12 represents some of the previous study related to the antibacterial activity of the PBE for different species of pine trees.

Table 2.12. Antibacterial Activity of the Pine Bark Species

Pine Species	Bateria	Results	References
<i>P. pinaster</i>	<i>S. aureus</i> <i>E. coli</i>	6.25, 12.5, 25 mg/ml (8.3-, 6.3-, and 4.0-log (CFU/ml) 25 mg/ml,	Ramos et al., (2022)
<i>P. pinea</i>	<i>S. aureus</i> <i>E. coli</i>	6.25, 12.5, 25 mg/ml (8.3-, 6.0-, and 4.6-log (CFU/ml)) 25 mg /ml	Ramos et al., (2022)
<i>P. eldarica</i>	- Inhibit the growth of <i>P. aeruginosa</i> - Did not inhibit the growth of <i>S. aureus</i> and <i>E. coli</i>	- Essential oil (1.5-2.0mm)-3mg/ml - Proanthocyanidins total (1.0-1.5mm)- 3mg/ml - Hydroalcoholic extract (1.5-2.0mm)- 3mg/ml	Sadeghi et al., (2016)
<i>P. pinea</i>	<i>S. aureus</i> (in red meat)	- Control: 13.7×10^2 detected 7.1×10^2 CFU/g after 9 days (1% concnecration of extracts	Kocabaş et al., (2008)
PYC	<i>S. aureus</i> (in red meat)	17.2×10^2 CFU/g 9.8×10^2	Kocabaş et al., (2008)

For further analysis and characterization of the PBE, heavy metal composition also focused in this study. ICP-OES was also used to determine the mineral composition of the PBE in *P. sylvestris* (Krutul et al., 2017). In the study of Coşkun (2006), he used *P. nigra* as a biomonitor for toxic elements such as As, Cd, Cu, Pb, and Zn. These toxic elements were discovered in the bark of *P. nigra*, but not in high concentrations or pollution levels. Pine bark is a passive absorbent of air pollutants and has been used to monitor atmospheric pollution because of its ability to absorb and accumulate airborne contaminants (Chrabąszcz and Mróz, 2017). Pine bark has a porous structure, which makes it an excellent pollutant collector. Not only that, but using pine bark as biomonitoring for heavy metals is less expensive than other biomonitoring methods (Hoodaji et al., 2012). Thus, it is critical to ensure the quality of the PBE and whether heavy metals can still be present in the extracts after the extraction process because WHO strongly advises evaluating the heavy metals present in the plants that will be used to form the other materials for the finished products. Furthermore, PBE can be used as a cosmetic and nutraceutical additive. Thus, determining the concentration is critical. In the study of Drózdź and Pyrzynska (2021), toxic metals such as Zn, Cu, Cr, Pb, Ni, and Cd were found in pine bark extracts of *P. sylvestris*, with a higher concentration of Cd and Zn. However, the extracts' Cd and Pb levels are less than 0.3 and 10 mg/kg, respectively, of the WHO-recommended standard contents for toxic Cd and Pb (Drózdź and Pyrzynska, 2021). Hence, pine bark extracts do not pose a risk to human health. Finally, to further assess the effects of extraction process, surface morphological changes also aimed and determined.

2.6 Surface Morphological Analysis of Pine Bark

SEM analysis is infrequently used to compare the surface morphology of pine bark before and after extraction to ascertain the impacts of the extraction procedure on the bark. However, one study by Aspé and Fernández (2011), they used SEM analysis to examine the cell damage caused by extraction methods such as MAC, SOX, MAE, and UAE. The evaluation was done on the differences between the structure of the bark before and after four different extraction processes. The extraction methods exhibited distinct cell alterations and degree damage when compared to the control sample. A little pore rupture in the bark sample obtained by MAC and MAE indicated that the bark product had deteriorated and that its soluble components had moved into the solvent. However, due to the employment of high heat and pressure during the extraction process, the bark structure from SOX and UAE techniques was severely harmed, and more significant modifications were detected. This resulted in the cellular matrix's profuse rupture, which damaged the channels and caused some of the smaller particles to break up and disperse. In these circumstances, the pressure inside the plant matrices' pores will continue to increase as the extraction process goes on. Cell rupture may occur if this pressure is greater than the pores' capacity to withstand it, which is obviously comparable to the control. This study was also parallel to the study of Guo and co-workers (2021) on the effects of ultrasound-enhanced subcritical H₂O extraction method to the bark of cinnamon before and after extraction. All this previous studies included and discussed here are parallel to the purpose of this current study.

3. MATERIALS AND METHODS

3.1 Materials

The solvents used in this study such as absolute EtOH (C_2H_5OH) ($\geq 99.9\%$), MeOH (CH_3OH) ($\geq 99.9\%$), and Ace (C_3H_6O) ($\geq 99.9\%$) were of analytical or HPLC grade from ACS-ISOLab Chemicals (Wertheim, Germany), 2,2-Diphenyl-1-picrylhydrazyl ($C_{18}H_{12}N_5O_6$) (DPPH) and Folin-Ciocalteu reagent (FCR) were from Sigma-Aldrich (Steinheim, Germany). Gallic acid monohydrate ($C_7H_8O_6$) was from Carlo Erba reagents, Daisti group (Carlo Erba, Milan, Italy). Sodium carbonate anhydrous (Na_2CO_3), potassium dihydrogen phosphate (KH_2PO_4), sodium hydrogen phosphate (Na_2HPO_4), potassium chloride (KCl), potassium ferricyanide ($C_6N_6FeK_3$), trichloroacetic acid ($C_2HCl_3O_2$), ferric chloride ($FeCl_3$), and ascorbic acid are examples of salts ($C_6H_8O_6$) were from Merck (Darmstadt, Germany). All additional substances and standards employed in this investigation were of the analytical quality from local companies

RP-HPLC-DAD (LC20-A Prominence, Shimadzu), UV-Vis Spectrophotometer (Hach Lange DR 6000), SEM (FEI-Quanta FEG 250), ICP-OES (SpectroBlue, Spectro), water bath (Nüve ST30), GC-MS (Shimadzu GCMS QP 2010 ULTRA) Weighing Scale, Rotary Vacuum Evaporator (Büchi, B-100 with DOA-P730-BN vacuum pump), Centrifuge (Hettich zentrifugen Rotina 380), Vortex, Soxhlet Apparatus, Incubator, Autoclave, and Magnetic Stirrer are some of the major equipment used in this study.

3.2 Methods

3.2.1 Preparation of *Pinus nigra* bark samples

Pinus nigra bark (Black Pine Bark) was obtained from trees of ages ~65 and ~25 years old from the village of Üyücek and Ahlatçık, Kastamonu, Türkiye as shown in Figure 3.1 created using Google Maps and Mapchart.net.

Sample Collection Areas:

1. Ahlatçık, 37210 Ahlatçık/Kastamonu Merkez/Kastamonu (41.35777°N, 33.58182°E)
2. Üyücek, 37210 Üyücek/Kastamonu Merkez/Kastamonu (41.30343°N, 33.62139°E)



Figure 3.1. Areas for Sample Collections

The collected bark samples were transported to the Central Research Laboratory of Kastamonu University, where they were left to air dry for a day at room temperature. Afterward, the bark samples were pulverized and sieved to select particle sizes ranging from 0.125 to 1 mm, as shown in Figure 3.2. The pulverized samples were stored in sealed bags and kept at four °C before the experiments to prevent the loss of phenolic and other bioactive components (Aspé and Fernández, 2011; Vieito et al., 2018).



Figure 3.2. Process for the Preparation of Bark Samples

3.2.2 Extraction Process

For the extraction process of pine bark, preliminary experiments were employed to select the important factors and their ranges in isolating PCs. EY and TPC of the extracts were used to evaluate the effect of individual factors. Maceration (MAC) extraction was used and carried out using the different such as extraction time (30-360 mins), temperature (20-60°C), solvent type (aqueous EtOH, MeOH, and Ace), solvent composition (30-90% v/v EtOH/H₂O), particle size (0.250-1 mm), and solid/liquid ratio (10-150 g/L). Their ranges were defined taking into account of the earlier findings from the literature. Table 3.1 shows the detailed parameters used in this study and Table 3.2 shows the codes used for labelling the samples, respectively. After the extraction, the extracts were filtered to separate from the solid material and concentrated in a rotary evaporator at 40°C. Then further dried at vacuum oven to obtained dried extracts as shown in Figure 3.3. The dried extracts were labelled as PNBE and stored at -20°C for future analysis. The extraction was carried out in three replicates. The EY% of the pine bark was measured using the formula (3.1) below:

$$\text{Extraction yield \%} = \frac{W_1}{W_2} \times 100 \quad (3.1)$$

where W_1 = weight of the dry extracts, W_2 = weight of the plant material.

Table 3.1. Levels and Factors that Affects the Extraction Process of PNBE

Code	Factors	Unit	Levels/Types
A	Time	min	30, 60, 120, 240, and 360
B	Temperature	°C	20, 30, 40, 50, and 60
C	Solvent type	-	H ₂ O, EtOH, MeOH, and Ace
D	EtOH Concentration	%	30, 50, 70, 80, and 90
E	S/L ratio	g/L	20, 50, 100, 150, and 200
F	Particle size	mm	0.125-0.250, 0.250-0.500, and 0.500-1.0

Table 3.2. Lables and Codes used for the Analysis of PNBE

A	B	C	D	E	F
	MAC-T30	MAC-TM20	MAC-C30	MAC-SL10	
EtOH (50%)	MAC-T60	MAC-TM30	MAC-C50	MAC-SL20	MAC-PA
MeOH (50%)	MAC-T120	MAC-TM40	MAC-C70	MAC-SL50	MAC-PB
Ace (50%)	MAC-T240	MAC-TM50	MAC-C80	MAC-SL100	MAC-PC
	MAC-T360	MAC-TM60	MAC-C90	MAC-SL150	

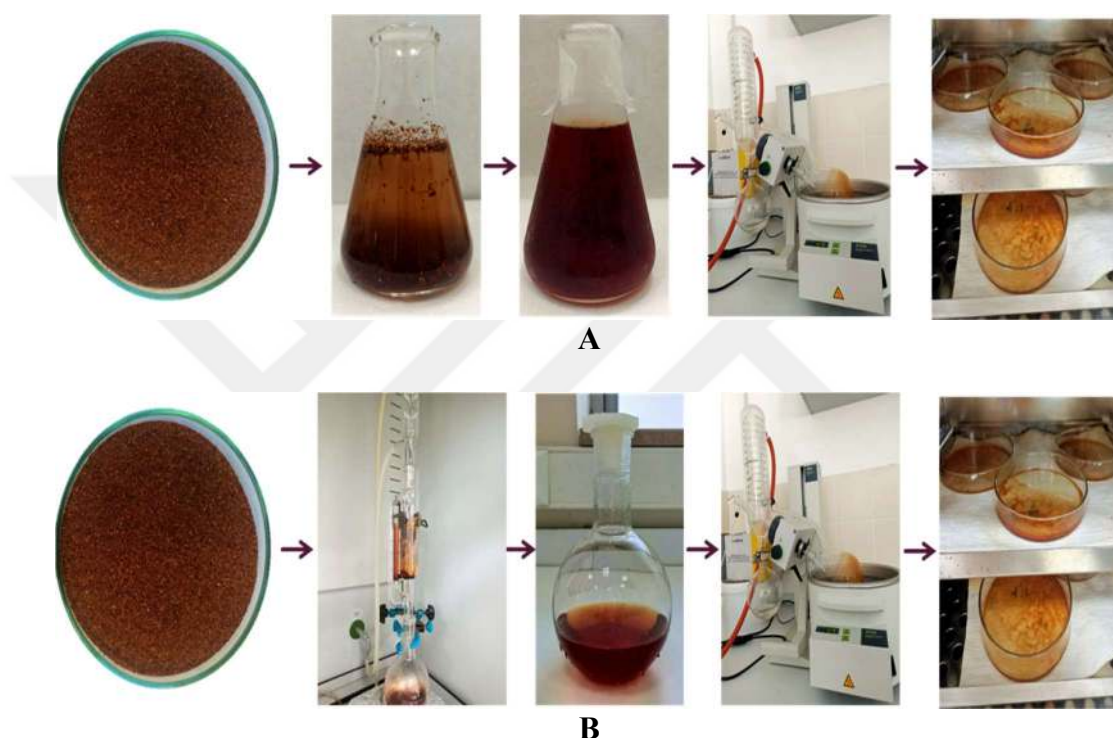


Figure 3.3. Extraction Process of PNBE by MAC (A) and SOX (B)

3.2.3 Optimization of PNBE by RSM

RSM was used to optimize the extraction parameter for PNBE. The experimental design was based on three factors/five levels in CCRD. The CCRD experimental design was chosen because it allows for more accurate factor impact estimation. Extraction time (minutes), temperature (°C), and ethanol concentration (%) toward the responses; TPC (mgGAE/100g) and DPPH anti-radical scavenging activity (% reduction) were chosen as independent factors in this study. The S/L ratio (100 g/L) and particle size (0.250-0.500 mm) were remained constant throughout the optimization process and were therefore excluded as one of the optimization factors

for RSM. The values obtained from the single-factor analysis performed prior to designing the experiments for the RSM were used to determine the levels of independent factors. These factors were chosen for the RSM design because of their significant effects on the responses. Table 3.3 lists the independent variables and their corresponding coded levels. There are 20 runs, including six replicates at the center points, which can be used to define experimental error and data reproducibility in the design given by RSM. To reduce bias in the data, all experiments were run at random.

Table 3.3. Factors and Levels of RSM Optimization for the Extraction of PNBE

Codes	Independent Factors	Units	Coded Levels				
			$-\alpha$	-1	0	$+1$	$+\alpha$
A	Time	mins	38.18	120.00	240.00	360.00	441.82
B	Temperature	°C	29.77	40.00	55.00	70.00	80.23
C	EtOH Concentration	v/v %	39.77	50.00	65.00	80.00	90.23

3.2.4 Mass Transfer: Diffusion Coefficient

The mass transfer, diffusion coefficient was calculated using the second Fick's law and assumed as the particles were having a spherical shape and the formulas for calculating D_{eff} were used as described in the literature review in formulas 2.1 through 2.5 following the methods used by Mosca and co-workers (2018) with slight modifications. The experimental data was the TPC of PNBE extracted using the optimized MAC method by single-factor analysis and RSM. The results were expressed as D_{eff} (m^2/s).

These are some of the following assumptions for the calculation of diffusion coefficients of PCs from PNBE. (i) The extracted PCs are regarded as a single compound and are represented by gallic acid equivalents, (ii) the solid material is spherical in shape and of the same size, (iii) the diameter of the particles corresponds to the average diameter of the sample, (iv) the diffusion is carried out in parallel, (v) the solute's diffusivity is constant, (vi) internal diffusion is the controlling stage of the extraction process.

3.2.5 Determination and Characterization of PNBE

3.2.5.1 Spectrophotometric analysis of phenolic compounds from PNBE

The method of Kupina and co-workers (2018) was followed with slight modification on the quantification of TPC. The TPC of PNBE was quantified by FC method where gallic acid was used as the reference standard. The PNBE with 1 mg/ml concentration was prepared. A series of test tubes containing 15 ml of H₂O, 1 ml of FCR, and 1 ml of sample were thoroughly mixed and set aside for 6 minutes. Then, 3ml of a 20% sodium carbonate solution was added to individual test tube and thoroughly mixed. The test tubes were then heated in a H₂O bath for 120 minutes. Afterward the absorbances of PCs was measured at 765 nm wavelength using UV-vis spectrophotometer as shown in Figure 3.4. The values for TPC using the formula (3.2) were expressed as gallic acid equivalents (mgGAE/100g d.w) and the standard curve is shown in appendix 1.

$$\text{TPC} = \frac{A-b}{m} \times \frac{V \cdot D}{W \cdot 1000} \times 100 \quad (3.2)$$

where A= absorbance of the sample at 765 nm, b= y-intercept, m= slope, W= weight of the test material (mg), V= the volume of the test sample test solution (ml), D= dilution factor.

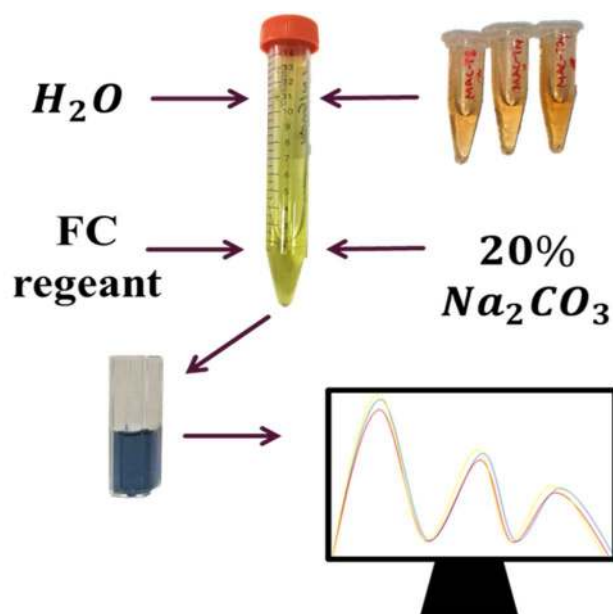


Figure 3.4. Process for the FC Assay for TPC of PNBE

3.2.5.2 Chromatographic analysis of phenolic compounds from PNBE

The dried PNBE extracts obtained from the optimized parameters were dissolved in EtOH at 1 mg/ml concentration. The extracts were analyzed using RP-HPLC-DAD. The system unit and parameters for RP-HPLC-DAD are shown in Table 3.4.

Table 3.4. RP-HPLC-DAD System Units and Parameters

RP-HPLC-DAD System Units and Parameters	
Solvents	3% acetic acid (solvent A) and methanol (solvent B)
Sample Volume	20 μ L
Flow Rate	0.6 ml/min
Column	Inertsil ODS-3 Reverse Phase (5 μ m-125x4.6mm)
Pump	LC-20 AT Prominence
Deaerator	DGU-20 A 5R Prominence
Autosampler	SIL-20AC HT
Column furnace	CTO-10AS VP
Detectors	Diode-Array SPD-M20A DAD
Wavelength	280, 320 or 360 nm

In addition, the gradient conditions for the mobile phases employed in the investigation are shown in Table 3.5. The standards for phenolic compounds used were pyrocatechol, catechin, taxifolin, myricetin, kaemferol, naringin, chrysin, ruten, butein, ellagic acid, caffeic acid, couramic acid, triacetin, 2-5 dihydroxybenzoic acid, and ferulic acid. All the standards used are HPLC grade.

Table 3.5. HPLC Gradient Elution Program

Time (min)	A Concentration (%)	B Concentration (%)
0	93	7
20	93	7
20	72	28
28	72	28
28	75	25
35	75	25
35	70	30
50	70	30
50	70	30
60	70	30
60	67	33
62	58	42
62	58	42
70	58	42
70	50	50
73	50	50
73	30	70
75	30	70
75	20	80
77	20	80
77	15	85
79	15	85
79	10	90
80	10	90
80	8	92
81	8	92
81	5	95
81	5	95
82	0	100
90	0	100
91	0	100
91	93	7
95	93	7

3.2.5.3 Chromatographic analysis of volatile organic compounds from PNBE

Using the optimized parameters for the extraction of PNBE, further evaluation of VOCs were evaluated using SPME GC-MS. The mode used for SPME was direct mode where the SPME fiber was dipped into the sample bottle and kept at 50°C for 30 mins. It is then directly injected to the GC-MS device. Table 3.6 shows the system units for the SPME GC-MS analysis of VOCs in PNBE.

Table 3.6. The System and Units for SPME GC-MS Analysis for VOCs in PNBE

SPME GC-MS System Units and Parameters	
SPME	
Fiber type	DVB/CAR/PDMS
Fiber size	50-30µm
GC-MS	
Column	RXI-5MS Capillary Column (30 m; 0.25 mm; 0.25 µm)
Carrier gas	Helium
Flow Rate	1 ml/min
Column temperature	50°C
Injection temperature	250°C
Pressure	100 kPa
Oven temperature program	50°C for 5 mins, from 50°C to 270°C in 5°C/min increment, 250°C at 5 mins. Total 54 mins
Interface temperature	250°C
Ion source temperature	200°C

3.2.5.4 Antioxidant capacity of PNBE.

The antioxidant activity of the PNBE was assessed using the DPPH and FRAP procedures to gauge the different mechanisms of action of the extracts. The DPPH assays for free radical scavenging were carried out in accordance with Yan and Jing (2018) and Tepe and co-workers (2021) with slight modifications. The DPPH process comprised dissolving the DPPH radical agents in EtOH to produce a 0.1mM stock solution. Then, the stock solution was covered with aluminum foil and stored at room temperature to avoid changes. Then, a 0.5 ml extracts and 0.5 ml DPPH solution were combined and mixed thoroughly and allowed to sit for 30 mins in a dark place at an ambient temperature for the DPPH analysis. Afterward, the

absorbance of the samples was determined using a UV-vis spectrophotometer in a 517 nm wavelength against the blank DPPH. The analysis was carried out in triplicates. The results obtained were expressed as % reduction for optimization and half maximal concentration (IC₅₀) ascorbic acid equivalents (µgAAE/ml) for optimized parameters and the calibration curve is shown in appendix 2. The scavenging activity of PNBE was measured using the equation (3.3) below:

$$\text{reduction \%} = \left(\frac{A_0 - A_1}{A_0} \right) * 100 \quad (3.3)$$

where A_0 = absorbance of the DPPH solution, A_1 = absorbance of the sample

The FRAP assay was carried out in accordance with the method of Vijayalakshmi and Ruckmani (2016). The FRAP test was carried out by combining the PNBE with 2.5 ml of 0.2M phosphate buffer solution (PBS-pH 6.6) and 2.5 ml of 1% potassium ferricyanide solution. A vortex shaker was used to thoroughly mix the reaction mixture before it was incubated for 20 mins at 50°C. 2.5 ml of 10% trichloroacetic acid was added to the mixture after the incubation, and then the mixture was centrifuged at 3,000 rpm for 10 mins. Then 2.5 ml deionized H₂O and 0.5 ml of 0.1% ferric chloride were added to the 2.5 ml supernatant. The colored solution was measured using a UV-vis spectrophotometer at 700 nm in comparison to a blank and a standard. Here, ascorbic acid was used as a reference standard (R²=0.96). Values for FRAP are represented as mgAAE/100g dry weight and the calibration curve is shown in appendix 3.

3.2.5.5 Antibacterial activity of PNBE

PNBE was tested for antibacterial activity against Gram positive and Gram negative bacteria such as *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Three bacteria were grown on Mueller Hinton Agar with 1 mg/ml concentrations of different PNBE extracted from different EtOH concentrations, time of extraction, temperature, S/L ratio, and particle size for 24 hours. The antibacterial activity of the PNBE was initially tested at 1 mg/ml concentration for antibacterial screening. 20µl of the extracts were pipetted onto the prepared nutrient agar and

allowed the ethanol to dry before adding the bacteria. After it dries, 10µl of cultured bacteria were pipetted over the marked area with the PNBE and placed in an incubator at 37°C for 24 hours. The results were observed after the incubation period. The aim of the analysis is to (i) determine whether PNBE has antibacterial effects, and (ii) determine whether the observed antimicrobial property of PNBE is dependent on the effects of the factors mentioned (Osonga et al., 2016).

3.2.5.6 Heavy metals composition of PNBE.

The PNBE after the optimization were analyzed for trace elements through ICP-OES (ICP-OES- SpectroBlue II, Spectro). The measurement is based on the emission of atoms excited by passing into the gas phase by spraying the sample into the quartz plasma torch at power 1200 W. The following are the operational criteria for the detection of trace elements in PNBE: The flow rates for the auxiliary gas, coolant, and nebulizer were 0.8 L/min, 13 L/min, and 0.8 L/min, respectively. The speed of the sample was 300 rpm. For the analysis, the PNBE was filtered through microfilters so that there are no particles left in it and is processed directly. The standard curves were created using a multi-element standard stock solution from Merck, Germany, which was provided for ICP-OES. Different common stock solutions exist depending on the necessary components. Prepared samples and calibration solutions were analyzed in SpectroBlue brand ICP-OES device. ICP-OES takes three measurements by itself (n=3) while determining the metal concentrations in the sample. The standard deviation is then determined by taking the average of these three observations. The concentrations obtained in this study were compared to the WHO permissible concentrations in plant materials (EU, 2015; WHO, 2007; Codex Stan, 1995).

Additional analysis was performed to support the study. These analyses were the following; the effects of extraction method on the EY, TPC, DPPH, and FRAP antioxidant potential, as well as the surface morphological changes in the bark of *P. nigra* following the extraction and the two distinct age ranges of the bark samples.

3.2.5.7 Effect of extraction method on PNBE

Following the optimization of modified MAC method (MAC-MD) of PNBE, it was compared to the normal MAC (MAC-NM) for 24 hours at room temperature (Korovkina et al., 2020) and SOX extraction using the MAC-MD's optimized parameters as summarized in Table 3.7 to determine if the difference between the optimized MAC and MAC for 24 hours and optimized MAC to SOX extraction are significant and to confirm the advantage of the optimized MAC to the two traditional methods. EY, TPC, DPPH and FRAP were used to compared. Additionally, the bark after the extraction was also examined for surface morphological changes.

Table 3.7. Effects of Extraction Method on PNBE

A	B	C	D	E	F	G
MAC-NM	EtOH	1440	Room temperature	60	100 g/L	0.250-0.500 mm
SOX	EtOH	240	Boiling point	60	100 g/L	0.250-0.500 mm
MAC-MD	EtOH	240	60	60	100 g/L	0.250-0.500 mm

*Type of extraction (A), solvent (B), time (C), temperature (D), solvent concentrations (E), S/L (F), and particle size (G)

3.2.5.8 Effect of bark age on PNBE

The *P. nigra* bark age of ~65 and the *P. nigra* bark age of ~25 were compared. The samples were prepared similarly. The optimal *P. nigra* bark age ~65 (PNBE-OB) extraction parameters were used for *P. nigra* bark age ~25 (PNBE-YB). The two distinct age of the tree were compared by their EY, TPC, and antioxidant capacity.

3.2.5.9 Comparison between the optimized parameters

The optimized parameters obtained from single-factor analysis and RSM were also compared in terms of their EY, TPC and antioxidant capacity such as DPPH and FRAP assay. Table 3.8 shows the optimized parameters of MAC extraction for PNBE by single-factor analysis and RSM.

Table 3.8. Comparison Between the Optimized Parameters

Code	A	B	C	D	E	F	G
OP1	SFA	EtOH	180	50	70	100 g/L	0.250-0.500 mm
OP2	RSM	EtOH	240	60	60	100 g/L	0.250-0.500 mm
OP3	SFA	EtOH	120	40	50	100 g/L	0.250-0.500 mm

*Type of optimization (A), solvent (B), time (C), temperature (D), solvent concentrations (E), S/L (F), particle size (G), single-factor analysis (SFA).

3.2.6 Surface Morphological Analysis of *P. nigra* Bark

The bark samples before and after the optimization were analyzed for surface morphological changes using SEM (Quanta FEG 250, FEI) to determine the surface structural changes caused by the extraction techniques. The operating voltage was 10.00 kV, and the vacuum pressure was 2.90×10^{-3} Pa. Samples were coated with Au-Pd to guarantee adequate electron refraction.

3.2.7 Statistical Analysis

ANOVA was employed in a single-factor analysis to identify the significant differences between the independent variables (one-way ANOVA). In RSM, ANOVA was also used, and the best-fitted model of response was obtained by emphasizing the following statistical parameters: regression p-value summarized at the 95% confidence level, multiple correlation coefficients (R^2), adjusted multiple correlation coefficients (adjusted R^2), lack of fit, coefficients variation (CV%), regression F-value were employed in analyzing the experimental data. Furthermore, for comparing the old and young tree T-test was used.

4. RESULTS AND DISCUSSIONS

4.1 Extraction Process

Prior to the extraction procedure for PNBE's final optimization, preliminary research was conducted to identify the relevant factors and their experimental range in order to isolate PCs from *P. nigra* bark. This section studied the impacts of variables including time, temperature, solvents, S/L ratio, and particle size. The influence of time on the EY and TPC are shown in Figure 4.1; it shows that when the time of extraction was varied from 30 to 60 minutes, the TPC reached a relative plateau (34.38 ± 7.45 mgGAE/100g to 40.04 ± 8.45 mgGAE/100g) and then steadily increased from 60 to 120 minutes (40.04 ± 8.45 mgGAE/100g to 49.03 ± 37.61 mgGAE/100g).

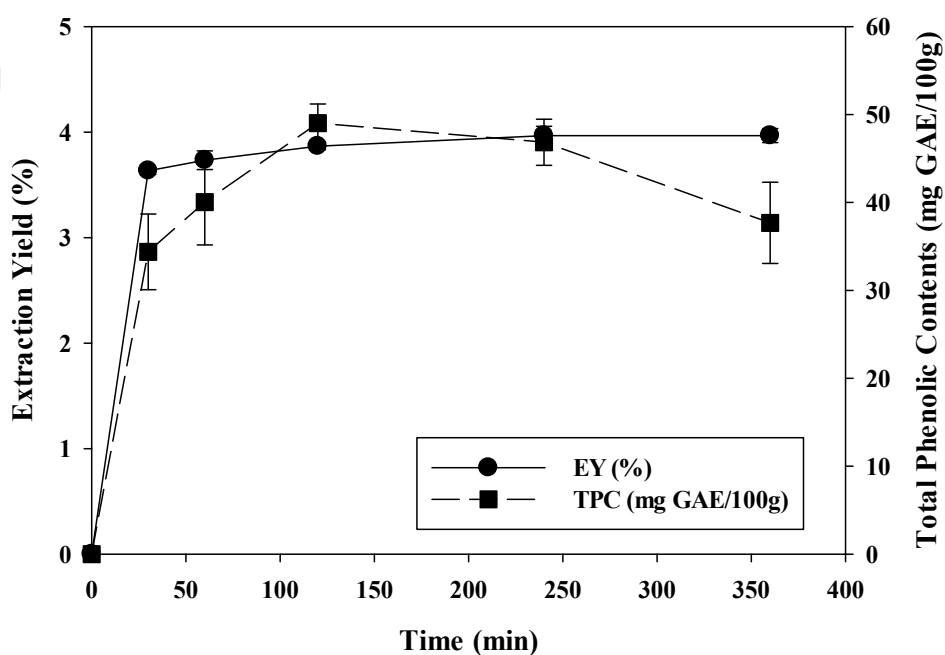


Figure 4.1. Effect of Extraction Time on PNBE

However, when the extraction time exceeded 120 minutes, the variation in TPC decreased; this could be due to the longer extraction time inducing more chemical reactions, which then cause oxidative conversion of the PCs. In contrast to that, the EY results indicates that the EY increases as the time increases from 30 to 240 min and obtained the highest yield (3.97 ± 0.15 %) at 240 min of extraction time. However, from the extraction time of 240 to 360 mins, changes on EY of *P. nigra* is no longer observed and already became constant in terms of equilibrium solubility. The statistical difference for EY ($p=0.154$) and TPC ($p=0.106$) were not significant. However, the extraction time of 180 minutes was chosen as the optimum point for the following step in order to prioritize the results of TPC and EY. Summary of the results obtained for EY and TPC are presented in Table 4.1.

Table 4.1. Summary of the Obtained Results for the Effect of Time

Effect of the Extraction Time (Mean $\pm$ Standard Deviation)		
Extraction Time (min)	EY (%)	TPC (mgGAE/100g)
30	3.63 ± 0.06	34.38 ± 7.46
60	3.73 ± 0.15	40.04 ± 8.45
120	3.87 ± 0.06	49.03 ± 3.76
240	3.97 ± 0.15	46.84 ± 4.55
360	3.97 ± 0.12	37.67 ± 8.00

The next factor evaluated was the factor of temperature. The effects of different extraction temperatures are shown in Figure 4.2, it shows that the TPC increases steadily as the temperature rises from 20 to 40°C (36.56 ± 1.59 mgGAE/100g to 49.03 ± 3.76 mgGAE/100g), and remained constant at 50°C (48.86 ± 1.69 mgGAE/100g) but when the temperature was at 60°C, the TPC decreased. Temperature increases the mass transfer which is why the TPC increases. However, further increase in the temperature promotes the oxidation of the TPC which resulted in the decrease and some will be lost because of their volatile's nature as mentioned in the literature. Whereas, the EY results, higher temperature resulted in higher EY (5.46 ± 0.25 %) because it accelerates mass transfer, which may then improve the extraction yield. The statistical difference for both EY ($p<0.001$) and TPC ($p<0.001$) were significant as shown in Table 4.2 in the summary of the results obtained. Thus, in the current study, the optimal extraction temperature was determined to be 50°C in

order to prioritize the high EY and TPC. Additionally, the findings of this study on the effects of temperature were consistent with those found in the literature, according to which an increase in temperature beyond a certain threshold might reduce the EY and TPC of the extracts.

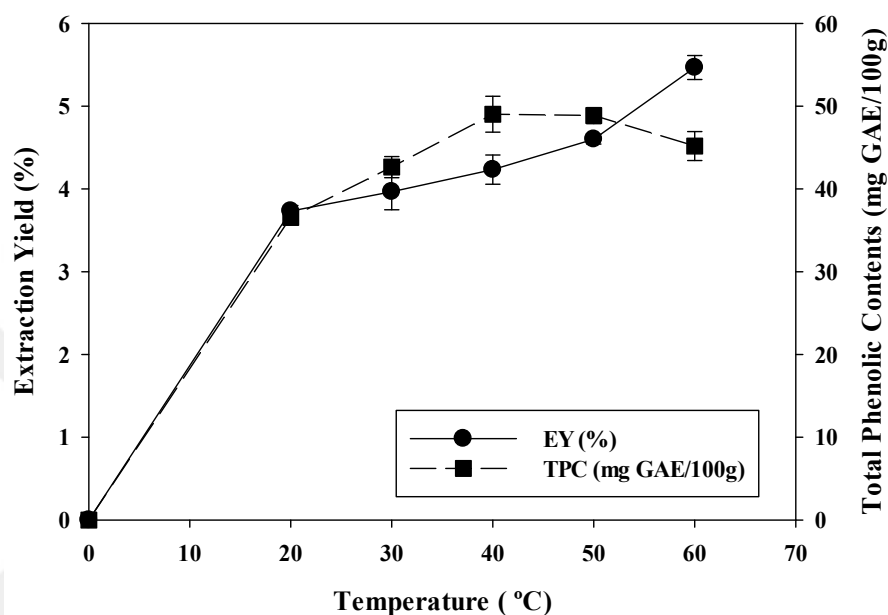


Figure 4.2. Effect of Extraction Temperature on PNBE

Table 4.2. Summary of the Obtained Results for the Effect of Temperature

Effect of the Extraction Temperature (Mean ± Standard Deviation)		
Extraction Temperature (°C)	EY (%)	TPC (mgGAE/100g)
20	3.73 ± 0.12 ^d	36.56 ± 1.59 ^a
30	3.97 ± 0.38 ^{cd}	42.64 ± 2.21 ^b
40	4.23 ± 0.31 ^{bc}	49.03 ± 3.76 ^a
50	4.60 ± 0.10 ^b	48.86 ± 1.69 ^b
60	5.47 ± 0.25 ^a	45.18 ± 3.02 ^{ab}

*same letters are not significant according to Duncan's multiple test

For the effects of solvent, the first evaluated was the effect of solvent types. The selected solvents were aqueous type, as discussed in the literature aqueous solvents is much better than the pure organic solvents. Hence, the effects of the solvent type aqueous: EtOH, MeOH, and Ace were compared in this study. Figure 4.3 shows the EY and TPC of various organic solvents.

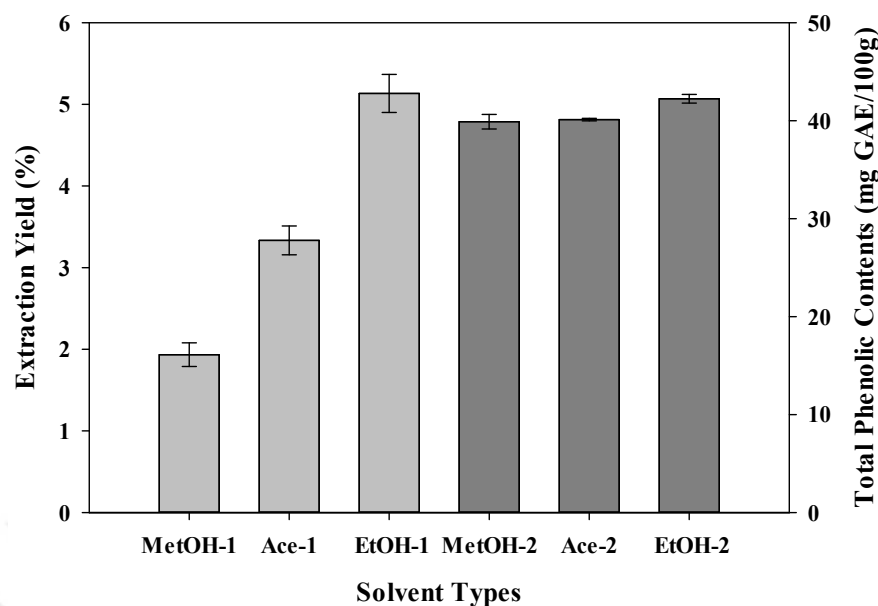


Figure 4.3. Effect of Solvent Type on PNBE

In Table 4.3 the EY and TPC decreased in order of decreasing capacity to extract soluble materials: 50% EtOH > 50% Ace > 50% MeOH. These findings agreed that polar solvents, such as EtOH, are more efficient for PCs extraction than other solvents tested. The statistical difference for both EY ($p < 0.001$) and TPC ($p = 0.031$) were significant. Hence, aqueous EtOH was chosen as the organic solvent for further analysis and optimizing the extraction process of PNBE.

Table 4.3. Summary of the Obtained Results for the Effect of Solvent Type

Effect of the Solvent Type (Mean ± Standard Deviation)		
Solvent Types	EY (%)	TPC (mgGAE/100g)
MeOH (50%)	1.93 ± 0.25	39.89 ± 1.29 ^b
Ace (50%)	3.33 ± 0.31	40.10 ± 0.21 ^b
EtOH (50%)	5.13 ± 0.40	42.23 ± 0.77 ^a

*same letters are not significant according to Duncan's multiple test

Further evaluation on the effect of solvents is the effect of different concentrations, EtOH was already chosen then the different concentrations were evaluated. The TPC and EY from *P. nigra* bark were influenced greatly by EtOH concentrations as shown Figure 4.4. In general, TPC with 40-50% EtOH is more effective than with higher EtOH concentrations. Increases in TPC (39.40 ± 2.61 mgGAE/100g to 49.03

± 3.76 mgGAE/100g) and EY ($3.33 \pm 0.31\%$ to $4.23 \pm 0.31\%$) were observed as EtOH concentration increased from 30 to 70%, This was probably brought on by the PCs' and other compounds' higher solubility in the EtOH and H₂O combination. In this study, the highest TPC were determined at 70% EtOH, as well as the highest EY, with only a slight difference when compared to 50% EtOH concentration. However, after 70% EtOH concentration, the TPC and EY decreased, this may be due to the solvent, which may influence cellular structures.

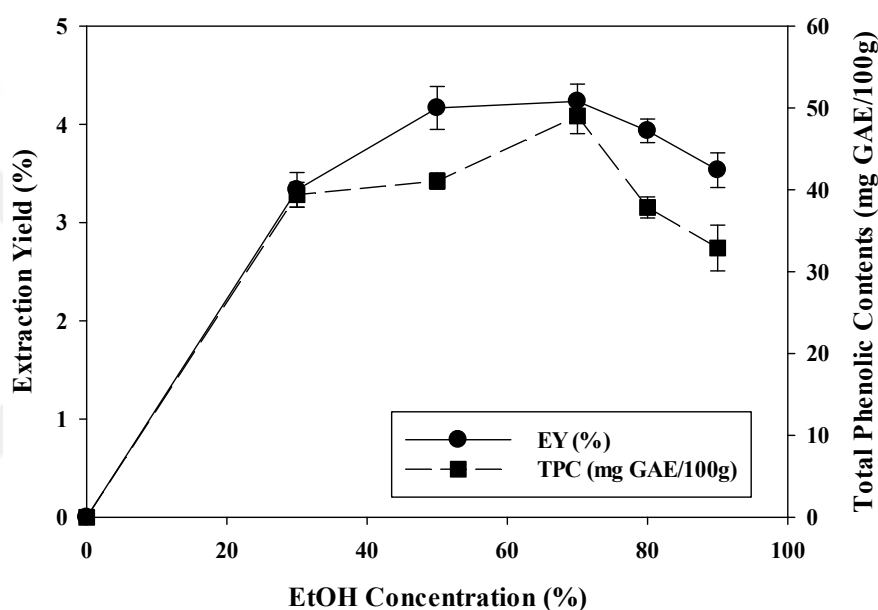


Figure 4.4. Effect of Ethanol Concentration on PNBE

In Table 4.4 the statistical difference for both EY ($p=0.04$) and TPC ($p=0.002$) were significant. Thus, in this study EtOH concentration of 70% was chosen for the subsequent experiments. Furthermore, the results obtained in this study is quite similar to results obtained in the literature, which stated that around 50% EtOH concentrations is good for the extraction of PNBE as compared to pure EtOH.

Table 4.4. Summary of the Obtained Results for the Effect of EtOH Concentration

Effect of the EtOH Concentration (Mean $\pm$ Standard Deviation)		
EtOH Concentration (%)	EY (%)	TPC (mgGAE/100g)
30	3.33 $\pm$ 0.31 ^b	39.40 $\pm$ 2.61 ^b
50	4.17 $\pm$ 0.38 ^a	41.06 $\pm$ 1.73 ^b
70	4.23 $\pm$ 0.31 ^a	49.03 $\pm$ 3.76 ^a
80	3.63 $\pm$ 0.40 ^{ab}	37.84 $\pm$ 2.23 ^{bc}
90	3.83 $\pm$ 0.25 ^a	32.89 $\pm$ 4.84 ^c

*same letters are not significant according to Duncan's multiple test

As shown in Figure 4.5, the TPC (30.43 $\pm$ 2.15 mgGAE/100g to 48.70 $\pm$ 3.29 mgGAE/100g) and EY (0.550 $\pm$ 0.132 % to 3.967 $\pm$ 0.153%) increases as the S/L ratio increases from 10 g/L to 100 g/L as summarized in Table 4.5. These findings were consistent with mass transfer principles, which state that mass transfer is driven by a greater concentration gradient when the S/L ratio is higher, resulting in faster chemicals diffusion from the extracted solid material into the solvent as previously discussed in the literature. However, if the S/L ratio increase further the EY and TPC also decreased because obviously the bark sample absorbed the solvents and after filtering only small amounts of extract were left. Thus, at 100 g/L was already the best and selected as the optimal ratio. Statistically, EY ($p < 0.001$) and the TPC ($p = 0.001$) results were significant.

Table 4.5. Summary of the Results Obtained for the Effect of Solid/liquid ratio

Effect of Solid/liquid ratio (Mean $\pm$ Standard Deviation)		
Solid/liquid ratio (g/L)	EY (%)	TPC (mgGAE/100g)
10	0.55 $\pm$ 0.13 ^c	30.43 $\pm$ 2.15 ^c
20	0.87 $\pm$ 0.06 ^c	37.96 $\pm$ 6.70 ^{bc}
50	1.97 $\pm$ 0.06 ^b	45.17 $\pm$ 2.39 ^{ab}
100	3.97 $\pm$ 0.15 ^a	48.70 $\pm$ 3.29 ^a
150	3.73 $\pm$ 0.15 ^a	49.22 $\pm$ 2.34 ^a

*same letters are not significant according to Duncan's multiple test

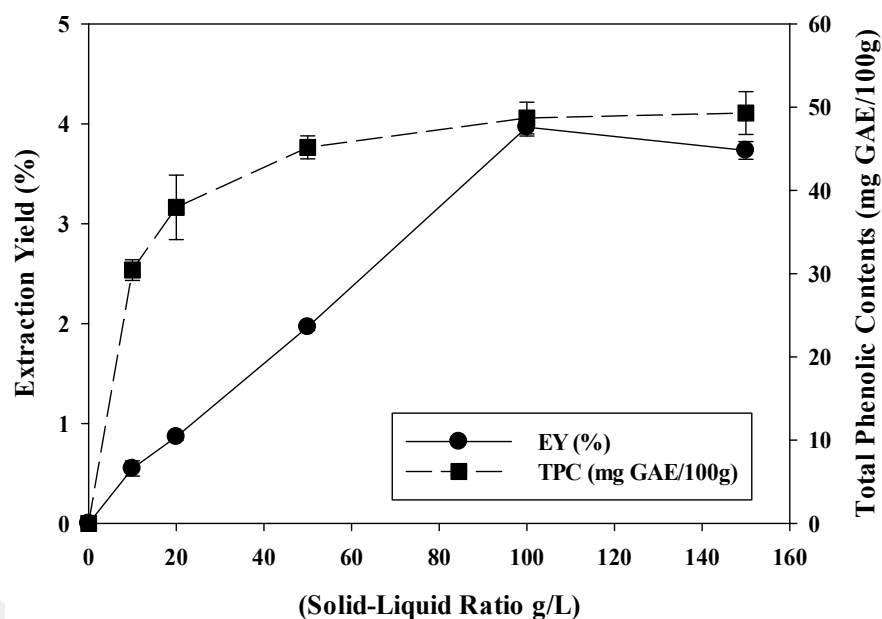


Figure 4.5. Effect of Solid-Liquid Ratio on PNBE

With regard to the impacts of particle size, it is expected that the EY and TPC will rise as the particle size decreases since doing so expands the available surface area for mass transfer. In this study, three different particle size ranges were evaluated. Figure 4.6 shows that at the smallest particle size (0.125-0.250 mm), both EY ($9.37 \pm 0.65\%$) and TPC (46.70 ± 1.56 mgGAE/100g) were high, followed by the next smaller size until the largest particle size tested. This means that the smaller the particle size, the greater the EY and TPC as shown in Table 4.6. This is because the specific surface area of small particles is greater, enabling greater penetration by increasing the solvent's interaction with the particles. Therefore, particle size has a direct impact on the amount of EY and TPC. The results for EY ($p < 0.001$) and TPC ($p = 0.032$) were statistically significant. However, for this study, the second smallest particle size was selected for the optimization.

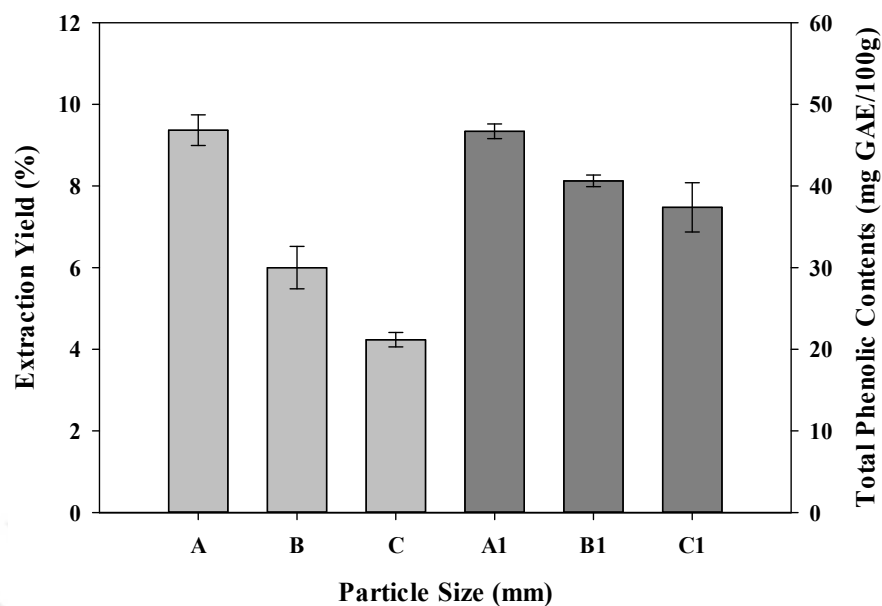


Figure 4.6. Effect of Particle Size on PNBE

Table 4.6. Summary of the Results Obtained for the Effects of Particle Size

Effects of Particle Size (Mean ± Standard Deviation)		
Particle Size (mm)	EY (%)	TPC (mgGAE/100g)
0.125-0.250 (A)	9.37 ± 0.65	46.70 ± 1.56 ^a
0.250-0.500 (B)	6.00 ± 0.90	40.62 ± 1.24 ^{ab}
0.500-1.000 (C)	4.23 ± 0.31	37.38 ± 5.22 ^b

*same letters are not significant according to Duncan's multiple test

Preliminary Optimization

Table 4.7 shows the optimal conditions determined by the results of the single-factor analysis. The RSM will be used to confirm the correlation of the methods for determining optimal conditions for the PNBE extraction process.

Table 4.7. Preliminary Optimization based on the Effects of Individual Factor

PNBE	A	B	C	D	E	F	EY %	TPC (mgGAE/100g)
MAC-MD	180	50	EtOH	70	100	0.250-0.500	5.7 ± 0.15	40.12 ± 1.13

* Time (A), temperature (B), solvent type (C), EtOH concentration (D), solid-liquid ratio (E), particle size (F), and optimize maceration method (MAC-MD)

4.2 Optimization of PNBE by RSM

Following the parameter ranges identified in the preliminary study, the values were fitted into the software to provide the design for the extraction process. In Table 4.8 demonstrates the experimental plan for the CCRD-based trials as well as the expected results for the response variables, such as TPC and DPPH anti-radical scavenging activity. TPC and DPPH anti-radical scavenging activity of PNBE ranged from 83.98-92.11% reduction and 36.06-40.42 mgGAE/100g, respectively.

Table 4.8. The Experimental and Predicted Values of TPC and DPPH as a Function of Time (A), Temperature (B), and Ethanol Concentration (C)

STD	RUN	A	B	C	TPC (mgGAE/100g)		DPPH (% reduction)	
					PRE	EXP	PRE	EXP
15	1	240	55	65	39.94	40.43	91.06	90.05
5	2	120	40	80	36.20	36.06	85.11	83.98
12	3	240	80.23	65	37.19	37.23	88.83	89.05
2	4	360	40	50	37.34	37.13	89.41	89.05
10	5	441.82	55	65	37.05	37.02	89.42	89.05
20	6	240	55	65	39.94	40.32	91.06	92.11
19	7	240	55	65	39.94	39.36	91.06	90.46
7	8	120	70	80	36.47	36.81	87.71	87.99
1	9	120	40	50	37.13	37.55	88.01	88.81
16	10	240	55	65	39.94	40.43	91.06	91.87
18	11	240	55	65	39.94	39.04	91.06	91.75
13	12	240	55	39.77	38.99	38.94	90.64	90.22
14	13	240	55	90.23	37.78	37.66	88.15	88.69
11	14	240	29.77	65	36.82	36.60	87.13	87.04
6	15	360	40	80	37.79	38.19	89.22	89.99
9	16	38.18	55	65	36.43	36.28	87.37	87.87
3	17	120	70	50	38.36	38.09	90.49	89.63
17	18	240	55	65	39.94	40.11	91.06	90.11
8	19	360	70	80	37.00	36.70	88.76	87.87
4	20	360	70	50	37.51	37.77	88.83	89.87

The following quadratic polynomial model was constructed for each of the two response using multiple regression analysis on the experimental data, as shown in Table 4.9. The derived equations showed the empirical link between the dependent and independent variables for each response. These equations were used to calculate

the predicted data in the statistical program and compared to the experimental data obtained from the actual analysis and gave a $y=0.93x + 2.51$ and $y=0.83x + 14.83$ for TPC and DPPH respectively as shown in Figure 4.7 and 4.8.

Table 4.9. Quadratic Polynomial Equations in terms of Coded Factors for the Responses

Responses	Equations
TPC	$Y = + 39.94 + 0.3115*A + 0.1843*B - 0.6049*C - 0.7524*AB + 0.9779*AC - 0.6774*BC - 3.20*A^2 - 2.94*B^2 - 1.55*C^2$
DPPH	$Y = + 91.06 + 1.03*A + 0.8498*B - 1.25*C - 2.17*AB + 1.92*AC + 0.0833*BC - 2.67*A^2 - 3.08*B^2 - 1.67*C^2$

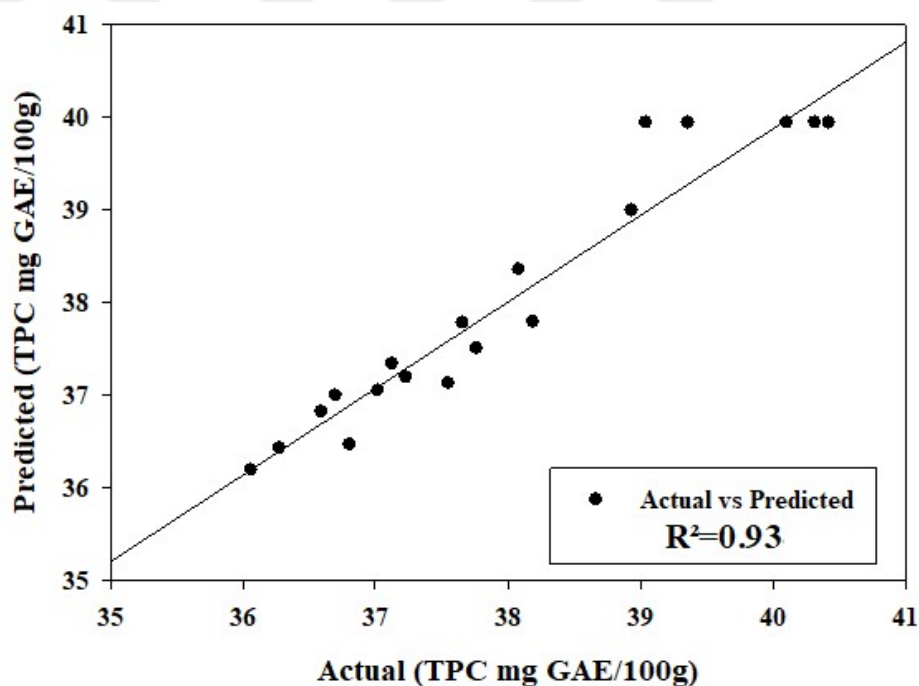


Figure 4.7. Plot for Predicted and Actual TPC

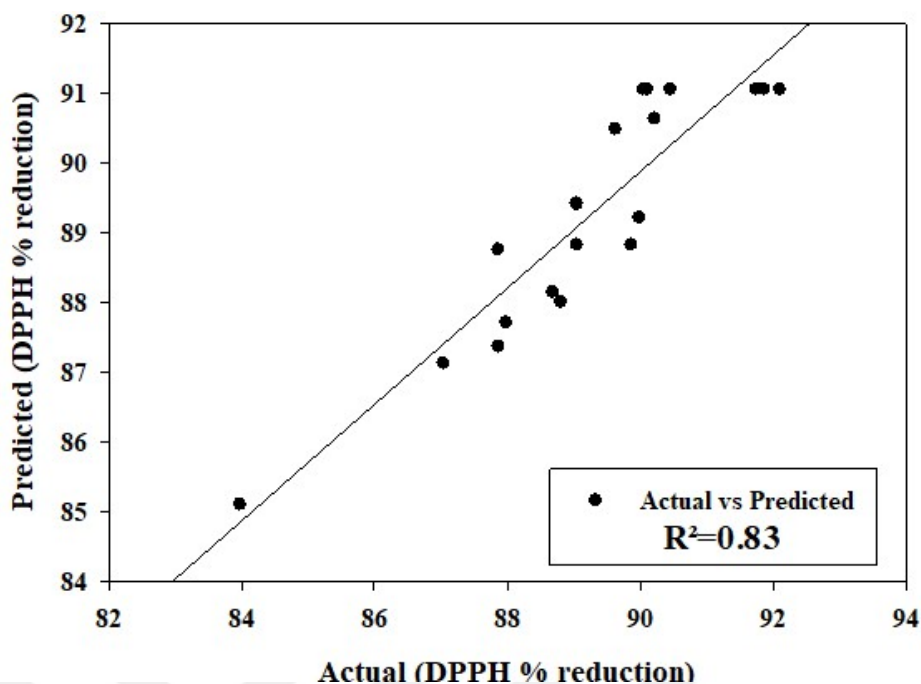


Figure 4.8. Plot for Predicted and Actual DPPH Antiradical Scavenging Activity

Even though it was carried out as an ANOVA research for a quadratic model, testing the model's significance and adequacy was important. Table 4.10 and 4.11 shows that the model of Prob>p for both TPC ($p<0.0001$) and DPPH ($p=0.0064$) were significant. The coefficient of determination (R^2) for the TPC and DPPH responses were 0.93, and 0.83, respectively. According to the R^2 values obtained, the RSM model could describe more than 99% of the response variables. The high R^2 values for each response showed that the developed quadratic polynomial models were a good fit for the CCRD design. The significance of the model terms is shown by p-values less than 0.05. In this case C, A^2 , B^2 , C^2 are significant model terms. Which means that concentration of EtOH had the significant effects on the TPC of PNBE. As for DPPH antiradical scavenging activity, similarly EtOH concentration had the significant effects for the antioxidant capacity of PNBE. Furthermore, as shown in Table 4.12, the predicted R^2 value was reasonably close to the adjusted R^2 value for TPC but not as close for DPPH, possible reasons might be some outliers data and others. As for the lack of fit of both responses, it shows that there were not statistically significant. Hence, these results confirmed the models' predictability in determining the best conditions for obtaining the highest TPC of PNBE but not much for the DPPH of PNBE.

Table 4.10. Analysis of Mean Square Deviation of Regression Equation for the TPC

TPC (mgGAE/100g)						
Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Model	37.14	9	4.13	15.71	< 0.0001	significant
Time (A)	0.47	1	0.47	1.78	0.21	
Temperature (B)	0.16	1	0.16	0.62	0.45	
EtOH Conc. (C)	1.77	1	1.77	6.73	0.03	
AB	0.57	1	0.57	2.16	0.17	
AC	0.96	1	0.96	3.64	0.09	
BC	0.46	1	0.46	1.75	0.22	
A ²	18.46	1	18.46	70.32	< 0.0001	
B ²	15.52	1	15.52	59.12	< 0.0001	
C ²	4.34	1	4.34	16.53	0.0023	
Residual	2.63	10	0.26			not significant
Lack of Fit	0.85	5	0.17	0.48	0.78	
Pure Error	1.78	5	0.36			
Cor Total	39.76	19				

Table 4.11. Analysis of Mean Square Deviation of Regression Equation for the DPPH

DPPH (% reduction)						
Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Model	54.09	9	6.01	5.58	0.01	significant
Time (A)	5.09	1	5.09	4.73	0.05	
Temperature (B)	3.49	1	3.49	3.24	0.10	
EtOH Conc. (C)	7.49	1	7.49	6.95	0.02	
AB	4.69	1	4.69	4.35	0.06	
AC	3.67	1	3.67	3.41	0.09	
BC	0.01	1	0.01	0.01	0.94	
A ²	12.82	1	12.82	11.90	0.01	
B ²	17.09	1	17.09	15.86	0.01	
C ²	5.00	1	5.00	4.64	0.06	
Residual	10.77	10	1.08			not significant
Lack of Fit	6.25	5	1.25	1.38	0.37	
Pure Error	4.52	5	0.90			
Cor Total	64.86	19				

Table 4.12 also shows the predicted R^2 of 0.75 for TPC are in reasonable agreement with the Adjusted R^2 of 0.87, the difference is less than 0.2. However, the predicted R^2 of 0.05 for DPPH are not so close to the Adjusted R^2 of 0.68, the difference is more 0.02. Thus, the RSM model obtained in this study can be used for the optimization of the TPC of PNBE, but not much as for antioxidant capacity of PNBE. The confirmation run should be used to evaluate all empirical models for DPPH.

Table 4.12. Fit Statistics for TPC and DPPH

Responses	R^2	Adjusted R^2	Predicted R^2	Adeq Precision	Std. Dev.	Mean	C.V. %
TPC	0.93	0.87	0.75	10.34	0.51	38.09	1.35
DPPH	0.83	0.68	0.05	8.11	1.04	89.27	1.16

The signal-to-noise ratio is measured by the Adeq Precision. A ratio greater than 4 is preferred. In this study, the ratio of 19.33 and 8.11 for TPC and DPPH indicates an adequate signal. Thus, this model can be used to explore the design space.

The Interaction Between the Factors

Figure 4.9 to 4.14 shows the interaction of factors for TPC and DPPH, respectively. In the Figure 4.9 shows the interaction between the factor time and temperature on TPC at a constant EtOH concentration of 65%. The TPC was at its lowest at the shortest extraction time and lowest temperature. The TPC increased initially and then decreased as the temperature increased from 20 to 80°C. When the temperature was set at a certain level, the TPC increased, but then decreased as the time increased.

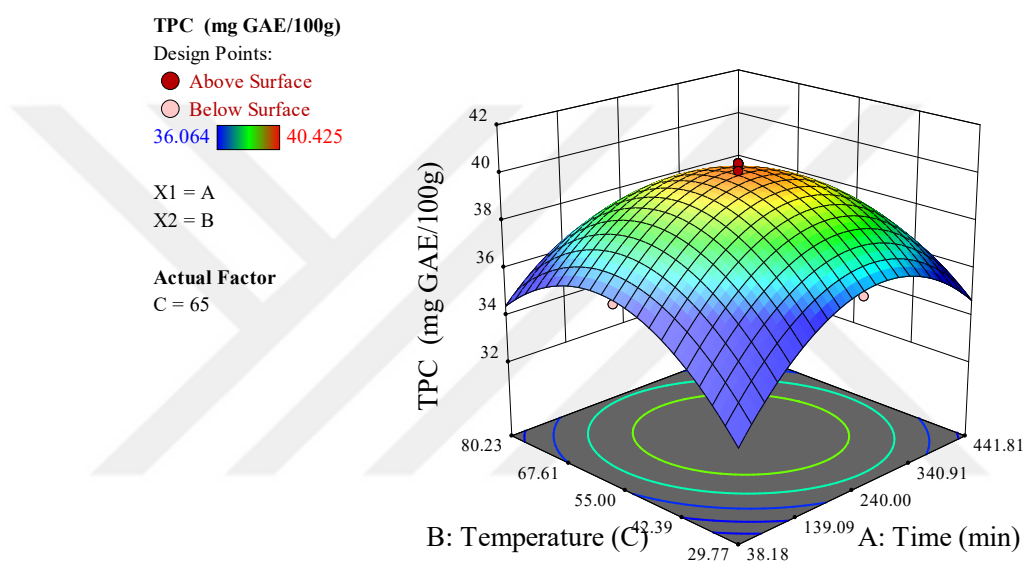


Figure 4.9. Correlative Effects of the Extraction Time and Temperature for TPC

The result from TPC is also similar to the results obtained from DPPH, where DPPH increases at first and then decreases when it reaches the optimum extraction time and temperature as shown in Figure 4.10. The reason for the decrease in TPC and DPPH after reaching the optimum value may be due to the effects of combined time and temperature, as there is a possibility for decomposition of the phenolic contents as also previously explained in the single-factor analysis. Thus, the optimal conditional parameters for extraction time and temperature would be distinct.

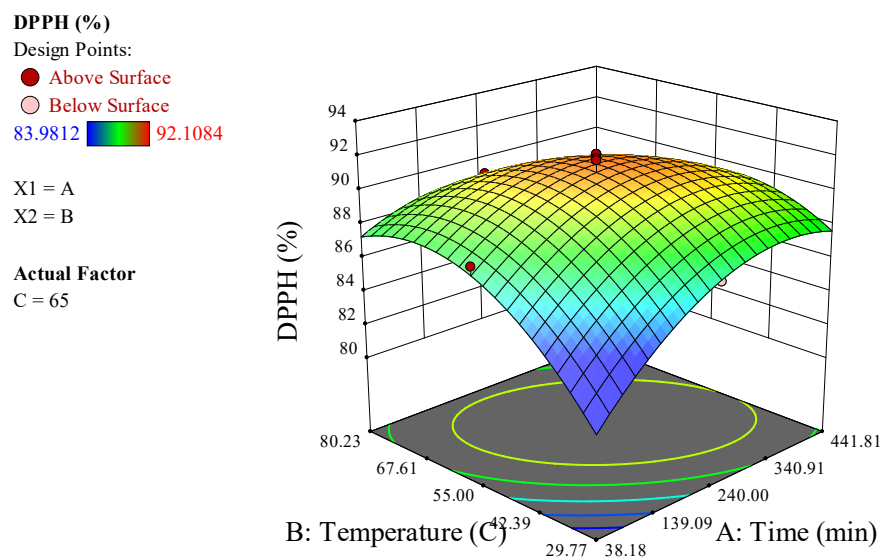


Figure 4.10. Correlative Effects of the Extraction Time and Temperature for DPPH

The relationships between EtOH concentration and temperature are shown in Figure 4.11. The TPC increased as the EtOH concentration and temperature increased. However, after a certain point, the TPC gradually decreases as the EtOH concentration increases. Temperature also improves TPC; an increase in temperature resulted in an increase in TPC. However, as the temperature further increased the TPC also decreases due to possibility of oxidation of TPC. This result is also similarly to the DPPH shown in Figure 4.12.

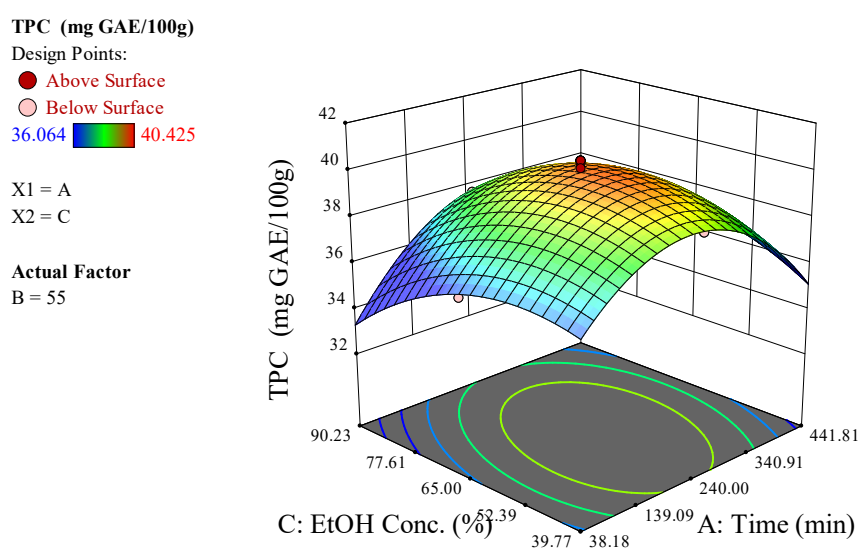


Figure 4.11. Correlative Effects of the Extraction Time and EtOH Concentration for TPC

DPPH (%)
 Design Points:
 ● Above Surface
 ○ Below Surface
 83.9812 92.1084

X1 = A
 X2 = C

Actual Factor
 B = 55

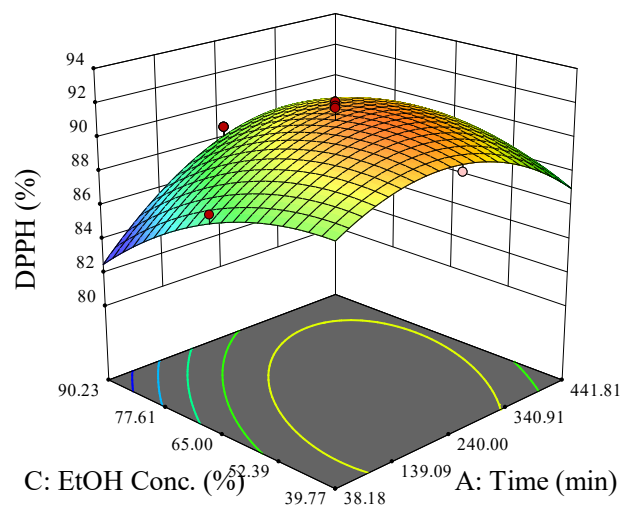


Figure 4.12. Correlative Effects of the Extraction Time and EtOH Concentration for DPPH

Figure 4.13 shows that, in the beginning, as the EtOH concentration and temperature increased, the TPC increased and once they reached a certain value, the TPC was then decreased. Similarly to the DPPH results as shown in Figure 4.14. Temperature increases favored extraction by increasing both solubility and the diffusion coefficient from a solid matrix to a liquid matrix. However, rising temperatures have an impact on the stability of phenolic compounds.

TPC (mg GAE/100g)
 Design Points:
 ● Above Surface
 ○ Below Surface
 36.064 40.425

X1 = B
 X2 = C

Actual Factor
 A = 240

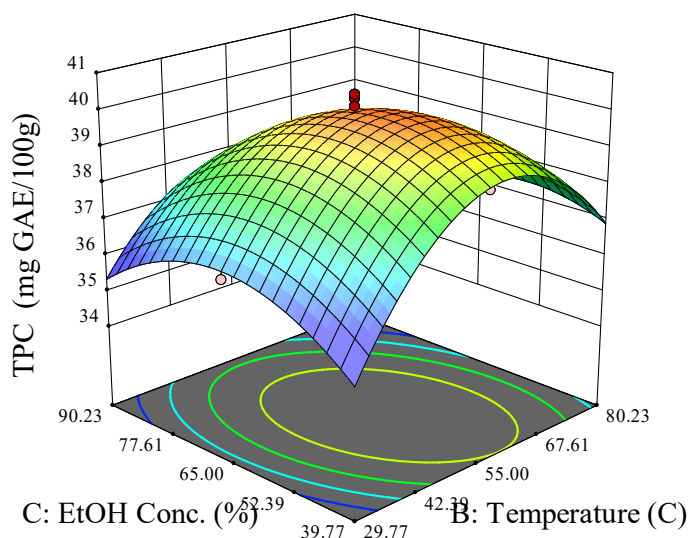


Figure 4.13. Correlative Effects of the EtOH Concentration and Temperature for TPC

DPPH (%)
 Design Points:
 ● Above Surface
 ○ Below Surface
 83.9812 92.1084

X1 = B
 X2 = C

Actual Factor
 A = 240

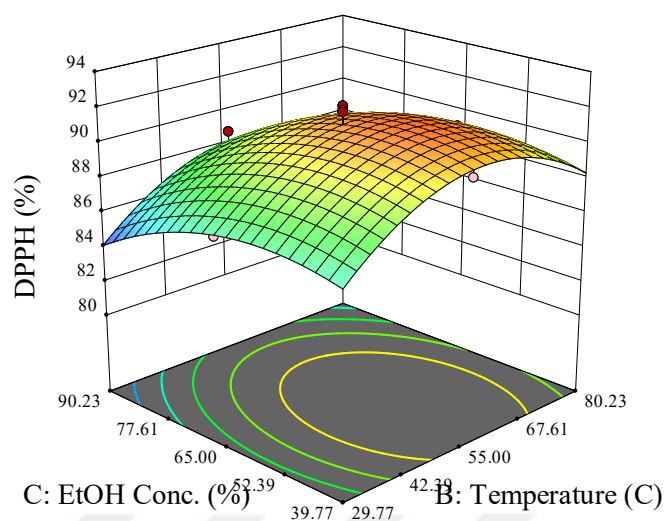


Figure 4.14. Correlative Effects of the EtOH Concentration and Temperature for DPPH

Validation of the Predictive Model

Using the analytical model program, the extraction experiment was conducted in order to further evaluate the validity of the experimental methodology. Table 4.13 displays the predicted and the adjusted conditions. The results showed that the experimental and predicted values for TPC and DPPH were not significantly different. Therefore, the RSM optimal conditions are close to being accurate.

Table 4.13. Verification of the Predictive Model

Term	Time	Temperature	EtOH conc.	TPC	DPPH
Predict	243.70	57.11	58.34	40.00 ± 0.51	91.33 ± 1.04
Experimental	240	60	60	42.56 ± 1.63	89.16 ± 0.98

Correlation between DPPH Antiradical Scavenging Activity and TPC

In this study, the correlations between TPC and DPPH assay were also performed. The results revealed a good correlation between TPC and DPPH assay ($R^2=0.93$) as shown in Figure 4.15. This means that some phenolic compounds in PNBE is responsible for the effective antioxidant capacity.

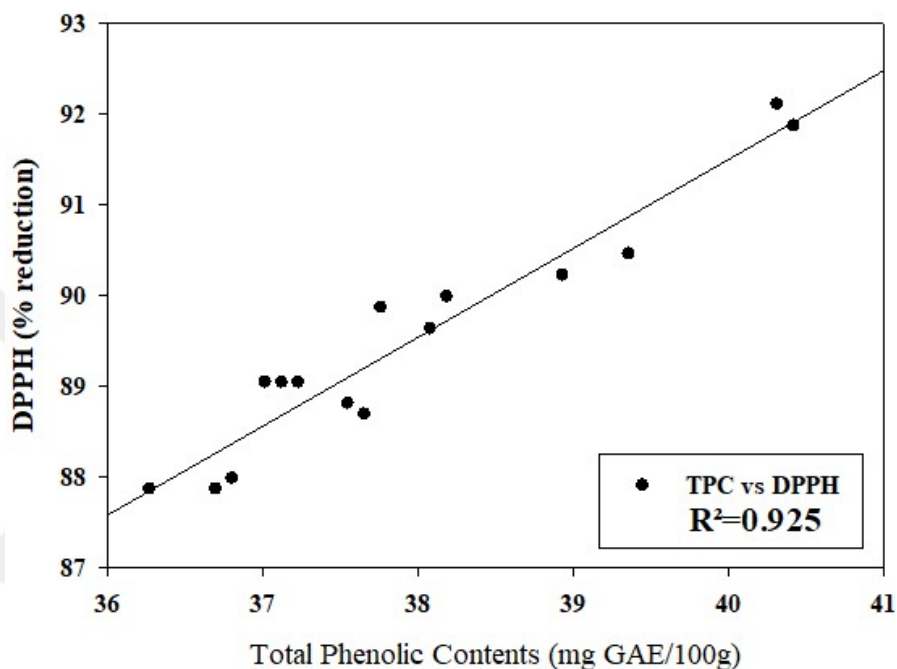


Figure 4.15. Correlation between TPC and DPPH Antiradical Scavenging Activity

4.3 Mass Transfer: Diffusion Coefficient

There is currently no literature on the calculation of mass transfer coefficients and diffusion coefficients of PCs from the bark of *Pinus* species, so in this study, the diffusivity of PCs from pine species, specifically the bark of *P. nigra*, was calculated using diffusion coefficients by Fick's Second Law. The calculated D_{eff} in this study was 1.01×10^{-12} (m^2/s) with $R^2=0.96$ using the optimized conditions of extraction of PNBE as shown in Table 4.14. The results showed that the diffusion of PCs in PNBE is similar to the other plant materials as discussed in Table 2.3 in the literature review of this study. Furthermore, the result of this study also under the range of typical diffusion coefficients for solid solutes in liquid solvents as discussed in the literature study. Figure 4.16 shows the experimental data with their natural numbers as linear

coefficients where the diffusion coefficient was calculated and in Figure 4.17, its shows the accuracy of the experimental data as compared to the predicted data obtained by using the diffusion coefficient value and linear coefficients

Table 4.14. Diffusion Coefficient of Phenolic Compounds from PNBE.

PNBE	A	B	C	D	E	D_{eff} (m ² /s)	R ²
MAC-MD	240	60	60	100	0.250-0.500	1.01×10^{-12}	0.96

* Time (A), temperature (B), EtOH concentration (C), solid-liquid ratio (D), particle size (E), and optimize maceration method (MAC-MD)

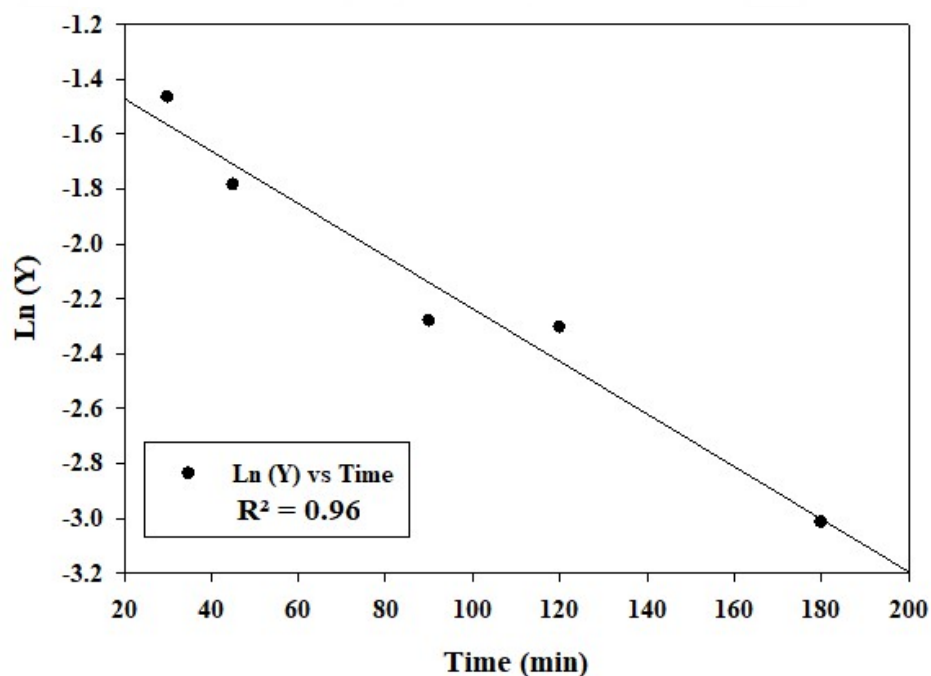


Figure 4.16. TPC Againsts Time for Diffusion Coefficients

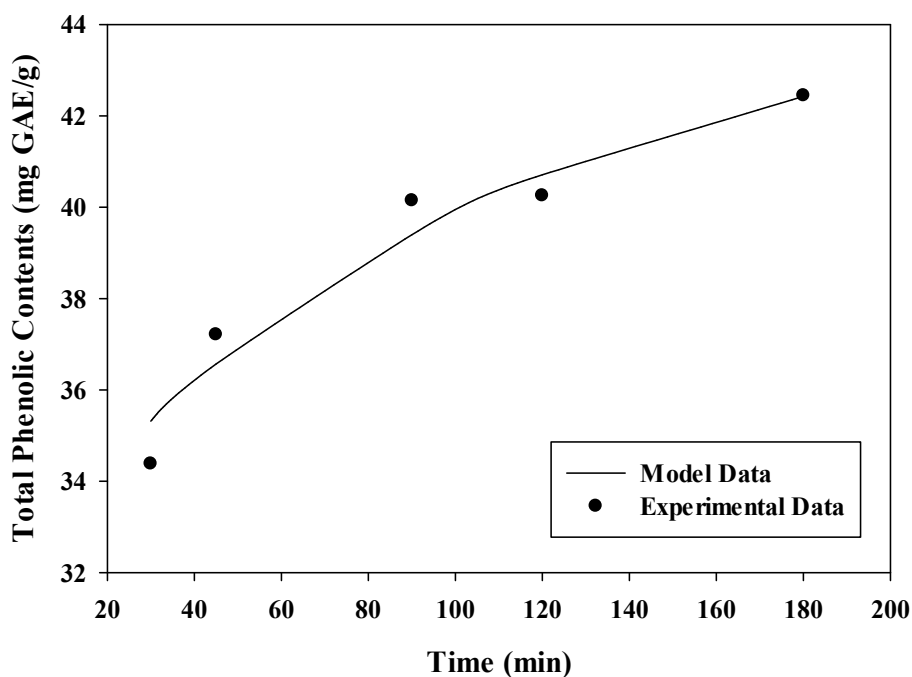


Figure 4.17. Experimental TPC and Predicted TPC using Diffusion Coefficients

4.4 Determination and Characterization of PNBE

4.4.1 Spectrophotometric Analysis of Phenolic Compounds from PNBE

The EY, TPC, and antioxidant capacity were assessed using the optimized extraction parameters with the used of spectrophotometric analysis. Table 4.15 provides information on EY and TPC, while, the information on te the two antioxidant capacities are provided succeeding Tables.

Table 4.15. Comparison Between the Optimal Conditions Obtained

Sol.	Time	Temperature	EtOH concentration	EY	TPC
OP1	180	50	70	5.70 ± 0.15^{ab}	40.12 ± 1.13
OP2	240	60	60	6.40 ± 0.66^a	42.56 ± 1.63
OP3	120	40	50	5.10 ± 0.40^b	41.87 ± 0.86

*same letters are not significant according to Duncan's multiple test

RSM and single-factor analysis both produced optimum results, and those results were compared. The EY of the three sets of optimal conditions differs significantly, as indicated in Table 4.15, as do other parameters. Only OP2 and OP3 exhibit a

discernible difference, whereas the rest do not. However, the difference between the TPC of the three sets of optimal conditions shows no statistically significant. Hence, OP2 from RSM was selected and used as the optimal conditions for further analysis of PNBE. OP1 is from the single-factor analysis, OP2 is from the RSM, and OP3 is the lowest levels in the range of optimization for the single-factor analysis.

4.4.2 Chromatographic Analysis of Phenolic Compounds from PNBE

The RP-HPLC-DAD was used to characterize the PCs from PNBE. Based on the results, only 12 PCs were discovered in PNBE out of the 15 PCs that were utilized as standards in this investigation. Seven flavonoids, one simple phenolic, one triglyceride, one hydroxybenzoic acid, three hydrocinnamic acid acids, and one were present in this investigation. These PCs are presented in Table 4.16. Figure 4.18 shows the chromatogram of the HPLC results. According to this study, PNBE has the most 2,5-hydroxybenzoic acid, which has the potential to be used for pharmaceutical applications, particularly for antioxidant activity. The amount of catechin in PNBE also aids in its ability to act as an antioxidant.

Table 4.16. Phenolic Compounds Found in PNBE

Chemicals	Ret. Time	Conc. (mg/L)
Pyrocatechol	19.753	0.502
Catechin	27.381	8.468
Kaempferol	79.700	0.949
Myricetin	74.915	9.698
Coumaric Acid	43.685	0.564
Ellagic Acid	ND	ND
Caffeic Acid	31.502	1.946
Taxifolin	46.714	3.727
Naringin	66.267	5.537
Chrysin	81.785	2.635
Triacetin	ND	ND
2-5 Dihydroxybenzoic Acid	25.669	9.743
Ferulic Acid	46.644	5.094
Rutin	ND	ND
Butein	77.970	1.301
ND: Not detected		

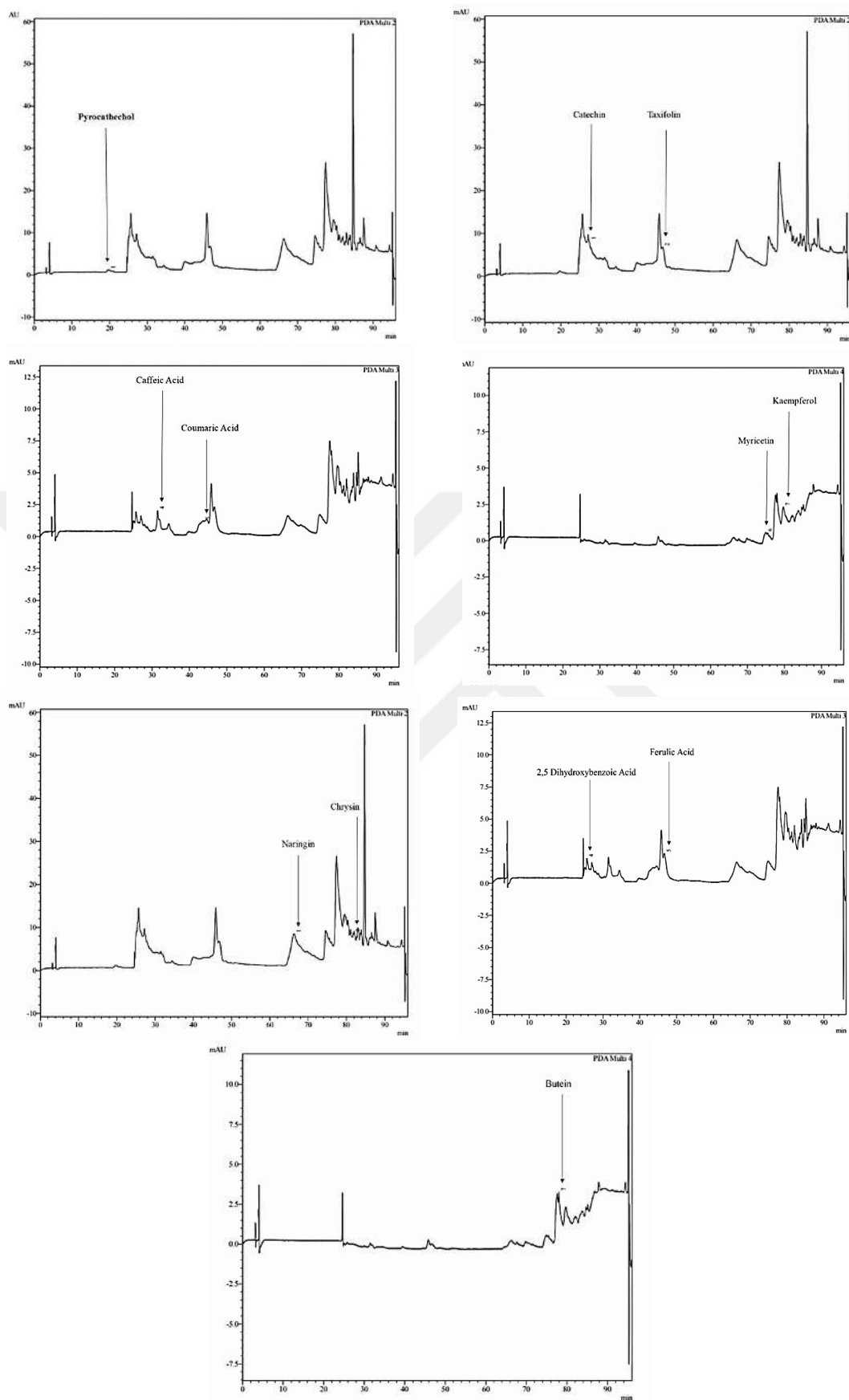


Figure 4.18. Chromatogram of Phenolic Compounds from PNBE

4.4.3 Chromatographic Analysis of Volatile Compounds from PNBE

Figure 4.19 displays the results of the chromatographic analysis of PNBE's VOCs conducted using SPME GC-MS. In the chromatogram, there are four peaks with respective retention time (RT) that are visible. These compounds are represented by mostly decane (RT=10.72), dodecane (RT=17.60), tetradecane (RT=23.45), and hexadecane (RT=28.54) with 8.30, 15.71, 19.00, and 7.92% total area detected in the SPME fiber, respectively. Other peaks also showed in the chromatogram are consisted of mostly alkanes, alkenes, alcohols, ethers, and esters. Furthermore, in SPME GC-MS analysis of PNBE, phenol compound (RT= 20.40) are also detected with 2.70% of the total area. This compound is categorized as simple phenolic which gives the pine trees a woody minty odor. Terpenes such as Δ -cadinene (RT=26.75) and (-)-alpha-amorphene (RT=25.56) are also detected with 0.98 and 0.24% of the total area detected. These terpenes are classified as sesquiterpenes and serve as plant metabolites that are added to foods as flavorings and fragrances.

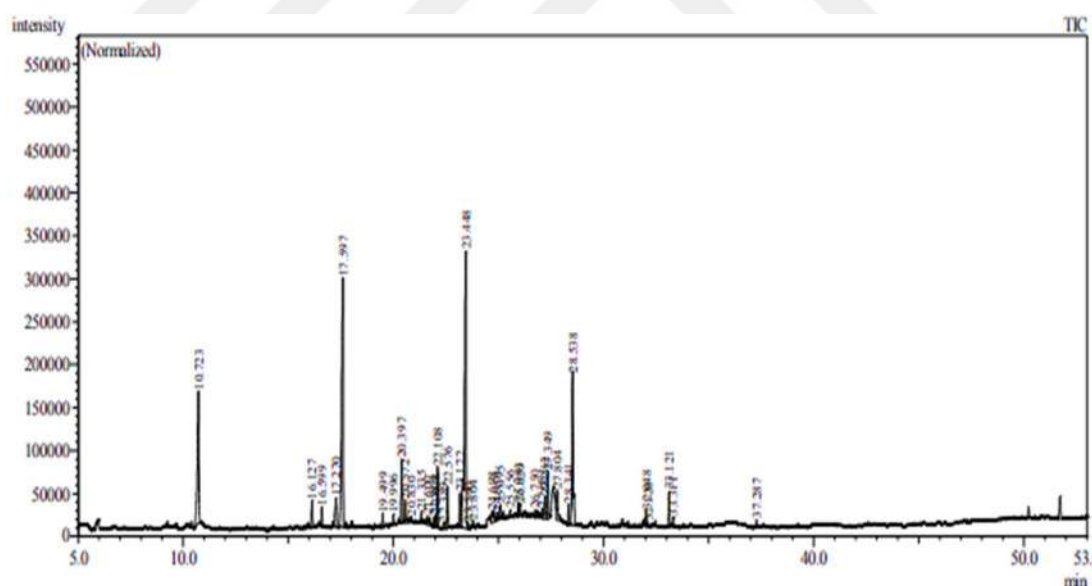


Figure 4.19. Chromatogram of the Volatile Organic Compounds from PNBE

4.4.4 Antioxidant Capacity of PNBE

In Figure 4.20 shows the percentage scavenging activity of the three optimized parameters against the reference standard, ascorbic acid. Results were presented as a percentage reduction (%) and the half-maximal inhibitory concentration (IC_{50}) in ascorbic acid equivalents ($\mu\text{g AAE/ml}$).

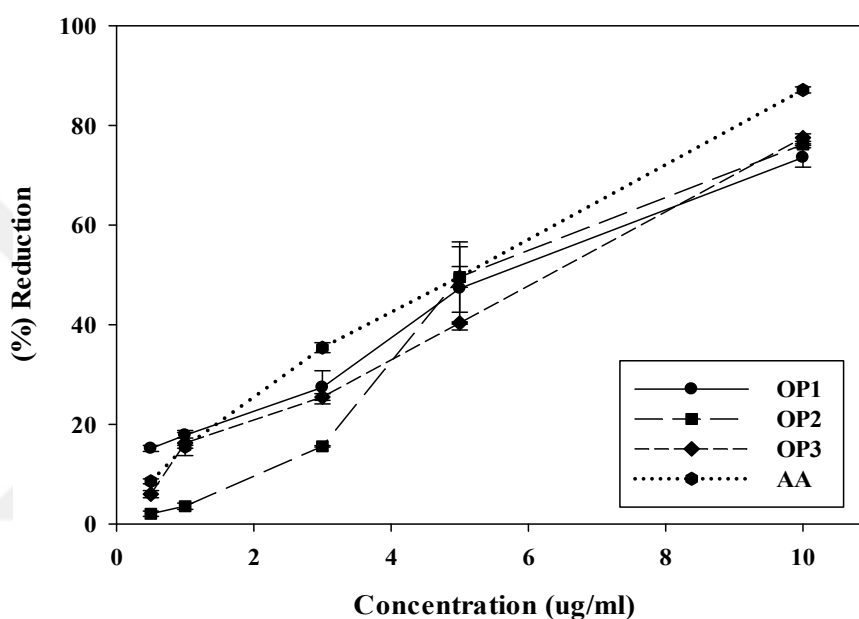


Figure 4.20. DPPH Radical Scavenging Assay of *P. nigra* Bark Extracts with Three Optimized Parameters and Ascorbic Acid

As shown in the Figure 4.20, when the three optimization parameters based on their IC_{50} value were compared to the reference standard, each of the optimized parameters showed a significant difference. However, when compared to one another, there was no significant difference. PNBE were found to be capable of capturing this radical, with IC_{50} values of 6.09 ± 0.46 , 6.41 ± 0.39 , and 6.24 ± 0.04 $\mu\text{g/ml}$ expressed as dried ethanolic extract for OP1, OP2, and OP3, respectively, and 5.23 ± 0.07 $\mu\text{g/ml}$ for ascorbic acid control. As summarized in Table 4.17, lower IC_{50} value means have high antioxidant capacity. Therefore, the antioxidant capacity of the PNBE is high but not as high as the ascorbic acid. However, it can be concluded that PNBE is a good source antioxidant potential chemicals.

Table 4.17. Percentage Scavenging Activity of the Three Optimized Parameters as well at Different Concentrations and their Respective IC₅₀ (μg/ml) Values.

DPPH Assay (Mean ± Standard Deviation)				
Concentration	OP1	OP2	OP3	AA
0.5	15.15 ± 1.05	5.98 ± 1.24	2.05 ± 0.91	8.56 ± 0.83
1	17.81 ± 0.97	16.24 ± 4.34	3.56 ± 1.09	15.50 ± 0.48
3	27.42 ± 5.77	25.48 ± 1.15	15.58 ± 0.25	35.39 ± 1.72
5	47.28 ± 14.47	40.34 ± 0.39	49.55 ± 12.21	49.58 ± 3.61
10	73.55 ± 3.35	77.54 ± 1.30	76.15 ± 0.29	87.11 ± 1.07
IC ₅₀	6.09 ± 0.46 ^a	6.24 ± 0.04 ^a	6.41 ± 0.39 ^a	5.23 ± 0.07 ^b

*same letters are not significant according to Duncan's multiple test

In this study, FRAP was also used to measure antioxidant capacity of the PNBE. Figure 4.21 shows the FRAP assay results for three optimized parameters presented as ascorbic acid equivalents (mgAAE/100g). The Figure below shows that OP2 had the higher ascorbic acid equivalents (18.40 ± 0.56 mgAAE/100g) than OP1 (13.30 ± 0.62 mgAAE/100g) and OP3 (15.20 ± 0.51 mgAAE/100g) as also shown in Table 4.18.

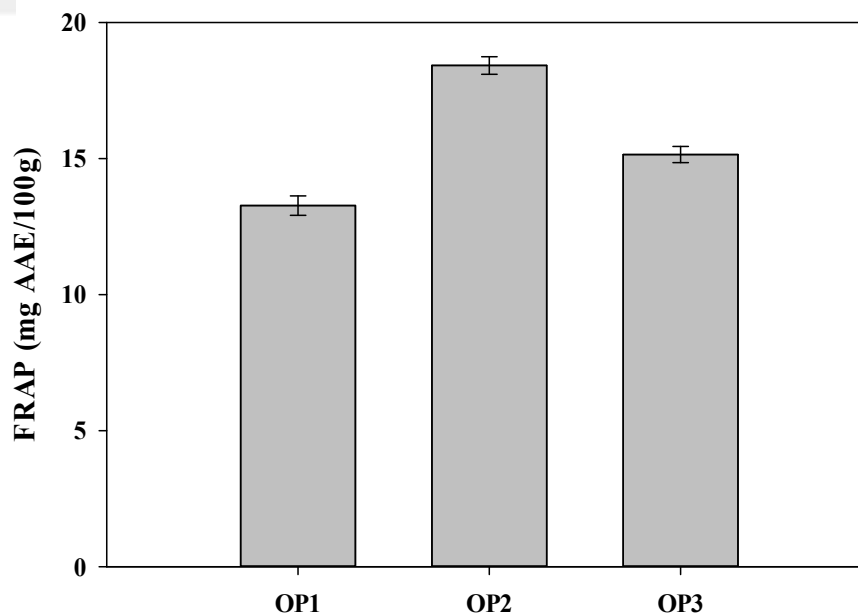


Figure 4.21. FRAP Assay of PNBE with Three Optimized Parameters as Ascorbic Acid Equivalents

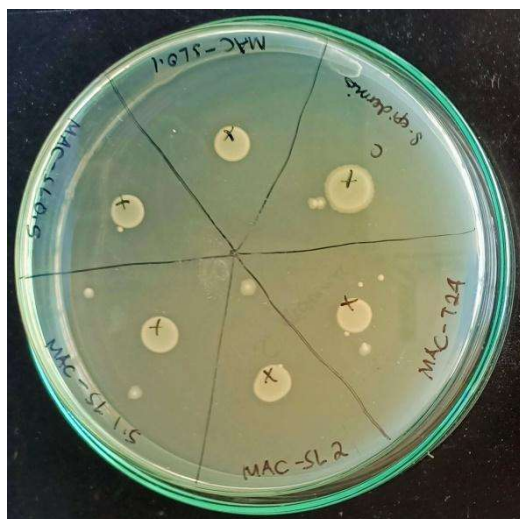
Table 4.18. Summary of the FRAP Assay of PNBE with Three Optimized Parameters as Ascorbic Acid Equivalents

FRAP Assay (Mean $\pm$ Standard Deviation)	
Optimized Parameters	FRAP (mgAAE/100g)
OP1	13.27 $\pm$ 0.62 ^{ac}
OP2	18.42 $\pm$ 0.56 ^{ab}
OP3	15.15 $\pm$ 0.51 ^{bc}

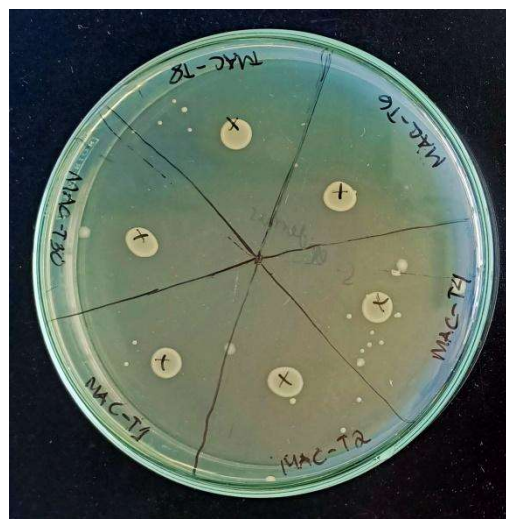
*same letters are not significant according to Duncan's multiple test

4.4.5 Antibacterial Activity of PNBE

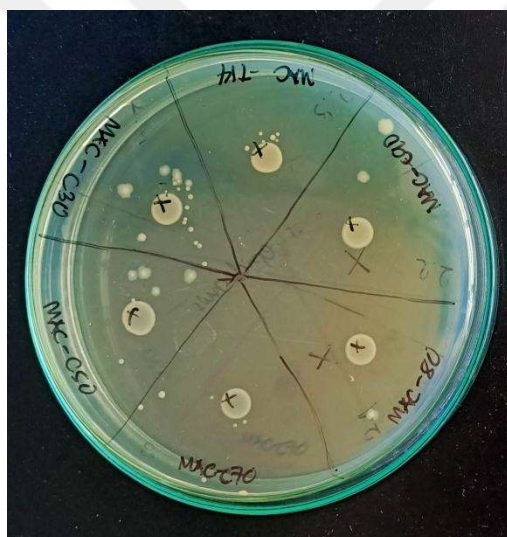
Antibacterial screening analysis revealed that PNBE had low antibacterial activity (20% reduction for 1mg/ml PNBE), resulting in only a small portion of the size being reduced when compared to the control. It also shows that difference in time, temperature, ethanol concentration, solid/liquid ratio, and particles sizes had no effects on the antibacterial activity of the extracts as represented in Figure 4.22, Figure 4.23, and Figure 4.24 respectively. *Staphylococcus epidermidis* (10mm), *Escherichia coli* (12mm), and *Pseudomonas aeruginosa* (11mm) were the control bacteria, and all of the bacteria with extracts ranged in size from 7-9mm. The reduction was only about 2-3mm. Thus, the antibacterial activity of *P. nigra* bark extracts at 1mg/ml is considered between 20-30%.



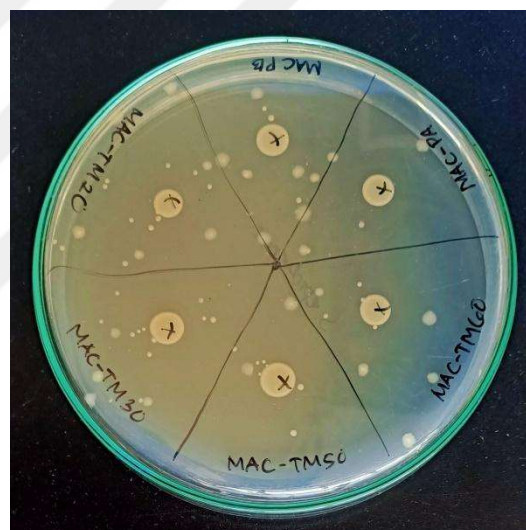
A



B

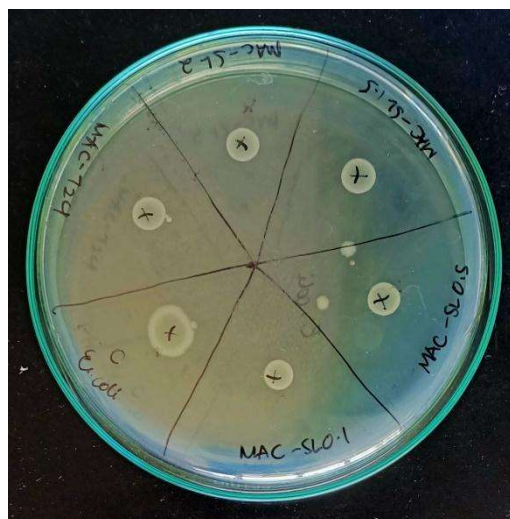


C

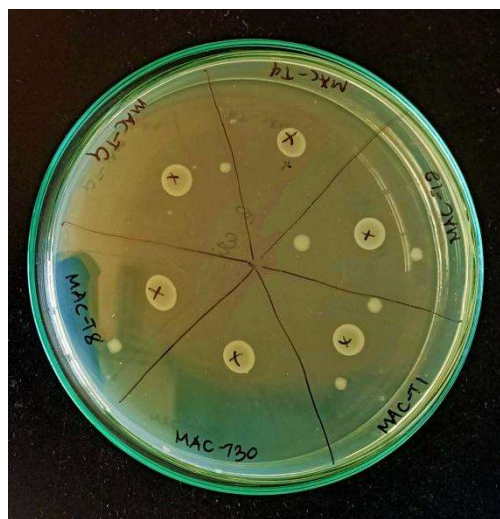


D

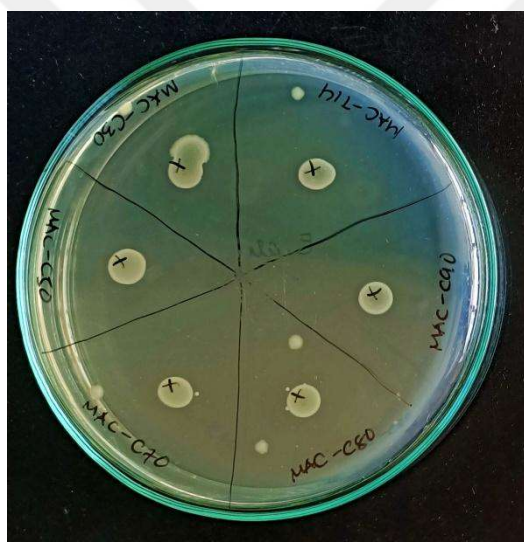
Figure 4.22. Antibacterial Screening of the PNBE Against *Staphylococcus epidermidis*. Control and Solid/liquid ratio (A), Time of Extraction (B), Ethanol Concentration (C), Temperature and Particle size (D)



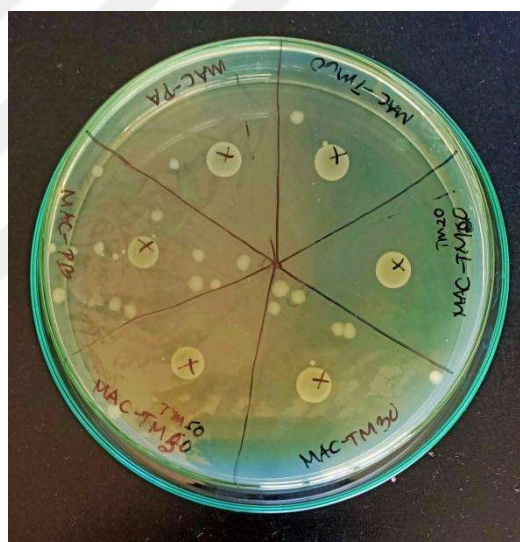
A



B

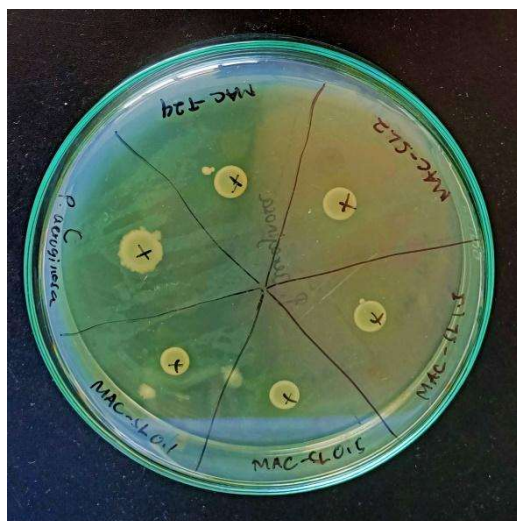


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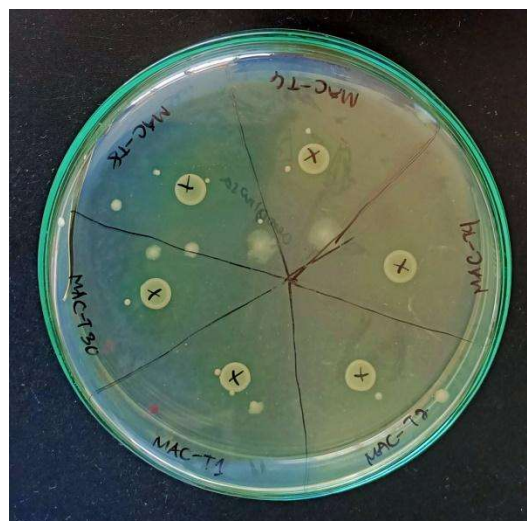


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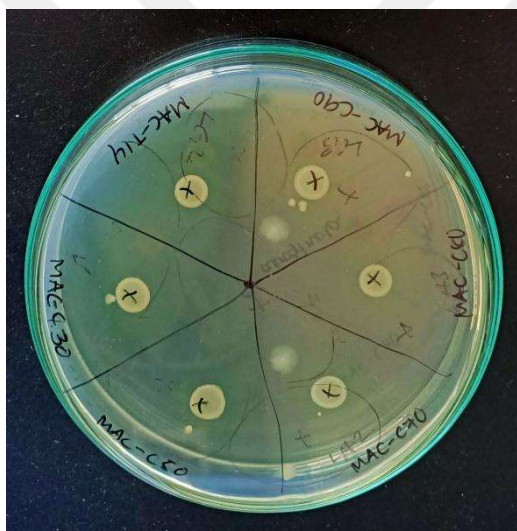
Figure 4.23. Antibacterial Screening of the PNBE Against *Escherichia coli*. Control and Solid/liquid ratio (A), Time of Extraction (B), Ethanol Concentration (C), Temperature and Particle size (D)



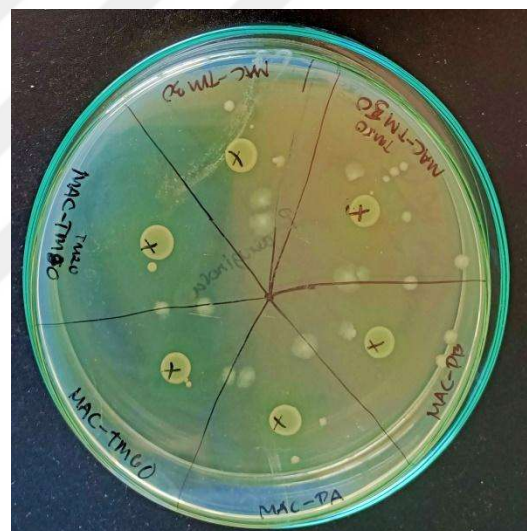
A



B



C



D

Figure 4.24. Antibacterial Screening of the PNBE Against *Pseudomonas aeruginosa*. Control and Solid/liquid ratio (A), Time of Extraction (B), Ethanol Concentration (C), Temperature and Particle Size (D).

4.4.6 Heavy Metals Composition of PNBE

To evaluate the presence of heavy metals in the extracts, the PNBE was also assessed and the results are shown in Table 4.19. When present in larger proportions in plants and foods, heavy metals, which are toxic, can be damaging to human health. Because it was crucial to guarantee the PNBE's quality and safety, a heavy metals study was conducted. In the study, the PNBE was used to evaluate the presence of heavy metals such as As, Pb, Cd, Cu, and Cr. Results revealed that these heavy metals are found in PNBE with certain concentrations that are below the permissible concentrations established by WHO, EU, and Codex Standard as seen in Table 2.4, page 49. In the results obtained, the highest heavy metal detected in PNBE was Pb, however the concentration is still within the allowable limits. So, in terms of heavy metal contents, PNBE is still safe.

Table 4.19. Heavy Metals Contents of PNBE

Heavy Metals	Min (Detection) ppb	Max (Detection) ppb	Concentrations (ppb)
As	8.00	120.00	192 ± 3.49
Cd	0.50	120.00	16.65 ± 0.05
Cr	8.00	96.00	94.85 ± 0.61
Cu	0.84	120.00	177.62 ± 1.22
Pb	12.64	120.00	215.82 ± 0.53

4.4.7 Effects of Extraction Method on PNBE

The current study also aimed to compares the two traditional methods in terms of the EY, TPC and antioxidant activity. In Table 4.20, SOX extraction obtained the highest yield of $11.07 \pm 0.09\%$ compared to normal MAC (MAC-NM) with only $5.50 \pm 0.36\%$, whereas the TPC are the opposite, with MAC-NM obtaining the highest TPC of 29.47 ± 1.064 mgGAE/100g than SOX extraction with only 24.22 ± 0.89 mgGAE/100g. It indicates that the high EY and low TPC for SOX extraction and the low EY and high TPC for MAC-NM are most likely caused by the difference in temperature. Furthermore, to confirm the preliminary results, another modified/optimized MAC (MAC-MD) method was employed with the same

extraction parameters as the previous methods: S/L ratio, particle size, and EtOH concentration. The only difference was that the temperature was increased to 50°C and the time was reduced to 120 minutes. In the same Table 40, the EY of $5.067 \pm 0.40\%$ for the MAC-MD method is the lowest compared to the two traditional methods.

Table 4.20. Summary of the Obtained Results for the Effects of Extraction Techniques

Effects of the Extraction Techniques (Mean $\pm$ Standard Deviation)				
Extraction Techniques	EY %	TPC (mgGAE/100g)	DPPH (%reduction)	FRAP (mgAAE/100g)
MAC-MD	5.07 ± 0.40^b	42.23 ± 0.77	83.43 ± 0.45	26.47 ± 0.51^a
MAC-NM	5.50 ± 0.36^b	29.47 ± 1.06	78.79 ± 0.98	18.82 ± 0.76^b
SOX	11.1 ± 0.09^a	24.22 ± 0.89^{ac}	86.16 ± 0.25	19.48 ± 1.34^b

*same letters are not significant according to Duncan's multiple test

However, it obtained the highest TPC (42.23 ± 0.077 mgGAE/100g). As for the antioxidant capacity of PNBE, the total antioxidant capacity resulted in FRAP assay, MAC-MD (26.47 ± 0.51 mgAAE/100g) obtained the highest followed by SOX extraction. For DPPH antiradical scavenging activity SOX obtained the highest followed by MAC-MD. Whereas, for MAC-NM, based on all the responses evaluated obtained the lowest. Thus, the results indicated that the additional temperature added to MAC-NM would increased the EY, TPC, and antioxidant capacity of the PNBE. The difference between the extraction methods for EY ($p < 0.001$), TPC ($p < 0.001$), DPPH antiradical scavenging activity ($p < 0.001$), and FRAP ($p < 0.001$) are all statistically significant.

4.4.8 Effects of Bark Age on PNBE

This study would also like to know if there is a difference between the age of the tree in terms of their EY, TPC, DPPH, and FRAP antioxidant capacity. Figure 4.25 shows the differences in EY, TPC, and antioxidant activity of two bark samples based on their age range and the summary of the results are shown in Table 4.21.

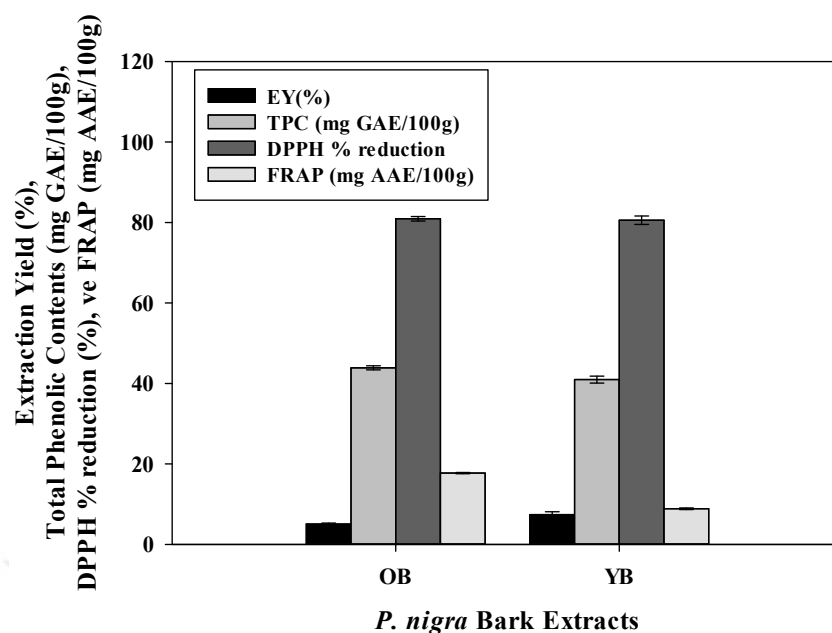


Figure 4.25. EY, TPC, and Antioxidant Activity of the two PNBE at Different Age Ranges.

Table 4.21. Comparison on the Age of Bark Samples

Type of Bark	Age of the Tree (Mean $\pm$ Standard Deviation)			
	EY	TPC	DPPH	FRAP
OB	5.07 $\pm$ 0.40	43.87 $\pm$ 0.89	80.92 $\pm$ 0.97	17.72 $\pm$ 0.27
YB	7.40 $\pm$ 1.21	40.96 $\pm$ 1.49	80.55 $\pm$ 1.82	8.86 $\pm$ 0.33

The young bark samples perform better than the older bark samples in terms of EY. However, when it comes to TPC, the old bark samples have higher phenolic contents. Furthermore, antioxidant activity also showed higher in the old bark than the young bark. This demonstrates good results; while young bark had a EY, old bark had a TPC. This leads to the conclusion that older trees cut down in the wood industry, as well as the waste generated after the trees are cut down, can be a rich source of PCs. Thus, waste from the pine wood industry could be managed.

4.5 Surface Morphological Analysis of *P. nigra* Bark

The two *P. nigra* bark samples contained both inner and outer bark, and the homogenized bark (0.250-0.500mm) from older trees had a higher outer and inner bark ratio and a darker brown appearance than the homogenized bark (0.250-0.500mm) from younger trees which had a brownish yellow appearance as shown in Figure 4.26.

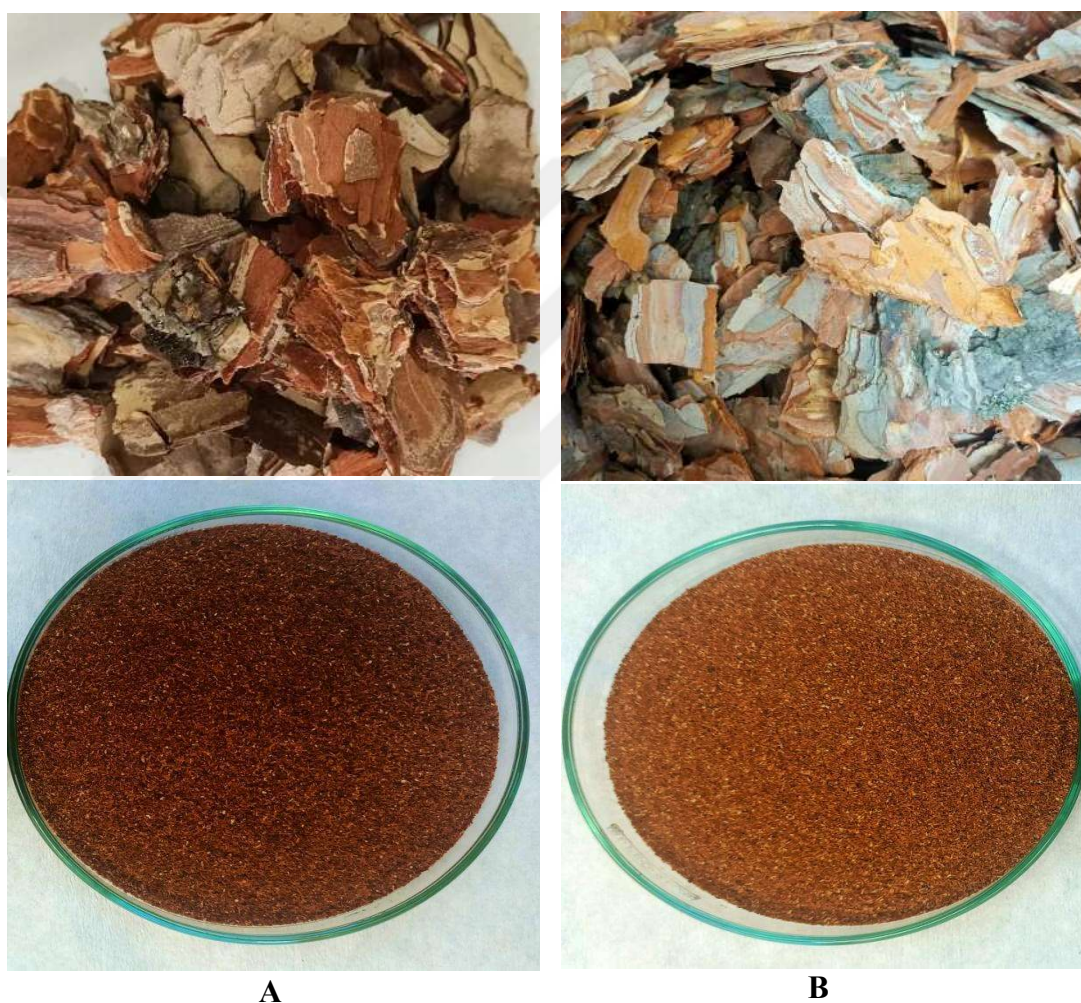


Figure 4.26. Differences in Appearance between the Two Bark Samples based on their Age Range. ~65 years Old Bark (A) and ~25 years Old Bark (B)

SEM analysis of *P. nigra* bark particles before and after extraction was performed to better understand the effect of the extraction process on the structure of the bark sample. The structural changes were determined at 2000x magnification. Figure 4.27 shows that the pores were layered and not damaged before the extraction; however, the layered pores were altered and damaged after the extraction. Figure 4.28 compares the three extraction techniques with the same EtOH concentration, particle size, and S/L ratio. The only distinction was one of time and temperature. Figure 4.28, A and B show particles from an optimized MAC with a temperature of 60°C and a time of 240 minutes. C and D are from the standard maceration, where the temperature was kept at room temperature for 24 hours. E and F are from a SOX extraction in which the temperature was held at the boiling point of aqueous EtOH for 120 minutes. The SEM analysis revealed that temperature caused changes in the bark structure. SOX extraction had the highest temperature, followed by optimized MAC, and then normal MAC. The layered pores in the Figure 4.28 were completely altered and damaged by the SOX extraction, whereas the layered pores in the normal MAC are still visible with minor damage for the young bark except for the old bark.

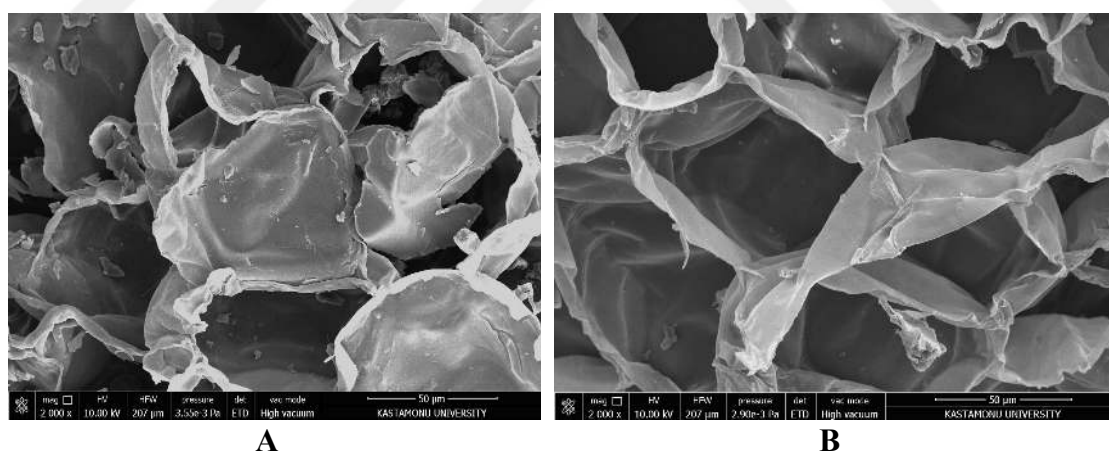
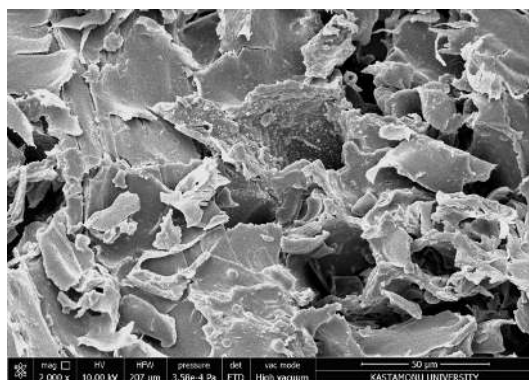
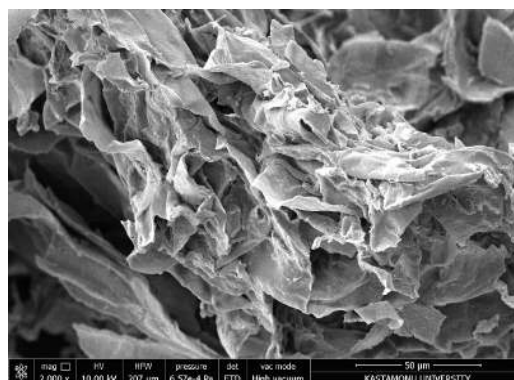


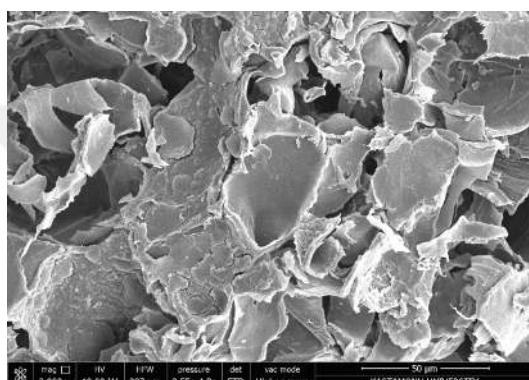
Figure 4.27. *P. nigra* Bark Structure Before the Extraction. Old Bark (A) and Young Bark (B)



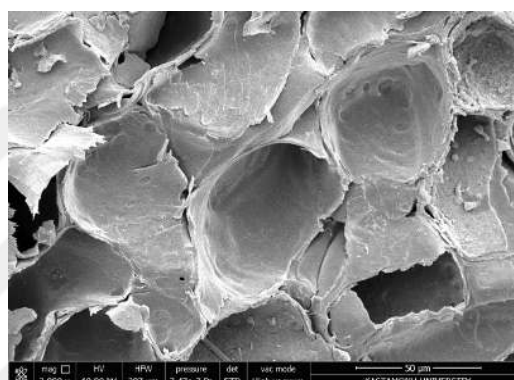
A



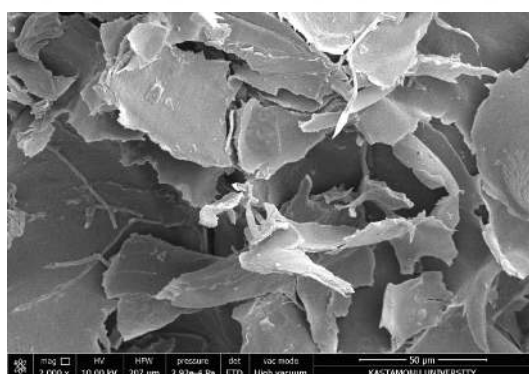
B



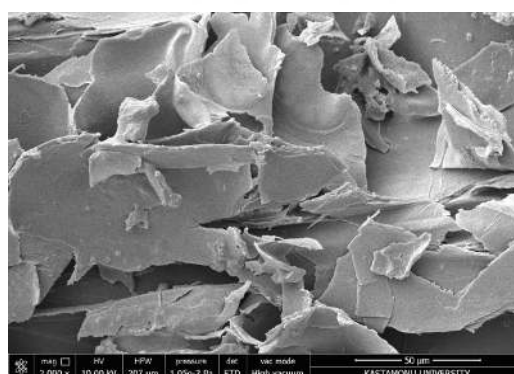
C



D



E



F

Figure 4.28. Structure of *P. nigra* at 2000x Magnifications. Optimized Maceration for Old and Young Bark (A and B), Normal Maceration for Old and Young bark (C and D), and Soxhlet Extraction for Old and Young Bark (E and F).

5. CONCLUSIONS

The characterization of antioxidant activity, antibacterial activity, heavy metal contents and phenolic profiles, as well as VOCs of PNBE, were carried out in this study. The phenolic compounds (PCs) were extracted from the bark of Black pine collected in *Üyücek* and *Ahlatçık*, Kastamonu, Türkiye in the summer of 2022. PNBE was extracted using MAC extraction. In the extraction procedure, the effects of time, temperature, and solvent concentrations on TPC and DPPH antioxidant capacity were optimized using RSM. Results showed that RSM is a great model for optimizing the extraction of PNBE. After the optimization, the EY ($6.40 \pm 0.66\%$ d.w), diffusion coefficients (D_{eff}) ($1.01 \times 10^{-12} \text{ m}^2/\text{s}$), TPC ($42.56 \pm 1.13 \text{ mgGAE}/100\text{g d.w}$), DPPH ($\text{IC}_{50} 6.24 \pm 0.04 \text{ } \mu\text{LAEE}/\text{ml}$), and FRAP ($18.42 \pm 0.56 \text{ mgAAE}/100\text{g d.w}$) antioxidant assays were determined using the optimized parameters at 60% EtOH, 240 mins, and 60°C, with constant S/L ratio (100 g/L) and particle size (0.250-0.500 mm). Analysis in phenolic profiling using RP-HPLC-DAD revealed that 2,5 dihydroxybenzoic acid, myricetin, catechin, naringin, and ferulic acid are the major components in PNBE, accounting for 19.42%, 19.33%, 16.88%, 11.04%, and 10.15% of the total amount measured, respectively. In contrast, others are less than 10%. For SPME-GC-MS, there four prominent peaks were detected such as decane (RT=10.72), dodecane (RT=17.60), tetradecane (RT=23.45), and hexadecane (RT=28.54) with 8.30, 15.71, 19.00, and 7.92% total area detected in the SPME fiber, respectively. Other peaks that are less prominent than the significant four are also present, primarily alkanes, alkenes, alcohols, ethers, esters, and terpenes, including the cadinene family of sesquiterpenes, which made up 0.98 and 0.24% of the total area observed. The antibacterial power of PNBE was also evaluated, showing that it only reduces the growth of control bacteria by 20–30%. However, at some points, its capability to stop bacterial development is less efficient than other pine species. Furthermore, heavy metal compositions were also evaluated to ensure the overall quality of PNBE. Heavy metals such as As, Cr, Cd, Cu, and Pb were found in PNBE with concentration levels of 192 ± 3.49 , 94.85 ± 0.61 , 16.65 ± 0.05 , 177.62 ± 1.22 , and $215.82 \pm 0.53 \text{ ppb}$, respectively. These concentrations are less than the acceptable levels established by the WHO, EU, and Codex Stan 193-

1995. In conclusion, this research shows that PNBE has the potential to recover PCs for applications in medicine, food, and other industries. Furthermore, the results of this study will now be used as a guide for further research on the phenolic compounds found in PNBE, including investigations into their many biological functions similar to other pine tree species that have received extensive research.



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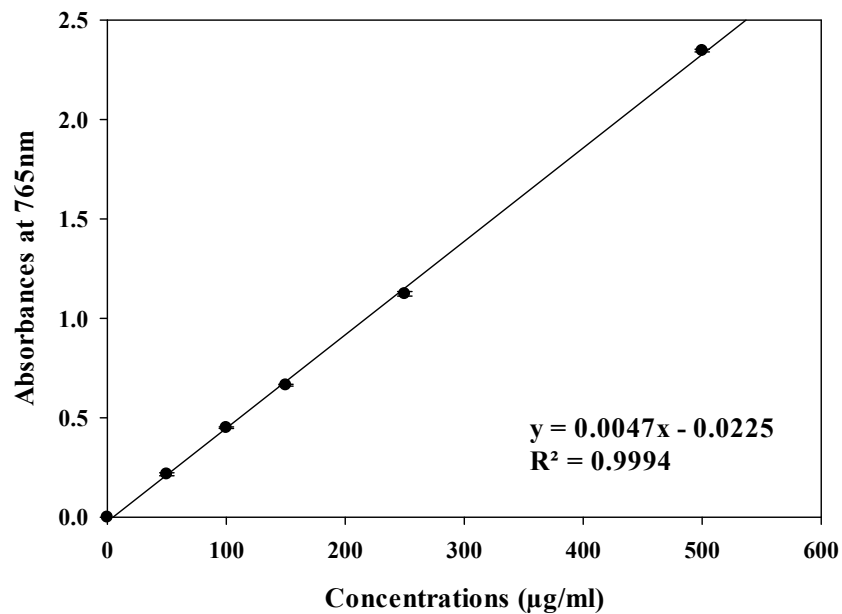
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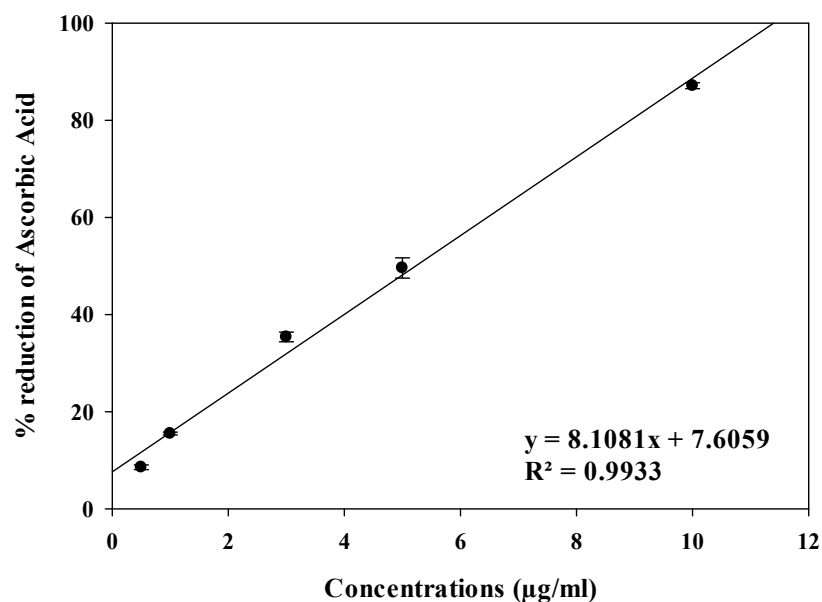


APPENDICES

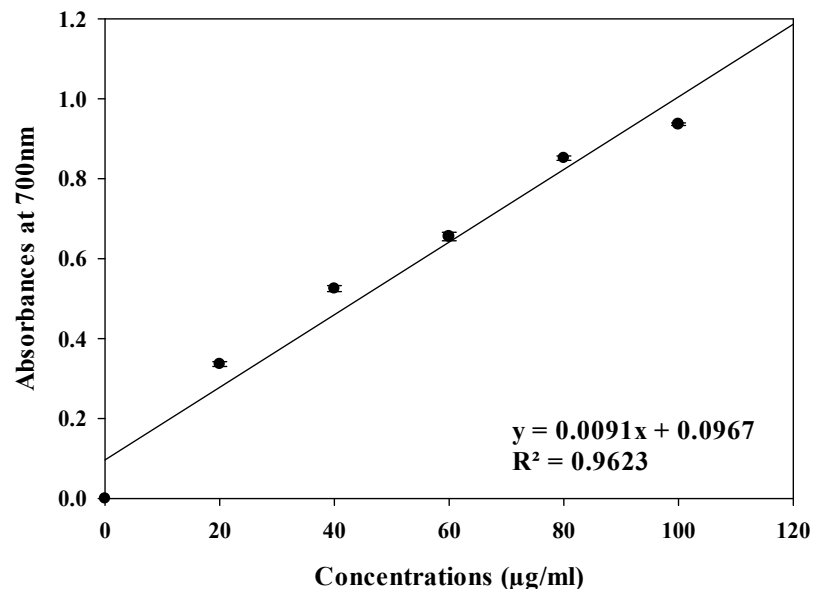
Appendix 1. Standard Curve of Gallic Acid as Standard for Total Phenolic Contents using Folin-Ciocalteu Method.



Appendix 2. Standard Curve of Ascorbic Acid as Standard for DPPH Scavenging Activity.



Appendix 3. Standard Curve of Ascorbic Acid as Standard for FRAP Antioxidant Capacity.



Appendix 4. Effects of Time

EY (%)					
Group Name	N	Missing	Mean	Std Dev	SEM
30	3	0	3.633	0.0577	0.0333
60	3	0	3.767	0.321	0.186
120	3	0	3.867	0.0577	0.0333
240	3	0	3.967	0.153	0.0882
360	3	0	3.667	0.0577	0.0333
Source of Variation	DF	SS	MS	F	P
Between Groups	4	0.231	0.0577	2.11	0.154
Residual	10	0.273	0.0273		
Total	14	0.504			

TPC (mgGAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
30	3	0	34.383	7.458	4.306
60	3	0	40.043	8.452	4.88
120	3	0	49.028	3.761	2.172
240	3	0	46.844	4.554	2.629
360	3	0	37.674	8	4.619
Source of Variation	DF	SS	MS	F	P
Between Groups	4	457.819	114.455	2.533	0.106
Residual	10	451.886	45.189		
Total	14	909.705			

Appendix 5. Effects of Temperature

EY (%)					
Group Name	N	Missing	Mean	Std Dev	SEM
20	3	0	3.733	0.115	0.0667
30	3	0	3.967	0.379	0.219
40	3	0	4.233	0.306	0.176
50	3	0	4.6	0.1	0.0577
60	3	0	5.467	0.252	0.145
Source of Variation	DF	SS	MS	F	P
Between Groups	4	5.513	1.378	21.314	<0.001
Residual	10	0.647	0.0647		
Total	14	6.16			
Comparison	Diff of Means	p	q	P	P<0.050
60 vs. 20	1.733	5	11.806	<0.001	Yes
60 vs. 30	1.5	4	10.217	<0.001	Yes
60 vs. 40	1.233	3	8.4	<0.001	Yes
60 vs. 50	0.867	2	5.903	0.002	Yes
50 vs. 20	0.867	4	5.903	0.003	Yes
50 vs. 30	0.633	3	4.314	0.015	Yes
50 vs. 40	0.367	2	2.497	0.108	No
40 vs. 20	0.5	3	3.406	0.044	Yes
40 vs. 30	0.267	2	1.816	0.228	No
30 vs. 20	0.233	2	1.589	0.287	No
TPC (mgGAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
20	3	0	36.56	1.586	0.916
30	3	0	42.638	2.208	1.275
40	3	0	49.028	3.761	2.172
50	3	0	48.858	1.686	0.973
60	3	0	45.177	3.019	1.743
Source of Variation	DF	SS	MS	F	P
Between Groups	4	319.355	79.839	11.918	<0.001
Residual	10	66.993	6.699		
Total	14	386.348			
Comparison	Diff of Means	p	q	P	P<0.050
40 vs. 20	12.468	5	8.343	<0.001	Yes
40 vs. 30	6.39	4	4.276	0.018	Yes
40 vs. 60	3.851	3	2.577	0.112	No
40 vs. 50	0.17	2	0.114	0.937	Do Not Test
50 vs. 20	12.298	4	8.23	<0.001	Yes
50 vs. 30	6.22	3	4.162	0.018	Yes
50 vs. 60	3.681	2	2.463	0.112	Do Not Test
60 vs. 20	8.617	3	5.766	0.003	Yes
60 vs. 30	2.539	2	1.699	0.257	No
30 vs. 20	6.078	2	4.067	0.017	Yes

Appendix 6. Effects of Solvent

EY (%)					
Group Name	N	Missing	Mean	Std Dev	SEM
MeOH (50%)	3	0	1.933	0.252	0.145
Ace (50%)	3	0	3.333	0.306	0.176
EtOH (50%)	3	0	5.133	0.404	0.233
Source of Variation	DF	SS	MS	F	P
Between Groups	2	15.44	7.72	72.375	<0.001
Residual	6	0.64	0.107		
Total	8	16.08			
Comparison	Diff of Means	p	q	P	P<0.050
EtOH (50%) vs. MeOH (50%)	3.2	3	16.971	<0.001	Yes
EtOH (50%) vs. Ace (50%)	1.8	2	9.546	<0.001	Yes
Ace (50%) vs. MeOH (50%)	1.4	2	7.425	0.002	Yes
TPC (mgGAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
MeOH (50%)	3	0	39.894	1.294	0.747
Ace (50%)	3	0	40.106	0.213	0.123
EtOH (50%)	3	0	42.234	0.767	0.443
Source of Variation	DF	SS	MS	F	P
Between Groups	2	10.05	5.025	6.529	0.031
Residual	6	4.617	0.77		
Total	8	14.667			
Comparison	Diff of Means	p	q	P	P<0.050
EtOH (50%) vs. MeOH (50%)	2.34	3	4.621	0.02	Yes
EtOH (50%) vs. Ace (50%)	2.128	2	4.201	0.025	Yes
Ace (50%) vs. MeOH (50%)	0.213	2	0.42	0.777	No

Appendix 7. Effects Ethanol Concentrations

EY (%)					
Group Name	N	Missing	Mean	Std Dev	SEM
30	3	0	3.333	0.306	0.176
50	3	0	4.167	0.379	0.219
70	3	0	4.233	0.306	0.176
80	3	0	3.633	0.404	0.233
90	3	0	3.833	0.252	0.145
Source of Variation	DF	SS	MS	F	P
Between Groups	4	1.683	0.421	3.778	0.04
Residual	10	1.113	0.111		
Total	14	2.796			
Comparison	Diff of Means	p	q	P	P<0.050
70 vs. 30	0.9	5	4.672	0.013	Yes
70 vs. 80	0.6	4	3.115	0.067	No
70 vs. 90	0.4	3	2.076	0.191	Do Not Test
70 vs. 50	0.0667	2	0.346	0.812	Do Not Test
50 vs. 30	0.833	4	4.326	0.017	Yes
50 vs. 80	0.533	3	2.769	0.091	Do Not Test
50 vs. 90	0.333	2	1.73	0.249	Do Not Test
90 vs. 30	0.5	3	2.595	0.11	No
90 vs. 80	0.2	2	1.038	0.48	Do Not Test
80 vs. 30	0.3	2	1.557	0.297	Do Not Test
TPC (mgGAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
30	3	0	39.397	2.614	1.509
50	3	0	41.064	1.733	1
70	3	0	49.028	3.761	2.172
80	3	0	37.837	2.228	1.286
90	3	0	32.894	4.841	2.795
Source of Variation	DF	SS	MS	F	P
Between Groups	4	414.529	103.632	9.893	0.002
Residual	10	104.758	10.476		
Total	14	519.288			
Comparison	Diff of Means	p	q	P	P<0.050
70 vs. 90	16.135	5	8.634	<0.001	Yes
70 vs. 80	11.191	4	5.989	0.003	Yes
70 vs. 30	9.631	3	5.154	0.006	Yes
70 vs. 50	7.965	2	4.262	0.013	Yes
50 vs. 90	8.17	4	4.372	0.016	Yes
50 vs. 80	3.227	3	1.727	0.271	No
50 vs. 30	1.667	2	0.892	0.543	Do Not Test
30 vs. 90	6.504	3	3.48	0.04	Yes
30 vs. 80	1.56	2	0.835	0.568	Do Not Test
80 vs. 90	4.943	2	2.645	0.091	No

Appendix 8. Effects of Solid Liquid Ratio

EY (%)					
Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	0.55	0.132	0.0764
20	3	0	0.867	0.0577	0.0333
50	3	0	1.967	0.0577	0.0333
100	3	0	3.967	0.153	0.0882
150	3	0	4.2	0.346	0.2
Source of Variation	DF	SS	MS	F	P
Between Groups	4	34.846	8.711	260.045	<0.001
Residual	10	0.335	0.0335		
Total	14	35.181			
Comparison	Diff of Means	p	q	P	P<0.050
150 vs. 10	3.65	5	34.541	<0.001	Yes
150 vs. 20	3.333	4	31.544	<0.001	Yes
150 vs. 50	2.233	3	21.134	<0.001	Yes
150 vs. 100	0.233	2	2.208	0.15	No
100 vs. 10	3.417	4	32.333	<0.001	Yes
100 vs. 20	3.1	3	29.336	<0.001	Yes
100 vs. 50	2	2	18.926	<0.001	Yes
50 vs. 10	1.417	3	13.406	<0.001	Yes
50 vs. 20	1.1	2	10.41	<0.001	Yes
20 vs. 10	0.317	2	2.997	0.06	No
TPC (mgGAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	30.433	2.152	1.242
20	3	0	37.957	6.707	3.872
50	3	0	45.17	2.39	1.38
100	3	0	48.695	3.294	1.902
150	3	0	49.291	4.456	2.573
Source of Variation	DF	SS	MS	F	P
Between Groups	4	773.09	193.272	11.232	0.001
Residual	10	172.066	17.207		
Total	14	945.156			
Comparison	Diff of Means	p	q	P	P<0.050
150 vs. 10	18.858	5	7.874	<0.001	Yes
150 vs. 20	11.333	4	4.732	0.011	Yes
150 vs. 50	4.121	3	1.721	0.272	No
150 vs. 100	0.596	2	0.249	0.864	Do Not Test
100 vs. 10	18.262	4	7.626	<0.001	Yes
100 vs. 20	10.738	3	4.484	0.013	Yes
100 vs. 50	3.525	2	1.472	0.323	Do Not Test
50 vs. 10	14.738	3	6.154	0.002	Yes
50 vs. 20	7.213	2	3.012	0.059	No
20 vs. 10	7.525	2	3.142	0.051	No

Appendix 9. Effects of Particle Size

EY (%)					
Group Name	N	Missing	Mean	Std Dev	SEM
0.125-0.250	3	0	9.367	0.651	0.376
0.250-0.500	3	0	6	0.9	0.52
0.500-1.000	3	0	4.233	0.306	0.176
Source of Variation	DF	SS	MS	F	P
Between Groups	2	40.807	20.403	46.138	<0.001
Residual	6	2.653	0.442		
Total	8	43.46			
Comparison	Diff of Means	p	q	P	P<0.050
0.125-0.250 vs. 0.500-1.000	5.133	3	13.37	<0.001	Yes
0.125-0.250 vs. 0.250-0.500	3.367	2	8.769	0.001	Yes
0.250-0.500 vs. 0.500-1.000	1.767	2	4.601	0.018	Yes
TPC (mgGAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
0.125-0.250	3	0	46.695	1.564	0.903
0.250-0.500	3	0	40.624	1.235	0.713
0.500-1.000	3	0	37.383	5.218	3.013
Source of Variation	DF	SS	MS	F	P
Between Groups	2	134.075	67.037	6.445	0.032
Residual	6	62.405	10.401		
Total	8	196.48			
Comparison	Diff of Means	p	q	P	P<0.050
0.125-0.250 vs. 0.500-1.000	9.312	3	5.001	0.014	Yes
0.125-0.250 vs. 0.250-0.500	6.071	2	3.26	0.061	No
0.250-0.500 vs. 0.500-1.000	3.241	2	1.741	0.265	No

Appendix 10. Comparison between Predicted and Optimized Results

TPC (mgGAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
Predict	3	0	91.330	1.040	0.600
Experimental	3	0	89.160	0.980	0.566
Difference	2.170				
t = 2.630 with 4 degrees of freedom. (P = 0.058)					
Group Name	N	Missing	Mean	Std Dev	SEM
Predict	3	0	40.000	0.510	0.294
Experimental	3	0	42.560	1.633	0.943
Difference	-2.560				
t = -2.592 with 4 degrees of freedom. (P = 0.061)					

Appendix 11. Difference between the Optimal Conditions based on EY and TPC

EY (%)					
Group Name	N	Missing	Mean	Std Dev	SEM
OP1	3	0	5.700	0.150	0.0866
OP2	3	0	6.400	0.660	0.381
OP3	3	0	5.100	0.400	0.231
Source of Variation	DF	SS	MS	F	P
Between Groups	2	2.540	1.270	6.164	0.035
Residual	6	1.236	0.206		
Total	8	3.776			
Comparison	Diff of Means	p	q	P	P<0.050
OP2 vs. OP3	1.300	3.508	0.038	Yes	
OP2 vs. OP1	0.700	1.889	0.204	No	
OP1 vs. OP3	0.600	1.619	0.157	No	
TPC (mgGAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
OP1	3	0	40.120	1.130	0.652
OP2	3	0	42.560	1.630	0.941
OP3	3	0	41.870	0.860	0.497
Source of Variation	DF	SS	MS	F	P
Between Groups	2	9.492	4.746	3.047	0.122
Residual	6	9.347	1.558		
Total	8	18.839			

Appendix 12. Difference between the Optimal Conditions based on DPPH

Group Name	N	Missing	Mean	Std Dev	SEM
OP1	3	0	6.09	0.46	0.27
OP2	3	0	6.41	0.39	0.23
OP3	3	0	6.24	0.04	0.03
AA	3	0	5.23	0.07	0.04
Source of Variation	DF	SS	MS	F	P
Between Groups	3	2.48	0.83	8.93	0.006
Residual	8	0.74	0.09		
Total	11	3.22			
Comparisons for factor:					
Comparison	Diff of Means	p	q	P	P<0.050
OP2 vs. AA	1.18	4	6.712	0.006	Yes
OP2 vs. OP1	0.32	4	1.809	0.599	No
OP2 vs. OP3	0.17	4	0.95	0.905	No
OP3 vs. AA	1.01	4	5.762	0.015	Yes
OP3 vs. OP1	0.15	4	0.859	0.927	No
OP1 vs. AA	0.86	4	4.902	0.035	Yes

Appendix 13. Difference between the Optimal Conditions based on FRAP

Group Name	N	Missing	Mean	Std Dev	SEM
OP1	3	0	13.27	0.62	0.36
OP2	3	0	18.42	0.56	0.32
OP3	3	0	15.15	0.51	0.30
Source of Variation	DF	SS	MS	F	P
Between Groups	2	40.71	20.36	63.48	<0.001
Residual	6	1.924	0.321		
Total	8	42.64			
Comparisons for factor:					
Comparison	Diff of Means	p	q	P	P<0.050
OP2 vs. OP1	5.15	3	15.74	<0.001	Yes
OP2 vs. OP3	3.27	3	10.01	0.001	Yes
OP3 vs. OP1	1.88	3	5.737	0.016	Yes

Appendix 14. Comparison between the Age Range of the Tree Sample

EY (%)					
Group Name	N	Missing	Mean	Std Dev	SEM
OB	3	0	5.07	0.40	0.23
YB	3	0	7.40	1.21	0.70
Difference	-2.333				
t = -3.162 with 4 degrees of freedom. (P = 0.034)					
TPC (mgGAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
OB	3	0	43.87	0.89	0.51
YB	3	0	40.96	1.49	0.86
Difference	2.908				
t = 2.906 with 4 degrees of freedom. (P = 0.044)					
DPPH (%)					
Group Name	N	Missing	Mean	Std Dev	SEM
OB	3	0	80.92	0.97	0.56
YB	3	0	80.55	1.82	1.05
Difference	0.367				
t = 0.308 with 4 degrees of freedom. (P = 0.773)					
FRAP (mgAAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
OB	3	0	17.72	0.27	0.16
YB	3	0	8.86	0.33	0.19
Difference	8.86				
t = 35.709 with 4 degrees of freedom. (P = <0.001)					

Appendix 15. Comparison between the Effects of Extraction Method

EY (%)					
Group Name	N	Missing	Mean	Std Dev	SEM
MAC-MD	3	0	5.070	0.400	0.231
MAC-NM	3	0	5.500	0.360	0.208
SOX	3	0	11.100	0.0900	0.0520
Source of Variation	DF	SS	MS	F	P
Between Groups	2	67.906	33.953	342.152	<0.001
Residual	6	0.595	0.0992		
Total	8	68.501			
Comparisons for factor:					
Comparison	Diff of Means	p	q	P	P<0.050
SOX vs. MAC-MD	6.030	3	33.155	<0.001	Yes
SOX vs. MAC-NM	5.600	2	30.791	<0.001	Yes
MAC-NM vs. MAC-MD	0.430	2	2.364	0.146	No
TPC (mgGAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
MAC-MD	3	0	42.230	0.770	0.445
MAC-NM	3	0	29.470	1.060	0.612
SOX	3	0	24.220	0.890	0.514
Source of Variation	DF	SS	MS	F	P
Between Groups	2	514.740	257.370	307.785	<0.001
Residual	6	5.017	0.836		
Total	8	519.757			
Comparisons for factor:					
Comparison	Diff of Means	p	q	P	P<0.050
MAC-MD vs. SOX	18.010	3	34.113	<0.001	Yes
MAC-MD vs. MAC-NM	12.760	2	24.169	<0.001	Yes
MAC-NM vs. SOX	5.250	2	9.944	<0.001	Yes
DPPH (% reduction)					
Group Name	N	Missing	Mean	Std Dev	SEM
MAC-MD	3	0	83.426	0.252	0.145
MAC-NM	3	0	78.788	0.451	0.260
SOX	3	0	86.164	0.975	0.563
Source of Variation	DF	SS	MS	F	P
Between Groups	2	83.415	41.708	102.711	<0.001
Residual	6	2.436	0.406		
Total	8	85.852			
Comparisons for factor:					
Comparison	Diff of Means	p	q	P	P<0.050
SOX vs. MAC-NM	7.376	3	20.048	<0.001	Yes
SOX vs. MAC-MD	2.737	2	7.441	0.002	Yes
MAC-MD vs. MAC-NM	4.639	2	12.608	<0.001	Yes
FRAP (mgAAE/100g)					
Group Name	N	Missing	Mean	Std Dev	SEM
MAC-MD	3	0	26.470	0.515	0.297
MAC-NM	3	0	18.821	0.769	0.444
SOX	3	0	19.483	1.340	0.774
Source of Variation	DF	SS	MS	F	P
Between Groups	2	107.761	53.880	60.940	<0.001
Residual	6	5.305	0.884		
Total	8	113.066			

Comparisons for factor:					
Comparison	Diff of Means	p	q	P	P<0.050
MAC-MD vs. MAC-NM	7.649	3	14.090	<0.001	Yes
MAC-MD vs. SOX	6.987	2	12.870	<0.001	Yes
SOX vs. MAC-NM	0.663	2	1.221	0.421	No

