DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

INVESTIGATION OF *NIGELLA SATIVA*, *DIOSPYROS LOTUS* AND *MESPILUS GERMANICA* EXTRACTS' PHENOLIC COMPOUNDS, ANTIOXIDANT PROPERTIES AND APOPTOTIC EFFECTS IN SOME CANCER CELL LINES

by
Umar Muazu YUNUSA

July, 2021
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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"INVESTIGATION OF *NIGELLA SATIVA, DIOSPYROS LOTUS* AND *MESPILUS GERMANICA* EXTRACTS' PHENOLIC COMPOUNDS, ANTIOXIDANT PROPERTIES AND APOPTOTIC EFFECTS IN SOME CANCER CELL LINES"** completed by **UMAR MUAZU YUNUSA** under supervision of **PROF. DR. RAZİYE ÖZTÜRK ÜREK** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Umar Muazu YUNUSA

INVESTIGATION OF *NIGELLA SATIVA, DIOSPYROS LOTUS* AND *MESPILUS GERMANICA* EXTRACTS' PHENOLIC COMPOUNDS, ANTIOXIDANT PROPERTIES AND APOPTOTIC EFFECTS IN SOME CANCER CELL LINES

ABSTRACT

This research aimed to extract and profile phenolic compounds from *Nigella sativa* seed, *Mespilus germanica* leaf and fruit, *Diospyros lotus* seed and fruit, evaluate and compare the antioxidants and cytotoxic potential of the extracts. Flavonoids (flavone, flavanone, flavonol and flavan-3-ols) and phenolic acids (FPA, BHPA, AHPA, BPBH-1, BPAH-2, BPAH-1 and BPBH-2) extracts were obtained following extractions with different solvent systems. The lyophilized extracts were prepared for HPLC analysis, antioxidants and cytotoxic activity determinations. The total phenolic/flavonoid/tannin contents (TPC/TFC/TTC) were also determined. Flavonoids: hesperidin, quercitrin, epicatechin, epigallocatechin and epigallocatechin gallate; and phenolic acids: benzoic, 4-hydroxybenzoic, rosmarinic, protocatechuic, syringic, gallic, vanillic, chlorogenic, ferulic, o-coumaric, sinapic, caffeic, p-coumaric and t-cinnamic acids were determined by HPLC. *M. germanica* leaf extract gave the highest TPC, TFC and TTC. In antioxidants assays, BPAH-1 extract of *D. lotus* fruit, BPBH-2 extract of *M. germanica* leaf, flavan-3-ol methanolic of *M. germanica* leaf showed the highest DPPH$^{\bullet}$, $^{\bullet}$OH, NO$^{\bullet}$ scavenging effects, respectively. The ferrous ion chelating effect of *N. sativa* seed flavone extract was the highest. Furthermore, the BPAH-1 extract of *D. lotus* fruit had the highest ferric ion reducing power and FRAP value. Most of the extracts showed good cytotoxic effects on HeLa and HepG2 cell lines (IC_{50} <100 µg/mL). Also, it was determined the cytotoxicity on HepG2 > HeLa. Significant antioxidant and cytotoxic activities determined from the different extracts could be originated as effects of phenolic compounds identified. This research revealed the potential plant biodiversity in terms of antioxidant and anticancer properties of three plants from Turkey.

Keywords: *Nigella sativa*, *Mespilus germanica*, *Diospyros lotus*, flavonoids, phenolic acids, HPLC, antioxidants, anticancer.

NIGELLA SATIVA, DIOSPYROS LOTUS VE *MESPILUS GERMANICA* EKSTRELERININ FENOLIK BILEŞENLERI, ANTIOKSIDAN ÖZELLIKLERI VE BAZI KANSER HÜCRE HATLARINDAKI APOPTOTIK ETKILERININ INCELENMESI

ÖZ

Bu araştırma, *Nigella sativa* tohumu, *Mespilus germanica* yaprağı ve meyvesi ile *Diospyros lotus* tohumu ve meyvesinden fenolik bileşenleri ekstrakte etme ve profilini çıkarma, ekstraktların antioksidanlarını ve sitotoksik potansiyellerini değerlendirmeyi ve karşılaştırmayı amaçlamıştır. Flavonoid (flavon, flavanon, flavonol ve flavan-3-oller) ve fenolik asit (FPA, BHPA, AHPA, BPBH-1, BPAH-2, BPAH-1 ve BPBH-2) ekstraktları, farklı çözücü sistemleri ile ekstraksiyon sonrasında elde edilmiştir. Liyofilize ekstreler HPLC analizi, antioksidanlar ve sitotoksik aktivite tayinleri için hazırlandı. Toplam fenolik/flavonoid/tanen içerikleri (TPC, TFC, TTC) de belirlendi. Flavonoidler: hesperidin, kersitrin, epikateşin, epigallokateşin ve epigallokateşin gallat ve fenolik asitler: benzoik, 4-hidroksibenzoik, gallik, protokateşik, rosmarinik, şiringik, vanilik, klorojenik, kafeik, ferulik, sinapik, o-kumarik, p-kumarik ve t-sinnamik asitler HPLC ile belirlendi. *M. germanica* yaprağı ekstresi en yüksek TPC, TFC ve TTC'yi verdi. Antioksidan analizlerde, *D. lotus* meyve BPAH-1ekstresi, *M. germanica* yaprak BPBH-2 ile flavan-3-ol metanolik ekstreleri, sırasıyla en yüksek DPPH•, •OH, NO• sönümleme etkileri göstermişlerdir. *N. sativa* tohum flavon ekstresi en yüksek demir iyonu şelatlama etkisi göstermiştir. Ayrıca, *D. lotus* meyvesinin BPAH-1 ekstresi en yüksek ferrik iyon indirgeme gücü ve FRAP değerine sahip bulunmuştur. Ekstraktların çoğu, HeLa ve HepG2 hücre hatları üzerinde iyi sitotoksik etkiler göstermiştir (IC_{50} <100 µg/mL). Ayrıca, HepG2 > HeLa sitotoksisite belirlenmiştir. Farklı ekstraktlardan saptanan önemli antioksidan ve sitotoksik aktiviteler, belirlenen fenolik bileşiklerin etkilerinden kaynaklanabilir. Bu araştırma, Türkiye'den üç bitkinin antioksidan ve antikanser özellikleri açısından potansiyel bitki biyoçeşitliliğini ortaya çıkarmıştır.

Anahtar Kelimeler: *Nigella sativa, Mespilus germanica, Diospyros lotus,* flavonoidler, fenolik asitler, HPLC, antioksidanlar, antikanser.

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CHAPTER ONE
INTRODUCTION

1.1 Background

Free radicals are molecules carrying a single electron. The radicals are grouped into reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive carbonyl species (RCS), and reactive sulfur species (RSS) (Miller et al., 1990; Losada-Barreiro & Bravo-Diaz, 2017). These free radicals are generated endogenously through an enzymatic pathway or nonenzymatic processes or exogenously from the surrounding environment. At optimal levels, radical species are required for normal physiological functions, for example, redox homeostasis, electron transporters in mitochondria, intracellular cell signalling, immune defence against invading pathogens, electron transporters in mitochondria and metabolic regulation (Gruhlke & Slusarenko, 2012; Lau & Pluth, 2019; Katerji et al., 2019). Excess amount of them in the cell oxidizes and damages several important biomolecules (e.g., carbohydrate, proteins, membranes, lipids, DNA, RNA) and therefore leads to oxidative stress (Giles et al., 2001; Semchyshyn & Lushchak, 2012).

Andersson (2018) defined oxidative stress/nitrosative stress/carbonyl stress "as the disproportion between the free radicals generations and a biological system's ability to detoxify or to mend the oxidative damage caused by the reactive species". Oxidative stress is linked to several cardiovascular (CVD) and neurodegenerative diseases (NDD), ageing, cancer, apoptosis, and necrosis (Semchyshyn, 2014; Pizzino et al., 2017). Antioxidants are substances that hinder or decelerate the oxidation of biological compounds caused by radical species (Halliwell, 2007). They are generally grouped into endogenous and exogenous antioxidants. Endogenous antioxidants were further categorised into enzymatic and non-enzymatic. Enzymatic antioxidants contained catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione peroxidase (GPx). Nonenzymatic antioxidants comprise glutathione, lipoic acid, L-arginine, and coenzyme Q10, vitamins A, C, and E (Birben et al., 2012; Pizzino et al., 2017). Exogenous antioxidants are predominantly isolated from plant species

and are classified into carotenoids (astaxanthin, cryptoxanthin, carotenes, xanthophylls and zeaxanthin), polyphenols (anthocyanins, flavonoids, lignans, phenolic acids and stilbenes) sterols, and vitamins (Xu et al., 2017).

Assessing the antioxidant status of a body's system is important in measuring the extent of oxidative stress and its implication in many diseases. Assessment of antioxidant status can be done through measurement of enzymatic and non-enzymatic antioxidants, and determination of total antioxidant capacity (TAC). Assessment by TAC can be inside the living cells (*in vivo*) and/or outside the living cell (*in vitro*) models (Alam et al., 2013). Similarly, the *in vitro* models of TAC determination include 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH$^{\bullet}$) scavenging, nitric oxide radical (NO$^{\bullet}$) scavenging, hydroxyl radical ($^{\bullet}$OH) scavenging, ferrous ion chelating activity (metal chelating activity), ferric ion reducing power (commonly reducing power), and ferric reducing antioxidant power (FRAP) assays (Katerji et al., 2019). The *in vivo* test procedures include the following assays: reduced glutathione (GSH) estimation, glutathione reductase (GR) estimation, GPx estimation, GST estimation, SOD estimation, CAT estimation, ferric reducing ability of plasma estimation, γ-glutamyl transpeptidase activity (γ-GGT) estimation, lipid peroxidation (LPO) estimation, and LDL estimation (Alam et al., 2013).

Flavonoids and phenolic acids are two larger groups of polyphenols widely distributed in plants. Flavonoids are the most diverse, are classified into flavones, flavanones, flavans, flavanols, flavonols, flavanonols, anthocyanidins, isoflavonoids, neoflavonoids and chalcones (Panche et al., 2016). Phenolic acids are categorized into benzoic acid and cinnamic acid derivatives (Heleno et al., 2015). Legumes, nuts, cereal grains, raspberries, cranberries, apples, grapes, pears, jams, leguminous plants, tea, citrus fruits, wine, strawberries and juices are rich sources of polyphenols (Velderrain-Rodríguez et al., 2014).

1.2 Free Radical

A free radical is an atom/molecule/ion possessing a lone electron in its outermost shell. The unpaired electron causes the species highly reactive and unstable (Wu et al., 2013). The free radical species include oxygen-centred radicals, ROS; nitrogen-centred, RNS; carbon-centred radicals, RCS; and sulfur-centred radicals, RSS (Miller et al., 1990; Losada-Barreiro & Bravo-Diaz, 2017). These radicals can attack and damage important biological molecules including RNA, DNA, protein, and lipids (Phaniendra et al., 2015). Other consequences of free radicals include neuronal death, LPO, DNA mutation, inactivation of enzymes, and the destruction of cell membranes (Toyokuni, 1999). LPO is implicated in inflammation, atherosclerosis, ageing, myocardial infarction, and cancer (Saiin et al., 2018). Free radicals can cause DNA damage through chemical modifications of sugar and bases, depurination and depyrimidination. Due to its single-stranded nature and proximity to the mitochondria, RNAs are more vulnerable to oxidative degradation than DNA.

1.2.1 Reactive Oxygen Species

ROS are among the most important classes of free radicals in the living system. ROS are produced from molecular oxygen through different varieties of physiological processes, including redox reactions. Families of ROS include charged species, such as $^{\bullet}OH$, superoxide ($O_2^{\bullet-}$), alkoxyl (RO^-), and peroxyl radicals (ROO^-); and uncharged species, for example, dioxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Losada-Barreiro & Bravo-Diaz, 2017). Generation of ROS through redox reactions in biological systems involves the monovalent reduction of molecular oxygen, in the presence of unoccupied electrons to yield $O_2^{\bullet-}$. The dismutation of $O_2^{\bullet-}$ by catalytic and/or spontaneous processes produced H_2O_2. Any metal-containing molecules present in the mixture could reduce H_2O_2 to generate highly reactive compounds (Touyz, 2004).

ROS are generated endogenously and exogenously. The endogenous sources in mammalian organisms comprise cellular organelles, cellular enzymes, and endogenous chemicals (Gilbert & Colton, 2002). Apart from mitochondria which are

major sources of ROS, other cellular organelles such as peroxisomes, neutrophils, macrophages, neurons also contribute to ROS production. The enzymes responsible for ROS production in mammalian organisms comprise the cytochrome P450 enzymes, the monoamine oxidase enzymes, xanthine oxidase (XOD)/dehydrogenase (XDH) enzymes, and the membrane NADPH enzymes. Arachidonic acid a member of polyunsaturated fatty acids (PUFA), catecholamines, prostaglandin and haemoglobin are known to generated ROS (Gilbert & Colton, 2002). ROS take part in gene expression, signal transduction, activation of cell signalling cascades, apoptosis, and as intracellular and intercellular messengers (Hancock et al., 2001; Held, 2012). The exogenous sources include narcotic drugs, ionization, anaesthetizing gases, UV radiation, drugs (e.g., bleomycin and adriamycin), chemicals (e.g., alcohol) and such as toxins, pesticides, xenobiotics (Halliwell, 1991).

Excessive generation of ROS has been known to cause necrosis, apoptosis and autophagic cell death. In the necrosis pathway, ROS formation triggers LPO, which can subsequently lead to the increase in intracellular calcium level, the opening of the mitochondrial permeability transition pore (MPTP), and damage of cellular integrity. These induce necrosis through cell swelling and/or cell rupturing. The increase in ROS generation can cause DNA damage through chemical modifications of sugar and bases, depurination and depyrimidination. The damage can cause apoptosis through activation of p53, the opening of the MPTP pore, and the caspase signalling cascade (Mani, 2015). Excessive generation of ROS can also induce the inactivation of the autophagy-related gene 4, which sequentially causes accumulation of the autophagy-related gene 8 that is needed for the commencement of autophagosome (Scherz-Shouval et al., 2007). An autophagosome is a spherical structure with a double-layer membrane. ROS can induce oxidation of amino acids, for example, arginine, lysine and threonine, and can cause the protein-protein cross-linkages. This may result in the damaging of protein structures, disrupt of enzymes, receptors, and also transport proteins (Phaniendra et al., 2015).

1.2.2 Reactive Nitrogen Species

RNS are free-radical species produced from nitric oxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet-}$) through different varieties of physiological processes. The families of RNS include nitric oxide or nitrogen monoxide ($NO^{\bullet}$) produced from nitric oxide synthases (NOS); nitrite (NO_2^-) produced from $NO^{\bullet}$; dinitrogen trioxide (N_2O_3) produced from $NO^{\bullet}$ and O_2; nitrogen dioxide ($NO_2^{\bullet}$) produced from $ONOO^-$ decomposition; nitronium cation (NO_2^+) produced from $ONOOCO_2^-$ decomposition; nitrosonium cation (NO^+) produced from $NO^{\bullet}$; nitrosoperoxycarbonate anion ($ONOOCO_2^-$) produced from $ONOO^-$ and CO_2; nitroxyl (HNO) produced from the one-electron reduction of $NO^{\bullet}$; nitryl chloride ($Cl\text{-}NO_2$) produced from NO_2^- and HOCl; peroxynitrite ($ONOO^-$) produced from $NO^{\bullet}$ and $^{\bullet}O_2^-$; and s-nitrosothiols (RSNOs) produced from addition of a $NO^{\bullet}$ group to a cysteine thiol/sulfhydryl (Martínez & Andriantsitohaina, 2009). These species participate greatly in $^{\bullet}O_2^-$ detoxification, thus avert the damage linked to the ROS. However, these radical species do play a significant role in the oxidation of important biological molecules including carbohydrate, protein, lipids, metal co-factors, DNA and RNA bases (Wink et al., 1996; Martínez & Andriantsitohaina, 2009).

An abnormal concentration of RNS in living cells increases sphingolipid formation of ceramide and its derivatives (i.e., sphingosine), which can lead to cellular oxidative damage and nitrosative stress via NADPH oxidase and NOS activations, and also mitochondrial functional distortions (Won & Singh, 2006). Nitrosative stress is an analogy to “oxidative stress” and it occurs when the generation of RNS outbalance the antioxidant defence system.

$NO^{\bullet}$, $NO_2^{\bullet}$, and $ONOO^-$ damage DNA and RNA through base and sugar lesions. The DNA damage-induced apoptosis, increased mutation rates and cell proliferation all of which are markers of tumorigenesis (Szabó & Ohshima, 1997; Sawa & Ohshima, 2006). $NO^{\bullet}$ is the principal RNS generated by living cells and it is the main source of other RNS. It can function as both a pro-inflammatory and anti-inflammatory agent (Bauerova & Bezek, 2000). $NO^{\bullet}$ stimulates proinflammatory cytokine production. The

$NO^{\bullet}$ anti-inflammatory properties include its capacity to hinder the prostaglandin E2, thromboxane, and interleukin 6 syntheses (Stadler et al., 1993; Amin et al., 1997), and its ability to suppress the generation of $O_2^{\bullet-}$ by neutrophils via directionally action on NADPH oxidase (Clancy et al., 1992).

$NO^{\bullet}$ is promptly produced in different tissue and its very small size nature help it to infiltrate speedily across cellular structures and diffuse via distances of several microns (Gonon et al., 2004). $NO^{\bullet}$ plays a significant role in neurotransmission, immune defences, inflammation, induces vasodilation in the cardiovascular system, and apoptosis (Sharma et al., 2007). With regards to apoptosis, $NO^{\bullet}$ does perform dual roles. Excessive abnormal concentration of $NO^{\bullet}$ induces apoptosis through the ceramide synthesis, while the normal concentration of $NO^{\bullet}$ suppresses apoptosis via the hindrance of ceramide production (Martínez & Andriantsitohaina, 2009).

1.2.3 Reactive Carbonyl Species

RCS are a class of free radicals possessing one or more highly extremely carbonyl groups. RCS is generally understood for their mutilating activity on biological molecules (e.g., carbohydrate, proteins, membranes, lipids, DNA, RNA) (Semchyshyn & Lushchak, 2012). RCS can be source endogenously or exogenously from the surrounding environment. The endogenous source can be through the enzymatic pathway or nonenzymatic processes. Semchyshyn (2014) summarized RCS generated through these processes. In the enzymatic pathway, RCS is generated through glycolysis (e.g., acetaldehyde, glyceraldehyde-3- phosphate, dioxyacetone phosphate, methylglyoxal) and through the polyol pathway (e.g., 3-deoxyglucosone, 3-deoxyfructose). Polyol pathway comprises of two-step process by which glucose is converted to sorbitol via reduction. The sorbitol is immediately transformed into fructose. The RCS produced through nonenzymatic processes include those produced through the oxidation of amino acids (e.g., acrolein, glycolaldehyde, glyoxal, methylglyoxal, 2-hydroxypropanal); LPO (e.g., acrolein, crotonaldehyde, glyoxal, hexanal, isolevuglandins, malondialdehyte (MDA), 4-hydroxy-2-nonenal/4-HNE, 4-oxo-2-nonenal/4-ONE); and glycation (e.g., acrolein, glyoxal, glucosone,

methylglyoxal, 3-deoxyglucosone). Semchyshyn (2014) further listed the exogenous source as smoke, browned food and additives, exhaust fumes, pharmaceutical, and other industrial pollutants.

An excessive level in the concentration of RCS in a living cell may lead to carbonyl stress. Miyata et al. (1999) described carbonyl stress as a complication "resulting from either increased oxidation of carbohydrates and lipids or inadequate detoxification or inactivation of reactive carbonyl compounds derived from both carbohydrates and lipids by oxidative and nonoxidative chemistry". Carbonyl stress is implicated in ageing, atherosclerosis, diabetes, obesity, renal failure and heart diseases (Semchyshyn & Lushchak, 2012). Other negative consequences of RCS include their roles in inflammation, apoptosis, and necrosis (Semchyshyn, 2014).

Apart from their damaging effect, a low level of RCS contributes immensely as cellular signalling messengers, maintenance of metabolic equilibrium, regulators of gene expression, immune response and adaptation to different stresses (Forman et al., 2008; Niki, 2009). Other beneficial roles of RCS, is their use as a potent antibacterial, antifungal, anticancer, antiprotozoal and antiviral agents, and their capacity to temper various biological events, for example, cell proliferation, cell differentiation and cell reproduction (Talukdar et al., 2009; Semchyshyn, 2014).

1.2.4 Reactive Sulfur Species

According to Giles et al. (2017) "RSS are molecules which contain at least one redox-active sulfur atom or sulfur-containing functional group in their structure and are capable of either oxidizing or reducing biomolecules under physiological conditions to trigger or propagate a noticeable cellular signal or wider biological event". Families of RSS include elemental sulfur, hydrogen sulfide (H_2S), hydrogen disulfide, disulfide, thiol, thiyl radical, hydropersulfide, polysulfide, hydropolysulfide, iron-sulfur cluster, polysulfane, nitrosothiol, sulfoxide, sulfinic acid, sulfenic acid, sulfonic acid, thiosulfinate, thiosulfonate, sulfate and thiosulfate (Mishanina et al., 2015; Lau & Pluth, 2019).

An excess amount of RSS in the cell oxidizes and damages several important biomolecules and therefore leads to oxidative stress (Giles et al., 2001). At low concentrations, RSS exerts beneficial effects. For example, H_2S plays a significant role in angiogenesis, anti-inflammation, cytoprotection and vasodilation (Olson & Straub, 2015). Thiols function as cellular redox buffers, decreasing ROS and so balanced the cell redox states (Giles et al., 2001). Other beneficial roles of RSS include their roles in cell signalling, redox homeostasis, electron transporters in mitochondria and metabolic regulation (Gruhlke & Slusarenko, 2012; Lau & Pluth, 2019).

1.3 Oxidative Stress and its Measurement

1.3.1 Oxidative Stress

Andersson (2018) defined oxidative stress "as the situation when there is an imbalance between the systemic generation of ROS, RNS, RCS and RSS, and a biological system's ability to detoxify the reactive intermediates caused by the species or to repair the resulting damage". In a typical normal cell, the production of free radical species, namely, ROS, RNS, RSS or RCS, is control by balancing systems comprising endogenous and exogenous antioxidants, and proteins (e.g., albumin, transferrin, haptoglobin and ceruloplasmin) (Maes et al., 2011). Antioxidants can scavenge ROS, RNS, RSS or RCS and repair the oxidative damage created by these species, thereby protecting target structures or molecules, such as lipids, carbohydrates, membranes, protein, lipoproteins, DNA and RNA from oxidative injuries (Halliwell, 2007). In the event of low antioxidant's concentrations and low functions of antioxidant enzymes inside a body, the rate of radical generation overpowers the antioxidant systems and subsequently cause oxidative stress and its analogies (Maes et al., 2011).

Pizzino et al. (2017) highlighted the oxidative stress role and free radicals in the aetiology of many illnesses, for example, cancer, CVD (i.e., hypertension, arteriosclerosis, ischemia), NDD (i.e., Parkinson's, Alzheimer's, etc.), kidney sicknesses (i.e., proteinuria, uremia, renal failure), respiratory diseases, and rheumatoid arthritis. Oxidative stress can contribute to tumour onset through base and

sugar lesions of DNA. The authors further highlighted the potential act of oxidative stress in causing late matureness.

1.3.2 Measurement Oxidative Stress Markers

Measurement of oxidative stress markers is important in understanding the pathogenicity of many illnesses. Oxidative stress can be measured: a) directly through direct measurement of ROS and RNS, b) indirectly through assessment of oxidative damage against biological molecules, and c) indirectly through assessment of antioxidant status (Katerji et al., 2019). Oxidative markers of ROS and RNS such as H_2O_2, $^{\bullet}OH$, and ROO^{-} can be measured directly following staining with fluorescent probe 5-(and -6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) (Ubezio & Civoli, 1994). Dihydroethidium fluorescent staining method can be employed to measure $O_2^{\bullet-}$ level in a living cell (Peshavariya et al., 2007). Derivative of reactive oxygen metabolites test was used to quantify hydroperoxides (R-OOH) levels (Trotti et al. 2002). Other direct methods used for estimation of oxidative stress include measurement of ROS/RNS ratio, ROS/$O_2^{\bullet-}$ ratio, and DCFDA–ROS levels can be used directly to measure oxidative stress in a living system (Ubezio & Civoli, 1994).

Oxidative stress could also be assessed indirectly by quantifying the extent of RNA/DNA injury, LPO, and protein oxidation/nitration. Measurement of oxidative injury produced by ROS, RNS, RCS and RSS against proteins, lipids and nucleic acids is an optimistic approach towards oxidative stress measurement. Techniques such as advanced oxidation protein products assay (Witko-Sarsat et al., 1996) and 2,4-dinitrophenylhydrazine (DNPH) assay (Levine et al., 1990) can be used to assess protein nitration. Zhang et al. (2013) defined protein oxidation “as the covalent modification of a protein-induced either by the direct reactions with ROS and RNS or indirect reactions with secondary byproducts of oxidative stress”.

Measurement of MDA concentration via thiobarbituric acid reactive substances (TBARS) assay is used to estimate oxidative stress caused by lipid damage. MDA is the most fortunately studied byproduct of the chemical reaction of PUFA and ROS.

Other approaches of measuring oxidative damage to the cellular lipids include measurement of 8-iso-prostaglandin F2 α via ultra-high-performance liquid chromatography (UHPLC), 4-hydroxy-2-nonenal (4-HNE) using HPLC, oxidized levels of LDL using sandwich ELISA assay, and lipid hydroperoxides (LOOH) using ferrous oxidation xylenol orange assay (Katerji et al., 2019). LPO is the term used to describe oxidative degradation of lipids by oxidants, for example, ROS and RNS (Ayala et al., 2014).

Measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG) level in a biological sample can be used to assess oxidative DNA damage. The 8-OhdG is the product addition of the OH group to the deoxyguanosine residues. Its levels could be measured using ELISA, HPLC incorporated with an electrochemical detector, immunohistochemical (IHC) analysis, and oxyDNA-FITC conjugate fluorescence assays (Katerji et al., 2019). Accumulation of thymidine glycol (TG) in tissues is another biomarker of oxidative DNA damage. TG level could be assessed immunohistochemically by the streptavidin-biotin-peroxidase complex method (Ito et al., 2012). Comet assay had been used to evaluate oxidative stress in DNA. The assay identified DNA damage formed by single- or double-stranded breaks (Wongworawat et al., 2016). The level of DNA repair enzymes, for example, human-8-oxoguanine-DNA-glycosylase and apurinic endonuclease, can be measured by IHC analysis (Li et al., 2001; Chaisiriwong et al., 2016) or HPLC (Park et al., 2001) to estimate the oxidative DNA damage in biological samples.

Assessing antioxidant status is another indirect approach to evaluating the extent of oxidative stress in living cells. Assessment of antioxidant status can be done through measurement of enzymatic and non-enzymatic antioxidants, and determination of TAC. The enzymes CAT, SOD, GPx, and GST play a significant role in oxidative stress by regulating ROS levels. CAT regulates ROS levels through the catalytic conversion of H_2O_2 into water and oxygen (Djordjevic, 2004). SOD regulates ROS by catalyzing the conversion of $O_2^{\bullet-}$ to H_2O_2 and O_2 (Halliwell & Gutteridge, 1986). GPx regulates ROS levels by catalyzing the H_2O_2 reduction and lipid peroxides to H_2O and their corresponding lipid alcohols through chemical oxidation of GSH into glutathione

disulfide (GSSG) (Arthur, 2001). GST quenches ROS through the addition of GSH and guards the cell against oxidative injury (Kumar & Trivedi, 2018).

Nonenzymatic antioxidants such as GSH, vitamins A, C and E play a protective effect in reducing oxidative damage by scavenging and/or neutralizing the detrimental effects produced by radical species (Birben et al., 2012). Therefore, assessing their level is paramount in measuring oxidative stress. The GSH levels are measured commonly using an Ellman reagent, 5,5′-dithiobis-(2-nitrobenzoic acid), GSH-400 method, and 2,3-naphthalenedicarboxaldehyde fluorometric assay (Ellman, 1959; Katerji et al., 2019). The levels of vitamins A, C, & E can be determined using reversed-phase HPLC.

Measurement of TAC is paramount to evaluating the oxidative state of a biological sample *in vivo* and *in vitro*. A prior study (Alam et al., 2013) summarized the two models of evaluation TAC. The *in vitro* procedures are as follows:

- DPPH$^{\bullet}$ scavenging activity
- $^{\bullet}$OH scavenging activity
- $^{\bullet}$OH averting capacity (HORAC)
- $O_2^{\bullet -}$ scavenging activity
- NO$^{\bullet}$ scavenging activity
- Peroxynitrite radical scavenging activity
- Total radical-trapping antioxidant parameter (TRAP)
- Biological antioxidant potential (BAP)
- Cupric ion reducing antioxidant capacity (CUPRAC)
- Oxygen radical absorbance capacity (ORAC)
- 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity/Trolox equivalent antioxidant capacity (TEAC)
- Ferrous ion chelating activity
- Ferric ion reducing power method
- FRAP assay
- H_2O_2 assay

- Phosphomolybdenum method
- Ferric thiocyanate (FTC) method
- Thiobarbituric acid (TBA) method
- N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) method
- β-carotene linoleic acid method/conjugated diene assay.

In *in vivo* methods of evaluating antioxidant activity, the sample under investigation is customarily injected or administered to the experimental subject/animals at a specific concentration. After a certain time, the animals are generally sacrificed and blood or tissues are taken for the investigation (Aydemir et al., 2000; Öztürk-Ürek et al., 2001). The *in vivo* test procedures used include:

- CAT test,
- The ferric reducing ability of plasma,
- GPx estimation,
- GR test,
- GST estimation,
- γ-GGT assay,
- LPO assay,
- LDL estimation,
- GSH estimation,
- SOD estimation.

1.4 Cancer

Suh et al. (2017a) explained that "cancer occurs through highly complex processes that involve the multiple coordinated mechanisms of carcinogenesis". Cancer-related deaths are increasing at a high rate, in 2018 alone 18.1 million incidences of cancer and 9.6 million cancer deaths happened (Cotas et al., 2020). Cancer incidences have been projected to increase globally by about 68% in 2030 (Hussein & Abdullah, 2020). There are more than a hundred cancer types. Cancers are customarily designated after the tissues/organs from which they were originated, even if it is later spread to other

body parts. The most common are the cancers of the adrenal glands, anal, bladder, bone, brain and spinal cord, breast, cervical, colorectal (colon), oesophagus, eye, gallbladder, kidney, liver, lung, mouth (oral), ovarian, prostate, throat, thyroid, pancreas, skin, small intestine, stomach, vaginal and vulvar.

1.4.1 Breast Cancers

Breast cancers are a group of cancers that develops from breast tissue as a result of the abnormal growth of breast cells. Although it occurs in Men, this type of cancer is affecting one in eight women on average and is usually found among women aged between 50 and 70 years (Tinoco et al., 2013). Breast cancers are originated from breast lobules, breast connective tissue and mammary ducts. Those starting from the mammary ducts goes by the name of ductal carcinomas, while those evolving from lobules goes by the name of lobular carcinomas. The common breast cancers comprised ductal carcinoma *in situ*, invasive ductal carcinoma-lobular carcinoma, lobular carcinoma *in situ*, inflammatory breast cancer, Paget's disease of the nipple, phyllodes tumours of the breast, and metastatic breast cancer (Wild, 2014).

The common risk factors of breast cancer include childbearing, short term breastfeeding, menopause at a late age, oral contraceptives, menarche and the menstrual cycle, hormonal therapy for the menopause, use of diethylstilbestrol during pregnancy, use of fertility drugs, high-fat diets, alcohol consumption, smoking, ionizing radiation, exposures to extremely-low-frequency electromagnetic fields and artificial light, chemicals such as dichlorodiphenyltrichloroethane and polychlorinated biphenyls, and family history (Key et al., 2001).

1.4.2 Skin Cancers

Skin cancer occurred due to the abnormal growth of skin cells in the epidermis, the outermost skin layer. The abnormal growth is triggered by DNA damage. Long exposure of skin to ultraviolet radiation from the Sun is the major cause of skin cancer (Gallagher et al., 2010). Skin cancer can occur from long exposure of skin to ultraviolet

rays from the Sun (Gallagher et al., 2010). The main skin cancers include melanoma, skin lymphoma, basal cell, squamous cell skin cancer Kaposi sarcoma, Merkel cell (carcinoma) (Marks, 1995).

1.4.3 Cervical Cancers

Cervical cancer is the fifth most deadly cancer among women. It occurs as a result of excessive and abnormal growth of cells in the cervix (Farzana et al., 2019). The cervix ties the vagina to the upper part of the uterus, a place where a conceived baby grows. There are three main types of cervical cancers: adenocarcinoma, squamous cell carcinoma, and adenosquamous carcinomas (mixed carcinomas) (Schiffman & Brinton, 1995; Farzana et al., 2019). The most common risk factor of cervical cancer is an infection caused by human papillomavirus (HPV). This infection has an estimated yearly case of 440,000 worldwide. Other common risk factors are herpes simplex virus (HSV-2), HIV and chlamydia infections, use of birth control pills (oral Contraceptives), smoking, lower educational and income levels, Immunosuppression, starting sex at a young age, multiple pregnancies, poor hygiene of the male partner, and having many sexual partners (Schiffman & Brinton, 1995).

1.4.4 Liver Cancers

Cancers in the liver are categorized into two: primary and secondary. Primary liver cancers are those types of cancers that start in the liver, and they comprise hepatocellular carcinoma, angiosarcoma, bile duct cancer (intrahepatic cholangiocarcinoma), hemangiosarcoma and hepatoblastoma. These types of liver cancers are rated worldwide as number fourth common origin of any death caused by cancer worldwide (Fernández-Palanca et al., 2021). Secondary liver cancers metastasize to the liver from other sites of the body such as the pancreas, stomach, lung, breast, or colon. The most common causes of liver cancer are cirrhosis due to persistent hepatitis B and C infections, illnesses of alcohol-related liver and non-alcoholic fatty liver, liver flukes, aflatoxin, smoking, obesity and type 2 diabetes mellitus (Pinheiro et al., 2019).

1.5 Antioxidants

Halliwell & Gutteridge (1995) defined antioxidants as ‘‘any substance that, when present at a relatively small amount in comparison to those of an oxidizable substrate, substantially prolongs or prevents the oxidation of that substrate by a free radical species or any other oxidant”. Antioxidants and their biological functions have become an important subject of discussion over the past years and also the topic of extensive research all over the world because of their functions in the food and pharmaceutical-related industries (Hamidi et al., 2020). Antioxidants are scavengers of ROS, RNS, RCS and RSS. They can protect, scavenge, and repair the oxidative damage caused by these species, thereby shielding biological molecules against oxidative injuries (Halliwell, 2007). Antioxidants are of two classes, those synthesized within the body are called endogenous antioxidants, and the other is called exogenous antioxidants.

The endogenous antioxidants are further classified into enzymatic and nonenzymatic (Pizzino et al., 2017). Although highly efficient, endogenous antioxidants in our body are inadequate to thwart the deleterious impacts of ROS, RNS, RCS and RSS generated in the body, hence the justification on the need for exogenous antioxidants (Sansone and Brunet, 2019). The exogenous antioxidants (sterols, carotenoids, polyphenols and also vitamins A, B, C, E) are customarily isolated from photosynthetic organisms as described earlier (Xu et al., 2017). Phenolics, flavonoids and carotenoids are major groups of natural compounds known to have strong antioxidant activity. Microalgae are known to be reached the source of these exogenous antioxidant molecules. Microalgal antioxidants include enzymes, such as CAT, GR and SOD; fat-soluble compounds, such as carotenoids and vitamin E; and water-soluble compounds, like other vitamins, phycobiliproteins, and polyphenols (Shalaby, 2014; Smerilli et al., 2017).

Like antioxidants compounds, antioxidants enzymes are known to destroy free radicals and repair the oxidative damage caused by oxidants. Phenolics acids (chlorogenic acids, gallic, protocatechuic and syringic acids) and flavonoids (catechin and epicatechin) were isolated from microalgae

(*Ankistrodesmus sp., Spirogyra sp., Euglena cantabria* and *Caespitella pascheri*). The polyphenols were later identified using RP-HPLC (Jerez-Martel et al., 2017). Fourteen polyphenolics possessing strong antioxidant activities were isolated from *Dunaliella tertiolecta,* and identified using RP-HPLC (López et al., 2015). Eight phenolic acids namely 3-hydroxybenzoic, 4-coumaric, 4-hydroxybenzoic, caffeic, protocatechuic, salicylic, sinapic, and syringic acids were obtained from the medlar fruit using HPLC–MS. The total contents and antioxidants capacities of most of these phenolic acids decreased as the ripening progressed (Gruz et al., 2011).

The mechanisms of antioxidant activity of the phenolic compounds include: a) inhibiting free radical generation, b) scavenging ROS, RNS or RSS such as $^{\bullet}OH$, $O_2^{\bullet-}$, RO^{-} and ROO^{-} by hydrogen atom donation, c) protection of antioxidant defences, d) metals chelating activity, e) reducing power potential, f) inhibition of microsomal monooxygenase, GST, mitochondrial succinate oxidase, NADH oxidase etc. (Brown et al., 1998; Kumar & Pandey, 2013).

Other important enzymes that are associated with antioxidant activity are polyphenol oxidases (PPO) and peroxidases (POD). PPO is a copper-containing metalloprotein found in many higher plants. The enzyme possesses three different functions connected as (a) catechol oxidase, (b) laccase and (b) cresolase (Sheptovitsky & Brudwig, 1996). The PPO is the main plant enzyme behind the enzymatic browning of raw fruits through the oxidation of phenolic compounds to their respective reactive quinones (Queiroz et al., 2008). Other functions of PPO include oxygen scavenging activity (Trebst & Depka, 1995), and shielding plants against biotic and abiotic stress and plant pathogens (Mohammadi & Kazemi, 2002).

In medlars, low PPO and POD activities were obtained in the early stages of fruits development, while their high activities in pre-and post- ripening phases (Aydin & Kadioglu, 2001). The activity of PPO extracted from medlar fruit was determined using substrates 4-methyl catechol, catechol, L-3,4-dihydroxyphenylalanine, epicatechin, 3-(3,4-dihydroxy phenyl) propionic acid (DHPPA), (p-hydroxyphenyl) propionic acid, tyrosine with the highest and lowest activity observed with 4-methyl

catechol and DHPPA respectively (Dincer et al., 2002; Ayaz et al., 2008).

1.6 Phenolic Compounds

Phenolics, also known as polyphenols, are the most abundant secondary metabolites generated by plants through pentose phosphate, phenylpropanoid and shikimate pathways (Lin et al., 2016). Phenolics are bioactive compounds containing benzene rings and one or more OH parts bounded to the ring(s). They are present in substantial amounts in plant foods (Velderrain-Rodríguez et al., 2014). Phenolics compounds from fruits and vegetables have health benefits and can contribute to flavour and taste properties. The polyphenols are subdivided into larger groups, namely, phenolic acids, flavonoids, tannins and stilbenes (Figure 1.1). These polyphenols are strong antioxidants and their biological potentials (antibiotic, antiviral, anticarcinogen, anti-inflammatory, and anti-ageing activities) were well documented. Taking phenolics compounds is linked to a reduced risk of CVD, NDD, cancer, type II diabetes, osteoporosis, modulation of signal transduction, and vasodilation effect (Velderrain-Rodríguez et al., 2014).

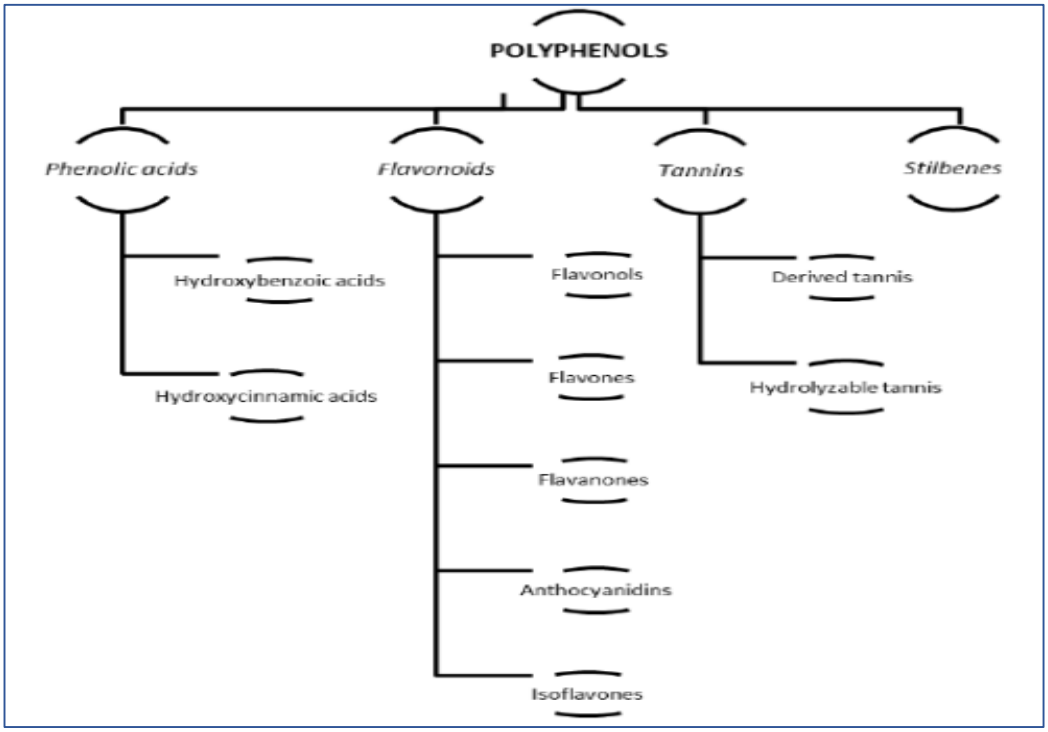


Figure 1.1 Main classes of phenolic compounds (Ozcan et al., 2014)

1.6.1 Flavonoids

Flavonoids are the most diverse groups of polyphenols, incorporating two or more aromatic rings, with one or more OH groups connected by a carbon bridge. Flavonoid is usually subdivided into flavones, flavanones (dihydroflavones), flavans, flavanols, flavonols, flavanonols (other names 3-hydroxyflavanone, 2,3-dihydroflavonol)**,** anthocyanidins, isoflavonoids, neoflavonoids and chalcones (Figure 1.2). The most studied flavonoids are flavones (e.g., luteolin, apigenin, tangeritin, tageretin, nobiletin, baicalein, luteolin glucosides, rpoifolin, chrysin, sinensetin, 7,8-dihydroxyflavone, 6,7,-dihydroxyflavone, apigenin 7,4'-dimethyl ether), flavanones (e.g., hesperitin, hesperidin, naringin, naringenin, eriodictyol, taxifolin, homoeriodictyol), flavans (e.g., flavan-3-ols, flavan-3,4-diols, flavan-4-ols), flavanols (e.g., catechin, gallocatechin, catechin-3-gallate, gallocatechin-3-gallate, epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate, theaflavin), flavonols (e.g., galangin, kaempferol, morin, datisetin, rhamnazin, quercetin, isoharmnetin, rutin, myricetin, rhamnetin, fisetin, pachypodol, quercetagetin, tamarixetin, pyranoflavonols, robinetin, furanoflavonols), flavanonols (e.g., dihydroquercetin, (+)-dihydrorobinetin, (-)-fustin, dihydrokaempferol), anthocyanins (e.g., cyanidin, delphinidin, malvidin, pelargonidin, apigenidin, peonidin), isoflavonoids (e.g., genistin, genistein, glycitein, daidzin, daidzein), neoflavonoids (e.g., calophyllolide), chalcones (e.g., phloridzin, arbutin, phloretin. chalconaringenin) (Mori et al., 1987; Ververidis et al., 2007; Kumar & Pandey, 2013; Panche et al., 2016; Patil & Masand, 2018).

These flavonoids are widely found in wheat, soybean, onions, cocoa, kale, grapefruit, lettuce, oranges, chamomile, tomatoes, lemons, black currants, apples, celery, tea, parsley, wine, red peppers, mint, peaches, ginkgo Biloba, bananas, buckwheat, bilberries, blueberries, blackberries, cranberries, strawberries, raspberries, red grapes, merlot grapes and pears (Panche et al., 2016). Flavonoids have potent antioxidative, anti-carcinogenic, anti-inflammatory, antimicrobial, anti-mutagenic properties, and are potent inhibitors of several enzymes, including XOD, lipoxygenases (LOX) enzyme and cyclooxygenases (COX) (Hayashi et al., 1988; Walker et al., 2000). The role of flavonoids in plants includes serving as antimicrobial

compounds, protecting the plants from biotic/abiotic stresses, UV filters, heat acclimatization, freezing tolerance, drought resistance, and are behind the colour and aroma of fruits and flowers (Panche et al., 2016).

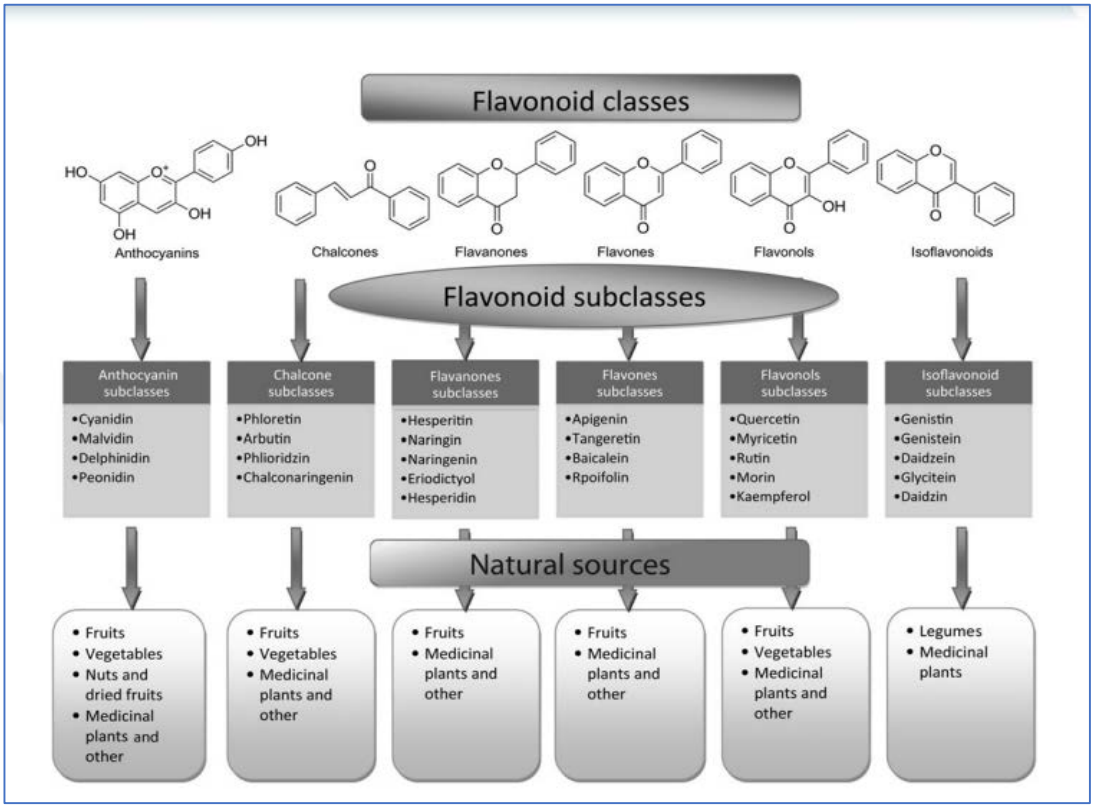


Figure 1.2 Flavonoid: classes, subclasses and natural sources (Panche et al., 2016)

1.6.2 Phenolic acids

The second largest group and the second widely distributed plant polyphenols are phenolic acids. They are described as aromatic acid compounds consisting of a phenolic ring and carboxylic group (-COOH) attached to the C_6-C_1 bridge. They are classified into hydroxybenzoic acid, hydroxycinnamic acid and hydroxyphenyl acetic acid derivatives (Figure1.3) (Chen et al., 2020). The phenolic acids derived from the hydroxybenzoic acid include chlorogenic acid, 2-hydroxybenzoic acid (another name, salicylic acid), 3-hydroxybenzoic acid (another name, m-hydroxybenzoic acid), 4-hydroxybenzoic acid (another name, p-hydroxybenzoic acid), neochlorogenic acid, vanillic acid, syringic, gallic acid, and ellagic acid. The hydroxycinnamic acid derivatives include p-coumaric acid, caffeic acid, α-cyano-4-hydroxycinnamic acid,

cichoric acid, ferulic acid, and sinapinic acid (another name, sinapic acid). The hydroxyphenyl acetic acid derivatives include 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, homovanillic acid, homoisovanillic acid and 4-hydroxy-3,5-dimethoxyphenylacetic acid (Heleno et al., 2015; Chen et al., 2020).

Phenolic acids can be sourced from many plant species including cereals, apple, cherry juice, blueberry, raspberry, cranberry, aloe vera, pear, artichoke, cherry, peanut orange, pineapple, grapefruit, lemon, mushroom, peach, sunflower, spinach, potato, lettuce, tea, coffee and cider (Ozcan et al., 2014). Phenolic acids have medicinal properties and evidence of their role in disease prevention are studied. They can act as therapeutic agents against oxidants, cancer, bacteria, fungi, virus etc.

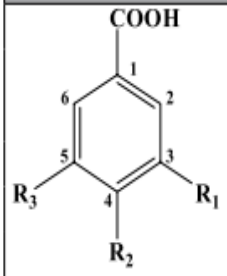
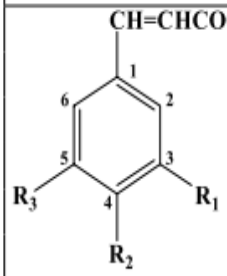
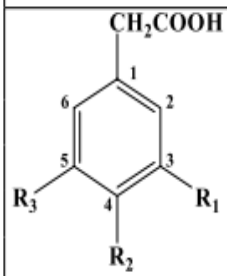
Basic structures	Abbreviations	Compounds	Substituents		
			R_1	R_2	R_3
	3-H-B	3-Hydroxybenzoic acid	OH	H	H
	4-H-B	4-Hydroxybenzoic acid	H	OH	H
	3,4-DH-B	Protocatechuic acid	OH	OH	H
	3-H-4-M-B	Isovanillic acid	OH	OCH_3	H
	4-H-3-M-B	Vanillic acid	OCH_3	OH	H
	4-H-3,5-DM-B	Syringic acid	OCH_3	OH	OCH_3
	3-H-C	3-Hydroxycinnamic acid	OH	H	H
	4-H-C	*p*-Coumaric acid	H	OH	H
	3,4-DH-C	Caffeic acid	OH	OH	H
	3-H-4-M-C	Isoferulic acid	OH	OCH_3	H
	4-H-3-M-C	Ferulic acid	OCH_3	OH	H
	4-H-3,5-DM-C	Sinapic acid	OCH_3	OH	OCH_3
	3-H-P	3-Hydroxyphenylacetic acid	OH	H	H
	4-H-P	4-Hydroxyphenylacetic acid	H	OH	H
	3,4-DH-P	3,4-Dihydroxyphenylacetic acid	OH	OH	H
	3-H-4-M-P	Homoisovanillic acid	OH	OCH_3	H
	4-H-3-M-P	Homovanillic acid	OCH_3	OH	H
	4-H-3,5-DM-P	4-Hydroxy-3,5-dimethoxyphenylacetic acid	OCH_3	OH	OCH_3

Figure 1.3 Molecular structures of hydroxybenzoic, hydroxycinnamic acid, hydroxyphenyl acetic derivatives (Chen et al., 2020)

1.6.3 Tannins

Tannins are astringent, heat stable and plant polyphenolics with sour/bitter tastes that either precipitate/binds alkaloids and proteins (Ashok & Upadhyaya, 2012). They are secondary metabolites that are rich in OH groups and were found in the inhibition of non-heme iron absorption, by coloured complex formation with iron in the gastrointestinal lumen (Lohlum et al., 2012) thus reducing the bioavailability of this important mineral.

Additionally, they are reported to hinder the oxidation of alkaloids & morphine and reduce protein digestibility in animals & humans by either making protein partially unavailable or hindering the role of digestive enzymes (Tadele, 2015). Tannins are divided into hydrolysable tannins commonly esters of gallic acid (i.e., gallotannins and ellagitannins), condensed tannins/proanthocyanidins (i.e., procyanidin A2 and B2), and phlorotannins (phloroglucinol). Tannins can be found in relative concentration in tea, berries, wine, coffee and chocolate (Ozcan et al., 2014).

1.6.4 Stilbenes

Stilbenes (1, 2-diphenylethylene) are phytoalexins compounds generated produced by plants against injury, disease or stressors. Stilbenes are principally found in grapes, wine, berries, soy and peanuts as cis and trans isomeric forms of resveratrol (Cassidy et al., 2000; Khoo & Falk, 2014).

1.7 Functional Properties of Phenolic Compounds

1.7.1 Antibacterial Activity

The worldwide rampant increase of bacterial resistance to commercially available antibiotics has called forth the investigation of naturally occurring candidate drugs from various plants (Shannon & Abu-Ghannam, 2016). Different phenolic compounds (phenolic acids, flavonoids, tannins and stilbenes) were found to have antibacterial properties. The potent antibacterial properties of the flavonoids on *Staphylococcus*

aureus and *Proteus vulgaris* was investigated. According to Mori et al. (1987), 7,8-dihydroxyflavone was effective against *S. aureus*; kaempferol, morin and quercetagetin were effective *against P. vulgaris*; robinetin, myricetin, dihydrorobinetin, & epigallocatechin were effective against both bacteria; and the activity was as a result of inhibiting of DNA synthesis in bacterial species by the flavonoids. The molecular actions of flavonoids involve the formation of complex with protein through hydrogen, covalent and hydrophobic bindings, disruption of microbial membranes, and inactivating microbial adhesions enzymes (Kumar & Pandey, 2013).

Polyphenol compounds from microalgae *Spirulina sp.* were determined to have an antibacterial effect (Kuntzler et al., 2018). Phenolics extracted from *Chlorella vulgaris* were found to be effective against G^- bacteria, namely, *Klebsiella pneumoniae, Escherichia coli, P. mirabilis, Salmonella typhi, Vibrio cholerae,* and G^+ bacteria viz. *S. aureus, Bacillus subtilis, Enterococcus sp., Clostridium botulini* and *Nocardia sp* (Dineshkumar et al., 2017). Dantas et al. (2019) examined the antibacterial activity of polyphenol extracted from *Scenedesmus subspicatus* against *E. subtilis, K. pneumoniae* and *E. coli.* Positive results were obtained. The compounds responsible for these properties as speculated by the authors were phenolic acids, flavonoids and tannins. Flavonoid compounds isolated from *C. vulgaris* and *Chlamydomonas reinhardtii* showed a highly efficient zone of inhibition against *Pseudomonas aeruginosa, E. coli* and *S. aureus* (Jayshree et al., 2016).

The antibacterial activity of the *Nigella* species was also reported. In one study, the seed extracts of *N. arvensis* indicates antibacterial activity with a varying range of minimum inhibitory concentration against six bacterial species namely, *B. cereus, B. subtilis, Bacteroides fragili, S. epidermidis, S. aureus* and *Enterococcus faecalis* (Landa et al., 2006). This activity could be on account of phenolics compounds (carvacrol, thymol and thymohydroquinone) and antimicrobial compounds (β-pinene) identified in the plant (Dorman & Deans, 2000). The amount of β-pinene reported in the plant was 21.4% (Havlik et al., 2006).

1.7.2 Antifungal Activity

The use of synthetic fungicides harms the environment and human health. This motivated researchers to search for alternative fungicides from plants that are environmentally friendly and safe for human health. The antifungal activity of phenolic compounds has been documented. Quercetin, a flavonol, and ferulic acid were reported to exhibited potent activity against *Alternaria alternata*, *Botrytis cinereae*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Phytophthora infestans* (Wianowska et al., 2016). This potency maybe as a result of the inhibition of fungal spores.

Polyphenolics compounds and carotenoid extracts of microalgae *Spirulina sp.* and *Nannochloropsis sp.* were found to lessen contamination caused by fungal pathogens *F. graminearum F. meridionale* and *F. asiaticum* (Scaglioni et al., 2019). The antifungal inhibition of *F. oxysporum* and *Colletotrichum gloeosporioides* by the phenolic acids (gallic, chlorogenic, syringic, p-hydroxybenzoic, caffeic, ferulic and p-coumaric) and flavonoids (phloridzin, phloretin and galangin) extracts of *Barkleyanthus salicifolius* were also reported (de Jesús Joaquín-Ramos et al., 2020).

1.7.3 Anti-Inflammatory Activity

Inflammation is the reaction of body tissues to microbial pathogens, damaged cells, chemicals and xenobiotics, harmful stimuli or irritants. In the process of inflammation, the inflammatory cells secret pro-inflammatory cytokines together with energizing the manifestation of NOS isoforms, iNOS and cyclooxygenase-2 (COX-2) (Sanjeewa et al., 2016). The usage of polyphenolics and other natural products from plant materials to treat inflammation or swelling have well-being documented. In one study, flavonol, flavone and flavanone classes of flavonoids were shown to hinder the function of COX-2 enzymes (D'Mello et al., 2011). COX-2 is one of the three isoforms of COX, the others are COX-1 and COX-3. These enzymes are responsible for the transformation of arachidonic acid into prostaglandins and thromboxanes (Smith et al., 2000).

COX-1 is linked to the development of ovarian cancer (Malerba et al., 2019). While the COX-2 was found to induces inflammation, angiogenesis, cancer stem cell-like activity, and promotes apoptotic resistance, and metastasis of cancer cells (Hashemi Goradel et al., 2019). Moreover, eriodictyol, fisetin, homoeriodictyol, pachypodol, rhamnetin, robinetin, tangeritin and theaflavin were reported to impede the activity of the LOX enzyme (Madeswaran et al., 2011). LOX is a non-heme iron-containing enzyme that catalyzes the peroxidation of PUFA to their corresponding hydroperoxy derivatives (Snodgrass & Brüne, 2019).

1.7.4 Antidiabetic Activity

Polyphenols, especially flavonoids, phenolic acids and tannins can regulate carbohydrate metabolism - by inhibiting α-glucosidase & α-amylase and weakened hyperglycemia. Polyphenols compounds were reported to ward off the progression of long-term diabetes complications, for example, CVD, diabetic neuropathy, diabetic nephropathy and diabetic retinopathy (Lin et al., 2016). Anthocyanins, flavonoids organic acids, phenolic acids and tannins extracted from *Punica granatum* (Pomegranate) were found to lower LPO and oxidative stress in type 2 DM via contrasting mechanisms, namely, boosting the antioxidant activity of various enzymes, scavenging ROS and inducing metal chelating activity (Lin et al., 2016). Flavonoids i.e., catechin, EGC, EGCG, isolated from *Diospyros kaki* have potent activity against α-amylase secreted by the pancreas (Kawakami et al., 2010).

Moreover, flavonoids (apigenin, catechin, daidzein, naringenin, luteolin glycoside, kaempferol, quercetin, pelargonidin-3-rutinoside, cyanidin-diglucoside), phenolic acids (caffeic acid, ellagic acid, ferulic acid), chebulagic acid and chebulinic acid extracted from *E. coracana*, *Rubus idaeus, C. anthelminticum* and *T. chebula* showed inhibitory activity against several enzymes including α-glucosidase, salivary α-amylase, sucrase, pancreatic α-amylase and maltase (Gao et al., 2007; Ani & Naidu, 2008, Shobana et al., 2009; Zhang et al., 2011).

1.7.5 Anticancer Activity

Even though cancers were reported as the leading cause of death worldwide (Cotas et al., 2020), finding an effective method to handle them remains a huge challenge. Therapeutic agents and drugs development for cancer treatment is a prerequisite for continued progress on it. Recent studies had shown the antiproliferative and cytotoxic effects of polyphenols on different cancer cell lines. Polyphenolics extracted from two microalgae species *C. sorokiniana* and *Dunaliella sp*. were found to have antioxidant and anticancer activities against four different human cancer cell lines viz. Caco2, MCF7, PC3 and HepG2 (Senousy et al., 2020). Suh et al. (2017b) investigated the anti-proliferative activity of polyphenol extracts of *Botryidiopsidaceae sp.* against tumorigenic cell lines and non-tumorigenic keratinocyte cells. The flavonoid extracts of *C. vulgaris* and *C. reinhardtii* were found to be effective against breast cancer, MCF7 (Jayshree et al., 2016). With the efficiently high amount of total phenol and flavonoid contents, these microalgae exhibited strong antioxidant and cytotoxic potency.

1.8 Plant Studies and Aim of the Study

1.8.1 Nigella sativa

N. sativa belongs to the genus *Nigella* which has around twenty different species usually found in the Middle East, Northern Africa, Western Asia and Southern European countries. Among these species, thirteen were reported to be cultivated or in wild form from several places in Turkey (Davis, 1988). These includes *N. lancifolia, N. segetalis, N. fumariifolia, N. latisecta, N. stellaris, N. nigellastrum, N. orientalis, N. damascena, N. elata, N. unguicularis, N. oxypetala, N. arvensis, and N. sativa* (Kökdil & Yılmaz, 2005; Nimet et al., 2015). Ranunculaceae are small shrubs with primitive polycarpellary characters (Da-Cheng et al., 2015).

N. sativa is an erect annual plant with soft leaves and bluish flowers of 20–30 mm diameter (Havlik et al., 2006). In Turkey, the herb is grown between June and September with its seeds reaching maturity between August and October. It grows in

moist well-drained soil over broad-spectrum pH. The hermaphrodite flower can propagate up to 0.3 m (Plants for a Future, 2021). The plant is called black cumin in English, Çörek Otu in Turkish, and Habbatus Sauda / Habet el Baraka / Kamun-Aswad in Arabic (Houghton et al., 1995). *N. sativa* is taxonomically described as follows:

- ✓ Domain: Eukaryota
- ✓ Kingdom: Plantae
- ✓ Phylum: Spermatophyta
- ✓ Subphylum: Angiospermae
- ✓ Class: Dicotyledonae
- ✓ Order: Ranunculales
- ✓ Family: Ranunculaceae
- ✓ Subfamily: Ranunculoideae
- ✓ Genus: *Nigella*
- ✓ Species: *Nigella sativa* (UniProt, 2021a).

So many studies on *N. sativa* led to the isolation of different classes of phytochemicals. Amongst which are alkaloids (Ali & Blunden, 2003; Ali et al., 2008; Avula et al., 2010), benzofurans and saponins (Bıçak et al., 2017). Additionally, the metabolomics profile of *N. damascene, N. nigellastrum, N. orientalis, N. hispanica, N. arvensis* and *N. sativa* indicated the species to contained different classes of alkaloids (10), flavonoids (10), saponins (8) and phenolics (6) (Farag et al., 2014). The seeds also contain 36–38% fixed & 0.4–2.5% essential oils, alkaloids, and proteins (Lautenbacher, 1997). The seeds were used for decades in the treatment of bronchitis, headache, rheumatism, eczema, and influenza (Burits & Bucar, 2000).

1.8.2 Mespilus germanica

M. germanica (medlar) belongs to the genus *Mespilus,* in the subfamily Maloideae of the family Rosaceae (Akbulut et al., 2016). The genus *Mespilus* (Medlar) has two species *M. canescens* and *M. germanica* (Shafi, 2014). *M. germanica* (English: medlar, Turkish: Muşmula, Beşbıyık, Ezgil, Döngel, Gelinboğan, Töngel, Arabic:

Almushmilat Shajara) is a small tree or spiny shrub that heighten up to 4–7 m in frost-free areas, poor acidic soils and rocks (Canbay et al., 2015). It has broadened leaves and flowers corresponding to that of apple (Gülçin et al., 2011). The brownish and sometimes reddish apple-shaped edible fruit of medlar has a weight and diameter ranging from 10 - 80 g and from 1.5 to 3 cm, respectively (Gruz et al., 2011). The fruit enclosed stony seeds which are subglobose. The elliptic-oblong dark green leaves of the medlar are eight-fifteen cm long and three-four cm wide (Safari & Ahmady-Asbchin, 2019). *M. germanica* is taxonomically classified as follows:

- ✓ Domain: Eukaryota
- ✓ Kingdom: Plantae
- ✓ Phylum: Spermatophyta
- ✓ Subphylum: Angiospermae
- ✓ Class: Dicotyledonae
- ✓ Order: Rosales
- ✓ Family: Rosaceae
- ✓ Genus: *Mespilus*
- ✓ Species: *Mespilus germanica* (Petrova & Barstow, 2017; UniProt, 2021b).

The plant is indigenous to Armenia, Azerbaijan, Bulgaria, Crimea, Caucasia, Georgia, Greece, Iraq, Iran, Italy, Southwest Russia, Turkey, Turkmenistan and Ukraine (Browicz, 1972; Khadivi et al., 2019). In Turkey, the wild and cultivated forms are usually found wild in the northern and western Anatolia, Karadeniz, Ege and Marmara regions (Yilmaz & Gerçekcioğlu, 2013). The flowers full bloom in May and the mature fruits are harvested around September – December. It is then stored in cold, dark and ventilated places for future uses (Gruz et al., 2011). The flowers bloom in May and the harvesting of mature fruits runs from the middle of September to the middle of December. The fruits are usually stored in cold, dark and ventilated places for future uses (Gruz et al., 2011).

The best-known varieties of medlars are Common medlar (with medium fruit), Dutch medlar (with big fruit), Nottingham medlar (with small fruit), Royal medlar (with small fruit) and Stoneless medlar (Glew et al., 2003). The fruit of medlar is rich

in lipid which is presumed to help in flavour and aroma during ripening (Ayaz et al., 2002).

1.8.3 Diospyros lotus

D. lotus L. (commonly, *lotus* persimmon) is a deciduous tree part of the Ebenaceae family. The tree belongs to the genus *Diospyros*, which has over 700 species. *D. lotus* grows in the semi-shaded area for up to 9 m height (Chittendon 1956). The trees are cultivated in various countries for their palatable fruits (Saral et al., 2016). It is native to the Middle East, Southeast Europe, East and Southwest Asia (Hedrick, 1972).

In Turkey, the tree is usually cultivated around the Black Sea region, northeast and southern Anatolia (Ayaz et al., 1997). *D. lotus* is called date-plum in English and Yabani Trabzon Hurması, Uvaz, Kara Hurma in Turkish. *D. lotus* is taxonomically classified as follows:

- Domain: Eukaryota
- Kingdom: Plantae
- Phylum: Spermatophyta
- Subphylum: Angiospermae
- Class: Dicotyledonae
- Order: Ebenales
- Family: Ebenaceae
- Genus: *Diospyros*
- Species: *Diospyros lotus* (UniProt, 2021c).

The fruits of *D. lotus* are spherical, one and half-two cm in diameter and bluish-black colour at maturation (Davis, 1978) and were used over the years in traditional medicine as an astringent, sedative, antitussive, antiseptic, antitumor, laxative, nutritive, febrifuge, and for the treatments of diarrhoea, constipation, dry coughs, fauvism, and hypertension (Cho et al., 2015). Chemical analysis on *D. lotus* fruits revealed sugar, fatty acids, phenolic compounds, terpenes, organic acids and

naphthoquinones (Ayaz et al., 1997; Loizzo et al., 2009; Rashed et al., 2012). Extracts of the fruits have been found to have strong antiproliferative activity against C32 and A375 cells (Loizzo et al., 2009).

The leaves are egg-shaped measuring six-twelve cm in length and three-six cm in width (Tian et al., 2020). Chemical profiling of the leaves leads the way to the identification and quantification of myricetrin, myricetin, hyperoside, isoquercitrin, quercetrin, quercetin, myricetin-3-O-β-d-glucoside, myricetin-3-O-β-d-galactoside, astragalus and vitamin C (Tian et al., 2020). The leaves of *D. lotus* have antitumor, analgesic, antipruritic, anti-inflammatory effects, hepatoprotective, and the amelioration of ultraviolet damage (Jeon et al., 2014; Kim et al., 2014; Tian et al., 2020). Extracts of *D. lotus* seeds were also reported to have antioxidant, anti-hemolytic and nephroprotective properties (Moghaddam et al., 2012).

1.8.4 Aim of the Thesis

This research aimed to extract flavone, flavanone, flavonol, flavan-3-ol and phenolic acids from *N. sativa, M. germanica* and *D. lotus;* profile and quantify the flavonoids and phenolic acids using HPLC; evaluate and compare the antioxidant potential of the extracts using total polyphenolic content, DPPH$^{\bullet}$ scavenging, $^{\bullet}$OH scavenging, NO$^{\bullet}$ scavenging, ferrous ion chelating, ferric ion reducing power, and FRAP assays; and determine the cytotoxic effects of the extracts on cell lines, namely, HeLa and HepG2 using MTT assay.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Chemicals, Reagents, Standards, Equipment and Cell Lines Collection

2.1.1 Chemicals and Reagents

The chemicals – ethanol (isolab, ≥99.9%), methanol ACS reagent (carlo erba, ≥99.9%), methanol HPLC grade (sigma, ≥99.9%), n-hexane (sigma), acetonitrile HPLC - GOLD - Ultragradient grade (carlo erba), diethyl ether (fisher scientific), ethyl acetate (carlo erba), ascorbic acid (sigma), formic acid (merck, 98-100%), orthophosphoric acid (isolab chemicals), deoxyribose, ferrozine, sodium acetate, potassium acetate, vanillin, deoxyribose, acetic acid, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), thiobarbituric acid (TBA), sodium nitroprusside (SNP), ethylenediaminetetraacetic acid (EDTA), phosphate-buffered saline (PBS), potassium ferricyanide [$K_3Fe(CN)_6$], Folin–Ciocalteu reagent (FCR), n-(1-naphthyl) ethylenediamine dihydrochloride (NEDA·2HCl), Gibco Dulbecco's Modified Eagle Medium (DMEM), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), dimethyl sulfoxide (DMSO), butylated hydroxytoluene (BHT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulfanilamide, HCl, NaOH, Na_2CO_3, NaCl, NaH_2PO_4, Na_2HPO_4, KCl, K_2HPO_4, KH_2PO_4, KOH, H_2O_2, $FeCl_2$, $FeCl_3$, $AlCl_3$ - used were of analytical and/or HPLC grade. All chemicals and reagents, unless other-wise specified, were purchased from Sigma.

2.1.2 Standards

Apigenin, luteolin, diosmin, chrysoeriol, eupatorine, quercetin, quercitrin, isoquercetin, rutin, kaempferol, isorhamnetin, myricetin, hesperetin, hesperidin, naringenin, catechin, epicatechin, epigallocatechin, epigallocatechin gallate, protocatechuic acid, vanillic acid, ferulic acid, sinapic acid, caffeic acid, sinapic acid, syringic acid, rosmarinic acid, tannic acid, benzoic acid, 4-hydroxybenzoic acid, o-coumaric acid, p-coumaric acid and t-cinnamic acid were the standard used for HPLC analysis. Ascorbic acid, quercetin, gallic acid, tannic acid, EDTA, BHT were the

standards used in antioxidant activity determinations. All standards were purchased from Sigma.

2.1.3 Equipment

UV-VIS spectrophotometer (UV-1800, Shimadzu), freeze-drying (Labconco), HPLC (1100 series, Agilent technologies), microplate shaker (fisher scientific), UV-VIS plate reader, evaporator (R-100, Buchi), centrifuge (universal 320R, Hettich zentrifugen), autoclave (Hirayama), ultrasonic bath (Elma elmasonic p), Soxhlet apparatus, scales (Sartorius), hemocytometer, CO_2 incubator (NuAire), microscope (CKX410, Olympus), heating magnetic stirrer (VELP scientifica).

2.1.4 Cell Lines Collection

HeLa (ATCC®, CCL-2™) and HepG2 (ATCC®, HB-8065™) cell lines utilized in the present research were acquired from the American Type Culture Collection (ATCC).

2.2 Samples Collection and Preparation

2.2.1 Nigella sativa

The seeds of *N. sativa* (Figure 2.1) were purchased from Gökçehan Baharatları Gida San. ve Tic. Ltd. Şti., Pınarbaşı, Izmir/Turkey. To remove any moisture from the seeds, a certain amount of it was lyophilized. Thereupon, the dry seeds were milled into a fine powder using a kitchen mill grinder.

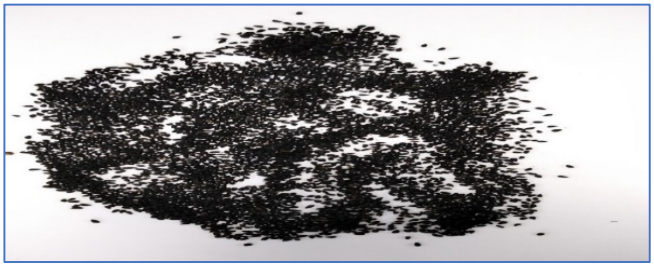


Figure 2.1 *N. sativa* seeds sample (Personal archive, 2021)

2.2.2 Mespilus germanica

The leaves and fruits of *M. germanica* (Figure 2.2) were collected from Denizli/Turkey, air dried, lyophilized until constant weight and ground into powder using a mill grinder.

Figure 2.2 *M. germanica* leaves and fruits samples (Personal archive, 2021)

2.2.3 Diospyros lotus

The fruits of the *D. lotus* (Figure 2.3) were gifted from Bizim Yöresel, Ordu/Turkey and washed thoroughly to remove any dust and impurities and then air-dried. Thereafter, seeds were removed from the fruit's pod. Both the seeds and fruit's pod were then shaded to dry for some days, and lyophilized until constant weight. The lyophilized sample was ground into powder using a mill grinder.

Figure 2.3 *D. lotus* seeds and fruits samples (Personal archive, 2021)

2.3 Extractions

2.3.1 Soxhlet Extraction

To remove liposoluble substances from the plant parts, certain amounts of the milled powders were defatted with n-hexane for 4 h using Soxhlet equipment (Figure 2.4). In this extraction, the milled sample was put into a cartridge paper, stapled and placed inside Soxhlet equipment. Solvent, n-hexane, was added to the round bottom flask and the extraction was performed under reflux. The defatted brans were allowed to dry at room temperature and kept at 4 °C for further analysis. The hexane extract was evaporated to dryness using a rotary evaporator, freeze-dried and lyophilized.

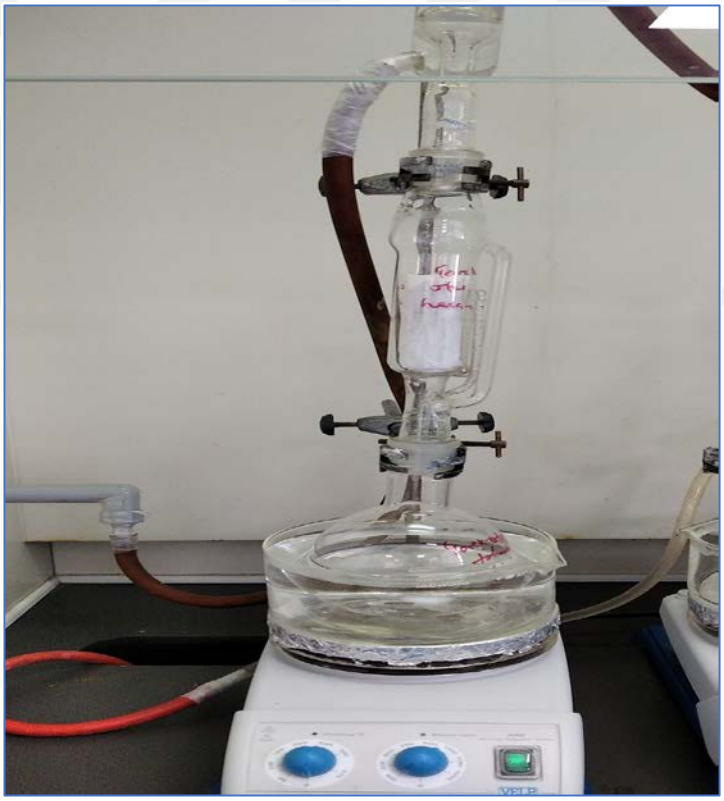


Figure 2.4 Soxhlet extraction (Personal archive, 2021)

2.3.2 Flavone Extraction

For flavone extraction, the method of Valentão et al. (1999) was adopted. Diethyl ether solvent (150 mL) was added to an Erlenmeyer flask containing 2 g of the defatted

bran. The Erlenmeyer flask was then kept on a thermo-shaker incubator (25 °C) for a period of 20 min. The slurry was filtered and the obtained residue was again extracted two more times with 75 mL diethyl ether for 10 min. The filtrates were combined, concentrated to dryness under vacuum in a fume hood, freeze-dried and lyophilized. The extraction yield was then determined.

2.3.3 Flavanone Extraction

Flavanone was extracted adopting the previous method of Pellati et al. (2004) with little changes. Defatted bran (2 g) of each of the five samples was extracted with 120 mL of ethanol-water (80:20%) for a period of 2 h at 90°C. The extract was centrifuged (5000 xg) for 10 min and filtered. The filtrate was evaporated with a rotavapor at reduced pressure. Flavanone extract was concentrated under vacuum in a fume hood, freeze-dried and lyophilized. The extraction yield was obtained from the lyophilized flavanone extract.

2.3.4 Flavonol Extraction

A Soxhlet extractor was used to extract flavonol from the different plant samples. Two (2) grams of the defatted bran was put into a cartridge paper, stapled and placed inside Soxhlet equipment. Thereupon, 200 mL of 95% aqueous methanol (v/v) and 60 mL of 25% HCl (v/v) were added to the round bottom flask and the extraction was carried out for 2 h under reflux. After cooling, the liquid extract was evaporated with a rotavapor at reduced pressure. The flavonol extract was concentrated under vacuum in a fume hood, freeze-dried and lyophilized. The extraction yield was then determined.

2.3.5 Flavan-3-ol Extraction

The defatted bran (2 g) of each of the five samples was suspended in 40 mL of absolute methanol, placed in ultrasonic baths (60 °C) and extracted for 2 h. The extract was centrifuged (5000 xg) for 10 min and filtered. The obtained residue was again

extracted two more times as described earlier. The final residue was dry and subjected to acid hydrolysis. The three filtrates were put together and the methanol was evaporated with a rotavapor at reduced pressure. Afterwards, the extract was concentrated under vacuum in a fume hood, freeze-dried, lyophilized and labelled as a methanolic fraction. The extraction yield was then determined.

In acid hydrolysis, the dried pellet of each of the five samples was firstly weighed and then extracted with 100 mL 2.5 M HCI-methanol (4:1, v/v) in a water bath for 2 h at 100 °C. The extract was centrifuged (5000 xg) for 10 min and filtered. The filtrate was subjected to liquid-liquid extraction (LLE) three times with 40 mL of diethyl ether and four times with 40 mL ethyl acetate. The ether phase was concentrated up to approximately 2 mL to dryness under vacuum in a fume hood. The ethyl acetate phase was evaporated with a rotavapor at reduced pressure for up to approximately 2 mL aliquot. The two aliquots of ether and ethyl acetate were combined further concentrated to dryness under vacuum in a fume hood, freeze-dried and lyophilized. The extraction yield of the combined lyophilized aliquot was eventually determined.

2.3.6 Phenolic Acids Extraction

Phenolic acids extractions were performed adopting the earlier method of Kim et al. (2006) with some minor changes. Analytical grade methanol (120 mL) was added to five Erlenmeyer flasks each containing 6 g of a defatted sample. The Erlenmeyer flask was then kept on a magnetic mixer at room temperature for a period of 1 h. The extract was centrifuged (5000 xg) for 10 min and filtered. The pellet was re-extracted following the same process mentioned earlier. The residue was allowed to dry before subjected to assays related to bound phenolic acids. The two filtrates were combined and the methanol was evaporated with a rotavapor at reduced pressure. The methanolic extract was again freeze-dried and lyophilized. The lyophilized crude methanolic extract (CME) was further extended for analysis to obtained extractable phenolic acids.

2.3.6.1 Extractable Phenolic Acids

The CME of each of the five samples was liquefied in 12 mL of acidified water (pH 2.0 with HCl) and subjected to LLE three times with 30 mL of ether. The three ether layers were combined, concentrated dryness under vacuum in a fume hood. The resultant was labelled as free phenolic acids (FPA) extract. The water phase was neutralized to pH 7.0 with 2 M NaOH, dried under vacuum and lyophilized. The lyophilized matter was liquefied in 12 mL of 2 M NaOH and stirred at room temperature for a period of 4 h. The obtained solution was acidified to pH 2.0 and subjected to LLE as stated earlier. The ether layers were combined, concentrated dryness under vacuum in a fume hood. The resultant was labelled as alkaline-hydrolysable phenolic acids (BHPA) extract.

The water layer obtained after this BHPA extraction was immediately treated with 12 mL of 6 M HCl and boiled at 95 °C for a period of 20 min. Like in FPA and BHPA extractions, the obtained solution subjected to LLE as stated earlier. The ether layers were combined, concentrated dryness under vacuum in a fume hood. The resultant was labelled as acid-hydrolysable phenolic acids (AHPA) extract. The FPA, BHPA and AHPA extracts were freeze-dried and lyophilized. The extraction yields of the three extracts were eventually determined.

2.3.6.2 Bound Phenolic Acids Extraction

The dried residue obtained following initial methanolic extraction was divide into two parts and subjected to hydrolysis to extract hydrolysable phenolic acids in the bran that were not extracted by the absolute methanol. The hydrolysis was carried out using two different protocols. Protocol I started with alkaline hydrolysis, in which 60 mL 2 M NaOH was added to an Erlenmeyer flask containing one part of the residue. The Erlenmeyer flask was put on a magnetic mixer and stirred at room temperature for a period of 4 h. The slurry was centrifuged and filtered. The supernatant was labelled as bound phenolic acids from basic hydrolysis (BPBH-1) extract. The pellet was subjected to acid hydrolysis with 6 M HCl at 95 °C for a 1 h period. The slurry was

centrifuged and filtered. The supernatant was labelled as bound phenolic acids from acid hydrolysis (BPAH-2) extract.

In protocol 2, acid hydrolysis was made first and then followed by alkaline hydrolysis. Two other extracts (BPAH-1 and BPBH-2) were obtained from this protocol. The BPBH-1, BPAH-2, BPAH-1 and BPBH-2 extracts were acidified to pH 2.0 and subjected to LLE. In the LLE, the four extracts were partitioned three times with 60 mL of ether. The ether phases were combined, concentrated dryness under vacuum in a fume hood, freeze-dried and lyophilized. The extraction yield of the BPBH-1, BPAH-2, BPAH-1 and BPBH-2 extracts were eventually determined.

2.3.7 Total Polyphenols Extraction

A total of 2 g of the defatted bran was extracted with 160 mL of aqueous methanol (methanol: water, 3:1, v/v) in a Soxhlet apparatus for 4 h (Figure 2.5). After cooling, the aqueous methanol was evaporated with a rotavapor at reduced pressure. Thereupon, the extract was concentrated under vacuum in a fume hood, freeze-dried and lyophilized. The extraction yield was then determined.

Figure 2.5 Total polyphenols extraction in Soxhlet extractor (Personal archive, 2021)

2.4 HPLC analysis

2.4.1 HPLC Equipment and Instrumentation

The HPLC analyses for flavonoids and phenolic acids were performed using an Agilent technologies 1100 series (Figure 2.6). The separation was accomplished by a C18 reverse-phase column (inner diameter: 4.6 mm, length: 150 mm, particle size: 5 μm). The chromatograms detection was achieved with a G1315B diode array detector. The column temperature was set at 25 °C and the injection volume was 20 μL.

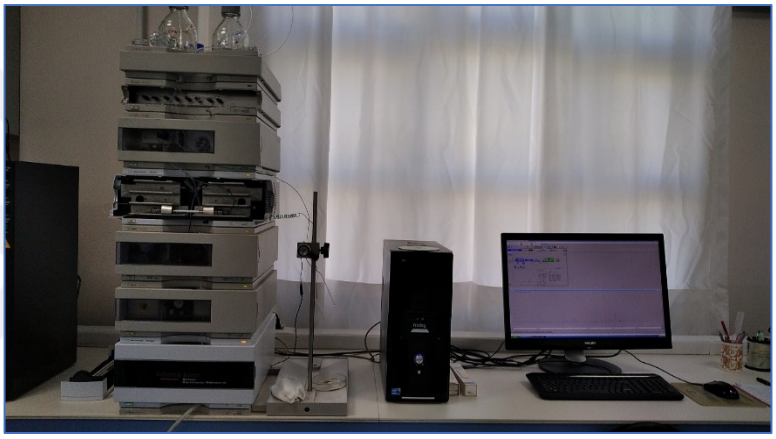


Figure 2.6 HPLC equipment (Personal archive, 2021)

2.4.2 Sample Preparation

The lyophilized flavonoids and phenolic acids extract of each of the five samples were reconstituted in methanol to 1000 ppm for HPLC analysis and antioxidant activity determination.

2.4.3 Chromatographic Analysis of Flavone

Valentão et al. (1999) method was adopted. The solvent phase consisted of A: (5% aqueous formic acid, v/v) and B: HPLC grade methanol. A volumetric flow rate of 1 mL/min was set for a total run time of 35 min. The sample was eluted with the following gradient: 0–5 min 50% A, 5–30 min 40% A and 30–35 min 20% A before returning to the initial conditions. The chromatograms were recorded at 350 nm.

2.4.4 Chromatographic Analysis of Flavanone

Pellati et al. (2004) method was adopted. The solvent system consisted of A: (0.6% aqueous acetic acid solution, v/v) and B: HPLC grade methanol. A volumetric flow rate of 0.4 mL/min was set for a total run time of 30 min. The sample was eluted with the following gradient: 0–5 min 80% A, 5–8min 60% A, 8–12 min 60% A, 12–25 min 40% A and 25–30 min 40% A before returning to the onset conditions. The chromatograms were recorded at 285 nm.

2.4.5 Chromatographic Analysis of Flavonol

Olszewska (2008) method was adopted. The solvent system consisted of the following solvents: A (0.5% aqueous orthophosphoric acid, v/v) and B (HPLC grade methanol). A volumetric flow rate of 1 mL/min was set for a total run time of 30 min. The sample was eluted with the following gradient: 0–10 min 60% A, 10–21 min 40% A, 21–23 min 40% A and 23–30 min 60% A before returning to the initial conditions. The chromatogram peaks were monitored simultaneously at 254 nm for isoquercetin and quercitrin, and at 370 nm for rutin, kaempferol, isorhamnetin, myricetin and quercetin.

2.4.6 Chromatographic Analysis of Flavan-3-ol

De Villiers et al. (2004) method was adopted. The solvent system comprised of A: (2% aqueous acetic acid solution, v/v) and B: (70% aqueous acetonitrile, v/v). A volumetric flow rate of 1.2 mL/min was set for a total run time of 33 min. The sample was eluted with the gradient as follows: 0–3 min 95% A, 3–8 min 95% A, 8–10 min 85% A, 10–12 min 80% A, 12–20 min 75% A, 20–30 min 60% A, 30–31 min 20% A and 31–33 min 95% A before returning to the onset conditions. The chromatograms were recorded at 280 nm.

2.4.7 Chromatographic Analysis of Phenolic Acids

Kim et al. (2006) method was adopted. The solvent system comprised of HPLC grade acetonitrile (A) and 2% (v/v) aqueous acetic acid solution (B). A volumetric flow rate of 1 mL/min was set for a total run time of 60 min. The sample was eluted with the following gradient: 0-30 min 100% B, 30-50 min 85% B, 50-55 min 50% B and 55-60 min 0% B before returning to the initial conditions. Chromatographic peaks were monitored concurrently at 280 for the benzoic acid derivatives and 320 nm for the cinnamic acid derivatives.

2.4.8 Qualitative and Quantitative Analysis of Phenolic Compounds

Flavonoids and phenolic acids were recognized from their retention time and observing similarities between the spectral features of their peaks and those of available standards. Quantitation was achieved from the calibration plots acquired by plotting peak areas versus the corresponding concentrations of standard solutions. The standards calibration curves of the phenolics compounds are shown in Table 2.1.

Table 2.1 Calibration curves of phenolics standards

Phenolic Compounds	**λ (nm)**	**Linear equation**	**R^2**	**Class**
Apigenin	350	y=64.676x	0.9951	Flavone
Chrysoeriol	350	y = 36.904x	0.9949	Flavone
Diosmin	350	y = 26.596x	0.9993	Flavone
Eupatorin	350	y = 57.035x	0.995	Flavone
Luteolin	350	y = 85.385x	0.9998	Flavone
Hesperidin	285	y = 143.06x	0.9968	Flavanone
Hesperetin,	285	y = 113.88x	0.9958	Flavanone
Naringenin	285	y = 185.05x	0.9938	Flavanone
Quercitrin	254	y = 37.91x	0.998	Flavonol
Isoquercetin	254	y = 20.507x	0.9988	Flavonol
Rutin	370	y = 19.207x	0.9983	Flavonol
Kaempferol	370	y = 50.421x	0.9965	Flavonol
Isorhamnetin	370	y = 45.253x	0.9985	Flavonol
Myricetin	370	y = 44.813x	0.9997	Flavonol
Quercetin	370	y = 45.406x	0.9902	Flavonol
Catechin	280	y = 12.30x	0.9984	Flavan-3-ol
Epicatechin	280	y = 12.481x	0.9995	Flavan-3-ol
Epigallocatechin	280	y = 2.2169x	0.9982	Flavan-3-ol
Epigallocatechin gallate	280	y = 18.728x	0.9992	Flavan-3-ol
Benzoic acid	280	y = 10.661x	0.9937	Phenolic acids
4-hydroxybenzoic acid	280	y = 36.063x	0.9964	Phenolic acids
Gallic acid	280	y = 44.016x	0.9949	Phenolic acids
Protocatechuic acid	280	y = 40.296x	0.997	Phenolic acids
Rosmarinic acid	280	y = 22.499x	0.9894	Phenolic acids
Syringic acid	280	y = 61.74x	0.9951	Phenolic acids
Vanillic acid	280	y = 43.058x	0.9999	Phenolic acids
Chlorogenic acid	315	y = 34.372x	1	Phenolic acids
Caffeic acid	320	y = 126.91x	0.9981	Phenolic acids
Ferulic acid	320	y = 127.37x	0.9914	Phenolic acids
Sinapic acid	320	y = 64.552x	0.9807	Phenolic acids
o-Coumaric acid	320	y = 51.672x	0.989	Phenolic acids
p-Coumaric acid	320	y = 110.3x	0.9747	Phenolic acids
t-Cinnamic acid	320	y = 2.7365x	0.993	Phenolic acids

2.5 Total Polyphenols Determinations

2.5.1 Total Phenolic Content

The total phenolic content (TPC) in each of the five plant samples was evaluated following the Folin–Ciocalteu method (FCM). The FCM is based on the transfer of electrons from a reducing agent (e.g., phenolic compound) that is energetically

oxidized in alkaline medium to phosphomolybdic/phosphotungstic acid complexes (present in Folin–Ciocalteu reagent, FCR) to form complexes that could be observed at 750–765 nm range (Singleton et al., 1999; Magalhães et al., 2008). The following reactions are believed to occur (Gülcin, 2012).

$$Na_2WO_4 / Na_2MoO_4 + \text{Phenol} \rightarrow (\text{Phenol} - MoW_{11}O_{40})^{4-} \quad (2.1)$$

$$Mo^{5+}_{(yellow)} + e^- \rightarrow Mo^{4+}_{(blue)} \quad (2.2)$$

Due to the simplicity, sensitivity and high precision of the FCM method, it is applicable in the identification, specification and standardization of biological specimens as long as interferences are minimized (Gülcin, 2012).

The experiment was done following Singleton et al. (1999) procedure with some minor changes. A 2N FCR solution was prepared by mixing 10 g of sodium tungstate ($Na_2WO_4.2H_2O$) 2.5 g of sodium molybdate ($Na_2MoO_4.2H_2O$, 25 g) in 70 mL of ddH_2O, with 5 mL of 85% H_3PO_4 and 10 ml of concentrated HCl, and heated under reflux for a long period of 10 h. After boiling, 15 g of lithium sulfate ($Li_2SO_4.4H_2O$) was added to give an intense yellow colour solution, followed by the addition of a drop of bromine, and finally made to 100 mL with ddH_2O. The resultant solution should be clear as a trace of other colours can cause an elevated blank reading. A 7% (w/v) Na_2CO_3 was also prepared.

Extract (30 µL), ddH_2O (282.5 µL) and FCR (62.5 µL) were combined in a test tube and vortexed. The mixture was allowed to react at room temperature for 6 min. Thereafter, 345 µL of Na_2CO_3 (7%, w/v) was supplemented to the test tube and vortexed. The reaction mixture was incubated at room temperature in the dark condition for 2 h. The absorbance was recorded at 760 nm using an ultraviolet–visible spectrophotometer. The obtained results of TPC are presented in mg GAE/g DW using a standard calibration plot generated from a gallic acid standard ($y = 0.0934x$; $R^2 = 0.9911$).

2.5.2 Total Flavonoid Content

The total flavonoid content (TFC) in each of the five plant samples were evaluated using the aluminium chloride method (Figure 2.7). The principle of the method is that $AlCl_3$ forms acid-stable complexes with the C_4 keto group and either the C_3 or C_5 OH groups of flavones and flavonols. Besides this, it also forms acid-labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids (Ahmed & Iqbal, 2018).

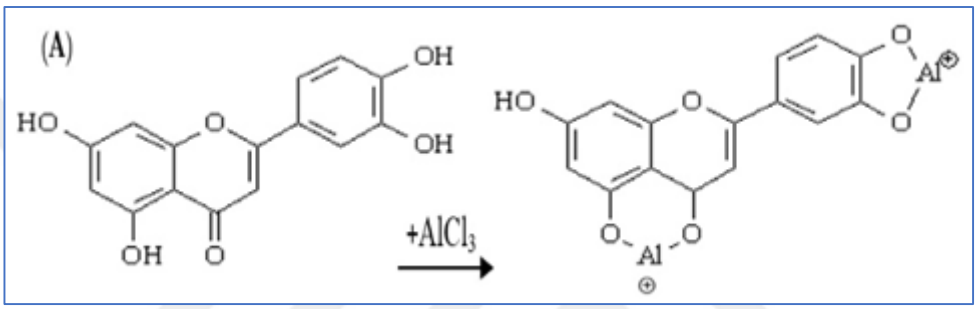


Figure 2.7 Formation of flavonoid complex with $AlCl_3$

The assay was done according to a modified Dowd method (Dowd, 1959). The working reagents included 10% (w/v) $AlCl_3$ (1 g/10 mL of 2% methanol) and 1 M sodium acetate (0.0203g/100 mL ddH_2O). Extract (200 µL), ddH_2O (1120 µL), 10% (w/v) $AlCl_3$ (40 µL) and 1 M sodium acetate (40 µL) were mixed in a test tube and vortexed. The reaction mixture was incubated in the dark condition at room temperature for a period of 30 min. Readings were then taken at 415 nm with a spectrophotometer. The results of TFC are expressed in mg QE/g DW. The results were extrapolated from a quercetin calibration plot ($y = 0.0572x$; $R^2 = 0.9964$).

2.5.3 Total Tannin Content

The total tannin content (TTC) in each of the five plant samples were evaluated following the modified vanillin/HCl method of Broadhurst and Jones (1978). To 100 µL of extract, 600 µL of 4% (v/v) vanillin (4 g in 100 mL methanol) solution and 300 µL of concentrated HCl were added. The mixture was immediately vortexed and incubated in the dark condition at room temperature for a period of 20 min. Readings

were then taken at 500 nm using a spectrophotometer. The amounts of TTC are expressed in mg TAE/g DW. The results were derived from a tannic acid calibration plot ($y = 0.0112x$; $R^2 = 0.950$).

2.6 *In vitro* Antioxidant Activity Determinations

2.6.1 DPPH• Scavenging Assay

A solution of stable DPPH• is reduced into a diphenylhydrazine (DPPH-H) molecule in the presence of an antioxidant compound that donates hydrogen atoms to the DPPH• (Figure 2.8). The change in colour of the DPPH• reagent from purple to yellow DPPH-H reflects the radical scavenging power of the antioxidant agent and can be assessed by measuring the decrease in absorbance at 517 nm (Guo, 2007). The DPPH• scavenging assay has high sensitivity and reproducibility and does not need highly specialized equipment or skills (Gülcin, 2012).

$$DPPH^{\bullet} + \text{A-H} \rightarrow \text{DPPH-H} + A^{\bullet} \qquad (2.3)$$

where A-H is antioxidant and A• is oxidized antioxidant

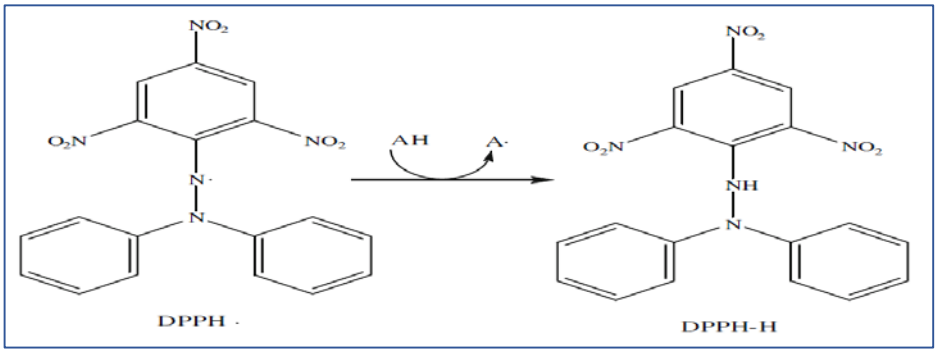


Figure 2.8 Scavenging effect of an antioxidant on the DPPH• (Gülcin, 2012)

The DPPH• scavenging assay was done according to a modified Brand-Williams et al. (1995) method. The DPPH• working reagent (1 mM) was obtained by dissolving 3.943 mg of a solute form of DPPH• with 10mL methanol. Each extract (at various concentrations) was mixed with DPPH• reagent (1 mM) to obtain a final volume of 1

mL and vortexed. The reaction mixture was incubated in the dark condition at room temperature for 30 min, and the absorbance was recorded at 517 nm using a spectrophotometer (Figure 2.9). A blank reading contained only DPPH$^{\bullet}$ reagent. The standard curve was plotted with vitamin C ($y = 2.6707x$; $R^2 = 0.9949$). The percentage inhibition (I) of DPPH$^{\bullet}$ was obtained from the below formula.

$$I\ (\%) = \frac{A0-A1}{A0}\ x\ 100 \qquad (2.4)$$

where A0 = blank absorbance and A1 = sample absorbance

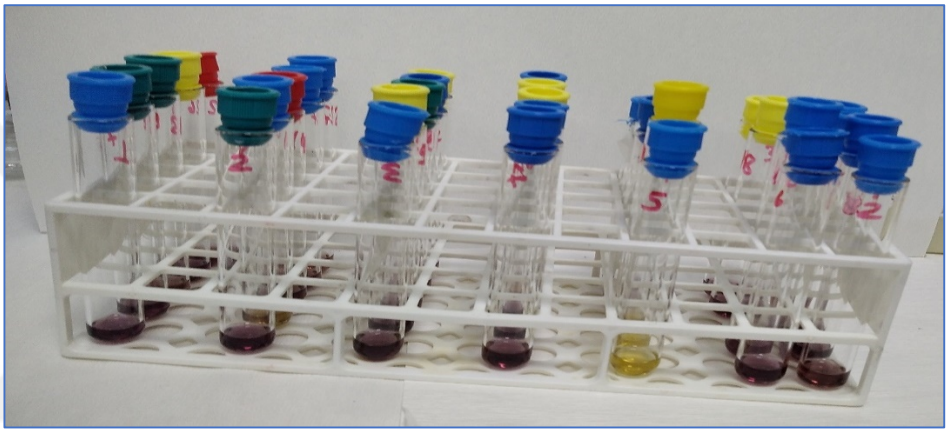


Figure 2.9 DPPH$^{\bullet}$ assay (Personal archive, 2021)

2.6.2 $^{\bullet}$OH Radical Scavenging Assay

The deoxyribose assay was employed to evaluate the extract $^{\bullet}$OH scavenging ability. In the chemistry, $^{\bullet}$OH are generated from a mixture consisting of ascorbic acid, Fe^{3+} (from $FeCl_3$) and H_2O_2 in the presence of a slight excess of EDTA over the Fe^{3+} salt. The $^{\bullet}$OH that was not scavenged by the EDTA will react with the deoxyribose and degraded it. The deoxyribose sugar is degraded on exposure to $^{\bullet}$OH to form an MDA product. On heating, the MDA formed a pink MDA-TBA complex with the TBA (λ_{max} 535 nm). Any other molecule added to the reaction mixture that is capable of reacting with $^{\bullet}$OH should compete with deoxyribose for $^{\bullet}$OH. The presence of ascorbic acid, a reducing agent, in the mixture may increase the rate of deoxyribose degradation. The presence of H_2O_2 in the mixture may accelerate the rate of $^{\bullet}$OH production (Halliwell & Gutteridge, 1981; Halliwell et al., 1987; Moorhouse et al., 1987).

$$Fe^{3+}\text{-EDTA} + \text{ascorbate} \rightarrow Fe^{2+}\text{-EDTA} + \text{oxidized ascorbate} \quad (2.5)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + OH^- + {}^{\bullet}OH \quad (2.6)$$

$${}^{\bullet}OH + \text{deoxyribose} \rightarrow \text{product} \xrightarrow{heat\ with\ TBA} \text{chromogen} \quad (2.7)$$

The deoxyribose assay was done following the method of Halliwell et al. (1987) with slight changes. The working reagents for the deoxyribose assay included 1mM EDTA (0.02922 g /100 mL ddH_2O), 1mM ascorbic acid (0.0044 g /25 mL ddH_2O), 1mM $FeCl_3$ (0.00405 g /25 mL ddH_2O), 28 mM deoxyribose (0.09387 g /25 mL ddH_2O), H_2O_2 (1mM), 25 mM KH_2PO_4-KOH buffer (0.3405g of KH_2PO_4 and 0.14 g of KOH in 100 mL ddH_2O), pH 7.4, 1% (w/v) TBA (1 g /100 mL ddH_2O) and 2% (w/v) TCA (2 g /100 mL ddH_2O). The reagents were prepared immediately before use.

Extract at different concentrations were added into a test tube containing KH_2PO_4-KOH buffer (25 mM), EDTA (1mM), ascorbic acid (1mM), $FeCl_3$ (1mM), deoxyribose (28mM), H_2O_2 (1mM), KH_2PO_4-KOH buffer (25 mM) to obtain a total volume of 1 mL. The reaction mixture was vortexed and incubated at 37 °C for a period of 1 h. Thereafter, 1 mL of 1% (w/v) TBA and 1 ml of 2% (w/v) TCA were added. A blank reading without an extract or standard was also prepared. The resulting reaction mixture was vortexed, boiled for 15 min and cooled. After cooling, the absorbency of the chromogen was obtained at 535 nm with a spectrophotometer (Figure 2.10). The standard curve was plotted with BHT ($y = 6.3131x + 20.099$; $R^2 = 0.9716$). Inhibition (I) of deoxyribose degradation by an extract was calculated in percentage from the below formula.

$$I\ (\%) = \frac{A0-A1}{A0}\ x\ 100 \quad (2.4)$$

where A0 = blank absorbance and A1 = sample absorbance

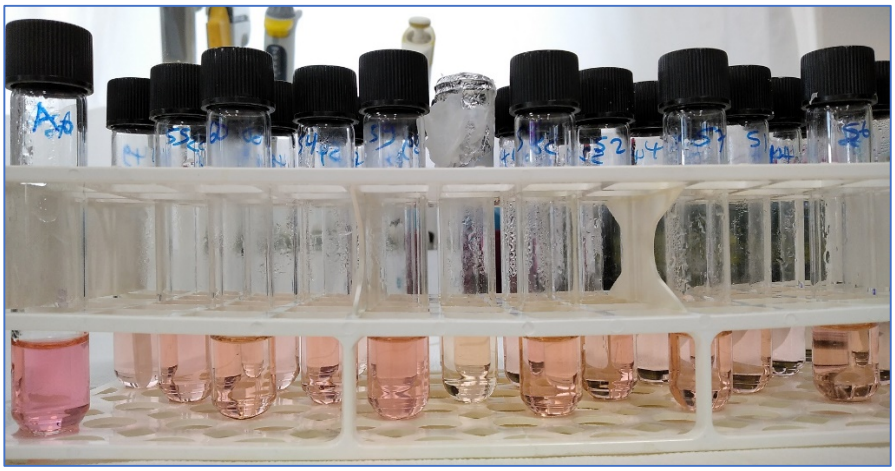


Figure 2.10 ˙OH assay (Personal archive, 2021)

2.6.3 NO˙ Scavenging Assay

NO˙ is an extremely unstable species, it readily combines with molecular O_2 to generate stable compounds, such as nitrate (NO_3^-) and nitrite (NO_2^-). NO˙ is synthesized in different mammalian cells (e.g., endothelium, neurons, macrophages, neutrophils, platelets) (Marcocci et al., 1994a). A method developed by German chemist Johann Peter Griess (1829–1888) is frequently used for the measurement of NO˙ generation in living cells. The reaction method is named after the chemist, the Griess reaction.

The chemistry of the Griess test is based on the principle that at physiological pH SNP in aqueous solution spontaneously produced NO˙, which will subsequently be combined with molecular O_2 to generate NO_2^- that can be measured by Griess reagent (Marcocci et al., 1994a and 1994b). Any scavenger of NO˙ in the reaction mixture compete with O_2 leading to the reduction of NO_2^- generation. Griess reaction involves two-step diazotization reactions (Figure 2.11). At the first step, NO_2^- generated from the interaction of NO˙ with O_2 combined with sulfanilic acid (4-aminobenzenesulfonic acid) to generate a diazonium ion (p-diazonium sulfanilamide). In the second step, the p-diazonium sulfanilamide reacts with the n-(1-naphthyl) ethylenediamine (NED) to generate a pink chromophoric azo product that has an absorption maximum at 543 nm (Fox, 1979 & 1985; Grisham et al. 1996).

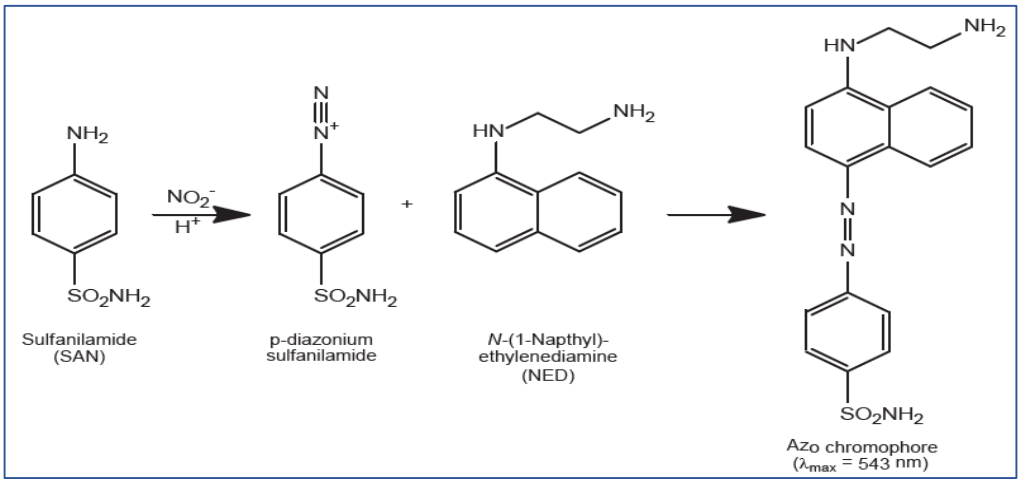


Figure 2.11 Chemistry of NO_2^- detection in a Griess reaction (Patton & Kryskalla, 2011)

The NO• scavenging ability of the extract was determined by using an SNP generating NO• system. The assay was done according to the method of Sreejayan & Rao (1997) with some modifications. The working reagents included 1 M PBS buffer (0.8 g NaCl, 0.02g KCl, 0.144 g Na_2HPO_4 and 0.0245 g KH_2PO_4 in 100 mL with ddH_2O), pH 7.4, 10 mM SNP dihydrate (0.0894 g $C_5FeN_6Na_2O.2H_2O$ in 30 mL ddH_2O), solution A: 2% (w/v) sulphanilamide solution (2 g in 100 mL 4% H_3P0_4) and solution B: 0.2% (w/v) NEDA·2HCl (0.2 g /100 mL ddH_2O). Griess reagent working solution was prepared by mixing an equal volume of solution A and B. The reagents were prepared immediately before use.

SNP solution (10 mM) was added to a mixture of PBS buffer (20 mM) and extract (at different concentrations) to obtain a total volume of 1875 µL. A blank experiment without an extract or standard but with the equivalent amount of PBS buffer solution was conducted identically. The solution was vortexed and incubated in light condition at 25 °C for 2 h 30 min. After incubation, 500 µL of an upper layer of solution was removed and diluted with 500 µL of Griess reagent. The absorbance of the chromophore produced during diazotization of nitrite with sulphanilamide and subsequent coupling with the NEDA·2HCl was taken at 542 nm using a UV-VIS spectrophotometer (Figure 2.12). The standard curve was plotted with vitamin C ($y = 1.360x + 20.099$; $R^2 = 0.950$). The scavenging activity of the plant extracts against NO• in percentage using the below formula.

$$I\ (\%) = \frac{A0-A1}{A0}\ x\ 100 \qquad (2.4)$$

where A0 = blank absorbance and A1 = sample absorbance

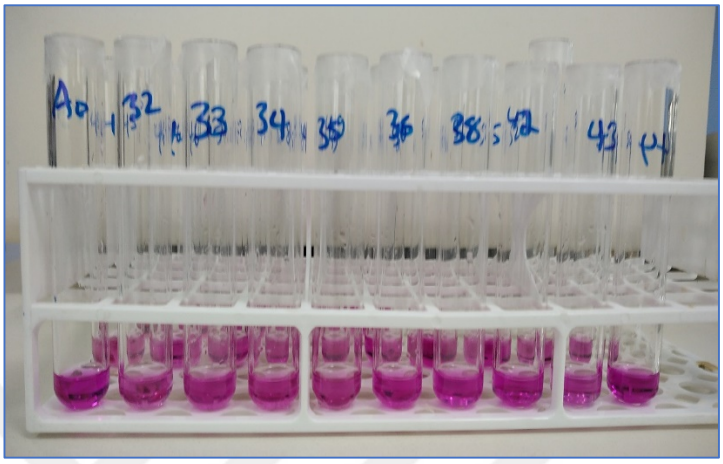


Figure 2.12 NO˙ assay (Personal archive, 2021)

2.6.4 Ferrous Ion chelating Activity

Ferrous ion chelating assay is based on the principle that Fe^{2+} can donate a single electron to too many other compounds, and therefore generate radical species. In the reaction mixture, Fe^{2+} react with ferrozine to form the Fe^{2+}–ferrozine complex. Any chelating agent (e.g., antioxidant) in the reaction mixture, will compete with ferrozine and thus disrupt the formation ferrous–ferrozine complex. This help reducing the formation of radical species by the Fe^{2+}. Measurement of colour reduction at an absorption maximum of 562 nm indicates the extent of binding of chelator, for example, antioxidant to the complex (Adjimani & Asare, 2015).

For the phenolic compounds, Fe^{2+} bind the phenolic structures at several coordination sites (Figure 2.13) (Aboul-Enein et al., 2003). The possible coordination sites of Fe^{2+} to the flavonoids include a) between 5-OH and 4-carbonyl group, b) between 3-OH and 4-carbonyl group, c) between 3', 4'-OH group in B ring. The possible coordination sites of Fe^{2+} to the phenolic acids include a) between 3-methoxy and 4-OH groups b) between 3-OH and 4-OH groups.

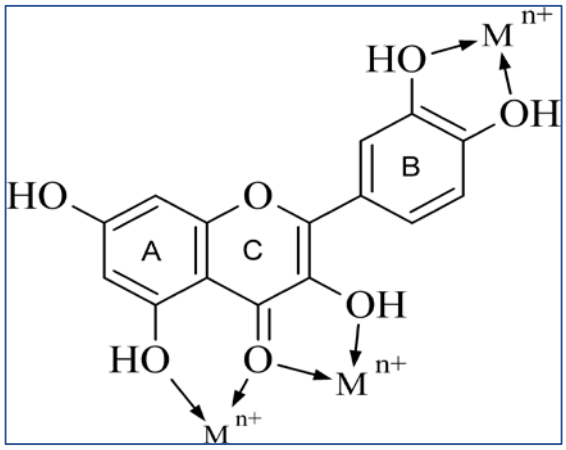


Figure 2.13 Possible coordination sites of metal (M^{n+}) to flavonoid (Kasprzak et al., 2015)

The binding of Fe^{2+} by the extract was measured following the method of Dinis et al. (1994) with minor changes. The working reagents for this assay included 1 mM $FeCl_2$ (0.00254 g / 10 mL ddH_2O) and 5 mM ferrozine (0.0616 g / 25 mL ddH_2O). Reagents were prepared immediately before use.

Extract at different concentrations was added to a solution of 1 mM $FeCl_2$. The resulting mixture obtained was vortexed and incubated in the dark at room temperature for 30 min. The reaction was initiated on the addition of ferrozine (5 mM) to obtain a final volume of 1000 µL, vortexed and incubated in the dark condition at room temperature. A blank reading without an extract or standard was also prepared. After the reaction mixture had equilibrated for a period of 10 min, the absorbance readings were taken using a spectrophotometer at 562 nm (Figure 2.14). The standard curve was plotted with EDTA ($y = 8.99x$; $R^2 = 0.9919$). The percentage of inhibition of Fe^{2+}–ferrozine complex was obtained using the below formula.

$$I\ (\%) = \frac{A0-A1}{A0}\ x\ 100 \qquad (2.4)$$

where A0 = blank absorbance and A1 = sample absorbance

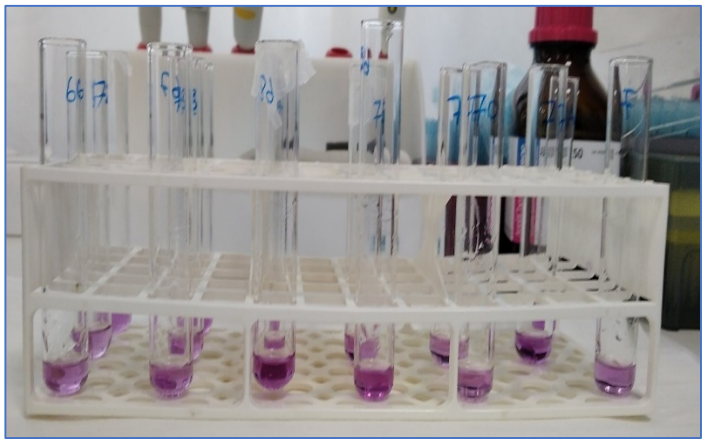


Figure 2.14 Ferrous chelating assay (Personal archive, 2021)

2.6.5 Ferric Ion Reducing Power

Ferric ion reducing power assay method is based on the principle that antioxidants, which have reduction potential, react with potassium ferricyanide ($K_3[Fe(CN)_6]$) to form potassium ferrocyanide ($K_4[Fe(CN)_6]$), which then reacts with ferric chloride to generate intense coloured Prussian blue complex ($Fe_4[Fe(CN)_6]_3$) that possess a strong absorbency at 700 nm (Gülcin, 2012). The IUPAC name of the complex is iron (III) hexacyanoferrate (II). The change in optical density at λ_{max} of 700 nm is strongly linked to the reduction potential of the electron-donating compound present in the reaction mixture. Higher absorbance of the reaction mixture indicated greater Fe^{3+} to Fe^{2+} transformation ability, thus, higher reducing power of a compound (Mathew & Abraham, 2006).

Ferric ion reduction to a ferrous ion by a reducing agent or an antioxidant compound has been used as an indicator of the electron-donating capacity of such compound (Yıldırım et al., 2001). The reducing ability of any compound could signify its potential antioxidant property.

$$K_3[Fe(CN)_6] + \text{antioxidant} \rightarrow K_4[Fe(CN)_6] + \text{oxidized antioxidant} \quad (2.8)$$

$$4FeCl_3 + 3\,K_4[Fe(CN)_6] \rightarrow Fe_4[Fe(CN)6]_3 + 12KCl \quad (2.9)$$

where $Fe_4[Fe(CN)6]_3$ is the Prussian blue complex.

A method of Oyaizu (1986) was adopted to evaluate the ferric ion reducing capability of the plant sample(s). For this analysis, the Fe^{3+} to Fe^{2+} reduction in the presence of an extract was investigated. Working reagents for this assay included 0.2 M phosphate buffer (1.6936 g of K_2HPO_4 and KH_2PO_4 in 100 mL ddH_2O), pH 6.6, 1% (w/v) $K_3Fe(CN)_6$ (0.25 g /25 mL ddH_2O), 10% (w/v) TCA (10 g /100 mL ddH_2O) and 0.1% (w/v) $FeCl_3$ (0.025 g /25 mL ddH_2O) solutions. The reagents were prepared immediately before use.

Sample extract (50 µL) was added to a mixture of 650 µL phosphate buffer solution (0.2 M) and 500 µL $K_3Fe(CN)_6$ solution (1%, w/v) to obtain a combined volume of 1200 µL. The mixture was vortexed and immediately incubated at 50 °C for a period of 20 min. Aliquots of 500 µL TCA (10%, w/v) was added to the mixture and centrifuged (3000xg) for 10 min. A portion (500 µL) of an upper layer of a centrifuged mixture solution was mixed with 500 µL ddH_2O and 100 µL $FeCl_3$ (0.1%, w/v). The final concentration of the extract in the reaction mixture was 29.41 µg/mL. The optical density was recorded at 700 nm using a spectrophotometer. An intense colour gave higher absorbance which indicated greater Fe^{3+} to Fe^{2+} transformation power, thus, greater reducing power (Figure 2.15). The standard curve was plotted with vitamin C ($y = 0.0848x$; $R^2 = 0.997$). Results are expressed in mg vitamin C equivalent (VCE) per g DW.

Figure 2.15 Reducing power assay (Personal archive, 2021)

2.6.6 Ferric Reducing Antioxidant Power (FRAP)

The FRAP is a colourimetric assay based on the capacity of antioxidants to reduce the ferric-2,4,6-tris(2-pyridyl)-s-triazine $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ complex to the ferrous form $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ at low pH (Figure 2.16). The end-product $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ has an intense blue colour and can be monitored by measuring the change in absorption at 593 nm (Figure 2.17) (Benzie & Strain, 1999). The intense colour gave higher absorbance, and the increase of absorbance is directly associated with the $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$- $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ reducing/transformation ability of the electron-donating compound inside the reaction mixture (Mathew & Abraham, 2006).

The FRAP test is inexpensive, simple, robust, and it does not need extensive skills or equipment (Gülcin, 2012). The assay is nonspecific, it can be applied to various biological samples and fluids, for example, cerebrospinal fluid, serum, plasma, saliva, and urine (Benzie & Strain, 1999).

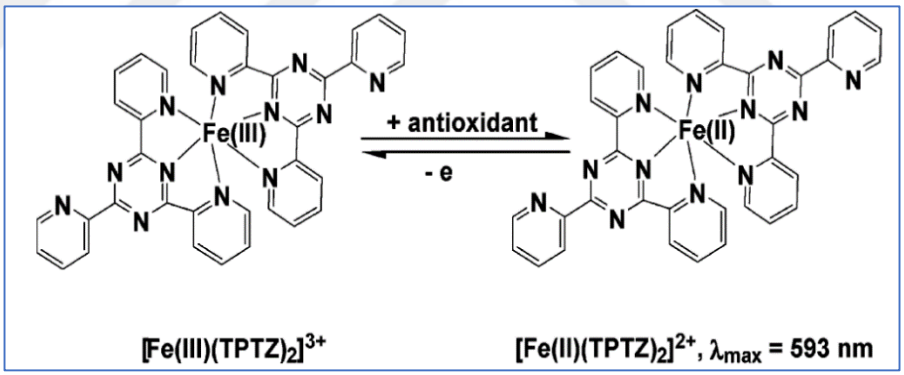


Figure 2.16 $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$- $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ complex transformation by an antioxidant (Huang et al., 2005)

The experiment was done following the method of Benzie and Strain (1996) as described by Thaipong et al. (2006) and adapted as follows. The prepared stock solutions comprised of 300 mM acetate buffer (2.4609 g $C_2H_3NaO_2$ and 1720 µL of concentrated glacial acetic acid, adjusted to a final volume of 100 mL with ddH_2O), pH 3.6, 10 mM TPTZ (0.03123 g TPTZ in 10 mL of 40 mM HCl), and 20 mM $FeCl_3$ (0.03244g/10 mL ddH_2O). The fresh working solution was prepared immediately before use and it was prepared by combining 25 mL acetate buffer, 2.5 mL TPTZ, and

2.5 mL $FeCl_3$ solutions earlier prepared and then warmed at 37 °C for 10 min before use.

Sample extract (10 µL) and methanol (65 µL) were mixed with 1425 µL of FRAP solution to obtained a final volume of 1500 µL. The final amount of the extract in the mixture was 6.67 µg/mL. Afterwards, the obtained reaction mixture was incubated in the dark condition away from light at 37 °C for 30 min. A blank reading without an extract or standard was also prepared. Readings of the intense coloured product $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ were then taken at 593 nm using a spectrophotometer. The standard curve was plotted with vitamin C ($y = 0.3982x$; $R^2 = 0.9883$). Results are expressed in mg VCE per g DW.

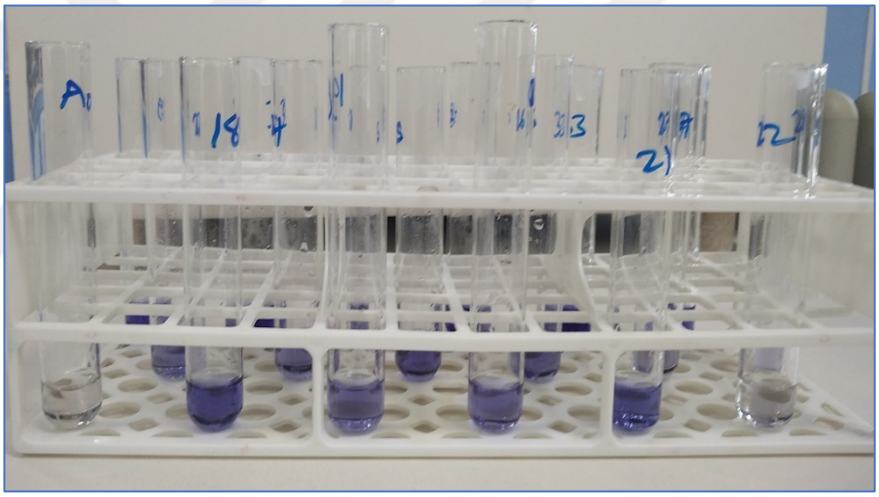


Figure 2.17 FRAP assay (Personal archive, 2021)

2.6.7 IC_{50} Determination

The percentage inhibition of DPPH$^{\bullet}$, $^{\bullet}OH$, $NO^{\bullet}$ or ferrozine–Fe^{2+} complex by the extract(s) was plotted against the corresponding concentration of a sample or standard to extrapolate the concentration of antioxidant needed to scavenge 50% of DPPH$^{\bullet}$, $^{\bullet}OH$, $NO^{\bullet}$, or to inhibit 50% ferrozine–Fe^{2+} complex (IC_{50}). IC_{50} stands for median inhibitory concentration. A low value of IC_{50} suggests greater antioxidant activity. The IC_{50} results are expressed in µg/mL. The average values were obtained from triplicate experiments from the same extract.

2.7 *In vitro* Anticancer Activity Determinations

2.7.1 Stock solutions

The stock solutions of the extracts for the cytotoxicity assay were prepared by dissolving 2.4 mg of lyophilized extracts in 3 mL of 10% (v/v) DMSO (90% ddH_2O) to obtain a concentration of 800 ppm. Working solutions of 20 ppm (0.25% DMSO), 40 ppm (0.5% DMSO) and 80 ppm (1% DMSO) were eventually obtained.

2.7.2 Cell Growth Conditions, Trypsinization and Hematocytometry

The HeLa, HepG2 cell lines were grown in DMEM inside a humidified incubator (5% CO_2, 37 °C). Adherent cells grown in a culture medium were detached from the plastic flask container by trypsinization. Firstly, the medium from the culture vessel was removed. Enough volume of trypsin was added to the plastic container to completely cover the monolayer of cells. The container was placed inside a humidified incubator and incubated at 37 °C for 5 min with 5% CO_2. The adherent cells that attached to the surface were mechanically detached from the wall of the container and centrifuged (750 xg) for 5 min. The supernatant was aspirated and discarded, and the pellets were re-suspended in a fresh medium. The resultant cell suspension was subjected to cell counting using a hemocytometer.

2.7.3 MTT assay

The MTT assay was used to test the cytotoxicity effects of the extracts against HeLa and HepG2 cell lines. In this assay, thiazolyl blue tetrazolium bromide (often called MTT), which is a yellow dye, is reduced to blue formazan crystals by cellular enzymes. The dye is used for assessing cell viability, proliferation and cytotoxicity. MTT assay is employed to determine the cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. The colourimetric assay is non-radioactive and it is chemistry involved the reduction of a water-soluble yellow MTT salt to purple formazan product by NAD(P)H-dependent oxidoreductase produced in the mitochondria of the living cells (Morgan et al., 1998).

The assay was carried out per the Mosmann et al. (1983) method as described by Bahuguna et al. (2017) and adapted as follows. Cell lines in suspension were seeded in a flat-bottom microtiter plate (96-well) at a density of ~ 1.5×10^5 cells/mL per well and allowed to adhere in a humidified incubator at 37 °C with 5% CO_2. After incubation, the culture medium was carefully removed from the plates. Then, the cells were treated with the various concentrations of the extract (20, 40 and 80 ppm). The control cell was treated with only 10% of DMSO. The microtiter plates were immediately incubated for 24 or 48 h at 37 °C in a 5% CO_2 incubator. Thereupon, 20 µL of MTT working solution (250 mg/ 50 mL in ddH_2O) was added to each well and the plates were further incubated for 3.5 h at 37 °C in a 5% CO_2 incubator. After that, the medium was carefully discarded and the formazan crystals were solubilized by adding 100 µL of DMSO per well. The plate was then put on a microplate shaker and run for 1 min. Lastly, the absorbance was measured using the microplate reader at 570 nm. The percentage inhibition (I) was obtained using the below formula.

$$\mathrm{I}\ (\%) = \frac{\mathrm{A0}-\mathrm{A1}}{\mathrm{A0}}\ x\ 100 \qquad (2.4)$$

where A0 = blank absorbance and A1 = sample absorbance

The percentage inhibition of cancer cells by the extract(s) was plotted against the corresponding concentration of the extract(s) to obtain the amount required to scavenge 50% of the cells. A low value of IC_{50} implies greater cytotoxicity activity. The IC_{50} results are expressed in µg/mL.

2.8 Statistical Analysis

Each assay, HPLC analysis, total polyphenols determinations and antioxidant activity determinations, was done in triplicate from the same extract to extrapolate their reproducibility. All values are expressed as mean ± S.D. The data obtained were analyzed by One-way Analysis of Variance (ANOVA) followed by a post hoc analysis for multiple comparisons using GraphPad InStat V3.05. Tukey-Kramer multiple comparisons test was used to determine the significant differences. A p-value of <0.05 was considered statistically significant.

CHAPTER THREE
RESULTS AND DISCUSSION

In this section the results of extractions yield of *N. sativa* seed, *M. germanica* leaf, *M. germanica* fruit, *D. lotus* seed and *D. lotus* fruit carried out using different reagents; the HPLC profile of flavonoids and phenolic acids; the total polyphenolic contents; the *in vitro* antioxidants activities carried out using DPPH$^{\bullet}$ scavenging, $^{\bullet}$OH scavenging, NO$^{\bullet}$ scavenging, ferrous ion (Fe^{2+}) chelating, ferric ion (Fe^{3+}) reducing power, and FRAP assays; and the *in vitro* anticancer activities of the five plant samples were presented and discussed.

3.1 Extractions Yield

Flavonoids and phenolic acids were extracted from the five plant samples using different reagents. The results of extractions yield are presented in percentage (%) and are shown in Table 3.1. Diethyl ether was used in the extraction of flavone from the plant samples. The decreasing order of flavone yield (highest to lowest) are as follows: *M. germanica* leaf > *N. sativa* seed > *D. lotus* fruit > *M. germanica* fruit. Eighty per cent aqueous ethanol (80%, v/v) solvent was used in the extraction of flavanone from the plant samples. The decreasing order of flavanone yield are as follows: *M. germanica* fruit > *M. germanica* leaf > *N. sativa* seed. Acidified methanol (25% HCL; 95% methanol–water, v/v) solvent system was used in the extraction of flavonol from the plant samples. The decreasing order of flavonol yield are as follows: *M. germanica* fruit > *M. germanica* leaf > *D. lotus* fruit > *N. sativa* seed.

The extraction of flavan-3-ol was carried out using two protocols. In protocol I, the flavan-3-ol was extracted with absolute methanol. The extracts obtained after this extraction were designated as flavan-3-ol methanolic extracts. The decreasing order of flavan-3-ol methanolic extracts yield are as follows: *M. germanica* fruit > *D. lotus* fruit > *M. germanica* leaf > *N. sativa* seed > *D. lotus* seed. In protocol II, the flavan-3-ol was extracted from the residue of methanolic extraction by acid hydrolysis with 2.5 M HCI-methanol (4:1, v/v). The extracts obtained after this hydrolysis was designated as

flavan-3-ol acidic extracts. The decreasing order of flavan-3-ol acidic extracts yield are as follows: *D. lotus* fruit > *M. germanica* fruit > *D. lotus* seed > *M. germanica* leaf > *N. sativa* seed. FPA, BHPA and AHPA extracts were from the CME of the five samples.

Table 3.1 Extraction yields (%)

Extracts	***N. sativa* seed**	***M. germanica* leaf**	***M. germanica* fruit**	***D. lotus* seed**	***D. lotus* fruit**
Flavone	0.80	2.12	0.40	-	0.68
Flavanone	16.75	34.05	66.07	-	-
Flavonol	13.13	68.73	69.20	-	51.88
Flavan-3-ol methanol	14.61	30.06	74.87	12.31	60.08
Flavan-3-ol acid	1.66	2.83	4.77	2.96	14.85
FPA	1.26	2.72	0.57	0.47	0.54
BHPA	0.18	1.09	1.36	0.53	0.39
AHPA	1.10	0.59	2.01	0.34	2.07
BPAH-1	1.42	2.36	4.59	0.66	1.55
BPAH-2	0.67	0.76	9.45	1.23	0.76
BPBH-1	1.33	2.59	2.41	1.10	1.70
BPBH-2	0.62	2.28	1.57	1.17	1.35
TPPC	19.18	34.63	80.88	14.71	66.10

The CME were obtained after the samples were extracted with methanol. The decreasing order of FPA extracts yield are as follows: *M. germanica* leaf > *N. sativa* seed > *M. germanica* fruit > *D. lotus* fruit > *D. lotus* seed. The decreasing order of BHPA extracts yield are as follows: *M. germanica* fruit > *M. germanica* leaf > *D. lotus* seed > *D. lotus* fruit > *N. sativa* seed. The decreasing order of AHPA extracts yield are as follows: *M. germanica* fruit > *D. lotus* fruit > *N. sativa* seed > *M. germanica* leaf > *D. lotus* seed. The residues after phenolic acids extraction with methanol were subsequently subjected to bound phenolic acids extractions. Four other fractions were obtained: BPAH-1, BPAH-2, BPBH-1 and BPBH-2. The decreasing order of BPAH-1 extracts yield are as follows: *M. germanica* fruit > *M. germanica* leaf > *D. lotus* fruit > *N. sativa* seed > *D. lotus* seed. The decreasing order of BPAH-2 extracts yield are as follows: *M. germanica* fruit > *D. lotus* seed > *M. germanica* leaf (= *D. lotus* fruit) > *N.*

sativa seed. The decreasing order of BPBH-1 extracts yield are as follows: *M. germanica* leaf > *M. germanica* fruit > *D. lotus* fruit > *N. sativa* seed > *D. lotus* seed. The decreasing order of BPBH-2 extracts yield are as follows: *M. germanica* leaf > *M. germanica* fruit > *D. lotus* fruit > *D. lotus* seed > *N. sativa* seed. The decreasing order of total polyphenol content (TPPC) yields are as follows: *M. germanica* fruit > *D. lotus* fruit > *M. germanica* leaf > *D. lotus* seed > *N. sativa* seed. However, it should be noted that these extraction yields do not represent in any way the actual amounts of phenolic acids and flavonoids contents of the plant samples as some other compounds could be extracted together with the phenolic compounds.

3.2 Phenolic Acids Profile

3.2.1 N. sativa Seed

Individual phenolic acids identified and quantified by HPLC in different extracts of *N. sativa* seed are shown in Table 3.2. HPLC is a chromatographic analytical technique used in analytical chemistry and related fields to identify and quantify the individual constituent of a mixture (Cannell, 1998). Phenolic acids were classified as hydroxybenzoic acid, hydroxycinnamic acid and hydroxyphenyl acetic acid derivatives (Chen et al., 2020). In this study, four benzoic acid derivatives (benzoic, 4-hydroxybenzoic, rosmarinic and vanillic acids) and four cinnamic acid derivatives (caffeic, ferulic, sinapic and p-coumaric acids) were identified and quantified from the five extracts (FPA, BHPA, AHPA, BPAH-1 and BPBH-2) of *N. sativa* seed. The chromatograms of the five extracts are presented in Figures 3.1-3.5.

Each phenolic acid was recognized by comparison of its R_t with that of commercially available standard. Chromatographic peaks in BPAH-2 and BPBH-1 extracts do not correspond to the available standard, hence not quantified. Different extracts of *N. sativa* seed contained different phenolic acids. The CME of the *N. sativa* seed contained four FPAs, five BHPAs and an AHPA. Acid and base hydrolysates are usually employed to break down the ester bond of phenolics conjugates and hence release phenolic compounds (Nuutila et al., 2002; Kim et al., 2006), however, the results obtained in the current study show these hydrolysates to affect the yield of

individual phenolic acids in the seed extracts of *N. sativa*. Only p-coumaric, 4-hydroxybenzoic caffeic and sinapic acids were identified in four acid and base hydrolyzed extracts. The 4-hydroxybenzoic acid was released in a relative amount in FPA, BHPA and BPBH-2 fractions. Ferulic acid was also identified in three fractions (FPA, BHPA and AHPA), however, in low concentration.

The individual phenolic acids identified in this study correspond with those identified by Toma et al. (2014) and Topcagic et al. (2017) in the seed extracts of *N. sativa*. The major phenolic acids identified in the five extracts of *N. sativa* seed were 4-hydroxybenzoic acid in FPA (15.69±1.37 µg/g), 4-hydroxybenzoic acid in BHPA (12.69±0.87 µg/g), ferulic acid in AHPA (1.71±0.02 µg/g), p-coumaric in BPAH-1 (7.59±0.53 µg/g) and 4-hydroxybenzoic in BPBH-2 (20.44±1.17 µg/g). Furthermore, the sum of mean values of phenolic acids in the different extracts of *N. sativa* seed were 20.84±1.43, 22.59±1.32, 1.71±0.02, 7.59±0.53 and 26.09±1.59 µg/g for FPA, BHPA, AHPA, BPAH-1 and BPBH-2 extracts, respectively. Moreover, the level of TPAC in the seed extracts of *N. sativa* determined by HPLC (78.82±4.89 µg/g DW) was lower than 109.80±1.69 mg GAE/g DW obtained by the FCM. The lower value obtained can be explained because some chromatogram peaks observed does not correspond to the available standard, thus were not identified.

The results of HPLC analyses phenolic compounds are expressed in µg/g DW and mean ± SD were obtained from triplicate analyses. The explanation of abbreviation are as follows: ΣBA: sum of benzoic acid derivatives, ΣCA: sum of cinnamic acid derivatives, ΣPHA: sum of phenolic acids in the individual extract, TPAC: total phenolic acids compounds, BE: benzoic acid, 4HB: 4-hydroxybenzoic acid, GA: gallic acid, RO: rosmarinic acid, SY: syringic acid, PC: protocatechuic acid, VA: vanillic acid, CHO: chlorogenic acid, CA: caffeic acid, FE: ferulic acid, SI: sinapic acid, OCO: o-coumaric acid, PCO: p-coumaric acid, TCI: t-cinnamic acid, HPD: hesperidin, QCTN: quercitrin, EC: Epicatechin, EGC: epigallocatechin, EGCG: epigallocatechin gallate.

Table 3.2 Phenolic acids in different extracts of *N. sativa* seed

Phenolic acids	**FPA**	**BHPA**	**AHPA**	**BPAH-1**	**BPAH-2**	**BPBH-1**	**BPBH-2**	**TPAC**
Benzoic	3.42±0.05	-	-	-	-	-	-	3.42±0.05
4-hydroxybenzoic	15.69±1.37	12.69±0.87	-	-	-	-	20.44±1.17	48.82±3.41
Gallic	-	-	-	-	-	-	-	-
Protocatechuic	-	-	-	-	-	-	-	-
Rosmarinic		2.83±0.05	-	-	-	-	-	2.83±0.05
Syringic	-	-	-	-	-	-	-	-
Vanillic	0.95±0.01	2.85±0.13	-	-	-	-	-	3.80±0.14
ΣBA	20.06±1.43	18.37±1.05	-	-	-	-	20.44±1.17	
Chlorogenic	-	-	-	-	-	-	-	-
Caffeic	-	-	-	-	-	-	2.45±0.14	2.45±0.14
Ferulic	0.78±0.01	3.19±0.22	1.71±0.02	-	-	-	-	5.68±0.25
Sinapic	-	1.03±0.05	-	-	-	-	3.20±0.28	4.23±0.33
o-Coumaric	-	-	-	-	-	-	-	-
p-Coumaric	-	-	-	7.59±0.53	-	-	-	7.59±0.53
t-Cinnamic	-	-	-	-	-	-	-	
ΣCA	0.78±0.01	4.22±0.27	1.71±0.02	7.59±0.53	-	-	5.65±0.42	
ΣPHA	20.84±1.43	22.59±1.32	1.71±0.02	7.59±0.53	-	-	26.09±1.59	**78.82±4.89**

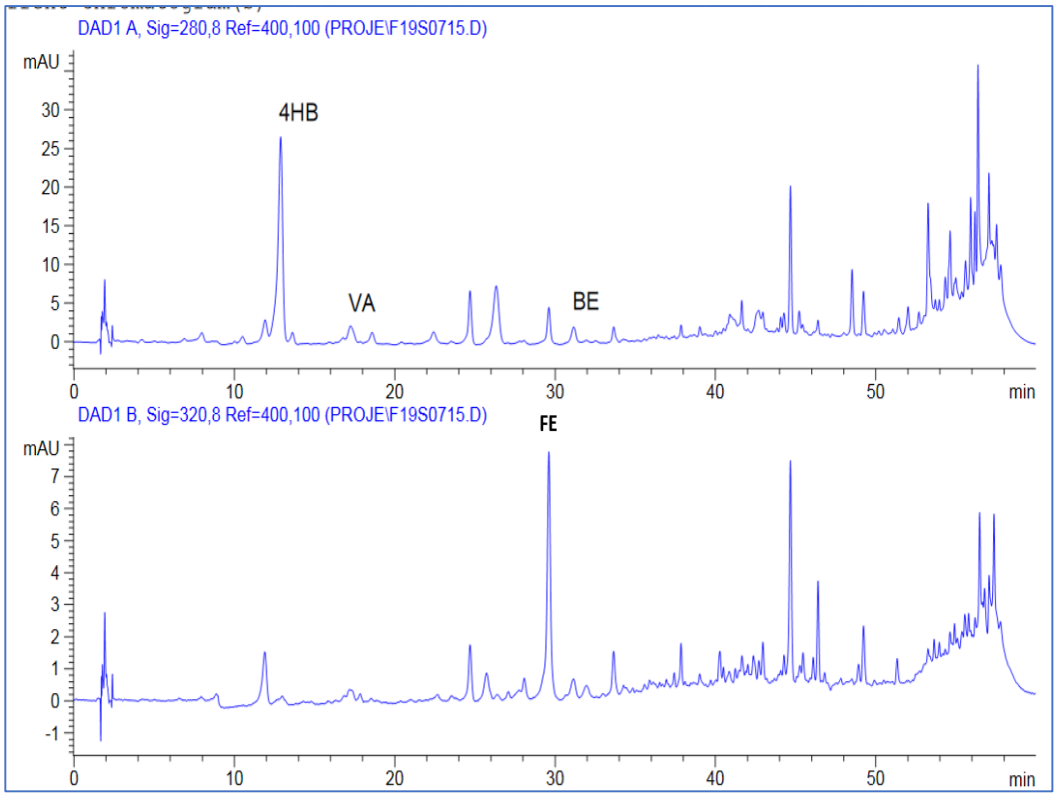


Figure 3.1 Chromatogram of *N. sativa* seed FPA extract

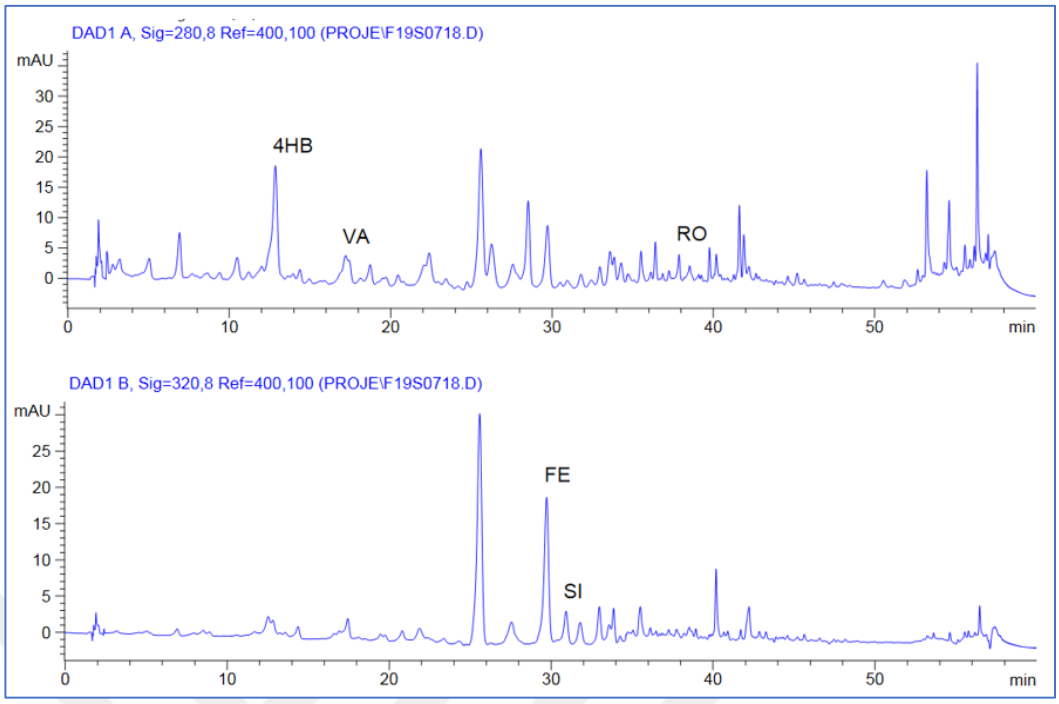


Figure 3.2 Chromatogram of *N. sativa* seed BHPA extract

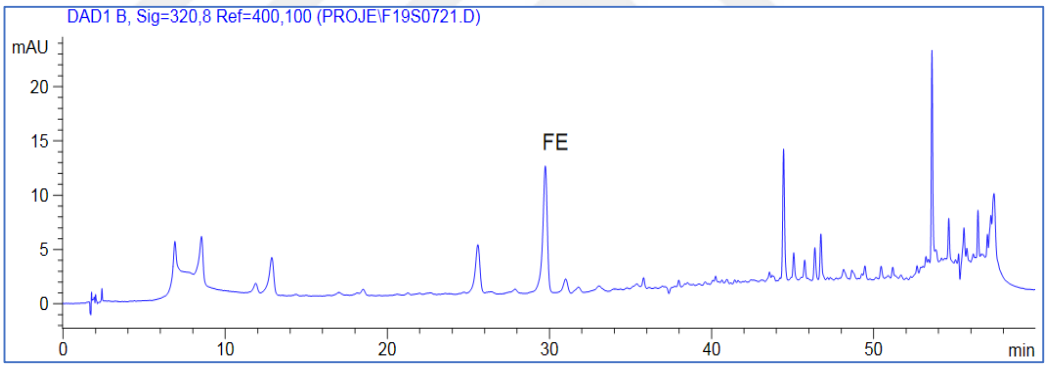


Figure 3.3 Chromatogram of *N. sativa* seed AHPA extract

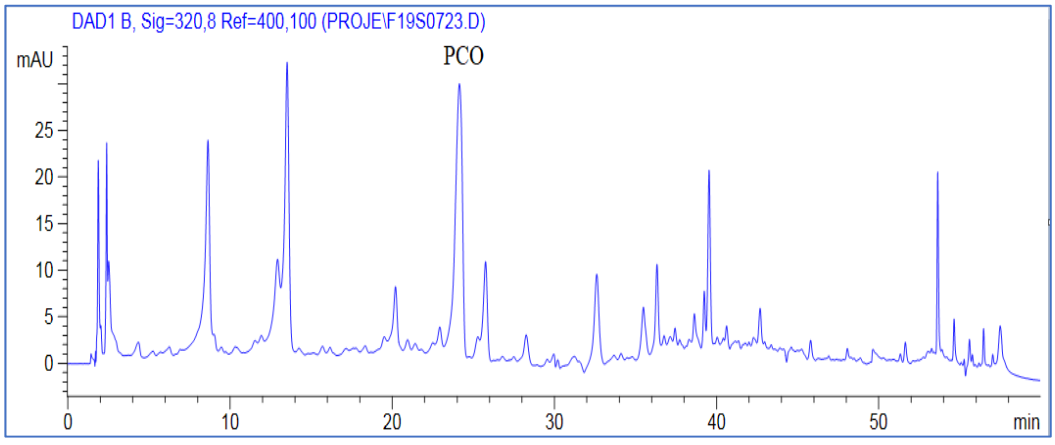


Figure 3.4 Chromatogram of *N. sativa* seed BPAH-1 extract

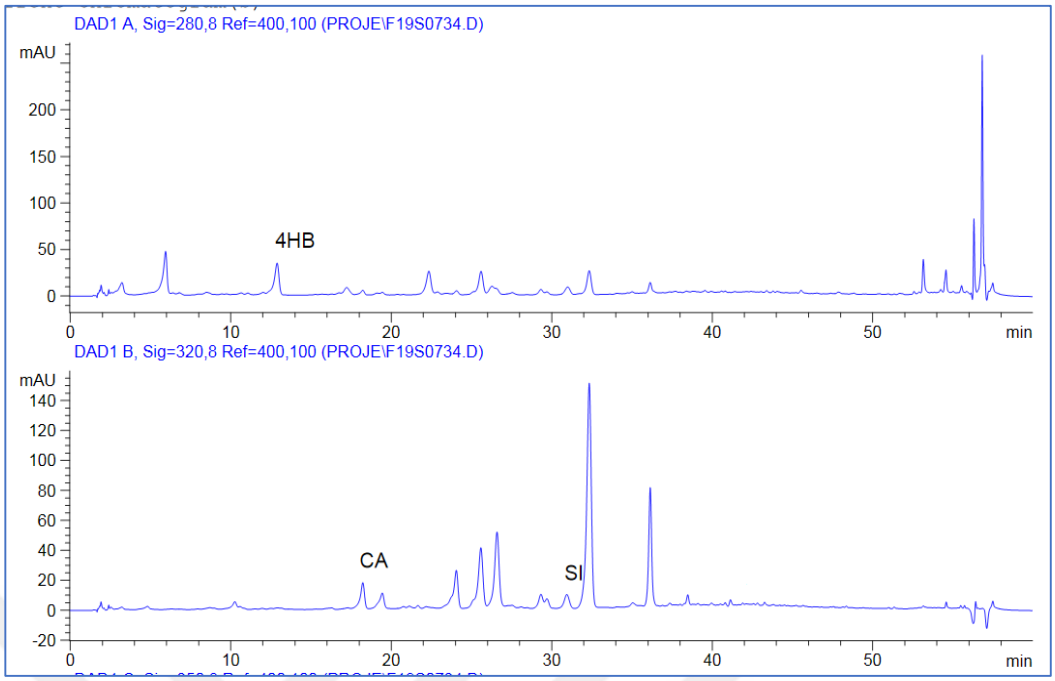


Figure 3.5 Chromatogram of *N. sativa* seed BPBH-2 extract

3.2.2 M. germanica Leaf

Individual phenolic acids identified and quantified in different extracts of *M. germanica* leaf are presented in Table 3.3. Seven phenolic acids comprising three benzoic acid derivatives (benzoic, 4-hydroxybenzoic and vanillic acids) and four cinnamic acid derivatives (caffeic, ferulic, sinapic and p-coumaric acids) were identified using HPLC. These compounds were detected in six extracts, and they have been recognized according to their R_t and observing similarities between the spectral characteristics of their peaks and those of available standards. The chromatograms of the six extracts of *M. germanica* leaf are shown in Figures 3.6-3.11. No phenolic acid was identified in the BPAH-2 extract.

About individual phenolic acid, ferulic acid was detected in five extracts, sinapic acid in two extracts, and the other phenolic acids were each detected in a single fraction. In extractable phenolic acids extraction from the CME; vanillic, ferulic, caffeic and p-coumaric acids were found in FPA; ferulic and sinapic in BHPA and AHPA fractions. With regards to bound phenolic acids extractions, base hydrolysis proved to be more effective in extracting the compounds from the leaf of *M.*

germanica. Ferulic acid was found in two fractions (BPBH-1 and BPBH-2), benzoic acid was identified in the BPBH-1 fraction and 4-hydroxybenzoic acid in BPBH-2 fraction. The obtained result of the current research is similar to the earlier studied of Kim et al. (2006). In that study, ferulic, vanillic, caffeic and p-coumaric acids were identified and quantified in FPA extract; and 4-hydroxybenzoic in bound phenolic basic hydrolysis extract.

With the respect to the individual extracts of *M. germanica* leaf the major phenolic acids identified were vanillic in FPA (15.68±0.92 µg/g), ferulic acid in BHPA (11.55±0.52 µg/g), ferulic acid in AHPA (0.90±0.02 µg/g), p-coumaric in BPAH-1 (4.27±0.20 µg/g), benzoic acid in BPBH-1 (43.83±1.85 µg/g) and 4-hydroxybenzoic in BPBH-2 (14.40±1.23 µg/g). Among all the individual phenolic acids identified, benzoic acid identified in BPBH-1 had the highest concentration and sinapic acid identified in AHPA had the lowest concentration. Furthermore, the sum of phenolic acids values in the different extracts of *M. germanica* leaf were 31.59±1.91, 15.00±0.66, 1.54±0.07, 4.27±0.20, 58.83±2.86 and 20.87±1.50 µg/g for FPA, BHPA, AHPA, BPAH-1, BPBH-1 and BPBH-2 extracts, respectively. The amount of TPAC in the *M. germanica* leaf determined by the HPLC (132.10±7.20 µg/g DW) was lower than 529.44±2.27 mg GAE/g DW obtained by the FCM.

Table 3.3 Phenolic acids in different extracts of *M. germanica* leaf

Phenolic acids	**FPA**	**BHPA**	**AHPA**	**BPAH-1**	**BPAH-2**	**BPBH-1**	**BPBH-2**	**TPAC**
Benzoic	-	-	-	-	-	43.83±1.85	-	43.83±1.85
4-hydroxybenzoic	-	-	-	-	-	-	14.40±1.23	14.40±1.23
Gallic	-	-	-	-	-	-	-	-
Protocatechuic	-	-	-	-	-	-	-	-
Rosmarinic	-	-	-	-	-	-	-	-
Syringic	-	-	-	-	-	-	-	-
Vanillic	15.68±0.92	-	-	-	-	-	-	15.68±0.92
ΣBA	**15.68±0.92**	-	-	-	-	**43.83±1.85**	**14.40±1.23**	
Chlorogenic	-	-	-	-	-	-	-	-
Caffeic	10.56±0.79	-	-	-	-	-	-	10.56±0.79
Ferulic	2.13±0.05	11.55±0.52	0.90±0.02	-	-	15.00±1.01	6.47±0.27	36.05±1.87
Sinapic	-	3.45±0.14	0.64±0.05	-	-	-	-	4.09±0.19
o-Coumaric	-	-	-	-	-	-	-	
p-Coumaric	3.22±0.15	-	-	4.27±0.20	-	-	-	7.49±0.35
t-Cinnamic	-	-	-	-	-	-	-	
ΣCA	15.91±0.99	15.00±0.66	1.54±0.07	4.27±0.20	-	15.00±1.01	6.47±0.27	
ΣPHA	**31.59±1.91**	**15.00±0.66**	**1.54±0.07**	**4.27±0.20**	-	**58.83±2.86**	**20.87±1.50**	**132.10±7.20**

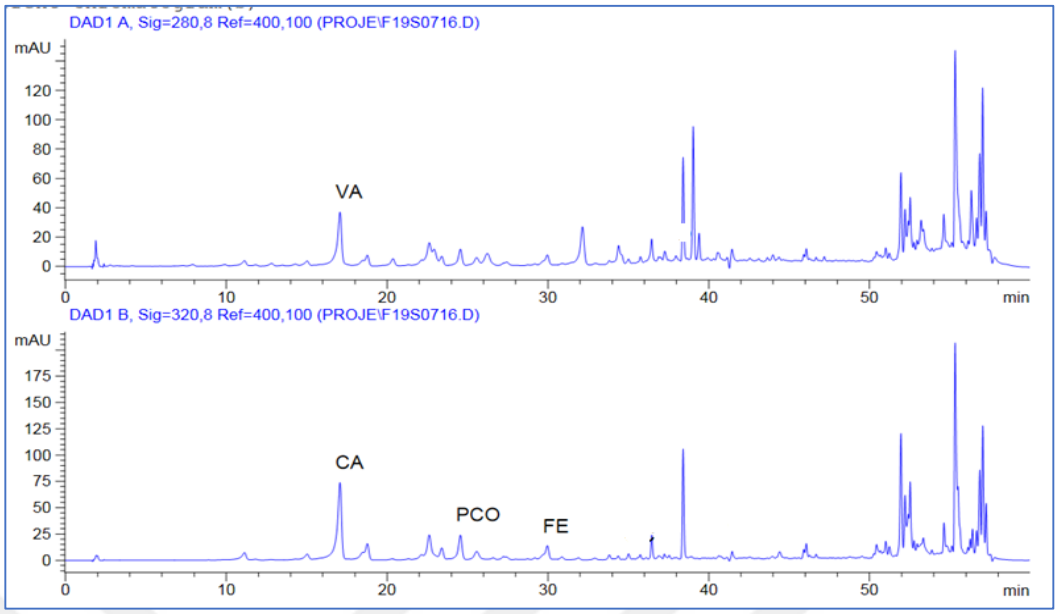


Figure 3.6 Chromatogram of *M. germanica* leaf FPA extract

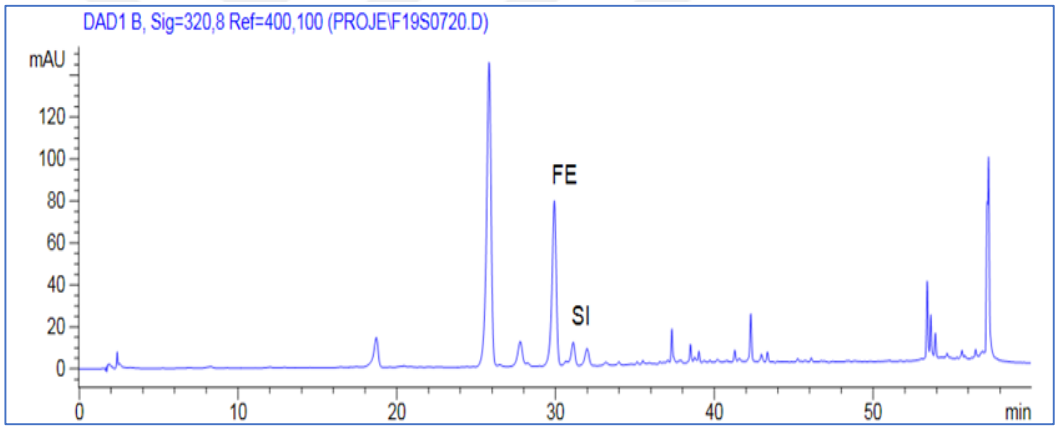


Figure 3.7 Chromatogram of *M. germanica* leaf BHPA extract

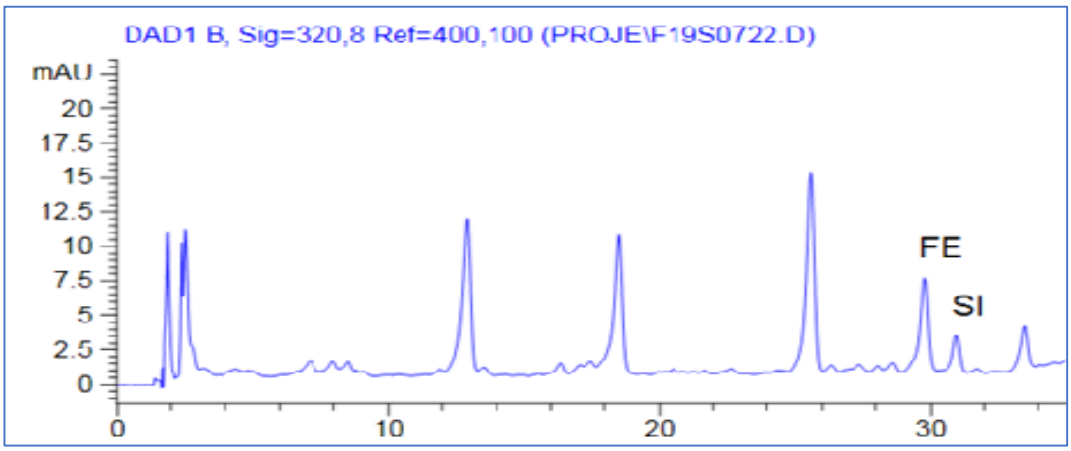


Figure 3.8 Chromatogram of *M. germanica* leaf AHPA extract

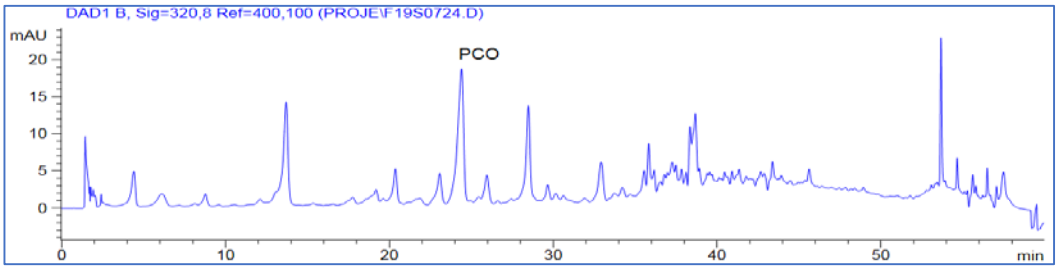


Figure 3.9 Chromatogram of *M. germanica* leaf BPAH-1 extract

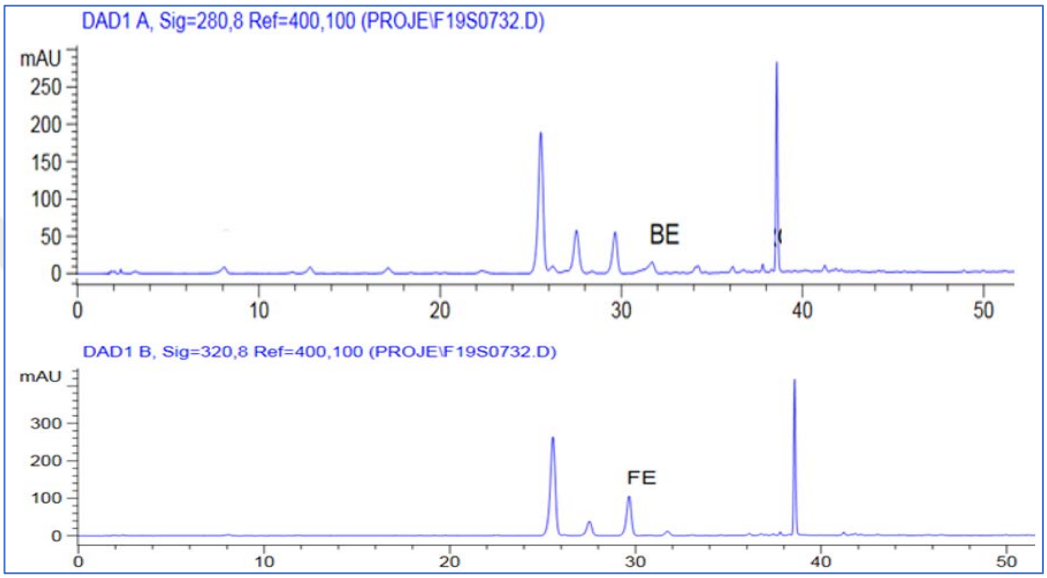


Figure 3.10 Chromatogram of *M. germanica* leaf BPBH-1 extract

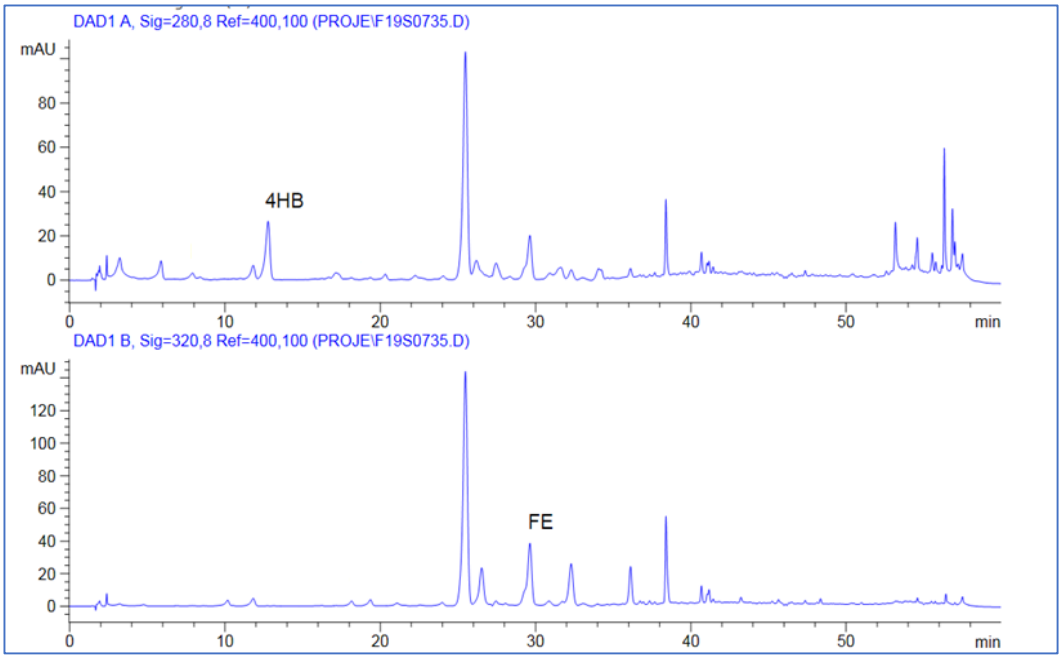


Figure 3.11 Chromatogram of *M. germanica* leaf BPBH-2 extract

3.2.3 M. germanica Fruit

The phenolic acids profile of different extracts of *M. germanica* fruit are shown in Table 3.4. Four phenolic acids comprising three hydroxybenzoic acids derivatives (syringic, protocatechuic and vanillic acids) and one hydroxycinnamic acid derivative (ferulic), with R_t similar to the available standards were detected and quantified in four different extracts; FPA, BHPA, BPBH-1, and BPBH-2. The protocatechuic, ferulic and vanillic acids identified in the current study were also identified in the *M. germanica* fruit by Rop et al. (2011). Similarly, Gülçin et al. (2011) identified ferulic, syringic, and vanillic acids in Turkish medlar. The chromatograms of the four extracts of *M. germanica* fruit are presented in Figures 3.12-3.15. The chromatographic peaks of acid hydrolysis fractions (AHPA, BPAH-1 and BPAH-2) does not correspond to that of available standards; thus, no phenolic acid was identified. About individual phenolic acid, ferulic acid was more pronounced, it was identified in four extracts; FPA, BHPA, BPBH-1, and BPBH-2. Protocatechuic acid was identified in FPA and BPBH-1; syringic acid was found in BHPA and BPBH-2; and vanillic acid in only BPBH-2 extract. The major phenolic acid recognized in the six extracts of *M. germanica* fruit were protocatechuic acid in FPA (1.90±0.10 µg/g), ferulic acid in BHPA (9.30±0.58 µg/g), protocatechuic acid in BPBH-1 (4.47±0.51 µg/g) and vanillic in BPBH-2 (2.30±0.10 µg/g). In addition, ferulic acid identified in BHPA and BPBH-2 had the highest and lowest concentration, respectively.

Moreover, the sum of phenolic acids in the different extracts of *M. germanica* fruit were 3.07±0.28, 17.65±0.93, 5.29±0.53 and 4.56±0.16 µg/g for FPA, BHPA, BPBH-1 and BPBH-2 extracts, respectively. The amount of TPAC in the *M. germanica* fruit determined by the HPLC (30.57±1.90 µg/g DW) was lower than 57.53±3.98 mg GAE/g DW obtained by the FCM. The obtained results from the current research showed base hydrolysis an effective method for phenolic acid extractions and hydrolysis with acid was not effective. According to Robbins (2003), hot acidic conditions can degrade phenolic acids, thus making them difficult to identify. The current research gave results that are similar to the work of Kim et al. (2006). In that study, syringic, vanillic and ferulic acids were identified in basic hydrolysis extract.

Table 3.4 Phenolic acids in different extracts of *M. germanica* fruit

Phenolic acids	**FPA**	**BHPA**	**AHPA**	**BPAH-1**	**BPAH-2**	**BPBH-1**	**BPBH-2**	**TPAC**
Benzoic	-	-	-	-	-	-	-	-
4-hydroxybenzoic	-	-	-	-	-	-	-	-
Gallic	-	-	-	-	-	-	-	-
Protocatechuic	1.90±0.10	-	-	-	-	4.47±0.51	-	6.37±0.61
Rosmarinic	-	-	-	-	-	-	-	-
Syringic	-	8.35±0.35	-	-	-	-	1.90±0.05	10.25±0.4
Vanillic	-	-	-	-	-	-	2.30±0.10	2.30±0.10
ΣBA	**1.90±0.10**	**8.35±0.35**	-	-	-	**4.47±0.51**	**4.20±0.15**	
Chlorogenic	-	-	-	-	-	-	-	-
Caffeic	-	-	-	-	-	-	-	-
Ferulic	1.17±0.18	9.30±0.58	-	-	-	0.82±0.02	0.36±0.01	11.65±0.79
Sinapic	-	-	-	-	-	-	-	
o-Coumaric	-	-	-	-	-	-	-	
p-Coumaric	-	-	-	-	-	-	-	
t-Cinnamic	-	-	-	-	-	-	-	
ΣCA	1.17±0.18	9.30±0.58	-	-	-	0.82±0.02	0.36±0.01	
ΣPHA	**3.07±0.28**	**17.65±0.93**	-	-	-	**5.29±0.53**	**4.56±0.16**	**30.57±1.90**

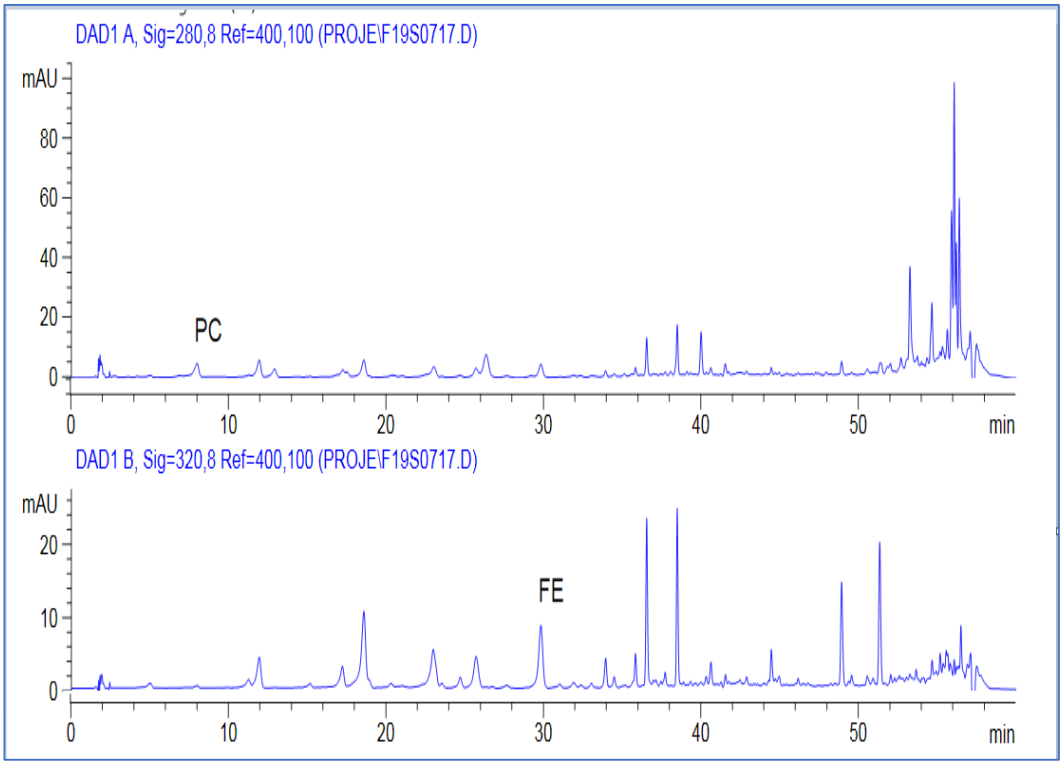


Figure 3.12 Chromatogram of *M. germanica* fruit FPA extract

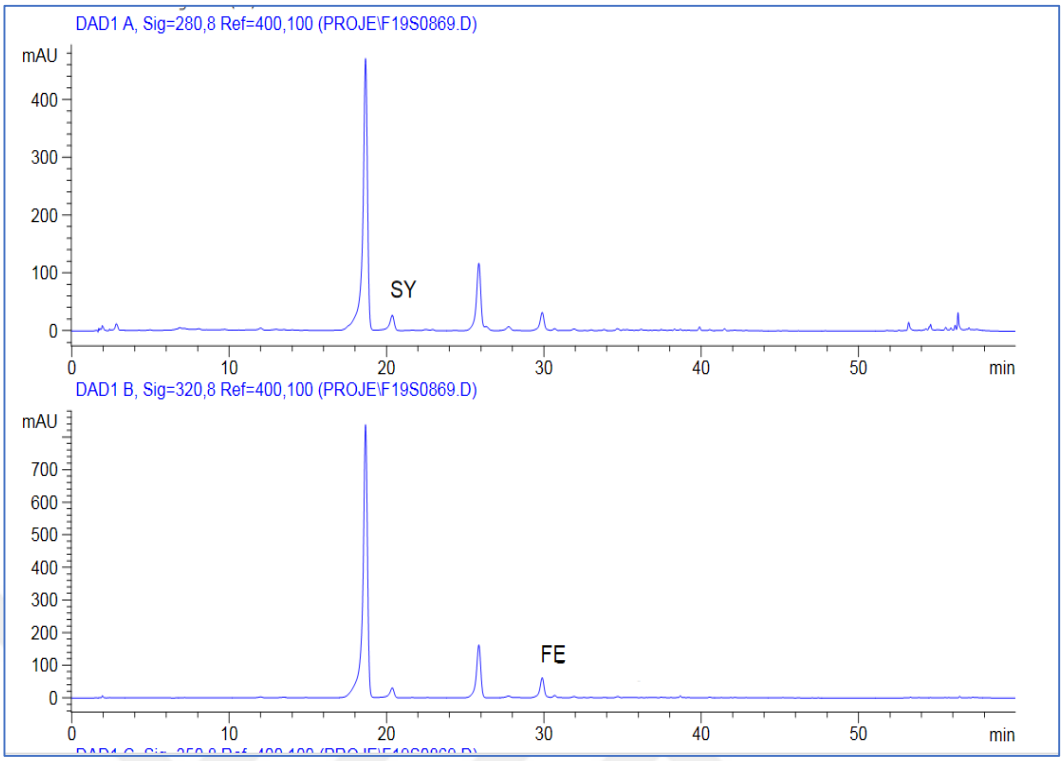


Figure 3.13 Chromatogram of *M. germanica* fruit BHPA extract

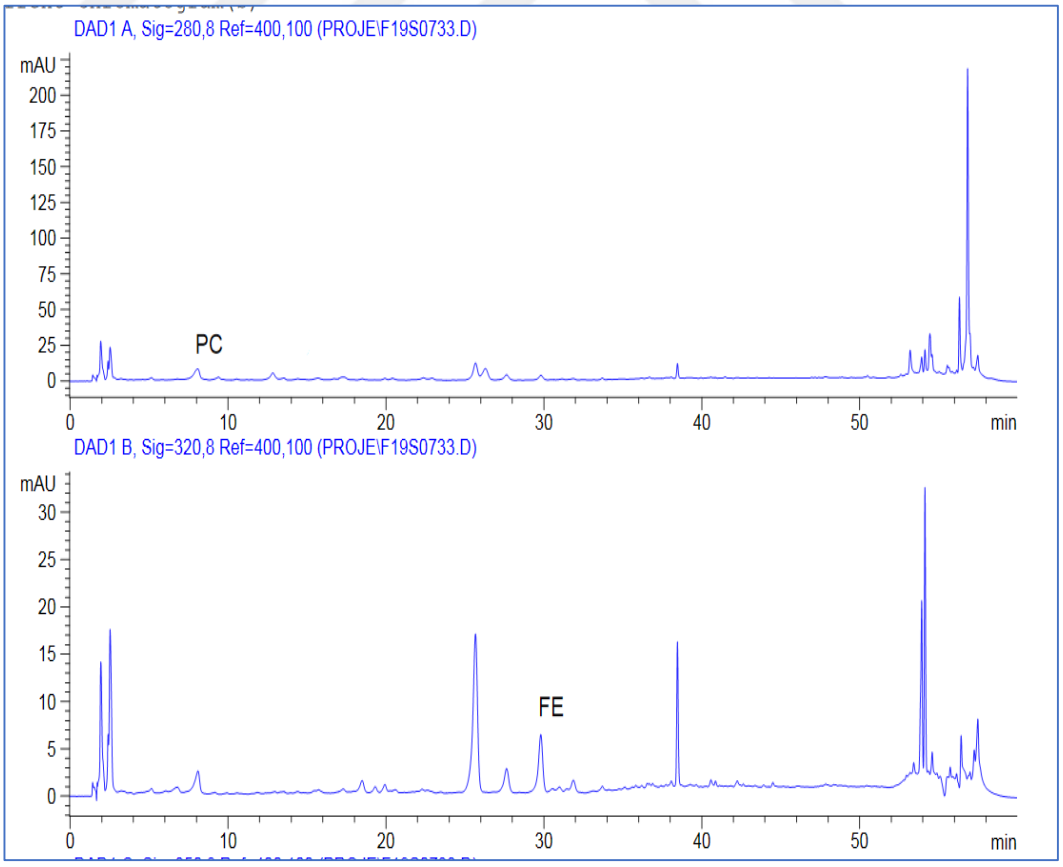


Figure 3.14 Chromatogram of *M. germanica* fruit BPBH-1 extract

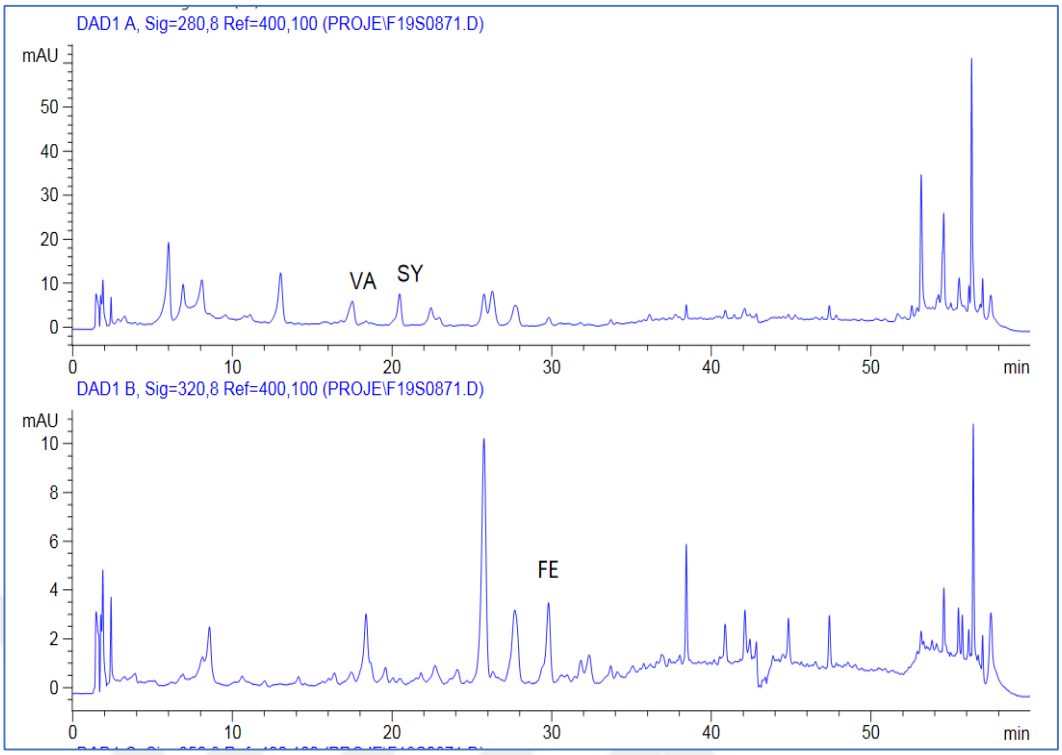


Figure 3.15 Chromatogram of *M. germanica* fruit BPBH-2 extract

3.2.4 D. lotus Seed

The contents of phenolic acids in various extracts of *D. lotus* seed are presented in Table 3.5. Thirteen phenolic acids, o-coumaric, gallic, caffeic, benzoic, syringic, protocatechuic, rosmarinic, ferulic, 4-hydroxybenzoic, vanillic, sinapic, p-coumaric and t-cinnamic acid, were identified and quantified. The chromatograms of the seven extracts of *D. lotus* seed are presented in Figures 3.16-3.22. Some of the phenolic acids (gallic, protocatechuic, caffeic, p-coumaric and t-cinnamic) identified in this study were also identified in *D. lotus* seed from an earlier study (Zeynep et al., 2020). Different extracts of *D. lotus* seed contained different phenolic acid profiles. Few phenolic acids were identified in the CME; three as FPAs, two as BHPAs and four as AHPAs. The individual bound phenolic acids were higher than the extractable phenolic acids identified in the CME. Four were identified in BPAH-1 and BPBH-1, six in BPAH-2 and eight in BPBH-2 fractions.

The most abundant phenolic acids identified in *D. lotus* seed were four benzoic acid derivatives, including vanillic, 4-hydroxybenzoic, protocatechuic and gallic acids, and

two cinnamic acid derivatives, including o-coumaric and t-cinnamic acids. These phenolic acids were identified in three or more extracts. In terms of quantity, gallic acid identified in FPA (94.10±1.67 µg/g), BHPA (27.13±0.61 µg/g) and BPAH-1 (233.34±3.84 µg/g) extracts; t-cinnamic acid identified in AHPA (244.45±3.61 µg/g), BPAH-2 (89.40±1.35 µg/g) and BPBH-2 (95.27±1.62 µg/g) extracts; and protocatechuic acid identified in BPBH-1 (20.70±0.35 µg/g) are the main phenolic acids quantified by HPLC in the six extracts of *D. lotus* seed.

Furthermore, the sum of phenolic acids in the different extracts of *D. lotus* seed were 107.98±2.03, 39.35±0.81, 315.52±5.26, 337.73±6.57, 203.55±3.71, 34.94±0.71 and 111.06±2.22 µg/g for FPA, BHPA, AHPA, BPAH-1, BPAH-2, BPBH-1 and BPBH-2 extracts, respectively. Moreover, the level of TPAC in the seed extracts of *D. lotus* measured by HPLC (1150.13±21.31 µg/g DW) was lower than 465.74±9.09 mg GAE/g DW obtained by the FCM.

Data obtained in the current study showed that most of the phenolic acids in *D. lotus* seed occur in conjugate/bound with other biomolecules, and can be released upon hydrolysis with acid or base. This is in agreement with the works of White and Xing (1997) and Kim et al. (2006) in which they opined that some phenolic acids occur as conjugates with carbohydrates, fatty acids, or proteins. Acidic and basic hydrolyses are usually employed to break down the ester bond (Nuutila et al., 2002; Kim et al., 2006). Phenolic acids can bind to the plant's cell walls and help them to defend themselves against invading pathogens (Gruz et al., 2011). The bound phenolics can also protect the plants against biotic and abiotic stresses, freezing tolerance, drought resistance (Panche et al., 2016). Moreover, the esters bond form by the bound phenolic acids in conjugation with other molecules can help to protect the cells against oxidative damage caused by radical species, such as ROS, RNS, RCS and RSS (Tamagnone et al., 1998; Gruz et al., 2011).

Table 3.5 Phenolic acids in different extracts of *D. lotus* seed

Phenolic acids	**FPA**	**BHPA**	**AHPA**	**BPAH-1**	**BPAH-2**	**BPBH-1**	**BPBH-2**	**TPAC**
Benzoic	-	-	-	-	-	-	5.60±0.27	5.60±0.27
4-hydroxybenzoic	-	-	-	36.37±1.13	9.24±0.21	2.44±0.09	1.50±0.07	49.55±1.50
Gallic	94.10±1.67	27.13±0.61	-	233.34±3.84	83.68±1.61	10.52±0.21	-	448.77±7.94
Protocatechuic	6.58±0.17	12.22±0.20	-	65.95±1.54	15.35±0.35	20.70±0.35	-	120.80±2.61
Rosmarinic	7.30±0.19	-	-	-	-	-	-	7.30±0.19
Syringic	-	-	-	-	-	-	1.19±0.05	1.19±0.05
Vanillic	-	-	11.33±0.16	-	3.37±0.12	-	2.00±0.04	16.70±0.32
ΣBA	**107.98±2.03**	**39.35±0.81**	**11.33±0.16**	**335.66±6.51**	**111.64±2.29**	**33.66±0.65**	**10.29±0.43**	
Chlorogenic	-	-	-	-	-	-	-	
Caffeic	-	-	1.58±0.07	-	-	-	-	1.58±0.07
Ferulic	-	-	-	-	-	1.28±0.06	2.38±0.08	3.66±0.14
Sinapic	-	-	-	-	-	-	2.02±0.05	2.02±0.05
o-Coumaric	-	-	58.16±1.42	-	2.51±0.07	-	1.10±0.04	61.77±1.53
p-Coumaric	-	-	-	2.07±0.06	-	-	-	2.07±0.06
t-Cinnamic	-	-	244.45±3.61	-	89.40±1.35	-	95.27±1.62	429.12±6.58
ΣCA	-	-	304.19±5.10	-	91.91±1.42	1.28±0.06	100.77±1.79	
ΣPHA	**107.98±2.03**	**39.35±0.81**	**315.52±5.26**	**337.73±6.57**	**203.55±3.71**	**34.94±0.71**	**111.06±2.22**	**1150.13±21.31**

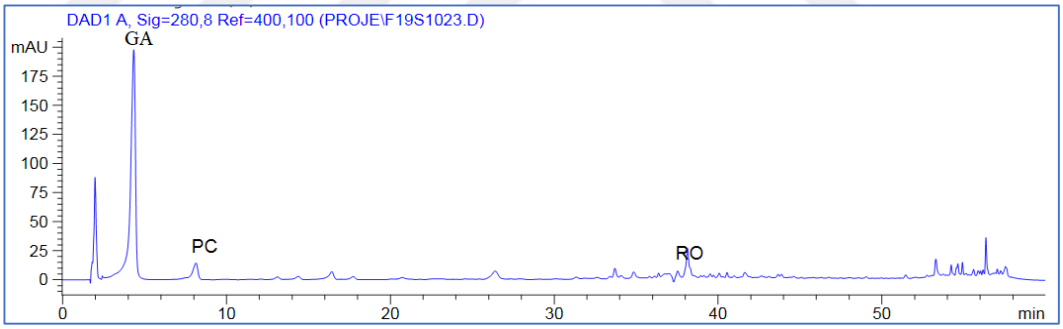


Figure 3.16 Chromatogram of *D. lotus* seed FPA extract

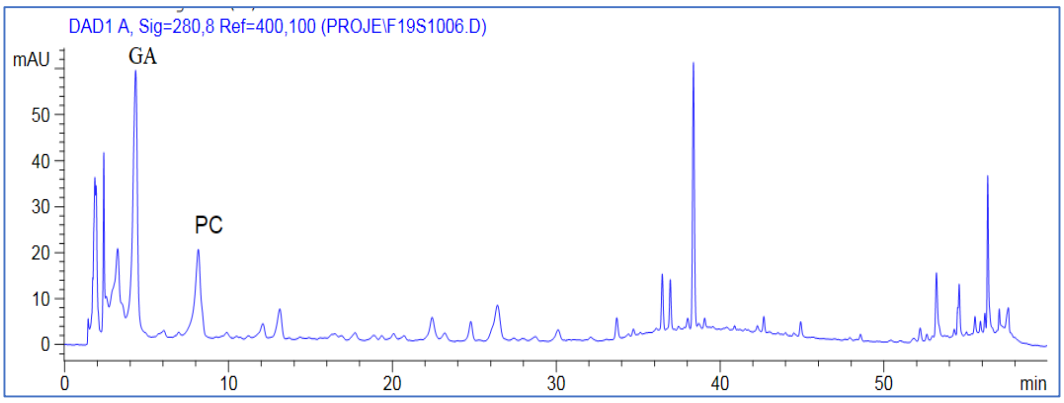


Figure 3.17 Chromatogram of *D. lotus* seed BHPA extract

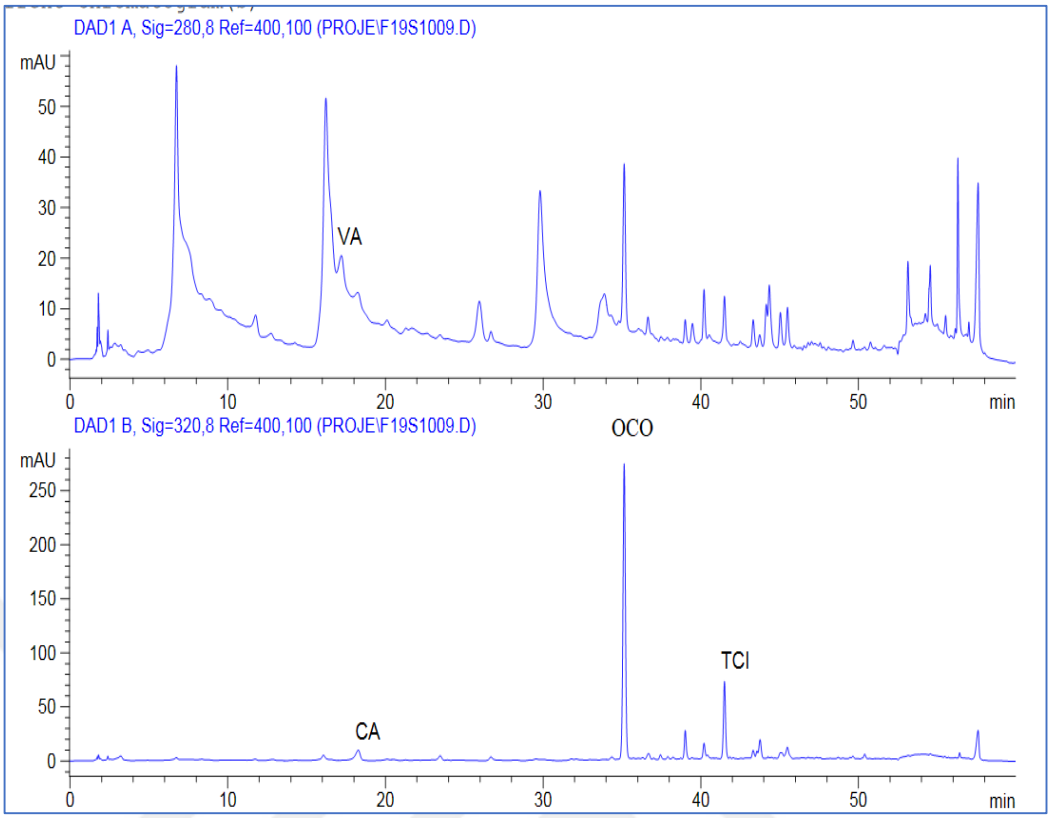


Figure 3.18 Chromatogram of *D. lotus* seed AHPA extract

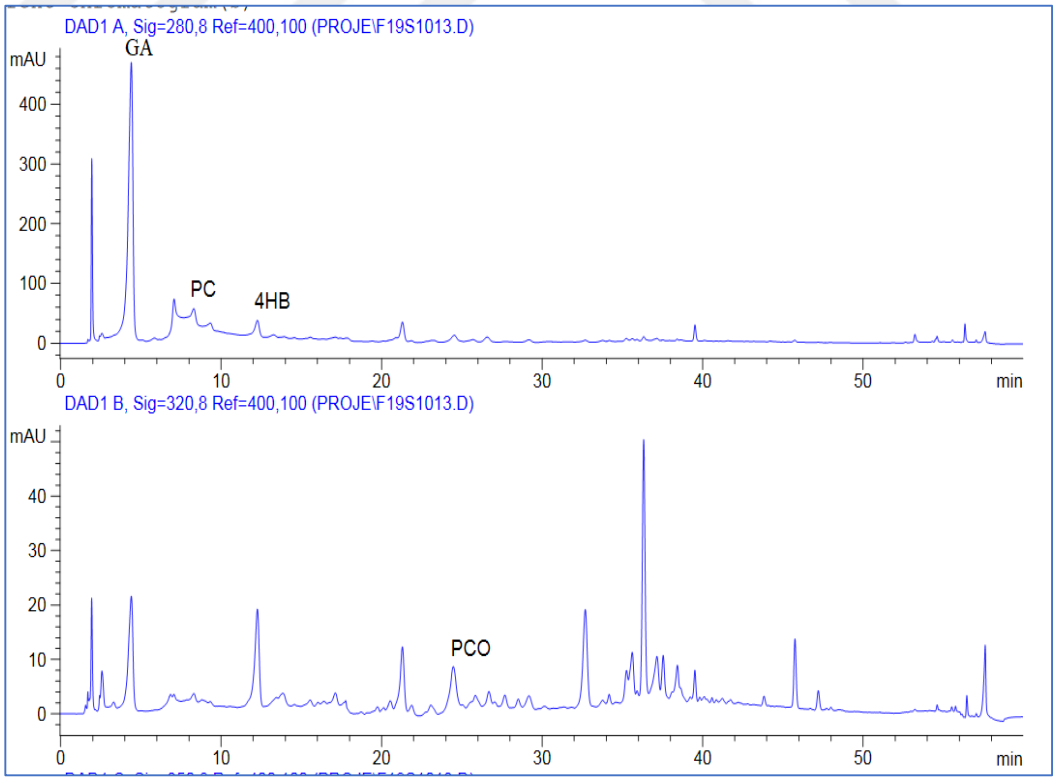


Figure 3.19 Chromatogram of *D. lotus* seed BPAH-1 extract

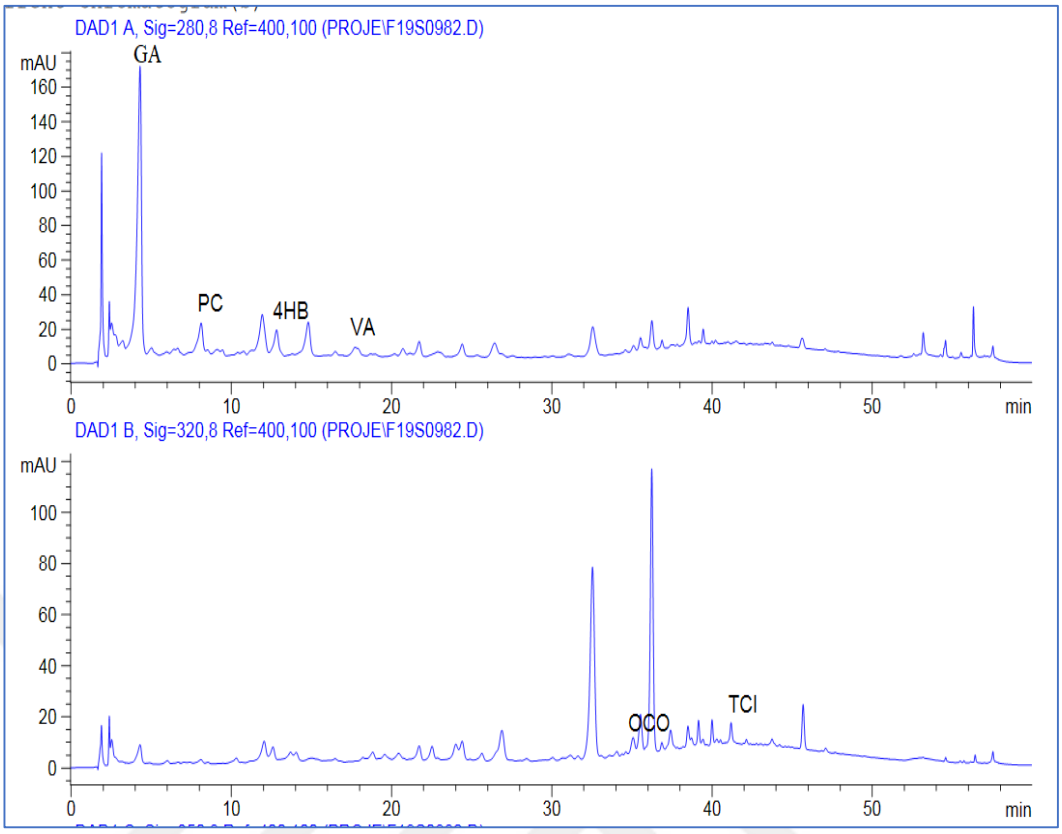


Figure 3.20 Chromatogram of *D. lotus* seed BPAH-2 extract

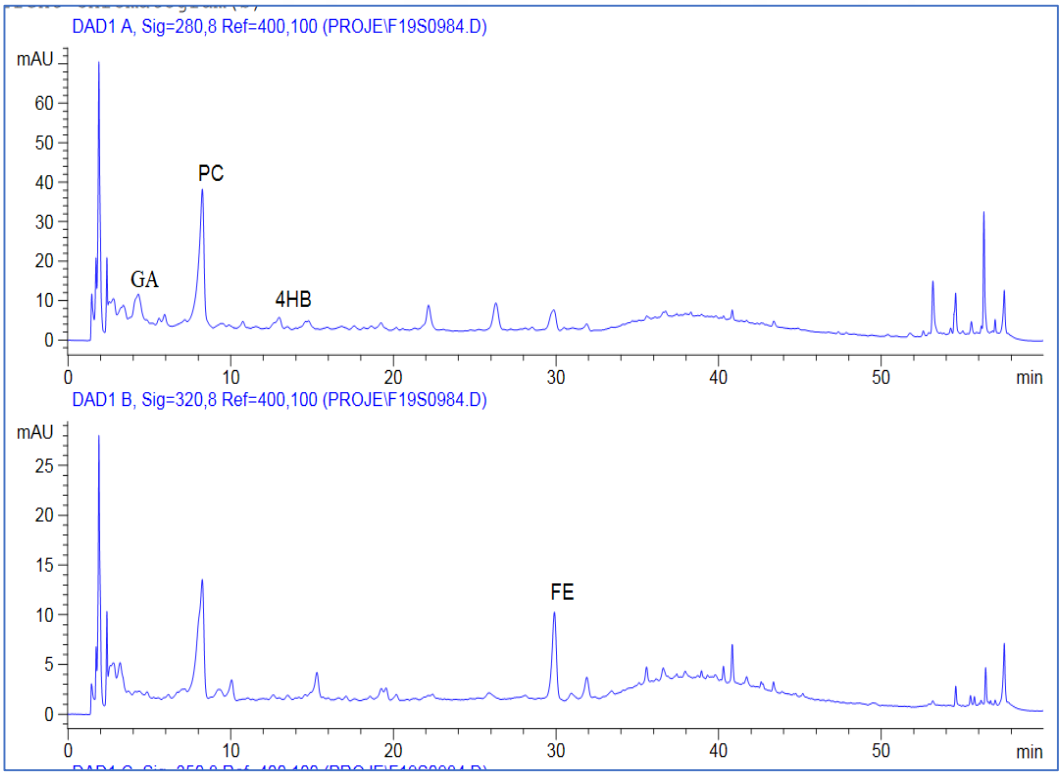


Figure 3.21 Chromatogram of *D. lotus* seed BPBH-1 extract

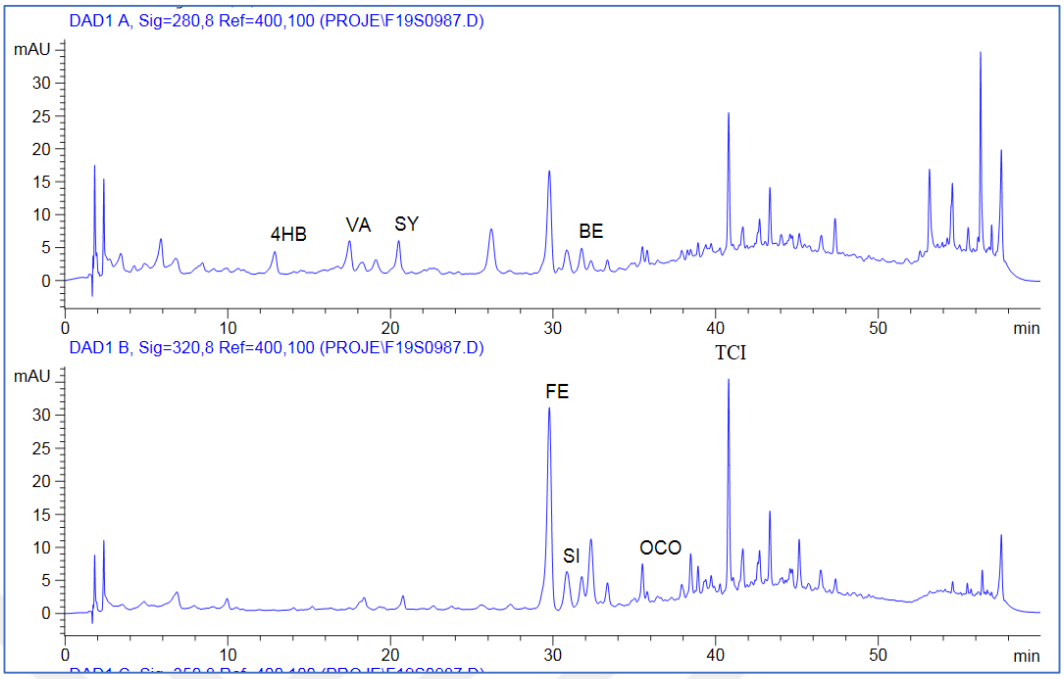


Figure 3.22 Chromatogram of *D. lotus* seed BPBH-2 extract

3.2.5 D. lotus Fruit

Table 3.6 shows the profile of individual phenolic acids identified in the different extracts of *D. lotus* fruit. Five benzoic acid derivatives, including 4-hydroxybenzoic, gallic, protocatechuic, syringic and vanillic acids, along with five cinnamic acid derivatives, including chlorogenic, ferulic, sinapic, o-coumaric and t-cinnamic acids, were recognised and quantified by HPLC. These phenolic acids were identified in six extracts (i.e., FPA, BHPA, BPAH-1, BPAH-2, BPBH-1, BPBH-2), by comparing their R_t and observing similarities between the spectral characteristics of their peaks and their respective standards. The chromatograms of the six extracts of *D. lotus* fruit are presented in Figures 3.23-3.28. Some of the phenolic acids (gallic, protocatechuic, t-cinnamic, syringic and ferulic) identified in this study were also identified in *D. lotus* fruit from earlier studies (Gao et al., 2014; Zeynep et al., 2020). The chromatogram peaks in the AHPA fraction do not possess spectral characteristics that are similar to the available standard, hence not identified.

The bound phenolic acids were quantified in significant concentrations than the extractable phenolic acids identified in the CME. *D. lotus* fruit is rich in gallic,

protocatechuic, vanillic, ferulic and o-coumaric acids; they were identified in relatively high amounts in three or more extracts. Except for the BPBH-2 extract, gallic acid appeared as the major component of the remaining five extracts, namely FPA, BHPA, BPAH-1, BPAH-2 and BPBH-1. The sum of phenolic acids values in the different extracts of *D. lotus* fruit were 30.79±0.93, 30.76±1.79, 1173.09±4.41, 622.66±4.32, 38.03±1.52 and 16.47±0.45 µg/g for FPA, BHPA, BPAH-1, BPAH-2, BPBH-1 and BPBH-2 extracts, respectively. The level of TPAC in the *D. lotus* fruit extracts determined by HPLC (1911.8±13.42 µg/g DW) was lower than 49.04±4.91 mg GAE/g DW obtained by the FCM. Edible fruits like persimmon (*D. lotus*) can synthesize different classes of phenolic compounds, including phenolic acids. The phenolic acids can appear in free form or conjugate with other compounds, such as organic acids, carbohydrates, proteins. The content of both free and bound forms of phenolic acids can be influenced by the biotic and abiotic stress conditions (Gruz et al., 2011).

Table 3.6 Phenolic acids in different extracts of *D. lotus* fruit

Phenolic acids	**FPA**	**BHPA**	**AHPA**	**BPAH-1**	**BPAH-2**	**BPBH-1**	**BPBH-2**	**TPAC**
Benzoic	-	-	-	-	-	-	-	-
4-hydroxybenzoic	-	-	-	-	3.92±0.11	2.90±0.10	-	6.82±0.21
Gallic	23.69±0.68	11.18±0.34	-	1167.11±4.04	569.23±2.91	16.90±0.72	3.23±0.09	1791.34±8.78
Protocatechuic	-	0.82±0.08	-	-	10.67±0.15	16.03±0.59	-	27.52±0.82
Rosmarinic	-	-	-	-	-	-	-	-
Syringic	-	0.82±0.06	-	-	-	-	4.29±0.11	5.11±0.17
Vanillic	2.76±0.09	1.81±0.06	-	-	3.21±0.10	1.28±0.06	5.11±0.13	14.17±0.44
ΣBA	**26.45±0.77**	**14.63±0.54**	-	**1167.11±4.04**	**587.03±3.27**	**37.11±1.47**	**12.63±0.33**	
Chlorogenic	-	-	-	5.98±0.37	-	-	-	5.98±0.37
Caffeic	-		-	-	-	-	-	
Ferulic	-	0.24±0.04	-	-	-	0.92±0.05	3.84±0.12	5.00±0.21
Sinapic	0.58±0.03	-	-	-	-	-	-	0.58±0.03
o-Coumaric	3.76±0.13	5.12±0.46	-	-	2.30±0.09	-	-	11.18±0.68
p-Coumaric	-	-	-	-	-	-	-	-
t-Cinnamic	-	10.77±0.75	-	-	33.33±0.96	-	-	44.10±1.71
ΣCA	4.34±0.16	16.13±1.25	-	5.98±0.37	35.63±1.05	0.92±0.05	3.84±0.12	
ΣPHA	**30.79±0.93**	**30.76±1.79**	-	**1173.09±4.41**	**622.66±4.32**	**38.03±1.52**	**16.47±0.45**	**1911.8±13.42**

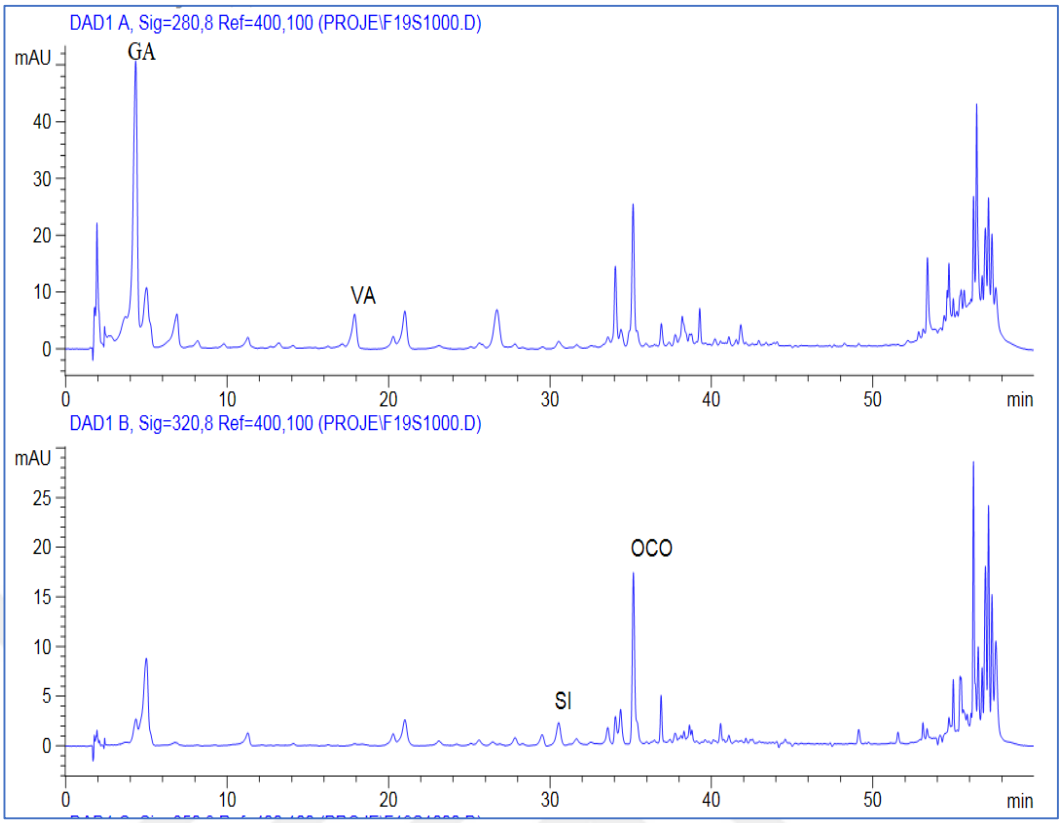


Figure 3.23 Chromatogram of *D. lotus* fruit FPA extract

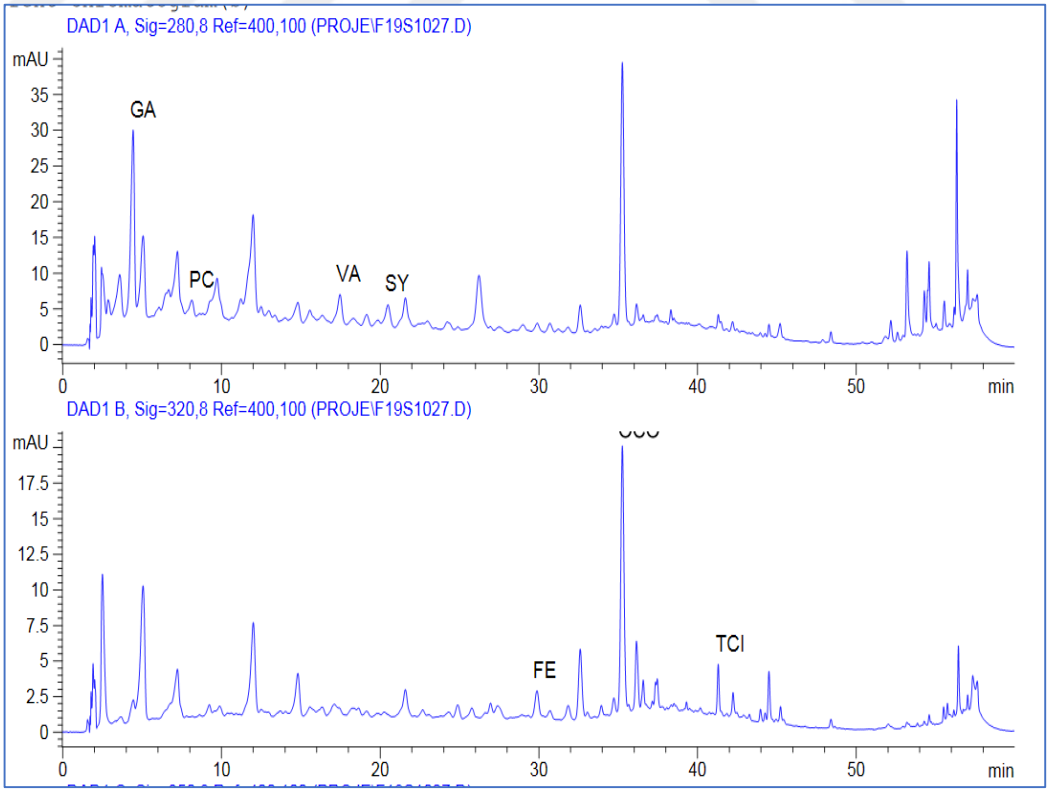


Figure 3.24 Chromatogram of *D. lotus* fruit BHPA extract

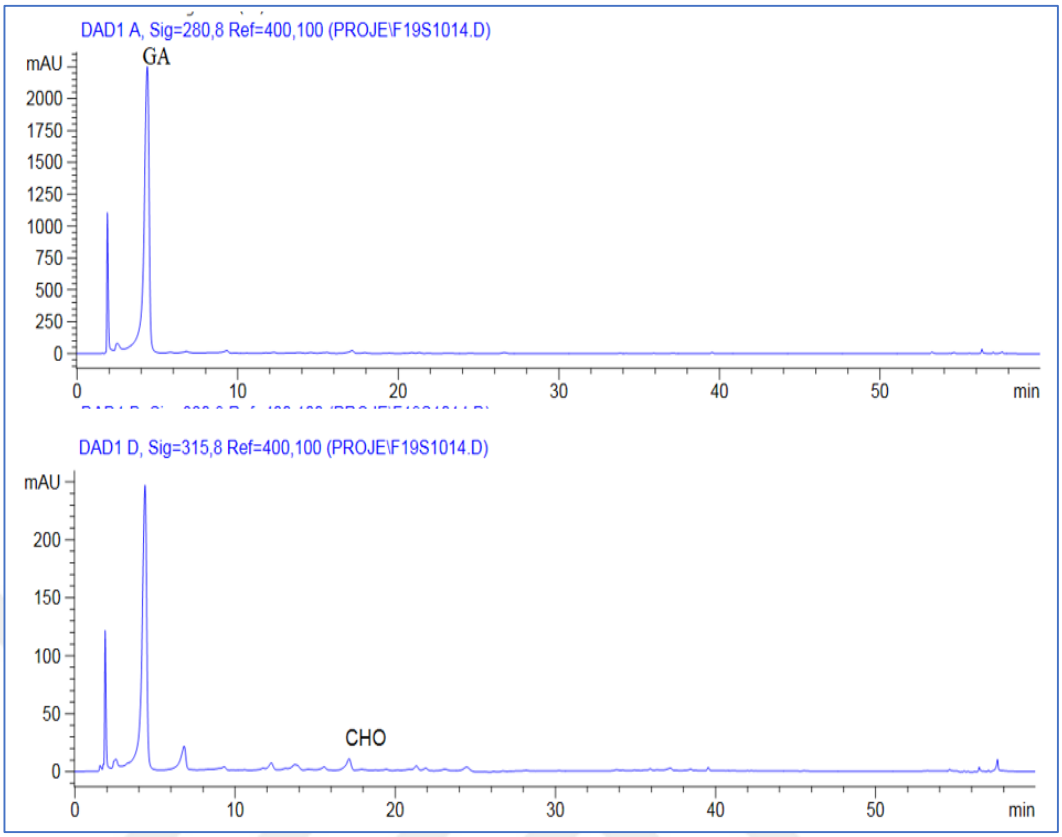


Figure 3.25 Chromatogram of *D. lotus* fruit BPAH-1 extract

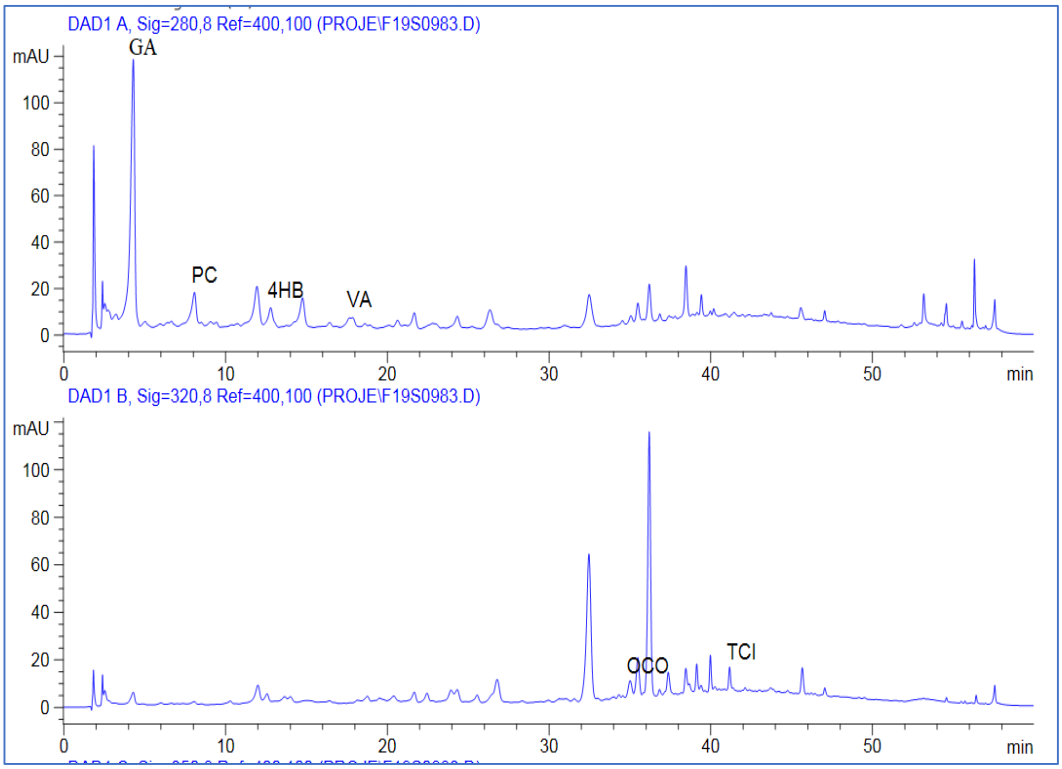


Figure 3.26 Chromatogram of *D. lotus* fruit BPAH-2 extract

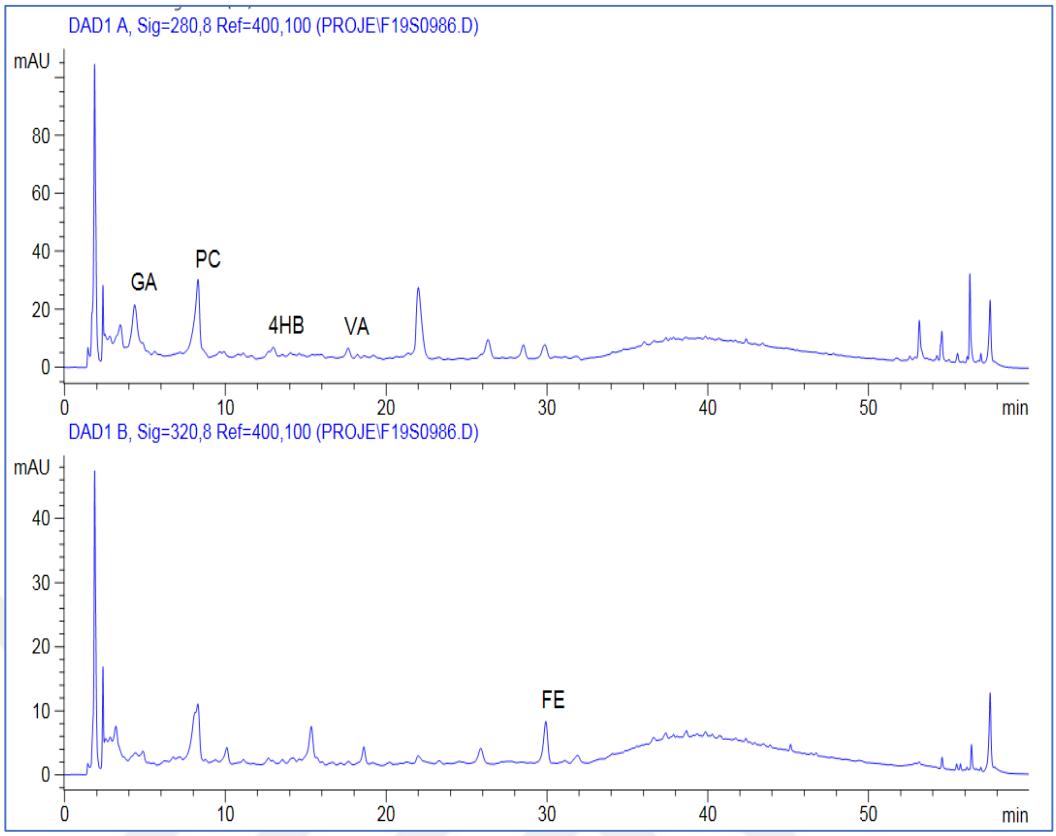


Figure 3.27 Chromatogram of *D. lotus* fruit BPBH-1 extract

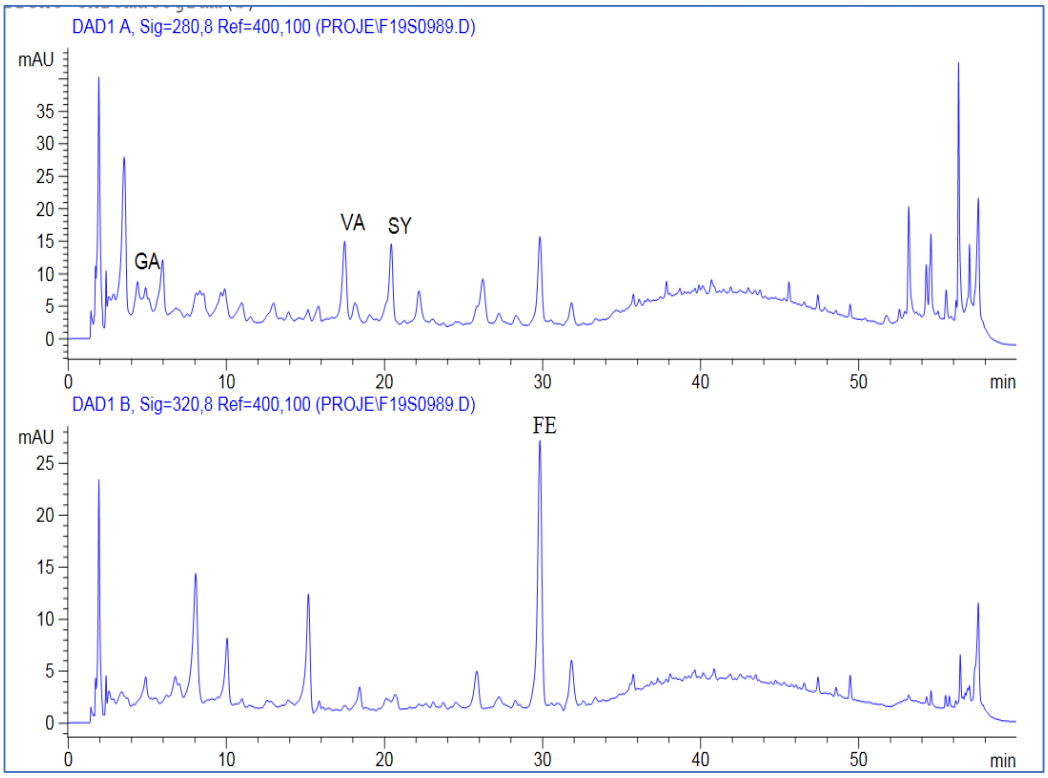


Figure 3.28 Chromatogram of *D. lotus* fruit BPBH-2 extract

3.3 Flavonoids Profile

Table 3.7 presents the profile of flavonoids determined by HPLC in the various extracts of the plant samples. Flavonoids were identified from the different extracts by comparison of their R_t and their chromatographic features with that of their standards. Hesperidin was identified in *N. sativa* seed, *M. germanica* leaf and *M. germanica* fruit extracts. Quercitrin was identified only in *N. sativa* seed extract. Epicatechin and epigallocatechin gallate (EGCG) were identified in the flavan-3-ol methanolic extract of *M. germanica* fruit and *N. sativa* seed, respectively. Epigallocatechin (EGC) was identified in high concentration in the flavan-3-ol methanolic extract of *M. germanica* fruit, and in the flavan-3-ol acidic extract of *N. sativa* seed, *D. lotus* seed and *D. lotus* fruit. The chromatograms of flavonoids compounds of the five samples are presented in Figures 3.29-3.37.

Flavonoids are the largest group of polyphenols. The biological properties of flavonoids are accredited to their configurational structure, the position of functional groups, and the total number of hydroxyl groups attached to the structure (Kalsi, 2021). Hesperidin, belong to the flavanone group of flavonoids. Hesperidin has been used over the years in the treatments of CVD, NDD and cancer (Li & Schluesener, 2017). Haggag et al. (2020) highlighted the potentiality of using hesperidin for the COVID-19 treatment.

Quercitrin is member of flavonol group of flavonoids. Quercitrin was reported to scavenge free radicals and prevents lipid peroxidation *in vitro* (Wagner et al., 2006). Epicatechin is a member of the flavan-3-ols family that contains a OH group in C_3 of the saturated ring. Epicatechin was found to modulate macronutrient metabolism, induced vasodilation, antioxidant activities by acting directly as a scavenger of free radicals, such as ROS and RNS and indirectly as a regulator of SOD and GPx enzymes (Calderón-Oliver & Ponce-Alquicira, 2018; Schwarz et al., 2018). EGCG is an ester of epigallocatechin and gallic acid. EGC & EGCG have been proved to have multiple pharmacological effects and have been used in the treatments of cancer treatment, oral diseases, CVD and NDD (Chu et al., 2017).

Table 3.7 Flavonoids content in different extracts of the *N. sativa* seed, *M. germanica* leaf and fruit, and *D. lotus* seed and fruit

Compound	Extracts	*N. sativa* seed	*M. germanica* leaf	*M. germanica* fruit	*D. lotus* seed	*D. lotus* fruit
Hesperidin	Flavanone	2.92±0.14	8.44±0.49	2.10±0.10	-	-
Quercitrin	Flavonol	1.10±0.10	-	-	-	-
Epicatechin	Flavan-3-ol methanol	-	-	7.68±0.51	-	-
EGCG	Flavan-3-ol methanol	127.85±4.73	-	-	-	-
EGC	Flavan-3-ol methanol	-	-	61.42±1.71	-	-
EGC	Flavan-3-ol acid	113.31±3.49	-	-	1460.80±10.74	933.17±6.11

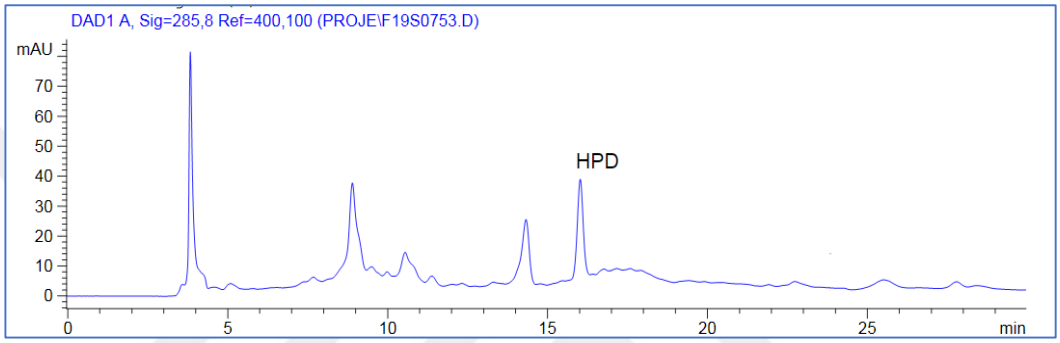


Figure 3.29 Chromatogram of *N. sativa* seed flavanone extract

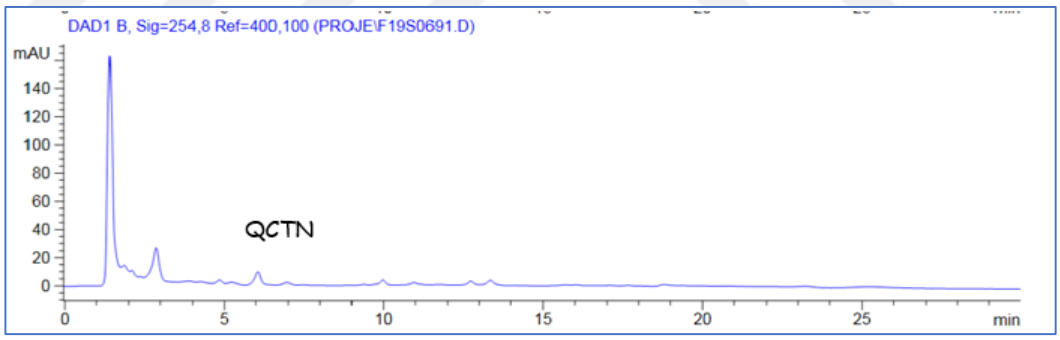


Figure 3.30 Chromatogram of *N. sativa* seed flavonol extract

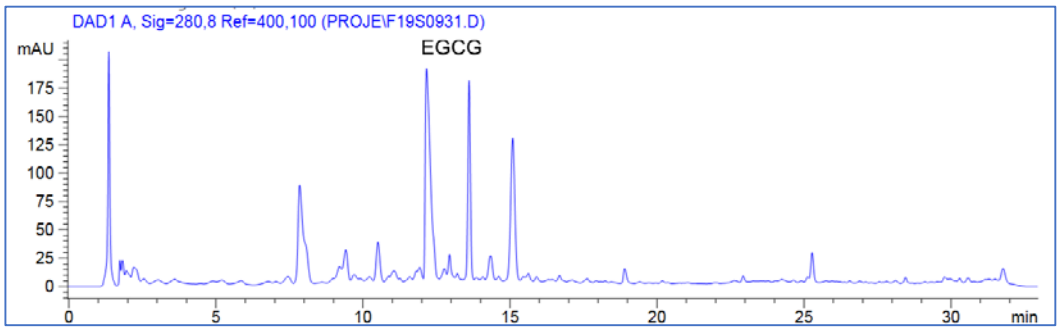


Figure 3.31 Chromatogram of *N. sativa* seed flavan-3-ol methanolic extract

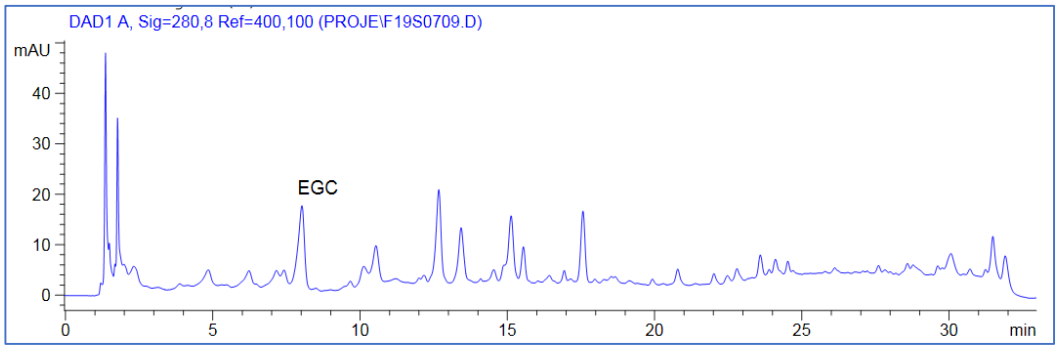


Figure 3.32 Chromatogram of *N. sativa* seed flavan-3-ol acidic extract

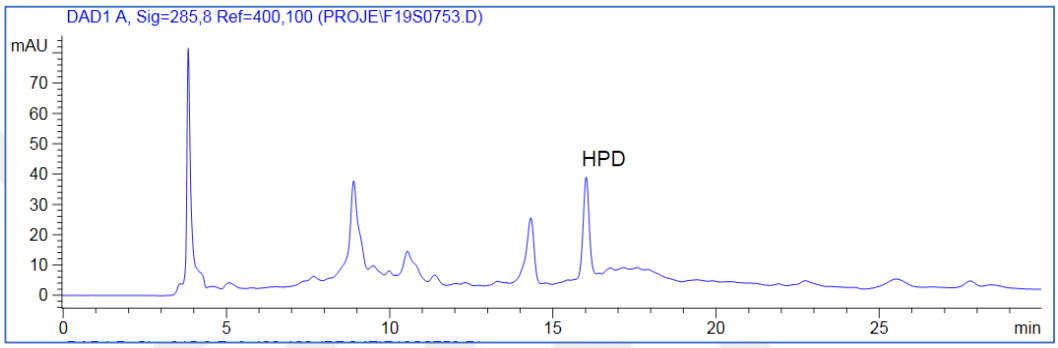


Figure 3.33 Chromatogram of *M. germanica* leaf flavanone extract

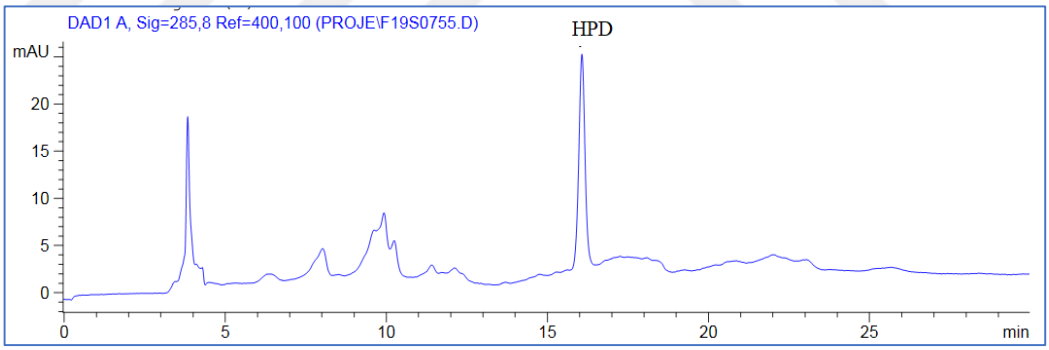


Figure 3.34 Chromatogram of *M. germanica* fruit flavanone extract

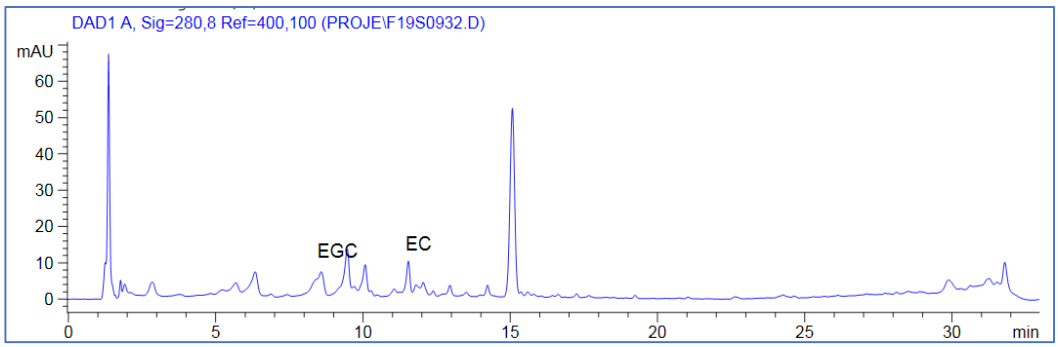


Figure 3.35 Chromatogram of *M. germanica* fruit flavan-3-ol methanolic extract

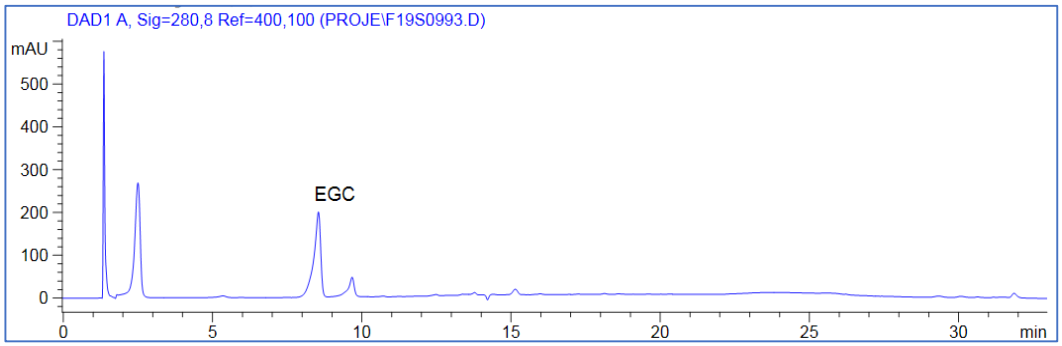


Figure 3.36 Chromatogram of *D. lotus* seed flavan-3-ol acidic extract

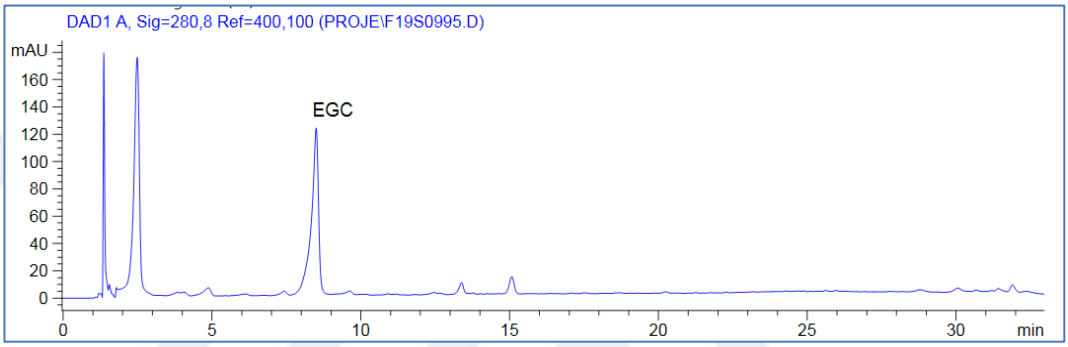


Figure 3.37 Chromatogram of *D. lotus* fruit flavan-3-ol acidic extract

3.4 Total Polyphenolic Content

The total polyphenol content of *N. sativa* seed, *M. germanica* leaf, *M. germanica* fruit, *D. lotus* seeds and *D. lotus* fruits are summarised in Table 3.8. Results are presented in mean ± SD and values with different superscripts within the same column are considered statistically significant ($P<0.05$). The TPC was evaluated by the FCM which is based on the transfer of electrons from a phenolic compound that is energetically oxidized in an alkaline medium to phosphomolybdic acid (a strong acid and oxidant compound present in FCR) (Singleton et al., 1999). The TFC was determined using the $AlCl_3$. In this assay, the $AlCl_3$ in the reaction mixture will bind with flavone and flavonol group in either the C_4 keto group, C_3 or C_5 OH group, or the ortho-dihydroxyl group in the A- and B-ring group of flavonoids and generate a stable complex (Ahmed & Iqbal, 2018). Measurement of the extent of complex formation would indicate the flavonoids contents in the extract. TPC, TFC and TTC are an indicator of the antioxidant activity of a plant. Medicinal herbs/plants with high

amounts of polyphenols compounds have potential antioxidant properties (Safari & Ahmady-Asbchin, 2019).

Table 3.8 Total polyphenol content of *N. sativa* seed, *M. germanica* leaf and fruit, and *D. lotus* seed and fruit

	TPC (mg GAE/g DW)	**TFC (mg QE/DW)**	**TTC (mg TAE/g DW)**
N. sativa seed	109.80±1.69[a]	16.94±0.71[a]	210.42±3.72[a]
M. germanica leaf	529.44±2.27[b]	66.74±5.31[b]	355.95±1.86[b]
M. germanica fruit	57.53±3.98[c]	5.97±0.05[c]	214.29±2.68[a]
D. lotus seed	465.74±9.09[d]	57.49±1.89[d]	348.51±3.61[b]
D. lotus fruit	49.04±4.91[c]	3.70±0.03[c]	216.37±6.46[a]

M. germanica leaf gave the highest TPC (529.44±2.27 mg GAE/g DW), TFC (66.74±5.31 mg QE/g DW) and TTC (355.95±1.86 mg TAE/g DW). It is closely followed by *D. lotus* seed: TPC (465.74±9.09 mg GAE/g DW), TFC (57.49±1.89 mg QE/g DW) and TTC (348.51±3.61 mg TAE/g DW). The decreasing order of TPC and TFC of the five samples are as follows: *M. germanica* leaf > *D. lotus* seed > *N. sativa* seed > *M. germanica* fruit > *D. lotus* fruit. However, the decreasing order of TTC are as follows: *M. germanica* leaf > *D. lotus* seed > *M. germanica* fruit > *D. lotus* fruit > *N. sativa* seed.

Statistical analysis revealed significant differences ($P<0.05$) between the TPC of *N. sativa* seed, *M. germanica* leaf and *D. lotus* seeds. However, no significant difference ($P>0.05$) was found between the TPC of *M. germanica* fruit and *D. lotus* fruits. Similar statistical results were obtained in the TFC of the five samples. In TTC assay, *N. sativa* seed, *M. germanica* fruit and *D. lotus* fruits showed insignificant differences ($P>0.05$) among their mean values. Furthermore, no significant difference ($P>0.05$) was found between the TTC of *M. germanica* leaf and *D. lotus* seed.

The TPC and TTC of *N. sativa* seed obtained in the current study are higher than what was documented by Dalli et al. (2021). In that study, the TPC and TTC were found as 31.16±0.57 mg GAE/g DW and 29.82±1.57 mg catechin equivalent per g DW, respectively. However, the same study obtained a slightly higher TFC (18.4±0.44

mg QE/g DW) than that of the current study. The TPC in *M. germanica* leaf extract is greater than 380.58±0.73 mg GAE/g DW documented by Safari & Ahmady-Asbchin (2019). However, the TFC (75.169±0.04 QE/g DW) obtained by these researchers is slightly higher than what was obtained in the present study. *M. germanica* fruit TPC and TFC obtained in this study, is higher than the 25.08 mg GAE/g DW and 2.39 mg QE/g DW for TPC and TFC, respectively, documented by Gülçin et al. (2011).

The amounts of TPC and TFC of *D. lotus* seed is higher than what was documented by Jeong et al. (2007). The amounts obtained by these researchers were 44.36±0.23 mg/g for TPC and 3.45±0.49 mg/g for TFC. In contrast to this, the current study yielded lower TPC in comparison with the recent work of Zeynep et al. (2020). This study revealed higher TPC and TFC than the earlier works of Gao et al. (2014) and Murathan (2020) from the *D. lotus* fruits. Gao et al. (2014) obtained 3.3±0.18 mg GAE/g DW and 2.8±0.10 mg rutin equivalent per g DW as the TPC and TFC, respectively, while Murathan (2020) documented 130.3±16.2 mg GAE/100 g DW and 12.7±1.9 mg rutin equivalent per 100 g DW as the TPC and TFC, respectively. The *D. lotus* seed yields a higher amount of TPC; however, this result is lower than what was reported recently (Zeynep et al., 2020).

3.5 *In vitro* Antioxidant Activities

3.5.1 Flavone Extracts

The antioxidant properties of flavonoids compounds originate from their properties of proton donation, chelate formation, Fe^{3+} to Fe^{2+} reducing capability, and dismutation of radicals such as $DPPH^{\bullet}$, $^{\bullet}OH$ and $NO^{\bullet}$ (Ebrahimzadeh et al., 2008). In antioxidant assays, the lower the IC_{50} value of $DPPH^{\bullet}$, $^{\bullet}OH$ and $NO^{\bullet}$ scavenging, and ferrous ion chelating assays, the higher the antioxidant potential. Also, the higher the value obtained from ferric ion reducing power and FRAP assays, the higher the antioxidant capacity of the extract. The results are presented in mean ± SD and values with different superscripts within the same column are considered statistically significant ($P<0.05$).

The flavone extracts of all tested plants showed a potential scavenging effect of DPPH•, •OH and NO•, ferrous ion chelation, and Fe^{3+} to Fe^{2+} transformation power (Table 3.9). Flavone extract of *M. germanica* leaf showed higher •OH and NO• scavenging power. Flavone extract of *M. germanica* fruit showed higher DPPH• scavenging power. The ferrous ion chelating property of the flavone extract of *N. sativa* seed was better. The antioxidant potential of the flavone extract of *D. lotus* fruit estimated through ferric ion reducing power and FRAP assays were superior.

Table 3.9 Antioxidant activity of flavone extracts

	DPPH•	**•OH**	**NO•**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	144.27±4.08^{a}	10.14±0.36^{a}	60.29±0.72^{a}	43.99±1.29^{a}	52.90±0.43^{a}	30.77±0.76^{a}
M. germanica leaf	138.40±1.72^{a}	9.46±0.24^{a}	40.57±0.49^{b}	53.01±1.02^{b}	56.08±0.94^{b}	20.65±0.62^{b}
M. germanica fruit	129.91±2.77^{c}	9.64±0.21^{a}	44.76±0.46^{c}	51.26±1.37^{b}	55.31±0.50^{b}	35.00±0.80^{c}
D. lotus fruit	133.31±3.43^{c}	10.55±0.23^{e}	102.14±1.14^{e}	330.41±3.12^{e}	86.94±1.11^{e}	112.91±1.35^{e}

In the DPPH• scavenging assay, the difference in the mean value of *N. sativa* seed and *M. germanica* leaf is not large enough to be considered significant ($P>0.05$) following Tukey-Kramer multiple comparisons test. Likewise, the mean value of *M. germanica* fruit and *D. lotus* fruit. In •OH scavenging of the flavone extracts, the difference in the mean of *N. sativa* seed, *M. germanica* leaf and *M. germanica* fruit was not significant ($P>0.05$), however, these data differed significantly ($P<0.05$) with that of *D. lotus* fruit. Furthermore, a statistically significant difference ($P<0.05$) was observed between the NO• scavenging and FRAP assays of the five samples. Moreover, the mean value of the antioxidant activity of *M. germanica* leaf and fruit as determined by Fe^{2+} chelating and Fe^{3+} reducing power assays is not significant ($P>0.05$). However, a significant difference ($P<0.05$) was observed between these mean values and those obtained from *N. sativa* seed and *D. lotus* fruit.

The DPPH• scavenging capacity of a phenolic extract is attributed to its proton donating ability. In this assay, hydrogen is donated by an antioxidant compound to a free stable DPPH• and converted it into DPPH-H. In doing so, the DPPH• reagent

colour is reduced. The decreased in optical density at 517 nm reflected the extent of the DPPH$^{\bullet}$ scavenging power of the antioxidant (Guo, 2007; Ebrahimzadeh et al., 2008).

3.5.2 Flavanone Extracts

Antioxidant activity of flavanone extracts of *N. sativa* seed, *M. germanica* leaf and *M. germanica* fruit as determined by the DPPH$^{\bullet}$, $^{\bullet}$OH, NO$^{\bullet}$ scavenging, Fe^{2+} chelating, Fe^{3+} reducing power and FRAP assays are shown in Table 3.10. All the three plant's parts showed the potential scavenging effect of DPPH$^{\bullet}$, $^{\bullet}$OH and NO$^{\bullet}$, ferrous ion chelation, and Fe^{3+} to Fe^{2+} transformation power. The highest $^{\bullet}$OH scavenging and ferrous ion binding activities were found in the flavanone extracts of *N. sativa* seed. Likewise, the *M. germanica* leaf presented the highest DPPH$^{\bullet}$ and NO$^{\bullet}$ scavenging activities, and ferric ion reducing ability.

Table 3.10 Antioxidant activity of flavanone extracts

	DPPH$^{\bullet}$	**$^{\bullet}$OH**	**NO$^{\bullet}$**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	111.28±1.26^{a}	9.07±0.14^{a}	32.06±0.56^{a}	49.79±1.23^{a}	95.79±1.33^{a}	117.83±1.36^{a}
M. germanica leaf	52.51±0.58^{b}	9.34±0.13^{a}	25.74±0.52^{b}	56.52±1.36^{b}	244.53±1.98^{b}	251.98±2.09^{b}
M. germanica fruit	123.94±1.33^{c}	10.02±0.13^{c}	45.02±0.78^{c}	55.29±1.17^{b}	69.74±1.27^{c}	29.36±0.66^{c}

The differences in the mean values of DPPH$^{\bullet}$, NO$^{\bullet}$, ferric ion reducing, and FRAP assays of all three samples are significant ($P<0.05$). For the $^{\bullet}$OH scavenging assay of the flavanone extract, the mean difference of *N. sativa* seed and *M. germanica* leaf is insignificant ($P>0.05$), likewise, the mean difference of *M. germanica* leaf and fruit of ferrous ion chelating assay. $^{\bullet}$OH, is a strong oxidant that is generated in a wide range of environments by Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + {}^{\bullet}OH$) (Lyngsie et al., 2018). This radical species can attack and damage the target structure and initiates LPO (Halliwell & Gutteridge, 1985). LPO is implicated in several CVD and NDD (Saiin et al., 2018). Scavengers of $^{\bullet}$OH such as phenolic compounds often show partial protective effects against this damage (Moorhouse et al., 1987). According to Pandey

& Rizvi (2012), the ˙OH scavenging activity of flavonoids is related to 3',4'-dihydroxy structure in the B ring, and the multiple OH groups in the A and B rings. Hesperidin identified in the flavone extracts of *N. sativa* seed, *M. germanica* leaf and *M. germanica* fruit may be responsible for the ˙OH scavenging activity observed in the three samples. Hesperidin is a flavanone glycoside that has multiple OH groups in its structure, it can scavenge ˙OH through proton donation.

3.5.3 Flavonol Extracts

The flavonol extracts of *N. sativa* seed, *M. germanica* leaf, *M. germanica* fruit and *D. lotus* fruit showed the potential scavenging effect of DPPH˙, ˙OH and NO˙, ferrous ion chelation, and Fe^{3+} reducing capabilities through ferric ion reducing and FRAP assays (Table 3.11). *M. germanica* leaf flavonol extract gave the highest DPPH˙ and NO˙ scavenging activity and exhibited a good ferric ion reducing power. Its FRAP value was also the highest. *N. sativa* seed flavonol extract was the highest quencher of ˙OH. The ferrous ion chelating activity of *D. lotus* fruit flavonol extract was also the highest. Except for the ferrous ion chelating assay, the differences in the mean values of the four samples in all the other assays are considered not significant ($P<0.05$).

Table 3.11 Antioxidant activity of flavonol extracts

	DPPH˙	**˙OH**	**NO˙**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	117.36±1.25^{a}	9.59±0.12^{a}	45.56±0.56^{a}	110.21±1.06^{a}	86.99±1.44^{a}	47.42±1.11^{a}
M. germanica leaf	65.53±1.11^{b}	12.44±0.17^{b}	30.37±0.46^{b}	84.63±0.90^{b}	243.34±2.21^{b}	252.74±2.27^{b}
M. germanica fruit	159.75±1.71^{c}	11.64±0.12^{c}	35.35±0.60^{c}	62.51±1.11^{c}	77.76±1.25^{c}	27.11±0.61^{c}
D. lotus fruit	108.25±1.34^{e}	10.08±0.16^{e}	124.52±1.48^{e}	62.32±1.08^{c}	86.58±1.34^{a}	29.74±0.52^{c}

NO˙ is the major RNS produced by cells and it is the main source of other RNS (Bauerova & Bezek, 2000). The Griess assay is usually employed for the measurement of NO˙ production in living systems (Marcocci et al., 1994a). NO˙ is a key cell signalling molecule and it has a significant role in neurotransmission, immune defences, vasodilation and apoptosis (Sharma et al., 2007). However, overproduction

of NO• in living cells can lead to nitrosative stress. The nitrosative stress could cause nitrosylation reactions that can affect the structure of proteins and so inhibit their normal function (Shahat et al., 2015). Furthermore, excess generation of NO• is known to cause inflammation, DNA and RNA lesions, cancer, and other pathological conditions (Moncada & Higgs, 2006; Sawa & Ohshima, 2006). Scavenging NO• could help to arrest the chain of reactions started by overproduction of NO• (Ebrahimzadeh et al., 2008). The result derived in the current study presented the *N. sativa* seed, *M. germanica* leaf, *M. germanica* fruit and *D. lotus* fruit as a potent scavenger of NO• generated through the Griess system. The NO• scavenging activity of phenolic compounds could be related to the methoxy and the phenolic groups attached to their structures (Sreejayan and RAO, 1997).

3.5.4 Flavan-3-ol Methanolic Extracts

The results of the antioxidant activity of flavan-3-ol methanolic and acidic extracts of the five samples as determined by the DPPH•, •OH, NO• scavenging, Fe^{2+} chelating, Fe^{3+} reducing power and FRAP assays are shown in Tables 3.12-3.13.

Table 3.12 Antioxidant activity of flavan-3-ol methanolic extracts

	DPPH•	**•OH**	**NO•**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	114.57±1.38^{a}	10.93±0.19^{a}	35.32±0.51^{a}	46.80±0.57^{a}	83.78±1.31^{a}	111.07±1.59^{a}
M. germanica leaf	48.60±0.59^{b}	12.15±0.15^{b}	22.53±0.52^{b}	56.23±0.60^{b}	400.88±5.17^{b}	417.28±5.36^{b}
M. germanica fruit	115.92±1.57^{a}	11.58±0.12^{c}	77.27±1.26^{c}	50.06±0.55^{c}	88.13±1.52^{a}	466.12±5.99^{c}
D. lotus seed	46.36±0.66^{b}	10.17±0.13^{d}	45.62±0.58^{d}	142.75±1.66^{d}	434.62±5.94^{d}	498.68±6.34^{d}
D. lotus fruit	122.91±1.54^{e}	10.27±0.12^{d}	119.05±1.46^{e}	104.41±1.52^{e}	86.19±0.61^{a}	64.03±1.35^{e}

From the results presented in Table 3.12, the flavan-3-ol methanolic extract of *D. lotus* seed gave the highest DPPH• and •OH scavenging activity, ferric ion reducing power, and had the highest FRAP value. *M. germanica* leaf showed the highest NO• scavenging activity, while the *N. sativa* seed presented the highest ferrous ion chelating activity. Statistical analysis showed significant differences ($P<0.05$) between the mean

values of NO•, ferrous ion chelating, and FRAP assays of all the tested samples. The DPPH• mean difference of *N. sativa* seed and *M. germanica* fruit was not significant (P>0.05), likewise, that of the ferric ion reducing power of *N. sativa* seed, *M. germanica* fruit and *D. lotus* fruit. Additionally, the •OH mean difference of *D. lotus* seed and *D. lotus* fruit was not significant (P>0.05).

3.5.5 Flavan-3-ol Acidic Extracts

The results of the antioxidant activity of flavan-3-ol acidic extracts are shown in Table 3.13. Like the result of antioxidant activity of flavan-3-ol methanolic extracts, *D. lotus* seed presents the greatest DPPH• scavenging activity, ferric ion reducing power, and had the highest FRAP value. *N. sativa* seed showed the highest NO• scavenging activity, *D. lotus* fruit showed the highest •OH scavenging activity, and the *M. germanica* leaf presented the highest ferrous ion chelating activity.

Table 3.13 Antioxidant activity of flavan-3-ol acidic extracts

	DPPH•	**•OH**	**NO•**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	82.51±1.18[a]	11.22±0.17[a]	34.31±0.43[a]	72.19±1.29[a]	230.05±2.63[a]	175.15±2.14[a]
M. germanica leaf	100.75±1.25[b]	11.44±0.19[a]	39.57±0.55[b]	64.87±1.17[b]	163.56±2.08[b]	210.88±2.58[b]
M. germanica fruit	93.68±1.15[c]	13.26±0.19[c]	58.35±0.62[c]	NA	139.88±1.60[c]	180.79±2.22[a]
D. lotus seed	47.07±0.45[d]	11.02±0.11[a]	34.91±0.49[a]	209.50±2.45[d]	572.22±5.93[d]	593.93±6.80[d]
D. lotus fruit	50.71±0.51[e]	10.96±0.15[a]	37.35±0.54[e]	NA	299.10±2.94[e]	253.06±2.47[e]

The differences in the mean values of DPPH•, ferrous ion chelating, and ferric ion reducing power assays of all five samples are considered significant (P<0.05). In the FRAP experiment, the mean difference of *N. sativa* seed and *M. germanica* fruit is statistically not significant (P>0.05), however, this average value differed significantly (P<0.05) with those of *M. germanica* leaf, *D. lotus* seed and *D. lotus* fruit. Furthermore, the statistical analysis on the results of NO• scavenging assay showed no significant difference (P>0.05) was observed between *N. sativa* seed and *D. lotus* seed.

Significant differences ($P<0.05$) were observed between this value and those of *M. germanica* leaf, *M. germanica* fruit and *D. lotus* fruit. Moreover, the mean value of *M. germanica* fruit in the $^{\bullet}OH$ scavenging deviated significantly ($P<0.05$) from the mean values of all the other samples.

Transition metal ion, Fe^{2+} can donate a single electron to several compounds, and therefore generate radical species (Adjimani & Asare, 2015). Taking this into account, the ferrous ion chelation is crucial in avoiding ROS and RNS generation that can cause oxidative damage to several compounds including lipids, carbohydrates, membranes, proteins, lipoproteins, DNA and RNA (Halliwell, 2007). Hence, the chelation of ferrous ions is of great significance and would provide an effective therapeutic approach in the management of NDD, CVD, cancer and diabetes that is caused by the radical species (Aparadh et al., 2012).

In the reaction mixture of ferrous ion chelating assay, Fe^{2+} react with ferrozine to form Fe^{2+}-ferrozine complex. However, in the presence of chelating agents, the complex formation is disrupted. Thus, the agent captures ferrous ion first before ferrozine, which in turn decrease the formation of radical species (Aboul-Enein et al., 2003). As described earlier, Fe^{2+} can bind to the phenolics structures at several coordination sites and therefore be chelated (Aboul-Enein et al., 2003). The possible coordination sites of Fe^{2+} to the flavonoids structures include a) in-between 5-OH and 4-carbonyl group, b) in-between 3-OH and 4-carbonyl group, c) in-between 3', 4'-OH group in B ring. The possible coordination sites of Fe^{2+} to the phenolic acids structures include a) in-between 3-methoxy and 4-OH groups b) in-between 3-OH and 4-OH groups.

Epicatechin, epigallocatechin and epigallocatechin gallate identified in flavan-3-ol methanolic and acidic extracts could be responsible for the ferrous ion chelating activity of the tested plants. These flavan-3-ol compounds have several metal coordination sites that could help them to chelate ferrous iron.

3.5.6 FPA Extracts

The results antioxidant activity of FPA extracts are shown in Table 3.14. The FPA extract of *D. lotus* seed gave the highest DPPH$^{\bullet}$ scavenging activity, Fe^{3+} reducing power, and had the highest FRAP value. *N. sativa* seed extract showed the highest $^{\bullet}OH$ scavenging and Fe^{2+} chelating activities. The *M. germanica* leaf had the highest NO$^{\bullet}$ scavenging activity.

The differences in the mean values of DPPH$^{\bullet}$, NO$^{\bullet}$, Fe^{3+} reducing power and FRAP assays of all the five samples were statistically significant ($P<0.05$). The $^{\bullet}OH$ scavenging assay showed no significant difference ($P>0.05$) in the mean values of *M. germanica* leaf and *D. lotus* fruit, but this mean difference differed significantly ($P<0.05$) with those of *N. sativa* seed, *M. germanica* fruit and *D. lotus* seed. In the ferrous ion chelating assay, no significant difference ($P>0.05$) was observed in the mean values of *N. sativa* seed and *M. germanica* fruit.

Table 3.14 Antioxidant activity of FPA extracts

	DPPH$^{\bullet}$	**$^{\bullet}OH$**	**NO$^{\bullet}$**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	145.21±1.81^{a}	11.08±0.13^{a}	64.57±1.56^{a}	47.72±0.69^{a}	92.20±1.30^{a}	64.00±1.29^{a}
M. germanica leaf	53.79±0.71^{b}	11.97±0.18^{b}	24.81±0.22^{b}	57.37±0.89^{b}	387.02±3.15^{b}	311.73±3.40^{b}
M. germanica fruit	118.46±1.59^{c}	13.72±0.21^{c}	41.19±0.56^{c}	48.67±0.83^{a}	99.79±1.22^{c}	103.89±1.39^{c}
D. lotus seed	45.60±0.50^{d}	12.53±0.19^{d}	45.19±0.62^{d}	78.09±1.08^{d}	477.51±4.49^{d}	484.38±4.24^{d}
D. lotus fruit	70.46±1.01^{e}	11.63±0.15^{b}	111.36±1.55^{e}	74.27±0.92^{e}	149.93±1.55^{e}	117.14±1.34^{e}

Phenolic acids are the second most abundant plant's polyphenols. They contain a phenolic ring and an organic carboxylic acid group and at least one OH group connected to the structure (Chen et al., 2020). Phenolic acids are considered an excellent natural antioxidants. Like its counterpart, flavonoids, the antioxidant properties of phenolic acids may originate from their radical (e.g., DPPH$^{\bullet}$, $^{\bullet}OH$ and NO$^{\bullet}$) scavenging activities, proton donation, chelate formation and Fe^{3+} to Fe^{2+} transformation power (Ebrahimzadeh et al., 2008; Chen et al., 2020).

Gallic, protocatechuic and rosmarinic acids identified in the FPA extract of *D. lotus* could be responsible for the strong DPPH$^{\bullet}$ scavenging activity and Fe^{3+} reducing power observed in the extract. These activities could be related to the electron-donating ability of free carboxylic acid groups attached to the phenyl groups of the gallic, protocatechuic and rosmarinic acids. According to Chen et al. (2020), the -COOH group of the hydroxybenzoic acid derivatives has the strongest electron-donating ability, followed by the -CH=CHCOOH group of the hydroxycinnamic acid derivatives, and -CH_2COOH group of the hydroxyphenyl acetic acid derivatives has the weakest electron-donating group. An electron-donating group can decrease the dissociation energy of the phenolic OH bond and then intensify its proton donation ability, free radical scavenging capability and chelate formation.

Conversely, *N. sativa* seed presented the highest $^{\bullet}$OH scavenging and Fe^{2+} chelating activities. These activities could be related to the benzoic, 4-hydroxybenzoic, vanillic and ferulic acids identified in its FPA extract. These phenolic acids could donate a proton from their -COOH group or any other -OH group that is attached to their structure to the $^{\bullet}$OH and therefore neutralize it. The binding sites of Fe^{2+} to the benzoic, 4-hydroxybenzoic, vanillic and ferulic acids could be in between the double bond and hydroxyl of the carboxyl acid group of their structure; or specifically in between 3-methoxy and 4-hydroxyl groups of the ferulic and vanillic acids. Furthermore, the highest NO$^{\bullet}$ scavenging activity of the FPA extract of *M. germanica* leaf could be as a result of the vanillic, caffeic and ferulic identified. These phenolic acids could scavenge NO$^{\bullet}$ through proton donation.

3.5.7 BHPA Extracts

The results antioxidant activity of BHPA extracts of all the tested plants are shown in Table 3.15. The BHPA extract of *M. germanica* fruit gave the highest DPPH$^{\bullet}$ and $^{\bullet}$OH scavenging activities, Fe^{3+} reducing power, and had the highest FRAP value. Like the FPA extract, the BHPA extract of *M. germanica* leaf had the highest NO$^{\bullet}$

scavenging activity. The Fe^{2+} chelating activity of *N. sativa* seed BHPA extract was the strongest.

Table 3.15 Antioxidant activity of BHPA extracts

	DPPH˙	**˙OH**	**NO˙**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	150.59±1.66[a]	11.56±0.15[a]	80.40±1.21[a]	47.53±0.49[a]	88.60±1.41[a]	74.90±1.35[a]
M. germanica leaf	93.25±1.24[b]	11.20±0.11[a]	26.05±0.26[b]	52.12±0.75[b]	155.52±1.88[b]	171.68±1.48[b]
M. germanica fruit	47.87±0.61[c]	9.28±0.12[c]	32.92±0.42[c]	151.00±2.08[c]	398.14±2.88[c]	390.61±3.43[c]
D. lotus seed	57.03±0.84[d]	10.36±0.16[d]	64.42±0.74[d]	98.75±1.37[d]	240.57±2.43[d]	234.30±3.16[d]
D. lotus fruit	67.91±1.13[e]	10.29±0.12[d]	120.97±1.88[e]	116.67±1.64[e]	226.57±2.20[e]	193.99±1.21[e]

Apart from the ˙OH scavenging assay, the differences in the mean values of the fives samples in all the other assays were statistically significant ($P<0.05$). Concerning ˙OH scavenging assay, no significant differences ($P>0.05$) were observed between the *N. sativa* seed and *M. germanica* leaf, and between the *D. lotus* seed and fruit. Syringic and ferulic acids identified in the BHPA extract of *M. germanica* fruit could be responsible for its exceptional DPPH˙ and ˙OH scavenging activities, and Fe^{3+} to Fe^{2+} transformation power. These activities could be through proton donation. Fe^{3+} reduction to Fe^{2+} by an antioxidant compound is used as an indicator of the electron-donating capacity of such compound (Yıldırım et al., 2001). In this chemistry, an antioxidant compound would donate an electron to $K_3[Fe(CN)_6]$ to form a reduced form of the complex (potassium ferrocyanide), that would subsequently react with $FeCl_3$ to generate Perl's Prussian blue complex of iron (III) hexacyanoferrate (II) (Gülçin, 2015).

The highest ferrous ion binding potential of the BHPA of *N. sativa* seed extract could be related to the 4-hydroxybenzoic, rosmarinic, vanillic, ferulic and sinapic acids identified by HPLC. The possible coordination sites of Fe^{2+} to the sinapic acid (also called sinapinic acid) could be in between 3-methoxy and 4-hydroxyl groups; and/or in between 4-hydroxyl and 5-methoxy groups.

3.5.8 AHPA Extracts

The results of the antioxidant activity of AHPA extracts of all the tested plants are presented in Table 3.16. The AHPA extract of *M. germanica* leaf gave the highest DPPH• and NO• scavenging activities, Fe^{3+} reducing power, and had the highest FRAP value. The AHPA extract of *M. germanica* fruit had the highest •OH scavenging activity. Like the FPA and BHPA extracts, the Fe^{2+} binding activity of *N. sativa* seed AHPA extract was the strongest.

The •OH scavenging assay showed no significant difference ($P>0.05$) in the mean values of *N. sativa* seed and *D. lotus* fruit, but this mean difference differed significantly ($P<0.05$) with those of *M. germanica* leaf, *M. germanica* fruit and *D. lotus* seed. Furthermore, no significant difference ($P>0.05$) was observed in the DPPH• mean values of *N. sativa* seed and *D. lotus* seed, and this value differed significantly ($P<0.05$) with the remaining plant samples.

Table 3.16 Antioxidant activity of AHPA extracts

	DPPH•	**•OH**	**NO•**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. saiva seed	100.02±1.74^{a}	13.52±0.20^{a}	69.27±1.25^{a}	54.61±0.73^{a}	111.49±1.30^{a}	200.39±1.64^{a}
M. germanica leaf	62.17±1.13^{b}	11.39±0.15^{b}	27.32±0.30^{b}	61.39±0.77^{b}	284.26±1.53^{b}	346.84±2.42^{b}
M. germanica fruit	124.89±1.80^{c}	10.71±0.12^{c}	73.09±1.14^{c}	85.65±1.57^{c}	141.12±1.11^{c}	117.50±1.63^{c}
D. lotus seed	95.99±1.17^{a}	12.62±0.19^{d}	80.46±1.12^{d}	220.78±2.22^{d}	151.16±1.41^{d}	68.89±0.67^{d}
D. lotus fruit	135.54±1.66^{e}	13.08±0.19^{a}	108.57±1.08^{e}	164.57±1.83^{e}	101.53±1.64^{e}	26.74±0.16^{e}

Ferulic and sinapic acids identified by the HPLC could be responsible for the highest DPPH• and NO• scavenging activities, and ferric ion reducing capability observed in the AHPA extract of *M. germanica* leaf. These activities could be due to the electron-donating potential of the ferulic and sinapic acids to the oxidants. The high •OH quenching power of the AHPA extract of *M. germanica* fruit could be related to syringic and ferulic acids identified. The two phenolic acids possess hydroxyl groups in their structure, so they can scavenge •OH through proton donation.

3.5.9 BPAH-1 Extracts

The results antioxidant activity of BPAH-1 extracts of all the tested plants are shown in Table 3.17. The BPAH-1 extract of *D. lotus* fruit had the greatest DPPH$^{\bullet}$ scavenging potential, Fe^{3+} reducing power, and had the highest FRAP value. The BPAH-1 extract of *D. lotus* seed had the highest $^{\bullet}OH$ and NO$^{\bullet}$ scavenging activities. The Fe^{2+} binding activity of the BPAH-1 extract of *M. germanica* fruit was superior.

The differences in the mean values of Fe^{2+} binding and FRAP assays of all the five samples were statistically significant ($P<0.05$). In the DPPH$^{\bullet}$ scavenging assay, no significant difference ($P>0.05$) was observed between *D. lotus* seed and fruit, and this value differed significantly ($P<0.05$) with the remaining plant samples. Similarly, no significant difference ($P>0.05$) was observed in the mean values of $^{\bullet}OH$ scavenging assay of *N. sativa* seed and *M. germanica* leaf. Likewise, no significant difference ($P>0.05$) was observed in the mean values of Fe^{3+} reducing power of *N. sativa* seed and *M. germanica* fruit. Furthermore, no significant difference ($P>0.05$) was observed between the fruits of *M. germanica* and *D. lotus* of NO$^{\bullet}$ scavenging assay.

Table 3.17 Antioxidant activity of BPAH-1 extracts

	DPPH$^{\bullet}$	**$^{\bullet}OH$**	**NO$^{\bullet}$**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	105.24±1.31^{a}	13.44±0.16^{a}	46.68±0.56^{a}	57.03±1.00^{a}	173.59±1.45^{a}	210.48±1.50^{a}
M. germanica leaf	112.39±1.26^{b}	13.37±0.18^{a}	70.97±0.73^{b}	70.55±1.41^{b}	162.62±1.59^{b}	162.33±0.88^{b}
M. germanica fruit	79.07±1.05^{c}	11.71±0.13^{c}	58.62±0.60^{c}	50.47±1.05^{c}	179.64±1.54^{a}	401.14±2.18^{c}
D. lotus seed	45.49±0.64^{d}	9.28±0.13^{d}	43.47±0.47^{d}	181.26±2.09^{d}	480.70±3.31^{d}	549.84±2.57^{d}
D. lotus fruit	45.42±0.68^{d}	11.76±0.15^{c}	57.07±0.54^{c}	195.64±2.52^{e}	1011.22±3.33^{e}	856.79±2.87^{e}

Gallic and chlorogenic identified in the BPAH-1 extract of *D. lotus* fruit, and 4-hydroxybenzoic, gallic, protocatechuic, p-coumaric identified in the BPAH-1 extract of *D. lotus* seed could be responsible for the highest radical scavenging activity and Fe^{3+} to Fe^{2+} transformation ability observed in the two samples. These compounds, particularly gallic acid, were found in relative amounts.

3.5.10 BPAH-2 Extracts

The results antioxidant activity of BPAH-2 extracts of all the tested plants are shown in Table 3.18. The BPAH-2 extract of *D. lotus* seed had the highest DPPH$^{\bullet}$ and NO$^{\bullet}$ scavenging activities, Fe^{3+} reducing power, and had the highest FRAP value. *D. lotus* fruit extract had the highest $^{\bullet}$OH scavenging activity. *M. germanica* had the highest ferrous ion chelating activity.

Table 3.18 Antioxidant activity of BPAH-2 extracts

	DPPH$^{\bullet}$	**$^{\bullet}$OH**	**NO$^{\bullet}$**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	98.14±1.27^{a}	13.95±0.14^{a}	73.18±0.93^{a}	57.09±1.06^{a}	149.54±1.42^{a}	190.57±1.46^{a}
M. germanica leaf	79.30±1.13^{b}	12.94±0.15^{b}	73.27±1.02^{a}	53.56±0.90^{a}	455.44±2.38^{b}	112.63±0.85^{b}
M. germanica fruit	129.84±1.36^{c}	12.74±0.10^{b}	117.35±1.34^{b}	56.36±1.10^{a}	64.90±1.32^{c}	17.33±0.07^{c}
D. lotus seed	47.68±0.62^{d}	13.53±0.14^{d}	24.16±0.15^{c}	225.02±1.43^{d}	465.51±2.59^{d}	533.36±3.29^{d}
D. lotus fruit	49.60±0.91^{d}	11.82±0.11^{e}	50.72±0.59^{d}	81.05±1.05^{e}	320.75±2.03^{e}	403.41±3.40^{e}

The mean values of ferric ion reducing power and FRAP assays of all the five samples were statistically significant ($P<0.05$). No significant differences ($P>0.05$) were observed between *D. lotus* seed and fruit of the DPPH$^{\bullet}$ scavenging assay, between the *M. germanica* leaf and fruit of the $^{\bullet}$OH scavenging assay, and between *N. sativa* seed and *M. germanica* leaf of the NO$^{\bullet}$ scavenging assay. Likewise, no significant difference ($P>0.05$) was observed in the mean values of the ferrous ion chelating of *N. sativa* seed, *M. germanica* leaf and fruit.

3.5.11 BPBH-1 Extracts

Antioxidant activity of BPBH-1 extracts of all the tested plants as determined by the DPPH$^{\bullet}$, $^{\bullet}$OH, NO$^{\bullet}$ scavenging, Fe^{2+} chelating, Fe^{3+} reducing power and FRAP assays are presented in Table 3.19. *D. lotus* seed indicated the highest DPPH$^{\bullet}$ and $^{\bullet}$OH, scavenging potential and ferric ion reducing potential. *M. germanica* leaf had the

highest FRAP value. *N. sativa* seed showed the highest NO• scavenging and ferrous ion chelating activities.

The differences in the mean values of NO• scavenging, Fe^{3+} reducing power and FRAP assays of all the five samples were statistically significant ($P<0.05$). In the •OH scavenging assay, the mean value of *M. germanica* leaf BPBH-1 extract differed significantly ($P<0.05$) with the remaining samples. Like the BPAH-2 extract, no significant difference ($P>0.05$) was observed in the mean values of the ferrous ion chelating of *N. sativa* seed, *M. germanica* leaf and fruit of the BPBH-1 extracts. Furthermore, the DPPH• result of *N. sativa* seed and *M. germanica* fruit showed no significant difference ($P>0.05$). Likewise, the DPPH• result of *M. germanica* leaf and *D. lotus* seed.

Table 3.19 Antioxidant activity of BPBH-1 extracts

	DPPH•	**•OH**	**NO•**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	131.82±1.21^{a}	12.31±0.19^{a}	24.14±0.15^{a}	46.80±0.70^{a}	107.45±0.86^{a}	69.31±1.08^{a}
M. germanica leaf	91.29±0.80^{b}	14.49±0.18^{b}	32.60±0.31^{b}	48.40±0.61^{a}	152.75±1.25^{b}	811.00±3.01^{b}
M. germanica fruit	133.82±1.40^{a}	12.17±0.08^{a}	54.93±0.66^{c}	48.32±0.52^{a}	102.63±0.93^{c}	87.02±0.78^{c}
D. lotus seed	88.86±0.52^{b}	11.65±0.07^{a}	71.64±0.59^{d}	71.55±0.95^{d}	168.00±1.28^{d}	118.64±1.37^{d}
D. lotus fruit	71.93±0.92^{e}	11.94±0.08^{a}	66.13±0.89^{e}	93.70±1.14^{e}	206.08±1.72^{e}	138.22±1.08^{e}

3.5.12 BPBH-2 Extracts

The results antioxidant activity of BPBH-2 extracts of all the tested plants are shown in Table 3.20. The BPBH-2 extract of *M. germanica* leaf had the highest •OH and NO• scavenging activities, Fe^{2+} chelating activity, and had the highest FRAP value. *D. lotus* fruit extract had the highest DPPH• scavenging activity and ferric ion reducing power.

FRAP assay on the BPBH-2 extracts showed a significant difference ($P>0.05$) among the mean of all five samples. DPPH• and NO• scavenging, and ferrous ion

chelating assays showed significant differences (P>0.05) among the mean of *D. lotus* seed and fruit, however, this value differed significantly with those of *N. sativa* seed, *M. germanica* leaf and *M. germanica* fruit. No significant difference (P>0.05) was observed in the mean values of the ferric ion reducing power assay of *N. sativa* seed, *M. germanica* leaf and fruit. In the ˙OH scavenging assay, the difference in the mean value of *N. sativa* seed and *M. germanica* leaf was not significant (P>0.05).

Table 3.20 Antioxidant activity of BPBH-2 extracts

	DPPH˙	**˙OH**	**NO˙**	**Ferrous ion chelating**	**Ferric ion reducing power**	**FRAP**
	IC_{50} (µg/mL)				mg VCE per g DW	
N. sativa seed	127.94±1.18^{a}	8.81±0.07^{a}	39.45±0.45^{a}	50.94±0.83^{a}	123.49±1.37^{a}	114.08±0.95^{a}
M. germanica leaf	92.76±1.05^{b}	8.60±0.06^{a}	34.38±0.46^{b}	49.43±0.57^{a}	127.08±1.23^{a}	156.33±1.33^{b}
M. germanica fruit	110.85±1.07^{c}	11.81±0.09^{c}	105.17±1.07^{c}	245.82±2.17^{c}	120.68±1.12^{a}	96.80±0.80^{c}
D. lotus seed	87.41±0.59^{d}	16.48±0.12^{d}	68.91±0.77^{d}	94.70±0.91^{d}	148.33±1.37^{d}	82.87±0.74^{d}
D. lotus fruit	86.10±0.70^{d}	11.77±0.08^{c}	68.67±0.63^{d}	93.83±0.83^{d}	180.81±1.59^{e}	145.77±1.35^{e}

3.6 *In vitro* Anticancer Activities

MTT assay was used to investigate the *in vitro* cytotoxicity of flavone, flavanone, flavonol, flavan-3-ol methanol, flavan-3-ol acid, FPA, BHPA, AHPA, BPAH-1, BPAH-2, BPBH-1 and BPBH-2 extracts of all the five samples were tested against HeLa and HepG2 cell lines. HeLa cells are cancer cells lines extracted from the human cervix, while HepG2 cells are extracted from the human liver. MTT assay is employed to evaluate the cellular metabolic activity and it is based on the ability of the cellular mitochondrial dehydrogenase enzyme to reduce the yellow MTT into purple formazan crystals (Morgan et al., 1998; Ogbole et al., 2017). The effect of the extracts at different concentrations on HeLa and HepG2 cell lines were tested for 24 h and 48 h. The IC_{50} was obtained after the percentage inhibition of cancer cells by the extract was plotted against its corresponding concentration. A lower IC_{50} value signifies higher cytotoxic effects. The IC_{50} results are expressed in µg/mL. The IC_{50} value of 200 µg/mL and above are not taken into account and are symbolized with (-) in the tables. Similarly, NA indicates “analysis was not carried out”.

The results of cytotoxicity of the various extracts on HeLa cell lines 24 h after treatments are shown in Table 3.21. The highest cytotoxic activities of *N. sativa* seed on HeLa cell lines 24 h after treatments were observed in AHPA (IC_{50} = 59.72 µg/mL) and BHPA (IC_{50} = 71.47 µg/mL) extracts. The highest cytotoxic activity of *M. germanica* leaf was observed in BPAH-1 extract (IC_{50} = 36.24 µg/mL). This activity was the highest observed among all the extracts on HeLa cell lines after 24 h. For the *M. germanica* fruit, BPAH-2 extract showed the highest effect (IC_{50} = 93.44 µg/mL). Furthermore, the highest cytotoxic activities of *D. lotus* seed and fruit were observed in FPA (IC_{50} = 72.47 µg/mL) and BPAH-1 (IC_{50} = 72.47 µg/mL) extracts, respectively.

Table 3.21 Cytotoxicity of plant extracts on HeLa cell lines after 24 h

Extracts	***N. sativa* seed**	***M. germanica* leaf**	***M. germanica* fruit**	***D. lotus* seed**	***D. lotus* fruit**
	IC_{50} (µg/mL)				
Flavone	145.65	-	-	NA	-
Flavanone	-	-	-	NA	NA
Flavonol	153.96	-	-	NA	-
Flavan-3-ol methanol	-	188.27	149.57	-	-
Flavan-3-ol acid	136.28	110.98	104.78	160.52	199.72
FPA	188.11	145.43	-	72.47	190.84
BHPA	71.47	166.39	-	120.06	-
AHPA	59.72	-	-	-	-
BPAH-1	-	36.24	142.99	-	86.57
BPAH-2	-	145.88	93.44	-	-
BPBH-1	156.16	142.15	-	-	-
BPBH-2	-	-	177.96	119.26	-

The cytotoxicity effects of various extracts of *N. sativa* seed, *M. germanica* leaf, *M. germanica* fruit, *D. lotus* seed and *D. lotus* fruit at different concentrations on HeLa cell lines 48 h after treatments are presented in Table 3.22. Flavan-3-ol acidic, FPA, BHPA and BPAH-2 extracts of *N. sativa* seed; flavone and FPA extracts of *M. germanica* leaf; flavonol extract of *M. germanica* fruit; flavan-3-ol acidic, FPA and BHPA extracts of *D. lotus* seed; flavan-3-ol acidic and BPAH-1 extracts of *D. Lotus* fruit showed the highest cytotoxicity effect on HeLa cell lines 48 h after treatments. The IC_{50} of these extracts were less than 100 µg/mL.

Different extracts of the five samples exhibited good cytotoxicity effects on HeLa cell lines 48 h after treatments in comparison with the earlier work (Puspitasari et al., 2015). Puspitasari et al. (2015) obtained an IC_{50} of 467 µg/mL 48 h after treating HeLa cell lines (~ 1.0 x 10^6 cells/mL per well) with the ethanolic extract of *Arcangelisia flava* leaves. Additionally, the current research showed a better IC_{50} than the previous work of Baharum et al. (2014). In comparison with the present study, the researchers obtained higher IC_{50} (low activities) from the *Theobroma cacao*. The researchers treated HeLa cell lines (~ 1.0 × 10^5 cells/mL per well) with the methanolic extracts of various parts of *Theobroma cacao* for 48 h. The obtained IC_{50} were as follows: root (321.7 µg/mL), husk (372.7 µg/mL), leaf (430.7 µg/mL), unfermented shell (468.3 µg/mL), bark (688.7 µg/mL) and pith (868.0 µg/mL).

Table 3.22 Cytotoxicity of plant extracts on HeLa cell lines after 48 h

Extracts	***N. sativa* seed**	***M. germanica* leaf**	***M. germanica* fruit**	***D. lotus* seed**	***D. lotus* fruit**
	IC_{50} (µg/mL)				
Flavone	-	40.00	-	NA	102.11
Flavanone	-	-	-	NA	NA
Flavonol	-	-	99.61	NA	-
Flavan-3-ol methanol	-	-	108.23	182.08	-
Flavan-3-ol acid	88.89	108.13	173.79	71.04	98.85
FPA	99.23	79.99	-	62.43	137.02
BHPA	77.45	-	158.83	87.03	-
AHPA	107.26	-	-	131.03	143.43
BPAH-1	-	-	110.31	142.48	53.37
BPAH-2	88.98	-	104.25	142.61	125.78
BPBH-1	-	-	-	114.28	159.26
BPBH-2	105.00	-	125.52	150.55	149.03

The results of *in vitro* cytotoxicity activities of the various extracts on HepG2 cell lines 24 h after treatments are presented in Table 3.23. Most of the extracts showed a good cytotoxic effect (IC_{50} < 100 µg/mL). The best IC_{50} values obtained from each of the five samples are as follows: 59.52 µg/mL in BPAH-1 extract of *N. sativa* seed, 50.94 µg/mL in AHPA extract of *M. germanica* leaf, 48.53 µg/mL in BHPA extract

of *M. germanica* fruit, 63.42 µg/mL in BPAH-2 extract of *D. lotus* seed, 44.79 µg/mL in BPBH-2 extract of *D. lotus* fruit.

Table 3.23 Cytotoxicity of plant extracts on HepG2 cell lines after 24 h

Extracts	***N. sativa* seed**	***M. germanica* leaf**	***M. germanica* fruit**	***D. lotus* seed**	***D. lotus* fruit**
	IC_{50} (µg/mL)				
Flavone	184.50	53.54	-	NA	66.57
Flavanone	87.82	-	-	NA	NA
Flavonol	80.86	113.61	161.92	NA	-
Flavan-3-ol methanol	66.68	67.79	61.81	130.41	-
Flavan-3-ol acid	148.85	150.38	133.30	-	85.90
FPA	66.82	53.14	-	95.11	59.64
BHPA	-	-	48.53	117.12	-
AHPA	63.12	50.94	54.38	207.04	101.44
BPAH-1	59.52	71.85	-	198.16	150.46
BPAH-2	68.68	65.47	69.53	63.42	44.79
BPBH-1	83.99	156.40	123.95	99.97	124.41
BPBH-2	104.60	71.06	52.16	66.73	46.32

The results of cytotoxicity assay of the various extracts on HepG2 cell lines 48 h after treatments are presented in Table 3.24. AHPA and BPBH-1 extracts of *N. sativa* seed; FPA, BHPA and AHPA extracts of *M. germanica* leaf; flavonol and flavan-3-ol methanolic extracts of *M. germanica* fruit; and flavonol, BPAH-2 and BPBH-2 extracts of *D. lotus* fruit did not show any cytotoxic effects at testing range (20-80 µg/mL). However, the remaining extracts of each of the five plant samples showed good cytotoxicity on HepG2 cell lines.

The current study showed an excellent cytotoxicity effect on HepG2 cell lines 48 h after treatments in comparison with the previous work of Baharum et al. (2014). The researchers treated HepG2 cell lines (~ 1.0×10^5 cells/mL per well) with the methanolic extracts of various parts of *Theobroma cacao* for 48 h and obtained the following IC_{50}: root (237.3 µg/mL), husk (396.0 µg/mL), cherelle (427.3 µg/mL), unfermented shell (464.3 µg/mL), leaf (493.3 µg/mL), bark (828.3 µg/mL) and pith (951.0 µg/mL).

Table 3.24 Cytotoxicity of plant extracts on HepG2 cell lines after 48 h

Extracts	***N. sativa* seed**	***M. germanica* leaf**	***M. germanica* fruit**	***D. lotus* seed**	***D. lotus* fruit**
	IC_{50} (µg/mL)				
Flavone	98.93	36.49	64.67	NA	48.91
Flavanone	72.74	76.29	86.34	NA	NA
Flavonol	50.36	40.00	-	NA	-
Flavan-3-ol methanol	70.97	92.00	-	33.89	33.62
Flavan-3-ol acid	24.91	61.10	79.49	80.00	40.00
FPA	47.87	-	46.36	80.00	40.00
BHPA	58.27	-	60.30	45.96	85.69
AHPA	-	-	69.59	41.94	111.28
BPAH-1	66.96	45.42	75.96	41.89	36.43
BPAH-2	31.25	84.40	42.12	32.46	-
BPBH-1	-	60.47	40.00	57.23	105.42
BPBH-2	52.13	84.30	80.00	120.60	-

CHAPTER FOUR
CONCLUSIONS

Turkey is a rich source of edible plants among which include *Nigella sativa, Mespilus germanica* and *Diospyros lotus*. These plants can be found wild in various regions of Turkey. *N. sativa, M. germanica* and *D. lotus* can be utilized to produce different natural products that have biological significance. Extracts of them were used over the years in traditional medicine. Taken this into consideration the current research aimed to extract and profile flavonoids and phenolic acids from *N. sativa* seed, *M. germanica* leaf and fruit, *D. lotus* seed and fruit, evaluate and compare the antioxidants and cytotoxic potential of the extracts.

In the first stage of this study, the dry samples of three plants were lyophilized to remove any moisture present in the samples, milled and defatted with n-Hexane in a Soxhlet extractor. The purpose of this extraction was to remove liposoluble substances from the samples. Afterwards, the defatted samples were subjected to flavonoids, phenolic acids and total polyphenol content extractions. Flavone, flavanone, flavonol, and flavan-3-ols were extracted with ether, 80% ethanol, acidified methanol, and methanol, ether and also ethyl acetate, respectively. Phenolic acids were extracted with methanol. The CME and residues were subsequently subjected to further extractions yielding seven extracts: FPA, BHPA, AHPA, BPBH-1, BPAH-2, BPAH-1 and BPBH-2. Total polyphenol content extractions were done using methanol-water (3:1, v/v) in a Soxhlet apparatus.

In the second stage, the obtained extracts were evaporated to dryness using a rotary evaporator at reduced pressure, concentrated in a fume hood, freeze-dried and lyophilized. The extractions yields were then determined. *M. germanica* leaf gave the highest yield from flavone, FPA, BPAH-2 and BPAH-2 extractions. *M. germanica* fruit gave the highest yield from flavanone, flavonol, flavan-3-ols methanolic, BHPA, BPAH-1, BPAH-2 and total polyphenol content extractions. *D. lotus* fruit gave the highest yield from flavan-3-ols acidic and AHPA extractions.

In the third stage, the lyophilized extracts were reconstituted in methanol (1000 ppm) and subjected to HPLC analysis. Five flavonoids (hesperidin, quercitrin, epicatechin, epigallocatechin and epigallocatechin gallate) and fourteen phenolic acids compounds (benzoic, 4-hydroxybenzoic, gallic, protocatechuic, rosmarinic, syringic, vanillic, chlorogenic, caffeic, ferulic, sinapic, o-coumaric, p-coumaric and t-cinnamic acids) were identified and quantified from the various extracts of *N. sativa*, *M. germanica* and *D. lotus.* Most of the phenolic acids identified were found in significant amounts in bound phenolic extracts.

In the fourth stage, the same extracts used in HPLC analysis were diluted to different concentrations and subjected to antioxidants activity determinations by the DPPH$^{\bullet}$, $^{\bullet}$OH, NO$^{\bullet}$ scavenging, ferrous ion chelating, ferric ion reducing power and FRAP assays. BPAH-1 extract of *D. lotus* fruit, BPBH-2 extract of *M. germanica* leaf, flavan-3-ol methanolic of *M. germanica* leaf showed the highest DPPH$^{\bullet}$ (IC_{50} = 45.42±0.68 µg/mL), $^{\bullet}$OH (IC_{50} = 8.60±0.06 µg/mL), NO$^{\bullet}$ scavenging effects (IC_{50} = 22.53±0.52 µg/mL), respectively. The ferrous ion chelating effect (IC_{50} = 43.99±1.29 µg/mL) of *N. sativa* seed flavone extract was superior. Furthermore, the BPAH-1 extract of *D. lotus* fruit had the highest ferric ion reducing power (1011.22±3.33 mg VCE/g DW) and FRAP value (856.79±2.87 mg VCE/g DW).

In the fifth stage, the lyophilized total polyphenol extracts were reconstituted in 80% methanol (1000 ppm) and subjected to total phenolic content, total flavonoid content and total tannin content analyses. The TPC, TFC and TTC were determined by FCM, $AlCl_3$ and vanillin/HCl methods, respectively. *M. germanica* leaf extract gave the highest TPC (529.44±2.27 mg GAE/g DW), TFC (66.74±5.31 mg QE/DW) and TTC (355.95±1.86 mg TAE/g DW).

In the final stage of this study, the lyophilized flavonoids and phenolic acids extracts were reconstituted in 10% (v/v) DMSO (90% ddH_2O) to obtain a stock concentration of 800 ppm. Working solutions of 20 ppm (0.25% DMSO), 40 ppm (0. 5% DMSO) and 80 ppm (1% DMSO) were then obtained. The working solutions were used in MTT assays to observed the *in vitro* cytotoxic effects of the extracts on HeLa

and HepG2 cell lines at 24 and 48 h after treatments. Most of the extracts showed good cytotoxic effect (IC_{50} < 100 µg/mL). Precisely, the cytotoxic activity of the extracts on the HepG2 cell line was much better than that of HeLa.

Significant antioxidant and cytotoxic activities observed from the different extracts could be attributed to the flavonoids and phenolic acids identified. This research revealed *N. sativa*, *M. germanica* and *D. Lotus* as an important sources of natural compounds with biological properties, and could also provide a valuable approach for developing novel antioxidants and anticancer agents from the three plants. In addition, it contributes to revealing the potential of plant biodiversity in terms of determining the phenolic components, evaluating their antioxidant and anticancer properties of three plants from Turkey.

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