Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i8.7536

PHYTOCHEMICAL EVALUATION OF THE ANTIOXIDANT POTENTIAL OF *OLEA FERRUGINEA* ETHYL ACETATE FRACTION IN OXIDATIVE STRESS-INDUCED CARDIAC HYPERTROPHY RAT MODEL

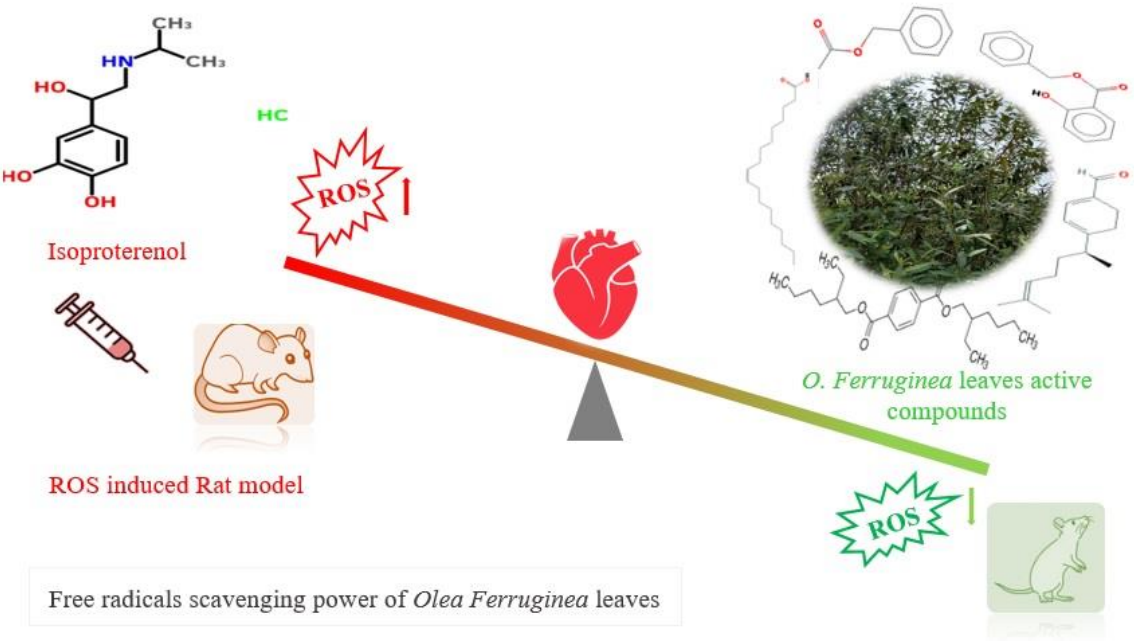
Noureen Sharif [1], Sobia Tabassum[1*], Iram Murtaza[2], Naseer Ahmed[3], Gul Shahnaz[4], Abbas Rahdar[5], Sadanand Pandey[6,7], Nagina Atlas[1]

[1*]Department of Biological Sciences, Faculty of Sciences, International Islamic University, Islamabad, Pakistan.
[2]Department of Biochemistry, Faculty of Biological Sciences, Quai-i-Azam University, Islamabad, Pakistan.
[3]Department of Pharmacology, Rehman College of Dentistry, Rehman Medical Institute, Peshawar, Pakistan
[4]Department of Pharmacy, Quaid-i-Azam University Islamabad, 45320, Pakistan,
[5]Department of Physics, Faculty of Sciences, University of Zabol, Zabol 538-98615, Iran,
[6]Department of Chemistry, College of Natural Science, Yeungnam University, 280 Daehak-Ro, Gyeongsan, Gyeongbuk, 38541, Republic of Korea.
[7]School of Bioengineering and Food Technology, Faculty of Applied Sciences and Biotechnology, Shoolini University, Solan 173229, Himachal Pradesh, India

***Corresponding Author:** Sobia Tabassum**:**
***Email:** *sobia.tabasum@iiu.edu.pk*

Abstract

Reactive oxygen species (ROS) are a common contributor to many diseases, particularly heart failure / cardiac hypertrophy. Phytochemicals are considered key components to counteract ROS and can be utilized in treatment without side effects. The study aimed at phytochemical screening of *O. ferruginea* ethyl acetate fraction (EAF) and evaluation of its antioxidant capacity using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2) antioxidant assay, and *in vivo* oxidative stress-induced Cardiac Hypertrophy (CH) rat model. Results of this study showed strong potential for scavenging ROS with IC_{50} value 43.89±1.23 µg/ml) with DPPH and H_2O_2 along with the presence of higher Total Phenolic Content (TPC) (560 ±8.3), and Total Flavonoid Content (TFC) (430±4.7) contents. Gas Chromatography-Mass Spectrometry (GC-MS) analysis translated most of the compounds. Further in vitro results were validated by *in vivo, an* antiradical scavenging power estimation study on the oxidative stress-induced CH rat model with a significant reduction in H_2O_2 levels (3.5 fold) versus the diseased control group (7 fold) ($p < 0.05$). This study concludes that *O. ferruginea* may serve as an anti-oxidant pool of natural active compou nds that can be utilized for further biochemical and molecular analysis to control Cardiac hypertrophy.



Graphical Abstract

Keywords: *Olea ferruginea,* Ethyl acetate fraction, Antioxidant activity, Gas Chromatography-Mass Spectrometry, Cardiac Hypertrophy,

Introduction

Phytochemicals, also referred to as plant-derived natural products, are compounds created by plants that have biological activity and are suitable for pharmacological investigations [1]. They include both primary as well as secondary components, like carbohydrates, and amino acids in addition to primary components like glucose. These phenolic chemicals, in particular polyphenols, are thought of as exogenous natural antioxidants because of their antioxidant capabilities [2-4]. These substances have enormous medicinal and antioxidant potential[5]. Several investigations have revealed the potential of antioxidants found in a variety of plant extracts high in polyphenolic components to mitigate ROS illnesses like cardiovascular, Alzheimer's, diabetes, and intestinal diseases[6-8]. Excess of ROS production in the heart can cause the formation and progression of maladaptive Cardiac hypertrophy (CH) leading to heart failure. ROS directly impairs the electrophysiology and contractile mechanism of cardiomyocytes. Additionally, ROS causes a deficit in energy by interfering with proteins involved in energy metabolism function[9].

Natural products containing bioactive compounds have shown promising results in preventing and treating cardiovascular diseases by fighting oxidative stress, inflammation, and cell death. Among the benefits of using natural compounds as nutraceutical agents are their inexpensive cost and relative safety[10]. Further research is needed to explore the therapeutic potential of phytochemicals and plant-based whole foods.

The family of olive trees and their relatives, known as *Oleaceae*, are deciduous trees and shrubs. This family of plants has long been valued for its traditional, medicinal, and nutraceutical applications. Antioxidants contained in these plants may also be used to both prevent and cure disease practice[11, 12].

Olea ferruginea also known as Kahoo locally or Indian olive in general is a species of broadleaved evergreen tree. The plant is found throughout Pakistan, including the Hindukush, the Salt Range, the Himalayas, the Suleiman Ranges, and the Kala Chitta Hills[13], Baluchistan[14], Khyber Pakhtoon Khawa[15] and lower hills of Azad Kashmir district Sudhnoti [16] and district Kotli[17].

Traditionally, tea was made from *O. ferruginea* leaves. It was specifically used to treat skin conditions, colds, flu, and coughs[18]. Chewing on young leaves helps prevent mouth sores and

toothaches. As Miswak (tooth cleaning), young branches are utilized. The wood is widely used and incredibly durable. Its long logs are employed in roof thatching as guards[17].

Pharmacological activities of *O. ferruginea* have been discovered including strong antioxidant activity in vitro[19, 18, 20]. Antibacterial [21], anti-viral [22] anti-diabetic[23] Anticancer activity[24], anti-oxidant activity[19, 18, 20], anti-inflammatory[23], anti-microbial[25], antifungal[26], Insecticidal[21].

In Pakistan, there are still plant species with limited phytochemical investigations. *O. ferruginea* usage in traditional medicine has been reported earlier but limited investigations are known regarding phytochemical screenings or *in vivo* antioxidant potentials. In the current study crude extract and ethyl acetate fractions of *O. ferruginea* plant were screened, characterized, and assessed for free radical scavenging capacity using DPPH and H_2O_2 antioxidant assays. The current study also examined the anti-oxidant impact of test plant EAF on isoproterenol (ISO) induced ROS in the CH rat model.

Methods

Olea Ferruginea Collection and Identification

Olea Ferruginea plant leaves (**Figure 1**) were collected from the District Poonch region Azad Jammu & Kashmir. A Botanist from the Department of Environmental Sciences, International Islamic University Islamabad, identified by the plant as *Olea Ferrugina* and granted voucher number IIUI/DES/EBL/002-2020.

Post Collection Treatment

For the extraction of phenolic compounds, leaves were washed, air dried under shade, and then processed via a grinder into tiny fragments using an electric grinder.

Plant Extraction

Extraction of medicinal plant's coarsely ground leaves has been carried out by the Maceration Method[18] (**Figure 1**) using water, 80% ethanol, 80% methanol, and n-hexane as solvent system. 50 g of dry plant material was weighed using a balance before being placed in a 500 mL beaker containing 450 mL of 80% methanol solvent. The same step was repeated for other solvents as well. After this, to macerate, the mixture was kept for nearly 5 days inside the shaking incubator at 37°C for 05 days for intermittent shaking. The filtrate solution was then made by filtering the *O. ferruginea* extract through muslin cloth and Whattman no. 1 filter paper. For additional examination, the filtrate was kept at 4°C and also filtrate was concentrated using a rotary evaporator (Heidholph Laborota 4000-efficient Germany and Buchi Rotavapor R-20) under reduced pressure and controlled temperature below 45 ^{0}C. After that, a thick, semi-solid, sticky mass that was brownish-black in color was obtained and put in the oven. (Memmert Beschichung Loading Model 100-800). The dried material was weighed and labeling of semi-solid material was carried out, and then stored in an airtight container in a refrigerator at 4 °C.T. The condensed extract % yield was computed and subjected further for additional testing.

Phytochemical Analysis of Extract

Plant extracts' phytochemical components were screened using the industry-standard method[27, 28].

Test for Phenols

1 ml of plant extract solution was mixed with 2 ml of distilled water. The dark green was observed with the addition of a few drops of 10% Ferric chloride. This indicates the presence of phenols.

Test for Flavonoids

2-3 drops of NaOH (10%) were added to 1 ml extract solution. Appearance of yellow color and disappearance of color on adding dilute acid with blackish red precipitates indicates flavonoids.

Test for Tannins

A solution of 1 milliliter of plant extract and 2 milliliters of ferric chloride was combined. The appearance of a black color suggested the tannin's presence.

Test for Terpenoids

2.5 milliliters of extract and 2 milliliters of chloroform were combined to create a mixture. 1.5 ml of conc. Sulphuric acid was mixed with it. The appearance of a reddish-brown interface indicated the presence of terpenoids.

Test for steroids

10 ml chloroform and 10 ml conc. Sulphuric acid was mixed with 1 ml of plant extract. The red color upper layer and yellow color layer with green fluorescence in the acid layer indicated the presence of steroids.

Fractionation and Analysis of ethyl acetate Fraction (EAF)

Fractionation

Methanol extract of *O. ferruginea* was used for liquid-liquid fractionation[29, 30] (**Figure 1**). The extract was dissolved in distilled water. Solvent systems based on polarity from non-polar to polar in the order of hexane, chloroform, ethyl acetate, and water were selected. Ethyl acetate fraction was collected and dried first in a rotary evaporator and then again dried in an oven to remove any moisture content, weighed, and stored for further analysis.

Plant Extract and Fraction FTIR Analysis

FTIR is the most effective analytical technique for functional group identification and the detection of chemical bond presence in a substance. The semisolid extract and desiccated powder of the *O. Ferruginea* leaves EAF was analyzed using FTIR (BRUKER Model ALPHA FTIR spectrophotometer) in the 400–4000 cm–1 region to illustrate the chemistry and functional groups of the plant's phytochemicals.

Gas Chromatography-Mass Spectrometry (GCMS) Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) analysis is a powerful analytical technique widely used for identifying and quantifying the chemical composition of complex mixtures, including plant extracts and fractions. The GC component divides the individual substances based on their volatility in the sample mixture, while the MS component identifies and quantifies these compounds based on their mass-to-charge ratios.

GC-MS analysis was performed utilizing a combined 7890A gas chromatograph system (Agilent Technologies, model-5977B, USA), and a mass spectrophotometer outfitted with an HP-5 MS fused silica column (five percent phenyl methyl siloxane, 30.0 m × 250 μm, sheet thickness 0.25 μm), interfaced with a 5675C Inert MSD including a Triple-Axis detector. The relative percent amount of each component was ascertained by comparing its average peak area to the overall areas.

Component identification was accomplished using retention indices, and the National Institute of Standards and Technology database was used to interpret the mass spectrum (NSIT). The standard mass spectra of known components kept in the NIST library were compared with the acquired spectra of the unknown components of the *O. ferruginea* EAF fraction (NISTII).

Total Phenolic Content (TPC) Estimation

The method [31] and [32] was slightly modified to estimate the phenolic content. Specifically, 1,000 μL of freshly prepared 2 percent sodium carbonate and 100 μL of different extracts and fractions were mixed with 100 μL of Folin-Ciocalteu reagent at a concentration of 1 mg/ml. After carefully mixing the mixture, it was left in the dark for 30 minutes. The absorbance of the resultant mixture was measured at 750 nm using a spectrophotometer and a microplate reader (Multiskan GO, Thermo, Germany). The data were expressed as mg GAE/g, and the standard curve was created using gallic acid (Gallic acid equivalent).

Total Flavonoid Content Estimation

The approach of Park et al. (2008) [33] was used to compute the total flavonoid content (TFC). In a 10 ml test tube, 0.3 ml of extracts, 3.4 ml of 30% methanol, 0.15 ml of NaNO2 (0.5 M), and 0.15 ml of AlCl3.6H2O (0.3 M) were mixed. After five minutes, 1ml of 1 M NaOH was added. After thoroughly mixing the solution, the absorbance was measured in a microplate reader (Multiskan GO, Thermo, Germany) using spectrophotometry against the reagent blank at 506 nm. Using a rutin standard solution (0 to 100 mg/l), the standard curve for total flavonoids was created using the same method as previously mentioned. The milligrams of rutin equivalents per gram of dried fraction were used to denote the total flavonoids.

Extract/Fraction Antioxidant Power

DPPH Assay

By employing the DPPH assay following method[34], antioxidant activity was determined. A mixture of Sample (EAF/ Blank AC-NG/ EAF-AC-NG) and Methanol solution of DPPH were mixed respectively and kept for 30 minutes in the dark at 37°C. Ascorbic acid and DPPH solution in methanol were used as the positive control, and negative controls respectively. Following a 30-minute dark incubation period optical density of all samples was recorded at 517nm in a UV spectrophotometer microplate reader (Multiskan GO, Thermo, Germany). The following formula was used to compute the percentage of inhibition:

% Inhibition = Abs. of Control-Abs. of Sample/Abs. (control)*100

Hydrogen Peroxide (H_2O_2) Scavenging Power Estimation

The ability of the samples to scavenge hydrogen peroxide was examined using the following method[35]. Each extract's stock solutions (1000 µg/mL) were diluted with sterile distilled water to yield concentrations ranging from 31.25 to 500 µg/mL. Ascorbic acid was selected as a positive control. A 40 mM hydrogen peroxide solution was made in a 50 mM phosphate buffer (pH 7.4). Samples (0.4 mL) were added to a hydrogen peroxide solution in distilled water (0.6 mL). After 10 minutes of incubation, the mixture's absorbance was measured at 230 nm against a blank solution that contained phosphate buffer but no hydrogen peroxide. The percentage of scavenging activity was computed using the following formula:

Scavenging activity (%) = (1 − absorbance of sample extract or fraction/ absorbance of control) × 100. (2)

In vivo Study for Assessing Radical Scavenging Power

We purchased 24-week-old Sparuge Dowely rats from Quaid-i-Azam University in Islamabad, with a weight range of 180 to 280 grams. The Quaid-i-Azam University's Bio-Ethics Committee gave the study approval and issued an approval number (No. #BEC-FBS-QAU2019-184). Rats were randomly assigned to five groups of six animals each after seven days of acclimatization. Rats were included to create the ROS in the CH model by Isoproterenol (CH inducer) s.c. injection[36]. For fourteen days, ISO was given on alternating days, and other dosages were given every day. Experimental groups were **(Group 1)** Control (n = 6), **(Group 2)** Isoproterenol (ISO) 5mg/kg (n= 6), **(Group 3)** ISO + Ascorbic Acid (A.A) 80mg/kg (n = 6), **(Group 4)** ISO + Ethyl Acetate Fraction (EAF) 50 mg/kg (n= 6), **(Group 5)** ISO + Ethyl Acetate Fraction (EAF) 100mg/kg (n= 6). The animals were kept at the departmental animal house facility for 14 days in a climate-controlled, light-regulated environment with a 12-hour cycle of light and dark. On the fifteenth day, the animals were sacrificed. After obtaining heart blood, the rat's heart was removed, and all parts were kept in storage at -80°C for further examination. After centrifuging the blood for ten minutes at 4000 rpm to extract the serum, the serum was stored at -20 °C. Each tissue sample weighed 100 mg, and then it was minced in 333ul of ice-cold extraction solution (HEPES buffer) that contained 1 mM PMSF. The mixture was centrifuged for ten minutes at four degrees Celsius at 10,000 rpm and the obtained supernatant was kept at -20 °C. Supernatants were used for H_2O_2 measurement [37].

Statistical Analysis

One-way ANOVA was applied on data to analyze data by employing Graph pad, Prism.

Results

***Olea Ferruginea* Phytochemical Investigation and Characterization**

Extract Yield %

Medicinal plants are rich in bioactive compounds/biopharmaceuticals. These can be isolated on a polarity basis by using different organic solvents. *O. ferruginea* plant leaf extract (OLE) was prepared from leave parts by using hexane, water, ethanol, and methanol **Figure 1**.

Figure 1 ***O. ferruginea*** **leaves extraction and EAF preparation**

Extract yield was different in different solvents based on the nature of solvents. A minimum yield (0.12%) was observed in n-hexane solvent and a maximum yield (12.2%) was produced from 80% methanol (**Table 1**). The extract with the highest yield was selected for further studies.

Phytochemical Analysis

O. ferruginea leaves were investigated for phytoanalysis of pharmacologically important phytochemicals e.g. phenols, flavonoids, alkaloids, terpenoids, glycosides, and sterols. Results indicated the presence of secondary metabolites (**Table 2**).

Percent Yield of Ethyl Acetate Fraction (EAF)

Following the crude extract's fractionation, the percentage yields for the hexane and chloroform fractions were 7.2% and 9%, respectively, as shown in **Table 1**.

Total Phenolic Content and Total Flavonoid Content Estimation

The method [31] was modified in order to determine the total phenolic content in leaf extracts. **Table 3** presents the estimated total phenolic and total flavonoid content profile of the extract and its various fractions. The total phenolic content is expressed as gallic acid equivalent per gram of extract.

The maximum quantity of TPC was observed in EAF (560 ±8.3 mg GAE/g of dry fraction) followed by 80% ME (399±5.5 mg GAE /g extract). Chloroform fraction showed 317 ±8.9 mg GAE/g of dry fraction while the lowest quantity was isolated from n-Hexane (112±9.6mg GAE/g dry fraction). All fractions indicated significantly ($P < 0.05$) different TPC. For crude extract, the phenolic content of EAF was significantly higher (< 0.05).

Table 1 Percent Yield of *O. ferruginea* leaves Extract and Fraction in Different Solvents

Sample	**Solvent**	**% Yields**
	80% Methanol	12.2
	80% Ethanol	11
Extract	Distilled H2O	10
	n-Hexane	0.12
	Ethyl acetate	67
Fraction	Chloroform	7
	n-Hexane	0.12

Table 2 Phytochemical Analysis of Extract for Secondary Metabolites

Phenols	+
Cardiac Glycosides	+
Alkaloids	+
Flavonoids	+
Tannins	+
Terpenoids	+
Saponins	+

+ (Presence)

Table 3 Total Phenolic (TPC) and Total Flavonoid Content (TFC) of extract and Fractions

Extract/Fraction	TPC (mg GAE /g Extract/Fraction	TFC (mg RE /g Extract/Fraction
80% Methanol Extract	399±5.5	320±3.6
Ethyl Acetate Fraction	560 ±8.3	430±4.7
n-Hexane Fraction	112±9.6	213±2.8
Chloroform Fraction	317 ±8.9	260±6.3
P < 0.05		

Total flavonoid content was denoted as rutin equivalent per gram of extract/fraction. This study showed the highest TFC in EAF (430±4.7RE/g dry fraction), followed by 80% ME (320±3.6mg RE/g dry extract) > Chloroform fraction (260±6.3 mg RE/g dry fraction) > n-hexane fraction (213±2.8 mg RE/g dry fraction).

Fraction Fourier Transform Infra-Red Spectroscopy (FTIR) Analysis

To ascertain the chemistry of the chosen plant's phytochemicals, the dried powder of the EAF of *O. ferruginea* plant leaves was exposed to FTIR between 400–4000 cm^{-1} range (**Figure 2**). The EAF of *O. ferruginea* leaves revealed the following frequencies of functional groups/compounds: at 3752 cm^{-1} OH Stretching Vibration is seen, at 3377 cm^{-1} Polyphenols (O-H) were indicated, at 2912 cm^{-1} C-H/terpenoids/ carboxylic acids are observed, C=O (Flavonoids) at 1672 cm^{-1}, at 1439 cm^{-1} C-H/Terpenes/alkanes, at 1026 cm^{-1} are C-O Vibration in alcohol hydroxyl group, Alkyl amines, and C-Cl are indicated at 1018 cm^{-1} and 736 cm^{-1} respectively.

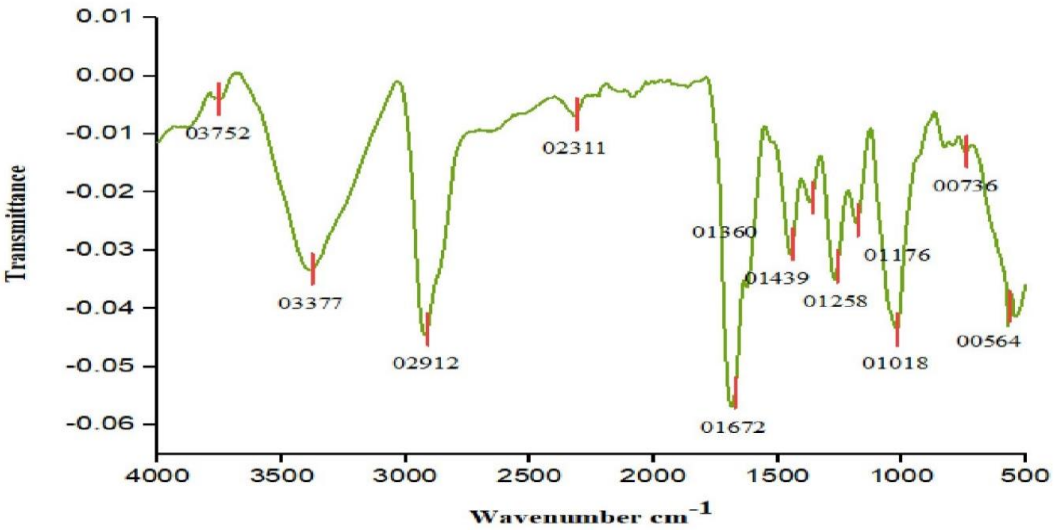


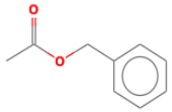
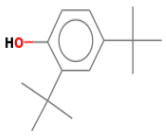
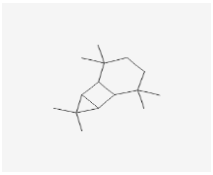
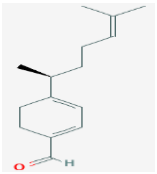
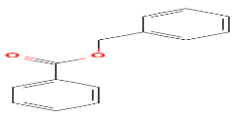
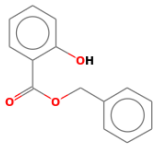
Figure 2 ***FTIR Analysis of Ethyl Acetate Fraction of O. ferruginea Leaves***

Gas Chromatography-Mass Spectroscopy (GCMS) Analysis

In the current study, EAF was subjected to GC-MS for the indication of volatile compounds in EAF. GCMS analysis is a powerful analytical technique widely used for identifying and quantifying the chemical composition of complex mixtures, including plant extracts. 15 compounds peaks including phenolic and alcoholic compounds, as well as fatty acids and esters.

The some of the identified bioactive substances in the EAF are as follows (also listed in **Table 4)**: 2,4-Di-tert-butyl-phenol, (-)-.gamma.-Curcumen-15-al (terpenoid), Ylangenal (terpenoid), Oleic Acid, 10E, Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, 12Z-Octadecadienoic acid (Linoleic acid), 9-Octadecenoic acid, (E) (eladic *acid),* Acetic acid, phenylmethyl ester, Glycerol 1-palmitate, 2,2,5,5,8,8-hexamethyl-, (1.alpha.,6.beta.,7.alpha.,9.alpha.), Benzyl

Table S4 GCMS Analysis Identified Compounds Ethyl Acetate Fraction (EAF) of *O. Ferruginea* leaves

S #	RT	Compound Name	Area %	CAS #	Structure	Medicinal properties	Reference
1	7.384	Acetic acid, phenylmethyl ester	4.49	00014 0-11-4		Antioxidant	[38] Nahar *et al.*, 2005
2	12.471	2,4-Di-tert-butylphenol	0.78	00009 6-76-4		Antioxidant	[39]Milaeva *et al.*, 2010
3	13.598	Tricyclo[4.3.0.0(7,9)]non-3-ene, 2,2,5,5,8,8-hexamethyl-, (1.alpha.,6.beta.,7.alpha.,9.alpha.)	1.66	05483 2-80-3		----------	----------
4	13.598	(-)-.gamma.-Curcumen-15-al Terpenoids	1.66	23542 1-68-8		Curcumin Family compound, Antioxidant	[40] Tuong *et al.*,· 2023
5	13.598	Ylangenal terpenoids	1.66	04161 0-68-8		Antioxidant	[41] Park *et al.*, 2020
6	14.088	Benzyl Benzoate	17.57	00012 0-51-4		antioxidant	[42] Ribeiro *et al.*, 2020
7	15.748	Benzoic acid, 2-hydroxy-, phenylmethyl ester	4.34	00011 8-58-1		Antioxidant	[43] Adeyemi *et al.*, 2023

Benzoate, Tricyclo[4.3.0.0(7,9)] non-3-ene, Benzoic acid, 2-hydroxy-, phenylmethyl ester, methyl ester, bis(2-ethylhexyl) ester, Cyclohexanone, Bis(2-ethylhexyl) phthalate, benzene-nitro, 1,4-Benzenedicarboxylic acid, Hexadecanoic acid, Benzyl Benzoate.

Reactive Oxygen Species Scavenging (ROS) Power

DPPH Assay

The results of testing extract and EAF fraction capacity to scavenge free radicals using the DPPH technique are shown in **Table 5**. Because of its fast rate of hydrogen acceptance, antioxidants react with DPPH, a nitrogen-centered radical with a distinctive absorption at 517 nm, to produce 1, 1-

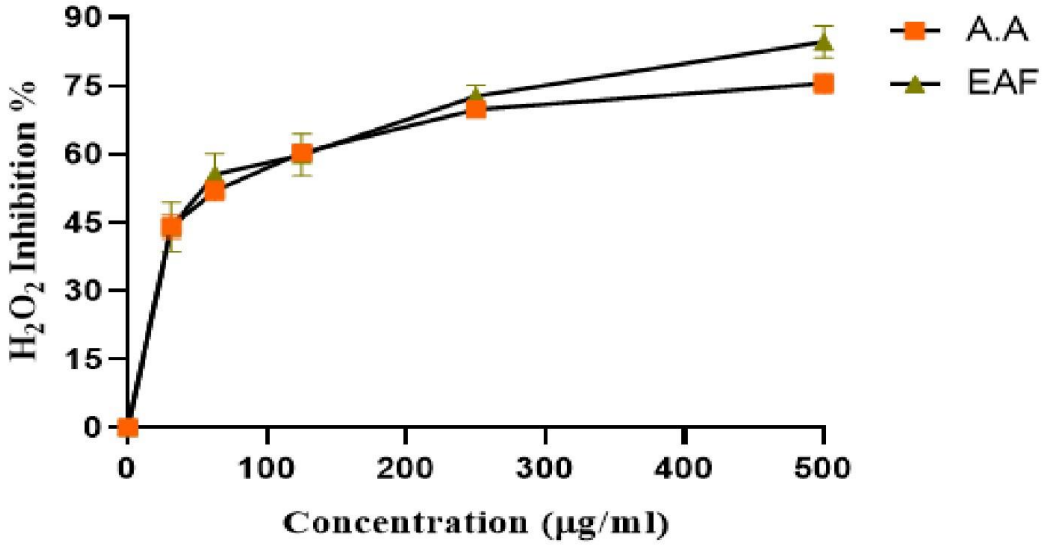
diphenyl-2-picryl hydrazine[44]. The reducing power of an alcoholic DPPH solution in the presence of a hydrogen-donating stimulant provides the basis for the DPPH assay[45]. Antioxidant scavenging potential is shown by the degree of discoloration. By using a stable free radical, this assay can reveal how reactive certain fractions are. When DPPH absorption is bleached, it indicates that a chemical has the ability to scavenge free radicals without the need for any enzymatic activity[46].
The 80%ME, 80%EE, ascorbic acid, and EAF, possessed the ability to use hydrogen-donating activity to neutralize DPPH-free radicals. IC_{50} value calculated for 80% ethanol and 80% Methanol was 100.40±2.676 µg/ml and 88.95±13.912 µg/ml respectively (**Table 5**). According to the findings, the concentration of inhibition (IC_{50}) of the fraction of olive leaf that produced 50% inhibition of the DPPH radical was found to be IC_{50} 43.90±1.24 µg/mL, a value that was less than that of ascorbic acid (55.34±3.07) (**Table 5**).

H_2O_2 scavenging assay
The human body, the air, water, plants, bacteria, and food all naturally contain low amounts of H_2O_2. It decomposes quickly into water (H_2O) and oxygen (O_2), and it may also generate hydroxyl

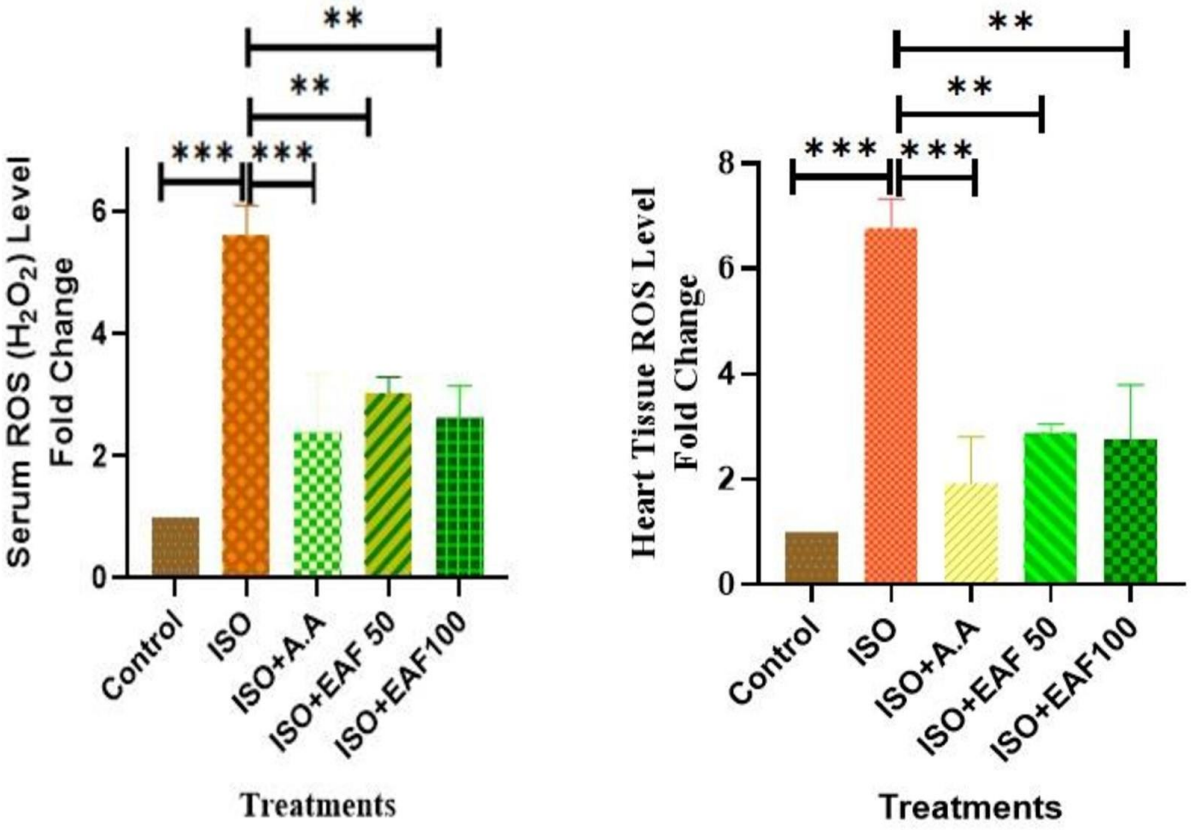
Table S5 DPPH Assay IC_{50} (µg/ml) different Extracts and EAF Fraction

	DPPH Assay	**H_2O_2 Assay**
Extract/Std/Fraction	***IC* $_{50}$ (µg/ml)**	
A.A	55.358±3.036	59.00279±3.89
80 % ME	88.95±13.912	----
80 % EE	100.40±2.676	----
Ethyl acetate Fraction	43.896±1.238	57.077±1.98

radicals (OH), which have the ability to damage DNA and start lipid peroxidation. The presence of phenolic groups in *O. ferruginea's* EAF fraction may have contributed to the fraction's ability to effectively scavenge hydrogen peroxide and neutralize it into H_2O by donating electrons to it. As demonstrated in **Figure 3a** the scavenging activity of several EAF fractions on hydrogen peroxide was concentration-dependent (31.25–500 µg/mL). The EAF showed a significant H_2O_2 scavenging activity (IC_{50} 57.077±1.98 µg/mL) whereas AA exhibited IC_{50} 59.003±3.89µg/mL (**Table 5**). There was no significant difference in the IC_{50} value of EAF with respect to standard ascorbic acid IC_{50} value $P < 0.05$. IC_{50} was used to express the scavenging potential of fraction (concentration of fraction having capability to scavenge 50 percent radical). The antioxidant activity of the samples increases with decreasing IC_{50}.



a H_2O_2 Assay, percentage inhibition of ascorbic acid and EAF



b i*n vivo O. ferruginea* EAF ROS scavenging power estimation

Figure 3 ***Estimation of Radical Scavenging Power of EAF****. **(a)** Measurement of H_2O_2 levels using H_2O_2 assay. **(b)** in vivo measurement of ROS levels in the serum and heart tissue in ISO-induced Cardiac Hypertrophy rat model (n = 6). Statistically significant differences are depicted as p-values & statistical analysis was conducted using one-way ANOVA followed by Tukey's test. (*): $p < 0.05$),*

(**): $p < 0.001$, (***): $p < 0.001$. Data are depicted as mean ± SE.

In vivo Measurement of EAF Antioxidant Potential

The ROS test was examined at 505 nm using the DEPPD method [47]. In the current *in vivo* study, ISO administration overproduced the ROS in serum and heart tissue homogenate **Figure 3b**. The heart H_2O_2 levels of rats treated with ISO showed a substantial increase ($p < 0.05$).

According to **Figure 3b,** the ISO group's serum ROS activity increased by 4.5 times compared to the Control. H_2O_2 levels in A.A., EAF50mg/kg, and EAF100mg/kg group were significantly reduced. Heart tissue ROS levels (**Figure 3b)** in Rats treated with ISO also demonstrated a significant enhancement ($p < 0.05$) of H_2O_2 levels. The H_2O_2 level was increased 5.5-fold in the ISO group when compared to the Control. In A. A and EAF, there was a significant decrease in cardiac H_2O_2 (folds) when compared to the ISO-induced CH group as depicted in **Figure 3b**. When comparing EAF and standard drug ascorbic acid there was no significant difference among H_2O_2 levels. EAF reversed the oxidative stress by 2.5 and 3.5 fold in serum and heart tissue respectively.

Discussion

Oxidative stress generated by Reactive Oxygen Species (ROS) is a notorious and common cause of different cardiovascular diseases including Cardiac Hypertrophy (CH). Due to side effects of conventional therapeutics, plant extracts, and fractions, a complex mixture of active compounds, are known to be safe antioxidant therapeutics in different diseases. In the current study underutilized tree, *Olea Ferruginea* leaves were selected for the characterization of active compounds and evaluated for *in vivo* antioxidant potential in ROS-induced cardiac Hypertrophy rat model.

In the current study, phytochemical screening results indicated the presence of phytochemicals e.g. phenols, flavonoids, alkaloids, terpenoids, glycosides, and sterols. Previous qualitative studies of the *O. ferruginea* plant also indicated the presence of flavonoids, phenolics, alkaloids, and terpenoids, glycosides, sterols, and saponins[48]. These substances have tremendous antioxidant and healing properties [5].

In the current study, 80% methanol extract has more radical scavenging as compared to 80 ethanol. Earlier studies reported that 80% Methanol antioxidative concentration is lower as compared to 80% ethanol [49]. In the current study *O. ferruginea* plant leaves 80% methanol extract was selected for further studies and was further subjected to fractionation with ethyl acetate, n-hexane, and chloroform. About 67% of the obtained fraction consisted of ethyl acetate. The ethyl acetate fraction of *O. ferruginea* Leaves (EAF) was chosen for further research because the percent yield of EAF is much higher. Another study reported the highest concentration and yield of ethyl acetate fraction in their study [46]. According to a prior study, extraction with the use of extremely polar solvents produced a higher yield of extract but with a lower phenolic & flavonoid content as compared to non-polar solvents. According to the study, using polar and nonpolar solvents in combination can improve phytochemical extraction efficiency with high antioxidant properties[50]. Ethyl acetate is a semi-polar solvent with both polar and nonpolar properties.

The results revealed that the EAF soluble fraction of plant leaves showed the highest total phenolic contents (560 ±8.3) when compared with other fractions. Our results correlate with previous scientific findings[48, 22]. This study showed the highest TFC in EAF (430±4.7RE/g dry fraction), followed by 80% ME (320±3.6mg RE/g dry extract). Our results tie back to other studies where extract and its derived fractions showed significant differences ($P < 0\ 05$) in terms of TFC[51]. The quantities of total phenolic and flavonoid components in natural product extracts are regarded as important factors for assessing the extract's quality and biological potential [52].

FTIR of fraction revealed the existence of primary amines, cardiac glycosides, nitriles, alkanes, polyphenols, and esters. These compound's presence has been reported in earlier studies[22]. Plants have a variety of pharmacological characteristics due to the existence of secondary metabolites including phenolic chemicals, steroids, glycosides, alkaloids, and terpenoids[53].

Different studies investigated the antioxidant properties of the bioactive compounds, also found in the

ethyl acetate fraction of *O. ferruginea e.g.* Ylangenal (terpenoid) [41], documented antioxidant properties of (-)-.gamma.-Curcumen-15-al (terpenoid) [40], Oleic Acid [54], 2,4-Di-tert-butylphenol[39], other compounds antioxidant properties documented in previous studies are listed in **Table 4.** Benzyl Benzoate esters benzyl benzoate, which is widely used in the food, cosmetics, agriculture, and pharmaceutical sectors. Our results suggested that *O. ferruginea* leaves are a valuable natural product source that possesses strong antioxidant capabilities. Phenolic compounds' ability to engage in metabolic oxidation-reduction reactions is mostly linked to their structural properties (benzene ring and number-position of hydroxyl groups) [2].

These Results showed that extract and EAF have potent antioxidant activity. Our results tie back to previous antioxidant profiling showing potent antioxidant activity [19, 18, 20]. EAF fraction higher percent radical scavenging power of EAF (93.93%) was observed more than extract and other fractions. According to another research study conducted ethyl acetate fractions depicted the highest percentage (92.96% ± 0.13) inhibition of DPPH radical when compared to other fractions[48]. IC_{50} value of EAF fraction (43.89±1.24 µg/ml) was lower as compared to extract (88.95±13.912 µg/ml) and other fractions. The low IC_{50} value of EAF accounts for the presence of high levels of flavonoids and phenolics[46]. Other studies also indicate the potent antioxidant activity of EAF of plants[48, 50], and different IC_{50} values are reported for *O. ferruginea* plant leaves in previous studies ranging from 12 µg/ml to 173 µg/ml [48 19, 18, 20, 22]. Results of the H_2O_2 assay obtained showed that EAF has potent antioxidant activity. Earlier studies have reported a positive correlation between phenolic components and plant extracts' ability to scavenge free radicals[55, 56].

In the current *in vivo* study, ISO administration overproduced the ROS (H_2O_2) in serum and heart tissue homogenate **Figure 3b**. Different investigations already reported also indicated that oxidative stress plays a crucial role in CH[57, 58].

In the current in *vivo* study when comparing EAF and standard drug ascorbic acid there was no significant difference among H_2O_2 levels. EAF reversed the oxidative stress by 2.5 and 3.5 fold in serum and heart tissue respectively. Ethyl acetate fraction's potent antioxidant activity was also reported in different studies[59, 60]. According to reports the risk of diseases brought on by reactive oxygen species is said to be reduced by these bioactive chemicals [61] in a number of ways, including oxidative enzyme inhibition, ROS quenching, and free radical scavenging.

Conclusion

Our phytochemical investigation results showed that plant leaves are rich in phenolic compounds. EAF TPC and TFC were higher as compared to extracts and different fractions. Our GCMS results showed 15 active compounds having potent radical scavenging potential. Antioxidant activity indicated low IC_{50} value of the EAF fraction was lower as compared to extract and other fractions. *In vivo, the* study of EAF delivery decreased ROS levels as compared to the diseased group. Hence, the results of the present study provided evidence of the high scavenging power of plants against free radicals. The overall study concludes that *O. ferruginea* is a promising candidate as an antioxidant agent for the medical industry to minimize the side effects and resistance of synthetic products for the treatment of oxidative stress-induced diseases. The study also recommends the commercial usage of the leaves of *O. ferruginea* since they are a rich source of natural antioxidants and can be utilized to produce nutraceuticals.

Acknowledgments

The authors pay their sincere thanks to the Signal Transduction Lab, Department of Biochemistry, Quaid-i-Azam University Islamabad, Pakistan, for providing the *in vivo* study facility.

Ethical approval

The Quaid-i-Azam University's Bio-Ethics Committee granted the *in vivo* study approval and issued an approval number (No. #BEC-FBS-QAU2019-184).

Author contributions

All authors contributed to the study's conception and design

Statements and Declarations

The authors declare that there are no conflicts of interest, whether financial or non-financial, among them.

References

1. Ahn K. The worldwide trend of using botanical drugs and strategies for developing global drugs. BMB reports 2017; 50(3):111.
2. Zeb A. Concept, mechanism, and applications of phenolic antioxidants in foods. J Food Biochem 2020. 44(9): e13394.
3. Porras-Loaiza AP, López-Malo A. Importancia de los grupos fenólicos en los alimentos. Temas selectos de Ingeniería de Alimentos 2009; 3(1): 121-134.
4. Singh RB, Fedacko J, Fatima G, et al. Why and how the Indo-Mediterranean diet may be superior to other diets: the role of antioxidants in the diet. Nutr 2022; 14(4): 898.
5. Swallah MS, Sun H, Affoh R, Fu H, Yu H. Antioxidant Potential Overviews of Secondary Metabolites (Polyphenols) in Fruits. Int J food sci 2020; 9081686. https://doi.org/10.1155/2020/9081686.
6. Stagos D. Antioxidant Activity of Polyphenolic Plant Extracts. Antioxidants (Basel) 2019; 9(1):19. https://doi: 10.3390/antiox9010019.
7. Ndjenda Ii MK, Nguelefack-Mbuyo EP, Atsamo AD, et al. Antihypertensive Effects of the Methanol Extract and the Ethyl Acetate Fraction from Crinum zeylanicum (Amaryllidaceae) Leaves in L-NAME-Treated Rat. J Evid Based Complement Altern med 2021; eCAM 2656249. https://doi.org/10.1155/2021/2656249.
8. Ghani A, Adib A, Ugusman A, et al. "Role of Terpenophenolics in Modulating Inflammation and Apoptosis in Cardiovascular Diseases: A Review" *Int J Mol Sci* 2023; 6: 5339. https://doi.org/10.3390/ijms24065339
9. Takimoto E, Kass DA. Role of oxidative stress in cardiac hypertrophy and remodeling. Hypertension 2007; 49(2):241-8.
10. Maurya A, Prasad J, Das S, Dwivedy AK. Essential oils and their application in food safety. Front Sustain Food Syst 2021; 5: 653420.
11. Paiva-Martins F, Gordon MH, Gameiro P. Activity and location of olive oil phenolic antioxidants in liposomes. Chem Phys Lipids 2003; 124(1):23-36. https://doi.org/10.1016/S0009-3084 (03)00032-X.
12. Khan, MN, Soherwardy R, Sarwar A. Nutritional assessment of olive fruit (Oleaeuropaea) available at local market. Int Food Res J. 2015; 22(2): 526. ISSN 1985-4668.
13. Ahmad K, Pieroni A. Folk knowledge of wild food plants among the tribal communities of Thakht-e-Sulaiman Hills, North-West Pakistan. J Ethnobiol Ethnomed 2016; 12: 17. https://doi.org/10.1186/s13002-016-0090-2
14. Ullah A, Anjum S, Masood A, et al. Nutritional prospects of some wild edible medicinal plants of District Harnai Balochistan, Pakistan. Food Sci Technol 2023; 43. e115922.https://doi.org/10.1590/fst.115922.
15. Hayat N, Ullah A, Parveen S, Ali SG. Diversity, utilization, and economic importance of fuel wood and fruit plants at Tehsil Dir, Pakistan. Pure Appl Biol 2023; 12(1):703-721
16. Liaqat I, Hussain S, Abid H, et al. An Ethnobotanical Survey of Medicinal Plants Used for Primary Health Care from Patan Sher Khan and Surrounding Areas of District Sudhnoti, Azad Jammu and Kashmir, Pakistan. J Appl Res Plant Sci 2023; 4(01): 518–528. https://doi.org/10.38211/joarps.2023.04.01.62.
17. Amjad MS, Arshad M. Ethnobotanical inventory and medicinal uses of some important woody plant species of Kotli, Azad Kashmir, Pakistan. Asian Pac J Trop Biomed 2014; 4:952-958

18. Mehmood A, Murtaza G. Phenolic contents, the antimicrobial and antioxidant activity of *Olea Ferruginea Royle* (Oleaceae). BMC Complement Altern Med 2018; 18:173.
19. Sharma RK, Sharma N, Samant SS, et al. Antioxidant activities in methanolic extracts of *Olea Ferruginea* Royle fruits. Int J Biosci Biochem Bioinforma 2013; 3(2): 154-156.
20. Sharma N, Kumar I, Sharma RK. Antioxidant activities in stem bark, leaves, and fruits of *Olea ferruginea* Royle grow in Himachal Pradesh, in relation to altitudinal changes. Int J Food Nutr Sci 2020; 9(4): 48-48
21. Yaseen M, Kamran M, Farid A, et al. Antibacterial, Hemagglutination, and Insecticidal Activity Studies on the Solvent Extracts of the Roots of *Olea ferruginea*. Makara J Sci 2022; 26(1): 8.
22. Rafique R, Din Z, Ilat S, et al. In Vitro Antiviral Activity of Stem and Leaf Extract of *Olea ferruginea* Royle against Foot and Mouth Disease Virus. Pak J Sci 2021; 73:364-377.
23. Rauf A, Rashid U, Shah ZA, et al. Anti-Inflammatory and Anti-Diabetic Activity of Ferruginan, a Natural Compound from *Olea ferruginea*. Processes 2023; 11(2): 545.
24. Hashmi MA, Shah HS, Khan A, et al. Anticancer and alkaline phosphatase inhibitory effects of compounds isolated from the leaves of *Olea ferruginea Royle*. Rec Nat Prod 2015; 9(1): 164-168.
25. Hassan HU, Raja NI, Abasi F, et al. Comparative study of antimicrobial and antioxidant potential of *Olea Ferruginea* fruit extract and its mediated selenium nanoparticles. Mol 2022; 27(16): 5194.
26. Amin A, Khan MA, Shah S, et al. Inhibitory effects of *Olea Ferruginea* crude leaves extract against some bacterial and fungal pathogen. Pak J Pharm Sci 2013; 26(2): 251-255.
27. Sofowora A. 1993. Phytochemical Screening of Medicinal Plants and Traditional Medicine in Africa Edition. Spectrum Books Ltd., Nigeria, 150-156.
28. Trease, G.E. and Evans, W.C. Pharmacognosy. 11th Edition, Bailliere Tindall, London, 1989:45-50.
29. Bhattacharjee S, Elancheran R, Dutta K, et al. Cardioprotective potential of the antioxidant-rich bioactive fraction of *Garcinia pedunculata Roxb. ex Buch.-Ham* against isoproterenol-induced myocardial infarction in Wistar rats. *Front Pharmacol*.2022; 4(13):1009023.
30. Ashagrie G, Abebe A, Umer S. Analgesic and Anti-Inflammatory Activities of 80% Methanol Extract and Solvent Fractions of *Ehretia cymosa Thonn* (*Boraginaceae*) Leaves in Rodents. *J Exp Pharmacol* 2023; **15**:63-79.
31. Folin O, Denis W. A New Colorimetric Method for the Determination of Vanillin in Flavoring Extracts. Ind Eng Chem 1912; 4(9): 670-672.
32. Sarma R, Das M, Mudoi T, et al. Evaluation of antioxidant and antifungal activities of polyphenol-rich extracts of dried pulp of *garcinia pedunculata roxb*. And *garcinia morella gaertn. (clusiaceae).* Trop J Pharm Res 2016; 15(1): 133-140.
33. Park YS, Jung ST, Kang SG, et al. Antioxidants and proteins in ethylene-treated kiwifruits. Food Chem 2008; 107(2): 640-648.
34. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT - Food Sci Technol 1995; 28(1):25-30.
35. Ruch RJ, Cheng SJ, Klaunig JE. Carcinogenesis, Rely. Chim Acta 1989; (10):1003-1008.
36. Khatua TN, Borkar RM, Mohammed SA, et al. Novel sulfur metabolites of garlic attenuate cardiac hypertrophy and remodeling through induction of Na+/K+-ATPase expression. Front Pharmacol 2017; 8: 18.
37. Mushtaq S, Ali T, Javed Q, et al. N-Acetyl Cysteine Inhibits Endothelin-1-Induced ROS Dependent Cardiac Hypertrophy through Superoxide Dismutase Regulation. Cell J 2015; 17(2):355-60.
38. Nahar L, Russell WR, Middleton M, et al. Antioxidant phenylacetic acid derivatives from the seeds of Ilex aquifolium. Acta Pharm. 2005; 55(2):187-93.
39. Milaeva E, Sofiya O, Meleshonkova N, et al. Antioxidative Activity of Ferrocenes Bearing 2, 6-Di-Tert-Butylphenol Moieties. Bioinorg chem appl 2010; 165482.

40. Tuong DTC, Moniruzzaman M, Smirnova E, et al. Curcumin as a Potential Antioxidant in Stress Regulation of Terrestrial, Avian, and Aquatic Animals: A Review. Antioxidants 2023; 12(9): 1700.
41. Park MS, Park MS, Lee KG. Antioxidative activities of volatile and non-volatile extracts of *Schisandra chinensis Baill* fruit. Flavor Fragr J 2020; 35(4): 435-442.
42. Ribeiro PRE, Montero IF, Saravia SAM, et al. Chemical composition and antioxidant activity in the essential oil of *Cinnamomum zeylanicum Nees* with medicinal interest. J Med Plant Res 2020; 14(7): 326-330.
43. Adeyemi MM. GC-MS Analysis of Methanol Extracts of *Phragmanthera incana* Leaves From Guava, Mango, Cashew, and Kolanut Trees. Med Health Sci E J. 2023; 7(1). ISSN: 2469-6774.
44. Yamaguchi T, Takamura H, Matoba T, Terao J. HPLC Method for Evaluation of the Free Radical-Scavenging Activity of foods by using 1, 1-diphenyl-2-picrylhydrazyl. Biosci Biotechnol Biochem 1998; 62(6):1201-4. http//doi: 10.1271/bbb.62.1201. PMID: 9692204
45. Sánchez-Moreno C. Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food science and technology international 2002; 8(3):121-37.
46. Sannigrahi S, Mazuder UK, Pal DK, et al. Antioxidant potential of crude extract and different fractions of Enhydra fluctuans Lour. Iran J Pharm Res 2010; 9(1): 75.
47. Hayashi I, Morishita Y, Imai K, et al. High-throughput spectrophotometric assay of reactive oxygen species in serum. Mutat Res 2007; 631(1): 55-61. http://doi: 10.1016/j.mrgentox.2007.04.006.
48. Siddiqui SZ, Abbasi MA, Aziz-ur-Rehman RT, et al. *Olea Ferrugenia*: a potential natural source of protection from oxidative stress. J Med Plant Res 2011; 5(17): 4080-4086.
49. Hafeez M, Hassan SM, Mughal SS, et al. Antioxidant, Antimicrobial and Cytotoxic Potential of *Abelmoschus esculentus*. Chem Biomol Eng 2020; 5(4): 69.
50. Nawaz H, Shad MA, Rehman N, et al. Effect of solvent polarity on extraction yield and antioxidant properties of phytochemicals from bean (*Phaseolus vulgaris*) seeds. Braz J Pharm Sci 2020; 56:e17129.
51. Masood A, Manzoor M, Anjum S, et al. Quantitative analysis of total phenolics, flavonoids, and antioxidant activity of olive fruits (*Olea Ferruginea*) based on geographical region and harvesting time in Zhob district, Pakistan. Appl Ecol Env Res 2021; 19(1): 13-25.
52. Moreira L, Dias LG, Pereira JA, Estevinho L. Antioxidant properties, total phenols, and pollen analysis of propolis samples from Portugal. Food Chem Toxicol 2008; 46(11): 3482-3485.
53. Mohammadi-Cheraghabadi M, Hazrati STerpenoids, steroids, and phenolic compounds of medicinal plants. Phytochemicals in Medicinal Plants: Biodiversity, Bioactivity and Drug Discovery. 2023; 105.
54. Wang J, Zhang Y, Fang Z, et al. Oleic acid alleviates cadmium-induced oxidative damage in rats by its radical scavenging activity. Biol Trace Elem Res 2019; 190: 95-100.
55. Sultana F, Imran-Ul-Haque M, Arafat M, Sharmin S. An overview of nanogel drug delivery system. J Appl Pharma Sci 2013; 3(8): S95-S105.
56. Safdar MN, Kausar T, Jabbar S, et al. Extraction and quantification of polyphenols from kinnow (*Citrus reticulate* L.) peel using ultrasound and maceration techniques. J Food Drug Anal 2017; 25(3):488-500. doi: 10.1016/j.jfda.2016.07.010.
57. Ramachandra CJ, Cong S, Chan X, et al. Oxidative stress in cardiac hypertrophy: From molecular mechanisms to novel therapeutic targets. Free Radic Biol Med 2021; 166:297-312.
58. Vashi R, Patel BM. NRF2 in cardiovascular diseases: a ray of hope! J Cardiovasc Transl Res 2021; 14: 573-586.
59. Jin M, Yu H, Jin X, et al. *Dracocephalum Moldavica L*. Extracts Protect H9c2 Cardiomyocytes against H_2O_2-Induced Apoptosis and Oxidative Stress. Biomed Res Int 2020; 2: 8379358. http://doi: 10.1155/2020/8379358

60. Zhang J, Yanfang Wu, Chenyu W, et al. The antioxidant, anti-inflammatory, and analgesic activity effect of ethyl acetate extract from the flowers of *Syringa pubescens* Turcz. J Ethnopharmacol 2023; 322: 117561. https://doi.org/10.1016/j.jep.2023.117561.
61. Zilani MNH, Sultana T, Asabur Rahman SM, et al. Chemical composition and pharmacological activities of *Pisum sativum*. BMC Complement Altern Med2017; 17: 1-9.

Figures legends:

Figure 1. *O. ferruginea* leaves extraction and EAF preparation

Figure 2. FTIR Analysis of Ethyl Acetate Fraction of *O. ferruginea* Leaves

Figure 3. Estimation of Radical Scavenging Power of EAF. **(a)** Measurement of H_2O_2 levels using H_2O_2 assay. **(b)** *in vivo* measurement of ROS levels in the serum and heart tissue in ISO-induced Cardiac Hypertrophy rat model (n = 6). Statistically significant differences are depicted as p-values & statistical analysis was conducted using one-way ANOVA followed by Tukey's test. (*): $p < 0.05$), (**): $p < 0.001$, (***): $p < 0.001$. Data are depicted as mean ± SE.