Phytochemical characterization of lipids from *Rivina humilis* L. plant parts

Internship Report Submitted for partial fulfillment of

Bachelor of Technology in Biotechnology
(B.Tech Biotechnology)

By

Akansha Bhandari

Under the supervision of
Dr. Giridhar Parvatam
Chief Scientist
Plant Cell Biotechnology Department

CSIR-Central Food Technological Research Institute,
Mysore-570020

Graphic Era (Deemed to be University), Dehradun
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DECLARATION

I, Ms. Akansha Bhandari declare that the internship report entitled **“Phytochemical characterization of lipids from *Rivina humilis* L. plant parts”** submitted to Graphic Era (Deemed to be University), Dehradun for partial fulfilment of the degree of Bachelor of technology in Biotechnology carried out under the esteemed supervision of **Dr P. Giridhar**, Chief Scientist, Plant Cell Biotechnology Department, CSIR-CFTRI, Mysore during the period 13 July 2023 to 28 August 2023.

I hereby declare that the results of the present investigations have not previously been submitted elsewhere for the award of the degree.

Date:

Place: Mysore

(Akansha Bhandari)

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At the very outset of this report, I would like to extend my sincere and heartfelt obligation to all the personages who have helped me in this endeavor. Without their active guidance, help, cooperation, and encouragement, I would not have made headway in this internship.

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Date:

Place: **Akansha Bhandari**

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LIST OF ABBREVIATIONS

Abbreviations	**Full Form**
AA	Ascorbic acid
ABTS	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
$A_{control}$	Absorbance of control
AMe	Acidified methanol
A_{sample}	Absorbance of sample
DLATGS	Deuterated L-Alanine Doped Triglycine Sulphate
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EC_{50}	Effective concentration 50
EOs	Essential oils
Eq.	Equivalent
FAs	Fatty acids
FC	Folin-Ciocalteu’s reagent
FRAP	Ferric Reducing Antioxidant Power
FTIR	Fourier-transform infrared spectroscopy
g	Gram
GA	Gallic acid
HPLC	High-Performance Liquid Chromatography
Me	Methanol
MCTs	Micro centrifuge tubes
mg	milligram
ml	Milliliter
Mo	Molybdenum
nm	Nanometer
RTO	Root oil
SDO	Seed oil
STO	Stem oil
TAA	Total antioxidant activity
TCA	Trichloroacetic acid
TFC	Total flavonoid content

TPC	Total phenolic content
UV	Ultraviolet
µl	Microliter

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INTRODUCTION

Lipids are one of the most important components of natural foods and many synthetic compounds and emulsions. They are classified as oils, greases, fats, and fatty acids (FAs). The compositional factors (levels of omega fatty acids, phytosterols, phospholipids, glycolipids etc.) of lipids directly play a major role in its contribution to health (Kozłowska et al., 2016).

Plant oils represent an important renewable resource from nature. They are used mainly for food and feed purposes and renewable sources of industrial feedstocks and fuel. Up to 2006, the world production of plant oils amounted to 127 million tonnes. The major sources of vegetable oil (79%) are oil palm, soybeans, rapeseed, and sunflower (Dyer et al., 2008). These oils are sources of major edible FAs (which play an important role in cellular metabolism and energy sources) as well as many nutraceutical compounds such as phenolics, flavonoids, tocopherols, phytosterols, oryzanol, lignans, carotenoids etc. (Kozłowska et al., 2016).

Oils have a variety of uses besides edible applications. More pieces of evidence are pointing to the importance of FAs in human nutrition, including their role in brain function, the growth and development of the human embryo, and the treatment and prevention of many serious diseases like cardiovascular disease and inflammation. Many FAs are now known to have anticancer potential. As more study is conducted, the significance of fats and FAs in human nutrition is coming to light. FAs are important in many industrial uses in addition to being a vital part of the human diet, including soaps and detergents, cosmetics, lubricants, ink, varnish, and paints, among others (Kumar et al., 2016).

Rivina humilis L. (Petiveriaceae), commonly called pigeon berry, is a wild herbaceous bushy perennial. The plant is found in colonies that grow on various types of shaded soils. It grows up to a height of 120 cm (4 ft). This plant is native to the Caribbean and tropical America and now widely naturalized in Indo-Malaysia and Pacific regions (Swarbrick 1997). The berries of the plant contain a high level of betalain pigments. A recent report on the dietary safety of *R. humilis* berries juice indicates that these berries could be a prospective dietary or industrial source of betalains (Khan et al., 2011). Recently the fatty acid composition of oils obtained from different plant parts of *R. humilis* has been investigated by Riya et al., 2023. oil obtained from leaf, stem, root, and seeds were used in this study. An oil yield of 17.66 % in the root, followed by 13.18 % in the stem, 11.25 % in seeds and 8% in the leaf was reported. Palmitic acid, stearic acid, oleic acid, and linoleic acid were the predominant fatty acids detected in all four plant parts.

In view of the available literature, we have observed that the physicochemical characterisation and bioactive potential of the lipids obtained from different plant parts of *Rivina humilis* are not yet done. Since, there is an ever-expanding market exists for oil crops from both nutritional and industrial perspectives, in the present study, we have investigated the nutraceutical composition and *in vitro* antioxidant potential of oils obtained from the stem, root, and seed of *R. humilis*.

REVIEW OF LITERATURE

Rivina humilis

Rivina humilis, commonly called as blood berry, pigeon berry, and rouge berry, belongs to the family Phytolaccaceae. Linnaeus recognised the genus Rivina for the first time in 1753. The name Rivina was given to pigeon berry in honour of A.Q. Rivinus, professor of Botany at Leipzig in 18th century. This is a wild herbaceous bushy perennial. The plant is found in colonies that grow on various types of shaded soils. It grows up to a height of 120 cm (4 ft). This plant is native to the Caribbean and tropical America and is now widely naturalized in Indo-Malaysia and Pacific regions (Swarbrick 1997). Flowers are pink/white, which blooms from May to October. Berries accumulate betalains in various shades of orange, red or purple (Khan et al., 2011). The berries are considered irresistible to birds. Hence the plant is recommended to attract birds to the garden. Southwestern Native Americans used the berries for a red dye. In Mexico, the leaves were employed to treat wounds. In Jamaica, this herb is used for cleaning block tubes, infertility, or any womb-related problem, and it is also used for menstruation flow problems. The herb *R. humilis* is boiled and drinks three times daily for infertility and other wombs-related problems (Bagga, 2017).

Table 1. Classification of *Rivina humilis*

Domain	Eukaryota
Kingdom	Plantae
Phylum	Spermatophyta
Subphylum	Angiospermae
Class	Dicotyledonae
Order	Caryophyllales
Family	Petiveriaceae
Genus	*Rivina*
Species	*Rivina humilis* L.

Morphology

R. humilis is a perennial herbaceous to woody erect plant that grows to a height of 2 to 4 feet. Stems are erect, dichotomous, branched, glabrous, angular, and pubescent at the nodes. The leaves are simple, exstipulate, oblong to elliptical, 10 -12 cm long, petiolate, with a rounded or attenuate base and a dark apex. Green to light green in tint, with a mainly glabrous surface that is sometimes pubescent. The margins range from smooth to wavy. The leaves are spirally arranged around the stem. Terminal or axillary racemes are 5-15.5 cm long and thin, producing 30-54 flowers. Flowers are tiny, white with pink shading, pedicelate, pedicel 5mm long, bracteate, bracteolate, bisexual, tetramerous, 4 tepals, 2-3 x 1-2.5 mm, green when young, white with pink tips when mature, persistent; somewhat hairy outside, 4 stamens alternating with tepals, dimorphic, dimorphic, dimorphic, dimorphic, dimorphic, dimorphic, Anthers are introse, dithecous, pollen grains are spheroidal, 24-30 m, psilate, pentazoniporate; ovary is superior, ovoid to globose, monocarpellary, and unilocular. The style is shorter than the ovary, persistent, and curved, with a capitate stigma. The fruit is a berry that is crimson red in colour, globose, 3-4 mm in diameter, with a glossy surface, persistent tepals, and a style. Berries are grouped freely in some and compactly in others. Black, tiny, 3 mm in diameter, lenticular, and hairy seeds (Bagga, 2017).

Fatty acids identified from different plant parts of *Rivina humilis*

Recently the fatty acid composition of oils obtained from different plant parts of R. humilis have been investigated by Riya *et al*., 2023.oil obtained from leaf, stem, root and seeds were used in this study. The major fatty acids identified are listed below:

Table 2. Major fatty acids identified in different plant parts

	Leaf oil	Stem oil	Root oil	Seed oil
Palmitic acid	22.69 ± 0.24	27.09 ± 0.10	39.25 ± 0.29	22.23 ± 0.31
Linoleic acid	17.65 ± 0.17	35.16 ± 0.44	22.38 ± 0.06	24.04 0.26
α Linoleic acid	47.83 ± 0.79	22.05 ± 0.86	4.46 ± 0.05	0.00
Stearic acid	2.93 ± 0.15	2.71 ± 0.11	5.33 ± 0.21	2.54 ± 0.016
Oleic acid	7.84 ± 0.18	11.55 ± 1.14	22.17 ± 0.11	44.48 ± 0.15
SFA	26.68 ± 0.44	31.24 ± 0.16	45.77 ± 0.20	30.39 ± 0.11
PUFA	65.48 ± 0.62	57.21 ± 1.30	26.83 ± 0.00	24.12 ± 0.14
UFA	73.32 ± 0.44	68.76 ± 0.16	54.23 ± 0.20	69.69 ± 0.01

*Source: (Riya *et al*., 2023)

Nutraceutical compounds present in plant oils

Phenolics

Around 8000 distinct phenolic structures make up the secondary metabolites known as phenolic chemicals, which are extensively distributed across the plant kingdom. When plants are exposed to stress circumstances like wounding, infection, or UV radiation, they are implicated in the adaption processes that take place. These compounds have at least one phenol group in their chemical structure. An aromatic ring with one or more hydroxyl groups makes up their structure. Although phenolic compounds can be found in plants in their free form, they are typically found linked to proteins or carbohydrates.

Due to their ability to act as antioxidants, phenolic compounds have gained more attention during the past ten years. Their capacity to scavenge free radicals aids in the prevention of oxidative stress-related chronic illnesses like cancer, cardiovascular, and neurological diseases (Shahidi and Ambigaipalan, 2015). Phenolic compounds also possess other biological effects related to their antioxidant capacity such as antimicrobial and anti-inflammatory properties (Cosme *et al*., 2020).

Flavonoids

Flavonoids constitute a massive family of water-soluble polyphenolic chemicals with about 9000 members in different classes such as flavanols, flavones, flavanones, anthocyanidins, catechins, and biflavans. They are widely distributed in the plant kingdom and are especially ubiquitous in vegetables, berries, and fruits A wide range of biological and antioxidant activites are also seen among flavonoids in the human and animal diet against infections, allergens, carcinogens, and other agents causing inflammation, in addition to the varied structures and functions of flavonoids in plants. Several of the functions are carried out by interacting with vital host enzymes such cytochromes P450 (CYPs). For instance, menopausal symptoms can be avoided as well as the risk of several hormones-dependent breast and prostate cancers being lowered (Hodek *et al*., 2002). Knowledge of flavonoid biosynthesis and the link between structure and function in plants and people will facilitate the manufacture of metabolic engineering was used to target flavonoids molecules for usage as more efficient drugs and/or chemo preventive agents (Dixon and Steele, 1999).

Carotenoids

Carotenoids are pigments that give many fruits and vegetables their colour. They are a family of lipid-soluble tetraterpenoids with a 40-carbon polyene hydrocarbon chain structure. Carotenes and xanthophylls make up most of the human diet. They can act as light harvesting pigments and are powerful antioxidants in lipid forms. Due to their physicochemical characteristics, they act as photo protectants and prevent retinal degeneration. A diet high in carotenoids has been epidemiologically linked to a lower risk for many diseases. Carotenoids have also shown antioxidant capabilities and singlet oxygen quenching capacity to prevent chronic disease *in vitro* and in animal models (Nishino *et al*., 2009). Intervention studies using carotene to protect various malignancies and cardiovascular problems have produced contradictory findings (Stahl *et al*., 2005).

Phytosterol

Vegetable oils include phytosterols, which cause hypocholesterolemia. In the human diet, phytosterols are significant micronutrients. According to evidence, phytosterols are crucial in lowering blood cholesterol levels, which reduces cardiovascular morbidity. The main sources of phytosterols are edible vegetable oils (46.3%), followed by cereals (38.9%), vegetables (9.2%), nuts (2.0%), fruits (1.5%), beans and bean products (1.4%), and tubers (0.8%). The Non saponifiable fraction of plant oils contains phytosterols, which are naturally occurring plant sterols. Although phytosterol absorption in humans is significantly lower than that of cholesterol, phytosterols are plant components with a chemical structure similar to cholesterol but with an additional methyl or ethyl group (Yang *et al*., 2019). When added to fat spreads and other food matrices, phytosterols have been found to be effective at lowering cholesterol. Phytosterols appear to have anticancer characteristics in addition to being crucial in the control of cardiovascular disease. The reduction of carotenoid levels in the blood is a side effect of phytosterol ingestion (Jones and AbuMweis, 2009).

Lignans

Lignans are a class of phytochemicals that are created when two phenylpropanoid units undergo oxidative dimerization (Sok *et al*., 2009). Although the biological function of lignans in plants is still up for debate, it is generally accepted that they aid in plant defence against pathogens and pests as well as in regulating plant growth. Lignans provide important pharmacological functions in addition to their natural roles, such as having anticancer, anti-inflammatory, immunosuppressive, cardiovascular, antioxidant, and antiviral effects. It has been proposed that diets high in lignans may also be preventive against illnesses linked to estrogen, such as osteoporosis (Dinelli *et al*, 2007).

Antioxidant activities of plant oils

Potential sources of antioxidant compounds have been searched in several types of plant materials such as vegetables, fruits, leaves, oilseeds, cereal crops, barks and roots, spices and herbs, and crude plant drugs (Kähkönen *et al*., 1999). It is common practice in the study of natural compounds to identify antioxidants as "molecules able to react with radicals" or provided of reducing power to counteract the oxidative stress caused by radicals. This approach is witnessed by the chemistry of several tests developed to assay the antioxidant activity of natural extracts or isolated phytochemicals, which are based on the reaction of the potential antioxidant with some colored persistent radical (e.g., DPPH or ABTS) or with some oxidizing nonradical species such as Fe3+ ions (e.g., FRAP assay) (Amorati *et al*., 2013). Different chemical molecules that have conjugated carbon double bonds and hydroxyl groups, which can donate hydrogen and suppress free radicals and reduce oxidative stress, are what make up essential oils (EOs). For evaluating the antioxidant activity of EOs and their natural or synthesised derivative molecules, many in vitro chemical-based approaches have been devised. DPPH, ABTS, and hydroxyl tests have often been used with extracts, EOs, and isolated chemical molecules to assess the radical scavenging activity of organic compounds. Additionally, the reducing power of the examined chemicals, EOs, or combinations was assessed using the ferric antioxidant power reduction (FRAP) (Amorati *et al*., 2013).

AIM OF THE STUDY

The aim of the present study is to understand the chemical and bioactive potential of lipids extracted from *Rivina humilis* plant parts (stem, root, and seed) by analysing the nutraceutical composition and *in vitro* antioxidant potential. This will provide insights into the various applications of the same in the food and pharma industries.

OBJECTIVES

1. Determination of nutraceutical composition of lipids obtained from stem, root, and seed.

2. Estimation of antioxidant potential of lipids through *in vitro* methods.

MATERIALS AND METHODS

1. Plant material

Rivina humilis L., whole plants were collected from shady environs of CSIR-CFTRI, Mysore, Karnataka, India. Tender stem, root, and seeds were collected separately and washed well with running tap water. The plant parts were kept in an oven at 45 °C for drying. Each sample was grounded separately into fine powders and stored at room temperature in polythene covers until further analysis.

2. Chemicals

HPLC grade hexane, HPLC grade methanol, Ethanol, HPLC grade chloroform, Acetic anhydride, Gallic acid, Folin Ciocaltean reagent, Sodium carbonate, Rutin, Aluminium chloride, Ascorbic acid, Sodium phosphate, Ammonium molybdate, Sulfuric acid, DPPH (2,2-Diphenyl-1-picrylhydeazyl), Potassium persulphate, ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), Potassium dihydrogen phosphate (KH_2PO_4), Dipotassium phosphate (K_2HPO_4), Potassium ferricyanide ($K_3Fe(CN_6)$, Trichloroacetic acid, Ferric chloride ($FeCl_3$).

3. Oil extraction

Oil extraction was carried out using Soxhlet apparatus. A known weight of each plant part were extracted in n-hexane for 8 h using the Soxhlet apparatus. The temperature was set at 85 °C. The obtained oil along with the solvent was subjected to rotary evaporator (Heidolph Instrument, Schwabach, Germany) for the complete evaporation of the solvent. The collected oil was stored at 4 °C until further use.

4. Fourier-transform infrared spectroscopy (FTIR) analysis

The FTIR spectra of the extracted oil were taken in duplicates using an FTIR instrument (Tensor II, M/s. Bruker, Germany). Wavelength was set in a mid-infrared range of 4000-400 cm^{-1} with a DLATGS (Deuterated L-Alanine Doped Triglycine Sulphate). A PC-based data acquisition method with OPUS Software (version 7.5) was used to compile the qualitative transmission molecular fingerprint of each oil. Prior to analysis, the silicon plate was wiped well with isopropyl alcohol. Hexane was used as the cleaning solution in between different oil samples. The data acquisition was set in wavelength against percentage transmittance. The obtained spectral data was interpreted using FTIR functional group identification references.

5. Determination of Nutraceutical composition

5.1. Preparation of methanolic extractives of oil

0.5 g of each oil was extracted with 10 ml of 80% aqueous methanol (80%Me) and 70% aqueous methanol (70%AMe) containing 0.1% hydrochloric acid (HCl) by continuously shaking using a vortex machine for about an hour at room temperature. Then the extracts were centrifuged at 7500 rpm for 10 min and the supernatant was collected and stored at -20 °C until further analysis.

5.2. Determination of total phenolics content (TPC)

Folin-Ciocalteu's (FC) reagent was used to determine the soluble total phenolics content in the methanolic extractives (80% aqueous methanol and 70% aqueous methanol containing 0.1% hydrochloric acid) of oil (Kumar *et al*., 2020). Gallic acid (0.1mg/ml) was used as the standard. Briefly, different volumes of standard and sample was pipetted out into test tubes and the volume was made up to 3 ml with distilled water. A 0.5 ml of FC reagent prepared by diluting it with distilled water in a 1:1 ratio was added to this. Reaction mixture was incubated for 3 min at room temperature in the dark, followed by adding 2 ml of 20% sodium carbonate solution. Tubes were vortexed well and placed in a boiling water bath for 1 min. After cooling, absorbance was measured at 650 nm using a single-beam UV-visible spectrophotometer (Thermo Scientific, Genesys 50).

5.3. Determination of total flavonoid content (TFC)

The total flavonoid content in the methanolic extractives of the oil was quantified using the standard curve obtained from one of the common flavonoid standards, rutin (0.1 mg/ml). The aluminium chloride method was used for the estimation (Riya *et al*., 2023). Briefly, different volumes of standard and sample were pipetted into a 96-well plate and the volume was made up to 100 µl using absolute ethanol. To this 100 µl of 2% methanolic aluminium chloride was added and the reaction mixture was incubated at room temperature in the dark for 15 min. Absorbance was recorded at 430 nm using an ELISA multimode plate reader (TECAN, SPARK 10M).

5.4. Estimation of total phytosterols content

Total phytosterol was estimated using the Liberman-Burchard method reported earlier by (Sabir *et al*., 2003) with slight modifications. Cholesterol (1mg/ml) was used as the standard compound. Briefly, a known volume of the sample dissolved in chloroform was taken in a test tube and volume was made up to 5 ml with chloroform. Then 2 ml of Liberman-Burchard reagent (0.5 ml H_2SO_4 and 10 ml acetic anhydride) was added to it. The test tubes were incubated in the dark for 15 min and later absorbance was read at 640 nm using a single-beam UV-visible spectrophotometer (Thermo Scientific, Genesys 50). The concentration of phytosterols was calculated using the standard curve obtained.

5.5. Total oryzanol content

A known weight (g) of the oil was taken in a centrifuge tube and 10 ml of n-hexane was added to it. This was vortexed well until the oil dissolves completely. The absorbance of this solution was measured using a single-beam UV-visible spectrophotometer (Thermo Scientific, Genesys 50) at 314 nm. Total oryzanol content was calculated using an extinction coefficient of 358.9 according to the following formula (Gopala Krishna *et al*., 2006).

$$\text{Oryzanol (g/100 g)} = \frac{\text{OD of the hexane solution}}{\text{Weight of oil (g)} \times 10} \times \frac{100}{358.9}$$

5.6. Estimation of lignan content

Lignan content was estimated by spectrophotometric method (Thermo Scientific, Genesys 50). 10 mg of each oil was weighed in a centrifuge tube and mixed with 10 ml of HPLC grade chloroform and n-hexane in a 7:3 v/v ratio. Absorbance was measured at 288 nm using the same extraction solution as the blank. The below formula was used for calculation (Bhatnagar *et al*., 2015).

$$\text{Lignans (g/100 g of oil Sesamol Eq.)} = \frac{\text{OD}}{\text{Weight (g)}} \times \frac{100}{230.1}$$

Where 230.1 is the specific extinction value of Sesamol.

5.7. Determination of carotene content

Carotene content of the extracted oil was estimated by taking 0.2 g of melted oil at 65 °C in 10 ml of n-hexane. The solution was homogenized well until the oil dissolves completely. 1 ml of this solution was further diluted to 10 ml with n-hexane. Absorbance was read at 446 nm using single-beam UV-visible spectrophotometer (Thermo Scientific, Genesys 50). Hexane was used as the blank. Concentration of carotene was quantified using the formula below (Chandrasekaram et al., 2009).

$$\text{Carotene (ppm)} = \frac{383 \times \text{OD} \times \text{volume}}{100 \times \text{weight (g)}}$$

Where 383 is the diffusion coefficient.

6. Determination of *in vitro* antioxidant activities

6.1. Total antioxidant activity (TAA)

The phosphomolybdenum method was used to determine the total antioxidant activity of the methanolic extractives of oil (Prieto et al., 1999). A reagent was prepared by adding 4 mM ammonium molybdate, 28 mM sodium phosphate, and 0.6 M H_2SO_4 in distilled water. Ascorbic acid (0.1mg/ml) was used to plot the standard curve. In short, a series of volumes of standard and a known volume of sample was pipetted into a test tube and the volume was made up to 300 µl with distilled water. To this, 3 ml of the prepared reagent was added and the test tubes were incubated at 95 °C for 90 min in the water bath. Absorbance was recorded at 695 nm after cooling.

6.2. DPPH free radical scavenging activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of the methanolic extractives was assessed according to the method reported earlier (Alshehri et al., 2020). A 0.1 mM DPPH solution was prepared by adding 3.94 mg of DPPH in methanol. This solution was vortexed well for a minimum of 30 min before use. The absorbance of this solution was adjusted between 0.95 - 1.1 at 517 nm using methanol as the blank. A series of volumes of 0.1 mg/ml of ascorbic acid standard and samples were used for analysis. This was treated with the DPPH solution in the dark for 15 min. Later the absorbance was noted at 517 nm using a single-beam UV-visible spectrophotometer (Thermo Scientific, Genesys 50). The percentage DPPH radical scavenging activity was calculated using the formula below.

$$\text{DPPH Scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Where A_{control} and A_{sample} are the absorbances of control and samples.

6.3. ABTS scavenging activity

Methanolic extractives of the oil were assessed for their ability to scavenge ABTS (2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) free radicals. A 7.4 mM ABTS solution was prepared using ABTS and potassium per sulphate in distilled water. This dark blue-green solution was considered the main stock and the same was incubated for 16 h in the dark. Later, the required quantity of ABTS working stock was prepared by mixing stock solution in methanol and the absorbance was adjusted to 0.7 ± 0.01 at 734 nm using methanol as the blank. A 0.1 mg/ml working stock of ascorbic acid was used as the standard. Briefly, a series of volumes of standard and samples (10 – 200 µl) were taken in 2 ml MCTs and the volume was made up to 200 µl using methanol. Then 1.8 ml of ABTS working stock was added to each tube and the reaction mixture was incubated at room temperature in the dark for 6 min. Then the absorbance was measured and the percentage of ABTS scavenging activity was calculated using the formula given below (Re *et al*., 1999).

$$\text{ABTS Scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Where A_{control} and A_{sample} are the absorbances of control and samples.

6.4. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power (FRAP) of the samples was quantified using the standard curve plotted against the ascorbic acid standard using the method detailed earlier (Kumar et al., 2015). To elaborate, a series of volumes of standard and a known volume of samples were taken in a 2 ml microcentrifuge tube (MCT) and the volume was made up to 200 µl using distilled water. To this 500 µl of 0.2 M phosphate buffer (pH 6.6) was added followed by 500 µl of 1% potassium ferricyanide. Vortexed well and the MCTs were incubated at 50 °C in the water bath for 30 min. After cooling, 500 µl of 10 % trichloroacetic acid (TCA) was added. This was centrifuged at 7000 rpm for 10 min. 500 µl of the upper layer of the supernatant was collected to fresh MCTs followed by the addition of an equal volume of distilled water. To this, a freshly prepared 0.1% ferric chloride was added and the absorbance was recorded immediately at 700 nm using a single-beam UV-visible spectrophotometer (Thermo Scientific, Genesys 50).

RESULTS AND DISCUSSION

1. Plant material

Fig.1. ***Rivina humilis*** **L., plant twig with its berries**

Fig.2. ***R. humilis*** **plant powders obtained after drying (a) stem, (b) root, and (c) seed.**

2. Oil extraction

As per the recent report (Riya et al., 2023), Oil extraction in the Soxhlet apparatus resulted in a yield of 17.66 % in the root, followed by 13.18 % in the stem, and 11.25 % in seeds. The Soxhlet apparatus used for extraction and the rota evaporator used for evaporation of the extraction solvent are shown in figure.3. The obtained stem oil (STO), root oil (RTO) and seed oil (SDO) after solvent evaporation are shown in figure.4.

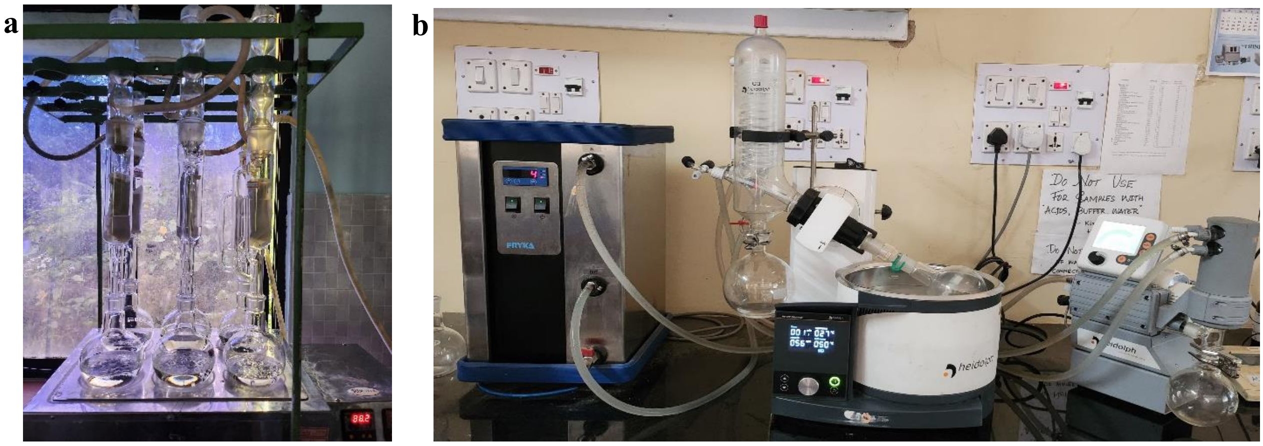

Fig.3. (a) Oil extraction in Soxhlet apparatus (b) Solvent evaporation in rota evaporator

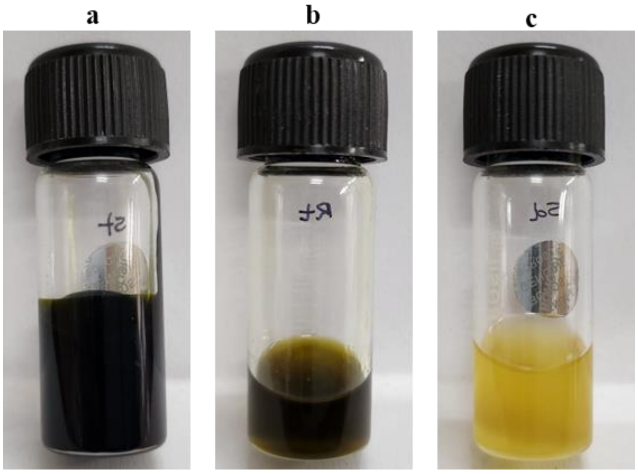

Fig.4. Oil obtained (a) STO (b) RTO (c) SDO

3. Fourier-transform infrared spectroscopy (FTIR) analysis

R. humilis plant part oil (Stem oil (STO), root oil (RTO), and seed oil (SDO)) was taken to FTIR analysis to identify the major functional groups. FTIR spectrum in the range of 4000-400 cm^{-1} is shown in Fig.5. and the major functional groups identified are listed in the table below (Table 3.). Almost ten major functional groups in all the different oils were detected. Peaks at 722 (Overlapping of methylene rocking vibration), 1375-1377 (Bending vibration of CH_2 group) 2854 (Asymmetric CH_2 Stretching mode of methylene chains in membrane lipids), and 2922-2924 (CH_2 acyl chains), and were the commonly identified functional groups in all the oils (Kumar et al., 2020; Movasaghi et al., 2008 & Rohman and Man 2010).

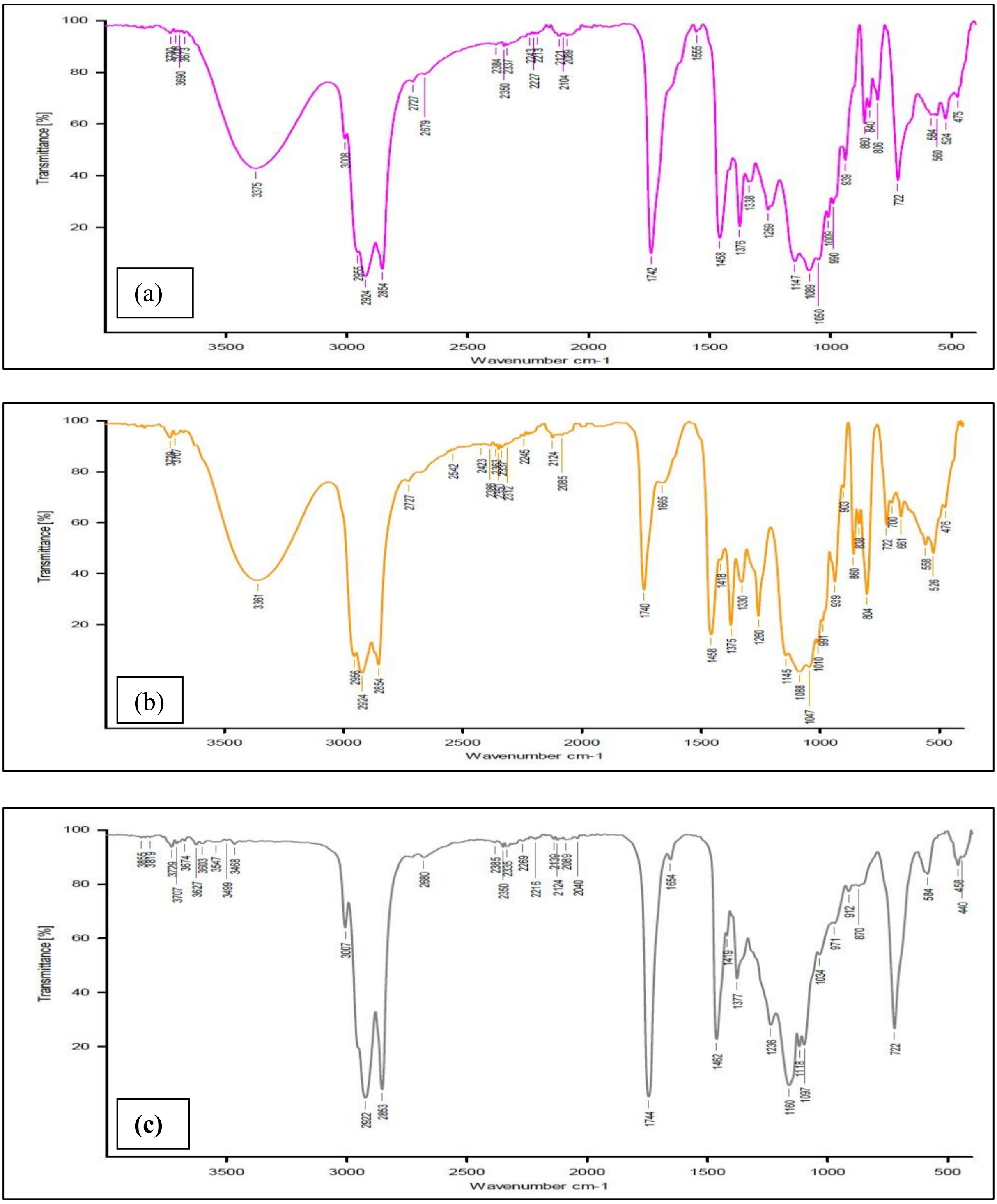

Fig.5. FTIR spectra showing the major functional group present in (a) STO, (b) RTO and (c) SDO.

Table 3. Major functional groups identified through FTIR in stem, root, and seed oil

1. Functional groups identified in stem oil (STO)	
722	Overlapping of methylene rocking vibration
1089	stretching PO_2 symmetric vibration
1147	C-O stretching vibration
1259	PO_2 Asymmetric (phosphate I)
1376	Bending vibration of CH_2 group
1458	C=O Carbonyl group
1742	C=O stretching mode of lipid
2854	Asymmetric CH_2 Stretching mode of methylene chains in membrane lipids
2924	CH_2 acyl chains
2. Functional groups identified in root oil (RTO)	
722	Overlapping of methylene rocking vibration
1260	PO_2 Asymmetric (phosphate I)
1375	Bending vibration of CH_2 group
1458	C=O Carbonyl group
1740	ester carbonyl functional group of the triglycerides
2854	Asymmetric CH_2 Stretching mode of methylene chains in membrane lipids
2924	CH_2 acyl chains
2956	Asymmetric stretching vibration of CH_3 of acyl chains
3361	Stretching N-H asymmetric O-H, N-H, C-H
3. Functional groups identified in seed oil (SDO)	
722	Overlapping of methylene rocking vibration
1097	stretching C-O-C of the ether group
1118	TAG derived from secondary alcohol
1160	CO stretching
1377	C-H Bending
1462	Bending vibration of the CH_2 and CH_3 aliphatic groups
1744	C=O stretching mode of lipids
2853	Asymmetric CH_2 Stretching mode of methylene chains in membrane lipids
2922	CH_2 acyl chains
3007	-C=CH cis double bond stretching

4. Determination of Nutraceutical composition

4.1. Determination of total phenolics content (TPC)

The results of total phenolic content (TPC) estimation were expressed in mg/100g oil GAEq. The standard curve obtained for gallic acid is shown in figure.6. TPC content in stem, root, and seed oil is shown in figure.7. The solvent used for extraction played a significant role in the extractability of the phenolics from the oil sample. 80% methanolic (80%Me) extract has the highest TPC content. Out of the three oils, 80% Me of root oil (RTO) showed the highest TPC (278.72 mg/100g oil.). Similar content was reported in Anise oil (252 mg/100g oil) (Kozłowska *et al*., 2016). The lowest TPC content was observed in 70% acidified methanolic extract (70% AMe) seed oil (31.06 mg/100g oil). Similar content of TPC was reported in *Basella rubra* seed oil (34.22 mg/100g oil) (Kumar *et al*., 2020). TPC in stem oil (119.31 mg/100g oil) was similar to nutmeg oil (119 mg/100g oil) (Kozłowska *et al*., 2016). In general, the content of natural polyphenols in vegetable oil might change depending on the conditions of extraction and processing and can be reduced more when using hot pressing techniques to produce commercial products (Garcia *et al*., 2006).

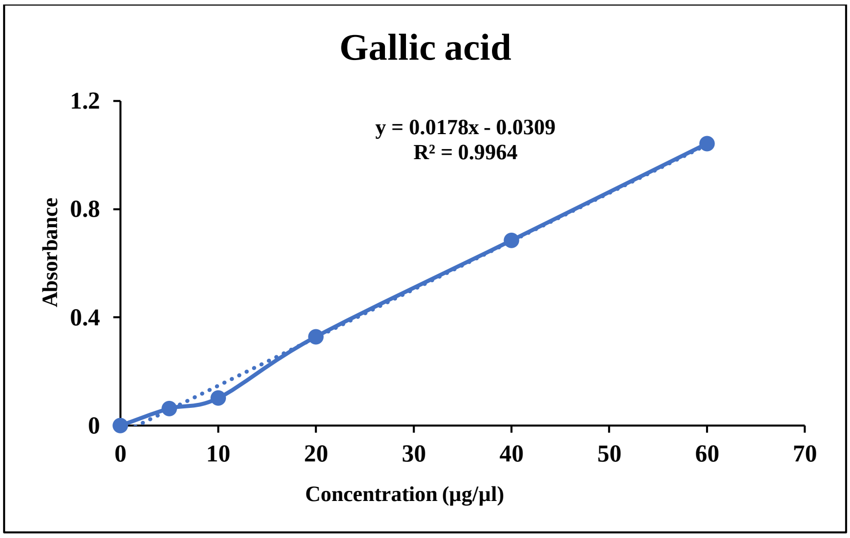

Fig.6. Standard curve of Gallic acid

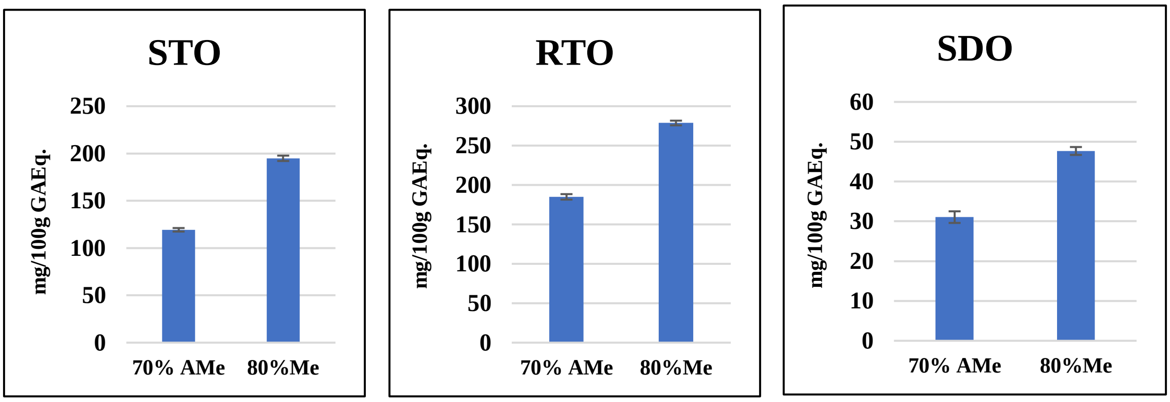

Fig.7. TPC in methanolic extractives of oils

(*AMe - acidified methanol, Me – methanol) *Values are mean ± SD

4.2. Determination of total flavonoid content (TFC)

Flavonoids belong to a class of low molecular weight phenolic compounds (over half of 8000 naturally occurring phenolic compounds) widely distributed in the plant kingdom. Health-promoting effects such as antioxidative, anti-inflammatory, and anti-mutagenic properties of flavonoids make them indispensable components in various nutraceutical, medicinal, and pharmaceutical applications (Panche et al., 2016). One of the major flavonoids, rutin, was used to plot the standard curve for total flavonoid content estimation (figure.8.) with an R^2 value of 0.9984. Figure.9. represents the TFC estimated in different oils. As in the case of TPC, the extractability of total flavonoids was also comparatively good at 80 %Me in all the oils. The maximum was reported in STO (763 mg/100g oil) followed by RTO (63.68 mg/100g oil) and SDO (2.28 mg/100 g oil). Similar content was reported in safflower and soybean oil (3mg/100g oil) (Xuan et al., 2018). Compared to STO and RTO, seed oil showed insignificant TFC content.

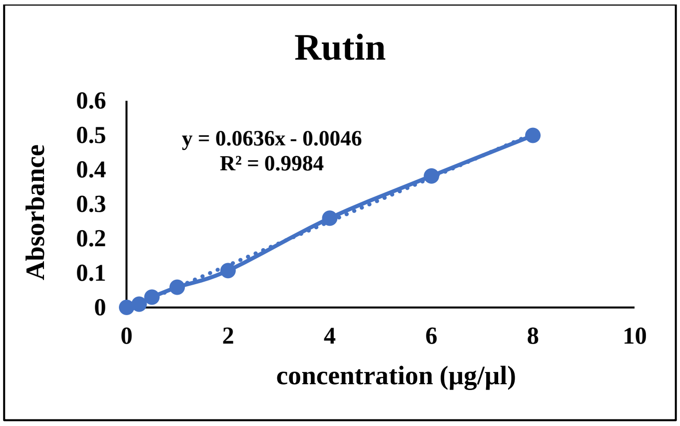

Fig.8. Standard curve of Rutin

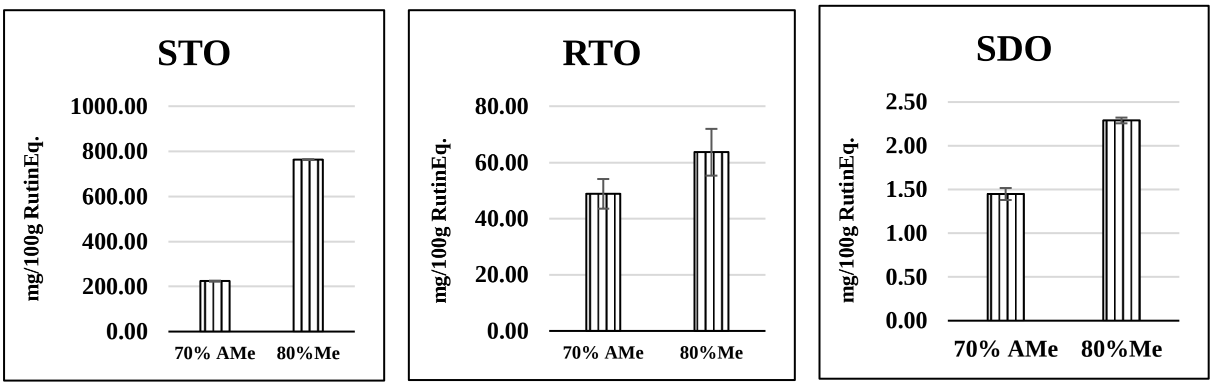

Fig.9. TFC in methanolic extractives of oils

(*AMe - acidified methanol, Me – methanol) *Values are mean ± SD

4.3. Estimation of total phytosterols content

Phytosterols are important micronutrient in human diets, which is known to exert hypocholesterolemic function. Besides, they are also known to exhibit other health-promoting effects such as anti-inflammatory, immune-modulatory, and anticancer properties. 43.6% of phytosterols are contributed by edible vegetable oils. (Yang *et al*., 2016). In the present study, phytosterol content in STO, RTO and SDO was estimated and the results are expressed in g/100g oil (Figure.10.a.). Cholesterol was used as the standard phytosterol (figure.10.b.). The highest content was estimated in STO (32.25g/100g), followed by RTO (9.98g/100g) and SDO (5.39g/100g). The phytosterol content obtained in seed oil was almost double the amount compared to *Basella rubra* seed oil (2.26 g/100g) reported recently (Kumar et al., 2020).

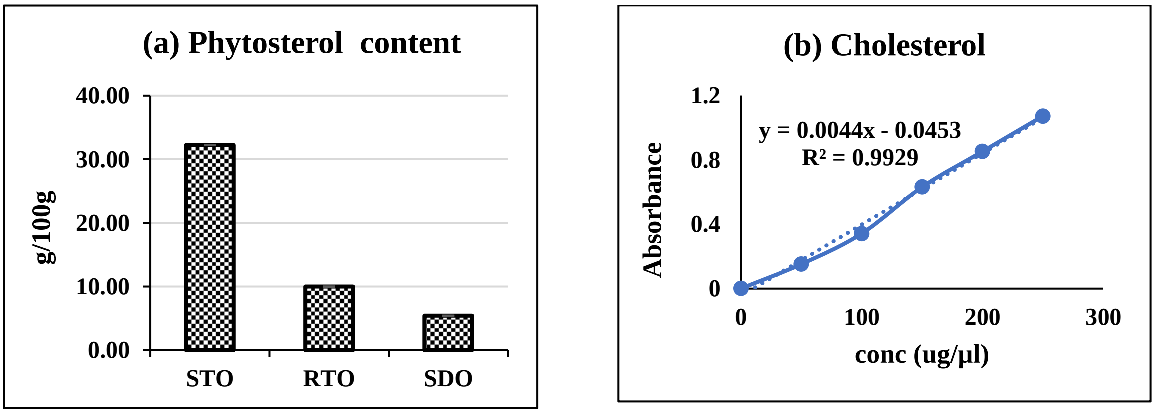

Fig.10. (a) Total phytosterol content in different oils (b) Standard curve of cholesterol

4.4. Total oryzanol content

Oryzanol, an antioxidant substance, is known to lower plasma cholesterol as well as to reduce levels of cholesterol absorption and platelet aggregation. Additionally, oryzanol has been used to treat hyperlipidaemia, and menopausal issues, and to build muscular strength (Patel and Naik 2004). A class of oryzanol called gamma oryzanol (a complex mixture of ferulate, esterified with sterols or triterpene alcohols) is present in rice bran oil (1-2%) (Lilitchan *et al*., 2008). In the present study, the results of oryzanol content estimated in different oils were expressed in g/100 g of oil. The highest oryzanol content was obtained in SDO (0.412 g/100) followed by RTO (0.146 g/100g) and STO (0.013 g/100g) as shown in the figure.11.

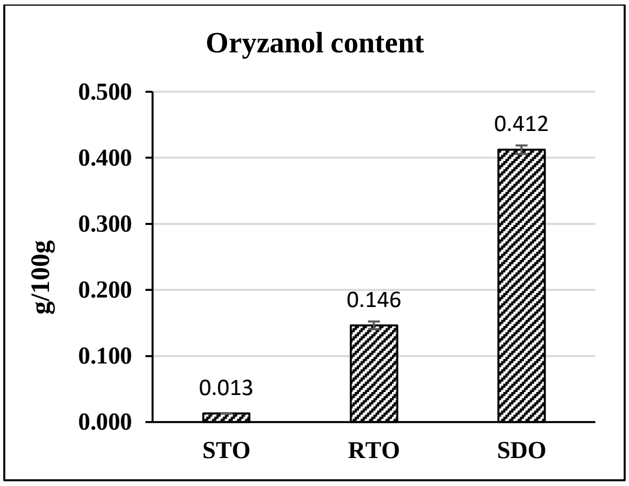

Fig.11. Oryzanol content in different oils

***Values are mean ± SD**

4.5. Estimation of lignan content

Lignans are plant secondary metabolites, implicated in protection against pathogens and UV radiations. They exhibit properties like anti-inflammatory, antitumor and antioxidant activities (Garcia et al., 2019). Lignans can be found in a wide variety of foods that are regularly consumed in the West, including flaxseed and other seeds, as well as vegetables, fruits, and drinks like coffee, tea, and wine (Landete, 2012). The results of the present study are shown in the figure.12. and the results are expressed in g/100g oil Sesamol Eq. As in the case of other general seed oils, lignan content in *R. humilis* SDO was also reported in lower quantity (0.298g/100g oil). This was comparatively low when compared to sesame oil with 1.08 g/100 g lignan content (Reshma et al., 2010). STO and RTO showed a higher content of lignan.

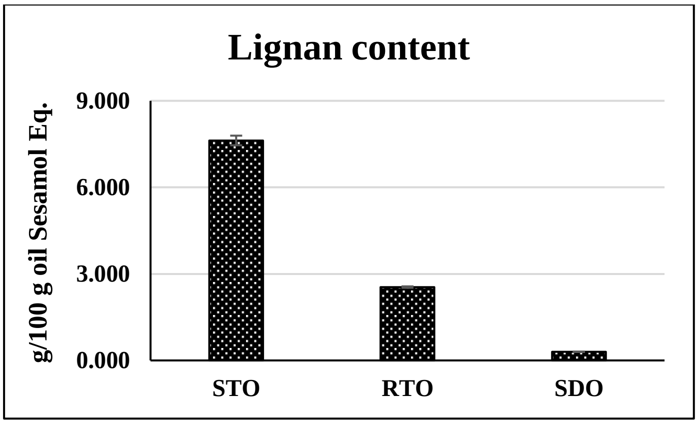

Fig.12. Lignan content in different oils

***Values are mean ± SD**

4.6. Determination of carotenoid content

The results of total carotenoid content were expressed in ppm. The highest content was in STO (7779.68± 56.42). RTO and SDO had 144.58 ± 5.68 and 15.24 ± 0.23 ppm respectively. Carotenoids are substances with unique properties that serve as a foundation for their wide range of activities and functions in various types of living organisms (Britton, 1995).

5. Determination of *in vitro* antioxidant activities

5.1. Total antioxidant activity (TAA)

Total antioxidant activity was assessed using the phosphomolybdenum method. Here, molybdenum (VI) is reduced to molybdenum (V) in the presence of potential bioactives present in the sample. This change is indicated by the formation of a green phosphate/Mo(V) complex at acidic pH (Riya et al., 2023). The results obtained for TAA in the present study in represented in figure.13.a. Ascorbic acid was used as the standard antioxidant compound with an R^2 value of 0.9984 (figure13.b.). The results are expressed as g/100g oil AA Eq. in all the oils, 80%Me showed highest activity. RTO exhibited the highest activity (1.69 g/100g) followed by STO (1.30 g/100g) and SDO (0.21 g/100g). A similar TAA was reported recently in *Basella rubra* seed oil (0.21 g/100g) (Kumar et al., 2020).

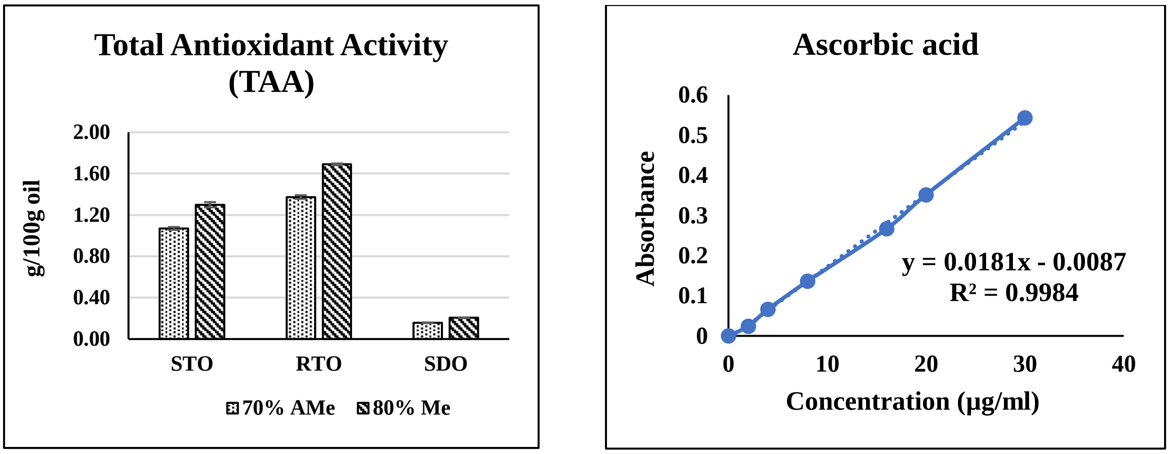

Fig.13. (a) TAA of oils (b) Standard curve obtained for Ascorbic acid

(*AMe - acidified methanol, Me – methanol) *Values are mean ± SD

6.2. DPPH free radical scavenging activity

DPPH, the stable free radical (purple colour) turns yellow when scavenged. So, the scavenging potential of the sample is directly indicated by the colour change measured at 517 nm. The antioxidant present in the sample can directly react with the DPPH free radicals produced in the suitable solvent. In the present study, we have compared this property of the oil sample to the ascorbic acid standard (EC_{50} 7.70 µg/mL) figure.14. The results are expressed in EC_{50}. The lower the value, the higher the antioxidant power of the samples. 70% AMe showed the lowest EC_{50} in all the samples. Stem and root oil extractives exhibited a similar range of EC_{50} values (12.77 and 10.44 mg/ml respectively). SDO extractive showed a comparatively higher value (58.35 mg/ml).

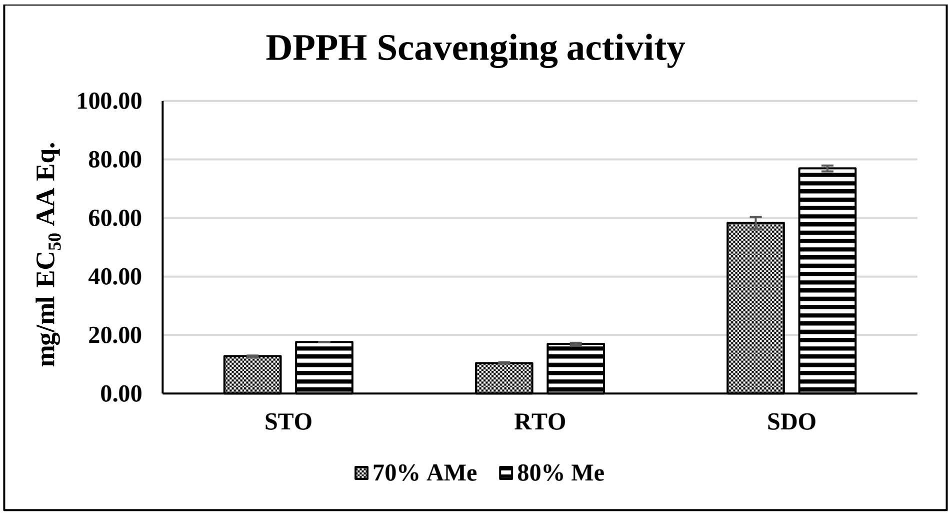

Fig.14. DPPH scavenging activity of methanolic extractives of oil

6.3. ABTS scavenging activity

Methanolic extractives of oil samples were subjected to ABTS scavenging activity assay, one of the extensively used method to determine antioxidant activity and the results were expressed in mg/ml EC50 AAEq. (fig.15.). There was a greater difference in the EC_{50} values in 70%AM and 80%M extractives. The lowest values were observed in 80%M extractives. STO (1.73 mg/ml) showed the highest activity followed by RTO (2.80 mg/ml). 80%M and 70%M extractives of seed oil showed EC_{50} values of 12.30 and 46.97 mg/ml respectively. This was comparable to the already reported EC_{50} value (56.19 mg/ml) of *B. rubra* seed oil (Kumar et al., 2020).

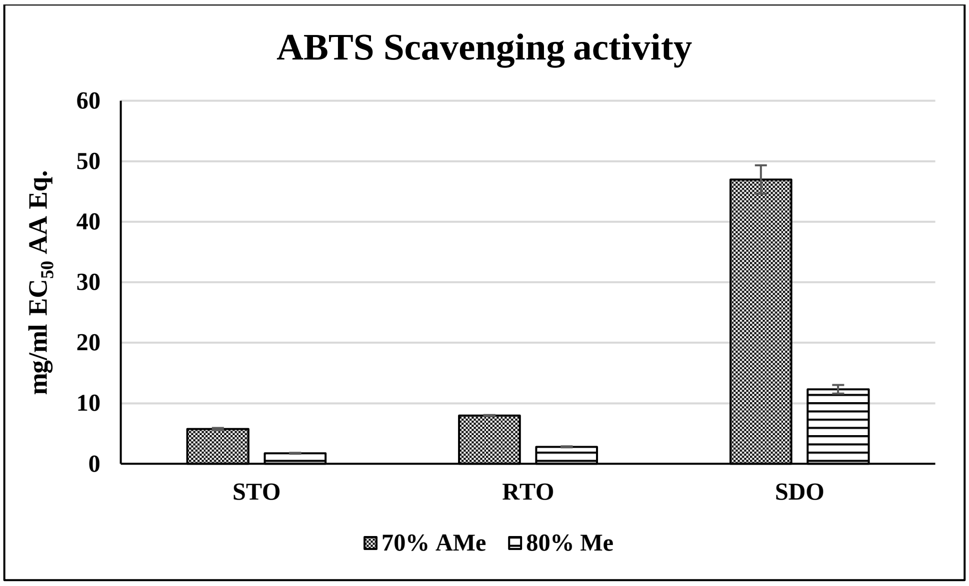

Fig.15. ABTS Scavenging activity of methanolic extractives of oil

(*AMe - acidified methanol, Me – methanol) *Values are mean ± SD

6.4. Ferric reducing antioxidant power (FRAP)

In FRAP, the reductive ability was measured in terms of the reduction of potassium ferricyanide to the ferrous form in the presence of different concentrations of the samples. This is one of the commonly used methods for the determination of the antioxidant activity of oil samples. Results are quantified using the standard curve plotted for ascorbic acid (R^2=0.9988) and expressed in mg/100g oil AAEq. (figure.16.). 80% Me extract showed higher activity in all the oils. STO showed a FRAP activity of 118.95mg/100g followed by RTO (90.10 mg/ml). compared to STO and RTO, seed oil reported a lower FRAP activity (12.54 mg/ml).

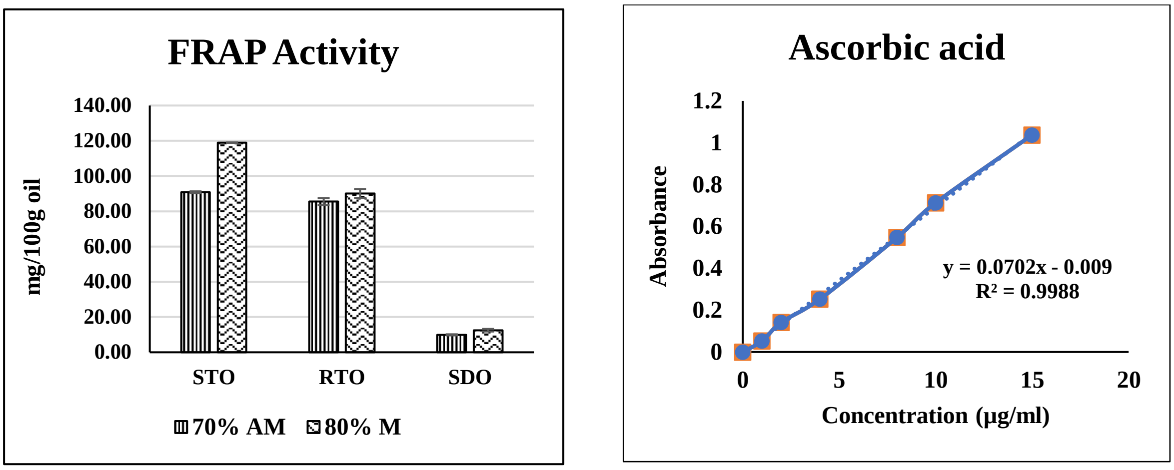

Fig.16. (a) FRAP activity of methanolic extractives of oil (b) Standard curve of AA

(*AMe - acidified methanol, Me – methanol) *Values are mean ± SD

CONCLUSIONS

The present study investigated the nutraceutical composition and in vitro antioxidant potential of oils obtained from the stem, root, and seeds of *Rivina humilis*. Since there is an ever-expanding market for oil crops from both nutritional and industrial perspectives, the present study can be considered as an insight into the oil characteristics and thus it will be helpful in identifying the scope for its future application in various industries. First, major functional groups present in stem oil, root oil, and seed oil were analysed using FTIR and we have identified functional groups in support of the class of lipids in all the oils. The nutraceutical composition of oils revealed that they are a good source of phytosterols, lignans, oryzanol, carotenoids and phenolics. STO and RTO were comparatively good in terms of their phenolics and flavonoid contents. In seed oil, both were reported in lower quantities. A similar trend was observed in phytosterol, carotenoids, and lignan content as well. In the case of oryzanol, an antioxidant compound, seed oil was reported the highest. Antioxidant activities of the oils were analysed using *in vitro* methods like TAA, DPPH, ABTS AND FRAP. The results obtained were totally in line with the nutraceutical composition. It is concluded that the good nutraceutical profile in STO and RTO could directly contribute to their good antioxidant activities obtained in all the assays. Though the antioxidant activities of SDO was lower when compared to the other two oils, the results were comparable to other seed oil. As oils have a wide range of applications in various industries, our present study establishes a platform for a new source of the same as well as their scope for further applications.

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