ANTHELMINTIC ACTIVITY OF THE SEED OIL OF *RICINUS COMMUNIS*

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A thesis will be submitted to the Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, College of Health Sciences, Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master in Pharmacognosy.

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This is to certify that the thesis prepared by Temesgen Berhanu, entitled: **"Anthelmintic Activity of the Seed Oil of *Ricinus communis*"** and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacognosy complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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List of abbreviations and acronyms

^{13}C-NMR: Carbon thirteen Nuclear Magnetic Resonance

^{1}H-NMR: Proton Nuclear Magnetic Resonance

ANOVA: Analysis of Variance

DEPT: Distortionless Enhancement by Polarization Transfer

DMSO: Dimethyl sulfoxide

LF: Lymphatic Filariasis

LB: Luria-Bertani

ESI- MS: ElectroSpray Ionization- Mass Spectrometry

NGM: Nematode Growth Media

RAF-3: Ricinoleic acid

RAME: Ricinoleic acid methyl ester

STH: Soil Transmitted Helminths

STN: Soil Transmitted Nematods

TLC: Thin Layer Chromatography

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Abstract

Anthelmintic Activity of the Seed Oil of *Ricinus communis*

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Helminthic infections are among the most widespread parasitic infections in humans. It is estimated that more than half of the world's population suffer from parasitic infections. It poses morbidity particularly in children and pregnant women. Helminthic infections are more common in developing countries with poorer personal and environmental hygiene. Plant-derived drugs and herbal remedies remain important resources to alleviate and cure diseases, especially in developing countries. Variety of plants from various families including *Ricinus communis* from the family Euphorbiaceae are used to treat helminthiasis across the world. *R.communis* is an annual oilseed crop commonly known as castor. Antimicrobial, antifungal, anticancer, antidiabetic, anti-inflammatory and antimalarial are some of the pharmacological activities that have been reported from extracts of *R. communis*. In the present study, the petroleum ether seed extract of *R. communis*, its base hydrolysate and the major constituent of the hydrolysate were tested for their anthelmintic activities against the model organism *Caenorhabditis elegans.* The dried and powdered seeds were extracted using petroleum ether and then the oil collected was subjected to base hydrolysis to obtain the hydrolysate. Fractionation of the hydrolysate by column chromatography packed with silica gel 60-G resulted in the isolation of the hydroxylated fatty acid (9Z)-12-hydroxyoctadec-9-enoic acid (ricinoleic acid). The isolated free fatty was esterified by 5% Potassium hydroxide solution in methanol to get ricinoleic acid methyl ester. Structural elucidation of the compounds was achieved by spectroscopic techniques including ESI-MS, ^{1}H and ^{13}C-NMR spectral data. Results of the anthelmintic assay revealed that

ricinoleic acid and its methyl ester are strongly active against *C. elegans* worms compared to the oil. Ricinoleic acid exhibited 97% mortality at the tested concentration of (1 mg/ml). In conclusion, the present study demonstrated that the oil of *R. communis* seeds possesses strong anthelmintic activity against the model nematode *C. elegans* worms and the activity of the oil would be attributed to the presence of ricinoleic acid as a major constituent.

Key words: Anthelmintic activity, Seeds of *Ricinus communis*, Fatty acids, *Caenorhabditis elegans* assay, Ricinoleic acid

1. Introduction

1.1 Helminthiasis

The term "helminths" is derived from the Greek word “meaning worms” that have plagued humans and animals since our earliest recorded history (Hotez *et al.,* 2004). There are two major species of helminths. The nematodes (known as roundworms) include the major intestinal worms (known as soil-transmitted helminths) and the filarial worms that cause lymphatic filariasis (LF) and onchocerciasis. The platyhelminths (also known as flatworms) include the flukes (known as trematodes), the tapeworms and the pork tapeworm that causes cysticercosis (Hotez *et al.*, 2004). Helminthic infections are among the most widespread parasitic infections in humans (Abdoli, 2020). It is estimated that more than half of the world’s population suffers from these infections. In particular, it poses greatest morbidity in children and pregnant women (Farrell *et al.*, 2018). They are more common in developing countries with poorer personal and environmental hygiene. It is also among the most important animal diseases inflicting heavy production losses (Pabalan *et al.*, 2018; Baker and Ensink, 2012; Asaolu and Ofoezie, 2003).

Human GIT is the residence of numerous helminths, but some also live in tissues. They harm the host by depriving food, causing blood loss, injury to organs, intestinal or lymphatic obstruction, and secreting toxins (King and Li, 2018). Many humans harbor helminths (worms) of various species that lead to malnutrition, pneumonia, eosinophilia, and anemia. Other clinical manifestations of helminthic infections include dysentery, dermatological disorders, loss of appetite, and loss of body weight (Pabalan *et al.*, 2018; Baker and Ensink, 2012).

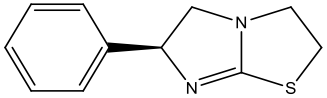
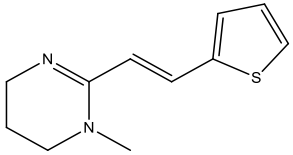
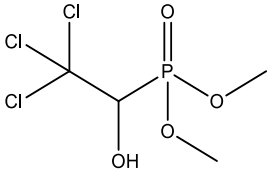
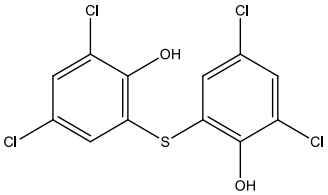
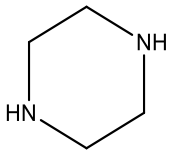
The most common helminthiasis are those caused by infection with intestinal helminths, ascariasis, trichuriasis, and hookworm, followed by schistosomiasis and lymphatic filariasis (Bethony *et al*., 2006). Transmission of most of these diseases involves environmental contamination with eggs and infective larvae (Pabalan *et al*., 2018; Lustigman *et al*., 2012; Bethony *et al*., 2006).

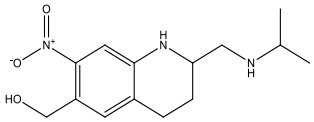
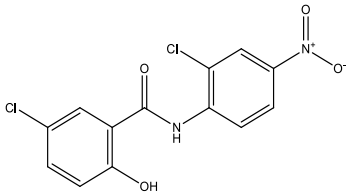
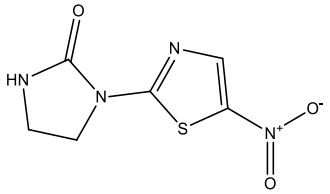
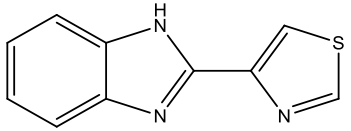
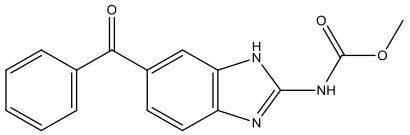
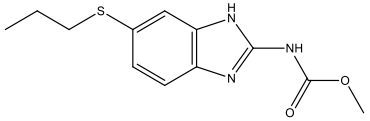
1.2 Treatment of helminth infections

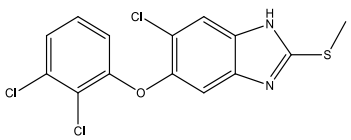
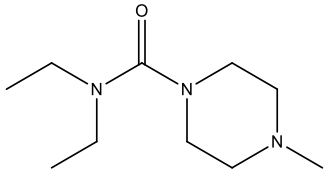
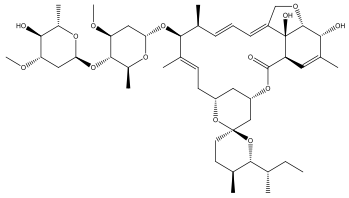
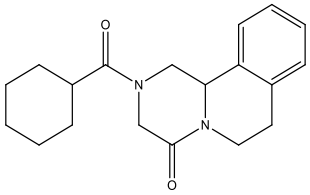
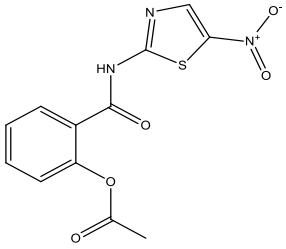
Worm infections result in an important burden on public health, leading to the loss of millions of disability-adjusted life years. In several countries, socio-economic development, sanitation, and health education combined with anthelmintic treatment are key factors in preventing new cases and curing existing worm infections (Vlaminck *et al*., 2019; Van Den Enden, 2009).

Anthelminthics either kill or expel intestinal parasitic worms. Their mechanisms include interfering with the parasite's carbohydrate metabolism, inhibiting respiratory enzymes, and blocking neuromuscular action. Preventive chemotherapy (PC) with benzimidazole drugs is the backbone of soil-transmitted helminths (STH) control programs (Vlaminck *et al*., 2019; Speich *et al*., 2016; Van Den Enden, 2009). Some of the most commonly used anthelmintics are listed in (Table 1).

Table 1: Current pharmacotherapeutic agents used to treat worm infections.

	Name	Chemical class	Mechanism of action	Structure	Reference
1	Levamisole	Imidazothiazole	Immunomodulation		(Albonico *et al.*, 2003)
2	Pyrantel	Pyrimidine derivative	Cholinergic depolarizing neuromuscular agent		(Albonico *et al.*, 2002; Reynoldson *et al.*, 1997)
3	Metrifonate	Organophosphorus	Cholinesterase inhibitor		(Doenhoff *et al.*, 2008)
4	Bithionol	Halogenated phenol	–		(Leonardi *et al.*, 2016)
5	Piperazine	Cyclic secondary amine	GABA receptor agonist		(Mohan *et al.*, 2014)

6	Oxamniquine	Tetrahydroquinoline derivative	–		(Köhler, 2001)
7	Niclosamide	Halogenated salicylanilide derivative	Inhibits energy production in the parasite		(Imperi *et al.*, 2013)
8	Niridazole	Nitrothiazole derivative			(Barakat *et al.*, 2014)
9	Thiabendazole	Benzimidazoles	Inhibit polymerization of tubulin		(Mottier and Prichard, 2008; Gottschall *et al.*, 1990)
	Mebendazole				
	Albendazole				

	Triclabendazole				
10	Diethylcarbamazine	Piperazine derivative	Interfere in arachidonic acid metabolism		(Gottschall *et al.*, 1990)
11	Ivermectin	Macrocyclic lactones	Hyperpolarization and paralysis of the somatic muscles		(Õmura and Crump, 2004)
12	Praziquantel	Isoquinoline derivatives	Spastic paralysis and tegumental disruption		(Cupit and Cunningham, 2015)
13	Nitazoxanide	Nitrothiazole derivative			(Somvanshi *et al.*, 2011)

1.3 Medicinal plants

Since prehistoric times, humankind has used natural products, such as plants, animals, and marine organisms as medicines to alleviate and cure diseases. According to fossil records, the use of plants in humans as medicines could be traced back to a minimum of 60,000 years (Yuan *et al*., 2016).

According to WHO, herbal medicines are plant-derived materials or preparations with therapeutic or other human health benefits, which contain either raw or processed ingredients from one or more plants (Pandey *et al*., 2013). Plant-derived drugs and herbal remedies remain important sources to alleviate and cure diseases, especially in developing countries. Approximately 60–80% of the world's population still relies on traditional medicines for the treatment of common illnesses (Pandey *et al*., 2013; World Health Organization, 2005). Medicinal plants are among natural source of secondary metabolites such as alkaloids, tannins, flavonoids, steroids, and terpenoids. Some have nutritional value and others have pharmacological benefits (Sen and Chakraborty, 2015).

1.4 Medicinal plants used to treat helminthiasis

Plants have played a central part in combating many ailments in humans and livestock in many indigenous communities (Tolossa *et al*., 2013) . The term medicinal plants include various types of plants used in herbalism and some of these plants have medicinal activity. These medicinal plants are rich source of ingredients that can be used in drug development and synthesis (Das and Choudhury, 2012).

Different plants from various families are used to treat helminthiasis across the world. For example, in Bangladesh *Clerodendrum viscosum* and *Tinospora cordifolia* are used for

combating helminthiasis by traditional healers (Uddin and Hassan, 2014). In India *Carica papaya, Cleome viscosa, Scopari adulcis, Vernonia cinerea* and *Withania somnifera* are common medicinal plants used to treat helmentiasis (Das and Choudhury, 2012; Alagesaboopathi, 2009). In Pakistan *Azadirachta indica, Citrullus colocynthis, Lamium amplexicaule, Mallotus philippinensis*, and *Withania somnifera* are used by the local community to treat helminthic infections (Khalid *et al*., 2010; Jabbar *et al*., 2006). In Africa some of the most common medicinal plants used to treat helminths include *Albizia anthelmintica, Embelia schimperi*, and *Myrsine africana* in Kenya (Muthee *et al*., 2016) and *Phytolacca dodecandra, Priva adhaerens* and *Vernonia amygdalina* in Uganda (Matovu *et al.*, 2020). Similarly, *Afromomum melegueta, Khaya senegalensis*, and *Xylopia aethiopica* are the main source of treatment of helminthiasis in endemic areas of Togo (Ataba *et al*., 2020). In different local communities of Ethiopia *Agati gratifola, Agrimonia eupatori, Butea fondosa, Carica papaya, Combretum mucoreatum, Cucurbita moschata, Hagenia abyssinica, Helleborus niger, Mangifera indica and Ricinus communis* are used to treat helminth infections in humans and livestock (Yirga *et al*., 2022; Scantlebury *et al.,* 2013; Mesfin *et al.,* 2009; Giday *et al*., 2009).

1.5 *Ricinus communis* L.

Ricinus communis is the sole species in the monotypic genus *Ricinus*. *R. communis* is an annual oilseed crop commonly known as castor also “Palma Christi” or “wonder tree.” The plant, belongs to the spurge family Euphorbiaceae (Figure 1). This family contains some 6,745 species in 218 genera (Singh *et al.,* 2020; Odunsi *et al.,* 2012). Many members of the family are important food sources. Others are useful for their waxes and oils and as a source of medicinal herbs. *R. communis* is found in tropical and subtropical regions of the world including

the Arabian Peninsula as a wild sprouting plant. The plant is mentioned in connection with different compositions for cosmetic and medical products. Parts of the plant as well as the castor oil itself are among the oldest drugs and have been used in traditional or folk remedies for rituals of sacrifice (Franke *et al*., 2019).

Figure 1. Aerial part of *Ricinus communis* (Photographed by Temesgen Berhanu around Melke shiti village, Negele Arsii, West Oromia Ethiopia- April, 2021)

1.5.1 Traditional uses

Castor plant has been cultivated as far back as 4000 A.D. *R. communis* has been a therapeutic agent for around 4000 years. It is used as a herbal medicine for treating many different diseases, disorders, and also many infections. Leaves, roots, bark, and various parts of *R. communis* have been used for medicinal purposes. There are numerous uses of *R. communis* plant which utilize every part of the plant (Franke *et al*., 2019; McKeon, 2016).

The powdered leaves are found to be effective in combating mosquitoes and repelling aphids, rust mites, and whiteflies. An infusion of the leaves is used as an eye lubricant and also for relieving stomach aches. The leaves as such are used as a decoction or poultice and applied to the breasts of females for an increase in milk secretion (lactation). Fresh juice obtained from the leaves has been reported for its use as an emetic in the poisoning of narcotics like opium. There is also a report in the literature that the leaves can be useful against jaundice and as an anthelmintic agent (Singh *et al*., 2020; Naz and Bano, 2012; Bhattarai, 1992). The roots are used for various purposes such as a powerful purgative, and for toothache (Rana *et al*., 2019). Seeds of *R. communis* are the primary source of oil which is in use both as herbal medicine and as a conventional therapy for various ailments (Franke *et al*., 2019).

1.5.2 Ethnopharmacology

Different pharmacological activities of *R. communis* are well documented. Extracts from different parts of the plant exhibited antimicrobial, antifungal, anticancer, antidiabetic, anti-inflammatory, antimalarial, antioxidant, central analgesic, anticonvulsant, antinociceptive, anthelminthic, antifertility, laxative, uterine contracting, anti-implantation, antiasthmatic, bone regeneration, molluscicidal, antiulcer, antihistamine, wound-healing, cytotoxic, insecticidal, anti-

arthritic, antidandruff and hepato-protective activities (Abdul *et al*., 2018; Rampadarath *et al*., 2014; Zarai *et al*., 2012). These biological activities are attributed to different primary and secondary metabolites found in this plant such as fatty acids, flavonoids, alkaloids and terpenes (Srivastava *et al.,* 2014).

1.5.3 Phytochemistry

As many as eighty compounds were identified from *R. communis* seeds, leaves, roots, and stem extracts. These include alkaloids, terpenoids, flavonoids, benzoic acid derivatives, coumarins, tocopherols, terpenoids, and fatty acids (Ribeiro *et al*., 2016). Some of the compounds identified in *R. communis* seeds, leaves, roots, and stem extracts are listed in Appendix I.

1.6 Statement of the problem

Helminths especially soil-transmitted nematodes (STNs) such as *Ascaris lumbricoides, Trichuris trichiura*, and hookworms affect one-third of the general world population. In developing countries, it is estimated that one out of every two people is infected with at least one STN. Helminth infection leads to a loss in millions of disability-adjusted life years, which is comparable to major killers like malaria and tuberculosis (TB) (Panda *et al*., 2020; Humphries *et al*., 2012).

Currently, four anthelmintic drugs, namely, albendazole, mebendazole, levamisole, and pyrantel pamoate are listed as essential medicines to combat helminth infections (Keiser and Utzinger, 2008). The downside of current anthelmintics especially benzimidazole (BZ) is drug resistance due to single dosage and similarity of the mechanism of action. Also, side effects of the listed medications include dizziness, nausea and vomiting, drowsiness, diarrhea, fatigue, pruritus,

headache, tinnitus, convulsions, neuropsychiatric disturbances, intrahepatic cholestasis, and hypersensitivity reactions (Van Den Enden, 2009; Coles 2006).

Since a limited number of medications are used to combat helminth infections, there is the danger of the emergence of resistance species. This is because when parasites are repeatedly exposed to the same drug, parasites that survive, may pass on genetic variants that make the offspring more resistant to the anthelmintic drug (Mutombo *et al*., 2019). There is, therefore, a need to find new drugs derived from natural products to help fight these parasitic worms. Traditional medicine would be a good place to look for anthelmintics as the custodians of indigenous knowledge know which plants to use against which diseases. It has been reported that plants can be used as a source of anthelmintic drugs which have fewer side effects than synthetic drugs (Cheraghipour *et al*., 2019). Natural products have been a reputable source for drugs as they have developed secondary metabolites due to various abiotic and biotic stresses (David *et al*., 2015). Thus, the present study dealt with anthelmintic activity of the oil of castor beans with the aim of discovering compound(s) with new mechanism of action and fewer or no side effects.

2. Objective

2.1 General objective

- To evaluate the *in vitro* anthelmintic activity of the oil of *Ricinus communis* seeds and its major compound against *Caenorhabditis elegans*

2.2 Specific objectives

- To study the anthelmintic l activity of the petroleum ether seed extract of *R. communis*;
- To determine the anthelmintic activity of the different fractions obtained from the petroleum ether seed extract of *R. communis*;
- To isolate compound(s) from the hydrolysate of the oil and determine the anthelmintic activity of the isolated compound(s); and
- To prepare the methyl ester derivative of the isolated compound and determine its anthelmintic activity.

3. Materials and Methods

3.1 Materials

3.1.1 Plant material

The seeds of *R. communis* were collected in April 2022 from Negelle Arsi town, west Arsi zone, Oromia regional state 225 Km South of Addis Ababa, Ethiopia which is located in the Great Rift Valley. The authenticity of the plant material was confirmed by Ato Melaku Wondafrash, the National Herbarium, Department of Plant Biology and Biodiversity Management, College of Natural Sciences, Addis Ababa University (AAU), where a botanical specimen was deposited (collection number TB-002) for future reference.

3.1.2 Chemicals, reagents and instruments

The following chemicals, solvents and drugs were used during this study: distilled water (AAU Department of Pharmaceutics and social pharmacy), Petroleum ether, ethyl acetate, acetone, *n*-hexane, chloroform, ethyl acetate, hydrochloric acid, sulfuric acid (LOBA-Chemie, Mumbai, India), methanol (Sheba Pharmaceutical PLC, Ethiopia), castor oil, palmitic acid and oleic acid (Amman Pharmaceutical Industries Co., Amman, Jordan); potassium hydroxide pellet, silica gel 60G/F254 (Carl Roth®, Karlsruhe, Germany); precoated analytical TLC, nematode growth media (NGM), Luria-Bertani (LB) media and agar, M9 buffer (Leibniz Institute of Plant Biochemistry (IPB) laboratory, Halle, Germany); dimethyl sulfoxide (DMSO) (Duchefa Biochemie, Haarlem, The Netherlands); penicillin-streptomycin solution (Capricorn Scientific GmbH, Germany). All the chemicals and solvents were used as received.

The following equipment and instruments were used to carry out the experiments. Rotavapor (Buchi Rota Vapor R-200, Switzerland), light Microscope (Olympus CKX41, Olympus Life Science, Waltham, Massachusetts, USA). CAMAG TLC visualizer (Lab Pilot Process Group, Switzerland). NMR spectra were recorded using Bruker Avance DM×400 FT-NMR 500 MHz for ^{1}H and 126 MHz for ^{13}C (Bruker, USA). Agilent LC-MS system with diode array detector, Agilent 11005 series system (Agilent system, USA), and ESI-API were used for the characterization of isolated compounds.

3.1.3 Test organisms

Caenorhabditis elegans worms were obtained from Leibniz Institute of Plant Biochemistry (IPB) laboratory, Halle, Germany. The parasites were subsequently maintained at 20 °C on 60 mm nematode growth medium (NGM) plates seeded with OP50 bacteria (*Escherichia coli* strain).

3.1.4 Reference drug

Ivermectin (Sigma-Aldrich, Sigma-Aldrich Chemie GmbH - Schnelldorf, Germany) supplied by the Ethiopian Pharmaceutical Manufacturing Factory (EPHARM, Ethiopia) was used as a reference drug.

3.2 Methods

3.2.1 Extraction

The seeds of *R. communis* were cleaned of dust and debris and washed gently with water, and dried under shade. Then the dried seeds were pulverized with a grinder. Dried seed powder (250 g) was macerated in petroleum ether in (1.5 L) with occasional shaking. The mixture was filtered using muslin cloth followed by Whatman No. 1 filter paper. To maximize yield, the marc was re-

macerated twice for 72 h. Then, the combined filtrates were evaporated under reduced pressure at a temperature not exceeding 40 °C in a rotary evaporator to obtain the oil. The oil was then transferred to an amber coloured glass vials and stored in a refrigerator at 4 °C until use.

3.2.2 Hydrolysis of oil

The oil (20 g) was hydrolyzed by refluxing with 12% ethanolic potassium hydroxide solution (25 ml) for 1 h on a hot plate. The organic solvent was evaporated and the residue re-dissolved in 300 ml of deionized water and then acidified with concentrated HCl to pH = 1. The liberated fatty acids were extracted with 600 ml of ethyl acetate. The organic solvent was dried in a rotary evaporator (Buchi Rota Vapor R-200, Switzerland) at 40 °C (Vaisman *et al*., 2008).

3.2.3 Fractionation and isolation of compounds

The hydrolyzed oil was fractionated over silica gel column into several fractions using ethyl acetate and hexane in different ratios. The column was initially eluted with 100 ml of *n*-hexane (F1), followed by *n*-hexane: ethyl acetate mixtures of increasing polarity (9:1, 8:2, 7:3 and 6:4) to obtain four more fractions (F2 - F5) each containing 100 ml of eluent. The different solvent fractions were concentrated under reduced pressure using a rotary evaporator (Buchi Rota Vapor R-200, Switzerland). The dried fractions were then transferred into vials and stored in a desiccator for further use. Fraction 3 (F-3) which showed reddish brown spot on TLC plate in day light was further partitioned between *n*-hexane and methanol in a ratio of 1:1. The methanol layer was then dried in a water bath at 50 °C to remove the organic solvent. The dried material was then treated with dry acetone and kept in refrigerator overnight. After 24 h, the sample was

taken out of the refrigerator and decanted to remove the solvent. The resulting white foggy solid substance coded RAF-3 was then analyzed by TLC.

3.2.4 Esterification of the isolated compound

RAF-3 (50 mg) was dissolved in *n*-hexane (5 ml) to which 10 ml of 5% potassium hydroxide in anhydrous methanol was added and heated at 50 ºC for 15 min. To the resulting mixture a few drops of glacial acetic acid was added followed by addition of 10 ml of water and 10 ml of *n*-hexane. The solution was vortexed vigorously and the upper organic layer taken and dried in an open air. The dried sample coded RAME was then analyzed by TLC (Aldai *et al*., 2005).

3.2.5 Chromatogram visualization and structural elucidation

TLC chromatogram development was carried out using *n*-hexane: ethyl acetate (4:1) solvent system then dried and visualized by spraying with 50% of sulfuric acid. Molecular masses of the compounds were analyzed by negative mode mass spectrometry-electron spray ionization (MS-ESI). 1D and ^{13}C NMR spectra were recorded by dissolving the compounds either in deuterated dimethyl sulfoxide or deuterated chloroform at 288 K. Chemical shifts are expressed in δ scale (ppm) using tetramethylsilane as an internal standard and coupling constants *J* are in hertz (Hz). Multiplicities of ^{1}H-NMR signals were indicated as singlet (*s*), broad singlet (*brs*), doublet (*d*), doublet of doublets (*dd*) and (*m*) multiplate.

3.2.6 *Caenorhabditis elegans* assay

Anthelmintic assay was carried out on the model nematode *Caenorhabditis elegans* using the method developed by Thomsen *et al*. (2012). The nematodes were cultured on NGM Petri plates using the uracil auxotroph *Escherichia coli* strain OP50 as a food source. After 4 days of

cultivation, the nematodes were transferred from the Petri plate to a 15 ml falcon tube by rinsing each plate twice with 2 ml M9 buffer. The worm suspension was then centrifuged for 1 min at 800 rpm. After removal of the supernatant, the nematodes were washed again with 2 ml M9 buffer under the same conditions and, depending on the number of nematodes, re-suspension was carried out in 2 to 8 ml M9 buffer. To this suspension, 10 µl penicillin-streptomycin solution (10 mg/ml) was added. The assay was performed in 384 well plates after adjusting the worm number to 20-30 per 20 µl. The outer wells were filled with 40 µl water to minimize evaporation prior to incubating 20 µl of worm suspension with 20 µl test solution. The number of living and dead nematodes in each well was then counted using an inverted cell culture microscope (Olympus CKX41, Olympus Life Science, Waltham, Massachusetts, USA) after 30 min. Dimethyl sulfoxide (2% DMSO) was used as a negative control, while ivermectin (10 µg/ml) was used as positive control. All the assays were done in triplicate.

3.2.7 Preparation of test substances

Test substances were dissolved in 2% DMSO and M9 buffer to give a concentration of 1 mg/ml. All test samples were tested at a concentration of 1 mg/ml.

3.2.8 Data analysis

Data is presented in terms of the average percentage of dead worms to the total number of nematodes. Data obtained from the experiments were processed by SPSS 25. The comparison was based on a one-way ANOVA analysis. Confidence interval 99% was used, and p values < 0.01 were considered significant.

4. Results and Discussion

4.1 Extraction yield

In the current study, the dried and powdered seeds of *R. communis* (250 g) were subjected to cold maceration with petroleum ether and yielded a yellow coloured oil (21.1 g). The percentage yield calculated from the dried matter was found to be 8.4% (w/w). However, prior studies showed that castor oil content vary from 34.6 to 56.6% (w/w) when extracted with *n*-hexane (Jolayemi *et al*., 2022). In the present study, the low oil yield obtained might be due to variation attributed to the variety of plant collection season, changes in the climatic conditions and geographical location where the seeds are collected.

4.2 *In vitro* anthelmintic activity of *Ricinus communis* seed oil

Plants are an important sources of novel pharmacologically active compounds from which many drugs being derived directly or indirectly (Fowler, 2006). Despite the current development of synthetic chemistry as a tool to discover and manufacture drugs, the contribution of plants to disease treatment and prevention is still enormous. Even at the dawn of the 21st century, 11% of the 252 drugs considered as basic and essential by the WHO were exclusively of flowering plant origin (Veeresham, 2012). Phytochemical research based on ethnopharmacological information is generally considered an effective approach to the discovery of new pharmacological agents from plants (Pandey *et al*., 2013). *R. communis* was selected for this study since it has many claims including anthelmintic properties (Yirga *et al*., 2022; Mesfin *et al*., 2009).

The oil isolated from the seeds of *R. communis* and commercially available castor oil exhibited significant activity ($p < 0.01$) killing 57% and 60% of *C. elegans* worms, respectively, at a concentration of 1 mg/ml compared to the negative control 2% DMSO (Figure 1).

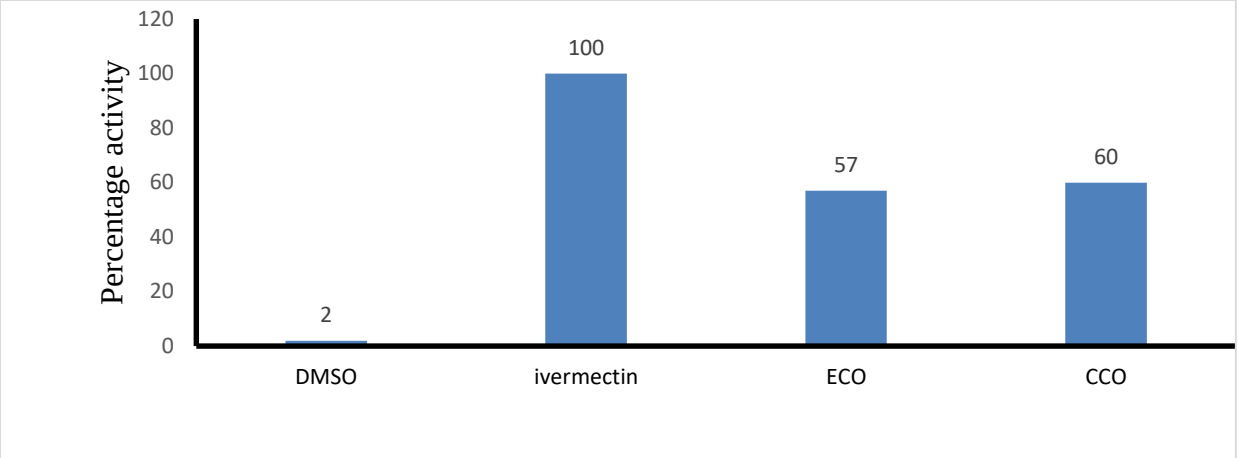


Figure 2: Anthelmintic activities of the oil extracted from the seeds of *Ricinus communis* (ECO) and commercial castor oil (CCO) against *Caenorhabditis elegans* at a concentration of 1 mg/ml (2% DMSO: Negative control; Ivermectin (10 μg/ml): Positive control).

Previous studies have demonstrated that fixed oils obtained from different plants possess activity against parasitic worms. For example, *Cucurbita pepo* seed oil displayed nematocidal activity against *Heligmosoides bakeri* causing 89% mortality (Dotto and Chacha, 2020). Anthelmintic properties of several fixed oils have been attributed to the presence of medium and long chain fatty acids. The oil extract of *Jatropha curcas* seeds which contains palmitic acid as a major constituent (55%) showed significant antihelmentic activity against *Haemonchus contortus* (Costa *et al*., 2015). These results indicated that the presence of free fatty acids in fixed oils could be a major reason behind the antihelmentic activity of fixed oil contining plants. Thus, the oil obtained from the seeds of *R. communis* was hydrolyzed further to obtain free fatty acids.

4.3 Free fatty acid from *R. communis* seed oil

Hydrolysis of *R. communis* seed oil was carried out by refluxing the oil with alcoholic solution of potassium hydroxide followed by acidification. Separation of the fatty acid was done by partitioning with ethyl acetate. As shown in Figure 2, the oil was detected by TLC. TLC is one of various analytical methods that is used for detection of mixtures of fatty acids and methyl ester derivatives. Fatty acids are not easy to detect on TLC when viewed under UV_{254} and UV_{366} nm as they lack a useful UV absorption chromophore. Therefore, 50% of sulfuric acid was used as a spraying reagent to visualize the oil, the hydrolysate and the major fatty acid (RAF-1).

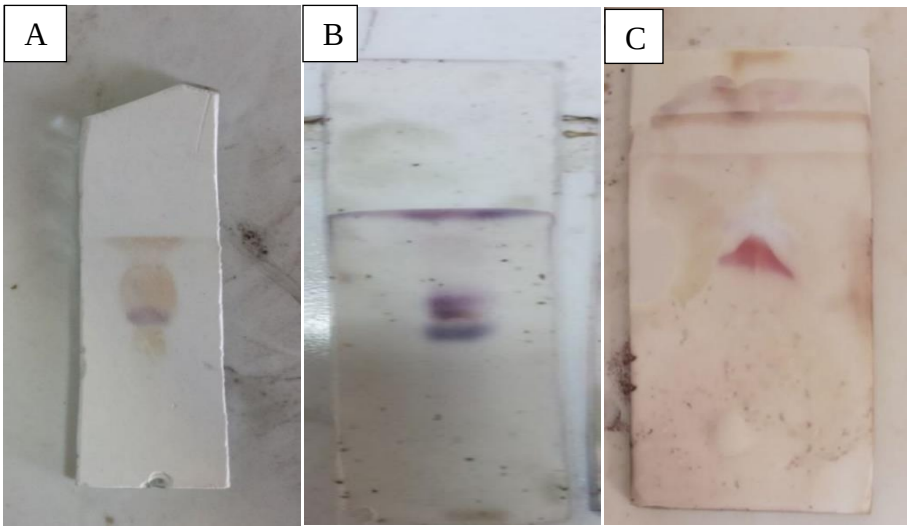


Figure 3: TLC chromatogram of the oil extracted from the seeds of *Ricinus communis* (A), hydrolyzed oil (B), and RAF-3 (free fatty acid) in day light after spraying with 50% H_2SO_4 followed by heating at 100 °C for 2 min on hot plate; Solvent system: *n*-Hexane: Ethyl acetate (4:1).

4.4 Anthelmintic activity of the hydrolysate

At a concentration of 1 mg/ml, the hydrolysate obtained from the oil of *R. communis* exhibited a significant anthelmintic activity ($p < 0.01$) killing 92% of *C. elegans* worms when compared to the negative control (Figure 3).

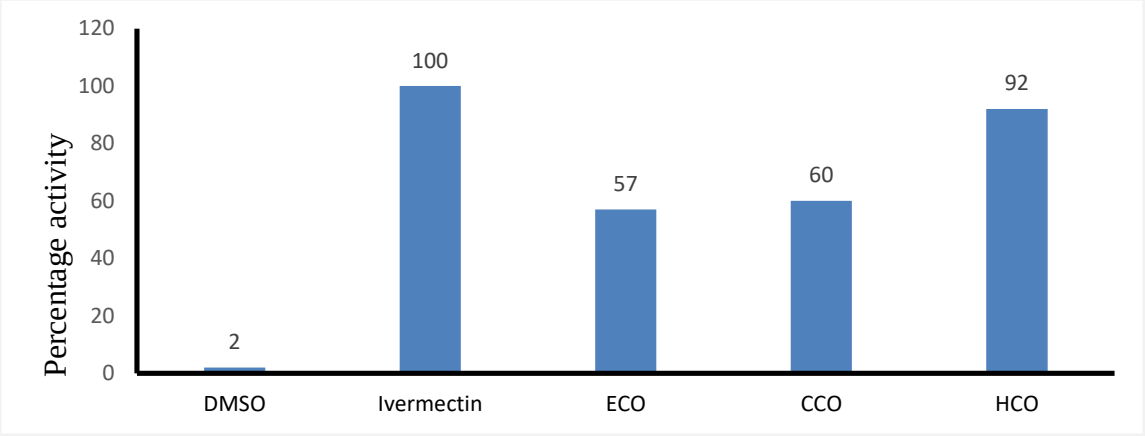


Figure 4: Anthelmintic activities of the oil obtained from the seeds of *Ricinus communis* (ECO), its hydrolysate (HCO), and commercial castor oil (CCO) against *Caenorhabditis elegans* at a concentration of 1 mg/ml (2% DMSO: Negative control; Ivermectin (10 μg/ml): Positive control).

4.5 Anthelmintic activities of the *n*-hexane and methanol fractions of *R. communis* oil hydrolysate

The hexane and methanol fractions of the hydrolysate exhibited significant activity ($p < 0.01$) at 1 mg/ml concentration compared to the negative control. However, the activity of the methanol fraction was better than the activity of the hydrolysate and the hexane fraction. At a concentration of 1 mg/ml, the hexane and methanol fractions killed 86% and 93% of *C. elegans* worms, respectively (Figure 4).

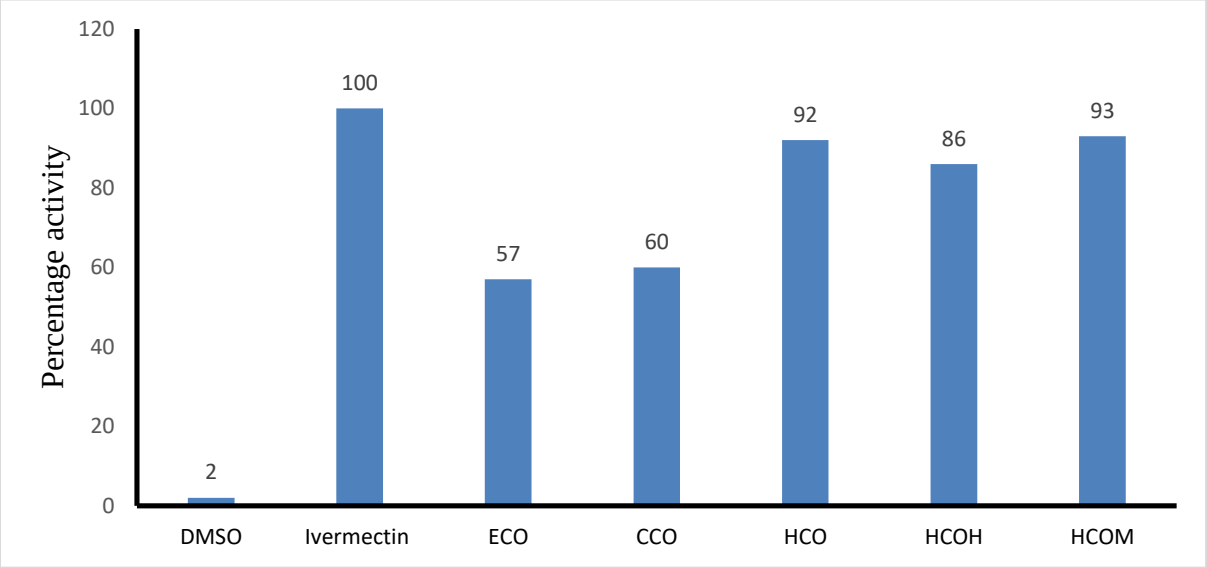


Figure 5: Anthelmintic activities of the oil extracted from the seeds of *Ricinus communis* (ECO), its hydrolysate (HCO), commercial castor oil (CCO), hexane fraction from the hydrolysate (HCOH) and methanol fraction from the hydrolysate (HCOM) against *Caenorhabditis elegans* at a concentration of 1 mg/ml (2% DMSO: Negative control; Ivermectin (10 μg/ml): Positive control)

4.6 Structural elucidation of RAF-3 and RAME

Owing to the highest activity demonstrated by the methanol fraction of the hydrolysate, it was treated with dry acetone and kept in a refrigerator overnight to facilitate crystallization. The resulting solid substance was designated RAF-3. RAF-3 was further esterified by a method described under section 3.2.4 to obtain a colourless oily substance coded RAME.

4.6.1 Structural elucidation of RAF-3

RAF-3 was isolated as a white waxy solid from acetone with R_f value of 0.6 in *n*-hexane/Ethyl acetate (4:1) solvent system. RAF-3 gave a pseudo-molecular ion at m/z = 297.4 $[M-H]^-$ in the

negative mode ESI-MS, indicating a relative molecular mass of 298 mu (Figure 5). The molecular formula $C_{18}H_{34}O_3$ was deduced for RAF-3 based on its ESI-MS, ^{1}H and ^{13}C-NMR spectral data.

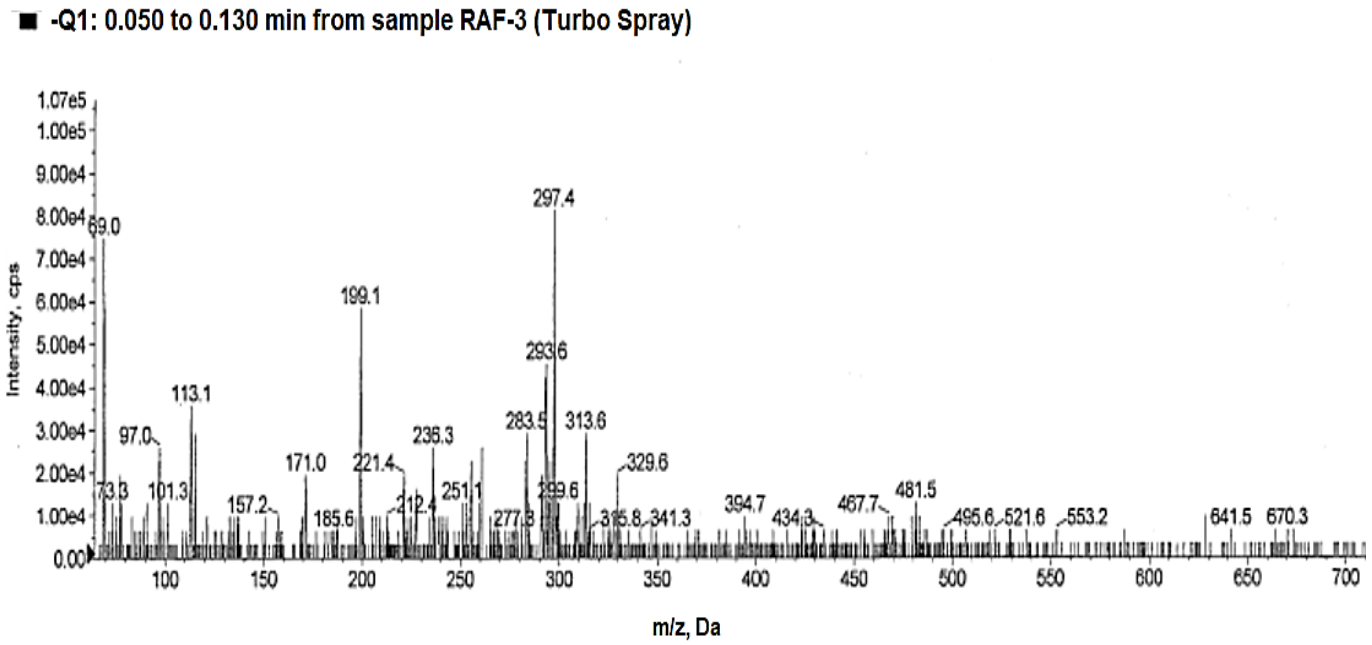


Figure 6: Negative-mode ESI-mass spectrum of RAF-3

The ^{13}C-NMR spectrum of RAF-3 showed the presence of 18 carbon atoms. The presence of carboxyl group was evident by a signal resonating at δ 174.93 (HO-C=O), while two signals at δ 128.22 and 130.16 were assigned to two olefinic carbons (carbon double bond, -CH=CH-). In addition to these, there was a signal resonating at δ 70.15 due to the carbon attached to the hydroxyl group, C-OH. Thirteen signals that appear between δ 22.44- δ 38.67 were assigned to the methylene groups, $-CH_2-$, in the hydrocarbon chain. Of these, the one resonating at δ 38.67

(CH_2) was assigned next to the carboxyl group. A peak at 14.37 ppm in the ^{13}C NMR spectrum was assigned to the methyl group at the end of the chain (Figure 6).

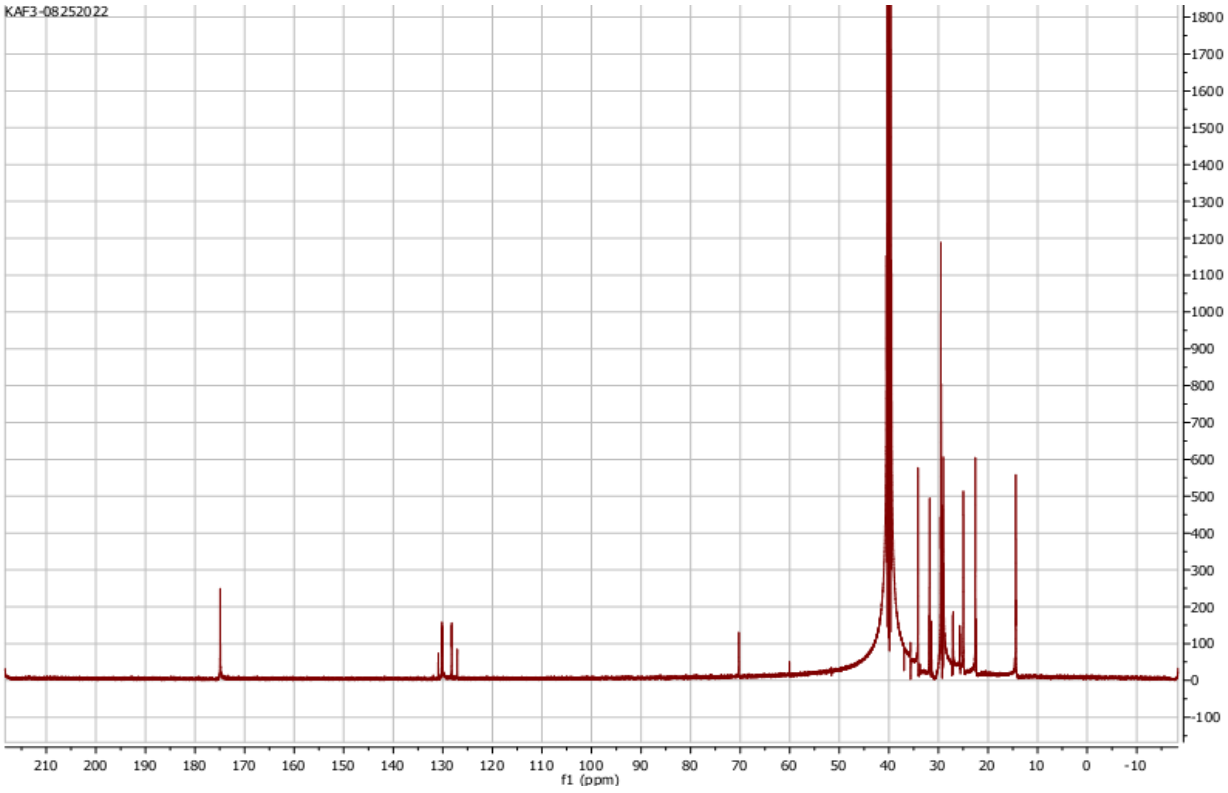


Figure 7: ^{13}C-NMR spectrum of RAF-3 in deuterated dimethyl sulfoxide.

The ^{1}H-NMR spectrum is shown in Figure 7. Each of the signals in the ^{1}H-NMR spectrum closely matched with those signals presented in the ^{13}C-NMR spectrum. Of all the key signals in the ^{1}H-NMR spectrum, the peak that appeared at 11.96 ppm was assignable to the hydroxyl proton of the carboxylic functional group. Other important signals resonating at δ 5.31 and δ 5.33 ppm were due to the presence of one olefinic double bond protons, -CH=CH-, while

oxymethine signal (-CHOH-) was indicated by the signal resonating at 3.52 ppm. All the remaining signals are shown in Table 2.

A close examination of ESI-MS, ^{13}C and ^{1}H NMR spectrum of RAF-3 led to identification of RAF-3 as (9Z)-12-hydroxyoctadec-9-enoic acid (ricinoleic acid), which was consistent with ^{1}H and ^{13}C–NMR spectral data reported in National Center for Biotechnology Information (NCBI), an online database. Previously, ricinoleic acid (Figure 8) was isolated from the seeds, roots and leaves of *R. communis* (Bataglion *et al.*, 2014; Wafa *et al.*, 2014). The seed oil of *Phyllantus niruri* (Euphorbiaceae) also contains ricinoleic acid (Ahmad *et al.*, 1981).

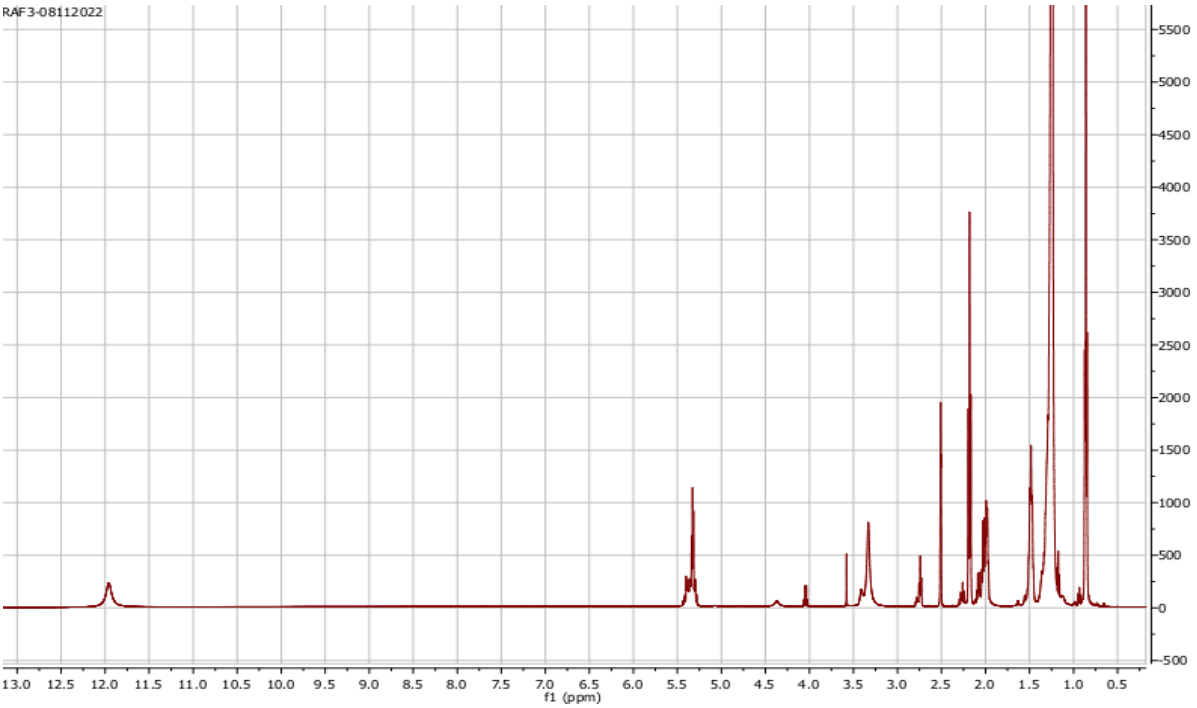


Figure 8: ^{1}H-NMR spectrum of RAF-3 in deuterated dimethyl sulfoxide

Table 2: Comparison of ^{1}H and ^{13} C-NMR data of RAF-3 and ricinoleic acid from National Center for Biotechnology Information (NCBI) online database. https://nmrshiftdb.nmr.uni-koeln.de/molecule/10009331

	^{1}H-NMR (ppm)		^{13}C-NMR (ppm)	
	RAF-3	Literature (ricinoleic acid)	RAF-3	Literature (ricinoleic acid)
1	OH-11.95	OH-12.27	174.93	177.13
2	2.18	2.40	34.12	36.64
3	1.48	1.61	24.96	24.81
4	1.33	1.42	28.96	28.93
5	1.29	1.34	28.55	28.96
6	1.34	1.36	28.23	28.73
7	1.34	1.36	29.57	29.25
8	2.18	2.25	31.38	28.28
9	5.33	5.49	130.16	132.33
10	5.31	5.48	128.22	124.73
11	1.90	1.89	40.58	37.52
12	3.52	3.54	70.15	71.91
13	1.47	1.49	38.67	37.21
14	1.39	1.41	24.96	25.31
15	1.31	1.33	29.91	29.32
16	1.30	1.32	31.83	31.65
17	1.32	1.36	22.94	22.94
18	0.87	0.99	14.37	14.02

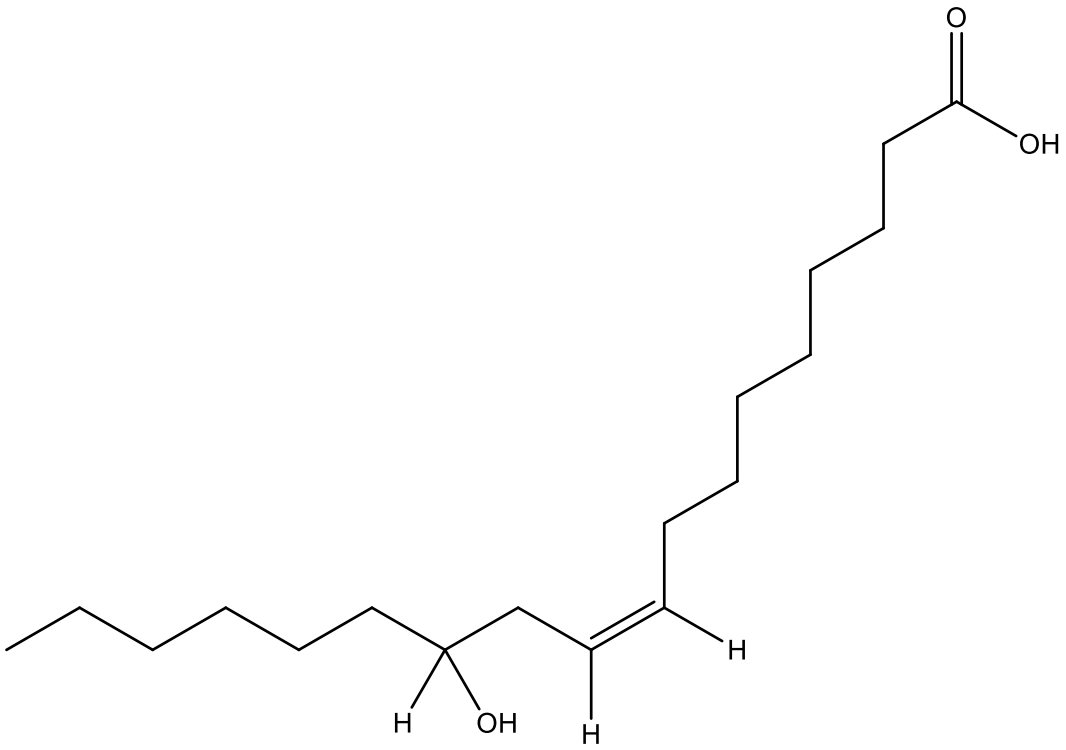


Figure 9: Structural formula of ricinoleic acid isolated from *Ricinus communis*

4.6.2 Structural elucidation of RAME

Ricinoleic acid was esterified to the corresponding methyl ester to check if anthelmintic activity of the fatty acid is affected by structural modification. The structure of RAME was confirmed by its spectral properties. RAME showed a pattern of signals in the ^{1}H, ^{13}C and DEPT-NMR spectra (Figures 9-11) similar to that of RAF-3, except for the presence of one additional signal in RAME due to a methoxy group moiety (OCH_3; 3.50 ppm in ^{1}H and 51.66 ppm in ^{13}C-NMR). All the remaining signals are shown in Table 3.

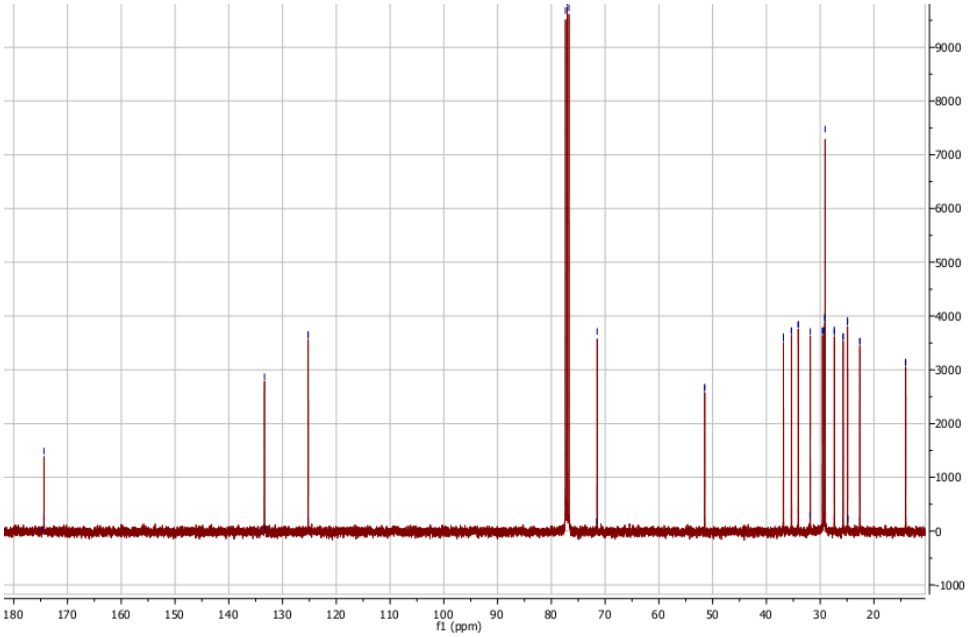


Figure 10: ^{13}C-NMR spectrum of RAME in deuterated chloroform

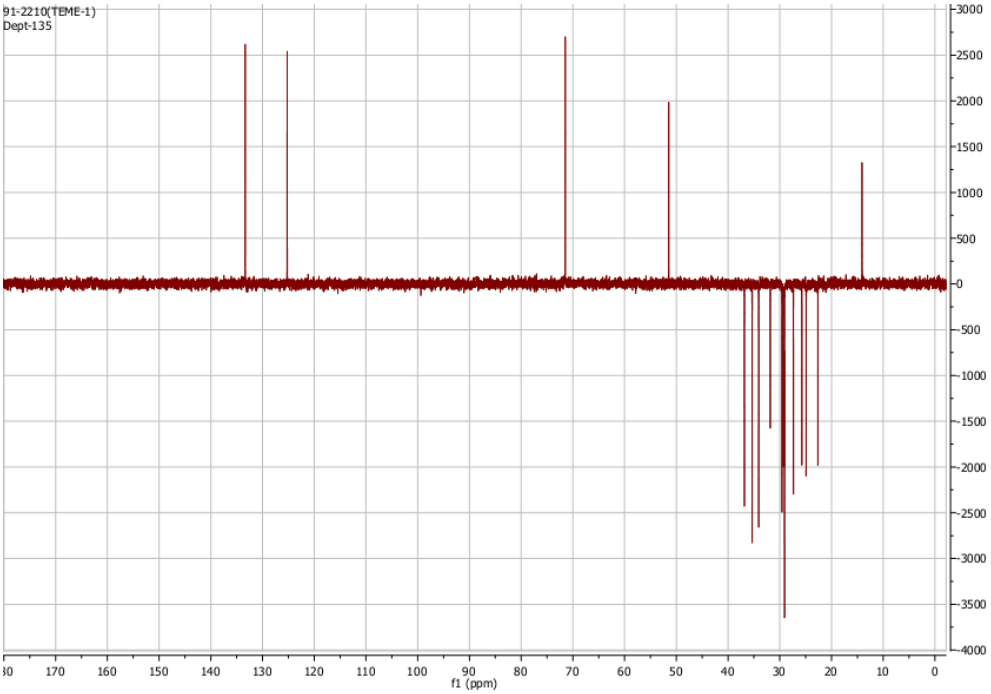


Figure 11: DEPT-135 spectrum of RAME in deuterated chloroform

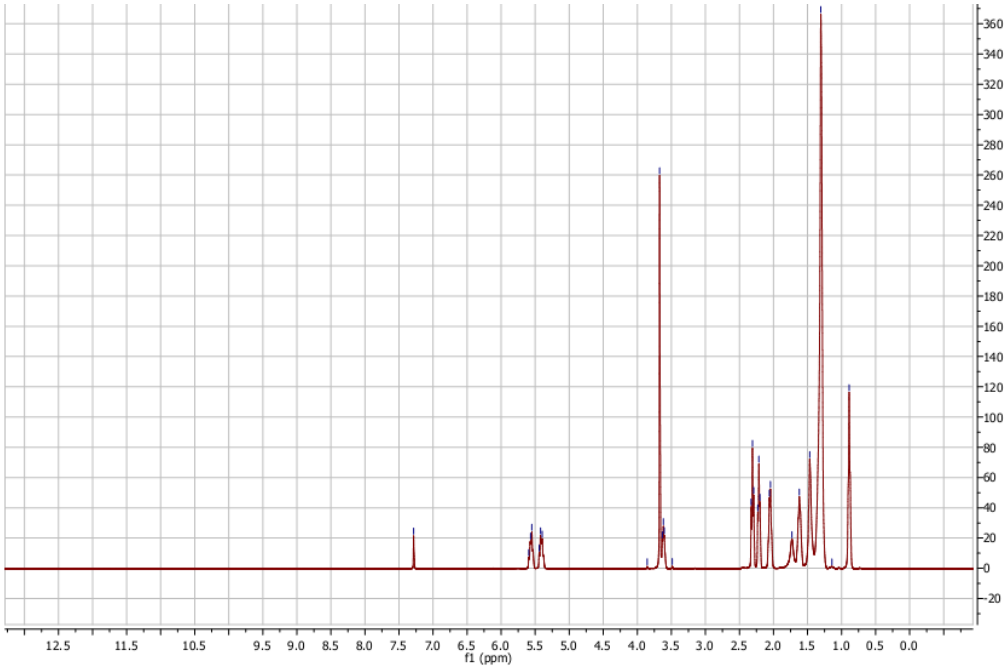


Figure 12: ^{1}H-NMR spectrum of RAME in deuterated chloroform

Table 3: Comparison of ^{1}H and ^{13}C-NMR data of RAME and ricinoleic acid methyl ester from National Center for Biotechnology Information (NCBI) online database.

	^{1}H-NMR (ppm)		^{13}C-NMR (ppm)	
	Ricinoleic acid methyl ester	RAME	Ricinoleic acid methyl ester	RAME
1	-----	-----	174.61	174.59
2	2.30	2.30	33.84	33.84
3	1.71	1.63	25.33	25.33
4	1.32	1.32	28.93	28.93
5	1.32	1.32	28.96	28.96
6	1.32	1.32	28.73	28.73
7	1.34	1.34	29.25	29.25
8	2.01	2.04	31.36	31.36
9	5.51	5.54	130.42	133.35
10	5.51	5.42	123.79	125.18

Table 3: continued

11	2.27	2.21	39.87	39.89
12	3.36	3.61	71.91	71.91
13	1.38	1.36	37.21	37.91
14	1.32	1.32	25.31	25.31
15	1.29	1.29	29.32	29.32
16	1.30	1.30	31.65	31.94
17	1.36	1.36	22.94	23.04
18	0.98	0.87	14.02	14.02
1′	3.82	3.67	51.82	51.66

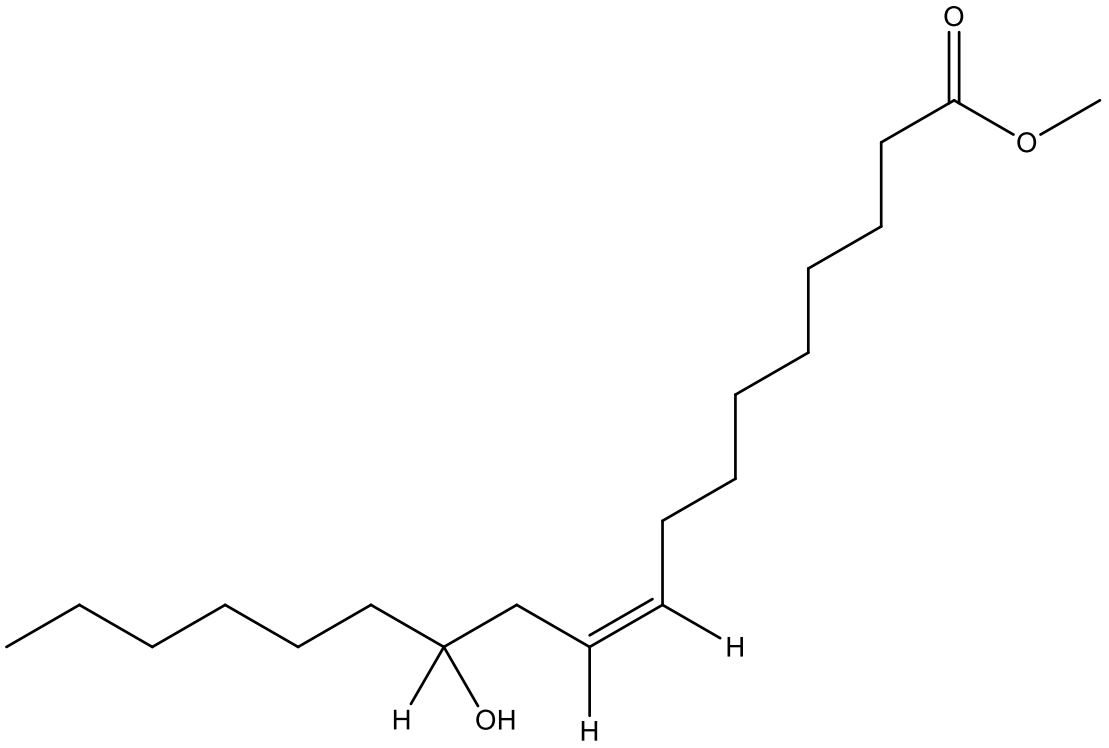


Figure 13: Structural formula of RAME (ricinoleic acid methyl ester)

4.7 Anthelmintic activity of ricinoleic acid and methyl ricinoleate

Ricinoleic acid exhibited strong anthelmintic activity killing 97% of *C. elegans* worms at a concentration of 1 mg/ml. Similarly, ricinoleic acid methyl ester caused percentage mortality of 97% at the same concentration. These activities were by far higher than the activities demonstrated by palmitic acid and oleic acid which showed 18% and 19% mortality, respectively, at the same concentration (Figure 13). This gives an indication that the hydroxyl group of ricinoleic acid is crucial in enhancing anthelmintic activity since its structure is different from oleic acid by the presence of one hydroxyl group at position 12. However, esterification of the carboxylic acid of ricinoleic acid does not seem to affect the anthelmintic activity of the fatty acid.

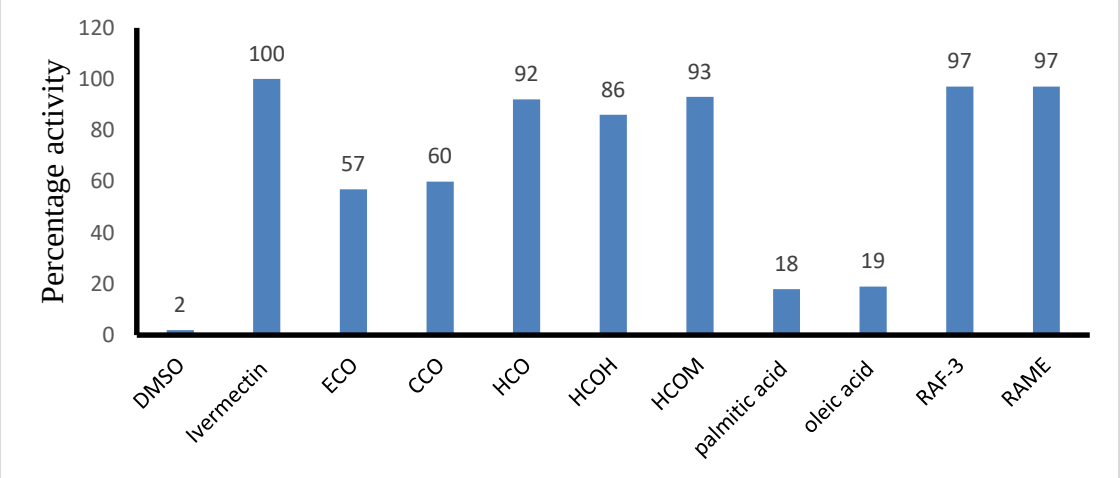


Figure 14: Anthelmintic activities of the oil extracted from the seeds of *Ricinus communis* (ECO), its hydrolysate (HCO), commercial castor oil (CCO), hexane fraction from the hydrolysate (HCOH), methanol fraction from the hydrolysate (HCOM), oleic acid, palmitic acid, ricinoleic acid and ricinoleic acid methyl ester against *Caenorhabditis elegans* at a concentration of 1 mg/ml (2% DMSO: Negative control; Ivermectin (10 µg/ml): Positive control).

Stadler *et al*. (1994) have shown that the hydroxylated fatty acid, S-coriolic acid [(9Z, 11E, 13S)-13-hydroxyoctadeca-9, 11-dienoic acid] possesses strong anthelmintic activity against *C. elegans.* Unsaturation and hydroxylation have been indicated to increase activity against this model organism. Hydroxylated fatty acids are known to have multiple roles, they have structural functions as constituents of phospholipids which are the "building blocks" of cell membranes; also serve as storage materials in cells, and fatty acid (FA) derivatives are involved in cell signaling (Pineda-Alegría *et al*., 2020; Carvalho and Caramujo, 2018).

A previous study by Hirazawa *et al*. (2001) demonstrated that medium-chain fatty acids (carbon numbers C_6–C_{10}) possess good activity against the monogenean worm *Heterobothrium okamotoi*. This finding was considered to be crucial since *H. okamotoi* has an obvious pathogenicity and its susceptibility to chemicals is low. Other studies have also shown that medium and long chain fatty acids inhibit growth *C. elegans* and parasitic worms. Caprylic acid, stearidonic acid, eicosapentaenoic acid, alpha-linolenic acid, docosahexaenoic acid, arachidonic acid and pelargonic acid are some of the fatty acids which have displayed significant anthelmintic activity against different helminths (Bonde *et al*., 2021; Davis *et al*., 1997). It has been asserted that fatty acids could cause a change in the fatty acid composition (saturated and unsaturated) of the phospholipids membranes of helminths. This would cause saturation or unsaturation of the membrane, damaging its fluidity and functionality leading to mortality (Pineda-Alegría *et al.,* 2020; Carvalho and Caramujo, 2018)

Nematodes, including *C. elegans,* are highly dependent on lipids for energy storage and energy metabolism (Kumarasingha *et al.,* 2019). These compounds are also described to be fundamental in *C. elegans* (Burns *et al*., 2015). Tegumental damages, cuticular distortion and interaction with

plasma membrane lipoproteins have been proposed as the possible mechanism of action of fatty acids (Lalthanpuii and Lalchhandama, 2020; Kumarasingha *et al.*, 2019). Hence disruption of one or more functions including disruption in lipid homeostasis could be a possible mechanism for the activity of ricinoleic acid and its methyl ester. It has also been suggested that the anthelmintic activity of fatty acids could be due to adverse interference with the nematode cuticle or hypodermis via a detergent (solubilization) effect and through direct interaction of the fatty acids with the lipophilic regions of target plasma membranes (Panda *et al*., 2020; Pineda-Alegría *et al*., 2020; Stadler *et al*., 1993).

5. Conclusion

In the present study, the anthelmintic activity of the oil isolated from the seeds of *R. communis*, a medicinal plant used in folk medicine for the treatment of several diseases including worm infection, was assessed for its anthelmintic activity against the model organism *C. elegans.* The results obtained demonstrated the *in vitro* anthelmintic potential of the oil. Hydrolysis of the oil liberated the hydroxylated unsaturated omega-9 fatty acid identified as ricinoleic acid. Evaluation of the anthelmintic properties of ricinoleic acid and its methyl ester prepared by esterification reaction revealed that both ricinoleic acid and methyl ricinoleate possess potent anthelmintic activity against *C. elegans*. Although in traditional medicine only the leaves of *R. communis* have been recorded to have anthelmintic property, the current study has established that the seeds of the plant also possess genuine activity against *C. elegans*. Results of the present study confirmed that the anthelmintic activity of the seeds of *R. communis*, in part or in full, is attributed to the presence of ricinoleic acid.

Recommendation

Based on the current findings, the following recommendations are forwarded:

- The activity of the oil of *R. communis* and its major constituent ricinoleic acid should be investigated against parasitic worms infecting humans.
- The potential anthelmintic activities of hydroxylated fatty acids must be studied.

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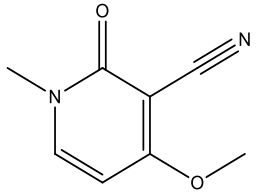
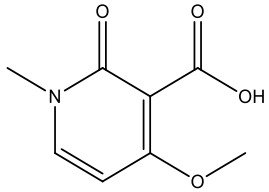
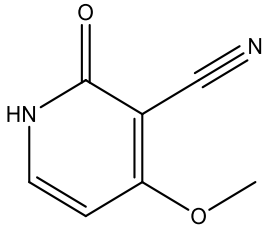
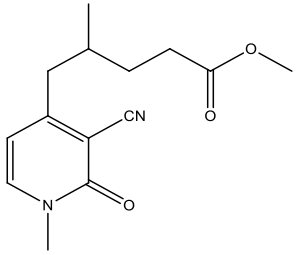
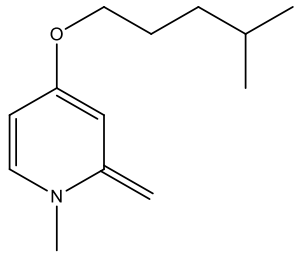
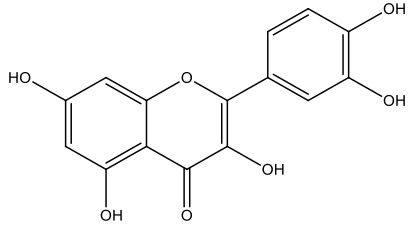
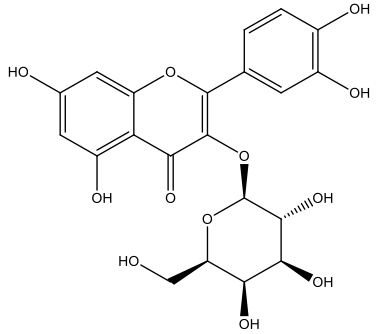
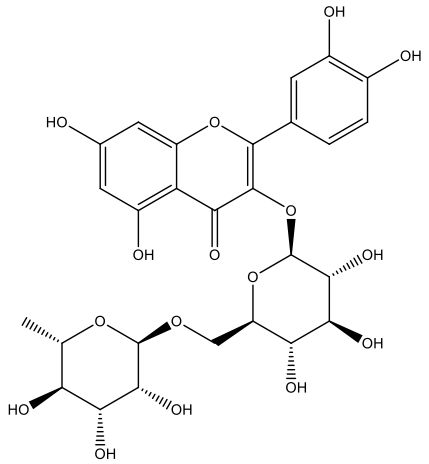
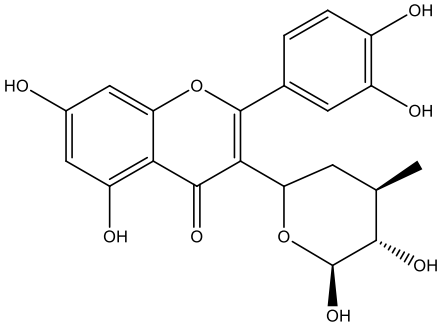
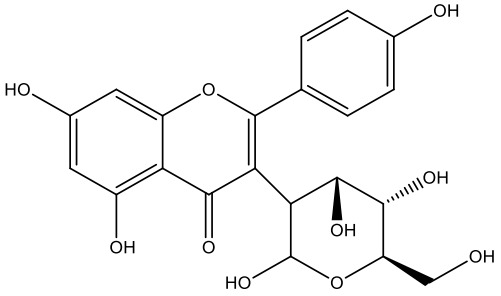
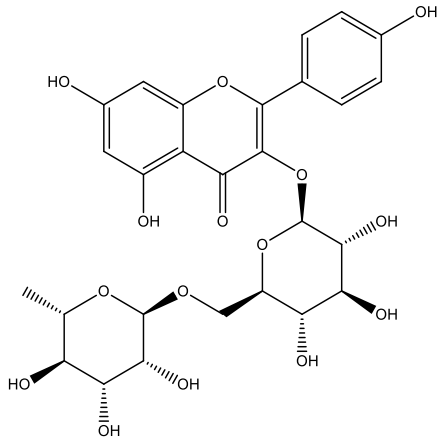
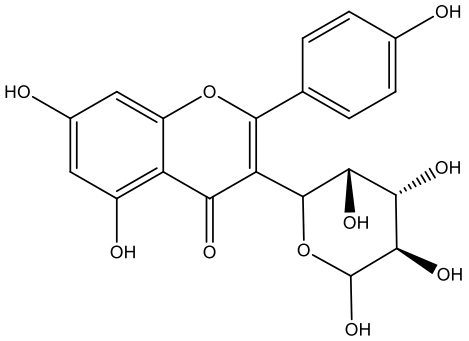
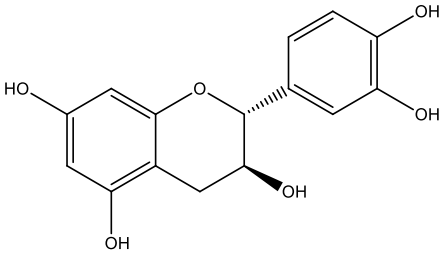
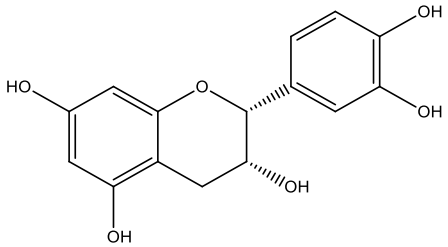
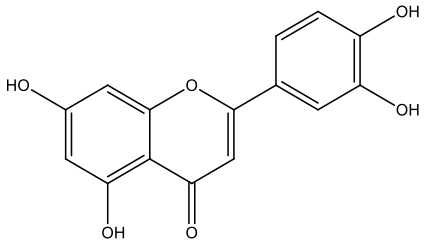
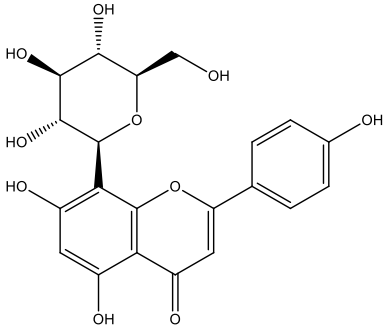
Appendix I: List of compounds identified from *Ricinus communis* seeds, leaves, roots, and stem extracts

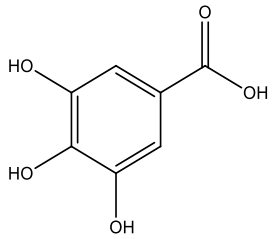
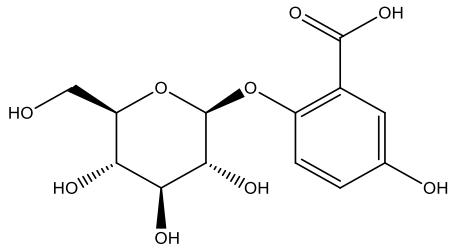
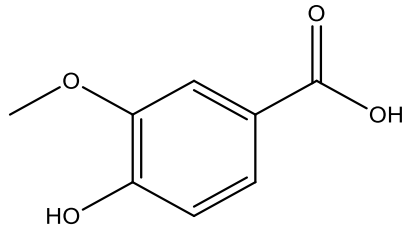
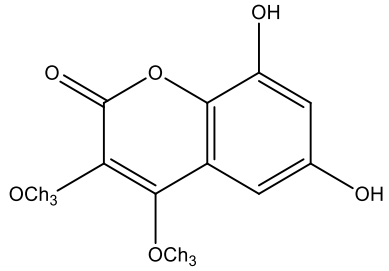
No	Class of the compound	Name of the compound	Structure of the compound	Reference
1	Alkaloids	Ricinine		(Liu *et al.*, 2018; Wachira *et al.*, 2014; Hussein *et al.*, 2015; Singh *et al.*, 2013)
		3-carboxy-4-methoxyN-methyl-2-pyridone		(Liu *et al.*, 2018; Wachira *et al.*, 2014; Singh *et al.*, 2013)
		N-demethylricinine		(Liu *et al.*, 2018; Wachira *et al.*, 2014; Singh *et al.*, 2013)
		Methyl 5-(3-cyano-1- methyl-2-oxo-1,2- dihydropyridine-4-il) pentanoate		(Liu *et al.*, 2018; Wachira *et al.*, 2014; Singh *et al.*, 2013)

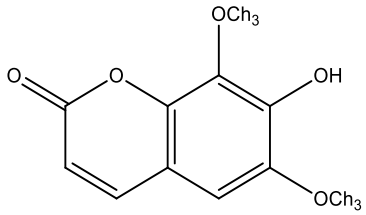
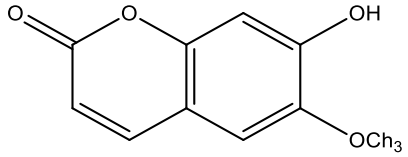
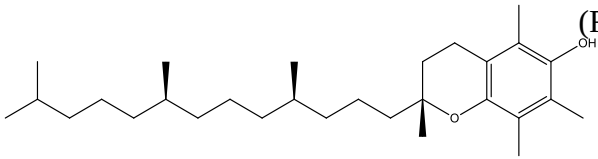
		1-methyl-4-(4- metilpentiloksi) pyridine-2 (1H)-on		(Liu *et al.,* 2018; Wachira *et al.,* 2014; Singh *et al.,* 2013)
2	Flavonoids	Quercetin		(Falcone *etal.,* 2012; Singh *et al.,* 2009)
		Quercetin-3-O-d-galactoside		(Falcone *etal.,* 2012; Singh *et al.,* 2009)

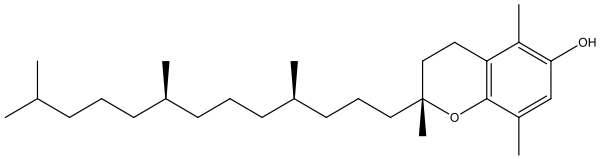
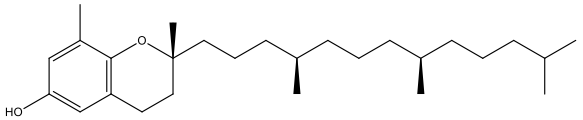
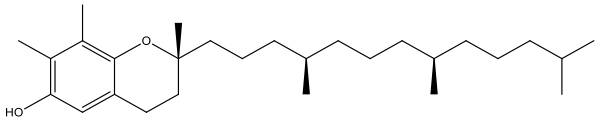
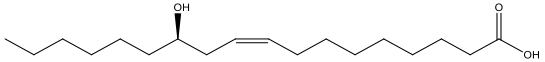
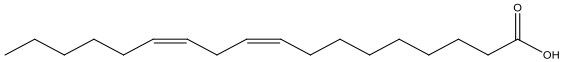
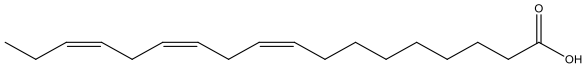
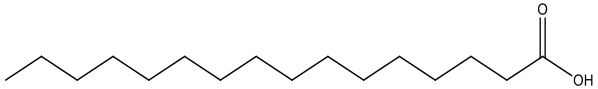
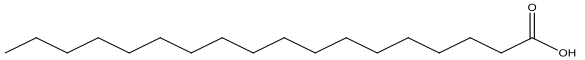
Quercetin-3-O-d-rutinoside		(Falcone *etal.*, 2012; Singh *et al.*, 2009)
Quercetin-3-O-d-xylopyranoside		(Falcone *etal.*, 2012; Singh *et al.*, 2009)
Kaempferol 3-O-d-glucopyranoside		(Falcone *etal.*, 2012; Singh *et al.*, 2009)

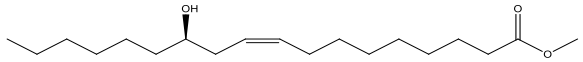
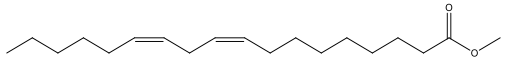
Kaempferol-3-O-B-D rutinosid		(Falcone *etal.,* 2012; Singh *et al.,* 2009)
Kaempferol-3-O-d-xylopyranoside		(Falcone *etal.,* 2012; Singh *et al.,* 2009)
Catechin		(Falcone *et al.,* 2012; Singh *et al.,* 2009)

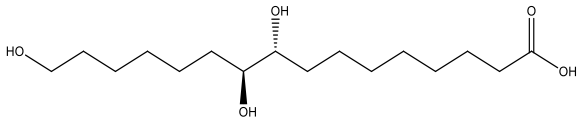
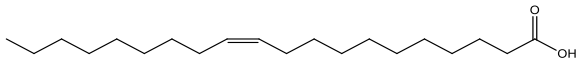
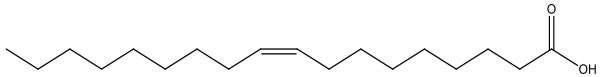
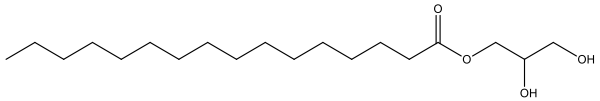
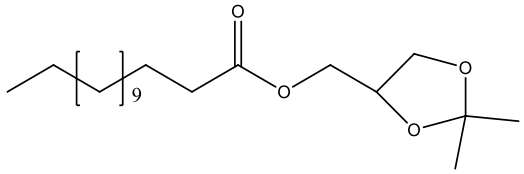
Epicatechin		(Falcone *et al.,* 2012; Singh *et al.,* 2009)
Luteolin		(Falcone *et al.,* 2012; Singh *et al.,* 2009)
Vitexin		(Falcone *et al.,* 2012; Singh *et al.,* 2009)

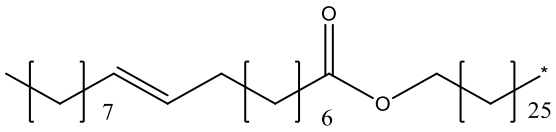
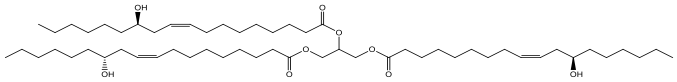
3	Benzoic acid derivatives	Gallic acid		(Singh *et al.*, 2009)
		Gentisic acid		(Singh *et al.*, 2009)
		Vanillic acid		(Singh *et al.*, 2009)
4	Coumarins	3,4- dimethoxy-6,8-dihydroxy coumarin		(Bigi *et al.*, 2004)

		Isofraxidine		(Bigi *et al.*, 2004)
		Scopoletin		(Bigi *et al.*, 2004)
5	Tocopherols	α tocopherol		(Ribeiro *et al.*, 2016).

		β tocopherol		(Ribeiro *et al.*, 2016).
		δ tocopherol		(Ribeiro *et al.*, 2016).
		γ tocopherol		(Ribeiro *et al.*, 2016).
6	Fatty acids	Ricinoleic acid		(Bafor *et al.*, 1991)
		Linoleic acid		(Ribeiro *et al.*, 2016)
		Linolenic acid		(Ribeiro *et al.*, 2016)
		Palmitic acid		(Ribeiro *et al.*, 2016)
		Stearic acid		(Ribeiro *et al.*, 2016)

Methyl ricinoleate		(Yeboah *et al.*, 2021; McKeon, 2016; Jena and Gupta, 2012)
Methyl linoleate		(Yeboah *et al.*, 2021; McKeon, 2016; Jena and Gupta, 2012)

Aleuritic acid		(Yeboah *et al.*, 2021; McKeon, 2016; Jena and Gupta, 2012)
Gondoic acid		(Yeboah *et al.*, 2021; McKeon, 2016; Jena and Gupta, 2012)
Oleic acid		(Yeboah *et al.*, 2021; McKeon, 2016; Jena and Gupta, 2012)
1-Palmitic acid glycerol ester		(Yeboah *et al.*, 2021; McKeon, 2016; Jena and Gupta, 2012)
1- palmitic acid glycerol-2,3-dimethyl ketal ester		(Yeboah *et al.*, 2021; McKeon, 2016; Jena and Gupta, 2012)

n-Heptacosanyl oleate		(Yeboah *et al.*, 2021; McKeon, 2016; Jena and Gupta, 2012)
Triricinolein		(Yeboah *et al.*, 2021; McKeon, 2016; Jena and Gupta, 2012)