Nematicidal Activity of the 80% Methanol Extract and Major Compounds Isolated from the Unripe Fruits of *Peponium vogelii* (Hook. f.) Engl.

By: Israel Mohamed (B. Pharm.)

Advisor: Professor Kaleab Asres

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacognosy

Addis Ababa, Ethiopia

November, 2022

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by Israel Mohamed, entitled: "**Nematicidal Activity of the 80% Methanol Extract and Major Compounds Isolated from the Unripe Fruits of *Peponium vogelii* (Hook. f.) Engl.**" 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.

Signed by the Examining Committee:

Name	**Signature**	**Date**
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Advisor: Professor Kaleab Asres	----------	----------

Declaration

This thesis has not previously been accepted for any degree and is not being concurrently submitted in candidature for any degree in any university. I declare that this thesis entitled "Nematicidal Activity of the 80% Methanol Extract and Major Compounds Isolated from the Unripe Fruits of *Peponium vogelii* (Hook. f.) Engl." is a result of my own investigation, except where otherwise stated. I have understated stated. I have undertaken the study independently with guidance and support of my research advisor. Other sources are acknowledged by citations giving explicit references. A list of references is appended.

Signature:_____

Israel Mohamed Helango

This thesis has been submitted for examination with my approval as university advisor.

Advisor's Signature:_____

Professor Kaleab Asres (PHD)

Abstract

Helminthiasis remain a major public health problem affecting an estimated 1.45 billion individuals worldwide, particularly in tropical developing countries. The aim of this study was to investigate the nematicidal activity of 80% methanol extract of unripe fruits of *Peponium vogelii* Hook. f. Engli. (Cucurbitaceae), a medicinal plant used to treat intestinal parasite infection in Southwest Ethiopia, and determine the compounds responsible for activity using bioassay-guided fractionation. 80% methanol extract of unripe fruit of *P. vogelii* was first fractionated by using solvents of different polarity followed by a silica gel column chromatography and reverse phase HPLC, while nematicidal assay was followed throughout on *Caenorhabditis (C.) elegans* as a model organism. Results of the study revealed that the 80% methanol extract of the unripe fruits of *P. vogelii* was active against *C. elegans* ($p < 0.001$) with an IC_{50} value of 23.5 μg/ml. Bioassay-guided fractionation led to the isolation of five active components designated PVMF2-4, PVMF2-5, PVMF2-7, PVMF2-8, and PVMF2-9, which exhibited percentage inhibition of 84%, 96%, 97%, 89% and 41%, respectively. Based on spectroscopic data (^{1}H and ^{13}C NMR and ESI-MS), PVMF2-8 and PVMF2-8 which were active and major components from active fraction were identified as linoleic acid and palmitic acid respectively. The results support the use of *P. vogelii* unripe fruits for the treatment of intestinal parasite infection by traditional healers in Southwest Ethiopia.

Keywords: *Peponium vogelii*, traditional medicine, nematicidal activity, palmitic acid, linoleic acid.

Acknowledgements

I feel very grateful to express my deepest appreciation to my advisor Prof. Kaleab Asres (PhD) for his guidance, encouragement and support throughout my work without whom this study would not be realized. I extend my sincere appreciation to Dr. Daniel Bisrat (PhD) for his guidance and support in interpreting the NMR data, and Ms. Eyael Tewelde for assisting me through nematicidal assay. Mr. Mthandazo Dube is acknowledged for HPLC running and acquiring NMR data, and Mr. Melaku Wondeafrash for identification of the plant material.

I would like to acknowledge staff members of the Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, College of Health Sciences, Addis Ababa University, for their support during the laboratory work. I would like to thank the School of Graduate Studies, Addis Ababa University for financial support and Hawassa University for sponsoring my study. Leibniz Institute of Plant Biochemistry is acknowledged for the provision of ESI-MS and NMR instruments. I am also very grateful to TRI-Sustain Project-DAAD for financial support.

Last but not least, I would like to forward my gratitude to my beloved family whose prayer, love and support have given me the chance to be here and the power to finish this study. I would also love to thank all my friends and colleagues for their constructive comments and support.

Thank you all!

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List of acronyms and abbreviations

^{13}C NMR	Carbon thirteen Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
ANOVA	Analysis of Variance
APCI	Atmospheric Pressure Chemical Ionization
CHS	College of Health Sciences
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl Sulfoxide
DoP	Department of Pharmacology
GABA	Gamma Aminobutyric Acid
HPLC-DAD	High-pressure liquid chromatography diode array detection
IC_{50}	Inhibitory concentration that suppresses 50% of nematodes
LD_{50}	Lethal Dose which cause death to 50% of mice
MS	Mass Spectrometry
ESI-MS	Electron Spray Ionization-Mass Spectrometry
NGM	Nematode Growth Medium
NMDA	N-Methyl-D-Aspartate
NMR	Nuclear Magnetic Resonance
OECD	Organization of Economic Cooperation and Development
RCF	Relative Centrifugal Force
SoP	School of Pharmacy
TLC	Thin Layer Chromatography

TMS	Tetramethylsilane
UV	Ultraviolet

1. Introduction

1.1 Intestinal parasite infection

Intestinal worms belonging to Phylum *Nematoda* can produce pathological manifestations in humans acting in either the small or large intestine (Raul N *et al*., 2011). Intestinal nematodes infection involves infestation with one or more intestinal parasitic worms that include hookworm (*Ancylostoma duodenale* and *Necator americanus*), roundworm (*Ascaris lumbricoides*), and whipworm (*Trichuris trichiura*), which are the most widespread species (Bethony *et al.*, 2006; Keiser and Utzinger, 2008).

Nematode infestations continue to pose problems in human and veterinary medicine (Manke *et al*., 2015; Jimenez *et al.*, 2019). These infections are associated with significant morbidity and mortality of an estimated 1.45 billion individuals worldwide (Becker *et al.*, 2018). Despite little decline in estimated burden of helmenth infection reported in some parts of Africa due to improvements in living conditions and expansion of major deworming efforts, sub-Saharan Africa remains highly endemic area (Pullan *et al.*, 2014).

Helminthes are the most abundant and ubiquitous multicellular organisms on earth, with an estimated 100,000 to 1,000,000 species (Liu, 2018). They are large, multicellular, invertebrates with well-developed organ systems with characteristic elongated, flat or round bodies. Majority of them are active feeders and microscopic while a few can be viewed with the naked eye in their adult stages (Coghlan, 2005). Their sizes range from <1 mm in length (e.g., *Strongyloides stercoralis*) to 30 cm or more (e.g., *Ascaris lumbricoides*). Over 25,000 nematode species have been described, and more than half of them are parasitic for humans, animals or plants (Coghlan, 2005). Adult worms live for years in the human gastrointestinal tract (Mascarini-Serra, 2011).

They are transmitted by eggs present in human faeces which in turn contaminate soil in areas where sanitation is poor (WHO, 2011). Infestation can occur from consuming fruits and vegetables that have not been thoroughly washed, peeled or cooked (Montresor *et al.*, 2002). Although light helminthic infections are often asymptomatic, heavy parasite infections cause a series of morbidities, including detrimental effects on human growth, nutrition, cognition, school performance, work productivity and pregnancy, which may severely impair the quality of life. Moreover, the infections also indirectly cause a considerable disease burden via impairment of the immune system, leading to increased susceptibility to malaria, HIV/AIDS and tuberculosis (Cross, 1996; Bethony *et al.*, 2006).

1.2. Medicinal plants for the treatment of intestinal parasite infection

Historically, medicinal plants have been used since ancient times and in folklore for the treatment of many diseases and illnesses including intestinal parasite infection (Khamesipour *et al*., 2021; Dias *et al*., 2012). Medicinal plants are proved to be good sources of new leads in view of success of the available chemotherapeutic agents (Yuan *et al*., 2016). They either provide drugs directly for array of diseases such as morphine from the latex of *Papaver somniferum* L. or they may provide template molecules on which to base further new structures by organic synthesis. For example, acetylsalicyclic acid (aspirin) is derived from the natural product salicin isolated from the bark of the willow tree *Salix alba* L. (Katiyar *et al.*, 2012; Yuan *et al*., 2016). Hence, owing to the successful achievements in the past, screening of medicinal plants for this objective with the greatest possibility of success is always needed (Chaachouay *et al*., 2020). Some medicinal plants with anthelminthic activity are listed in Appendix I.

1.2.1 Ethiopian medicinal plants with anthelminthic activity

Ethiopia is a center of diversity for a number flora and fauna and it is the sixth center of biodiversity in the world. The country is endowed with rich flora, having more than 6,500 species of vascular plants out of which an estimated 12% of them are endemic and about 887 species are used as medicinal plants (Demissew *et al*., 2021). The majority (80%) of Ethiopian people depends on traditional medicine for their primary health care, and more than 95% of traditional medicinal preparations are of plant origin (Kassaye *et al.*, 2007; Demie *et al*., 2018). However, their ethnobotanical uses and potential as sources of anthelminthic remedies has not been sufficiently explored (Asres *et al.*, 2017; Tadele, 2017).

Despite the limited works done on evaluation of Ethiopian medicinal plants for their anthelminthic activity, some reports have illustrated that they can be sighted as potential sources for future anthelminthic drugs. The few Ethiopian medicinal plants which proved to have anthelminthic activity in Ethiopia include: *Acacia nilotica*, *Artemisia herba-alba*, *Cissus quadrangularis*, *Croton macrostachyus*, *Ekebergia capensis*, *Embelia schimperi*, *Hagenia abyssinica*, *Mimusops kummel*, *Punica granatum* and *Schinus molle* (Karumi *et al*., 2003; Eguale *et al.*, 2006; Debebe *et al.*, 2015; Zenebe, 2017; Ahmed *et al.*, 2020, Tewelde, 2021,).

1.3 The genus *Peponium*

The genus *Peponium* Engl. belongs to the flowering plants family Cucurbitaceae, which is represented by around 98 genera and 975 species (Bodine and Rogers, 2009; Xu and Chang, 2017). The genus has received little attention in the literature and about 21 species are described so far with a high proportion of endemics adapted to coastal habitats, but members of the genus

also grow in dense forest along rivers wherever there is an adequate water supply (Page J and Jeffrey, 1975; Mutie *et al*., 2020).

Peponium is a dioecious genus whose members are climbing perennial herbs with tuberculate roots, simple leaves, and simple or bifid tendrils (Page J and Jeffrey, 1975). *Peponium* is distinguished vegetatively by the absence of characteristic stalked, paired petiole glands located just below the leaf lamina and the flowers are showy and always 5-petaled. The fruits are thin-walled, indehiscent, and bacciform with many small, dark seeds, which are ovoid or cylindric, fleshy and many-seeded (Jeffrey, 1978).

Peponium species are native to Angola, Benin, Burundi, Cameroon, Cape Provinces, Central African Repu, Congo, Cote d'Ivoire, Ethiopia, Gabon, Ghana, Guinea , Ivory Coast, Kenya, KwaZulu-Natal, Lesotho, Madagascar, Malawi, Mozambique, Nigeria, Northern Provinces, Rwanda, Seychelles, Somalia, Sudan, Tanzania, Uganda, South Africa, Zaïre and Zimbabwe (Jeffrey, 1967). In Ethiopia members of the genus remain relatively poorly identified except for the widespread African species *P*. *vogelli* (Bodine and Rogers, 2009).

Perusal of literature revealed that the crude extract of *P. vogelii* leaf possesses antimalarial (Asnake *et al*., 2016) and antibacterial (Yemata *et al*., 2019) activities. To date, there appears to have been no chemical report published on *Peponium* species with the exception of phytochemical screening of non-protein amino acids such as citrulline, pyrazol-l-ylalanine and γ-glutamyl-β-pyrazol-l-ylalanine from the seeds of P. hirtellum and P. mackenii, and secondary metabolites such as alkaloids and saponins from leaf crude extracts of P. vogelii (Dunnill and Fowden, 1965; Yemata et al., 2019).

1.4 *Peponium vogelii* (Hook. f.) Engl.

1.4.1 Botanical description

Peponium vogelii is a herbaceous climber or trailer with tuberculate roots, simple leaves, and simple or bifid tendrils. It produces stems up to 10 m long that scramble over the ground or climb into nearby vegetation, supporting themselves by means of tendrils. The receptacle forms an elongated and sub cylindrical tube with 5 distinct lobes, and the flowers are showy and always 5-petaled (Bodine and Rogers, 2009). The fruits are very bitter while unripe but when ripe are sweet and are much appreciated by Kipsigi and Masai children in the Narok area of Kenya (Njoroge and Newton, 1994). The plant is native to Ethiopia and commonly known by its vernacular names such as *surupa* (Sidama), *tojo* (Sheka), *entach* (Meinit) Asnake *et al*., 2016; Garedew and Abebe, 2018).

Figure 1. Aerial part of *Peponium vogelii* (Photographed by Israel Mohamed around Ramada village, Shebedino, Sidama South Ethiopia- July, 2021)

1.4.2 Ethnobotanical uses *Peponium vogelii*

In Ethiopia the fruits of *P. vogelii* are taken orally and are said to be good for stomach ache in Sidama region of Southern Ethiopia (Busse H and Tefera G 2013; Gebre and Chinthapalli, 2021). The ripe fruits of *P. vogelii* are also taken orally to treat febrile malaria, gonorrhea and

jaundice (Asnake *et al.*, 2016; Tuasha *et al*., 2018). In Yeki District, Sheka Zone, Southwest Ethiopia, the unripe fruits of *P. vogelii* are eaten to expel intestinal parasites (Garedew and Abebe, 2018). In Congo the pulped-up leaves are used to maturate abscesses and furuncles (Bouquet, 1969). In Tanzania the ripe fruit pulp of *P. vogelii* is sweet and eaten raw, especially by children and herdsmen. The dried powdered leaves are rubbed into scarifications for leprosy and used to alleviate menstrual problems (Olarewaju *et al.,* 2021).

1.5 Statement of the problem

Intestinal parasite infections remain a major public health problem affecting hundreds of millions of people, particularly in tropical developing countries, where the technical expertise and financial resources necessary for new drug development are scarce (Tagboto and Townson, 2001). Control strategies of these infections rely mostly on a limited number of synthetic anthelminthic drugs. In addition to lack of safe and effective currently available anthelminthic drugs and an increasing drug resistance have limited the usefulness of some existing drugs (Sangster and Gill, 1999; James *et al*., 2009). Therefore, there is an urgent need for new, affordable, safe, and effective drugs with novel mechanism of action (Geary *et al.*, 2012; Becker *et al.*, 2020; Jayawardene *et al*., 2021).

Medicinal plants have been known to be good sources of pharmacologically active compounds against several ailments, including nematodes infections (Bahmani1 *et al.*, 2014; Rajasree *et al.*, 2016; Ishnava and Patel, 2020). Despite the anthelminthic claims of *P. vogelii*, to date, there appears to have been no report in the literature concerning the phytochemical composition and anthelminthic activity of the plant. Therefore, the aim of present work was to investigate the anthelminthic activity of the unripe fruits of *P. vogelii*.

2. Objectives

2.1 General objective

- ✓ To investigate the nematicidal activity of the unripe fruit 80% methanol extract and major compounds isolated from *Peponium vogelii*

2.2 Specific objectives

- ✓ To carry out the acute oral toxicity test of the fruit extract.
- ✓ To investigate nematicdal activity of the unripe fruit extract of *P. vogelii*.
- ✓ To isolate compounds from the most active fraction.
- ✓ To elucidate the structures of the isolated compounds.
- ✓ To evaluate nematicidal activity of the isolated compounds.

3. Materials and Methods

3.1 Materials

3.1.1 Plant material

Unripe fruits of *P. vogelii* were collected from Ramada village located in Shebedino woreda, Leku town administration, Sidama region, located in the Rift Valley above the south shore of Lake Hawassa at 6°52'0" North and 38°27'0" East (295 km south of Addis Ababa, Ethiopia) in the year 2022. The plant material was authenticated 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 IS-001) for future reference.

3.1.2 Chemicals

Methanol, acetone, chloroform and *n*-hexane (LOBA-Chemie, India), 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 100× (Capricorn Scientific GmbH, Germany) were all used as received. Ivermectin (Sigma-Aldrich, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) was kindly donated by the Ethiopian Pharmaceutical Manufacturing Factory (EPHARM, Ethiopia).

3.1.3 Instruments

The following instruments were used to carry out the experiments. Rotavapor (Heidolph Instruments GmbH and Co., Germany), Primo Star normal light microscope (Carl Zeiss,

Germany), HPLC, TLC visualizer (Lab Pilot Process group, Switzerland), Jasco V-770 UV-Vis/NIR spectrophotometer (JASCO, Germany), Agilent Technologies 7000 series Mass spectrometer, Agilent DD2 400 MHz NMR spectrometer and Agilent VNMRS 600 MHz NMR spectrometer (Agilent Technologies Inc., USA), light Microscope (Olympus CKX41, Olympus Life Science, Waltham, Massachusetts, USA).

3.1.4 Test organism

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

3.1.5 Experimental animals

Female white Swiss albino mice weighing 22 - 28 g and age 5 - 6 weeks were employed throughout the acute oral toxicity test. The mice were obtained from the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy (SoP), College of Health Sciences (CHS), Addis Ababa University (AAU). The animals were housed in stainless steel cages at room temperature and a 12 h light/12 h dark cycle. They were provided with water and food pellets *ad libitum* in the animal house of the SoP, CHS, and AAU. The experiment were conducted in accordance with the internationally accepted Laboratory Animal Use and Care Guideline (ILAR, 1996) and were approved by the Institutional Review Board of the SoP, AAU.

3.2 Method

3.2.1 Preparation of plant material

The unripe fruits of *P. vogelii* (4 kg) were collected from its natural habitat and rinsed with tap water to remove dirt. Then, they were sliced carefully with knife, cut to small pieces and the seeds separated from the flesh. The fleshy part was dried under shade for four weeks, powdered and stored at room temperature.

3.2.2 Extraction

3.2.2.1 Maceration

Dried fruit powder of *P. vogelii* (400 g) was macerated with 1200 mL of 80% methanol at room temperature for 72 h with continuous agitation and filtered first with sterile gauze and then using Whatman no. 1 filter paper. This was repeated three times and the combined filtrate was concentrated in a Rota vapor (Büchi R-215, BÜCHI Labortechnik AG, Switzerland) with rotation speed 30 rotation per minute at a temperature not exceeding 40 °C. The remaining aqueous solution was dried in an oven at 40 °C and the dried extract was transferred to an amber-coloured bottle and stored in a refrigerator at 4 °C until use.

3.2.3 Bioassay guided fractionation and isolation

First, 24.8 g of dried 80% methanol extract (PV-H) was exhaustively extracted by vigorously shaking with 100 ml of *n*-hexane in a conical flask and filtered. This was repeated two times and the solvent from the combined filtrate was removed under reduced pressure. The residue was further extracted in a similar manner using solvents of increasing polarity (chloroform, acetone, methanol and water). The aqueous fraction was dried in an oven at a temperature not exceeding 40 °C. The following amounts of residues were obtained: *n*-hexane (1.2 g), chloroform (5.2 g),

acetone (0.6 g), methanol (4.3 g) and water (3.2 g). After the solvent fractions were tested for anthelminthic activity the active fraction was further sub-fractionated by gradient elution silica gel column chromatography using chloroform and methanol. The column was initially eluted with 300 ml of chloroform (PVMF-1), followed by chloroform: methanol mixtures of increasing polarity (9.5:0.5, 9:1, 8.5:1.5, 8:2, 7.5:2.5) to obtain five more sub-fractions (PVMF-2 - PVMF-6) each containing 150 ml of eluant. All the fractions were concentrated and tested for bioactivity.

3.2.4 HPLC-DAD analysis

The most active sub-fraction (120 mg) obtained from silica gel column chromatography (PVMF2) was dissolved in 1 ml of solvent was injected and separated at a flow rate of 20 ml/min on C-18 reversed phase HPLC column with an internal diameter of 250 mm x 4.6 mm packed with 5 µm diameter particles. The mobile phase was composed of solvent (A): H_2O with 0.1% trifluoroacetic acid (0 – 20%), and solvent (B): acetonitrile (LC-MS Chromasolv®, Fluka) with 0.1% trifluoroacetic acid (80 – 100%). Eluate fractions were collected every 30 sec. A total of 91 sub-fractions were collected. Fractions showing similar chromatographic profiles were mixed to obtain nine fractions (PVMF2-1 – PVMF2-9) of volume 100 ml, 156.7 ml, 20 ml, 120 ml, 20 ml, 30 ml, 10 ml, 34.7 ml and 320 ml respectively. After drying the following amounts were obtained: PVMF2-1 (1.4 mg), PVMF2-2 (2.5 mg), PVMF2-3 (1.3 mg), PVMF2-4 (2.3 mg), PVMF2-5 (0.9 mg), PVMF2-6 (2.2 mg), PVMF2-7 (1.3 mg), PVMF2-8 (14.3 mg) and PVMF2-9 (41.6 mg). All fractions dissolved in DMSO were tested for nematicidal activity. Then, active and major fractions (PVMF2-8 and PVMF2-9) were analyzed by using mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy to elucidate the chemical structures of the

compounds. The UV absorption spectra of the compounds within the sample were recorded at 215 nm, 254 nm, 365 nm and 215-600 nm.

3.2.5 Structural elucidation

Molecular masses of the active compounds were analyzed by negative mode mass spectrometry-electron spray ionization (MS-ESI) on Agilent Technologies 1200 series Mass spectrometer. 1D NMR spectra were recorded on a Varian DD2 400 and VNMRS 600 FT-NMR spectrometers operating at a proton NMR frequency of 399.92 MHz and 599.83 MHz, respectively, using a 5 mm inverse detection cryoprobe. ^{13}C spectra were recorded on VNMRS 600 NMR spectrometer operating at 150.84 MHz. All experiments were performed in deuterochloroform solution and 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 Acute oral toxicity tests

Acute oral toxicity study was conducted as per the internationally accepted protocol of OECD Guideline 425 (OECD, 2008). Six female non-pregnant and nulliparous mice 6 - 8 weeks, weighing 22 - 28 g were selected. All mice were fasted (food only) for 4 h before and 2 h after administration of the test substances. The test substances were dissolved in distilled water. One mouse was randomly selected and orally administered 2000 mg/kg of the hydroalcoholic extract of unripe fruits of *P. vogelii*. Then the mice were observed for general signs and symptoms of toxicity and mortality within 24 h. The mouse which received 2000 mg/kg of the extract died after 30 min of administration. Therefore, main test was conducted for acute oral toxicity study. Since there was no information regarding the 50% lethal dose (LD_{50}) and the slope of the dose-

response curve for the extract, dosing was initiated at 175 mg/kg (OECD, 2008). One mouse was randomly selected and given a dose of 175 mg/kg of 80% methanol extract. Since the experimental animal survived for 48 h, the dose of the test substance was increased by a factor of 3.2 to 560 mg/kg for second experimental animal. Similarly, since the second mouse survived for 48 h, the dose of the test substance was increased by a factor of 3.2 to 1760 mg/kg for the third experimental animal. However, following administration of 1760 mg/kg of the extract resulted in the death of the mouse 1 h after administration. Therefore, the remaining three mice received 560 mg/kg of the extract. Experimental animals were observed individually for general signs and symptoms of toxicity, physical or behavioral changes such as loss of appetite, ruffled fur, lacrimation, mortality, and other signs of toxicity continuously for 4 h with 30 min interval and then for 14 consecutive days with an interval of 24 h (OECD, 2008).

3.2.7 Preparation of test substances

The hydroalcoholic extract of *P. vogelii* fruits was dissolved in 2% DMSO to prepare 1 mg/ml and 5 mg/ml concentrations. Solvent fractions and sub-fractions from column silcagel chromatography were tested with a concentration of 1 mg/ml, while a concentration of 10 µg/ml was used for the isolated compounds.

3.2.8 Nematicidal assay

Nematicidal assay was carried out on model nematode *Caenorhabditis elegans* using the method developed by Thomson *et al*. (2012). The nematodes were cultured on nematode growth media (NGM) petri plates using the uracil auxotroph *Escherichia coli* strain OP50 as food source. The preparation of NGM medium shown in Appendix II. After 4 days of cultivation, L4 larvae 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 RFC. 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 organisms, resuspension in 2 to 8 ml M9 buffer was carried out. To this suspension, 10 µl penicillin-streptomycin solution (10 mg/ml) was added. The assay was performed in 96 and 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 for 30 mnutes. The number of living and dead animals in each well was then counted using inverted cell culture microscope (Olympus CKX41, Olympus Life Science, and Waltham, Massachusetts, USA). Two percent of dimethyl sulfoxide (DMSO) and the standard anthelminthic drug ivermectin (10 µg/ml) were used as negative and positive controls, respectively. All the assays were done in triplicate.

3.2.9 Data analysis

Data is presented in terms of average percentage of dead nematodes to the total number of worms. Data obtained from the experiments was processed by SPSS 22. The comparison was based on one way ANOVA analysis. Confidence interval 95% was used and values $p < 0.05$ were considered significant. The IC_{50} for each growth condition was calculated by fitting the data to a non-linear least-squares sigmoid regression curve, fixing the top and bottom of the curve at 100 and 0 percent, respectively. The IC50 corresponds to the concentration that would yield an inhibition of 50%.

4. Results and Discussion

4.1 Extraction yield

In the current study, unripe fruits of *P. vogelii* were used to prepare extracts. The powdered plant material subjected to cold maceration with 80% methanol yielded a brown coloured amorphous material. The percentage yield calculated from the dried matter was found to be 6.2% (w/w).

4.2 Bioassay-guided fractionation of the 80% methanol extract

As shown in Figure 2, the 80% methanolic extract of unripe fruits of *P. vogelii* was found to be active against *C. elegans* (>50% inhibition) at concentrations of 1 mg/ml and 5 mg/ml. From solvent fractions only the methanol fraction displayed marked nematicidal activity against *C. elegans* is shown in Figure 3. Which was further sub-fractionated on silica gel column chromatography which resulted six sub-fractions among these sub-fractions, sub-fraction 2 (PVM-F2) which was eluted by a mixture of chloroform and methanol in a ratio of 9.5:0.5 showed appreciable nematicidal activity against *C. elegans* (Figure 4). Finally, PVM-F2 which is a white sticky amorphous substance, was further subjected to HPLC with the aim of isolating the compound(s) responsible for bioactivity.

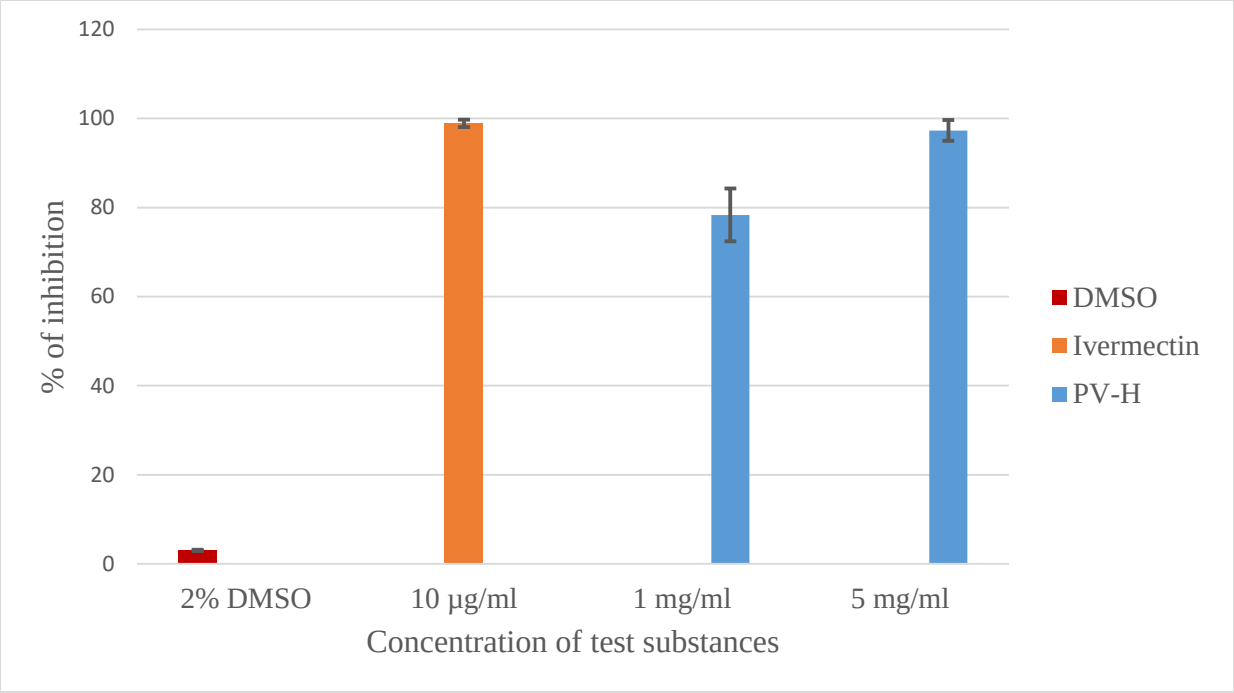

Figure 2. Nematicidal activity of the 80% methanolic extract of unripe fruits of *Peponium vogelii* (PV-H) against *Caenorhabditis elegans* at concentrations of 1 mg/ml and 5 mg/ml. DMSO (2% dimethylsulfoxide).

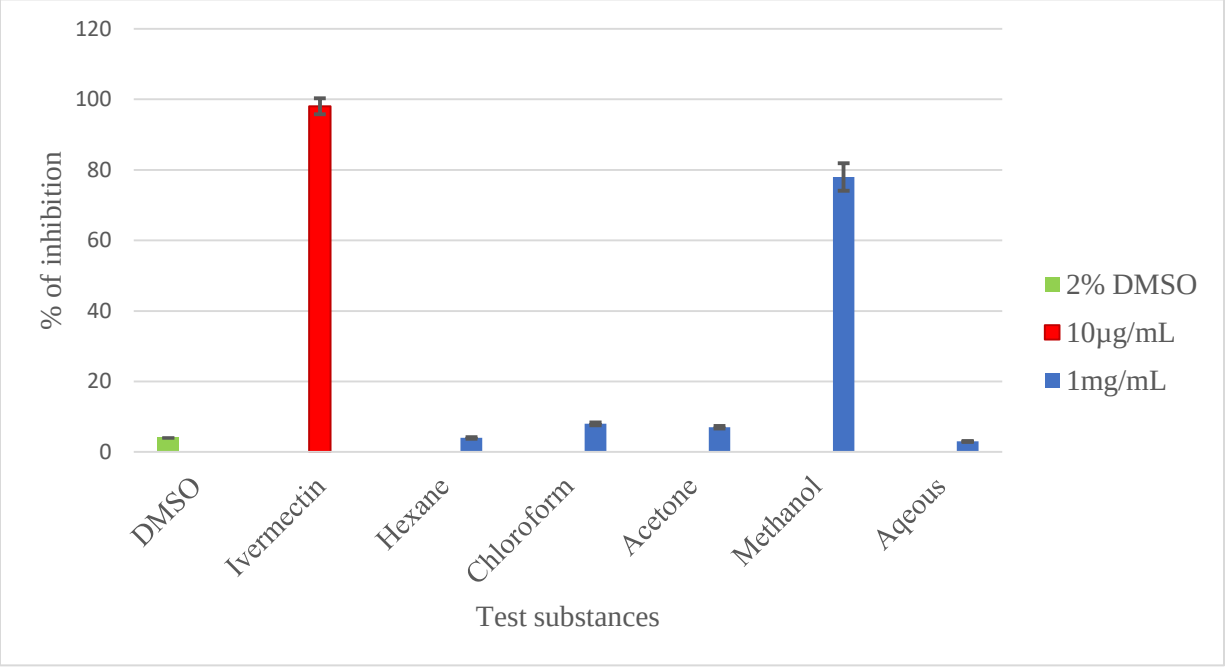

Figure 3. Nematicidal activity of solvent fractions obtained from the 80% of methanolic extract of unripe fruits of *Peponium vogelii* against *Caenorhabditis elegans*.

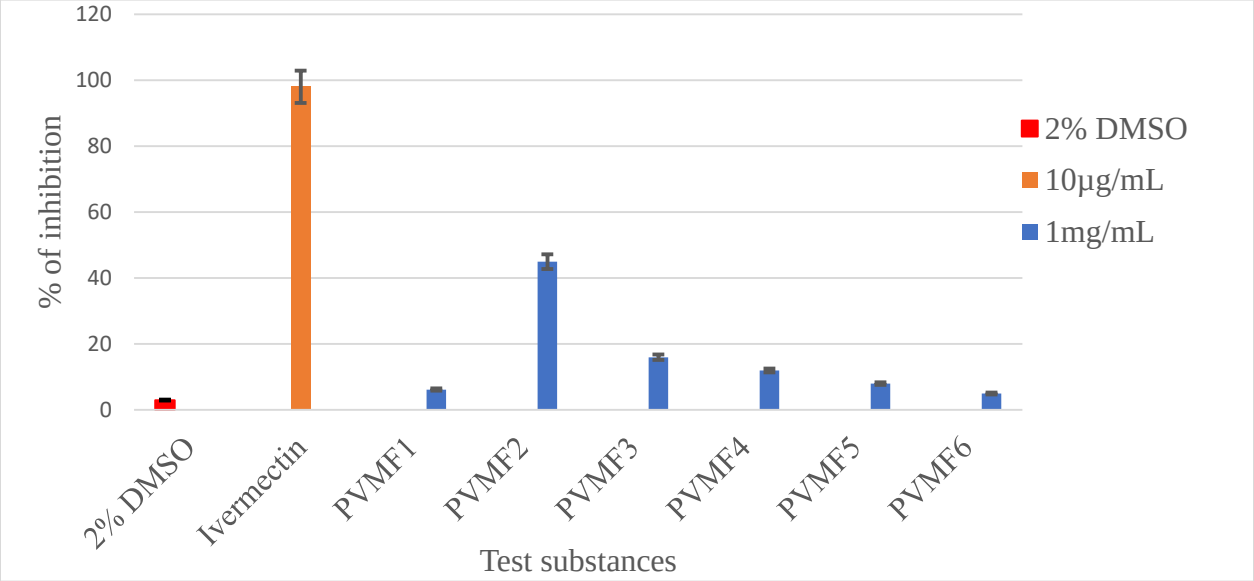

Figure 4. Nematicidal activity of sub-fractions obtained from the methanolic fraction of 80% methanolic extract of unripe fruits of *Peponium vogelii* against *Caenorhabditis elegans*.

4.3 HPLC analysis of PVMF-2

Owing to the marked nematicidal activity demonstrated by PVMF-2, it was further subjected to HPLC to isolate the compounds responsible for bioactivity. As shown in figure 5, nine fractions designated as PVMF2-1 to PVMF2-9 were obtained from reverse phase HPLC column. Each of these fractions was isolated, dried, dissolved in DMSO, and tested for nematicidal activity against *C. elegans*. Among these fractions only five of them labeled as PVMF2-4, PVMF2-5, PVMF2-7, PVMF2-8 and PVMF2-9 showed remarkable activity as shown in Figure 6. Retention time, percentage compositions and percentage of inhibition of sub-fraction from reverse phase HPLC summarized in Table 1.

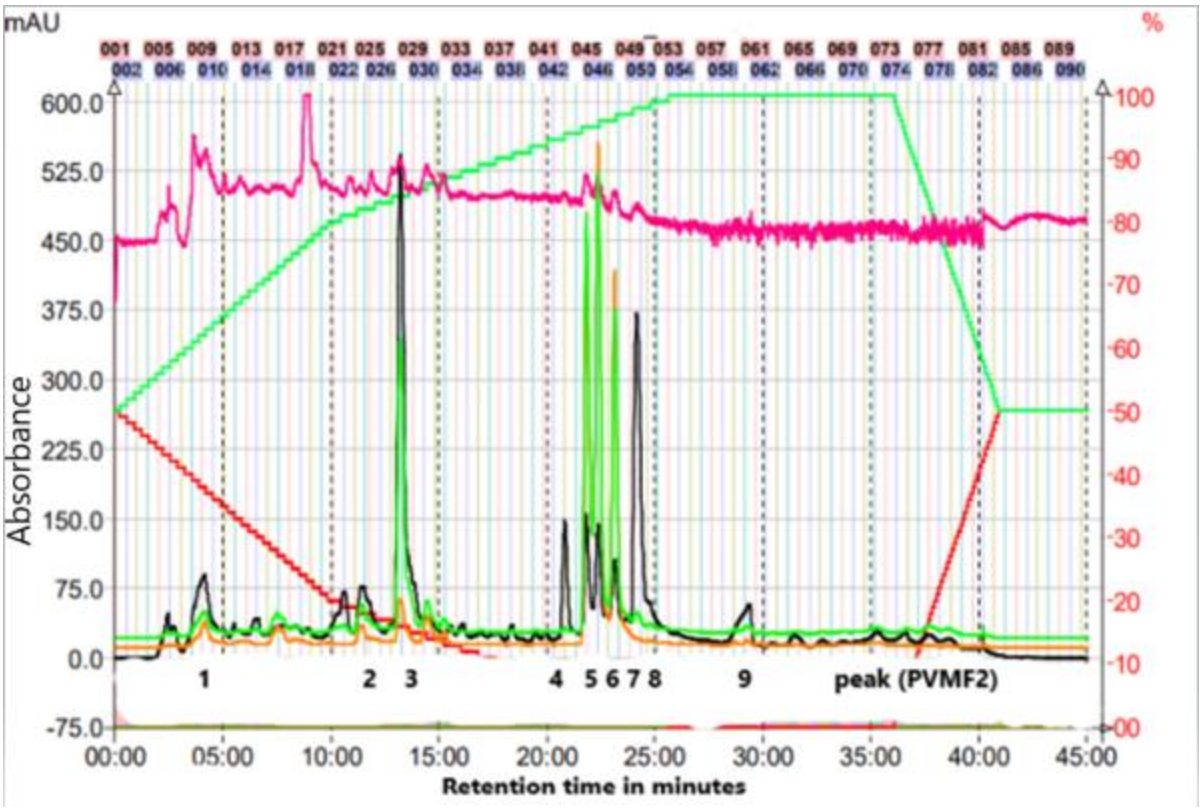

Figure 5. HPLC chromatogram of the active fraction (PVMF2) obtained from silica column chromatography [Developed with acetonitrile and water; detection wavelength 215 nm (black signal), 254 nm (gold), 365 nm (pink), 215-600 nm (green); flow rate 20 ml/min; other chromatographic conditions as described in text].

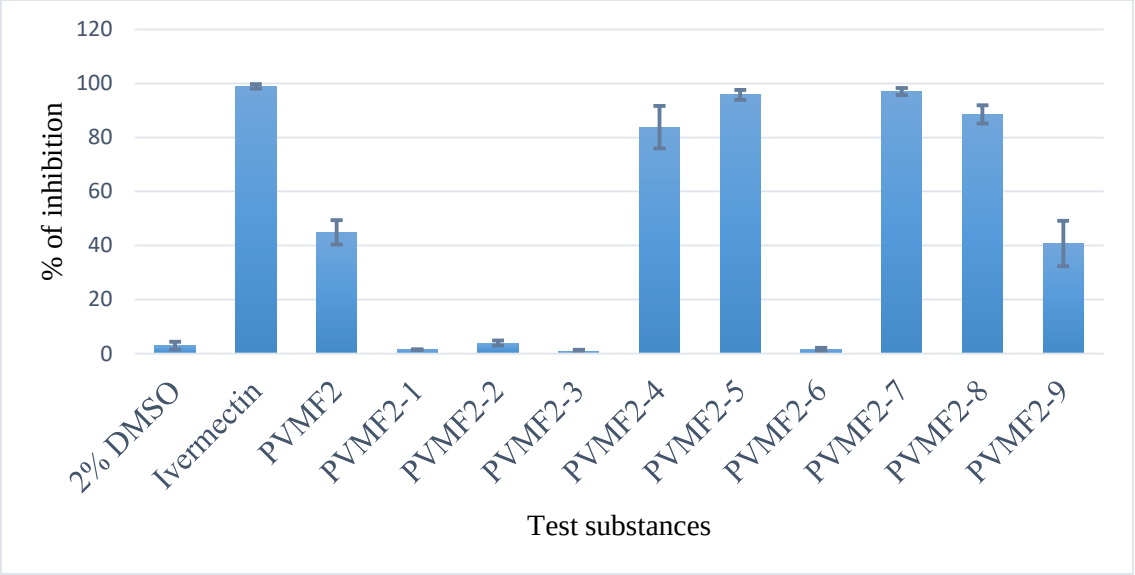

Figure 6. Nematicidal activity of PVMF2-1 to PVMF2-9 collected from HPLC against *Caenorhabditis elegans* at a concentration of 10 µg/ml.

Table 1. Retention time and percentage composition of active compounds

Identified active compounds	Retention time (min)	Percent composition (w/w)*	Percentage of inhibition
PVMF2-1	4	2.1	1.49
PVMF2-2	12	3.6	3.90
PVMF2-3	14	1.9	0.84
PVMF2-4	21	3.39	83.8
PVMF2-5	22	1.32	95.8
PVMF2-6	22.5	3.2	1.62
PVMF2-7	23	1.91	97.5
PVMF2-8	24	21.09	88.54
PVMF2-9	29	61.35	40.7

* Quantification was done by normalization method

4.4 Structural elucidation of the active compounds

Among the nine components obtained from preparative HPLC, PVMF2-9 (41.6 mg) and PVMF2-8 (14.3 mg) were major compound found in high amounts. PVMF2-9 was collected by combining sub-fractions 51 to 82, whilst PVMF2-8 was obtained by mixing sub-fractions 47 to 50. Structural elucidation of the two major components was carried out as presented below.

4.4.1 PVMF2-9

As shown in Figure 7, PVMF2-9 gave a pseudomolecular ion at m/z = 255.6 $[M-H]^-$ in the negative mode ESI-MS, corresponding to a relative molecular weight of 256.5 amu. A molecular formula of $C_{16}H_{30}O_2$ was proposed for PVMF2-9 based on its ESI-MS, ^1H, ^{13}C NMR and DEPT-135 spectral data.

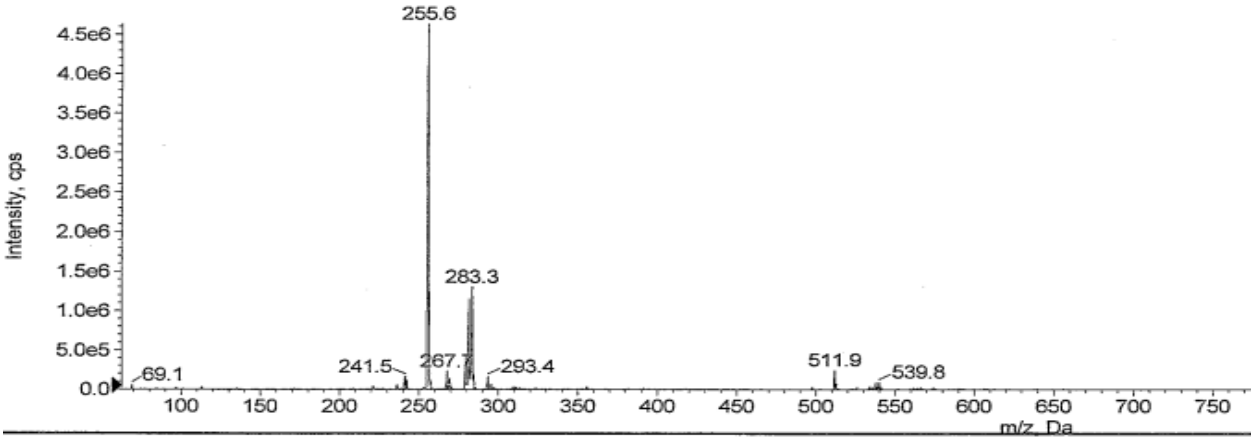

Figure 7. Negative mode ESI-MS spectrum of compound PVMF2-9

The ^{13}C NMR (Figures 8) and DEPT-135 (Figure 9) spectra of PVMF2-9 showed the presence of sixteen carbon atoms, of which one is a methyl carbon (δ 14.10), fourteen are methylene carbon atoms (δ 22.60-33.88) and one is a carbonyl of carboxylic acid group (δ 179.17). In the ^1H NMR spectrum of PVMF2-9 (Figure10), the presence of a carboxylic OH group was evident by the

presence of a signal that resonates at δ 7.50. A typical of aliphatic methyl group was also apparent due to the presence of a triplet at δ 0.88. The presence of relatively deshielded methylene protons (δ 2.35, *t*, 2H) is an indication for the presence of a CH_2 group adjacent to a carbonyl group. In addition to this, there were thirteen methylene protons (δ 1.26-1.63, m, 26H) in the ^{1}H NMR spectrum of PVMF2-9.

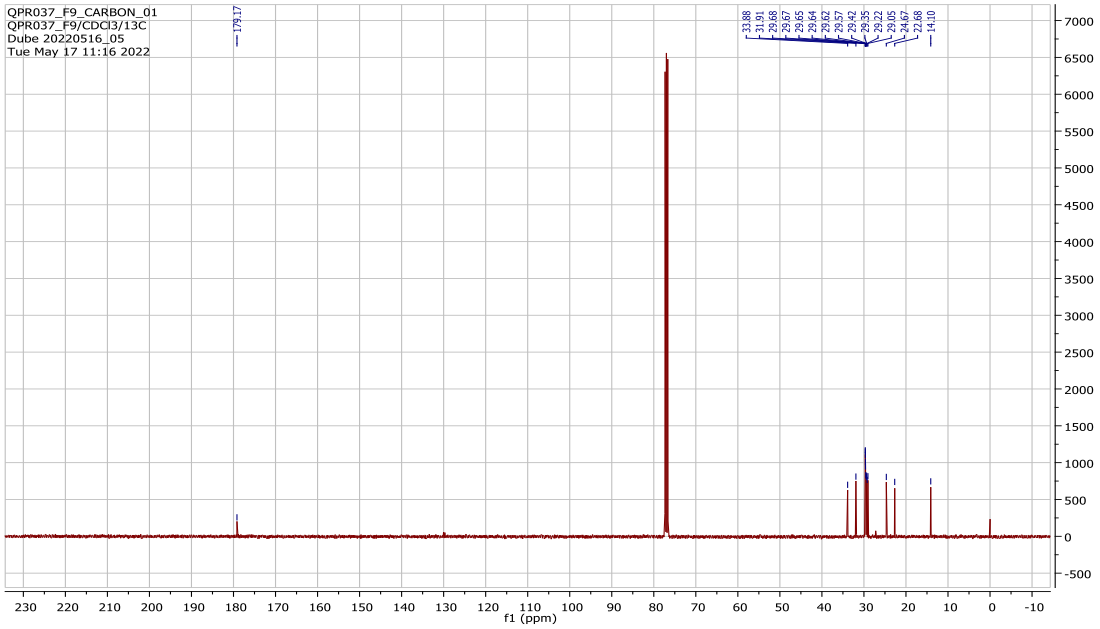

Figure 8. ^{13}C NMR spectrum of PVMF2-9

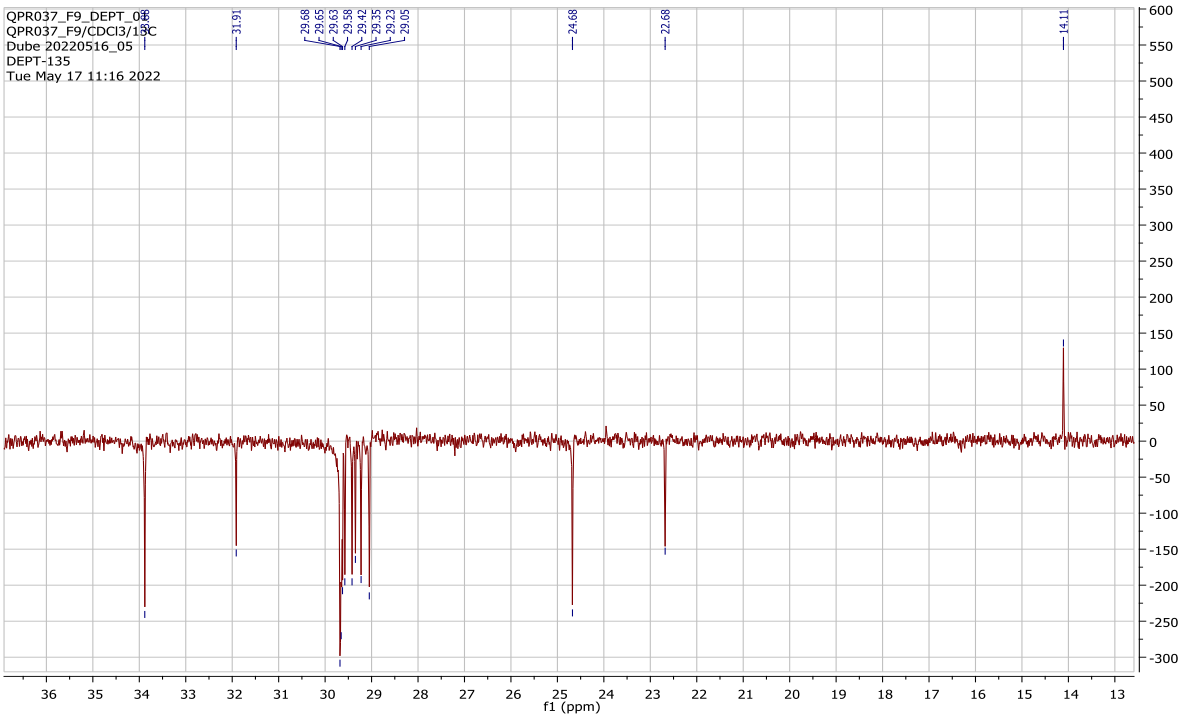

Figure 9. DEPT-135 spectrum of PVMF2-9

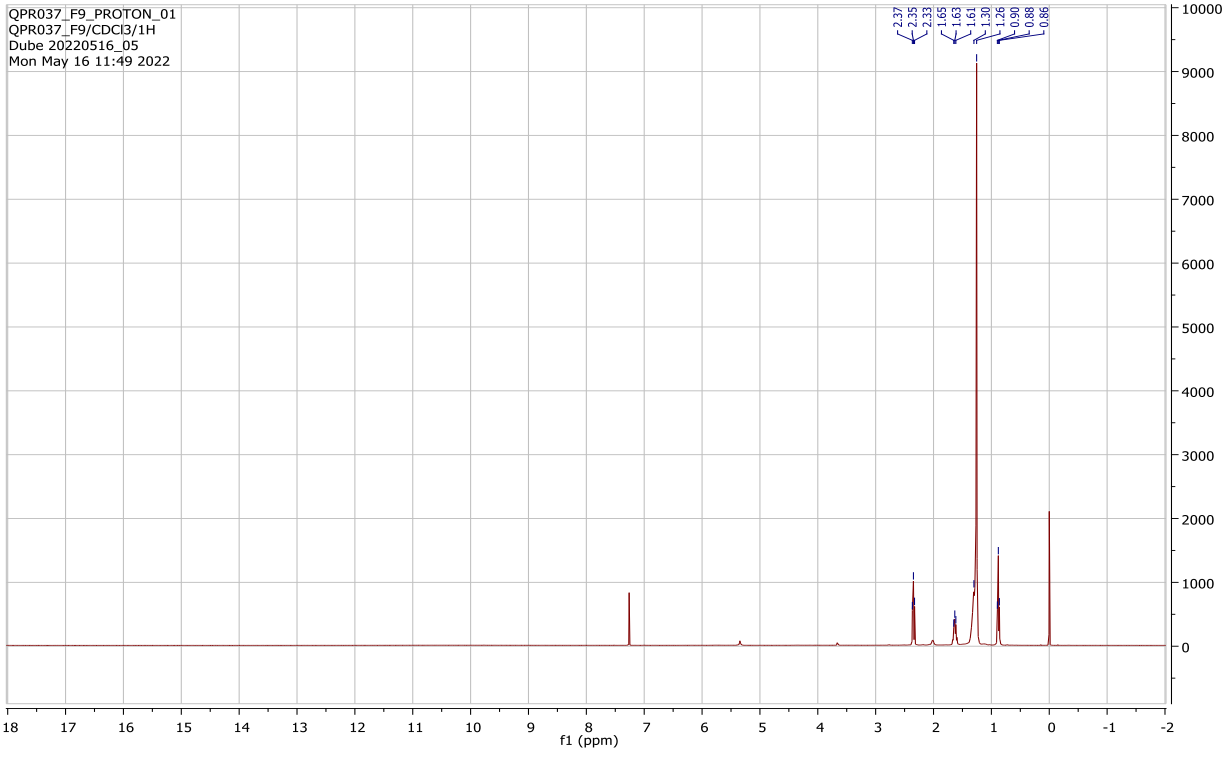

Figure 10. ^{1}H NMR spectrum of PVMF2-9

Thus, based on the above assignments obtained from the experimental results of ESI-MS, ^{13}C NMR, DEPT-135 and ^{1}H NMR, and by comparing these results with the ^{13}C and ^{1}H spectral data of palmitic acid reported in Human Metabolome Database (HMDB) as shown in Table 2, PVMF2-9 was identified as palmitic acid which is otherwise known as hexadecanoic acid (Figure 11).

Table 2. Comparison of the ^{1}H NMR and ^{13}C NMR spectral data of PVMF2-9 and palmitic acid reported in HMBD darabase. https://pubchem.ncbi.nlm.nih.gov/compound/palmitic-acid

Assignment	^{13}C NRM (ppm)		^{1}H NMR (ppm)	
	Pamitic acid	PVMF2-9	Palmitic acid	PVMF2-9
1	180.58	179.17	-----	-----
2	34.23	33.88	2.35	2.35
3	24.80	24.67	1.64	1.63
4	29.21	29.05	1.26	1.26
5	29.49	29.35	1.26	1.26
6	29.37	29.22	1.26	1.26
7	29.57	29.57	1.26	1.26
8	29.81	29.68	1.26	1.26
9	29.81	29.62	1.26	1.26
10	29.81	29.65	1.26	1.26
11	29.81	29.64	1.26	1.26
12	29.81	29.67	1.26	1.26
13	29.49	29.42	1.26	1.26
14	32.05	31.91	1.26	1.26
15	22.79	22.68	1.26	1.26
16	14.14	14.10	0.89	0.88

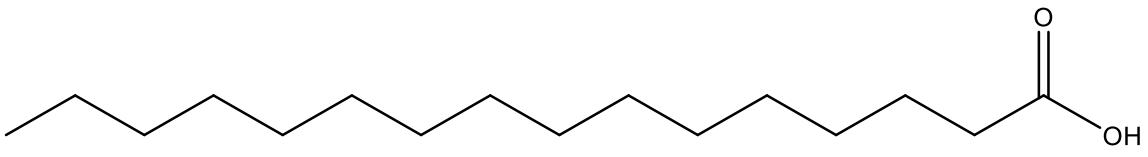

Figure 11. The structural formula of PVMF2-9 (palmitic acid)

4.4.2 PVMF2-8

As shown in Figure 12, the negative ESI-MS spectrum of PVMF2-8 gave a psudeuomolecular ion at m/z = 279.3 [M-H], corresponding to a relative molecular weight of 280 amu. This along with the ^{1}H NMR, ^{13}C NMR and DEPT-135 spectral data established that PVMF2-8 has a molecular formula of $C_{18}H_{32}O_{2}$.

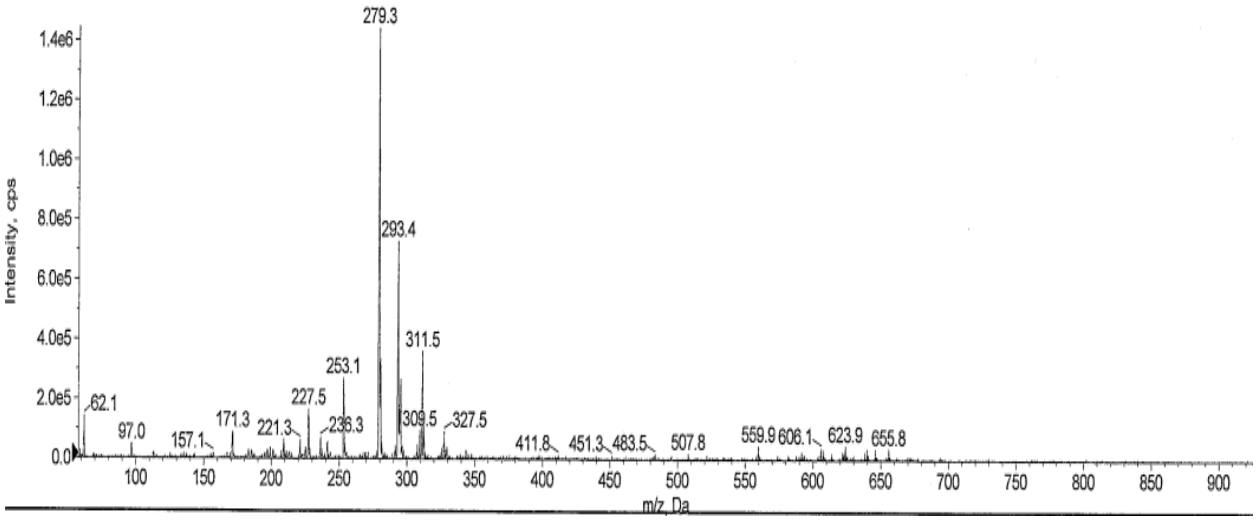

Figure 12. Negative mode ESI-MS spectrum of PVMF2-8

The ^{1}H NMR spectrum of PVMF2-8 showed a multiplet at δ 5.34-5.37 which is assignable to four olefinic protons as shown in Figure 13. A CH_2 group adjacent to a carbonyl was evident from a triplet signal resonating at δ 2.77 and integrated for 2H in the ^{1}H NMR spectrum. The ^{1}H NMR spectrum of PVMF2-8 showed a further multiplet at δ 2.35 which is assignable to allylic (H-6) protons. Moreover, a triplet resonating at δ 0.89 was typical of aliphatic methyl protons.

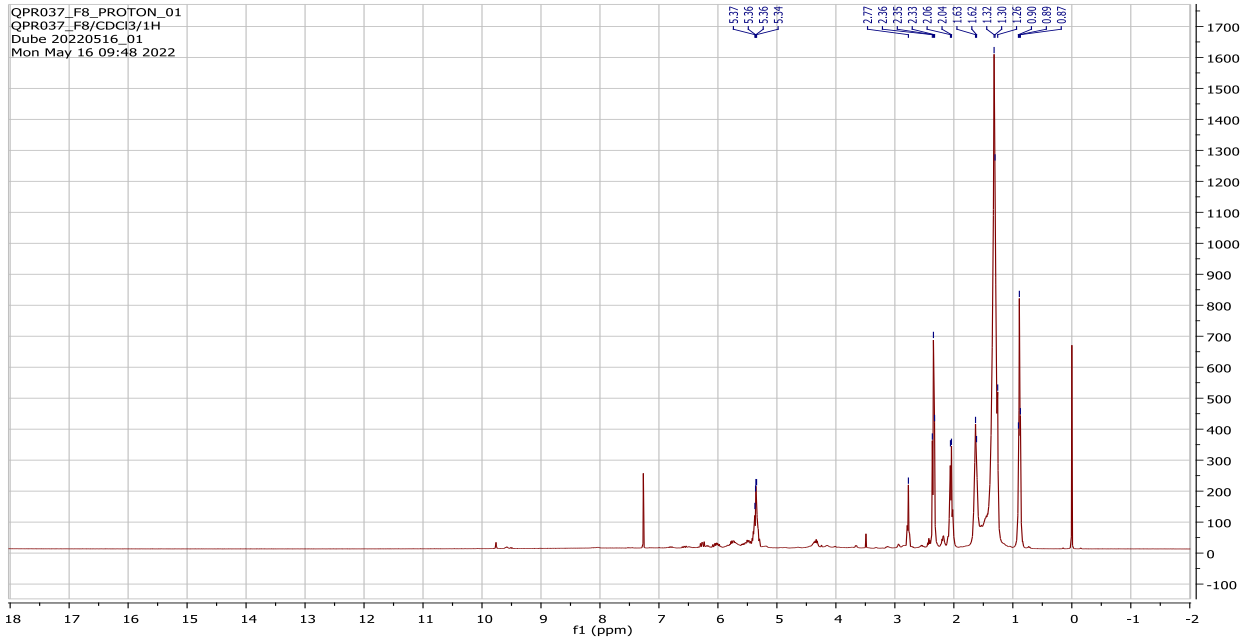

Figure 13. ^{1}H NMR spectrum of PVMF2-8

The ^{13}C NMR spectrum of PVMF2-8 (Figure 14) corroborated that the compound contains 18 carbons. As shown in Figure 15, the DEPT-135 revealed the presence of one methyl carbon (δ 14.05), twelve methylene carbons (δ 33.95, 31.50, 29.56, 29.32, 29.12, 29.05, 29.01, 27.18, 27.16, 25.61, 24.64, and 22.55) and four olefinic methine carbons (δ 130.19, 130.00, 128.04, and 127.87) and a carbonyl carbon (δ 179.56).

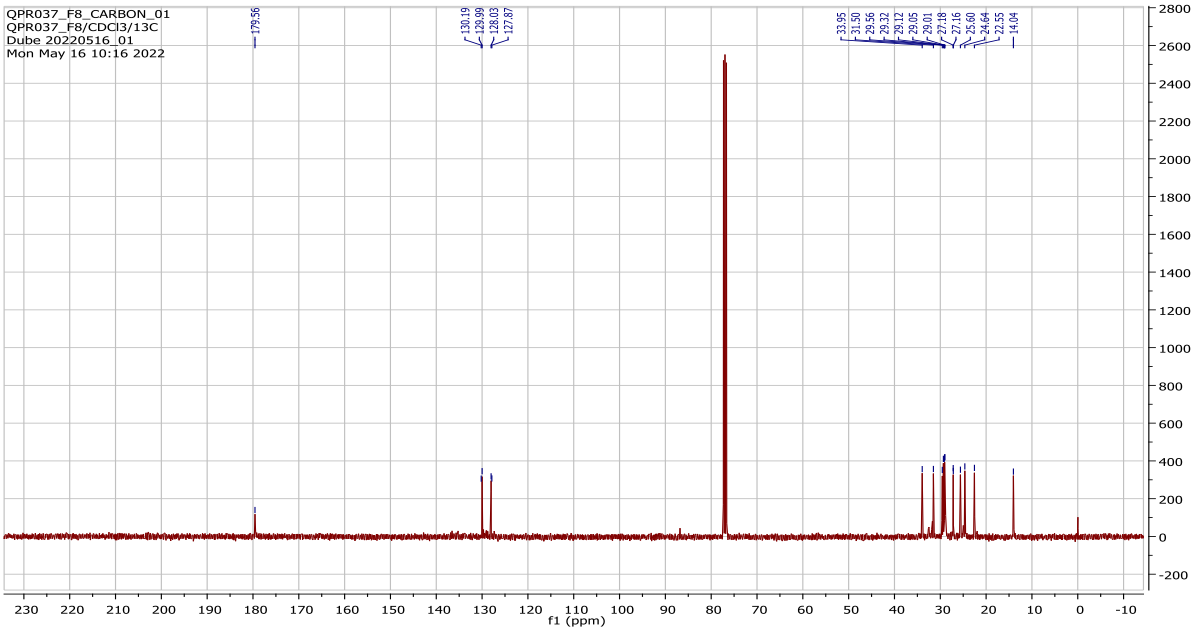

Figure 14. ^{13}C NMR spectrum of PVMF2-8

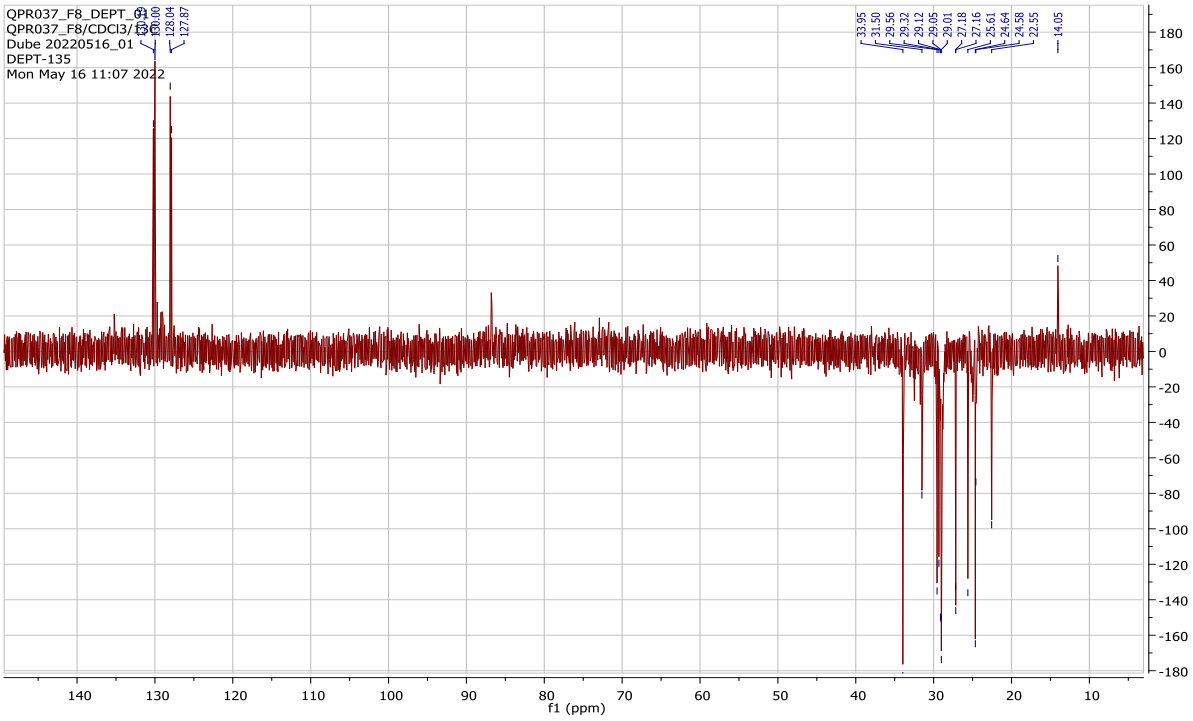

Figure 15. DEPT-135 spectrum of PVMF2-8

The complete assignments of ^{1}H and ^{13}C chemical shifts of PVMF2-8 along with the literature values for linoleic acid are listed in Table 3. From the chemical shifts presented and by comparing the ^{1}H and ^{13}C-NMR spectral data of linoleic acid reported in HMB Database, PVMF2-8 was identified as linoleic acid [(9Z, 12Z)-octadeca-9, 12-dienoic acid] **(**Figure 16).

Table 3. Comparison of ^{1}H NMR and ^{13}C NMR spectral data of PVMF2-8 and linoleic acid from HMBD database

Assignment	^{13}C NRM (ppm)		^{1}H NMR (ppm)	
	Literature (linoleic acid)	PVMF2-8	Literature (linoleic acid)	PVMF2-8
1	179.77	179.56	----	----
2	33.96	33.95	2.36	2.35
3	25.58	25.60	1.63	1.62
4	27.18	27.18	1.33	1.30
5	29.00	29.05	1.33	1.30
6	24.63	24.64	1.33	1.30
7	29.34	29.32	1.33	1.30
8	27.17	29.12	2.04	2.04
9	130.02	129.99	5.34	5.34
10	128.00	128.03	5.35	5.36
11	29.57	29.56	2.78	2.77
12	127.84	127.87	5.36	5.36
13	130.21	130.19	5.33	5.34
14	29.15	29.12	2.05	2.05
15	29.07	29.05	1.33	1.30
16	31.51	31.50	1.33	1.30
17	22.58	22.55	1.33	1.30
18	14.12	14.04	0.90	0.89

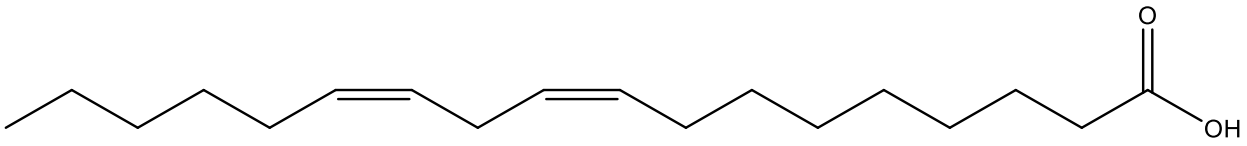

Figure 16. Structural formula of PVMF2-8 (linoleic acid)

4.5 Biological activity tests

4.5.1 Acute oral toxicity of the extract

Acute toxicity test involves an estimation of a dose which has proved to be lethal to 50% of the tested group of animals during a period not exceeding 24 h. Acute toxicity test results of the present study revealed that the 80% methanolic extract of the fruits of *P. vogelii* after oral dose of 2000 mg/kg showed severe toxicity leading to death of the experimental animal. Therefore, in order to determine the LD_{50} of the extract, main test was employed. This test revealed that the LD_{50} of the hydroalcoholic extract of the fruits of *P. vogelii* is above 550 mg/kg but less than 1750 mg/kg.

4.5.2 *In vitro* anthelminthic activity of the hydroalcoholic extract

In the present study a positive linear correlation was established between percent of inhibition (activity based on movement of *C. elegans*) and concentrations of test substances ($R_2 = 0.99$) in anthelminthic assays. The hydroalcoholic extract of the fruits of *P. vogelii* displayed significant ($p < 0.001$) nematicidal activity with an IC_{50} value of 23.5 µg/ml against *C. elegans* with percentage inhibition of 76.66% and 97.566 % at 1 mg/ml and 5 mg/ml, respectively, as shown in Figure 2.

Traditional medicine practitioners and local people in southern parts of Ethiopia widely use the fruits and leaves of *P. vogelii* for the expulsion of intestinal parasites and stomach trouble (Heidi,

2013; Garedew and Abebe, 2018). It was on the basis of this ethnobotanical claim that the plant was selected for nematicidal assay. In the current study, anthelminthic assay was carried out on adult *C. elegans* which is an attractive assay model for study of nemanticidal properties. This model organism has many features that make it an outstanding experimental system such as easy and inexpensive to grow, its simple anatomy, including its small size, rapid life cycle, transparency, well-annotated genome and ease of genetic analysis allow for studies of diverse biological processes, including those related to human nutrition and disease (Corsi *et al*., 2015).

4.5.3 Nematicidal activity of PVMF2-9 (palmitic acid)

The present study unveiled that palmitic acid is one of major compounds responsible for anthelminthic property of the 80% methanol extract of *P. veogelii* fruits. As shown in Figure 6, incubation of *C. elegans* with 10 µg/ml of PV-F9 (palmitic acid) inhibited movement of *C. elegans* in the observation period. This is consistent with earlier studies which reported that palmitic acid is one of constituents of the seed extracts of *Cucurbita pepo* L. which demonstrated strong activity against the intestinal roundworm of rodents (*Heligmosoides bakeri*) (Grzybek *et al.*, 2016). Pineda-Alegría *et al.* (2020) demonstrated that palmitic acid and stearic acid, in some combinations, inhibited *Heligmosoides contortus* egg hatching by 100%. It was also reported that palmitic acid is one of the major constituents of the methanol extract of *Acmella oleracea* which showed a broad-spectrum nematicidal activity against cestode and nematode parasites (Lalthanpuii, *et al*., 2020). In addition, palmitic acid is one of the several fatty acids isolated from the larvicidal fractions of *Areca catechu* L., which were found to be active against *Toxocara canis.* Furthermore, larvicidal activity of fatty acids against *T. canis* was found to depend markedly on the chain length (Nakamura *et al.*, 1988).

4.5.4 Nematicidal activity of PVMF2-8 (linoleic acid)

Results of the current study disclosed that linoleic acid is one of the major compounds responsible for the strong nemanticidal effect of the hydroalcoholic extract of *P. vogelii* fruits against *C. elegans*. As shown in Figure 6, at a concentration of 10 µg/ml the percent inhibition of linoleic acid against *C. elegans* was 88.5%. This is consistent with the findings of Panda *et al.* (2020) who reported that linoleic acid isolated from the ethanol extract of fruits of *Holigarna caustica* possesses nematicidal activity. Similarly, Ayers *et al.* (2010) found that linoleic acid from *Clonostachys candelabrum* has significant nematicidal activity against *Haemonchus contortus*. Linoleic acid was shown to be the only detectable nematicidal agent in the mycelial extracts of several predacious fungi of the genus *Arthrobotrys* (Pineda-Alegría *et al.,* 2020). In another study fatty acids including linoleic acid extracted from the cultures of *Basidiomycetes, Pleurotus pulmonarius* and *Hericium coralloides* were shown to have nemanticidal properties towards the saprophytic nematode *C. elegans* (Stadler *et al.,* 1994). It was also reported that medium chain fatty acids have a larvicidal activity against *Toxocara cani* and *Heterobothrium okamotois* in an *in vitro* assay (Liu *et al.,* 2018). Structural activity relationship analysis of fatty acids against the cist nematode *Heterodera zeae* revealed that nematocidal activity of fatty acids depends on different factors including chain length, the number and position of double bond (Faizi *et al.,* 2011). And also these free living nematodes are known to be affected by fatty acids with chain lengths C_8-C_{12} and longer (Stadler *et al.*, 1994; Panda *et al.*, 2020).

Nematicidal mechanism of action of fatty acids is not known. But it has been suggested that their effect against nematodes is due to adverse interference with the nematode cuticle or hypodermis via a detergent (solubilization) effect, or through direct interaction of the fatty acids with the lipophilic regions of target plasma membranes (Davis *et al.*, 1997; Panda *et al.*, 2020). Fatty

acids like linoleic acid can affect neuromuscular physiology of parasites particularly ionotropic neurotransmitter receptors, like gamma-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA) receptors, which are targets of some common anthelmintics like Ivermectin®, piperazine, levamisole and tetramisole (Aoshima, 1996; Frayha *et al.*, 1997; Liu *et al.*, 2018).

To the best of our knowledge, this is the first report on the nematicidal activity of fruits of *P. vogelii*. The current investigation on the anthelminthic potential of *P. vogelii* extract is of special relevance for the countries like Ethiopia, as it may help to obtain indigenous sources of drugs to combat infections caused by intestinal helminths (Alemu *et al.*, 2018).

5. Conclusion

Results of the present study confirmed that the unripe fruit extract of *P. vogelii* possess genuine anthelmintic activity against *C. elegans*. LD_{50} of the 80% methanol extract of the fruits of *P. vogelii* is above 550 mg/kg but less than 1750 mg/kg. The findings further established that palmitic acid and linoleic acid are the major compounds present in the hydroalcoholic extract of *P. vogelii*, which significantly contribute to the nematicidal activity of the plant. The results also support the use of unripe fruits of *P. vogelii* for the treatment of intestinal parasites in traditional medicine. The genuine nematicidal activity observed in the present study could make the fruit extract of *P. vogelii* and isolated compounds to be a potential addition to the current drugs used for the treatment of intestinal parasites.

Recommendations

Based on the findings of the present study, the following recommendations are suggested.

- ✓ Evaluate the effects of linoleic acid and its analogues on common parasites in *in vivo* antihelmenthic studies;
- ✓ Determine mode of action of the isolated compounds;
- ✓ Use palmitic and linoleic acid as lead compound for the development of safe and cost-effective anthelminthic drugs;
- ✓ Elucidate the structures of other compounds that are responsible for the anthelmintic activity of *P. vogelii*; and
- ✓ Sub-acute and chronic cytotoxicity test of the extract and isolated compounds.

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Appendices

Appendix I. List of some medicinal plants used anthelminthic

Name of plant	Family	Part used	Method of preparation	References
Abrus precatorius L.	Fabaceae	Root	Decoction	(Barbosa *et al.*, 2020)
Adenia gummifera (Harv.) Harms	Passifloraceae	Root	Decoction	(Barbosa *et al.*, 2020)
Ajuga integrifolia Buch-Ham.	Lamiaceae	Leaf	Crush, filter and drink	(Teklay, Abera and Giday, 2013)
Aloe macrocarp.	Aloaceae	Leaf	Fresh leaves chewed and swallow the juice	(Mengesha, 2019)
Aloe megalacantha	Aloaceae	Latex	Squeeze latex, filter and drink	(Wondimu, Asfaw and Kelbessa, 2007)
Artabotrys Brachypetalus Benth.	Annonaceae	Root	Decoction	(Barbosa *et al.*, 2020)

Bridelia cathartica Bertol.	Phyllanthaceae	Root	Decoction	(Barbosa *et al.*, 2020)
Brucea antidysentrica J.F. Mill.	Simaroubaceae	Leaf	Fresh leaves crushed given for a person in need	(Mengesha, 2019)
Carca papaya L.	caricaceae	Fruit		(Mengesha, 2019)
Cineraria abyssinica Sch. Bip. ex A. Rich	Asteraceae	Leaf	Squeeze the leaf	(Garedew and Abebe, 2018)
Clutia abyssinica Jaub. & Spach.	Euphorbiaceae	Leaf	Crushed and drunk the fluid	(Teklay, Abera and Giday, 2013)
Cyanoglassum lanceolatum Forssk.	Boranginaceae	Bark	Ground and squeeze	(Garedew and Abebe, 2018)
Dovyalis abyssinica (A.Rich.) Warb.	Flacourtiaceae	Fruit	Eat the fruit or drink its juice	(Teklay, Abera and Giday, 2013)
Embelia schimper Vatke	Musaceae	Fruit		(Mengesha, 2019)
Embelia schimperi Vatke	Myrsinaceae	Fruit		(Garedew and Abebe, 2018)
Euphorbia abyssinica J.F.Gmel.	Euphorbiaceae	Latex	Mix part with locally made beer and drink it	(Teklay, Abera and Giday,

				2013)
Ficus vasta Forssk.	Moraceae	Bark	Crush and it with honey	(Teklay, Abera and Giday, 2013)
Gymnanthemum coloratum (Willd.) H.Rb. & B. Kahn	Asteraceaa	Root	Decoction	(Barbosa *et al.*, 2020)
Halothamnus somalensis	Chenopodiacea e	Root	Crushed and mixed with water and taken oral.	(Debebe *et al.*, 2015b)
Heinsia crinita (Afzel.) G. Taylor	Rubiaceae	Root	Decoction	(Barbosa *et al.*, 2020)
Hypoxis hemerocallidea Fisch., C.A.Mey. & Avé-Lall.	Hypoxidaceae	Corm	Cold infusion	(Barbosa *et al.*, 2020)
Jasminum floribundum R.Br	Oleaceae	Leave	Leave are crushed and mixed with water	(Seyoum and Zerihun, 2014)
Jatropha curcas L	Euphorbiaceae	Pod	Pounded and prepared in the form of tablets	(Debebe *et al.*, 2015b)
Kageneckia lanceolata R.	Rosaceae	Bark	Decoction	(Macía, García and Vidaurre, 2005)

Lantana trifolia L.	Verbenaceae	Leaf	Boil it with milk or tea and drink	(Teklay, Abera and Giday, 2013)
Lepidium cf. bipinnatifidum Desv	Rosaceaea	Whole plant	Infusion	(Macía, García and Vidaurre, 2005)
Maclura africana (Bureau) Corner	Moraceae	Root	Decoction	(Barbosa *et al.*, 2020)
Merendra bengalensis (Roxb.) Benth	Lamiaceae	Leaf	Crush, filter and drunk the fluid	(Teklay, Abera and Giday, 2013)
Myrisin African L.	Myricinaceae	Fruit	The fruit crushed and taken as juice	(Seyoum and Zerihun, 2014)
Otostegia integrifolia Benth.	Lamiaceae	Leaf	Crush, filter and drink the fluid	(Teklay, Abera and Giday, 2013)
Oxalis corniculata L.	Oxalidaceae	Bulb	Peel the external part and eat it	(Teklay, Abera and Giday, 2013)
Peponium vogelii (Hook.f) Engl.	Cucurbitaceae	Fruit	Fresh fruit is eaten	(Garedew and Abebe, 2018)
Pittosporum virdiflorum Sims.	Pittosporaceae	Leaf	The fresh leaves grinded and juice	(Garedew and Abebe, 2018)

			drunken	
Plumbago zeylanica L	Plumbaginacea e	Seed	Pounded and eaten in empty stomach.	(Debebe *et al.*, 2015b)
Rumex abyssinicus Jacq.	Polygonaceae	Leaf	Crush, filter and drink the fluid	(Teklay, Abera and Giday, 2013)
Saba comorensis (Boj.) Pichon	Apocynaceae	Fruit	Fruit are eaten directly (chewing)	(Mengesha, 2019)
Senna occidentalis (L.) Link	Fabaceae	Root	Decoction	(Barbosa *et al.*, 2020)
Solanum mariginatum L.f	Solanaceae	Root	Crush, filter and drink the fluid	(Teklay, Abera and Giday, 2013)
Verbena officinalis L	Verbenaceae	Root	Crush , filter and drink the fluid	(Teklay, Abera and Giday, 2013)
Vernonia amygdalina Del.	Asteraceae	Leaf	Juice is extracted from fresh leaf and taken one cup	(Mengesha, 2019)
Ximenia americana L.	Olacaceae	Fruit	Ripen fruits are directly eaten (chewing	(Mengesha, 2019)
Zehneria scabra (L.f.)	Cucurbitaceae	Root	Crush by mixing it with	(Teklay, Abera

Sond.	Verbena officinalis, filter and drink the juice	and	Giday, 2013)		

Appendix II: Preparation of NGM medium

Nematode growth medium (NGM medium) is prepared by 3 g NaCl, 17 g agar, and 2.5 g peptone were weighed, mixed and dissolved in 975 ml of distilled water in a 2 litre Erlenmeyer flask by boiling in hot plate at 55^{o}C with repeated shaking until clear solution is obtained. Then it was autoclaved at 121℃ for 50 min. and 1 ml 1 M $CaCl_2$, 1 ml 5 mg/ml cholesterol in ethanol, 1 ml 1 M $MgSO_4$, 25 ml 1 M KPO_4 buffer and autoclaved solution were mixed by swirling until clear solution is obtained. Then NGM solution transferred to petri plates (2/3 full of agar plate) using a peristaltic pump. Then the plates stored at room temperature for 2-3 days before use to allow for detection of contaminants, and to allow excess moisture to evaporate and stored at room temperature until use.