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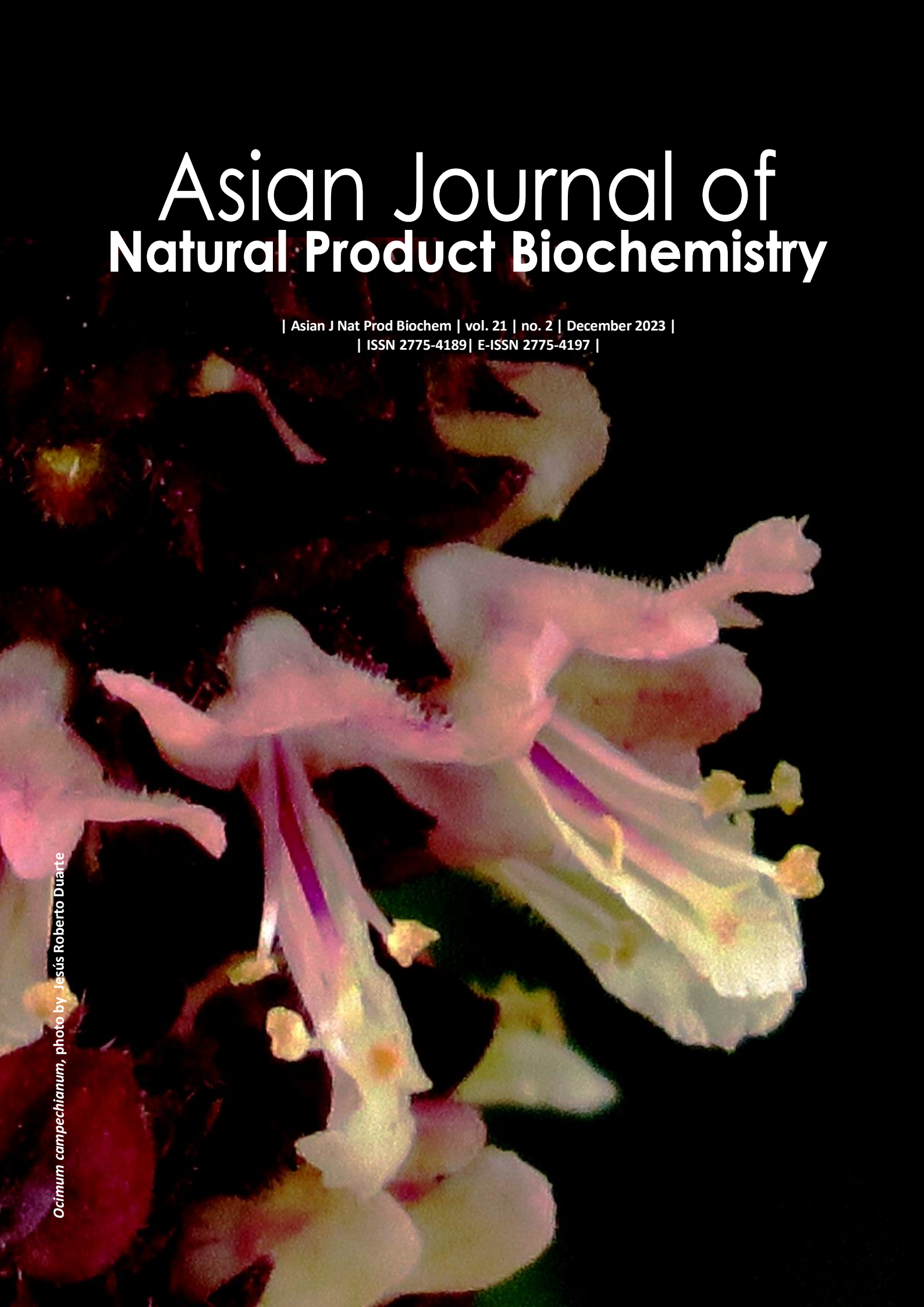

Ocimum campechianum, photo by Jesús Roberto Duarte

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Introduction is about 600 words, covering the aims of the research and provide an adequate background, avoiding a detailed literature survey or a summary of the results. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for a manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgements** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

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Journal:

Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158. DOI: 10.13057/biodiv/d070213.

The usage of "et al." in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al. 1999. Future of health insurance. N Engl J Med 965: 325-329. DOI: 10.10007/s002149800025.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in the book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds.). Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta*. 50th annual symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.). Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from the internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. DOI: 10.1038/msb.2008.24. www.molecularsystembiology.com.

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In vitro evaluation of antifungal potential of selected plant species against *Fusarium solani*

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Abstract. *Pant D, Tapwal A, Tiwari B, Thakur JS. 2023. In vitro evaluation of antifungal potential of selected plant species against* Fusarium solani. *Asian J Nat Prod Biochem 21 (2): 51-57.* The hazardous effect of synthetic fungicides is a major concern. Therefore, the development of safe and eco-friendly fungicides is momentarily imperative. The Himalaya is considered a rich repository of floristic diversity, endowed with several medicinally important plant species exhibiting antimicrobial potential. The aim of this study was to evaluate the anti-mycotic potential of five selected Himalayan plant species viz. *Berberis lycium* Royle, *Zanthoxylum armatum* DC., *Valeriana jatamansi* Jones, *Ageratina adenophora* (Spreng.) R.M. King & H. Rob. and *Polystichum squarrosum* (D. Don) Fee by poisoned food technique against *Fusarium solani* which is considered as one of the major causes of Shisham. Crude extract of *B. lycium* exhibited maximum growth inhibition (51.58%) of *F. solani*, followed by *Z. armatum* (21.94%) and *V. jatamansi* (21.45%). However, the inhibitory effect of *A. adenophora* and *P. squarrosum* extracts on fungal growth was insignificant. Furthermore, the potentials of three solvents (aqueous, ethanol and acetone) were evaluated for the extraction of antimycotic phytochemicals from the leaf of *B. lycium* and ethanol was found to be comparatively more effective. Phytochemical analysis of a crude extract of *B. lycium* revealed the presence of different groups of bioactive compounds, such as alkaloids, flavonoids, terpenoids, steroids, phenolics and tannins. Thus, the presence of diverse groups of phytochemicals in *B. lycium* makes it more effective in controlling the growth of *F. solani* and can be used in the development of eco-friendly fungicides.

Keywords: Antifungal, *Berberis lycium*, *Fusarium solani*, Himalayan plant species, phytochemicals

INTRODUCTION

Fungi are the most common pathogens that cause severe damage to agricultural crops and forest tree species. Forests endure serious harm from fungal pathogens, which cause prominent diseases like wilting, scab, moldy coatings, rusts, blotches, rotting tissue, anthracnose, leaf spot, blight, mildew, coils, gall, canker, damping-off, root rot, dieback, etc. (Iqbal et al. 2018). Most fungal pathogens are opportunistic in nature, invading the host plant during physiological stress or through isolated regions of damaged or diseased tissue (Lilja et al. 2010). On a global scale, *Fusarium* is one of the top ten most common genera of plant pathogenic fungi (Dean et al. 2012). *Fusarium solani* is one of such destructive pathogens that cause wilt disease in *Dalbergia sissoo* Roxb. ex DC. (Shisham). *Fusarium* wilt disease has been linked to Shisham population declines in Uttar Pradesh, Bihar, Punjab, and Himachal Pradesh. The *F. solani* is usually found on roots, and the disease appears during the humid months of July to September. Pathogen hyphae and jelly-like substances plug the vessels, causing wilt symptoms (Bakshi and Singh 1959). The *D. sissoo* is an important timber tree species of India, and it is especially susceptible to root pathogens, which cause wilting and root rot (Bhandari et al. 2014). Dieback due to wilting is a serious problem in this species. In severe conditions, the dieback is identified by thinning leaves and crowns, drying of branch tips, table roofing, and a stag-head (Khan and Khan 2000). Infected trees show signs of wilting, and as the leaves fall off, the branches become clear, and the plants die within a few months (Kumar and Khurana 2016). The *F. solani* is a soil-dwelling fungus that can survive in the soil for several years without active contact with the host. Mycotoxins, which are secondary metabolites produced by *F. solani*, pose a serious threat to plants and animals (Prasad et al. 2018). The management wilt (caused by *F. solani*) by applying fungicides is cumbersome and can pollute underground water resources. Therefore, eco-friendly strategies like applying biocontrol agents or botanicals in disease management are much preferred.

Biological control refers to disease management by eco-friendly means like plant extracts, plant products, and microorganisms such as bacteria, viruses, fungi, or other living creatures that suppress pathogens (Dawood 2015). Due to a lack of mobility, plants have evolved alternate defensive systems, compromising an extensive range of chemical metabolites as instruments to handle stress circumstances (Munuswamy et al. 2013). The basic role of plant secondary metabolites is in defense against various diseases and herbivores. In other words, secondary metabolites are released by plants as a defense strategy (Netala et al. 2015). The secondary metabolites of plants, such as phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins, are quite effective against various phytopathogens. These

metabolites are known to exhibit antimicrobial properties and are used by plants to defend themselves against harmful microorganisms (Das et al. 2010). Tapwal et al. (2011) evaluated the phyto-extract of five plants for antimicrobial potential against phytopathogens under laboratory conditions and observed that the extract of *Cannabis sativa* L. was most effective against *Curvularia lunata* (Wakker) Boedijn. Singh et al. (2018) screened nine plant extracts at varied concentrations on the growth of *F. solani*, and *Curcuma longa* L. rhizome extract was the most effective. Similarly, Seepe et al. (2020) evaluated the antifungal activity of eight plants against *Fusarium* species inoculated on maize seeds and the maximum inhibitory effect was shown by acetone extract of *Melia azedarach* L. against *F. proliferatum*, followed by mixed acetone extracts from *Combretum erythrophyllum* (Burch.) Sond. and *Quercus acutissima* Carruth. Exhibited antifungal activity on *F. verticilloides*, *F. proliferatum* and *F. solani*. Sheel et al. (2022) tested five weed extracts on the mycelial growth of *Botryodiplodia palmarum* (Cooke) Petr. & Syd. and observed growth inhibition ranging from 2.77 to 85.71%. Wilt has been associated with *F. solani* (Bajwa et al. 2003) and threatens the production and post-harvest storage of numerous fruits and vegetables (Meenu and Kaushal 2017), causing large economic losses due to stem rotting and softness in bananas (Prihatna et al. 2018; Pegg et al. 2019), tomato (Ravikumara et al. 2022), pigeon pea, and nearly any plant species. Bhardwaj (2012) evaluated the antifungal efficacy of aqueous extracts from 20 different plants against *F. solani*, the fungus that causes dry rot disease in potatoes. Aznabakieva et al. (2023) diagnosed the disease as root and stem rot of pepper plants caused by *F. solani* by comparing the symptoms on wilted pepper plants with those of *Fusarium* wilt. Furthermore, it has been noted that *F. solani*, which causes dry root rot, can affect the most prevalent citrus rootstocks (Menge and Nemec 1997). In many nations where soybeans are cultivated, *F. solani* damages soybean roots in the early stages of the growing season (Roy et al. 1997) and produces Sudden Death Syndrome (SDS) (Wrather et al. 2010). Plant extracts in disease management are environment-friendly, inexpensive, safer than synthetic pesticides and fungicides, and are currently classified as "green agrochemicals" with their growing use (Karunamoorthi 2012). Himalaya is considered a rich resource of medicinally important plants. Traditionally, the Himalayan flora has been utilized to cure various diseases of bacterial and fungal origin in humans and animals. The antimicrobial property of these medicinal plants could also be used to control the plant pathogenic fungi. Therefore, this study aimed to evaluate the antimycotic potential of five plants (three medicinal plants, one fern and one weed) against *F. solani.*

MATERIALS AND METHODS

Study area

The leaves of five plant species viz., *Berberis lycium* Royle, *Zanthoxylum armatum* DC., *Valeriana jatamansi* Jones, *Ageratina adenophora* (Spreng.) R.M. King & H. Rob. and *Polystichum squarrosum* (D. Don) Fee were collected from Conifer Forest near Himalayan Forest Research Institute, Shimla, Himachal Pradesh, India (N 31° 04.066' E077° 10.493' E, 1898 masl). The leaves of these plants were picked, washed and shade-dried for 2-3 weeks. The dried leaves were ground to powder, stored in airtight containers at 4°C and utilized within two weeks.

Fungal culture

A pure culture of *F. solani* (FRI NTCC-1146) was procured from National Type Culture Collection (NTCC), Forest Protection Division, Forest Research Institute (FRI), Dehradun, Uttarakhand, India.

Preparation of crude extracts

Powdered dried leaves were mixed with sterile distilled water in 1:16 (w/v) and kept in a water bath shaker at 180 rpm for three days. The extract was filtered through Whatman filter No. 1 paper and stored under refrigeration (Pandey and Tripathi 2014).

Assessment of antifungal activity of crude extracts

Plant extracts were evaluated for their antimycotic efficacy against *F. solani* following the poisoned food technique (Nene and Thapliyal 1993). Potato Dextrose Agar (PDA) was autoclaved for 15 minutes at 15 psi, 121°C and amended with the requisite quantity of crude extracts before pouring to get desired concentrations (10%, 20% and 30%). The crude extracts of selected plant species were passed through syringe filters (pore size: 0.45 µm) to avoid contamination. After the solidification of the media, the 5 mm diameter mycelial disc of the pathogen was cut from the actively growing culture and inoculated. PDA plate without amendment served as control. The radial growth of the pathogen was recorded after 5 days of incubation at 25±1°C, and percent growth inhibition was determined by the following formula:

$$\text{Percent growth inhibition} = \frac{\text{Colony diameter in control} - \text{Colony diameter in treatment}}{\text{Colony diameter in control}} \times 100$$

Preparation of solvent extracts

Dried powdered leaves were mixed separately in water, ethanol and acetone, and then metabolites were extracted for about five hours in a soxhlet extraction unit. After that, extracts were filtered through Whatman No. 1 paper and solvents were evaporated at temperature (40°C) using a rotatory evaporator to get the final volume of 30 mL. The extracts were then stored at 4°C for further use (Sheel et al. 2022).

Assessment of antifungal activity of solvent extracts

Each extract (0.5 mL) was pipetted into a previously weighed watch glass, kept in a hot air oven at 80°C overnight and weighed again to determine the dry content of extracts. Following the poisoned food technique (Nene and Thapliyal 1993), PDA media was mixed with aqueous, ethanol and acetone extracts to make different concentrations, i.e., 500 ppm, 1000 ppm and 1500 ppm and

PDA plates without extracts severed as control. A 5 mm disc of the pathogen was inoculated, and growth was recorded on the fifth day of incubation and compared to determine growth inhibition.

Phytochemical screening of plants extracts

The shade-dried leaves of selected plant species were evaluated for the presence or absence of phytochemical constituents, such as alkaloids, flavonoids, steroids, terpenoids, carbohydrates, amino acids, saponins (Morsy 2014), proteins, phenolics and tannins (Shah et al. 2014).

Test for alkaloids

The presence of alkaloids in the extract was analyzed by Mayer's reagent Test, in which 2 mL of filtrate was taken in a test tube, and 1-2 drops of Mayer's reagent were added. The presence of alkaloids was confirmed by the appearance of a yellow-colored precipitate.

Test for flavonoids

The presence of flavonoids in the extract was detected by a lead acetate test. Two (2) mL of extract and three (3) drops of 10% lead acetate solution were added to a test tube. The presence of flavonoids was indicated by an intense yellow color.

Test for steroids and terpenoids

The Salkowski test was performed to detect the presence of steroids and terpenoids. Five (5) mL of extract was taken, and 2 mL of chloroform was added. After that, 3 mL of concentrated sulphuric acid was added from the sides of the test tube. The presence of steroids was indicated by a cherry red color in the chloroform layer, while the formation of a reddish-brown color at the interface confirms the presence of terpenoids.

Test for carbohydrates

To confirm the presence of carbohydrates in the extract, Fehling's test was performed. An equal volume of Fehling's solution A and B were mixed, and 2 drops of the extract were added and gently heated, a brick-red precipitate of cuprous oxide confirms the presence of both carbohydrates and reducing sugars.

Test for amino acids

A sulphur test was performed to detect the presence of amino acids. Five (5) mL of extract was taken in a test tube, and 2 mL of 40% sodium hydroxide solution was added. This was followed by adding 2 drops of 10% lead acetate solution, and the preparation was gently heated. Brown or black color indicates the presence of cysteine amino acid.

Test for proteins

The Presence of proteins in the extract was analyzed by the biuret test. 2 mL of extract was taken, 1 drop of 2% copper sulphate solution was added, followed by 1 mL ethanol (95%) and potassium hydroxide pellets. The presence of proteins was indicated by the appearance of pink color in an ethanolic solution (Shah et al. 2014).

Test for phenolics and tannins

The presence of phenolics and tannins in the extract was determined by following the ferric chloride Test. Two (2) mL of extract were taken in a test tube, and a few drops of 5% ferric chloride solution were added. The appearance of dark green color confirms the presence of phenolics and tannins (Shah et al. 2014).

Test for saponins

The presence of saponins was confirmed by the Froth Test. The extract powder was blended with 2 mL of distilled water and left to stand for 10 minutes. The presence of saponins was indicated by the appearance of stable froth (Morsy 2014).

Statistical analysis

All the experiments were carried out in triplicates, and data was analyzed by Analysis of Variance (ANOVA) using Blue Sky Statistic software to determine significant differences. Post-hoc test was applied at the level of $p<0.05$.

RESULTS AND DISCUSSION

Morphology of *Fusarium solani*

On PDA, the colony of *F. solani* appeared as fluffy or cottony with abundant aerial mycelium, whitish-yellow to pale pink-reddish color. The average colony diameter on the fifth day of incubation was 6.9±1.07 cm. Hyphae were septate, branched, and hyaline, 2.32-3.87 µm in diameter, and produced conidia in 3-6 days (Figure 1).

In vitro antimycotic activity of crude extracts

The crude extracts of selected five botanicals inhibited the growth of *F. solani* in the range of 3.32% to 51.58%. The inhibitory effect shown by the plant extracts was found to be dose-dependent and maximum inhibition was observed at 30% concentration. At 30% concentration of crude extracts, maximum growth inhibition was exhibited by *B. lycium* (51.58%), followed by *Z. armatum* (21.94%), *V. jatamansi* (21.45%), *A. adenophora* (11.21%) and minimum by *P. squarrosum* (10.12%) (Figure 2). The inhibitory effect of *A. adenophora* and *P. squarrosum* extracts on *F. Solani* was insignificant at $p<0.05$ level. In the case of *Z. armatum* and *V. jatamansi*, substantial inhibition of fungal growth was observed at 10 and 20% concentration, but statistically significant inhibition was only noticed at 30% concentration. Nevertheless, of the tested plant species, the highest antimycotic activity against *F. solani* was observed in the crude extract of *B. lycium*, followed by *V. jatamansi* and *Z. armatum*. Inhibition of fungal growth by 10.7%, 50% and 53.57%, respectively, was observed at 10, 20 and 30% concentrations of *B. lycium* compared to control. In a similar study, Raturi et al. (2014) recorded growth inhibition of *F. oxysporum* by ethanol extract of *Z. armatum* from 10.88% to 17.55%. Likewise, Guleria et al. (2013) evaluated the efficacy of essential oil extracted from the leaves of *Z. armatum*, which exhibited extraordinary

antifungal activities against *Alternaria alternata* (Fr.) Keissl. (35.6±1.49%), *A. brassicae* (Berk.) Sacc. (14.5±0.36%), *C. lunata* (42.0±1.63%), respectively. Similarly, Joseph et al. (2008) evaluated antifungal efficacy of various plant extracts viz., *Azadirachta indica* A. Juss., *Artemisia annua* L., *Eucalyptus globulus* Labill., *Ocimum sanctum* L. and *Rheum emodi* Wall. at different concentrations (5, 10, 15 and 20%) against *F. solani* and observed maximum growth inhibition by the leaf extract of *A. indica*, followed by *R. emodi*, *E. globulus*, *A. annua* and *O. sanctum*.

In vitro antimycotic activity of solvent extracts

Of the tested plant species, *B. lycium* was further selected to evaluate the efficacy of selected solvents for extracting antimycotic compounds from leaf samples. This was due to the significantly higher growth inhibitory potential associated with *B. lycium*.

The growth of *F. solani* was inhibited in the range of 2.35-73.17% by all solvent extracts at all the tested concentrations. Maximum growth inhibition was recorded by the aqueous extract of *B. lycium* at 1500 ppm concentration (37.03%), followed by 1000 ppm (23.54%) and minimum inhibition at 500 ppm (21.97%) (Figure 3 and Table 1). Likewise, the maximum growth inhibition by ethanol extract of *B. lycium* was also recorded at 1500 ppm concentration (73.17%), followed by 1000 ppm (18.00%) and minimum inhibition was recorded at 500 ppm (2.35%). Furthermore, acetone extract showed 18.82% growth inhibition at 1500 ppm concentration, but the colony diameter reduction was insignificant compared to the control. However, the highest statistically significant inhibition was observed only in 1500 ppm ethanol extract, followed by 1500 ppm aqueous extract.

Ethanol has been used to extract metabolites like phenolic derivatives and several antioxidant compounds from plants (Hikmawanti et al. 2021). Abu-Taleb et al. (2011) used ethanol as a solvent to extract metabolites of *Alhagi maurorum* Medik., *Calotropis procera* (Aiton) Aiton fil. and *Datura innoxia* Mill. And recorded 35-85% growth inhibition of *F. solani*. Shukla and Dwivedi (2012) evaluated the antimicrobial efficacy of ethanolic extracts of four plants against *F. udum* and *F. oxysporum*. Their results revealed that at a 15% concentration of extract, maximum growth inhibition of *F. udum* was exhibited by turmeric (89.2%), followed by garlic (88.26%) and black pepper (82.22%) and at the same concentration. Garlic, turmeric and black pepper reduced the growth of *F. oxysporum* up to 94.63%, 87.96% and 77.74%. Respectively. Hussain et al. (2011) investigated the antibacterial activity of aqueous, petroleum ether, and ethanolic extracts of the root of *B. lycium* against *Candida albicans*. The results revealed that ethanolic extract exhibited maximum growth inhibition (27.00±0.65), followed by aqueous extract (27.00±0.31), whereas petroleum ether extract had no significant effect against *C. albicans*.

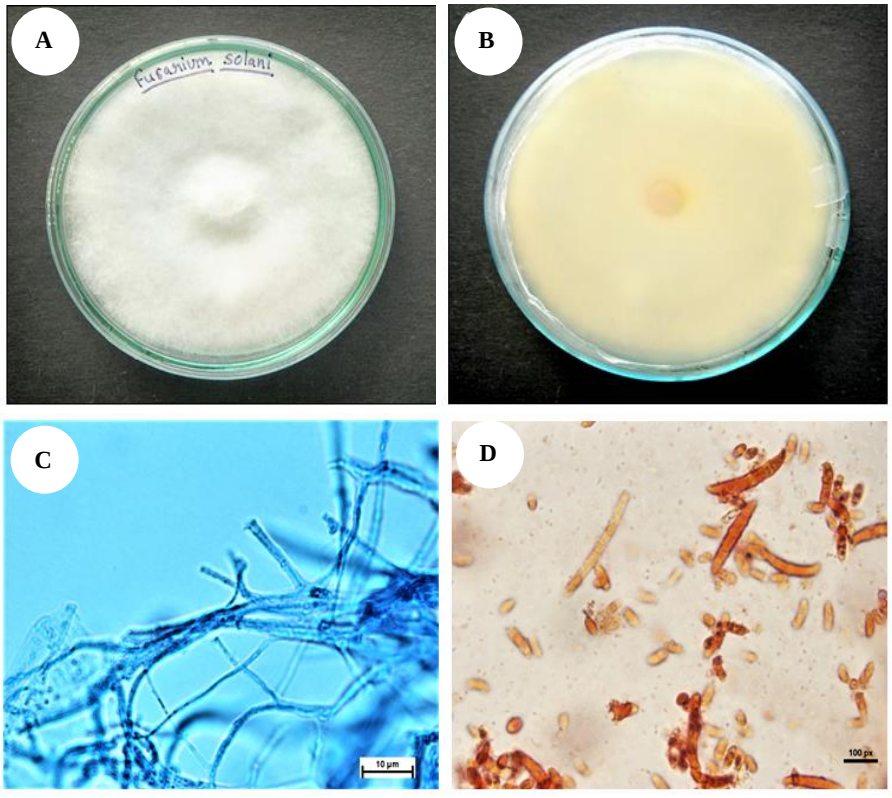

Figure 1. A. Top view of five days old culture of *F. solani*, B. Reverse view of *F. solani*, C. Hyphae of *F. solani* (at scale 10 µm), D. Macro and micro conidia of *F. solani* (at scale 10 µm)

Table 1. Growth inhibition of *F. solani* by the extracts of *B. lycium* at different concentrations

Type of extracts	Growth inhibition (%) of *F. solani* at different concentrations of extracts		
	500 ppm	1000 ppm	1500ppm
Aqueous	21.97±3.27[a]	23.54±3.87[a]	37.03±13.42[a]
Ethanolic	2.35±1.54[a]	18.00±9.60[a]	73.17±8.08[b]
Acetone	4.31±1.33[a]	10.71±2.85[a]	18.82±4.56[a]

Note: At α = 0.01 and 0.001 (Post Hoc Tukey's HSD), the mean sharing common alphabet in a row are not significantly different for each other

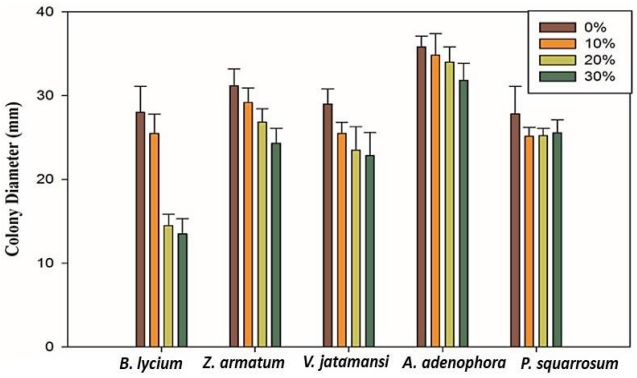

Figure 2. Colony diameter of *F. solani* at 0 (control), 10, 20 and 30% concentration of crude extracts of selected plant species

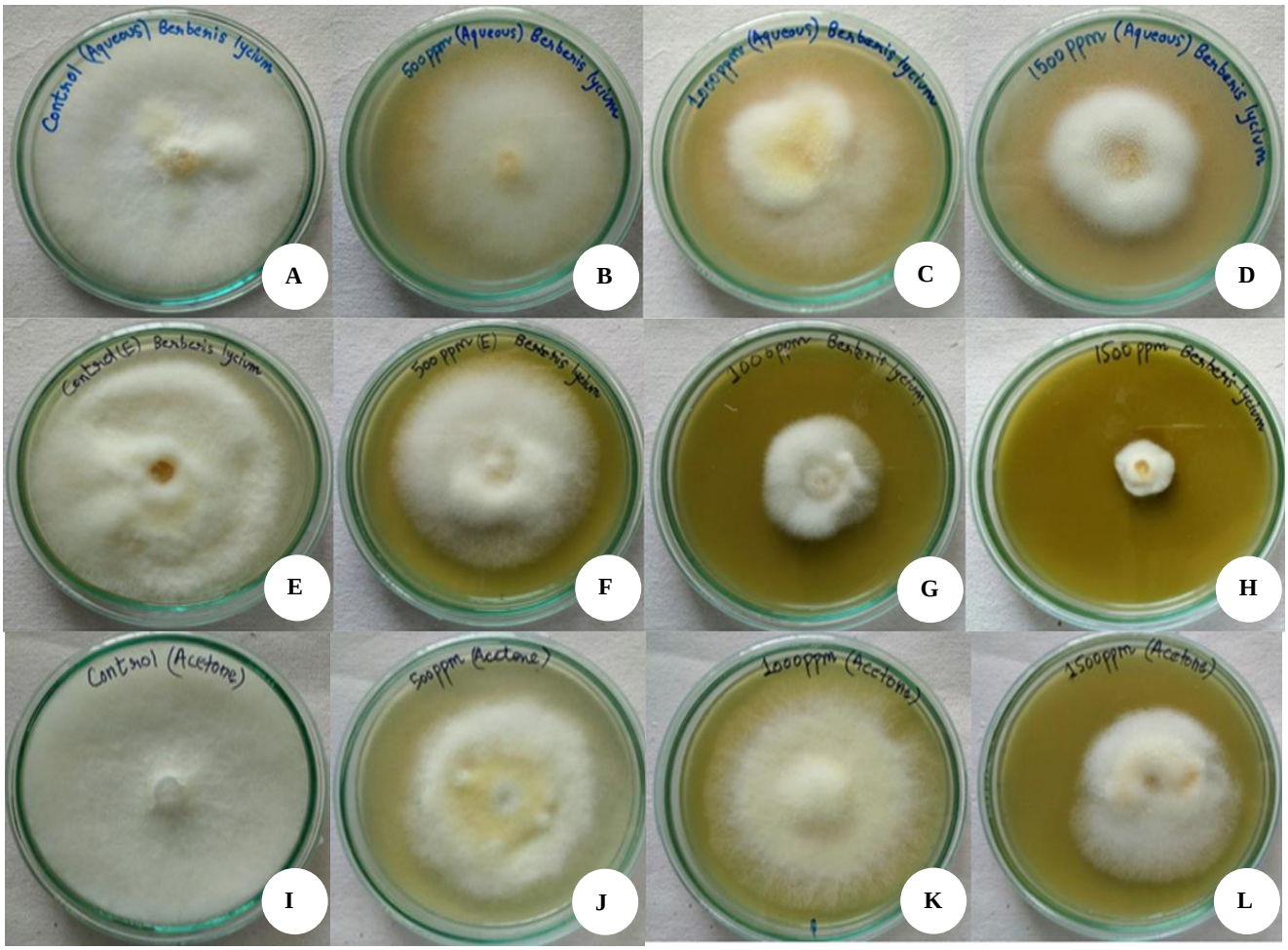

Figure 3. A. Control of *F. solani* in *B. lycium* (Aqueous extract), B-D. Growth inhibition of *F. solani* by aqueous extracts of *B. lycium* at different concentrations, E. Control of *F. solani* in *B. lycium* (Ethanol extract), F-H. Growth inhibition of *F. solani* by ethanol extracts of *B. lycium* at different concentrations, I. Control of *F. solani* in *B. lycium* (Acetone extract), J-L. Growth inhibition of *F. solani* acetone extracts of *B. lycium* at different concentrations

Phytochemical screening

Many phytopathogens are believed to be inhibited by phytochemicals belonging to different groups, such as alkaloids, flavonoids, terpenoids, steroids, phenolics and tannins. The need for eco-friendly and cost-effective means of controlling plant diseases was the driving force behind assessing the antifungal activity of native plant species. In the present study, crude extracts of five plant species were screened against the plant pathogenic fungus *F. solani,* and *B. lycium* extract was found to be relatively more effective in inhibiting fungal growth. *B. lycium* is a shrubby species belonging to the Berberidaceae family that has traditionally been used to cure various ailments in the Indian Himalayan region. It is used to cure jaundice, diabetes, internal wounds, rheumatism, diarrhea, stomachache, eye infections and fractured bones (Ali et al. 2015).

Extracts of this species effectively limit microbial growth, including *Bacillus cereus, Bacillus subtilis, Micrococcus luteum, Escherichia coli, Enterobactor aerogenus, Klebsiella pneumonia, Pseudomonas aeruginosa, Proteus mirabilis, Streptococcus pneumonia, Staphylococcus aureus, and Salmonella typhimurium, C. albicans etc.* (Malik et al. 2017). Inhibitory effect of fruit and root extracts of *Berberis orthobotrys* Bien. ex Aitch. Against *F. solani* has been documented by Dad et al. (2017).

Selecting a suitable solvent is critical for extracting bioactive components from plants with the desired activity. The chemical character (polarity) of the intended bioactive molecule heavily influences solvent selection. Based on the polarity, three major solvent systems, i.e., polar, intermediate polar and nonpolar solvents, are generally used to extract the plant metabolites (Abubakar and Haque 2020). In earlier studies, bioactivity activity against pathogenic microorganisms was reported in extracts of *B. lycium* using polar solvents like water and alcohol (Malik et al. 2017). Therefore, this study observed maximum growth inhibitory activity with ethanol. Among most organic solvents, ethanol is considered safer and widely used, either in pure form or combined with water for extraction.

The present study evaluated the presence of phytochemicals like alkaloids, flavonoids, steroids, terpenoids, carbohydrates, reducing sugars, phenolics, tannins, amino acids, and proteins in the crude extract of *B. lycium.* All tested phytochemical groups were present in the extracts of five plants, except for protein, which was present only in the extract of *V. jatamansi* (Table 2).

Plants harbor a variety of phytochemicals, which play an important role in defense and stress management. Phytochemicals are categorized as primary or secondary metabolites based on their involvement in plant metabolism (Wadood et al. 2013). Pathogenic fungi growth is inhibited by a variety of secondary metabolites derived from plants and other natural sources (Elgharbawy et al. 2020). Based on their chemical structures, these phytochemicals belong to different groups such as terpenes, phenolics, polyketides, and alkaloids.

Table 2. Phytochemical screening of selected plant species

Phytochemicals	Presence or absence of phytochemicals in selected botanicals				
	B. lycium	***Z. armatum***	***V. jatamansi***	***A. adenophora***	***P. squarrosum***
Alkaloids	+	+	+	+	+
Phenolics and tannins	+	+	+	+	+
Flavonoids	+	+	+	+	+
Steroids and terpenoids	+	+	+	+	+
Reducing sugars	+	+	+	+	+
Saponins	+	+	+	+	+
Amino acids	+	+	+	+	+
Proteins	-	-	+	-	-

Several phenolics, including flavonoids, are generally produced by plants to defense against pathogenic microbes (Cushnie and Lamb 2005). Terpenoids are involved in the breakdown of membranous tissue and the weakening of microorganism's cell walls (Hernandez et al. 2000). Saponins are specialized terpenoids that possess a wide range of bioactivities. Many have anti-herbivore and/or antimicrobial plants use in their defense mechanisms (da Silva Magedans et al. 2021).

In conclusion, the antimycotic activity of crude extracts of 5 plants species was investigated against *F. solani.* Out of five, the highest inhibition activity was observed in the ethanolic extract of *B. lycium*. Therefore, *B. lycium* could be used to develop potential botanical fungicides to manage virulent and destructive plant pathogenic fungi like *F. solani.* Among the tested solvents, ethanol was found to be most suitable for extracting antifungal phytochemicals from *B. lycium,* and it is essential to isolate and characterize these phytochemicals to utilize their potential in pest management.

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Medicinal syrup for children from the association of significant parts of anti-COVID-19 medicinal plants from the Centre, Cameroon

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Abstract. *Lamy GML, Nnanga LS, Mahama, Ossongo AF, Elomo LB, Tagne RS, Mbo JA, Likeng JLN, Nga EN, Harmsen K. 2023. Medicinal syrup for children from the association of significant parts of anti-COVID-19 medicinal plants from the Centre, Cameroon. Asian J Nat Prod Biochem 21: 58-66.* In Africa, Improved Traditional Medication (ITM) has been promoted since 1970 and classified into four categories. The import-substitution policy under the "Made in Cameroon" label encourages local production. Globally, there is an urgent need for anti-COVID-19 drugs, especially for children. The mechanisms of action of SARS-CoV-2 can be attenuated by combination therapy of the parts of anti-COVID-19 medicinal plants. A plant has several parts whose therapeutic properties generally differ. From 2020 to 2022, the most cited anti-COVID-19 plants in the literature were *Allium sativum* L., *Citrus ×limon* (L.) Burm.fil., *Cymbopogon citratus* (DC.) Stapf, and *Zingiber officinale* Roscoe. Unfortunately, a gap exists in the names of the significant parts of these plants. For example, were the barks, leaves, roots, or flowers used? In addition, the significant association of said plants and the majority of traditional preparation methods are not specified. The objective was to determine the significant parts of 4 anti-COVID plants, their significant association, and the majority traditional method of preparation allowing the pre-formulation of an infant anti-COVID ITM syrup. An ethnomedicinal survey, ANOVA, and the pre-formulation protocol of a medicated syrup were used. Altogether, 180 informants from several ethnic groups participated. The bulb and rhizome parts for *A. sativum* and *Z. officinal* showed a significant difference ($P<0.05$) between plants. The leaves and fruits of *C. citratus* and *C. limon* showed a highly significant difference ($P<0.000$) between plants. The association of four significant plants and infusion, the majority traditional mode of preparation, allowed the pre-formulation of the infant syrup named COVID-Med, an ITM category 2. The bulbs, rhizomes, leaves, and fruits were significant parts of *A. sativum*, *Z. officinale*, *C. citratus*, and *C. limon*.

Keywords: Coronavirus, ethnomedicine survey, improved traditional medication, medicated syrup protocol, traditional extraction method

INTRODUCTION

The import-substitution policy is one of the three fundamental orientations of Cameroon's national development for strategy 2020-2030 (SND30) (MINEPAT 2020). In import-substitution, it is a question of producing locally and promoting the use of local products through the label "Made in Cameroon." In the 1970s and at the very beginning of the 1980s, most African states implemented policies to promote traditional therapies under the impetus of various international organizations (like the World Health Organization, African Organization, African and Malagasy Council for Higher Education). These policies are based on a common premise: the biological effectiveness of traditional treatments; their development as Improved Traditional Medication (ITM). Official recommendations encourage traditional health practitioners to change some practices to achieve rigorous botanical identification, precise dosage, better hygiene, and the development of galenic forms and packaging (Simon and Egrot 2012).

For a long time, there has been a discussion that children cannot get COVID-19. It is now established that children are indeed affected (Zimmermann and Curtis 2020). COVID-19 is returning worldwide following its resurgence in China (Cowling 2023; Dyer 2023; Huizhong and Aniruddha 2023). The clinical manifestations of COVID-19 are multifactorial with comorbidities (Oladele et al. 2020). A combination therapy, such as the association of parts of two or more plants with specific therapeutic actions on SARS-CoV-2, is recommended to combat the mediators of the disease. Recently, in India, it has been

demonstrated that for greater effectiveness, anti-COVID-19 plants must be put together and then transformed into bio-medicines (Pattanayak 2021). However, there are almost no proposals yet to transform the combinations of the parts of several anti-COVID-19 plants into dosage forms such as ITM syrups for children that are easily accessible to them. Between 2020 and 2022, among the most cited anti-COVID-19 plants in the literature were *Allium sativum* L. (Oladele et al. 2020; Alhazmi et al. 2021; Pattanayak 2021; Ugwah-Oguejiofor and Adebisi 2021; Odebunmi et al. 2022), *Citrus ×limon* (L.) Burm.fil. (Pattanayak 2021; Odebunmi et al. 2022), *Cymbopogon citratus* (DC.) Stapf. (Oladele et al. 2020; Ugwah-Oguejiofor and Adebisi 2021; Odebunmi et al. 2022) and *Zingiber officinale* Roscoe (Oladele et al. 2020; Alhazmi et al. 2021; Pattanayak 2021; Ugwah-Oguejiofor and Adebisi 2021; Odebunmi et al. 2022).

Unfortunately, information is lacking on the names of the exact parts (roots, bark, leaves, flowers, and the other parts) of these plants used against the symptoms of this disease. Therefore, to understand these shortcomings, hypotheses can be made. The first hypothesis concerns the objectives targeted by the authors. Perhaps the names of the parts of these plants were not part of the author's intentions. In this case, these authors keep readers in the dark because the latter do not know which specific part(s) of the plants mentioned should be used against the disease.

Consequently, a reader can use a whole plant through ignorance, thus neutralizing the desired therapeutic effect. However, only part of the said plant was needed. The second hypothesis relates to the ignorance of the authors. Perhaps the authors were unaware that not all medicinal plant parts have the same curative, palliative, and preventive therapeutic properties. In this case, there is a risk of adverse effects linked to the choice of part of the plants mentioned instead of another part of the same plants. As a result, a reader may randomly select a part of a plant that is acutely toxic, and the worst ensues.

The objective of this study was to determine the parts significant of the 4 anti-COVID-19 plants *A. sativum*, *C. limon*, *C. citratus*, and *Z. officinale*, the significant association of these plants and the traditional method of preparation allowing the pre-formulation of an infant anti-COVID-19 ITM syrup in Centre Province, Cameroon.

MATERIALS AND METHODS

Study area

The study took place in the Mfoundi District, Centre Province, Cameroon (Figure 1), precisely in four sub-districts (Yaoundé 1, Yaoundé 2, Yaoundé 3, and Yaoundé 4) (Oyono 2015). The climate is punctuated by two dry seasons alternating with two wet seasons, with rainfall of 1600 mm on average per year. The temperature fluctuates between 18°C and 28°C in the wet seasons, 16°C and 31°C in the dry seasons. The vegetation of the Mfoundi district is of the intertropical type with a predominance of the southern humid forest. The soils are mostly red lateritic.

Identification of botanical samples

Botanical samples of plant parts (leaves, rhizomes, pods, fruits, flowers) collected in the study area were identified at the Cameroon National Herbarium (CNH), Yaoundé District. The collected plant specimens were compared with those from the related collections.

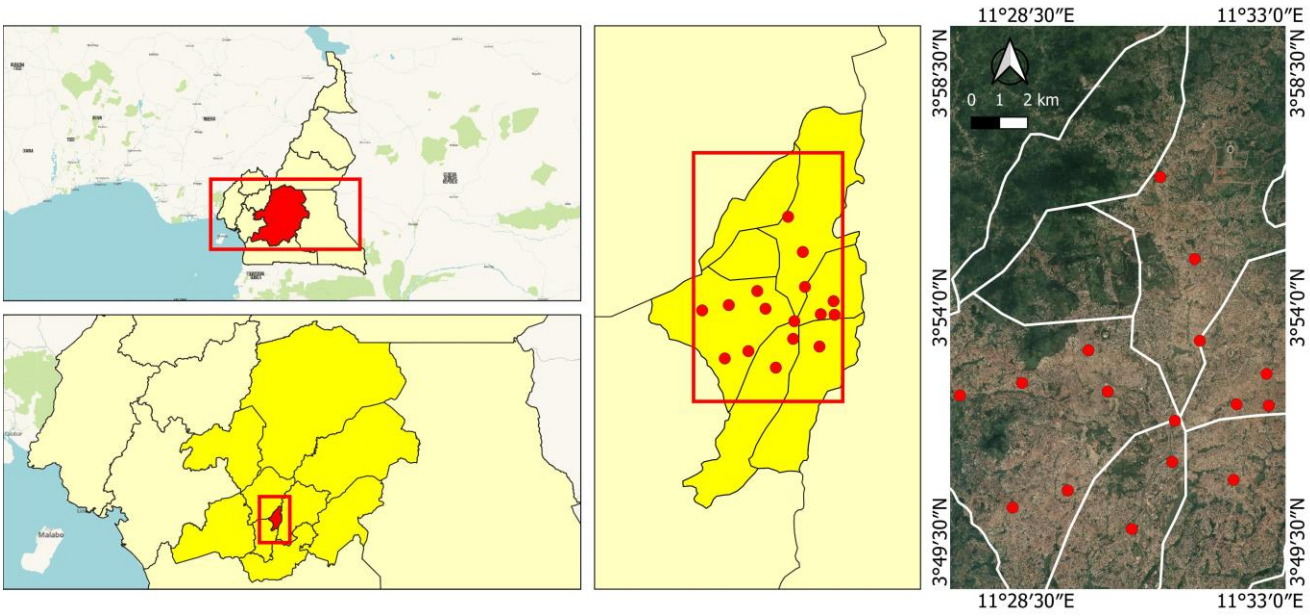

Figure 1. Location of the study area in the Mfoundi District, Centre Province, Cameroon

Ethnomedicine

Data was collected based on the availability of informants. Sampling was carried out on a non-probability (random) basis. All informants voluntarily gave their informed consent either by name or anonymously. Three markets were visited per Sub-district. Fifteen informants were interviewed per market, that is, forty-five per borough. The informants had to name the most used part for each of the four plants *A. sativum*, *C. limon*, *C. citratus*, and *Z. officinale*. Following the recommendations of the Nagoya Protocol on access and benefit-sharing from local knowledge, informants who agreed to participate by name were cited as co-authors (Maxime et al. 2020).

Syrup preparation

It is a question of preparing a medicated syrup for ten bottles of 125 mL each, i.e., totaling 1,250 mL. According to the European Pharmacopoeia, the medicated syrup is prepared in two stages: preparing the simple syrup and incorporating the medicinal and aromatic active ingredients (Dénou et al. 2021). Moreover, for a final 100% medicated syrup, the value of the extract is 4.32%, that of the simple syrup 95.25%, that of the preservative 0.4%, and that of the aroma 0.03%.

Traditional extraction method by infusion

The goal is to obtain an infused extract obtained from a concentrated infusion of 119.5 g of *C. limon* fruit juice; 28.3 g of bulbs of *A. sativum*; 80 g of fresh *Z. officinale* rhizomes then 86.4 g of *C. citratus* leaves for 2 L of water.

Operating mode

Measure 2 L of distilled water using the Beaker. Then, this water is introduced into a stainless steel container. Then, carefully wash some fresh fruits of *C. limon*, cut them in half, squeeze the juice into the Beaker, and weigh 119.5 g. The next step is to clean, wash and weigh 28.3 g of fresh bulbs of *A. sativum* and grind them in a porcelain mortar. The next step is cleaning, washing, and weighing 80 g of fresh *Z. officinale* rhizomes, then grinding them in the mortar. Subsequently, wash and weigh 86.4 g of fresh leaves of *C. citratus*, then keep them. The next step is to boil the 2 L of distilled water in the stainless steel container on the hot plate at 90 to 100°C. Then, stop the plate once the steam has escaped from the stainless steel container, and remove the container from the heating plate. By systematically closing the lid after adding each component, Lift the lid of the container and gradually introduce the *C. limon* juice, the crushed *Z. officinale* rhizomes, and the already crushed *A. sativum* bulbs. Finally, introduce the previously washed *C. citratus* leaves and close the container hermetically. Leave the active substances of the plants in contact with the hot solvent (distilled water) to be released under heat for 10 to 20 minutes. It should be left to cool for about 30 minutes. Filter or sieve the extract using the funnel and cotton, then keep the extract cool in tinted containers or covered with aluminum foil to protect the extract from light. The infused product is put in a tray and brought to evaporation in an oven calibrated at 60°C. After 24 hours, the vegetable matter devoid of water is removed from the oven and kept away from light. Following the European Pharmacopoeia, only 4.32% of all the final infused was taken for the rest, the infused, that is to say, the extract composed of the active substances (researched active principles) of all the plants used without water.

Preparation of simple syrup according to the European Pharmacopoeia

The ingredients are taken for an amount of 100%. Remember that 100% = 100 ÷ 100 = 1 = 1 L (liquid) or 1 g (powder, solid) = 1000 mL or 1000 mg. Hence the following proportions: sucrose (powder): 65% = 65 / 100 = 0.650 g = 650 mg; distilled water (liquid): 35% = 35 / 100 = 0.35 L = 350 mL. Thus, for a quantity of 3.5 L or 3,500 mL, the necessary ingredients will be: sucrose (powder, solid): 650 x 3,500 = 2,275,000 mL = 2,275 L; distilled water (liquid): 350 x 3,500 = 1,225,000 mL = 1,225 L

Operating mode

Preparing 3,500 g of simple syrup was discussed by heating sufficient water. The sucrose was dissolved at 80-85°C and then filtered immediately while hot with a previously heated filter. Then, the whole was homogenized and completed at 3,500 g (Dénou et al. 2021).

Preparation of the syrup composed of several plants according to the European Pharmacopoeia

The proportion of ingredients for 100% = 100 / 100 = 1 L or 1 g. The following proportions are adopted: extract (containing the active principle): 4.32%=4.32/100=0.0432 g; simple syrup: 95.25% = 95.25 / 100 = 0.9525 L; preservative (sodium benzoate): 0.4% = 0.4 / 100 = 0.004 g (powder) and flavorings (vanilla + mint): 0.03% = 0.03 / 100 = 0.0003 g hence vanilla (0.02%) and mint (0.01%).

Preparation of the final syrup composed of several medicinal plants

Since we have ten bottles of 125 mL each, it will be necessary to prepare 125 x 10 = 1250 mL = 1,250 L of syrup composed of several medicinal plants, hence the following proportions: extract: 1250 x 4.32% = 54 mg = 0,054 g; simple syrup: 1250 x 95.25% = 1190,625 mL = 1,190.625 L; preservative (sodium benzoate): 1250 x 0.4% = 5 mg =0,005 g and flavorings (vanilla (0.02 g) + mint (0.01 g)): 1250 x 0.0003 = = 0.375 mg = 0.000375 g

Operating mode

It is necessary to weigh 1190.6 L of simple syrup and then introduce it into the blender. Then, weigh 54 g of extract containing the desired active ingredients then add to the simple syrup. The next step is to mix the extract and the simple syrup. Then, weigh 5 g of preservative (Sodium Benzoate). Next, weigh the aromas, particularly 0.25 g of vanilla and 0.125 g of mint. Then, stir everything until complete dissolution (disappearance of the particles).

Packaging and labeling

It is packaged in tinted bottles with a capacity of 125 mL. The common is to use the white label for readability and a pleasant presentation.

Data processing and analysis

The data was processed using Word and Excel 2013 software. Then, an Analysis of Variance (ANOVA) to compare the different data was performed using the STATGRAPHICS Plus 5.0 software.

RESULTS AND DISCUSSION

Identification of informants

Therefore, 180 informants participated in the study, i.e., 80 women and 100 men (Table 1). Men were in the majority over women.

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Table 2 presents all the anti-COVID-19 medicinal plants cited in the literature. According to the informants, Each plant has a significantly more used part. For *A. sativum*, the bulbs vary from 0.41 ± 0.19 to 1.08 ± 0.19. This variation is significant (P <0.05 since 0.03 <0.05) between said plants; this result reflects that the bulbs represent the most used part of the plant, according to the informants. Concerning *C. limon*, the variation of the fruit means ranges from 0.5 ± 0.17 to 2.08±0.17. This variation is highly significant (P <0.05 because 0.000 <0.05) between all the plants; this result reflects that the fruits are the most used part of the plant, according to the informants. For *C. citratus*, mean leaf responses ranged from 0.75±0.16 to 2.08±0.16. According to the informants, there is a highly significant difference (P <0.05 because 0.000 <0.05) between all these medicinal plants; this result reflects that the leaves are the most used part of the plant. Regarding *Z. officinale*, a variation of 0.5±0.22 to 1.33±0.22 is observed at the level of the rhizomes. According to the informants, this variation is significant (P <0.05 because 0.02 <0.05) between said plants; this result reflects that the rhizomes represent the most used part of the plant.

The analysis of Table 3 shows that for all the associations of medicinal plants, a variation of garlic (*A. sativum*) ranges from 0.5 (association of 2 plants) to 1.08 (association of 4 plants) with an average of 0.49±0.19. This variation is significant (P <0.05 because 0.04 <0.05) between said associations. This result reflects that garlic is used in combinations of 2 and 3 medicinal plants according to the same number of informants, but this number differs from the higher number who think that garlic is used in the combination of 4 plants. Regarding lemon (*C. limon*), the average is 0.63±0.17. The maximum is found in the association of 4 medicinal plants (0.9±0.17), and the minimum in 2 medicinal plants (0.5±0.17). There is no significant difference (P >0.05 because 0.14 >0.05) between the different combinations of medicinal plants in using lemon.

Table 1. Names of sub-district, gender, and total informants

Sub-districts	Women	Men	Total
Yaoundé 1	21	24	45
Yaoundé 2	19	26	45
Yaoundé 3	18	27	45
Yaoundé 4	22	23	45
Total	80	100	180

Table 2. Anti-COVID-19 medicinal plants from the literature, averages, p-values , and most used parts cited by informants

Anti-COVID-19 Medicinal Plants from the Literature	Most Used Parts Cited by informants			
	Bulbs	Fruits	Leaves	Rhizomes
Allium sativum L.	1.08±0.19c	0.5±0.17a	0.75±0.16a	0.5±0.22a
Citrus ×limon (L.) Burm.fil.	0.5±0.19ab	2.08±0.17b	0.83±0.16a	0.5±0.22a
Cymbopogon citratus (DC.) Stapf	1.0±0.19bc	0.91±0.17a	2.08±0.16b	1.0±0.22ab
Zingiber officinale Roscoe	0.41±0.19a	0.5±0.17a	0.91±0.16a	1.33±0.22b
Means	0.75±0.18	0.99±0.17	1.33±0.16	0.83±0.22
P-values	0.03	0.000	0.000	0.02

Note: At the 5% significance level (0.05), the means followed by the same letter on the vertical are statically identical

Table 3. Association of medicinal plants, averages, p-values , and medicinal plants most cited by informants

Association of Medicinal Plants	Informants Most Cite Medicinal Plants			
	Garlic	Lemon	Lemongrass	Ginger
Association of 2 plants	0.5±0.19a	0.5±0.17a	0.83±0.16a	0.5±0.19a
Association of 3 plants	0.41±0.19a	0.5±0.17a	0.75±0.16a	0.5±0.19a
Association of 4 plants	1.08±0.19b	0.9±0.17a	2.08±0.16b	1.0±0.19a
Means	0.49±0.19	0.63±0.17	1.22±0.16	0.67±0.19
P-values	0.04	0.14	0.00	0.11

Note: At the 5% significance level (0.05), the means followed by the same letter on the vertical are statically identical

This result reflects that lemon is used in all associations of medicinal plants according to the same number of informants. Concerning lemongrass (*C. citratus*), the variation between the associations of medicinal plants ranges from 0.83±0.16 to 2.08±0.16. A highly significant difference (P <0.05 because 0.000 <0.05) exists between all these plant associations. This result reflects that lemongrass is used in combinations of 2 and 3 medicinal plants according to the same number of informants. However, this number differs from the high number that thinks lemongrass is used in the four-plant combination. Regarding ginger (*Z. officinale*), a variation of 0.5±0.19 to 1.0±0.19 is observed. There is no significant difference (P >0.05 because 0.11 >0.05) between the different combinations of medicinal plants. These results reflect that ginger is used in all associations of medicinal plants according to the same number of informants.

All parts of *A. sativum*, *C. limon*, *C. citratus*, and *Z. officinale* plants contain secondary metabolites (Table 4). Moreover, each of these parts reveals particular therapeutic properties and medicinal uses. These results reflect that the consumption of the combination of these plants is complementary.

Figure 2 shows that infusion is the majority traditional preparation method regardless of the district. This result reflects that concerning the objective of this study, the infusion is the traditional method of preparation for the pre-formulation of the traditional improved anti-infant COVID-19 medicinal syrup.

Results of the preparation of the medicinal compound syrup

Figure 3 shows the labeled vials containing the ITM "COVID-Med" syrup. The presentation of the label reveals: name of the syrup (COVID-Med); extract containing the active ingredient (4.32%); simple syrup (95.25%); preservative (0.4%); aroma (0.03%); place of manufacture (Galenics and Pharmaceutical Legislation Laboratory); date of manufacture (2022.09.16); expiration date (2026.09.15) and lot number (001). These results show that this syrup is derived, among other things, from the combination of the significant parts (bulbs, fruits, leaves, and rhizomes) of the anti-COVID-19 medicinal plants cited in the literature (*A. sativum*, *C. limon*, *C. citratus*, and *Z. officinale*).

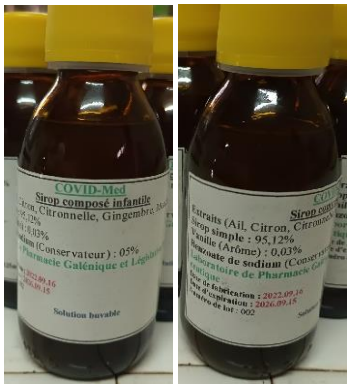

Figure 3. Illustration of labeled bottles containing the syrup named "COVID-Med" Picture. LAMY, October 2022

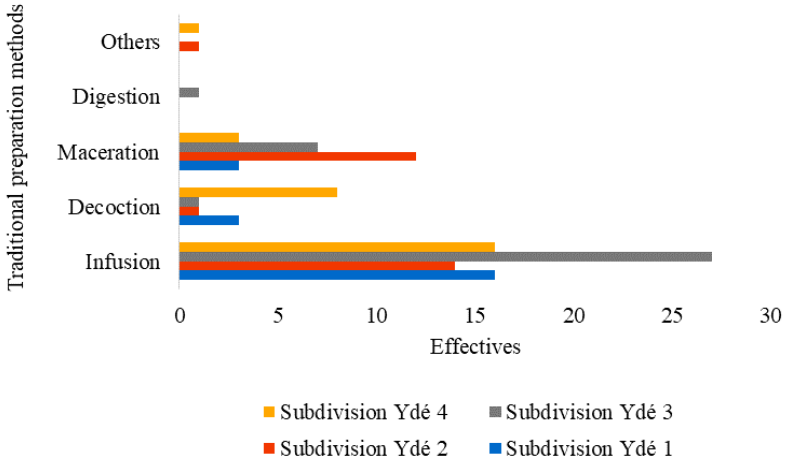

Figure 2. Traditional preparation methods according to numbers

Table 4. Anti-COVID-19 medicinal plants cited in the literature, nomenclature, and characteristics of these plants

Plant most used in association	Nomenclature				Plants characteristics		
	Scientifical name/ family	Common names (French)	Local names	Herbarium vouchers numbers	Secondary metabolites	Therapeutic properties	Medicinal uses
Plant 1	*Allium sativum* (L.) Amaryllidaceae	*Ail*	*Anyon (Bulu)*	44810/NHC	Pods: • Carbohydrates • Proteins • Fibers • Prostaglandins (phenol acid, phytosteroids, polyphenols, flavonoids (apigenin, myricetin), etc.) • Vitamines (B1, B2, C, A, etc.) • Mineral (potassium, sulfur, phosphorus, calcium, magnesium, sodium, chlorine, etc.) • Trace elements (iron, zinc, manganese, boron, copper, nickel, molybdenum, iodine, selenium) • Essential oil (diallyl disulfides, allicin (antibiotic), alliin, alliinase, inulin) (Naganawa et al. 1996; Hussain et al. 2021)	Antiviral (influenza B virus) Hypo-allergenic Digestion Anticoagulant Hypolipidemic Antihypertensive Chelator Antioxidant (Fleischauer and Arab 2001; Ammarellou et al. 2022)	Flu, cold, etc.
Plant 2	*Citrus ×limon* (L.) Burm.fil. / Rutaceae	*Citron*	*Ngoumbang (Bulu)* *Ngnofiang (Ngoumba)* *Lemoune (Fulfulde)*	0565/SRF/CAM	Fruit: • Trace elements: calcium; iron; magnesium; phosphorus; potassium; copper; sodium; zinc • Vitamines: C, B1, B2, B5, B6, A, E, furanocoumarins (psoralen, bergapten) • Fatty acids: saturated; monounsaturated; polyunsaturates (Nicolosi et al. 2000; Ramón-Laca 2003; Ollitrault et al. 2020)	Antioxidant Antiinflammatory Diuretic Antiseptic Healing (Carvalho et al. 2005; Klein 2014)	Flu, cold, etc.
Plant 3	*Cymbopogon citratus* (DC.) Stapf / Poaceae	*Citronnelle* or *Verveine des Indes*	*Ossang (Bulu)* *Maguianga (Ngoumba)* *Fiber grass (Pidjin)* *Haco tie (Fulfulde)*	48536/SFR/CAM	Leaves: • Tannins (catechic tannins and phlobotanin), • Phenolic acids (derivates of caffeic and p-coumaric acid), • Glycosides • Flavonoids (apigenin and derivatives of luteolin), • Saponin (Kiani et al. 2022)	Antimicrobial, Hypoglycemic, Hypolipidemic, Hypotensive, Cardioprotective, Anti-inflammatory, Analgesic, Antitumor, Insecticide (Shah et al. 2011)	Cough, headache, fever, etc.
Plant 4	*Zingiber officinale* Roscoe / Zingiberaceae	*Gingembre*	*Cocobianco (Bulu) Ndjindja (Ngoumba)*	43125/NHC	Rhizomes: starch; oleoresin; essential oil; proteins; vitamins; mineral; gingerols; zingerone; esters (Emmanuel et al. 2021)	Anti-inflammatory (Mustafa and Srivastava 1990)	Flu, fever, headache, dry cough, sore throat, etc.

Discussion

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The results of Tables 1, 2, 3, and 4 could be explained as follows; the male informants could have more knowledge on the most used parts (rhizomes of ginger, fruits of lemon, bulbs of garlic, and leaves of lemongrass) of 4 anti-COVID-19 medicinal plants (*C. limon*, *A. sativum*, *Z. officinale,* and *C. citratus*). As well as on the majority association, which is that of 4 plants. And on the infusion, the majority of traditional methods of preparation. This result could be explained by the fact that infusion has more advantages than other traditional preparation methods (Oborah 2022). It is a method in which the active ingredient sought is quickly released into the solvent (a few minutes). It is less expensive regarding materials to use, with instructions accessible to all levels of education. This method requires using easily accessible plant parts such as the leaves (Devgun et al. 2010). In short, the infusion does not contribute to the disappearance of medicinal plants. This considers the mainly used aerial parts, which can regenerate naturally. According to Titanji (2021), all of these results could be explained by the positive contribution of the parts of these medicinal plants to the health of the populations in the study area. Indeed, the parts of all anti-COVID-19 plants used in combination contain secondary metabolites (alkaloids, flavonoids, etc.). These secondary metabolites have therapeutic properties (antimicrobial, anti-inflammatory, etc.), making it possible to treat many illnesses (cough, flu, etc.). Consumption of the combination of parts of these plants would allow the body to fight against foreign bodies such as bacteria, microbes, and viruses while strengthening the immune system.

These plant ingredients would therefore have a protective and fortifying role (Rafieian-Kopaei 2012; Sofowora et al. 2013). Many researchers have studied medicinal plants' protective and fortifying or invigorating role. According to Manvitha and Bidya (2014), the leaves of *C. citratus* have a toning role in the body. Rafique et al. (2020) claim that *C. limon* fruits have a protective role, while Emmanuel et al. (2021) report that *Z. officinale* rhizomes have a protective role. Ammarellou et al. (2022) indicate that *A. sativum* bulbs are invigorating. Thus, combining these plants in the form of syrup by children may benefit them for several reasons. From the fruits of *C. limon*, the human body will benefit from trace elements, vitamins, and fatty acids; the leaves of *C. citratus* will provide humans with tannins, phenolic acids, glycosides, flavonoids, and saponins. The rhizomes of *Z. officinale* will enrich their organism with starch, oleoresin, essential oil, proteins, vitamins, minerals, gingerols, zingerone, and esters. Finally, the *A. sativum* pods will provide them with carbohydrates, proteins, fibers, prostaglandins, vitamins, minerals, trace elements, and essential oils.

The results in Table 4 could also be explained by in-depth studies on each of the different parts of these plants. Botany has contributed to describing and identifying each plant and its different parts (Botineau 2010). Phytochemistry has, for example, made it possible to determine the chemical compounds (alkaloids, flavonoids, etc.) present in the extracts of the parts of said plants after laboratory studies (Obame 2009). The behavior of the bacterial, microbial, or viral strains in the face of the extracts of the parts of the said plants made it possible to conclude.

Thus, microbiology or pharmacology has made it possible to highlight the antibacterial, antimicrobial, or antiviral activities of the parts of said plants (Loufoua et al. 2015). These in-depth studies justify the uses in traditional medicine (against the flu, colds, coughs, headaches, and fever) reported by informants in the field. Therefore, it could also heal the symptoms of COVID-19, including fever, dry cough, general fatigue, headache, and sore throat (Li et al. 2020)

Preparing the medicated syrup

Infusion has been a significant traditional method of preparation. This result can be explained by the advantages offered by this method. According to Stéphane et al. (2021), the infusion requires light parts such as the leaves. It is a method of extracting active substances that lasts 10 to 20 minutes. It is simple and very quick to make. However, it has the disadvantages of losing volatile chemicals, degradation of fragile chemicals, etc. The procedure used in this study was to reduce the loss of volatile chemicals from *C. citratus* leaves. Indeed, instead of transferring the boiled water into the container containing the plant matter, which leads to a loss of compounds, the reverse was done. After opening the lid, these plant materials were poured into the boiling water container. *C. citratus* leaves were added last, then the container was closed until cool. This technique reduces the losses revealed by the drawbacks of the process. The choice of the extraction method for the active substances contained in the plants is made according to the duration of extraction, the operating mode, the advantages, the disadvantages, the part of the plants to be used, etc. For example, maceration is another traditional extraction method whose advantages are: simple and less expensive, limiting the release of volatile chemical substances into the air, etc. Its disadvantages are bacterial proliferation, long preparation time, etc. It requires the use of the hard parts of the plant, such as bark, roots, etc. (Rasul 2018; Abubakar and Haque 2020). The "COVID-Med" ITM syrup obtained is a category 2 ITM (MINSANTE 2020) with numerous characteristics. It must be prepared in advance. It must be packaged with a batch number. The raw materials used in its composition are well-known to the population. Its production is made according to methods that guarantee its stability and standardization. If deemed necessary by the competent authority, its safety and efficacy are guaranteed by ethnomedical evidence from long-use experience or open clinical trials. The active ingredients that compose it are raw materials. The main chemical groups of the raw materials are known; stability tests determine their shelf life. For developing countries, ITMs constitute an alternative of primary importance to health expenditure devoted to pharmaceutical specialties imported from industrialized countries (Guedje et al. 2012).

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Effect of *Lactuca sativa* supplemented diet on Poloxamer 407 induced hyperlipidemic albino rats (*Rattus norvegicus*)

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Abstract. *Ayo VI, Adondua MA, Morayo AE, Ekele JU, Amilo D, Ochuele DA, Ayantse LM, Barrah C, Abdulsalam IO, Eya SB, Iheanacho CC, Tibile ST, Mohammed RI, Barde CE. 2023. Effect of* Lactuca sativa *supplemented diet on Poloxamer 407 induced hyperlipidemic albino rats (*Rattus norvegicus*). Asian J Nat Prod Biochem 21: 67-78.* Cardiovascular diseases (CVDs) have primarily contributed to the global disease burden. They represent the leading cause of mortality and healthcare expenditures in developed and third-world nations, responsible for approximately 30% of global deaths and 10% of global diseases annually. This study investigated the effects of a *Lactuca sativa* L. (lettuce) supplemented diet on Poloxamer 407-induced hyperlipidemic albino rats. Twenty-four (24) rats were grouped into six groups of treatments, i.e., four rats in each treatment. Treatments applied in this study were: control treatment (feed and water only), a P-407 induced without Atorvastatin, a P-407 induced treated with Atorvastatin, and P-407 induced with 10%, 30%, and 50% *L. sativa* supplemented diet. P407 was administered intraperitoneally at 1000 mg/kg body weight. Body weight was measured every three days for 14 days. Blood sample collection was carried out for the analysis of lipid profiles (High-Density Lipoprotein Cholesterol (HDL-C), Low-Density Lipoprotein Cholesterol (LDL-C), Very Low-Density Lipoprotein (VLDL), Triacylglycerides (TAG), and Total Cholesterol (TC)) and liver function parameters (ALP, ALT, AST, GGT, TP, ALB and GLB). The liver and brain tissues were analyzed for lipid peroxidation levels. Results showed that induction of P407 resulted in a higher body weight gain ($p<0.05$) compared to other treatments. The treatment groups other than the P-407 treatment showed a significant decrease in Total Cholesterol (TC), triacylglycerides (TAG), and LDL cholesterol levels and a significant increase in HDL cholesterol. The TC, TAG, LDL-C, and HDL-C levels in treating P-407 with a 50% *L. sativa*-supplemented diet did not differ ($P>0.05$) compared to the control treatment. The atherogenic risk prediction indices indicated a decreased risk in the treated groups with Atorvastatin or *L. sativa*-supplemented diet. Furthermore, liver function parameters were better in the treatment groups with Atorvastatin or *L. sativa*-supplemented diet, including decreased liver function parameters and increased total protein, albumin, and globulin levels. The *L. sativa*-supplemented diet also exhibited anti-lipid peroxidation activity, as indicated by reduced malondialdehyde (MDA) levels. In conclusion, the *L. sativa*-supplemented diet had hypolipidemic effects, anti-lipid peroxidation activity, and hepatoprotective effects, suggesting its potential as an antihyperlipidemic agent.

Keywords: Albino rats, hyperlipidemia, *Lactuca sativa*, Poloxamer 407, supplemented diet

INTRODUCTION

Cardiovascular diseases (CVDs) have emerged as the primary contributor to the global disease burden (Shukr et al. 2019). CVDs are the leading cause of mortality and increasing healthcare expenditures in developed and third-world nations, responsible for approximately 30% of global deaths and 10% of global diseases annually (Bhatnagar et al. 2008). It is projected over 24 million people will be affected by cardiovascular ailments by 2030 (Wang et al. 2015). Hyperlipidemia, characterized by elevated levels of plasma lipids such as Total Cholesterol (TC) and triglycerides (TAG), is a well-known determinant of cardiovascular disease (CVD) (Zhang et al. 2014; Ayo et al. 2023a). Hyperlipidemia is typically defined as increased cholesterol or triglyceride-carrying lipoproteins in the blood above a defined standard limit (Avogaro and Cazzolato 1975). Hyperlipidemia, or dyslipidemia, can lead

to severe complications associated with atherosclerosis, including cardiovascular disease, cerebrovascular disease, peripheral vascular disease, and strokes (Shattat 2015). The accumulation of lipids, particularly cholesterol, in the arterial walls causes narrowing and reduced blood flow, leading to atherosclerosis. The risk of morbidity and mortality increases if hyperlipidemia occurs with other prevalent conditions such as hypertension, diabetes mellitus, and renal disorders (Amanda et al. 2013). Various factors contribute to the development of hyperlipidemia, including genetic abnormalities, type II diabetes mellitus, obesity, thyroid dysfunction, alcoholism, hormonal therapy, renal diseases, jaundice, lipoprotein lipase mutations, and certain medications (Santamarina-Fojo et al. 2004).

Lactuca sativa L., commonly known as lettuce, is the *Asteraceae* plant family and native to the Mediterranean region. It is known globally as a vegetable crop, consumed as fresh in salads or occasionally cooked (Lebeda et al. 2007). It has a low-calorie, low-fat, and low-sodium, making it a popular choice for salads. It is also a good source of dietary fiber, folate, vitamin C, and essential minerals, such as iron (Kim et al. 2016). The nutritional composition of *L. sativa* includes moisture (93.4g), protein (2.1g), fat (0.3g), minerals (1.2g), fiber (0.5g), carbohydrates (2.5g), calcium (310mg), phosphorus (80mg), iron (66mg), vitamin A (1650 IU), thiamine (0.09mg), riboflavin (0.13mg), and vitamin C (10mg) (Gopalan and Balaraman 1966). The bioactive compounds in *L. sativa*, such as phenolic compounds, are attributed to their antioxidative activity and various pharmacological effects, such as cardio-protective, antihyperlipidemic, anti-cancer, and antidiabetic activities (Hedges and Lister 2005). Several epidemiological studies have provided evidence that the consumption of vegetables, particularly green leafy vegetables like *L. sativa* (lettuce), is linked to the treatment of cardiovascular diseases (CVDs) (Nicolle et al. 2004a). A study on rats by Nicolle et al. (2004b) showed that a diet containing 20% lettuce had a cardio-protective effect by improving cholesterol metabolism and enhancing plasma antioxidant capacity. The cholesterol-lowering effect of *L. sativa* can be attributed to its fiber content. Soluble fibers such as pectin in lettuce affect lipid metabolism and reduce dietary cholesterol absorption in animals and humans (Nishimura et al. 2000; Abu et al. 2023). The mechanisms underlying the inhibition of cholesterol absorption are the disruption of micelle formation and the slowing down of cholesterol transfer through the unstirred layer (Stedronsky 1994). Furthermore, polyphenols present in lettuce have been proven to effectively inhibit the oxidation of Low-Density Lipoprotein (LDL), a significant contributor to atherosclerosis and hyperlipidemia (Aviram et al. 2000; Yakubu et al. 2019; Ekele 2023).

The use of plants, including vegetables such as *L. sativa* (lettuce), to treat hyperlipidemia is crucial due to their abundance of bioactive and nutritional substances (Ejeh et al. 2022; Edogbanya et al. 2023). Research reports indicate that *L. sativa* used in this study demonstrated minimal or no adverse effects compared to synthetic drugs commonly used to treat hyperlipidemia. The *L. sativa* is readily available in Nigeria, which benefits cost and accessibility. Therefore, this study aimed to assess the antihyperlipidemic effect of an *L. sativa*-supplemented diet in Albino rats with hyperlipidemia induced by Poloxamer 407.

MATERIALS AND METHODS

Study area

The study was conducted at the Central Research Laboratory, Federal University Wukari, Taraba state, Nigeria, from October 2022 to March 2023.

Sample collection and preparation

The study utilized mature, fresh, healthy *L. sativa* from Jos, Plateau State, Nigeria. The leaves were carefully rinsed with tap water to ensure cleanliness from soil and dust particles. Subsequently, the leaves were air-dried under the sun for 6 days to reach a brittle state, followed by crushing the leaves into fine fragments using a mortar and pestle. The powdered leaves were labeled correctly and stored in dry containers until they were required for further use.

Experimental animals

The rats used in the study were procured from the Animal House located in the Department of the Central Research Laboratory, Federal University Wukari, Taraba State, Nigeria. Their weight ranged from 110g to 145g, over 3 weeks old. They were mixed-sexed (males and females). They were kept in cages with a 12-hour light-dark cycle, following animal standard laboratory protocols approved by the University's Faculty and Ethics Committee (Approval number: 1765BG). The rats were given free access to water and feed throughout the experiment. Feed was obtained from Wukari Central Market, Taraba State, and constituted wheat maize, sugar, and calcium carbonate. Feed was given ad-lib.

Treatment of animal

Twenty-four (24) Albino rats were grouped into six groups. Each group consisted of four animals. The rats were subjected to the following treatment: (i) Treatment 1: Water and feed only. (ii) Treatment 2: Induced by an intraperitoneal injection of P-407 without treatment. (iii) Treatment 3: Induced and treated with Atorvastatin. (iv) Treatment 4: Induced and treated with 90% feed with 10% *L. sativa*. (v) Treatment 5: Induced and treated with 30% feed with 70% *L. sativa*, (vi) Treatment 6: Induced and treated with 50% feed with 50% *L. sativa.*

Diet preparation

The powdered *L. sativa*, which had been air-dried and pulverized, was mixed with the Albino Rats' feed following the experimental treatments. Treatment group IV received a diet of 90% Albino Rat feed and 10% *L. sativa*. Treatment group V received a diet with 70% Albino Rats feed and 30% *L. sativa*, while treatment group VI received a diet comprising 50% Albino Rats feed and 50% *L. sativa*.

Induction of hyperlipidemia

Poloxamer 407 was utilized as the inducing agent to trigger the hyperlipidemic effect. The method outlined by Megalli et al. (2005) was employed to induce hyperlipidemia. Poloxamer 407 was administered intraperitoneally at 1,000 mg/kg BW.

Record of body weight

The body weight of the experimental animal was recorded before the experiment began. The rats' body weights were measured individually every three days throughout the experiment.

Collection of blood samples

After fourteen (14) days of treatment, the rats were subjected to a fasting period of twelve (12) hours following their last day. They were then anesthetized using chloroform. Sterilized syringes and needles were utilized to obtain whole blood samples from the heart via cardiac puncture. The blood samples were collected into lab bottles and immediately sealed with corks. The collected blood samples were centrifuged at 3,000 revolutions per minute for 10 minutes to obtain serum. The serum was used to analyze lipid profiles and liver function tests.

Lipid profile analysis

The serum samples were used for the analysis of several lipid parameters, including Very Low-Density Lipoprotein (VLDL), Low-Density Lipoprotein Cholesterol (LDL-C), High-Density Lipoprotein-Cholesterol (HDL-C), triacylglycerol (TAG), and Total Cholesterol (TC). These parameters were determined to assess the rats' lipid profile and evaluate the effects of treatment on their cholesterol and lipid levels.

Determination of total cholesterol

Total cholesterol in the serum was determined using the method described by Allain et al. (1974) using an Agappe reagent kit. It contains specific reagents and chemicals necessary for accurately measuring total cholesterol levels.

Determination of triglycerides

Triglycerides were analyzed using the enzymatic colorimetric method by Allain et al. (1974) and an Agappe reagent kit.

Determination of HDL-cholesterol

HDL-cholesterol was determined using the method described by Assmann (1979), which involves an Agappe reagent kit.

Estimation of LDL-cholesterol

LDL-cholesterol was determined by the Friedewald equation, as described by Friedewald et al. (1972). The equation is presented as follows:

LDL-C = TC – HDL-C – TG/5

Where: LDL-C: Low-Density Lipoprotein Cholesterol, TC: Total Cholesterol, HDL-C: High-Density Lipoprotein-Cholesterol, TAG: Triacylglycerol

Liver function test

The serum samples were also analyzed for various liver function parameters, including Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Gamma-glutamyl transferase (GGT), Total protein (TP), and Albumin (ALB) using a UV/visible spectrophotometer.

Assessment of Aspartate Aminotransferase (AST) activity

AST activity in the serum was measured using the procedures described by Reitman and Frankel (1957). A Randox reagent kit was used for this analysis.

Assessment of Alanine Aminotransferase (ALT) activity

ALT activity in the serum was investigated using the method described by Reitman and Frankel (1957) using a Randox reagent kit.

Assessment of Alkaline Phosphatase (ALP) activity

The Alkaline Phosphatase (ALP) serum activity was assessed using the Agappe reagent kit, following the procedure by Schlebusch et al. (1974).

Assessment of Gamma-Glutamyl Transferase (GGT)

The method by Szasz (1976), utilizing the Agappe reagent kit, was employed to determine the serum activity of Gamma-Glutamyl Transferase (GGT).

Determination of Total Protein (TP)

The Randox reagent kit was utilized to determine the total protein concentration, following the method outlined by Weichselbaum (1946).

Determination of Albumin (ABL)

The Agappe kit was employed to determine the serum albumin concentration, following the method described by Doumas et al. (1971).

Estimation of Globulin

The estimation of globulin was performed using the following formula:

Globulin = Total protein – Albumin

Preparation of liver and brain homogenates

0.3 grams (0.3g) of liver and brain tissues were homogenized in 1.5mL of Tris HCL, resulting in 1.5% (w/v) homogenates to assess the extent of lipid peroxidation in the experimental animals. The homogenates were centrifuged for 10 minutes, and the supernatants were utilized to determine the activity of Thiobarbituric Acid Reactive Substance (TBARS).

Determination of Thiobarbituric Acid Reactive Substance (TBARS)

Estimating Thiobarbituric Acid Reactive Substances (TBARS) in the tissues was conducted using the method outlined by Fraga et al. (1988). Rat liver homogenates, adjusted to 10 mg protein/mL in 120 mM KCl, 50 mM

phosphate buffer, pH 7.4, were incubated with 1000, 100, and 10 µg dry weight/mL of plant extract at 37°C for 15 min. Following incubation, Sodium dodecyl sulfate (0.2 mL of 3% (w/v)) and 0.05 mL of BHT 4% in ethanol were added. After mixing, the mixture was added with 2 mL of 0.1N HCl, 0.3 mL of 10% (w/v) phosphotungstic acid, and 1 mL of 0.7% (w/v) 2-thiobarbituric acid and then heated for 60 min in boiling water, and TBARS were extracted using 5 mL n-butanol. After centrifuging at 10,000 rpm for 10 min, the fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission using a Hitachi F-3010 fluorescence spectrophotometer. The values are expressed as the ratio of TBARS formed in the presence of plant extracts compared to control.

Statistical analysis

The obtained biochemical results were analyzed statistically using One-Way Analysis of Variance (ANOVA), followed by Duncan multiple comparisons using Statistical Package for Social Science (SPSS) version 21. Significance between means was determined at a p-value of less than 0.05 ($p<0.05$). The results for each treatment were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Impact of *Lactuca sativa* supplemented diet on weights of P407-induced hyperlipidemic rats

Table 1 and Figure 1 illustrate the results of the statistical analysis. The control treatment showed that the body weight from day 0 to 7 was not significantly different ($p>0.05$). However, the body weight in the control group from days 10 to 14 increased significantly ($p<0.05$) compared to days 0-7. The P407 treatment showed that the body weight was not significantly different ($p>0.05$) from day 0-1, but the body weight was increased constantly and significantly ($p<0.05$) from day 4. The body weight on days 10 and 12 did not differ; however, it differed significantly ($p<0.05$) compared to that of days 4, 7, and 14. Body weight on day 14 showed a significant difference ($p<0.05$) compared to days 0, 1, 4, 7, 10, and 12.

In the P407+STD treatment, the body weight on day 1 differed significantly ($p<0.05$) compared to days 0, 4, 7, 10, 12, and 14. In the treatment group of P407 with a 10% *L. sativa* supplemented diet, the body weight on days 1, 4, 7, 10, 12, and 14 differed significantly ($p<0.05$) compared to day 0. Day 1 showed a significant difference ($p<0.05$) compared to days 0, 4, 7, 10, 12, and 14. Body weight on day 4 differed significantly ($p<0.05$) compared to days 0, 1, 7, 10, 12, and 14. The body weight on days 12 and 14 was significantly higher ($p<0.05$) than on days 0, 1, 4, 7, and 10.

The treatment group of P407 with a 30% *L. sativa*-supplemented diet showed that the body weight from day 0 to 14 was not different ($p>0.05$). The treatment group of P407 with a 50% *L. sativa*-supplemented diet showed that the body weight increased significantly from day 1 ($p<0.05$). The body weight was stable until day 12 and increased significantly on day 14 ($p<0.05$).

Impact of *Lactuca sativa* supplemented diet on lipid profile of P407 induced hyperlipidemic rats

The administration of P407 increased total cholesterol levels compared to the control treatment. The P407 treatment had a significant increase ($p<0.05$) in cholesterol levels compared to the P407 with STD, P407 with 10% *L. sativa* supplemented diet, P407 with 30% *L. sativa* set, and P407 with 50% *L. sativa* treatments. However, the cholesterol levels of P407 with STD, P407 with 10% *L. sativa* set, and P407 with 30% *L. sativa* treatments did not differ ($p>0.05$). Still, it is significantly lower ($p<0.05$) than the P407 treatment and higher than the control treatment. On the other hand, the cholesterol level of P407 with a 50% *L. sativa*-supplemented diet showed no significant difference ($p>0.05$) compared to the control treatment. However, it is significantly lower ($p<0.05$) than the P407, P407 with STD, P407 with 10% *L. sativa* supplemented diet, and P407 with 30% *L. sativa* supplemented diet (Table 2 and Figure 2).

The administration of P407 increased triglyceride levels significantly ($p<0.05$) compared to the control treatment. The triglyceride level in the P407 treatment was significantly higher ($p<0.05$) than the P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa* and P407 with 50% *L. sativa*. The P407+STD treatment showed a significant difference ($p<0.05$) in triglyceride levels compared to the control treatment, P407, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*

The triglyceride levels in the P407 with 10% *L. sativa* and P407 with 30% *L. sativa* did not differ. Still, they significantly differed ($p<0.05$) from the control treatment, P407 with STD and P407 with 50% *L. sativa*. The triglyceride level in P407 with a 50% *L. sativa*-supplemented diet did not differ ($p>0.05$) from the control. However, it differed significantly ($p <0.05$) compared to the P407, P407 with STD, P407 with 10% *L. sativa*, and P407 with feed with 30% *L. sativa*. Additionally, the induction of P407 resulted in a significant difference ($p<0.05$) in HDL-C levels compared to the control.

The HDL-C level in the P407 treatment was significantly reduced ($p<0.05$) compared to the P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. The HDL-C level in the P407 with 10% *L. sativa* significantly differed ($p<0.05$) compared to the P407 treatment. It significantly differed ($p<0.05$) compared to the P407 with STD, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. The HDL-C levels of P407 with STD treatment, P407 with 30% *L. sativa*, P407 with 50% *L. sativa*, and control treatment did not differ ($p>0.05$). However, the HDL-C of the P407 treatment significantly differed from that of P407 with a 10% *L. sativa*-supplemented diet.

The induction of P407 increased significantly ($p<0.05$) in LDL-C level compared to the control treatment, P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. The LDL-C levels in the treatments of P407 with STD, P407 with 10% *L. sativa*, and P407 with 30% *L. sativa* were higher ($p<0.05$) compared to the control, P407 with 50% *L. sativa*. P 407

with 50% *L. sativa* showed no significant difference (P>0.05) in HDL-C level compared to the control. Still, it had a significant difference (p<0.05) in HDL-C level compared to P 407, P407 with STD, P407 with 10% *L. sativa*, and P407 with 30% *L. sativa*.

The induction of P407 increased significantly (p<0.05) in the VLDL level compared to the control treatment. The VLDL level in the P407 treatment was significantly higher (p<0.05) than the P407 with STD, P407 with 10% *L. sativa*, P407 with Feed with 30% *L. sativa*, and P407 with 50% *L. sativa*, P407 with STD, P407 with 10% *L. sativa*, P407 with Feed with 30% *L. sativa* and P407 with 50% *L. sativa* showed no significant difference (P>0.05) in VLDL level compared to the control.

Table 1. The effect of diet supplemented with *Lactuca sativa* on the weights of hyperlipidemic rats induced by P407

Treatment	Day 0 (g)	Day 1 (g)	Day 4 (g)	Day 7 (g)	Day 10 (g)	Day 12 (g)	Day 14 (g)
Control	$119.7^{a}\pm2.51$	$124.7^{a}\pm4.51$	$130.3^{a}\pm7.02$	$134.0^{a}\pm2.64$	$140.7^{b}\pm2.89$	$143.0^{b}\pm3.00$	$149.0^{b}\pm1.00$
P 407	$124.3^{a}\pm5.69$	$127.7^{a}\pm3.06$	$150.0^{b}\pm5.57$	$167.0^{bc}\pm7.94$	$172.7^{c}\pm8.50$	$179.0^{c}\pm8.89$	$214.0^{d}\pm9.64$
P407 with STD	$136.7^{a}\pm5.51$	$148.3^{ab}\pm9.29$	$159.7^{b}\pm8.02$	$158.7^{b}\pm13.20$	$160.7^{b}\pm2.31$	$158.7^{b}\pm1.53$	$158.0^{b}\pm2.65$
P407 with 10% *Lactuca sativa*	$119.7^{a}\pm8.96$	$129.3^{ab}\pm8.08$	$138.0^{b}\pm7.55$	$149.7^{bc}\pm4.73$	$146.0^{bc}\pm1.73$	$156.7^{c}\pm4.62$	$156.3^{c}\pm7.51$
P407 with 30% *Lactuca sativa*	$115.3^{a}\pm8.50$	$124.3^{a}\pm3.06$	$126.3^{a}\pm3.21$	$131.7^{a}\pm7.23$	$129.7^{a}\pm9.02$	$128.7^{a}\pm12.50$	$127.3^{a}\pm11.37$
P407 with 50% *Lactuca sativa*	$107.7^{a}\pm7.09$	$121.0^{ab}\pm1.0$	$123.3^{bc}\pm4.16$	$131.0^{bc}\pm7.55$	$134.7^{bc}\pm9.66$	$136.3^{bc}\pm17.04$	$139.0^{c}\pm23.58$

Note: Each value represents mean ± SD, n=3; mean values with different superscripts are significantly different (P<0.05) across the row

Table 2. The effect of *Lactuca sativa* supplemented diet on the lipid profile of P407-induced hyperlipidemic rats

Treatment	TC (mg/dL)	TAG (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	VLDL-C (mg/dL)
Control	$122.17^{a}\pm1.96$	$113.23^{a}\pm2.60$	$45.00^{a}\pm0.90$	$54.62^{a}\pm0.97$	$22.65^{a}\pm0.52$
P 407	$231.57^{b}\pm6.64$	$210.73^{b}\pm2.02$	$13.78^{b}\pm2.78$	$157.64^{b}\pm3.98$	$42.15^{b}\pm0.40$
P407 with STD	$151.77^{c}\pm0.83$	$127.43^{ac}\pm4.50$	$43.34^{a}\pm2.61$	$82.70^{c}\pm3.89$	$25.49^{a}\pm0.90$
P 407 with 10% *Lactuca sativa*	$154.97^{c}\pm1.33$	$144.00^{c}\pm14.43$	$43.23^{ac}\pm1.91$	$82.77^{c}\pm2.01$	$28.77^{a}\pm2.92$
P 407 with 30% *Lactuca sativa*	$152.83^{c}\pm0.21$	$133.13^{c}\pm0.40$	$41.95^{a}\pm3.21$	$82.59^{c}\pm0.56$	$26.63^{a}\pm0.08$
P 407 with 50% *Lactuca sativa*	$132.97^{a}\pm0.96$	$110.03^{a}\pm2.57$	$44.74^{a}\pm0.3$	$66.22^{a}\pm0.90$	$22.01^{a}\pm0.51$

Note: Each value represents mean ± SD, n=3; mean values with different superscripts are significantly different (P<0.05) in the same column. LDL-C: Low-Density Lipoprotein Cholesterol; TC: Total Cholesterol; HDL-C: High-Density Lipoprotein-Cholesterol; TAG: Triacylglycerol; VLDL-C: Very Low-Density Lipoprotein- Cholesterol

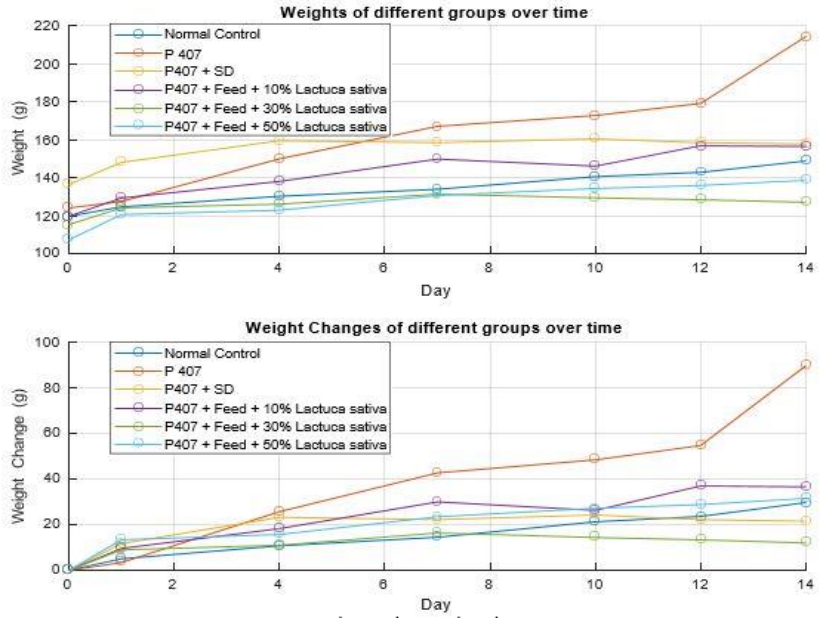

Figure 1. The effect of treatment on the body weight of rats over time

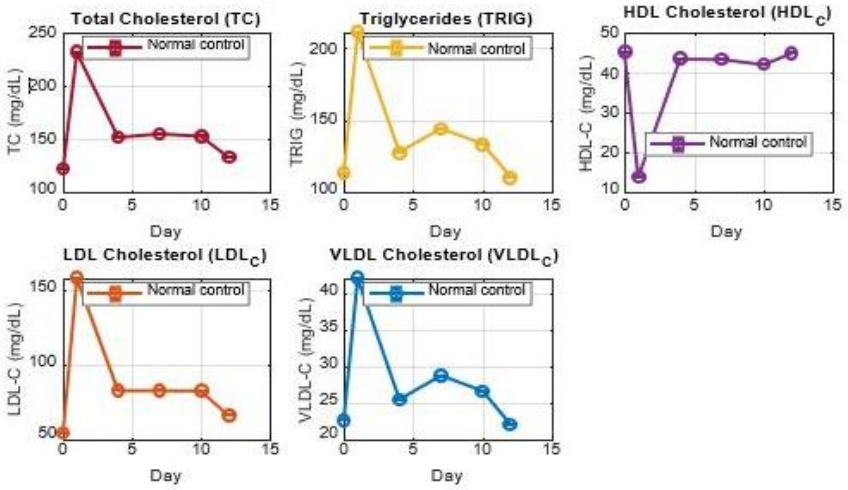

Figure 2. Lipid profile of P407-induced hyperlipidemic rats with *Lactuca sativa*-supplemented diet

The effect of *Lactuca sativa* supplemented diet on total cholesterol/HDL and LDL/HDL ratios in P407-induced hyperlipidemic albino Rats

Table 3 and Figure 3 show that the induction of P407 increases significantly ($p<0.05$) in the total cholesterol/HDL ratio compared to the control. The total cholesterol/HDL ratio in P407 treatment was also higher ($p<0.05$) than the P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. The total cholesterol/HDL ratio in the P407 with STD, P407 with10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa* set were significantly lower ($p<0.05$) than P407 treatment, and no significant difference ($P>0.05$) compared to control. The induction of P407 increased ($p<0.05$) the LDL/HDL ratio in the P407 treatment compared to the control. The LDL/HDL ratio in the P407 treatment also significantly differs ($p<0.05$) compared to the P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa* set, and Feed with 50% *L. sativa* set.

P407 with STD set, P407 with Feed with 10% *L. sativa* set, P407 with Feed with 30% *L. sativa* set showed a significant difference ($p<0.05$) in LDL/HDL ratio compared to the control treatment, and P407 with 50% *L. sativa* supplemented diet. The treatment of P407 with a 50% *L. sativa* supplemented diet set showed no significant difference ($P>0.05$) in LDL/HDL ratio in comparison to the control treatment but showed a significant difference ($p<0.05$) in LDL/HDL ratio compared to P407 set, P407 with STD set, P407 with Feed with 10% *L. sativa* set, and P407 with Feed with 30% *L. sativa* set.

The effect of *Lactuca sativa* supplemented diet on parameters of liver function in hyperlipidemic rats induced by P407

Table 4 and Figure 4 show the analysis results of parameters for liver function: AST, ALP, ALT, GGT, TP, ALB, and GLB after treatment with a *L. sativa*-supplemented diet. The induction of P407 causes a significant increase in AST level in the P407 treatment compared to the control. The treatment of P407 caused an increase significantly ($p<0.05$) in ASP level compared to the P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*, P407 with STD and P407 with 10% *L. sativa* had significantly higher ($p<0.05$) ASP levels compared to the control treatment, P407 set, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. The AST level of the treatments of P407 with a 30% *L. sativa* supplemented diet and 50% *L. sativa* supplemented diet did not differ compared to the control but showed a significant difference ($p<0.05$) compared to the P407 P407 with STD and P407 with 10% *L. sativa*.

The ALP level of the P407 treatment was significantly higher ($p<0.05$) than the other treatments. The treatment of P407 had a higher ALT level ($p<0.05$) than the control. The ALT level of the P407 treatment significantly differed ($p<0.05$) from the P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa* set. The ALT level of the treatments P407 with STD and P407 with 10% *L. sativa* diet showed a significant difference ($p<0.05$) compared to the control treatment, P407, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. The ALT level in the treatment of P407 with a 30% *L. sativa* supplemented diet was significantly different ($p<0.05$) compared to the control and a significant difference ($p<0.05$) compared to P407, P407 with STD, P407 with 10% *L. sativa* and P407 with 50% *L. sativa*.

The ALT level in the treatment of P407 with a 50% *L. sativa*-supplemented diet shows no significant difference ($P>0.05$) compared to the control treatment. It showed a significant difference ($P>0.05$) compared to the P407, P407 with STD, P407 with 10% *L. sativa*, and P407 with 30% *L. sativa*. The GGT level in the P407 treatment was significantly different ($p<0.05$) compared to the control treatment, the P407with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. The GGT level in the P407 with STD, P407 with 10% *L. sativa*, and P407 with 30% *L. sativa* did not differ

(P>0.05); however, their GGT levels significantly differ (p<0.05) compared to the control, P407 and P407 with 50% *L. sativa*. The treatment P407 with a 50% *L. sativa* supplemented diet showed no significant difference (p<0.05) in GGT level compared to the control. It showed a significant difference (p<0.05) in the GGT level compared to the P407, P407 with STD, P407 with 10% *L. sativa*, and P407 with 30% *L. sativa* set.

The P407 treatment resulted in a lowered and significantly different (p<0.05) TP level compared to the control and P407 compared to the P407 with STD P407 with 10% *L. sativa*. P407 with 30% *L. sativa*, and P407 with Feed with 50% *L. sativa*. The TP level in the P407 with STD treatment was slightly lower (p<0.05) compared to the control. It also differed significantly (p<0.05) compared to the P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. Furthermore, the treatment of P407 with a 10% *L. sativa* supplemented diet differed significantly (p<0.05) on TP level compared to the control, P407, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. The TP levels in the treatments P407 with 30% *L. sativa* and P407 with 50% *L. sativa* and control treatments showed no significant difference (P>0.05); however, their TP levels differed significantly (p<0.05) compared to P407, P407 with STD and P407 with 10% *L. sativa*.

The ALB level in the P407 treatment was lower (p<0.05) than the control. Also, it differed significantly (p<0.05) compared to P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa* set. The ALB levels in the treatment of the P407 with STD and P407 with a 30% *L. sativa*-supplemented diet did not differ (P>0.05). They differed significantly (p<0.05) compared to the control, P407, P407 with 10% *L. sativa*, and P407 with 50% *L. sativa*. The ALB level in the treatment of P407 with 10% *L. sativa* supplemented diet differed significantly (p<0.05) compared to the control, P407, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. P407 with feed with 50% *L. sativa* set showed no significant difference (P>0.05) on the ALP level compared to the control. It differed significantly (p<0.05) on the ALP level compared to the control, P407, P407 with 10% *L. sativa* and P407 with 30% *L. sativa*.

The GLB level in the P407 treatment differed significantly (p<0.05) compared to the control and P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. The GLB levels in the treatments of the P407 with STD, P407 with 10% *L. sativa*, and P407 with 30% *L. sativa* did not differ (P>0.05). However, they differed significantly (p<0.05) compared to the control, P407, and P407 with 50% *L. sativa*. The GLB levels in the treatments of P407 with 50% *L. sativa* and the control did not differ (P>0.05). However, they differed significantly (p<0.05) compared to the P407, P407 with 10% *L. sativa*, and P407 with 30% *L. sativa*.

The effect of *Lactuca sativa* supplemented diet on lipid peroxidation in the liver and brain of P407-induced hyperlipidemic rats

The induction of P407 results in a significant increase (p<0.05) in lipid peroxidation in the liver compared to control. The lipid peroxidation in P407 treatment also differed significantly (p<0.05) compared to the P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*. The lipid peroxidation in the treatments of the P407 with STD, P407 with 10% *L. sativa*, and P407 with 30% *L. sativa* did not differ (p<0.05), they differed significantly (p<0.05) compared to the control, P407 and P407 with 50% *L. sativa* as shown in Figure 5.

The induction of the P407 caused a significant increase (p<0.05) in lipid peroxidation in the brain compared to the control. Also, it differed significantly (p<0.05) compared to the P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa* (Figure 6). P407 with STD, P407 with 10% *L. sativa*, P407 with 30% *L. sativa*, and P407 with 50% *L. sativa*, and the control treatment had no significant difference (P>0.05) in lipid peroxidation. However, the lipid peroxidation of these treatment groups differed significantly (p<0.05) compared to the P407 treatment.

Table 3. The effect of *Lactuca sativa* supplemented diet on total cholesterol/HDL and LDL/HDL ratios in P407-induced hyperlipidemic rats

Treatment	TC/HDL-C ratio	LDL-C/HDL-C ratio
Normal control	2.71^{a}±1.96	1.21^{a}±0.97
P 407	16.80^{b}±6.64	11.44^{b}±3.98
P407 with STD	3.48^{a}±0.83	1.91^{c}±3.89
P 407with 10% *L. sativa*	3.58^{a}±1.33	1.91^{c}±2.01
P 407 with 30% *L. sativa*	3.64^{a}±0.21	1.97^{c}±0.56
P 407 with 50% *L. sativa*	2.97^{a}±0.96	1.48^{a}±0.90

Note: Each value represents mean ± SD, n=3; mean values with different superscripts are significantly different (P<0.05) in the same column. Atorvastatin is denoted as STD.

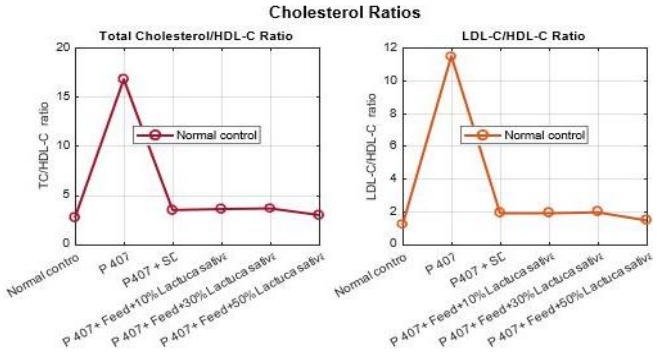

Figure 3. The effect of *Lactuca sativa* supplemented diet on total cholesterol/HDL and LDL/HDL ratios in P407-induced hyperlipidemic rats

Table 4. The effect of *Lactuca sativa* supplemented diet on the liver parameters of P407-induced hyperlipidemic rats

SETS	AST (U/L)	ALP(U/L)	ALT(U/L)	GGT (U/L)	TP(g/dL)	ALB(g/dL)	GLB (g/dL)
Normal control	21.85^{a}±2.07	41.37^{a}±1.56	9.33^{a}±4.62	22.30^{a}±3.77	5.62^{a}±0.02	2.04^{a}±0.02	2.58^{a}±0.04
P 407	52.70^{b}±2.88	79.83^{b}±5.15	19.67^{b}±2.31	38.49^{b}±1.77	2.72^{b}±0.01	0.68^{b}±0.41	1.06^{b}±0.06
P 407 with STD	30.77^{c}±3.37	38.78^{a}±1.34	13.33^{c}±2.31	30.94^{c}±0.48	4.79^{ac}±0.06	1.64^{ac}±0.03	2.13^{c}±0.08
P 407 with 10% *L. sativa*	30.37^{c}±3.00	32.23^{a}±1.02	13.67^{c}±2.3	29.56^{c}±0.94	4.66^{c}±0.05	1.43^{c}±0.21	2.23^{c}±0.20
P 407 with 30% *L. sativa*	23.93^{a}±3.81	32.56^{a}±0.80	11.00^{ac}±5.20	31.47^{c}±2.23	5.74^{a}±0.06	1.67^{ac}±0.08	2.05 ^{c}±0.11
P 407 with 50% *L. sativa*	22.06^{a}±4.22	32.90^{a}±1.84	6.67^{a}±2.89	26.34^{a}±1.06	5.67^{a}±0.11	1.75^{a}±0.02	2.93 ^{a}±0.13

Note: Each value represents mean ± SD, n=3; mean values with different superscripts are significantly different (P<0.05) in the same column. AST: Aspartate Aminotransferase; ALP: Alkaline Phosphatase; ALT: Alanine Aminotransferase; GGT: Gamma-Glutamyl Transferase; TP: Total Protein; ALB: Albumin; GLB: Globulin

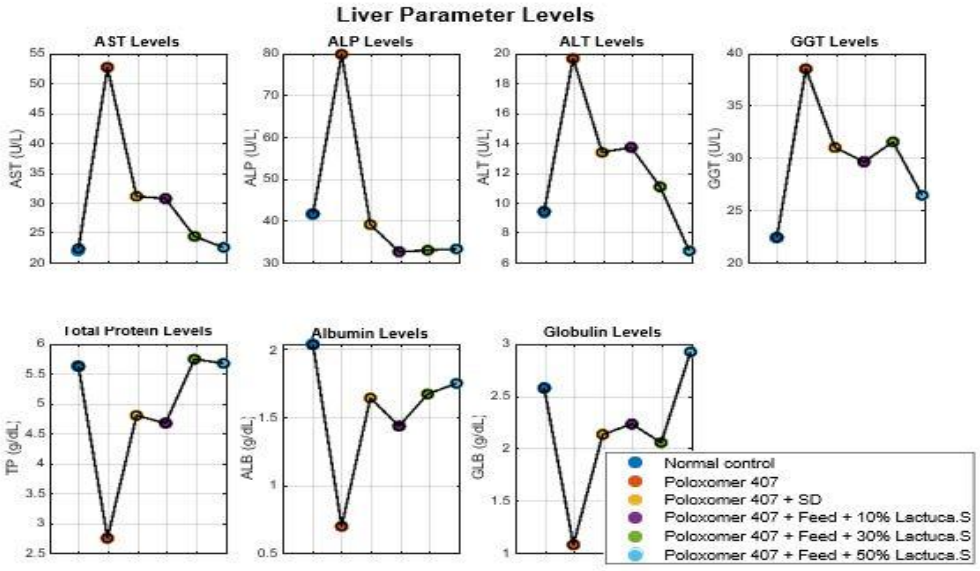

Figure 4. The effect of *Lactuca sativa* supplemented diet on the liver parameters of P407-induced hyperlipidemic rats**.** Atorvastatin is denoted as STD.

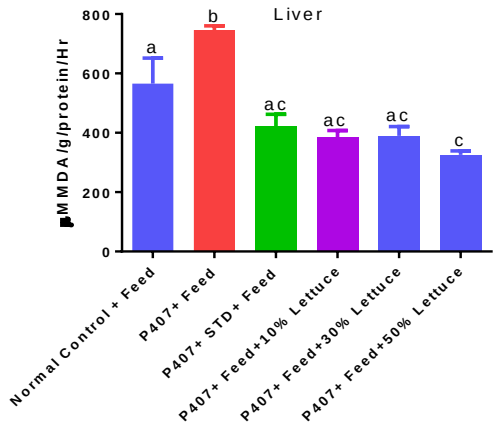

Figure 5. The effect of *Lactuca sativa* supplemented diet on lipid peroxidation in the liver of P407 induced hyperlipidemic rats. Each value represents mean ± SD, n=3, and mean values with different superscripts are significantly different (p<0.05)

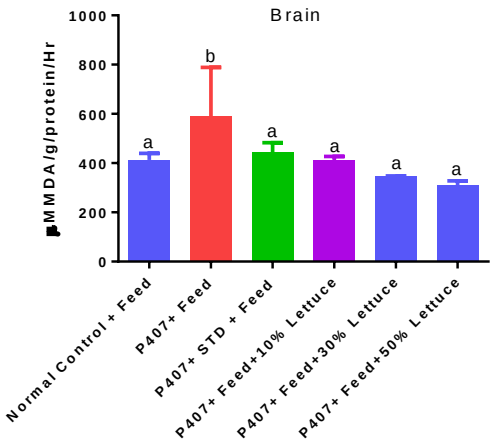

Figure 6. The effect of *Lactuca sativa* supplemented diet on lipid peroxidation in the brain of P407 induced hyperlipidemic rats. Each value represents mean ± SD, n=3, and mean values with different superscripts are significantly different (p<0.05). Atorvastatin is denoted as STD.

Discussion

Hyperlipidemia is a crucial risk factor for the emergence of Coronary Heart Diseases (CHDs), including ischemic heart disease, myocardial infarction (Ayo et al. 2023b), and stroke (Vaziri and Morris 2011). Consequently, it has become one of the greatest threats to public health (Ayo et al. 2023c). Cardiovascular risk assessment has helped reduce and manage different CVDs (Shukr et al. 2019). Plants are used to treat dyslipidemia in various ways worldwide because they have broad pharmacological effects with multiple mechanisms. The standard medicine for CVDs was Atorvastatin at 10mg/kg dose. The induction of Poloxamer 407 was carried out for the hyperlipidemia model in experimental animals. Poloxamer 407 is a non-ionic surfactant that is harmless to cellular membranes and has previously been used to cause hyperlipidemia successfully (Hyeung et al. 2006) by stimulating HMG-CoA and decreasing the activity of lipoprotein lipases. Poloxamer 407 was used in the hyperlipidemic model because of its ease of use, high repeatability, and lack of undesired underlying clinical conditions (Kim et al. 2008).

Induction of P407 increases body weight significantly ($p<0.05$), indicating the accumulation of fats in the body. However, the hyperlipidemic rats that received a *Lactuva sativa*-supplemented diet and Atorvastatin as the standard drug showed that the increased body weight was lower than the P407 treatment (Table 1). The results indicate that the *L. sativa*-supplemented diet significantly ($p<0.05$) reduced the concentrations of Total Cholesterol (TC), triglycerides (TAG), Very Low-Density Lipoprotein (VLDL), and Low-Density Lipoprotein Cholesterol (LDL-C). These reductions suggest the antihyperlipidemic effects of the *L. sativa*-supplemented diet. The *L. sativa* (lettuce) prevents weight gain by reducing fat mass accumulation and increasing energy expenditure. Consumption of lettuces enhances glucose homeostasis, increases insulin sensitivity, and improves lipid profile in albino rats. It might be attributable to the plant's abundance of bioactive molecules like esculin and chlorogenic acid (Yokozawa et al. 2006). Conversely, the P407 set significantly increased TC, TAG, LDL, and VLDL levels (Table 2).

The increase in TC levels due to the induction of P407 was likely caused by indirect activation of HMG-CoA reductase after intraperitoneal administration of P407 (Johnston 2004). The possible reduction in Total Cholesterol (TC) levels in *Lactuva sativa*-supplemented diet may be attributed to the decreased function of hepatic HMG-CoA reductase and/or the increased cholesterol-7-alpha-hydroxylase, an enzyme responsible for the conversion of cholesterol into bile acids. Furthermore, the standard drug, Atorvastatin, inhibits HMG-CoA reductase, a key enzyme in cholesterol biosynthesis. These findings are consistent with a prior study conducted by Beckmann et al. (2009) that polyphenols had lipid-lowering properties. The increase in triglyceride (TAG) levels due to the induction of P407 primarily arises from the inhibition of TAG breakdown. P407 directly inhibits the metabolism of capillary lipoprotein lipase (LPL), the enzyme that controls plasma triglyceride breakdown (Johnston 2004). Atorvastatin decreases triglyceride (TAG) concentrations by activating lipoprotein lipase. Supplementation of *L. sativa* in the diet might lead to a reduction in TAG levels either by stimulating endothelium-bound lipoprotein lipase activity (Sikarwar and Patil 2011) or lipolysis inhibition to prevent the conversion of triglycerides into fatty acids.

The present study showed a significant ($p<0.05$) decrease in LDL cholesterol (LDL-C) and Very Low-Density Lipoprotein (VLDL) levels in all treatment groups, with the 50%:50% *L. sativa* treatment showing the most significant reduction effects. These findings are in line with the study conducted by Baum et al. (1998) and Beckmann et al. (2009), which suggested that phenolic compounds could enhance the density of LDL-C receptors in the liver, thereby facilitating the binding of apolipoprotein B and improving the liver's ability to remove LDL-C from the bloodstream efficiently.

HDL cholesterol (HDL-C) functions as a cholesterol scavenger that efficiently collects excess cholesterol and cholesterol esters from the bloodstream and peripheral tissues, transporting them to the liver for breakdown into bile acids. This process is important in lowering blood and peripheral cholesterol levels and inhibiting atherosclerotic plaque development in the aorta (Karmarkar 2008; Kim et al. 2008). The results in this study show an increase ($p<0.05$) in HDL-C after administration of the standard drug Atorvastatin and the *L. sativa*-supplemented diets. The increased HDL-C levels may be attributed to increased Lecithin-Cholesterol Acyltransferase (LCAT) activity. According to Geetha et al. (2011), LCAT is an enzyme that integrates free cholesterol into HDL-C. According to Yokozawa et al. (2006), this process increases the reverse cholesterol transport, reduces LDL-C absorption by endothelial cells, and helps prevent the production of oxidized LDL-C.

Table 3 and Figure 3 show the effect of a *L. sativa* supplement diet on total cholesterol/HDL and LDL/HDL ratios in Poloxamer 407-induced hyperlipidemic albino rats. Atherogenic risk prediction indices (TC/HDL-c and LDLc/HDL-c) are numerical correlations between TC, LDL-c, and HDL-c that were effectively used as indicators of measuring atherosclerosis progression and the risk possibility of CHDs (Nicholls et al. 2007). An LDL-c/HDL-c ratio of less than 2.3 and an HDL-c/TC ratio of more than 0.3 means a lower risk of peripheral artery disease (Ojiakor and Nwanjo 2005). The *L. sativa*-supplemented diet substantially ($p<0.05$) increased the TC/HDL-c ratio and decreased the LDL-c/HDL ratio compared to the P-407 treatment without the *L. sativa*-supplemented diet. In addition, compared to rats in the induced untreated group, the standard medicines revealed a significant ($p<0.05$) increase in the TC/HDL-c ratio with a decrease in the LDL-c/HDL ratio. Increasing the TC/HDL-c ratio and decreasing the LDL-c/HDL ratio in the *L. sativa*-supplemented diet indicated that the *L. sativa*-supplemented diet has anti-atherogenic activity, lowering the possibility of coronary atherosclerosis (Dobiasova and Frohlich 2001).

Table 4 and Figure 4 show the effect of *L. sativa* supplementation on liver function parameters in P407-

induced hyperlipidemic albino rats. Hyperlipidemia is one of the diseases that harm the liver; it can cause fatty infiltration in the liver, resulting in non-alcoholic fatty liver disease (Assy et al. 2000). Fatty liver is a buildup of triglycerides and lipids in the liver cells that, if left untreated, leads to liver inflammation. Liver damage could be distinguished from steatosis to steatohepatitis, fibrosis, and necrosis (Assy et al. 2000). The increased concentrations of AST, ALT, GGT, and ALP seen in the serum of the P-407 treatment without receiving Atorvastatin may be related to liver damage caused by the buildup of triglycerides along with additional lipids in the liver cells (Hyeung et al. 2006). Supplementation of *L. sative* in the diet resulted in a substantial ($p<0.05$) recovery of liver enzyme concentrations. The reversal of these liver enzymes approaching normal concentrations may be due to polyphenols in the lysates with their membrane stabilizing effect, preventing intracellular enzyme leaking (Muthu et al. 2008). The *L. sativa*-supplemented diet reduced the serum transaminase level to normal due to the hepatic parenchymal repair and hepatocyte regeneration (Chavan et al. 2012). It indicated that the *L. sativa*-supplemented diet possesses curative effects on the liver, particularly the diet with 50% supplementation of *L. sativa*, which has the highest impact.

Total protein, albumin, and bilirubin concentrations are also decreased in hyperlipidemic rats with fatty liver lesions. Total protein, albumin, and bilirubin are all indicators of liver function; the liver releases bilirubin and hence interferes with proper liver function, affecting the process of conjugation or excretion. Total protein and bilirubin levels assess liver function and bile excretion efficiency (Usha et al. 2008). The current study showed a significant ($p<0.05$) decrease in total protein, albumin, and bilirubin levels in the P407 treatment compared to the control. These alterations might be an indication of hyperlipidemia-induced fatty liver damage. However, the *L. sativa*-supplemented diet restores total protein, albumin, and bilirubin levels to normal, with the 50%:50% *L. sativa*-supplemented diet group exhibiting the most benefit. The *L. sativa*-supplemented diet appears to improve liver function. The treatment of Atorvastatin also increased total protein, albumin, and globulin concentrations.

Figure 5 shows the effect of *L. sativa* supplementation on lipid peroxidation in the liver and the brain of P407-induced hyperlipidemic rats. Aldehydes, hydrocarbon gases, and chemical leftovers such as malondialdehyde (MDA) are the end products of the lipid peroxidation process. MDA, a prominent lipid peroxidation product, is one of the most important indicators for investigating oxidative damage on lipids (Maryam et al. 2014). MDA is a significant reactive carbon molecule often utilized as a lipid peroxidation biomarker (Karataş et al. 2006). Abnormally increased amounts of lipid peroxidation and reduced antioxidant defense systems can cause cellular organelle damage and oxidative stress (Mahboob et al. 2005). The extent of tissue damage caused by free radicals is determined by the balance between free radical formation and the endogenous antioxidant defense mechanism (Sanilkumar and Muthu 2013). The oxidative degradation of Polyunsaturated Fatty Acids (PUFA), which are abundant in cell membranes, sets off a self-perpetuating chain reaction that produces a variety of harmful products such as malondialdehyde (MDA) and 4-hydroxynonenal. Lipid peroxidation is a spontaneous radical-related process that has the potential to be damaged due to the destruction of membranes, lipids, and additional cell components when uncontrolled and self-enhancing (Mahboob et al. 2005). An increase in MDA showed that oxidative stress might result in free radical-mediated lipid peroxidation in cell membranes. MDA is an excellent marker for evaluating oxidative stress in degenerative diseases, including hyperlipidemia and diabetes mellitus (Padalkar et al. 2012).

In this present study, the induction of Poloxamer 407 significantly increased the MDA concentration. The significant ($p<0.05$) increase in lipid peroxidation in the poloxamer 407 treatment might be due to a reduction in antioxidant defense or increased free radical generation (Ceretta et al. 2012). The findings agreed with a previous study that reported increased lipid peroxidation in hyperlipidemic rats (Gopalakrishnan and Dhanapal, 2014). However, the administration of 10 mg atorvastatin, which served as the positive control, also significantly decreased the MDA concentration compared to the control treatment. Compared to the control, the MDA levels in the *L. sativa*-supplemented diet treatment groups were significantly lower. The ability of the *L. sativa*-supplemented diet to inhibit lipid peroxidation in hyperlipidemic rats may be attributed to the presence of steroids, flavonoids, phenols, and tannins, as found in the preliminary phytochemical screening. Steroids and flavonoids have antihyperlipidemic activity (Ghule et al. 2009; Patel et al. 2009).

In conclusion, this study revealed that a *L. sativa*-supplemented diet has a significant hypolipidemic effect, anti-lipid peroxidation activity, and hepatoprotective effects, with the best result obtained in the treatment of 50% *L. sativa*-supplemented diet which could be used as an antihyperlipidemic agent. The *L. sativa* could be added to the diet of those with high blood lipids. Further research must identify and isolate the active compounds with antihyperlipidemic compounds in *L. sativa*, as they have many therapeutic properties. Pharmaceutical industries should also study *L. sativa* leaves so that derivative drugs have minimal side effects.

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Effectiveness of *Passiflora foetida* (Baby Semitoo) and *Ocimum campechianum* extracts (Married Man Pork) against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*

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Abstract. *Scott S, Daniel R, Kalicharran L. 2023. Effectiveness of* Passiflora foetida *(Baby Semitoo) and* Ocimum campechianum *extracts (Married Man Pork) against* Pseudomonas aeruginosa *and* Klebsiella pneumoniae. *Asian J Nat Prod Biochem 21: 79-87.* Medicinal plants have long been used to treat illnesses. They contain many secondary metabolites, which have antibiotic properties, and can treat antimicrobial resistance in bacteria. The aim of this study was to investigate the phytochemicals in *Passiflora foetida* L. (Baby Semitoo) and *Ocimum campechianum* Mill. (Married Man Pork) and to analyze the antimicrobial potential against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Plant extracts were prepared using hexane, methanol, and water, with a rotary evaporator. The extracts were tested using zone of inhibition, Minimum Inhibitory Concentration (MIC), and Minimum Bacterial Concentration (MBC) methods. The extract of *O. campechianum* produced the highest percent yield for each solvent, while methanol produced the highest yield of all solvents. The results of the phytochemical test showed that, *O. campechianum* contained flavonoids, tannins, saponins, glycosides, and phenols; while flavonoids, tannins, glycosides, and saponins were found in *P. foetida* extract. Similarly, *O. campechianum* also showed higher antimicrobial potential than *P. foetida*. The *P. aeruginosa* proved more susceptible than *K. pneumoniae* to both plants.

Keywords: Antibiotic resistance, *Klebsiella pneumonia, Ocimum campechianum, Passiflora foetida, Pseudomonas aeruginosa*

Abbreviations: MBC: Minimum Bacterial Concentration, MDR: Multidrug-Resistant, MIC: Minimum Inhibitory Concentration

INTRODUCTION

The ability of bacteria to withstand the antagonizing effect of an antibacterial agent upon reproduction prevention or bactericidal is known as resistance. Antibiotic resistance develops because of inappropriate and unregulated usage of antibiotics (Cesur and Demiröz 2013). The intense use of antibiotics has resulted in the evolution of resistant microorganisms over the years. When antibiotics fail to eliminate all pathogens, those surviving become stronger and more resistant. The resultant resistance is then spread rapidly between the organisms either by horizontal transfer or cell division (Rossiter et al. 2017).

Before the advent of antibiotics, illnesses were treated with natural, home remedies. Treatment spanned from the mending of minor scrapes and bruises to serious ailments like pneumonia and asthma. The effectiveness of home treatments is evident because humans have continued to survive plagues, and all other ailments that occurred in the past (Ionescu 2018).

The two plants used in this study were *Passiflora foetida* L. (Baby Semitoo) and *Ocimum campechianum* Mill. (Married Man Pork). The *P. foetida* commonly called Baby Semitoo, belongs to the family Passifloraceae (DeFilipps et al. 2004). *Passiflora*, also known as passion flower or passion vine, consists of an average 500 species that can be found in temperate and tropical regions. It includes dicotyledonous shrubs, climbers and herbs. Species of Passiflora may contain indole alkaloids such as harmane, harmaline, and harmol; flavonoids such as apigenin, luteolin, and scopoletin; or benzoflavone. The flower and fruit have only traces of these chemicals, however, the leaves and the roots are potent (Tiwari et al. 2015). The *P. foetida* contains cyanogenic glycosides, gynocardin, and flavonoids.

The *O. campechianum* commonly known as Married Man Pork, belongs to the family Lamiaceae (DeFilipps et al. 2004). Lamiaceae (mint), includes 236 genera and over 7,000 species. Species of mint may contain flavonoids and sterols. They can also be classified into several biological categories such as antioxidant, cytotoxic, antibacterial, antifungal and antiviral (Nousiba et al. 2020). The *O. campechianum* leaves can soothe colic, reduce tumors, remedy swollen groin, and treat red sediments in urine, while the seeds may be used to treat the irritated eyes of children. The plant contains three chemical compounds: eugenol, methyl eugenol, and sesquiterpenes (DeFilipps et al. 2004).

The aforementioned plants were tested against two known resistant microbes, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, which can cause hospital-acquired and respiratory infections respectively. The *P. aeruginosa* is a gram-negative bacillus that can be found in freshwater and community reservoirs. Infections include folliculitis,

pneumonia, and otitis externa (Wilson and Pandey 2023). The *P. aeruginosa* is opportunistic and causes infections, such as ventilator-associated pneumonia and catheter-associated urinary tract infections. Infections are usually severe, and treatment can be difficult because the bacterium has a limited susceptibility to antimicrobials (Reynolds and Kollef 2021). Its antibiotic resistance is expressed by restricting membrane permeability, efflux systems, and antibiotic-inactivating enzymes (Wilson and Pandey 2023).

In contrast, *K. pneumoniae* contributes to 11.8% of all hospital-acquired pneumonia worldwide. It is highly resistant and poses a significant threat to immunocompromised patients. The *K. pneumoniae* is a gram-negative bacterium of the Enterobacteriaceae family. The pathogen has a high infection and low recovery rate. Its antibiotic resistance is expressed by mechanisms of evasion, such as usage of porins and efflux pumps (Paczosa and Mecsas 2016; Ashurst and Dawson 2023).

The study investigates possible native plants that can be used to treat two pathogens that cause great harm to human health. Respiratory infections are infectious diseases of the upper and lower respiratory tract, and are transmitted by bacteria, viruses, and fungi (Saleri and Ryan 2019). These pathogens have a great impact on human health because they can cause illnesses and also increase the effects of chronic lung diseases. In 2015, respiratory infections were the cause of 3.2 million deaths, and in low-income countries they are one of the leading causes of deaths (Jose 2018). In contrast, hospital-acquired infections are nosocomial infections that occur while receiving health care. Worldwide about 1.7 million persons acquire these infections and over 98,000 persons die yearly. Generally, immunocompromised patients are most susceptible to infections (Haque et al. 2018).

This study investigated the effectiveness of phytochemicals extracts in *P. foetida* and *O. campechianum* and analyzed their antimicrobial potential against *K. pneumoniae* and *P. aeruginosa*.

MATERIALS AND METHODS

Experimental design

The experiment was based on the randomized block design, whereby the two medicinal plants *P. foetida* and *O. campechianum* were tested against two pathogens *P. aeruginosa* and *K. pneumoniae* at concentrations of 0.05g/mL, 0.1g/mL, and 0.15g/mL.

Sample collection

The two medicinal plants, namely *P. foetida* and *O. campechianum* were bought from the local market and microbes *P. aeruginosa* and *K. pneumoniae* were obtained from the George Town Public Hospital Corporation (GPHC) laboratory and sub-cultured weekly to maintain the colonies. The experiments were conducted in the Microbiology and Quality Control Laboratories of the New Guyana Pharmaceutical Company, and the Plant Pathology Laboratory at the National Agricultural Research and Extension Institute (NAREI). Experimentation began in May and was concluded in June 2021.

Preparation of plant extract

The leaves of each plant were rinsed carefully to remove dust and soil particles. Leaves were cut into small pieces using a sterile pair of scissors and 50 g of each plant sample was soaked in 500 mL of each solvent (distilled water, methanol, and hexane). This mixture was left at room temperature for eleven days, during which occasional shaking was done to increase extraction. The mixtures were filtered using Whatman No.1 filter paper and methanol and hexane extracts were dried using a rotary evaporator. The aqueous *P. foetida* and *O. campechianum* extracts were dried using a hot plate until a small quantity of liquid remained, then further drying was done in a hot air oven. After drying all extracts were carefully labeled and stored in a dry place until further investigation (Shahid et al. 2013). The percentage yield of each extract was calculated using the formula:

Yield (%) = $(X_1*100)/X_0$ where X_1 refers to the weight of extract after evaporation of the solvent and X_0 refers to the dry weight of plant before extraction (Gonelimali et al. 2018).

Phytochemical analysis

Phytochemical analysis, included terpenoids, flavonoids, glycosides, tannins, saponins, phlobatannins, coumarins, phenols, quinones, triteroids, and steroids (Table 1).

Microbial analysis

The zone of inhibition test was done in triplicates, while the other two tests were performed in duplicates. In these three tests, methanol and distilled water were used as the negative controls for methanolic and aqueous extracts, respectively. For the positive control, the antibiotic ciprofloxacin was used.

Sterilization

All equipment was sterilized in an autoclave before use, such as the test tubes, borer, and tweezers. The agar plates were sterilized before use for two and a half hours in a hot air oven.

0.5 McFarland standard

The solution was prepared by mixing 9.95 ml of 1% H_2SO_4 with 0.05 ml of 1% $BaCl_2$. The solution was stored in an Erlenmeyer flask with a glass stopper and used to standardize the bacterial suspensions (De Zoysa 2019).

Broth preparation

Tryptic soy broth was prepared according to the instructions on the container. Then after autoclaving, broth was pipetted into the required test tubes for usage.

Agar preparation

Mueller Hinton agar was prepared according to the instructions on the container. Then after autoclaving, agar was poured into sterilized agar plates. Plates were stored in the refrigerator until ready to use.

Table 1. Procedures of testing phytochemical compounds present in the plants (Thilagavathi et al. 2015; Roghini and Vijayalakshmi 2018)

Phytochemical	Methodology	Indicator
Terpenoids	0.8 g of each plant extract was transferred to a test tube and 10 mL of methanol was added to the tube. The mixture was filtered, and 5 mL of the filtrate was removed. 5 mL of the solution was transferred to another test tube and 2 mL of chloroform and 3 mL of sulfuric acid were added.	Reddish-brown color
Flavonoids	0.5 g of each plant extract was added to a test tube with 10 mL of distilled water. After mixing, 5 mL of dilute ammonia solution, then 1 mL of concentrated H_2S0_4 were added.	Yellow color
Glycosides	A few drops of glacial acetic acid and ferric chloride were added to 1 mL of each extract. Then 3-4 drops of concentrated sulfuric acid were also added to each extract.	Blue-green color
Tannins	A few drops of 10% lead acetate were added to 2 mL of each extract.	White precipitate
Saponins	9 mL of distilled water was added to 1 mL of each extract and shaken vigorously for 15 seconds. The extract was then allowed to stand for 10 minutes.	Stable foam
Triteroids	To 2 mL of each extract was added 10 mL of chloroform. After which 1 mL of acetic anhydride and 2 mL of concentrated sulfuric acid were also added.	Red, pink, or violet color
Phlobatannins	A few drops of 2% hydrochloric acid were added to 1 mL of each extract.	Red precipitate
Coumarins	To 1 mL of each extract was added 1 mL of 10% sodium hydroxide.	Yellow color
Phenols	To 1 mL of each extract, 2 mL of distilled water and a few drops of 10% ferric chloride were added.	Blue or green color
Quinones	1 mL of concentrated sulphuric acid was added to 1 mL of each extract.	Red color
Steroids	To 2 mL of each extract was added 10 mL of chloroform. After which 1 mL of acetic anhydride and 2 mL of concentrated sulfuric acid were also added.	Blue-green color

Inoculum preparation

The inoculum was prepared using the direct colony suspension method. 5 mL of saline was poured into a test tube and sterilized for 15 minutes in an autoclave. After cooling, bacterial suspension was prepared by transferring 2-3 small colonies from the 24-hour culture to the test tubes. The inoculum was standardized using the 0.5 McFarland standard prepared previously.

Agar well-diffusion test

Extracts were made using 0.15 g/mL, 0.1 g/mL and 0.05 g/mL crude extracts in the respective solvents. The Mueller Hinton agar plates were inoculated by swabbing the organism on the surface of agar plate using sterile cotton swabs (six plates for each organism). After which 6 mm wells (three wells per plate) were made using a sterile laboratory cork borer and tweezers. The wells were then filled with 30-40 µL of each extract using 1 mm syringes (one per well). The agar plates were incubated for 18-24 hours, and the zone of inhibition for each well was measured (Balouiri et al. 2016).

Minimum Inhibitory Concentration (MIC) using agar dilution

Plant extracts were tested for MIC using the highest concentration of extracts (0.15 g/mL). For this 1:9 dilution was made for each of the four extracts. 2 mL of the extract was added to test tube 1 and 1 mL of sterile Tryptic Soy Broth (TSB) was added to the other nine tubes. Using a 1 mL glass pipette and pipette filler, 1 mL of the extract from test tube 1 was transferred to test tube 2. Then after mixing, 1 mL of test tube 2 was added to test tube 3. The dilutions were continued until test tube 9 where 1 mL was discarded. From a 0.5 McFarland standard TSB culture suspension of the organism, 1 mL of the culture suspension was added to each test tube. This test was then repeated for the other organism. After which the tubes were incubated for 18-24 hrs and the turbidity of each test tube was observed. Any turbidity in the tubes suggested growth. The smallest dilution with no growth was the MIC (Rollins 2000, unpublished data).

Minimum Bacterial Concentration (MBC)

The MBC was determined by inoculating the dilutions used for MIC, showing no growth on the surface of agar plates. This was done using Mueller Hinton agar plates and the plates were inoculated for 18-24hrs at 37°C. The plates were divided into quarters and for each test tube that didn't display visible growth, streaking was done using an inoculating loop. Any growth of colonies indicated that plant extract was bacteriostatic but not bactericidal, while the absence of growth indicated it was bactericidal. The test was performed for both pathogens using both plant extracts (Rollins 2000).

Data analysis

The results obtained consisted of both quantitative and qualitative variables. Descriptive and inferential statistics were conducted to analyze the results. Using descriptive statistics, histograms and bar charts were generated, along with the means and standard deviations. While using inferential statistics, one way ANOVA and the Tukey test were conducted on the raw data. Analysis was done using SPSS and Excel software.

RESULTS AND DISCUSSION

Yield preparation

The amount and percentage yield from each plant using each solvent is shown in Table 2. For *P. foetida*, water extract produced the highest yield while for *O. campechianum*, methanol produced the highest yield. Hexane produced no yield for both plant samples.

Table 2. Calculation of the % yield of the extracts of *P. foetida and O. campechianum*

Plants	Solvents	Weight before extraction (g)	Weight after drying (g)	Yield (%)
P. foetida	Methanol	50	1.411	2.82
	Water	50	1.97	3.94
	Hexane	50	0	0
O. campechianum	Methanol	50	2.606	5.21
	Water	50	1.225	2.45
	Hexane	50	0	0

Phytochemical analysis

The phytochemicals present in methanolic, and aqueous extracts of each plant are shown in Table 3. Glycosides were present in aqueous and methanolic extracts of both plants. Flavonoids, tannins, glycosides, and phenols were present in the methanolic extract of *O. campechianum*, while flavonoids, tannins, and glycosides were present in methanolic extract of *P. foetida*. The aqueous extract of *O. campechianum* was found to contain tannins, glycosides, and saponins, while aqueous extract of *P. foetida* only contained glycosides and saponins.

Table 3. Plant compounds present in *P. foetida* and *O. campechianum* extracts

Plant compounds	*O. campechianum*		*P. foetida*	
	Methanolic	Aqueous	Methanolic	Aqueous
Terpenoids	-	-	-	-
Flavonoids	+	-	+	-
Glycosides	+	+	+	+
Tannins	+	+	+	-
Saponins	-	+	-	+
Phlobatannins	-	-	-	-
Coumarins	-	-	-	-
Phenols	+	-	-	-
Quinones	-	-	-	-
Steroids	-	-	-	-
Triteroids	-	-	-	-

Note: (-) Absence; (+) Presence

Microbial analysis

Agar well diffusion for P. aeruginosa

The zone of inhibition recorded for *P. foetida* was shown at three concentrations (0.15 g/mL, 0.05 g/mL, and 0.1 g/mL) against *P. aeruginosa*. Zones of inhibition were observed for two concentrations (0.1 g/mL and 0.15 g/mL) of methanolic extract indicating only methanolic *P. foetida* extract can inhibit growth of *P. aeruginosa*. The largest zone of inhibition occurred at 0.15 g/mL for methanolic *P. foetida* extract (Table 4).

The zone of inhibition recorded for *O. campechianum* was shown at three concentrations (0.15 g/mL, 0.05 g/mL, and 0.1 g/mL) against *P. aeruginosa*. Zones of inhibition were observed for both extracts at all tested concentrations indicating *P. aeruginosa* is susceptible to *O. campechianum*. The largest zone of inhibition occurred at 0.15 g/mL for aqueous extract and 0.1 g/mL for methanolic extract (Table 4).

The mean zone of inhibition for each extract at the three concentrations is shown in Figure 1. The results show that aqueous *O. campechianum* extract produced the largest zone of inhibition for all extracts against *P. aeruginosa*. The results of aqueous *P. foetida* can be seen at the blue bars (0 mm) indicating no zone of inhibition was observed.

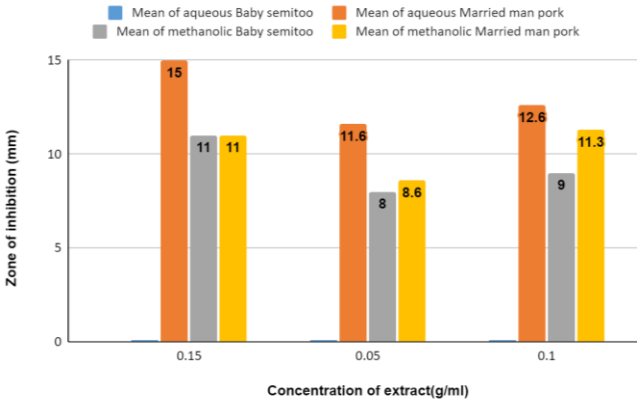

Figure 1. Mean zone of inhibition for plant extracts against *P. aeruginosa*

Table 4. Zone of inhibition in *P. foetida* and *O. campechianum* extracts

Concentrations of extract (g/mL)	Methanol				Aqueous			
	Trial 1 (mm)	Trial 2 (mm)	Trial 3 (mm)	Mean±SD	Trial 1 (mm)	Trial 2 (mm)	Trial 3 (mm)	Mean±SD
P. foetida								
0.05	8	8	-	8±5.6	-	-	-	-
0.1	9	10	8	9±0.7	-	-	-	-
0.15	11	10	12	11±0.7	-	-	-	-
O. campechianum								
0.15	17	15	13	15±2.8	12	11	10	11±1.4
0.05	13	10	12	11.6±0.7	10	8	8	8.6±1.4
0.1	13	11	14	12.6±0.7	11	11	12	11.3±1.4

Note: (-) no zone of inhibition

Agar well diffusion for K. pneumoniae

The zone of inhibition for *P. foetida* was recorded at three concentrations (0.15 g/mL, 0.05 g/mL, and 0.1 g/mL) against *K. pneumoniae*. Zones of inhibition were not observed for neither methanolic nor aqueous extracts indicating *K. pneumoniae* was resistant to *P. foetida* (Table 5).

The zone of inhibition for *O. campechianum* was recorded at three concentrations (0.15 g/mL, 0.05 g/mL, and 0.1 g/mL) against *K. pneumoniae*. Zones of inhibition were observed for two concentrations (0.1 g/mL and 0.15 g/mL) of aqueous extract indicating aqueous *O. campechianum* extract can inhibit the growth of *K.pneumoniae*. The same zone of inhibition was observed at both concentrations (Table 5).

The mean zone of inhibition for each extract at the tested concentrations is shown in Figure 2. Results indicate that only aqueous *O. campechianum* extract can inhibit *K. pneumoniae*.

The zone of inhibition for the antibiotic ciproflaxacin at 0.1 g/mL against *P. aeruginosa* and *K. pneumoniae* is shown in Table 6.

Minimum Inhibitory Concentration (MIC)

The results of minimum inhibitory concentration for the extracts of *O. campechianum* against *K. pneumoniae* and *P. aeruginosa* are presented in Table 7. The results below indicate that methanolic extract cannot inhibit growth of *K. pneumoniae* and the pathogen is resistant to the extract. The MIC of methanolic extract against *P. aeruginosa* was 38 mg/mL (10^{-3}). The MIC of aqueous extract against *P. aeruginosa* was19 mg/mL (10^{-4}), while MIC against *K. pneumoniae* was 150 mg/mL (10^{-1}).

The Minimum Inhibitory Concentration (MIC) of methanolic and aqueous extracts of *P. foetida* against *P. aeruginosa* and *K. pneumoniae* is presented in Table 7. The results indicate that neither methanolic nor aqueous extract can inhibit growth of *K. pneumoniae* and the pathogen was resistant to *P. foetida*. The MIC of methanolic *P. aeruginosa* was 38 mg/mL (10^{-3}), while no inhibition was observed for aqueous *P. foetida* extract.

Minimum Bacterial Concentration (MBC)

The results of Minimum Bacterial Concentration (MBC) of methanolic and aqueous extract of *P. foetida* are shown in Table 8. It was observed that methanolic extract can kill *P. aeruginosa*, while aqueous extract cannot. The MBC of methanolic extract was 75mg/mL(10^{-2}). It was observed that neither methanolic nor aqueous extract can kill *K. pneumoniae.*

The Minimum Bacterial Concentration (MBC) of methanolic and aqueous extracts of *O. campechianum* are shown in Table 8. The results indicate that *O. campechianum* cannot kill either *P. aeruginosa* or *K. pneumoniae*. Neither methanolic nor aqueous extracts can kill both pathogens.

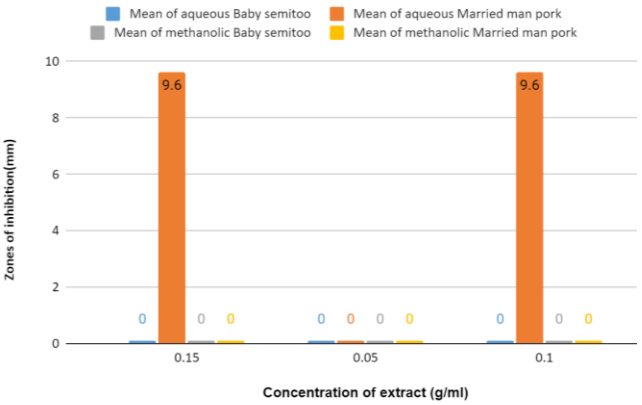

Figure 2. Mean zone of inhibition in plant extracts against *K. Pneumoniae*

Table 6. Zone of inhibition obtained from antibiotic ciprofloxacin

Microbes	Trial 1 (mm)	Trial 2 (mm)	Trial 3 (mm)	Mean±SD
K. pneumoniae	41	38	42	40.3±2.8
P. aeruginosa	52	50	48	50 ±2.8

Table 5. Zone of inhibition for *P. foetida* and *O. campechianum* extracts

Concentrations of Extract (g/mL)	Aqueous				Methanol			
	Trial 1 (mm)	Trial 2 (mm)	Trial 3 (mm)	Mean±SD	Trial 1 (mm)	Trial 2 (mm)	Trial 3 (mm)	Mean
P. foetida								
0.05	-	-	-	-	-	-	-	-
0.1	-	-	-	-	-	-	-	-
0.15	-	-	-	-	-	-	-	-
O. campechianum								
0.05	-	-	-	-	-	-	-	-
0.1	11	10	8	9.6±2.1	-	-	-	-
0.15	10	10	9	9.6±2.1	-	-	-	-

Note: (-) no zone of inhibition.

Table 7. MIC of methanolic and aqueous extracts of *P. foetida* and *O. campechianum*

Dilutions	Concentrations (mg/mL)	*P. aeruginosa*		*K. pneumoniae*	
		Methanolic Extract	Aqueous Extract	Methanolic Extract	Aqueous Extract
P. foetida					
10^{-1}	150	-	+	+	+
10^{-2}	75	-	+	+	+
10^{-3}	38	-	+	+	+
10^{-4}	19	+	+	+	+
10^{-5}	9	+	+	+	+
10^{-6}	5	+	+	+	+
10^{-7}	2	+	+	+	+
10^{-8}	1	+	+	+	+
10^{-9}	0.5	+	+	+	+
O. campechianum					
10^{-1}	150	-	-	-	+
10^{-2}	75	-	-	+	+
10^{-3}	38	-	-	+	+
10^{-4}	19	+	-	+	+
10^{-5}	9	+	+	+	+
10^{-6}	5	+	+	+	+
10^{-7}	2	+	+	+	+
10^{-8}	1	+	+	+	+
10^{-9}	0.5	+	+	+	+

Note: (-) No growth; (+) Growth

Table 8. MBC of methanolic and aqueous extracts of *P. foetida* and *O. campechianum*

Dilutions	Concentrations (mg/mL)	*P. aeruginosa*		*K. pneumoniae*	
		Methanolic Extract	Aqueous Extract	Methanolic Extract	Aqueous Extract
P. foetida					
10^{-1}	150	-	+	+	+
10^{-2}	75	-	+	+	+
10^{-3}	38	+	+	+	+
10^{-4}	19	+	+	+	+
O. campechianum					
10^{-1}	150	+	+	+	+
10^{-2}	75	+	+	+	+
10^{-3}	38	+	+	+	+
10^{-4}	19	+	+	+	+

Note: (-) No growth; (+) Growth

Post Hoc Tukey test for *P. aeruginosa*

The methanolic *P. foetida* extract showed a significant difference between the aqueous *P. foetida* and *O. campechianum* extracts. The methanolic extract of *O. campechianum* showed a significant difference between the aqueous extracts of *P. foetida* and *O. campechianum*. In addition, the aqueous extract of *P. foetida* showed a significant difference from the aqueous *O. campechianum* extract (Table 9). No significant difference was observed between methanolic *P. foetida* and methanolic *O. campechianum* extract

In Table 9, methanolic *P. foetida* extract showed a significant difference between the aqueous *P. foetida* and *O. campechianum* extracts. The methanolic extract of *O. campechianum* showed a significant difference from the aqueous extracts of *P. foetida*. In addition, the aqueous extract of *P. foetida* showed a significant difference from the aqueous *O. campechianum* extract. No significance difference was observed between methanolic *P. foetida* and methanolic *O. campechianum* extract.

Post-Hoc Tukey test for *K. pneumoniae*

In Table 10, significance was observed between the aqueous *O. campechianum* extract and methanolic *P. foetida* extract; methanolic and aqueous *O. campechianum* extract, and aqueous *P. foetida* and *O. campechianum* extracts. In Table 10, significance existed between the aqueous *O. campechianum* extract with methanolic *P. foetida* extract; methanolic and aqueous *O. campechianum* extract, and aqueous *P. foetida* and *O. campechianum* extracts.

Table 9. Post Hoc statistical analysis of 0.15, 0.1, 0.05 concentration *P. foetida* and *O. campechianum* extracts

Tukey HSD	***P. foetida* and *O. campechianum* extract**		**Significance**
0.15 concentration	Methanolic extract of *P. foetida*	Methanolic extract of *O. campechianum*	1
		Aqueous extract of *P. foetida*	<.001
		Aqueous extract of *O. campechianum*	0.017
	Methanolic extract of *O. campechianum*	Aqueous extract of *P. foetida*	<.001
		Aqueous extract of *O. campechianum*	0.017
	Aqueous extract of *P. foetida*	Aqueous extract of *O. campechianum*	<.001
0.1 concentration	Methanolic extract of *P. foetida*	Methanolic extract of *O. campechianum*	0.085
		Aqueous extract of *P. foetida*	<.001
		Aqueous extract of *O. campechianum*	0.007
	Methanolic extract of *O. campechianum*	Aqueous extract of *P. foetida*	<.001
		Aqueous extract of *O. campechianum*	0.305
	Aqueous extract of *P. foetida*	Aqueous extract of *O. campechianum*	<.001
0.05 concentration	Methanolic extract of *P. foetida*	Methanolic extract of *O. campechianum*	0.414
		Aqueous extract of *P. foetida*	0.115
		Aqueous extract of *O. campechianum*	0.058
	Methanolic extract of *O. campechianum*	Aqueous extract of *P. foetida*	0.012
		Aqueous extract of *O. campechianum*	0.496
	Aqueous extract of *P. foetida*	Aqueous extract of *O. campechianum*	0.002

Table 10. Results of Post Hoc statistical analysis of 0.15 and 0.1 concentration *P. foetida* and *O. campechianum* extracts

Tukey HSD	***P. foetida* and *O. campechianum* extract**		**Significance**
0.15 concentration	Methanolic extract of *P. foetida*	Methanolic extract of *O. campechianum*	1
		Aqueous extract of *P. foetida*	1
		Aqueous extract of *O. campechianum*	<.001
	Methanolic extract of *O. campechianum*	Aqueous extract of *P. foetida*	1
		Aqueous extract of *O. campechianum*	<.001
	Aqueous extract of *P. foetida*	Aqueous extract of *O. campechianum*	<.001
0.1 concentration	Methanolic extract of *P. foetida*	Methanolic extract of *O. campechianum*	1
		Aqueous extract of *P. foetida*	1
		Aqueous extract of *O. campechianum*	<.001
	Methanolic extract of *O. campechianum*	Aqueous extract of *P. foetida*	1
		Aqueous extract of *O. campechianum*	<.001
	Aqueous extract of *P. foetida*	Aqueous extract of *O. campechianum*	<.001

Discussion

The use of medicinal plants to treat diseases is an old practice. It is the main source of care for approximately 85% of the world and the derivative for 80% of all synthetic drugs (Fitzgerald et al. 2020). The emergence of antimicrobial resistance has resulted in increased interest in these medicinal plants. They produce secondary metabolites which are not essential for their normal growth but aid in reproduction and act as defense mechanisms against bacteria, viruses, fungi, etc. Secondary metabolites have proven to be effective against both gram -ve and +ve bacteria, additionally, plant-derived drugs break down the cell wall and membranes of microbes, resulting in cell death (Anand et al. 2019).

In the experiment conducted above, extracts were made by soaking the leaves of both plants in hexane, methanol, and water. Results showed that only methanol and water produced yield, which may be due to the polarity of solvents. Hexane is a nonpolar solvent and as the plant may contain few or no non-polar molecules, no yield was produced. The methanolic extract of *O. campechianum* produced the highest percent yield (5.21%), followed by the aqueous *P. foetida* extract (3.94%), then the methanolic *P. foetida* (2.82%), and finally the aqueous *O. campechianum* extract (2.45%). The *O. campechianum* methanolic extract had the largest yield of all solvents. Although, yields of the extracts varied. This may be a result of spills when handling the dried extract and also spillage of concentrated liquid during filtration. According to Azwanida (2015), on the extraction methods of medicinal plants, a particle size smaller than 0.5 mm is ideal for efficient extraction. As such, the smaller the size of the leaves the better the extraction. The leaves used for maceration were small strips and as such the particle size may have also hindered extraction of the plant compounds.

The phytochemical analysis results of *P. foetida* and *O. campechianum* showed a wider range of compounds were present in *O. campechianum* than in *P. foetida.* Of the two solvents used, methanolic extracts were found to have more plant compounds. Flavonoids, tannins, glycosides, and phenols were found to be present in the methanolic extract of *O. campechianum,* while flavonoids, tannings, and

glycosides were found in the methanolic extract of *P. foetida*. The aqueous extracts of *O. campechianum* were found to contain tannins, glycosides, and saponins, while the aqueous extract of *P. foetida* only contained glycosides and saponins. The results of *P. foetida* are dissimilar to those in Birudu et al. (2015) and Prasanth et al. (2018) which displayed a wider range of compounds inclusive of flavonoids, tannins, and phenols in the aqueous extract. This difference may be because of the area in which the plants were grown. Secondary metabolites such as flavonoids combat resistance by inhibition of virulence factors, efflux pump, biofilm formation, membrane disruption, cell envelope synthesis, nucleic acid synthesis, and bacterial motility inhibition (Biharee et al. 2020). While phenols combat resistance excluding flavonoids, combat resistance by cell membrane disruption, inhibition of DNA gyrase, inhibition of helicase activity, multi-drug efflux pump inhibitors, dehydratase, and protein kinase inhibition (Rempe et al. 2017). The *O. campechianum*, unlike *P. foetida*, contains both flavonoids and phenols which may explain its greater antibiotic activity against the pathogens. In addition, the administration of flavonoids to *P. aeruginosa* alters transcription of quorum sensing-controlled target promoters and suppresses virulence factor production (Paczkowski et al. 2017). This confirms the effectiveness of both *O. campechianum* and *P. foetida* against *P. aeruginosa*.

In plants, the accumulation of secondary metabolites varies with a number of factors, such as light, temperature, soil water, soil fertility, and salinity (Yang et al. 2018). Any change in either of the factors may cause an alteration in the secondary metabolites present. Kumar et al. (2019) analyzed the effect of geographical and seasonal variations on the phenolic contents of antioxidant activity from different plant parts. It was found that altitude and seasonal variations significantly affect the secondary metabolites in plant parts. Research on *P. foetida* in Guyana is limited so further similarities could not be made. The present study showed that results of methanolic extract are similar to those obtained in a previous study on methanolic root extract of *P. foetida* by Emin et al. (2010). Research of *O. campechianum* is limited so no comparison to previous research was made.

The antimicrobial analysis unearthed that *O. campechianum* showed better antimicrobial activity than *P. foetida*, and *P. aeruginosa* isolate was more susceptible to both plants than *K. pneumoniae*. The aqueous extract proved to have greater antibiotic properties than methanolic extract of *O. campechianum* as larger zones were seen. The methanolic extract of *P. foetida* had more antibiotic content than aqueous extract. Zones of inhibition were only observed in the methanolic extract of *P. foetida* against *P. aeruginosa*.

The results obtained for *P. foetida* were unlike those seen in previous studies in different regions. A study done by Emin et al. (2010) revealed significant antibiotic activity of *P. foetida* against both *K. pneumoniae* and *P. aeruginosa*. The concentration of extract from 250-1250 mcg obtained zones of 7-13 mm and 14-22 mm for *K. pneumoniae* and *P. aeruginosa*, respectively. In contrast, no antimicrobial activity was observed against *K.pneumoniae*. Inhibition zones of 8 mm, 9 mm, and 11 mm from 50 mg/mL, 100 mg/mL, and 150 mg/mL concentrations respectively were observed for *P.aeruginosa*. No previous research with *O. campechianum* was found regarding the pathogens.

Ciprofloxacin, the positive control in this experiment, used a concentration of 0.1 g/mL and obtained areas above 30 mm for both pathogens. This is much different compared to the results obtained using the extracts of both plants. Results obtained for the two plants also displayed that there was a significant difference between the means of the extracts against *K. pneumoniae* and *P. aeruginosa*.

The MIC of *O. campechianum* against *P. aeruginosa* observed was 38 mg/mL and 19 mg/mL in methanolic and aqueous extracts respectively. While the MIC of *O. campechianum* against *K. pneumoniae* was 150 mg/mL for the aqueous extract. The MIC of *P. foetida* against *P. aeruginosa* was 38 mg/mL for the methanolic extract. The only plant which displayed bactericidal properties was *P. foetida* which had an MBC of 75 mg/mL against *P. aeruginosa*. This suggests that although *O. campechianum* is better at inhibiting bacterial growth of *P. aeruginosa* and *K. pneumoniae* than *P. foetida*, the plant cannot kill *P. aeruginosa*, unlike *P. foetida*.

Statistical analysis results of one-way ANOVA for *P. aeruginosa* revealed a significant difference between *P. foetida* and *O. campechianum* extracts. All concentrations (0.15 g/mL, 0.l g/mL and 0.05 g/mL) showed significance with P-values <0.001, <0.001 and 0.002. In contrast, statistical analysis of *K. pneumoniae* revealed a significant difference between *P. foetida* and *O. campechianum* extracts. Significance was seen at 0.15 mg/mL and 0.1 mg/mL with P-values <0.001 and <0.001. At 0.05mg/mL the P value was >0.05.

In conclusion, the phytochemicals present in *O. campechianum* were flavonoids, tannins, saponins, glycosides, and phenols, while flavonoids, tannins, glycosides, and saponins were found in *P. foetida* extract. The *O. campechianum* can inhibit but not kill both *P. aeruginosa* and *K. pneumoniae*, while *P. foetida* can kill and inhibit the growth of *P. aeruginosa*. The *P. foetida* displayed no antibiotic activity against *K. pneumoniae*. The *O. campechianum* was statistically significant compared to *P. foetida* against *K. pneumoniae*, whereas *P. foetida* was statistically significant compared to *O. campechianum* against *P. aeruginosa*. The *O. campechianum* can be used to inhibit and control the growth of both pathogens.

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Anticancer activity of secondary metabolite isolated from the rhizospheric fungus *Fusarium oxysporum* isolate-*ABRF1*, 2-propenoic acid, pentadecyl ester

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Abstract. *Sahu MK, Suthakaran S, Ghosh SC, Singh D, Das A, Jha H. 2023. Anticancer activity of secondary metabolite isolated from the rhizospheric fungus* Fusarium oxysporum *isolate-ABRF1, 2-propenoic acid, pentadecyl ester. Asian J Nat Prod Biochem 21: 88-100.* The rhizospheric fungus *Fusarium oxysporum ABRF1* from the Achanakmar Biosphere Reserve, Chhattisgarh, India, was evaluated for its anticancer potency using various cellular and molecular assays. A differential cytotoxic profile of the different fractions of the *ABRF1* isolate was observed against various cancer cell lines. DNA fragmentation analysis revealed the aqueous and toluene fractions were effective and correlated well with apoptotic gene expression in several breast cancer cells. The fractions showed markedly increased expression of pro-apoptotic protein markers – BAX and cleaved caspase 3 in the breast cancer cells. Structural and functional characterization of potential secondary metabolite from the fungal isolate *ABRF1* was carried out using Gas Chromatography with Mass Spectroscopy and Nuclear Magnetic Resonance that characterized as 2-propenoic acid, pentadecyl ester. The underlying mechanisms responsible for the anticancer property were confirmed by molecular docking analysis of 2-propenoic acid, pentadecyl ester, and positive controls such as Doxorubicin and Noscapine. The results showed an efficient binding with molecular targets such as protein kinases (EphA2) and epithelial-to-mesenchymal transition markers (Vimentin) in breast cancer cells. The active metabolite 2-propenoic acid, pentadecyl ester, has a potential anticancer property that needs to be taken further for in vivo studies and drug development in the future.

Keywords: Anticancer activity, *Fusarium*, molecular docking, rhizospheric range, secondary metabolite

INTRODUCTION

The quest and search for therapeutic agents have encouraged researchers on their expedition to the most unexplored spots of nature. Fungi are the most preferred source because of the production of secondary metabolites, which have the potential as medicines for various diseases (Demain and Fang 2000). Secondary metabolites provide greater structural diversity and opportunities to discover new molecules (Arnott and Planey 2012). The proportion of natural products or their derivatives is approximately 30% of currently available drugs (Gkarmiri et al. 2017). The ease of extraction of significant fungal metabolites has paved the way for their utilization and rigorous exploration along with herbal plants. The screening, identification, and isolation of bioactive compounds are essential to meet the increasing demand for natural medicines. The microbiome in the rhizospheric region plays diverse functions in plant nutritional uptake and recycling (Berg and Koskella 2018), coping with biotic and abiotic stresses (Bakker et al. 2018; Compant et al. 2019), activation of plant defense system during the onset of infection (Castillo-González and Zhang, 2018), nitrogen mineralization (Leach et al. 2017), attracting insect pollinators (Kaiser 2006), etc. The microbiota develops competitive strategies and cooperative behaviors at intra- and inter-species levels for fulfilling nutritional requirements. They withstand ecological turbulence when cohabitating in the phyllosphere, endorhizosphere, and ectorhizosphere of soil (Hibbing et al. 2000).

Rhizospheric fungi are microorganisms that spend the whole or part of their lifecycle residing symbiotically within the healthy root tissues of higher host plants and mimic the chemistry of their respective hosts. Fungi produce various extracellular enzymes such as cellulases, amylases, pectinases, laccases, lipases, and proteinases (Meligy et al. 2014; Sahu et al. 2023a,b). These fungal enzymes play a crucial role in biodegradation and hydrolysis processes and are important against pathogenic infection and in fulfilling the nutritional needs of the host plants (Pinton et al. 2010). The rhizosphere range of the fungus produces many secondary metabolites with a biological activity that may be harmful to other organisms, such as mycotoxins and phytotoxins, or beneficial, such as antibiotics and other pharmaceuticals. These compounds interact with microorganisms, plant cells, and other fungi (Miranda et al. 2010). Secondary metabolites can regulate the prokaryotic cells and the metabolism of other fungus and plant cells. Synthesis of these low molecular weight

compounds is not required for normal growth of the fungus; however, these compounds may provide several benefits to the organism (Sahu and Jha 2020a,b). Volatile organic compounds synthesized by fungi possess antimicrobial properties and thus regulate the diversity and population of the microbiome (Dreher et al. 2019). Metabolic exchange in regulating microbial interaction involves complex regulatory mechanisms that drive the secondary metabolite biosynthesis. Bioactive secondary metabolites produced by the rhizospheric fungal communities are fundamental to various processes, such as *Trichoderma harzianum* used as a biocontrol agent, aids transport metals, antifungal, stimulates plant growth, and enhances plant biomass (Vinale et al. 2012; Braga et al. 2016). They also boost systemic disease resistance against phytopathogens, suppress necrosis in leaves, alter the plant hormone metabolism, etc. (Sarsaiya et al. 2019). Progress in the characterization of these secondary metabolites for the benefit of humanity has been progressing very slowly. The use of fungal-derived bioactive compounds as vital natural pharmaceutical biomolecules, specifically targeting anticancer properties, has been established in this study. Despite their importance and benefits, research on these fungal metabolites is limited.

Recent studies showed several bioactive compounds, including antifungal and antibacterial agents, have been isolated from fungi (Sahu et al. 2020). The promotion of plant growth is the significant contribution of fungal symbiosis. This study examined a Fusarium species isolated from untapped soil of the Achanakmar forest of Chhattisgarh (India) for its various biological properties. The crude extract of pure isolates was chosen for purification of their secondary metabolites using chromatography techniques (TLC, HPLC, GC–MS) that were then subjected to nuclear magnetic resonance (NMR) to test them for their in-vitro anticancer properties along with in-silico molecular docking was performed to strengthen the study.

MATERIALS AND METHODS

Chemicals and reagents

The solvents were of analytical grade and purchased from Merck, Germany. Potato Dextrose Agar (PDA), Czapek Dox Agar, and Potato Dextrose Broth were obtained from HiMedia, India. Doxorubicin, 2,2'azinobis (3-ethyl benzothiazole-6-sulphonic acid), and other molecular biology reagents were procured from Sigma-Aldrich, USA. Deionized water (Millipore, USA) was used throughout the experiment.

Fungal isolation and identification

Rhizospheric fungi were collected from the rhizospheric region of soil from the Achanakmar Biosphere (Bilaspur) in the central mainland of the Indian subcontinent renowned as "Herbal State-Chhattisgarh" by the method of (Radhika and Rodrigues 2010). The fungus was identified and characterized as described previously by (Sahu et al. 2020).

Extraction

Fusarium oxysporum (Isolate *ABRF1*) was initially incubated at 25 ±2°C for 15-20 days to obtain the maximum biomass of the fungus. After incubation, to obtain the promising secondary metabolites, the cultured biomass was treated with 1.5% Triton X-100 (v/v) and incubated under shaking conditions at 100 rpm for 30 min. It helped disrupt the cell wall to enhance the release of intracellular secondary metabolites. (Lin et al. 1976; McGhee et al. 1982; Robinson et al. 2001; Soccol et al. 2017). The culture media was filtered to separate the secondary metabolites from the fungal biomass (Alkhulaifi et al. 2019; Sharma et al. 2016). The collected filtrate was concentrated up to 20% of the original volume (v/v) using a vacuum rotary evaporator at 50±5°C. The concentrated extract was stored at 4°C. The working solution was prepared for subsequent experiments by diluting the concentrated extract in sterilized distilled water.

Solvent extraction process

Fungal biomass was subjected to solvent extraction using ethanol to obtain secondary metabolites. Dried ethanolic extract was purified using column chromatography with a silica gel mesh size of 60-120. One g of fungal crude extract was dissolved in ethanol (1:1 w/v) and was loaded on the column. Increasingly, polar solvents were used for elution. Every fraction was collected and spectrophotometrically evaluated. Crude ethanolic extract was purified using various eluents ranging from polar to non-polar solvents, i.e., acetonitrile, ethyl acetate, methanol, chloroform, and toluene. The fraction with maximum activity was purified further to obtain a pure compound. The ethanol used in the Soxhlet extractor was of analytical grade, and the fraction obtained was concentrated and dried in a sterilized condition.

Biochemical assay

The dried extract was dissolved in 1% DMSO (Dimethyl sulphoxide) in PBS (Phosphate buffer saline) and vortex for 1 min. Phytochemical analysis was conducted using the supernatant obtained after centrifugation at 100 g for 2 min., as described by Sahu et al. (2023a,b).

Purification of the ethanolic extract

Thin-Layer Chromatography (TLC)

TLC was used to separate the compounds of *F. oxysporum* ethanolic extract. Different column fractions of the ethanolic extract were loaded on thin-layer silica gel plates, and separation was carried out in an ascending TLC manner (Azerang et al. 2019). The ethanolic crude extract and its relatively pure column fractions from isolate *ABRF1* were spotted on a silica TLC and visualized using UV - 366 nm. The varying concentration of the mobile phase of the solvent system, i.e., Acetonitrile: Water: Acetic acid at a ratio of 18:80:2 v/v, accelerated the release of different compounds from the extract. The air-dried chromatogram was subjected to an iodine chamber to evaluate the TLC profile. Fractions with similar TLC profiles were pooled together, and forming a single spot suggested the relative purity of the compound.

High-Performance Liquid Chromatography (HPLC) analysis

The purity of the secondary metabolites was validated by HPLC using a C_{18} column (Shimadzu Liquid Chromatography LC10A; Shimadzu Corp. Kyoto, Japan). HPLC-grade methanol was used as a solvent for dissolving the extract in a ratio of 1:1 w/v. 20µl sample was injected, and the elution flow rate was set at 1mL/min through mobile (an isocratic) phase, i.e., Acetonitrile: Water: Acetic acid at the ratio 18:80:2 v/v. The chromatogram profile was analyzed at 280 nm. A calibration curve was prepared using standard catechin and phloroglucinol (200 ppm/10 µl of injection volume) as described in (Shen et al. 2007).

Spectroscopic method of compound identification

Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectroscopy was carried out (Bhat 2013; D'Souza and Kamat 2017) to determine functional groups present in the extract. FTIR spectrometer was used to record the infrared transmittance at a wavenumber ranging from 4000-400 cm^{-1} of the purified compounds (FTIR, I05 Nicolet Avatar 370, Thermo Scientific, USA). Next, 5 mg of the pure compound was mixed with 95 mg KBr spectroscopic grade for pellet preparation. The pellet and control KBr pellets were used for analysis.

Gas Chromatography-Mass Spectroscopy (GC-MS)

The qualitative and quantitative analysis of the compounds in the extract was carried out using Shimadzu GC-MS-QP2020, Kyoto, Japan. The electron impact ionization was taken at 70eV, and the mass ranged from 40-500 amu. Comparison between the relative index and mass spectra obtained to that of the available standards in the GCMS library of NIST, Gaithersburg, Maryland, USA, led to identifying compounds in the extract (Leiss et al. 2011).

Nuclear Magnetic Resonance (NMR) spectroscopy

An NMR spectrometer (Bruker advance III 400 MHz) was used to obtain the ^1H NMR spectra of the isolated molecule at the Vellore Institute of Technology, Vellore, India. The solvent used for analysis was DMSO. Chemical shifts were expressed as parts per million (į scale) (Hoeksma et al. 2019).

Bioactive efficacy of the isolated secondary metabolites

Anticancer activity

MTT assay was used to determine anticancer activity as described previously by (Geesala et al. 2016). Anticancer analysis of the fungal extracts and fractions were studied by using different human cancer cell lines such as prostate cancer (*DU-145*), liver cancer (*HepG2*), primary control cell line (*HEK-293*), breast cancer (*MDA-MB-231, MDA-MB-468*, and *MCF-7*), and lung cancer (*A-549*). Cells were briefly trypsinized, and 5×10^3 cells per well were seeded in a 96-well plate. After 24h of incubation, cells were treated with increasing concentrations (0.1, 1, 10, and 100 mg/mL) of all the column fractions, viz. water, chloroform, toluene, ethyl acetate, acetonitrile, and methanol for 48 h followed by fixation of the treated cells along with their respective vehicle controls. The optical density was measured at 510 nm using a multimode reader (Perkin Elmer, Germany), and percent inhibition with IC_{50} was calculated using the Graph pad prism described by Manupati et al. (2019).

DNA fragmentation apoptosis assays

Luminal and Triple Negative Breast Cancer cells, *MCF-7* and *MDA-MB-231*, respectively, were seeded at a density of 0.5×10^6 cells for 24h in complete media to assess the degree of apoptosis induced by the various fractions of *F. oxysporum ABRF1*. Fractions B and C were excluded from further assays as they exhibited cytotoxicity against control *HEK293* cells. The cells were then treated for 24 h with the fractions (50 µg/mL) and positive control, doxorubicin (0.543 µg/mL). After incubation, DNA was extracted from the apoptotic cells using a standard protocol as described previously (Matassov et al. 2004). The concentration of DNA was quantified using a NanoDrop-1000 spectrophotometer (NanoDrop, Wilmington, USA), and 5 µg of DNA was loaded in 1% agarose gel containing ethidium bromide, electrophoresed, and examined under UV radiation to determine DNA fragmentation.

Gene expression analysis

MCF-7 and *MDA-MB-231* were seeded at a density of 0.25×10^6 cells for 24h in a 6-well plate, followed by treatment using the fractions and/or doxorubicin for 24h. Total RNA was extracted using RNA iso plus (Takara Bio, Shiga, Japan), and the mRNA present in the total RNA was used as a template for cDNA synthesis (Puregene, Delhi, India). qRT-PCR analysis of various apoptotic genes was performed using human-specific primers and eukaryotic 18S rRNA as an internal control.

Immunoblot analysis

MDA-MB-231 cells were seeded with 0.5×10^6 cell density and exposed to various fractions and/or doxorubicin for 48h. Total protein was extracted using RIPA lysis buffer supplemented with a protease inhibitor cocktail (Puregene, Delhi, India) and subjected to SDS-PAGE. The protein was then transferred to a PVDF membrane and probed for the presence of apoptotic markers BAX, BCL2, and cleaved Caspase3 (Cell Signaling Technologies, Boston, USA). The β-tubulin expression served as the loading control. Bands were visualized using HRP-based chemiluminescence in a G: Box (Syngene, USA). The density of bands in the obtained images was analyzed using NIH ImageJ software after normalization against β-tubulin.

Molecular docking in silico studies

The interaction of 2-Propenoic acid, pentadecyl ester with anticancer signaling pathways was analyzed by the Molecular Operating Environment (MOE) software compendium (licensed and owned by Chemical Computing Group, Montreal, Canada) (Naik et al. 2011). The targeted protein structures were retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB PDB). Molecular modeling was performed using the docking package *Induced Fit*, a refinement method of MOE (Huo et al. 2020). Protonate 3D application of the software-enabled addition of hydrogen and partial charges to the system

eliminated the bond length and angle biases. A Gaussian surface was generated by Surface and Maps, which was available in the Compute feature, which rendered the binding site and removed the backbone.

MOE/Compute/Simulations/Dock was the location for performing docking. Still, it was based on specific parameters, which were set manually. Hence, the residues close to the ligand atoms defined the docking site, and refinement in the force field minimized the energy in the receptor pocket. The database browser was generated with an S field with the calculated binding free energy identical to the E_refine score. RMSD (root mean square deviation) score was also developed, which quantitatively measured the similarity between two superimposed atomic coordinates. The ligand interaction feature generated the interaction map between the ligand and the receptor's active site, deciphering the participating amino acids and involving chemical bonds.

Focal Adhesion Kinase (RCSB PDB ID: 1MP8), C-Myc promoter (RCSB PDB ID: 6AU4), Vimentin coil 1A/1B (RCSB PDB ID: 3SSU), and TOM1 protein (RCSB PDB ID: 1ELK) were selected as targets for anticancer analysis as these are major components of cellular growth and division and can be modulated by metabolites to regulate their activity.

Statistical analysis

Data were presented as the mean ± standard deviation (SD, n = 3). Statistical difference between various groups was determined by One-Way Analysis of Variance (ANOVA) followed by Duncan multiple range tests. P values ≤ 0.05 were considered statically significant.

RESULTS AND DISCUSSION

Taxonomic, phylogenetic, and biochemical properties of the isolated fungus

Based on morphological and molecular characterization, the fungal isolate ABRF-1 was identified as *F. oxysporum.* The crude extract from the *F. oxysporum* (Figure 2.A) was further analyzed for its chemical compounds using spectroscopic techniques. Biochemical analysis of the crude extract and ethanol acetate, toluene, methanol, chloroform, and acetonitrile fractions was performed. The alkaloid, carbohydrate, cardiac glycosides, coumarins, cyanogenic glycosides, emodins, polyose, polyuronide, and anthraquinones were observed in respective fractions. Chloroform and acetonitrile fractions were rich in biomolecules, as represented in Table 1. One potent compound detected using the standard mass spectrometry databases (Mass Centre and Xcalibur software) (Figure 2.B) was 2-propenoic acid, pentadecyl ester.

Characterization of fungal compounds

Analysis of secondary metabolites using TLC

Fraction on TLC separation has fluorescent spots (spots A and B) with the Rf value of 0.281 (A1) and 0.351 for spot A2. Spots B3-B5 had retention times of 0.233, 0.252, and 0.334. respectively (Figure 2.C).

Identification of fungal secondary metabolites using HPLC

The HPLC analysis of *F. oxysporum* fungus with gradient elution showed nine peaks in spectra of ethanolic extract (Figure 1.A). The peaks with the voltage of 0.5167, 2.5833, 2.8333, 3.55, 3.9, 4.6667, 4.9167, 5, and 9.1333 were observed, and the compound with Rf values of 2.8667 and 3.0833 (Table 2).

GC-MS analysis of the compound isolated from *Fusarium oxysporum ABRF1*

GC-MS analysis of the ethanolic fungal extract and its purified fractions indicated the presence of six major peaks (Figure 1.B). Components and their respective peaks are presented in Table 3. 2-propenoic acid, pentadecyl ester, was the metabolite that was selected to test for its biological activity l. The molecular weight of the compound 2-propenoic acid, pentadecyl ester, was 282 kDa (Figure 1.C).

FTIR analysis of pure compounds

Spectral windows of the aqueous and toluene fractions had chemically significant regions corresponding to C-H (3450- 2850cm-1), amide region dominated by C=O amide I and N-H amide II (Table 4). Furthermore, it also included protein and peptides (1800-1500 cm^{-1}), mixed region (1500-1200 cm^{-1}), polysaccharides region (1200-900 cm^{-1}), and true fingerprint region (900-700 cm^{-1}) (Figure 1.D-E).

Table 1. Secondary metabolites in the fractions of *F.oxysporum* and their respective rich column fraction

Test	Toluene	Chloroform	Ethyl acetate	Methanol	Acetonitrile
Alkaloid Test	+	++++	+	-	++++
Carbohydrate	-	++++	+	-	++++
Cardiac glycosides	-	+++	++	++	++
Coumarins	+	++++	++	+	+++
Cyanogenic glycosides	-	-	-	-	-
Emodins	-	++++	+	+	+++
Polyoses	-	+++	++++	+	+++++
Polyuronoids	+	+++++	++	++	+++++
Anthraquinones	-	-	-	-	-

Note: -: Absent, +: Present, ++: Low concentration, +++: High concentration, ++++: Very high concentration, +++++: Very very high concentration

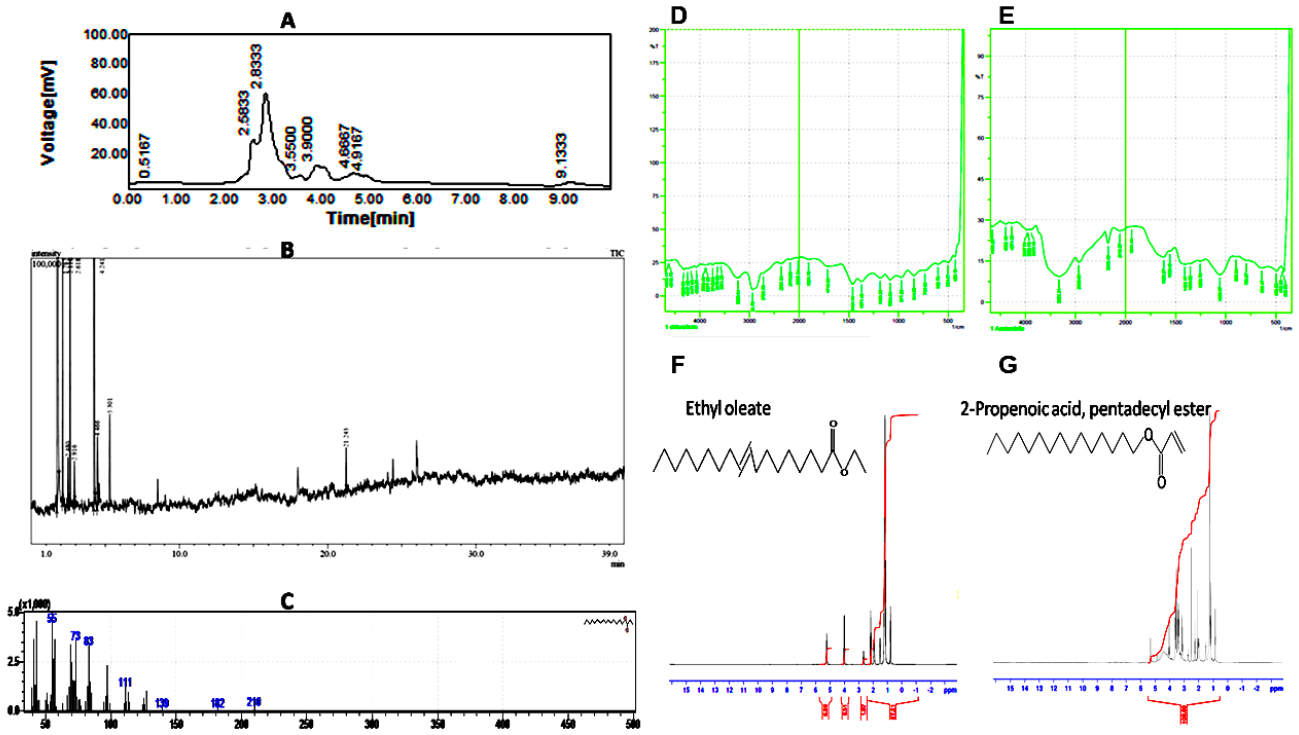

Figure 1. Structure elucidation of 2-propenoic acid, pentadecyl ester with different spectroscopic methods (A) High-Performance Liquid Chromatography (HPLC) chromatogram profile of *Fusarium oxysporum* extract, (B) Chromatogram profile of the *F. oxysporum* extract using GC/MS analysis, (C) Mass spectrum depicting the molecular weight of 2-propenoic acid, pentadecyl ester, (D) FTIR spectrum of chloroform, (E) acetonitrile fractions of column fraction of strain *ABRF1*, and (F) NMR spectrum of the standard ethyl oleate compared with (G) the obtained compound 2-propenoic acid, pentadecyl ester

Table 2. Representing retention time of different secondary metabolites by HPLC analysis

Peak no	RT [min]	Area[mV*sec]	Area%	Height[mV]	Height%
1	0.51	2.90	0.14	0.059	0.05
2	2.58	314.92	14.79	29.40	24.55
3	2.83	1,206.00	56.62	60.34	50.37
4	3.55	68.09	3.20	5.54	4.63
5	3.90	272.76	12.81	11.63	9.71
6	4.66	152.82	7.18	6.21	5.19
7	4.91	50.061	2.35	4.48	3.74
8	9.13	62.36	2.93	2.10	1.76
		2,129.95		119.78	

Table 3. Showing retention time of obtained secondary metabolites and suggesting their possible name by GCMS library of NIST

Peak	R. Time	Area	Height	Name
1	1.78	34,815,263.00	10,119,374.00	Ethanol
2	2.11	114,479.00	90,047.00	Allyl fluoride
3	2.48	25,042.00	17,032.00	Ethyl acetate
4	2.61	248,635.00	171,088.00	1-propanol, 2-methyl
5	2.91	20,774.00	14,772.00	Ethanol, 2-nitro
6	4.24	468,393.00	225,979.00	Ethane, 1, 1-diethoxy
7	4.46	62,871.00	24,547.00	1-butanol, 3-methyl
8	5.30	70,597.00	31,554.00	Toluene
9	21.24	21,897.00	14,406.00	2-propenoic acid, pentadecyl ester
		35,847,951.00	100.00	

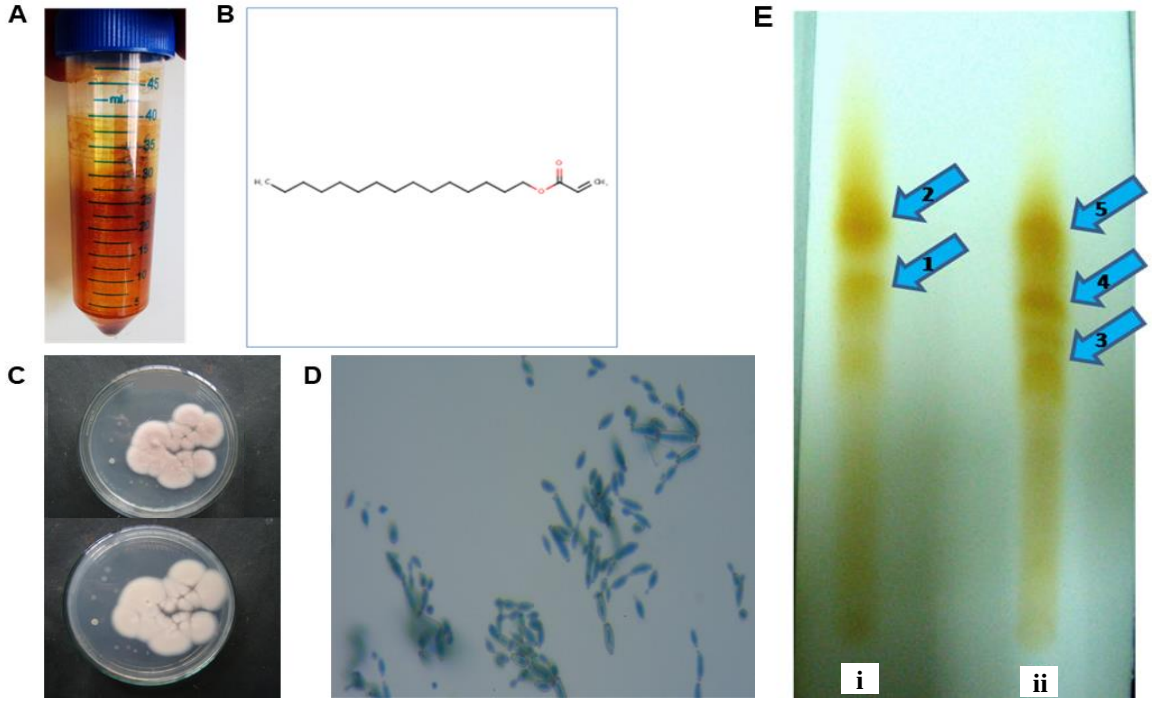

Figure 2. Isolation and Characterization of Secondary Metabolites from *Fusarium oxysporum* (A) Crude extract obtained from the fungus *Fusarium oxysporum* ABRF1 (B) Predicted structure of 2-propenoic acid, pentadecyl ester (C) Colony morphology of the isolated *Fusarium* sp. ABRF1 (both front and back views) (D) Micrograph illustrating the morphological characteristics of *Fusarium* sp. ABRF1 (E) Chromatogram of the (i) chloroform and (ii) acetonitrile soluble fractions obtained from the ethanolic extract of *Fusarium oxysporum*

^{1}H NMR

Column fractions were subjected to structural characterization using the NMR spectrum, and the following peaks were observed at a mixture of į values of proton ^{1}H. The antibacterial activity compound of the secondary metabolite may be due to the presence of methyl, ketone, and hydroxyl functional groups, as predicted from the data. ^{1}H NMR spectrum exhibited the presence of functional groups such as CH2, CH3, OH, and C-H protons. Primary data of the compound of the isolated compound showed the presence of Carbon, Hydrogen, and Hydroxyl groups from the ^{1}H NMR spectrum, which are indicative of ester 2-propenoic acid, pentadecyl ester being (Figure 1.F). This compound also showed the presence of a wide peak at 0.9, 1.3, and 1.5δ, which are primary, secondary, and tertiary aliphatic clusters, respectively. The carbonyl groups at 2.2δ and one of the hydroxyl groups were intact at 3.5, and a few other chemical shifts were also observed. The esters group at 4.1δ confirmed the presence of 2-propenoic acid, pentadecyl ester (Figure 1.G).

Anticancer activity

The intracellular and extracellular secondary metabolites of *ABRF1* and the ethanolic fraction were screened for cytotoxicity against *MCF-7*, *MDA-MB-231*, *DU-145*, *HepG2*, and *A549* cell lines. The extracellular fraction was more potent than the intracellular fraction in inhibiting all cell lines except HepG2 (Table 5). This finding corroborates that most microbes use their extracellular metabolites to communicate with their environment and release these metabolites in response to stress. The ethanolic fraction was highly effective in controlling the proliferation of *DU-145* but did not against *MCF-7*, *MDA-MB-231*, *HepG2*, and *A549* cells (Table 5).

Fraction A (aqueous fraction) had moderate anticancer potential against the *DU-145* prostate cancer and HepG2 liver cancer cell lines with IC_{50} values of 26.2 and 38.8 µg/mL, respectively. However, fraction B (methanol fraction) was moderately potent against the *MCF-7* breast cancer cell line with an IC_{50} value of 24.2 µg/mL. Fraction C (chloroform fraction) had an IC_{50} of 9.4 µg/mL against MDA-*MB-468.* Fraction D (toluene fraction) was moderately active against *MCF-7* with an IC_{50} value of 33.4 µg/mL). The results showed the presence of anticancer components in the fraction of *F. oxysporum ABRF1* with low toxicity against *HEK-293* normal primary cells, except fractions B and C (Table 6).

Apoptosis assays

DNA fragmentation analysis

DNA fragmentation indicates early apoptosis in which cellular endonucleases cleave genomic DNA into fragments between the nucleosomes. After treatment with various fractions, the DNA integrity was obtained in MCF-7 and MDA-MB-231 cells. Fractions D and E caused DNA damage in *MCF-7* cells, while fractions A, D, and E caused DNA cleavage in *MDA-MB-231* cells (Figure 3).

Gene expression analysis

TP53 expression was significantly upregulated by fraction A in *MCF-7* and fractions A and E in *MDA-MB-231* cells (Figure 4.A). Fractions A, D, and F significantly downregulated the expression of a *BCL2* anti-apoptotic gene (Figure 4.B) in *MDA-MB-231* cells. In the *MCF-7* cells, fractions A and D treatment resulted in significant upregulation of both *BID* (Figure 4.C), *BAD* (Figure 4.D), and *BAX* (Figure 4.E). Fractions A and D stimulated the expression of *CASP9* in both *MCF-7* and *MDA-MB-231* (Figure 4.F). Interestingly, significant upregulation of the expression of pro-apoptotic genes was observed in the *MDA-MB-231* after treatment of positive control, doxorubicin.

Protein markers

The gene expression results were further corroborated using immunoblot analysis (Figure 5.A). The anti-apoptotic marker, BCL2 (Figure 5.B), was downregulated in all fractions. In contrast, the pro-apoptotic marker, BAX (Figure 5.C), was significantly upregulated in *MDA-MB-231* cells treated with fraction D. Fraction D also exhibited upregulation of cleaved Caspase3 compared to untreated cells normalized against control β-tubulin (Figure 5.D).

In silico anticancer analysis by Molecular Docking

The binding energy score mimics the potential energy change when the protein and ligand come together. This means that a very negative score corresponds to a strong binding, and a less negative or even positive score corresponds to a weak or non-existing binding. The binding energy or ΔG_{bind} value for target 1MP8 (specific for *MCF-7*) was classified as transferase, i.e., a protein kinase was found to be -10.2313 KCal/mol with 2-propenoic acid, pentadecyl ester specifically at Site 2, and with doxorubicin, it showed the best ΔG_{bind} i.e., -26.9348 kCal/mol (Fig. 4A). The direct bond-forming residues on the active site were Ala 549, Leu 507, Lys 561, Val 554, Gly 563, Asp 546 Trp 588, Glu 614, and Ala 548.

The binding energy for target 1ELK was observed to be -11.3677 KCal/mol with 2-propenoic acid, pentadecyl ester specifically for Site 1, and that of doxorubicin (maximum affinity) and noscapine had lower binding energy scores of -26.9388 KCal/mol and -19.5897 KCal/mol, respectively. Hydrogen bond formation involved direct interaction with Thr A102, Ile A103, Asn A108, and Lys A62. However, Glu B44, Arg B94, and Asn B91 interacted with molecules via water hydration (Fig. 4B).

The binding energy or ΔG_{bind} value for target 6AU4 (specific against cell line *DU-145*) which forms DNA quadruplex in c-Myc promoter's NHE III1 region was -10.7394 KCal/mol with 2-propenoic acid, pentadecyl ester specifically at Site 2 (Tables 7, 8, and Figure 5.C) as compared with Doxorubicin (-15.8666 Kcal/mol) and Noscapine (-10.9021 KCal/mol). The amino acids located at the active site of the receptor-ligand interaction were DG B2, DG B11, DG B13, DG B14, DG B15, DG B17, DG B18, DG B19, and DA A22 (Figure 6.C).

The binding energy or ΔG_{bind} value for target 3SSU was observed to be -7.9592 KCal/mol with 2-propenoic acid, pentadecyl ester specifically for Site 1. In comparison, Doxorubicin and Noscapine showed the lowest binding energy score of -10.5860 KCal/mol and -8.6372 KCal/mol, respectively, and maximum affinity with Site 1 (Figure 6.D). Hydrogen bond formation involved direct interaction with residues Leu A164, Asp A167, Asp A162, and Arg A159 (Figure 6.A).

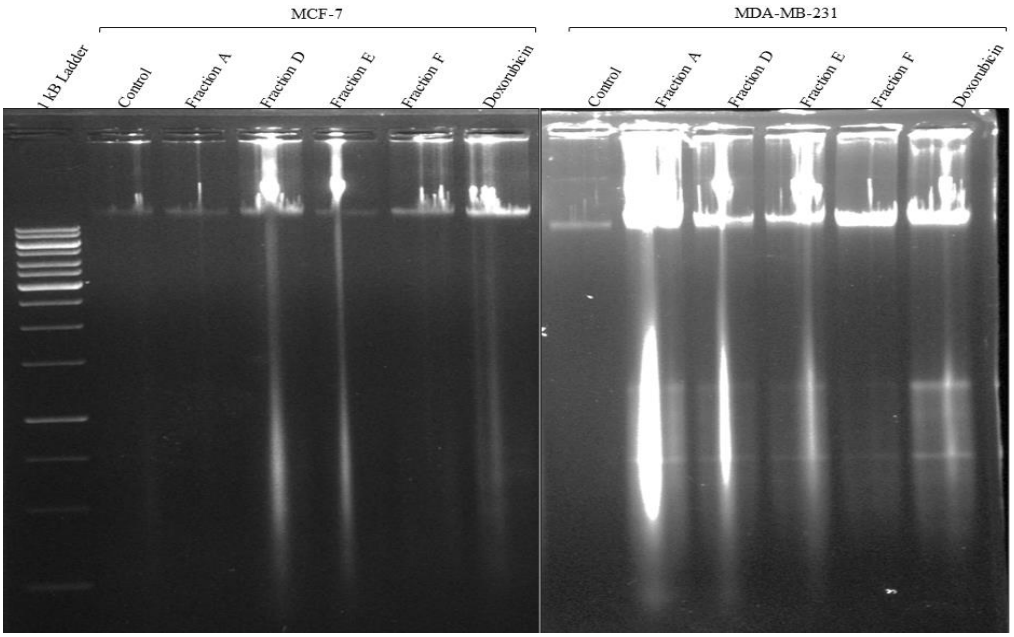

Figure 3. Differential apoptosis of breast cancer cells using DNA fragmentation analysis. Representative photomicrographs depicting DNA fragmentation of MCF-7 (left panel) and MDA-MB-231 (right panel) in presence of various solvent fractions of the isolate, ABRF1. Fraction D and E caused DNA fragmentation in both the types of breast cancer cells. Additionally, fraction A showed marked increase in DNA fragmentation in the MDA-MB-231 cells. Data represented are results of three independent experiments

Table 4. The wavenumber ranges for common functional groups

S. No.	Column Fraction	Wavenumber (cm^{-1})	The Functional Group Identified/Peak Description
1	Obtained peak from acetonitrile fraction of isolate ABRF1	1058.97	CH_2 rocking
2		1058.97	C-O stretching
3		1253.78	C-Oh rocking C-C stretching due to carboxylic acid, ether alcohol, and esters
4		1356.02	C-H scissoring and bending vibrations
5		1406.17	COO- symmetric stretching
6		1620.27	NH_3^+ asymmetric deformation
7		1941.44	NH_3^+ asymmetric deformation
8		2349.4	C-O bond
9		2933.85	CH_2 Asymmetric stretching
10		3328.31	O-H stretching vibration of hydroxyl groups
11		3875.16	O-H stretching-free hydroxyl
12		3939.78	O-H stretching-free hydroxyl
13		3967.74	O-H stretching-free hydroxyl
1	Obtained peak from chloroform fraction of isolate ABRF1	1084.04	C-N symmetric stretching
2		1183.38	C-O bond
3		1371.45	O-H bending polysaccharide
4		1461.14	COO- symmetric stretching
5		1710.93	C=O carbonyl stretching of esters
6		1904.79	NH_3^+ asymmetric deformation
7		2025.34	C≡C alkyne stretching
8		2179.66	C≡C stretch due to alkyne
9		2349.4 2361.94	C-O bond
10		2725.53	Not useful
11		2936.75	OH stretch due to carboxylic acid
12		3229.94	OH stretch due to carboxylic acid
13		3574.25	O-H stretching-free hydroxyl
14		3640.8	O-H stretching-free hydroxyl
15		3742.06	O-H stretching-free hydroxyl
16		3877.09	O-H stretching-free hydroxyl
17		3893.48	O-H stretching-free hydroxyl

Table 5. IC_{50} value of intracellular, extracellular metabolites and ethanolic extract of isolate *Fusarium oxysporum ABRF1* against several cancer cell lines

Fungal Extract	IC_{50} (µg/mL)				
	MCF-7	MDA-MB-231	DU-145	HEPG2	A549
Extracellular secondary metabolites	26.09 ± 1.3	23.22 ± 1.15	15.66 ± 0.78	1807.5 ± 375.57	31.75 ± 1.5
Intracellular secondary metabolites	≥1000	101 ± 5	≥1000	830.45 ± 1.13	≥1000
Ethanolic extract	NI	NI	2.3695±0.156	NI	NI

Table 6. IC_{50} value of *F. oxysporum ABRF1* fractions against various cancer cell lines

Fungal Fraction	IC50[a] (µg/mL)						
	MCF-7[b]	MDA-MB-468[c]	MDA-MB-231[d]	DU-145[e]	HepG-2[f]	A-549[g]	HEK-293[h]
Fraction A	150.23±7.25	136.5± 6.86	106.41±14.61	26.21±4.29	38.8±5.43	89.13±3.16	NI
Fraction B	24.17±6.94	107.03±7.79	82.68±19.24	60.98±11.18	103.86±9.61	58.92±19.81	135.4±9.636
Fraction C	176.16±23.9	9.37±3.66	NI	192.46±17.02	441.56±72.6	149.79± 35.20	173.16±14.67
Fraction D	33.43±6.55	163.45±5.99	67.45±17.90	67.45±17.90	130.13±8.62	47.96±4.19	NI
Fraction E	54.04±8.02	66.81±5.32	61.19±10.92	987.33±104.14	334.56±144.76	56.64± 13.49	NI
Fraction F	110.34±12.8	75.53±4.53	59.95±9.61	85.63±22.94	252.63± 48.97	51.80±11.49	NI

Note: NI: No inhibition; Fractions A: Water; B: Methanol; C: Chloroform; D: Toluene, E: Ethyl acetate, F: Acetonitrile. Note: [a]50% inhibitory concentrations and mean ± SEM of IC50 (µg/mL) values of different fractions represent the mean of three individual experiments: [b]Luminal-A (ER+/PR+/Her2−) breast cancer; [c]Basal (low claudin) triple-negative (ER−/PR−/Her2−) breast cancer; [d]Basal triple-negative (ER−/PR−/Her2−) breast cancer; [e]Moderate metastatic potential (PSA+) androgen-independent prostate cancer; [f]Liver hepatocellular carcinoma; [g]Adenocarcinoma human alveolar basal epithelial cells lung cancer; [h]Non-cancerous primary human embryonic kidney cells

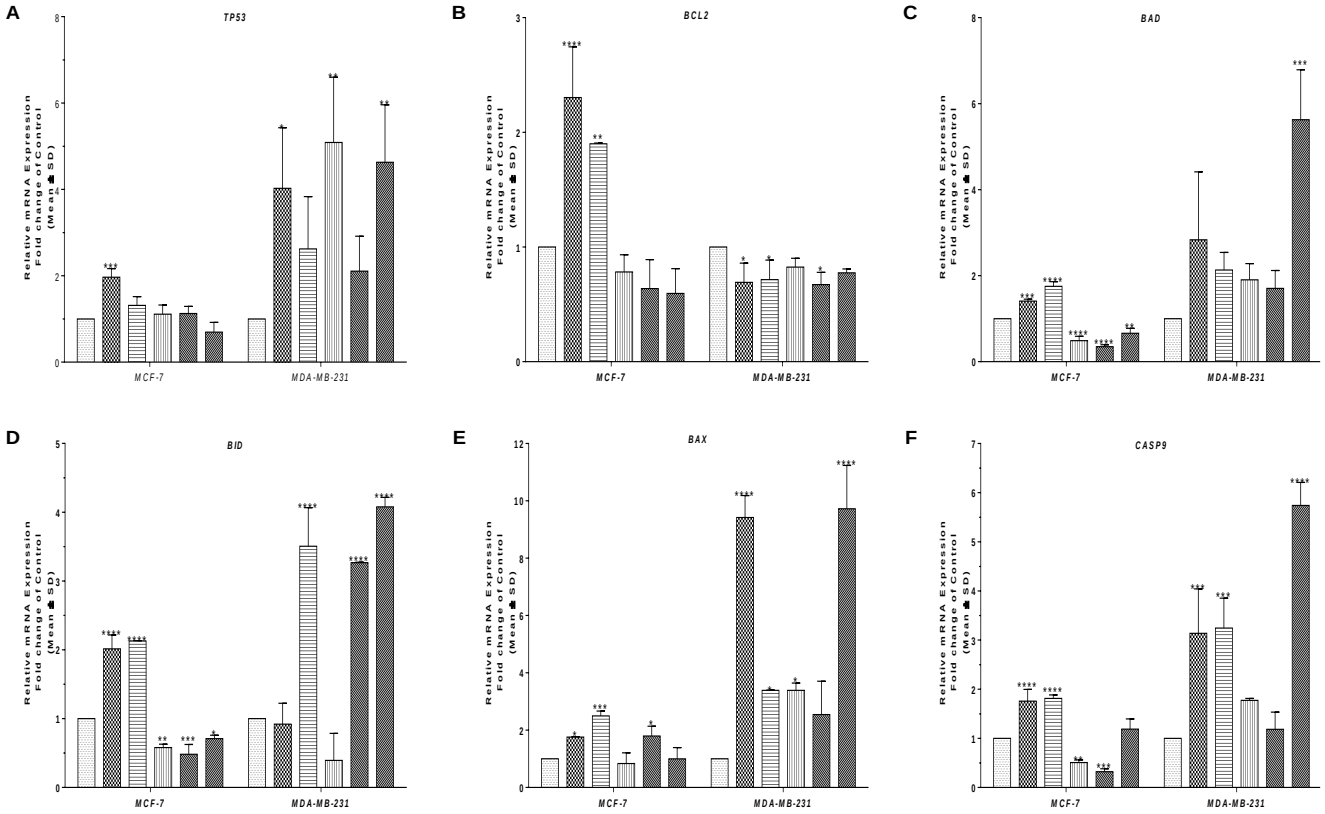

Figure 4. Differential apoptotic marker expression profile. Graphs show the gene expression profile of (A) TP53, (B) BCL2, (C) BAD, (D) BID, (E) BAX, and (F) CASP9 in luminal MCF-7 and TNBC MDA-MB-231 breast cancer cell lines upon treatment with various fractions of ABRF1. Data represented are results of experiments repeated thrice (*p ≤ 0.05, **p ≤ 0.01 ***p ≤ 0.001 and ****p ≤ 0.0001 as compared with their respective control)

Table 7. *In silico* anticancer studies of the compound with different targets in respect of binding energy and number of direct contacts (all polar, non-polar interactions)

Compound Name	Therapeutic Studies	Max Affinity with Site	Binding Energy (kCal/mol)	No. of Direct Contacts (All Polar, Non-Polar Interactions)
2-propenoic acid, pentadecyl ester with 6AU4: DNA	Anticancer study	Site-2	-10.7394	DG B2, DG B11, DG B13, DG B14, DG B15 DG B17, DG B18, DG B19, DA A22 (direct contacts).
2-propenoic acid, pentadecyl ester with 1MP8: Transferase		Site-2	-10.2313	Ala 549, Leu 507, Lys 561, Val 554, Gly 563, Asp 546 Trp 588, Glu 614, Ala 548 (direct contact).
2-propenoic acid, pentadecyl ester with 3SSU		Site-1	-7.9592	Leu A164, Asp A167, Asp A162, Arg A159 (direct contact).
2-propenoic acid, pentadecyl ester with 1ELK		Site-1	-11.3677	Glu B44, Arg B94, Asn B91 (water hydration). Thr A102, Ile A103, Asn A108, Lys A62 (direct contact).

Table 8. A comparison between binding energy score in kCal/mol for the compound 2-propenoic acid, pentadecyl ester in anticancer targets active site and to identify the probable mechanism of action

Molecule	Binding Energy (kCal/mol) of Different Targets for Anticancer Molecular Docking			
	6AU4 (SITE 2)	1MP8 (SITE 2)	3SSU (SITE 1)	1ELK (SITE 7)
Doxorubicin	-15.8666	-26.9348	-10.5860	-26.9388
Noscapine	-10.9021	-18.8507	-8.6372	-19.5897
Pentadecyl acrylate	-10.7394	-10.2313	-7.9592	-11.3677

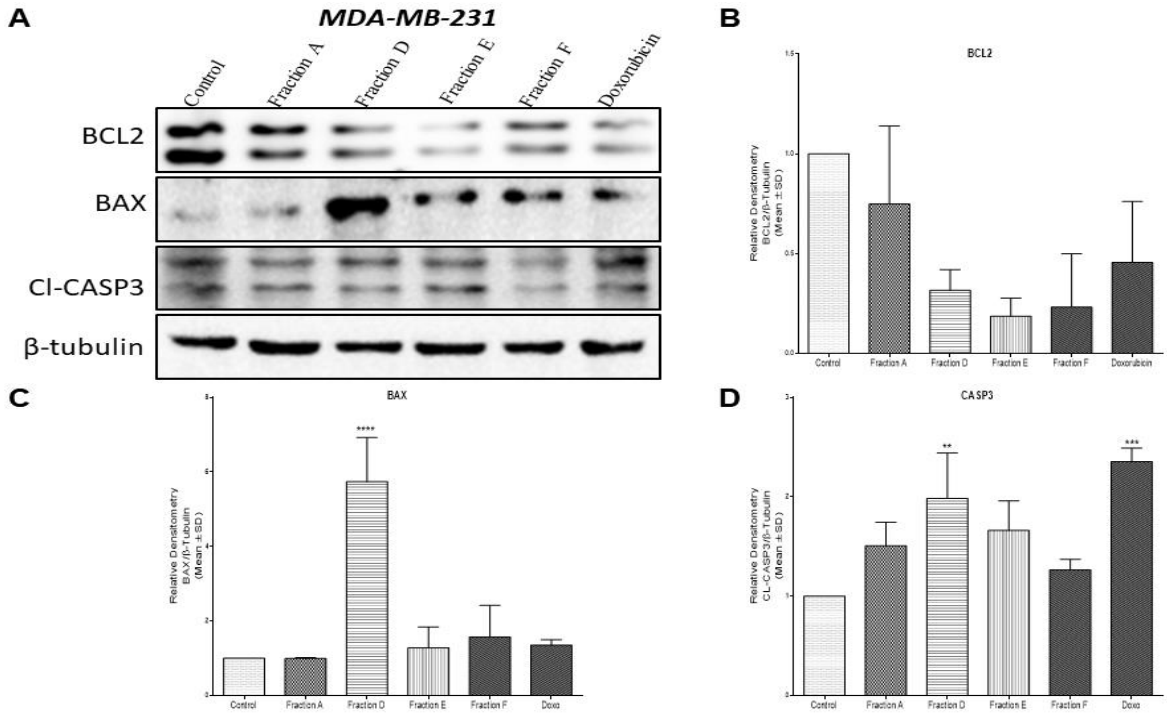

Figure 5. Apoptotic protein expression analysis. (A) Representative image of immunoblot analysis of MDA-MB-231 cells treated with various fractions of ABRF1 and positive control doxorubicin. Quantification of protein bands (B) BCL2, (C) BAX, and (D) Cl-CASP3 using NIH ImageJ software normalized against internal loading control β-tubulin. Data are represented as mean ± SD from three replicates, $^{**}p \leq 0.01$ $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$ compared to control

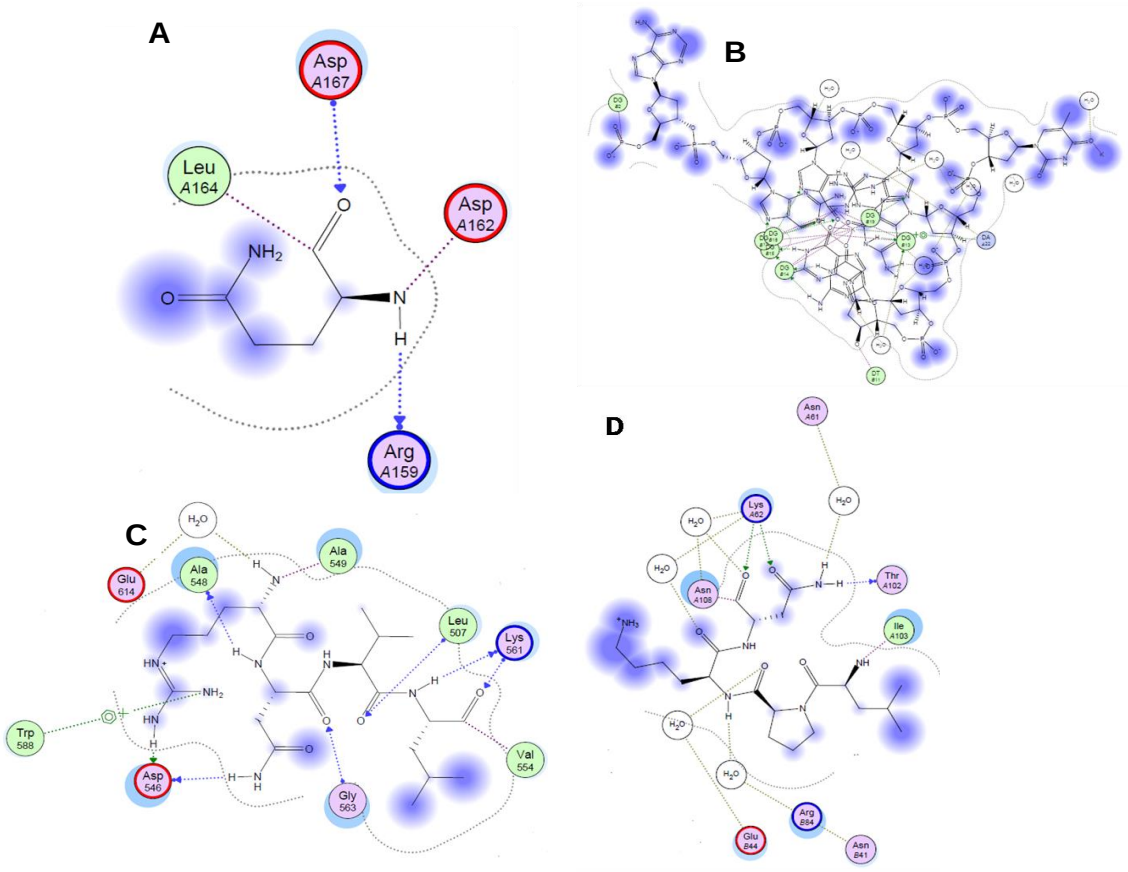

Figure 6. Molecular docking and 2D interaction diagram of 2-propenoic acid, pentadecyl ester.Interactions of 2-propenoic acid, pentadecyl ester with (A) major quadruplex formed in the human c-MYC promoter (RCSB ID:6AU4), (B) Focal Adhesion Kinase (FAK) (RCSB ID:1MP8), (C) central helical domain of vimentin (RCSB ID:3SSU), and (D) VSH domain of TOM1 (RCSB ID:1ELK).

Discussion

The present study isolated and characterized bioactive secondary metabolites from *F. oxysporum ABRF1)* (Wyatt et al. 2013; Gautier et al. 2016). The bioactive metabolites of the crude extract from the *F. oxysporum* were identified using TLC, HPLC, and FTIR. The structure of the bioactive molecule was identified as 2-propenoic acid, pentadecyl ester, using the standard mass spectrometry databases viz. GC-MS and NMR. The secondary metabolite was hypothesized to have drug-likeliness based on SWISS ADME and moderate solubility in water. Based on the BOILED-Egg model, the 2-propenoic acid pentadecyl ester was predicted to be actively absorbed through the gastrointestinal tract and penetrate the blood-brain barrier. The molecule is a lead candidate with a carrier required for drug targeting.

The isolated bioactive metabolite was evaluated for anticancer properties mainly focused on DNA fragmentation apoptosis assay, gene and protein expression analysis, and *in silico* validation with molecular docking studies. The secondary metabolite from *F. oxysporum ABRF1)* led to DNA fragmentation that was observed as a smear and not in the distinctive ladder pattern of 180-200 bp fragments as the cells underwent apoptosis asynchronously (Matalová and Španová 2002). Fragmentation of nuclear DNA triggers the intrinsic apoptotic pathway in the cell. The interactions between various members of BCL2, anti-apoptotic - BCL2, and pro-apoptotic markers – p53 that initiate oligomerization sensitizer – BAD, activator - BID, and pore-former – BAX cause increased mitochondrial outer membrane permeability and release of cytochrome-c into the cytosol. Cytochrome C initiates the caspase cascade that digests proteins needed for cellular functioning and triggers apoptosis (Kale et al. 2018). Cyt C oligomerizes with APAF1 in the cytoplasm to form the apoptosome that activates Caspase9, activating the executioner Caspase3 and triggering cell death (Ledgerwood and Morison 2009).

Several diseases, such as inflammation, carcinomas, and metabolic pathways, have been targeted using inhibitors of protein kinases (Bhullar et al. 2018). EphA2 (Ephrin receptor A2), Aurora-A, and FAK (focal adhesion kinase) are protein kinases reported to be involved in cancer. High expression profiles of these protein kinases in human tumors suggest that their inhibitors may be a potent candidate for cancer treatment (Gross et al. 2015). TOM1 protein or Target of Myb protein 1 is reported to play a vital role in processes such as intracellular trafficking, the dilapidation of GFR (growth factor receptor) complexes through translocation into the lysosome, endocytosis, neutrophil degranulation, and endosomal & protein transport (Keskitalo et al. 2019). TOM1 is a potential target against the *MCF-7* breast cancer cell line (Chevalier et al. 2016).

Fusarium species are a significant source of cytotoxic metabolites, e.g., camptothecin, taxol, baccatin III, phelligridin B, and metacytofilin (Noman et al. 2021; Tapfuma et al. 2019). The initial screening of the fractions of *F. oxysporum* isolate-*ABRF1* showed anticancer potential. Subsequently, a differential cytotoxic profile of the various fractions was observed against several cancer cell lines. The suspected mechanism of cytotoxic activity of these fractions was assessed by analyzing their impact on DNA fragmentation, apoptosis-related genes, and protein expression. DNA fragmentation indicates early apoptosis, wherein endonucleases break down genomic DNA into fragments between the nucleosomes. Fragmentation of nuclear DNA triggers the intrinsic apoptotic in the cell. One of the common therapeutic fungi, the *Ganoder macurtisii strain* (GH-16-015), showed anticancer activity against a wide range of cancer cell lines - *A549*, *HBL-100*, *HeLa*, and *T-47D* (Serrano-Márquez et al. 2021). Altersolanol B from *Stemphylium solani* induced intrinsic apoptosis in *MCF-7* cells through downregulation of anti-apoptotic BCl-2 and upregulation and activation of caspase 9, poly (ADP-ribose) polymerase (PARP) and pro-apoptotic Bax (Siraj et al. 2022). The results of this study showed that the identified secondary metabolite, 2-propenoic acid, pentadecyl ester downregulated the expression of an anti-apoptotic marker, BCL2, and upregulated the pro-apoptotic markers - *BID*, *BAD*, *BAX*, and *Caspase9*. The anticancer properties of the metabolites may be attributed to the binding of these molecules with specific targets and their consequent modulation to regulate metabolic and molecular processes.

The present study further validated the proposed mechanism by protein marker assay and molecular docking analysis. The protein markers corroborated the gene expression results, showing the downregulated BCL2 and significantly upregulated BAX and activation by cleavage of Caspase 3 in *MDA-MB-231* cells. The identified secondary metabolite, 2-propenoic acid, pentadecyl ester, was subjected to molecular docking on selected targets and compared to standard drugs, Doxorubicin and Noscapine. An effective interaction between the secondary metabolite and these cancer-related molecular targets exists. We hypothesize that 2-propenoic acid, pentadecyl ester, interacts with c-myc and other cell cycle regulatory targets and controls DNA double-strand break repair gene expression (Chen et al. 2018). It renders cancer cells prone to DNA damage, consequently inducing apoptosis. The active metabolite 2-propenoic acid, pentadecyl ester, suppresses anti-apoptotic genes and activates pro-apoptotic genes, leading to cytotoxic activity. The molecule may act by a multipronged mechanism on multiple targets. This secondary metabolite is an effective anticancer agent with minimal adverse effects; therefore, it needs further study.

In conclusion, a fungal species isolated from the Achanakmar forest reserve, *F. oxysporum ABRF*1, had high cytotoxicity. We identified a novel secondary metabolite, 2-propenoic acid, pentadecyl ester, with therapeutic potential as an anticancer agent. Further studies are required to evaluate this secondary metabolite's molecular mechanism of action.

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