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The effect of fractionation of the essential oil from pinus syvestris on the synergistic and antagonistic medicinal activities

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ABSTRACT

Crude and fractionated essential oils from the seed of *Pinus sylvestris* were analysed by gas chromatography-mass spectrometry (GC-MS) and was evaluated for their synergistic and antagonistic medicinal activities against *E.coli*, *Klebsiella pneumonia* and *Bacillus cereus*. Column Chromatographic revealed that highest yield was obtained from fraction P1. The GC-MS analysis revealed major chemical constituents like isoborneol, Fenchol, δ-terpineol, Terpine-1-ol and Diisooctyl phthalate across the samples. The phytochemicals obtained showed the present of alkaloids, phenolic acid, tannin, saponin, flavonoids, terpenoids and cardiac glycosides in all the samples except alkaloids that was absent in the crude essential oil. The values for antioxidant and the ABTS activities varied across the samples with fraction P3 having the lowest IC_{50} value in both assays. For the α-amylase assay, least value was recorded for fraction P2 while for α-glucosidase assay, fraction P4 recorded the least value. The crude essential oil with a MIC value of 6.20 mg/mL was most effective against E.coli, while fractions P1, P2 and P4 and the crude essential oil were most effective against K. *pneumonia*, and fraction P2 was most effective against B. cereus. All the fractions and crude essential oil showed antioxidant, antidiabetic, and antibacterial activities, the activities of the crude essential oil were synergic only in relation to antibacterial activity on *E.coli*, while all the fractions proved the antagonistic activity of some components in the crude essential oil.

Keywords: *Pinus sylvestris, medicinal, antagonistic, synergistic, essential oil*

Introduction

A major contentious issue that man has been faced with over the years has to do with the consumption or usage of synthetic chemical items, which include food, insecticides, preservatives, and medications (Hayes *et al.*, 2006). As a result, the health of man is affected as well as the environment. Several efforts are put up by scientist, by the introduction of natural products to make products used as drugs to contain more natural or organic. According to popular belief, Africa is blessed with a wide variety of plant species, many of which have a great potential for use as drugs (Manach *et al.*, 2004).

Pinus sylvestris (Scots pine) is of the family Pinaceae that originated in Eurasia and is primarily found in Northern and Eastern Europe. It has now been found in some parts of Africa as a widespread coniferous plant (Sonibare and Olakunle, 2008; Zafar *et al.*, 2010). The leaves are evergreen and fragrant; it is the most varied pine species in the world, with a variety of uses (Maciag, 2007). Considering that it has anti-inflammatory, anti-parasitic, anti-viral, anti-spasmodic, anti-hyperglycemic, and expectorant properties, *P. sylvestris* oil has a wide medical application (Silori *et al.*, 2019). *P. sylvestris* oil has also been observed to have insecticidal and larviciding effects (Fayemiwo *et al.*, 2014).

Synergistic effect is said to be observed when the effect of the combine components of the crude essential oil is higher than what is observed when the crude essential oil is separated into different fractions that contain components different from what was observed in the crude essential oil (Zainol *et al.*, 2017).

While there have been numerous studies on the therapeutic properties of *P. sylvestris* essential oil, there are some schools of thought that contend different plant extract constituents may be the cause of synergic activity. Other schools of thought contend that certain compounds may have the opposite effect and have anti-synergic properties. This study was conducted to ascertain whether the crude essential oil or the fractions obtained by column chromatography will possess synergic or antisynergic activities, in terms of some biomarkers.

Materials and Methods

Materials

Pinus sylvestris L. twigs were collected at Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria, between Latitudes 3.90 and 7.45. The twig was identified by a herbarium specimen, IFE-17939, at the Botany Department of Obafemi Awolowo University. The twigs were broken up into tiny pieces and dried by air.

Extraction of the Essential Oil

Twigs of Pinus sylvestris L. were extracted by the method of hydrodistillation for a period of four hours. With the use of anhydrous sodium sulfate, the extract was dried. The obtained essential oils were kept in an airtight container and chilled to 4 °C.

Fractionation

The crude extract (22.81 g for *P. sylvestris*) of essential oil was separated into various fractions using a 3 x 100 cm column size. A range of 0.063 to 0.200 mm-sized particles of silica gel were used as the stationary phase. Hexane and ethyl acetate were used to elute the sample using a polarity gradient (100/00, 80/20, 60/40, 40/60, 20/80); and an additional ethyl acetate/methanol combination system was also used (100/0, 80/20, 100/0). Each time, the column was filled with a measured volume (100 mL) of each solvent mixture using a separating funnel. The eluted fractions were collected in test tubes in aliquots of 10 mL. Fractionation monitoring was done on a thin layer chromatographic (TLC) aluminum plate. The fractions were spotted on pre-coated silica gel (F254) and developed using the solvent ratio that was used for the elution. The plate was taken out and dried with a hot air dryer. The TLC plate was visualized by spraying with H_2SO_4 reagent and subjecting the plate to heat in a hot oven at 110 °C for 5 minutes. After solvent evaporation on a rotary evaporator at 40 °C, the resulting fractions were categorized based

on their chromatographic profiles, and a total of three fractions (P1, P2, P3, and P4) were obtained.

GC-MS analysis

The Agilent 6890N instrument containing a flame ionization detector and a capillary column HP-5MS (30 m, 0.25 mm, 0.25 m), coupled with an Agilent Technologies mass spectrometer, model number 5973N, was used. The temperature in the GC was 60 ˚C for one minute, ramped up to 180 ˚C for ten minutes at a rate of 10 ˚C per minutes, and then increased to 280 ˚C for fifteen minutes. The temperature of the injector was left at 270 ˚C, and 1 µL 1:10 split ratio injections were used for the samples. Helium, the carrier gas, flowed at 1.0 mL min-1. The scans were done at 20 to 550 m/z at 2 scans per seconds. MS was used to identify the various compounds, and their constituents' identities were verified by comparing their Kovat's retention indices with respect to C8–C32 n-alkanes and making use of samples or information from NIST 2008, as well as publications and the library.

Determination of Total Phenolic Acids

The Folin-Ciocalteu method was used (Elshafie *et al.*, 2017). A 125 µL extract was combined with Folin-Reagent, Ciocalteu's, and distilled water. Six minutes were given for the mixture to stand before 7% sodium carbonate was added. After 90 minutes of standing time, the mixture was read for absorbance at 760 nm on a SpectrumLab70 spectrophotometer, and the outcome was presented as equivalents of gallic acid (GAE).

Determination of Saponin

Spectrophotometric method (Elshafie *et al.*, 2017) was employed. The sample (2 g) was measured with but-2-ol added. A beaker containing a 40% magnesium carbonate solution after filtering through No. 1 Whatman filter paper held the mixture. One mL of the solution was added to two mL of $FeCl_3$. At a nanometer of 380 nm, the absorbance was determined using a SpectrumLab70 spectrophotometer after being left to stand for 30 minutes to allow the color to develop.

Determination of Tannins

The sample (0.5 mL) was dissolved in 80% ethanol, and 2 mL of Folin-Ciocalteu reagent was added to 0.1 mL of the diluted sample. The solution was left for 8 minutes, after which 7.5 mL of 7% Na_2CO_3 was added and incubated for two hours. The tannic acid curve was used as a reference to determine the absorbance at 760 nm and the tannin concentration (Elshafie *et al.*, 2017).

Determination of Flavonoid

A 1 mL of distilled water was used to dissolve approximately 0.25 g of the extract; the resulting mixture was added to 0.150 mL of freshly made AlCl3, 5% $NaNO_2$ solution, and 1 M NaOH solutions. A SpectrumLab70 was used to measure the absorbance at 510 nm after five minutes. The result was expressed as milligrams per gram (mg/g).

Determination of Alkaloid

To 0.3 g of ethanol, 200 mL of 10% acetic acid in ethanol was added 0.3 g of the sample; it was left for four minutes and filtered. The filtrate obtained was concentrated in a water bath, after which dropwise addition of NH4OH was added till precipitation was complete. Washing of the precipitate was done with dilute NH4OH. The residue obtained was weighed after drying (Elshafie *et al.*, 2017).

Determination of Cardiac Glycosides

Chloroform was mixed with 2 mL of the extract in a conical flask and filtered. Into the filtrate obtained, a mixture of 2 mL 29% sodium nitroprusside and pyridine was added and stirred for ten minutes, after which a solution of 20% NaOH was added for color formation. Absorbance was then read at 510 nm on a SpectrumLab70.

Quantitative Test for Terpenoids

An extract of 100 mg was added to 9 mL of ethanol and left for 24 hours (Elshafie *et al.*, 2017). A separating funnel was used to extract the filtrate obtained using 10 mL of petroleum ether. The ether extract was split into glass vials that were precisely weighed and allowed to dry completely(W_f). The total terpenoids' contents were measured by the formula:

$$(W_I - W_f / W_I \times 100)$$

Where W_I is the initial weight and W_f is the final weight.

DPPH scavenging assay

Method described by Gonçalves *et al.*, 2011 was adapted. The change in color of the reaction mixture was monitored at 517 nm. Briefly, an aliquot of 1 mL, 0.3 mM DPPH ethanol solution was mixed with 2.5 mL of different concentrations of the sample and standards ranging from 50, 100, 150, 200, and 250 µg/mL). This was allowed to incubate at room temperature in dark conditions, where the absorbance was measured after 30 min at 517 nm. DPPH served as the negative control, while ethanol was used as a blank. The degree of DPPH color change from purple to yellow revealed the studied samples' level of scavenging effectiveness. All tests were performed in triplicate (n - 3), and average were obtained. Inhibitory concentration at 50% (IC50) was calculated. The following equation was used to determine the DPPH free radical scavenging activity percentage.

DPPH radical scavenging activity (%) = [(Abs control – Abs sample)]/(Abs control) ×100
Where Abs control is the absorbance of DPPH radicals + methanol; Abs sample is the absorbance of DPPH radical + sample or standard.

ABTS radical scavenging activity

ABTS activity was assessed using (Gonçalves *et al.*, 2011). $K_2S_2O_8$ (5 mL) solution of 4.9 mM and 5 mL of a 14 mM ABTS solution were combined and stored in the dark f or 16 hours at room temperature. This solution was diluted with ethanol before use to achieve an absorbance of 0.700 at 734 nm. After homogenizing the crude essential oil and fractions with 1 mL of the ABTS solution at varying concentrations, the absorbance at 734 nm was measured. Each test included running ethanol blanks, and all measurements were completed at least six minutes later. The IC50 (g/mL) value for ABTS scavenging was used. Using the following formula, the inhibition percentage of the ABTS radical was determined:

ABTS scavenging activity (%) = $\{(A_0 - A_1) / A_0\} \times 100$
Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

Anti-Diabetic Activity Assay

2.14.1 Alpha-Amylase Inhibitory Assay

The method of Ali *et al.*, (2006) was used to determine the α-amylase activities of *P. sylvestris* crude essential oil and fractions was determined by. Porcine pancreatic α-amylase (EC 3.2.1.1) (0.5 mg/mL) was added to 500 mL of the sample, and 500 mL of a 0.02 mol/L sodium phosphate buffer (pH 6.9 with 0.006 mol/L NaCl) before being incubated at 25°C for ten minutes. The reaction mixture was then given 500 mL of a 1% starch solution in a 0.02 mol/L sodium phosphate buffer (pH 6.9 with 0.006 mol/L NaCl). The reaction mixture was stopped with the aid of 1.0 mL of dinitrosalicylic acid after incubation at 25 °C for ten minutes. It is then incubated for five minutes in a boiling water. The absorbance was measured at 540 nm with the Spectrumlab 752S. The % inhibition of the α -amylase inhibitory activity was measured. The same technique was used to create a reference, but distilled water was used in place of the extract.

The α-amylase inhibitory activity was calculated as percentage inhibition:

$$\% \text{ inhibition} = \frac{[Abs_{ref} - Abs_{sam}] \times 100}{Abs_{sam}}$$

Where Abs_{ref} is the absorbance of the reference and Abs_{sam} is the absorbance of the sample

Alpha-glucose Inhibitory Assay

The sample (50 µL) was combined with a 100 L solution of -glucosidase (1.0 U/mL) and a 0.1 mol/L phosphate buffer (pH 6.9). These mixtures were then mixed with 0.1 mol/L of phosphate buffer (pH 6.9), 5 mmol/L of a solution of p-nitrophenyl-d-glucopyranoside, and 50 L of phosphate buffer (pH 6.9) after being incubated at 25 °C for 10 minutes. After the mixture was incubated for 5 minutes at 25 °C, an absorbance measurement at 405 nm was performed using a UV-visible spectrophotometer (Spectrumlab 752S). The % inhibition of the α-glucosidase inhibitory action was reported (Apostolidis *et al.*, 2007). Percentages of the blank control were used to express the results.

Percentage inhibition is calculated as follows:

$$\% \text{ inhibition} = \frac{[Abs_{ref} - Abs_{sam}] \times 100}{Abs_{sam}}$$

Where Abs_{ref} is the absorbance of the reference and Abs_{sam} is the absorbance of the sample

Antibacterial Screening

The three bacteria that were employed in this study were Escherichia coli (NCIB 1161], Klebsiella pneumoniae (NCIB 1161) and Bacillus cereus (NCIB 1161), which were obtained from the Central Research Laboratories, University of Ilorin. Isolates were inoculated into the nutrient broth and cultured at 37°C for 24 hours to generate the bacteria used in the study.

Antimicrobial Sensitivity Profiling

The zone of inhibition was identified using the Agar well diffusion technique, where Mueller-Hinton agar was employed. On 150 mm pre-sterilized plates, 20–25 mL of the molten media were applied after cooling it to 45 °C. Following this, bacterial species cultures grown for 24 hours were dispersed with

a clean cotton swab, with each microbe distributed equally over the whole surface of the agar plate to achieve consistent plate surface increase. The Petri plates were left to dry. With the aid of a sterile borer, 3–4 wells of 5 mm of depth and 6 mm of diameter were made on each plate's surface to hold the sample. A 50 L of the undiluted extract was added to the appropriate wells, along with 10 mcg of gentamicin as a positive control and dimethyl sulfoxide (DMSO) as a negative control. The plates were then placed in an incubator for bacterial growth for 24 hours at 37°C after being kept at room temperature for 30 minutes. After incubation, a ruler was used to measure the zones of inhibition, and the results were reported in millimetres (Sadeghi-Nejad *et al.*, 2010).

Establishing Minimum Inhibition Concentration (MIC)

The minimum inhibitory concentration was established using the agar dilution technique. Inhibitory concentration. The MIC was assessed on extracts that displayed antibacterial action on any organism in the agar-well diffusion assay. Different concentrations of each extract were used in this test. Each organism was prepared as an overnight incubated suspension in nutrient broth, and 50 µL was added to each test tube. It was then incubate24 hours at 37 °C. Each tube's suspension was injected into nutrient agar after incubation to determine whether or not bacterial growth was inhibited. Bacterial growth on solid media suggested that a certain concentration of the extract was ineffective at inhibiting the bacteria (Andualem *et al.*, 2014).

Table 1: Major constituents of the crude and fractions, P1, P2, P3 and P4 of the essential oil of P. sylvestris and the yield obtained.

	Crude			P1			P2			P3			P4		
Yield (g)	22.81			9.33			2.47			4.70			6.31		
COMPOUNDS	%	RI_C	RI_L	%	RI_C	RI_L	%	RI_C	RI_L	%	RI_C	RI_L	%	RI_C	RI_L
Fenchol	10.45	1000.3	1100.7	34.62	994.0	1100.7	-	-	-	-	-	-	-	-	-
Terpine-1-ol	11.28	1123.3	1134.0	-	-	-	-	-	-	17.84	1128.3	1134.0	-	-	
Terpinene-4-ol	8.82	1157.4	1164.5	-	-	-	-	-	-	-	-	-	-	-	-
Isoborneol	11.82	1140.5	1147.8	15.71	1140.2	1147.8	-	-	-	-	-	-	-	-	-
P-cymen-7-ol	4.18	1263.1	1270.1	4.94	1264.3	1270.1	-	-	-	-	-	-	-	-	-
α-terpineol	10.50	1168.3	1175.6	13.89	1170.0	1175.6	-	-	-	-	-	-	-	-	-
Longifolene	8.84	1390.5	1404.0	-	-	-	6.78	1400.1	1404.0	-	-	-	-	-	-
Fenchyl acetate	-	-	-	14.59	1204.1	1209.0	-	-	-	-	-	-	-	-	-
δ-terpineol	-	-	-	-	-	-	31.55	1145.0	1148.1	-	-	-	-	-	-
Verbenol	-	-	-	-	-	-	7.93	1127.3	1133.7	-	-	-	-	-	-
α-terpinylacetate	-	-	-	-	-	-	5.94	1328.7	1332.8	-	-	-	-	-	-
Sabinol cis	-	-	-	-	-	-	-	-	-	13.89	1130.1	1135.6	-	-	-
Diisooctyl phthalate	-	-	-	-	-	-	-	-	-	-	-	-	40.98	2514.0	2519.0
Isobornylacetate	-	-	-	-	-	-	-	-	-	-	-	-	6.02	1265.2	1271.0
γ-terpinene	-	-	-	-	-	-	-	-	-	-	-	-	4.85	1045.3	1050.3

RI_C is the retention indices calculated, while RIL is the retention indices found in the literature, as reported by Babushok *et al.*, 2011. Retention indices of components for dimethyl silicone stationary phase.

Table 2: Quantitative phytochemical parameters in *P. sylvestris*.

Components	P1	P2	P3	P4	Cru	Crude extract
Phenolic acid (GAE/g)	5.50 ± 0.1^{b}	5.64 ± 0.01^{b}	5.61±0.02^{b}	5.30 ± 0.2^{a}		5.21 ± 0.02^{a}
Saponin (mg/g)	6.00 ± 0.15^{c}	4.50 ± 0.09^{b}	4.09± 0.01^{a}	4.53 ±0.01^{b}		4.48 ± 0.01^{b}
Tannin (mg/100g)	4.40 ± 1.79^{c}	2.40 ± 0.2^{b}	5.70 ± 5.84^{d}	2.10 ± 0.1^{a}		2.30 ± 0.5^{b}
Flavonoid (mg/g)	2.75 ± 0.04^{b}	2.28 ± 0.12^{a}	5.21 ± 0.02^{c}	5.30 ± 0.06^{c}		5.32 ± 0.07^{c}
Alkaloid (mg/g)	1.20 ± 0.07^{a}	2.63 ± 0.01^{c}	4.21 ± 0.03^{d}	2.40 ± 0.05^{b}		ND
Cardiac Glycoside (mg/g)	5.32 + 0.02^{c}	4.30 ± 0.02^{a}	5.00 ± 0.05^{c}	4.78 ±0.06^{b}		4.75 ± 0.05^{b}
Terpenoid(mg/g)	5.10 ± 0.06^{b}	4.60 ± 0.02^{a}	5.04 ± 0.58^{b}	5.72 ± 0.02^{c}		5.30 ± 0.22^{b}

The table's values are each represented as mean SD (n = 3). A different letter after a value in the same row indicates a significant difference ($p< 0.05$). ND= Not determined is ND.

Table 3: Radical scavenging and In-vivo anti-diabetic activities of the fractionated and crude essential oil of *P. sylvestris*.

Sample	Radical scavenging IC_{50} (µg/mL)		In-vivo anti-diabetic IC_{50} (µg/mL)	
	DPPH	ABTS	α-amylase	α-glucosidase
P1	875.00±7.28^{g}	506.00±6.30^{f}	4.32 ± 0.03^{c}	3.92 ± 0.01^{d}
P2	835.00±6.10^{f}	368.00±6.30^{g}	4.08 ± 0.54^{b}	3.00 ± 0.06^{c}
P3	508.00±3.30^{b}	235.00±6.30^{a}	4.62 ± 0.01^{d}	4.21 ± 0.02^{de}
P4	761.00±5.10^{e}	265.00±6.30^{c}	4.34 ± 0.03^{b}	2.57 ± 0.02^{b}
Crude essential oil	700.00±8.30^{c}	251.00±6.30^{b}	4.90 ± 0.01^{e}	4.22 ± 0.02^{e}
Ascorbic	734.20±6.40^{d}	342.20±7.30^{e}	-	-
Gallic acid	482.86±5.20^{a}	321.10±4.23^{d}	2.32± 0.01^{a}	2.02 ± 0.02^{a}
Acarbose	-	-		

The table's values are each represented as mean SD (n = 3). A different letter after a value in the same column indicates a significant difference ($p< 0.05$).

Table 4: Anti-bacterial activities of fractionated and crude essential oils of *P. sylvestris*.

Essential Oil	Concentration / MIC (mg/mL)														
	Escherichia coli					*Klebsiella pneumonia*					*Bacillus cereus*				
	50	25	12.5	6.25	MIC	50	25	12.5	6.25	MIC	50	25	12.5	6.25	MIC
	Diameter of zone inhibition (mm)														
P1	3.1	2.2	0.0	0.0	12.0	5.0	4.0	2.1	2.0	10.0	3.0	3.0	2.0	0.0	15.2
P2	4.2	4.1	2.2	0.0	9.5	4.8	3.8	2.0	2.0	10.0	4.1	2.3	2.0	0.0	13.2
P3	4.1	4.1	2.0	0.0	9.5	4.3	2.8	1.8	0.0	12.5	3.0	2.0	0.0	0.0	20.0
P4	4.0	4.0	2.2	0.0	9.5	5.1	4.0	2.0	1.9	10.0	3.0	2.2	0.0	0.0	20.0
Crude	6.0	4.4	3.0	2.0	6.2	5.0	4.0	3.0	2.0	10.0	3.0	2.1	0.0	0.0	20.0

Results and Discussions

Results

Column chromatography was used to fractionate the crude essential oil of *P. sylvestris,* the elute collected was grouped into four fractions: P1, P2, P3, and P4. Fraction P1 yielded the highest fraction of 9.33 g (Table 1).

Table 1 presents the major components from GC-MS analysis of the crude essential oil and fractions P1, P2, P3, and P4 of *P. sylvestris*. The analysis revealed isoborneol (11.82%) as the major component of

the crude essential oil, followed by terpine-1-ol (11.28%), while p-cymen-7-ol (4.18%) had the lowest composition. Fenchol (34.62%) was the highest component observed in fraction P1, closely followed by isoborneol (15.71%), but of the major components observed, P-cymen-7-ol had the least composition of 4.94%. As seen in fraction P2, the composition of δ-terpineol was the highest (31.55%), followed by verbenol (7.93%), and α-terpinylacetate (5.94%) was the least of the major components. Terpine-1-ol was the highest component (17.84%) observed in fraction P3, but of the major components observed in the GC-MS analysis, Sabinol cis (13.89%) was found to have the lowest composition. The main constituent identified in fraction P4 was diisooctyl phthalate, which had a composition of 40.98%, followed by isobornylacetate, which had a composition of 6.02%.

The results of a quantitative investigation of the phytochemical parameters of *P. sylvestris* fractions and crude essential oil are shown in Table 2. Both the crude essential oil and all the fractions obtained contained phenolic acid; the crude essential oil had the lowest concentration of phenolic acid (5.21 GAE/g), which is significantly similar to the value obtained in fraction P4. Fraction P2 had the highest (5.64 GAE/g), but the value was not significantly different from the values obtained in fractions P1 and P3. Fraction P1 had the highest value of saponin content (6.00 mg/g), whereas fraction P3 had the lowest value of 4.09 mg/g. The concentration of tannin was found to be higher in fraction P3 (5.70 mg/100g), and least in fraction P4 (2.10 mg/100g). The value of flavonoids was highest in the crude essential oil of *P. sylvestris* (5.32 mg/g) and lowest in fraction P2 (2.28 mg/g). Alkaloid had the highest value in fraction P3 (4.21 mg/g), but was not determined in the crude essential oil. Cardiac glycoside was highest in fraction P1 (5.32 mg/g) and lowest in fraction P2 (4.30 mg/g). The highest concentration of terpenoids was found in fraction P4 (5.72 mg/g), while the lowest concentration was found in fraction P2 (4.60 mg/g).

The results of the antibacterial activities of the crude essential oil and fractions of P. sylvestris were examined using the disc diffusion method against *E. coli*, *K. pneumoniae*, and B .cereus. The antibacterial activities of the crude and factions against three bacterial species were shown in Table 4. The crude essential oil recorded zone of inhibition of 2.00 mm with a dose of 6.25 mg/mL while no activity was recorded with all the fractions. The concentration of 50 mg/mL produced the highest activity; fraction P2 recorded a zone of inhibition of 4.20 mm, which was lower than the zone of inhibition of the crude essential oil, which was 6.00 mm. The crude essential oil recorded a minimum inhibitory concentration (MIC) of 6.20 mg/mL, which indicated a higher activity against E.coli than all of the fractions. The result of the antibacterial activities of the crude and fractions of *P. sylvestris* against K. pneumonia revealed that all samples recorded a zone of inhibition with a dose of 6.25 mg/mL except fraction P3 with a dose of 50 mg/mL. Fraction P4 recorded a zone of inhibition of 5.10 mm which was the greatest observed zone of inhibition while the least value of a zone of inhibition was observed in fraction P3 with a value of 4.30 mm. The MIC values recorded for crude and fractions P1, P2, and P4 were 10.00 mg/mL indicating fraction P3 had the least activity against K. pneumonia.

B. cereus is the only gram-positive bacterial species included in the study. The crude, fractions P3 and P4 recorded no activity against B. cereus with concentrations of 6.25 mg/mL and 12.5 mg/mL. The highest activity of zone of inhibition was recorded with concentration of 50 mg/mL with fraction P3 recording highest inhibition of 4.10 mm with MIC value of 13.20 mg/mL indicating that it had the highest activity against B. cereus.

The antioxidant activities of the crude essential oils and fractions were determined using the DPPH (2,2-diphenyl-1-picryl) and ABTS (2,2-azobis-2-aminopropane hydrochloride) radical scavenging assays. Table 3 displayed the values of the inhibitory concentration at 50% (IC_{50}) for DPPH, ABTS, and two positive standards (vitamin C and garlic acid). The highest radical scavenging activity for DPPH and ABTS was reported in fraction P3 (508 and 235 µg/mL, respectively), but the least activity was recorded in fraction P1 with IC_{50} values recorded to be 875 and 506 µg/mL for DPPH and ABTS respectively. Garlic acid, with an IC_{50} value of 482.86 µg/mL for DPPH and 321.10 µg/mL for ABTS had a higher radical scavenging activities than the crude as well as the fractions. The IC_{50} values for the crude essential oil and fraction P3 both showed that they have a greater capacity for radical scavenging in DPPH and ABTS than vitamin C. The radical scavenging activities were higher in the ABTS assay than in the DPPH assay for the fractions, crude essential oil, and standards.

Table 3 demonstrated the anti-diabetic activities of crude essential oil and fractions of P. sylvestris using the α-amylase and α-glucosidase assays. Fraction P2 had the lowest IC50 value for -amylase (4.08 µg/mL), indicating that it was more effective in inhibiting -amylase than other fractions and crude essential oil of P. sylvestris. The higher IC50 value for α-amylase found in crude essential oil (4.90 µg/mL) suggests less antidiabetic efficacy. In the α-glucosidase test, fraction P2 with the lowest IC50 (2.57 µg/mL) value inhibited α-glucosidase more effectively, but crude essential oil with the highest value of 4.22 µg/mL had the least anti-diabetic activity based on the IC50 value observed.

Discussions

Essential oils consist of numerous components that are responsible for the extracts' activities. In some situations, some constituents may work against the extracts' desired effects, which could be responsible for the reduced activity of the extract (Manindra *et al.*, 2011). The research identified isoborneol (11.82%) and terpine-1-ol (11.28%) as the major components of the crude essential oil. Isoborneol (0.8%) and terpine-1-ol (10.80%) were also identified by Sarin *et al.*, (2015). The constituent of the essential oil from *P. sylvestris* needle has been determined by a number of studies. According to Oyewole *et al.*(2021), the three major components of a Nigerian sample of *P. sylvestris* needle essential oil were α-terpineol (27.17%), borneol (6.72%), and fenchol (1.74%). α-terpineol (20.50%), Terpinolene (26.7%), 1-terpineol (5.3%), α-pinene (6.0%) terpineol (10.8%), and γ-terpineol (5.3%) were listed as the primary constituents by Sarin *et al.*, (2015). The main constituents, according to Judzentiene and Kupcinskiene (2008), were α-pinene (16.1%) and -3-carene (14.3%), with 3-Cyclohexene-1-methanol, alpha.,.alpha. 3-Cyclohexen-1-ol,1-methyl-4-(1-methylethyl) (21.82%), 4-trimethyl (27.1%), and Cyclohexanol, 1-methyl-4-(1-methylethenyl) (14.07%) are the main compounds found in another sample from Nigeria, according to Fayemiwo *et al.*, (2014). Fatbardhë *et al.* (2019) reported that β-caryophyllene, (4.50%), β-pinene (13.65%), α-pinene (32.45%), δ-cadinene (7.05%) limonene (9.35%) and 3-carene (6.45%) and were the main constituents of the essential oil of P. sylvestris . Fenchol (10.45%), which was also found in the crude essential oil, was also present in fraction P1, but the quantity was higher in fraction P1 (34.62%) while Fenchyl acetate, which was not present in the crude essential oil, was also observed in fraction P1. Fenchol was also reported by Oyewole *et al.* (2021). Hisashi (2021) confirmed that the primary component identified in fraction P2 was -terpineol (31.55%). According to Sarin *et al.* (2015), the major components of fraction P3 were terpine-1-ol (17.84%).

Diisooctyl phthalate (40.98%), which was reported to be the highest component of fraction P4, has been reported earlier by Rafal *et al*. (2012). According to Owolabi *et al*. (2009), a significant factor responsible for the differences in the components of the essential oil observed from different locations may be attributed to the treatments the needles received after harvesting or the origin of the needle. The different components and the variability in the quantity of the component could be a result of the crude essential oil fraction. The research reported flavonoids (5.32 mg/g), terpenoids (5.30 mg/g), and phenolic acid (5.20 GAE/g) as the major phytochemicals from the crude essential oil of *P. sylvestris*. This was similar to what was reported by Skrypnik *et al*. (2019), who reported flavonoids (5.20 mg/g) and phenolic acid (4.0 mg/g) for the essential oil of *P. sylvestris*. The presence of phenolic acid was noted in all fractions and the crude essential oil with no significant difference; Skrypnik *et al*. (2019) likewise reported the same phytochemical for the essential oil of *P. sylvestris*. The highest amount of saponin was observed in fraction P1 (6.00 mg/g). This was also reported by Jia *et al*. (2018) to be present in the essential oil of P. sylvestris . Tannin was also found to be highest in fraction P3 (5.70 mg/100g) and lowest in fraction P4 (2.10 mg/100g), this same component was reported by Berit *et al*.(2021) to be present in the essential oil of *P. sylvestris*. Flavonoid was found in all of *P. sylvestris* fractions and crude essential oil, with the crude essential oil containing the greatest concentration (5.32 mg/g), however these findings were equivalent to those of Skrypnik *et al*., (2019). Alkaloid was present in all *P. sylvestris* fractions, with fraction P3 having the highest value (4.21 mg/g), although it was not present in the crude essential oil.

This is comparable to the findings of Virjamo and Riitta (2018). The highest concentration of cardiac glycoside was found in fraction P1 (5.32 mg/g); Achiano and Giliomee (2007) also identified this component in the essential oil of *P. sylvestris*. The highest concentration of terpenoid was found in fraction P4 (5.72 mg/g), which was comparable to other fractions and the crude essential oil of P. sylvestris but did not differ significantly in value. Gürkan *et al*. (2007) also noted this phytochemical to be present in the essential oil of *P. sylvestris*.

Isoborneol was reported to have antibacterial properties (Micaela *et al*., 2021). The existence of isoborneol, which was discovered to be present in abundance in the crude essential oil but not significantly different from fraction P4, may have greatly contributed to the flavonoid's antibacterial property. Plant extracts with a high flavonoid content from a variety of species have antibacterial activities, according to Mishra *et al*. (2013).

Tannin was found in the crude essential oil and fractions, but in greater abundance in fraction P3. Tannin has been reported to possess antibacterial properties (Cotas *et al*., 2020). The antibacterial activity of tannin in the crude essential oil and fractions could be attributed to the presence of components such as fenchol, δ-terpineol, and diisooctyl phthalate, all of which were found in varying amounts in the crude and fractions of *P. sylvestris*. Kotan *et al*. (2007) revealed that fenchol demonstrated broad-spectrum antibacterial action. Huang *et al*. (2020) found that δ-terpineol had a good inhibitory effect on a variety of gram-positive and gram-negative bacteria, whereas Waheed *et al*. (2018) discovered that diisooctyl phthalate inhibited antibacterial activity. The presence of phenolic acid in extracts has been linked to antibacterial properties (Obonga *et al*., 2017). Although all samples contained phenolic acids, the antibacterial activity demonstrated by fraction P2 could be attributed to the presence of δ-terpineol, which has been shown to have antibacterial properties (Huang *et al*., 2020).

Antioxidant properties of alkaloid have been reported (Aminah *et al*., 2018). Fraction P3 had one of the highest values of alkaloids, which may due to the presence of terpine-1-ol in the extract. Christina *et al*. (2018) showed that terpine-1-ol had a variety of biological uses as an antioxidant. According to Raina *et al*. (2019), longifolene, one of the constituents of fraction P2, exhibits antidiabetic properties. This constituent may have been linked to the phenolic component since phenolic acid has strong antidiabetic properties (Gowd *et al*., 2017) and is present in fraction P2. Terpenoid, which was shown to have its largest constituent in fraction P4, was noted to be a good antidiabetic drug (Suchitra *et al*., 2020). Diisooctyl phthalate has been linked to anti-diabetic properties (Kumar *et al*., 2017). It can be inferred that the higher terpene content in fraction P4 may be responsible for the antidiabetic activity exhibited by fraction P4, and the diisooctyl phthalate may have contributed to the quality of terpenoids.

The antibacterial activity was determined against two grams positive and one gram negative bacteria. The results showed that crude and fractions were active against the three bacterial strains, but at varying degrees of activity, as indicated by the zone of inhibition and MIC values. The large spectrum of the crude essential oil and fractions is as reported by Keeratirathawat *et al*. (2013); Kaániová *et al*. (2018); Kurti *et al*. (2019); Fayemiwo *et al*. (2014); and Oyewole *et al*. (2021).

The crude essential oil with the highest efficacy against E. coli was reported to have a minimum inhibitory concentration of 6.20 mg/g, (Oyewole *et al*., 2021) also reported that the highest activity against bacterial species tested by the essential oil of P. sylvestris was found with E. coli. The crude essential oil, fractions P1, P2, and P4, had the lowest MIC values of 10.00 mg/mL against K. pneumoniae, and fraction P2 was shown to have the highest antibacterial activity against B. cereus with a MIC of 13.20 mg/mL. Research has shown that extracts with high contents of phenolic acid, tannin, and flavonoids (Obonga *et al*., 2017; Cotas *et al*., 2020; Mishra *et al*., 2013) are beneficial. Antibacterial properties are common. The varied actions seen may be due to the varying flavonoid, tannin, and phenolic contents. The diverse components present at varying percentages in each fraction and the crude essential oil of P. sylvestris may also be responsible for the various activities. DPPH and ABTS assays were used to assess the antioxidant activity of the fraction and crude essential oil of P. sylvestris. The difference in the antioxidant activity observed in the values of IC50 was a result of the different components observed in the crude and fractions. Some components, such as terpine-1-ol, fenchol, and α-terpineol, have been linked to antioxidant activity (Christina *et al*., 2018). The anti-diabetic activities of the crude essential oil and fraction of P. sylvestris were determined using an -amylase and α-glucosidase assay. The crude essential oil of P. sylvestris fractions exhibited -amylase and -glucosidase activity. This activity can be attributed to the presence of flavonoids, tannins, saponins, phenolic acid compounds, and steroids. Tadesse *et al*. (2017). The presence of longifolene in fraction P2 could explain the higher anti-diabetic activity observed in the -amylase assay. This was reported by Raina *et al*. (2019). The increased activity observed in the -glucosidase assay can be attributed to the high terpenoid value observed, which confirmed the findings of Suchitra *et al*. (2020), who discovered that terpenoid compounds have anti-diabetic properties. The high antidiabetic activity of -glucosidase can also be attributed to the high value of diisooctyl phthalate found in fraction P4. Kumar *et al*. (2017) reported the effect of diisooctyl phthalate as an antidiabetic agent.

Conclusions

The result of the research showed that the chemical components observed in the crude essential oil were different from those observed in the fractions, and those present were of a different percentage. This is evident in the different values obtained in the phytochemical analysis of all the activities determined; the crude essential oil had the highest activity only in the antibacterial activity against *E. coli,* which indicated a synergic activity. The higher activities observed by the fractions on other biomarkers show the antagonistic activity exhibited by some of the components in the crude essential oil. This study may lead to additional studies that will help researchers determine the fractions needed to increase the activity of essential oils.

References

Mishra, A., Kumar, S., & Pandey, A. K. (2013). Scientific validation of the medicinal efficacy of Tinospora cordifolia. *The Scientific World Journa*l, vol. 2013. https://doi.org/10.1155/2013/292934

Agarwal, C., Hofmann, T., Visi-Rajczi, E., & PÃ¡sztory, Z. (2020). Low-frequency, Green Sonoextraction of Antioxidants from Tree Barks of Hungarian Woodlands for Potential Food Applications. *Chemical Engineering and Processing - Process Intensification*, 159, 108221.

Aminah, D., Poppy A. Z. H., Jansen S., Siti, F. S., & Denny S. (2018). Antioxidant Activity of Alkaloid Compounds from Litsea cubeba Lour. *Oriental Journal of Chemistry,* 34(2), 1149-1152.

Sadeghi-Nejad, B., Shiravi, F., Ghanbari, S., Alinejadi, M., & Zarrin, M. (2010). Antifungal activity of Antifungal activity of Satureja khuzestanica (Jamzad) leaves extracts. *Jundishapur Journal of Microbiology*, 3(1), 36-40.

Berit, M. B., Heidi L. E., Øivind, Ø., Håvard, S., Inga, M., A., Karl-Christian, M., Kristin, M. S., Spiridoula, A., Stig, M. T., & Ian, D. W. (2021). Extracts of pine bark (Pinus sylvestris) inhibit Cryptosporidium parvum growth in cell culture. *Parasitology Research*, 120(8), 2919-2927.

Christina, K., Nurhayat, T., & Gerhard, B. (2018). α-Terpineol, a natural monoterpene: A review of its biological properties. *Open Chemistry*, 16(1), 349–361.

Joao, C., Adriana, L., Pedro, M., Diana P., Artur, F., Ana, M. M., Gabriela, J. S., & Leonel, P. A. (2020). Seaweed phenolics: from extraction to applications. *Mar Drugs*, 18(8), 384.

Apostolidis, E., Kwon, Y. I., & Shetty, K. (2007). Inhibitory potential of herb, fruit, and fungal-enriched cheese against key enzymes linked to type 2 diabetes and hypertension. *Innovative Food Science and Emerging Technologies*, 8(1), 46–54.

Fatbardhë, K., Annamaria, G., Giangiacomo, B., Behxhet, M., Fabrizio, G., Cristian, T., Stefania, A., Luca, G., Emanuela, Z., Daniela, P., & Avni, H. (2019). Chemical composition, antioxidant and antimicrobial activities of essential oils of different Pinus species from Kosovo. *Journal of Essential Oil Researc*h, 13(4), 263-275.

Fayemiwo, K. A., Adeleke, M. A., Okoro, O. P., Awojide, S. H., & Awoniyi, I. O. (2014) Larvicidal efficacies and chemical composition of essential oils of Pinus sylvestris and Syzygium aromaticum against mosquitoes. *Asian Pacific Journal Tropical Biomedice*, 4(1), 30–34.

Gizachew, A., Tamrat, A., Nigatu, K., Solomon, G., Adane, M., & Haile, A. (2014). A comparative study of Widal test with blood culture in the diagnosis of typhoid fever in febrile patients. *BMC Research Notes*, 7, 653.

Gowd, V., JiaZ, Q., & Chen, W. (2017). Anthocyanins as promising molecules and dietary bioactive components against diabetes—a review of recent advances. *Trends Food Science Technology*, 68, 1-13.

Gürkan, S., Juha, H., Kani, I., Jarmo, K., & Holopainen, J. K. (2007). Variation in needle terpenoids among Pinus sylvestris L. (Pinaceae) provenances from Turkey. *Biochemical Systematics and Ecology,* 35(10), 652–661.

Elshafie, H. S., & Camele, I. (2017). An Overview of the Biological Effects of Some Mediterranean Essential Oils on Human Health. *BioMed Research International*. 2, 1-14

Tyrone, B. H., Paola, C., Sarah, C., & Duc, C. (2006). Pesticide Mixtures, Endocrine Disruption, and Amphibian Decline: Are we underestimating the impact? *Environmental Health Perspectives*, 1, 40-50

Hisashi, K. (2021). Bioactive Compounds Involved in the Formation of the Sparse Understory Vegetation in Pine Forests. *Current Organic Chemistry*, 25, 1731-1738.

Huang, J., Yang, L., Zou, Y., Luo, S., Wang, X., Liang, Y., Du, Y., Feng, R., & Wei, Q. (2020). Antibacterial activity and mechanism of three isomeric terpineols of Cinnamomum longepaniculatum leaf oil. *Folia Microbiologica*, 66(1), 59-67.

Jia, X., Liu, T., Li, X., & Yonghua, Z. (2018). Needles Resistance in Pinus sylvestris L. var. mongolica Litv. Exposed to Elevated Air Temperature and Cadmium-Contaminated Soils for 3 Years. *Water, Air, & Soil Pollution*, 229(6), 188.

Judzentiene, A., & Kupcinskiene, E. (2008). Chemical Composition on Essential Oils from Needles of Pinus sylvestris L. Grown in Northern Lithuania. *Journal of Essential Oil Research*, 20(1), 26–29.

Achiano, K, A., & Giliomee, J. H. (2007). Rearing the house fly predator Carcinops pumilio (Erichson) (Coleoptera: Histeridae) using eggs and larvae of Drosophila melanogaster (Meigen) (Diptera: Drosophilidae) as prey. *African Journal of Biotechnology*, 6(17), 15.

Kačániová, M., Gasper, J., Terentjeva, M., Kunová, S., Kluz., M, Hanus, P., & Puchalski, C. (2018). Antimicrobial Activity and Resistance of Microorganisms Isolated from Honey Bees. *Scientic Papers: Animal Science Biotechnologies* 51(1):133–138.

Keeratirathawat, S., Nair, J., & Levitan, J. (2013). The antibacterial ecacy of pine oils on pathogens commonly found in biosolids applied to pine plantations. *International Journal of Environment and Waste Management*, 12(2):146–153.

Oyewole, K. A., Oyedara, O. O., Awojide, S. H., Olawade, M. O., & Adetunji, C. O. (2021). Chemical Constituents and Antibacterial Activity of Essential Oils of Needles of Pinus Sylvestris (Scots Pine) from South West Nigeria. *Research Square*, doi:10.21203/rs.3.rs-635195.

Kotan, R., Kordali, S., & Cakir, A. (2007). Screening of Antibacterial Activities of Twenty-One Oxygenated Monoterpenes. *Zeitschrift für Naturforschung* C, 62, 7-8.

Kumar, K. M, Chandrappa, C. P., Channabasava, R., Ramachandra, Y. L., Padmalatha, R. S., Ravishankar, R. V., & Govindappa, M. (2017). Anti-diabetic activity of endophytic fungi, Penicillium species of Tabebuia argentea; in silico and experimental analysis. Research. *Journal Phytochemistry*, 11: 90-110.

Kurti, F., Giorgi, A., Beretta, G., Mustafa, B., Gelmini, F., Testa, C., Angioletti, S., Giupponi, L., Zilio, E., Pentimalli, D., & Hajdari, A. (2019) Chemical composition, antioxidant and antimicrobial activities of essential oils of different Pinus species from Kosovo. *Journal of Essential Oil Research*, 31(4), 263–275.

Gonçalves, M., Ribeiro, A., Mendes, I., Matos, M., & Santos, A. (2011). Tracking novelties in psychotherapy process research: The innovative moments coding system. *Psychotherapy research: journal of the Society for Psychotherapy Research*, 21, 497-509.

Maciąg, A., Milaković, D., Christensen, H. H., Antolović, V., & Kalemba, D. (2007). Essential oil composition and plant-insect relations in Scots pine (Pinus sylvestris L.). *Food Chemistry Biotechnology*, 71:71–95.

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: food sources and bioavailablity. *American Journal Of Clinical Nutrition*, 79, 727-747.

Manindra, M., Kumar, A. A., Zafar, H. S., & Akash, S. (2011). Essential oil composition and antimicrobial activity of three ocimum species from uttarakhand (Índia). *International Journal of Pharmacy and Pharmaceutical Sciences*, 3, 223.

Micaela, M. Q., Ivo, P., Sriram, H., Daniel, R., Cristiana, C. P., Nuno, B., João, M. B., João, P. T., & Rui, G. S. (2021). Auto-Disinfectant Acrylic Paints Functionalised with Triclosan and Isoborneol—Antibacterial Assessment, *Polymer*s, 13(13), 2197.

Mitić, Z. S., Jovanović, B., Jovanović, S., Mihajilov-Krstev, T., Stojanović-Radić, Z. Z., Cvetković, V. J., Mitrović, T. L., Marin, P. D., Zlatković, B. K., & Stojanović, G. S. (2018). Comparative study of the essential oils of four Pinus species: Chemical composition, antimicrobial and insect larvicidal activity. *Industrial Crops & Products*, 111, 55–62.

Obonga, W. O., Omeje, E. O., & Ocheme, W. G. (2017). Antimicrobial Evaluation of crude Extracts, Fractions and Oil from Monodora myristica Seeds (Gaertn.) Dunal. *African Journal of Pharmaceutical Research & Development*, 9(1), 18-23.

Owolabi, M. S., Oladimeji, M. O., Lajide, L., Singh, G., Mariwuthu, P., & Isidorov, V. A. (2009). *EJEAF Chem.*, 8(9), 828.

Rafal, S., Marek, C., Krzysztof, J. R., & Barbara, M. (2012). Identification of volatiles from Pinus silvestris attractive for Monochamus galloprovincialis using a SPME-GC/MS platform. *Environmental Science Pollution Research*, 19:2860–2869.

Raina, R., Verma, P. K., Peshin, R., & Kour, H. (2019). Potential of Juniperus communis L. as a nutraceutical in human and veterinary medicine. *Heliyon*, 5(8), e02376.

Ali, S., Stone, M. A., Peters, J. L., Davies, M. J., & Khunti, K. (2006). The prevalence of co-morbid depression in adults with Type 2 diabetes: systematic review and meta-analysis. *Diabetic Medicine*, 23(11), 1165-1173.

Sarin, T., Narisa, K., Rith, W., & Nijsiri, R. (2015). Chemical Components of Four Essential Oils in Aromatherapy Recipe. *Natural Product Communications*, 10(6), 1091 - 1092.

Silori, G. K., Kushwaha, N., & Kumar, V. (2019). *Essential Oils from Pines: Chemistry and Applications*. Springer, 275-297

Skrypnik, L., Grigorev, N., Michailov, D., Antipina, M., Danilova, M., Pungin, A. (2019). Comparative study on radical scavenging activity and phenolic compounds content in water bark extracts of alder (Alnus glutinosa (L.) Gaertn.), oak (Quercus robur L.) and pine (Pinus sylvestris L.). *European Journal of Wood and Wood Products*, 77, 879-890.

Sonibare, O. O., & Olakunle, K. (2008). Chemical composition and antibacterial activity of the essential oil of Pinus caribaea from Nigeria. *African Journal Biotechnology*, 7(14), 2462–2464.

Suchitra, K. P., Renu, B., & Awanish, K. (2020). Targeting type II diabetes with plant terpenes: the new and promising antidiabetic therapeutics. *Springer*, 76(1), 241-254.

Tadesse, B. T., Ariaya, H., Yalemtsehay, M., & Mekuria, T. (2017). Antidiabetic activity and phytochemical screening of extracts of the leaves of Ajuga remota Benth on alloxan-induced diabetic mice. *BMC Complementary and Alternative Medicine*, 17:243.

Virjamo, V., & Julkunen-Tiitto, R. (2018). Quality and quantity of piperidine alkaloids in needles and bark of Scots pine (Pinus sylvestris) seedlings. *Phytochemistry Letters*, 26, 106–109.

Vyas, P., & Patil, S. (2011). Antimicrobial activity of essential oils against multidrug resistant enterobacterial pathogens. *Trends in Biosciences*, 4(1):23–24.

Waheed, A., Chohan, M. M., Ahmed, D., & Ullah, N. (2018). The first report on the in vitro antimicrobial activities of extracts of leaves of Ehretia serrata. *Saudi Journal of Biological Sciences*, 26(6), 1253-1261.

Zafar, I., Fatima, A., Khan, S. J., Rehman, M., & Mehmud, S. (2010). GC-MS studies of needles essential oils of Pinus roxburghaii and their antimicrobial activity from Pakistan. *Electronic Journal of Environmental Agricultural Food Chemistry*, 9(3), 468–473.

Zainol, S. N., Said, S. M., Abidin, Z. Z., Azizan, N., Majid, F .A. A., & Jantan, I. (2017). Synergistic benefit of eugenia caryophyllata l. and cinnamomum zeylanicum blume essential oils against oral pathogenic bacteria, *Chemical Engineering Transactions*, 56, 1429-1434.