EVALUATION OF ANTIBACTERIAL ACTIVITY AND ISOLATION OF SOME ORGANIC CONSTITUENTS FROM SEEDS OF *Myristica fragrans* Hott. (ZADEIK-PO)

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Abstract

The seeds of *Myristica fragrans* Hott. (Zadeik-po) are the chief ingredients in a variety of Myanmar Traditional Medicine Formulations (TMF). These formulations are generally used for the treatment of asthma, dysentery, diarrhoea, pneumonia, typhoid fever, hemolytic anemia and urinary tract infection. The Zadeik-po seeds were selected as plant material and collected from Mudon Township, Mon State. The research was focused on antibacterial activity of various extracts and isolation, identification of some organic constituents from Zadeik-po seeds. The polar, non-polar extracts and essential oil of Zadeik-po seeds were screened for antibacterial activity by agar disc diffusion method. The essential oil of Zadeik-po exhibited the pronounced antibacterial action against all tested 20 microorganisms. In addition, Minimum Inhibitory Concentration (MIC) values of active essential oil of Zadeik-po were also determined by microplate dilution method on five bacterial strains. The lowest MIC values of essential oil was found to be 0.0625 mg mL^{-1} with *Escherichia coli* LT. α-Pinene (0.5 %), Myristicin (0.03 %) and Eugenol (0.01 %) were isolated from essential oil of Zadeik-po by column and preparative thin layer chromatographic methods. They were identified by UV, FT IR, ^{1}HNMR and GC MS spectroscopic methods. The isolated compounds also showed antibacterial property against the *Staphylococcus aureus, Escherichia coli, Shigella boydii, Samonella typhi* and *Vibrio cholera*. From these scientifically observations, it can be inferred that Zadeik-po seeds may be used in the formulation of medicine especially for the treatment of diseases related to bacterial infections.

Keywords : *Myristica fragrans* Hott. (Zadeik-po), antibacterial activity, agar disc diffusion method, microplate dilution method, α-pinene, myristicin, eugenol

Introduction

Medicinal plants are abundant in Myanmar. Eighty five percent of the population in Myanmar lives in rural areas. Most people use the traditional medicinal plants for the treatment of diseases. There are numerous indigenous medicinal plants which are reputed to be effective against the disease of bacterial origin (Boyd and Horel, 1981). *Myristica fragrans* Hott. (Zadeik-po) commonly known as Nutmeg is one of the well-known Myanmar indigenous medicinal plant for making the traditional medicine formulations. It belongs to family Myristicaceae, spreading aromatic evergreen tree usually growing to about 5 to 13 m high, occasionally 20 m. Zadeik-po seeds are ovoid, about 20 to 35 mm long and 15 to 25 mm wide. They are grayish brown in colour with minute reddish brown spots, lines and furrowed. The odour is strong and aromatic with agreeable taste (Peter, 2001). The seeds are native of Moluccas, now cultivated in many tropical countries of both hemispheres, India, Indonesia and Srilanka. They have been introduced into Myanmar about 20 years ago presumably from Indonesia and acclimatized in Kyoneka and Mudon Agricultural farms near Mawlamyine.

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Zadeik-po seeds are popular as spices and also possess various therapeutic properties (Copalakrishnan, 2002). In eastern countries they are employed more as a drug than as condiment. For a long time, they have been used as a folklore medicine for treating diarrhea, mouth sores and insomnia (Assa *et al*., 2014). Oil of Zadeik-po is employed for flavouring food products. It is also used for scenting soaps, tobacco and dental creams and also in perfumery. The seeds contain volatile oil (5-10 %), fat or nutmeg butte (30-40 %), proteins, carbohydrates, starch, calcium, phosphorus, iron and colouring matter. The main chemical components of volatile oil are α -pinene, borneol, eugenol, and myristicin. α-Pinene, camphene and borneol show allergenic, antibacterial, anti-inflammatory and antiviral activities. In addition, eugenol and myristicin also have anticeptic, antiulcer, antitumor, anti-inflammatory and antibacterial properties (Asgarpanah and Kazemivash, 2012).

The present work deals with the antibacterial activity of crude extracts and essential oil of Zadeik-po seeds, isolation and identification of some organic constituents from active extract. The photograph of Zadeik-po tree, fruits and seeds are shown in Figure 1.

(a) (b) (c)

Figure 1 Photographs of (a) tree (b) fruits and (c) seeds of Zadeik-po

Materials and Methods

All chemicals were obtained from British Drug House (BDH). Trypticase soy broth from Difco, Trypticase soy agar from Becton and Triple sugar iron agar from Difco, column (3×60 cm), silica gel (40-60 μm, Wakogel), TLC precoated plates (GF_{254} aluminium plates, Merck) were employed. The following instruments were used for the determination of physical data: melting point; Gallenkamp melting point apparatus, UV spectra; Shimadzu UV-240 UV-Visible spectrophotometer, IR spectra; JEOL JNM-GX FT IR spectrophotometer, NMR spectra; ^{1}HNMR (300 MHz) and mass spectra; GC MS spectrometer at the Goettingen University in Germany and Shimadzu GC MS-QP 5050A at Myanmar Indigenous Medicine Research and Development Department, Yangon.

Zadeik-po seeds were collected from Mudon Township, Mon State. They were cut into pieces and ground in a grinding machine. The powdered sample was stored in air-tight container.

Preliminary Phytochemical Tests

A few grams of dried powdered sample was subjected to the tests of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, saponins, steroids, cyanogenic glycosides, tannins and terpenoids as the preliminary phytochemical test according to reported methods (Robison, 1983 ; M - Tin Wa, 1972).

Preparation of Various Crude Extracts by Successive Solvent Extraction Method

The powdered sample (300 g) was first percolated with pet-ether (60-80 °C) for one week per three times. After removal of pet-ether soluble matter, the defatted marc was then extracted with ethanol. The solvent was removed under reduced pressure in a rotary evaporator. The concentrated alcohol soluble matter was partitioned between water and dichloromethane followed by ethyl acetate. The ethyl acetate and aqueous layer were separately concentrated. The condensed pet-ether, ethanol, dichloromethane, ethyl acetate and aqueous extracts were kept for screening of antibacterial activity.

Extraction of Essential Oil by Steam Distillation Method

The powdered sample (100 g) and distilled water (500 mL) were placed in the 1 L round-bottomed flask. The flask was fitted to Clevenger essential oil apparatus which was joined to water condenser. When the flask was heated, the condensed oil and water coming out from condenser were collected in the receiver flask. The oil was then extracted with pet-ether in a separating funnel. The pet-ether extract was dried over anhydrous sodium sulphate. After filtering, the pet-ether was evaporated to get the essential oil which was then weighed until to be constant weight and kept in air tight bottle.

Antibacterial Screening of Crude Extracts and Essential Oil by Agar Disc Diffusion Method

Paper discs (6 mm) were used to impregnate the extract to obtain approximately 20 μg/disc and allowed to dry at 42 °C in an incubator. The bacterial suspension from trypticase soy broth was streaked evenly onto the surface of the trypticase soy agar plates with sterile cotton swab. After the inoculum had dried for 5 min, the dried discs were placed on the agar with flamed forceps and gently pressed down to ensure proper contact. After inoculations, the plates were incubated immediately or within 30 min. After over-night incubation at 37 °C, the zone of inhibition diameter including 6 mm discs were measured (Mar Mar Nyein *et al*, 1991). In the present work, the crude extracts and essential oil were tested on 20 strains of bacteria.

Determination of Minimum Inhibitory Concentration (MIC) of Essential Oil of Zadeik-po

For the determination of MIC values of the tested sample, the positive (medium and inoculum) and negative (medium and sample) controls were always included. Prior to the performing of MIC, the 100 μ dm^3 of trypticase soy broth was first introduced in 96 wells. Then 100 μ dm^3 of sample was introduced into the first well. By using multi-channel pipette (8–channels) and Titertek micro-titration equipment, 100 μ dm^3 of the mixture was used for downstream serial dilutions up to 12 consecutive wells, each already containing 100 μ dm^3 of media. The last 100 μ dm^3 was discarded. While transferring the content of each well, the mixture was mixed thoroughly with a multi-channel pipetter. Then 20 μ dm^3 of the already prepared inoculum was introduced to its respective wells and the microplates were incubated at 37 °C for 18 h. Growth of the microorganisms was determined by absorbance at 450 nm on an automated microplate reader (Bio Rad) as well as confirming by culturing onto trypticase soy agar was subjected to incubation at 37 °C for overnight. The last well with no growth of the

microorganisms was taken to represent the MIC of the extract. The essential oil of Zadeik-po was tested with organisms: *E. coli* ETEC, *E. coli* LT, *E. coli* EHEC, *S. aureus* MLW 96, *S. aureus* KMM by microplate dilution method.

Isolation of Phytoconstituents from Essential Oil by Column and Preparative Thin Layer Chromatographic (PTLC) Methods

A chromatographic column was packed with silica gel (120 mL) using toluene as the solvent. The essential oil was carefully placed on the top of the silica gel, using a pasteur pipette by allowing it to flow down the walls of the column just above the surface of silica gel. The tap was opened just to let the sample enter into the gel. The column was then filled with solvent. Gradient elution was performed with toluene, toluene:EtOAc (49:1, 19:1 and 9:1 v/v) and 20 fractions were collected. The fractions were monitored by TLC. Finally four main fractions: FV, FVI, FVII and FVIII were obtained after combining the fractions giving similar behaviour on TLC chromatogram. From fractions FV, FVI and FVIII, compound **I** (0.5 %), compound **II** (0.03 %) after purified by PTLC with tol : EtOAc – 29:1 v/v and compound **III** (0.01 %) were isolated respectively. Fraction VII occurred as a mixture.

Identification of Isolated Compounds by Spectroscopic Techniques

By using silica gel column and PTLC techniques, three compounds were isolated from essential oil of Zadeik po. The isolated compounds were characterized by modern spectroscopic techniques such as UV, FT IR, ^{1}HNMR and GC MS spectroscopic techniques.

Determination of Antibacterial Activity of Isolated Compounds

The antibacterial activity of isolated compounds from essential oil was determined by agar disc diffusion method. The isolated compounds were screened by *S. aureus, E. coli*, *Shigella boydii*, *Samonella typhi* and *Vibrio cholera* microorganisms.

Results and Discussion

Preliminary Phytochemical Inverstigation of Zadeik-po Seeds

The phytochemical tests revealed that α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, saponins, steroids, tannins and terpenoids were present in Zadeik-po seeds but alkaloids and cyanogenic glycosides were not detected.

Different Crude Extracts of Zadeik-po

The powdered sample of Zadeik-po seeds was successively extracted with solvents of different polarity: pet-ether (60-80 ºC), dichloromethane, ethyl acetate ethanol and water. The soluble matter contents of pet-ether, dichloromethane, ethyl acetate, ethanol and aqueous extracts were observed to be 36.48 %, 4.07 %, 3.21 %, 10.25 % and 2.98 % respectively. However, pet-ether soluble matter content of Zadeik-po seeds was the highest, indicating that they possessed more non-polar phytoconstituents than polar constituents.

Extraction of Essential Oil by Steam Distillation Method

Essential oil of Zadeik-po seeds was extracted by using the steam distillation method. The yield percent of essential oil was found to be 3.01 % based on the powdered sample. The essential oil was kept for screening the antibacterial activity.

Antibacterial Screening of Crude Extracts and Essential Oil by Agar Disc Diffusion Method

Screening of antibacterial activity of crude extracts and essential oil has been done by agar disc diffusion method on 20 bacterial strains. According to the results presented in Table 1, it was found that pet-ether, dichloromethane, ethyl acetate and aqueous extracts of Zadeik-po did not show any antibacterial activity against all species tested. Ethanol extract of Zadeik-po showed mild antibacterial activity. However, essential oil of Zadeik-po exhibited the pronounced antibacterial action against all tested 20 bacterial strains. It was also observed that most of the active extract showed more remarkable inhibition zone. The effects of essential oil and different extracts from Zadeik-po on *Staphylococcus aureus* MLW 96 and *Escherichia coli* LT are shown in Figure 2.

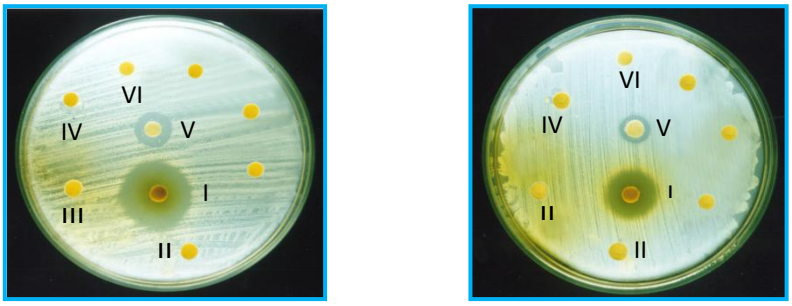
Minimum Inhibitory Concentration (MIC) of Essential Oil

The MIC values of active essential oil of Zadeik-po on five bacteria strains were determined by micro plate dilution method using Falcon 3072 sterile pack containing 96 wells (Figure 3). The active essential oil was tested on 3 species of *E. coli* and 2 species of *S. aureus*. The MIC values of essential oil of Zadeik-po determined by micro plate reader at the wavelength 450 nm are shown in Table 2 and Figure 4. It was found that the lowest MIC values of essential oil of Zadeik-po were 0.0625 mg mL^{-1} obtained with *E. coli* LT and 0.125 mg mL^{-1} with *S. aureus* MLW 96. In addition, the MIC values of essential oil of Zadeik-po on five bacterial strains determined by micro plate reader at the wavelength 450 nm are shown in Table 3.

Table 1 Antibacterial Activity of Crude Extracts and Essential Oil of Zadeik-Po on Different Species of Bacteria

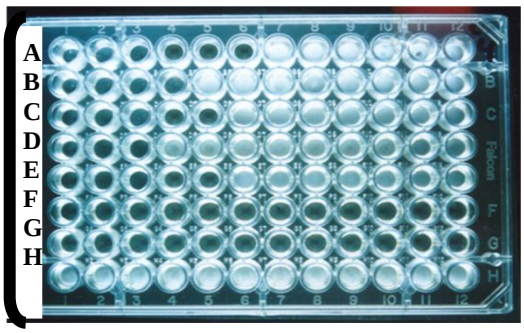
No.	Type of Bacteria	Diameter of inhibition zone (mm)					
		I	II	III	IV	V	VI
1	*Staphylococcus aureus* MLW 96	24	–	–	–	14	–
2	*Staphylococcus aureus*	20	–	–	–	10	–
3	*Staphylococcus aureus* D 25	24	–	–	–	12	–
4	*Staphylococcus aureus* MOE 20	18	–	–	–	12	–
5	*Escherichia coli* LT	20	–	–	–	12	–
6	*Escherichia coli* ETEC	22	–	–	–	12	–
7	*Escherichia coli* ATCC	18	–	–	–	12	–
8	*Escherichia coli* STLT	20	–	–	–	14	–
9	*Samonella typhi*	22	–	–	–	–	–
10	*Samonella stanley*	20	–	–	–	–	–
11	*Vibrio cholerae*	24	–	–	–	16	–
12	*Vibrio cholera Inaba*	20	–	–	–	14	–
13	*Vibrio cholera Ogawa*	20	–	–	–	14	–
14	*Bacillus subtilis*	22	–	–	–	12	–
15	*Bacillus pumilus*	18	–	–	–	12	–
16	*Pseudomonas aeruginosa*	18	–	–	–	–	–
17	*Proteus morganii*	20	–	–	–	–	–
18	*Shigella sonnei*	16	–	–	–	–	–
19	*Shigella boydii*	18	–	–	–	–	–
20	*Shigella flexneri*	16	–	–	–	–	–

I = Essential oil, II = Pet-ether extract, III = Dichloromethane extract, IV = Ethyl acetate extract, V = Ethanol extract, VI = Aqueous extract (Disc diameter = 6 mm)



Staphylococcus aureus MLW 96 *Escherichia coli* LT

Figure 2 Effects of essential oil and different extracts from Zadeik-po on *Staphylococcus aureus* MLW 96 and *Escherichia coli* LT



A = medium + sample + *E.coli* LT
B = medium + sample + *E.coli* EHE*C*
C = medium + sample + *E.coli* ETE*C*
D = medium + sample + *S. aureus* KMM
E = medium + sample + *S. aureus* MLW 96
F = nutrient agar
G = negative control (medium + sample)
H = positive control (medium + inoculum)

Figure 3 Falcon 3072 sterile pack containing 96 wells used for the determination of MIC values of essential oil of Zadeik-po

Table 2 Optical Density of Various Concentrations of Essential Oil from Zadeik-po on *E.coli* LT and *S. aureus* MLW 96 by Micro Plate Dilution Method at the Wavelength 450 nm

Essential Oil Conc. ($mg\ mL^{-1}$)	Optical Density			
	A	**E**	**G**	**H**
2	0.143	0.151	0.135	0.263
1	0.152	0.167	0.146	0.274
0.5	0.161	0.214	0.151	0.270
0.25	0.169	0.232	0.142	0.271
0.125	0.234	0.255	0.143	0.271
0.0625	0.251	0.310	0.144	0.275
0.0312	0.297	0.359	0.143	0.276
0.0156	0.299	0.362	0.154	0.276
0.0078	0.305	0.382	0.148	0.287
0.0039	0.313	0.399	0.146	0.275
0.0019	0.325	0.401	0.149	0.279
0.0009	0.333	0.412	0.145	0.285

A = medium + sample + *E. coil* LT E = medium + sample + *S.aureus* MLW 96
G = negative control (medium + sample) H = positive control (medium + inoculum)

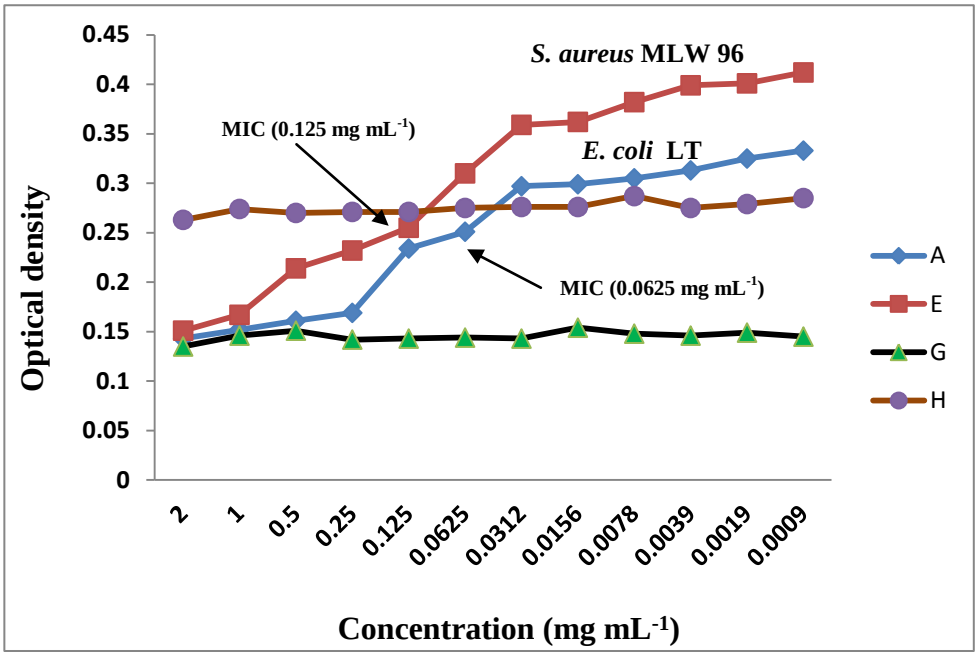


Figure 4 A plot of optical density and various concentrations of essential oil of Zadeik-po tested on *E.coli* LT and *S. aureus* MLW 96 by using micro plate dilution method

Table 3 MIC Values of Essential Oil of Zadeik-po by Micro Plate Dilution Method at the Wavelength 450 nm

No.	Bacterial strain	MIC value (mg mL^{-1})
A	*E.coli* LT	0.0625
B	*E.coli* EHE*C*	0.25
C	*E.coli* ETE*C*	0.125
D	*S. aureus* KMM	0.5
E	*S. aureus* MLW96	0.125

Isolation of Phytoconstituents from Active Essential Oil

The active essential oil of Zadeik-po was separated by column and preparative thin layer chromatographic (PTLC) methods using toluene and toluene: EtOAc with various ratios: 49:1, 19:1, 9:1 v/v as eluent. Three compounds, **I, II and III** at R_f values of 0.87 (toluene), 0.65 (toluene: EtOAc – 19:1) and 0.52 (toluene: EtOAc – 4:1) were isolated, resulting 0.5 %, 0.03 % and 0.01 % of yields respectively.

Identification of Isolated Compounds

The isolated compounds from essential oil of Zadeik-po were identified by using the modern spectroscopic methods: UV, FT IR, ^{1}HNMR and GC MS spectroscopic techniques.

α-Pinene (I): Pale yellow oil (0.5 % yield); UV-visible, λ_{max} (nm) in MeOH : 230; FT IR υ (cm^{-1}) (Neat), 2959, 2869 ($\upsilon_{asym\text{-}C\text{-}H}$ and $\upsilon_{sym\text{-}C\text{-}H}$), 1630 ($\upsilon_{C=C}$) of olefinic group, 1458, 1375 ($\delta_{C\text{-}H}$), 891, 800 (δ_{oop} of alkene); GC MS, (m/z) : 136 [M$^+$], 121, 105, 93, 79, 53, 39.

Myristicin (II): Light yellow oil (0.03 % yield);); UV-visible, λ_{max} (nm) in MeOH : 230, 240 (sh), 280; FT IR υ (cm^{-1}) (Neat), 3077, 3003 ($\upsilon_{=CH}$), 2975, 2896 ($\upsilon_{asym\text{-}C\text{-}H}$ and $\upsilon_{sym\text{-}C\text{-}H}$), 1632 ($\upsilon_{C=C}$) in vinyl compound, 1612, 1508, 1432 ($\upsilon_{C=C}$) in aromatic ring, 1357, 1317 ($\delta_{C\text{-}H}$), 1282, 1131 ($\upsilon_{asymC\text{-}O}$ of ar C–O), 1090, 1045 ($\upsilon_{C\text{-}O\text{-}C}$) in ether, 994, 966 ($\delta_{oop)}$ in vinyl compound, 827, 806 (δ_{oop} of C–H ar); ^{1}HNMR ($CDCl_3$, 300 MHz), δ_H (ppm) : 6.36 (d, *J*=8.57 Hz, 2H-1, 3), 5.81-5.98 (m,1H-8), 5.92 (s, 2H-10), 5.05-5.09 (m, 2H-9), 3.86 (s, 3H-11), 3.27 (d, *J*=5.8 Hz, 2H-7); GC MS, (m/z) : 192 [M^+], 177, 165, 161, 133, 119, 91, 71, 65.

Eugenol (III): Pale yellow oil (0.01 % yield); UV-visible, λ_{max} (nm) in MeOH : 220, 230, 280, in MeOH + NaOH : 228, 246, 296; FT IR υ (cm^{-1}) (Neat), 3513 ($\upsilon_{O\text{-}H}$ of phenolic O-H group) 3076, 3003 ($\upsilon_{=CH}$), 2975, 2896 ($\upsilon_{asym\text{-}C\text{-}H}$ and $\upsilon_{sym\text{-}C\text{-}H}$), 1637 ($\upsilon_{C=C}$) in vinyl compound, 1611, 1513, 1464, 1432 ($\upsilon_{C=C}$) in aromatic ring, 1367 (δ_{OH}), 1268, 1234 ($\upsilon_{asymC\text{-}O}$ of ar–C–O), 1149, 1034 ($\upsilon_{C\text{-}O\text{-}C}$) in ether, 995, 914 ($\delta_{oop)}$ in vinyl compound, 850, 817 (δ_{oop} of C–H ar); ^{1}HNMR ($CDCl_3$, 300 MHz), δ_H (ppm) : 6.88 (d , *J*=7.50 Hz, 1H-6), 6.69-6.72 (m,2H-3, 5), 5.91-6.02 (m, 1H-8), 5.76 (m, 1H-1 OH), 5.05-5.14 (m, 2H-9), 3.86 (s, H-10), 3.33-3.35 (d, *J*=5.8 Hz, 2H-7); GC MS, (m/z) : 164 [M^+], 149, 137, 131, 121, 91, 77.

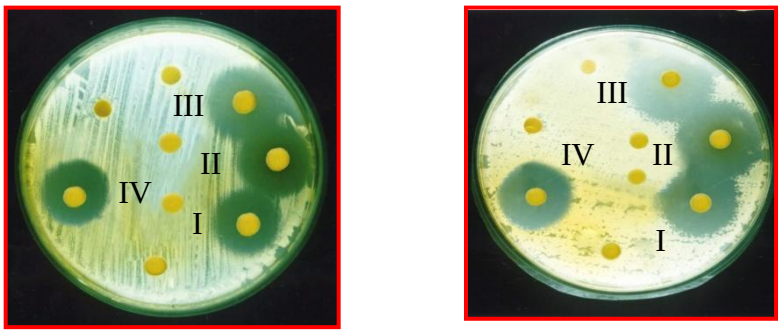
Antibacterial Activity of Isolated Compounds

The antibacterial activity of isolated compounds was determined by agar disc diffusion method. The antibacterial activity of α-Pinene, Myristicin and Eugenol isolated from essential oil of Zadeik-po were also screened on five main bacterial strains: *S. aureus, E. coli, Shigella boydii, Samonella typhi* and *Vibrio cholera*. Myristicin and Eugenol showed antibacterial activity against all five bacterial strains. But α- Pinene is active against *S. aureus, E. coli, Shigella boydii* and *Vibrio cholera* and inactive against *Samonella typhi* (Table 4 and Figure 5).

Table 4 Antibacterial Activity of the Isolated Compounds Compared with Tetracyclin

Isolated compound	Inhibition zone diameter (mm)				
	A	**B**	**C**	**D**	**E**
α-Pinene (I)	16	16	14	18	-
Myristicin (II)	16	18	18	20	16
Eugenol (III)	24	22	24	22	20
Tetracyclin (IV)	20	24	30	24	22

A = *E.coli*, B = *S.aureus*, C = *Shigella boydii*, D = *Vibrio cholerae*, E = *Samonella typhi* (Disc diameter = 6 mm)

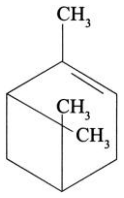


Escherichia coli *Vibrio cholerae*

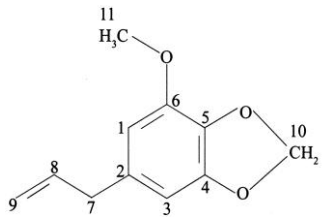
Figure 5 Effects of isolated compounds from essential oil on *Escherichia coli* and *Vibrio cholerae*

Conclusion

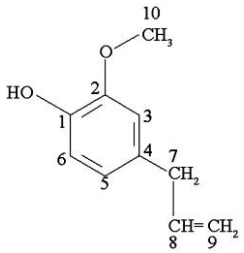
From the research work, it could be concluded that preliminary phytochemical tests were revealed the presence of α-amino acids, carbohydrates, flavonoids, glycosides, phenolic compounds, saponins, steroids, tannins and terpenoids. Among the extracts, pet-ether soluble matter content was found to be highest. Essential oil (3 %) was also extracted from Zadeik po seeds by steam distillation method. By using agar disc diffusion method, essential oil exhibited pronounced antibacterial action against all tested 20 bacterial strains. MIC values of active essential oil of Zadeik-po were determined by microplate dilution method on five bacterial strains. The lowest MIC values of essential oil was found to be 0.0625 mg mL^{-1} with *E. coli* LT. α- Pinene (0.5%), Myristicin (0.03%) and Eugenol (0.01%) were isolated from essential oil of Zadeik-po by column chromatographic and PTLC methods. In addition, α- Pinene, Myristicin and Eugenol also showed antibacterial property. From the scientifically obervation, it can be inferred that Zadeik-po seeds have been used in the formulations of medicine for the treatment of diseases: namely pneumonia, urinary tract infection, diarrhoea, dysentery, cholera and typhoid fever.



α – Pinene



Myristicin



Eugenol

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