Isolation of Bioactive Compounds from the Fruits of *Zanthoxylum acanthopodium* DC. (Mat-Khar)

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Abstract

Zanthoxylum acanthopodium DC. is well known as "Mat-Khar" in the Kayah state, Myanmar. Compound MKC-1 (R_f = 0.30 (n-Hex : EtOAc 15:1 v/v) and compound MKC-2 (R_f = 0.19 (n-Hex : EtOAc 15:1 v/v) were isolated from ethyl acetate extract of *Zanthoxylum acanthopodium* DC. by using column chromatography. Functional groups of these pure compounds were identified by FT IR spectroscopic method. According to the data of R_f values, chemical properties, and UV and FT IR spectra, these two compounds may be α, β - unsaturated compounds. Finally, antimicrobial activity of compound MKC-2 showed higher activity (22.01- 27.50 mm) against six human pathogens (gram positive and gram negative) than compound MKC-1 showed activity (16.77 - 22.40 mm). In the investigation of minimum inhibitory concentration, the MICs value of MKC-1 was 50 mg/ 2 mL against *Candida albicans* and 25 mg/ 2 mL against *Pseudomonas aeruginosa.* MICs values of MKC-2 was 6.25 mg/ 2 mL against *Candida albicans* and 12.50 mg/ 2 mL against *Pseudomonas aeruginosa.* According to the above results, *Zanthoxylum acanthopodium* DC. (Mat-Khar) fruits possessed bioactive substances. Therefore, *Zanthoxylum acanthopodium* DC. (Mat-Khar) fruits should be applied as the local health for producing antimicrobial substances as evidence in this research work.

Keywords: Mat-Khar, α, β - unsaturated compounds, human pathogens, minimum inhibitory concentration, antimicrobial substances

Introduction

In Myanmar, *Zanthoxylum acanthopodium* DC. called as Mat-Khar, is a species that only grows in Kayah State and is also called as Ei-San-Maw by Kayah vernacular in Kayah state. Plants of Mat-Khar are grown in Kayah state at 899 meters (2950 feet) above sea level. Especially, Mat-Khar fruits are mainly used as spice in making popular Kayah sausage. Previous studies showed the potency of *Z. acanthopodium* as an antioxidant, antibacterial against human pathogens, anti-inflammatory, and antiacne (Julistiono, *et al.*, 2018; Wijaya, *et al.*, 2019). Andaliman (*Zanthoxylum acanthopodium* DC.) fruits are known as the Batak society spicy and are used to eliminate the smell of fish and raw meat (Siregar, 2002). Cahyana and Mardiana, 2003 also stated that Mat-Khar possessed an antimicrobial activity.

Medicinal plants have shown significant activity to control various infectious diseases including bacterial and fungal infections (Arora *et al.*, 2013). These diseases account for approximately one-half of all the deaths in most developing countries of the world (Madhumitha and Saral, 2011). This problem has increased to such a recorded level, partly due to the observed resistance cases to most of the currently in use antimicrobial drugs (Abdallah, 2011). Therefore, the research aims to analyze antimicrobial agents from mat-khar fruits and to isolate bioactive compounds from the extract of mat-khar fruits at the local sources of Kayah State.

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Materil and Methods

Collection and Preparation of Sample

The fresh fruits of *Zanthoxylum acanthopodium* DC. (Mat-Khar) were locally collected from Min Su Quarter, Loikaw Township (N 19° 40′ 49.188″ E 97° 13′ 28.910″), Kayah State during October, 2021 and taxonomically identified by the Department of Botany, Loikaw University.

Extraction of Crude Extracts from the fruits of *Zanthoxylum acanthopodium* DC.

The collected samples were cleaned and shade dried at room temperature. The dried fruitsplant sample (200 g) were pulverized by using the blender and macerated with 1000 mL of ethyl acetate at the ratio of 1:5 w/v at room temperature for one month. After maceration, the solvent was evaporated by using water bath at 70 ˚C. Finally, the extracts were weighed and stored in a refrigerator.

Isolation of Organic Compounds by Column Chromatography

According to thin layer chromatographic analysis, the ethyl acetate crude extract of *Zanthoxylum acanthopodium* DC. was developed to isolate the active compounds by silica gel column chromatography with n-hexane : ethyl acetate (9 : 1, 8 : 2, 7 : 3, 1 : 1 v/v) as the eluting solvent. The column was sealed with a piece of cotton wool on the bottom. The silica gel (60-120 MESH) 90 g was dissolved in petroleum ether and the column was packed by the wet method (Simon and Gray,1998). Each fraction was checked with TLC (Silica gel 60 F_{254}, Germany) plates and combined the fractions with the same R_f value. Firstly, fractions (10- 35) with the same R_f value were combined and PTLC was performed.

Identification of pure compounds

Two compounds were observed under 254 and 365 nm wavelength of UV lamp (GL- 9406, China) at Loikaw University, UV-visible(Shimadzu) spectrophotometer and functional group of obtaining two compounds were analyzed by using FT IR (Shimadzu, FT IR - 8400, Japan) spectrophotometer at Department of Chemistry in Pathein University.

Characterization, solubility and some chemical properties of isolated compounds

The isolated compounds were characterized by the determination of their physical properties: R_f values, solubility in various solvents and some chemical properties: reaction with I_2 vapour, 5 % H_2SO_4, 2,4- DNP (2,4 - dinitrophenyl-hydrazine), $KMnO_4$, $FeCl_3$.

Antimicrobial Activity of Isolated Compounds

Isolated compounds were tested by agar well diffusion method (Dubey and Maheshwari, 2002) for the antimicrobial activities. Cork borer was used to make the wells (8 mm in diameter) in the autoclaved basal antimicrobial test-medium. Wells impregnated with compounds (20 µL/ well) were incubated at room temperature for 24hours. After incubation, the clear zones were measured. Therefore, the diameter of clear zones had been observed as potent activity as shown by compounds. Clear zones surrounding the wells indicated the presence of antimicrobial activities which inhibit the growth of the test organisms selectively. The test organisms used for this experiment were *Bacillus pumilus, Bacillus subtilis* IFO 90571, *Candida albicans* NITE 09542, *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi.* The organisms were obtained from National Institute of Technology and Evaluation (NITE, Japan), and Pharmaceutical Research Department, Yangon, Myanmar.

Determination of Minimum Inhibitory Concentrations (MICs) of Isolated Compounds

Minimum Inhibitory concentrations (MICs) were carried out by a two-fold serial dilution method (Demain, 1986). Test organisms of *Candida albicans* and *Pseudomonas aeruginosa* were used. GYP broth (glucose 1.0 g, yeast extract 0.3 g, peptone 0.3 g, distilled water 100 mL) was prepared and labeled A-F. Two-fold serial dilutions of the compounds were prepared to give a decrease in concentration ranging 50 mg/ 2 mL, 25 mg/ 2 mL, 12.5 mg/ 2 mL, 6.25 mg/ 2 mL, 3.13 mg/ 2 mL, 1.56 mg/ 2 mL, respectively. This concentration was achieved by weighting and dissolving 100 mg/ 2 mL of the compound in a test tube labeled S containing 1 : 1 (v/v) (MeOH: H_2O). one milliliter was transferred from test tube S to test tube A containing 1 mL of GYP to give 50 mg/ 2 mL. This procedure continued until a concentration of 1.56 mg/ 2 mL was obtained in the six test tube labeled F. Homogenous mixture was obtained by vigorously shaking each tube for at least 5 seconds. And, 0.2 mL of test organisms was inoculated in each assay plate. A 20 µL of different dilutions of the compound was dropped into the agar well. After incubation for 24 hours, the MICs were determined by selecting the lowest concentration of metabolite which caused complete inhibition of test (fungi and bacterial) growth. Experiments were done in triplicate. The MICs were determined by the lowest concentration of the compounds that prevented visible growth (Andrews, 2005, Ewansiha, *et al.*, 2016).

Results and Discussion

Identification of isolated compounds

When the fruits of *Zanthoxylum acanthopodium* DC. was extracted with ethyl acetate solvent (by using maceration method), 10.0 g, 5 % of extract was obtained. Compound MKC-1 and compound MKC-2 were isolated from ethyl acetate extract of *Zanthoxylum acanthopodium* DC. by using column chromatography. Isolated compounds were characterized by determination of their R_f values, solubility, chemical properties, and UV and FT IR spectra.

Determination of solubility and R_f value of isolated compounds

Fractions (10 - 35) with the same R_f value were combined and PTLC was performed. Finally, two compounds MKC-1 and MKC-2 (Figures 1) were obtained. Isolated compounds MKC-1 and MKC-2 were soluble in petroleum ether, n-hexane, ethyl acetate, ethanol, and methanol solvent (Table 1). Their R_f values were determined with n-hexane: ethyl acetate (15:1) solvent system (Tables 1 and Figures 2).

Compound MKC-1 **Compound MKC-2**

Figure 1 Isolated compounds from ethyl acetate crude extract of *Zanthoxylum acanthopodium* DC.

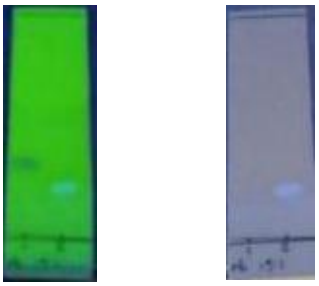
Table 1 Solubility and R_f value of isolated compounds, MKC-1 and MKC-2

Isolated compounds	PE	n-Hexane	EtOAc	EtOH	MeOH	R_f values
MKC- 1	+	+	+	+	+	0.30(n-Hex: EtOAc, 15 : 1)
MKC- 2	+	+	+	+	+	0.19 (n-Hex: EtOAc, 15 : 1)

Table 2 Characterization of isolated compounds by color reagents test

Reagents	MKC-1	Remark	MKC-2	Remark
Iodine vapour	ND	-	ND	-
5 % H_2SO_4	ND	-	ND	-
2,4-DNP	ND	-	ND	-
$FeCL_3$	ND	-	ND	
$KMnO_4$	Decolored	Unsaturated compounds	Decolored	Unsaturated compounds

ND- Not Detect



Under 254 nm Under 365 nm

Figure 2 TLC Photographs for isolated compounds MKC-1 and MKC-2 under UV Lamp (15 : 1 n-Hex : EtOAc)

Figure 3 $KMnO_4$ test for isolated compounds, MKC-1 and MKC-2

Compound MKC-1

Compound MKC-1 was isolated as a white powder and its R_f value was 0.30 in the solvent system of n-Hexane: EtOAc (15:1 v/v). Compound MKC-1 was UV active due to the presence of a conjugated double bond. The maximum absorption wavelength at 208, 226 and 274 nm are due to the n-π* transition (Figure 4 and Table 3). The FT IR spectrum of compound MKC-1 showed the band at 2957 cm^{-1}, 2915 cm^{-1} and 2847 cm^{-1} indicating the presence of C-H stretching vibration of $–CH_2$- and $–CH_3$ groups. The absorption band at 1692cm^{-1} indicates the C=O stretching vibration of aldehyde and ketone. The absorption band appeared at 1463 cm^{-1} due to the presence of C-H bending vibration. Absorption bands appeared at 1059 cm^{-1} appeared due to the presence of C-O stretching vibration. Absorption bands appeared at 802 cm^{-1} and 719 cm^{-1} due to the presence of C-H out of plane bending vibration (oop or

aromatic C-H) shown in Figure 5 and Table 4. Additionally, Compound MKC-1 was observed decolorized in $KMnO_4$ (Figure 3). Compound MkC-1 was soluble in MeOH, EtOH, EtOAc, n-Hexane and PE. According to the results of chemical properties, R_f value, UV and FT IR spectral analyses, compound MKC-1 may be α, β-unsaturated compound.

Table 3 UV spectral data of isolated compound MKC-1

Solvent used	Observed λ_{max} (nm)	Remark
MeOH	208, 226, 274	n- π* transition

Table 4 FT IR data of isolated compound MKC-1

Wave number (cm^{-1})	Literature* Wave number (cm^{-1})	Band assignment
2957, 2915 2847	3050~2800	C-H stretching vibration of alkane
1692	1710 ~1665	C=O stretching vibration (α,β-unsaturated compound)
1463	1470 ~1450	C-H bending vibration
1059	1280~1020	C-O stretching vibration
802,719	900 ~ 675	C-H out of plane bending vibration (“oop or aromatic C-H”)

*(Andronie *et al*., 2016 and Smith, 1998)

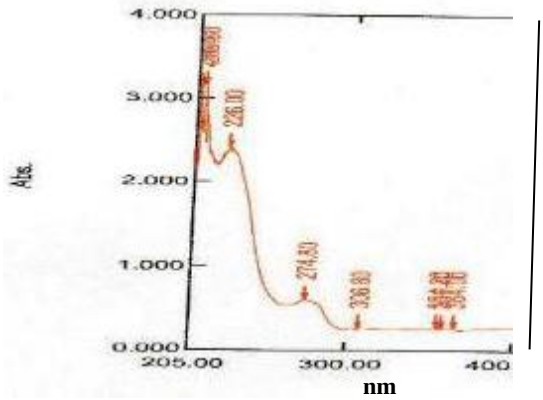


Figure 4 UV spectrum of the isolated compound, MKC-1

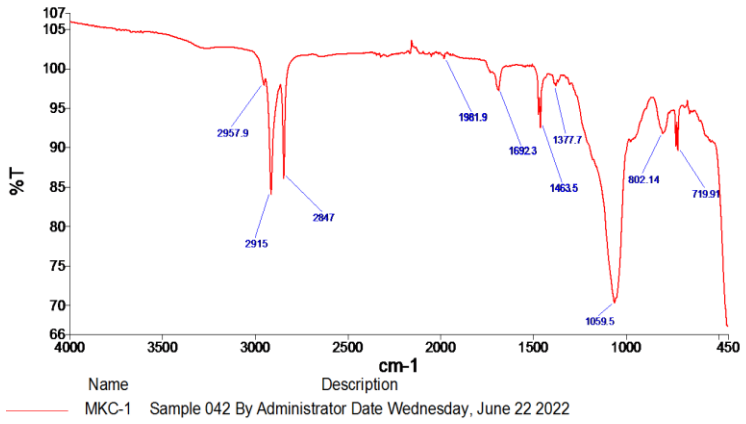


Figure 5 FT IR spectrum of the isolated compound, MKC-1

Compound MKC-2

Compound MKC-2 was isolated as a white powder and its R_f value was 0.19 in the solvent system of n-Hexane: EtOAc (15:1 v/v). Compound MKC -2 was UV active due to the presence of a conjugated double bond. The maximum absorption wavelength at 200, 202, 274 nm is due to the n-π* transition (Figure 6 and Table 5). The FT IR spectrum of compound MKC -2 showed the band at 2954 cm^{-1}, 2918 cm^{-1} and 2847 cm^{-1} indicating the presence of C-H stretching vibration of $–CH_2$- and $–CH_3$ groups. The absorption band at 1688 cm^{-1} indicates the C=O stretching vibration of aldehyde and ketone. The absorption band appeared at 1463 cm^{-1} due to the presence of C-H bending vibration. Absorption bands appeared at 1056 cm^{-1} appeared due to the presence of C-O stretching vibration. Absorption bands appeared at 802 cm^{-1} and 719 cm^{-1} due to the presence of C-H out of plane bending vibration (oop or aromatic C-H) shown in Figure 7 and Table 6. Additionally, Compound MKC-2 was observed decolorized in $KMnO_4$(Figure 3). Compound MKC-2 was soluble in MeOH, EtOH, EtOAc, n-Hexane and PE. According to the data of chemical properties, R_f value, UV and FT IR spectral analyses, compound MKC-2 may be α, β-unsaturated compound.

Table 5 UV spectral data of the isolated compound MKC-2

Solvent used	Observed λ_{max} (nm)	Remark
MeOH	200, 202, 274	n- π* transition

Table 6 FT IR data of the isolated compound MKC-2

Wave number (cm^{-1})	Literature* Wave number(cm^{-1})	Band assignment
2954, 2918 2847	3050~2800	C-H stretching vibration of alkane
1688	1710 ~1665	C=O stretching vibration (α,β-unsaturated compound)
1463	1470 ~1450	C-H bending vibration
1056	1280~1020	C-O stretching vibration
802, 719	900 ~ 675	C-H out of plane bending vibration (“oop or aromatic C-H”)

*(Andronie *et al*., 2016 and Smith, 1998)

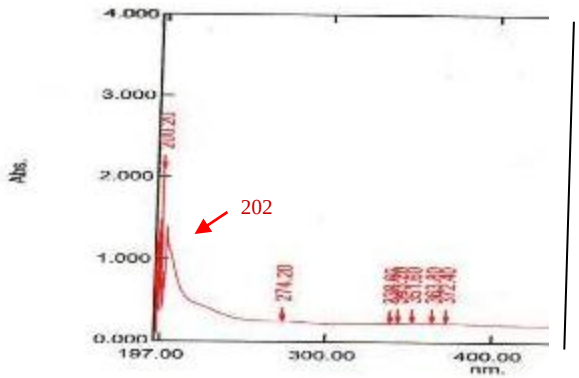


Figure 6 UV spectrum of the isolated compound, MKC-2

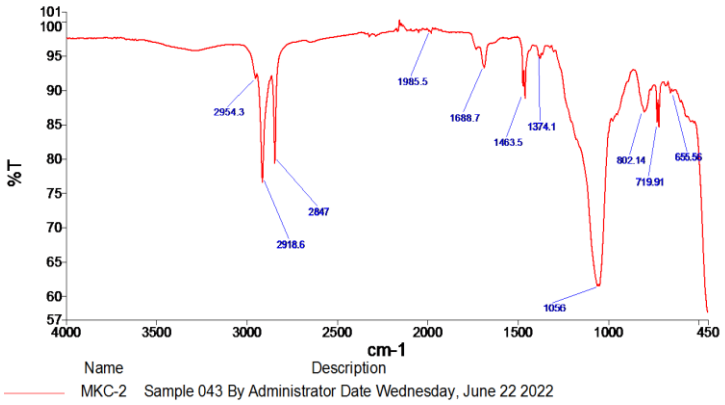


Figure 7 FT IR spectrum of the isolated compound, MKC-2

Investigation of Antimicrobial Activities of Isolated Compounds

Antimicrobial activities of isolated compounds (MKC-1 and MKC-2) were performed by agar well diffusion method with six selected organisms. Compound MKC-1 showed activity of 19.40 mm against *Bacillus pumilus*, 16.77 mm against *Bacillus subtilis*, 17.50 mm against *Candida albicans*, 18.72 mm against *Escherichia coli*, 22.40 mm against *Pseudomonas aeruginosa*, 17.27 mm against *Salmonella typhi.* and compound MKC-2 showed activity at 23.20 mm against *Bacillus pumilus*, 22.64 mm against *Bacillus subtilis*, 22.10 mm against *Candida albicans*, 22.72 mm against *Escherichia coli*, 27.50 mm against *Pseudomonas aeruginosa*, 22.01 mm against *Salmonella typhi* (Table-7 and Figure-8). In the investigation of the antimicrobial activity of the isolated compounds, the antimicrobial activity of MKC-2 showed higher than MKC-1.

Table 7 Investigation of Antimicrobial Activities from Isolated Compounds

	Microorganisms and Inhibition zone diameters (mm)					
Compounds	**1**	**2**	**3**	**4**	**5**	**6**
MKC-1	19.40 (++)	16.77 (++)	17.50 (++)	18.72 (++)	22.40 (+++)	17.27 (++)
MKC-2	23.20 (+++)	22.64 (+++)	22.10 (+++)	22.72 (+++)	27.50 (+++)	22.01 (+++)

1.Bacillus Pumilus *4. Escherichia coli* (-) No Activity
2. Bacillus subtilis *5. Pseudomonas aeruginosa* (+) 9 - 14 mm = Low Activity
3. Candida albicans *6. Salmonella typhimurium* (++) 15 - 20 mm = Medium Activity
Agar well= 8 mm 20 µL / well (+++) 21 mm-above= High Activity

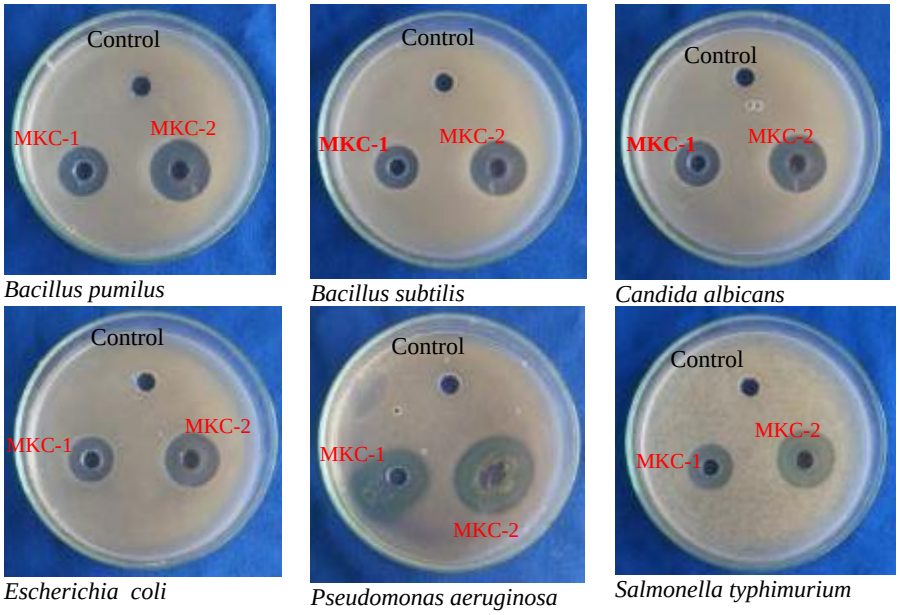


Figure 8 Investigation of antimicrobial activities of isolated compounds, MKC-1 and MKC-2

Minimum Inhibitory Concentrations (MICs) of isolated compounds

MICs of compounds were determined by two-fold serial dilution method and were ranging from 50 mg/ 2 mL to 1.56 mg/ 2 mL. MICs were read in mg/ 2 mL after overnight incubation. It was observed that the MICs value of the isolated compound (MKC-1) was50 mg/2 mLwhich can inhibit the growth of *Candida albicans*, any further increase in its concentration above 50 mg/ mL resulted in a more suppressive effect on the growth of *Candida albicans.* MICs value of the isolated compound (MKC-1) was 25 mg/ 2 mL which can inhibit the growth of *Pseudomonas aeruginosa*, any further increase in its concentration above 25 mg/ 2 mL resulted in a more suppressive effect on the growth of *Pseudomonas aeruginosa.* (Table -8 and Figure 9-10).

MICs value of the isolated compound (MKC-2) was 6.25 mg/ 2 mL which can inhibit the growth of *Candida albicans*, any further increase in its concentration above 6.25 mg/ 2 mL resulted in a more suppressive effect on the growth of *Candida albicans.* MICs value of the isolated compound (MKC-2) was 12.50 mg/ 2 mL which can inhibit the growth of *Pseudomonas aeruginosa*, any further increase in its concentration above 12.50 mg/ 2 mL resulted in a more suppressive effect on the growth of *Pseudomonas aeruginosa.* In the observation of MICs values of compounds, MKC-1, and 2 act as bactericidal and fungicidal substances (Table 9 and Figure 11-12).

Table 8 Minimum inhibitory concentrations of compound MKC-1 on *Candida albicans* and *Pseudomonas aeruginosa*

MIC values (mg/2mL)	Antimicrobial activity (mm)	
	Candida albicans	***Pseudo. aeruginosa***
Control	-	-
50	13.50	17.90
25	-	13.84
12.50	-	-
6.25	-	-
3.13	-	-
1.56	-	-

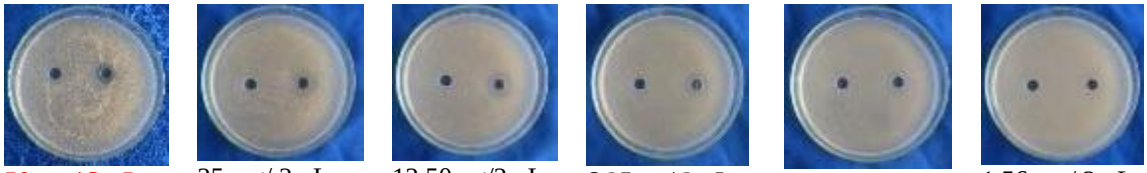


Figure 9 Minimum inhibitory concentrations of compound MKC-1 on *Candida albicans*

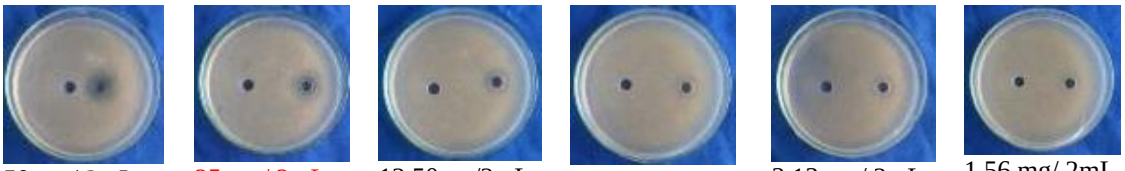


Figure 10 Minimum inhibitory concentrations of compound MKC-1 on *Pseudomonas aeruginosa*

Table 9 Minimum inhibitory concentrations of compound MKC-2 on *Candida albicans* and *Pseudomonas aeruginosa*

MIC values (mg/2mL)	Antimicrobial activity (mm)	
	Candida albicans	***Pseudo. aeruginosa***
Control	-	-
50	12.55	12.30
25	11.30	11.11
12.50	10.82	**10.00**
6.25	**10.00**	-
3.13	-	-
1.56	-	-

Figure 11 Minimum inhibitory concentrations of compound MKC-2 on *Candida albicans*

Figure 12 Minimum inhibitory concentrations of compound MKC-2 on *Pseudomonas aeruginosa*

Conclusion

In the research work, *Zanthoxylum acanthopodium* DC. (Mat-Khar) fruits possessed antimicrobial activities. It can be concluded that the isolated compounds and extracts of *Zanthoxylum acanthopodium* DC. (Mat-Khar) fruits are effective against human pathogens. Thus, it can be suggested that *Zanthoxylum acanthopodium* DC. (Mat-Khar) fruits should be used for biomedical applications due to it has wide spectrum of biological activities, such as antifungal, antibacterial and antimicrobial. However, further examination is required to identify the structure of the compound by using NMR, Mass Spectrometry and to determine the dose, toxicity and bioavailability of the active compounds of *Zanthoxylum acanthopodium* DC. (Mat-Khar) fruits to analyze its efficacy as a therapeutic agent and hence provide a platform for further research and development of new therapeutic agents from a local source.

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