*In vitro*and*invivo*Screening of Antibacterial Activities of Different Crude Extracts and Isolated CompoundLupeol from *Tamarindus indica* L. (Ma-gyi) StemBark

Thin Thin Win[1], Myint Myint Khine[2], Saw Hla Myint[3]

Abstract

The stem bark of *Tamarindusindica* L. (Ma-gyi) is used as folk medicine for the treatment of jaundice, malarial fever, laxative, diarrhoea and boils. The Ma-gyi stem bark was chosen for determination of antibacterial activity and organic constituents. The plant sample was collected from Kamayut Township, Yangon Region. Lupeol was isolated from ethyl acetate extract of ma-gyi stem bark by column and preparative thin layer chromatographic methods. It was identified by UV, FT IR, ^{1}H NMR and ^{13}C NMR spectroscopic methods. Then, *in vitro* screening of antibacterial activity was investigated on four crude extracts (PE, EtOAc, EtOH and H_2O) of Ma-gyi stem bark against 3 species of *Staphylococcus aureus*and 2 species of *Escherichia coli*, one species of *Bacillus subtilis*, one species of *Proteus morganii*and one species of *Vibrio cholera* by employing agar disc diffusion method. Except PE extract, all of the crude extracts were found to exhibit the inhibition zones against all of the organisms tested. Moreover, *in vivo* screening of antibacterial activity of 70% ethanol extract, ethyl acetate extract and lupeol was also carried out by using S*taphylococcus aureus*induced albino rat model. Among them, ethyl acetate extract of Ma-gyi stem bark was found to exhibit the most effective anti-inflammatory and wound healing properties on the rats.

***Keywords**:* *Tamarindusindica*L. (Ma-gyi), antibacterial activity, lupeol, agar disc diffusion method, anti-inflammatory, wound healing properties

Introduction

Tamarindus indica L. (Figure 1) belongs to the dicotyledonous family Leguminosaesub family Caesalpiniaceae, which is the third largest family of flowering plants with a total of 727 genera and 19,327 species (Lewis *et al*., 2005). It is found in virtually all tropical climatic regions, from India through Africa to the Caribbean and South America and up to Southern Florida (Morton, 1987). 26 genera and 124 species including *T. indica* grow in Myanmar. Scientific classification of *Tamarindus indica* (Kress *et al*., 2003) is described as follows:

Family	:	Leguminosae
Genus	:	*Tamarindus*
Species	:	*T. indica*
Scientific Name	:	*Tamarindusindica*L.
English Name	:	Tamarind
Myanmar Name	:	Ma-gyi

An infusion of the leaves is said to be cooling and useful in bilious fever of the fresh leaves is applied to swellings and boils, and for relieving paid of the flowers in inflammatory affection of the conjunctiva. The bark is astringent and is given in diarrhoea; in lotions and poultices, it is also applied to sores boils. In some countries, the bark is reported to be prescribed in asthma, amenorrhoea and as tonic and febrifuge.

[1] Associate Professor,Dr, Department of Chemistry, Kyaukse University
[2] Professor and Head, Dr, Department of Chemistry, Kalay University
[3] Professor and Head (Rtd.), Dr, Department of Chemistry. University of Yangon



Tamarind tree Tamarind stem bark Close up to tamarind flower

Figure 1. Images of the different parts of *Tamarindusindica*L.

Materials and Methods

Sample Collection

Tamarindus indica L. (ma-gyi) was collected from Kamayut Township, Yangon Region. After collection, the sample was identified by authorized botanist at the Department of Botany, University of Yangon. The collected samples were washed with water and dried at room temperature for two weeks. It was then powdered in a grinding machine. The dried powdered samples were then stored in air-tight containers so as to prevent from fungus attack. 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; Perkin Elmer GX FT IR spectrophotometer, NMR spectra; Bruker 600 spectrometer were used to record the spectra.

Preparation of Crude Extracts

The dried powdered samples (ca.100 g) were extracted with 70 % EtOH (200 mL × 4) at room temperature for two weeks. After two weeks, the extract was filtered and concentrated by rotatory evaporator at 45 °C. The concentrated extracts were combined and then partitioned with PE (60-80 °C) to remove the fat. In this way two layers, namely PE soluble layer (upper layer) and PE insoluble layer (lower layer) (or) aqueous layer were obtained. Then the aqueous layer was partitioned with ethyl acetate in a separating funnel. The EtOAc extract was dried over anhydrous Na_2SO_4, filtered and the total filtrate was concentrated under vacuum rotatory evaporator to obtain EtO Accrude extract. The EtOAc crude extract (1 g) was kept for isolation of organic substituents.

The dried powdered samples (100 g) were extracted with (250 cm^3) of, pet-ether, ethyl acetate, 70 % ethanol and water in separate conical flask, respectively for at least 7 days and then filtered. The filtrates were evaporated by using rotatory evaporator and desiccated. Then the dried extracts were weighed. Each extract was stored in refrigerator for screening of antibacterial activity and wound-healing effect.

Isolation of Organic Compound from EtOAc Crude Extract of *T. indica* Stem Bark

The EtOAc crude extract (1 g) was fractionated on a silica gel column (PE:CH_2Cl_2) (99:1)-(CH_2Cl_2:MeOH) (1:2) as eluant. After combining together similar fractions, F_1, F_2, F_3 and F_4 fractions were obtained. F_3 upon further evaporation on silica gel column with CH_2Cl_2:MeOH (6:1) developing solvent, yielded pure compound (0.009 %, yield) as white crystal.

Structural Elucidation

The structure of isolated compound was elucidated and identified by modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR and ^{13}C NMR spectroscopies.

Screening of Antibacterial Activity

(i) *in vitro* Screening of Different Crude Extracts by Agar Disc Diffusion Method

The actibacterial activity of different crude extracts (PE, EtOAc, EtOH and H_2O) was determined by agar disc diffusion method (Mar MarNyein*et al*., 1991) at DMR, Yangon.

Procedure

The filter discs (6 mm diameter) were made by punched No.1 Whatmann filter paper. The disc were sterilized by autoclaving and following by dry heating at 60 °C for 1 hr. It was then impregnated with concentrated extracts to obtain approximately 20 μg/disc. Prior to adherence on the culture plates, the discs were allowed to dry at 42 °C in incubator. The bacterial suspension from trypticase soy broth was streak evenly onto the surface of the trypticase soy agar plate with sterile cotton swab. After the inoculums had dried (5 min), the dried discs were placed on the agar with flamed forceps and gently pressed down to ensure proper contact. One disc, impregnated individually with solvent was placed along the test disc for control and comparing purpose. The plates were incubated immediately or within 30 min after inoculation. After overnight incubation at 37 °C, the zones of inhibition diameter including 6 mm discs were measured.

(ii) *in vivo* Screening of Different Crude Extracts and Isolated Compound Lupeol by using *S. aureus*Induced Albino Rats Model

i*n vivo* anti-bacterial effect of 70 % ethanol extract, ethyl acetate extract and isolated compound lupeol from ethyl acetate extract were examined using *Staphylococcus aureus*induced albino rats model.

Procedure

Twelve albino rats were taken and divided into four groups (3 in each group) (I, II, III & IV). All albino rats were then anaesthetized. Anaesthetized rats were shaved on the back area of about 3 cm diameter with blades. Incised wound about 1 cm in length was made on shaved area of rats. 8 CFU of *Staphylococcus aureus*of each rat in each group were subcutaneously injected to the incised wound of each rat in each group at four times hourly (Figure 6).

The inflammatory and exudates was found within one day. No treatment was taken in control group (I). The wounds were treated with ethanol extract, ethyl acetate extract and isolated compound (lupeol), observation for possible as wound healing during one day, three days and two days. The wounds of rats A II, B II, C II were treated with 3 mg of each ethanol extract respectively. Similarly, the wounds of rats in groups III and IV were treated with ethyl acetate extract and the isolated compound (lupeol), respectively. In case of isolated compound (lupeol) 3 mg of each were supplied.

The doses used and the observation of wound healing of the rats before and after treated with samples are described in Table 6 and Figures7-10 (Goodman *et al.*, 1995).

Results and discussion

Identification of Isolated Compound fromEtOAc Extract of*T. Indica* Stem Bark

Fraction 3 of the ethyl acetate extract of *T. indica* stem bark separated by column chromatography on silica gel (CH_2CL_2: MeOH, 6:1) gave compound known as lupeol. It possessed the molecular formular $C_{30}H_{50}O$, and melting point 218 °C ($CHC1_3$) which is coincident with that of lupeol (217 °C) (Merck index, 2001). It is inactive under UV light and also gave a dark violet spot of TLC after spraying and heating with anisaldehyde and sulphuric acid reagent. This colour test suggested that the isolated compound is a terpenoid

compound.

Isolated compound showed an absorption maximum at 209.8 nm (1.6, MeOH) in the UV spectrum (Table 1 and Figure 2). Absorptions at 3500 (OH), 3060 (C=CH_2 stretching for vinylidine), 2927, 2854 (CH-stretching for CH_3& CH_2) and 1659 (C=C stretching of alkene) cm^{-1} were found in the IR spectrum (Table 1 and Figure 2).

In ^1H NMR spectrum (in $CDC1_3$), the olefinic protons showed as doublets at 8 4.56 and 4.68 ppm. The 7 methyl signals as singlets at δ 1.69, 1.03, 0.97, 0.94, 0.83, 0.78 and 0.76 ppm were coincident with that of authentic lupeol and also agreed with literature value of lupeol. The H-3 signal also appeared as a doublet of doublet at 3.19 ppm (Table 3and Figure 3).

The ^{13}C NMR spectrum showed the signals of one hydroxy (δ 79.0 ppm), a disubstituted double bond (δ 150.9, 109.3 ppm), five methine carbons (δ 38.0, 47.9, 48.2, 50.4 and 55.3 ppm) and 7 methyl groups (δ 14.5, 15.4, 15.9, 16.1, 18.0, 19.3 and 28.0 ppm) (Table 4and Figure4).

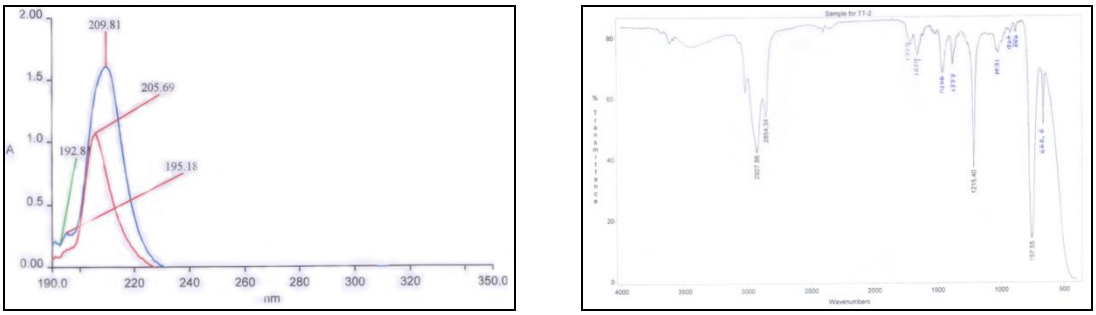


Figure 2. UV and FTIR spectra of isolated compound (lupeol)

Table 1. UV spectral Data (λ_{max}, nm) of Isolated Compound (lupeol) from *T. indica*Stem Bark

Solvent Used	Observed (λ_{max}, nm)
MeOH	209, 195, 192
MeOH + NaOH	205

Table 2. Assignment of Absorption Bands in FT IR Spectrum of Isolated Compound (lupeol) from *T. indica*Stem Bark

Wavenumber (cm^{-1})	Band Assignments
3500	OH - stretching for OH group
3060	C = CH_2 stretching for vinylidine
2927, 2854	CH = stretching for CH_3& CH_2
1659	C = C stretching of alkene

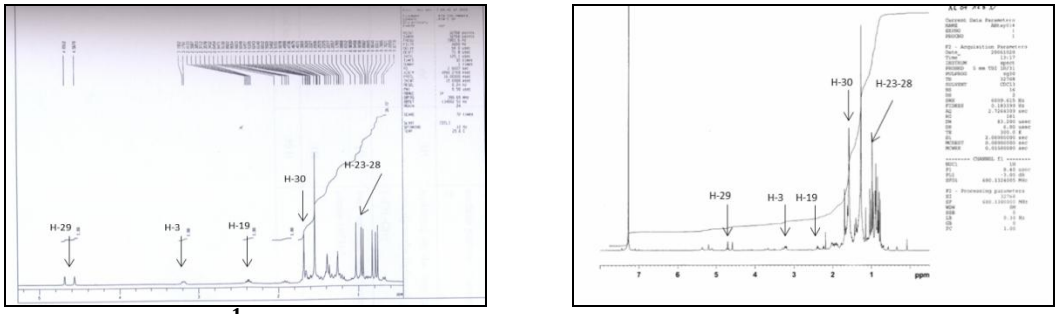


Figure 3. The ^{1}H NMR spectrum of isolated compound compared with that of lupeol from their reported spectrum

Table 3. Assignment of Signals^{1}H NMR (600 MHz, $CDCL_3$) Spectrum of Isolated Compound from *T. indica*Stem Bark

Atom	δ_H (ppm)	Multiplicity	Assignment
H-1A	1.70 - 1.60	m	- CH_2 (methylene)
H-1B	1.00 - 0.90	m	- CH_2 (methylene)
H-2	2.00 - 0.80	m	- CH_2 (methylene)
H-3	3.30	dd	- CHOH
H-5	1.00 - 0.80	m	- CH (methine)
H-6, 7	1.60 - 0.70	m	- CH_2 (methylene)
H-9	1.30	m	- CH (methine)
H-11	1.60 - 1.20	m	- CH_2 (methylene)
H-12	1.40 - 0.90	m	- CH_2 (methylene)
H-13	1.68	m	- CH (methine)
H-15	1.70 - 0.80	m	- CH_2 (methylene)
H-16	1.40 - 1.30	m	- CH_2 (methylene)
H-18	1.70 - 1.50	m	- CH (methine)
H-19	2.40	m	- CH (methine)
H-21	1.60 - 1.20	m	- CH_2 (methylene)
H-22	1.40 - 1.20	m	- CH_2 (methylene)
H-23	0.83 - 0.76	s	- CH_3 (methyl)
H-24	0.83 - 0.76	s	- CH_3 (methyl)
H-25	0.83 - 0.76	s	- CH_3 (methyl)
H-26	1.03 - 0.94	s	- CH_3 (methyl)
H-27	1.03 - 0.94	s	- CH_3 (methyl)
H-28	0.83 - 0.76	s	- CH_3 (methyl)
H-29	4.56 - 4.69	brd s	C = CH_2 (olefinic)
H-30	1.70	s	- CH_3 (methyl)

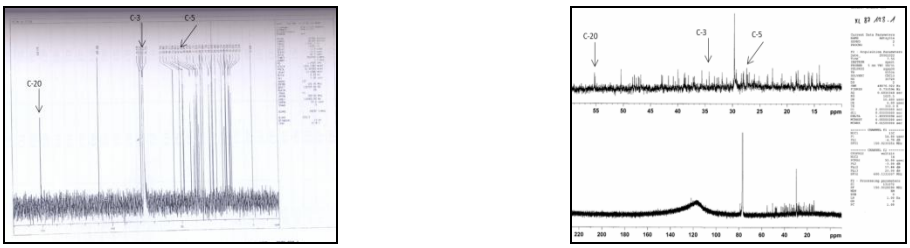


Figure 4. The ^{13}C NMR spectrum of isolated compound compared with that of lupeol from their reported spectrum

***invitro* Screening of Antibacterial Activity of Different Crude Extracts from *T. indica*Stem Bark**

The inhibition zone diameters for different crude extracts of *Tamarindusindica*stem bark are illustrated in Table 5 and Figure 5. It can be obviously seen that PE extract (inhibition zone diameters ranged 0 mm), EtOAc extract (inhibition zone diameters ranged between 15 mm - 25 mm), EtOH extract (inhibition zone diameters ranged between 15 mm - 23 mm) and H_2O crude extract (inhibition zone diameters ranged between 15 mm - 22mm) exhibited the antibacterial activity against 3 species of *Staphylococcus aureus*, 2 species of *Escherichia coli*, one species of *Bacillus subtilis*, one species of *Proteus morganii*and one species of *Vibrio cholerae*. It was observed that pet-ether extracts of stem bark did not show any antibacterial activity against all organisms tested. Therefore, EtOAc, EtOH and H_2O crude extracts of stem bark can be considered to be biologically active.

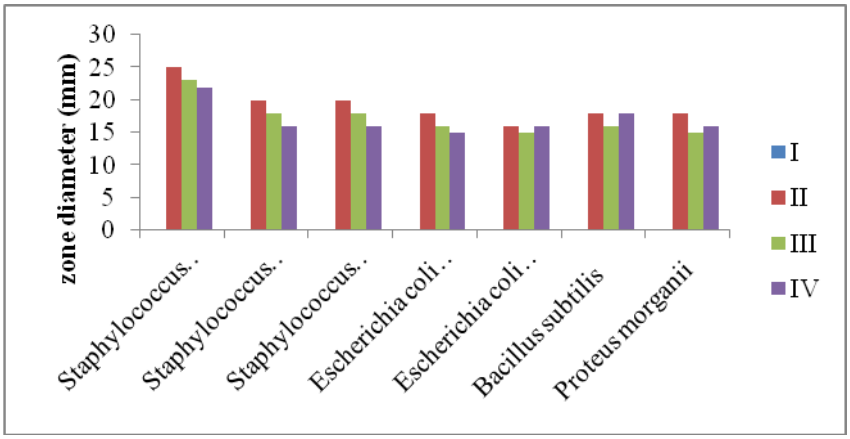
Table 4. ^{13}C NMR (600 MHz, $CDCl_3$) Spectral Data of Isolated Compound (Lupeol) and Literature Values of Reported Lupeol

No.	Carbon No	Isolated Compound Observed Values -/ppm	Literature Values -/ppm
1.	C-1	38.7	38.7
2.	C-2	27.4	27.4
3.	C-3	79.0	79.0
4.	C-4	38.8	38.8
5.	C-5	55.3	50.3
6.	C-6	19.3	18.3
7.	C-7	34.2	34.2
8.	C-8	40.8	40.8
9.	C-9	50.4	50.4
10.	C-10	37.2	37.2
11.	C-11	20.9	20.9
12.	C-12	25.1	25.9
13.	C-13	38.8	38.8
14.	C-14	42.8	42.8
15.	C-15	27.4	27.4
16.	C-16	35.5	35.6
17.	C-17	43.0	43.0
18.	C-18	48.2	48.3
19.	C-19	47.9	48.0
20.	C-20	150.9	150.9
21.	C-21	29.8	29.8
22.	C-22	40.0	40.0
23.	C-23	28.0	28.0
24.	C-24	15.4	15.3
25.	C-25	16.1	16.1
26.	C-26	15.9	16.0
27.	C-27	14.5	14.5
28.	C-28	18.0	18.0
29.	C-29	109.3	109.3
30.	C-30	19.3	19.3
	Total Carbons	30 C	

Table 5. Antibacterial Activities of Different Crude Extracts of *T. indica*on Different Species of Bacteria by Agar Disc Diffusion Method

No.	Type of Bacteria	Inhibition Zone Diameter (mm)			
		I	II	III	IV
1.	*Staphylococcus aureus*ws	-	25	23	22
2.	*Staphylococcus aureus*ns	-	20	18	16
3.	*Staphylococcus aureus*w sepsi	-	20	18	16
4.	*Escherichia coli* (STLT)	-	18	16	15
5.	*Escherichia coli* (ATTC)	-	16	15	16
6.	*Bacillus subtilis*	-	18	16	18
7.	*Proteus morganii*	-	18	15	16
8.	*Vibrio cholerae*	-	20	18	16

I = PE extract of *T. indica* III = EtOH extract of *T. indica*
II = EtOAc extract of *T. indica* IV = H_2O extract of *T. indica*



I = PE extract of *T. indica* III = EtOH extract of *T. indica*
II = EtOAc extract of *T. indica* IV = H_2O extract of *T. indica*

Figure 5. Bar graph of bacterial inhibition zone diameters (mm) of different crude extracts of *T. indica*on eight microorganisms

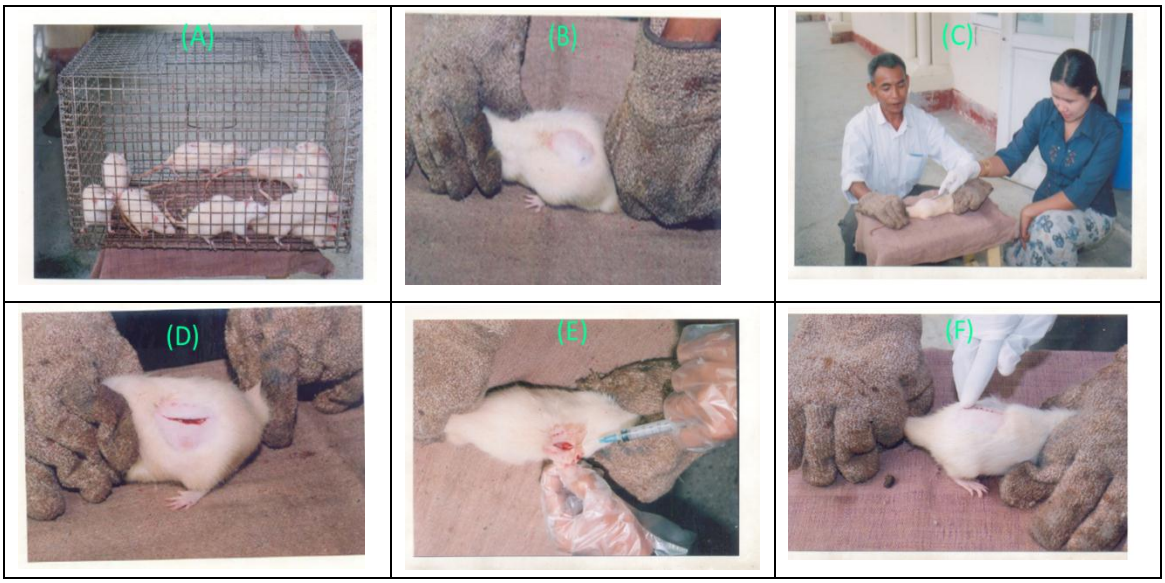


Figure 6. *in vivo* antibacterial activity testing of the different crude extracts and isolated compound (lupeol) from *T. indica*stem bark on experimental albino rats

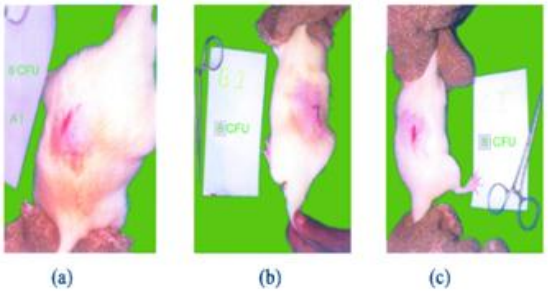


Figure 7. Observation of inflammation and exudation of *S. aureus*induced rats (control group) (a) 8 CFU/ml (b) 8 CFU/ml (c) 8 CFU/ml

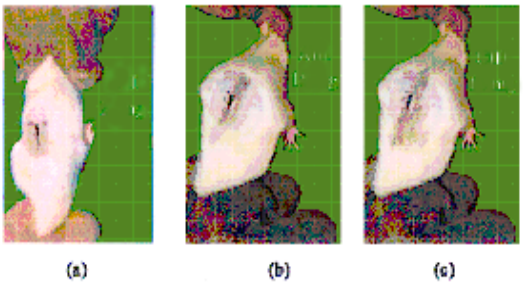


Figure 8. Wound healing observed after treated with ethanol extracts(a) 3 mg (b) 3 mg (c) 3 mg

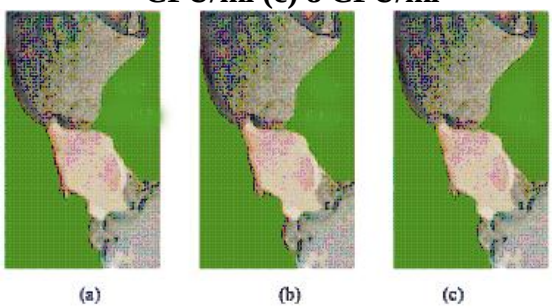


Figure 9. Wound healing observed after treated with ethyl acetate extracts (a) 3 mg (b) 3 mg (c) 3 mg

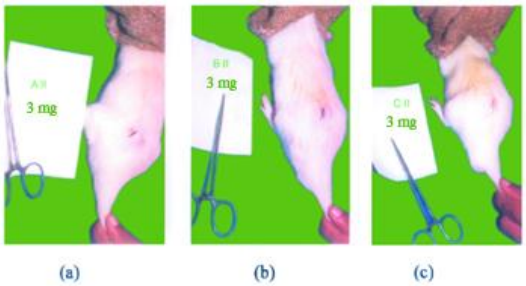


Figure 10. Wound healing observed after treated with isolated compound (lupeol) (a) 3 mg (b) 3 mg (c) 3 mg

***in vivo* Screening of Antibacterial Activity of Different Crude Extracts and Compound Isolated from Ethylacetate Extract of *T. indica*Stem Bark**

In order to highlight the usefulness of *T. indica*stem bark in the treatment of boil, *in vivo* antibacterial activities of ethyl acetate crude extracts, 70 % ethanol crude extracts and isolated compound (lupeol) were examined using *S. aureus* induced albino rat model.All the experimental results are demonstrated in Figure 6.

Figure 7 shows that rats (control group) inflammatory and exudated were found within one day.

Figure 8 shows the rats treated with different amount of EtOH extract (3 mg of each). Wound healing was observed on three days and inflammatory was also found to be reduced.

Figure 9 shows the effect of ethyl acetate extract on wound healing properties. Ethyl acetate extract took one day to heal the wound together with reducing inflammation.

Figure 10 shows the effect of isolated compound (lupeol) on wound healing properties of on rats. In the case of isolated compound, two days were needed to heal the wound and also to reduce inflammation. All of the results are summarized in Table 6.

From this experiment, it was investigated that isolated compound (lupeol) and EtOAc extract had the most potency in wound healing on *S.aureus*induced albino rats. It is because of a bioactive compound (lupeol) has antiinflammatory properties present in ethyl acetate extract of ma-gyi bark.

Table 6. The Doses of Sample and Observation of Wound Healing of *Staphylococcus aureus*Induced Rats in *in vivo*

Dose	Rats												Observed	Remark
	A I	B I	C I	A II	B II	C II	A III	B III	C III	A IV	B IV	C IV		
S.aureus/ CFU/ml	8	8	8	8	8	8	8	8	8	8	8	8	inflamma-tory and exudated was found	Control group (A I, B I, C I)
EtOH extract/mg	-	-	-	3	3	3	-	-	-	-	-	-	wound healing after 3 days	Group II
EtOAcextract/ mg	-	-	-	-	-	-	3	3	3	-	-	-	wound healing after 1 day	Group III
Isolated Compound (Lupeol)/mg	-	-	-	-	-	-	-	-	-	3	3	3	wound healing after2 days	Group IV

CFU = Colony Forming Unit
Staphylococcus aureus = Septic Wound, Microbiology Department, Institute of Medicine (2), Yangon

Conclusion

The following inferences could be deduced from the overall assessment of the chemical investigation on the stem bark of *Tamarindusindica*L. (Ma-gyi). On silica gel column chromatographic separation, one compound was isolated from ethyl acetate crude extract of the *T. indica*stem bark. This compound was identified to be (lupeol, 0.009 % yield, m.pt. 218 °C). The isolated compound lupeol was characterized by some physical and chemical properties and structurally identified by the combination of UV, FT IR, ^{1}H NMR and ^{13}C NMR spectroscopic methods and also by comparing with the reported data.

The antibacterial activity of crude extracts (PE, EtOAc, EtOH and H_2O) of *T. indica*was screened by using agar disc diffusion method on eight bacterial strains including 3 species of *S. aureus*, 2 species of *E. coli*, one *B. subtilis*, one*P. morganii*andone*V. cholerae*. Except PE extracts, all of the crude extracts were found to exhibit antibacterial activity against the organisms tested.

in vivo antibacterial tests performed on *S. aureus*induced albino rat models indicated that ethyl acetate crude extract has most potent wound healing (after one day) properties when compared to isolated compound (lupeol) from ethyl acetate extract (after two days) and 70 % EtOH extract (after three days) from *T. indica*stem bark.

Among them, ethyl acetate extract from *T. indica*stem bark was found to exhibit the most effective anti-inflammatory and wound healing properties on the rats. Thus, ethyl acetate extract of *T. indica* stem bark may be used in treatment of boil. Likewise, isolated compound (lupeol) may be used to treat boil or skin abscess, owing to its effective antibacterial action against *Staphylococcus aureus.*

In conclusion, it was found that crude extracts (EtOH, EtOAc and H_2O) of *T. indica*stem bark can be effective in the formulation of medicine for the treatment of diseases such as diarrhoea, fever, inflammation, laxative and boils.

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