Investigation of Some Biological Activities and Isolation of Organic Constituents from Aerial Parts of *Centella asiatica* (L) Urban (Myin-khwa)

Than Than Nu[1], Yin Yin Htwe[2], Daw Hla Ngwe[3]

Abstract

Centella asiatica (L) Urban (Myin-khwa) is well known traditional medicinal plant because it has valuable medicinal properties such as anti-inflammatory, wound healing, diuretic, antibiotic and antioxidant properties. The research is focused on investigation of antimicrobial activity as well as in vivo wound healing activity and isolation of some organic constituents from aerial parts of *Hydrocotyle asiatica* L. (Myin-khwa). They were collected from Mudon Township, Mon State. The crude extracts of aerial parts of Myin-khwa were prepared by using solvent extraction method. The antimicrobial activity of non-polar and polar crude extracts: pet-ether, chloroform, ethyl acetate, ethanol and watery extracts of Myin-khwa was investigated against six microorganisms such as *Bacillus subtilis*, *Staphytococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli* by agar well diffusion method. By silica gel column chromatographic separation technique, three compounds: Asiatic acid (0.006 %, m.pt 325–327 °C), salicylic acid (0.005 %, m.pt 156–158 °C) and kaempferol (0.05 %, m.pt 277–279 °C) were isolated from the active ethyl acetate extract of Myin-khwa. The isolated compounds were structurally identified by modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR, ^{13}C NMR and EI MS spectroscopic techniques. In addition, *in vivo* wound healing activity of ethanol and watery extracts as well as isolated compounds was determined on the wound induced on rats by *Staphylococcus aureus*.

Keywords: *Centella asiatica* (L) Urban (Myin-khwa), antimicrobial activity, agar well diffusion method, wound healing activity, Asiatic acid, salicylic acid, kaempferol

Introduction

Myanmar is one of the South East Asian countries which have a large number of indigenous medicinal plants. Medicinal plants are reputed to be useful for treatment of various diseases **(**Gupta *et al.***,** 2008). The study of indigenous medicinal plants and their therapeutics play a very important role in health care system of Myanmar. *Centella asiatica* (L) Urban (Myin-khwa) is a slender, creeping plant and rooting at the nodes. It is a perennial, slightly aromatic herb inhabitation damp and moist place of tropical and sub-tropical regions of India. It is found in Southeast Asia, Sri Lanka and parts of China. It is native to countries like Sri Lanka, Madagascar, South Africa and Malaysia. The chemical constituents of Myin-khwa are classified into main groups including essential oil, flavone derivatives, triterpenic steroids, triterpenic acids and triterpenic acid sugar ester or saponin (Brainkhaus *et al.*, 2000). It also contains various important constituents for clinical and pharmaceutical uses. Asiatic acid, asiaticoside, madecossoside and madecassic acid are the biologically active constituents that have a potential to be promoted as commercial product (Bonte *et al.*, 1994). Myin-khwa is used in the Ayurvedic system of medicine to treat various diseases. The fresh extract of this plant has been used by the people of Java and the Malay Peninsula for many years, as both topical and internal agents, for healing of wounds. In Malaysia, although this herb is commonly eaten fresh as a vegetable (salad), especially among the Malay communities, it is also said to have beneficial effects in improving memory and in treating mental fatigue, anxiety and eczema (Chauhan *et al.*, 2010).

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The present work focused on investigation of antimicrobial activity as well as *in vivo* wound healing activity of Myin-khwa and also deal with isolation of some organic constituents and identification of isolated compounds by melting point determination, UV, FT IR, ^{1}H NMR, ^{13}C NMR and EI MS spectroscopic techniques. The photographs of plants, flowers and fruits of Myin-khwa are shown in Figure 1.

(a) (b) (c)

Figure 1. Photographs of Myin-khwa (a) plants (b) fowers and (c) fruits

Materials and Methods

All chemicals used in this research work were obtained from British Drug House (BDH). All other reagents and solvents used were analytical grade. Instruments employed in the work consist of lab ware, glass ware and other supporting facilities. For isolation and identification of organic compounds, column (3 × 60 cm), silica gel (40 – 60 μm, Wakogel), TLC precoated plates (GF_{254} Aluminium plates, Merck), Melting point (Gallenkamp), UV lamp (365–254 nm), FT IR spectrometer (Perkin Elmer), UV-visible spectrometer-Shimadzu, Japan, 600 MHz ^{1}HNMR, 125 MHz ^{13}C NMR and EI-MS spectrophotometers at the Department of Organic and Biomolecular Chemistry, University of Göettingen, Germany were used.

Myin-khwa was collected from Mudon Township, Mon State. The plant was identified by authorized botanist at Botany Department, Yangon University. The collected fresh sample was washed and air dried at room temperature for two weeks and then they were ground into powder by a grinder. The dried powdered sample was stored in the air-tight containers to prevent the moisture and other contaminations.

Preliminary Phytochemical Tests

A few grams of dried powder sample of Myin-khwa was subjected to the test of alkaloids, cyanogenic glycosides (Trease and Evans, 1980), flavonoids, organic acids, glycosides, phenolic compounds, α-amino acids (Marini *et al.*, 1981), reducing sugars, carbohydrates, saponin glycosides, steroids, tannins and terpenoids (Shriner *et al*., 1980) as the preliminary phytochemical test according to reported methods.

Preparation of Various Crude Extracts from Myin-khwa

The dried powdered sample (100 g) was extracted with 500 cm^3 of pet-ether (PE) for one week by solvent extraction method and filtered. This procedure was repeated for three times. Then the filtrate was concentrated by a vacuum rotatory evaporator to get respective pet-ether extract. Similarly, chloroform ($CHCl_3$), ethyl acetate (EtOAc) and ethanol (EtOH) extracts of dried powdered sample were prepared according to the above procedure.

In the preparation of watery extract, 100 g of dried powdered samples were soaked in 500 cm^3 of distilled water in the conical flask. These samples were boiled on a water bath for 6 hr and filtered. This process was carried out for three times. The combined filtrate was evaporated to dryness over a water bath at 100 °C to get the corresponding watery extract.

Antimicrobial Activity of Crude Extracts of Myin-khwa

The antimicrobial activities of different crude extracts such as pet-ether, chloroform, ethyl acetate, ethanol and watery extracts from Myin-khwa were determined against six strains of microorganisms such as *Bacillus subtilis*, *Bacillus pumilus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans* and *Escherichia coli* by employing agar well diffusion method at Pharmaceutical Research Department (PRD), Ministry of Industry, Yangon.

Screening of Antimicrobial Activity of Crude Extracts of Myin-khwa

Meat extract (0.5 g), peptone (0.5 g) and sodium chloride (0.25 g) were mixed with distilled water and the solution made up to 100 cm^3 with distilled water. The pH of this solution was adjusted at 7.2 with 0.1 M sodium hydroxide solution and 1.5 g of agar was added. The nutrient agar medium was put into sterilized conical flask and plugged with cotton wool and then autoclaved at 121°C for 15 minutes. After cool down to 40 °C, one drop of suspended strain was inoculated to the nutrient agar medium with the help of a sterilized disposable pipette near the burner. About 20 cm^3 of medium was poured into the sterilized petri dishes and left 10-15 mins in order to set the agar. After that the agar wells were made with a 10 mm sterilized cork bare and the wells were filled with 0.1 cm^3 of each extract sample to be tested. And the plates were incubated at 37 °C for 24 hours. After incubation, the diameters of inhibition zones including 10 mm wells were measured.

Isolation of Compounds from Crude Extract of Myin-khwa

The 95 % EtOH extract was extracted with 250 mL of pet-ether (60 – 80 °C) by using separating funnel, the soluble matter of pet-ether was obtained. The defatted alcohol soluble portion was then partitioned between ethyl acetate and water by using separating funnel. After removal of the solvent, ethyl acetate soluble extract was obtained. The ethyl acetate extract (5 g) was separated by column chromatographic separation techniques. Gradient elution was performed successively using $CHCl_3$:MeOH in the ratios of 19:1, 15:1, 9:1, 7:1, 5:1, 3:1 and 1:1 v/v. From this separation, seven main fractions F_1 to F_7 were collected. The condensed fraction F_3 was washed with ethyl acetate and crystallized from ethanol to yield 0.006% of compound **I**. The compound **II** was isolated from fraction F_5 to yield 0.005% and compound **III** was isolated from fraction F_7. The isolated compounds were characterized by melting point determination and structurally identified by modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR, ^{13}C NMR and EI MS spectroscopy.

***In vivo* Screening of Wound Healing Activity of Myin-khwa by Using *Staphylococcus aureus (S. aureus)* Induced Albino Rats Model**

(a) Samples

95 % EtOH extract, watery extract, isolated compounds **I**, **II** and **III** from Myin-khwa and clindamycin.

(b) Animals used

21 numbers of albino rats (200-250 g body weight)

(c) Procedure

Twenty one rats were divided into seven groups (Group I to VII, 3 rats in each group). All of these rats were anaesthetized. Anaesthetized rats were shaved on the back area of about 5 cm diameter with a scalpel blade. The area was then cleaned with spirit to maintain aseptic condition. An incised wound of about 1 inch in length was made on shaved area of rats by using a sterile scalpel blade. *Staphylococcus aureus* (1×10^6 CFU/mL) were injected to the incised wound of each rat in each group of test (Figure 2).

After one day, inflammation of wound was found in all of the rats. No treatment was taken in control group (Group I). In other groups, the wounds were treated daily with respective 4 mg/day applied doses of 95 % EtOH, watery extracts and isolated compounds **I**, **II** and **III** from Myin-khwa. Clindamycin (4 mg/day application dose) was used as standard. The observations of wound healing of the rats before and after treated with samples were recorded.

Experimental work

Group I - Control (No treatment of wound)

Group II - Wound treated with 4 mg/day dose of 95 % EtOH extract

Group III - Wound treated with 4 mg/day dose of watery extract

Group IV - Wound treated with 4 mg/day dose of compound **I**

Group V - Wound treated with 4 mg/day dose of compound **II**

Group VI - Wound treated with 4 mg/day dose of compound **III**

Group VII - Wound treated with 4 mg/day dose of clindamycin

(a) (b) (c) (d)

Figure 2. Photographs showing inflammation of wound on albino rats

(a) Incising the wound on albino rat

(b) *S.aureus* used to induce wound on rat

(c) Injecting the *S. aureus* to wound

(d) Inflammation of the wound occurred after 24 hours

Results and Discussion

Preliminary Phytochemical Tests

Preliminary phytochemical tests indicated the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponin glycosides, tannins, steroids and terpenoids. However cyanogenic glycosides were not detected in Myin-khwa.

Soluble Matter Content of Myin-khwa

In this experiment, the crude extracts were successively extracted with different polarity of pet-ether, chloroform, ethyl acetate, ethanol and water by employing percolation method. From the experiment, 21.5 % of ethanol, 10.35 % of water, 7.3 % of ethyl acetate, 4.48 % of chloroform and 2.98 % of pet-ether extract were obtained and these were kept for further works. It was observed that the ethanol soluble matter content was found to be the highest. It indicated that most of plant constituents are polar compounds.

Antimicrobial activity of the crude extracts of Myin-khwa

In the present study, antimicrobial activity of crude extracts such as pet-ether, chloroform, ethyl acetate, ethanol and watery extracts from Myin-khwa was determined by agar well diffusion method. In this investigation, the extracts were tested against six microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomnas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli* species. The inhibition zone diameters resulted from antimicrobial activity of Myin-khwa are shown in Table 1. According to the results, it was found that ethyl acetate extract of Myin-khwa exhibited the most pronounced antimicrobial action against all tested microorganisms with the inhibition zone diameter ranged between 38 mm-40 mm (Figure 3). Ethanol and watery extracts of Myin-khwa showed the antimicrobial activity with inhibition zone diameters ranged between 20 mm-25 mm against all six microorganisms. But chloroform extract did not show antimicrobial activity against *Escherichia coli* and pet-ether extract also did not show any activity. Therefore, it can be observed that ethyl acetate extract of Myin-khwa significantly exhibited antimicrobial activity when compared with the other extracts.

Isolated Compounds

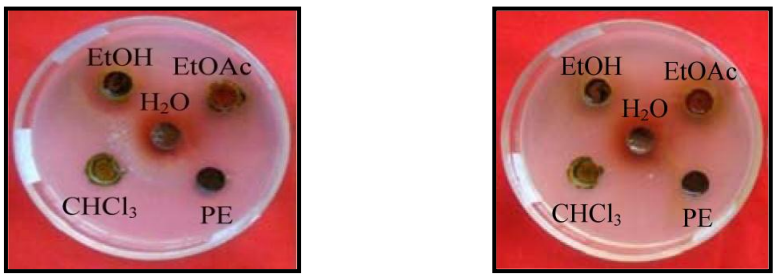
By silica gel column chromatographic separation, three compounds were isolated from ethyl acetate extract of Myin-khwa. They were identified by melting point, UV, FT IR, ^{1}H NMR, ^{13}C NMR and EI MS spectroscopic techniques.

Asiatic acid (I) : White crystal (0.006 % yield, m.pt 325 – 327 °C) (Lit. 325 – 330 °C, Merck Index, 2001); FT IR υ(cm^{-1}), 2500 ~ 3408 (υ_{O-H}), 2924, 2854 ($\upsilon_{asym-C-H}$), 1697 ($\upsilon_{C=O}$), 1618 ($\upsilon_{C=C}$) of olefinic group), 1464, 1377 (δ_{C-O-H}), 1230 (υ_{-C-COO}), 1148 (υ_{C-O}), 1048 (δ_{O-H}), 964 (δ_{oop} of alkene); ^{1}H NMR (d_6-DMSO, 600MHz), δ_H (ppm) : 0.62 (s, 3H – 24), 0.75 (s, 3H – 26), 0.78 (d, *J* = 6Hz, 3H – 29), 0.88 (d, *J* = 6Hz, 3H – 30), 1.03 (s, 3H – 27), 2.21 (d, *J* = 11.2Hz, 1H – 18), 3.20 (d, *J* = 11.2Hz, 1H – 23), 3.30 (d, *J* = 9.6 Hz, 1H – 3), 3.46 (d, *J* = 10.8Hz, 1H – 23), 3.64 (m, 1H – 2), 5.16 (br s, 1H – 12); ^{13}C NMR (d_6-DMSO, 125 MHz), δ_C (ppm) : 47.62 (C-1), 68.71 (C-2), 77.77 (C-3), 43.51 (C-4), 48.21 (C-5), 18.54 (C-6), 32.76 (C-7), 40.01 (C-8), 47.64 (C-9), 38.30 (C-10), 23.62 (C-11), 125.72 (C-12), 139.54 (C-13), 42.17 (C-14), 28.61 (C15), 24.76 (C-16), 48.01 (C-17), 53.45 (C-18), 39.44 (C-19), 39.32 (C-20), 31.05 (C-21), 37.41 (C-22), 65.55 (C-23), 14.31 (C-24), 17.42 (C-25), 17.42 (C-26), 23.79 (C-27), 179.73 (C-28), 17.34 (C-29), 21.35 (C-30); EI MS (m/z) : 488 [M^+], 443, 426, 320, 262, 203, 189 , 133, 64.

Table 1. Inhibition Zone Diameters of Some Crude Extracts from Myin-khwa Against Six Microorganisms

organisms	Gram	Inhibition Zone Diameters (mm)				
		PE	$CHCl_3$	EtOAc	EtOH	Watery
Bacillus substilis	Gram (+)ve	ND	12 (+)	40 (+++)	20 (+++)	20 (+++)
Staphylococcus aureus	Gram (+)ve	ND	13 (+)	40 (+++)	20 (+++)	20 (+++)
Pseudomonas aeruginosa	Gram (-)ve	ND	13 (+)	40 (+++)	20 (+++)	20 (+++)
Bacillus pumilus	Gram (+)ve	ND	13 (+)	40 (+++)	20 (+++)	23 (+++)
Candida albicans	Gram (+)ve	ND	13 (+)	38 (+++)	20 (+++)	25 (+++)
Escherichia coli	Gram (-)ve	ND	ND	39 (+++)	20 (+++)	20 (+++)

Agar well diameter - 10 mm , 10 mm – 14 mm (+), 15 mm -19 mm (+ +)
20 mm - above (+ + +), ND = not detected



Bacillus substilis ***Staphylococcus aureus***

Figure 3. The photographs showing inhibition zones of myin-khwa extracts against *Bacillus substilis* and *Staphylococcus aureus* microorganism

Salicylic acid (II) : White amorphous powder (0.005 % yield, m.pt 156 – 158 °C) (Lit. 157 – 159 °C, Merck Index, 2001)); UV-visible, λ_{max} (nm) in MeOH : 211, 232, 292, 357, in MeOH + NaOH : 212, 235, 297, 386; FT IR υ (cm^{-1}), 3471, 3240 (ν_{O-H} of phenolic O-H group) 3500 ~ 2500 (ν_{O-H} of -COOH), 3062 (ν_{C-H} of =CH), 1697 ($\nu_{C=O}$ of α-β unsaturated –COOH), 1620, 1542, 1450 ($\nu_{C=C}$ of aromatic C=C group), 1334 (δ_{O-H} of hydroxyl group), 1271 – 1240 (ν_{C-O-C}), 1026 (ν_{C-O} in C-OH group), 864 (δ_{OOP} O-H in benzene), 771 – 547 (δ_{OOP} C-H deformation in benzene); ^{1}H NMR ($CDCl_3$, 600 MHz), δ_H (ppm) : 7.94 (dd, *J* = 8.0, 1.6 Hz, 1H-6), 7.50 (m, 1H-4), 7.01 (d, *J* = 8.4 Hz, 1H-3), 6.95 (m, 1H-5); ^{13}C NMR ($CDCl_3$, 125MHz), δ_C (ppm) : 130.5 (C-6), 137.2 (C-4), 117.3 (C-3), 119.6 (C-5), 111.6(C-1), 162.5 (C-2), 175.2 (C-7).

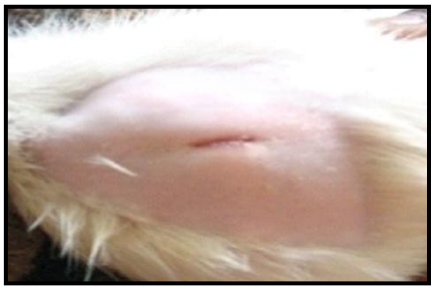
Kaempferol (III) : Yellow powder (0.05 % yield, m.pt 277 – 279 °C) (Lit. 276 – 278 °C, Merck Index, 2001)); UV-visible, λ_{max} (nm) in MeOH: 255, 294, 370, in MeOH / NaOMe : 247, 320, 428, in MeOH / $AlCl_3$: 269, 330, 458, in MeOH / $AlCl_3$ / HCl : 265, 304, 359, 425, MeOH / NaOAc : 261, 325, 389, in MeOH / NaOAc / H_3BO_3 : 261, 301, 388; FT IR υ (cm^{-1}), 3425 ,3371 (υ_{O-H} of ar-OH), 3015 ($\upsilon_{=CH}$), 1654 ($\upsilon_{C=O}$), 1612, 1519, 1458 ($\upsilon_{C=C}$), 1381 (δ_{O-H}), 1265 (υ_{C-O} of –C–O–H), 1135 ($\upsilon_{asymC-O}$ of ar–C–O), 1018 ($\upsilon_{sym\ C-O}$ of ar–C–O), 825 (δ_{oop} of C–H ar), 786 (δ_{oop} of C–H ar); ^{1}H NMR ($CDCl_3$, 600MHz), δ_H (ppm) : 6.15 (d, *J*=2.1 Hz, 1H-6), 6.35 (d, *J*=2.1 Hz, 1H-8), 7.81 (dd, *J*=9.1, 1.8 Hz, 1H-2′), 6.79 (dd, *J*=8.9, 2.1 Hz, 1H-3′), 6.79 (dd, *J*=8.9, 2.1Hz, 1H-5′), 7.81 (d, *J*=9.1, 1.8 Hz, 1H-6′); ^{13}C NMR ($CDCl_3$, 125MHz), δ_C (ppm) : 158.7 (C-2), 135.22 (C-3), 175.30 (C- 4), 163.75 (C-5), 98.36 (C-6), 163.75 (C-7), 93.77 (C-8), 156.74 (C-9), 103.17 (C-10), 122.14 (C-1′), 129.38 (C-2′), 115.24 (C-3′), 160.43 (C-4′), 115.24 (C-5′), 129.38 (C-6′); EI MS (m/z) : 286 [M^+], 258, 229 , 213, 184 , 121, 69.

Wound healing activity

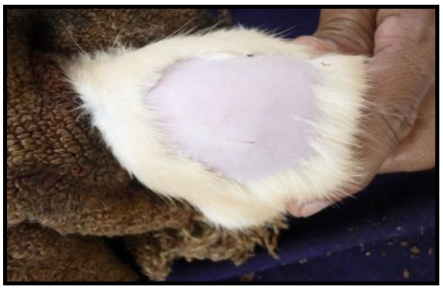
In vivo wound healing activities of 95 % EtOH extract, watery extract and isolated compounds were also determined on the wound induced by using *Staphylococcus aureus* on albino rats. From this experiment, the complete wound healing was observed after 3 days and 5 days by treating with 95 % EtOH and watery extracts of Myin-khwa in 4 mg/kg.bw/day doses respectively. Therefore, 95 % EtOH extract was more effective than watery extract in complete wound healing. And also the infected rats were treated with isolated compounds: Asiatic acid, salicylic acid and kaempferol. It was found that the wounds of rats were completely healed after 6, 3 and 3 days when treated with Asiatic acid, salicylic acid and kaempferol in 4 mg/kg.bw/day doses, respectively. Among these compounds, salicylic acid and kaempferol were also found to have higher potency than Asiatic acid in wound healing activity. The results are described in Table 2 and Figure 4.

Table 2. Wound Healing Effect of Crude Extracts from Myin-khwa on Albino Rats Infected with *Staphylococcus aureus*

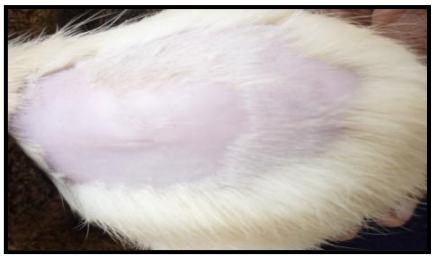
Group	Extracts	Dose (mg/kg/day)	Time needed for total wound healing (day)
I	Control	-	10
II	Ethanol	4	3
III	Watery	4	5
IV	Asiatic acid	4	6
V	Salicylic acid	4	3
VI	Kaempferol	4	3
VII	Clindamycin	4	6



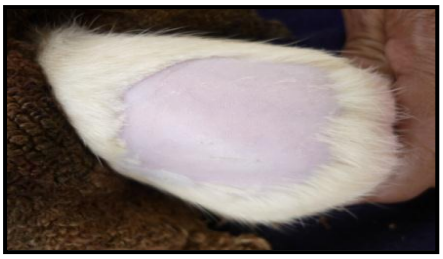
(a) Control group (10 days)



(d) After treatment with Asiatic acid (6 days)

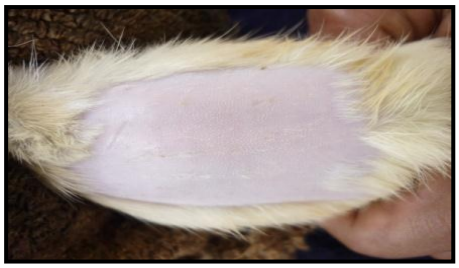


(b) After treatment with ethanol extract (3days)

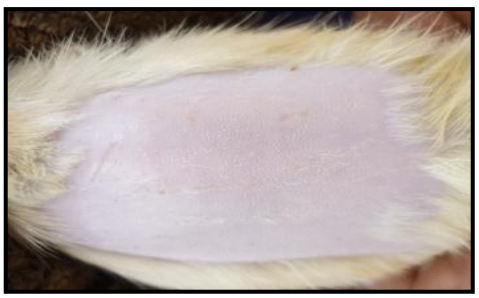


(e) After treatment with Salicylic acid (3 days)

(c) After treatment with watery extract (5 days)



(f) After treatment with Kaempferol (3 days)



(g) After treatment with Clindamycin (6 days)

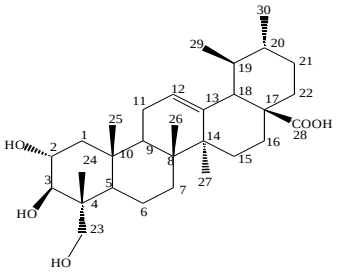
Figure 4. Images showing the wound healing effects of extracts, isolated compounds and clindamycin on albino rats infected by *Staphylococcus aureus*

Conclusion

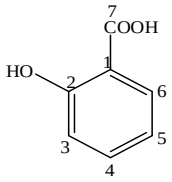
From overall assessment of the present work concerning with the investigation of antimicrobial activity as well as *in vivo* wound healing activity and isolation of some organic constituents from aerial parts of *Hydrocotyle asiatica* L. (Myin-khwa). The preliminary phytochemical tests indicated the presence of alkaloids, α-amino acids, carbohydrates, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponin

glycosides, tannins, steroids and terpenoids but cyanogenic glycosides were absent in Myin-khwa. Screening of antimicrobial activity by agar well diffusion method showed that ethyl acetate extracts of Myin-khwa significantly inhibited antimicrobial activity. Chloroform extract of Myin-khwa showed mild activity. But pet-ether extract of Myin-khwa did not show any activity. Asiatic acid (0.006 %, m.pt 325–327 °C), salicylic acid (0.005 %, m.pt 156–158 °C) and kaempferol (0.05 %, m.pt 277–279 °C) were isolated from the ethyl acetate extract of Myin-khwa by column chromatographic separation method.

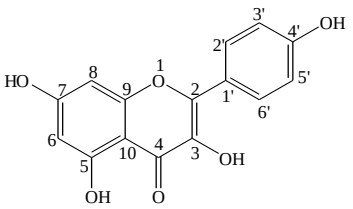
In vivo wound healing activity of crude extracts and isolated compounds was determined on the wound induced on rats by *Staphylococcus aureus.* It was found that 95 % EtOH extract (3 days) was more healing than watery extract (5 days) in wound healing activity. In addition, it was also observed that salicylic acid (3 days) and kaempferol (3 days) are more potent than Asiatic acid (6 days) in complete wound healing. Therefore, the present work will contribute to scientific development of Myanmar traditional medicine formulation, especially in the area concerned with diseases related to bacterial infections.



Asiatic acid



Salicylic acid



Kaempferol

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