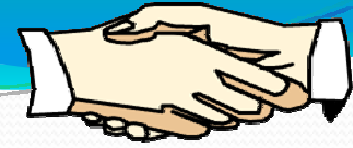


Elemental Composition, Phytochemical Screening and Antibacterial Activity of *Aegle marmelos* (L.) Correa (ဥချုံ)


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Introduction



- ❖ using herbs and medicinal plants is the universal phenomenon
- ❖ ingredients in traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, bioactive principles and lead compounds in synthetic drugs ⁽¹⁾
- ❖ using traditional medicine as part of primary health care is applied in 75% of world population - WHO ⁽²⁾
- ❖ many people in Myanmar use traditional medicines as these are minimal toxicity, affordable and easily available
- ❖ absorb & accumulate minerals, essential to human nutrition
- ❖ trace elements play pivotal role for human and against various diseases ⁽³⁾

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- ❖ **microorganisms are closely associated with the health and welfare of human beings**
 - ❖ **some are beneficial and some are detrimental⁽⁴⁾**
 - ❖ ***Aegle marmelos* (L.) Correa, Ok-shit, Bael tree, Rutaceae widely distributed and found in India, China, Bangladesh, Myanmar and Thailand**
 - ❖ **edible and possess many medicinal properties^(5,6)**
 - ❖ **crude extracts of Ok-shit is proved to have antimicrobial, antidiabetic, anti-hyperlipidemic, antioxidant, anticancer and anti-inflammatory effects against various animal models⁽⁴⁾**

- 
- ❖ ***A. marmelos* leaves extract against some clinical pathogens reported by Karumaran 2016, Ibrahim 2015 and Senthilkumaran 2014 ^(1,4,7)**
 - ❖ **young fruit - Traditional Medicine Formulation No 41 (Wunmeetauk Hsay) ⁽⁸⁾**
 - ❖ **antihyperglycemic activity by Soe Sandar Phyo *et al.*, 2015**
 - ❖ **lipid lowering effect on Wistar Albino rats by Aye Aye Mya, 2016 ^(9,10)**
 - ❖ **anti-diarrhoeal activity of unripe fruit of ဥချွန် was studied by Khin Tar Yar Myint *et al.*, 2017 ⁽¹¹⁾**
 - ❖ **in Myanmar, scientific evaluation of elemental composition and antibacterial activity of Ok-shit leave has not been published yet**

General objective

- ❖ To study elemental composition, phytochemical screening and antibacterial activity of leaves of *Aegle marmelos* L. Correa. (Ok-shit) (ဥချွန်) collected in Upper Myanmar

Specific objectives

- ❖ To determine the amount of macrominerals; Ca, Mg, Na, K in Ok-shit leaves
- ❖ To measure the amount of microminerals; Cu, Fe, Mn, Zn content in Ok-shit leaves
- ❖ To investigate the phytochemical constituents of leaves of Ok-shit
- ❖ To evaluate the antibacterial activity of aqueous and ethanolic extracts of leaves of Ok-shit on some bacteria
- ❖ To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of crude extracts of Ok-shit leaves

Materials and Methods

Reagents and Chemicals

analytical grade reagents of

- ❖ Ca, Mg, K, Na, Cu, Fe, Mn and Zn standard
- ❖ 70% Nitric acid (HNO₃)
- ❖ 69% Hydrochloric acid (HCl)
- ❖ Ethanol
- ❖ Mueller-Hinton agar
- ❖ Muller-Hinton broth (Hi Media, India)
- ❖ Ceftriaxone 30 µg
- ❖ Double de - ionized water (DDW)
- ❖ *S. aureus* (ADCC-25923)
- ❖ *P. aeruginosa* (ADCC-25923)
- ❖ *E. coli* (ADCC-27853)

Instruments

**Atomic absorption spectrophotometer
AA 6650**



Muffle furnace



Soxhlet



Rotary evaporator



Plant authenticity

- ❖ **identified and confirmed for specific botanical name by competent taxonomist^(12,13)**

Sample Collection

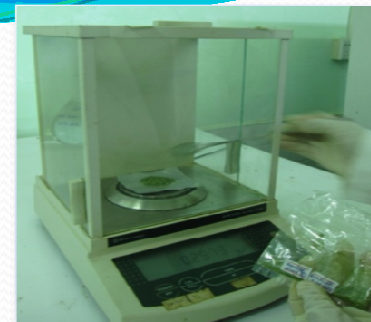
- ❖ **Pyin Oo Lwin township, Mandalay Region, in May 2016**

Sample preparation

- ❖ **thoroughly washed with tap water and rinsed with distilled water to remove the dust and particles**
- ❖ **air dried in shade at room temperature**
- ❖ **crushed, powdered and homogenized**
- ❖ **dried in oven at 60 °C to obtain constant weight and ground to powder for further analysis**

Digestion of Ok- shit Sample⁽¹⁴⁾

Ok-shit powder
(2.5) g

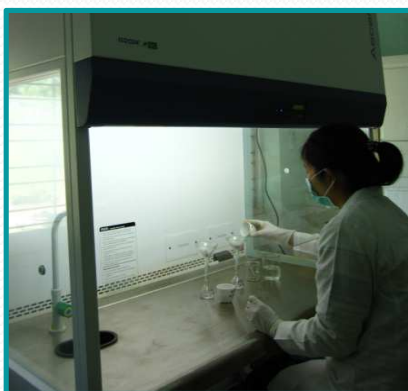



Heating 110 °C, 2 hours, in an oven
to remove moisture

furnace, at 550 °C for 4 hr,
to obtain grey ash, & cool

5 ml of 6M HNO₃ ,
to dissolve & digest, filter

Made up with DDW
(50 ml Volumetric flask)



- 
- ❖ **blank control was carried out in the same way of sample preparation using solvent only**
 - ❖ **standard solutions of each metal were separately prepared from their respective concentration of 1000 mg/ml stock solutions, from which further serial dilutions were made to cover the optimum absorbance range for standard calibration curve**
 - ❖ **all samples were measured in three times**

Preparation of extracts⁽¹⁵⁾

100 g of Ok-shit 500 ml in solvent

60°C for 6 hours,
three time

liquid extract

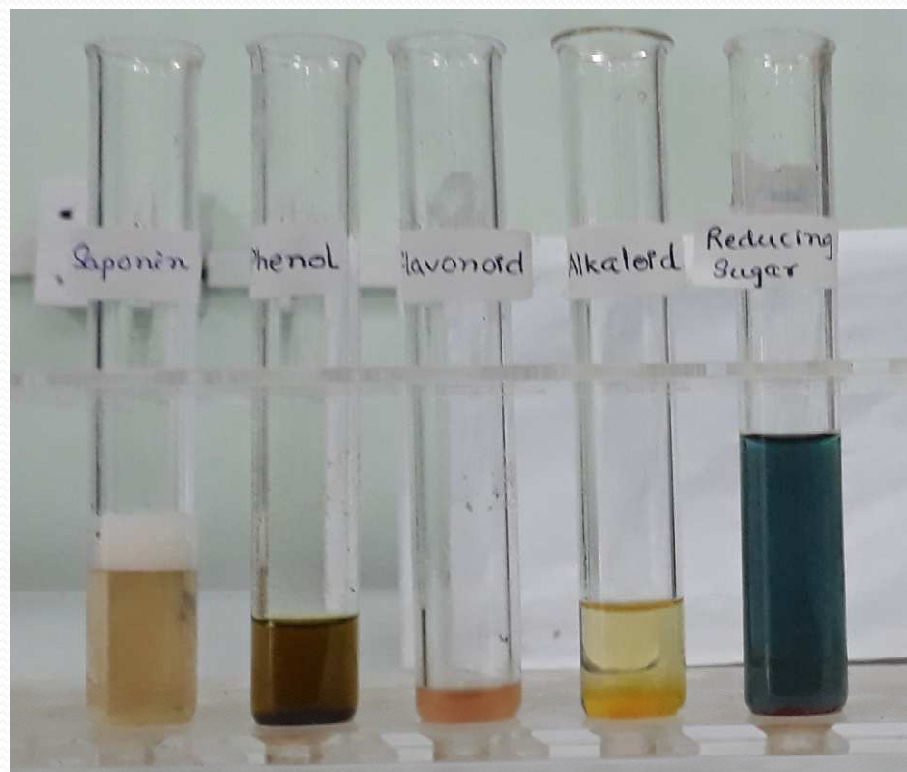
electromantle, 50°C

dry extract



Phytochemical tests for types of compounds

Harborne J.B (1998), Raaman (2006) Phytochemical Methods^(16,17)

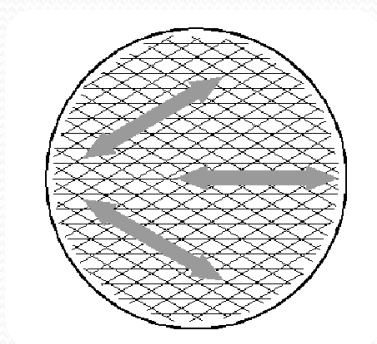


Determination of antibacterial activity

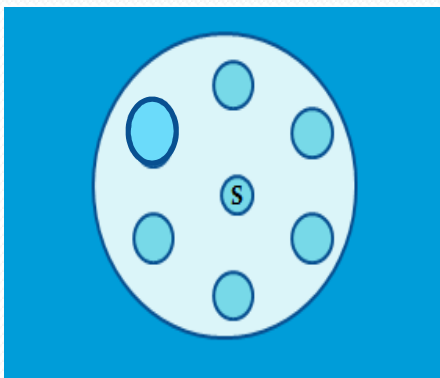
- ❖ **determined by agar well diffusion technique according to modified Kirby and Bauer method (WHO, 2003) ⁽¹⁸⁾**



test pathogens were seeded over the Mueller Hinton agar plates with a sterile swab



the Mueller Hinton plate were swabbed over the entire surface of the medium 3 times, rotating the plate 60° after each application



- ❖ **seven uniform wells of 8 mm diameter were made in the medium by sterile borer**
- ❖ **20 μ l of each extracts (30, 60, 90, 120, 150 mg/300 μ l) were transferred into separate wells**
- ❖ **positive control - Ceftriaxone (30 μ g/disc)**
- ❖ **negative control - same solvent only**
- ❖ **plates were incubated - 37 °C for 24 hrs**
- ❖ **plates were observed for zone inhibition, (CLSI) zone size interpretative chart**

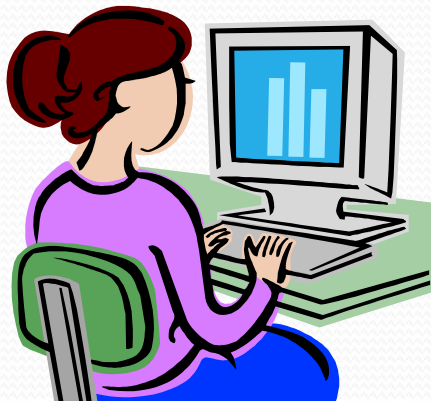
Determination of Minimum Inhibitory Concentration (MIC) & Minimum Bactericidal Concentration (MBC)

- ❖ **zone of inhibition >10 mm were proceeded for minimum inhibitory concentration by broth dilution method**
- ❖ **different concentrations of ranging 0.25 -10 mg/ml were tested**
- ❖ **series of 12 tubes for each test organisms was prepared**
- ❖ **contains 20 µl of test organisms, 1 ml of different concentration of Ok-shit extract and 1 ml of Mueller-Hinton broth**
- ❖ **control tube - broth only and inoculum only**
- ❖ **incubated at 37° C for 24 hours**
- ❖ **determination of MBC, one loopful from each tube of above dilutions was streaked on Muller Hinton agar plate and incubated at 37° C for 24 hours**

Statistical analysis



- ❖ Microsoft Excel v. 2007
- ❖ results were presented as mean \pm SE



Results and Discussions

Plant Authenticity

morphology, taxonomy & anatomy – taxonomist



Aegle marmelos (L.) Correa

Table 1. Minerals content (ppm) of Ok-shit leaves

Present/ Other Study	Macrominerals				Microminerals				Reference
	Ca	Mg	K	Na	Cu	Fe	Mn	Zn	
OK-shit	1994.39 ± 8.6	42.60 ± 0.12	724.12 ± 8.30	69.32 ± 0.58	3.82 ± 0.22	95.53 ± 1.27	15.89 ± 1.29	13.68 ± 0.26	
India, June 2017	94.9	243	1596	184	1.34	18.24	–	1.39	19
India, 2009					43.40 ± 1.46	799.87 ± 1.05	30.10 ± 5.66	66.32 ± 3.79	20
WHO/FAO 2001	–	–	–	–	73	425	500	100	21
WHO 1996	3600	–	10-100	400-500	–	–	–	–	22
Ajasa, 2004	44 -614	2000	6380 - 36600	2610 - 51340	–	–	–	–	23

Yield percent of different Extracts of Ok-shit leaves

Sr. No	Solvent	Yield (%)
1	Distilled water	40.69
2	50% Ethanol	32.38
3	70% Ethanol	33.4



Table 2. Phytochemical constituents of Ok-shit leaves

No.	Phytochemical	Reagents	Observation	Result
1.	Alkaloids	Dragendorff's solution	Orange ppt	+
2.	α amino acid	Ninhydrin	Pink color	+
3.	Carbohydrate	α -naphthol, Con: H_2SO_4 solution	Pink ring color	+
4.	Flavonoids	Con: HCl , Mg turning	Reddish brown	+
5.	Glycosides	10 % lead acetate solution	Yellow ppt	+
6.	Phenols	10% Fe Cl_3 solution	Blue	+
7.	Protein	10 % Na OH , 10 % CuSO_4 solution	Red or Violet	+
8.	Reducing sugar	Benedict's solution	Brick red ppt	+
9.	Saponins	H_2O , Shaken 15 minutes	2 cm foam	+
10.	Starch	Iodine solution	Blue	—
11.	Steroids	acetic anhydride, Con: H_2SO_4 solution	Greenish blue Solution	—
12.	Tannins	1% Fe Cl_3 , Dil: H_2SO_4 solution	Yellowish brown	+
13.	Tri-terpene	CHCl_3 , acetic anhydride, Con: H_2SO_4 solution	Reddish brown coloration	—

(+) = Detected

(-) = Not detected

Table 3. Antibacterial activities of Ok-shit extracts

Extracts/ Ceftriaxone standard	Diameter of inhibition zone (mm) of Ok-shit extracts and Standard		
	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>
Aqueous/ Std	14 -20mm /31 mm	13-19 mm /32 mm	12-18 mm /30 mm
50% EtOH/ Std	20-25 mm /35 mm	16-25 mm /35 mm	15-20mm /32 mm
70% EtOH/ Std	18-24 mm /34mm	15-24 mm /29 mm	14-18 mm /31 mm

Agar well – 8 mm, Standard antibiotic – Ceftriaxone 30 µg



(1 mg to 5 mg/ well)

Fig 2: Antibacterial activities of Ok-shit leaves extracts on tested organisms

Table 4. MIC and MBC of Ok-shit extracts


Ok-Shit Extract	<i>Staphylococcus aureus</i>		<i>Pseudomonas aeruginosa</i>		<i>Escherichia Coli</i>	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	(mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Aqueous	>7	>8	>7	>8	>7	>8
50% EtOH	1	2	2	3	4	5
70% EtOH	2	3	3	4	5	6





- 1 = Test organism + Ethanolic extract (0.25 mg/ml)
- 2 = Test organism + Ethanolic extract (0.5 mg/ml)
- 3 = Test organism + Ethanolic extract (1 mg/ml)
- 4 = Test organism + Ethanolic extract (2 mg/ml)
- 5 = Test organism + Ethanolic extract (3 mg/ml)
- 6 = Test organism Only

MIC (1 mg/ml)

MBC (2 mg/ml)

- 
- ❖ **minerals content were comparable with other studies**
 - ❖ **macrominerals content of Ok-shit were within permissible limit of Ajasa, (2004) except calcium**
 - ❖ **calcium content in Ok-shit leaves - 1994.39 ± 8.6 ppm**
 - ❖ **within permissible limit (3600 ppm) of WHO 1996**
 - ❖ **may be foliar absorption from surroundings air, exposed to rock mining work**
 - ❖ **calcium plays essential function in bone, teeth, nerve transmission, muscle contraction, etc ^(3,19)**
 - ❖ **microminerals content were within permissible limit of vegetables set by FAO/WHO, 2001(Table 1)**
 - ❖ **minerals contents in plants depends on climatic factors, plant species, air pollutions and other environmental factors**

- 
- ❖ **Ok-shit - alkaloids, α amino acid, carbohydrate, flavonoids, glycosides, phenols, protein, reducing sugar, saponins and tannins (Table 2)**
 - ❖ **alkaloids, flavonoids, glycosides, phenol, saponins , tannins, - antimicrobial, antidiabetic, hypocholesterolemic, antidiarrheal, anticancer activities etc and also inhibited the growth of *S. aureus* and *E. coli* ^(19,22,24,25,26)**

- 
- ❖ Ok-shit leaves have varying degrees on inhibition of growth of tested organisms (Table 3 and 4)
 - ❖ comparable to other studies
 - ❖ Ibrahim N. A., *et al.*, **Egypt**, 2015 - essential oils of Ok shit leaves observed 23 mm and 25 mm on *S. aureus* and *E.coli*
 - ❖ MIC value was 100 µg/ml against on *E.coli*
 - ❖ Senthilkumaran R., *et al.*, **India**, 2014 – Ok shit leaves extracts possessed 26mm and 24 mm on *S. aureus* and *E. coli*
 - ❖ MIC and MBC of crude extracts
3.9 mg/ml and 7.8 mg/ml against *S. aureus*
7.8 mg/ml and 15.6 mg/ml against *E.coli*

- ❖ Pandey A. *et al.*, **India**, 2011 - ethanolic extract of Ok-shit fruit inhibited 19 mm, 13.5 mm and 13 mm against on *S. aureus*, *P. aeruginosa* and *E.coli*
- ❖ MIC values of ethanolic extract of fruits were obtained 1.98 mg/ml against *S. aureus*
- ❖ differences in antibacterial activity of same plant extracts have been reported by various investigators might be due to microorganisms used, sample collection time, part used of plant, method of extractions, types of solvent, different geography, climate and habitat of plant samples



Conclusions

- ❖ **good source of essential macro and micro minerals**
- ❖ **has quite a number of chemical constituents which may be involved in many pharmacological activities in accordance with literature review**
- ❖ **Ok-shit possess antibacterial activities on tested bacterial strains**
- ❖ **provide nutrients for human beings, preventive properties against selected pathogens**
- ❖ **helpful for many herbal medicine user, by using Ok-shit leaves for different types of ailments**




Acknowledgement

The authors would like to respectfully acknowledge

- ❖ **Director General**
- ❖ **Dr. Khin Phyu Phyu, Director and**
- ❖ **Board of Directors (Department of Medical Research)**
- ❖ **Dr. Kay Thwe Thwe Maung , Sineor Officer
(Public Health Laboratory)**
- ❖ **Dr Soe Myint Aye, Professor,
Department of Botany (University of Mandalay)**
- ❖ **All of my colleagues, who have willingly helped me
throughout with their ability**

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THANK YOU

