Elemental Composition, Phytochemical Screening and Antibacterial Activity of *Aegle marmelos* (L.) Correa (အိုင်း)
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)
- 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, Ibrahimm 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 ဥသစ was studied by Khin Tar Yar Myint et al., 2017 (11)
- in Myanmar, scientific evaluation of elemental composition and antibacterial activity of Ok-shit leaf 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) 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

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
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

20µl of each extracts (30, 60, 90, 120, 150 mg/300 µl) were transferred into separate wells

positive control - Ceftriaxone (30µg/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 ± SE
Results and Discussions

Plant Authenticity

morphology, taxonomy & anatomy – taxonomist

*Aegle marmelos* (L.) Correa
## Table 1. Minerals content (ppm) of Ok-shit leaves

<table>
<thead>
<tr>
<th>Present/Other Study</th>
<th>Macrominerals</th>
<th>Microminerals</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca</td>
<td>Mg</td>
<td>K</td>
</tr>
<tr>
<td>OK-shit</td>
<td>1994.39 ± 8.6</td>
<td>42.60 ± 0.12</td>
<td>724.12 ± 8.30</td>
</tr>
<tr>
<td>India, June 2017</td>
<td>94.9</td>
<td>243</td>
<td>1596</td>
</tr>
<tr>
<td>India, 2009</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHO/FAO 2001</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>WHO 1996</td>
<td>3600</td>
<td>_</td>
<td>10-100</td>
</tr>
<tr>
<td>Ajasa, 2004</td>
<td>44 -614</td>
<td>2000</td>
<td>6380 - 36600</td>
</tr>
</tbody>
</table>
Yield percent of different Extracts of Ok-shit leaves

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Solvent</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distilled water</td>
<td>40.69</td>
</tr>
<tr>
<td>2</td>
<td>50% Ethanol</td>
<td>32.38</td>
</tr>
<tr>
<td>3</td>
<td>70% Ethanol</td>
<td>33.4</td>
</tr>
</tbody>
</table>
Table 2. Phytochemical constituents of Ok-shit leaves

<table>
<thead>
<tr>
<th>No.</th>
<th>Phytochemical</th>
<th>Reagents</th>
<th>Observation</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td>Dragendorff’s solution</td>
<td>Orange ppt</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>$\alpha$ amino acid</td>
<td>Ninhydrin</td>
<td>Pink color</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Carbohydrate</td>
<td>$\alpha$-naphthol, Con: $\text{H}_2\text{SO}_4$ solution</td>
<td>Pink ring color</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Flavonoids</td>
<td>Con: $\text{HCl}$, Mg turning</td>
<td>Reddish brown</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>Glycosides</td>
<td>10% lead acetate solution</td>
<td>Yellow ppt</td>
<td>+</td>
</tr>
<tr>
<td>6.</td>
<td>Phenols</td>
<td>10% $\text{Fe Cl}_3$ solution</td>
<td>Blue</td>
<td>+</td>
</tr>
<tr>
<td>7.</td>
<td>Protein</td>
<td>10% $\text{Na OH}$, 10% $\text{CuSO}_4$ solution</td>
<td>Red or Violet</td>
<td>+</td>
</tr>
<tr>
<td>8.</td>
<td>Reducing sugar</td>
<td>Benedict's solution</td>
<td>Brick red ppt</td>
<td>+</td>
</tr>
<tr>
<td>9.</td>
<td>Saponins</td>
<td>$\text{H}_2\text{O}$, Shaken 15 minutes</td>
<td>2 cm foam</td>
<td>+</td>
</tr>
<tr>
<td>10.</td>
<td>Starch</td>
<td>Iodine solution</td>
<td>Blue</td>
<td>-</td>
</tr>
<tr>
<td>11.</td>
<td>Steroids</td>
<td>acetic anhydride, Con:$\text{H}_2\text{SO}_4$ solution</td>
<td>Greenish blue Solution</td>
<td>-</td>
</tr>
<tr>
<td>12.</td>
<td>Tannins</td>
<td>1% $\text{Fe Cl}_3$, Dil: $\text{H}_2\text{SO}_4$ solution</td>
<td>Yellowish brown</td>
<td>+</td>
</tr>
<tr>
<td>13.</td>
<td>Tri-terpene</td>
<td>$\text{CHCl}_3$, acetic anhydride, Con: $\text{H}_2\text{SO}_4$ solution</td>
<td>Reddish brown coloration</td>
<td>-</td>
</tr>
</tbody>
</table>

(+)= Detected  (-)= Not detected
### Table 3. Antibacterial activities of Ok-shit extracts

<table>
<thead>
<tr>
<th>Extracts/ Ceftriaxone standard</th>
<th>Diameter of inhibition zone (mm) of Ok-shit extracts and Standard antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Aqueous/ Std</td>
<td>14-20 mm /31 mm</td>
</tr>
<tr>
<td>50% EtOH/ Std</td>
<td>20-25 mm /35 mm</td>
</tr>
<tr>
<td>70% EtOH/ Std</td>
<td>18-24 mm /34 mm</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Ok-Shit Extract</th>
<th><em>Staphylococcus aureus</em></th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Escherichia Coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (mg/ml)</td>
<td>MBC (mg/ml)</td>
<td>MIC (mg/ml)</td>
</tr>
<tr>
<td>Aqueous</td>
<td>&gt;7</td>
<td>&gt;8</td>
<td>&gt;7</td>
</tr>
<tr>
<td>50% EtOH</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>70% EtOH</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

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
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References


8. Myanmar Traditional Medicine Formulary, Department of Medical Research (Lower Myanmar), Ministry of Health, The government of the Republic of the Union of Myanmar, 2013, 280-285


THANK YOU