An *In Vitro* Evaluation of the Effectiveness of Gotu Kola (*Centella asiatica*) on Inhibiting the Growth of Selected Microorganisms in Human Saliva

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ABSTRACT

Background: Gotu Kola (*Centella asiatica*) has been used as a traditional medicine for many years to cure different kinds of diseases. Studies have been reported that Gotu Kola extracts might be used as a cure for oral diseases such as periodontal disease. In the present study, Gotu Kola leaves extracted with water will be used to evaluate its effect on some microorganisms living in the human saliva using minimum inhibitory concentration (MIC) method.

Material and Method:Gotu Kola fresh leaves extract have been used with water as a solvent, a rotary evaporator was used to separate the solvent from the extract. The following microorganisms: Streptococci, Lactobacilli, and *Staphylococcus aureus* have been isolated fromthe Saliva of ten volunteers participated in the present study. Nutrient broth tubes have been prepared for MIC test, where various concentrations of the Gotu Kola extracts (0.5mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml, and 5 mg/ml) were added, respectively. The tubes incubated at 37^{0}C for 48h.

Results:The MIC test shows that a concentration of 4 mg/ml have the ability to inhibit the growth of oral Lactobacilli and 8 mg/ml has the ability to inhibit the growth of *S. aureus*which may be due to asiaticoside and asiatic acid which are active ingredients that the leaves extracts consists of. There was no MIC for Streptococci.

Conclusion:The Gotu Kola leaves extract can be used to inhibit the growth of some oral microorganisms at certain concentration.

Keywords:*Centella asiatica*, oral microorganisms, antimicrobial activity, minimum inhibitory concentration. (J Bagh Coll Dentistry 2016; 28(1):174-178).

INTRODUCTION

Gotu Kola which is also known in Malaysia as (Pegaga) is one of the most well-known herbs in the world that have been used as a folk medicine, especially in South East Asia. The plant is a slender-stemmed delicate perennial creeping herb, belonging to the Umbelliferae family. Originally, the plant was identified botanically as *Hydrocotyle asiatica* Linn, but subsequently, it was named Centella asiatica [(1)].

Gotu Kola, *C. asiatica* is also known as (Vallarai in India, Di Chien Tsao in China and Navelwort in Europe). All are believed to have the same active compounds and possibly good antimicrobial activity to inhibit the growth of some microorganisms that are believed to be etiologic microorganisms in oral diseases [(1-4)].

Many microorganisms including Staphylococcus species, Streptococcus species, and Lactobacillus species are found in human saliva [(5)]. Dental caries has been known to be caused by acidogenic and aciduric bacteria, such as mutans streptococci and lactobacilli [(6,7)]. These bacteria are involved in the fermentation of dietary carbohydrates producing organic acids that are responsible for decalcification and decay of teeth [(8,9)]. As the main reservoir for *S. aureus* are the nares, it is reasonable that the organism will occasionally be present in saliva and on the oral mucosa which cause the infection [(10)].

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This gives a great motive for the researchers to evaluate the effect of Gotu Kola extracts on these microorganisms.

Several methods have been used for preparation of medicinal plants extract such as maceration, infusion, digestion, decoction, and percolation. The extraction process of medicinal plant extracts including freeze drying and rotary evaporator. Different solvents have been used for extraction of medicinal plants including 95% ethanol and n-hexane [(11,12)], according to Dash*et al.*[(12)], water can be used as another solvent for extraction process.

In the present study, an experiment will be performed to see how effective is the Gotu Kola fresh leaves extracted with water as a solvent on some isolated microorganisms in human saliva using minimum inhibitory concentration (MIC).

MATERIALS AND METHODS

Gotu Kola source

Gotu Kola fresh plants which is also known as (Pucuk Pegaga) in local Malaysian markets were obtained. The plants were washed with distilled water and sterile scissor was used to cut the leaves from the stem to cut into pieces (figure. 1).

Figure 1. *Centella Asiatica* Fresh Leave

Extraction Method

The extraction method of Gotu Kola was performed by using water as solvent; the procedure followed Dash with some modifications[12].

The Gotu Kola fresh contained 262.41 g of leaves were weighed using electronic balance (monobole inside, weighting technology, PB 3002-S), then were chopped and placed inside a blender (Fiamma SDN BHD, EBM-9182FG, Kuala Lumpur, Malaysia) with 300 ml distilled water for 2-3 minutes.

The blended leaves were then poured in a sterile flask inside the reflux ® (Copnes scientific Sdn Bhd, Selangor, Malaysia). The reflux consists of an Allihn condenser (Cone 24/29, 250 mm effective length, Copnes scientific Sdn Bhd, Selangor, Malaysia) and a conical flask (1000 ml, socket 24/29, Copens scientific Sdn Bhd, Selangor, Malaysia). An anti-bumping granule (BDH Prolabo ® (Batch no. 08A110018, UK) was added to the flask to prevent the liquid from being bumped during the boiling process (figure. 2).

Figure 2. The Design of the Reflux Containing the Gotu Kola Fresh Leaves

The fresh leaves were boiled under reflux for one hour. After the boiling process, the flask containing the extract solution is kept for 20 minutes to cool at room temperature. Then the solution was filtered using a sterile handkerchief, folded, and squeezed to obtain a red colored solution (figure. 3).

Figure 3. Filtered Gotu Kola Extracts Solution

The filtered extract solution was placed in the rotary evaporator (Heidolph, Laborota 4011-digital) for 3-4 h which was run at 87^0C, 15^0C vacuum cooler, 300 mbar and 62 rpm to remove water solvent from the extract (figure 4).

Figure 4. Rotary Evaporator Containing the Extract Solution

After the evaporation process, the concentrated extract was poured in a crucible and placed in a drying oven for 24h at 105^0C. The dried Gotu Kola was first broken into smaller pieces by using a pair of sterile forceps then it was ground by a mortar and pestle to obtain the fine powder.

The Gotu Kola powder is put in a sterile glass petri dish and placed in a drying oven overnight at 105^0C to remove all the moistures from the powder. Then, the glass petri dish placed in desiccators to cool for 1 hour. Then, the powder is stored in small container for use (figure 5).

Figure 5. Gotu Kola fine Powder in a Sterile Container

Media Preparation for MIC

Mixing 8g of nutrient broth from Merk ® (Cat no. 1.0543.0500, Darmstadt, Germany) with 1L of distilled water. The media poured into six test tubes and autoclaved at 121^0C for 15 min.Mouthwash containing Chlorhexidine 0.12% w/v (Oradex, Malaysia) was used as positive control and deionized water was used as negative control. Then, the tubes were stored in the fridge for use.38g of Mueller Hinton agar (Sigma Aldrich, Malaysia) mixed with 1L of distilled water. The media was autoclaved at 121^0C for 15 min, the media poured into the plates and kept in the fridge.

Saliva Samples

Fresh saliva samples from 10 clinically healthy volunteers without any obvious signs and symptoms of systemic and oral diseases were collected in the morning. The volunteers were university students aged (18-24) and previously informed not to wash their mouth for 24 hours.

Bacterial Culture

Three types of bacteria species were isolated from the saliva of the volunteers: Streptococci, Lactobacilli, and *Staphylococcus aureus*. Mitis salivary agar Sigma aldrich, Malaysia was used to culture oral Streptococci. Rogosa agar (HiMedia Labs. India) was used to culture oral lactobacilli. Blood agar (HiMedia Labs., India) was used to culture *S. aureus*. Both Streptococci and *S. aureus* were incubated aerobically at 37^0C for 24h while Lactobacilli were incubated anaerobically at the same temperature. The isolated microorganisms were subcultured on Mueller Hinton agar and inoculated into nutrient broth as stock cultures for use. The cultures were identified on the basis of gram staining characters and biochemical tests.

The Method of Minimum Inhibitory Concentration (MIC)

Extracting 0.5g (500mg) of Gotu Kola leaves powder dissolved in 5 ml distilled water in a small beaker to get a concentration of 100 mg/ml.A series of different concentrations (10 mg/ml, 8mg/ml, 4 mg/ml, 2mg/ml, 1 mg/ml, and 0.5 mg/ml) have been added aseptically into the broth tubes, respectively. The microbial inoculum was standardized at 0.5 McFarland. 0.1 ml of the broth containing the isolated microorganisms from the saliva had been added to each one of the eight tubes including positive and negative controls, respectively. The tubes were then incubated at 37^0C for 24h. 0.1 ml from each tube have been added to Mueller Hinton agar plates to assist the MIC results and incubated at 37^0C for 48h. The procedure was done in triplicate.

RESULTS

The results showed that none of the concentrations inhibit the growth of oral streptococci (table 1). A concentration of 4 mg/ml showed antimicrobial activity towards oral lactobacilli except for 3 out of 10 (30%) volunteers (table 2). A concentration of 8 mg/ml showed antimicrobial activity towards *S. aureus*which can be consider to be MIC (table 3).

Table 1. Test Performance for Minimum Inhibitory Concentration (MIC) on Oral Streptococci

No. of volunteers	10 mg/ml	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml	+ ve CHl0.12%	-ve DW
1	+	+	+	+	+	+	-	+
2	+	+	+	+	+	+	-	+
3	+	+	+	+	+	+	-	+
4	+	+	+	+	+	+	-	+
5	+	+	+	+	+	+	-	+
6	+	+	+	+	+	+	-	+
7	+	+	+	+	+	+	-	+
8	+	+	+	+	+	+	-	+
9	+	+	+	+	+	+	-	+
10	+	+	+	+	+	+	-	+

+: Microbial growth, -: No microbial growth

Table 2. Test Performance for Minimum Inhibitory Concentration (MIC) on Oral Lactobacilli

No. of volunteers	10 mg/ml	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml	+ ve CHl 0.12%	-ve DW
1	-	-	-	+	+	+	-	+
2	-	-	-	+	+	+	-	+
3	-	-	-	+	+	+	-	+
4	-	-	-	+	+	+	-	+
5	-	-	-	+	+	+	-	+
6	-	-	+	+	+	+	-	+
7	-	-	-	+	+	+	-	+
8	-	-	-	+	+	+	-	+
9	-	-	+	+	+	+	-	+
10	-	-	+	+	+	+	-	+

+: Microbial growth, -: No microbial growth

Table 3. Test Performance for Minimum Inhibitory Concentration (MIC) *S. Aureus*

No. of volunteers	10 mg/ml	8 mg/ml	4 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml	+ ve CHl 0.12%	-ve DW
1	-	-	+	+	+	+	-	+
2	-	-	+	+	+	+	-	+
3	-	-	+	+	+	+	-	+
4	-	-	+	+	+	+	-	+
5	-	-	+	+	+	+	-	+
6	-	-	+	+	+	+	-	+
7	-	-	+	+	+	+	-	+
8	-	-	+	+	+	+	-	+
9	-	-	+	+	+	+	-	+
10	-	-	+	+	+	+	-	+

+: Microbial growth, -: No microbial growth

DISCUSSION

Methanol and ethanol were used previously to obtain extracts from *Centella asiatica* while in the present study water was usedto obtain plant extracts. Few factors contribute in influencing the rate of extraction and quality of extracted bioactive phenolic compounds, including type of extraction method, particle size of medicinal plants, type of solvent, temperature and extraction time [13,14].

The results showed that *C. asiatica* has no antimicrobial activity on oral Streptococci which is compatible with other studies [15] and incompatible with others [16] which it might due to the type of extraction method and the antimicrobial susceptibility test they used. Antimicrobial activity of *C. asiatica* towards oral Lactobacilli appeared at 4 mg/ml which is relevant to previous studies on the same microorganism [16].

The results also showed positive antimicrobial activities towards*S. aurues* at 8 mg/ml, the results similar to previous studies[11,17-19]. It was reported that Gotu Kola leaves are rich in asiaticoside and Asiatic acid which are considered as the active ingredients in the herb itself that proves its efficacy towards microorganisms such as *S. aureus*, *E. coli*, *S. pneumonia*, and *H. pylori*[11,13,14]. Analytical methods such as TLC and HPLC are needed for further confirmation.

As a conclusion, the Gotu Kola leaves extract using water as solvent with MIC of4 mg/ml and8 mg/ml can inhibit the growth oforal lactobacilli and *S. aureus*, respectively. The crude extract couldn't inhibit the growth of oral Streptococci. Further *in vitro* studies evaluating the effectiveness of *C. asiatica* on different types of oral microorganisms are recommended to accomplish this study.

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