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Ultrasound-microwave Assisted Extraction (UMAE) of Andrographolide from Sinta (*Andrographis paniculata*) with its Bioactivity Assessment

ABSTRACT

Andrographolide (AG) is known to possess some pharmacological properties such as anti-inflammatory, antioxidant, anti-dengue, anti-tumor and anti-tuberculosis. Green extraction techniques such as ultrasound and microwave have shown the effectiveness in extracting high purity AG from Andrographis paniculata or Sinta. Ultrasound-assisted extraction (UAE), which operates at non-thermal conditions, prevents the thermal degradation of AG while microwave-assisted extraction (MAE) allows an increased extraction yield. This study aimed to determine the effect of sequential ultrasound- microwave-assisted extraction (UMAE) in the yield of AG and its bioactivity assessment. The UAE obtained its highest AG yield of 539.24 mg L^{-1} at 10 minutes sonication time and MAE with 781.65 mg L^{-1} at 5 minutes irradiation time with 420 W microwave power. The UMAE obtained the highest AG yield of 1,066.49 mg L^{-1} when sequentially exposed to 10 minutes sonication and 10 min irradiation with a microwave power of 280 W. Cytotoxic activity testing of Sinta extract containing AG from UMAE confirmed a lethal concentration (LC_{50}) with value at 76.02 mg L^{-1}. Furthermore, it has an intermediate susceptibility to Escherichia coli but resistant to both Bacillus clausii and Klebsiella spp., highlighting the potential of its valuable medicinal applications.

Keywords: *Andrographolide, Ultrasound-microwave-Assisted Extraction (UMAE), bioactivity assessment*

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INTRODUCTION

Extraction methods are classified as conventional and non-conventional. Conventional extraction methods or the traditional methods of extracting bioactive compounds proceed at prolonged exposure of the sample to elevated temperatures, longer extraction time, low extraction selectivity, and greater solvent requirements (*Swami et al. 2008*). Non-conventional extraction methods or modern green extraction techniques such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) have been explored to answer these limitations.

Ultrasound-assisted extraction creates cavitation bubbles, which then implode immediately. This phenomenon causes micro-fractures, perforation in cell membranes, and cell wall rupture causing a greater transfer of the desired extract from the solid to the solvent. Intensification of inter-particle collisions increase liquid circulation and thus, turbulence (*Vinatoru 2001*). Typically, UAE is mostly utilized for bioactive compound extractions (*Tao and Zhang 2014*). It was proven that ultrasonication is an important pre-treatment to get high extracts of oils from apricot, almond and rice bran (*Luque-Garcia and De Castro 2003*).

Microwave-assisted extraction is also among the effective modern techniques of extraction. It is advantageous due to its faster heating process for extraction, smaller equipment size, lesser use of an organic solvent, which makes it recognized as a green technology. Importantly, MAE increases extract yield at a short extraction time (*Alupului and Calinescu 2012*). The microwave energy penetrates the sample matrix and influences the polar constituent of the sample and the solvent, freeing the chemicals desired (*Chan et al. 2011*). This microwave heating aimed the microscopic moisture traced in the plant cell and evaporates it causing the plant cell itself to swell, thus creating great pressure from the inside on the cell walls. This initiates the rupture of the plant cell wall causing the desired compounds to escape (*Mandal et al. 2007*).

A modification of MAE is the Ultrasound microwave-assisted extraction (UMAE). The UMAE method proceeds by first subjecting the sample to UAE, considering it as a pretreatment, before letting it undergo MAE. Similar extraction mechanisms occur- the UMAE offers higher yields with

these combined mechanisms of UAE and MAE. UAE provides the solvent access into the cell through cell wall rupture, which increases diffusion and facilitates the release of the active compounds. Then for a short time, MAE subjects the UAE-treated solution to microwaves, intensifying the solvent's penetration into the plant matrix (*Pingret et al. 2012*). The UMAE operates at a faster time, with lesser use of solvent and higher yields, and it can extract low diffusing and hardly extracted active compounds. The drawback of UMAE is only the additional set-up. Furthermore, UMAE continues without the risk of thermal ruin of the compounds present (*Chan et al. 2011*). Advantages can be derived from both UAE and MAE, in which the former proceeds at non-thermal conditions and the latter at a very short extraction time (*Liew et al. 2016*).

Andrographis paniculata, generally known as *Sinta*, is an annual herb belonging to the Acanthaceae family. *Sinta* is used traditionally in herbal medicine in different parts of the world. In the traditional Chinese medicine system, it is ordinarily known as "King of Bitters". *Sinta* has a very bitter flavor and it can remove heat from the body. This herb prefers sunny conditions that make it abundant in Southeast Asian countries. *Sinta* plant grows well in all types of soil, which explains its wide distribution and is found in various habitats of habitat such as plains, hill slopes, wastelands, farms, dry or wetlands, seashore, and even on the roadside (*Benoy et al. 2012*). In Malaysia, it was traditionally used to treat hypertension and diabetes, while in India, it was known for treating colds and dysmenorrhea (*Xu 2009*). Its use in herbal medicine is linked to the bioactive compounds present in it including Neoandrographolide, 14-Deoxyandrographolide, and Onysilin. One particular bioactive compound of concern is the Andrographolide (AG).

Andrographolide is documented to have many interesting properties. Some of which are anti-inflammatory (*Levita et al. 2009*), antioxidant (*Mittal et al. 2016*), anti-dengue (*Edwin et al. 2016*), anti-tumor (*Guo et al. 2016*), and anti-tuberculosis (*Prabu et al. 2015*). Considering its many beneficial effects, various methods of extracting AG have been studied to identify which among them is most advantageous in terms of effectivity and efficiency.

This study aimed to extract AG from *Sinta* using UMAE and compare the yields obtained using Soxhlet, UAE, MAE and UMAE as well as to identify the effects of sonication time on UAE, irradiation time and microwave power on MAE, and sonication time, irradiation time and microwave power on UMAE. Furthermore, this study aimed to assess the bioactivity of the *Sinta* extract containing AG obtained via UMAE, specifically its cytotoxicity and antibacterial sensitivity.

MATERIALS AND METHODS

Materials and reagents

The leaves and stems of *Sinta* in its post-flowering stage were harvested from Novaliches, Quezon City, Philippines. It was washed and the adhering dirt was removed. Taxonomical identification of the plant was done by the Botany Division of the National Museum, Manila, Philippines. The cleaned biomass samples were cut and dried using a freeze dryer (Scanvac, Labogene, Allerød, Denmark) at Department of Science and Technology-Food and Nutrition Research Institute (DOST-FNRI), Philippines for 3 days and finely powdered. The prepared samples of *Sinta* were stored in an air-tight container and kept in the refrigerator maintained at 1.7-3.3°C before use. Ethanol (95%, analytical grade, Belman Laboratories, Quezon City, Philippines) was used as the solvent. A mobile phase of 48% v/v methanol (Aishite Trading Lab. Chemicals, Quezon City, Philippines) and 52% v/v distilled water was prepared for the quantification of Andrographolide. Standard Andrographolide (99% purity, Sigma-Aldrich, Singapore) was used for standard calibration.

Extraction of Andrographolide

Soxhlet extraction as a standard method was done and compared to UAE, MAE and UMAE in terms of AG yield. The prepared samples of *Sinta* were placed in a thimble made from filter paper and placed in the 500 mL Soxhlet apparatus equipped with a bulbed condenser (DXY Corporation, Hangzhou Shi, China). Extraction operation was maintained at 80°C using 50% v/v ethanol-water as the solvent. The extract was collected after 8 hours.

Samples for the UAE, MAE and UMAE were prepared using 8 grams of the prepared samples of *Sinta* mixed with 120 ml of 50% ethanol in an Erlenmeyer flask. For UAE, a specific hot spot in the bath was used for the sonication time of 5, 10 and 15 min. The mixture was exposed to ultra sonication by submerging it to the sonicator bath maintained at 40°C with the top of the mixture an inch below the water level. For MAE, the mixture was placed in a modified domestic microwave oven and irradiated for different irradiation times of 5, 10 and 20 min and microwave power of 120, 280 and 420 W. For UMAE, the mixture was subjected to a

sequential and the MAE following the operating conditions.

Characterization of Andrographolide

The presence of AG on the extract was measured using high-performance liquid chromatography (HPLC, PerkinElmer, PerkinElmer Inc., Massachusetts, USA) equipped with UV/Vis detector (Flexar, PerkinElmer Inc., Massachusetts). Standard solutions of AG via serial dilution were prepared for the calibration curve. The components of the extract were analyzed using a C18 reversed-phase column (Merck Millipore, Millipore Corporation, Massachusetts, USA). The samples were prepared for HPLC analysis using a filtration system consisting of a hydrophilic PTFE 0.45 µm filter (Samplicity, Sigma-Aldrich Corp., Missouri, USA). A mobile phase of 48% v/v methanol and 52% v/v water was used for all the runs with a cycle time of 240 seconds, a flow rate of 2 mL min^{-1}, and UV detector set at 255 - 220 nm and 38 sec retention time.

Brine Shrimp Lethality Assay Test of Sinta Extract

To assess the cytotoxicity of the *Sinta* extract, brine shrimp lethality assay test was used in this study (*Baravalia et al. 2012*). The ethanol solvent of the *Sinta* extract from the identified UMAE with highest AG yield was evaporated using a rotary evaporator at 58 kPa, 160 rpm, and 40°C. Hatching of brine shrimp eggs was done in artificial seawater (3.8% w/v sea salt in distilled water) for 48 hours. It was then dissolved in the artificial saltwater at five sample concentrations: 10 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, 1,000 mg L^{-1}, and 10,000 mg L^{-1} with three replicates each. Positive control was also prepared with 0 mg L^{-1} of the extract. Pasteur pipette was used to transfer live shrimps from the aquarium to the test tubes then after 20 hours, the remaining alive nauplii were counted. The lethal concentrations (LC^{50}) were calculated from the mortality results through probit analysis.

Kirby-Bauer Disc Method for Antibiotic Susceptibility Testing of Sinta Extract

To assess the antibiotic susceptibility of the obtained *Sinta* extract, the Kirby-Bauer Disc method was used (*Hudzicki 2009*). Here, the Agar medium was prepared by dissolving 20.36 g of Mueller-Hinton Agar in 500 mL distilled water using a hotplate for heating with frequent agitation using a magnetic stirrer. The autoclave was used to sterilize it at 121°C for 15 min. After the agar medium was cooled to 40-50°C, it was poured into a sterile petri dish at a depth of 4 mm and was allowed to solidify. Inoculation of the plates was done by streaking the inoculum on the agar ensuring even distribution. The filter paper disc was impregnated with the extract at a dose of 5 µL per disc and then placed onto the agar medium. The petri dishes were kept in the refrigerator at 4°C for 2 hr, then incubated at 37°C for 24 hr. The diameter of the zone of inhibition was measured in millimeters using a digital caliper. Streptomycin (5 µL per disc) was used in the positive control, while water was used in the negative control.

Analysis of data

One-way ANOVA was applied to assess the effect of the extraction methods, Soxhlet, UAE, MAE and UMAE on the obtained yield of AG in the extract. Similarly, this was also used to identify the effect of sonication time for UAE, irradiation time and microwave power, and their respective interactions for MAE on the AG yield. ANOVA of the general factorial model was used to identify the effect and interactions of the parameters, sonication time, irradiation time and microwave power on the UMAE concentration yield.

RESULTS AND DISCUSSION

Yield of Andrographolide using ultrasound-assisted extraction

The AG was extracted using the UAE method varied at 5, 10 and 15 min sonication time with 40°C as the operating temperature (**Figure 1**). The highest AG yield obtained was 539.24 mg L^{-1} at 10 min. This is in agreement with the results *Rao and Rathod (2015*). On the other hand, prolonging the sonication time to 15 min resulted in the lowest AG yield at 298.5 mg L^{-1} due to degradation of the extracted compounds (*Dong et al. 2010*). During sonication, aqueous solvents containing the target compounds subjected to ultrasound facilitated homolytic cleaving of H-O in water, forming hydroxyl radicals. This formation of excess hydroxyl radicals in the extract solutions promoted oxidative degradation of the target compound and provided a possible route for thermal decomposition (*Bremner et al. 2011*).

Yield of Andrographolide using microwave-assisted extraction

When the microwave power was at 280 W, an increasing trend of AG yield was obtained as the irradiation time is increased (**Figure 2**). However, when the microwave power is at 420 W, while prolonging the irradiation time, a decreased AG yield was observed. This

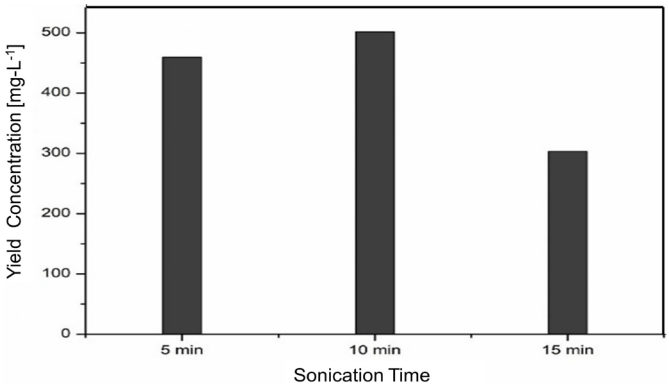

Figure 1. Yield of Andrographolide using ultrasound-assisted extraction at varying sonication time.

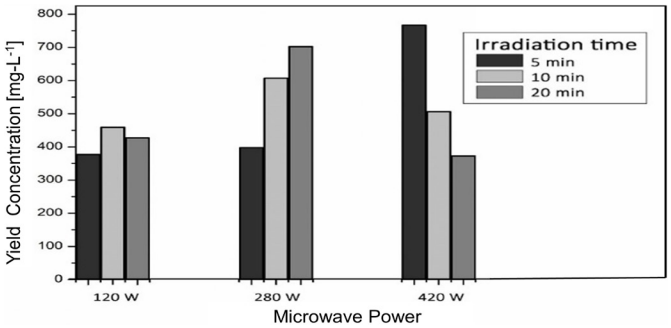

Figure 2. Yield of Andrographolide using microwave-assisted extraction at varying microwave power.

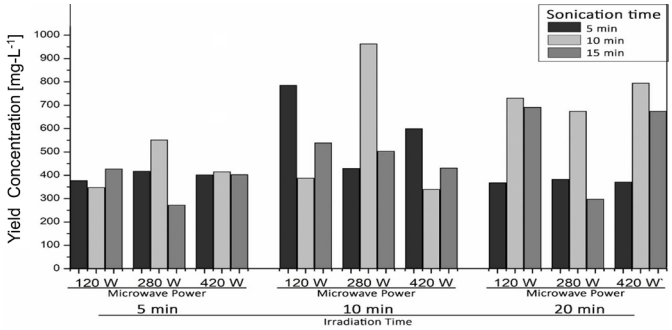

Figure 3. Yield of Andrographolide using ultrasound microwave-assisted extraction at different sonication time and microwave power.

relationship between irradiation time and microwave power exhibited that one parameter must be kept at lower conditions and the other parameter at higher conditions to achieve a high AG yield. This was confirmed by *Hartati et al.* (*2015*) in which higher concentrations of solute were obtained in the first five minutes of extraction for a microwave power of 30%; but as the extraction time progressed, higher solute concentrations were obtained for only 10% of microwave power. It was observed that AG yield decreased due to overexposure to microwave radiation determined by both the irradiation time and the microwave power.

Yield of Andrographolide using ultrasound-assisted extraction

At the condition of UMAE, the AG yield obtained at 961.88 mg L^{-1} was higher compared to the AG yield of UAE at a sonication time of 10 min, which was at 501.57 mg L^{-1} and MAE at an irradiation time of 10 min and a microwave power of 280 W, which was at 606.65 mg L^{-1} (**Figure 3**).

In similar studies of the extraction of natural compounds, UMAE was identified as the method, which exhibits the highest yield and purity as compared with UAE and MAE in the extraction of lycopene (*Lianfu 2008*) and polysaccharide (*Chen et al. 2007*). Similar trends as in UAE and MAE were obtained for UMAE since higher yield can be obtained when one of the parameters, either time of radiation and sonication or the microwave power, was kept at lower conditions.

Comparison of the Andrographolide yield obtained from the different extraction methods

It was confirmed that at $p<0.05$, the differences in AG yield obtained from the extraction methods were significant with p-value ≤ 0.0001 (**Table 1**), validating that the extraction method which obtained the highest AG yield was UMAE (**Figure 4**). The graph shows that the sequential approach of UMAE gives the highest AG yield as compared to the other extraction techniques Soxhlet, UAE and MAE. This was due to the combined effect of ultrasound and microwave irradiation that took place in the UMAE resulting in the cell wall disruption and increased solvent's diffusivity. The amount of AG yield obtained after 8 hr of extraction was 265.83 mg L^{-1}.

Effect of the parameters varied in ultrasound-assisted extraction, microwave-assisted extraction and Ultrasound microwave-assisted extraction on the Andrographolide yield

The result of the effect of sonication time varied at 5, 10 and 15 min in UAE was revealed to be of significant effect on the AG yield with $p= 0.0049$ wherein the highest AG yield was obtained at sonication time of 10 min (**Table 1**).

Also at $p<0.05$ level, the One-way ANOVA result of the effect of irradiation time and microwave power on MAE showed that there was a significant difference in the AG yield obtained at the different set conditions: irradiation time taken for 5, 10 and 20 min with a $p<0.0001$; and microwave power at 140 W, 280 W and 420 W with a $p<0.0001$. Furthermore, there was a

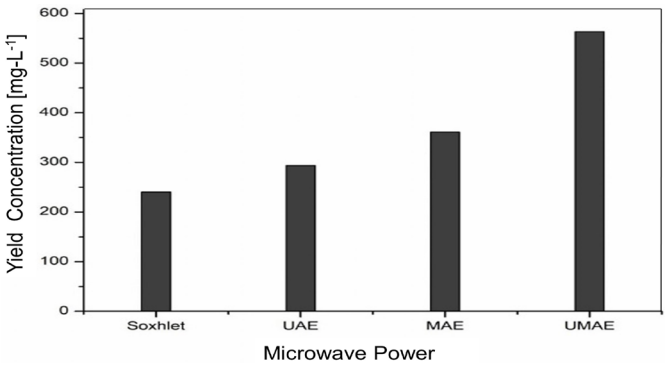

Figure 4. Comparison of the Andrographolide yield using different extraction methods.

significant interaction between the irradiation time and the microwave power with a p<0.0001. The highest AG yield obtained for this extraction method was at an irradiation time of 5 min and microwave power of 420 W.

The UMAE general factorial model at p<0.05 revealed that there was a significant AG yield difference in the variation of both sonication and irradiation time only. A p <0.0001 was obtained for the model term sonication time. Similarly, there was also a significant effect in varying the irradiation time, with p = 0.0101 while there was no significant effect gathered from the microwave power variations. This general factorial analysis also showed that there is a significant interaction between sonication and irradiation time only, with a p <0.0001. The highest AG yield for UMAE was obtained at sonication time of 10 min and an irradiation time of also 10 min with a microwave power of 280 W.

Table 1. ANOVA result of the effect parameters varied in ultrasound-assisted extraction, microwave-assisted extraction and ultrasound-assisted extraction on the Andrographolide yield.

Source	Degrees of Freedom	F-Value	P-Value	
aUAE				
Sonication Time	2	204.88	0.0049	Significant
aMAE				
Irradiation time	2	4181.99	< 0.0001	Significant
Microwave power	2	346.77	< 0.0001	Significant
bUMAE				
Sonication time	2	133.91	< 0.0001	Significant
Irradiation time	2	9.00	< 0.0001	Significant

a- One-Way ANOVA b- ANOVA of the general factorial model

Bioactivity Assessment of Sinta extract obtained via Ultrasound microwave-assisted extraction

The *Sinta* extract obtained from UMAE was subjected to rotatory evaporation to remove the solvent. The dried *Sinta* extract was tested for cytotoxicity activity through brine shrimp lethality test (**Figure 5**).

The probit analysis revealed that the LC_{50} of the UMAE extract of *Sinta* was at 76.02 mg L^{-1} (**Table 2**). This is lower than the LC_{50} reported by *Ayesha et al*. (*2015*) with 600 mg L^{-1} after conducting a cytotoxicity assessment of *Sinta* ethanolic extract via Soxhlet. The cytotoxic activity of the *Sinta* extract obtained from UMAE can be attributed to the presence of bioactive compounds such as AG; and has potential anti-tumor, anti-cancer and/or pesticide properties (*Okhuarobo et al. 2014*). Furthermore, compounds that possess brine shrimp toxicity, in general, also have cytotoxic properties against cells of solid tumors found in humans. Also, *Sinta* ethanolic extract containing AG obtained via maceration showed a biologically active response to the brine shrimp lethality test (*Moazzem et al. 2014*).

The antibacterial sensitivity of the *Sinta* extract containing AG from UMAE was determined using *Escherichia coli, Bacillus clausii* and *Klebsiella* spp. in Mueller Hinton agar (**Table 3**). The *Sinta* extract containing AG from UMAE was of intermediate susceptibility to *Escherichia coli* as its zone of inhibition was within the range 15-20 mm, while it is resistant to both *Bacillus clausii* and *Klebsiella* spp. This means that the *Sinta* extract via UMAE contains bioactive compound with antibacterial activity for the *Escherichia coli* only. More variation of the parameters used in the extraction method can be used in future studies to obtain the optimum yield of AG. Further

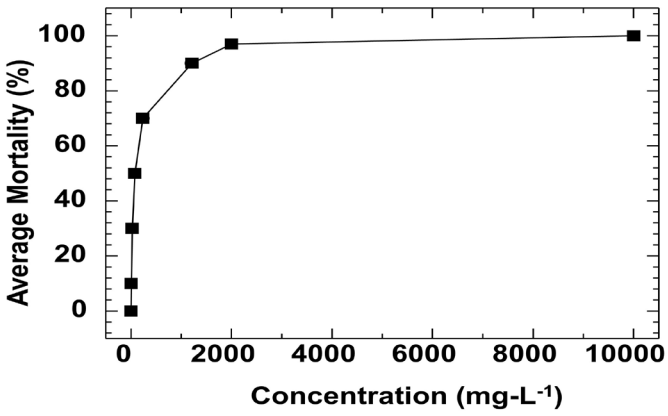

Figure 5. Cytotoxic activity of *Sinta* extract from Ultrasound microwave-assisted extraction at different concentrations of brine solution.

Table 2. Probit Analysis for the LD_{50} or LC_{50} calculation.

Lethal Dose/ Lethal Concentration	LC_{10}	LC_{30}	LC_{50}	LC_{70}	LC_{90}
Concentration (mg L^{-1})	4.772	24.488	76.016	235.968	1211.009

Table 3. Zone of Inhibition of *Sinta* extract from Ultrasound microwave-assisted extraction and Streptomycin against *Escherichia coli, Bacillus clausii* and *Klebsiella* spp.

Organism	Zone of Inhibition (mm)			Streptomycin (Positive Control)
	Trial 1	Trial 2	Trial 3	
Escherichia coli	16	18	18.52	38.01 mm
Bacillus clausii	12.7	10	9	30.00 mm
Klebsiella spp.	0.00	0.00	0.00	27.0 mm

research and compound isolation and identification are also necessary. Other antimicrobial properties can also be studied for future research to fully utilize the beneficial effects of *Sinta*.

CONCLUSION AND RECOMMENDATIONS

The absorption peaks detected by UV-Vis Spectrophotometer during HPLC analysis at 38 s retention time showed the presence of AG in the extracts via UAE, MAE, and UMAE. The UMAE showed the highest AG yield as compared to Soxhlet, UAE, and MAE due to the sequential effect of ultrasound and microwave irradiation. There was a significant concentration yield difference for the sonication times for UAE in which the best time was at 10 min. Similarly, there was a significant difference in varying the irradiation time and microwave power; and with significant interaction with each other for MAE. Also, it was identified that the highest AG concentration was extracted at an irradiation time of 5 min and 420 W microwave power. Consequently, there was a significant AG yield difference in the variation of both sonication and irradiation time and no significant effect from the variation of microwave power for UMAE. The highest AG yield was obtained at a sonication time of 10 min and an irradiation time also of 10 min.

The deaths of the brine shrimp during cell toxicity testing with an LC_{50} value of 76.02 mg L^{-1} using *Sinta* extract suggests potential antitumor, anticancer and/or pesticide properties of the extract. Also, the *Sinta* extract produced using UMAE is of intermediate susceptibility to *Escherichia coli* but it is resistant to both *Bacillus clausii* and *Klebsiella* spp.

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