Identification of DNA barcode sequences, chemical composition and antibacterial activities from ethanol extracts of *Kaempferia champasakensis* Picheans. and Koonterm (Zingiberaceae) in Vietnam

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Abstract

Kaempferia is a genus which contains the medicinal plants of the Zingiberaceae family, and Kaempferia champasakensis has been recently recorded for the flora of Vietnam. However, there is a little of information about DNA barcode, chemical composition and bioactivity of this species. In this study, we have successfully amplified and sequenced ITS and trnL-F regions of K. champasakensis. Furthermore, chemical compositions of ethanol extracts from leaves and rhizomes of K. champasakensis also have been elucidated via liquid chromatography-mass spectrometry. We also determined antibacterial activity of ethanol extracts using the disk diffusion method. The results showed that there were differences in ITS and trnL-F sequences between K. champasakensis and K. angustifolia; two species are similar in morphological characteristics.

Furthermore, six compounds in ethanol extracts of K. champasakensis rhizomes and leaves were determined including Kaempulchraol A, C, G, I, L and M. Additionally, the ethanol extracts could inhibit the growth of 6 tested bacteria including Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella enteritidis, and Samonela typhimurium.

Keywords*: Kaempferia champasakensis*, ethanol extract, chemical composition, antibacterial activities.

Introduction

Kaempferia genus consists of 60 species occurring primarily in India, South China and Malaysia[1]. In Vietnam, the genus was known to include ten species: *K. galanga, K. marginata, K. fallax, K. fissa, K. angustifolia, K. cochichinensis, K. elegans, K. candida, K. harmandiana,* and *K. pulchra*[2-4]. *Kaempferia* is a genus which contains the medicinal plants of the Zingiberaceae family and has been used extensively in traditional Vietnamense medicine and other countries. Several studies have been researched about the phytochemical constituents and biological activities such as antimicrobial and antioxidant activities of bioactive compounds isolated from leaves and rhizomes of several species of this genus[5-10].

Kaempferia champasakensis Picheans. and Koonterm were described by Picheansoonthon and Koonterm.[11] The type specimens were collected in Ban Lad Suea, Xanasomboon Town, Champasak Province, Lao PDR. Recently, Nguyen et al[3] and Tran et al[4] recorded *K. champasakensis* for the flora of Vietnam, whose distributions were identified in Binh Chau–Phuoc Buu Nature Reserve, Ba Ria-Vung Tau Province and Tanh Linh District, Binh Thuan Province, Vietnam.

During a field trip to the Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng ward, Xuyen Moc District, Ba Ria-Vung Tau Province in 2019, we encountered a flowering population of *Kaempferia* species. By comparison between its morphological attributes and those of reference species, it can be indicated that the *Kaempferia* species is *K. champasakensis.*

The ITS and *trn*L-F regions were previously used as molecular markers for DNA barcoding of considerable number of species belonging to the family Zingiberaceae and genus *Kaempferia*[12-15]. However, there is no information on DNA barcoding of *K. champasakensis*. In present study, ITS and *trn*L-F were used for DNA barcoding and phylogenetic tree construction.

Furthermore, we determined the phytochemical composition and antibacterial activity of ethanol extract from rhizomes and leaves of *K. champasakensis* and provided more information for further application of this species.

Material and Methods

Plant material: The fresh of rhizomes and leaves of *K. champasakensis* were collected from from Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng ward, Xuyen Moc District, Ba Ria–Vung Tau Province, location of about $10^0$36’18”N; $107^0$30’43”E, 25th August 2019, 31 m in elevation (Figure 1).

Additionally, we used the ITS and *trn*L-*trn*F sequences from Genbank database which were presented in table 1 to determine the taxonomic relationship of some species belonging to the *Kaempferia* genus in this study.

Bacterial strains: The antibacterial activity of ethanol extracts was evaluated by six bacterial strains comprised of two gram-positive bacteria, *Bacillus cereus* (ATCC 11774) and *Staphylococcus aureus* (ATCC 25923), and four gram-negative bacteria, *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enteritidis* (ATCC 13976) and *Samonela typhimurium (ATCC 13311)*. These bacterial strains were kindly provided by Department of Biotechnology, Institute of Food and Biotechnology, Industrial University of Ho Chi Minh city, Viet Nam. All bacterial strains were maintained at -20^{o}C in 20% glycerol solution and inoculated into Luria-Bertani broth at 37^{o}C for 24h to be re-activated again before using in further experiments.

Total genomic DNA extraction and PCR amplification: DNA samples were obtained from fresh leaf material using Gene Jet Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA) following the manufacture's instructions. Amplification of the ITS and trnL-F regions was performed using the primers following White et al[16] and Taberlet et al[17] including ITS region (forward: 5'TCCGTAGGTGAACCTGCGG3', reverse: 5'TCCTCCGCTTATTGATATGC3'), and trnL-F region (forward: 5'CGAAATCGGTAGACGCTACG3', reverse: 5'ATTTGAACTGGTGACACGAG3'). The PCR reactions were performed in an Mastercycler machine (Eppendorf, Germany) using a volume of 25µl reaction mixture: 12.5µl Go-Taq green master mix (Promega, USA), 1.25µl of each forward and reverse primers (10 µM), 9.5µl nuclease-free deionized water and 0.5µl DNA template (25µg/ml).

The PCR program consisted of 3 min at 95°C; 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 55^{o}C) and extension (2 min at 72°C); and a final extension at 72°C for 10 min. The PCR products were purified and sequencing using ABI 3130 XL Sequencer.

Sequencing data analysis: For multiple alignments, the ClustalW[18] was used to recognize the homology between sequences. Phylogenetic trees were generated using PAUP*4.0a146[19] with *Alpinia galanga* as outgroup[20]. Bootstrap values of 50% or higher were performed to obtain cluster supports.

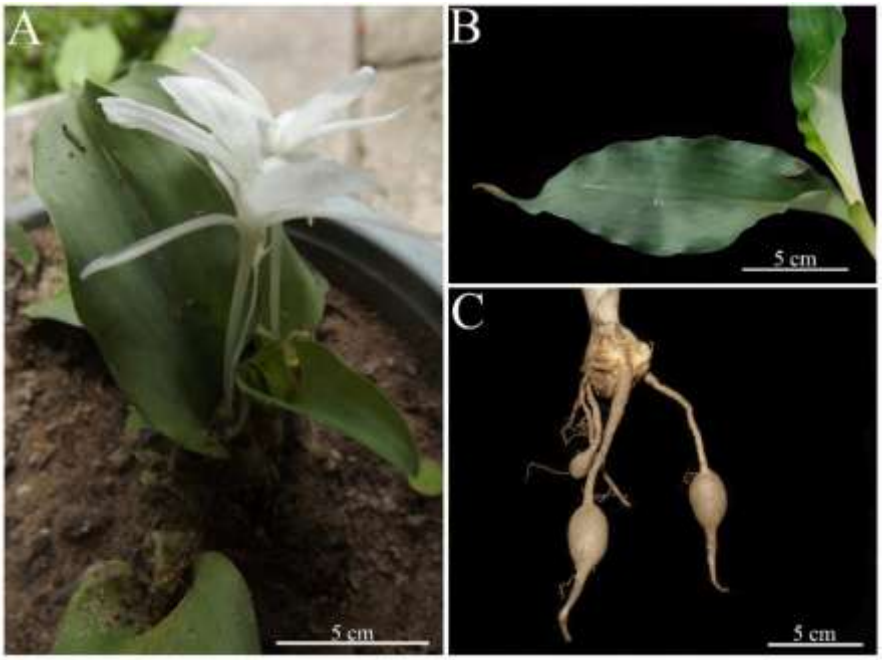

Figure 1: *K. champasakensis.* A. Habitat, B. Leaf. C. Rhizome.

Table 1
Sequences of eight *Kaempferia* species and *Alpinia galangal* from GenBank database used in this study

Taxa	Accession number (ITS/*trn*L-*trn*F)	Taxa	Accession number (ITS/*trn*L-*trn*F)
K. galangal	KM887413/KX559399	*K. elegans*	AY424764/AY424790
K. angustifolia	AB552949/KX559397	*K. pulchra*	KY454697/KX559404
K. parviflora	KY701332/KX559406	*K. marginata*	KY454700/KX559401
K. roscoeana	AJ388290/KX559407	*Alpinia galangal*	AF478715/AY424775
K. rotunda	AF478767/AY424791		

Extraction procedure: Fresh sliced rhizomes and leaves of *K. champasakensis* were dried at 50^0C until constant weight and ground into medicinal powder. 100g of the resulting powder was soaked in 1000 ml of 99% ethanol for 4 weeks at room temperature and filtered to collect the extract. The extract was then condensed under vacuum pressure at 60^0C to obtain the brown ethanolic paste[21]. To ensure the absolute absence of ethanol in the paste, sublimation dryer was utilized.

Liquid chromatography mass spectrometry (LC/MS): For determination of the chemical components in the ethanol extracts, LC-MS analysis was performed at the Central Laboratory for Analysis, University of Science, Vietnam National University of Ho Chi Minh City. An aliquot of ethanol extract was injected into HPLC Agilent 1200 infinity liquid chromatography system (Agilent Technologies, CA, USA) coupled with MicroTOF-QII mass spectrometer (Bruker Daltonics, Germany). The chromatographic separation was accomplished using an ACE3- C_{18} analytical column (4.6 ×150mm, 3.5 µm) as stationary phase at 40°C. The mobile phase used consisted of deionized water with 0.1% formic acid and acetonitrile (solvent B) with 0.1% formic acid (solvent A), 0.3mL/min flow rate.

The gradient elution program was summarized in table 2. In mass spectrometer, the extract was then ionized using electrospray ionization source (ESI) at positive mode and the mass spectra data were recorded on mode for a mass range 50-2000 m/z. Data Analysis software (Bruker, Germany) was used for data analysis.

Table 2
Gradient elution program for the chromatographic separation

Time (min)	Solvent A*	Solvent B*
0	90	10
15	0	100
30	0	100
31	90	10
40	90	10

(*): presented as the percentage of volume of mobile phase

Antibacterial activities: The method by Bauer et al[22] was used to study the antibacterial activity of the ethanol extract from rhizomes and leaves of *K. champasakensis*. Briefly, bacteria were inoculated into LB Broth until the turbidity of 0.5 McFarland standards. 100 µl of culture was then spread on the surface of Mueller Hinton plate and sterile 6mm diameter discs were subsequently placed on.

Each disc was added with 15 µl of ethanol extract and the plates were maintained at 4°C for 2 hours for the extract diffusing into the medium. The plate was incubated at 37°C for 24h and the diameter of the zone of inhibition of tested bacteria was determined. Sterilize distilled water and Gentamycin antibiotic discs (Nam Khoa BioTek, Viet Nam) were used as negative and positive control respectively. The average and standard deviation of measurements were calculated using the Excel 2010 software. The data of experiments were expressed as mean ± standard deviation (SD).

Results and Discussion

Molecular phylogenetic analysis: The PCR products of *trn*L-F and ITS sequences of the studied sample were clear bands about 1000 bps and 700 bps respectively (Figure 2). These bands had the similar sizes with predicted bands of White et al[16] and Taberlet et al[17] works in which the authors used the same primers. The PCR products were subsequently purified and analyzed for sequences. The ITS and trnL-F sequences of *K. champasakensis* after editing had the sizes about 643 and 777 bps respectively. These sequences were submitted to GenBank and the accession numbers of ITS and trnL-F sequences were MN905561 and MN883827 respectively.

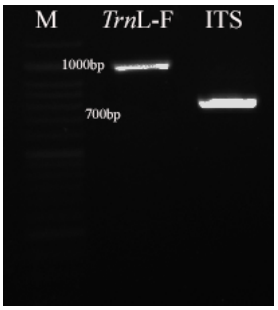

Figure 2: PCR amplification result of trnL-F and ITS regions of studied sample. M: ladder

To date, most of studies about *trn*L-F region of species of the *Kaempferia* genus in GenBank have focused into the *trn*L-*trn*F IGS, a fragment of *trn*L-F IGS with the length about 400 bps, whereas we analyzed *trn*L-F sequence with the length about 1000 bps in this study. Therefore, we only aligned *trn*L-*trn*F IGS of *K. champasakensis* with other species belonging to *Kaempferia* genus in GenBank to establish the genetic relationship of species in this study.

By using ITS and *trn*L-*trn*F IGS sequences in GenBank, the phylogenetic trees showed relationship among some species of *Kaempferia* genus (Figure 3). Of them, one species (*K. roscoeana*) grown in Thailand and Laos belongs to the white-flowered group, same group with *K. champasakensis*[11].

However, there were far distances between *K. roscoeana* and *K. champasakensis* in phylogenetic trees presented in figure 3A and 3B which demonstrate that they have a long distance in genetic relationship, especially in ITS and *trn*L-F sequences. Other species also belong to the white-flowered group in *K. angustifolia*, which grows in Southern region of Vietnam and has similar morphological characteristics with *K. champasakensis*[2,3].

In figure 3A and 3B, *K. champasakensis* and *K. angustifolia* were classified in the same group with the bootstrap values about 100% and 92% (ITS and *trn*L-*trn*F IGS sequences respectively). The results showed that the distances in genetic relationship (ITS and *trn*L-*trn*F IGS sequences) between *K. champasakensis* and *K. angustifolia* were smallest. We subsequently performed comparison of ITS and *trn*L-*trn*F IGS sequences of *K. champasakensis* and *K. angustifolia* using Bioedit software and global alignment technique.

Alignment results elucidated that there were 15 differences between *K. champasakensis* and *K. angustifolia* in ITS sequence (Table 3) whereas there were only 2 differences (similarity about 99%) at position 208 (Thymine) and position 419 (Thymine) in *trn*L-*trn*F IGS sequence of *K. champasakensis* as compared to those of *K. angustifolia*, both of them were Cytosine. Recently, several studies demonstrated the important role of DNA barcodes on facilitation for classification and establishment of revolution system of the Zingiberaceae family and the *Kaempferia* genus.

Kress et al[20] established the phylogenetic trees and re-classified the taxonomic relationship of some species of for 4 tribes of the Zingiberaceae family including *Globbeae, Hedychieae, Alpinieae, and Zingibereae*, using molecular analysis of ITS và matK sequences. Moreover, Techaprasan et al[12] used psbA-trnH *and* petA-psbJ sequences to determine the genetic diversity of some *Kaempferia* species in Thailand. Recently, Labrooy et al[14] also compared the morphological characteristics and ITS sequences of some *Kaempferia* species in Malaysia.

Ethanol extract composition: Based on comparison of mass of compounds (m/z) identified in other *Kaempferia* species, we determined 6 compounds belonging to Kaempulchraol group in rhizomes and leaves of *K. champasakensis* including Kaempulchraol A, C, G, I, L and M. Among them, Kaempulchraol C, G, I, L and M were only found in ethanol extract of rhizomes whereas Kaempulchraol A was found in both rhizomes and leaves of *K. champasakensis* (Table 4 and figure 4). Numerous bioactivities of extracts of plant species of the *Kaempferia* genus such as anti-cancer, anti-inflammatory, anti-allergenic, anti-bacterial activity, neuroprotective effects, and enhancement of wound healing have been documented[10].

Furthermore, some bioactive compounds which we identified in this study also have some pharmaceutical benefits. For example, Kaempulchraol G can inhibit the expression of viral protein R, an accessory protein of HIV which plays important role in controlling viral replication and pathogenesis[6].

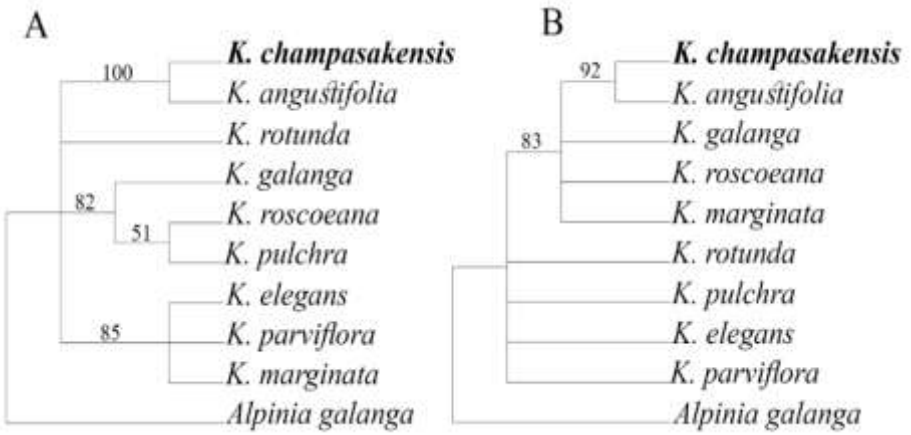

Figure 3: One of most-parsimonious tree obtained based on ITS region (A) and *trn*L-F region (B). Gaps treated as missing data. The bootstrap values of 50% or more than from 1000 replicates are shown above the nodes.

Table 3
The pairwise alignment of ITS region between *K. champasakensis* and *K. angustifolia.*

Sample	Positions								Differential nucleotides	Similarity (%)
	12	44	50	86	90	100	131	158	15	93
K. champasakensis	T	T	C	G	C	T	G	C		
K. angustifolia	G	C	T	A	A	C	A	G		
	Positions									
	163	388	415	421	460	462	457			
K. champasakensis	C	C	T	G	G	A	A			
K. angustifolia	T	A	C	C	C	G	G			

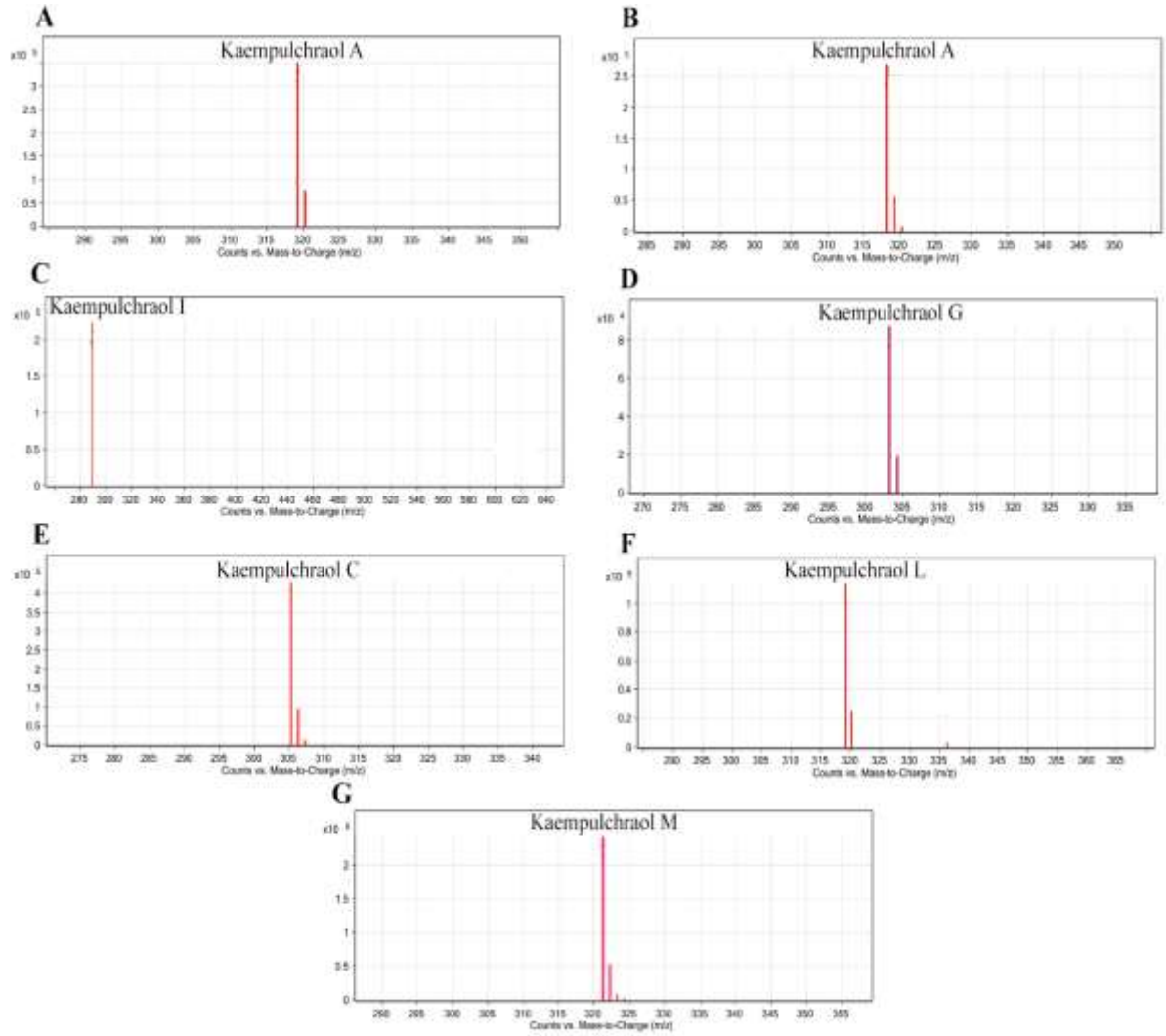

Figure 4: Mass spectrometry diagrams of 6 compounds of ethanol extracts of leaf and rhizome of *K. champasakensis*. A, C, D, E, F, G are compounds of rhizomes; B is compounds of leaf.

Table 4
Phytochemical composition of ethanol extracts of *K. champasakensis* rhizomes and leaves

Compounds		m/z	References
Rhizomes	Leaves		
Kaempulchraol I	-	288	23
Kaempulchraol G	-	302	24
Kaempulchraol C	-	305	24
Kaempulchraol A	Kaempulchraol A	318	24
Kaempulchraol L	-	318	25
Kaempulchraol M	-	321	25

Furthermore, Kaempulchraol I and L were proved as anticancer agents against the human cervical cancer (Hela cell line) and mouth squamous cell carcinoma (HSC-2 cell line). The presence of these bioactive compounds suggests the ethanol extracts of *K. champasakensis* as new resources for isolation of anticancer and antiviral agents as well as application of these extracts in ethnomedicine[9].

Antibacterial activity: The antibacterial activity of ethanol extracts of *K. champasakensis* rhizomes and leaves was presented in table 5 and figure 5 and the ethanol extract of *K. champasakensis* rhizomes exhibited stronger antibacterial activity as compared to that of *K. champasakensis* leaves. Accordingly, the diameter of the zone of inhibition of ethanol extract of *K. champasakensis* rhizomes against *S. enteritidis* was 40.3 ± 1.5 mm followed by *P. aeruginosa* (37.0 ± 2.6 mm), *E. coli* (32.3 ± 2.5 mm), *B. cereus* (28.3 ± 1.5 mm), *S. typhimurium* (16.0 ± 2.0 mm), and *S. aureus* (7.6 ± 1.2mm).

On the other hand, the ethanol extract of *K. champasakensis* leaves exhibited a strong antibacterial activity against *B. cereus* and *E. coli* with the diameters of the zone of inhibition about 16.3 ± 1.5 mm và 16.0 ± 2.6 mm respectively followed by *S. typhimurium* (13.3 ± 0.6 mm), *P. aeruginosa* (12.6 ± 2.5mm), *S. enteritidis (*11.6 ± 2.1 mm), and *S. aureus* (11.6 ± 2.1 mm). These results demonstrated that the antibacterial activity of ethanol extract of *K. champasakensis* leaves was weaker than that of *K. champasakensis* rhizomes (Table 5).

Table 5
The inhibition zone of ethanol extracts of *K. champasakensis* rhizomes and leaves against five tested bacteria.

Tested bacteria	**Growth inhibition zone (mm)**	
	Rhizomes	**Leaves**
Bacillus cereus	28.3±1.5	16.3±1.5
Escherichia coli	32.3±2.5	16.0±2.6
Pseudomonas aeruginosa	37.0±2.6	12.6±2.5
Samonela enteritidis	40.3±1.5	11.6±2.1
Samonela typhimurium	16.0±2.0	13.3±0.6
Staphylococcus aureus	7.6±1.2	11.6±2.1

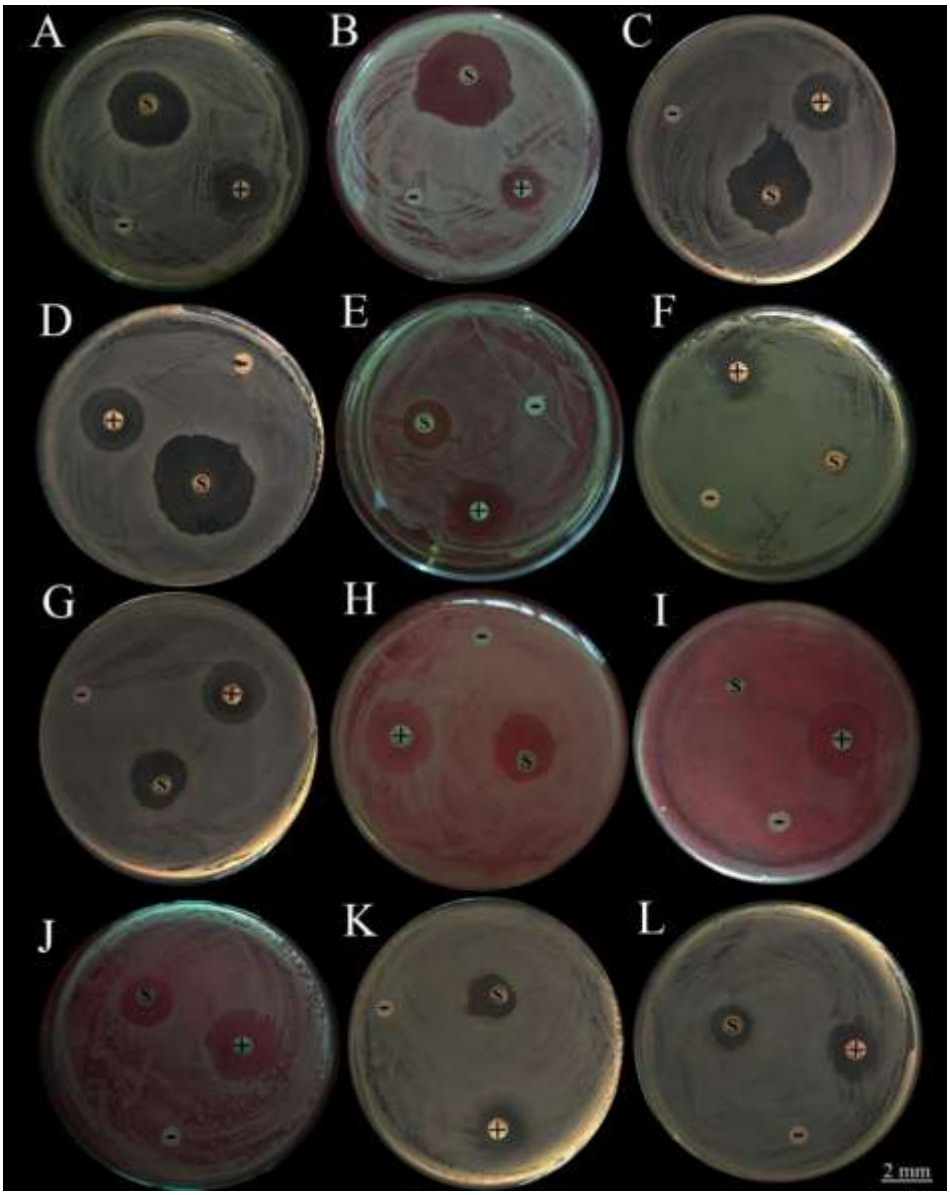

Figure 5: Antibacterial activities of ethanol extracts of *K. champasakensis* rhizomes and leaves against tested bacteria. Rhizome: A-*B. cereus*, B-*E. coli*, C-*P. aeruginosa*, D-*S. enteritidis*, E-*S. typhimurium*, F-*S. aureus*. Leaves: G- *B. cereus*, H- *E. coli*, I- *P. aeruginosa*, J- *S. enteritidis*, *K-S. typhimurium*, L-*S. aureus*. (-) Negative control with sterilized distilled water, (+) Positive control with discs containing gentamicin, (S) sample of ethanol extract.

Both ethanol extracts of *K. champasakensis* leaves and rhizomes could inhibit the growth of 6 tested bacteria, especially the extract of *K. champasakensis* rhizomes. These results were in line with the previous studies in which the antibacterial activities of some species of the *Kaempferia genus* were elucidated. For example, acetone, petroleum ether, chloroform, and methanol extracts of rhizomes of *K. galangal, K. roscoeana, K. elegans* could hinder the growth of some pathogenic gram positive and negative bacteria[5,7,8]. In previous study, Kumme et al[26] proved that ethanol extract of *K. parviflora* rhizomes could inhibit some pathogenic fungal strains, such as Trichophyton rubrum, *Trichophyton mentagrophytes* and *Microsporum gypseum.*

Additionally, antimicrobial activity of methanol extract of *Kaempferia angustifolia* rhizomes against *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella choleraesuis* and *Bacillus subtilis, Candida albicans, Saccharomyces cerevisiae* and *Aspergillus ochraceus* also has been elucidated[27]. Recently, Jeong et al[28] suggested that ethanol extract *K. parviflora* exhibited a strong antimicrobial activity against *E. coli và Cronobacter* spp.

Conclusion

In this study, the ITS and trnL-F regions of *K. champasakensis* have successfully sequenced. Moreover, the results of phylogenetic tree revealed the relationship and differences of ITS and trnL-F sequences between *K. champasakensis* and *K. angustifolia*, both of this spieces have similar morphological and genetic characteristics. Chemical constituents of ethanol extracts of *K. champasakensis* rhizomes and leaves were also determined including Kaempulchraol A, C, G, I, L and M. Ethanol extracts of *K. champasakensis* exhibited a strong antibacterial activity against 6 tested bacteria such as *B. cereus, S. aureus, E. coli, P. aeruginosa, S. enteritidis* and *S. typhimurium.* These findings suggest ethanol extracts of *K. champasakensis* as the potential resource to isolate the bioactive compounds and antibacterial agents for application in ethnomedicine.

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