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Antimicrobial Evaluation and GC-MS Analysis of *Strobilanthes crispus* Ethanolic Leaf Extract

Vuanghao Lim[1*], Chuan Seng Yap[2], Hui Wen Chong[1], Mohamed Saleem Abdul Shukkoor[2] and Madhavan Priya[3]

[1]Integrative Medicine Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam 13200 Kepala Batas, Pulau Pinang, Malaysia.
[2]Department of Pharmaceutical Biology, Faculty of Pharmaceutical Sciences, UCSI University, 56000 Kuala Lumpur, Malaysia.
[3]School of Medicine, Taylor's Lakeside Campus, Taylors University, 47500 Subang Jaya, Selangor, Malaysia.

Authors' contributions

This work was carried out in collaboration between all authors. Authors VL and MP designed the study. Authors CSY and HWC performed the statistical analysis. Authors VL and MP wrote the protocol and authors VL and CSY wrote the first draft of the manuscript. Author MSAS managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to assess the potential antimicrobial activities of the ethanolic leaf extract of *Strobilanthes crispus* (*S. crispus*) by determining the susceptibilities of various strains of microbes to the extract and to profile the bioactive compounds in the extract using GC-MS.
Study Design: *In vitro* assays, chromatography and spectrometry analysis.
Place and Duration of Study: This study was carried out at Integrative Medicine Cluster Lab, AMDI, USM and Faculty of Pharmaceutical Sciences, UCSI University, from July 2012 to December 2013.
Methodology: Anti-microbial activity was assessed using disc diffusion on *Aspergillus brasiliensis*,

**Corresponding author: E-mail: vlim@usm.my;*

Candida albicans, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumonia.* GC-MS analysis was used to profile the bioactive components of the plant part.
Results: The extract exhibited inhibitory activity against *Staphylococcus aureus* and *Streptococcus pneumoniae* at 200 mg/ml concentration, whereas no visible inhibition was observed against *Aspergillus brasiliensis*, *Candida albicans*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. GC-MS analysis of the ethanolic extract revealed the presence of few constituents which include acetic acid, butyrolactone, and hexanedioic acid that have been described in the literature as proven antimicrobial agents.
Conclusion: These results suggest that the ethanolic leaf extract of *Strobilanthes crispus* (*S. crispus*) can be used as a nutraceutical against *S. aureus* and *S. pneumoniae*.

Keywords: Strobilanthes crispus; GC-MS; ethanol extract; antimicrobial; disc diffusion.

1. INTRODUCTION

The search for new antimicrobial agents from medicinal plant products has been intense in recent decades [1-2], in part due to the emergence of antibiotic-resistant pathogens and the consequent need for new antimicrobial agents [2-3]. Plants produce many aromatic secondary metabolites that serve as natural defence mechanisms against pathogens. *Strobilanthes crispus* (*S. crispus*) is a traditional medicinal plant used by Sundanese villagers in west java to treat hepatitis and as a postpartum remedy [4]. Its leaf extracts have been reported to have high antioxidant activity [5-6] as well as anticancer [7-11] and antidiabetic activities [12]. Enhanced wound healing and reduced hepatocarcinogenesis properties of *S. crispus* extracts also have been reported [12-17]. To date, limited studies of the antimicrobial activity of *S. crispus* leaf extracts have been conducted, despite reports that these extracts have high antioxidant activity, which correlates well with toxicity [18] against microorganisms [1]. Hence, the aims of this study were to evaluate the effectiveness of *S. crispus* ethanolic leaf extract against selected strains of bacteria, fungus, and yeast and to identify the active constituents of the extract using gas chromatography-mass spectrometry (GC-MS).

2. EXPERIMENTAL DETAILS

2.1 Plant Materials

The plant, *S. crispus was* authenticated by Mr Shanmugam, from Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia (Voucher No. SK 1980/11) and three kilograms of leaves were dried under natural shade, were bought from Yik Poh Ling Herbal Farm, Seremban, Malaysia. The *S. crispus* leaves were manually separated from stalks and pulverised into dried powder using a miller.

2.2 Extraction Procedure

The powdered dried leaves were macerated with 95.0% ethanol in conical flasks at a ratio of 2 to 8 (200 gram of dried leaves per 800 mL of ethanol). The dried leaves were macerated for 4 d at room temperature (25±2℃) before the mixture was filtered. This extraction process was repeated until further macerations yielded transparent filtrates. Filtrates were collected and dried at 40ºC using a rotary evaporator (BUCHI Rotavapor R-200, Flawil, Switzerland) to evaporate off the solvent, leaving the dried crude extract. The dried crude extract was placed in a desiccator tank along with silica desiccant to absorb any remaining moisture. Throughout the extraction process, the containers were covered with aluminium foil to minimise photo-degradation of the extracts.

2.3 Test Microorganisms

All microorganisms used in this study were from the American Type Culture Collection (ATCC), Manassas, Virginia, US and purchased from Bioscientific Company, Kirrawee, Australia. Strains of bacteria used in this study were *Klebsiella pneumoniae* (ATCC 70063), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213), and *Streptococcus pneumoniae* (ATCC 49619). The fungi and yeast tested were *Aspergillus brasiliensis* (ATCC 16404) and *Candida albicans* (ATCC 90028) respectively. The cultures were stored in glycerol [20% (v/v)] stocks at -20℃ prior to reconstitution.

2.4 Inoculum Preparation

The concentration of each bacterial inoculum was standardised to 1-2 x 10^8 colony forming units (CFU) per mL [19-20]. A recommended method to achieve this concentration is to use McFarland standard as a reference to compare bacterial densities in a liquid medium [21]. A 0.5 McFarland standard is comparable to 1.5 x 10^8 CFU/mL of bacteria, 1-5 x 10^6 CFU/mL of *Candida* spp., or 0.4-5 x 10^6 CFU/mL of *Aspergillus* spp.

2.5 Antimicrobial Activity (Disc Diffusion Assay)

To prepare the *S. crispus* leaf extract for use in experiments, 1.6 g of the dried extract were carefully transferred into a new container and reconstituted with 5% (v/v) sterile dimethyl sulfoxide (DMSO) to make a stock solution of 100 mg/mL plant extract. The dried leaf extract of *S. crispus* was reconstituted to various concentrations (0.01, 0.1, 1, 10, 100, 150, 200, and 250 mg/mL) and 20 µL of each of these concentration was pipetted into blank sterile discs in petri-dishes and were allowed to dry. The dried discs were then used to determine antimicrobial activity of the plant part for the antimicrobial screening test. The *S. crispus* ethanolic leaf extracts were filtered using filter paper (Double Ring number 102, Fujian, China) to remove insoluble materials. After reconstitution, the resulting solutions were filtered to remove any undissolved extracts. The universal bottle with the stock solution was sealed with airtight paraffin film and stored in refrigerator at 4℃. A blank disc was treated with 10% (v/v) DMSO and placed on each agar medium as a negative control. Oxacillin was used as the positive control. Diameter of the resulting zone of inhibition was measured and the average values were recorded.

2.6 GC-MS Analysis

For GC-MS analysis, 1 µg of plant extract was dissolved in 10 mL of ethanol (HPLC grade). The mixture was sonicated for 2 minutes and centrifuged at 5000 g for 10 min. The supernatant was filtered using microfiltration syringe. The filtered supernatant was collected inside vial chamber. The injected volume was 1 µL per sample. GC-MS analysis was performed using an Agilent 6890 system (Santa Clara, CA, US) equipped with a fused silica capillary column (60 m x 0.25 mm, i.d.) coated with HP 5 methysilicone (film thickness 0.25 µm) [Scientific Glass Engineering, Melbourne, Australia]. The operating conditions were as follows: injector temperature, 250℃; carrier gas, helium; column flow rate, 0.9 mL/min; splitting ratio, 10:1; injection volume, 2 µL. Oven temperature was programmed from 70℃ to 320℃ at 22.5℃/min. Total ion chromatograms (TICs) and mass spectra were recorded using an Agilent Network 5973N mass engine with enhanced chemstation in the electron impact ionisation mode at 2200 eV. The transfer line was maintained at 280ºC, the source temperature was 230℃ (maximum 250℃), and the quadrupole temperature was 150℃ (maximum 200℃). The bioactive components were identified by comparing the mass spectrum NIST mass spectral library.

2.7 Statistical Analysis

Each experiment was run in triplicate. The zones of inhibition for each test were recorded and tabulated as mean ± standard error of the mean (SEM). Graphpad Prism 5 (San Diego, CA, USA) was used to statistically analyse the results using one-way analysis of variance (ANOVA), with Tukey's post-hoc multiple comparison test to identify any differences between samples. Results with $P < 0.05$ were considered to be statistically significant. ANOVA was previously used to compare significance of the antimicrobial activities of crude plant extracts in other studies [22-24].

3. RESULTS AND DISCUSSION

3.1 Extraction and Antimicrobial Activity

The comprehensive ethanolic leaf extract obtained was 9.6% in powdery form. Ethanol provides a particularly effective way of maximising the bioavailability of the actives extracted from the plant. In antimicrobial study, in comparison with the oxacillin (1 µg per disc) positive control, the relative antimicrobial effect of *S. crispus* ethanolic leaf extracts against *S. aureus* increased as the concentration of extract increased. The maximum antimicrobial action of the extract was 64.40% that of oxacillin (1 µg/ml), which illustrates the potential of this extract as an antimicrobial agent against *S. aureus* (Table 1). A previous study reported that *S. crispus* aqueous extracts did not have any antimicrobial activity against *S. aureus* [25]. This suggests that the active antimicrobial agent is not very

hydrophilic; instead, it is considerably lipophilic in nature. A previous study reported that verbascoside was the active antimicrobial agent of semi-purified *S. crispus* butanolic leaf extract [26-27].

S. pneumonia was also susceptible to *S. crispus* extracts. Both *S. aureus* and *S. pneumoniae* are Gram-positive bacteria. Another Gram-positive bacterium, *Bacillus cereus*, was also found to be susceptible to methanolic *S. crispus* leaf extracts in a recent study [28]. Furthermore, two species of bacteria from the *Streptococcus* genus, *Streptococcus sobrinus* and *Streptococcus mutans*, were reported to be susceptible to *S. crispus* leaf extract [25]. Additional studies reported that Gram-positive bacteria were more vulnerable than Gram-negative bacteria to the tested plant extracts [29-31]. Gram-negative bacteria generally have higher resistance due to the lipopolysacharide component of their outer membrane, which selectively prevents entry of hydrophilic solutes of 600 Da or larger and lipophilic solutes [31,32].

At 72 h of incubation, visible colonies were observed within the vicinity of the initial inhibition zones for discs impregnated with 100, 150, and 250 mg/mL of the extract (Table 2). Only one clear inhibition zone was observed, and it was from one of the discs treated with 250 mg/mL of *S. crispus* leaf extract. The lesser of effect on *S. pneumoniae* may be due to various mechanisms of resistance, such as modification of target site, decreased uptake of the antimicrobial agent, and an active efflux pump to remove the antimicrobial agent [33]. This active efflux action is the main mechanism of resistance for *S. pneumoniae* against many clinically used antibiotics.

The extract did not visibly inhibit the tested strain of *C. albicans*, even at the extract concentration of 250 mg/mL. Sainath et al. reported that the ethanolic extract of *Saraca indica* stem barks did not exhibit any inhibitory action against *C. albicans*, even though its aqueous and methanolic extracts had good antifungal activity against *C. albicans* [34]. In another study, aqueous and methanolic *Terminalia mollis* extracts exhibited anticandidal activity, whereas ethanolic extract was ineffective against *C. albicans* [35]. Hence, the antifungal effects of aqueous and methanolic extracts of *S. crispus* towards *C. albicans* should be explored. This is of particular interest because *S. crispus* is usually taken as a traditional medicine in the form of tea [7,12,36]. A study of the anticandidal effect of aqueous *S. crispus* leaf extract might be helpful for evaluating the potential of *S. crispus* tea as a way to prevent oral thrush.

None of the *S. crispus* ethanolic leaf extracts (0 to 250 mg/mL) had a visible inhibitory effect on the tested strain of *A. brasiliensis.* However, antifungal properties of other species of *Strobilanthes* have been reported in previous studies [22,24]. For example, Singh et al. [22] found that the petroleum ether, benzene, and chloroform extract of *Strobilanthes callosus* exhibited *in vitro* antifungal activity against *Aspergillus niger* and *Aspergillus flavus*, and the benzene extract was the most effective. The stems and roots of *Strobilanthes* spp. might be of interest, as antifungal properties have been reported previously [24]. Reneela [24] reported that the ethanolic *Strobilanthes ciliatus* stem extract resulted in an 18 mm inhibition zone for *Aspergillus* spp. and the root extract resulted in a 16 mm inhibition zone.

Table 1. Comparison of inhibitory action of *Strobilanthes crispus* (*S. crispus*) ethanolic leaf extracts and the positive control (oxacillin) used in the antimicrobial assay of selected Gram-positive bacteria

Concentration	Inhibition zone (%)					
	Oxacillin (1 µg)	50 mg/mL extract	100 mg/mL extract	150 mg/mL extract	200 mg/mL extract	250 mg/mL extract
S. aureus	100%[a]	31.91%[b]	42.87%[c]	49.40%[d]	57.85%[e]	64.40%[f]
S. pneumoniae	100%[g]	0%[h]	24.08%[i]	25.20%[i]	25.79%[i]	24.67%[i]

Results are reported as percentage of the mean diameter of the inhibition zone of the positive control (oxacillin, 1 µg per disc). Values with superscripts a–f are for the S. aureus antimicrobial assay, and values with superscripts g–i are for the S. pneumoniae antimicrobial assay. Values with different superscript letters indicate significantly different values (P< 0.05). Values with the same superscript letters indicate no significant difference (P > 0.05)

In this study, the *S. crispus* ethanolic did not visibly inhibit *K. pneumoniae*. Siew and Wong (2010) found that the aqueous *S. crispus* leaf extract did not exert any inhibitory action against *K. pneumoniae*. Reneela [24] reported that both the ethanolic stem and root extracts from *S. ciliatus* inhibited *Klebsiella sp*., although *S. aureus* was more susceptible to the extracts. Similarly, Siew and Wong [25] found that aqueous *S. crispus* leaf extracts did not inhibit *P. aeruginosa*. In contrast, Reneela [24] reported that extracts of roots and stems of *Strobilanthes spp*. may have antibacterial activity against *Pseudomonas*.

It is likely that there is a huge diversity of active antimicrobial compounds in members of the *Strobilanthes* genus and in different parts of the plants. *Pseudomonas spp*. has been known to be resistant to a wide range of antibacterial agents. Mechanisms of resistance such as mutation of the target site and production of beta-lactamase are not uncommon in *Pseudomonas spp*. [34]. These mechanisms may have contributed to the resistance of *P. aeruginosa* to our *S. crispus* ethanolic leaf extract.

3.2 GC-MS Analysis

Nineteen constituents of the *S. crispus* ethanolic extract were identified by GC-MS analysis (Fig. 1). Few identified compounds were associated with antimicrobial activity and have been reported previously. Acetic acid has antimicrobial properties and has been used as an alternative to common local antiseptics [37]. Based on the literature review, butyrolactone is active against the phytopathogenic bacteria *Erwinia carotovora*, with IC_{50} values of 5 and 4–18 µg/mL, respectively. The inhibition (IC_{50}) of streptomycin was reported to be 1.9 µg/mL, and inhibition of the germination of the dicot *Lactuca sativa* (IC_{50} of 5×10^{-5} M) was reported under the same experimental conditions [38]. Hexanedioic acid was found to inhibit growth and proliferation of both Gram-positive and Gram-negative bacteria and to have antibacterial effects on *K. pneumonia* [39]. Those identified compounds that matched 85% and above in the chromatogram are listed in Table 3.

Table 2. Zone of inhibition (mm) of *S. pneumoniae* when subjected to different concentrations of *S. crispus* ethanolic leaf extract during the initial screening test for over 120 h of incubation

Incubation period	Zone of inhibitions (mm)				
	24 h	48 h	72 h	96 h	120 h
Oxacillin (1 µg)	22.47±0.09[a]	22.43±0.12[a]	22.50±0.10[a]	22.50±0.10[a]	22.50±0.10[a]
0 mg/mL	0[b]	0[b]	0[b]	0[b]	0[b]
0.01 mg/mL	0[b]	0[b]	0[b]	0[b]	0[b]
0.1 mg/mL	0[b]	0[b]	0[b]	0[b]	0[b]
1 mg/mL	0[b]	0[b]	0[b]	0[b]	0[b]
10 mg/mL	0[b]	0[b]	0[b]	0[b]	0[b]
100 mg/mL	9.33±0.22[c]	9.27±0.34[c]	9.33±0.32[c]	9.40±0.25[c]	9.40±0.20[c]
200 mg/mL	13.00±0.26[d]	13.00±0.32[d]	12.90±0.26[d]	12.90±0.16[d]	12.90±0.26[d]
250 mg/mL	14.17±0.22[e]	14.53±0.23[e]	14.47±0.22[e]	14.50±0.20[e]	14.50±0.20[e]

Results are reported as mean ± SEM (mm) (n = 3). Values with different superscript letters indicate significantly different values (P < 0.05). Values with the same superscript letters indicate no significant difference (P > 0.05)

Table 3. Phytocomponents identified in the ethanolic extract of *S. crispus* by GC-MS

Peak	Retention time, Rt	Area (%)	Constituents
2	2.11	4.93	Histamine dichloride
3	2.26	4.63	Formic acid
4	2.45	4.93	Acetic acid
5	2.72	4.14	Glycolaldehyde
6	3.11	0.72	2-methoxy-1-propanol
9	3.83	0.65	Hexamethyl-cyclotrisiloxane,
10	4.42	1.92	4-hydroxy-4-methyl- 2-pentanone
13	12.01	14.35	Butyrolactone
14	12.27	1.80	3-cyclohexene-1-carboxylic acid
15	13.53	4.71	Acetic acid
16	18.35	5.23	Octamethyl-cyclotetrasiloxane
18	21.67	4.31	Hexanedioic acid
19	24.94	1.65	Benzoic acid

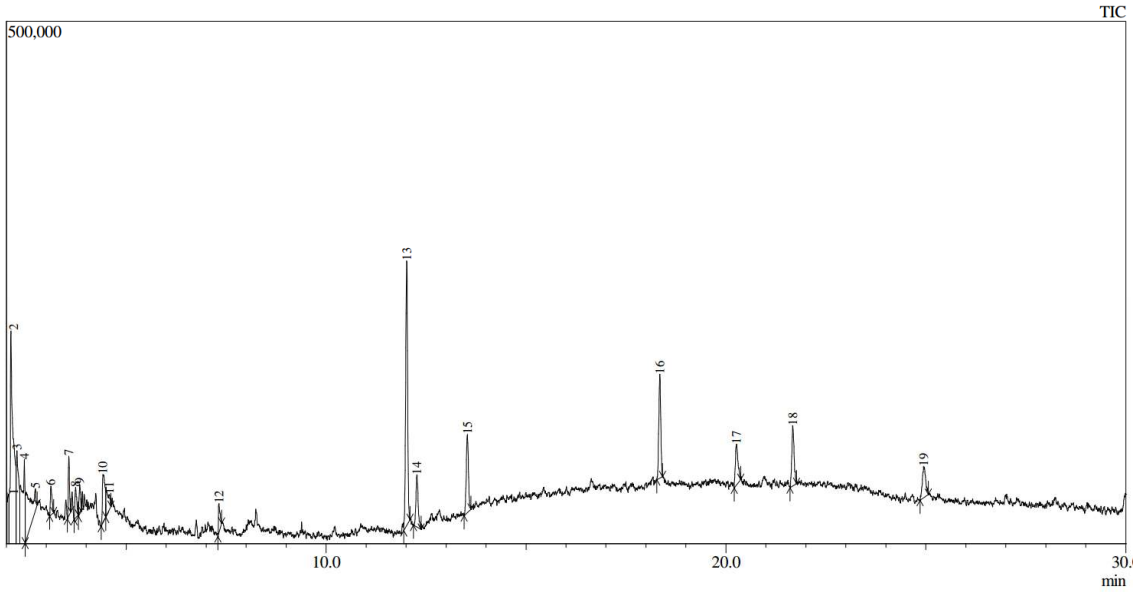

Fig. 1. GC-MS chromatogram of the *S. crispus* ethanolic extract

4. CONCLUSION

In the current investigation, *S. aureus* was most susceptible to the *S. crispus* ethanolic leaf extract identifying bioactive compounds analysed by GC-MS; the originality from this work is that the *S. crispus* ethanolic leaf extract may prove useful as an antimicrobial agent against *S. aureus*, and it will be helpful to carry out other data with minimum inhibition concentration and other studies. The claimed use of this plant in the traditional system of medicine has been justified in treating various disease-causing microbes. Diverse compounds were found in *S. crispus*, and structure-activity relationships would be conducted on pharmacologically active compounds to explore the pharmacological activities of this plant. Nevertheless, further studies are needed for the isolation of antibacterial active compounds from the plant.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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