พฤกษเคมี ฤทธิ์ต้านอนุมูลอิสระ และความเป็นพิษต่อเซลล์มะเร็งของสารสกัดหญ้าพันงูเขียว

Phytochemicals, Antioxidant and Cytotoxicity of *Stachytarpheta jamaicensis* (L.) Vahl Extracts

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บทคัดย่อ

หญ้าพันงูเขียว (*Stachytarpheta jamaicensis* (L.) Vahl) เป็นไม้ล้มลุกที่พบในเขตร้อนทั่วไปรวมถึงประเทศไทย งานวิจัยนี้ได้นำส่วนเหนือดินของหญ้าพันงูเขียวมาสกัดด้วยตัวทำละลายได้แก่ เฮกเซน ไดคลอโรมีเทน เมทานอล ตามลำดับและแยกสกัดด้วยน้ำ จากนั้นนำสารสกัดหยาบมาทดสอบฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH ABTS$^{\bullet+}$ และ FRAP พบว่าสารสกัดหยาบเมทานอลมีปริมาณสารประกอบฟีนอลิกและฤทธิ์ต้านอนุมูลอิสระ แบบ DPPH ABTS$^{\bullet+}$ และ FRAP สูงที่สุด และพบว่าสกัดหยาบไดคลอโรมีเทนมีปริมาณฟลาโวนอยด์ทั้งหมดสูงที่สุดคือ 61.93 mg CE/g ของสารสกัด จึงนำสารสกัดไดคลอไรมีเทนมาทดสอบความเป็นพิษต่อเซลล์มะเร็งช่องปากที่เป็นเซลล์มะเร็งไม่ดื้อยาและเซลล์มะเร็งดื้อยา (CLS-354/WT และ CLS-354/DX) ด้วยวิธี MTT พบว่าสารสกัดหยาบไดคลอโรมีเทนมีความเป็นพิษต่อเซลล์มะเร็งทั้งสองสายพันธุ์ โดยมีค่า IC_{50} เท่ากับ 197.7 ± 38.6 และ 143.4 ± 21.2 µg/mL ในเซลล์มะเร็งไม่ดื้อยาและเซลล์มะเร็งดื้อยาตามลำดับ งานวิจัยชี้ให้เห็นถึงฤทธิ์ทางชีวภาพของสารประกอบในหญ้าพันงูเขียวซึ่งเป็นข้อมูลที่เป็นประโยชน์สำหรับการศึกษาและพัฒนาสารต้านมะเร็งช่องปากต่อไป

คำสำคัญ: หญ้าพันงูเขียว สารประกอบฟีนอลิก ฟลาโวนอยด์ ฤทธิ์ต้านอนุมูลอิสระ เซลล์มะเร็งช่องปาก

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Abstract

Stachytarpheta jamaicensis (L.) Vahl is a traditional herb found across tropical regions, including Thailand. The aerial part of this plant was extracted with solvents : hexane, dichloromethane, methanol, and water, respectively. Then, crude extracts were tested antioxidant activities using DPPH, $ABTS^{\bullet+}$, and FRAP assay. The research results showed that methanol crude extract had the highest antioxidant activities whereas, dichloromethane crude extract had the highest total flavonoid content (61.93 mg CE/g extract). Therefore, it was further used for cytotoxicity test against human oral squamous carcinoma cell lines (CLS-354/WT and CLS-354/DX) and cell viability using MTT assay. It also found that CH_2Cl_2 crude extract exhibited a potent cytotoxicity against both cancer cell lines, and the IC_{50} concentrations of CH_2Cl_2 crude extract in the CLS-354/WT and CLS-354/DX were 197.7 ± 38.6 and 143.4 ± 21.2 µg/mL. This research results confirmed that the biological activity of phytochemicals from *Stachytarpheta jamaicensis* (L.) Vahl will probably be useful for the research and development of anti-oral cancer agents in the future.

Keywords: *Stachytarpheta jamaicensis* (L.) Vahl, Phenolic, Flavonoid, Antioxidant Activities, Oral Cancer cells

Introduction

Stachytarpheta jamaicensis (L.) Vahl **(***S. jamaicensis***)** is a well-branched plant belonging to the family of Verbenaceae, which also called as Brazillian tea, rooter comb, or blue snakeweed. This plant species is a medicinal herb found across tropical regions; Latin-America, Africa, Southeast Asia including Thailand [1-2]. Ethnobotanically, *S. jamaicensis* has been used in folk medicine for treating several ailments such as allergic diseases, cold, and digestive problems [3-4]. It also has been possesses anti-microbial, anti-diarrheal, anti-diabetic, and wound-healing activities [5-6]. The major groups of bioactive compounds found in the *S. jamaicensis* extracts are alkaloids, glycosides, quinones, tannins, terpenoids and steroids [1, 5-7]. Studies have suggested that phenolics, terpenoids and alkaloids are linked to antioxidant properties [8-9]. However, there is little knowledge about their anti-oral cancer effect. Therefore, this study assessed antioxidant activities and anti-oral cancer effects of the crude extracts of *S. jamaicensis*. The content of phenolics and flavonoids were determined. The cytotoxicity against oral cancer cells was carried out. Hence, the findings could provide information of antioxidant potential and the evident of anti-oral cancer property of *S. jamaicensis*, which might be useful for anti-oral cancer agent development.

Research methodology

Chemicals and Plant Materials

All solvents and chemicals e.g. hexane, dichloromethane, dimethyl sulfoxide, $ABTS^{\bullet+}$, DPPH were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Aerial parts of *S. jamaicensis* was collected from Nakornsawan province, Thailand, plants identity and voucher specimens were carried out at the Division of Biology, Department of Science, Faculty of Science and Technology, Prince of Songkla University. Then, the plant material was washed, air-dried and blended**.**

Plant extraction

3.5 L of hexane was macerated with the powder sample (485 g). The sample was soaked undisturbed at room temperature for 48 hours and then filtered**.** Extraction was repeated using 3.5 L of the same organic solvent for another 48 hours. Then the residue was further extracted using dichloromethane and methanol, then evaporated to dryness. For water extraction, 25.0 g of powder sample was extracted with 2.4 mL of water at 100 °C. The percentage extract yield is calculated. Crude extracts (0.01 g) were re-dissolved with 10 mL of dimethyl sulfoxide (DMSO). The extract solution was diluted at concentration ranging from 15.625 – 5,000 mg/L for further analysis.

Determination of total phenolic content (TPC) and total flavonoid content (TFC)

The amounts of TPC were determined using the Folin-Ciocalteu colorimetric method [8]. The absorbance was measured at 765 nm. Gallic acid was used as a standard. TPC was expressed as milligrams of gallic acid equivalents per gram of crude extract (mg GAE/g extract). TFC present in *S. jamaicensis* was analyzed following colorimetric method [9] which described by Egharevba *et al*., (2019). The absorbance was measured at 510 nm. The standard used was catechin. TFC was expressed as milligrams of catechin equivalents per gram of crude extract (mg CE/g extract).

Antioxidant activities test

Crude extract solution (100 µL) was mixed with 900 µL of 0.2 mM DPPH solution for DPPH radical scavenging assay test. The absorbance (A) was measured at 515 nm, after incubating in dark for 30 min. Trolox was used as a standard. The percentage of scavenging activity (%) was calculated as the following:

$$\% \text{ scavenging activity} = [1-(A_{sample}/A_{blank})] \times 100 \quad (1)$$

where A_{blank} is the absorbance of the control and A_{sample} is the absorbance of the tested sample.

The half maximal effective concentration (EC_{50}) was calculated from the equation obtained from a linear regression curve of the crude extract at various concentrations levels. The $ABTS^{\bullet+}$ radical scavenging activity [10] was determined according to the method described by Hidayati1 et al. (2017) with slightly modified. The solution of $ABTS^{\bullet+}$ radical cation was prepared by mixing 7 mM $ABTS^{\bullet+}$ solution with 4.9 mM potassium persulfate aqueous. The mixture was incubated in the dark at 4°C for 12–16 hours before used. The absorbance was measured at 734 nm. Trolox was used as a standard. Percentage of $ABTS^{\bullet+}$ radical cation scavenging was calculated. The ferric reducing antioxidant power (FRAP) method was conducted according to Sherikar *et al*. (2015) [11]. Freshly prepared FRAP reagent was incubated at 37°C for 10 min. The absorbance was measured at 593 nm and $FeSO_4$ was used as a standard.

Cell culture and Cytotoxicity test

The human oral squamous carcinoma cell line CLS-354 (CLS Cell Lines Service GmbH, Eppelheim, Germany) at 45 – 55 passages were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin and 2 mM stable L-glutamine.

The cells were maintained in an atmosphere of 95 % humidity and 5 % CO_2 at a temperature of 37 °C. All reagents for cell culture were obtained from Gibco, Life Technologies, Carlsbad, CA, USA. CLS-354 cells were plated in a 96-well plate at a density of 4.68×10^4 cells/cm^2 and allowed to grow for 24 h in an incubator. Cells were treated with each crude extract (1-256 mg/L) which was diluted in culture RPMI-1640 for 24 hours in the CO_2 incubator. After the 24 hours treatment, the culture medium was removed. 200 µL of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Gibco, Life Technologies, Carlsbad, CA, USA.) was added to each well and incubated for 4 hours. The MTT solution then removed. 200 µL of dimethyl sulphoxide was added to each well in order to dissolve the formazan crystals metabolized from the viable cells. The absorbance of the violet solution (A_{sample}) was read at 560 nm using a microplate reader and the absorbance at 670 nm as background ($A_{control}$) subtracted. Cell viability was calculated as the following:

$$\text{Cell viability (\%)} = (A_{sample}/A_{control}) \times 100 \quad (2)$$

Statistical analysis

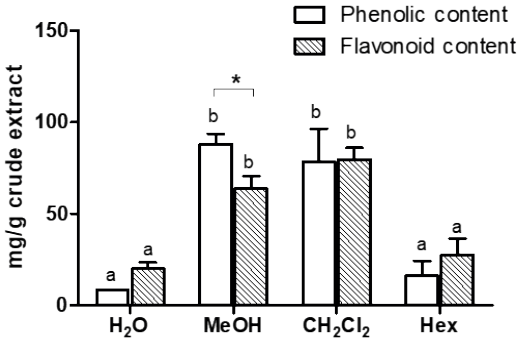
All experiments were analyzed from three individual samples (n = 3), which were run in duplicate. Values obtained were expressed as mean ± S.D. The difference between the mean of the sample was analyzed by one-way ANOVA following by all-pairwise multiple comparisons using GraphPad Prism 5 software. Significant differences were considered at $p < 0.05$.

Results and Discussion

Plant extraction and phytochemicals

In this study, different solvents were used as the extractant for *S. jamaicensis* extraction, including hexane (Hex), dichloromethane (CH_2Cl_2), methanol (MeOH) and water (H_2O). The results showed that Hex yielded the lowest mass of *S. jamaicensis* extraction, while the highest mass was obtained from MeOH extraction. The percentage yield ranged from 0.99 to 4.0 % w/w. The result of the extracted mass obtained is described as follows; the MeOH extract had the highest yield (4.01%), followed by CH_2Cl_2 (1.35 %) and Hex (0.99 %). Since, phenolics and flavonoids are the major groups of bioactive compounds with a variety of biological effects [12]. Thus, TPC and TFC of all crude extracts, including the water extract were also evaluated in this study. As shown in Figure 1, the phenolic and flavonoid contents of MeOH and CH_2Cl_2 extracts were significantly higher than those of H_2O and Hex extract, respectively ($p > 0.05$). The TPC of water, MeOH, CH_2Cl_2, and Hex extracts were 8.3 ± 0.30, 87.9 ± 5.8, 78.3± 18.1, and 16.07 ± 8.1 mg GAE/g extract, respectively. The TFC of water, MeOH, CH_2Cl_2, and Hex extracts were 19.9 ± 3.4, 63.6 ± 6.7, 79.8 ± 6.2, and 27.6 ± 8.9 mg CE/g extract, respectively. The content of flavonoid accompanied the content of phenolic, except in the MeOH extract, which had low flavonoid level than phenolic content ($p < 0.05$). The result indicated that all fractions were rich in flavonoids, while the MeOH extract might contains the non-flavonoid compound. Many secondary metabolites have been discovered in this plant

species, e.g. leaves of *S. jamaicensis* contain saponins, tannins and terpenoids [13-14] which may be non-flavonoid compounds in the MeOH extract.

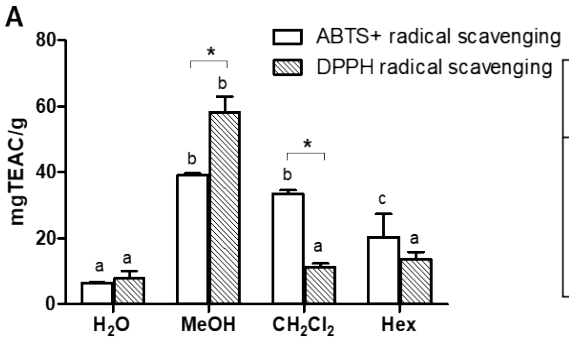


Crude extracts	Phenolic (mg GAE)	Flavonoid (mg CE)
H_2O	8.3 ± 0.30	19.9 ± 3.4
MeOH	87.9 ± 5.8	63.6 ± 6.7
CH_2Cl_2	78.3 ± 18.1	79.8 ± 6.2
Hex	16.07 ± 8.1	27.6 ± 8.9

Figure 1 Total phenolic content (TPC) and Total flavonoid content (TFC) of each crude extract of *S. jamaicensis*. The displayed values are mean ± SD (n = 3). The different lowercase letters indicate significant differences among crude extracts; * indicate a significant difference between TPC and TFC in the same concentration ($p < 0.05$).

Antioxidant activities

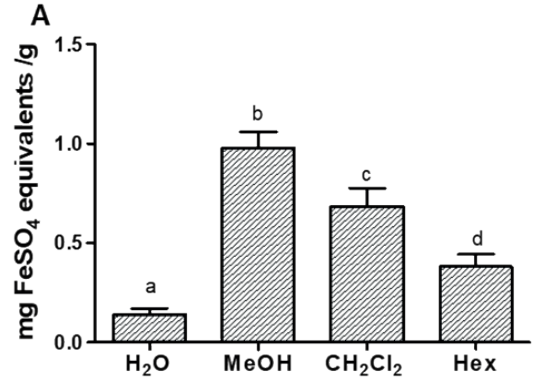
$ABTS^{\bullet+}$ and DPPH assays were used for assessing total antioxidant activity. Among four crude extracts, the total antioxidant activity of the MeOH extract was 39.07 ± 0.7 and 58.23 ± 4.8 mg of Trolox Equivalent Antioxidant Capacity per gram of crude extract (mg TEAC/g extract) using $ABTS^{\bullet+}$ and DPPH assay, respectively, whiles was greater than that of others, in which CH_2Cl_2 > Hex > water (Figure 2A). The half maximal effective concentration (EC_{50}) was calculated to compare the scavenging efficacy. The EC_{50} values against $ABTS^{\bullet+}$ and DPPH of the MeOH extract were 286.7 ± 21.1 and 184.4 ± 29.3 mg/L, respectively, which were significantly lower than those of water and Hex ($p < 0.05$). The CH_2Cl_2 exerted similar EC_{50} against $ABTS^{\bullet+}$ to the MeOH crude extract, while the EC_{50} against DPPH was weaker than the MeOH extract (Figure 2B), which may be due to $ABTS^{\bullet+}$ being more reactive than DPPH. The estimated EC_{50} was in the same trend with the total antioxidant activity. In order to investigate the reducing power, the extracts were further subjected to FRAP assay. The extracts from S. jamaicensis had a reducing power, on average, between 0.14 - 0.98 mg Fe^{2+} equivalents per gram of crude extract (mg Fe^{2+}/ g extract) and the strongest reducing power was found in MeOH extract (Figure 3A) with the EC_{50} value of 569.00 ± 18.66 mg/L (Figure 3B).



B

Crude extract	EC_{50} (mg/L)	
	ABTS assay	DPPH assay
H_2O	1,700.2 ±537.5[a]	2,646.1 ±189.9[a]
MeOH	286.7 ±21.1[b]	184.4 ±29.3[b]
CH_2Cl_2	265.5 ±22.8[b]	424.8 ± 136.8[c]
Hex	832.5 ±33.2[c]	476.2 ± 29.1[c]

Figure 2 Total antioxidant activities of each crude extracts of *S. jamaicensis*. (A) Scavenging activities against $ABTS^{\bullet+}$ and DPPH radicals. (B) EC_{50} at which of 50 % $ABTS^{\bullet+}$ and DPPH. The displayed values are mean ± SD (n = 3). The different lowercase letters indicate significant differences between $ABTS^{\bullet+}$ and DPPH in the same concentration ($p < 0.05$).



B

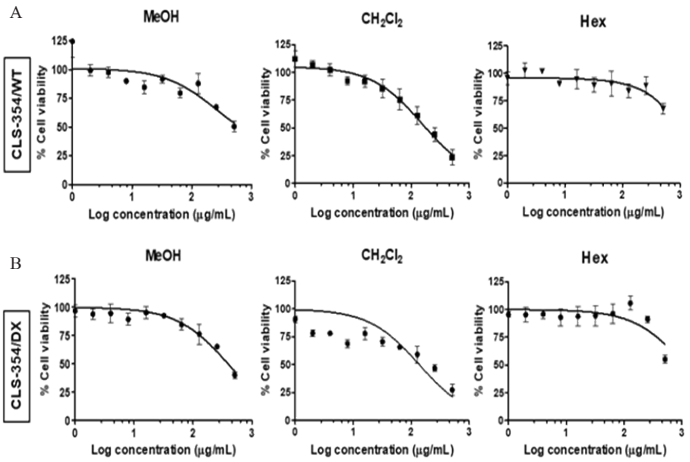
Crude extracts	EC_{50} (mg/L)
H_2O	3182.0 ± 529[a]
MeOH	569.0 ± 18.66[b]
CH_2Cl_2	725.7 ±131.9[b]
Hex	1451.0± 15.10[c]

Figure 3 FRAP results of crude extracts of *S. jamaicensis*. (A) The antioxidant potential was determined using a ferrous iron standard curve and expressed as mg Fe^{2+}/g extract. (B) EC_{50} of FRAP of crude extract (mg/L). The different lowercase letters indicate significant differences among crude extracts ($p < 0.05$).

Hence, the MeOH extract was the best in free-radical scavenging. It was suggested that the antioxidant ability of *S. jamaicensis* extract was from flavonoid and non-flavonoid compounds, which often originate from phenolic compounds. Most of them exist in MeOH extract, which was consistent with a previous report [15]. The main mechanisms of action of flavonoids including the polyphenols were as a chelate, stopping the chain reaction in the removal of free radicals by providing hydrogen to those radicals. The hydroxyl group of flavonoids not only scavenge radicals by oxidization reaction but also directly react with the other reactive compounds of the radicals, such as superoxide and peroxynitrite, which help to increase the stabilization of the reactive compounds [16].

Cytotoxicity against oral cancer cells

The MeOH, CH_2Cl_2 and Hex extracts were used to investigate cytotoxicity in an *in vitro* human cell line model of human mouth squamous cell carcinoma .The cell lines were CLS-354/WT and CLS-354/DX. The CLS-354/WT cell line is a less aggressive phenotype, while CLS-354/DX cell line is more aggressive by which the cells are multidrug resistant and invasive [17]. Upon treating the cells with *S .jamaicensis* crude extracts, the MeOH and CH_2Cl_2 exhibited a dose-dependent inhibitory effect on the growth of both cell lines, whereas the Hex extract had no inhibitory effect (Figure 4A, B). The half-maximal inhibitory concentration (IC_{50}) of the MeOH and CH_2Cl_2 were calculated and shown in Figure 4C. The IC_{50} concentrations of the MeOH in the CLS-354/WT and CLS-354/DX were 530.1 ± 77.5 and 402.7 ± 61.2 µg/mL, respectively .Interestingly, the IC_{50} concentrations of the CH_2Cl_2 in the CLS-354/WT and CLS-354/DX were 197.7 ± 38.6 and 143.4 ± 21.2 µg/mL, respectively, which were remarkably lower than that of the MeOH ($p < 0.05$). This result indicated a potential specificity for the CH_2Cl_2 crude extract in cytotoxicity against the cancer cells. However, the cytotoxic potentials of CLS-354/WT and CLS-354/DX were not significantly different. The observations suggest that the cytotoxic evident in the CH_2Cl_2 extracted-treated cell is not dependent, in part, on the antioxidant activity. As shown, the CH_2Cl_2 extract also contains a moderate level of phenolic content, flavonoid content and free-radical scavenging activities in comparison to the MeOH crude extract. Several studies have reported the cytotoxic effect of the CH_2Cl_2 crude extract of medicinal plants. For example, CH_2Cl_2 crude extract from *Murraya koenigii* leaves exerted stronger cytotoxicity against CLS-354 cells than that of MeOH crude extract, and carbazole alkaloids were the major active compounds responsible for anticancer activity [18]. CH_2Cl_2 crude extract from *Calea pinnatifida* is more potent than the EtOH extract in cytotoxicity against various cancer cell lines [19]. It is suggested that CH_2Cl_2 crude extract retains compounds with lyobipolar properties such as steroidal and terpenoid glycosides or aglycones. These compounds can interact with the lipid bilayer of cell membranes, whereas some compounds (e.g., saponin) are also able to reduce the surface tension of an aqueous solution, which contributes to antioxidant and anticancer properties. Although the substances involved in the anticancer effect of *S. jamaicensis* are unknown, some isolated pure compounds such as lanostane triterpenoid and steroidal glycosides [20] could explain this result. Meanwhile, either MeOH or EtOH crude extract retains a high number of phenolic compounds, which are soluble in water and other polar solution. So, they can form complexes with proteins and carbohydrates, which preferentially contribute to antioxidant and antibacterial activity [21].



C

Crude extracts	IC_{50} (μg/ml)	
	CLS-354/WT	CLS-354/DX
MeOH	530.1 ± 77.5[a]	402.7 ± 61.2[a]
CH_2Cl_2	197.7 ± 38.6[b]	143.4 ± 21.2[b]
Hex	ND	ND

Figure 4 Cytotoxicity of each crude extract with increasing concentration (1 – 512 μg/mL) of *S. jamaicensis* on the two human oral squamous cell carcinoma cell lines, (A) CLS-354/WT and (B) CLS-354/DX. (C) IC_{50} against the human oral squamous cell carcinoma cells were analyzed. The different lowercase letters indicate significant differences among crude extracts ($p < 0.05$).

Conclusion

The MeOH extract of *S. jamaicensis* significantly exhibited high antioxidant substances as well as antioxidant activity. Meanwhile, the CH_2Cl_2 crude extract interestingly inhibited cell proliferation of low and high aggressive human oral squamous cell carcinoma cells. This study provides evidence of the anti-oral cancer potential of *S. jamaicensis.* The observation in the present study should activates future research to identify the active substances involved in anti-oral cancer activity along with the understanding mechanism.

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