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Phytochemical analysis and correlation of total polyphenol content and antioxidant properties of *Symplocos cochinchinensis* leaves

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Abstract:

This study screened phytochemicals to determine their total polyphenols content (TPC) by Folin-Ciocalteu reagents and to evaluate antioxidant activity using DPPH scavenging, ABTS cation decolorization, and reducing power assays of various extracts of *Symplocos cochinchinensis* leaves. A correlation between TPC and antioxidant activity was analysed by Pearson's method. The results indicated that *S. cochinchinensis* leaves contained triterpenoids, alkaloids, anthraglycosides, flavonoids, anthocyanosides, proanthocyanidins, polyphenols, tannins, saponins, polyuronics, and reducing agents. All extracts had antioxidant properties with the ethyl acetate fraction exhibiting the highest antioxidant capacity, which had the lowest IC_{50} in DPPH (83.33 µg/ml), an ABTS of 46.21 µg/ml, and EC_{50} of 69.10 µg/ml in reducing power. There was a significant negative correlation between the TPC in *S. cochinchinensis* leaf extracts and their IC_{50} and EC_{50} values of antioxidant activity. It was suggested that *S. cochinchinensis* leaves have a great potential for antioxidant activity based on its high total polyphenol content and potential for use as a traditional treatment.

Keywords: **antioxidant activity, correlation, phytochemicals, polyphenols, *S. cochinchinensis* leaves.**

Classification number: **3.3**

Introduction

Oxidative damage is initiated by free radicals and reactive oxygen species (ROS) or reactive nitrogen species (RNS) that are produced by the normal aerobic metabolism of organisms. The oxidative reaction causes protein and lipid oxidation as well as DNA damage related to various diseases such as age-associated degenerative psychological disorders, atherosclerosis, cirrhosis, cancer, arthritis, diabetes, and haemorrhagic shock [1]. Molecular antioxidants have the ability to inhibit oxidation and these natural antioxidant sources have been receiving much attention not only because they lack the toxicity of synthetic ones such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) but also they bring positive health effects [2]. Therefore, antioxidants not only protect ourselves against the products of oxidative damage to DNA, proteins, or lipids; but also play a vital role in the prevention of diseases such as cancer, cardiovascular disease, Alzheimer's disease, and muscular degeneration by scavenging free radicals [3]. Recently, there has been increasing interest in plant-derived extracts or compounds with antioxidant capacity for an alternative treatment with minor side effects. Medicinal plants that are especially rich in their polyphenol content have been widely recognized as potent antioxidants [4, 5]. Polyphenols, a secondary metabolite found abundantly in plants, play an important role in the defence against free radicals. Several parts of medicinal plants, such as flavonoids, tannins, stilbenes, and coumarins, are rich in phenolic compounds. The antioxidant properties of polyphenols are due to their redox properties, which allow them to act as reducing agents, hydrogen donators, metal chelators, and single oxygen quenchers [6]. Polyphenols possess a wide range of biological activities including antioxidant, anti-inflammatory, antidiabetic, antiallergic, hepatoprotective, anticancer, and other effects and many

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of these biological functions have been attributed to their free radical quenching and antioxidant activity. Because of these beneficial effects for health, research on natural antioxidants derived from plants has intensified [7, 8].

Symplocos cochinchinensis (Lour.) Moore ssp. *Laurina* (Retz.) Nooteb. Symplocos laurina (*S. cochinchinensis*) belonging to the family Symploceaceae is widely distributed in many tropical and subtropical areas around the world including Vietnam. The plant is commonly used in folk medicine for the treatment of various disorders like leprosy, tumours, diarrheal, dysentery, menorrhagia, inflammation, and uterine problems [9]. Many plants in the *Symplocos* species have been used to treated various diseases based on their biological activities that include anti-diabetic, anti-HIV, antimicrobial, anti-inflammatory, antioxidant, and antitumor applications [4]. However, scientific reports on the biological potential of this indigenous Vietnamese plant are not widely available. Therefore, the present study was conducted to comprehensively study both chemical composition and biological activities of *S. cochinchinensis* collected from Cham island, Quang Nam province, Vietnam. Specifically, this study systematically evaluated *in vitro* antioxidant activities as well as determined the total polyphenol content of crude extracts and different fractions of *S. cochinchinensis* extracts to screen for potential medicinal application. Furthermore, the present study is the first to investigate the correlation between polyphenol content and the antioxidant activities of various extracts from *S. cochinchinensis* leaves.

Materials and methods

Plant materials

The plant samples were collected in August 2019 from Cham island, Quang Nam province, and identified and authenticated by BSc. Tran Ngoc Toan of the Greenviet Biodiversity Conservation Centre and checked again by the Southern Institute of Ecology, Vietnam Academy of Science and Technology where a voucher specimen was deposited for *Symplocos cochinchinensis* (Lour.) Moore ssp. *Laurina* (Retz.) Nooteb. Before grinding into the fine powder, the leaves were cleaned with tap water followed by distilled water, then they were air dried to the standard of loss on drying (LOD) following the Vietnam Pharmacopoeia 5th edition. The samples were stored in a sealed bag (Sample code: TTS-SC-0819) at the Research Center of Ginseng and Medicinal Materials in Ho Chi Minh city, Vietnam.

Chemicals

Gallic acid, Folin-Ciocalteu's phenol reagent, DPPH reagent (1,1-diphenyl-2-picrylhydrazyl), ABTS reagent (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), trichloroacetic acid, and ascorbic acid were purchased from Sigma-Aldrich® Co. Ltd (USA). Potassium ferricyanide and sodium carbonate were purchased from China of the highest grade. *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and methanol were obtained from Chemsol, Co. Ltd, Vietnam. Ethanol 96% was collected from OPC Pharmaceutical Company, Vietnam.

Extraction and fractionation

The crude extract (SCT) was obtained by using 45% ethanol to extract dried to a powdered material (Moisture is 9.73±0.41%) with a material/solvent ratio of 1/15 (g/ml) at room temperature for 24 h in a percolator apparatus. Then, the extract was collected at a rate of 2 ml/min and concentrated using a rotary evaporator at 60°C under reduced pressure.

To prepare fractionated extracts, the crude extract (moisture 15.50±0.34%) was solubilized in distilled water and sequentially extracted with solvents of increasing polarity (*n*-hexane, chloroform, ethyl acetate, and *n*-butanol). Then, the extract solutions were evaporated under reduced pressure conditions to dry and recover fractionated extract including *n*-hexane (SCH), chloroform (SCC), ethyl acetate (SCE), *n*-butanol (SCB), and water (SCW) extracts were obtained as fractional extracts. The percentage yield of crude and fractionated extracts of *S. cochinchinensis* leaves was 23.04% (SCT), 10.91% (SCH), 12.64% (SCC), 25.46% (SCE), 23.97% (SCB), 17.17% (SCW). The crude and fractionated extracts were preserved in sterilized vials and stored at 4°C.

The extraction yield of SCT was estimated using the expression: $H(\%)=(m\times(1-a))/(M\times(1-A))\times100$, where H is the extraction yield (%), m is the mass of the crude extract (g), M is the mass of the dried powder (g), a is the moisture of the crude extract (%), and A is the moisture of the dried powder (%). The extraction yield of fractionated extracts was estimated using the expression: $H(\%)=m/(M\times(1-A))\times100$, in which H is the extraction yield (%), m is the mass of the fractionated extract (g), M is mass of the crude extract (g), and A is the moisture of the crude extract (%).

Preliminary qualitative phytochemical screening

Preliminary qualitative phytochemical screening proceeded to identify the presence of phytochemicals in *S. cochinchinensis* leaves. The preliminary screening was carried out by Ciulei's method (1982) [10] with some modifications. The extracts of *S. cochinchinensis* leaves including diethyl ether, ethanol, and aqueous extracts were tested for secondary compounds like alkaloids, flavonoids, tannins, triterpenoids, saponins,

coumarins, anthraquinones, anthocyanosides, proanthocyanidins, lipids, volatile oils, carotenoids, reducing agents, polyuronics, and organic acids.

Determination of total polyphenol content

The total polyphenol content (TPC) of *S. cochinchinensis* leaf extracts were determined by a previously described method using Folin-Ciocalteu's reagent and gallic acid was used as a standard [11]. Briefly, 200 µl of test extract was mixed with 500 µl of Folin-Ciocalteu's reagent in 6 ml of double-distilled water. After 5 min, 1.5 ml of 20% *w/v* sodium carbonate solution was poured into this mixture and the volume was topped off to 10 ml with distilled water. The reaction was kept for 2 h at room temperature in the dark. The absorbance was measured at 758 nm and all measurements were made in triplicate. The TPC was calculated using the linear regression equation of gallic acid and expressed as mg gallic acid equivalent per 1 gram of dry weight (mg GAE/g d.w.).

In vitro antioxidant activity assay

DPPH radical scavenging assay: a DPPH free radical quenching assay was used to evaluate the antioxidant activity of *S. cochinchinensis* leaf extracts based on a previously described method [12]. Briefly, 4 ml of reaction mixture consisting of 0.5 ml of different concentrations of test extract or positive control and 0.5 ml of 0.6 mM DPPH reagent in methanol was incubated at room temperature for 30 min in the dark. The absorbances were measured at 515 nm using a Spectro UV-2550 spectrophotometer. DPPH inhibitory activity was expressed as the percentage inhibition (I%) of DPPH and calculated as (1-B/A)×100, where A and B are the absorbance of the blank (without test extract) and the sample (with test extract), respectively. IC_{50} (inhibitory concentration, 50%) values were calculated from the mean values of data from three trials. Ascorbic acid (vitamin C) at various concentrations (62.5, 125, 250, 500, 1000 µM) was used as a positive control.

ABTS radical cation decolorization assay: the ABTS antioxidant test was carried out according to the following description [13]. To start, a 7 mM ABTS solution was added to a 2.45 mM potassium persulfate solution and the mixture was incubated in the dark for 16 h at room temperature. Then, the solution was diluted by mixing 1 ml of ABTS solution with 50 ml of methanol to obtain an absorbance of 0.70±0.05 units at 734 nm using a spectrophotometer (Spectro UV-2550). Next, 20 µl of the test sample at various concentrations or the positive control was mixed with 980 µl of ABTS solution. The absorbance was measured at 734 nm after 6 min at room temperature. All samples were done in triplicate and the average of each sample was calculated. The results were expressed as an IC_{50} value for each sample from the proportion of the radical quenching activity, which was calculated by the formula: (%ABTS quenching activity)=(1-B/A)×100, where A and B are the absorbance of the blank (without test extract) and the sample (with test extract), respectively. Again, ascorbic acid at various concentrations (125, 250, 500, 750, 1000 µM) was used as a positive control.

Reducing power assay: the reducing property of all extracts was determined by assessing the ability of the extracts to reduce a $FeCl_3$ solution as described by Oyaizu [14]. The mixture including 0.2 ml aliquot, 0.5 ml of 200 mM sodium phosphate buffer (pH 6.6), and 0.5 ml potassium ferricyanide 1% was incubated at 50°C for 30 min, then 0.5 ml trichloroacetic acid 10% was added and centrifuged at 3000 rpm for 10 min. A volume of 0.5 ml of supernatant was mixed with equal volume double-distilled water and 0.1 ml ferric chloride 0.1%. The absorbance was measured at 700 nm. Ascorbic acid at various concentrations (31.25, 62.5, 125, 250, 500 µM) was used as a positive control. The reducing power activity was subsequently analysed via optical density and EC_{50} values. The lower the optical density value, the weaker the reduction activity of the sample. The EC_{50} value is the concentration of effective antioxidants for an absorbance of 0.5, which was calculated through the equation illustrating the correlation between the concentration of the sample and its optical density.

Data analysis

All experiments were carried out in triplicate. The obtained results were expressed in terms of mean ± SD (standard deviation) and the data were analysed by Graphpad Prism software (version 8, Inc., La Jolla, CA, USA) using the T-test and one-way ANOVA. The correlation coefficient (r) and coefficient of determination (R^2) were determined by the Pearson test, using Graphpad Prism software (version 8, Inc., La Jolla, CA, USA). P values <0.05 were considered statistically significant.

Results and discussion

Phytochemical analysis of S. cochinchinensis leaves

Phytochemical screening gives an idea concerning the chemical nature of the constituents that have biological effects present in extracts of medicinal materials. Therefore, it is useful for predicting the existence of crude products and also valuable for the detection of constituents present in samples. The presence of such essential phytochemical groups in this medicinal herb indicates its therapeutic properties and validates some its wide range of ethnomedicinal uses. Three extracts consisting of diethyl ether, ethanol, and aqueous were screened for qualitative chemical tests to identify various secondary

metabolites presenting in the leaf of *S. cochinchinensis*. The results in Table 1 showed that *S. cochinchinensis* leaves collected from Cham island, Quang Nam province, Vietnam contains secondary metabolites including triterpenoids, alkaloids, anthraglycosides, flavonoids, anthocyanosides, proanthocyanidins, tannins, saponins, polyuronics, and reducing agents.

Table 1. Preliminary phytochemical analysis of *S. cochinchinensis* leaves.

No.	Metabolites	Diethyl ether	Ethanol	Aqueous
1	Lipids	–	–	–
2	Carotenoids	–	–	–
3	Volatile oils	–	–	–
4	Triterpenoids	+	+	+
5	Alkaloids	+	+	–
6	Coumarins	–	–	–
7	Anthraglycosides	+	+	+
8	Flavonoids	+	+	+
9	Anthocyanosides	–	+	+
10	Proanthocyanidins	–	+	+
11	Polyphenols	+	+	+
12	Tannins	–	+	+
13	Saponins	–	+	–
14	Organic acids	–	–	–
15	Reducing agents	–	+	+
16	Polyuronics	–	–	+

Note: (+) indicates the presence and (−) the absence of a metabolite.

Determination of TPC in S. cochinchinensis leaves

Phytochemicals could contribute to potential biological activities, which is promising to the production of natural, new drugs. Important medicinal and pharmacological properties have been demonstrated in recent research that could be attributed to the phenolic compounds present in many plant extracts, for instance, anti-oxidant, anti-inflammatory, anti-microorganism, anti-carcinogenic, cardiovascular protection, improvement of endothelial function, inhibition for angiogenesis, and cell proliferation activities [8]. Our results show that the crude extract and its fractions of *S. cochinchinensis* leaves in Vietnam contain high levels of polyphenols, which is higher than the previously reported results [4], especially for the ethyl acetate fraction.

In detail, TPC was expressed as mg of gallic acid equivalent (GAE)/g of dry weight (d.w.) that was calculated using an equation obtained from a standard gallic acid graph ($y=0.0024x–0.2135$, $R^2=0.9907$). The results showed that the highest concentration of TPC was in the ethyl acetate fraction (852.74±13.28 mg GAE/g SCE d.w.), followed by the *n*-butanol fraction at 757.51±21.24 mg GAE/g SCB d.w., and then the total extract with 738.47±15.28 mg GAE/g SCT d.w., which are nearly double compared to that of the chloroform fraction (397.08±8.86 mg GAE/g SCC d.w.). By contrast, the *n*-hexane fraction was recorded as containing the lowest content of TPC (320.90±10.19 mg GAE/g SCH d.w.). Meanwhile, the TPC of the aqueous fraction is 511.35±8.86 mg GAE/g SCW d.w. Thus, these inconsistent results seem to imply that different extraction solvents can affect total phenolic content and the composition of extracts.

Antioxidant activity of crude and fractionated extracts of S. cochinchinensis leaves

A couple of methods had been utilized to determine the antioxidant property *in vitro* and using more than one method to evaluate antioxidant properties will accommodate more comprehensive information. In the present study, the antioxidant ability of different *S. cochinchinensis* leaf extracts was evaluated via an array of assays including a DPPH radical scavenging assay, ABTS radical cation decolorization assay, and a reducing power assay with ascorbic acid used as a positive control.

The DPPH method has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, where a purple-coloured solution bleaching acts like an indicator to evaluate antioxidant capacity [15]. At a concentration of 100 µg/ml, the ethyl acetate fraction had the highest activity with an inhibition percentage of 58.15% (Table 2). Of the six total extracts, five showed IC_{50} values ranging from 100 to 200 µg/ml. The SCE exhibited the strongest capacity compared with the lowest IC_{50} value (83.33±1.26 µg/ml). The order of the IC_{50} values in this test was VitC<SCE<SCB<SCW<SCT<SCC<SCH (Table 3).

The ABTS assay measures the loss of colour when an antioxidant is added to the blue-green chromophore $ABTS^{\bullet+}$. The antioxidant reduces $ABTS^{\bullet+}$ to ABTS and decolorizes it. Table 2 shows that the SCE also exhibited the highest activity with an inhibition percentage of 82.45% at 100 µg/ml. The scavenging of ABTS by the studied extracts increased with increasing content of the samples. When extracts were assessed at various concentrations, IC_{50} values in these assays were also calculated and the *S. cochinchinensis* leaf extracts exhibited IC_{50} values in the following order: VitC<SCE<SCT<SCB<SCW<SCC<SCH (Table 3).

In the reducing power assay, the ability to change colour will depend on the reducing capability of the test extract. The existence of reducing agents in the extract will react with Fe^{3+}/ferricyanide complex to its ferrous specimen. Thus, Fe^{2+} is determined by measuring the absorbance of the mixture after the reaction at a wavelength of 700 nm. In this test, antioxidant activity is generated via the reduction ability by giving away a hydrogen atom from the reducing compounds present in the investigated sample thereby inhibiting the propagation of a free radical chain. The reducing power assay of all studied *S. cochinchinensis* leaf extracts at 100

μg/ml is given in Table 2 and shows that all extracts had a potent reducing power. Similar to the two aforementioned activities, the reducing power also depended on the content of extracts and increased with increasing amounts of extracts. Besides, for the IC_{50} value, there were significant differences between SCB (75.76 μg/ml) compared to SCE (69.10 μg/ml) and SCW (80.75 μg/ml). Accordingly, this belongs to the extract group with high reducing power and are nearly 2.5 times higher than those of SCC and SCH (Table 3). The ranking order for reducing power was similar to the DPPH scavenging activity.

All values are expressed as mean ±SD (*n*=3). SCT, SCH, SCC, SCE, SCB, and SCW indicate crude ethanol extract, *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and aqueous fractions, respectively.

Table 2. Antioxidant capacity of crude extract and its fractions of *S. cochinchinensis* leaves at a concentration of 100 μg/ml.

No.	Samples	% Scavenging		Absorbance at 700 nm
		DPPH assay	*ABTS assay*	*Reducing power assay*
1	SCT	41.46±2.13	76.73±1.92	0.422±0.023
2	SCH	33.46±0.51	31.38±0.80	0.306±0.003
3	SCC	34.88±0.59	50.80±0.54	0.310±0.003
4	SCE	58.15±0.67	82.54±0.22	0.611±0.006
5	SCB	49.90±0.51	64.05±2.73	0.579±0.006
6	SCW	42.29±0.45	49.15±0.69	0.555±0.008

Table 3. The IC_{50} and EC_{50} values for antioxidant activities of crude extract and its fractions of *S. cochinchinensis* leaves.

No.	Samples	IC_{50} and EC_{50} (μg/ml)		
		DPPH assay	*ABTS assay*	*Reducing power assay*
1	SCT	174.33±3.12^{c}	60.67±1.09^{c}	138.16±5.69^{c}
2	SCH	244.23±1.94^{a}	127.53±0.9^{a}	175.64±2.86^{a}
3	SCC	195.50±7.49^{b}	118.93±0.3^{b}	169.43±0.65^{b}
4	SCE	83.33±1.26^{f}	46.21±0.70^{f}	69.10±2.28^{f}
5	SCB	114.23±2.56^{e}	84.30±2.13^{d}	75.76±1.27^{e}
6	SCW	148.70±2.84^{d}	106.03±0.47^{c}	80.75±0.39^{d}
7	VitC	4.20±0.07^{g}	9.59±1.57^{g}	4.20±0.19^{g}

All values are expressed as mean ±SD (*n*=3). SCT, SCH, SCC, SCE, SCB, SCW, and VitC indicate crude ethanol extract, *n*-hexane, chloroform, ethyl acetate, *n*-butanol, aqueous fractions, and vitamin C, respectively. Means with different letters in each column indicate significant differences (p<0.05).

Correlation between total polyphenols content and antioxidant activities

The results of this study also showed that there was a significant correlation between the studied antioxidant properties (IC_{50}/EC_{50} values) and the estimated total polyphenol content of *S. cochinchinensis* leaves. The total antioxidant capacity determined through ABTS, DPPH, and reducing power assays and TPC was significantly correlated with R^2 values of 0.7495, 0.9091, and 0.5510, respectively (Table 4). These correlation studies indicated that the evaluated antioxidant properties may be related to the presence of antioxidant bioactives such as polyphenols.

Table 4. The results of regression analysis between total polyphenol content and antioxidant activity (IC_{50}/EC_{50}).

Antioxidant values	Total polyphenol content			
	Linear regression	R^2	*r*	*p*
IC_{50} of DPPH assay	y=-0.2306x+297.6	0.7495	-0.8657	<0.0001****
IC_{50} of ABTS assay	y=-0.1433x+176.0	0.9091	-0.9534	<0.0001****
EC_{50} of reducing power assay	y=-0.1674x+217.9	0.5510	-0.7423	0.0004***

Note: ***: p<0.001 and ****: p<0.0001 are statistically significance.

The present study showed that TPC in *S. cochinchinensis* leaf extracts had significant and negative correlation with their IC_{50} of DPPH, ABTS, and EC_{50} of reducing power assays (r=-0.8657, r=-0.9534, and r=-0.7423, respectively, p<0.05) (Table 4). It can be predicted that phenolic compounds are the main contributor in antioxidant activities of *S. cochinchinensis* leaves by DPPH, ABTS, and reducing power assays.

In this study, the crude and fraction extracts were found to contain polyphenols with various concentrations, which resulted in different antioxidant levels. The ethyl acetate fraction (SCE) was recorded as the extract having the strongest antioxidant properties in all three assays including radical scavenging (DPPH and ABTS) potency and reducing power when compared to the lowest (the *n*-hexane fraction (SCH)), which were consistent with the highest and lowest polyphenol content of SCE and SCH, accordingly. Other findings also proved that there was a significant correlation between antioxidant activities and the polyphenol level of extracts, which could present a great number of polyphenols contributing to the evaluated antioxidant properties of *S. cochinchinensis* leaves. These obtained results were in agreement with the research of Sunil, et al. (2011) [4] which also supported the vital role of polyphenols derived from *S. cochinchinensis* leaf and bark extracts in India. The antioxidant activity was measured through reductive ability, scavenger of DPPH, nitric oxide, or inhibition of lipid peroxidation.

Phenolic compounds are secondary metabolites that are ubiquitously found in plants. The high phenolic content of *S. cochinchinensis* leaf extracts may be a good parameter of their antioxidant proficiency. The antioxidant properties of polyphenol compounds have been investigated, which is mainly owing to their redox characteristics and chemical structure allowing the neutralization of free

radicals, chelating metal ions, and singlet and triplet oxygen quenching activity [7, 8]. In this study, Pearson's method was applied to investigate *in silico* the correlation between polyphenols and the antioxidant activity of *S. cochinchinensis* leaf extracts, thereby providing additional information about the relationship between them. The highly remarkable correlations attained in the present study is consistent with the proven hypothesis that polyphenol composition significantly contribute to the antioxidant efficiency of plant extracts. Similar to some previous studies, a linear correlation between polyphenols content in the total extract and the antioxidant capacity was detected of some plants [16, 17]. Hence, polyphenol compounds could make a substantial contribution in the antioxidant activity of *S. cochinchinensis* leaves.

Conclusions

In this study, *S. cochinchinensis* leaves were proven to be a potential source of natural antioxidants. A linear correlation between polyphenol content and antioxidant capacity was demonstrated, which suggests that polyphenols could be applied as a marker for antioxidant properties of the *S. cochinchinensis* leaves. However, for a clearer view, further studies should be conducted on polyphenol composition and the *in vivo* effects of the *S. cochinchinensis* leaves.

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COMPETING INTERESTS

The authors declare that they have no conflict of interest regarding the publication of this article.

AUTHORS' CONTRIBUTION

Hai Ly Trieu and Vu Khanh Trang Le contributed equally as co-first authors to this work.

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