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# The Nitric Oxide Inducing Activity of *Aquilaria Subintegra* Tea Extract and Its Microencapsulated Form in Human Vascular Endothelial Cells

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**Abstract**

*Aquilaria subintegra* leaves are rich in polyphenols and flavonoids with antioxidant properties. *Aquilaria* spp. leaf tea is gaining attention for its health benefits; however, its effects on cardiovascular health remain underexplored. This study examined the effects of *A. subintegra tea* extract (AS extract) and microencapsulation on nitric oxide (NO) production in human umbilical vein endothelial cells (HUV-EC-C). Dried leaves were extracted using hot water, freeze-dried, and tested for cytotoxicity (MTT assay), NO production (Griess assay), and expression of endothelial nitric oxide synthase (eNOS and phospho-eNOS) (western blot). The extract was microencapsulated in maltodextrin-DE10 and freeze-dried. The results showed that a 30-minute extraction yielded the highest phenolic and flavonoid contents. AS extracts (10 - 100 µg/mL) were non-toxic and significantly enhanced NO production at 40, 80 and 100 µg/mL ($p < 0.05$). AS extract (80 and 100 µg/mL) increased eNOS expression and phosphorylation at Ser-1177 within 15 min. Microencapsulation at a 20:1 extract-to-coating ratio had > 80% encapsulation efficiency and induced NO production 1.8 times higher than the control. These findings elucidate the mechanisms underlying the vascular endothelial promotion of *A. subintegra* tea extract through NO synthesis. This suggests that *A. subintegra* tea extract and its microencapsulated form effectively promote NO synthesis, supporting its potential as a functional ingredient for cardiovascular health.

**Keywords:** *Aquilaria subintegra*, Human umbilical vein endothelial cells, Endothelial nitric oxide synthase, Nitric oxide

## Introduction

Cardiovascular disease (CVD) remains a significant public health issue as it is the leading cause of mortality in numerous nations globally. The likelihood of developing this category of diseases is associated with consumer habits and disregard for blood pressure management, leading to sustained hypertension in the population, a primary contributor to vascular degeneration, and ensuing cardiovascular disease [1,2]. Research on vascular cells is essential for the development of effective preventive and treatment approaches. Understanding the molecular mechanisms underlying vascular cell injury can facilitate the discovery of innovative treatments and drive the development of interventions to improve blood vessel health.

One of the most important mediators affecting blood vessel function is nitric oxide (NO), which is synthesized by endothelial nitric oxide synthase (eNOS) [3]. Endothelial dysfunction is a significant factor that leads to pathological alterations in vascular walls. Under normal conditions, endothelial cells function as barriers and selective agents between intravascular and extravascular fluids and significantly contribute to the production of various mediators essential for maintaining vascular homeostasis. Research indicates that the bioavailability of nitric oxide in individuals with cardiovascular disease is diminished, frequently due to

inflammation and oxidative stress [4]. Consequently, enhancing NO production in endothelial cells among the elderly or individuals prone to cardiovascular disease is an alternative approach for treatment and prevention. Despite the regular expression of the eNOS enzyme, its expression level can be modulated by naturally occurring phytochemicals. The natural products that are gaining attention for their efficacy in vasodilation are flavonoids, which are found in vegetables, fruits, herbs, wine, and tea [5,6].

Agarwood (*Aquilaria* spp.) is a sought-after economic wood valued by the fragrance industry both locally and internationally. In addition to its unique fragrance, agarwood possesses medicinal properties attributed to its chemical constituents, including flavonoids, alkaloids, terpenes, and phenolic acids [7]. *Aquilaria subintegra* is a plant native to Thailand that is predominantly cultivated in the districts of Songkhla and Pattani. It possesses interesting therapeutic efficacy but generates fewer aromatic chemicals than other species [8]. Leaf extracts have been reported to demonstrate antioxidant [9], acetylcholine esterase inhibitory activities [10], and anticancer activities [11]. Furthermore, it has been shown to exhibit low oral subacute toxicity in animal models [12]. Hence, the leaves have been used as herbal tea products locally. However, there is a lack of experimental evidence regarding the direct effects of *A. subintegra* tea on vascular endothelial function, particularly NO synthesis and eNOS activation. Furthermore, no previous research has evaluated whether microencapsulation can preserve the cardiovascular-related biological activity. To address this gap, a more thorough investigation is necessary to explore the biological mechanisms underlying the effects of *A. subintegra* tea and its microencapsulated form on the vascular cells. These findings would provide mechanistic insights into the role of *A. subintegra* tea in promoting vascular health, establish a scientific basis for its use as a functional ingredient, and support its development into nutraceutical products that could benefit both public health and the local herbal tea industry in southern Thailand.

## Materials and methods

### Preparation of extracts from *Aquilaria subintegra*

Tea samples from *Aquilaria subintegra* leaves were provided by the Thai Rung Agriculture-Sriwichai Kritsana Wood Processing Community Enterprise, Sadao District, Songkhla Province. Plant specimens were identified using external morphological characteristics of the plants before preparing plant specimens for reference in research (Voucher Specimens) and kept at the Faculty of Thai Traditional Medicine, Prince of Songkla University, Hat Yai Campus, Songkhla Province [12].

The tea leaves were ground into powder using a grinder. The optimum extraction conditions were studied by adding 100 g of sample powder to 1 L of distilled water at 95 °C for 15, 30 and 60 min. The extracted substance was filtered through filter paper No. 1 using a vacuum pump and centrifuged at 3,000 rpm for 10 min at 25 °C. The remaining substance was extracted again by boiling in distilled water, as described above. The filtrate was evaporated using a rotary evaporator under vacuum and dried in a freeze-dryer to obtain AS extracts. All extracts (AS extracts) were weighed, stored at –20 °C, and protected from light until analysis.

### Phenolic content and flavonoid content

The total phenolic content in the extracts under different conditions was determined by mixing 60 µL of each extract at a concentration of 1 mg/mL with diluted Folin-Ciocalteu solution (1:9 v/v), incubating in the dark for 2 min, and adding sodium carbonate (7.5% w/v; 2.0 mL). The mixtures were then incubated in a hot water bath at 50 °C for 15 min, followed by measurement of absorbance at 760 nm. The TPC was calculated by comparing it with the graph of the gallic acid standard solution [13].

For flavonoid content determination, the extracts obtained under different conditions at a concentration of 1 mg/mL (250 µL) were mixed with 1.25 mL of sodium nitrate (5% w/v) and incubated in the dark for 5 min before incubation with $AlCl_3$ (10% w/v) for another 5 min. Sodium hydroxide (0.5 mL of sodium hydroxide (1 M) was added and mixed thoroughly, and then the absorbance at a wavelength of 510 nm was measured. The total flavonoid content was calculated by

comparison with the standard curve of the quercetin solution [14].

**Cell culture**

The HUV-EC-C cell line (CRL- 1730TM, ATCC, USA) was grown in F-12K Medium (Kaighn's Modification of Ham's F-12 Medium, Hyclone) containing fetal bovine serum (10%), penicillin/streptomycin (1%), endothelial cell growth supplement from bovine neural tissue (30 mg/mL, Sigma catalog # E2759), and heparin (0.1 mg/mL, Sigma catalog #H3393). The cells were then grown in a carbon dioxide incubator with 5% carbon dioxide and 80% relative humidity. Subcultures were performed when the cells reached 80% - 90% confluency.

**Cytotoxicity test**

Before testing with the AS extract, HUV-EC-C cells were cultured in a 96-well plate ($1\times10^4$ cells/well) to approximately 80% confluency. The cells were treated with AS extract at a concentration of 5 - 500 µg/mL for 24 h. After the cells were incubated with the extract in a 96-well plate for the specified period, the cell culture medium was pipetted out and replaced with 0.5 mg/mL Thiazolyl blue tetrazolium bromide (MTT) solution and incubated in a $CO_2$ incubator for 4 h to induce the conversion of MTT to intracellular formazan crystals, which were further dissolved in 200 µL of dimethyl sulfoxide (DMSO) per well. The absorbance at 560 and 670 nm was measured, and the percentage of cell viability was calculated by comparison with the untreated samples.

**Total nitric oxide and nitrate/nitrite assay**

The total nitric oxide and nitrite concentrations were determined using an Assay Kit (R&D Systems, USA). Nitrite levels in cell culture media were assessed using Griess reagents I and II. To determine the nitrate concentration in the samples, endogenous nitrate concentration was evaluated using the Nitrite Assay Procedure. The samples (50 µL) were subjected to reductase and NADH assays, and incubated at 37 °C for 30 min. Following incubation, Griess reagents I and II were added to detect nitrates. The absorbance was measured at 540 nm with wavelength correction at 690 nm, and the concentrations were calculated relative to the nitrite or nitrate standard curves.

**Protein expression studies**

Protein expression in HUV-EC-C cells was studied using western blot analysis. Cells ($8.0\times10^4$ cells) were grown in $60\times15$ mm$^2$ culture dishes for 48 h, then the cells were incubated with various concentrations of AS for 24 h. The cell culture medium was aspirated and the cells were lysed with lysis buffer containing 1% Halt protease inhibitor cocktail (Thermo Scientific™, USA). The cells were centrifuged at 13,000 rpm at 4 °C for 10 min, and the supernatant was collected in test tubes for protein concentration determination using a detergent-compatible protein assay (Bio-Rad, USA). The protein sample (50 µg) was mixed with 2X Laemmli sample buffer and the proteins were separated by electrophoresis using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electricity was used to transfer the proteins from the gel to the plate. Nitrocellulose membranes were blocked with 5% non-fat dry milk for 1 h and incubated with primary antibodies (Cell Signaling Technology, USA) specific for eNOS (1:1000) or p-eNOS (1:500) at 4 °C for 16 h. The membranes were then incubated with secondary antibodies (1:5,000) at room temperature for 1 h and washed with TBST. The expression was examined by incubating the membranes in Immobilon Forte Western HRP substrate (Millipore, Germany) for 5 min, and band visualization was performed on Cytiva Amersham™ Hyperfilm™ to monitor the expression level of the protein bands on the films.

**AS-maltodextrin encapsulation**

AS powder (0.5 g) was dissolved in 500 mL of distilled water. The AS solution was then mixed with maltodextrin DE-10 in the proportions of AS:coating material of 5:1, 10:1, and 20:1, and stirred until dissolution. The mixture was stirred at 200 rpm for 10 min at room temperature using a magnetic bar for 15 min and then left at room temperature for 90 min. The mixtures were then poured into a stainless-steel tray at –40 °C and subjected to freeze-drying for 2 days. The dried samples were ground into a powder and stored at –20 °C until testing. The percentage yield of the AS-encapsulated powder was calculated using Eq. (1).

$$\text{Percentage of production (\% yield)} = \frac{\text{Dry weight of all samples after drying}}{\text{Dry weight of the initial sample before drying}} \times 100 \quad (1)$$

**Encapsulation efficiency**

Total phenolic content (TPC) was determined in the AS encapsulates (En-AS) by taking 0.1 g) dissolved in a solvent containing ethanol, acetic acid, and distilled water at a ratio of 50:8:42, which were mixed and shaken with a vortex mixer for 1 min. The solid fraction was then filtered through a 0.45-micron filter. The liquid fraction was analyzed for phenolic compounds in the microcapsules using the Folin-Ciocalteu assay. For the phenolic compounds on the surface, 0.1 g, En-AS was dissolved in a solvent consisting of ethanol and methanol at a ratio of 1:1 and shaken well with a vortex mixer for 1 min. The solid parts were then filtered through a 0.45-micron filter. Folin-Ciocalteu assay was used to measure the amount of phenolic groups in the microcapsules in the liquid part, and the encapsulation efficiency (EE) was calculated as the percentage of extract encapsulated using Eq. (2). The differences in the total phenolic content in the microcapsules and the phenolic content on the microcapsule surface were analyzed [15]. The encapsulation efficiency measurements were performed in triplicate (n = 3), and the results are expressed as mean ± standard deviation.

$$\% \text{ EE} = \frac{(\text{Total phenolic content in microcapsules} - \text{Phenolic content on microcapsule surface})}{\text{Total phenolic content in microcapsules}} \times 100 \quad (2)$$

**Scanning electron microscopy**

The particle morphology of the encapsulated powders was observed using scanning electron microscopy FESEM (FEI, model Apreo, Netherlands). The sample powders were fixed on scanning electron microscopy (SEM) stubs using adhesive tape and coated with a gold layer under vacuum; the samples were then observed under SEM at 5.00 kV.

**Statistical analysis**

Data from the experiments are expressed as mean ± standard deviation from 3 experiments, and the differences in means between samples were compared using 1-way ANOVA and Duncan's or Dunn's multiple comparison test, with $p < 0.05$, using GraphPad Prism 5.

## Results and discussion

**AS extract yield and phytochemical contents**

Dried agarwood leaves were extracted in hot water at 95 °C for different times (15, 30 and 60 min). The extracts (AS extract) were concentrated and powdered using vacuum evaporation and freeze-drying techniques. The dry weights of the tea extract from Agarwood leaves after hot water extraction at 15, 30 and 60 min were 7.02, 6.29 and 6.28 g, respectively, with yields of 14.05%, 12.58% and 12.55%, respectively, as shown in **Table 1**. The extracts were fine brown powders and were well soluble in water.

**Table 1** Percentage yield, phenolic content, and flavonoid content of AS extract.

| Extraction time (min) | Dried weight after freezing dry (g) | Yield (%) | Total phenolic content (mg GAE/g extract) | Total flavonoid content (mg QE/g extract) |
|---|---|---|---|---|
| 15 | 7.02 | 14.05 | 109.0 ± 2.095 [a] | 191.9 ± 5.36 [a] |
| 30 | 6.29 | 12.58 | 132.1 ± 1.539 [b] | 246.9 ± 13.9 [b] |
| 60 | 6.28 | 12.55 | 124.6 ± 2.341 [ab] | 204.7 ± 5.85 [ab] |

Different letters indicate statistically significant differences at $p < 0.05$ when compared in the same column.
GAE: Gallic acid equivalent (equivalent to gallic acid)
QE: Quercetin equivalent (Quercetin equivalent)

Previous studies have reported that agarwood leaves contain high levels of phenolic compounds including flavonoids, which are major bioactive constituents [10]. Therefore, we investigated the total phenolic and flavonoid content in the AS extract. As shown in **Table 1**, the AS extracts after heating for 15, 30 and 60 min had total phenolic content of 109.0 ± 2.095, 132.1 ± 1.539 and 124.6 ± 2.341 mg GAE/g extract, respectively, and total flavonoid content of 191.9 ± 5.36, 246.9 ± 13.9 and 204.7 ± 5.85 mg QE/g extract, respectively. These findings align with those of Zainal *et al.* [16], who reported that the total flavonoid content in agarwood leaf extracts ranged from 191 to 247 mg QE/g extract. LC-MS/MS QTOF analysis of the phytochemicals of AS tea leaves (**Table S1**) supported the finding that various compounds in AS extract were

classified in the subclass of flavonoid glycosides, including Isoorientin 6”-O-glucoside, Kaempferol 3 rhamnoside-(1->2) rhamnoside, Vitexin 4’-O-galactoside, Hesperetin-7-O-glucuronide, and Dihydrogenistin. Notably, certain compounds, including flavonoids, benzopyrans, and triterpenoids, have also been identified in other *Aquilaria* species, such as *A. sinensis* and *A. malaccensis*, as reported by Hashim *et al.* [7]. This suggested that *A. subintegra* leaves are a valuable source of flavonoids. However, the chemical composition of flavonoids in the leaf extracts remains less well documented than in other *Aquilaria* species.

The phytochemical content of the extracts processed at different times was compared. The extracts processed in hot water for 30 min had the highest amounts of phenolics and flavonoids, which were much higher than those extracted for 15 and 60 min. It is possible that prolonged extraction times negatively affected the phytochemical composition, likely due to the thermal degradation of certain bioactive compounds. This degradation results in a reduction in both the quantity and biological activity of these phytochemicals. Although the decline in total phenolic and flavonoid content at prolonged extraction (60 min) suggests possible thermal degradation, this was not validated through compound-specific degradation profiling or kinetic studies. Our LC-MS/MS phytochemical analysis (**Table S1**) confirmed the presence of multiple flavonoid glycosides in *A. subintegra* tea extract. Prolonged thermal exposure can cleave glycosidic bonds in flavonoid glycosides, releasing free aglycones that are chemically less stable. These aglycones are more prone to oxidative reactions, polymerization, and structural rearrangements, which collectively diminish their antioxidant potential and other biological activities [17]. This mechanism aligns with the observed decline in phenolic and flavonoid contents after extended heating time in the present study and further supports the importance of optimizing extraction parameters to preserve bioactive compounds. Future work should include time-course degradation profiling and kinetic modeling to confirm the thermal stability of individual compounds and correlate these changes with biological activity. Therefore, factors such as temperature, extraction duration, and brewing method significantly influence the phenolic compound content, flavonoid levels, and antioxidant activity of hot water extracts [18]. Therefore, the AS extract at 30 min was selected for further study of its biological activity.

**Effect of AS extract on endothelial cell viability**

In this study, the HUV-EC-C cell line was selected as a representative model of endothelial cells to investigate the biological activity of AS extract and its induction of NO production *in vitro*. The cytotoxicity study of the AS extracts is essential for determining the appropriate concentration range of extracts in endothelial cells. In this study, the toxicity of AS extract at 30 min was tested in HUV-EC-C cells. It was found that AS extracts at concentrations of 10 - 100 µg/mL were nontoxic to HUV-EC-C cells, and there was no statistically significant difference compared to the control group (0 µg/mL). However, AS extracts at concentrations of 200 and 300 µg/mL significantly reduced cell viability by only 50% (**Figure 1**). Furthermore, upon elevating the concentration to 400 µg/mL, we observed that the cell viability percentage was 51.44% ± 3.5%, which was comparable to the result at 300 µg/mL. This indicates that the AS extract at concentrations of 200 µg/mL and above was toxic to endothelial cells *in vitro*. However, the mechanisms of cell death, were not investigated in detail, as the present study design focused on examining non-cytotoxic conditions. These results indicate that the optimal concentration for evaluating biological activity in HUV-EC-C cells should not exceed 100 µg/mL. This limitation is particularly relevant because certain flavonoids can act as pro-oxidants, potentially inducing cellular apoptosis. Consequently, these compounds have been explored for their potential in anticancer drug development [19]. However, it is important to note that *in vitro* toxicity studies may not fully reflect toxicity in humans. A previous study assessed the toxicity of *A. subintegra* leaf extracts in experimental mice. The extract was administered orally at doses of 5, 50 and 300 mg/kg body weight over 28 days. The findings demonstrated that *A. subintegra* leaf extract was non-toxic to the tested animals, with a safe dosage of up to 40 mg/kg body weight for human consumption without adverse effects [12]. The discrepancy between toxicity findings in cultured cells and experimental animals can be attributed to differences in bioaccessibility and bioavailability, which influence the absorption,

metabolism, and overall biological effects of compounds *in vivo* [13].

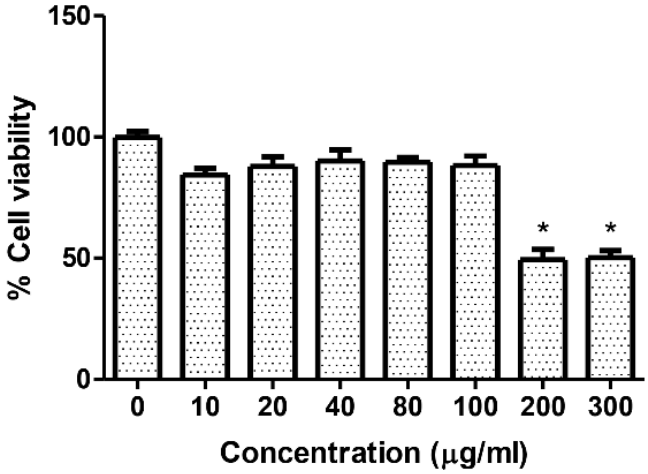


**Figure 1** Cell viability of endothelial cells. HUV-EC-C cells at passage NO.3 were treated with AS extract at various concentrations for 24 h. Cell viabilities were determined by MTT assay. Values are the mean ± SEM from 4 experiments, where *indicates statistically significant difference at $p < 0.05$ when compared to the control group (0 μg/mL).

**Effect of AS extract on nitric oxide production in endothelial cells**

NO is a crucial molecule that promotes vasodilation and maintains vascular homeostasis. It is synthesized by endothelial cells, and a reduction in NO levels is associated with vascular wall degeneration, contributing to conditions such as hypertension and atherosclerosis [3]. The quantification of NO can be evaluated by measuring nitrite, which is a stable byproduct of NO oxidation. As shown in **Figure 2**, analysis of nitrite content in HUV-EC-C cell culture medium revealed that control HUV-EC-C cells not incubated with the extract (0 μg/mL) produced 3.8 ± 0.92 μM nitrite. When HUV-EC-C cells were co-incubated with AS extract at concentrations of 10, 20 and 40 μg/mL for 24 h, the nitrite levels in the cell culture medium increased to 4.4 ± 0.39, 5.2 ± 0.46 and 5.9 ± 0.47 μM, respectively. At a concentration of 80 μg/mL, nitrite levels remained relatively high, with a slight decline observed at 100 μg/mL, indicating a plateau and a possible shift of the detectable nitrite pool toward nitrate formation due to further oxidation (**Figure 2(A)**). The oxidation process converts the nitric oxide produced by cells into nitrate and nitrite. Consequently, the levels of nitrate and nitrite were measured using the nitrate reductase test with Griess reaction. **Figure 2(B)** shows that the level of nitrite produced by endothelial cells exceeded the standard Griess reaction test (**Figure 2(A)**) by approximately 20-fold. The concentration of AS extract at 20 - 100 μg/mL promoted total nitrite production, in accordance with the higher concentration of the extract. The AS extract at concentrations of 80 and 100 μg/mL markedly increased the total nitrite production relative to the control group (0 μg/mL). A23187, a calcium ionophore that stimulates NO production in cell culture by activating calmodulin-dependent constitutive nitric oxide synthase (positive control), significantly increased the total nitrite level (**Figure 2(B)**). Analysis of endogenous nitrite and nitrate revealed that AS extract induced endothelial cells to produce endogenous nitrite (**Figure 2(C)**) and nitrate (**Figure 2(D)**), exhibiting an increasing trend, but the results were not statistically significant. The findings of this study demonstrated that AS extracts at concentrations of 80 and 100 μg/mL stimulated NO generation in endothelial cells. This was supported by the total nitrate/nitrite results obtained using the enzymatic reduction method, as the total nitrate/nitrite assay accounts for both nitrate and nitrite, it provides a more comprehensive and accurate estimation of NO production than nitrite measurement alone. For this reason, the total nitrate/nitrite data (**Figure 2(B)**) were selected for subsequent molecular-level analyses.

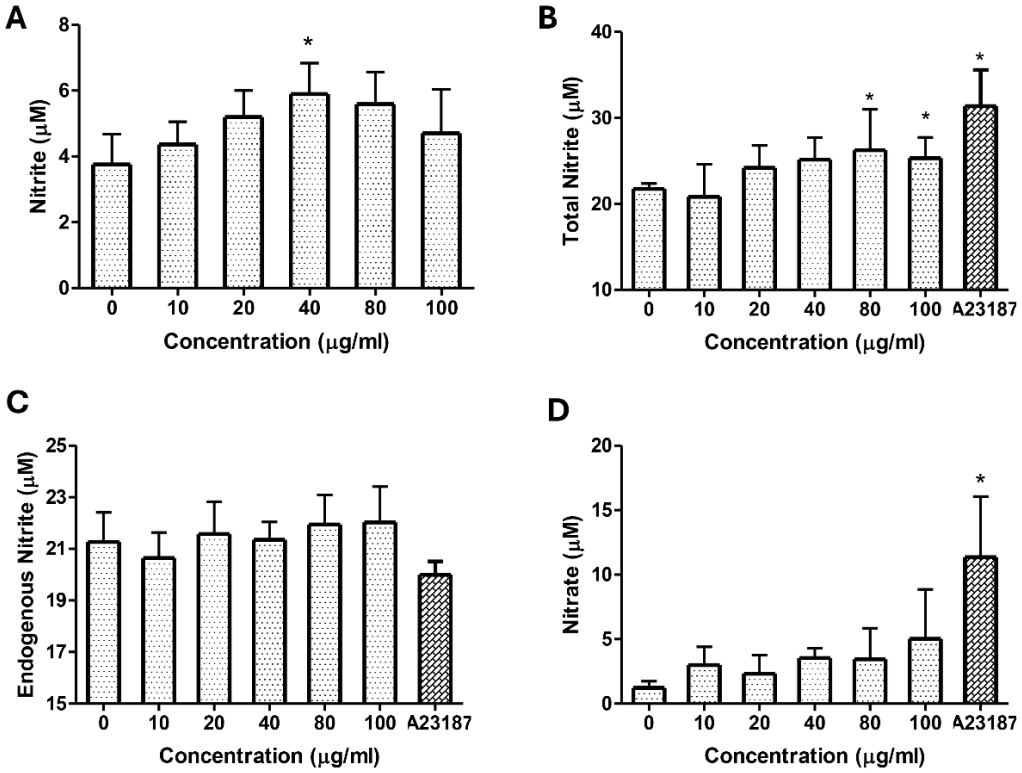


**Figure 2** The effect of the AS extract on the production of nitric oxide. HUV-EC-C cells were treated with the AS extract for 24 h. After the treatment, cell culture supernatants were collected to examine nitric oxide and its derivatives. (A) Nitrite levels detected by standard Griess reactions. (B) Total nitrite levels detected by a nitrate reduction test with the Griess reaction. (C) Endogenous nitrite levels detected by a nitrate reduction test with the Griess reaction. (D) Nitrate levels detected by a nitrate reduction test with the Griess reaction. A23187 (10 μM) was used as a positive control substance. Values are expressed as mean ± SD from 3 experiments, where * indicates a statistically significant difference at $p < 0.05$ when compared to the control group (0 μg/mL).

**Effect of AS extract on the expression of endothelial nitric oxide synthase (eNOS)**

NO is synthesized by the enzyme endothelial nitric oxide synthase (eNOS) [3], and it has been reported that some natural products can induce the expression of eNOS genes and proteins [20]. This study investigated the expression of eNOS protein upon stimulation with the AS extract. The results showed that eNOS protein expression in HUV-EC-C cells under normal conditions (without AS extract) was low. When the cells were treated with the AS extract at concentrations of 80 and 100 μg/mL for 15 min, the expression level of eNOS protein increased by approximately 2-fold and was statistically significant when compared to cells not treated with the extract (0 μg/mL). In this study, A23187 (10 μM) or $Ca^{2+}$ ionophore, a positive control in eNOS activation, highly stimulated eNOS protein expression, but the difference was not statistically significant from the AS extract treatment (**Figure 3(A)**). However, when the incubation time of HUV-EC-C cells with the AS extract and A23187 was increased for 20 h, the results showed that the expression level of eNOS protein in cells treated with the AS extract did not differ from that in the control group, except that the expression of eNOS protein in cells treated with the positive control A23187 was significantly reduced, as shown in **Figure 3(B)**. Thus, the results of this study demonstrate that the AS extract accelerates NO synthesis in endothelial cells by increasing the expression of eNOS protein with a short-term effect.

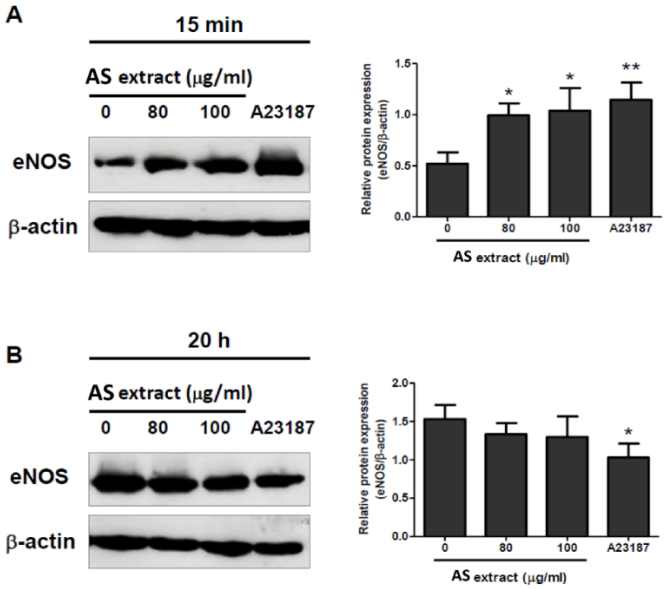


**Figure 3** The effects of AS extract on the expression of eNOS proteins in endothelial cells. HUV-EC-C cells passage NO. 5 - 10 were treated with the AS extracts (80 and 100 μg/mL) or positive control A23187 (10 μM) for 15 min (A) and 20 h (B). The protein expression levels were studied by Western blot analysis. Band intensities of eNOS are expressed relative to β-actin and displays as mean ± SD of 3 experiments. * and ** indicate statistically significant differences at $p < 0.05$ and $p < 0.01$, respectively, when compared to the control group (0 μg/mL).

Another important mechanism for NO synthesis is stimulation of enzyme phosphorylation. Certain drugs that activate proteins in the AKT signaling pathway can accelerate eNOS phosphorylation [21], resulting in increased nitric oxide production by endothelial cells. In this study, the expression of eNOS phosphorylated at Ser-1177 was investigated. The results showed that the expression level of eNOS protein phosphorylated at the Ser-1177 position tended to increase when treated with the AS extract at concentrations of 80 and 100 μg/mL for 15 min, but there was no statistically significant difference when compared with the negative control (μg/mL), whereas the positive control A23187 significantly increased the expression level of eNOS protein phosphorylated at the Ser-1177 position, as shown in **Figure 4**.

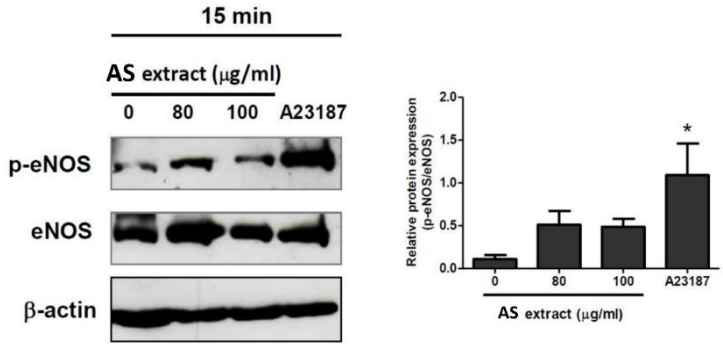


**Figure 4** The effects of AS extract on the phosphorylation of eNOS. HUV-EC-C cells passage NO. 5 - 10 were treated with AS extract at concentrations of 80 - 100 μg/mL or positive control substance A23187 (10 μM) for 15 min. The levels of phosphorylated eNOS at Ser1177 (P-eNOS) were investigated by Western blot analysis. Band intensities of P-eNOS are expressed relative to eNOS and displays as mean ± SD from 3 experiments. * Indicates a statistically significant difference at $p < 0.05$ when compared to the control group (0 μg/mL).

The mechanism underlying NO synthesis is attributed to certain flavonoids with pro-oxidant properties that enhance calcium ion ($Ca^{2+}$) influx into cells, thereby activating the signaling pathway [5]. This mechanism resembled the effect of A23187, a calcium ionophore used as a positive control in this study. Upon exposure to the extract, intracellular calcium levels increase rapidly (within approximately 5 min) [22]. The accumulation of $Ca^{2+}$ subsequently activates kinases such as protein kinase C delta (PKCδ) and serine/threonine protein kinase Akt (protein kinase B), which phosphorylate endothelial nitric oxide synthase (eNOS) at Ser1177. This phosphorylation enhances eNOS activity, facilitating the conversion of L-arginine into NO and L-citrulline [23,24]. Experimental observations revealed that eNOS protein expression levels changed relatively quickly within 15 min of exposure to AS extract. However, when the cells were incubated with the extract for 20 h, no significant changes in eNOS protein expression were detected (**Figure 3(B)**). This suggested that the increased total eNOS content observed at 15 min was due to elevated phosphorylated eNOS rather than de novo protein synthesis.

Consistent with other reports, several bioactive compounds have been reported to mediate nitric oxide synthase-induced eNOS phosphorylation in endothelial cells. These include the flavonoid luteolin [25], polyphenols extracted from black tea [26], and epigallocatechin-3-gallate (EGCG) extracted from green tea [27]. Additionally, some flavonoids can upregulate eNOS gene expression in endothelial cells. Previous studies have shown that resveratrol, a phenolic compound found in red wine, enhances eNOS gene expression by activating its promoter within 24 - 72 h. This represents a long-term regulatory effect that may occur following the initial phosphorylation process [28]. Although the present study did not evaluate eNOS mRNA levels, the rapid onset of increased eNOS protein at 15 min strongly suggests a post-translational regulatory mechanism rather than transcriptional upregulation. This interpretation is supported by the concurrent increase in phosphorylated eNOS at Ser-1177 and the absence of sustained elevation in total eNOS after 20 h. These findings are in line with previous studies reporting that flavonoid-rich extracts can activate upstream kinases such as Akt and PKCδ, which enhance eNOS catalytic activity via phosphorylation without requiring new protein synthesis. Therefore, future investigations will include gene expression analysis to determine whether AS extract also modulates eNOS gene expression during longer incubation periods.

**Microencapsulation of AS extract and its functional properties**

Herbal microencapsulation is a technique used to encapsulate herbs or plant extracts within a protective coating or shell, incorporating substances such as drugs, flavors, fragrances, vitamins, enzymes, or other active ingredients to enhance their stability, safety, and efficacy [29,30]. The encapsulating agent, maltodextrin DE-10, is a carbohydrate derived from starch and is often used as an encapsulating agent in powdered beverages because of its low cost, neutral odor and taste, and ability to protect sensitive bioactive compounds from temperature variation and oxidative degradation. Additionally, maltodextrin is non-toxic [31]. It is particularly effective in encapsulating anthocyanins and phenolic compounds, which are highly susceptible to oxidation [32]. In this study, the proportion of maltodextrin DE-10 and AS extract was analyzed for the benefit of further product development, with maltodextrin acting as the coating material and tea extract as the core material.

The results of this study showed that the microencapsulated powder of Agarwood tea leaves was a light, dry yellow powder that was soluble in water (**Figure 5(A)**). **Figure 5(B)** shows the surface morphology of En-AS under a scanning electron microscope (SEM) at 1,000× magnification. The particles appeared spherical with a relatively smooth surface, suggesting effective encapsulation. Uniformity in size and shape may contribute to the controlled release and stability of the extract. The encapsulation efficiency (%EE) was in the range of 84% - 90%, which was at a good level, indicating that maltodextrin DE-10 had the ability to encapsulate the bioactive compounds of agarwood tea leaves. The change in the proportion of the core-wall ratio affected the encapsulation yield (%EY) and encapsulation efficiency (%EE). The highest proportion of maltodextrin-DE10 gave the

highest yield weight owing to the weight of the wrapping material, but it had the lowest retention efficiency (84.77% ± 0.70%). Decreasing extract: coating material to 10:1 and 20:1 provided the %EE value of 89.55% ± 0.81% and 88.97% ± 0.53%, respectively, with similar efficiencies (**Table 2**). The findings of this study align with those of Muangrat *et al.* [33], who reported that a 10:1 extract-to-coating-material ratio resulted in the highest microencapsulation retention efficiency. This is because an excessive amount of encapsulated substance may lead to reduced extract loading or hinder its controlled release. Conversely, when the coating material exceeded a 20:1 ratio, the retention efficiency decreased, and the particle size increased. Therefore, achieving an optimal balance between the extract and encapsulating material is essential, with most studies identifying an appropriate range of 10:1 to 20:1 [34].

**Table 2** Encapsulation yield (%EY) and encapsulation efficiency (%EE) of AS extract.

| Extract: coating material (W/W) | %EY | %EE |
|---|---|---|
| 5:1 | 95.89 | 84.77 ± 0.70 [a] |
| 10:1 | 98.27 | 89.55 ± 0.81 [b] |
| 20:1 | 97.46 | 88.97 ± 0.53 [b] |

Different letters indicate statistically significant differences at $p < 0.05$ when compared in the same column. Encapsulation efficiency measurements were performed in triplicate (n = 3), and the results are expressed as mean ± standard deviation.

In addition, the total phenolic contents in the microencapsules were 57.11, 89.40 and 146.03 μg GAE/100 g, respectively, indicating that using coating materials had the bioactive compound content. En-AS with an extract ratio of 20:1 was tested for its effect on NO production in HUV-EC-C cells to assess its bioactivity by dissolving En-AS in distilled water at concentrations of 5, 10, 20 and 40 mg/mL. The findings indicated that En-AS stimulated NO production in cells at concentrations of 20 and 40 mg/mL, leading to an increase in NO levels from the control group by 1.5 and 1.8 times, respectively (**Figure 5(C)**). The microencapsules at a concentration of 40 mg/mL had a total phenolic content of 30 μg GAE. The use of this concentration was justified because the nominal weight includes the maltodextrin coating material, and the corresponding phenolic content is comparable to that found in 100 - 200 μg/mL of crude AS extract. Using this level allowed for a fair comparison of bioactive load between the crude and encapsulated forms. Higher concentrations of En-AS (> 40 mg/mL) were excluded due to cytotoxic effects observed in HUV-EC-C cells. Furthermore, by incorporating the extract into maltodextrin-based microencapsulation, we demonstrated that the extract remained bioactive and retained its ability to induce NO production. This finding suggests that the AS extract can be successfully formulated into microencapsulated products while preserving its biological properties, making it a promising candidate for functional food and nutraceutical applications. However, the use of maltodextrin alone may be insufficient for effective microencapsulation because it lacks emulsification and film-forming properties. When combined with other encapsulating agents such as gum arabic, the encapsulation efficiency (%EE) was significantly enhanced [34]. This suggests that future studies should explore the potential of composite encapsulating systems to improve stability and release profiles of bioactive compounds.

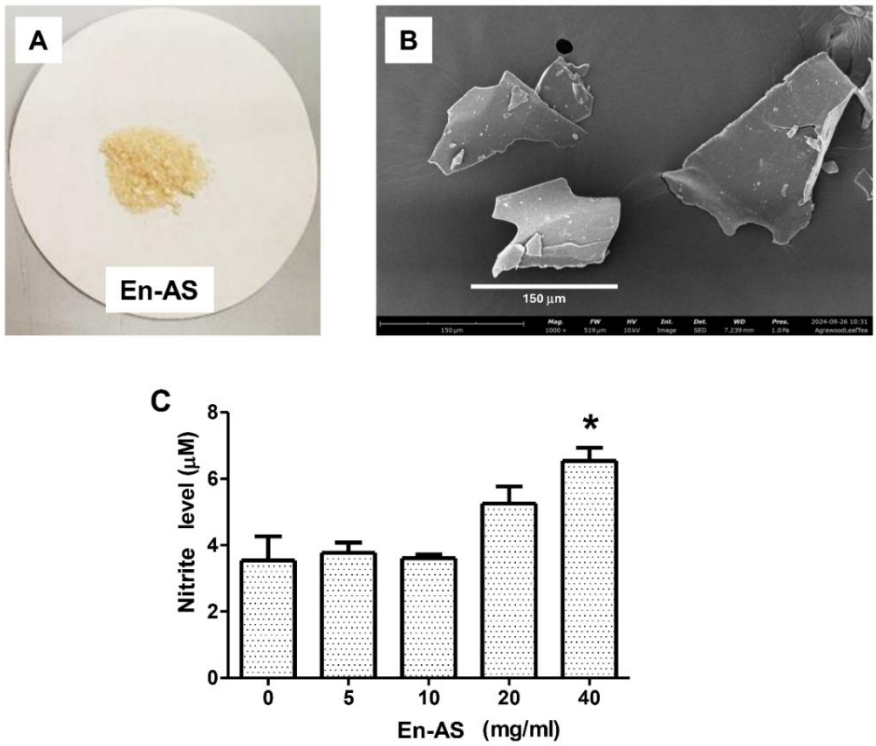


**Figure 5** Morphological characteristics, and nitric oxide-inducing activity of microencapsulated AS extract. (A) Appearance of microencapsulated AS extract (En-AS) after microencapsulation process (B) Particle characteristics of En-AS analyzed using SEM at magnification of 1,000×. (C) Effect of En-AS on nitric oxide production in endothelial cells (HUV-EC-C) cells passage NO.4 - 5 after treatment with En-AS at concentrations of 0 - 40 mg/mL for 24 h, and nitrite levels were detected by Griess reaction. Values are expressed as mean ± SD from 3 - 4 experiments, where * indicates a statistically significant difference at $p < 0.05$ when compared to the control group (0 mg/mL).

## Conclusions

These findings indicate that the hot water extract from *Aquilaria subintegra* leaf tea significantly enhanced NO production in endothelial cells by elevating the expression of eNOS protein and promoting its phosphorylation at the Ser-1177 site following exposure to the extract. Microencapsulation preserved the bioactive components while still demonstrating NO-stimulating actions. The AS extract was effective in stimulating NO production at concentrations of 80 - 100 µg/mL without cytotoxic effects, whereas the microencapsulated form (En-AS) was effective at 40 mg/mL, corresponding to approximately 30 µg GAE of phenolics and concentrations above this threshold induced cytotoxicity. This study demonstrates the efficacy of microencapsulation as a delivery mechanism for AS extract, assuring stability and bioavailability, which could benefit product development aimed at improving vascular function and circulatory health.

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## Declaration of generative AI in scientific writing

The authors acknowledge the utilization of AI-assisted technologies in the writing process. QuillBot were used to assist with language refinement and grammar correction to improve the clarity of the manuscript. All conceptualization, data analysis, interpretation of results, and critical discussion were conducted independently by the authors without using AI.

## CRediT author statement

**Piyawan Boonyanuphong**: Conceptualization; Investigation; Methodology; Formal analysis and

Visualization. **Thammarat Kaewmanee**: Resources; Conceptualization and Visualization. **Tanyarath Utaipan**: Conceptualization; Methodology; Investigation; Formal analysis; Writing - Review & Editing; Project administration and Funding acquisition.

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# Supplementary Materials

**Table S1** Identification of phytochemicals of *Aquilaria subintegra* tea leaves by LC-MS/MS QTOF.

| Compound Identification Name | Classification of Phytochemicals (Class - Sub class) |
|---|---|
| Quinic acid | Organooxygen compounds - Alcohols and polyols |
| Isoorientin 6"-O-glucoside | Flavonoids - Flavonoid glycosides |
| 3-(a-Naphthoxy)lactic acid glucuronide | Hydroxy acids and derivatives -Alpha hydroxy acids and derivatives |
| Chrysoeriol 7-[feruloyl-(->2)-Glucuronyl -(1->2)-glucuronide] | Flavonoids - Flavonoid glycosides |
| Dihydrocaffeic acid 3-O-glucuronide | Cinnamic acids and derivatives - Hydroxycinnamic acids glycosides |
| Mangiferin | Benzopyrans - 1-benzopyrans |
| Vitexin 4'-O-galactoside | Flavonoids - Flavonoid glycosides |
| Mangiferin | Benzopyrans - 1-benzopyrans |
| 3-(a-Naphthoxy)lactic acid glucuronide | Hydroxy acids and derivatives -Alpha hydroxy acids and derivatives |
| Hesperetin-7-O-glucuronide | Flavonoids - Flavonoid glycosides |
| Tagetiin | Flavonoids - O-methylated flavonoids |
| Eriodictyol 7-O-glucoside | Benzene and substituted derivatives - Benzoic acids and derivatives |
| Dihydroprudomenin | Flavonoids - Flavonoid glycosides |
| b-D-Glucopyranuronic acid, 1-(6-methoxy-2-naphthaleneacetate) | Organooxygen compounds - Carbohydrates and carbohydrate conjugates |
| Isochinomin | Benzopyrans - 1-benzopyrans |
| Mangiferin | Benzopyrans - 1-benzopyrans |
| Eriodictyol 7-glucoside | Benzene and substituted derivatives - Benzoic acids and derivatives |
| Urolithin A-3-O glucuronide | Organooxygen compounds - Carbohydrates and carbohydrate conjugates |
| Vitexin 4'-O galactoside | Flavonoids - Flavonoid glycosides |
| Mangiferin | Benzopyrans - 1-benzopyrans |
| Quercetin 3-galactoside | Flavonoids - Flavonoid glycosides |
| Dihydrogenistin | Isoflavonoids - Isoflavonoid O-glycosides |
| (+)-7-epi-Syringaresinol 4'-glucoside | Lignan glycosides – subclass not available |
| Kaempferol 3 rhamnoside-(1->2) rhamnoside | Flavonoids - Flavonoid glycosides |
| Naringenin 5,7-di-O-glucoside | Flavonoids - Flavonoid glycosides |
| 4-(4-Hydroxyphenyl) 2-butanone O-[2,6 digalloylglucoside] | Flavonoids - Flavonoid glycosides |
| Dihydrogenistin | Isoflavonoids - Isoflavonoid O-glycosides |
| Cucurbitacin I 2 glucoside | Flavonoids - Flavonoid glycosides |
| (+)-Syringaresinol | Furanoid lignans |
| Ganoderic acid epsilon | Prenol lipids - Triterpenoids |
| Ganoderic acid K | Prenol lipids - Triterpenoids |
| Biochanin A | Isoflavonoids - O-methylated isoflavonoids |
| Gingerol | Phenols - Methoxyphenols |