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Agarwood Tea Leaves: Uncovering the Antioxidant Phytochemicals and Their Protective Role Against Hepatocyte Oxidative

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Abstract

Liver cells play a key role in metabolizing nutrients, drugs, and foreign substances, during which reactive oxygen species (ROS) are generated. Excess ROS can lead to oxidative stress, contributing to liver diseases. Thus, controlling ROS is important for liver cell protection. This study aimed to investigate the chemical composition and antioxidant potential of *Aquilaria subintegra* (agarwood) leaves extract and its protective effects against hydrogen peroxide (H_2O_2)-induced oxidative stress in HepG2 cells. Phytochemical profiling was performed using LC-MS/MS QTOF. HepG2 cells were pre-treated with non-toxic concentrations of the extract for 24 h, followed by exposure to H_2O_2. Cell viability was assessed by MTT assay. Intracellular ROS was measured using 2',7'-dichlorofluorescein (DCF), while antioxidant enzyme activities, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), were determined using commercial kits. The results of the study showed that the phytochemicals found in the leaves of *A. subintegra* are diverse. It is mainly a substance in the group of flavonoids, benzopyrans, and organooxygen. The major antioxidants classified include quercetin 3-galactoside, mangiferin, isoorientin 6''-O-glucoside, vitexin 4'-O-galactoside, quinic acid, ganoderic acids and biochanin A. Agarwood tea leaves extract showed an effect in preventing H_2O_2-induced oxidative stress in HepG2 cells at concentrations ranging from 1.95 to 15.63 µg/mL, with a significant reduction in ROS levels at concentrations ranging from 1.95 to 7.81 µg/mL compared to the control group. The extract did not influence the activity of SOD and CAT enzymes; however, it significantly enhanced the activity of GPx enzyme within the concentration range of 7.81 to 62.50 µg/mL. In conclusion, the results of the study suggest that agarwood tea leaves extract may have an antioxidative effect on liver cells. This may be due to the presence of high amounts of antioxidants, which may be a promising strategy for mitigating oxidative damage in liver-related diseases.

Keywords: Agarwood tea leaves, HepG2, Reactive oxygen species, Cytoprotective

Introduction

Oxidative stress, a condition resulting from an imbalance between reactive oxygen/nitrogen species (ROS/RNS) and the body's antioxidant defense mechanisms, poses a significant threat to human health. These highly reactive species, originating both externally (from sources such as food, environmental pollutants, sunlight, and cigarette smoke) and internally within the body (through metabolic processes and inflammation), catalyze oxidation reactions that target vital biomolecules like DNA, lipids, and proteins [1,2]. This oxidative imbalance has been associated with the onset and progression of various human diseases, including cancer, diabetes, heart diseases, and notably, several liver-related disorders. The liver, a crucial metabolic organ, is particularly vulnerable to oxidative stress due to its multifaceted functions, including drug

metabolism and alcohol detoxification, leading to the generation of free radicals and reactive species [3]. Consequently, this oxidative insult results in cellular damage, fibrosis, and ultimately hepatocellular carcinoma, the most common form of liver cancer. To counteract the detrimental effects of oxidative stress, the body employs a defense system involving enzymatic and non-enzymatic antioxidants to maintain a delicate balance of free radicals.

As a strategy to mitigate the impact of oxidative stress-related diseases, the use of antioxidants derived from plants, including fruits, vegetables, and herbs, has garnered considerable attention. These plant-derived compounds, rich in antioxidants such as vitamins C and E, polyphenols, flavonoids, and tocopherols, serve as vital components in defending against oxidative damage and maintaining cellular integrity [4-7]. Among these natural sources, agarwood, renowned for its valuable essential oil, has emerged as an object of interest. Extracts from various parts of the agarwood tree, notably its leaves, have exhibited promising pharmacological properties, including anti-diabetic, anti-inflammatory, antimicrobial, and notably, antioxidant effects [8]. These extracts, containing diverse bioactive compounds like phenolics, steroids, fatty acids, benzofurans, santonoids, flavonoids, terpenoids, tannins, and alkenes [9,10], have shown potential in mitigating oxidative stress-related liver diseases through mechanisms such as lipid peroxidation reduction, DNA damage protection, and enhancement of antioxidant enzyme activity [4-7]. Despite extensive research on agarwood extracts, much attention has focused on chemical analyses using various assays, such as DPPH, TAC, and CUPRAC, with limited exploration of their biological effects, particularly on liver cells. The disparity between chemical analysis and biological studies arises due to the complexity of cellular mechanisms influencing antioxidant capacities, warranting a more comprehensive investigation. Given the liver's pivotal role in metabolic processes and xenobiotic metabolism, hepatocytes serve as an excellent model for studying the biological antioxidant properties against oxidative stress [11]. Hence, this study aims to identify bioactive compounds using LC-MS/MS QTOF and to assess the hepatoprotective potential of agarwood tea leaves extract against oxidative stress induced by hydrogen peroxide. This evaluation involves measuring cell viability, quantifying intracellular ROS levels, and evaluating the activities of key antioxidant enzymes like SOD, CAT, and GPx. Thus, this research will provide deeper insights into the biological antioxidant properties of agarwood leaves extracts, specifically in combating oxidative stress-related liver damage, and contributing to the understanding and potential therapeutic applications of natural antioxidants in mitigating oxidative stress-induced diseases.

Materials and methods

Chemicals and reagents

Hepatocellular carcinoma (HepG2) cells (ATCC® HB-8065™) were obtained from the American Type Culture Collection (ATCC). All cell culture reagents were purchased from Gibco, Thermo Fisher Scientific, Inc. All chemical reagents used for the analysis of antioxidant content were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific, Germany. SOD, CAT and GPx activities levels in cell extracts were measured using Cayman Chemicals assay kits (Ann Arbor, MI).

Preparation of agarwood tea leaves extract

The sample of agarwood leaves tea was obtained from the community enterprise involved in the processing of Thai agarwood wood, "Rueng Kaset-Sriwichai" (Sadao), located in Sadao district, Songkhla province. Twenty-four g of agarwood leaves were infused in 400 mL of boiling water for 30 min, repeated twice. The resulting extracts were combined and filtered, then concentrated using a rotary evaporator under vacuum at a temperature of 40 °C. Subsequently, the concentrated extracts were dried using a freeze-drying apparatus. The total yield of the extracts was weighed and stored at –20 °C in light-protected containers until further analysis.

Determination of total phenolic contents (TPC)

The Folin-Ciocalteu reagent was used to determine the total phenolic content of the extracts following the method of Aguilar-Garcia *et al*. [12]. A 2.5 mL volume of diluted Folin-Ciocalteu reagent, combined with 60 µL of concentrated agarwood leaves tea extract at 1 mg/mL, was initially kept shielded from light for 2 min. Then, 2.0 mL of 7.5% sodium carbonate

solution was added to the mixture, thoroughly combined, and subsequently incubated in a water bath at 50 °C for 15 min. Following incubation, the absorbance was measured at a wavelength of 760 nm. The results were expressed as mg gallic acid equivalent (GAE)/100 g extract.

Determination of total flavonoid contents (TFC)

The total flavonoid content of the crude extract was assessed using the aluminum chloride colorimetric assay, with some modification, based on the method described by Adusei *et al*. [13]. Briefly, the agarwood tea leaves extract at a concentration of 1 mg/mL with a volume of 250 µL was mixed with deionized water at a volume of 1.25 mL and 5% Sodium Nitrate at a volume of 75 µL, kept for 5 min. Subsequently, 10% Aluminum chloride at a volume of 150 µL was added and kept for an additional 5 min, followed by the addition of 1 M Sodium hydroxide at a volume of 0.5 mL. The resulting solution was thoroughly mixed and then its absorbance was measured at a wavelength of 510 nm.

LC-MS/MS QTOF analysis of the agarwood tea leaves extract

One mL of the agarwood tea leaves extract was dissolved in 0.5 mL of hot water, and the bioactive compounds were analyzed using an HPLC system (1290 Infinity II liquid chromatography) connected to a 6545 quadrupole-time-of-flight mass spectrometer. The condition of analysis was adapted according to Ito *et al*. [14]. Chromatography was set with a binary solvent composition (LC-MS grade water and 0.1% acetic acid; phase A and acetonitrile and 0.1% acetic acid; phase B) at a flow rate of 0.2 mL/min at 40 °C for column compartment stability. The gradient method was as follows: 30 min, 50% B; 40 min, 50% B; 42 min, 10% B. A Q-TOF mass spectrometer with Dual AJS Electrospray Ionization (ESI) in negative ion mode was used. The acquisition mode of the instrument is set to AutoMS2, with a range from 100 to 1200 m/z for both MS and MS/MS. For MS/MS data acquisition, the isolation width with collision energies was fixed at 10, 20, and 40 eV. Instrument parameters include a gas temperature of 325 °C with a flow rate of 13 L/min, a sheath gas temperature of 275 °C, and a nebulizer pressure of 35 psig. Injections are conducted with a volume of 10 µL. Mass Hunter Workstation software (Agilent Technologies, Santa Clara, CA, USA) was used for analysis.

HepG2 cell culture

The human hepatoma cell line (HepG2) HB-8065TM was purchased from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin at 37 °C in 5% CO_2 and 95% relative humidity.

Cell viability assay

HepG2 cells were seeded at a density of 1×10^5 cells per well in 100 µL of medium on 96-well plates and allowed to adhere for 24 h before treatment. The cells were then incubated with agarwood tea leaves extract at concentrations ranging from 15.63 to 2000 µg/mL for another 24 h, while complete medium was used as the control. Following incubation, the culture medium was discarded, and 100 µL of MTT solution (0.5 mg/mL) was added to each well, followed by a 3 h incubation period. Afterward, 100 µL of DMSO was introduced into each well to fully dissolve the formazan crystals. The absorbance was measured at 570 nm, with a reference wavelength of 630 nm, using a microplate reader. The results were expressed as percentage cell viability relative to the control.

Cytoprotective effect of agarwood tea leaves extract on H_2O_2-induced oxidative stress in HepG2 cells

HepG2 cells were cultured in 96-well plates at a density of 1×10^5 cells per well in 100 µL of medium and left to adhere for 24 h before treatment. Cells were exposed to varying non-toxic concentrations of the extract (0.98 - 62.50 µg/mL, 100 µL) for 24 h. Following this incubation, 100 µL of 1 M H_2O_2 was introduced, and the cells were further incubated for an additional 3 h. Cell viability was subsequently determined using the MTT assay. For the positive control, cells were treated with varying non-toxic concentrations of NAC (125 - 1000 µg/mL, 100 µL) for 24 h, followed by the addition of 100 µL of 1 M H_2O_2 and further incubation for 3 h. For the negative control,

the extract was excluded and replaced with culture media containing 1 mM H_2O_2.

The impact of agarwood tea leaves extract on intracellular reactive oxygen species (ROS) production

The measurement of intracellular ROS levels was performed using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), following the method of Renis *et al*. [15]. Cells were seeded at a density of 3×10^5 cells per well in a 6-well plate and cultured for 24 h. Cells were treated with different non-toxic concentrations of the extract (0.98 - 62.50 µg/mL, 100 µL) for 24 h. After incubation, 100 µL of 1 M H_2O_2 was added, followed by an additional 3 h incubation. Following incubation, the medium was removed, and cells were rinsed with PBS. Subsequently, 100 µL of 10 µM DCFH-DA was added and incubated for 30 min. The fluorescence intensity was measured using a spectrofluorometer (λ excitation at 488 nm, λ emission at 525 nm). For the positive control, cells were treated with NAC and 1 mM H_2O_2 for 3 h, whereas in the negative control, the extract was replaced with cell culture media containing 1 mM H_2O_2.

The effects of agarwood tea leaves extract on the activities of antioxidant enzymes SOD, CAT, and GPx

Cells were seeded at a density of 3×10^5 per well in a 6-well plate and treated with agarwood tea leaves extract (0.98 - 62.50 µg/mL) for 24 h. After incubation, 1 mM H_2O_2 was introduced for 3 h to induce oxidative stress. Lysis was performed using an ice-cold solubilization buffer, followed by centrifugation at 10,000× g for 15 min at 4 °C. The supernatant was collected and analyzed for SOD, CAT, and GPx enzyme activities using Cayman Chemicals assay kits.

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM), derived from 3 independent experiments. Statistical analysis was conducted using GraphPad Prism 6.0 (GraphPad Software, USA). Group differences were assessed through one-way ANOVA, with statistical significance set at $P < 0.05$ (95% confidence level).

Results and discussion

Total phenolic and flavonoid content in agarwood tea leaves extract

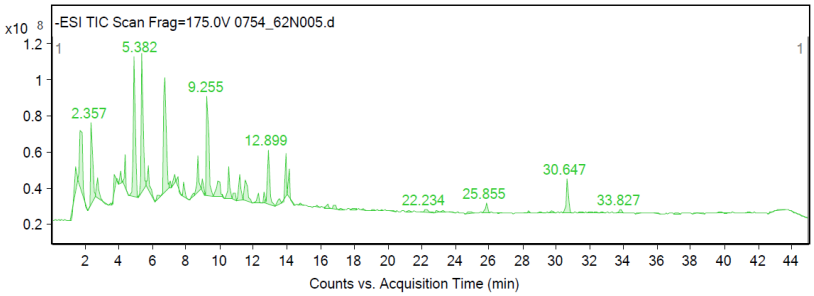
The analysis aimed to determine the phenolic and flavonoid content of agarwood tea leaves extract obtained from hot water extraction. The outcomes, as depicted in **Table 1**, revealed a total phenolic content measuring 18.30 ± 0.69 mg GAE/g of dry weight and a total flavonoid content of 22.90 ± 0.10 mg QE/g of dry weight. These quantities showed variability based on multiple factors, including the extraction solvent, agarwood species, leaves maturity, and extraction method. In a related study by Rashid *et al*. [16], *Aquilaria malaccensis* leaves were extracted using various solvents. The findings revealed that water extraction resulted in a significantly higher total phenolic content of 30.76 ± 7.63 mg GAE/g. However, the highest phenolic content was observed with 70% ethanol extraction, measuring 39.99 ± 11.95 mg GAE/g, compared to other solvents. Despite the superior phenolic content observed with 70% ethanol, the adoption of water as a solvent remains favorable due to its appropriateness for consumption and environmental compatibility compared to ethanol, methanol, or hexane solvents.

Table 1 Total phenolic and flavonoid content in agarwood tea leaves extract.

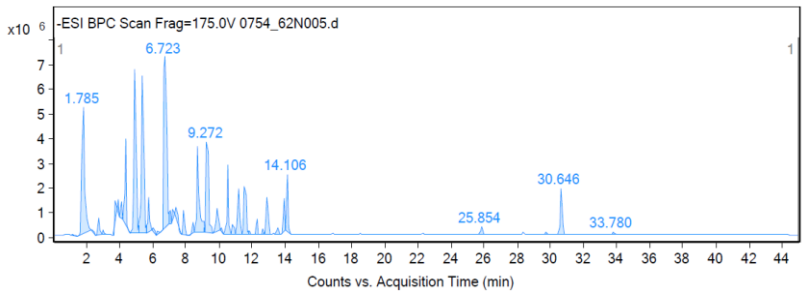
Sample	Total phenolic content (TPC) (mg GAE/g dry weight)	Total flavonoid content (TFC) (mg QE/g dry weight)
Agarwood tea leaves extract	18.30 ± 0.69	22.90 ± 0.10

Identification of Phytochemicals in agarwood (*A. subintegra*) tea leaves extract using LC-ESI-QTOF-MS/MS

Agarwood is highly valued for its unique fragrance and therapeutic properties, which are attributed to its rich composition of bioactive compounds. Identifying and quantifying these compounds using liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers high sensitivity and specificity, allowing precise detection of key bioactive constituents [17]. Additionally, it is useful for distinguishing agarwood from different sources [18,19]. Quantification of bioactive compounds also helps in understanding their pharmacological potential, supporting scientific research on agarwood's medicinal properties, such as its antioxidant and cytotoxic activity against human cell lines [19]. Thus, in this study analyzed the phytochemical profile of agarwood (*A. subintegra*) tea leaves using LC-MS/MS QTOF. **Figure 1** shows the total ion current (TIC) chromatograms and selective ion monitoring (SIM) mode in negative ionization. Identified compounds with molecular formulas, key ions (m/z), masses, and retention times are listed in **Table 2**. The 26 bioactive compounds in agarwood tea leaves were found and classified into different types of phytochemicals using online databases (https://foodb.ca & https://pubchem.ncbi.nlm.nih.gov). Flavonoids were the most common class of phytochemicals found in this study, followed by benzopyrans and organooxygen compounds (**Table 2**). Flavonoid glycosides, such as isoorientin 6"-O-glucoside, quercetin 3-galactoside, and vitexin 4'-O-galactoside, have been found to be important antioxidants. These glycosides are well-known for their potent ability to scavenge free radicals and provide protection against oxidative damage. These mechanisms are critical in preventing chronic conditions like cardiovascular diseases and cancer [20,21]. Among these bioactive constituents, mangiferin stands out as a primary bioactive compound in *A. subintegra* tea leaves, belonging to the benzopyran group (**Figure 2**). Consistent with findings by Ito *et al*. [14], mangiferin was also detected in *Aquilaria crassna* extracts using different solvents. Mangiferin, with its abundance of hydroxyl (-OH) groups, acts as a potent antioxidant by transferring hydrogen atoms or electrons to neutralize free radicals, effectively reducing oxidative stress [22]. Its C-glucoside linkage further enhances stability and antioxidant efficacy in biological systems, while its hepatoprotective effects include reducing liver inflammation, preventing oxidative damage to hepatocytes, and improving liver function [23,24]. Quercetin 3-galactoside, another key flavonoid, enhances water solubility through its sugar moiety, improving bioavailability and distribution in aqueous environments. The combination of these properties explains why *A. subintegra* tea may protect liver cells from H_2O_2-induced oxidative stress by reducing ROS levels. Additionally, quinic acid, a known antioxidant, was identified, further enhancing the tea's liver-protective and anti-inflammatory profile [25,26]. Ganoderic acids, triterpenoids typically found in Ganoderma species, were also detected. These compounds neutralize free radicals, reduce lipid peroxidation, and enhance antioxidant enzymes such as SOD, strengthening cellular defenses against oxidative stress [27,28]. Lastly, biochanin A, an isoflavone with antioxidant and estrogenic properties [29], was identified (**Table 2**). Its synergistic effects with other flavonoids further contribute to the potential of the tea to combat oxidative stress. Together, the unique phytochemical profile of *A. subintegra* tea suggests its potential in reducing oxidative stress and supporting liver health.

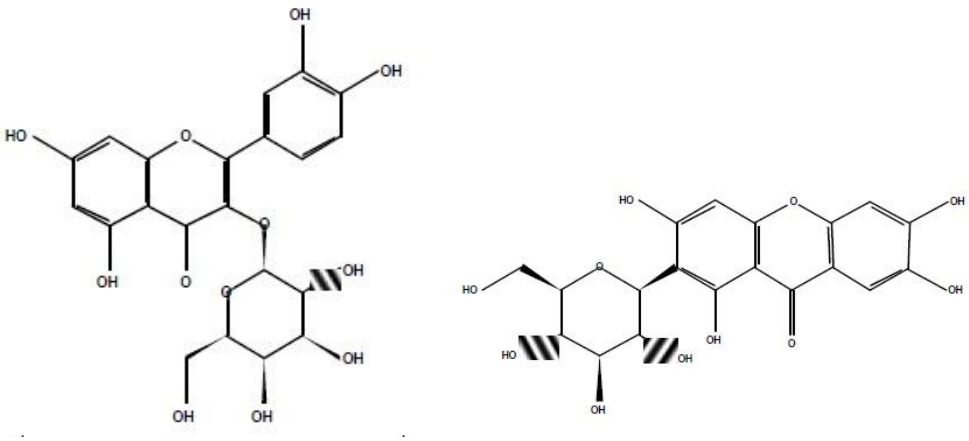


(A)



(B)

Figure 1 The chromatograms of (A) the MS TIC and (B) the SIM mode in negative mode of agarwood tea leaves.



(A) (B)

Figure 2 Structure of (A) Quercetin 3-galactoside and (B) Mangiferin found in agarwood tea leaves.

Table 2 Identification of phytochemicals of agarwood (*A. subintegra*) tea leaves by LC-MS/MS QTOF.

General					Compound identification	Classification of phytochemicals
Formula	**m/z**	**Mass**	**RT**	**Cpd.**	**Name**	**(Class - Sub class)**
C7 H12 O6	191.0560	192.0633	1.785	59	Quinic acid	Organooxygen compounds - Alcohols and polyols
C26 H28 O16	595.1305	596.1373	3.815	215	Isoorientin 6"-O-glucoside	Flavonoids - Flavonoid glycosides
C19 H20 O10	407.0993	408.1065	5.382	304	3-(a-Naphthoxy)lactic acid glucuronide	Hydroxy acids and derivatives -Alpha hydroxy acids and derivatives
C38 H36 O21	827.1691	828.1758	5.939	349	Chrysoeriol 7-[feruloyl-(->2)-Glucuronyl -(1->2)-glucuronide]	Flavonoids - Flavonoid glycosides

General					Compound identification	Classification of phytochemicals
Formula	**m/z**	**Mass**	**RT**	**Cpd.**	**Name**	**(Class - Sub class)**
C15 H18 O10	357.0828	358.09	6.386	361	Dihydrocaffeic acid 3-O-glucuronide	Cinnamic acids and derivatives - Hydroxycinnamic acids glycosides
C19 H18 O11	421.0778	422.0851	6.724	376	Mangiferin	Benzopyrans - 1-benzopyrans
C27 H30 O15	593.1519	594.1592	7.041	398	Vitexin 4'-O-galactoside	Flavonoids - Flavonoid glycosides
C19 H18 O11	421.0779	422.0852	7.243	404	Mangiferin	Benzopyrans - 1-benzopyrans
C19 H20 O10	407.0990	408.1062	7.400	419	3-(a-Naphthoxy)lactic acid glucuronide	Hydroxy acids and derivatives - Alpha hydroxy acids and derivatives
C21 H22 O12	465.1051	466.1122	8.592	474	Hesperetin-7-O-glucuronide	Flavonoids - Flavonoid glycosides
C21 H20 O13	479.0830	480.0911	8.866	488	Tagetiin	Flavonoids - O-methylated flavonoids
C21 H22 O11	449.1089	450.1161	8.874	489	Eriodictyol 7-O-glucoside	Benzene and substituted derivatives - Benzoic acids and derivatives
C25 H30 O14	553.1569	554.1641	8.903	492	Dihydroprudomenin	Flavonoids - Flavonoid glycosides
C19 H20 O9	391.1042	392.1115	9.269	517	β-D-Glucopyranuronic acid, 1-(6-methoxy-2-naphthaleneacetate)	Organooxygen compounds - Carbohydrates and carbohydrate conjugates
C20 H20 O11	435.0936	436.1008	9.756	545	Isochinomin	Benzopyrans - 1-benzopyrans
C19 H18 O11	421.0784	422.0857	9.872	549	Mangiferin	Benzopyrans - 1-benzopyrans
C21 H22 O11	449.1104	450.1173	10.154	566	Eriodictyol 7-glucoside	Benzene and substituted derivatives - Benzoic acids and derivatives
C19 H16 O10	403.0678	404.075	10.521	581	Urolithin A-3-O glucuronide	Organooxygen compounds - Carbohydrates and carbohydrate conjugates
C27 H30 O15	593.1513	594.1584	11.168	615	Vitexin 4'-O galactoside	Flavonoids - Flavonoid glycosides
C19 H18 O11	421.0783	422.0855	11.533	639	Mangiferin	Benzopyrans - 1-benzopyrans
C21 H20 O12	463.0888	464.096	11.767	646	Quercetin 3-galactoside	Flavonoids - Flavonoid glycosides
C21 H22 O10	433.1142	434.1215	12.268	659	Dihydrogenistin	Isoflavonoids - Isoflavonoid O-glycosides
C28 H36 O13	579.2087	580.2157	12.635	672	(+)-7-epi-Syringaresinol 4'-glucoside	Lignan glycosides - subclass not available
C27 H30 O14	577.157	578.1641	12.983	691	Kaempferol 3 rhamnoside-(1->2) rhamnoside	Flavonoids - Flavonoid glycosides
C27 H32 O15	595.168	596.1749	13.547	708	Naringenin 5,7-di-O-glucoside	Flavonoids - Flavonoid glycosides
C30 H30 O15	629.1513	630.1585	13.827	724	4-(4-Hydroxyphenyl) 2-butanone O-[2,6 digalloylglucoside]	Flavonoids - Flavonoid glycosides
C21 H22 O10	433.1151	434.1224	14.106	743	Dihydrogenistin	Isoflavonoids - Isoflavonoid O-glycosides
C36 H52 O12	735.3593	676.3453	16.775	811	Cucurbitacin I 2 glucoside	Flavonoids - Flavonoid glycosides
C22 H26 O8	417.1556	418.1628	18.513	837	(+)-Syringaresinol	Furanoid lignans
C30 H44 O7	515.3018	516.3088	25.851	898	Ganoderic acid epsilon	Prenol lipids - Triterpenoids
C32 H46 O9	573.3068	574.3139	25.86	908	Ganoderic acid K	Prenol lipids - Triterpenoids
C16 H12 O5	283.0612	284.0684	29.728	920	Biochanin A	Isoflavonoids - O-methylated isoflavonoids
C17 H26 O4	293.1766	294.1838	30.645	928	Gingerol	Phenols - Methoxyphenols

Cytotoxicity testing of agarwood tea leaves extract

The objective of this study was to evaluate the impact of agarwood tea leaves extract on the viability of HepG2 cells, aiming to establish safe concentrations for future investigations into the extract's ability to protect against oxidative stress induced by H_2O_2. HepG2 cells were exposed to varying concentrations of agarwood tea leaves extract (ranging from 0 to 2000 µg/mL) in a 100 µL solution for a duration of 24 h. Viability was assessed through the application of the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, a method wherein viable cells, utilizing the succinate dehydrogenase enzyme in their mitochondria, transform MTT into formazan, visibly altering its color to purple, thereby facilitating absorbance measurement. **Figure 3** illustrates a notable decline in cell viability at concentrations ranging from 125 to 2000 µg/mL in comparison to the control group ($P < 0.05$). Conversely, concentrations at or below 62.5 µg/mL exhibited no significant impact on cell viability. The IC_{50} value was determined to be 701.28 µg/mL. Hence, concentrations of the extract lower than 62.5 µg/mL were identified as non-cytotoxic and deemed suitable for subsequent experimental analyses.

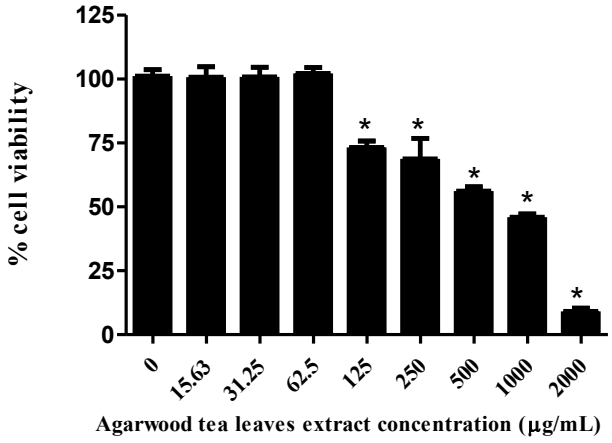


Figure 3 Cytotoxic effect of agarwood tea leaves extract (0 - 2000 µg/mL) on HepG2 cells. Cell viability was measured using the MTT assay. Data are presented as mean ± SEM (N = 3).
*Indicates a statistically significant difference ($p < 0.05$) compared to the control.

Cytoprotective effect of agarwood tea leaves extract on H_2O_2-induced oxidative stress in HepG2 cells

The protective effect of agarwood tea leaves extract against H_2O_2-induced cell injury was evaluated by pre-treating HepG2 cells with non-cytotoxic concentrations of the extract (0.98 - 62.50 µg/mL) for 24 h, followed by exposure to 1 mM H_2O_2 for 3 h. Cell viability was assessed using the MTT assay. This study aimed to investigate the potential of agarwood tea leaves extract in mitigating H_2O_2-induced oxidative stress in HepG2 cells. After the 24 h extract exposure, cells were then exposed to 1 mM H_2O_2 for 3 h. Subsequently, survival rates were compared to a control group (cells exposed solely to 1 mM H_2O_2). Results highlighted the significant protective effect of agarwood tea leaves extract at concentrations ranging from 1.95 to 15.63 µg/mL ($p < 0.05$). These concentrations displayed elevated survival rates of 61.20%, 73.10%, 73.02%, and 63.58% at 1.95, 3.90, 7.81, and 15.63 µg/mL, respectively (**Figure 4**). NAC served as a positive control in the range of 125 - 1000 µg/mL. Extract concentrations of 3.90 - 7.81 µg/mL exhibited cell protection similar to NAC against H_2O_2-induced damage. The extract's protective potential against cellular injury from H_2O_2 likely arises from its antioxidant compounds, like flavonoids, tannins, and saponins, found in agarwood tea leaves extract [10,30-32]. This protection might involve both direct scavenging of free radicals and indirect modulation of antioxidant enzymes. Furthermore, the extract notably decreased reactive oxygen species (ROS) production

compared to the control (**Figure 5**), attributed to its antioxidant capacity. Nonetheless, at higher concentrations (31.25 - 62.50 µg/mL), the extract failed to display significant differences in cell survival compared to the control without extract, implying diminished protective effects against oxidative stress. Elevated levels of flavonoids, particularly above 15.63 µg/mL, might provoke free radical generation, known as the pro-oxidant effect. This reaction aligns with ROS level findings (**Figure 5**), potentially explaining the extract's reduced protective capabilities against oxidative stress at higher concentrations.

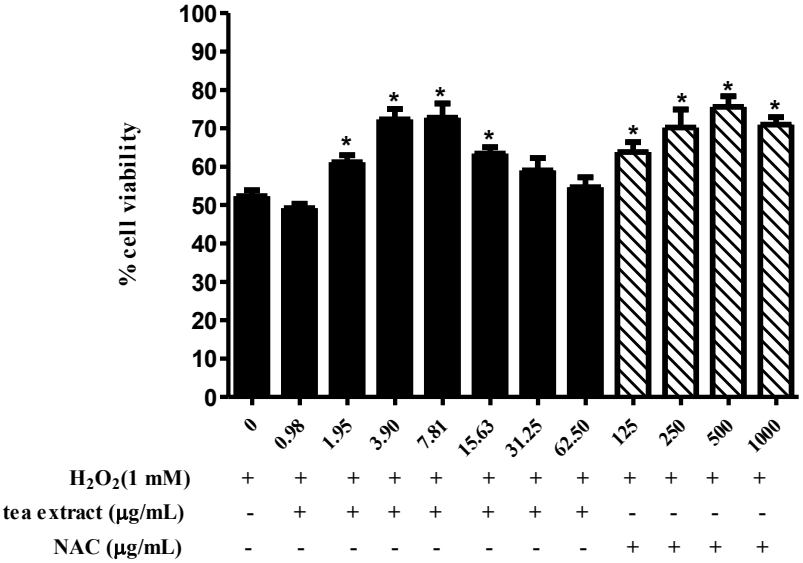


Figure 4 Protective effect of agarwood tea leaves extract on H_2O_2-induced cytotoxicity. HepG2 cells were pre-treated with non-toxic extract concentrations (0.98 - 62.50 µg/mL) for 24 h, followed by 1 mM H_2O_2 for 3 h. Cell viability was assessed via MTT assay. Positive control: NAC (125 - 1000 µg/mL) with 1 mM H_2O_2. Negative control: Culture media with 1 mM H_2O_2. Data are presented as mean ± SEM (N = 3).
*Indicate statistically significant differences ($p < 0.05$) compared to negative control.

Effect of agarwood tea leaves extract on intracellular reactive oxygen species (ROS) level

HepG2 cells were seeded at 3×10^5 cells per well in 6-well plates and treated with agarwood tea leaves extract (0.98 - 62.50 µg/mL) for 24 h, followed by 1 mM H_2O_2 for 3 h. After incubation, cells were rinsed with PBS and incubated with 100 µL of 10 µM DCFH-DA for 30 min. ROS levels were measured using a spectrofluorometer (excitation: 488 nm, emission: 525 nm). As shown in **Figure 5**, the extract significantly reduced ROS production at concentrations between 1.92 and 7.81 µg/mL ($p < 0.05$) compared to untreated controls. This highlights the extract's potential antioxidants in inhibiting ROS generation, mitigating cellular damage similar to the positive control (NAC). However, the concentration increase from 15.63 to 62.50 µg/mL did not significantly decrease ROS levels compared to the control. This suggests the potential pro-oxidant tendencies of higher extract concentrations, consistent with previous discussions. These findings corroborate Kong *et al*. [33] observations on the declining efficacy of *Barringtonia racemosa* (L.) flower extract in reducing ROS production as the extract concentration rises.

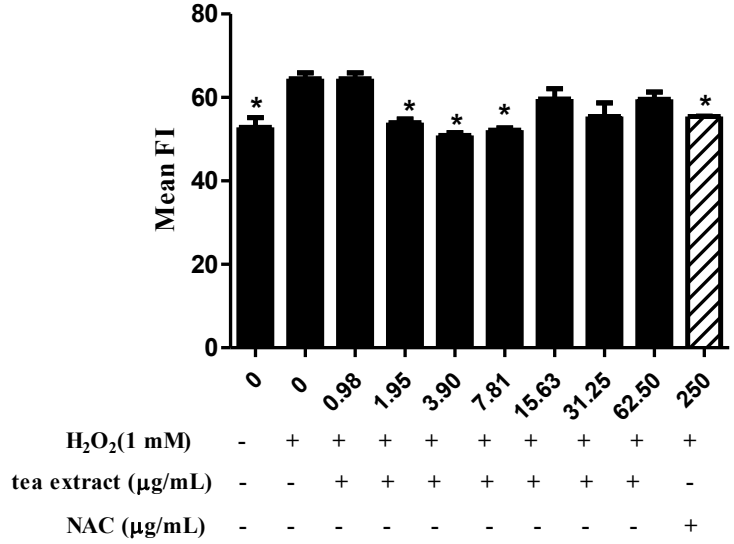


Figure 5 Effect of agarwood tea leaves extract on intracellular ROS levels. HepG2 cells were pre-treated with various concentrations of extract (0.98 - 62.50 µg/mL) for 24 h, followed by exposure to 1 mM H_2O_2 for 3 h. Intracellular ROS levels were measured using 10 µM DCFH-DA. The relative ROS levels (%) are presented as mean ± SEM (N = 3). $^*p < 0.05$ compared to the control (no extract).

Effect of agarwood tea leaves extract on antioxidant enzymes (SOD, CAT and GPx)

Antioxidants not only scavenge free radicals but also regulate antioxidant enzymes, helping mitigate oxidative stress [6]. To assess enzyme activity (SOD, CAT, and GPx), cells were treated with agarwood tea extract (0.98 - 62.50 µg/mL) for 24 h, followed by 1 mM H_2O_2 for 3 h. Enzyme activity in cell lysates was measured using Cayman Chemical kits. Results are shown in **Figures 6** - **8**. It was observed that the activities of SOD, CAT, and GPx enzymes in cells incubated with H_2O_2 alone were significantly reduced compared to control cells that were not treated with the extract and H_2O_2, showing reductions of 55.64%, 45.67%, and 72.03%, respectively. This decrease is attributed to oxidative stress within the cell caused by H_2O_2 passing through the cell membrane and initiating the Fenton reaction. The generation of the free radical hydroxyl radical upon encountering metal ions within the cell [34] may therefore contribute to a decrease in antioxidant enzyme activity. This aligns with previous studies showing a decrease in antioxidant enzyme activity under oxidative stress conditions [35-37]. However, when cells were incubated with non-toxic concentrations of the extract, no statistically significant effect on SOD enzyme activity (**Figure 6**) and CAT enzyme activity (**Figure 7**) was observed compared to cells treated with H_2O_2 alone. Agarwood tea extract failed to fully restore the decreased antioxidant activity in stressed cells to the same level as normal cells. Notably, a trend of increased SOD enzyme activity was observed within the concentration range of 1.95 - 7.81 µg/mL, which coincides with the concentration range protecting cells from induced H_2O_2 (**Figure 6**). Salla *et al*. [37] investigated the effect of papaya seed extract on SOD, CAT, and GPx enzyme activities in HepG2 cells subjected to oxidative stress with H_2O_2. They observed an increase in these enzyme activities under oxidative stress conditions with papaya seed extract. Furthermore, NAC, used as a positive control antioxidant at concentrations of 500 - 1000 µg/mL, significantly restored SOD and CAT activities, showing statistical significance at $p < 0.05$ compared to cells treated with H_2O_2 alone. Regarding the effect of agarwood tea leaves extract on GPx enzyme activity (**Figure 8**), our findings demonstrate that agarwood tea leaves extract significantly increased GPx activity in a dose-dependent manner, particularly at 7.81 - 62.50 µg/mL, restoring levels comparable to untreated control cells. In contrast, NAC at higher concentrations (250 - 1000 µg/mL) caused a persistent elevation in GPx activity. This sustained increase is likely due to GPx's unique

regulation, which relies on reduced glutathione (GSH) as a substrate rather than coordinated activity with SOD and CAT [38,39]. As a precursor to GSH, NAC boosts intracellular GSH, thereby enhancing GPx function. The irreversible activation observed may result from prolonged GSH elevation, keeping GPx continuously active. These findings highlight the distinct regulatory role of GPx and the importance of balanced antioxidant enzyme activity in combating oxidative stress.

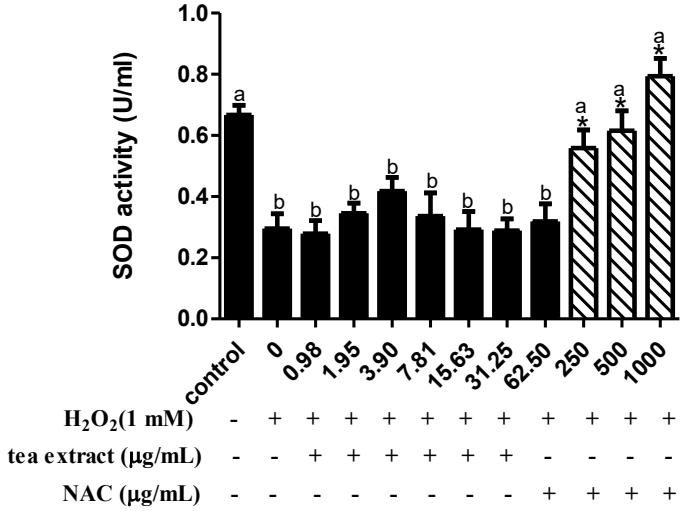


Figure 6 Effects of agarwood tea leaves extract on SOD activity in HepG2 cells. Cells were treated with varying concentrations (0 - 62.50 µg/mL) of extract for 24 h, followed by 1 mM H_2O_2 for 3 h. SOD activity was measured using Cayman chemical assay kits. Data are presented as mean ± SEM (n = 3).

*Indicates a significant difference ($p < 0.05$) compared to cells treated with H_2O_2 alone.

a-bIndicates a significant difference ($p < 0.05$) compared to the control without H_2O_2.

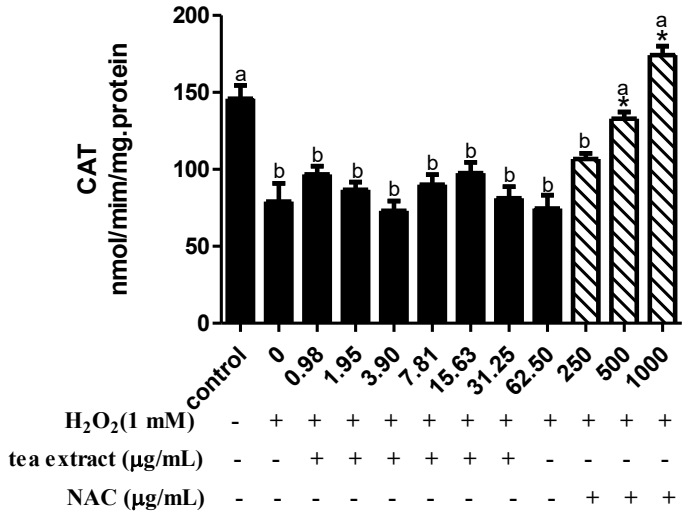


Figure 7 Effects of agarwood tea leaves extract on CAT activity in HepG2 cells. HepG2 cells were treated with varying concentrations of the extract (0 - 62.50 µg/mL) for 24 h, followed by exposure to 1 mM H_2O_2 for 3 h. CAT activity was assessed using Cayman chemical assay kits. Data are presented as mean ± SEM (n = 3).

*Indicates a significant difference ($p < 0.05$) compared to cells treated with H_2O_2 alone.

a, bIndicates a significant difference ($p < 0.05$) compared to the control without H_2O_2.

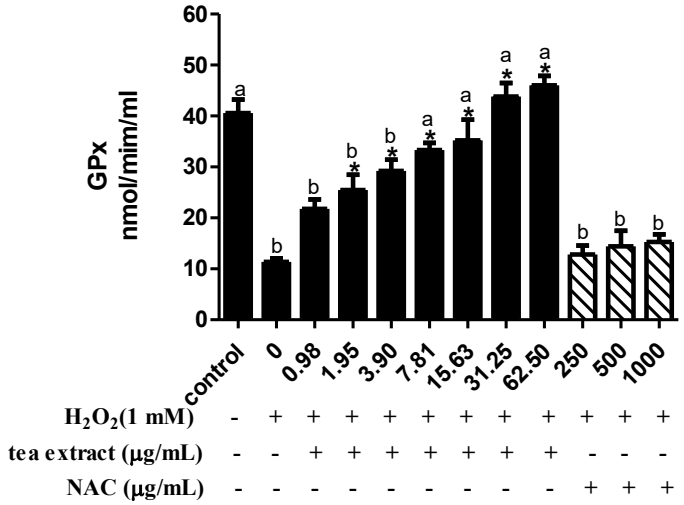


Figure 8 Effect of agarwood tea leaves extract on GPx activity in HepG2 cells. Cells were incubated with the extract (0 - 62.50 µg/mL) for 24 h, followed by 1 mM H_2O_2 for 3 h. NAC (250 - 1000 µg/mL) was used as a positive control. GPx activity was assessed using Cayman chemical assay kits. Data are presented as mean ± SEM (n = 3).
*Indicates a significant difference ($p < 0.05$) compared to cells treated with H_2O_2 alone.
a, bIndicates a significant difference ($p < 0.05$) compared to the control without H_2O_2.

Conclusions

The main phytochemicals identified in the agarwood tea leaves extract were quercetin 3-galactoside, mangiferin, isoorientin 6"-O-glucoside, vitexin 4'-O-galactoside, quinic acid, ganoderic acids and biochanin A, which are likely responsible for reducing reactive oxygen species (ROS) and oxidative stress. Agarwood tea leaves extract exhibited toxicity to HepG2 cells with an IC_{50} value of 701.28 µg/mL, while concentrations below 62.5 µg/mL were non-toxic. Our findings indicate that the extract can effectively protect cells against H_2O_2 induction at concentrations ranging from 1.95 to 15.63 µg/mL. This protection was associated with a significant decrease in ROS levels, particularly evident at concentrations of 1.95 - 7.81 µg/mL. While the extract did not alter the activities of antioxidant enzymes SOD and CAT, it did show a concentration-dependent increase in GPx enzyme activity within the range of 7.81 - 62.50 µg/mL. These results suggest that both direct free radical scavenging by phytochemicals in the extract and the upregulation of GPx activity contribute to the reduction in ROS, highlighting the potential of agarwood tea leaves extract to protect HepG2 cells from H_2O_2-induced oxidative damage.

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Declaration of Generative AI in Scientific Writing

Generative AI tools, namely QuillBot and ChatGPT (OpenAI), were employed exclusively for grammar correction and refinement of language expression. The conception of the study, literature synthesis, data analysis, interpretation of findings, and all aspects of critical discussion were entirely conducted by the author without reliance on AI.

CRediT Author Statement

Tanyarath Utaipan: Investigation, Data analysis, Visualization and Review of manuscript. **Patthamawadee Tongkaew:** Data analysis, Writing – review and editing. **Thammarat Kaewmanee:** Resources and Review of manuscript. **Piyawan Boonyanuphong:** Conceptualization, Methodology, Investigation, Data analysis, Writing – original draft, Supervision, Funding acquisition and Project administration.

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