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Short communication

Antioxidant activity of *Caesalpinia sappan* heartwood extracts against oxidative stress induced by hydrogen peroxide in MRC-5 cell lines

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Abstract

Background: *Caesalpinia sappan* (*C. sappan*) heartwood extract is a potentially effective antioxidant against oxidative stress. A few studies have been published on cell-based assay, especially in normal human lung fibroblast cells.
Objectives: To investigate how the concentration of ethanol used for extraction influence the antioxidant capacity of *C. sappan* heartwood extracts of against oxidative stress in human lung fibroblast MRC-5.
Methods: The antioxidant activity of crude extracts of *C. sappan* heartwood extracts were analyzed by chemi-based assay and cell-based assay (*in-vitro* lipid peroxidation), catalase (CAT), and superoxide dismutase (SOD) in MRC-5 cells.
Results: The results showed that the antioxidant effect of *C. sappan* heartwood extracts significantly depended on the percentage concentration of ethanol used for extraction. The crude extract prepared with 75% ethanol had the highest antioxidant effect. This extract had a total phenolic content of 741.8 mg GAE/ g CS extract, and it could reduce activities of CAT and SOD in H_2O_2-treated MRC-5 cells. The *C. sappan* extract could also inhibit lipid peroxidation in MRC-5 cells treated with H_2O_2.
Conclusion: These findings indicate that *C. sappan* heartwood extracts have a good antioxidative potential to reduce oxidative stress and could be used as a dietary supplement for maintaining health.

Keywords: Antioxidant activity, *Caesalpinia sappan,* human lung fibroblast MRC-5 cells, hydrogen peroxide

Reactive oxygen species (ROS) are produced normally in all aerobic organisms as by-products of metabolic pathways. The major source of ROS is the mitochondrial respiratory chain reaction. In normal cells, excess ROS is eliminated by the antioxidant defense system, including antioxidant enzymes—such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px)—and non-enzyme antioxidants to maintain balanced oxidative processes. However, an imbalance between ROS production and antioxidant defenses that leads to oxidative stress and damage occur in many pathogenic conditions including ischemic /reperfusion and exposure to exogenous chemical toxicants, such as particulate matter 2.5 (PM 2.5) and ultraviolet (UV). These overproduced ROS can attack protein, lipid, and DNA, which could lead to cell/tissue damage. [1-3] Oxidative damage is associated with many chronic diseases and pathological conditions including cancer, diabetes, neuro-degenerative, myocardial infarction, and pulmonary disease. [4, 5]

To date, many studies have been conducted in search of natural plant-derived antioxidants for preventing oxidative damage. [6] Plants contain polyphenols, such as phenolic compounds, and flavonoids, which are key antioxidants to attenuate oxidative stress directly via ROS scavenging and inhibiting ROS production. The antioxidant capacities of plant polyphenols have been demonstrated by activating enzyme expression and increasing enzyme

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activity.[7, 8] Additionally, polyphenols can suppress the inflammation response and activate the cell survival mechanism. [9]

Caesalpinia sappan (C. sappan) L. is a plant in the Leguminosae family, commonly known as Sappan wood. *C. sappan* is natively planted in Southeast Asia and its dried heartwood has long been used in traditional medicine, such as Ayurveda, Thai folk medicine, and Chinese traditional medicine to treat wounds, diarrhea, skin infection, cardiovascular disease, ulcer diarrhea, and cerebrovascular diseases. In Thailand, it is used as natural red coloring agent in food, herbal drink, natural fabric dye and cosmetics. [10-13] *C. sappan* wood contains various types of water-soluble flavonoids, such as brazilin, isoflavone, coumarin, protosappanin, and xanthone. Brazilin is the major active compound in *C. sappan* heartwood, and it has strong antioxidant activity, anticancer, antibacterial, anti-inflammatory, and anticomplementary activity. [14-18] Various extraction conditions of *C. sappan* heartwood have been used to enhance efficiency of extracts. Several studies have demonstrated antioxidant activities of *C. sappan* crude extracts with various extraction solvents, such as boiling water and 70%–95% ethanol. [10, 18] The crude ethanolic extracts were partially purified by various solvents including ethyl acetate and water, and the investigation of antioxidant capacity was primarily performed using chemistry-based assays. [19] Only, a few studies have used cell-based assays for measuring antioxidant capacity.

Therefore, the purpose of this study was to characterize the antioxidant activity of *C. sappan* heartwood extracts prepared using various ethanol concentrations. Both chemical-based and cellular-based assays were performed to measure the antioxidant activity of *C. sappan* extracts. In cell-based assay, MRC-5 cells, a normal human lung fibroblast cell line, were used as cellular oxidative stress model.

Materials and methods

Preparation for crude extract

The *C. sappan* heartwood was dried using hot air oven at 60°C for 48 h, before it was grounded to a coarse powder and macerated with ethanol at various concentrations (95%, 75%, and 50% ethanol) for 24 h, three times. Ethanol was evaporated at 40°C under vacuum. The crude extract was recovered and kept at –20°C.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay

The free radical scavenging of the crude extract was analyzed by DPPH antioxidant assay according to the method described in Gamez *et al.* [19] In 96 well plates, 100 µL of standard butylated hydroxytoluene (BHT 1–10 µg/mL), sample, or blank (distilled water) was added and mixed with 100 µL of DPPH solution. The mixture was left in the dark at room temperature for 30 min, before its absorbance was measured at 520 nm. The percentage of DPPH inhibition was calculated according to the equation shown below. The half maximal inhibitory concentration (IC_{50}) value was determined using the Graphpad Prism program based on the following formula:

% Inhibition of DPPH = $[(A - B) - (C - D)/ (A - B)] \times 100$

where A; absorbance of DPPH and ethanol, B; absorbance of ethanol, C; absorbance of a DPPH and sample, and D; absorbance of sample and ethanol.

Total phenolic content

Total phenolic content (TPC) was determined by the Folin-Ciocalteu method with slight modifications. [20] In brief, 70 µL of the crude extract (1 mg/mL) was mixed with 525 µL of 0.2 M Folin-Ciocalteu's reagent, vortexed, and left at room temperature for 5 min. A volume of 525 µL of 7.5% (w/v) $NaCO_3$ was added to the mixture. The reaction was left for 30 min at room temperature. Absorbance was measured at 725 nm. TPC was calculated from the calibration curve equation and expressed as milligram of gallic acid equivalents (GAE) per gram dry weight of crude extract.

Cell culture

The lung fibroblasts MRC-5 cells, purchased from American Type Culture Collection (ATCC), were maintained in Eagle's minimum essential medium (EMEM), and supplemented with 10% fetal bovine serum (FBS) in an incubator under 37°C and 5% CO_2 conditions.

Cell viability assay

The effect of crude extract on cell viability was measured by the 3-4, 5-dimethylthiazol-2, 5-diphenyltetrazolium bromide (MTT) assay. A volume of 100 µL of cell suspension was seeded in 96 well (6.4×10^4 cell/mL) and incubated for 24 h. After incubation, the cells were exposed to various concentrations of the crude extract (1, 10, 50, and

100 µg/mL, final concentration). The plates were incubated for an additional 24 h and 100 µL of 1 mg/mL MTT solution was added to each well, before incubation for 4 h at 37°C. Then, 100 µL of DMSO was added to each well. Absorbance was measured at 570 nm. The percentage of cell viability was calculated using untreated control cells as control (100% cell viability). The concentration of the *C. sappan* extract that gave a cell viability of about 70% was regarded as the optimum concentration

Cell antioxidant assay (CAA)

CAA determines the occurrence of ROS within cytosol. Cells were stained with 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA), as redox-sensitive fluorophore. The active fluorescent DCF product is generated in the presence of intracellular ROS. Briefly, MRC-5 cells were seeded (6.4×10^4 cells/mL) in a 96-well plate and cultured for 24 h. The medium was removed, before 100 µL of crude extract (1.25, 2.5, and 5 µg/mL, final concentration) was added. The plate was incubated at 37°C and 5% CO_2 for 24 h. The crude extract was removed and 100 µL of 300 µM H_2O_2 was added. The plates were incubated for an additional 24 h at 37°C. in 5% CO_2. After washing, 500 µM DCFH-DA was added, the mixture was incubated for 1 h. Finally, the DCFH-DA was removed. The cells were washed with PBS, before 100 µL EMEM medium was added. Fluorescence intensity was measured using excitation and emission wavelengths at 485 nm and 535 nm, respectively. The CAA value was expressed as the inhibition percentage of the generation of ROS as follows:

$$\% \text{ inhibition} = [(A - B) / A] \times 100$$

where A; absorbance of solution of H_2O_2, B; absorbance of H_2O_2, and crude extract

Activity of cellular antioxidant enzymes and lipid peroxidation

The MRC-5 cells were seeded at a density of 5×10^4 cells/mL. After treatment with various concentrations of crude extract (1.25, 2.5, and 5 mg/mL) for 24 h, the crude extract was removed, and 2 mL of 700 µM H_2O_2 was added before incubation for 24 h. The cells were collected and resuspended in ice-cold cell lysis buffer (20 mM Tris at pH 6.5, 150 mM NaCl, 10% Triton X-100). The cell lysates were centrifuged at 10,000xg at 4°C for 5 min. The supernatants were used to measure the SOD, CAT, and malondialdehyde (MDA) content. SOD activity was measured by the SOD assay kit-WST. The CAT activity was estimated by the method described by Aebi. [21] The MDA content was measured by the thiobarbituric acid reactive substances (TBARS) assay. [22]

Statistical analysis

All experiments were conducted in triplicate. The results were expressed as means ± standard deviation (SD)/standard error of the mean (SEM). Statistically significant differences between groups were tested for by one-way analysis of variance (ANOVA), followed by the Duncan test for multiple comparison test when necessary. The Pearson's correlation coefficient was used to test for significant correlation. The significance level was set at $P < 0.05$.

Results

Evaluation of antioxidant activity of C. sappan extracts by chemical-based assay

C. sappan heartwood was extracted with ethanol at three concentrations (95%, 75%, and 50%) to obtain three samples of crude extracts. The results showed that extraction with 75%, ethanol gave the highest yield (12.1%), followed by extractions with 50% (11.6%) and 95% (10.1%) ethanol (**Table 1).** Also, total polyphenol content in the extract with 75.0% ethanol was significantly higher than in those extracted with 95% and 50% ethanol. To investigate the free radical scavenging activity in each extract, the DPPH radical scavenging capacity was assessed. The extract with 75% ethanol exhibited the highest radical scavenging activity with an IC_{50} value of 2.2 µg/mL. The IC_{50} values of extracts with 95% and 50% ethanol were 2.8 and 3.9 µg/mL, respectively.

Evaluation of antioxidant activity of C. sappan extracts by cell-based assay

Effects of C. sappan extract on cell viability and intracellular ROS

To assess the protective effects of *C. sappan* extracts on H_2O_2-induced ROS generation in MRC-5, the MTT assay was performed. The results showed that at 1–10 µg/mL *C. sappan* extract concentration, the percentages of viable cells were above 83.0%. These concentrations of extracts were not toxic to the cells. In contrast, H_2O_2 at concentrations over 1500 µM severely decreased MRC-5 cell viability after 24-h

Table 1. Results of extraction yield, total polyphenol content, and antioxidant properties of different extracts.

Extract	Yield (%)	Total polyphenol content (GAE)	Radical scavenging activity DPPH IC_{50} (ug/mL)
95% ethanol	10.1	662.0 ± 2.9^{a}	$2.8 \pm 0.2^{b,c,d}$
75% ethanol	12.1	741.8 ± 3.2^{b}	$2.4 \pm 0.2^{a,b}$
50% ethanol	11.6	616.7 ± 2.5^{c}	3.9 ± 0.2^{e}
BHT			13.2 ± 0.3^{f}

Values are means ± SEM of experiment in triplicate. Different superscript letters (a - e) within the same column are statistically significant at $P < 0.05$, using one-way ANOVA following by Duncan multiple comparison test. BHT, butylated hydroxytoluene; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; GAE, gallic acid equivalents; IC_{50}, half maximal inhibitory concentration; SEM, standard error of the mean.

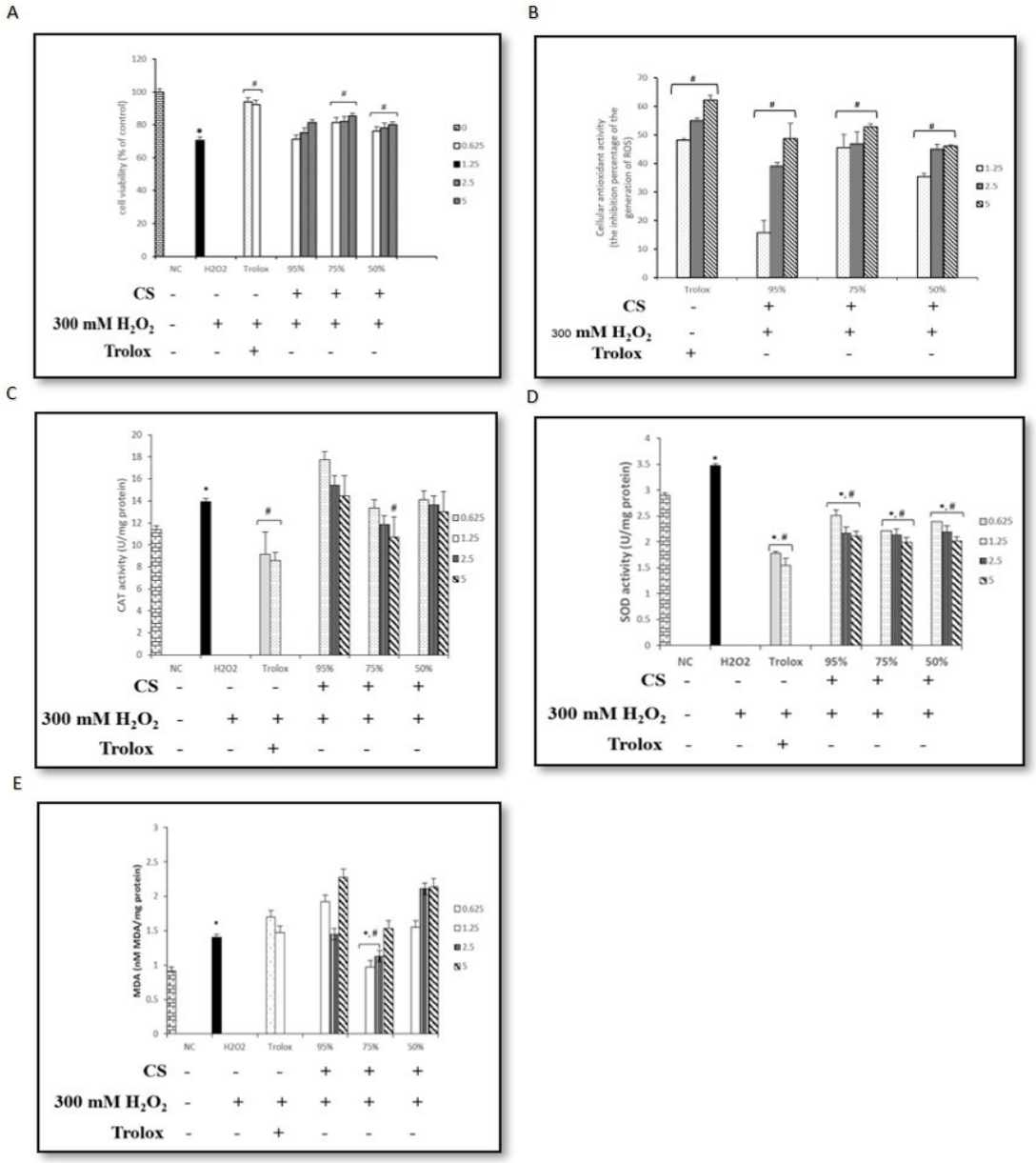

Figure 1. The effects of *C. sappan* extracts on H_2O_2-induced oxidative stress in MRC-5 cells. **(A)** Cell viability; **(B)** ROS generation; **(C)** CAT (U/mg protein); **(D)** SOD (U/mg protein); **(E)** MDA (nM MDA/mg protein). Values are means ± SD, are significantly different ($P < 0.05$); * compared with control; compared with H_2O_2-treated cells. CAT, catalase; MDA, malondialdehyde; ROS, reactive oxygen species; SD, standard deviation; SOD, superoxide dismutase.

treatment (data not shown). Therefore, it was used for subsequent experiments. Exposure of cells to 300 μM H_2O_2 was used to investigate the protective effect of *C. sappan* extracts. The viability of MRC-5 cells after treatment with 300 μM H_2O_2 decreased to 74.0% (**Figure 1A).** However, pretreatment of MRC-5 cells with *C. sappan* extracts at the concentrations of 2.5 and 5.0 μg/mL of 75% ethanolic extracts significantly increased viability of H_2O_2-treated MRC-5 cells to 80.0% – 83.0%, with 5.0%–10.0% recovery of cell viability relative to H_2O_2 alone. The 50% ethanolic *C. sappan* extracts also showed a similar cytoprotective effect. Additionally, pretreatment with 5.0 μg/mL of 95% ethanolic *C. sappan* extracts significantly increased the viability of MRC-5 cells relative to H_2O_2-treated cells.

The inhibitory effect of *C. sappan* extracts against ROS generation was explored, as shown in **Figure 1B**. At the concentration of 5.0 μg/mL of 75% ethanolic extracts, *C. sappan* extracts had the strongest inhibition of ROS generation (up to 54.0%). However, 2.5 and 5.0 μg/ml of 50% and 75% ethanolic extracts exhibited a similar capacity of ROS scavenging.

Effects of C. sappan extract on the activities of antioxidant enzyme and lipid peroxidation

To investigate the ability of *C. sappan* extracts to regulate activity of antioxidant enzymes (SOD and CAT) in MRC-5 cells, cells were pretreated with *C. sappan* extracts at concentrations of 1.25–5.0 μg/mL for 24 h, before they were treated with 700 μM H_2O_2 for 24 h and SOD and CAT enzyme activities were measured. The results showed that SOD and CAT activities significantly increased in H_2O_2-treated MRC-5 cells (**Figure 1C - 1D)**. Pretreatment of cells with extracts resulted in a significant decrease in SOD and CAT activities in a dose dependent manner. Specifically, the SOD activity was significantly lower in cells pretreated with ethanolic extracts at all concentrations than in H_2O_2-treated MRC-5 cells. However, the activity of CAT was significantly reduced only in H_2O_2-treated MRC-5 cells pretreated with ethanolic extracts at 0.5 μg/mL of 75% ethanolic extracts, and the reduction level was close to that of the trolox treatment.

MDA was measured as an indicator of oxidative damage caused by lipid peroxidation in H_2O_2-treated MRC-5 cells. As shown in **Figure1E**, MDA levels were significantly higher in H_2O_2-induced MRC-5 cells than in the control, indicating that cellular lipids were oxidized due to H_2O_2. Pretreatment of cells with 1.25 and 2.5 μg/mL of 75% ethanolic extracts significantly decreased MDA level in a dose-dependent manner relative to that of H_2O_2-treated MRC-5 cells.

Discussion

In this study, the antioxidant capacity of *C. sappan* extracts was evaluated using both chemical-based and cell-based assays. The TPC was evaluated in all extracts by the Folin-Ciocalteu method. The results revealed that high TPC levels in the extract trended to increase the scavenging activity, suggesting that the antioxidant activity of the extracts might be primarily due to the phenolic content. Additionally, it was found that IC_{50} values of all extracts obtained in this study were less than those of the standard antioxidant, BHT used as positive control, indicating that all *C. sappan* heartwood extracts had higher scavenging activity for DPPH radical than BHT. Notably, the *C. sappan* extracts obtained using 75% ethanol gave the highest free radical scavenging activity and the highest TPC.

Measurement of the free radical scavenging effect of antioxidants by the DPPH assay, a chemical-based method, is commonly used in evaluating antioxidant activities of phytochemicals. However, a more appropriate biologically relevant method to measure the antioxidant activity of phytochemicals has been developed based on cell culture. The cell-based assay is cost-effective, relatively fast, and provides a biologically relevant protocol addressing issues of uptake, distribution, and metabolism of antioxidants. [23] Additionally, the same antioxidant can exhibit different properties in chemical- and cell-based assays. Therefore, in this study, we investigated the antioxidant potential of *C. sappan* extracts in protecting human cells exposed to H_2O_2. Generally, H_2O_2, a ROS representative, is widely used as an exogenous oxidizing agent to induce oxidative stress in cell-based antioxidant assays. [24] Exogenous H_2O_2 readily enter cells because it has high membrane permeability, and it induces oxidative stress and damage. Furthermore, H_2O_2 can generate toxic ROS and the hydroxyl radical (•OH), via the Fenton reaction, causing much oxidative damage and cell injury. [25] Therefore, in this study, H_2O_2 was used to induce oxidative stress in an MRC-5 cell model. To the best of our knowledge, this is the first study to investigate

the scavenging capacity of *C. sappan* extracts in a cell-based assay using normal human lung fibroblast cells (MRC-5).

To examine the protective effect of *C. sappan* extracts on ROS generation, the capacity of extracts to inhibit intracellular ROS production after the extracellular addition of H_2O_2 in MRC-5 cells was investigated by the DCFH-DA assay. Intracellular ROS production was measured by a DCFH-DA fluorescent probe. DCFH-DA diffuses through the cell membrane and is hydrolyzed by intracellular esterase into DCFH in the cytosol. The DCFH is oxidized by intracellular ROS to form the highly fluorescent DCF. Therefore, the fluorescent intensity of DCF directly indicates the cellular ROS status. The findings of this study indicate that 50%–75% ethanolic extracts exhibited scavenging capacity to attenuate oxidative stress induced by H_2O_2. These ethanolic extracts showed consistent results in both cell viability and inhibition of ROS generation. The reduction in ROS generation in cells pretreated with *C. sappan* extracts might be attributed to the free radical scavenging activity of endogenous antioxidant systems. The antioxidant effect of *C. sappan* extracts to attenuate intracellular ROS can be attributed to the presence of brazilin and braziliein in *C. sappan* extracts. These two compounds contain phenolic hydroxyl that has antioxidant activity. [26] We further investigated whether the reduction in ROS generation by *C. sappan* extracts was also mediated by cellular antioxidant enzymes.

Oxidative stress is caused by an imbalance between ROS generation and antioxidant defense. The cellular defense system involves both antioxidant enzymes and free radical scavengers. The main antioxidant enzymes that combat harmful oxidants include SOD, CAT, and GPX. SOD detoxifies superoxide radicals into hydrogen peroxide, which is then destroyed by CAT. CAT is an important antioxidant enzyme in ROS scavenging, as it breaks down H_2O_2 into oxygen and water. [27,28] In this study, an oxidative stress environment was induced in MRC-5 cells by exposure to H_2O_2. A significant increase in SOD and CAT activities was observed in H_2O_2-induced MRC-5. This was probably due to the activation of the mechanism of cell protection against superoxide radical. [29] These results corroborated those of Radu M, *et al*.[30] who reported an increase in SOD and CAT activities in MRC-5 cells exposed to hematite nanoparticles. [30] Our results further showed that pretreatment of MRC-5 cells with *C. sappan* extracts prior to oxidative stress induction caused a significant reduction in SOD and CAT activities. These results suggest that *C. sappan* extracts contained abundant potent antioxidants. Additionally, the *C. sappan* extracts effectively inhibited lipid peroxidation in MRC-5 cells. These findings were associated with the alteration of SOD

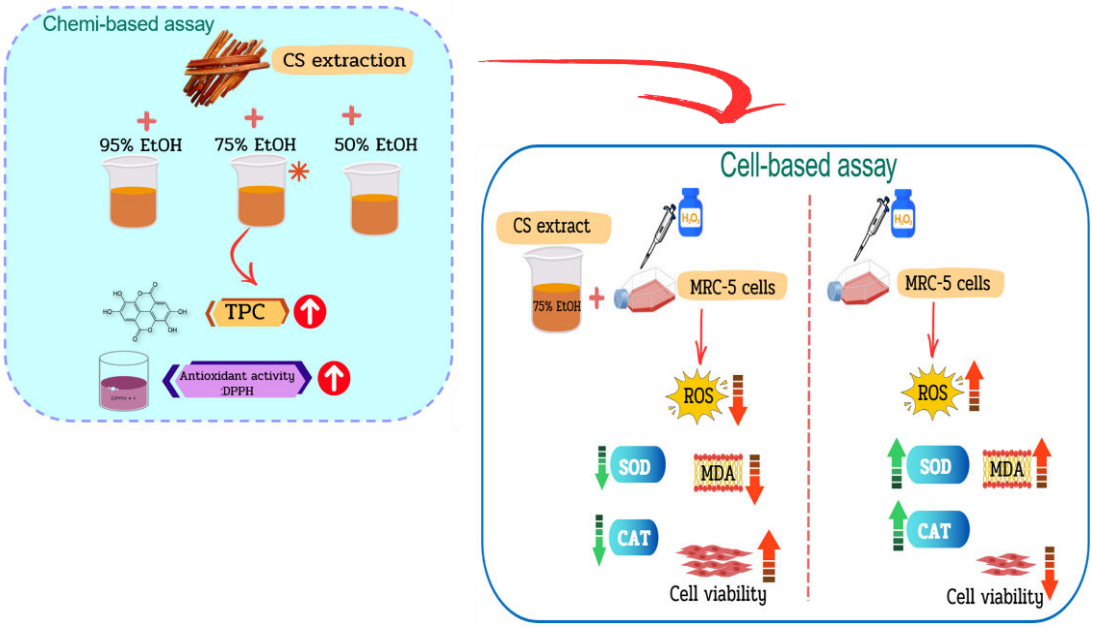

Figure 2. Graphical abstract illustrating the main concept and results of this study.

activity and inhibition of ROS production, suggesting that 75% ethanolic extract was responsible. However, pretreatment of MRC-5 cells with *C. sappan* extracts obtained from other ethanolic extractions increased MDA levels, indicating that these *C. sappan* extracts had a pro-oxidant effect at certain concentrations. It has been reported in many previous studies that coumaric, quercetin, and caffeic acids, at high doses, can induce intracellular ROS generation and have pro-oxidant capacity in cell culture model. [31-33] However, this study looked only at the antioxidant effect of *C. sappan* extracts against oxidative damage in MRC-5 cells induced by H_2O_2 **(Figure 2).** Therefore, there is need for further studies to investigate the protective effect of *C. sappan* extracts on human lung fibroblast (MRC-5) cells exposed to particular substances, such as PM2.5, to induce oxidative stress.

Conclusion

This study demonstrated the antioxidant activity of *C. sappan* extracts using both chemical and cellular-based assays. To the best of our knowledge, this study was the first to test cellular antioxidant activity of *C. sappan* extracts in a normal lung fibroblast MRC-5 cell line. Pretreatment of MRC-5 cells with the 75% ethanolic *C. sappan* extracts prior to H_2O_2 exposure, exhibited higher antioxidant activity than the other extracts. Our findings indicate that this *C. sappan* extract contained potent antioxidant compounds that proficiently protected normal human lung fibroblast cells from oxidative stress.

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Conflict of interest statement

None of the authors declare any potential or actual relationship, activity, or interest related to the content of this article.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for non-commercial purposes from the corresponding author on reasonable request.

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