The in Vitro Anti-Inflammatory and Antioxidant Activitites of the Bark Extracts from *Castanea Mollissima* in Vietnam

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Abstract
Evaluation results of anti-inflammatory effects through the inhibition of NO production in RAW264.7 macrophages and antioxidant activity using the DPPH method of the crude extract (TK); fractional extracts: *n*-hexane (TKH), ethyl acetate (TKE) and methanol (TKM) of the bark *Castanea mollissima* in Vietnam as follows: The crude extract (TK) exhibited good anti-inflammatory and antioxidant activities *in vitro* with SC_{50} value of 29.13 μg/mL and IC_{50} value of 19.6 μg/mL, respectively. Fractionated extract, ethyl acetate (TKE) exhibited the best anti-inflammatory and antioxidant activities *in vitro* with SC_{50} value of 21.67 μg/ml and IC_{50} value of 17.4 μg/ml, respectively. The remaining fractions showed moderate and weak activity. The above results guide further research on chemical composition and anti-inflammatory and antioxidant activities in the future of the *Castanea mollissima* in Vietnam.

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Keywords: *Castanea*, *Castanea mollissima*, anti-inflammatory, antioxidant

Introduction

Chestnut (*Castanea*) has 12 species, primarily distributed in the temperate regions of the Northern Hemisphere. In Vietnam, there are 2 species. Among them, *Castanea phansipanensis* is an endemic species in Lao Cai, used by the Red Dao people in Sapa, but research on its chemical composition and biological effects has hardly been conducted [1]. Additionally, the *Castanea mollissima* is widely cultivated in Son La, Lai Chau, Lang Son, and mainly in Zhongqiang - Cao Bang [2]. Chinese chestnuts are one of the famous specialties of Cao Bang, known for their delicious, sweet, creamy flavor, and the quality of Chinese chestnuts is distinctly different from those grown in other regions in terms of water content, glucide, and protein, which create the unique quality of Chinese chestnuts [3, 4]. The tree is easy to plant and care for, with high economic value; however, for many years, there has been no scientific research on post-harvest processing into commercial products, and the nuts are mainly used as gifts and for food preparation [5, 6].

Research by scientists has shown that extracts and some isolated compounds (including flavonoids, phenolic derivatives, tannins, phenolic acids, lignans, alkaloids, polysaccharides, glycosides, etc.) from species of the genus Castanea exhibit very good biological activities [11-15]. This paper evaluates the biological effects of the *Castanea mollissima* in Vietnam to further elucidate the anti-inflammatory and antioxidant activities of this valuable medicinal plant.

Notably, the leaves and stems of the chestnut (*Castanea mollissima*) are used to treat asthma, bone fractures, pustular boils, and otitis media [16]. This indicates that *Castanea mollissima* has good anti-inflammatory and antioxidant effects [17]. Moreover, studies on *Castanea mollissima* in Vietnam have mostly focused on identification, listing, description, or summarizing traditional usage experiences, with no research on both its chemical composition and biological activities, especially its anti-inflammatory and antioxidant activities. This paper conducts the first in vitro evaluation of the anti-inflammatory and antioxidant activities of the bark extract of the Castanea mollissima in Vietnam.

Study subjects and methods

Study Subjects

The subjects are samples of bark harvested in Chi Vien commune, Trung Khanh district, Cao Bang province in March 2023. They were named by Dr. Truong Ba Phong, from the Department of Biology, Faculty of Natural Sciences and Technology, Tay Nguyen University. The prototype specimen is stored at the Organic Chemistry Laboratory, Tay Nguyen University (Figure 1).

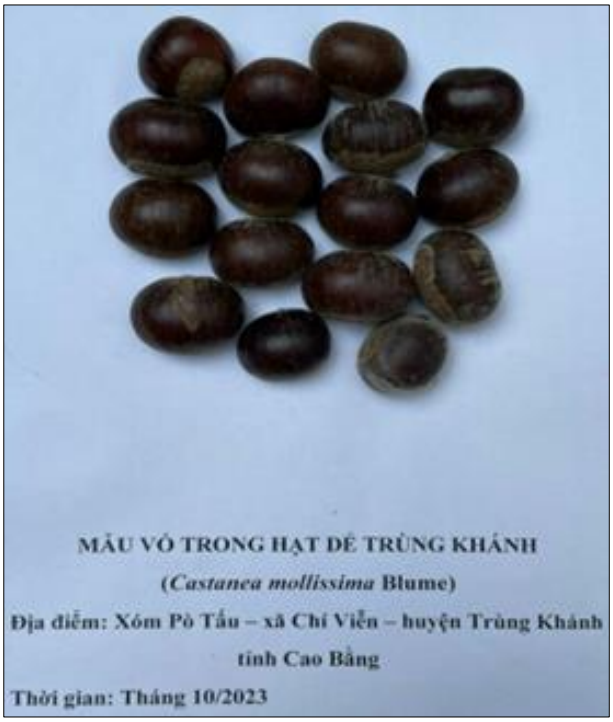

Fig 1: The bark harvested in Trung Khanh district, Cao Bang province.

Methods

Assessment of anti-inflammatory activity via nitric monooxide (NO) production inhibition.

Tested at the Institute of Chemistry of Natural Compounds, VAST

In vitro anti-inflammatory activity test: The in vitro anti-inflammatory activity was evaluated by the inhibition of NO production in RAW264.7 macrophage cells (American Type Culture Collection, Manassas, VA, USA). RAW264.7 cells were cultured using the method of Fumio Amano *et al*. The anti-inflammatory activity on RAW264.7 cells was determined using the Griess method as described by Verena M. Dirsch *et al*.

The specific procedures are as follows: RAW264.7 cells (murine macrophages) were cultured for 48 hours in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), at 37°C, 5% CO_2. Subsequently, the cell suspension was seeded into 96-well plates at a density of 2.5x10^5 cells/well. The cells were stimulated with 2 µl of control sample (-) LPS (0.1 mg/ml) for 24 hours and treated with various concentrations of test drugs or compounds. Cardamonin was used as a positive control sample (+). The cell culture supernatant was incubated with Griess reagent and $NaNO_2$ at different concentrations to establish a standard curve. The absorbance of the reaction mixture was measured at a wavelength of λ = 570 nm. Higher levels of NO corresponded to greater optical density, which was quantified based on the $NaNO_2$ standard curve and expressed as a percentage compared to the control sample (-) LPS. The ability to inhibit NO production was determined using the following formula:

$$\%\ \text{Inhibition} = 100\% - \frac{\text{NO sample concentration}}{\text{NOLPS concentration}} \times 100\%$$

Assessment of in vitro antioxidant activity.

Analysis of the ability to scavenge free radicals generated by DPPH (1,1-diphenyl-2-picrylhydrazyl; Brand-Williams *et al*., 1995, Shela *et al*., 2003, Kumar *et al*., 2013) is a recognized method for quickly determining antioxidant activity. The test compound is dissolved in 100% dimethyl sulfoxide (DMSO) and DPPH is dissolved in 96% ethanol. The absorption of DPPH at λ = 515 nm is measured after adding DPPH to the sample solution in a 96-well microplate. Results of the experiments are expressed as the mean value of at least 3 repeated tests ± standard deviation ($p \leq 0.05$).

The samples were dissolved in 100% DMSO at a concentration of 4 mg/ml for crude extract and 1 mg/ml for purified sample. Flavonoid at 1 mM or ascorbic acid at 5 mM in 10% DMSO served as positive controls. Samples were applied onto a 96-well microplate with DPPH solution to achieve final concentrations ranging from 200 µg/mL to 12.5 µg/mL (for crude extract) and from 50 µg/mL to 3.1 µg/mL (for purified sample) in the reaction mixture.

Incubate at 37°C for 30 minutes and measure the optical density (OD) at a wavelength of λ = 515 nm using a spectrophotometer (Infinite F50, Tecan, Switzerland).

The scavenging capacity (SC%) of free radicals at various sample concentrations is calculated using Excel software according to the formula:

$$SC\% = [\frac{OD_{test} - OD_{DMSO}}{OD_{control(-)}} \times 100\%] \pm \sigma$$

The standard deviation σ is calculated using the formula by Duncan as follows:

$$\sigma = \sqrt{\left(\Sigma\left(xi - \bar{x}\right) \wedge 2\right) / \left(n - 1\right)}$$

Determining SC_{50}: The sample (test substance) is diluted into decreasing concentrations, repeated three times at each concentration. The scavenging efficiency against DPPH free radicals for each sample is calculated based on the percentage of free radical neutralization compared to blank and negative control samples. Samples showing antioxidant activity against the DPPH system undergo further steps to determine the IC_{50} value (µg/mL, µM/mL). The SC_{50} value is the concentration of the test substance at which 50% of free radicals are neutralized, determined using Table Curve AISN Software (Jandel Scientific, USA) based on SC% values and the range of corresponding test substance concentrations.

Results

Sample Processing

The dried bark of *Castanea mollissima* were finely ground and soaked three times with methanol at room temperature. The combined methanol extracts were then concentrated by rotary vacuum evaporation at low temperature and reduced pressure to yield the totalextract (TK, 167 g). Theextract was subsequently fractionated with n-hexane and ethyl acetate.

After solvent removal, the respective fractions obtained were *n*-hexane extract (TKH, 58 g), ethyl acetate extract (TKE, 45 g), and methanol extract (TKM, 43 g). The masses of the obtained extract residues from Castanea mollissima leaves are presented in Table 1.

Table 1: The mass of extracts obtained from the *Castanea mollissima* plant

Component	Weight of dried sample	Weight of the extract (g)			
		Total extract	*n*-hexan	EtOAc	MeOH
Brak	3,9 kg	167 (**TK**)	58,0 (**TKH**)	45,0 (**TKE**)	43,0 (**TKM**)

Results of antioxidant activity

The DPPH free radical scavenging activity of the total extract (TK) and segmented extracts: *n*-hexane (TKH), ethyl acetate (TKE), and methanol (TKM) from *Castanea mollissima* leaves is shown in Table 2 and Figure 2.

Table 2: Results of in vitro antioxidant activity tests from bark of the *Castanea mollissima* plant

No.	Sample identifier	Free radical scavenging capacity (SC, %)	SC_{50} (µg/ml)
	Control (+) [axid ascorbic]	81,12 ± 0,8	11,54
	Control (-) [DPPH/EtOH + DMSO]	0,00 ± 0,0	-
1	TK	59,30 ± 0,9	36,12
2	TKH	60,41 ± 1,2	51,35
3	TKE	76,28 ± 0,6	20,65
4	TKM	31,22 ± 1,6	> 100

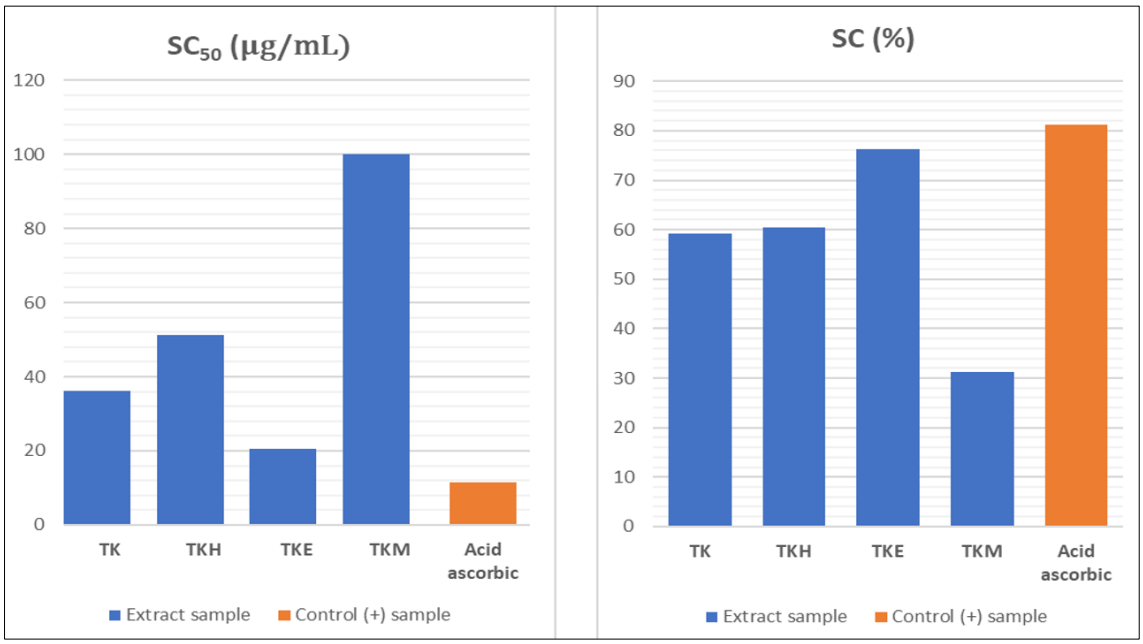

Fig 2: In vitro antioxidant activity from bark of *Castanea Mollissima*

The results indicate that the totalextract (TK) from *Castanea mollissima* bark exhibited a DPPH free radical scavenging capacity (SC) of 499.20 ± 0.8%, corresponding to an SC_{50} value of 36.12 µg/ml. Among the segmented extracts, ethyl acetate extract (TKE) demonstrated the highest antioxidant activity with a SC of 73.24 ± 0.5%, and an SC_{50} value of 10.62 µg/ml. Following TKE, the *n*-hexane extract (TKH) showed a SC of 56.45 ± 1.11%, with an SC_{50} value of 41.37 µg/ml. However, the methanol extract (TKM) did not exhibit significant activity with an SC of 28.21 ± 1.3% and an SC_{50} value >100 µg/ml. Therefore, the active compounds primarily reside in the less polar and moderately polar fractions of the extracts.

Results of anti-inflammatory activity

Anti-inflammatory activity through inhibition of NO production in RAW264.7 macrophage cells in vitro by the total extract (TK) and segmented extracts: *n*-hexane (TKH), ethyl acetate (TKE), and methanol (TKM) from *Castanea mollissima* leaves is depicted in Table 3 and Figure 3.

Table 3: Results of in vitro anti-inflammatory activity tests from bark of the *Castanea mollissima*

No.	Sample identifier	Highest concentration tested	Percentage of NO production (%)	Percentage of survived cells (%)	IC_{50} value
	Control (-)	-	100,0 ± 0,7	102,45 ± 0,28	
	Control (+): Cardamonin	3,0 μM	83,21 ± 1,1	71,33 ± 0,5	9,7 μM
	LPS	-	0,0 ± 0,3	100,0 ± 0,6	
1	TK	50 μg/ml	72,97 ± 1,4	79,41 ± 1,2	20,2 μg/ml
2	TKH	50 μg/ml	61,87 ± 1,0	72,31 ± 0,7	50,8 μg/ml
3	TKE	50 μg/ml	78,96 ± 1,3	74,98 ± 1,1	18,5 μg/ml
4	TKM	50 μg/ml	33,26 ± 0,9	67,81 ± 1,5	-

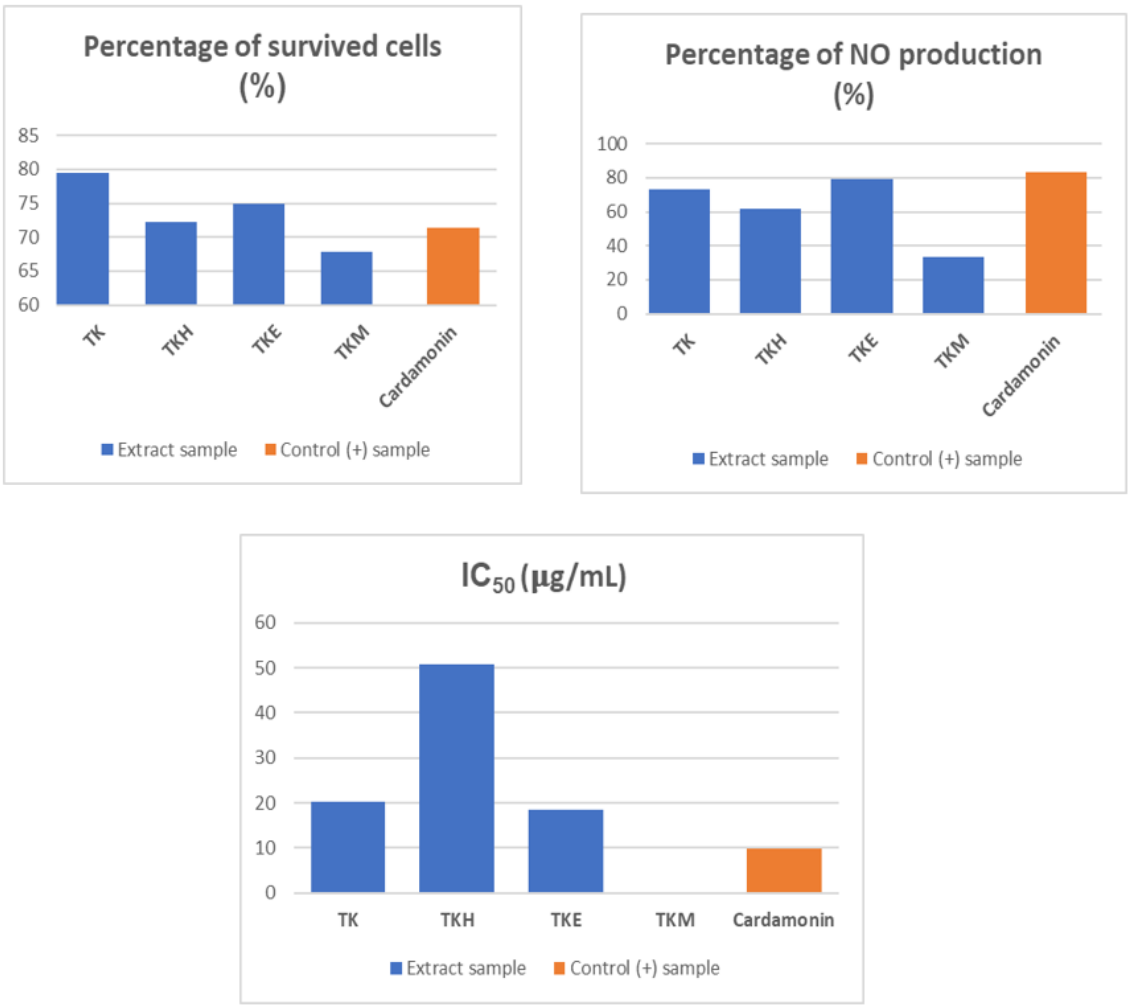

Fig 3: In vitro anti-inflammatory activity from bark of *Castanea Mollissima*

The results in Table 3 and Figure 3 demonstrate that both the total and segmented extracts from Castanea mollissima leaves exhibited significant inhibition of NO production in RAW264.7 cells at various tested concentrations ($p < 0.05$). The total extract (TK) showed strong inhibition of NO production with an IC_{50} of 20.2 µg/ml and exhibited non-toxic effects on cells (cell viability rate of 79.41 ± 1.2%). The ethyl acetate extract (TKE) showed the highest anti-inflammatory activity with an IC_{50} of 18.5 µg/ml and no cytotoxicity on cells (cell viability rate of 74.98 ± 1.1%). Following TKE, the n-hexane extract (TKH) had an IC_{50} value of 50.8 µg/ml and showed no cytotoxicity on cells (cell viability rate of 72.31 ± 0.7%). However, the methanol extract (TKM) showed minimal activity.

Thus, the anti-inflammatory activity of *Castanea mollissima* leaves is primarily concentrated in the less polar and moderately polar fractions, specifically TKH and TKE. Therefore, the TKH and TKE extracts are prioritized for further investigation into their chemical constituents.

Conclusion

The evaluation of anti-inflammatory and antioxidant activities of these extracts from the bark of *Castanea mollissima* showed that the total extract (TK) exhibited strong inhibition of NO production without cytotoxicity to cells, with an IC_{50} of 20.2 µg/ml. As for the segmented

extracts, TKH and TKE segments demonstrated good anti-inflammatory activity with IC_{50} values of 50.8 µg/ml and 18.5 µg/ml.

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