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Bioactive Properties of *Castanea mollissima* Leaf Extracts: Anti-Inflammatory, Antioxidant and Cytotoxic Activities

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Abstract:. *This study evaluated the antioxidative, anti-inflammatory and cytotoxic activities of* ***Castanea mollissima*** *leaf extract collected in Cao Bang – Viet Nam , determining the most bioactive fractions for sequential isolation. Anti-inflammatory activity was assessed by measuring the inhibition LPS-induced NO production in RAW264.7 macrophages, antioxidant capacity assessed using DPPH free radical scavenging method and SC_{50} estimation, and anticancer activity indicated by MTT cytotoxicity estimation against the KB, HepG2, A549, and MCF-7 cell lines at IC_5 concentrations. The findings illustrate that every fraction apart from TKM repressed NO production with minimal cytotoxicity, while TKE had the strongest effect (IC_{50} = 18.5 µg/mL) followed by TK (20.2 µg/mL) and TKH (50.8 µg/mL). TKE also had the strongest results in the DPPH assay again outperforming the other fractions (SC = 76.28 ± 0.6%; SC_{50} = 20.65 µg/mL) while TK and TKH had moderate activities (SC_{50} = 36.12 and 51.35 µg/mL, respectively) and TKM had no activity (SC_{50} > 100 µg/mL). The cytotoxicity exhibited by the crude extract was the strongest against the A549 cancer line (IC_{50} = 8.96 ± 1.83 µg/mL), followed by KB (10.58 ± 1.66 µg/mL), HepG2 (11.02 ± 1.51 µg/mL), and MCF-7 (15.12 ± 1.94 µg/mL).* ***Castanea mollissima*** *leaves have anti-inflammatory, antioxidant and anti-cancer properties simultaneously with the active constituents concentrated in the ethyl acetate and n-hexane fractions. Future research should be directed towards the mechanistic study of* ***Castanea mollissima*** *leaves extract, the NO/NF-κB and oxidative stress pathways, and the in vivo evaluation of its safety and efficacy to support its pharmaceutical or cosmetic usage.*

Keywords: Castanea mollissima; antioxidant; anti-inflammatory; cytotoxicity; DPPH; RAW264.7.

1. Introduction

The genus *Castanea* has 12 species distributed mainly in the northern temperate zone. In Vietnam, there are 2 species, of which the Chestnut tree (*Castanea phansipanensis*) is endemic in Lao Cai, used by the Red Dao people in Sapa as a bathing leaf [1]. In addition, the Chestnut tree (*Castanea mollissima*) is widely grown in Son La, Lai Chau, Lang Son and mainly in Trung Khanh - Cao Bang [2]. Trung Khanh Chestnuts have a delicious, sweet, fatty flavor, a characteristic rich taste, and a clearly different quality compared to other types of Chestnuts grown in other regions such as water content, gluside, and protein, creating the unique quality of Trung Khanh Chestnuts [3, 4], this is one of the famous specialties of Cao Bang that has been geographically indicated. However, its biological activities have not been systematically characterized in Vietnam, to date the seeds are mainly used as gifts and for food processing [5, 6]. Some studies conducted on species of the genus Castanea have shown that the extracts and some isolated compounds (belonging to the group of flavonoids, phenolic derivatives, tannins, phenolic acid, lignans, alkaloids, polysaccharides, glycosides...) show very good biological activity [11-15]. In traditional medicine, the stems and leaves of the *Castanea mollissima* species treat asthma, broken bones, boils, otitis media [16], suggesting that the Castanea mollissim tree has good anti-inflammatory and antioxidant effects [17]. However, studies on *Castanea mollissima* in Vietnam have mostly focused on identifying, listing, describing, or summarizing folk experience, but there have been no studies on both chemistry and biological activities, especially activities such as anti-inflammatory, antioxidant, anti-cancer. In this study, we evaluated the above biological activities of the extract of *Castanea mollissima* in Vietnam.

2. Materials and Methods

2.1. Materials

The stem and leaf specimens of the Trung Khanh Chestnut tree were collected in Chi Vien commune, Trung Khanh district, Cao Bang province in March 2023. The specimens were identified by Dr. Truong Ba Phong, Department of Biology, Faculty of Natural Sciences and Technology, Tay Nguyen University. The specimen specimen (DTK-L-01) is stored at the Organic Chemistry Laboratory, Department of Chemistry - USTH University.

***Figure 1**. Chestnut tree leaves (Castanea mollissima) collected in Trung Khanh district, Cao Bang province*

2.2. Evaluation of anti-inflammatory activity through inhibition of NO production

In vitro anti-inflammatory activity was evaluated through the ability to inhibit NO production on RAW264.7 macrophages (American Type Culture Collection, Manassas, VA, USA). The study was conducted at the Institute of Chemistry of Natural Compounds, Vietnam Academy of Science and Technology RAW264.7 cells were cultured according to the method of Fumio Amano et al. Determination of anti-inflammatory activity on RAW264.7 cell line according to the analytical method Griess of Verena M. Dirsch et al. Procedure: RAW264.7 cells (mouse macrophages) were cultured for 48 hours in Dulbecco's Modified Eagle Medium (DMEM) at 37° C, 5% CO_2, 10% fetal bovine serum (FBS). The cell suspension was then transferred to a 96-well plate at a density of 2.5x105 cells/well. The cells were stimulated with 2µl of LPS (0.1mg/mL) control (-) for 24 hours and various concentrations of drugs or reagents were added. Cardamonin was used as a (+) control. The cell suspension was incubated with Griess reagent and NaNO2 at various concentrations to construct a standard curve. The reaction mixture was measured at λ = 570nm. The higher the NO content, the greater the optical density and was determined based on the NaNO2 standard curve, comparing % with the control sample (-) LPS. The ability to inhibit NO production of the sample was determined according to the following formula:

$$Inhibition(\%) = 100 - \left(\frac{NO\ content\ of\ sample}{NO\ content\ of\ LPS\ control} \times 100\right)$$

in where:

- *NO content of sample* is the nitric oxide concentration measured in the treated sample;
- *NO content of LPS control* is the nitric oxide concentration in the LPS-induced control group.

2.3. Evaluation of in vitro antioxidant activity

Analysis of the ability to scavenge free radicals generated by DPPH (1,1-diphenyl-2-picrylhydrazyl) is a recognized method for rapid determination of antioxidant activity. The test substance was dissolved in dimethyl sulfoxide (DMSO 100%) and DPPH was dissolved in ethanol 96%. The absorbance of DPPH at λ = 515nm was determined after adding DPPH to the test solution on a 96-well microtiter plate. The results of the tests are expressed as the average value of at least 3 replicate tests ± standard deviation ($p \leq 0.05$). The sample was diluted in DMSO 100% at a concentration of 4 mg/mL for crude extract and 1 mg/mL for purified sample. Flavonoid 1 mM or ascorbic acid 5 mM in DMSO 10% was used as a positive control. The sample was dropped on a 96-well microplate with DPPH solution to obtain a final concentration of the sample in the reaction ranging from 200 µg/mL to 12.5 µg/mL (crude extract) and from 50 µg/mL to 3.1 µg/mL (purified sample). The sample was incubated at 37oC for 30 min and the optical density (OD) was measured at λ = 515 nm on a spectrophotometer (Infinite F50, Tecan, Switzerland). The ability to neutralize free radicals (Scavenging capacity, SC%) at sample concentrations is entered into the Excel data processing program according to the formula:

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

in where:

- OD_{sample} represents the optical density of the tested sample;
- OD_{DMSO} is the optical density of the DMSO control;
- $OD_{control(-)}$ denotes the optical density of the negative control.

Standard deviation σ is calculated according to Ducan's formula as follows:

$$\sigma = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}}$$

in where:

- x_i represents the individual measured value;
- $\bar{x}$ is the mean value of the dataset;
- n is the number of replicates.

Determination of SC_{50}: The sample (test substance) is diluted to decreasing concentrations, repeated 3 times at each concentration. The efficiency of trapping free radicals created by DPPH of each sample is calculated based on the % of free radical neutralization compared to the blank sample (Blank) and the negative control. Samples that showed antioxidant activity on the DPPH system were subjected to the next steps to find the IC_{50} value (µg/mL, µM/mL). The SC_{50} value is the concentration of the test substance at which 50% of the free radicals are neutralized, determined by TableCurve AISN Sofware (Jandel Scientific, USA) through the SC% value and the series of corresponding test substance concentrations.

2.4. Evaluation of Anticancer Activity

The anticancer activity of the sample was evaluated on four cancer cell lines including: KB epithelial cancer (CCL-17™), HepG2 liver cancer (HB-8065™), MCF-7 breast cancer (HTB-22™) and A549 lung cancer (CCL-185™), obtained from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 7 - 10% FBS (Fetal Bovine Serum) and necessary nutrients after thawing from the liquid nitrogen storage. The thawed cells were cultured in an incubator with 37% humidity and 5% CO_2. Cells in the log growth phase were used for further experiments. The viability of the four cell lines tested was evaluated by MTT (3- (4,5-dimethylthiazol-2 - yl)-2, 5 - diphenyltetrazolium) assay as described by Mosmann (1983). The experiment was carried out on 96 well plates with 190 µL of cell suspension mixed with extract diluted into a concentration range of 4, 16, 64 and 256 µg/mL incubated for 72 hours. After incubation, each well was added 10 µL of MTT solution (5 mg/mL) and incubated for 4 hours. The formazan crystals formed were dissolved in 100 µL DMSO and the absorbance of the sample was measured using an ELISA (Enzyme-linked Immunosorbent assay) plate reader at 540 nm. The experiment was repeated 3 times. The IC_{50} value was determined as the percentage inhibition of cell growth and Rawdata software. The percentage inhibition was calculated using the following formula:

$$\%\ Cell\ inhibition = \frac{OD_{control(+)} - OD_{sample}}{OD_{control(+)} - OD_{control(-)}} \times 100$$

in where:

- $OD_{control(+)}$: Optical density of the positive control;
- $OD_{control(-)}$: Optical density of the negative control;
- OD_{sample}: Optical density of the tested sample.

$$IC_{50} = High_{Conc} - \frac{(High_{Inh\%} - 50) \times (High_{Conc} - Low_{Conc}}{High_{Inh\%} - Low_{Inh\%}}$$

in where:

- HighConc: The highest concentration of the tested sample
- LowConc: The lowest concentration of the tested sample
- HighInh%: Percent inhibition at the highest concentration
- LowInh%: Percent inhibition at the lowest concentration

3. Results

3.1. Sample processing

The collected leaves *Castanea mollissima* (3.5kg) were dried, crushed, and soaked three times with methanol (3 x 6.0L) at room temperature. The total extract was distilled to remove the solvent using a rotary vacuum at low temperature and reduced pressure to obtain the total extract (TK, 53.0g).

The methanol extract was extracted with n-hexane and ethyl acetate, respectively. After removing all the solvent, the corresponding extracts were obtained, namely n-hexane (TKH, 46g), ethyl acetate (TKE, 40g) and methanol (TKM, 62g). The mass of the extracts obtained from the leaves of the Chongqing Chestnut tree *Castanea mollissima* is presented in Table 1.

***Table 1**. Extract yields obtained from leaves of Castanea mollissima*

Plant part	Dry sample weight (kg)	Extract yield (g)			
		Crude extract **(TK)**	n-Hexane fraction **(THK)**	EtOAc fraction **(TKE)**	MeOH fraction **(TKM)**
Leaves	2.9	53.0	46.0	40.0	62.0

3.2. Results of anti-inflammatory activity

The in vitro anti-inflammatory activity through inhibition of NO production on RAW264.7 macrophages of total extract (TK); fractional extracts: n-hexane (TKH), ethyl acetate (TKE) and methanol (TKM) of Chestnut tree are shown in table 2 and figure 2.

The results in table 2 and figure 2 show that total extracts and fractional extracts from leaves of Chestnut tree *Castanea mollissima* all showed inhibitory activity on NO production on RAW264.7 macrophages at the investigated concentration ranges ($p < 0.05$) at

different levels. Total extract (TK) strongly inhibited NO production with IC_{50} of 20.2µg/mL and was not toxic to cells (cell survival rate was 79.41 ± 1.2). The ethyl acetate fraction extract (TKE) exhibited the best anti-inflammatory activity with an IC_{50} of 18.5µg/mL and was not cytotoxic (cell survival rate was 74.98 ± 1.1%), followed by the n-hexane fraction extract (TKH) with an IC_{50} of 50.8µg/mL and was not cytotoxic (cell survival rate was 72.31 ± 0.7%), while the methanol fraction extract (TKM) showed almost no activity.

***Table 2**. In vitro anti-inflammatory activity of Castanea mollissima extracts*

No.	Sample code	Highest tested concentration	Inhibition of NO production (%) *	Cell viability (%) *	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	−

** Values are expressed as mean ± SD (n = 3).*

Thus, the anti-inflammatory activity of *Castanea mollissima* leaves is mainly concentrated in the low polarity and medium polarity TKH and TKE. Therefore, the high polarity TKH and TKE are given priority for further research on chemical composition.

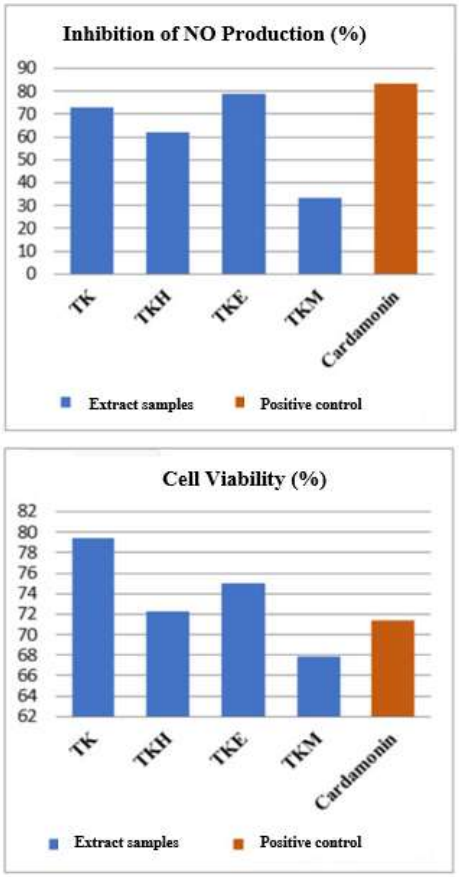


***Figure 2.** Anti-inflammatory activity of Castanea mollissima leaf extracts.*
(A) Inhibition of nitric oxide (NO) production (%); **(B)** Cell viability (%); **(C)** IC_{50} values (µg/mL).

3.3. Results of antioxidant activity

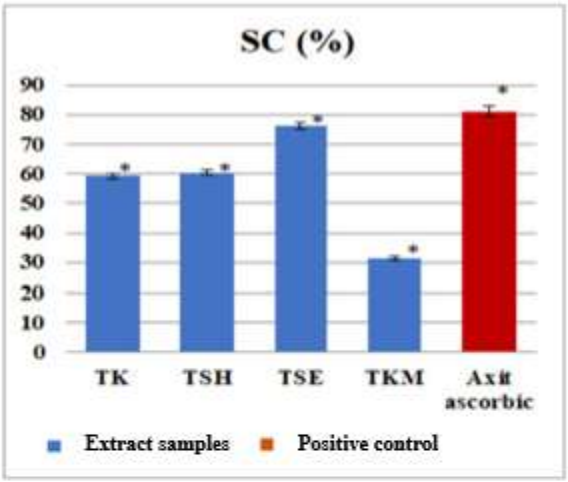
PPH free radical scavenging activity of total extract (TK); fractional extracts: n-hexane (TKH), ethy acetate (TKE) and methanol (TKM) of Chestnut leaves are shown in table 3 and figure 3.

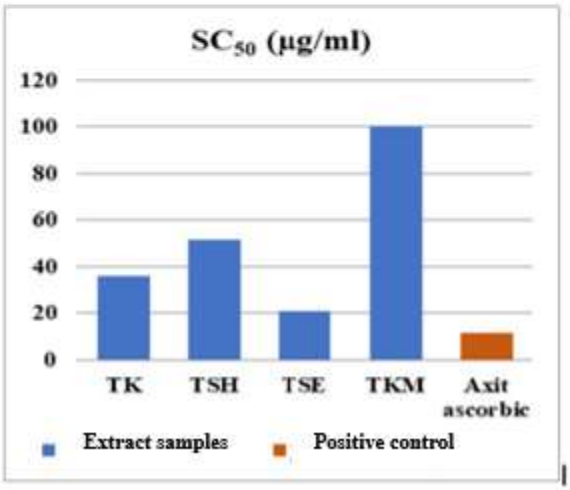
***Table 3.** In vitro antioxidant activity of Castanea mollissima leaves*

No.	Sample	Free radical scavenging capacity (SC, %) *	SC_{50}(µg/m)

	Positive control [ascorbic acid]	81.12 ± 0.8	11.54
	Negative 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

** Values are expressed as mean ± SD (n = 3).*





** Values are expressed as mean ± SD (n = 3).*

***Figure 3**. In vitro antioxidant activity of Castanea mollissima leaf extracts. (A) Free radical scavenging capacity (SC, %) of the extracts (TK, TSH, TSE, TKM) compared with ascorbic acid (positive control). (B) SC_{50} values (µg/mL) of the same samples. Data are expressed as mean ± SD (n = 3).*

The results showed that the total extract (TK) of *Castanea mollissima* leaves had a free radical neutralization capacity (SC) of 59.30 ± 0.9%, corresponding to a SC_{50} value of 36.12µg/mL according to the DPPH method. Among the fractional extracts, ethyl acetate extract (TKE) showed the best antioxidant activity with a SC free radical neutralization capacity of 76.28 ± 0.6%, corresponding to a SC_{50} value of 20.65µg/mL, followed by n-hexane extract (TKH) with a SC free radical neutralization capacity of 60.41 ± 1.12%, corresponding to a SC_{50} value of 51.35µg/mL and finally methanol extract (TKM) did not show activity with a SC free radical tneutralization capacity of 30.21 ± 1.5%, corresponding to a SC_{50} value >100µg/mL. Thus, the active ingredients showing antioxidant activity mainly concentrated in the less polar and medium polar fractions.

3.4. Results of anticancer activity

In this study, the MTT method was used to evaluate the cytotoxicity of the methanol extract of *Castanea mollissima* leaves on four cancer cell lines: KB (epithelial carcinoma), HepG2 (liver cancer), A549 (lung cancer) and MCF-7 (breast cancer), the results are shown in Table 4.

***Table 4**. Cytotoxic activity against four human cancer cell lines*

Sample	Concentration (µg/mL)	Cell growth inhibition (%)			
		KB	HepG2	A549	MCF7
Crude extract	256	76	79	79	77
	64	71	75	74	63
	16	30	30	37	28
	4	5	3	6	9
	IC_{50}	10.58±1.66	11.02±1.51	8.96±1.83	15.12±1.94
Ellipticine	IC_{50}	0.45±0.02	0.45±0.02	0.44±0.02	0.44±0.02

The results showed that *Castanea mollissima* leaf extract had cytotoxic effects on all four cancer cell lines tested, with IC50 values of 10.58 ± 1.66 µg/mL (KB), 11.02 ± 1.51 µg/mL (HepG2), 8.96 ± 1.83 µg/mL (A549) and 15.12 ± 1.94 µg/mL (MCF- 7), respectively. Although the IC50 values on the cell lines did not differ much, the best cytotoxicity was recorded on the lung cancer cell line (A549) and the lowest on the breast cancer cell line (MCF- 7). The results on two epithelial cancer (KB) and hepatoma (HepG2) lines showed insignificant differences. This suggests that the methanol extract of *Castanea mollissima* has potential against cancer cell lines, although the IC50 value was lower than that of the positive control Ellipticine. The comparison of anticancer activity with other species showed that the methanol extract from leaves of *Castanea mollissima* showed anticancer activity against ascites cancer cells (EAC) with IC50 = 71.50 ± 6.25 µg/mL (Dolai et al., 2012). In contrast, *Castanea mollissima* did not inhibit A549, SMMC-7721, MGC-803, HepG2 and MCF-7 cell lines (Huang et al., 2017). This suggests a difference in anticancer activity between species within the Castanea genus. In this context, the results of *Castanea mollissima* on A549, HepG2 and MCF-7 lines are considered to be relatively promising.

4. Conclusion

The results of the evaluation of the anti-inflammatory and antioxidant activities of the extracts obtained from the stems and leaves of *Castanea mollissima* showed that the total methanol extract (TK) had a strong inhibitory effect on NO production and was not toxic to the cells with an IC50 of 20.2µg/mL. Regarding the fractions, the TKH and TKE fractions exhibited good anti- inflammatory activity with IC50 values of 50.8µg/mL and 18.5µg/mL, respectively. The above results provide direction for further research on the chemical composition and anti-inflammatory, antioxidant activities, and anticancer activity of Vietnam in the future.

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