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UPLC/HR-MS/MS and GC/MS Metabolome Profiling of *Castanea mollissima* Blume Fruit Extracts and its Anti-inflammatory Potentials

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Abstract

Background: Chinese chestnuts (*C. mollissima* Blume fruit) are valuable nutritional resources grown worldwide, They have multiple health-promoting benefits and promising nutraceutical applications due to their enriched phytochemical contents and functional ingredients. In this study, fractions of different polarities were prepared from the Chinese chestnut to investigate their antioxidant, anti-inflammatory and phytochemical content. **Methods:** The total ethanol extract (TEE, 55g) of the peeled chestnut fruit was prepared by maceration with 80% ethanol, 10 g of TEE was further fractionated with dichloromethane to produce non polar fraction (NPF, 0.65 g) and polar fraction (PF, 9.3 g). TEE, PF and NPF were screened for their *in vitro* antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) scavenging assays, the most active of which (TEE) proceeded to screen its anti-inflammatory activity by inhibition of cyclooxygenase enzymes (COX-1 and COX-2). The chemical composition of TEE and NPF was studied by UPLC/HR-MS/MS and GC/MS analyses respectively. **Results**: TEE showed the most potent DPPH and NO scavenging activity with IC_{50} (µg/mL) = 120.69± 0.54, and 230.02± 0.321, respectively, compared to vitamin C (213.5± 0.121 and 211.56 ± 0.181 µg/mL, respectively). TEE of *C. mollissima* showed a selective anti-inflammatory effect against COX-2 not COX-1 with IC_{50} (µg/mL) 1.867 ± 0.115 and 38.25±0.68, respectively compared to indomethacin (0.13 ± 0.1; 27.031± 0.006 µg/mL) and celecoxib (4.18 ± 0.1; 92.52 ± 0.01 µg/mL) as reference drugs. UPLC-MS/MS analysis of TEE tentatively identified 41 metabolites that included 4 organic acids, 12 flavonoids, 4 phenolic compounds, 5 polyphenol, 9 fatty acids, 2 phospholipids and 1 triterpene, several of which are described for the first time in Chinese chestnut. GC/MS analysis of the non-polar fraction (NPF) revealed 32 unsaponifiable compounds (75.19%) including 25.98%, oxygenated, 20.92% oxygenated nitrogenous. and 28.29% non-oxygenated compounds in addition to 17 fatty acids as methyl esters (96.30%) that including 32.22% unsaturated and 64.08%. saturated fatty acids. **Conclusion***:* The *in vitro* bioactivity study suggested that total ethanol extract had a synergistic effect among its constituents that was relatively alleviated upon fractionation into polar and non-polar fractions. Chinese chestnut is an enriched resource of diverse phytochemicals having great nutraceutical potential.

Keywords**:** *C. mollissima* Blume, DPPH, NO, COX, UPLC-MS, GC/MC

1. Introduction

Chestnut species (family Fagaceae) are grown worldwide as valuable nutritional resources containing myriad functional ingredients, thus have promising applications in the nutraceutical and pharmaceutical industry [1, 2]. It includes *Castanea mollissima* Blum. (Chinese chestnut), *C.crenata* Sieb. & Zucc. (Japanese chestnut), *C.dentata* (Marsh.) Borkh. (American chestnut), and *C. sativa* Mill. (European or sweet chestnut) [3,4]. The Chinese chestnut is a multifunction food crop with good nut quality and strong resistance against stress allowing it to become an important germplasm resource for edible chestnuts [2]**.** *C. mollissima* nuts can be eaten dried, fried
or cooked to benefit from their high starch, dietary fiber, protein, vitamins, minerals and essential fatty acid contents [2]**.** It has been long used in Chinese folk medicine for various health-promoting properties that could be attributed to its high phenolic contents *viz.* phenylpropanoids, flavonoids, tannins, phenolic acids, anthocyanins and procyanidins, in addition to alkaloids and terpenes [5,2]**.** The burs and shell extracts from *Castanea* species were reported for their antioxidants, antibacterial, apoptotic, antilipemic and antiproliferative effects toward different human cancer cell lines [6,7,8,4,9]. Ellagic acid and chestanin are among the main constituents of *Castanea sp*. that exhibited anti-fungal activity [10]**.** Glucan extracted from *C. mollissima* fruit possesses anti-proliferative effect while castanol B extracted from its shells is reported to induce cell apoptosis [11,12]. The current study aimed to evaluate the antioxidant and anti-inflammatory potential of *Castanea mollissima* fruit extracts of different polarities and investigate its phytochemical composition *via* UHPLC/ HR- MS/MS and GC/MS for a comprehensive overview, thus aiding to further highlight the nutraceutical value of chestnut fruit.

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2. Material and methods

2.1. Plant material

C. mollissima fruits (Chinese chestnut) were obtained from local market (exported from China and authenticated by Fruit Department, Agricultural Institution, National Research Centre(Egypt).

2.2. Experimental

2, 2-diphenyl-1-picrylhydrazyl (DPPH), Ascorbic acid (vitamin C), celecoxib, indomethacin were purchased from Sigma-Aldrich, USA. Sodium nitroprusside (s.d.Fine-Chem.Ltd,Mumbai), *In vitro* anti-inflammatory activity was assessed using a kit provided by Cayman Chemical Company (USA). All solvents used are of analytical grade ADWIC (Egypt).

2.3. Preparation of total ethanol extract (TEE)

One Kg of Chinese chestnut was peeled, ground and extracted with 80% ethanol till exhaustion, the combined filtered extract was evaporated to dryness under vacuum at 55°C in a rotary evaporator to give the total ethanol extract of peeled fruit (TEE 55g), which is analyzed by UPLC-HR/MS-MS.

2.4. Preparation of the polar and non-polar fractions

Ten g of TEE extract of the fruit was defatted repeatedly with dichloromethane in a separating funnel until complete exhaustion, filtered, and concentrated under reduced pressure to dryness at 55°C using a rotary evaporator (Heidolph, Germany) to yield defatted polar fraction (PF, 9.35 g) and non-polar fraction (NP, 0.65 g), the later was further subjected to chemical investigations of its saponifiable and non-saponifiable components by GC/MS analysis.

2.5. In vitro bioactivity assays

2.5.1. Antioxidant activity

TEE, NPF and PF of *C. mollissima* were screened for free radical scavenging using DPPH and NO scavenging assays.

2.5.1.1. DPPH radical scavenging assay

The DPPH assay was performed according to the reported method of Ibrahim *et al.*,**[13]**. The absorbance was measured at 517 nm.

2.5.1.2. NO radical scavenging assay

The test is predicated on the concept that at physiological pH, nitrite ions, as detected by the Greiss reagent, are generated when sodium nitroprusside (SNP) is dissolved in an aqueous solution[14].The method was carried out as described by Ibrahim *et al.*, [13]. The absorbance of these solutions was measured at 540 nm against a blank solution.

2.5.2. Anti-inflammatory activity cyclooxygenase inhibition

The TEE of *C. mollissima* was tested for *in vitro* anti-inflammatory activity *via* inhibition of cyclooxygenase enzymes procedures were followed as per manufacturer's instructions [15]. Indomethacin and celecoxib were used as standard anti-inflammatory compounds.

2.6. Phytochemical study

2.6.1. Estimation of total phenolic content (TPC).

TPC was assayed using the Folin–Ciocalteu assay[16]. The absorbance was measured against the prepared reagent blank at 750 nm using a spectrophotometer (Jasco V630 spectrophotometer, Guangdong, China (Mainland)). The TPC was expressed as mg gallic acid equivalents GAE/100g extract.

2.6.2. Estimation of total flavonoid content (TFC)

TFC was determined using the aluminium chloride colorimetric assay method [17]. The absorbance of the solution was measured at 510 nm. The results were expressed as mg quercetin equivalents (QE) /100g extract. All samples were analyzed in triplicate.

2.6.3. Lipoidal content investigation in non-polar fraction via GC/MS analysis

One gram of the dichloromethane extract was refluxed for 6 h with 0.5 N alcoholic KOH (200 ml) in a boiling water bath. The procedure was conducted as reported [18] to obtain unsaponifiable fraction (USF) & fatty acid K salt residues. The residue of fatty acids was dissolved in 100 ml absolute methanol, mixed with 0.5 ml sulphuric acid, refluxed for 3 h in a boiling water bath, then cooled and evaporated to dryness to obtain fatty acid methyl ester fraction (FAME) [19]. The USF and FAME fractions of *C. mollissima* were subjected to GC/MS analysis adopting the following conditions. Capillary column of fused silica, 30m length, 0.32mm ID. and 0.25µm thickness, with TR-5MS (5% phenylpolysil phenylene siloxane as stationary phase, the carrier gas is Helium at 1ml/min, 13 psi. The Temperature programming is at 60°C isothermal for 3min 60°C- 280°C at a rate of 5°C/min at 260°C isothermal for 10 min. Ion source temperature 200°C, ionization voltage 70 eV, detector, Mass spectrometer. The constituents were identified by matching their mass fragmentation spectra and retention times analyzed by Gas / Mass tools with those of the library (Wiley Int.USA) and NIST (Nat. Inst. St. Technol., USA) and / or published data [20].

2.6.5. UPLC/HR-MS/MS analysis

The sample for LC-MS/MS analysis was prepared by adding 1 mL of the mobile phase working solution (acetonitrile: methanol: water, 25: 25: 50) to 50 mg of TEE of *C. mollissima* and proceeded as reported by [21]**.** LC-MS/MS analysis of the TEE in both positive and negative ionization modes was carried out on Exion LC High flow LC (Sciex hardware) coupled with triple TOF 5600 + IDA acquisition for LC-QTOF control according to [17]. The peaks and spectra were processed using the Analyst TF 1.7.1 and PeakView®1.2 Softwares (SCIEX, Framingham, MA, USA). The tentative identification of the compounds was carried out by comparison of their masses, fragmentation pattern, and molecular formula with those in the literature.

2.6.6. Statistical Analysis

The significance of variation in *C. mollissima* was analyzed by ANOVA one-way and post hoc for multiple comparisons using the IBM-SPSS statistics program (version 25) at $p \leq 0.05$, a t-test (n=3 replicates) and the significance of difference among means was determined at $p \leq 0.05$**.** The data is presented as mean± SD.

3. Results and discussion

3.1. In vitro bioactivity assays

3.1.1. Antioxidant activity

Antioxidant capacity of *C. mollissima* fruit TEE, PE and NP fractions were determined by DPPH and NO assays at concentration range of 62-1000 µg/mL[13] **.** Results showed a concentration-dependent scavenging activity (**Fig. 1 & 2**) where TEE showed the most potent effect against both DPPH and NO radicals with IC_{50} (µg/mL) values of 120.69 ± 0.54 and 230.02± 0.321, respectively, compared to vitamin C (213.5±0.121 and 211.56 ± 0.181 µg/mL respectively). While polar and non-polar fractions showed IC_{50} values (µg/mL) of 183.302± 0.165 and 358.68 ± 0.243 for the DPPH assay and 316.94 ± 0.432 and 449.26 ± 0.233 for the NO scavenging assay. Thus, from the above results it was concluded that TEE had better antioxidant activity than its fractions inferring a synergistic effect among its constituents that was partly lost upon fractionation.

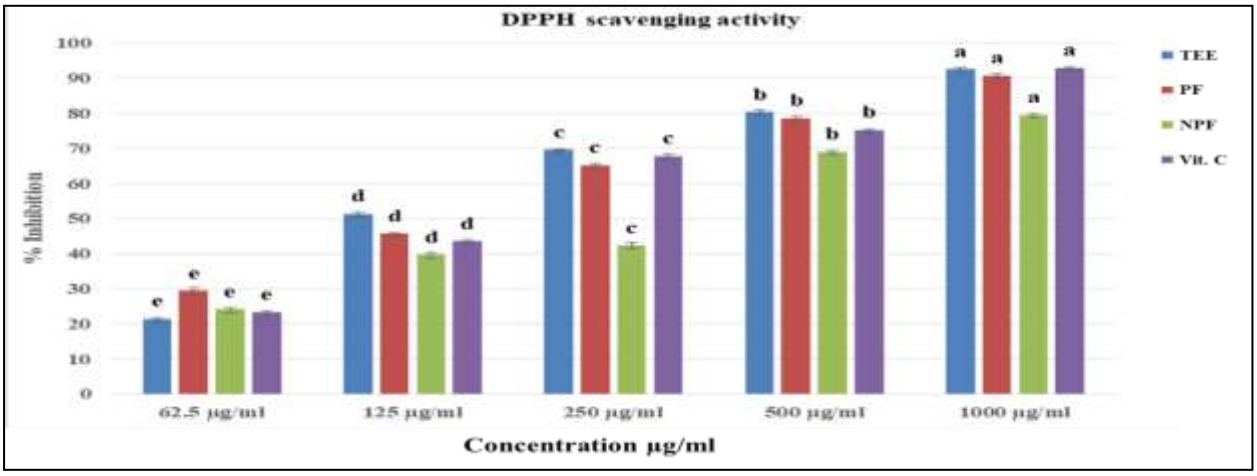

Fig: 1. The percentage of inhibition of DPPH free radicals by Chinese chestnut fractions at different concentrations against vitamin C. TEE: total ethanol extract, PF: polar fraction, NPF: non polar fraction. Values represent the mean ±SD. Different letters mean significant differences between concentration of the same extract $p<0.05$

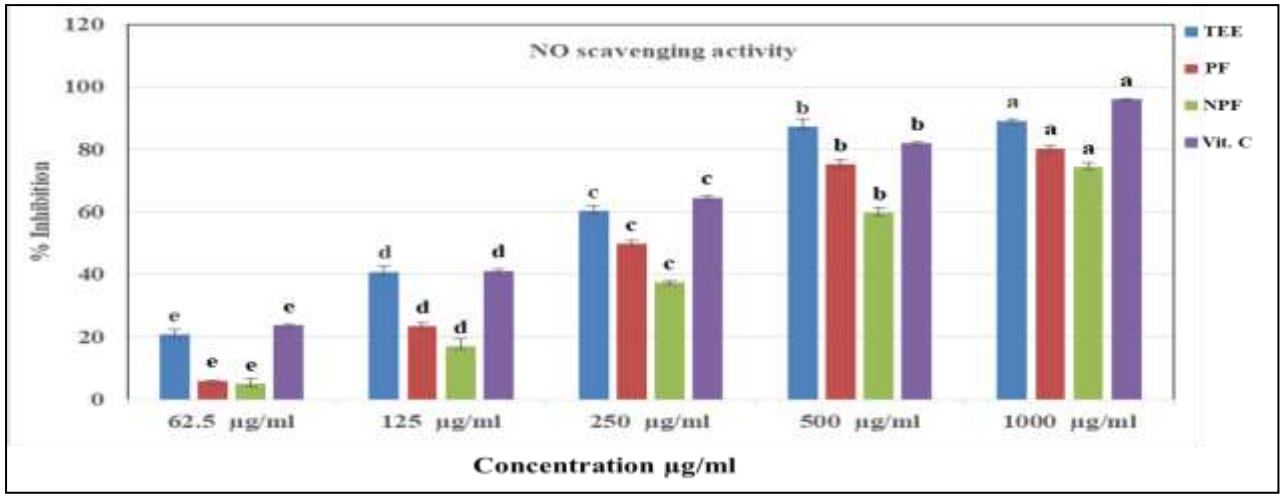

Fig 2: The percentage of inhibition of Nitric oxide (NO) free radicals by Chinese chestnut fractions at different concentrations. TEE: total ethanol extract, PF: polar fraction, NPF: non polar fraction. Values represent the mean ±SD. Different letters mean significant differences between concentration of the same extract $p<0.05$

3.1.2. **Anti-inflammatory activity**

C. mollissima fruit TEE proceeded to assay its anti-inflammatory activity against COX-2 not COX-1 enzymes. It exhibited a promising activity as COX-2 inhibitor with IC_{50} (µg/mL) (1.867 ± 0.115 and 38.25±0.68, for COX2 and COX1, respectively compared to indomethacin (0.13 ± 0.1; 27.031± 0.006 µg/mL) and celecoxib (4.18 ±0.1; 92.52 ± 0.01 µg/mL) as reference drugs, respectively. The observed anti-inflammatory activity *via* COX inhibition could be attributed to the presence of tannins, flavonoids and phenolic acids [22].

3.2. Phytochemical Study

3.2.1. Total phenolic and flavonoids contents

Total phenolic and flavonoid contents (TPC and TFC) were quantified as gallic acid and quercetin equivalent (GAE/100 g or QE/100 g extract), respectively, in TEE, PF, and NPF. It was found that TEE had the highest TPC (45 ± 0.0028 mg GAE/100 g), and TFC (29±0.006 mg QE/100 g), followed by

PF (39±0.003 mg GAE/100 g and 15±0.007 mg QE/100 g), then NPF (21± 0.001mg GAE/100g, TFC: undetected) (**Fig. 3**). This finding is consistent with the observed higher antioxidant activity of TEE superior to other PF and NPF since phenolic and flavonoid compounds are well reported for their radical scavenging activity as well as other health promoting properties [23]**.** Phenolic compounds from natural resources are recommendable as food additives in food processing more than the artificial antioxidants butylated hydroxyanisole and butylated hydroxytoluene [23,24]

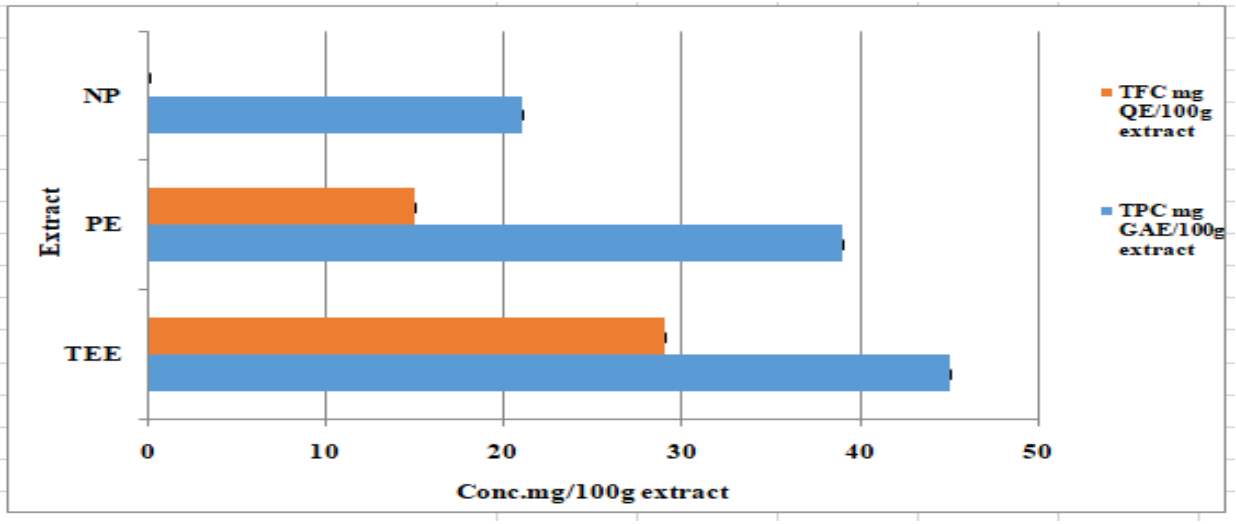

Fig. 3: Total phenolic content (TPC) and total flavonoid content (TFC) in Chinese Chestnut *C. mollissima* fruit. Values represent the mean ±SD.

3.2.2. Lipoidal content investigation in non-polar fraction *via* GC/MS analysis

GC/MS analysis of the Chinese chestnut non polar fraction extracted with dichloromethane revealed the identification of 32 unsaponifiable compounds constituting 75.19% of the total fraction. The oxygenated compounds accounted for **25.98%,** the oxygenated nitrogenous **20.92%** and the non-oxygenated compounds represented **28.29% (Table 1).** The major compounds were 2,2-Diethoxy ethanamine (**20.92**%), butylated hydroxytoluene (**7.34**%) in addition to stigma-5-en-3-ol **(3.50%)** and kaur-16-en-19-ol terpenoid **(0.81%).** On the other hand, analysis of the FAME resulted in the identification of 17 compounds constituting (**96.30**%) of the total fraction among which **32.22**% were unsaturated and **64.08**% were saturated fatty acids (**Table 2**). The major constituent was methyl hexadecanoate **(61.64%)** and 9,12- octadecadienoate **(28.13%).** The later major compound 9,12- octadecadienoic acid methyl ester is **ω-6** fatty acid , studies suggest that PUFA (polyunsaturated fatty acids) can help to reduce the risk of various health conditions, including heart disease, inflammation, atherosclerosis, autoimmune disorders, and diabetes [25].

Table 1: GC/MS analysis of the unsaponifiable fraction of Chinese chestnut *Castanea mollissima* fruit.

No	R_t	Compounds	BP	M^+	Area %	Molecular formula
1	9.43	1-Octene	55	112	3.02	C_8H_{16}
2	13.33	2,2-Diethoxy ethanol	103	134	0.88	$C_6H_{14}O_3$
3	13.91	1-Dodecene	55	168	0.94	$C_{12}H_{24}$
4	14/14	2,2-Diethoxy ethanamine	103	133	20.92	$C_6H_{15}NO_2$
5	19.19	Tetradecene	55	196	2.47	$C_{14}H_{30}$
6	19.39	1,1,3,3-tetraethoxy propane	103	220	2.84	$C_{11}H_{24}O_4$
7	21.31	3-Isopropyl-6,10-dimethyl-cyclodecene-1,4-dione (Neocurdione)	57/165	236	2.40	$C_{15}H_{24}O_2$
8	22.27	Butylated hydroxyl toluene	205	220	7. 34	$C_{15}H_{24}O$
9	22.41	2-Tert Butyl-4-isopropyl-5-methyl phenol	191	206	3.28	$C_{14}H_{22}O$
10	23.96	Hexadecene	224	55	3.73	$C_{16}H_{32}$
11	24.12	Hexadecane	226	57	0.69	$C_{16}H_{34}$
12	25.46	β- Eudesmol	79/149	222	0.63	$C_{15}H_{26}O$
13	25.53	α-Eudesmol	59	222	1.77	$C_{15}H_{26}O$
14	26.46	2-phenyl undecane	105	232	1.18	$C_{17}H_{28}$
15	27.82	3-Phenyl dodecane	91	246	0.85	$C_{18}H_{30}$
16	28.27	3-Octadecene	55	252	4.65	$C_{18}H_{36}$
17	28.39	Octadecane	57	254	1.14	$C_{18}H_{38}$
18	28.61	2-Phenyl dodecane	105	246	1.08	$C_{18}H_{30}$
19	29.05	6-Phenyl tridecane	91	260	0.74	$C_{19}H_{32}$
20	29.19	5-Phenyl tridecane	91	260	0.64	$C_{19}H_{32}$
21	29.37	6,10,14-Trimethyl pentadecane -2-one	58	268	2.53	$C_{18}H_{36}O$
22	30.66	2-Phenyl tridecane	105	260	0.70	$C_{19}H_{32}$
23	32.17	3-Eicosene	55	280	3.84	$C_{20}H_{40}$
24	35.74	Docosene	55	308	2.40	$C_{22}H_{44}$
25	35.83	Docosane	57	310	0.90	$C_{22}H_{46}$
26	37.50	Tricosane	57	324	0.83	$C_{23}H_{48}$
27	38.72	Kaur-16-en-19-ol	55	288	0.81	$C_{20}H_{32}O$
28	39.03	Tetracosene	57	236	1.09	$C_{24}H_{48}$
29	39.10	Tetracosane	57	238	1.04	$C_{24}H_{50}$
30	40. 64	Pentacosane	57	252	2.70	$C_{25}H_{52}$
31	42.12	Hexacosane	57	366	1.00	$C_{26}H_{54}$
32	51.88	Stigma-5-en-3-ol	414	414	3.50	$C_{29}H_{50}O$
Total identified compounds					**75.19**	
Non oxygenated compounds					28.29	
Oxygenated compounds					25.98	
Sterol					3.50	
Terpenoids					0.81	
Oxygenated nitrogenous compounds					**20.92**	

Table 2: GC/MS analysis results of fatty acid methyl esters detected in Chinese chestnut *C. mollissima* fruit non polar fraction

No	R_t	Compounds	BP	M^+	Molecular formula	Area %
1	6.42	Undecanoic acid methyl ester	74	200	$C_{12}H_{24}O_2$	0.05
2	6.60	Dodecanoic acid methyl ester	74	214	$C_{13}H_{26}O_2$	0.04
3	10.30	Pentadecanoic acid methyl ester	74	256	$C_{16}H_{32}O_2$	0.04
4	10.66	12-Methyl-tetradecanoic acid methyl ester	0.16	256	$C_{16}H_{32}O_2$	0.16
5	12.48	9-Hexadecenoic acid methyl ester	55	268	$C_{17}H_{32}O_2$	2.40
6	12.99	Hexadecanoic acid methyl ester	74	270	$C_{17}H_{34}O_2$	61.64
7	15.20	14-Methyl-pentadecanoic acid methyl ester	74	270	$C_{17}H_{34}O_2$	0.15
8	15.24	14-Methyl- hexadecanoic acid methyl ester	74/87	284	$C_{18}H_{36}O_2$	0.19
9	15.68	Hexadecanoic acid-2-hydroxy-methyl ester	55/83	286	$C_{17}H_{34}O_3$	0.67
10	16.76	9,12- Octadecadienoic acid methyl ester	67	294	$C_{19}H_{34}O_2$	28.13
11	17.28	9,12,15- Octadecatrienoic acid methyl ester	79	292	$C_{19}H_{32}O_2$	1.38
12	17.52	14-Methyl- heptadecanoic acid methyl ester	74/87	298	$C_{19}H_{36}O_2$	0.46
13	21.53	9-Eicosaenoic acid methyl ester	55	324	$C_{21}H_{40}O_2$	0.31
14	21.01	18-Methyl- nonadecanoic acid methyl ester	87	326	$C_{21}H_{42}O_2$	0.18
15	23.50	Heneicosanoic acid methyl ester	74	340	$C_{22}H_{44}O_2$	0.11
16	25.44	Docosanoic acid methyl ester	74	354	$C_{23}H_{46}O_2$	0.34
17	29.02	Tetracosanoic acid methyl ester	74	382	$C_{25}H_{50}O_2$	0.05
Total identified compounds						**96.30**
Saturated fatty acids						**64.08**
Unsaturated fatty acids						**32.22**

2. UPLC/HR-MS/MS analysis of the Chinese chestnut TEE

UPLC/HRMS/MS analysis was used to examine the active TEE of the edible part of *C. mollissima* Blume fruit. Metabolite elution occurred in a run time of 30 min, with metabolites eluted according to their polarity, from most polar to least polar. According to available literature data, they were identified using

retention time (Rt), MS data (molecular ion, fragmentation pattern and predicted formulae), compared to reported literature data (MS databases (FooDB, HMDB and Massbank) [26,27]**.** In this study, 41 metabolites were tentatively identified that included 9 fatty acids, 12 flavonoids, 4 organic acids, 4 phenolic compounds, 5 polyphenol, 2 phospholipids and 1 triterpene (**Table 3**).

Table 3. UPLC/HR-MS/MS analysis results of the Chinese chestnut *C. mollissima* Blume fruit TEE in both negative and positive ionization modes

No.	Rt. (min.)	Identification	$[M-H]^-$/ $[M+H]^+$	Elemental composition	Error (*ppm*)	MS_n ions *m/z* (+/-)
1	1.1	Malic acid	133.01315	$C_4H_5O_5^-$	1.8	115, 89, 71
2	1.13	Citric acid	191.01766	$C_6H_7O_7^-$	5	173, 129, 111, 87
3	1.2	Monogalloyl glucose	331.06788	$C_{13}H_{15}O_{10}^-$	5	313, 211, 169, 125
4	1.22	Isopropylmalic acid	175.05995	$C_7H_{11}O_5^-$	0.9	131, 115, 87, 71
5	1.27	Sucrose	341.10745	$C_{12}H_{21}O_{11}^-$	1.1	179, 161, 143, 131, 101, 89

6	2.4	Ellagic acid	300.99778	$C_{14}H_5O_8^-$	0.4	284, 257, 201, 145
7	2.5	Pantothenic acid	220.11605	$C_9H_{18}NO_5^+$	2.3	202,184, 124, 90, 72
8	2.9	Hydroxymethyl-methoxyphenyl-*O*-hexoside	315.10742	$C_{14}H_{19}O_8^-$	0.1	297, 179, 153, 123
9	3.45	Epigallocatechin-glucuronide	481.09884	$C_{21}H_{21}O_{13}^-$	2.4	463, 453, 437, 395, 305, 287, 277, 255
10	5.7	Hydroxybenzaldehyde	121.02902	$C_7H_5O_2^-$	2.4	108, 93, 77
11	5.9	Methyl Ellagic acid	315.01315	$C_{15}H_7O_8^-$	1.3	300, 247,
12	6.17	Quercetin-*O*-rhamnosylhexoside	609.14595	$C_{27}H_{29}O_{16}^-$	1.5	447, 301
13	6.43	Quercetin-*O*-hexoside	463.0866	$C_{21}H_{19}O_{12}^-$	1.1	301, 286, 270, 151
14	6.55	Gibberellin A-*O*-hexoside	509.20317	$C_{25}H_{33}O_{11}^-$	2.8	347, 289, 237, 163
15	6.8	Kaempferol-*O*-hexoside	447.09185	$C_{21}H_{19}O_{11}^-$	0.8	285, 151
16	6.86	Scopoletin	191.0339	$C_{10}H_7O_4^-$	0.1	176, 163, 148
17	6.9	Isorhamntein-*O*-rhamnosylhexoside	623.15952	$C_{28}H_{31}O_{16}^-$	1.8	461, 315
18	7.3	Dihydrokaempferol	287.05622	$C_{15}H_{11}O_6^-$	4	259, 243, 219, 201, 151
19	7.8	Phlorizin	435.12748	$C_{21}H_{23}O_{10}^-$	2.5	273, 167, 122
20	8	Eriodictyol	287.05622	$C_{15}H_{11}O_6^-$	4	151, 135, 125
21	8.9	Castacrenin A/B/C	613.04424	$C_{27}H_{17}O_{17}^-$	2.9	493, 299
22	9.31	Dimethyl Ellagic acid	329.02986	$C_{16}H_9O_8^-$	2	314, 299, 285, 271
23	9.5	Luteolin	285.03943	$C_{15}H_9O_6^-$	0.2	268, 151, 133
24	10	Gingerol	293.17537	$C_{17}H_{25}O_4^-$	2.2	236, 221, 192, 177
25	10.3	Naringenin	271.06112	$C_{15}H_{11}O_5^-$	3.8	253, 227, 177, 151, 119, 107
26	10.5	Methoxy-cinnamic acid	177.05477	$C_{10}H_9O_3^-$	0.8	162, 145, 133, 117
27	11.4	Hydroxy-Dimethoxyflavone	297.0755	$C_{17}H_{13}O_5^-$	0.8	265, 251, 191, 151, 147
28	12.1	Trimethylellagic acid	343.04573	$C_{17}H_{11}O_8^-$	2.6	328, 313, 297, 285, 270
29	16.13	Lysophosphatidylcholine(18:3) 9,12,15	518.32412	$C_{26}H_{49}NO_7P^+$	3.3	184,124,104
30	17.1	Hydroxy-octadecadienoic acid	295.22786	$C_{18}H_{31}O_3^-$	3.5	277
31	17.22	2-Linoleoyl-lysophosphatidylcholine: LPC [0:0/18:2(omega-6)]	520.33977	$C_{26}H_{51}NO_7P^+$	0.3	502,184,104
32	20	Oxooctadecatrienoic acid	291.19641	$C_{18}H_{27}O_3^-$	3.2	247
33	20.5	Oxooctadecanoic acid (oxostearic acid)	297.24396	$C_{18}H_{33}O_3^-$	3.2	253
34	20.67	Linolenic acid	277.21683	$C_{18}H_{29}O_2^-$	2.2	259
35	22.4	Linoleic acid	279.2312	$C_{18}H_{31}O_2^-$	2.5	261, 71

36	22.45	Digalloyl-hydroxyphloretin-*O*-hexoside	755.14472	$C_{35}H_{31}O_{19}^-$	0.9	737, 603, 435, 415, 347, 249
37	22.8	Ursolic/ Oleanolic acid	455.35166	$C_{30}H_{47}O_3^-$	0.7	409, 341, 317, 251, 161, 89
38	23.8	Palmitic acid	255.23241	$C_{16}H_{31}O_2^-$	2	211
39	24.1	Stearic acid	283.26236	$C_{18}H_{35}O_2^-$	2.8	239
40	24.23	Methyl Stearic acid	297.278	$C_{19}H_{37}O_2^-$	2.7	282
41	24.6	Oleic acid	281.24837	$C_{18}H_{33}O_2^-$	3.1	237

Organic acids

Four organic acids were detected in peaks 1,2,4 & 26 identified as malic acid, citric acid, isopropylmalic acid and methoxy-cinnamic acid (**Table 3**) matching reported literature on *C. mollissima* [5] except peak 26 that is the first time to be reported herein according to our knowledge. Organic acids mass spectra were recognized by the neutral loss of water (-H_2O, 18 amu) and carboxyl (-COO, 44 amu) moieties in addition to the alkyl substituent.

Phenolic compounds

Four phenolic compounds (in peaks 3, 8, 10, 24) detected at $[M-H]^-$ 331.06788; $C_{13}H_{15}O_{10}^-$ 315.10742; $C_{14}H_{19}O_8^-$, 121.02902; $C_7H_5O_2^-$ and 293.17537; $C_{17}H_{25}O_4^-$ (**Table 3**) were identified as monogalloyl glucose, hydroxymethyl-methoxyphenyl-*O*-hexoside (known as vanilloloside), hydroxybenzaldehyde, and gingerol, respectively, matching reported literature on Chinese chestnut [5].

Polyphenols

Chinese chestnuts are known for their enriched polyphenolic content, particularly ellagic acid and their derivatives in burs and shells are known for their potent antioxidant and anti-inflammatory effects [2]. Herein, we report the presence of 5 polyphenolic compounds in peaks 6, 11, 21, 22, and 28 (**Table 3**) identified as ellagic acid and its mono-/di-/tri-methyl derivatives recognized by neutral loss of methyl and water moieties matching literature. The methoxylated derivatives of ellagic acids, detected here for the first time, are reported for various bioactivities, e.g., antimicrobial and anticancer [28 ,29]. In peak 22, castacrenin A/B/C is an ellagitannin detected for the first time in the Chinese chestnut at $[M-H]^-$ 613.04424; $C_{27}H_{17}O_{17}^-$ despite previously reported in the Japanese and European chestnuts [30, 31].

Flavonoids

This is the major class detected in this study, where 12 compounds, including 2 chalcones (peaks 19, 36), 3 flavanones (peaks 9, 20, 25), 2 flavones (peaks 23, 27), and 5 flavonols (peaks 12, 13, 15, 17, 18) were tentatively identified (**Table 3**). Fragments included deglycosilation, dehydration, and RDA, matching previously reported literature on Chinese chestnut [5] except peak 36 digalloyl-hydroxyphloretin-*O*-hexoside which is reported herein for the first time. The metabolite was detected at $[M-H]^-$ 755.14472, $C_{35}H_{31}O_{19}^-$ and showed product ions corresponding to dehydration and sequential loss of galloyl moieties at *m/z* 737, *m/z* 603, and *m/z* 435 that is phlorizin, a previously reported chalcone in *C. mollissima* [5].

Fatty acids (FA)

In this study, 9 fatty acids were detected at Rt 17-24 min in peaks 30, 32-35, 38-41 (**Table 3**) including saturated and unsaturated FA, several of which are oxygenated FA matching literature [5]. The metabolites are recognized by dehydrated and decarboxylated product ions. Chinese chestnut is known to contain significant levels of mono- and poly-unsaturated FA, promoting it as a good functioning food resource [5, 2].

Phospholipids

Two lysophosphatidylcholine (LPC) in peaks 29 and 31 were identified as LPC18:3 (9,12,15) & 2- LPC 0:0/18:2(omega-6) recognized by the diagnostic product ion at *m/z* 184 of the phosphocholine head group as previously reported in Chinese chestnuts [32, 5].

Discussion

Inflammation and oxidative stress are connected to cellular processes. Excessive ROS production can lead to lipid peroxidation and stimulate macrophages, which in turn activate inflammatory transcription factors like NF-κB. These results in the release of pro-inflammatory cytokines like IL-6, IL-1β, and TNF-α, which can further contribute to ROS production [33, 34].

ROS are associated with the inflammatory response and frequently contribute to the tissue-damaging effects of inflammatory reactions. Chestnut may exert anti-inflammatory effects *via* reduction of the pro-inflammatory mediators through suppression of the NF-κB-mediated signaling pathways [35]**.** Chestnut extracts possessed DPPH radical scavenging activity and reduced the intracellular H_2O_2-induced ROS accumulation. It is used as an anti-irritant in cosmetic products to prevent or treat skin irritations [36]. It was reported that chestnut extract reduces oxidation of low density lipoproteins (LDL) and cholesterol, so it can help in

the prevention of coronary heart disease [37]**.** Chestnuts have high amounts of healthy bioactive metabolites of nutritional value, capable of improving the performance of the human body [38]**.** It had high contents of starch, polyphenols, antioxidants, vitamins (C, E) and minerals as K&P. The lipid fraction of chestnuts was rich in unsaturated fatty acids such as oleic acid and α-linolenic acid. These characteristics make chestnuts a valuable and healthy source of phytoconstituents and nutritional value for consumers [35]**.** Ellagic acid is metabolized to urolithine (A, B, D, etc.), a metabolic compound that plays an essential role in cardiovascular protection, acting as an anti-inflammatory. Gallic acid has also shown various biological properties, including antioxidant, anti-inflammatory, anti-viral, and anti-cancer activities [39]**.** Chestnut fruits can be consumed in their fresh state, raw, boiled, or roasted, or as a processed ingredient, such as chestnut flour, flakes, purée, yogurts, or candied chestnuts [40]. Roasted chestnuts are well accepted for their sweetness, coming from the sucrose content [41]**.** Chestnut fruits are gluten-free, making them a suitable alternative to wheat or cereal-based products. As a result, many new chestnut and chestnut flour-derived products have been developed, including pasta [42]**.** Nutritional analysis shows that chestnut fruits are rich in essential dietary nutrients and minerals, the low fat content and high unsaturated fatty acid content make them a healthy food option. Additionally, the high starch content and moderate sugar levels make chestnuts an energy-dense food crop, deserving more attention as a nutritious food source[43]**.**

Conclusion

The phytochemical study identified 41 metabolites *via* UPLC/HR-MS/MS, 32 unsaponifiable compounds, and 17 fatty acids as methyl esters, several of which are the first to be reported in Chinese chestnut. The *in vitro* bioactivity study recommended that fruit total ethanol extract (TEE) possesses a synergistic effect among its constituents, evidenced by its superior free radical scavenging activity *via* DPPH and NO assays compared to its polar and non-polar fractions. TEE has a selective anti-inflammatory effect against COX-2, not COX-1 enzymes, compared to indomethacin and celecoxib as reference drugs. The obtained results corroborate the great potential of Chinese chestnut in nutraceuticals and recommend further *in vivo* studies on their bioactivities.

5. Conflicts of interest

There are no conflicts to declare

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