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•Original article•

Isolation and microbial transformation of tea sapogenin from seed pomace of *Camellia oleifera* with anti-inflammatory effects

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[ABSTRACT] In the current study, tea saponin, identified as the primary bioactive constituent in seed pomace of *Camellia oleifera* Abel., was meticulously extracted and hydrolyzed to yield five known sapogenins: 16-*O*-tiglogycamelliagnin B (**a**), camelliagnin A (**b**), 16-*O*-angeloybarringtogenol C (**c**), theasapogenol E (**d**), theasapogenol F (**e**). Subsequent biotransformation of compound **a** facilitated the isolation of six novel metabolites (**a1**−**a6**). The anti-inflammatory potential of these compounds was assessed using pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns molecules (DAMPs)-mediated cellular inflammation models. Notably, compounds **b** and **a2** demonstrated significant inhibitory effects on both lipopolysaccharide (LPS) and high-mobility group box 1 (HMGB1)-induced inflammation, surpassing the efficacy of the standard anti-inflammatory agent, carbenoxolone. Conversely, compounds **d**, **a3**, and **a6** selectivity targeted endogenous HMGB1-induced inflammation, showcasing a pronounced specificity. These results underscore the therapeutic promise of *C. oleifera* seed pomace-derived compounds as potent agents for the management of inflammatory diseases triggered by infections and tissue damage.

[KEY WORDS] *Camellia oleifera*; Seed pomace; Tea sapogenin; Microbial transformation; Damage-associated molecular patterns and Pathogen-associated molecular patterns; Anti-inflammatory activity
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Introduction

Camellia oleifera Abel. is extensively cultivated in China, Japan, India, and other Southeast Asian countries, ranking alongside olive (*Olea europaea*), coconut (*Cocos nucifera*), and oil palm (*Elaeis guineensis* Jacq) as one of the world's four largest woody oil plants [1]. It is projected that by 2025, the total area cultivated with *C. oleifera* in China will reach 6 million hectares, with an expected annual Camellia oil output of 2.5 million tons [2]. The seed cake, a byproduct of*Camellia* oil production, accounts for approximately 50% of the seed's weight [3]. Currently, this seed cake is largely underutilized, often being discarded as industrial waste or used in low-value applications such as fertilizer, animal feed, or organic compost [4]. This practice not only wastes valuable resources but also contributes to environmental pollution. The *C. oleifera* seed cake still harbors significant quantities of bioactive compounds, including polyphenols, flavonoids, and polysaccharides, and is particularly rich in tea saponins, comprising 10%−20% of its content [5, 6]. Tea saponins, naturally occurring oleanane-type triterpenoid saponins found in oil

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These authors have no conflict of interest to declare.

crops like *C. oleifera* and its defatted seed cake [7], have the potential for commercial exploitation. These isolated saponins could be utilized in the development of functional foods, pharmaceuticals, natural preservatives, detergents, or as foaming agents and stabilizers in the beer industry [8, 9]. Thus, capitalizing on the tea saponin content in *C. oleifera* seed cake through green and efficient microbial fermentation processes to produce value-added products holds significant importance.

Inflammation serves as a physiological, protective response by the body. However, prolonged and excessive chronic inflammation can lead to damage across multiple organ systems, imposing a significant burden on individuals and society [10]. Two critical triggers, damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), mediate sterile and infectious inflammation, respectively [11]. PAMPs are unique to microorganisms, such as lipopolysaccharide (LPS)—a bacterial cell wall component [12]. Upon encountering exogenous PAMPs, effector cells of the innate immunity can actively release DAMP molecules, such as high-mobility group box 1 (HMGB1), *via* nonclassical pathways [13]. Acting as "a danger signal", an abundance of extracellular HMGB1 through the activation of the TLR4 receptor is implicated in the pathogenesis of diseases characterized by chronic inflammation, including rheumatoid arthritis, osteoarthritis, multiple sclerosis, chronic asthma, and inflammatory bowel disease [14, 15]. Notably, dietary intake of tea saponin has been reported to exert significant immunomodulatory effects on DAMP- and PAMP-involved inflammatory diseases [16, 17], highlighting its potential therapeutic application in mitigating these conditions.

Aligned with our longstanding interest in identifying potent pentacyclic triterpenoids, we have previously conducted biotransformation studies on constituents derived from olive pomace and soybean residues [18, 19]. In this research, we successfully extracted five known tea sapogenins from the seed pomace of *C. oleifera*. Among these, 16-*O*-tiglogycamelliagnin B (**a**), characterized by its unique substituents at the C-16 and C-23 positions, was chosen for subsequent microbial transformation. The structural elucidation of the resulting metabolites was accomplished through comprehensive Nuclear magnetic resonance (NMR) spectroscopy, high-resolution electrospray ionization mass spectrometry (HR-ESI-MS), and X-ray crystallography analyses. Given the acknowledged therapeutic benefits of tea sapogenins, it became imperative to explore their potential in treating inflammatory diseases. To this end, we assessed their inhibitory effects on both LPS- and HMGB1-activated RAW 264.7 cells. Furthermore, a preliminary structure-activity relationship (SAR) analysis of various tea saponins and their derivatives was conducted. This analysis revealed critical structural determinants, offering valuable insights for the development of novel drug candidates.

Results and Discussion

Given the intricate physicochemical properties of tea saponin, conventional extraction methodologies typically necessitate substantial organic solvent use and protracted extraction durations and incur high costs. To address these challenges, we adopted an approach combining water extraction with macroporous resin purification, setting the extraction parameters at a temperature of 75 °C, duration of 6 h, and a liquid-to-solid ratio of 10 mL·g^{-1}. This method yielded a maximum extraction efficiency of 5.73% for tea saponins from *C. oleifera* seed pomace (Fig. 1A). Subsequent hydrolysis under conditions of 80 °C for 5.5 h with 2.5

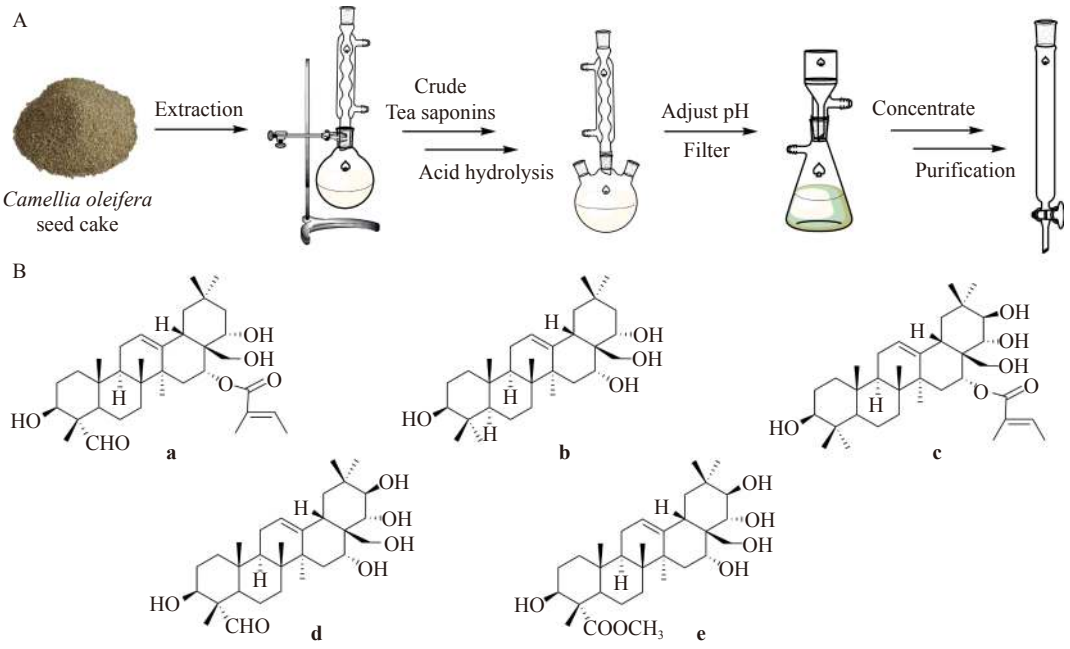

Fig. 1 Procedure of extraction and acid hydrolysis of *C. oleifera* (A) and chemical structures of compounds a–e (B).

mol·L^{-1} HCl concentration resulted in an 18.56% yield of crude sapogenin. Through this process, we isolated and identified five hydrolysis products: 16-*O*-tigloyl-camelliagnin B (**a**, 8.56% yield), camelliagnin A (**b**, 7.35%) [20, 21], 16-*O*-tigloyl barringtogenol C (**c**, 2.88%) [22], theasapogenol E (**d**, 0.67%) [23], and theasapogenol F (**e**, 0.54%) [24] employing 1D NMR, HR-MS and literature comparisons for structural verification (Fig. 1B). TThese compounds vary by the presence of a tigoyl group at the C-16 position and by their degrees of oxidation, particularly at the C-21 and C-23 positions. Notably,naturallyoccurringtriterpenoidsencompassover100distinctskeletal types [25], with the seeds of the tea plant primarily containing oleanane-type pentacyclic triterpenoids [26]. These are structurally akin to soyasaponins, a dietary group of triterpenoids found in soybeans and other legumes. In summary, the methodologies developed for the extraction, isolation, and identification of tea sapogenin from *C. oleifera* seed cake lay a foundational basis for subsequent pharmacological activity studies and potential industrial applications.

Compound **a** was isolated as an amorphous powder. HR-ESI-MS analysis determined its molecular formula as $C_{35}H_{54}O_6$, evidenced by the ion at *m/z* 571.3965 $[M + H]^+$ (calculated for $C_{35}H_{55}O_6$, 571.3993), indicating the presence of nine degrees of. The ^{13}C NMR spectra revealed the presence of one carbonyl carbon signal (δ_C 207.86), four oxygenated aliphatic carbon signals (δ_C 69.69, 72.12, 72.17, 72.84), and two olefinic carbon signals (δ_C 123.63, 142.98), consistent with an oleanane-type triterpenoid skeleton (Table S1). The tigloyl group's characteristic signals were identified at δ_C 167.68, 137.53, 130.48, 14.69, and 13.12, *as per* the literature. The ^{1}H NMR spectra displayed singular peak signals for six angular methyl groups at 1.45 (s, 3H), 1.34 (s, 3H), 1.13 (s, 3H), 1.10 (s, 3H), 0.92 (s, 3H), 0.83 (s, 3H), and a proton signal associated with the carbonyl carbon at δ_H 9.63 (s, 1H). The tigloyl group's proton signals were observed at δ_H 7.10 (qd, J = 7.0 Hz), δ_H 1.95 (s, 3H), δ_H 1.70 (d, J = 6.5 Hz), aligning with literature-reported data [26]. Consequently, compound **a** was identified as 16*α*-*O*-tiglogycamelliagnin B. Detailed structural elucidation of additional compounds is provided in the supplementary materials (Figs. S1−S16).

Compared to other constituents, compound **a** distinguishes itself with a tigloyl group at the C-16 position and an aldehyde group at C-23, contributing to its unique structural features. Being the most prevalent component extracted from the seeds of *C. oleifera*, compound **a** was selected for biotransformation experiments using four microbial strains: *Bacillus megaterium* CGMCC 1.1741, *Streptomyces griseus* ATCC 13273, *S. olivaceus* CICC 23628, and *Penicilium griseofulvum* CICC 40293. This process yielded six novel derivatives (Fig. 2), identified as 3*β*,22*α*,28-trihydroxy-16*α*-*O*-tigloylolean-12-en-23-oic acid (**a1**, yield 15.00%), 3*β*,22*α*,28-trihydroxy-16*α*-*O*-tigloylolean-12-en-23-oic acid methyl ester (**a2**, yield 14.25%), 3,4-*seco*-4,22*α*,23, 28-tetrahydroxy-16*α*-*O*-tigloylolean-12-en-3-oic acid (**a3**, yield 0.85%), 3,4-*seco*-4,28,29-trihydroxy-16*α*-*O*-tigloyl-12-en-22-oxo-23-norolean-3-oic acid (**a4,** yield 1.12%), 3,4-*seco*-22*α*,28-dihydroxy-16*α*-*O*-tigloyl-4-oxo-12-en-23-norolean-3-oic acid (**a5**, yield 1.75%), 3*β*,22*α*,28-trihydroxy-16*α*-*O*-tigloyl-23-norolean-12-ene (**a6**, yield 2.25%). The structural identification of these metabolites was meticulously achieved through comprehensive analysis using 1D and 2D NMR spectroscopy, HR-ESI-MS, and X-ray crystallography, thereby expanding the chemical diversity derived from C. *oleifera* seed components.

Compound **a1** was isolated as a white powder, with its molecular formula established as $C_{35}H_{54}O_7$ via the HR-ESI-MS, showing a peak at *m/z* 609.3746 $[M + Na]^+$ (calculated for $C_{35}H_{54}O_7Na$, 609.3762). This represents a 16 atomic mass unit (amu) increase from compound **a**, indicative of an additional oxygen atom incorporation. The ^{1}H NMR spectrum revealed six angular methyl signals at δ_H 1.63, 1.36, 1.12, 1.07, 0.97, and 0.83. The ^{13}C NMR spectrum displayed a novel carbon signal at δ_C 180.32 and the disappearance of the aldehyde carbon signal at δ_C 207.86 from the original C-23 position, suggesting the transformation of the aldehyde group into a carboxyl group. Additional carbon signals at δ_C 75.10, 72.15, 71.41, and 69.03, and olefinic carbon signals at δ_C 122.72 and 142.30 were observed (Table 1). In the HMBC spectrum, the signal at δ_H 4.64 (dd, J = 5.72, 10.40 Hz) exhibited long-range correlations with carbon signals at δ_C 54.18 (C-4), 39.00 (C-1), and 51.62 (C-5), indicating attachment to C-3. Furthermore, the signal at δ_H 1.63 (s, 3H) displayed correlations with carbon signals at δ_C 75.10 (C-3), 54.18 (C-4), and 51.62 (C-5), assigning this proton signal to H-24. The correlation of δ_C 180.32 with δ_H 1.63 (s, 3H, H-24) and 4.64 (dd, J = 5.72, 10.40 Hz, 1H, H-3) in the HMBC spectrum suggested that δ_C 180.32 corresponds to C-23. In the NOESY spectrum, a strong correlation was observed between δ_H 1.63 and δ_H 4.64 (dd, J = 5.72, 10.40 Hz), supporting the placement of this methyl group at the C-24 position. Additionally, δ_H 4.60 (dd, J = 5.45, 12.19 Hz, 1H) showed correlations with δ_C 31.46 (C-20) and 69.03 (C-28), assigning it to H-22. The signal at δ_H 6.33 (s, 1H) correlated with carbon signals at δ_C 41.38 (C-14), 44.11 (C-17), 72.15 (C-22) and 69.03 (C-28), indicating its position at H-16. The correlation of δ_H 6.33 (H-16) with δ_C 166.93 in the HMBC spectrum indicated the attachment of the tigloyl group to C-16, with further NOESY correlations suggesting a β-configuration for H-16. The correlation between δ_H 4.60 (dd, J = 5.45, 12.19 Hz, H-22) and δ_H 1.12 (s, 3H, H-30) indicated a *β*-configuration for H-22 (Fig. 3). Based on this comprehensive analysis, the structure of compound **a1** was elucidated as 3*β*,22*α*,28-trihydroxy-16*α*-*O*-tiglogyolean-12-en-23-oic acid. The detailed structural elucidation of compound **a2**−**a6** is provided in the Supplementary Information (Figs. S17−S58).

The biocatalysis of compound **a** with *P. griseofulvum* resulted in a singular product, **a1**, characterized by the oxidation of the aldehyde group at the C-23 position to a carboxyl group. Previous research has highlighted the capability of *S. griseus* for excellent site-selective oxidation of angular

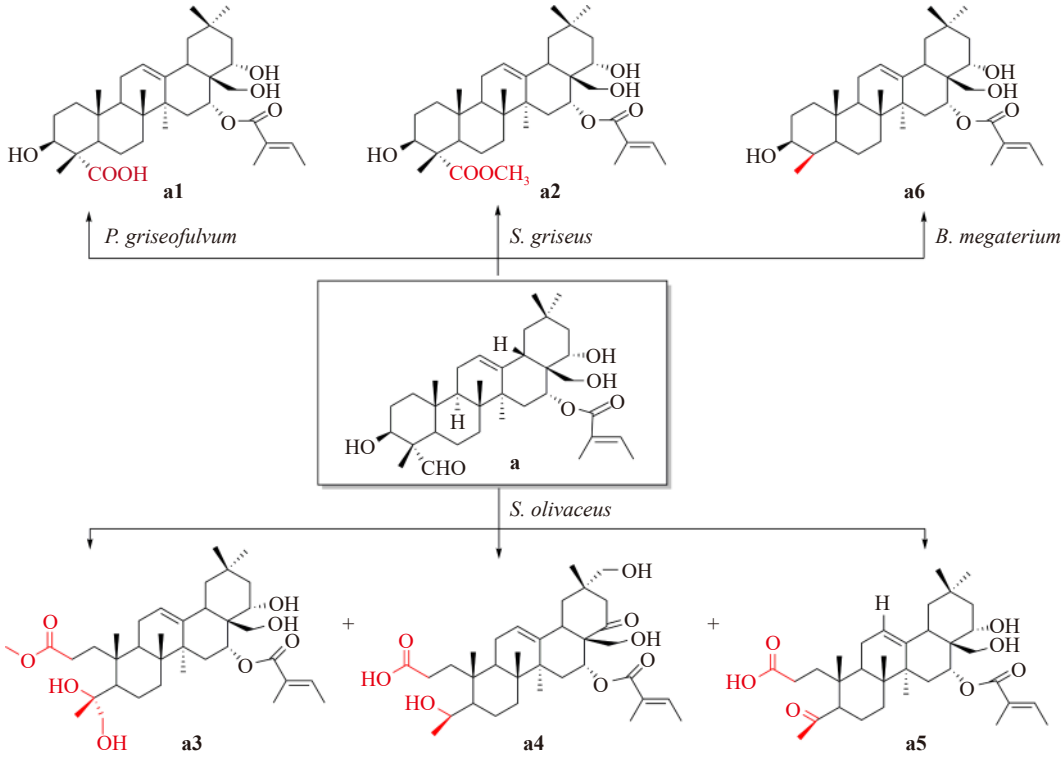

Fig. 2 Microbial transformation products of compound a.

methyl groups [27]. In this study, *S. griseus* was observed to catalyze the conversion of the aldehyde group at the C-23 position directly to a methyl ester, resulting in product **a2**. Intriguingly, *S. olivaceus* was found to transform compound **a** into three A-ring cleavage metabolites (**a3**−**a5**) through Bayer-Villiger oxidation at the C-3 position of compound **a**. Additionally, the removal of the C-23 aldehyde group was also removed in compounds **a4** and **a5**, suggests a series of continuous oxidation and elimination reactions targeting the aldehyde group. The absolute configurations of compound **a5** were ascertained through CuKα X-ray crystallography (Fig. 4). The selective functionalization of the inert C−H bond at C-29 position in compound **a4** highlights the potential of accessing a diverse array of drug derivatives, which may be challenging to synthesize through traditional methods or *de novo* synthesis. Biotransformation experiments with *B. megaterium* yielded a new metabolite, **a6**, featuring the removal of the aldehyde group at the C-23 position as well. These findings demonstrate that the aldehyde group at the C-23 position of the substrate is readily targeted by all four microbial strains for catalysis. *P. griseofulvum* and *S. griseus* effectively oxidized the aldehyde group to a carboxyl group, whereas *S. olivaceus* and *B. megaterium* facilitated the removal of the C-23-CHO group through a multistep continuous reaction mechanism. This de-aldehyde process mirrors reactions observed in the biosynthesis of steroids in nature, which are essential for the elimination of the two methyl groups at the C-4 position [28].

In this study, the anti-inflammatory effects of tea sapogenin and its metabolites were assessed in LPS- and HMGB1-activated RAW264.7 cells using the Griess method. The cytotoxicity of these compounds on RAW 264.7 cells was first evaluated through MTT assays, revealing that compounds **a1** and **a2** exhibited minimal cytotoxicity at the tested concentrations (Table 2). When examining the response to LPS stimulation, compound **b** demonstrated a dose-dependent inhibitory effect on nitric oxide (NO) release, with an IC_{50} value of 5.42 μmol·L^{-1}, which was more effective than the positive control, carbenoxolone. Compounds **c** and **e** yielded IC_{50} values of 50.17 and 55.31 μmol·L^{-1}, respectively, indicating moderate inhibitory activities. In contrast, compounds **a** and **d** did not exhibit significant inhibition of LPS-induced NO release (Fig. 5). Regarding HMGB1-induced inflammation, compounds **c**, **d**, and **e** showed moderate inhibitory effects on NO release, with IC_{50} values of 50.23, 27.82, and 27.55 μmol·L^{-1}, respectively. Notably, compounds **b** and **a2** could significantly inhibi HMGB1-induced NO release at low concentrations, exhibiting dose-dependent activities with IC_{50} values of 3.68 and 1.93 μmol·L^{-1}, respectively.

The structure-activity relationship (SAR) analysis in two inflammatory cell models revealed significant insights into the molecular features influencing the anti-inflammatory activity of tea sapogenin derivatives. The analysis highlighted that replacing the methyl group (-CH_3) at the C-23 position with an aldehyde group (-CHO), in conjunction with substituting a hydroxyl group (OH) at C-16 with a tigloyl moiety, as seen in compounds **a** and **b**, substantially diminished the inhibitory effects. This suggests that specific structural modifications at these positions can critically impact the compounds' bioactivity. Interestingly, the presence of the al-

Table 1 ^{13}C NMR (150 MHz) spectral data of compounds **a1**−**a6** (in pyridine-d_5)

No.	a1	a2	a3	a4	a5	a6
1	39.00	38.78	35.80	37.71	37.55	38.96
2	27.57	27.37	29.97	29.94	30.37	27.50
3	75.10	74.88	175.70	176.59	167.54	76.03
4	54.18	54.61	77.50	72.50	212.03	39.50
5	51.62	51.83	46.50	51.87	56.47	52.22
6	21.25	21.24	22.71	22.30	22.29	24.60
7	32.65	32.51	32.70	31.27	32.11	31.88
8	40.19	40.15	40.27	40.05	40.02	42.15
9	47.13	47.05	38.83	39.33	39.31	45.37
10	36.38	36.36	35.80	36.59	36.29	36.95
11	23.57	23.52	23.95	22.30	22.28	23.52
12	122.72	122.83	122.43	123.39	123.40	123.47
13	142.30	142.27	142.53	142.82	142.79	143.00
14	41.38	41.34	42.57	42.48	42.48	45.20
15	31.16	31.15	31.79	31.90	31.89	32.13
16	71.41	71.43	72.13	71.96	71.95	72.75
17	44.11	44.11	42.07	42.05	42.07	44.83
18	41.34	41.31	45.25	45.20	45.20	42.10
19	47.33	47.31	47.86	47.88	47.86	47.97
20	31.46	31.46	31.79	32.13	32.13	31.97
21	44.55	44.56	44.85	44.80	44.79	44.83
22	72.15	72.10	72.66	214.17	72.53	72.12
23	180.32	178.36	71.76	-	-	-
24	11.97	11.61	23.26	25.71	23.76	14.45
25	15.92	15.89	21.11	19.98	20.01	16.28
26	16.48	16.41	17.42	17.07	17.10	17.29
27	26.89	26.85	27.34	27.48	27.45	25.76
28	69.03	68.98	69.60	69.42	69.44	69.63
29	31.46	33.16	33.83	70.52	33.82	33.81
30	25.11	25.10	25.70	33.82	25.70	25.76
Tig						
1'	166.93	166.96	167.59	167.54	167.62	167.62
2'	129.82	129.82	130.48	130.45	130.45	130.47
3'	136.72	136.79	137.37	137.42	137.41	137.40
4'	13.96	13.97	14.62	14.60	14.61	14.58
5'	12.40	12.41	13.04	13.02	13.02	13.05
-OCH_3		51.54				

dehyde group at the C-23 position appeared to enhance the selectivity of compound **d** for inhibiting HMGB1-induced NO release compared to compound **e**. This finding indicates that the functional group at the C-23 position plays a role in the selectivity towards HMGB1-driven inflammation. Moreover, replacing the 23-CHO group in compound a with a methoxy carboxyl group (–$COOCH_3$), as in compound **a2,** significantly improved its inhibitory activity against both LPS and HMGB1-induced inflammatory responses, surpassing even that of carbenoxolone, a known anti-inflammatory agent. This suggests that the -$COOCH_3$ substitution at C-23 favorably influences the compound's anti-inflammatory efficacy. Furthermore, the removal of either the -CHO or -COOH group in compounds **a** or **a1**, leading to compound **a6**, resulted in enhanced inhibition of HMGB1-induced inflammation. This observation suggests that the presence of these functional groups may negatively impact the anti-inflammatory activity, pointing towards a detrimental effect of these groups on

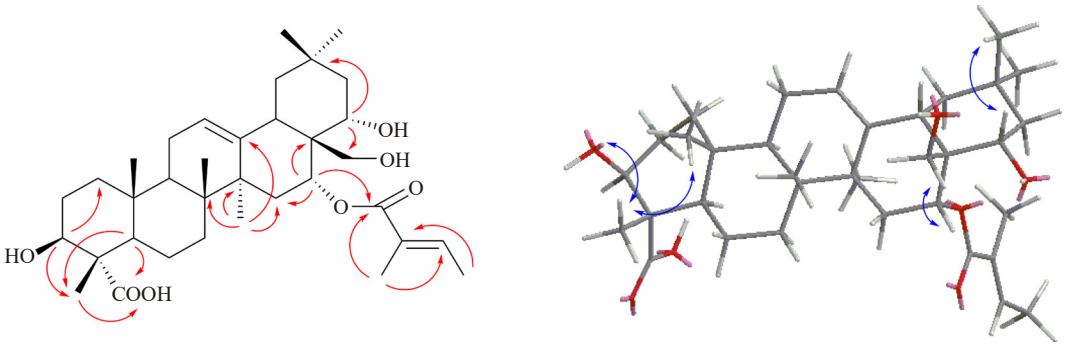

Fig. 3 Key HMBC (red arrows) and NOE (blue arrows) correlations of a1.

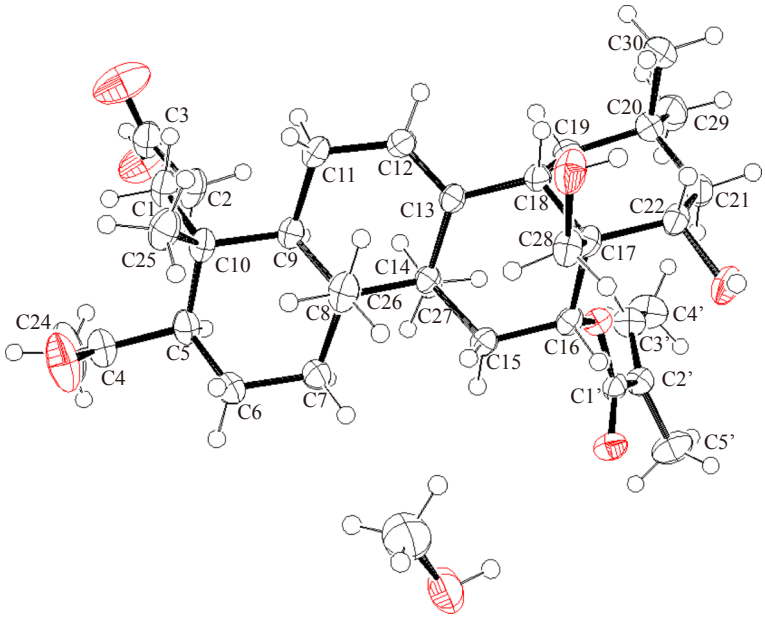

Fig. 4 X-ray crystallographic analysis of compound a5.

the compound's bioactivity. Additionally, the SAR analysis did not observe significant improvements in anti-inflammatory activity with A-ring cleavage products in either cell model, indicating that the integrity of the A-ring might be crucial for maintaining anti-inflammatory effects. Overall, these findings underline the importance of the types and configurations of polar moieties at the C-16 and C-23 positions in determining the anti-inflammatory potential of tea sapogenin and its derivatives.

Conclusions

C. oleifera cake, a byproduct of oil extraction, remains a rich source of tea saponins, compounds known for their remarkable biological activities. In this study, tea sapogenins were extracted from defatted *C. oleifera* seed cake and subjected to microbial transformation. This approach not only yielded modified sapogenins but also provided valuable insights into the *in vivo* metabolic pathways of these compounds. The conducted cellular assays highlighted compounds **b** and **a2** for their potent inhibitory effects on LPS and HMGB1-activated RAW 264.7 cells, underscoring their potential as therapeutic agents for treating inflammatory diseases triggered by infections and injuries. Furthermore, compounds **d**, **a3**, and **a6** demonstrated selective inhibition of HMGB1-induced inflammatory mediator production, offering a strategic advantage by sparing PAMPs-mediated innate immunity. This selectivity holds promise for the clinical management of endogenous, injury-elicited inflammatory conditions. The detailed molecular mechanisms by which active tea sapogenins and their derivatives exert protective effects against human diseases warrant further investigation to fully elucidate their therapeutic potential.

Experimental

Materials

Seed cake of *C. oleifera* was purchased from Ankang Tea Oil Co., Ltd. (20211210), Ankang City, Shaanxi Province, China. The defatted seed cake of *C. oleifera* was processed into a fine powder, which was then passed through an 80-mesh sieve for uniformity. A tea saponin standard with a purity of at least 98% was sourced from Shanghai Yuanye Biological Technology Co., Shanghai, China. AB-8 macro-

Table 2 LPS and HMGB1 inhibitory activity of compounds (mean ± SD, n = 3)

Compound	IC_{50}/μmol·L^{-1} [a]		Cell viability (%) [b]
	LPS	HMGB1	
a	> 50[c]	NA[d]	93.85 ± 19.5
b	5.42 ± 0.85	3.68 ± 0.71	92.38 ± 3.04
c	50.17 ± 1.93	50.23 ± 2.87	106.63 ± 2.54
d	NA	27.82 ± 8.11	101.08 ± 2.92
e	55.31 ± 0.63	27.55 ± 3.74	101.30 ± 4.01
a1	NA	> 50	55.00 ± 6.22
a2	2.29 ± 0.08	1.93 ± 0.08	72.01 ± 2.94
a3	> 50	31.10 ± 1.99	108.56 ± 1.94
a4	NA	> 50	100.97 ± 1.25
a5	NA	> 50	105.10 ± 1.11
a6	> 50	39.31 ± 3.80	99.56 ± 0.06
Carbenoxolone	6.639 ± 0.98	7.62 ± 0.86	97.42 ± 3.69

[a]: Concentration necessary for 50% inhibition (IC_{50}); [b]: Cell viability of a2 at 10 μmol·L^{-1}, and other compounds at 25 μmol·L^{-1} concentration; [c]: Effects showed NO inhibition activity was greater than 50 μmol·L^{-1}; [d]: There was no inhibitory activity under experimental concentrations.

porous resin, utilized for purification steps, was provided by the same company. LPS was acquired from Sigma–Aldrich (catalog number L2880), and recombinant human HMGB1 protein with a histidine tag was obtained from Sino Biological Inc., Beijing, China (cat. No. 10326-H08H). The Griess reagent kit, essential for nitric oxide detection, was purchased from the Beyotime Institute of Biotechnology. Hydrochloric acid (HCl), acetone, and other analytical or chemical reagents required for the experiments were procured from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Chromatography mediums such as Sephadex LH-20 were supplied by GE Healthcare, USA, while high-performance thin-layer chromatography (HPTLC) analyses were conducted using precoated silica gel GF_{254} plates, and the silica gel obtained from Qingdao Marine Chemical Group Co., China. For preparative reversed-phase high-performance liquid chromatography (RP-HPLC) separations, an Agilent 1100 instrument equipped with an Alltech 3300 ELSD detector and an Ultimate® XB-C18 column (250 mm × 21.2 mm, 5 μm) was used, operating with elution mixtures of acetonitrile (CH_3CN) and water. The separations and purifications of compounds were efficiently performed using column chromatography on silica gel (200–300 mesh), ensuring the high quality and purity of isolated substances for further analysis and testing. NMR spectra were recorded on a Bruker AV-600 spectrometer in pyridine-d_5 wiht TMS as the internal standard, and the chemical shifts are expressed in δ.

Acid hydrolysis of the extracted tea saponins and isolation

In this study, the process of extracting crude tea saponin from the seed pomace of *C. oleifera* involved grinding the seed pomace into a fine powder. A quantity of 5.0 g of this powder was measured and combined with 50 mL of water, establishing a liquid-to-solid ratio of 10 mL·g^{-1}. The pH of the mixture was adjusted to 9 using a 0.1 g·mL^{-1} NaOH solution, followed by reflux heating at a controlled temperature of 75 °C for 6 h. After heating, the mixture was centrifuged, and the supernatant was concentrated to obtain the crude extract. The crude tea saponins extracted were further purified using AB-8 macroporous resin, employing water and ethanol as the mobile phases. The fraction eluted with 75% ethanol was collected, concentrated, and dried for further analysis. The content of total tea saponin post-purification was quantified

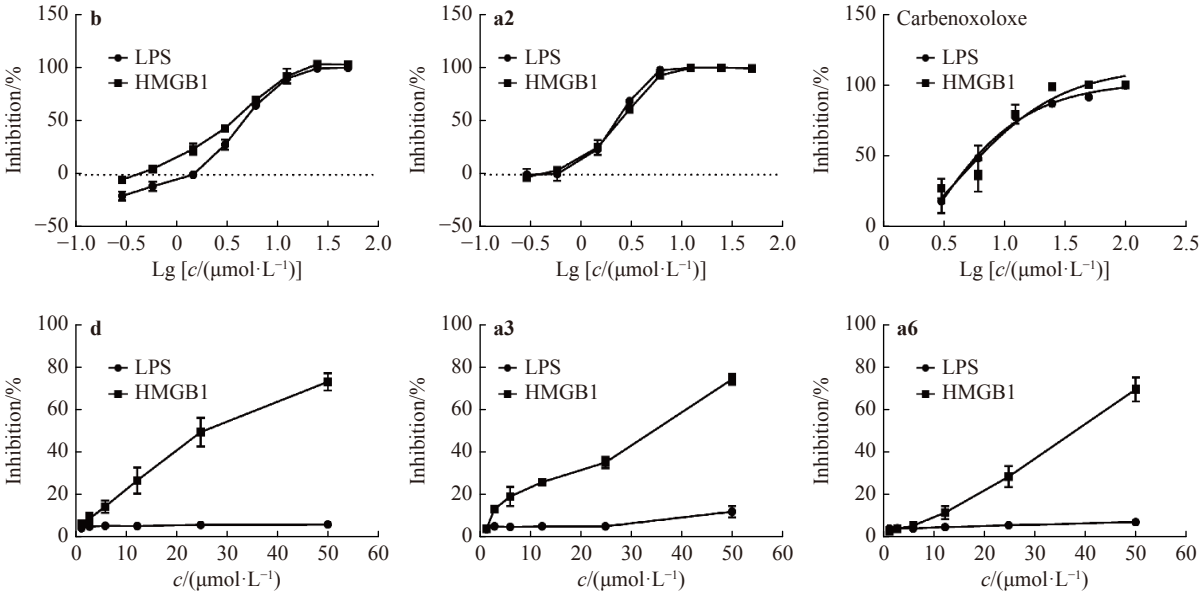

Fig. 5 Inhibitory effect of bioactive compounds on LPS- and HMGB1-induced RAW264.7 NO release.

through vanillin-sulfuric acid colorimetry [29].

To derive tea sapogenin, 6.0 g of the crude tea saponin was dissolved in 50 mL of methanol-water solution (*V*/*V*, 3 ∶ 1) containing 2.5 mol·L^{-1} HCl. This mixture was maintained at 80 °C with stirring for 5.5 h. The resulting hydrolysate was filtered while still hot, and its pH was neutralized using a 0.1 g·mL^{-1} NaOH solution. The subsequent addition of a substantial volume of pure water facilitated the removal of water-soluble components through filtration, collecting the residue. Given tea sapogenin's lower polarity and solubility in acetone (in contrast to tea saponin's water solubility), the hydrolysate was dissolved in acetone for further filtration. The acetone solution was then evaporated under reduced pressure to yield the crude tea sapogenin product. As a result, 500.0 g of crude tea saponin was hydrolyzed to obtain 106.3 g of products. This crude sapogenin underwent further purification via silica gel column chromatography, utilizing dichloromethane (CH_2Cl_2) and methanol (CH_3OH) as mobile phases to achieve a refined product for subsequent analyses and applications.

Biotransformation procedures

In this study, over 30 microbial strains preserved in our laboratory were screened for their capacity to biotransform 16-*O*-tiglogycamelliagnin B (**a**) as the substrate, utilizing thin-layer chromatography (TLC) to monitor the transformation process. Among these, *B. megaterium* CGMCC 1.1741, *S. griseus* ATCC 13273, *S. olivaceus* CICC 23628, and *P. griseofulvum* CICC 40293 demonstrated significant catalytic activity towards compound **a** compared to the control group, thereby being selected for further experimentation. The chosen microorganisms were cultivated in 50 mL of soybean meal glucose medium using a two-stage procedure in soybean meal glucose medium within 250 mL culture flasks. The composition of this medium included 20 g glucose, 5 g soybean meal, 5 g yeast extract, 5 g NaCl, and 5 g K_2HPO_4 per liter of distilled water, with the pH adjusted to 7.0 prior to sterilization at 121 °C for 20 min. The cultures were incubated at 28 °C with a shaking speed of 180 r·min^{-1}. A 10% inoculum from the 24-hour-old stage I culture was utilized to initiate the stage II culture, which underwent a further 24-hour incubation period. Subsequently, 1 mL of acetone containing 10 mg of the substrate was added to each culture. The cultures were then incubated for an additional four days, followed by extraction with ethyl acetate (EtOAc) in equal volumes performed three times to recover the transformed products. The extracts were analyzed using silica gel HPTLC plates for separation. The detection of the transformed products on the HPTLC plates was facilitated by spraying with a 10% sulfuric acid solution (H_2SO_4) in ethanol, enabling visualization of the catalytic conversion of compound a by the selected microorganisms.

Isolation and identification of biotransformation metabolites

The crude extracts resulting from the biotransformation of compound **a** by various microorganisms were independently processed through a silica gel column chromatography (20.0 g, 15 mm × 400 mm), utilizing a stepwise gradient of CH_2Cl_2/MeOH from 100 ∶ 1 to 5 ∶ 1 as the elution system. This procedure yielded three distinct fractions for each microbial transformation. The fractions, upon analysis by HPTLC, were combined based on their similarity, and further purification was achieved using preparative RP-HPLC employing a mobile phase of 60%−70% CH_3CN in water (H_2O). NMR spectra were acquired on a Bruker AV-600 spectrometer, using pyridine-d_5 as the solvent and tetramethylsilane (TMS) as the internal standard. The chemical shifts are reported in δ (parts per million, ppm), providing detailed structural information about the biotransformed products. HR-ESI-MS experiments were conducted on an Agilent 6210 ESI-TOF spectrometer.

HMGB1 and LPS-driven inflammatory response

To evaluate the anti-inflammatory activities of tea sapogenins and their derivatives, experimental models were established to assess NO release induced by HMGB1 and LPS, building upon methodologies outlined in our previous research [27]. Macrophage RAW 264.7 cells, acquired from the Peking Union Medical College (PUMC) Cell Bank, Beijing, China, were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, catalog number C11965500BT) supplemented with 10% fetal bovine serum (FBS, Gibco, catalog number 10099141), penicillin G (100 units/mL), and streptomycin (100 μg·mL^{-1}) (BI, catalog number 03-031-1B). The culture environment was maintained at 37 °C with 5% CO_2 and 95% humidity. For the experiments, tested compounds were initially dissolved in dimethyl sulfoxide (DMSO, Sigma, catalog number D2650) to a stock concentration of 100 mmol·L^{-1} and subsequently diluted to the required testing concentrations (ranging from 100 μmol·L^{-1} to 3.125 μmol·L^{-1}). In the HMGB1 model, these compounds, at various concentrations, were co-incubated with HMGB1 (1 μg·mL^{-1}) for 1 h *in vitro* [30] before being administered to the RAW 264.7 cells at a seeding density of 5.0 × 10^4 cells/well. For the LPS model, the cells were treated simultaneously with the small molecules and LPS (1 μg·mL^{-1}). Following a 24-hour incubation period, the concentration of NO in the culture medium was quantified using a Griess reagent kit (Beyotime, catalog number S0023) by measuring the optical density (OD) at 540 nm. The results were then calculated against a standard curve generated using sodium nitrite, allowing for precise quantification of NO production and, thereby, the assessment of the anti-inflammatory properties of the tested compounds.

Statistical analysis

All data presented in this study were obtained from a minimum of three independent experiments and are expressed as the mean ± standard deviation. Statistical analyses to compare differences among groups were conducted using one-way Analysis of Variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. Differences were considered statistically significant at a P-value of less than 0.05 ($P < 0.05$).

Supplementary Materials

Supplementary data to this article can be obtained by sending an E-mail to the corresponding author.

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