In recent years, studies focused on Chaenomeles species having diverse biological properties have opened new horizons for their potential utilization in traditional medication systems, as well as in functional foods. Various parts of Chaenomeles species have been reported to possess a diverse range of chemical constituents, numbering over 150 compounds so far. Triterpenes were previously reported as the main constituents of the genus Chaenomeles, and 23 triterpenes have been identified from this genus, including oleanane and ursane types, which are frequent metabolites in all 5 Chaenomeles species, viz., C. speciosa, C. sinensis, C. cathayensis, C. thibetica, and C. japonica. Moreover, both oleanolic and ursolic acids are regarded as markers for Chaenomeles species to identify, classify, and evaluate them. There are many literature reports on the strong anti-inflammatory actions of Chaenomeles extracts in vivo and in vitro, which are thought to be mainly due to the presence of glucosides, saponins, and multiple monomer compounds detected in the fruits.

Chaenomeles speciosa Lindley, also known as Chaenomeles lagenaria or Chinese Mugua, belongs to the family Rosaceae. It is a well-known herbal and edible plant enriched with health-promoting nutrients. In order to explore the anti-inflammatory constituents from C. speciosa, this study was designed; 20 compounds were isolated (1-20) from the fruits (Figure 1), including 5 flavonoids (1-5), 5 phenylpropanoids (6-10), 3 benzoic acid derivatives (11-13), 2 phloroglucinols (14 and 15), 2 purines (16 and 17), and 3 terpenoids (18-20). Their structures were elucidated by nuclear magnetic resonance analyses and from mass spectrometry data. These compounds were confirmed as catechin (1), epicatechin (2), catechin-5-O-β-D-glucoside (3), procyanidin B1 (4), quercetin-3-O-β-D-glucoside (5), p-coumaric acid (6), ferulic acid (7), caffecoic alcohol (8), 1-O-p-coumaroyl-β-D-glucose (9), 1-O-cinnamoyl-β-D-glucose (10), p-hydroxybenzoic acid (11), protocatechuic acid (12), benzoic acid-β-D-gentiobioside (13), phloracetophenone 4′-glucoside (14), 3,5-dihydroxyxylphenyl β-D-glucopyranoside (15), adenine (16), adenosine (17), betulalbuside A (18), vomifoliol (19), and rososide (20). Compounds 3-5, 8, 10, and 13-18 were isolated from the genus Chaenomeles and C. speciosa for the first time. Out of all these, compound 17 showed the best anti-inflammatory properties, comparable with those of the already known minocycline.

Keywords
Chaenomeles speciosa, phytochemical, anti-inflammatory activity, phloroglucinols, purines

Received: November 19th, 2019; Accepted: February 20th, 2020.
studies on Chaenomeles species. The compounds were determined and confirmed as catechin (1), epicatechin (2), catechin-5-O-β-D-glucoside (3), procyanidin B1 (4), quercetin-3-O-β-D-glucoside (5), p-coumaric acid (6), ferulic acid (7), caffeic alcohol (8), 1-O-p-coumaroyl-β-D-glucose (9), 1-O-cinnamoyl-β-D-glucose (10), β-hydroxybenzoic acid (11), protocatechuic acid (12), benzoic acid-β-D-gentiobioside (13), phloracetophenone 4′-glucoside (14), 3,5-dihydroxyphenyl β-D-glucopyranoside (15), adenine (16), adenosine (17), betulalbuside A (18), vomifoliol (19), and roseoside (20). This is the first report of the isolation of 3 constituents, 3,4-dihydroxybenzoic acid, quercetin, and methyl 3-hydroxybutanedioic ester, which showed suppression of TNF-α levels.

The current phytochemical screening of C. speciosa fruits led to the isolation and characterization of 20 compounds (1-20) (Figure 1), but we focused primarily on the polar constituents, so triterpenes were not isolated so far. However, in the isolation procedure, we found that large quantities of triterpenes existed in the lower polar fractions. Flavonoids are also frequent and typical bioactive constituents found in Chaenomeles species. Phloroglucinols have been reported in many Rosaceous plants and are widely distributed in Guttiferales, including members of Asteraceae, Clusiaceae, Lauraceae, Rutaceae, Myrtaceae, Euphorbiacea, and Aspidiaceae. In addition to these phloroglucinols, a terpenoid, betulalbuside A (18), was also found for the first time in a Chaenomeles species.

The ethanolic extracts (70%) of C. speciosa fruits had higher anti-inflammatory activity than that of water extracts and the former significantly inhibited the production of nitric oxide (NO), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). The 10% ethanol elution fraction showed the highest anti-inflammatory activity, which further confirmed that the higher polar fractions bear higher anti-inflammatory activities. The studies reporting the anti-inflammatory components of C. speciosa led to the isolation of 3 constituents, 3,4-dihydroxybenzoic acid, quercetin, and methyl 3-hydroxybutanedioic ester, which showed suppression of TNF-α levels.

The anti-inflammatory activities of C. speciosa extracts were tested at a 5.0 µg/mL concentration (Table 1), at which compounds 3, 4, 12, 17, and 18 showed anti-inflammatory activity with an inhibition rate of over 30%. Compound 17 showed the best anti-inflammatory activity, which was comparable with that of the positive drug minocycline (Table 1). These results are consistent with previous reports, which stated that procyanidin B1 (4) can significantly inhibit phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p38 and mitogen-activated protein kinase (MAPK) at translational levels, while inhibited NF-κB, TNF receptor associated factor 6 (TRAF-6), and myeloid differentiation-2 (MD-2) at transcriptional levels. Procyanidin B1 actively competes for the binding sites of toll-like receptor 4–MD-2 heterodimer with lipid polysaccharides (LPS) and then suppresses the downstream activation of NF-κB and MAPK (p38) signaling pathways. Apple procyanidin

*Figure 1. Structures of compounds 1-20 isolated from fruits of Chaenomeles speciosa.*
Table 1. Anti-Inflammatory Activity of Isolates Tested by Inhibition of Nitric Oxide Production Assay.

| Compounds (5.0 µg/mL) | Inhibition rate (%) |
|-----------------------|---------------------|
| 1                     | 9.4 ± 2.9           |
| 2                     | 3.9 ± 6.3           |
| 3                     | 33.0 ± 4.0          |
| 4                     | 40.0 ± 2.1          |
| 9                     | 25.0 ± 2.0          |
| 12                    | 35.8 ± 5.3          |
| 13                    | 25.6 ± 3.3          |
| 15                    | 18.6 ± 2.2          |
| 16                    | 11.2 ± 3.7          |
| 17                    | 51.6 ± 4.2          |
| 18                    | 33.9 ± 1.8          |
| 19                    | 18.3 ± 3.8          |
| 20                    | 26.1 ± 3.7          |
| Minocycline            | 59.5 ± 3.1          |

Results expression as mean ± standard deviation; 3 replicates were tested.

constituents are the most potent inhibitors of the NF-κB signaling pathway and hence reduce inflammation to a greater extent. Protocatechuic acid (12) showed promising anti-inflammatory activity in different animal models, whereas adenosine (17) was reported as an endogenous anti-inflammatory agent.

Materials and Methods

Plant Material

The fruits of *C. speciosa* were bought during March 2018 from a local market in Zhangshu city, Jiangxi Province, China. A voucher specimen (No. CS-201805) was deposited in the herbarium of the College of Agronomy (Jiangxi Agricultural University, Nanchang city, Jiangxi Province, China).

Equipment and Reagents

$^1$H-NMR and $^{13}$C-NMR spectral results were obtained from a Bruker 600 MHz spectrometer (Bruker Co., Rheinstetten, Germany), with tetramethylsilane as internal standard. $^{18}$C electrospray ionization-MS were recorded on an Agilent 1290-6420 Triple Quadrupole LC-MS spectrometer (Santa Clara, CA, USA). Silica gel medium pressure liquid chromatography was conducted on a BUCHI C-605 system (BUCHI, Flawil, Switzerland). For high-performance liquid chromatography (HPLC), a Hitachi Elite Chromaster system (5210 autosampler, 5110 pump, 5430 DAD, and 5310 column oven) was used, which was operated by EZChrom Elite software. Two Luna C18 columns (5 µm, 4.6 × 250 mm and 5 µm, 10 × 250 mm) were used for analysis and semipreparative HPLC, respectively. C18 columns were bought from Phenomenex Inc. (Torrance, CA, USA). HPLC grade methanol was purchased from Sigma (Sigma, St. Louis, MO, USA). Silica gel (100-200 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), Sephadex LH-20 (GE Healthcare Biosciences AB, Uppsala, Sweden), Amberlite XAD 16HP (H&E Co., Ltd, Beijing, China), and ODS-A (8-50 µm, 12 nm, YMC Co., Ltd., Kyoto, Japan) were used for column chromatography.

Extraction and Chromatography

The fruits of *C. speciosa* (5 kg) were ground and extracted exhaustively 3 times with 80% ethanol (3 × 30 L) at 90°C for 30 minutes. The combined extracts were evaporated to remove the solvent, yielding the dried ethanol extract (567 g), which was subjected to XAD macro resin column chromatography, eluting with water, 30% ethanol (v/v), 50% ethanol, and absolute (100%) ethanol, respectively, to yield 4 fractions (CSPA1–CSPA4).

The 30% ethanol fraction (CSPA2) was subjected to silica gel column chromatography eluting with dichloromethane (CH$_2$Cl$_2$)/methanol (MeOH) (from 2:1 to 1:1, v/v) to yield 4 fractions (CSPA2A–CSPA2D). Fraction CSPA2A was separated by silica gel column chromatography again to give 3 subfractions (CSPA2A1–CSPA2A3). Fraction CSPA2A2 was passed through a C$_{18}$ silica gel column (40% MeOH) and then subjected to preparative HPLC (34% MeOH) to yield compounds 1 and 2; fraction CSPA2B2 was fractionated by silica gel column chromatography (CH$_2$Cl$_2$/MeOH 10:1) again to give three sub-fractions (CSPA2B1–CSPA2B3). Fraction CSPA2B2 was treated by C$_{18}$ silica gel column chromatography (40% MeOH) and preparative HPLC (30% MeOH) to yield compounds 12, 14, 15, and 16. Fraction CSPA2C2 was subjected to silica gel column chromatography eluting with CH$_2$Cl$_2$/MeOH (from 7:1 to 1:1, v/v) to yield 3 subfractions (CSPA2C1–CSPA2C3). CSPA2C2 was fractionated on Sephadex LH-20 and C$_{18}$ silica gel columns (30% MeOH) to yield compounds 3, 4, 13, and 17; CSPA2C3, after separation on a Sephadex LH-20 column (MeOH), yielded compound 9.

The 50% ethanol fraction, CSPA3, was subjected to silica gel column chromatography eluting with CH$_2$Cl$_2$/MeOH (from 25:1 to 1:1, v/v) to yield 4 fractions (CSPA3A–CSPA3D). Fraction CSPA3A was separated on LH-20 to give 3 subfractions (CSPA3A1–CSPA3A3). Fraction CSPA3A1 was fractionated by C$_{18}$ silica gel column chromatography (55% MeOH) and preparative HPLC (40% MeOH) to yield compound 10; CSPA3A2 was separated by preparative HPLC (40% MeOH) to yield compound 19; CSPA3A3, after passage through a series of C$_{18}$ silica gel columns (55%-65% MeOH) yielded compounds 5, 6, 7, 8, and 11; fraction CSPA3B was separated by Sephadex LH-20 to give 2 subfractions (CSPA3B1–CSPA3B2), one of which, CSPA3B1, was subjected to C$_{18}$ silica gel column chromatography (20% MeOH) to yield compounds 18 and 20.

Anti-Inflammatory Activity Assay

The anti-inflammatory activity of the test isolates on the generation of inflammatory cytokines (IL, TNFα) in LPS-activated
RAW264.7 cells was evaluated by a previously described method. 34 Briefly, RAW264.7 cells were cultured in Dulbecco’s modified Eagle medium (Life Technologies, Grand Island, NY, USA) and then seeded in 96-well plates at a density of 4 × 10^4 cells per well. After incubation for 24 hours, the cells were treated with isolated compounds at an optimal concentration (5.0 µg/mL) for 1 hour. Then the treated cells were incubated with LPS (1.0 µg/mL) for another 24 hours. Nitrite concentration in the supernatant of the cell culture medium was determined spectrophotometrically using a nitrate/nitrite assay kit (Beyotime Biotechnology, Shanghai, China). Dimethyl sulfoxide at a final concentration of 0.1% was used as a negative control and minocycline as a positive control.

Conclusions

Phytochemical screening of the fruits of C. speciosa led to the isolation of 20 compounds, including 5 flavonoids (1-5), 5 phenylpropanoids (6-10), 3 benzoic acid derivatives (11-13), 2 phloroglucinols (14 and 15), 2 purines (16 and 17), and 3 terpenoids (18-20). Ten compounds, 3, 5, 8, 10, and 13-18, are reported for the genus Chaenomelis for the first time. Among all the isolated compounds, adenosine (17) showed the best anti-inflammatory activity.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article. This research was funded by scientific and technological research project of department of education of Jiangxi province, grant number GJJ180694; and Traditional Chinese Medicine project of Jiangxi province, grant number 2019A280.

ORCID ID

Chunpeng Wan https://orcid.org/0000-0001-6892-016X

References

1. Zhang R, Li S, Zhu Z, He J. Recent advances in valorization of Chaenomeles fruit: a review of botanical profile, phytochemistry, advanced extraction technologies and bioactivities. Trends in Food Science & Technology. 2019;91:467-482.
2. Huang W, He J, Nisar MF, Li H, Wan C. Phytochemical and pharmacological properties of Chaenomeles speciosa: An edible medicinal chinese mugua. Evid Based Complement Alternat Med. 2018;2018(11):1-10.
3. He H-B, Li X-Q, Li X-M, et al. Therapeutic effect of total terpenoids of Chaenomeles speciosa combined with omeprazole on gastric ulcer induced by indomethacin in rats. Zhongguo Zhong Yao Za Zhi. 2019;44(11):2338-2347.
4. Zheng X, Wang H, Zhang P, et al. Chemical composition, antioxidant activity and α-glucohydase inhibitory activity of Chaenomeles speciosa from four production areas in China. Molecules. 2018;23(10):2518.
5. Ma J, He W, Gao C, Yu R, Xue P, Niu Y. Glucosides of chaenomeles speciosa attenuate ischemia/reperfusion-induced brain injury by regulating NF-κB P65/TNF-α in mouse model. Zhejiang Da Xue Xue Bao Yi Xue Ban. 2019;48(3):289-295.
6. Chen Q, Wei W. Effects and mechanisms of glucosides of chaenomeles speciosa on collagen-induced arthritis in rats. Int Immunopharmacol. 2003;3(4):593-608.
7. Zhang L, Cheng Y-X, Liu A-L, Wang H-D, Wang Y-L, Du G-H. Antioxidant, anti-inflammatory and anti-influenza properties of components from Chaenomeles speciosa. Molecules. 2010;15(11):8507-8517.
8. Dai M, Wei W, Shen Y-Q, Zheng Y-Q. Glucosides of Chaenomeles speciosa remit rat adjuvant arthritis by inhibiting synoviocyte activities. Acta Pharmacol Sin. 2003;24(11):1161-1166.
9. Zhu L, Fang I, Li Z, Xie X, Zhang L. A HPLC fingerprint study on Chaenomelis Fructus. BMC Chem. 2019;13(1):7.
10. Xiao Y, Wang L, Jin G, Sun R, Hu X, Yang C. Studies on phenolic acid constituents of Acanthopanax sessiliflorus fruits. Chin Tradit Herbal Drugs. 2015;46:1583-1588.
11. Raab T, Barron D, Vera FA, Crespy V, Oliveira M, Williamson G. Catechin glucosides: occurrence, synthesis, and stability. J Agric Food Chem. 2010;58(4):2138-2149.
12. Wan C, Yuan T, Cirello AL, Seeram NP. Antioxidant and α-glucosidase inhibitory phenolics isolated from highbush blueberry flowers. Food Chem. 2012;135(3):1929-1937.
13. Meng LJ, Liu BL, Zhang Y, Zhou GX. Chemical constituents in root barks of Lycium chinense. Chin Tradit Herbal Drugs. 2014;45:2139-2142.
14. Jansen F, Gillessen B, Mueller F, Commandeur U, Fischer R, Kreuzaler F. Metabolic engineering for p-coumaryl alcohol production in Escherichia coli by introducing an artificial phenylpropanoid pathway. Biotechnol Appl Biochem. 2014;61(6):646-654.
15. Strack D, Heilemann J, Wray V, Dirks H. Structures and accumulation patterns of soluble and insoluble phenolics from Norway spruce needles. Phytochemistry. 1989;28(8):2071-2078.
16. Polturak G, Heinig U, Grossman N, et al. Transcriptome and metabolic profiling provides insights into betalain biosynthesis and evolution in Mirabilis jalapa. Mol Plant. 2018;11(1):189-204.
17. Xiao Y, Wang LB, Jin G, Sun R, Hu X, Yang CJ. Studies on phenolic acid constituents of Acanthopanax sessiliflorus fruits. Chin J Med Chem. 2012;22:223-226.
18. Chosson E, Chaboud A, Chulia AJ, Raynaud J. A phloracetophenone glucoside from Rhododendron ferrugineum. Phytochemistry. 1998;47(1):87-88.
19. Ersöz T, Harput UŞ, Saracoğlu İ, Çalış İ, Ogihara Y. Phenolic compounds from Sentelaria pontica. Turk J Chem. 2002;26:581-588.
20. Zheng B, Peng X, He Y, et al. Chemical constituents from Osaxis corinodula. J Chin Med Mat. 2018;41:1883-1886.
21. Peng W, Fu X, Li Y, et al. Phytochemical study of stem and leaf of Clausena lanisium. Molecules. 2019;24(17):3124.
22. Morikawa H, Kasai R, Otsuka H, et al. Terpenic and phenolic glycosides from leaves of *Breynia officinalis* HEMS.L. *Chem Pharm Bull.* 2004;52(9):1086-1090.

23. Wan C, Chen C, Li M, Yang Y, Chen M, Chen J. Chemical constituents and antifungal activity of *Ficus birta* Vahl. fruits. *Plants.* 2017;6(4):44.

24. Liu C, Tai Z, Feng S, Fang Y, Cai L, Ding Z. Chemical constituents from the seed coat of *Juglans regia*. *Zhongguo Zhong Yao Za Zhi.* 2012;37(10):1417-1421.

25. Li S, Guo L, Liu C, Fu ZA, Zhang Y. Combination of supercritical fluid extraction with counter-current chromatography to isolate anthocyanidins from the petals of *Chaenomeles sinensis* based on mathematical calculations. *J Sep Sci.* 2013;36(21-22):3517-3526.

26. Adamu M, Awouafack MD, Ahmed AS, McGaw LJ, Naidoo V, Eloff JN. Anthelmintic phloroglucinol derivatives and antifungal activity of fractions from *Leucosidea sericea* (Rosaceae). *S Afr J Bot.* 2013;86:171-171.

27. Pal Singh I, Bharate SB. Phloroglucinol compounds of natural origin. *Nat Prod Rep.* 2006;23(4):558-591.

28. Han Y-K, Kim Y-S, Natarajan SB, et al. Antioxidant and anti-inflammatory effects of *Chaenomeles sinensis* leaf extracts on LPS-stimulated RAW 264.7 cells. *Molecules.* 2016;21(4):422.

29. Li X, Yang Y-B, Yang Q, Sun L-N, Chen W-S. Anti-Inflammatory and analgesic activities of *Chaenomeles speciosa* fractions in laboratory animals. *J Med Food.* 2009;12(5):1016-1022.

30. Xing J, Li R, Li N, et al. Anti-inflammatory effect of procyanidin B1 on LPS-treated THP1 cells via interaction with the TLR4-MD-2 heterodimer and p38 MAPK and NF-κB signaling. *Med Cell Biochem.* 2015;407(1-2):89-95.

31. Andre CM, Greenwood JM, Walker EG, et al. Anti-inflammatory procyanidins and triterpenes in 109 apple varieties. *J Agric Food Chem.* 2012;60(42):10546-10554.

32. Kakkar S, Bais S. A review on protocatechuic acid and its pharmacological potential. *ISRN Pharmacol.* 2014;2014(12):1-9.

33. Cronstein BN. Adenosine, an endogenous anti-inflammatory agent. *J Appl Physiol.* 1994;76(1):5-13.

34. Ma Q, Han L, Bi X, et al. Structures and biological activities of the triterpenoids and sesquiterpenoids from *Alisma orientale*. *Phytochemistry.* 2016;131:150-157.