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RESEARCH ARTICLE

Organic Synthesis

Synthesis and biological evaluation of gallic acid esters as phagocyte oxidative burst inhibitors

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Abstract: Several degenerative diseases, including cancer, are caused by oxidative stress, which is caused by the overproduction and accumulation of free radicals. The purpose of the study was to synthesize gallic acid (GA or 3,4,5-trihydroxybenzoic acid) esters and evaluate their anti-inflammatory potential through the inhibition of reactive oxygen species (ROS). The compounds methyl gallate (2), *sec*-butyl gallate (3), ethyl gallate (4), isopropyl gallate (5), 2-methoxyethyl gallate (6), 4-methoxybutyl gallate (7), 2-methylbutyl gallate (8) and pentan-3-yl gallate (9) were synthesized. ^{1}H NMR, MS and IR data are reported for compounds 2-9, and ^{13}C NMR data for compounds 2, 3, 5, and 6. The molecular formulae of compounds 3 and 7-9 were established by HREI-MS spectroscopic data. All the synthesized compounds were tested for their anti-inflammatory and cytotoxic activities by chemiluminescence and MTT cytotoxicity assay respectively. The results revealed the anti-inflammatory potential of compounds 2-8 with an IC_{50} range between (13.3 – 54.3 μM) as compared to the standard anti-inflammatory drug, Ibuprofen ($IC_{50} = 54.3 \pm 9.2$ μM). The most potent inhibitors were found to be compound 3 (ROS $IC_{50} = 15.0 \pm 6.6$ μM) and compound 7 (ROS $IC_{50} = 13.3 \pm 0.8$ μM). All compounds were found to be non-cytotoxic in the NIH-3T3 fibroblast cell line. Compounds 3, 7- 9 were identified as new compounds.

Keywords: Anti-inflammatory, cytotoxicity, ester derivatives, gallic acid, ROS Inhibitors.

INTRODUCTION

Oxidative stress, is a result of an overproduction and accumulation of free radicals, and among the main source of degenerative diseases like cancer, atherosclerosis, ageing, and cardiovascular and inflammatory diseases (Badhani *et al*., 2015). Inflammation is the body's defence system that protects it from harmful changes and speeds up the healing process. The lack of a healing process for injuries or any other dysfunction will result in chronic inflammation. It is characterized by redness, pain, warmth, swelling, and lack of function in the injured region (Krishnaraju *et al*., 2009; Ho *et al*., 2010; Ali *et al*., 2019). Certain natural and synthetic drugs have been produced for the treatment of chronic inflammation and related diseases. These are classified as steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) (Crofford, 2013). Short-term use of steroidal drugs is linked with side effects, including cutaneous effects, electrolyte abnormalities, hypertension, hyperglycaemia, and neuropsychological effects, while their long-term use is associated with more serious consequences, such as osteoporosis, aseptic joint necrosis, adrenal insufficiency, growth suppression, and possible congenital malformations (Brown & Chandler, 2001).

The side effects related to these drugs create a need for the development of new and powerful anti-inflammatory drugs. Antioxidants decrease oxidative stress and neutralize ROS before they damage the tissues (Roots & Okada, 1975; Thadhani *et al*., 2011). By augmenting the natural antioxidant defence system with diverse exogenous antioxidants such as vitamins and synthetic agents, oxidative damage and disease development could be slowed down in the body (Lee *et al*., 1998). Tissue damage can be caused by an imbalance between antioxidant defences and repair mechanisms (Davies, 2000). This imbalance can also be a factor in tissue injuries. The impact of ROS on tissues is devastating regarding oxidative stress-induced cell death (Valko *et al*., 2007).

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Some naturally occurring phenolic acids and their analogs, such as gallic acids, have a wide range of essential pharmacological effects. Gallic acid (GA, 3,4,5-trihydroxybenzoic acid) possesses anti-inflammatory capabilities and regulates many pharmacological and biochemical pathways (Kroes *et al*., 1992). It also has anti-mutagenic and anticancer effects (Gichner *et al*., 1987; Inoue *et al*., 2000), in addition to its primary antioxidant activity (Golumbic & Mattill, 1942; Heo *et al*., 2007; Kumar *et al*., 2012). GA and its esters are used in the food and pharmaceutical industries as antioxidant additives (Locatelli *et al*., 2013). E-310 (propyl gallate) and E-311 (octyl gallate) are known to protect against oxidative damage induced by reactive oxygen species (ROS), such as hydroxyl radicals or hydrogen peroxide, and reactive sulphur species (RSS) (Klein & Weber, 2001; Fiuza *et al*., 2004). Synthetic galloyl esters were found to be effective and selective enzyme inhibitors, as well as synergistic peroxyl radical protectors in membranes (Lü *et al*., 2010).

A total of eight ester derivatives of gallic acid were synthesized during this study. Among them, 3, 7, 8, and 9 were identified as new compounds. Synthesized compounds were purified and characterized by spectroscopic techniques, such as EI-MS, IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopic data. The results indicated the effects of test compounds 2-9 on innate immune response phagocyte oxidative burst. All the compounds were found to be non-cytotoxic when checked against the mouse fibroblast NIH-3T3 cell line.

MATERIALS AND METHODS

General experimental conditions

Bruker Avance 400 and 500 MHz instruments were used for NMR experiments. ^{1}H NMR spectra were recorded at 400 or 500 MHz, while ^{13}C NMR spectra were recorded at 125 MHz. As per the international standard, the chemical shift (δ) was in ppm relative to tetramethylsilane (TMS) and coupling constants J in Hz. Precoated ALUGRAM, SIL G/UV254 aluminum plates (Kieselgel 60, 20 x 20, 0.5 mm thick, E. Merck, Germany) were used for thin layer chromatography (TLC) analysis. The reagents and solvents were purchased from Aldrich (St. Louis, Missouri, USA), E. Merck Darmstadt, (Germany), and Fluka (Buchs, Switzerland). They were used without purification. Developed chromatograms on TLC plates were visualized under ultraviolet light at 254 nm for fluorescence quenching spots, and 365 nm for fluorescent spots. FTIR-8900 (Shimadzu, Japan) was used to perform IR spectrophotometry of the compounds using KBr discs. Buchi 535 (Japan) melting point apparatus was used to measure melting points. JEOL JMS-600H mass spectrometer with a MASPEC data system was used to record electron impact mass spectra (EI-MS).

The general reaction for the synthesis of compounds 2–9 is given below.

Chemicals: Gallic acid purchased from Sigma Aldrich, India; Methanol, ethanol, isopropanol, 2- butanol, 4-methoxy-1-butanol, 2-methoxyethanol, 2- methyl-1-butanol, and pentane-3-ol from Aldrich, Poland.

General Reaction Procedure of Compounds 2-9

Chemical Synthesis

Concentrated sulphuric acid (0.5 mL) was carefully added to gallic acid (170 mg, 1 mmol) in the corresponding alcohols (2 mmol) in THF (5 mL) and the mixture was refluxed at 60-80 °C for 18-20 h in an oil bath with continuous stirring. The progress of the reaction was monitored by TLC (DCM : Methanol = 9:1). Once the reaction was complete, the reaction mixture was cooled to room temperature, and excess alcohol and solvent were removed by using a rotary evaporator. The resulting mixture was then poured into cold water (25 mL), extracted with ethyl acetate (3×25 mL), and washed with a saturated sodium bicarbonate solution (10 mL). It was then dried with Na_2SO_4, the solvent evaporated off under reduced pressure and the residue purified by column chromatography [Silica-gel 60 (230–400 mesh), isocratic elution with Hex–EtOAc (65:35)].
The following scheme describes the synthesis of all of the compounds (Figure 1).

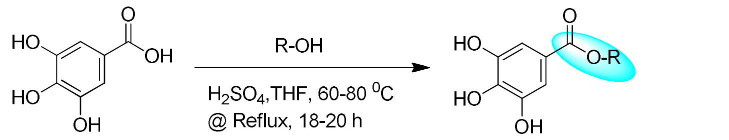


Figure 1: Synthesis of Gallic acid esters (**2-9**).

***Methyl 3,4,5-trihydroxybenzoate* (2).** Yield: 80-82%; m.p 209–210 ˚C; TLC (DCM: MeOH, 7:3 v/v) $R_f = 0.80$; ^{1}H-NMR (400 MHz, CD_3OD): δ_H 7.03 (s, 2H, H-2' /H-6'), 3.80 (s, 3H, C$\underline{H}_3$); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 166.2 (C-7'), 145.5 (C-3' / C-5'), 138.4 (C-4'), 119.2 (C-1'), 108.4 (C-2' / C-6'), 51.5 (C-1); EI-MS *m/z* (% rel. abund.): 184.1 [M$^+$, 96.7], 153.0 (100).

***Sec-butyl 3,4,5-trihydroxybenzoate* (3).** Yield: 50-52 %; TLC (DCM: MeOH, 7:3 v/v) $R_f = 0.70$; IR (KBr, cm^{-1}): 3548 (O-H stretching), 1743 (C=O stretch, ester), 1513 (C–C stretch (in–ring), 1062 (C-O stretch); UV/Vis (MeOH): λ_{max} nm 229, 276; ^{1}H-NMR (400 MHz, DMSO-d_6): δ_H 6.92 (s, 2H, H-2' /H-6'), 4.86 (m, 1H, H-1), 1.61 (m, 2H, H-2); 1.21 (d, $J_{4,1}$ = 6.1 Hz, CH_3-4), 088 (t, $J_{3,2}$ = 7.4 Hz, CH_3-3); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 165.4 (C-7'), 145.5 (C-3' / C-5'), 138.2 (C-4'), 119.8 (C-1'), 108.4 (C-2' / C-6'), 71.4 (C-1), 28.3 (C-2), 19.3 (C-4), 9.5 (C-3); EI-MS *m/z* (% rel. abund.): 226.1 [M$^+$,30], 170.0 (100), 153.0 (100); HREI-MS: Calculated for $C_{11}H_{14}O_5$: 226.0842, Observed m/z: 226.0841.

***Ethyl 3,4,5-trihydroxybenzoate* (4).** Yield: 78-80 %; m.p: 150-152 ˚C; TLC (DCM: MeOH, 7:3 v/v) $R_f = 0.80$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ_H 6.92 (s, 2H, H-2' /H-6'), 4.18 (q, $J_{1,2}$ = 7.1 Hz, 2H, H-1), 1.25 (t, $J_{2,1}$ = 7.1 Hz, 3H, CH_3), EI-MS *m/z* (% rel. abund.): 198.1 [M$^+$,53], 152.9 (100).

***Isopropyl 3,4,5-trihydroxybenzoate* (5).** Yield: 75-77 %; TLC (DCM: MeOH, 6:4 v/v) $R_f = 0.60$;^{1}H-NMR (400 MHz, DMSO-d_6): δ_H 6.92 (s, 2H, H-2' /H-6'), 5.01 (septet, $J_{1,2/1,3}$ = 6.2 Hz, 1H, H-1), 1.25 (d, $J_{2,1/3,1}$ = 6.0 Hz, 2CH_3); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 165.2 (C-7'), 145.4 (C-3' / C-5'), 138.2 (C-4'), 119.9 (C-1'), 108.4 (C-2' / C-6'), 67.1 (C-1), 21.7 (C-2 / C-3); EI-MS *m/z* (% rel. abund.): 212.1 [M$^+$,79], 170.0 (100), 153.0 (100).

2-*methoxyethyl 3,4,5-trihydroxybenzoate* (06). Yield: 60-63 %; TLC (DCM: MeOH, 6:4 v/v) $R_f = 0.60$; ^{1}H-NMR (400 MHz, DMSO-d_6): δ_H 6.94 (s, 2H, H-2' /H-6'), 4.26 (t, $J_{1,2}$ = 4.6 Hz, 2H, H-1), 3.59 (t, $J_{2,1}$ = 4.7 Hz, 2H, H-2), 3.15 (s, 3H, CH_3); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 165.7 (C-7'), 145.5 (C-3' / C-5'), 138.4 (C-4'), 119.2 (C-1'), 108.5 (C-2' / C-6'), 69.9 (C-1), 63.2 (C-2), 58.1 (C-3); EI-MS *m/z* (% rel. abund.): 228.2 [M$^+$,46], 170.0 (94), 153.0 (100).

***4-methoxybutyl 3,4,5-trihydroxybenzoate* (07).** Yield: 55-58%; TLC (DCM: MeOH, 6:4 v/v) $R_f = 0.70$; IR (KBr, cm^{-1}): 3394 (O-H stretching), 1685 (C=O stretch, ester), 1614 (C–C stretch (in–ring), 1248 (C–O stretch); UV/Vis (MeOH): λ_{max} nm 230, 262, 273; ^{1}H-NMR (500 MHz, DMSO-d_6): δ_H 6.93 (s, 2H, H-2' /H-6'), 4.15 (t, $J_{1,2}$ = 6.2 Hz, 2H, H-1), 3.31-3.21 (overlapped, 5H, H-4 & H-5), 1.67 (quintet, $J_{2,1/3}$ = 6.2 Hz, 2H, H-2), 1.59 (quintet, $J_{3,2/4}$ = 6.4 Hz, 2H, H-3); EI-MS *m/z* (% rel. abund.): 256.0 [M$^+$, 6], 170.0 (50), 153.0 (100); HREI-MS: Calculated for $C_{12}H_{16}O_6$: 256.0946, Observed m/z: 256.0947.

2-methylbutyl 3,4,5-trihydroxybenzoate **(08).** Yield: 70-72%; TLC (DCM: MeOH, 7:3 v/v) $R_f = 0.80$; IR (KBr, cm^{-1}): 3468, 3358 (O-H stretching), 1696 (C=O stretch, ester), 1612 (C–C stretch (in–ring), 1038 (C–O stretch); UV/Vis (MeOH): λ_{max} nm 230, 273; ^{1}H-NMR (500 MHz, DMSO-d_6): δ_H 6.93 (s, 2H, H-2' /H-6'), 4.05-3.95 (m, 2H, H-1), 1.77-1.71 (m, 1H, H-2), 1.48-1.40 (m, 1H, H-3), 1.25-1.16 (m, 1H, H-3), 0.93 (d, $J_{5,2}$ = 6.7 Hz, 3H, H-5), 0.89 (t, $J_{4,3}$ = 7.4 Hz, 3H, H-4); EI-MS *m/z* (% rel. abund.): 240.1 [M^+,58], 170.0 (100), 153.0 (92); HREI-MS: Calculated for $C_{12}H_{16}O_5$: 240.0997, Observed m/z: 240.0998.

Pentan-3-yl 3,4,5-trihydroxybenzoate **(09).** Yield: 60-63 %; TLC (DCM: MeOH, 7:3 v/v) $R_f = 0.50$; IR (KBr, cm^{-1}): 3358 (O-H stretching), 1680 (C=O stretch, ester), 1612 (C–C stretch (in–ring), 1032 (C–O stretch); UV/Vis (MeOH): λ_{max} nm 220, 228, 275; ^{1}H-NMR (400 MHz, DMSO-d_6): δ_H 6.92 (s, 2H, H-2' /H-6'), 4.79 (quintet, $J_{1,2/4}$ = 6.9 Hz, 1H, H-1), 1.64-1.52 (m, 4H, H-2, H-4), 0.85 (t, , $J_{3,2/5,4}$ = 7.4 Hz, 6H, H-3, H-5); EI-MS *m/z* (% rel. abund.): 240.2 [M^+,40], 170.1 (100), 153.1 (99); HREI-MS: Calculated for $C_{12}H_{16}O_5$: 240.0997, Observed m/z: 240.0998.

Anti-Inflammatory Assay

The anti-inflammatory activity of the synthesized compounds (2–8), and gallic acid (**1**) was evaluated by the following method reported by Helfand. *et al*. (Helfand *et al*., 1982; Mbiantcha *et al*., 2017). Initially all the compounds were evaluated at a single dose of 25 µg/mL, each in triplicate. The compounds having >50% inhibition were further evaluated at three different concentrations, 1, 10, and 100 µg/mL, to determine IC_{50} values. A compound that failed to inhibit the production of ROS from zymosan-activated whole blood cells at the highest used dose (100 µg/mL) was considered inactive.

Cytotoxicity Assay

The cell line used for the cytotoxicity assay was obtained from the Biobank facility, The Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi. It was purchased from ATCC, Manassas, USA. The cytotoxicity of the synthesized compounds (2–9), and gallic acid (1) was evaluated by the method reported by Pauwels. *et al*. (Pauwels *et al*., 1988; Choudhary *et al*., 2010). In this experiment, all the compounds were evaluated at the dose of 30 µM each, in triplicate. A compound having <50% inhibition at 30 µM was considered inactive. Cycloheximide was used as a standard drug (Siddiqui *et al*., 2021). Cycloheximide was used as a positive control in this assay. The percent inhibition was calculated by using the following formula:

$$\%\text{ inhibition} = \frac{100\text{- (mean of O.D of test compound - mean of O.D of negative control)}}{\text{(mean of O.D of positive control - mean of O.D of negative control)}} \text{ X } 100$$

The results (% inhibition) were processed by using Soft- Max Pro software (Molecular Device, USA).

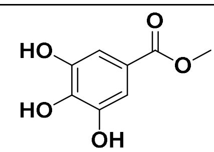
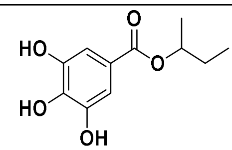
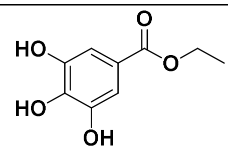
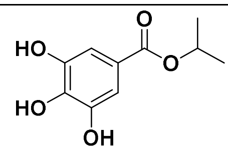
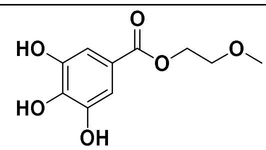
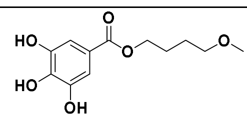
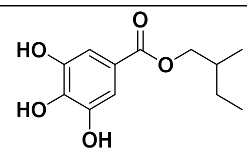
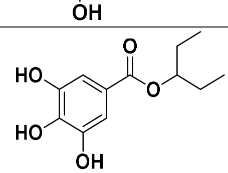
RESULTS AND DISCUSSION

The chemical structures of the synthesized gallic acid esters were deduced with the help of ^{1}H, ^{13}C NMR, IR, EI-MS, and HR-EIMS techniques. All the synthesized compounds were tested for their anti-inflammatory and cytotoxic activities by chemiluminescence and MTT cytotoxicity assay, respectively.

Anti-inflammatory activity

Oxidative burst assay was used to determine the anti-inflammatory properties of seven synthesized compounds. Among them, all the tested compounds (**2-8**) revealed potent to moderate inhibitory activity with IC_{50} values in the range of 13.3-54.3 µM, when compared to the standard drug Ibuprofen (IC_{50} = 54.3 ± 9.2 µM) (Table 1).

Table 1: Anti-inflammatory activity and Cytotoxicity of compounds 1-9.

Compound no	Structures	Oxidative burst inhibition IC_{50} (μM)	Cytotoxicity (3T3 Cell line)
2		51.6 ± 2.1	>30
3		15.0 ± 6.6	>30
4		30.2 ± 0.5	>30
5		26.8 ± 3.2	>30
6		28.4 ± 8.5	>30
7		13.3 ± 0.8	>30
8		54.3 ± 9.2	>30
9		N/T	>30a
1	Gallic acid	N/A	>30
Ibuprofen (Standard)	-	54.3 ± 9.2	-
Cycloheximide (Standard)	-	-	0.8 ± 0.14

*SD = Standard deviation, N/A = Not active, N/T= Not tested

Structure-activity relationship of various gallic acid esters

The standard drug used was ibuprofen (IC_{50} = 54.3 ± 9.2 µM). Among the test compounds, seven exhibited anti-inflammatory activities with IC_{50} values in the range of 13.3-54.3 µM. The new compounds 3 (IC_{50} = 15.0 ± 6.6 µM), 7 (IC_{50} = 13.3 ± 0.8 µM), and 8 (IC_{50} = 54.3 ± 9.2 µM) along with the known compounds 2 (IC_{50} = 51.6 ±

2.1 μM), 4 (IC_{50} = 30.2 ± 0.5 μM), 5 (IC_{50} = 26.8 ± 3.2 μM) and 6 (IC_{50} = 28.4 ± 8.5 μM) were found to be more potent inhibitors than ibuprofen (IC_{50} = 54.3 ± 9.2 μM). The parent gallic acid was found to be inactive (Table 1). This implies that *sec*-butyl gallate and 4-methoxybutyl gallate, which have four carbon atoms in the aliphatic chain, are potent anti-inflammatory agents. As a result, the new compounds 3 and 7 which were esterified with *sec*-butyl and 4-methoxybutyl alcohols, respectively, possessed potent anti-inflammatory activity.

Cytotoxic activities on NIH-3T3 cell line

The tested compounds 2–9 and gallic acid (1) were tested for their cytotoxic activity on NIH-3T3 mouse fibroblast cell line where all esters were found to be inactive (Table 1).

CONCLUSION

The ester derivatives of GA were synthesized, among which compounds 3 and 7–9 were new. Our findings suggest that seven compounds showed promising anti-inflammatory activity; among them, the new compounds 3 and 7 were found to be the most potent inhibitors of ROS. Therefore, these compounds must be further investigated through detailed *in vivo* studies to evaluate their anti-inflammatory potential.

Acknowledgments

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Ethical approval

The *in vitro* studies on human blood cells was conducted as per approval of the Independent Ethics Committee, UoK No: ICCBS/IEC-008-BC-2015.

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