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Antibacterial, Prooxidative and Genotoxic Activities of Gallic Acid and its Copper and Iron Complexes against *Escherichia coli*

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Abstract – *In this study, gallic acid and its complexes with aluminum and iron were investigated for their antibacterial, pro-oxidative, and genotoxic properties at alkaline pH. At 4.0µmol/mL, gallic acid displayed bacteriostatic property while aluminum-gallic acid and iron-gallic acid complexes showed bactericidal property against Escherichia coli ATCC 25922. A higher antibacterial activity was observed in the turbidimetric assay compared to the well-diffusion assay. The metal complexes of gallic acid also generated a higher concentration of malondialdehyde and hydrogen peroxide compared to gallic acid alone at $\geq$ 0.50µmol/mL. Using the SOS response of the DNA repair-deficient Escherichia coli PQ37, the metal complexes of gallic acid resulted to a significantly higher SOS Induction Factors ($\rho<0.01$) at $\geq$0.25µmol/mL. In addition, gallic acid and its metal complexes decrease the cell surface hydrophobicity of E. coli ATCC 25922 in a dose-dependent manner. The present study suggests that the antibacterial property of gallic acid and its metal complexes against Escherichia coli was caused by its pro-oxidative and genotoxic properties. Since metals are involved in the synthesis of the metal complexes of gallic acid, further tests should be conducted to determine their stability and effects to human health.*

Keywords – *gallic acid, genotoxicity, metal complexes, pro-oxidative property, SOS Chromotest*

I. INTRODUCTION

A significant number of studies on phenolic compounds in plants have been conducted, emphasizing on their several applications in the biomedical sciences. The focus has been geared towards understanding their mechanism of action since they are tapped as promising pharmaceutical agents. Of particular interest in most studies on phenolic acids is gallic acid or 3, 4, 5-tryhydroxybenzoic acid, a naturally occurring compound found in several food sources such as gallnuts, tea, tree barks, herbs, fruits and flowers [1-5]. In plant extracts, gallic acid exists as free molecular phenolic acid or as a component of tannic acid [6]. The antimicrobial property of gallic acid against several bacterial strains has been highlighted in several studies and the mechanism of its bactericidal action has also been described [7]. Gallic acid has other several pharmacologic applications ranging from antioxidant to anticancer agents [2].

There are conflicting reports on the ability of gallic acid to chelate metals and recent studies classified the compound as a poor metal chelator [8-11] although phenolic compounds were explained to form complexes with metals, preventing them in participating in oxidative processes [12]. Gallic acid was also reported in another study as an efficient biological reductant [13, 14], which allows it to of converting transition metals into forms which allows them to participate in Fenton reactions. The weak chelating property and strong reducing property of gallic acid has received attention as a potential mechanism on how gallic acid promotes Fenton-type reactions when it is mixed with redox metals such as iron and copper [13-16].

There were reports that gallic acid autooxidize in alkaline pH forming negatively charged radicals (17, 18) although few literatures have studied their effects on biological systems. Autooxidation of gallic acid could also be induced by photo-irradiation [4]. Recent literature reported the antibacterial property of gallic

acid and other trihydroxylated compounds are caused by their pro-oxidative property [8, 12]. Furthermore, gallic acid was reported to induce radical oxygen species (ROS)-mediated oxidative stress and promote the production of hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet-}$) and hydroxyl radicals ($\bullet OH^-$) that damages DNA, proteins, lipids and carbohydrates [14, 20].

Not much has been reported on the effects of gallic acid and its complexes with aluminum and iron in bacterial systems. In addition, alkaline pH influences metal speciation, forming anionic hydroxo complexes which could influence their over-all interaction with gallic acid radicals. It is interesting to know then whether the chelating activity of gallic acid can be increased by alkaline pH since gallic acid radicals are anionic [18] making the compound more suitable for metal complexation. Theoretically, chelation decrease metal-induced toxicity and form less toxic metal complexes in biological systems [21]. However, the net effects of the complexes of gallic acid and metals need to be investigated since several by-products can be formed especially in conditions which promote auto-oxidation of gallic acid. To verify this hypothesis, the effects of aluminum and iron on the antibacterial, pro-oxidative and genotoxic properties of gallic acid under alkaline condition were investigated using *Escherichia coli* as the representative microorganism. This bacterial strain was chosen as the representative microorganism in the study because of their high resistance against crude plant extracts [3] and their adaptive mechanisms against oxidative stress.

II. MATERIALS AND METHODS

Reagents and Materials

Anhydrous gallic acid was purchased from Merck, Germany. Aluminum chloride hexahydrate ($AlCl_3.6H_2O$, MW = 241.4322g/mol), Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$, MW = 270.2957g/mol), yeast extract, tryptone water, sodium chloride (NaCl), sodium hydroxide (NaOH), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate dehydrate ($Na_2HPO_4.2H_2O$), nutrient agar, 2-thiobarbituric acid (TBA), *o*-dianisidine, horseradish peroxidase, *n*-hexadecane and distilled water were obtained from Sigma Aldrich, Singapore through Chemline Scientific Inc. The SOS-Chromotest kit was purchased from Environmental Bio Detection Products Inc. (EBPI, Mississauga, Ontario). Soya lecithin and hydrogen peroxide were purchased commercially. All other reagents were of analytical grade.

Bacterial Strains and Growth Media

The bacterial strain *Escherichia coli* ATCC 25922 was obtained from the Department of Science and Technology-Cordillera Administrative Region (DOST-CAR) and stored at 4^{o}C until used. In a sterile Erlenmeyer flask, a bacterial suspension was prepared by inoculating a loopful of bacteria to sterile Luria Bertani broth (LB broth, pH = 7.1 to 7.3) and incubated for 24 hours at 37^{o}C. LB broth was prepared by mixing 5 g yeast extract, 10 g tryptone, and 9 g of NaCl in 1000mL of distilled water [2]. The optical density (OD_{600}) of *E. coli* was measured after 24 hours incubation using the sterile LB broth as the blank solution. Bacterial density was measured using serial dilution of the bacterial suspension and expressed as colony forming units/mL (CFU/mL). All assays were performed using the adjusted optical density of the bacteria in both LB broth and sterile phosphate buffered solution (PBS, pH = 7.1 to 7.3).

Preparation of Metal-Gallic Acid Solution

Gallic acid solution was prepared by dissolving 1.701 g in 100 mL of distilled water and adjusted to pH 8.5 using NaOH to yield a 100mM stock solution. Alkaline pH was used to accelerate the production of gallic acid radicals [18]. Using the same procedure, 100mM stock solutions of iron (III) chloride hexahydrate and aluminum chloride hexahydrate were also prepared in alkaline distilled water. To prepare a 500mL of 100mM concentration of the metal-gallic acid solutions, 250mL of gallic acid solution was mixed with 250mL of the metal solution using a magnetic stirrer for fifteen minutes at room temperature until the reagents were completely dissolved. Aluminum-gallic acid and iron-gallic acid solutions were diluted to 0.031 to 4.0µmol/mL and used in different assays. The abrsorption spectra of the metal complexes were obtained spectrophotometrically from 340nm to 800nm.

SOS Chromotest Assay

In order to evaluate the genotoxicity of gallic acid, aluminum-gallic acid solution and iron-gallic acid solution, the SOS Chromotest kit was utilized [23]. The kit contains growth medium for the bacteria, 10% dimethylsulfoxide in normal saline solution (10% DMSO in NSS, adjusted to pH 8.5), O-nitrophenyl-β-D-galactopyranoside (ONPP), p-nitrophenyl phosphate disodium (PNPP), 4-nitroquinoline-1-oxide (4-NQO),

Escherichia coli PQ37, stop solution, and 96-well microplates. The SOS Chromotest assay utilizes a bacterial strain using the SOS response as an indicator for DNA damage (24). The genotype of the *E. coli* PQ37 is: *F – thr leu his – 4 pyrD thi galE galK lacΔU169 Srl300::Tn10 rpoB rpsL uvrA rfa trp::Muc + sfiA::Mud (Ap, lac) cts* [25]. The SOS Induction Factor represents the normalized induction of *sul A* gene in each treatment and a direct indicator of primary DNA damage.

Using aseptic technique, bacterial cells were mixed with the growth medium and incubated for 18 hours at 37°C. The bacterial density (OD_{600}) was adjusted to 0.05 using additional growth medium. A volume of 10µL of sterile saline solution was dispensed to all wells which were used for the assay. Using double dilution technique, 10 µL of the genotoxic 4-NQO was added to the first well in the microplate. Gallic acid, aluminum-gallic acid solution and iron-gallic acid solution were also dispensed in a similar method in three replicates. A volume of 100µL of the adjusted bacterial suspension of *E. coli* PQ37 was added to each well containing the dispensed compounds. The microplate was incubated at 37°C for two hours. After incubation, 100µL of the blue chromogen mixed with alkaline phosphatase substrate was introduced to the wells and incubated at 37°C for 90 minutes. Lastly, 50µL of the stop solution was added. The absorbance was measured at 415nm and at 615nm to determine the genotoxicity of the samples. SOS induction potential was calculated using the equation:

$$\textbf{SOS Induction Potential} = \frac{10 * (OD_1 - OD_3)}{C_1 - C_3}$$

SOS Induction factor (SOSIF) was computed using the Excel Template provided with the SOS Chromotest kit. The formula used is shown below:

$$\textbf{SOSIF} = \frac{\textbf{[β-galactosidase / alkaline phosphatase]}_T}{\textbf{[β-galactosidase / alkaline phosphatase]}_{NC}}$$

*T = treatment, NC = negative control

A substance is classified as not genotoxic if SOSIF was < 1.5, inconclusive if SOSIF was between 1.5 and 2.0, and genotoxic if SOSIF was > 2.0 and a clear concentration-response relationship was observed. All treatment groups were compared to 4-nitroquinoline-1-oxide (positive control) and 10% DMSO in 0.9% NaCl solution (negative control, pH = 8.5). All other procedures were done aseptically.

Microbial Adhesion to Hydrocarbon Assay (MATH Assay)

The change in cell surface hydrophobicity of *E. coli* ATCC 25922 was evaluated using a modified microbial adhesion to hydrocarbon (MATH) assay [26]. After incubating the bacterial suspensions (OD_{600} = 0.30) with 0.031 to 4.0µmol/mL of gallic acid solution, aluminum-gallic complex solution and iron-gallic acid complex solution, 5 mL of the incubated bacterial suspension was washed three times with cold sterile PBS until the supernatant was clear. The pellets were finally suspended in 5mL of sterile phosphate buffer solution (pH = 7.1 to 7.3). An equal volume of n-hexadecane was mixed to the bacterial suspension, vortexed for one minute then kept still for thirty minutes to allow the polar and non-polar phases to separate. The hydrophilic phase was carefully removed using a micropipette and transferred to a quartz cuvette. Absorbance was measured at 400 nm using sterile PBS as the blank solution. The procedure was performed in triplicate. The change in hydrophobicity was expressed as mean hydrophobicity index (MHI, %) using the following formula:

$$\textbf{MHI (\%)} = \frac{(OD_b - OD_a)}{OD_b}$$

*OD_a = optical density after, OD_b = optical density after

Antimicrobial Assays

Turbidimetric and well diffusion assays were utilized to evaluate the antimicrobial properties of gallic acid, aluminum-gallic solution and iron-gallic acid solution. In the well diffusion assay, a 6mm well in nutrient agar was loaded with 50µL of the minimum genotoxic doses of the compounds of interest which were obtained using the SOS Chromotest kit. The agar plates were incubated at 37°C for 24 hours and the zone of inhibition was measured.

Using the turbidimetric assay, the bacterial suspension was adjusted to a starting OD_{600} of 0.05 using sterile Luria Bertani broth based on a similar method described in other literature using *Helicobacter pylori* [27]. A volume of 50mL of bacterial suspension was mixed with an equal volume of iron-gallic acid solution in concentrations of 0.031µmol/mL to 4.0µmol/mL of bacterial suspension. A negative control was prepared using alkaline water. The treatment and control groups were incubated for 24 hours at 37°C. After incubation, 5 mL of the treated bacterial suspension was centrifuged at 2500g for 10 minutes and

washed twice with cold PBS (pH=7.3). Bacterial pellets were suspended in PBS and the optical densities were obtained at 400nm using PBS as the blank. The procedure was done in three replicates. Treated bacterial cells were transferred to nutrient agar plates to determine their viability.

Thiobarbituric Acid Reactive Species (TBARS) Assay

In order to assess oxidative stress, thiobarbituric acid reacting species (TBARS) assay was performed based from literature [28]. In a test tube, 2mL of TBA reagent (20% trichloroacetic acid and 0.67% 2-thiobarbituric acid in 0.25N HCl) was mixed to 0.5mL of soya lecithin and 0.5mL of iron-gallic acid solution ranging from 0.031 - 4.0 µmol/mL. For the blank solution, alkaline water was utilized instead of soya lecithin since the colored iron-gallic acid complexes might interfere with the absorbance of the product. The mixture was heated in a water bath for 30 minutes, cooled, then centrifuged at 2500g for 10 minutes. The supernatant was collected and transferred to a quartz cuvette. Absorbance was measured at 532 nm and compared with the blank solution. TBARS concentration was obtained using the molar extinction coefficient of thiobarbituric acid-malondialdehyde adduct (TBA-MDA) expressed as µM.

Modified o-dianisidine Assay

To determine the ability of the metal-gallic acid complexes to generate H_2O_2, the modified o-dianisidine (3, 3-dimethoxybenzidine) assay was performed concurrently with the other assays. In separate test tubes, 500µL of gallic acid solution, aluminum-gallic acid complex and iron-gallic acid complex were mixed with 2.0 mL of phosphate buffered solution, 250µL of o-dianisidine, and 250µL of horseradish peroxidase and incubated for twenty minutes at 37°C. The blank solution was also prepared by replacing horseradish peroxidase with phosphate buffered solution. After incubation, the mixture was centrifuged at 5000g for 10 minutes. The absorbance of the supernatant was obtained at 460nm and compared to the blank solutions. All procedures were performed in three replicates in concentrations ranging from 0.031to 4.0 µmol/mL. The concentration of hydrogen peroxide was obtained using the molar coefficient of the oxidized o-dianisidine product and expressed as nM.

Statistical Test

Data on genotoxicity, malondialdehyde-thiobarbituric acid adduct production, and hydrogen peroxide production were presented as mean ± standard deviation (n=5).The results of hydrophobicity assay and antimicrobial assays were presented using bar graphs. One way analysis of variance (ANOVA) with post hoc Tukey HSD and Student's T-test were utilized to determine significant differences of the means. Statistical analysis was performed using SPSS 20.0 at α=0.01.

III. Results and Discussion

Characteristics of Metal Complexes of Gallic Acid

At pH 8.5, gallic acid forms colored complexes with metals. Upon observation, the complexes appear homogeneous, implying that the complexes are colloidal by nature. Aluminum and gallic acid forms a yellow colored solution while iron and gallic acid forms a dark colored solution (Figure 1), which indicate complexation with gallic acid. Spectrophotmometric data shows that the λ_{max} of aluminum-gallic acid complex and iron-gallic acid complex are 335nm and 400nm, respectively.

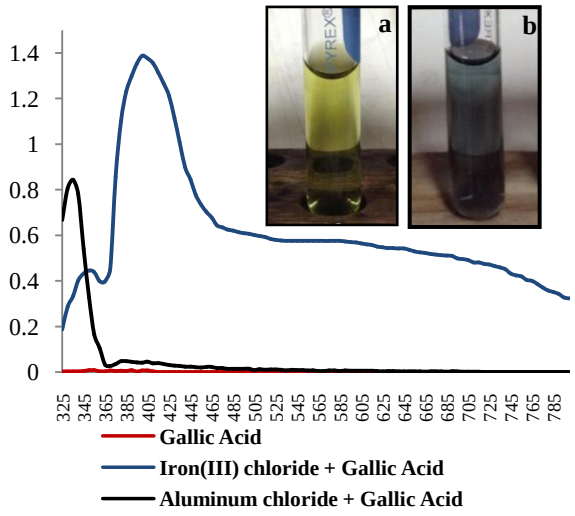

Fig. 1. Absorption Spectra of Aluminum-Gallic Acid Complex and Iron-Gallic Acid Complex
(Inset: a = Aluminum-Gallic Acid Solution, b = Iron-Gallic Acid Solution)

Antibacterial Property of Gallic Acid and Metal-Gallic Acid Complexes against E. coli ATCC 25922

Turbidimetric and well-diffusion assays were performed to compare the antibacterial properties gallic acid and its metal complexes. Similar to the reported literature data in Table 1, gallic acid showed bactericidal properties against *E. coli*. In Figure 2, *E. coli* cells grown in Luria Bertani broth supplemented with aluminum-gallic acid and iron-gallic acid complexes had significantly lower optical density (OD_{600}) compared to bacterial suspensions treated with

gallic acid and metal solutions ($\rho<0.01$) while the bacterial density of cells exposed to gallic acid, iron chloride, and aluminum chloride do not differ significantly ($\rho>0.01$). The antibacterial property of the tested compounds against *E. coli* increases in the following order: alkaline water < gallic acid < metal solutions < metal complexes of gallic acid.

Table 1. Mean Inhibitory and Bactericidal Concentration of Gallic Acid against Bacteria in Selected Literature Data

Bacterial Strain	Concentration of Gallic Acid	References
Enterococcus faecalis	62.5 mg/mL* (inhibitory)	[1]
Escherichia coli	1500.0 µg/mL (inhibitory)	[49]
	5000.0 µg/mL (bactericidal)	[49]
	125.0 mg/mL* (inhibitory)	[1]
Helicobacter pylori	0.2 – 1.0 mg/mL (inhibitory)	[27]
Pseudomonas	500 µg/mL (inhibitory)	[49]
aeruginosa	500 µg/mL (bactericidal)	[49]
	125.0* mg/mL (inhibitory)	[1]
Salmonella typhi	4.0 µg/mL (inhibitory)	[50]
Staphylococcus aureus	4.0 mM (bactericidal)	[4]
	7.81* mg/mL (inhibitory)	[1]
	1750 µg/mL (inhibitory)	[49]
Staphylococcus	5250 µg/mL (bactericidal)	[49]
epidermidis	62.5* mg/mL (inhibitory)	[1]
Vibrio cholera	31.3* mg/mL (inhibitory)	[1]

**ethanolic extract of gallic acid*

To determine the viability of bacterial cells in the turbidimetric assay, 25µL of the bacterial suspensions were inoculated in Nutrient Agar and incubated for 24 hours at 37°C. Bacterial cells exposed to aluminum-gallic acid and iron-gallic acid complexes were unable to grow in nutrient agar, implying the bactericidal effect of the metal-gallic acid complexes. In contrast, bacterial cells which were exposed to alkaline water, metal solutions, and gallic acid were still viable after 24 hours incubation, implying a bacteriostatic effect. In the well-diffusion assay (Figure 3), only iron-gallic acid complex produced a zone of inhibition (12.00 ± 0.25mm, n=5).

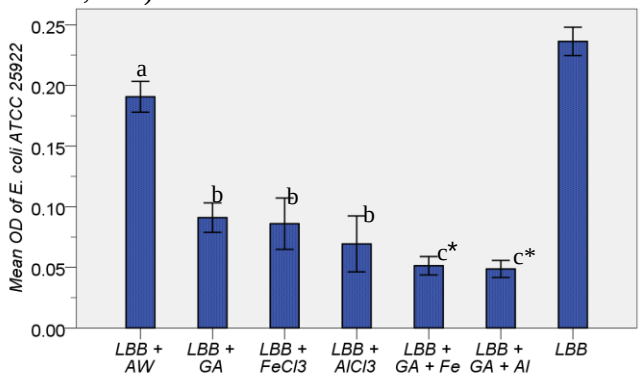

Figure 2. Optical Density of *Escherichia coli* ATCC 25922 in Luria Bertani Broth treated with gallic acid(GA), metals, metal-gallic acid complexes and alkaline water (AW) after 24 hours of incubation (n=5)

** Bacterial cells were not viable; Means with different letters are significantly different at α=0.01 in Tukey HSD test*

In addition, there was an observable darkening of gallic acid in nutrient agar which indicates oxidation. Apparently, an increasing the alkalinity of LB broth also inhibits bacterial growth although the pH of the bacterial suspensions varied as shown in Table 2.

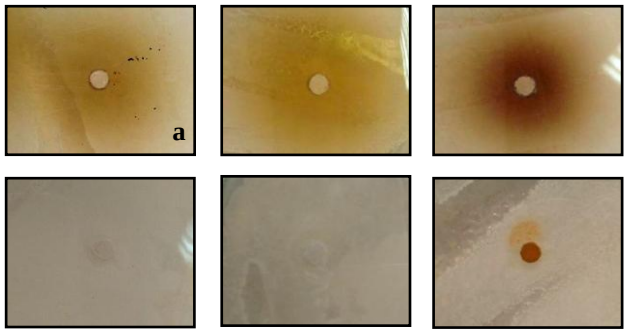

Fig. 3. Antimicrobial activity of gallic acid, metal solutions and metal-gallic acid complexes against *Escherichia coli* ATCC 25922 using well-diffusion assay (a = gallic acid, b = aluminum-gallic acid complex, c = iron-gallic acid complex, d = alkaline water, e = aluminum chloride, f = iron (III) chloride)

Pro-oxidative Properties of Gallic Acid and Metal-Gallic Acid Complexes

The results in Table 3 show that all the tested compounds promote MDA production. In addition, aluminum and iron increased the pro-oxidative property of gallic acid. The highest detectable concentration of MDA-TBA adducts was noted in the iron-gallic acid solution. Hydrogen peroxide (H_2O_2) was also detected in iron-gallic acid system at concentrations $\leq$0.25µmol/mL using the modified peroxidase assay. Alkanals, proteins, sucrose and urea also reacts with thiobarbituric acid to form colored compounds [29]. Since soybean lecithin was utilized in the assay, no interference from other TBA reacting compounds were expected and results of the assay can only be associated to the oxidation of unsaturated fatty acid. Previous literature [3] stated that gallic acid prevents lipid oxidation, although our results show that gallic acid is capable of causing lipid peroxidation. Since metals and hydrogen peroxide are involved, it is possible that the mechanism of oxidative damage is through Fenton-type reaction.

Genotoxic Property of Gallic Acid and Metal-Gallic Acid Complexes

Table 4 shows that at pH 8.5, gallic acid, aluminum-gallic acid and iron-gallic acid have significantly higher genotoxicity against *E. coli* PQ37 compared to gallic acid in concentrations ≥ 2.0 μmol/mL ($\rho<0.01$). The minimum genotoxic concentration for the alkalinized solution of gallic acid (pH = 8.5) is higher (4.0μmol/mL) compared to the minimum genotoxic concentration of aluminum-gallic acid and iron-gallic acid (1.0μmol/mL). Apparently, the positive control (4-NQO) has significantly higher genotoxicity in all concentrations compared to gallic acid and metal-gallic acid complexes ($\rho<0.01$).

Table 3. Malondialdehyde and H_2O_2 Production of Gallic Acid, Aluminum-Gallic Acid Complex and Iron-Gallic Acid Complex

Concentration (μmol/mL)	Gallic Acid		Aluminum + Gallic Acid		Iron + Gallic Acid	
	MDA-TBA (μmol/mL)	H_2O_2 (nmol/mL)	MDA-TBA (μmol/mL)	H_2O_2 (nmol/mL)	MDA-TBA (μmol/mL)	H_2O_2 (nmol/mL)
62.50	*ND*	*ND*	*ND*	*ND*	0.52 ± 0.03	*ND*
125.00	*ND*	*ND*	*ND*	*ND*	0.54 ± 0.13	*ND*
250.00	*ND*	*ND*	0.51 ± 0.12	*ND*	0.56 ± 0.01	260.85 ± 82.30
500.00	0.52 ± 0.32	*ND*	0.54 ± 0.12	*ND*	0.79 ± 0.04	262.12 ± 85.34
1000.00	0.58 ± 0.12	*ND*	0.85 ± 0.15	251.34 ± 58.92	0.81 ± 0.06	265.49 ± 88.50
2000.00	0.65 ± 0.05	202.54 ± 44.34	0.89 ± 0.06	258.86 ± 76.51	1.06 ± 0.08	589.97 ± 135.18
4000.00	0.78 ± 0.03	206.49 ± 51.09	1.24 ± 0.76	383.48 ± 51.09	1.37 ± 0.12	707.96 ± 88.50

ND = not detected, n = 5

Table 4. Mean SOS Induction Factor of Gallic Acid, Aluminum-Gallic Acid Complex and Iron-Gallic Acid Complex without S9 activation

C(%)	S9(-)					
	10% DMSO in NSS (pH = 8.5)	4-NQO (10μg/mL)	Gallic Acid (pH = 7.3) (4.0μmol/mL)	Gallic Acid (pH=8.5) (4.0μmol/mL)	Aluminum + Gallic Acid (4.0μmol/mL)	Iron + Gallic Acid (4.0μmol/mL)
1.5625	1.05 ± 0.11	2.23 ± 0.04[a,b]	1.00 ± 0.04	1.00 ± 0.01	1.03 ± 0.05	1.15 ± 0.16
3.125	1.04 ± 0.12	3.27 ± 0.13[a,b]	1.05 ± 0.06	1.00 ± 0.04	1.05 ± 0.02	1.31 ± 0.01[a,b]
6.25	1.04 ± 0.04	3.17 ± 0.12[a,b]	1.08 ± 0.08	1.00 ± 0.19	1.42 ± 0.01[a,b]	1.74 ± 0.11[a,b]
12.5	1.06 ± 0.14	3.33 ± 0.41[a,b]	1.11 ± 0.19	1.15 ± 0.08	1.89 ± 0.05[a,b]	1.90 ± 0.03[a,b]
25	1.06 ± 0.11	3.85 ± 0.34[a,b]	1.15 ± 0.08	1.23 ± 0.08	2.02 ± 0.03[a,b]	2.03 ± 0.10[a,b]
50	1.05 ± 0.12	7.14 ± 0.15[a,b]	1.33 ± 0.08[a]	1.77 ± 0.18[a,b]	2.12 ± 0.07[a,b]	2.18 ± 0.77[a,b]
100	1.05 ± 0.11	8.24 ± 0.23[a,b]	1.57 ± 0.18[a]	2.18 ± 0.05[a,b]	2.27 ± 0.06[a,b]	2.90 ± 0.54[a,b]

[a]*Means (n=5) are significantly different compared to the respective negative control (10%DMSO in NSS, pH = 12) using Student's T-test* ***(ρ < 0.01)***
[b]*Means are significantly different compared to the respective Gallic Acid solution (pH = 7.3) using Student's T-test* ***(ρ<0.01)***

Effect of Aluminum and Iron Complexes of allic Acid on Bacterial Cell Surface Hydrophobicity

To determine whether gallic acid and its metal complexes damage bacterial cell surfaces, the change in the cell surface hydrophobicity of *Escherichia coli* ATCC 25922 was evaluated. Changes in cell surface hydrophobicity imply a direct effect of radicals on the bacterial cell membrane. As shown in Figure 4, the cell surface hydrophobicity of *E. coli* decreases in a dose-dependent manner in all treatment groups.

Not much has been reported on the antibacterial properties of gallic acid and its metal complexes in alkaline pH although several studies claim that the toxicity of gallic acid is related to oxidative processes. In this study, it was hypothesized that alkaline autooxidation of gallic acid increases the net negative charge of gallic acid (Figure 5), making it more compatible to form complexes with metals such as aluminum and iron. In addition, it was hypothesized that gallic acid can chelate aluminum and iron in alkaline pH. Based from the results, metal complexes of gallic acid have higher antibacterial activity against *E. coli* ATCC 25922 compared to gallic acid in alkaline pH. The metal complexes of gallic acid are also more pro-oxidative and genotoxic compared to gallic acid. In a realted study, metal complexes of ferulic acid have higher antibacterial activities compared to ferulic acid [47]. This suggests that the antimicrobial property of

phenolic compounds can be increased by metal complexation.

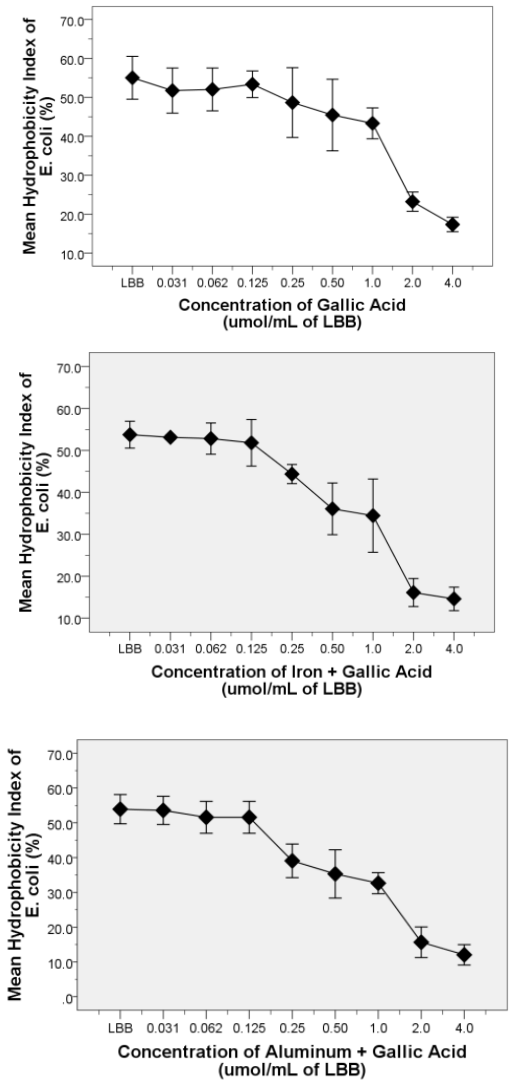

Figure 4. Comparison of the effect of gallic acid and metal-gallic acid complexes on the hydrophobicity of *Escherichia coli* ATCC 25922 (n = 5)

Alkaline autooxidation of gallic acid [18, 30] produces gallate radicals which bears a net negative charge (Figure 5). Theoretically, the negative charge of gallate radical is electrostatically compatible with cations thereby increasing the chelating property of the ligand and decreasing the deleterious effects of metals. However, the results obtained in the study suggests that alkaline pH and the presence of aluminum and iron increase the toxicity of gallic acid in a mechanism related to oxidative stress.

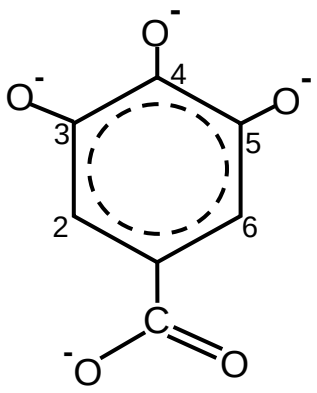

Figure 5. Molecular Structure of Gallate Radical

Alkaline pH promotes complexation of gallate radicals with aluminum and iron. Similar to other studies, the iron-gallic acid complex in Figure 2 appears dark blue [10, 11, 31, 32] while the color of aluminum-gallic acid complex resembles the yellow complex formed between aluminum with catechol-bearing groups such as flavonoids. In order to avoid complexation of other ions with gallate radical, phosphate buffered solution was not utilized. In addition, phosphate ions also promote autooxidation of iron in alkaline pH which could interfere with the complexation of iron and gallate radical. The differences in the spectrum of the metal-gallic acid solutions and gallic acid suggest the formation of complexes between gallic acid and metal ions.

Iron has been previously documented to form complexes with other phenolic compounds in a metal to ligand (M:L) ratio of 1:1 [33]. The complexation of gallic acid with iron is facilitated by the hydroxyl groups of catechol or galloyl moieties [10, 34] although protonated hydroxyl groups may also promote intramolecular hydrogen bonding, making gallic acid a poor entering group [33]. Gallic acid radicals were explained to cause one-electron reduction of Fe^{3+}, since the electronic configuration of the metal ion is d^6, with five up electrons and one down electron, implying the complexation to be bridged by one electron of gallic acid delocalized among deprotonated hydroxyl groups [32]. Generally, the studies agreed that the hydroxyl group in the *para* position relative to the carboxyl group of gallic acid is the bridging group to cations. On the other hand, aluminum chloride can form a quasi-tetrahedral complex of $AlCl_3$•L where L is a potential coordination site for gallic acid [35]. Complexation of aluminum by gallic acid was previously reported to

occur at alkaline pH although its pro-oxidative property was not described [36]. Aluminum is not a redox metal but it exhibits pro-oxidative property due to the formation of aluminum superoxide semireduced radical ion [37]. The complexation of aluminum and iron with gallate radicals in aqueous solutions therefore are influenced by the compatibility of the net charges of the cations and gallate radicals, pH, presence of hydroxide ions, and presence of other charged monoatomic or polyatomic ions. Complexation between metals and gallic acid occurs when gallate radicals successfully outcompete hydroxo ions in forming complexes with metals. Of greater emphasis is the effect of metal-gallic acid complexes in biological systems. Metals play a role in promoting oxidative stress [20] although other studies claim that some metals prevent the autooxidation of gallic acid in alkaline pH such as magnesium [30].

Gallic acid possesses broad-spectrum antibacterial properties against several Gram positive and Gram negative strains of bacteria (Table 2) in physiologic conditions although its association with oxidative damage was not discussed as a potential mechanism. Standard solutions of gallic acid seem to exhibit higher antibacterial properties compared to plant-derived gallic acid extracts. The minimum inhibitory and bactericidal concentration of gallic acid solution against *Escherichia coli* were reported to be 1500µg/mL and 5000 µg/mL, respectively [7] while the minimum inhibitory concentration of extracted gallic acid reported previously [1] was higher (250 mg/mL or ~1.47M). Compared to literature data, the minimum inhibitory concentrations presented in our results are lower if metals were complexed with gallic acid in alkaline pH.

Alkaline pH inhibits *E. coli* growth in Luria Bertani broth although the availability of carbon sources plays a bigger role in inhibiting the growth of the bacteria [22]. In order to associate the observed results in the turbidimetric assay, the pH of all supplemented bacterial suspensions were measured after 24 hours incubation. It was hypothesized that the pH of the supernatant should be highly alkaline since gallic acid and its metal complexes were synthesized at pH 8.5. However, there was only slight alkalinization of Luria Bertani (LB) Broth (dat not shown) even in the bacterial suspension supplemented with alkaline water. The pH of bacterial suspensions which were treated with metal-gallic acid solutions was slightly acidified. It is possible that proteins and amino acids present in Luria Bertani broth may have acted as buffers to prevent drastic pH changes in the culture broth although the acidification of the media could also be related to the deprotonation of gallic acid.

To determine the comparative antibacterial activities of gallic acid and metal-gallic acid complexes, two antibacterial assays were performed based from published protocols. The results in Figure 2 and Figure 3 revealed a higher antibacterial activity of the metal complexes of gallic acid at a concentration of 4.0µmol/mL. No bacterial growth was noted when the bacterial suspensions exposed to metal-gallic acid complexes were incubated for 24 hours in nutrient agar. In the well diffusion assay, only iron-gallic acid resulted to a zone of inhibition (12.0 ± 0.20mm). The cause of the negative antibacterial properties of gallic acid could be attributed to a low concentration in the assay. However, it has to be noted that anhydrous gallic acid solidifies easily, forming a pasty consistency when higher concentrations are prepared, making it difficult for the solution to diffuse in solid media. However, our results suggest that gallic acid and its metal complexes have higher antibacterial activities in bacterial suspensions than in solid agar media.

Complexation of galloyl-bearing compounds to metals is reflects their chelating action, which is supposed to decrease the oxidative damage caused by metals to cellular structures [10, 11]. However, gallic acid and its metal complexes are pro-oxidative since they promote lipid peroxidation and production of hydrogen peroxide (Table 3). In the TBARS assay, malondialdehyde (MDA) forms adducts with thiobarbituric acid in a ratio of 1:2, producing a pink color which can be measured at 532 nm [38]. Malondialdehyde is a decomposition product of polyunsaturated fatty acids caused by hydroxyperoxidation [39]. Hence, the production of malondialdehyde indicates the pro-oxidative property of the tested compounds due to the production of radical oxygen species.

The results in our study support the previously reported pro-oxidant property of gallic acid. Gallic acid was also reported to exhibit pro-oxidative effects due to its autooxidation, which results to the generation of reactive oxygen species [19] such as superoxide anions and hydrogen peroxide which participates in Fenton-type reactions. It was reported that the pro-oxidative property of gallic acid was attributed to the production of hydroxyl (•OH) radicals [4] although hydroxyl radicals are short-lived [17]. Gallic acid, when combined to peroxidases and H_2O_2, behaves as an antioxidant while in the absence of H_2O_2, it becomes an oxidant [16]. Since plant extracts contain hydrogen

peroxide (0.67µmol/g to 3.63µmol/g fresh weight) [40], gallic acid acts primarily as an antioxidant when obtained in plant samples.

In a system where iron and H_2O_2 are present, the concentration of gallic acid and iron affects its over-all activity. A molar ratio of >2:1 between the gallic acid and iron promotes antioxidant activity while the over-all effect is pro-oxidative if gallic acid: iron molar ratio <2:1 [41]. Since equimolar concentrations of metal and gallic acid were used, the metal complex used in the study is expected to be pro-oxidative. Gallic acid has high reducing power [13, 14] and poor metal chelating ability [8, 9, 10, 11]. Instead of chelating iron, gallic acid was reported to convert Fe^{3+} to Fe^{2+} [31], causing the reduction of oxygen to superoxide radical anions ($O_2^{\bullet-}$). When Fe^{2+} ions react with hydrogen peroxide formed from two superoxide radicals in the solution, highly reactive hydroxyl radicals are formed. Similarly, hydroxyl ion generation was also explained as the main mechanism of toxicity of photoirradiated gallic acid since semiquinone radicals were not detected [4]. Hydroxyl radicals cause damage to damage DNA, carbohydrates, proteins and lipids [13,14]. If hydroxyl radicals were the main cause of the pro-oxidant effects of iron-gallic acid, the reaction should occur in a relatively short period of time because hydroxyl radicals are short-lived [17].

In the absence of a biological reductant such as gallic acid, metals can also induce DNA damage through oxidative stress. Aluminum ions were reported to be genotoxic against lymphocytes [42] and plant cells [43] while ferric ions were genotoxic to human colon cancer cells [44] and *Escherichia coli* PQ37, PQ300 and OG400 [45]. In biological solutions, aluminum participates in the production of hydroxyl radicals by forming aluminum-superoxide complex which enhances the concentration of Fe^{2+}, which in turn, breaks down H_2O_2 into **.**OH radicals [37]. In literatures, gallic acid causes genotoxicity by causing oxidation and conformational changes of DNA and damage to deoxyribose or nitrogenous base [46]. The results shown in this study supports the notion that the genotoxic properties of aluminum and iron are related to ROS-induced DNA damage since the metals increased the generation of MDA and H_2O_2 caused by gallic acid (Table 3). Iron was acknowledged as a primary cause of reactive oxygen species generation and oxidative stress *in vivo* [31]. Aside from direct DNA damage, the products of lipid peroxidation caused by gallic acid and metal-gallic acid solutions indirectly caused genotoxicity. MDA is mutagenic in bacterial and mammalian cells and promote carcinogenesis in rats [13].

Our results, however, seem to agree with previous reports that gallic acid promotes Fenton reaction with redox metals such as ferric ions due to Fe^{3+}/Fe^{2+} recycling, thereby maintaining a constant generation of hydroxyl radicals which consequently damages biomolecules [14] although it is also possible that the poor chelating ability of gallic acid is unable to prevent the damaging effects of iron. This mechanism is supported by the results in our results since iron-gallic acid complex showed higher pro-oxidative properties compared to aluminum-gallic acid. Of particular interest is the interaction of aluminum and gallic acid in alkaline pH. Since aluminum is not a redox metal, a different mechanism appears to be involved in aluminum-gallic complexes.

Gallic acid has a high affinity to the bacterial cell membrane [4]. In addition, the metal complexes of gallic acid exhibit lipophilicity since metal complexation promotes electron delocalization in the chelate ring system such as in the case of ferulic acid [47]. Increased lipophilicity allows metal complexes to interact with bacterial cell membreanes more effectively.

It was surmised in this study that a pro-oxidative activity of gallic acid, aluminum-gallic acid complex and iron-gallic acid complex alters the bacterial cell surface properties of *E. coli* ATCC 25922 as evidenced by a dose-dependent decrease in cell surface hydrophobicity (Figure 5). To validate whether lipid peroxidation occurs in bacterial cells, TBARS assay was also conducted to 2 x 10^9CFU/mL *E. coli* cells. After 20 minutes, there was no MDA production. However, when the length of exposure was extended until 40 minutes, there was positive MDA generation in concentrations ≥2.0µmol/mL in suspensions containing gallic acid (pH=8.5), aluminum-gallic acid complex and iron-gallic acid complex (data not shown). No MDA was generated in suspensions mixed with gallic acid (pH = 7.3) and alkaline water (pH=8.5). Similar to the report of Aubron *et al*. (2012), the cell membrane of *E. coli* can be damaged by gallic acid. Our data suggests that a change in the cell surface hydrophobicity could be an indicator of oxidative damage. Probably, the change in the hydrophobicity of *E. coli* was initially caused by non-specific interactions with gallic acid and its metal complexes. Further changes in the hydrophobicity of the bacterial cell membrane could be a consequence of the pro-oxidative effects of gallic acid and its metal complexes. Since the assays utilized whole

bacterial cells, the observed genotoxic properties of gallic acid and its metal complexes could be a caused by the byproducts of the damaged bacterial cell membrane.

The results of our study suggest that the mechanism of the inhibitory and bactericidal property of gallic acid and its metal complexes is associated with the ability of the compounds to cause oxidative damage to the bacterial cell membrane and DNA. But it has to be noted that in *E. coli*, transcriptional activators OxyR and SOxR systems protect the bacterium from ROS-mediated or metal-induced oxidative stress [48]. Since gallic acid and its metal complexes display high affinity to the bacterial cell membrane due to their hydrophobic property, it is suggested that oxidative damage occurred in the phospholipid bilayer first, causing hydrophobicity changes. In addition, the damage to the cell membrane was reported to be irreversible, highlighting the potential of gallic acid as an efficient antibacterial agent [49].

IV. CONCLUSION AND RECOMMENDATION

In alkaline pH, aluminum and iron increases the antibacterial, pro-oxidative, and genotoxic properties of gallic acid. The antibacterial property of gallic acid and its metal complexes caused by their pro-oxidative and genotoxic properties as evidenced by the generation of malondialdehyde and hydrogen peroxide, induction of DNA repair response in DNA repair-deficient *E. coli* PQ37, and alteration of cell surface hydrophobicity of *E. coli* ATCC 25922 *in vitro*. The results suggest that the tested compounds can be used as disinfectants although further investigations should be done to determine their hazardous effects to human health since metals are involved. Future studies should also determine the stability of the complexes in different conditions.

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