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Favourable Drug-Lead Pharmacokinetic Features for Designing Gallic Acid-Standardized *Syzygium Polyanthum* Aqueous Extract-Based Product

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ABSTRACT

In this study, *Syzygium polyanthum* was standardized against gallic acid (GA), and a complete pharmacokinetic test was conducted using *in vitro* and *in vivo* models against this phytochemical. High-performance liquid chromatography showed that GA is a major phytochemical in aqueous extract of *S. polyanthum*. It exhibited a low equilibrium solubility and physiochemical stability at pH 2.0 and 7.4, but it deteriorated rapidly at pH 9.2. It showed low permeability toward Caco-2 intestinal absorption with eight times slower absorption in oral than intravenous administration. GA was unstable in mouse, rat, and dog plasma sera with *in vitro* half-lives (t1/2) of 60, 53, and 56 min, respectively, but was relatively stable in human plasma serum (t1/2 = 185 min). Approximately 5.6% of GA (10 µM) bound to the human plasma proteins. GA was stable in mouse, rat, dog, and human microsomal extracts with *in vitro* microsomal intrinsic clearance values of 72, 68, 6, and 22 µL/min/mg, respectively. GA selectively inhibited or stimulated the activity of the tested CYP450 enzymes. The in vivo oral bioavailability of GA was 54%, with short elimination half-life and a high volume of distribution. Thus, the mention pharmacokinetic features of GA must be considered during the development of GA-based products to yield the optimum dosage and pharmacological effect.

Keywords: Gallic acid, *Syzygium polyanthum*, aqueous extract, pharmacokinetic.

INTRODUCTION

Syzygium polyanthum or *Eugenia polyantha* is distributed in Malaysia, India, Vietnam, Indonesia, Thailand, and Singapore. In Malaysia, it has a common name of "Salam." It has been traditionally used as remedies for various types of illnesses. The leaves of this plant are traditionally used for treating itchy skin, abdominal pain, high blood pressure, diabetes, and gastritis. In Indonesia and India, the leaves of this plant can be used as an ingredient in curry and soups and can even be eaten raw. Roots and fruits of *S. polyanthum* are traditionally used to counteract hangover symptoms (Lelono *et al*., 2009; Sumono & Sd, 2008; Grosvenor *et al*., 1995; Ismail & Ahmad, 2019). These traditional claims were scientifically justified through numerous studies on the leaves, fruits, roots, stem, and barks of *S. polyanthum*. Many studies focused on the leaves because of their numerous bioactivities, such as antimicrobial (Kusuma *et al*., 2011; Hamad *et al*., 2017; Grosvenor *et al*., 1995; Fitri, *et al*., 2017; Mohamed *et al*., 1996), antioxidant (Kusuma *et al*., 2011; Wong, *et al*., 2006; Perumal *et al*., 2012; Har & Intan, 2012; Darusman *et al*., 2013; Othman *et al*., 2014; Safriani, *et al*., 2015; Widyawati *et al*., 2016; Hidayati *et al*., 2017), antihypertensive (Ismail *et al*., 2013; Ismail & Ahmad, 2017; Ismail *et al*., 2018), antidiarrheal

(Fitri, *et al*., 2017; Malik, 2013), antidiabetic (Lelono *et al*., 2009; Widyawati *et al*., 2015a; Widyawati *et al*., 2015b; Widharna *et al*., 2015), anti-cholinesterase (Darusman, *et al*., 2013), anti-plaque (Avriliyanti, *et al*., 2017), anti-lipase (Alias *et al*., 2017, Kato *et al*., 2013), anticancer (Sulistiyani *et al*., 2014), and antitumor (Ali *et al*., 2000). Water, methanol, and ethanol extracts of *S. polyanthum* are active extracts reported in these bioactivity studies.

S. polyanthum leaves contain various phytochemical groups, including phenols, flavonoids, tannins, triterpenoids, and alkaloids, as detected through high-performance liquid chromatography (HPLC) (Ismail, & Ahmad, 2019; Ismail *et al*., 2018; Ismail *et al*., 2000). 3,4,5-Trihydroxy benzoic acid or gallic acid (GA) is a major non-polar phenolic compound in *S. polyanthum* leaf extract (Ismail *et al*., 2018) obtained using methanol or water as solvent. GA exhibits strong antioxidant activity toward reactive oxygen species and inhibits lipid peroxidation and metal ion chelation (Badhani, *et al*., 2015). Other therapeutic activities of GA include anti-cancer (Wang *et al*., 2016; Khorsandi, *et al*., 2020), anti-mutagenic (Abdelwahed *et al*., 2007), anti-microbes (Sorrentino *et al*., 2018), anti-inflammatory (Karimi-Khouzani, *et al*., 2017), cardioprotective (Priscilla, & Prince, 2009), gastroprotective (Zhou *et al*., 2020), and neuroprotective (Maya, *et al*., 2018). In this study, the physicochemical ADME and pharmacokinetics attributes of GA under in vitro and *in vivo* conditions are addressed. The objectives are to establish the 1) aqueous and equilibrium stability of GA using buffers of various pH levels; 2) permeability potential of GA using Caco-2 cell monolayer cultured for 21 days; 3) plasma stability and binding in mouse, rat, dog, and human; 4) metabolic stability and CYP450 inhibition; and 5) in vivo bioavailability of GA in rats.

MATERIALS AND METHODS

HPLC-grade dimethyl sulfoxide (DMSO) was purchased from Spectrochem (Mumbai, India). Acetonitrile and methanol were purchased from Lab scan (Thailand). Methylcellulose was purchased from Methocel (Colorcon, India). Sodium bicarbonate, carbamazepine, telmisartan, furosemide, celecoxib, erythromycin, chlorambucil, Dulbecco phosphate-buffered saline (pH 7.4) (D8537), estriol, propranolol HCl, DMEM media powder (D5648), sodium bicarbonate (S5761), fetal bovine serum (FBS) (F2442), sodium pyruvate (S8636), lucifer yellow (L-0259), trypan blue (T6146), penicillin–streptomycin solution (PS) (P0781), non-essential amino acids (M7145), trypsin-EDTA solution (T4174), HEPES (H-4034), dimethyl sulfoxide (D-8779), vinblastine sulfate (V1377), enalapril, verapamil, di-potassium hydrogen phosphate, potassium dihydrogen phosphate, ticlopidine, quercetin, sulfaphenazole, quinidine, ketoconazole, and GA were purchased from Sigma–Aldrich (USA). Disodium hydrogen phosphate and potassium hydrogen phosphate were purchased from Qualigens (India). NADPH was purchased from SRL (India). Potassium chloride and sodium chloride were purchased from SD (India). Ethyl acetate, tween-80, and dimethylacetamide were purchased from Merck (India). Phosphoric acid was purchased from Lobachemie (India). Caco-2 cell line was obtained from ATCC, USA (Cat No. CRL-2102™). Blood plasma of mouse (CD-1), rat (Wistar) and dog (Beagle) was obtained from in-house stock (Aurigene, India). Human blood plasma was obtained from life care voluntary blood bank (Aurigene, India). Liver microsomes were purchased from Xenotech LLC (USA). HPßCD was purchased from Roquette (France).

Preparation of *S. polyanthum* Aqueous Extract

Extraction of *S. polyanthum* was carried out as previously described by Ismail *et al*. (2017). Raw course of dried *S. polyanthum* was extracted using filtered water (1.5:10 ration) for 3 h at 60°C and spray dried at the Institute of Bioproduct Development (Johor, Malaysia). Extracts were stored at 4°C for HPLC quantification.

HPLC Quantification of GA in *S. polyanthum*

HPLC analysis was conducted according to Ismail *et al*. (2018) with some modification, using a Waters 2690 Alliance Separation Module with Zorbax Eclipse XDB-C18 (4.6 mm × 150 mm × 5µm). *S. polyanthum* and GA standard of 2.0 mg each were dissolved in 50% methanol, vortexed, and then filtered. The mobile phase was methanol (80%) and deionized water (20%) with a flow rate of 1 mL/min. The chromatograms were monitored at 280 nm.

Good Laboratory Practice (GLP) Compliance

This study was conducted in compliance with "Organization for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice" (as revised in 1997)

ENV/MC/CHEM (98)17 concerning the Mutual Acceptance of Data in the Assessment of Chemicals [C (81)30(Final)] and [C(89)87(Final) / Revised in C(95)8(Final].

Physicochemical and Equilibrium Stability Test

Three buffers were prepared: Dulbecco's phosphate buffer saline (pH 7.4), simulated gastric fluid (pH 2.0), and sodium bicarbonate buffer (pH 9.2). The stock solution of GA and positive controls (erythromycin and chlorambucil) were prepared at a concentration of 10 mM in DMSO. Assay buffer (1.47 mL) was preincubated at 37°C for 5 min with shaking. Samples (30 µL) were added and incubated at 37°C for 120 min with shaking. An aliquot of 250 µL was withdrawn at 0, 30, 60, and 120 min and transferred into tubes containing 250 µL of acetonitrile and internal standard (celecoxib, carbamazepine, telmisartan, and furosemide). Samples were vortexed for 2 min, centrifuged at 13,000 rpm for 10 min at room temperature, and the supernatant was transferred into LC-MS/MS vials for analysis. Samples were analyzed by LC-MS/MS API- 4000 (Shimadzu) equipped with an ACE 3 C18, 4.0 × 150 mm.

Aqueous stability was assessed by the compound's disappearance at various time points based on the change in analyte to internal standard peak area ratio. Results are expressed as mean ± SD of test compound remained at any given time point. Aqueous stability was calculated using Equation 1:

$$\text{Aqueous stability} = \frac{A}{B} \times 100 \quad \ldots\ldots \text{(Eq. 1)}$$

A = Peak area ratio of compound at X hr; B = Peak area ratio of compound at 0 hr

For the equilibrium stability test, GA and controls (estriol and propranolol HCl) were dispersed in phosphate-buffered saline (PBS) using a vortex to achieve a final working concentration of 10 mM. The samples were incubated at 25°C with shaking (200 rpm) for 24 h, filtered (0.45 µm PVDF), and then analyzed for dissolved content using HPLC (Waters, 2695, HPLC) equipped with a Waters: Xterra, 4.6 × 150 mm, 5 µm column. In parallel, a 50-fold dilution of the 10 mM was prepared (theoretical concentration 200 µM) and labeled as a standard solution. The equilibrium solubility (µM) was calculated using Equation 2:

$$\text{Equilibrium solubility} = \frac{C \times D}{E} \quad \ldots\ldots \text{(Eq. 2)}$$

C= Standard solutiom Concentration (µM), D=Peak area of compound in PBS, E=Mean area of Compound in 100% DMSO

***In Vitro* Permeability Test**

Caco-2 cells were cultured in DMEM containing 10% FBS, 1 mM sodium pyruvate, 1 mM non-essential amino acids, and 1% PS. The cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity.

Caco-2 cells were seeded into the Millicell inserts with a cell density of 40,000/insert and incubated for 21 days. Prior to treatment, grown cells were washed with HBSS, and the Trans Epithelial Electrical Resistance (TEER) was measured to check monolayer integrity. TEER value of >320 ohms cm^2 was considered acceptable for Caco-2 monolayer.

For the apical part, 0.3 mL of HBSS was added to the basal chambers along with 0.2 mL of freshly prepared 10 µM GA and positive control (Vinblastine) to all inserts. The inserts were then incubated for 1 h in a humidified chamber maintained at 37°C. For the basolateral part, 0.3 mL of GA and positive control were added to the basal chambers and 0.2 mL of 1× HBSS to all inserts that were then incubated for 40 min at 37°C. The plate was removed from the humidified chamber, and both apical and basolateral solutions were submitted for LC-MS/MS analysis. Lucifer Yellow rejection test was carried out to counter check monolayer integrity after permeability assay. Only those inserts with cell monolayer with LYR of ≥98% were considered for the analysis. Papp values were calculated using Equation 3.

$$app\left(\frac{cm}{sec}\right) = \frac{dQ}{dt} \text{ X } \frac{Vd}{C0} \text{ X } \frac{1}{A} \quad \ldots\ldots\ldots\ldots \text{(Eq. 3)}$$

where dQ/dt = permeability rate in µg/s; C0 = initial concentration in µg/mL; A = membrane surface area (0.6 cm^2 for 12 mm inserts); Vd = volume in donor well (0.3 mL)

***In Vitro* Plasma Stability Test of GA In Mouse, Rat, Dog, And Human Plasma**

Plasma of mouse, rat, dog, and human (297 µL) was dispensed into microcentrifuge tubes and incubated at 37°C for 5 min in a shaking water bath. The reaction was initiated by adding 3 µL of 1.0 mM GA and positive control (enalapril) then and incubated at 37°C for 120 min.

At respective intervals of 0, 30, 60, and 120 min, 50 µL of the samples was aliquoted and transferred to pre-labeled microcentrifuge tubes containing 200 µL of acetonitrile, internal standard (celecoxib and furosemide), and 1.5% v/v phosphoric acid. The samples were vortexed and then centrifuged at 13,000 rpm for 10 min at room temperature, and the supernatant was transferred to LC-MS/MS vials for analysis.

Plasma stability is defined as the amount of substrate metabolized by the incubation with plasma and assessed by the disappearance of the compound at various time points based on the change in analyte to internal standard peak area ratio as measured in MRM mode using LC-MS/MS. Plasma stability was calculated using Eq. 1.

In Vitro Plasma Protein Binding of GA In Human Plasma

In vitro protein binding study was conducted using the rapid equilibrium dialysis (RED) method. RED base plate wells were activated by incubating with 20% v/v ethanol for 10 min. The incubation base plate was rinsed with MilliQ water twice and allowed to dry at room temperature.

GA was added into the human plasma at a final concentration of 10 µM. The mixture was aliquoted (200 µL) into the matrix (red) chamber, and DPBS buffer (350 µL) was added into the buffer (white) chamber of RED inserts that were assembled in a Teflon base plate. The base plate was incubated at 37°C for 5 h with continuous shaking. Then, 50 µL of post-dialysis samples from the matrix and buffer chamber were aliquoted, and an equal volume of buffer was added to the samples from the matrix chamber. In addition, an equal volume of plasma was added to buffer samples to yield identical sample composition between the buffer and non-buffer samples. The samples were mixed, and the reaction was stopped by adding 200 µL of acetonitrile, internal standard (celecoxib and furosemide), and 1.5% v/v phosphoric acid. The samples were then centrifuged at 13,000 rpm for 20 min, and the supernatant was analyzed by LC-MS/MS. The percentage bound of GA was calculated using Equation 4:

$$P\,app\left(\frac{cm}{sec}\right)=\frac{dQ}{dt}\ \mathrm{X}\ \frac{Vd}{C0}\ \mathrm{X}\ \frac{1}{A}\quad\ldots\ldots\ldots\ldots(\mathrm{Eq.\ 4})$$

In Vitro Metabolic Stability of GA In Mouse, Rat, Dog, And Human Liver Microsomes

A working stock solution of 4.0 µM GA and 1.0 mM of NADPH was prepared in potassium phosphate buffer. In brief, a 25 µL working solution of GA and positive control (verapamil) was dispensed into tubes containing 55 µL of microsomal protein. The mixture was incubated at 37°C for 5 min in a shaking water bath. The reaction was initiated by adding 20 µL of NADPH into all tubes except the T0 tubes, and the mixture was further incubated at 37°C for 5, 15, and 30 min. For T0, acetonitrile containing internal standard was added before the addition of pre-warmed NADPH. At respective time intervals, the reaction was stopped with 100 µL of acetonitrile containing the internal standard. After being vortexed for 2 min, the mixture was centrifuged at 13,000 rpm for 5 min at room temperature, and the supernatant was transferred to LC-MS/MS vials for analysis. The test compound's disappearance upon incubation with liver microsomes over a 30 min incubation time was measured in MRM mode using LC-MS/MS. The peak area ratios of analyte versus internal standard were used to calculate the % remaining at the end of 30 min in Microsoft Excel using Equation 5.

$$\%\text{Parent remained at X time}\ =\ \frac{\text{Time X}}{\text{T0}}\text{x}100.(\text{Eq. 5})$$

Time x= Time X peak area ratio, T0= T0 peak area ratio

In Vitro CYP450 Isomers Inhibition Assay

In brief, 20 µL of each CYP450 isoform substrate was mixed with 80 µL of microsomal protein. Then, 2 µL of GA (1 and 10 µM) and positive control (10 µM) was added to the assay mixture. The whole mixture was pre-incubated at 37°C in shaking water bath for 5 min. Control incubations contained all the above solutions except the inhibitor/test compound for each reaction. The reaction was initiated by adding preincubated 100 µL of 2.0 mM of NADPH into all tubes. After 20 min of incubation at 37°C, the reaction was stopped by adding 200 µL of quenching solution (acetonitrile with internal standard) to all tubes. The samples were vortexed for 2 min and then centrifuged at 13.000 rpm for 10 min at room temperature, and the clear supernatant was transferred to LC-MS/MS vials for analysis.

Percentage of inhibition was calculated for test and positive inhibitor considering the metabolite formed for each isoform compared with the control group using Equation 6.

$$\%\ \text{Inhibition}\ =\ \left[-\left(\frac{M_1-M_2}{M_3}\right)\right]\text{x}100\quad\ldots(\mathrm{Eq.\ 6})$$

M_1=Mean test peak area; M_2= Mean Blank area ratio; M_3=Mean control peak ratio

***In vivo* Pharmacokinetics Test**

This study was performed under the Animal License "812/PO/RcBi/S/04/CPCSEA" of Animal Welfare Division, Ministry of Environment, Forest and Climate Change, India. Male Wistar rats (230–250 g) used in the study were obtained from the in-house breeding facility. Six animals were housed in a cage (n = 3/cage). Each cage was numbered and labeled according to the group. The IV group was fed standard rodent chow Harlan feed (T.2014.15 - Teklad Global 14% Protein Rodent Diet, USA). The oral group was fasted overnight with only water ad-libitum, and feed was provided at 4 h post-dose. Blood samples (0.3 mL), including the pre-dose, were collected at different time points in microcentrifuge tubes containing 15 µL of Na2 EDTA as an anticoagulant centrifuged to separate the plasma. The plasma samples were stored at −80°C until analysis. After the last sampling, the animals were euthanized by CO_2 overdose.

Animal grouping: -Animal 1–3 for Group I for oral (50 mg/kg); -Animal 4–6 for Group II for intravenous (5 mg/kg)

Quantitative bio-analysis of GA in the plasma samples was performed using LC-MS/MS. Plasma samples were analyzed following protein precipitation of plasma with acetonitrile containing the internal standard (Celecoxib and Furosemide) and 0.15% v/v phosphoric acid. The lower limit of quantitation was 5 ng/mL. Plasma pharmacokinetic parameters were calculated from individual plasma concentration vs. time profiles using a non-compartmental analysis tool of Phoenix WinNonlin Professional software (Pharsight Corporation, USA, Version 6.4.0). Absolute oral bioavailability was calculated following Equation 7.

$$\textit{Absolute oral bioavailability} = \frac{AUC_{oral} \times Dose_{iv}}{AUC_{iv} \times Dose_{p0}} \quad \text{.....(Eq. 7)}$$

RESULT AND DISCUSSION

GA has been used as an active ingredient in some commercial products, especially in the cosmetic industry, because of its anti-oxidant property. For instance, a cosmetic product developed by Phace Bioactive Company contains GA, which was derived from oak bark and tea leaves to reduce hyper-pigmentation (Phacebioactive, 2021). Other cosmetic products that contain GA were developed by Orveda to improve the skin tone with skin-brightening effect (Orveda, 2021). Triphala is a health supplement containing GA derived from *E. officinalis* (Indian gooseberry), *T. belerica* (Belleric myrobalan), and *Terminalia chebula* (Chebulic myrobalan). This herbal mixture has been recommended for treating various diseases, including microbial infections, constipation, anemia, fatigue, tuberculosis, and pneumonia. Triphala has also been used in the wound healing of albino rats (Watson, & Preedy, 2019; Peterson, *et al*., 2017). In a recent update, GA is used as a biomarker in Synacinn™, a standardized polyherbal product for diabetes formulated from five Malaysian herbs, including *S. polyanthum* (Fahmi *et al*., 2020).

Despite its wide range of biological activities, the physiological impact and efficiency of GA are strictly dependent on its bioavailability, biochemical integrity, and interaction with the target tissue. ADME (absorption, distribution, metabolism, and excretion) aims to describe how a drug functions in the human body. It is a prerequisite to establish and optimize the early stage of drug discovery and development for further preclinical investigation to produce potent and less harmful compounds. An effective drug-lead nominee must have favorable features, including efficacy and selectivity to the site of action, minimal adverse effect, and stable plasma and liver microsomal with desirable absorption, delivery, metabolism, and excretion characteristics (Hefti, 2008). Prior to the ADME test, the physicochemical properties must be established for chemical stability, lipophilicity, aqueous solubility, hydrogen bonding, and molecular size (Jing *et al*., 2019). The administration route is also crucial, which influences absorption and distribution. For oral administration, Caco-2 or MDCK cell-based models are common in vitro models used to imitate intestinal permeability (Youhanna, & Lauschke, 2021). Once drugs enter the bloodstream, they will be distributed to the targeted organ by binding to the blood plasma components (serum albumin, lipoprotein) (Smith, Di, & Kerns, 2010) or as free drug substance (unbound) (Calvo *et al*., 2006). These drugs will pass through the liver, where they will be metabolized by cytochrome P-450 (CYP450) into other active metabolites or inactive forms (Zanger, & Schwab, 2013). Metabolic stable drugs will be slowly metabolized and circulated back to the bloodstream, whereas unstable drugs are excreted through urine. The pharmacokinetic study was performed on GA, which is the most abundant compound identified in *S. polyanthum*. This study is important in linking the pharmacological data to the effectiveness of herbal formulation containing GA.

HPLC quantification of GA in *S. polyanthum*

HPLC analysis was conducted to identify and quantify GA in *S. polyanthum*. As shown in Fig. 1, the highest content of phytochemical in *S. polyanthum* aqueous extract was GA with a concentration of 0.019 µg/mL at 1.259 min. The shouldering of peaks was observed between 0.8 - 1.3 minutes which may be due to the complex mixture of compounds in the crude extract (Atanasov *et al*., 2015).

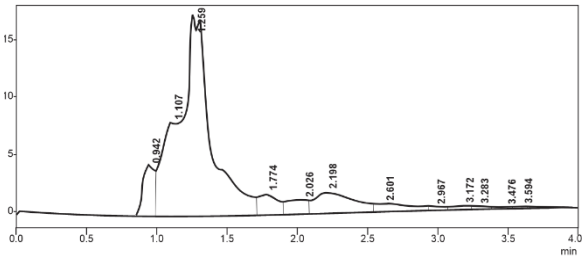

Figure 1. HPLC chromatogram of aqueous extract of *S. polyanthum* at 280 nm. The x-axis represents the retention time (min) while the y-axis represents the abundance (AU).

GA equilibrium solubility and stability in different pH levels

The solubility and physicochemical stability of drugs are primary factors affecting the movement of drugs from the administration site into the bloodstream. These factors influence drug performance, particularly via oral administration. In this study, the equilibrium solubility of GA was very low at 18 µM (3.2 µg/mL) in Dulbecco's Phosphate Buffered Saline, pH 7.4. Estriol and propranolol used as quality controls have mean solubilities at 66 µM and >200 µM, respectively. Furthermore, GA chemical stability was assessed in different pH levels to mimic the human body's pH. As presented in Figure 2 (% remained at 60 min: >70 as stable; 50–70 moderately stable; < 50 unstable), GA remained stable for 120 min at pH 2.0 (93.9%) and pH 7.4 (90.1%). However, GA was very unstable at pH 9.2 as it completely degraded during the measurement at 0 min. Erythromycin as a positive control showed time-dependent degradation at pH 2.0 and chlorambucil degradation at pH 7.4 and 9.2.

In a biological system, the pH of body fluids, including blood plasma, is balanced (weakly basic, pH 7.4) and the upper stomach has pH 4–6.5, whereas the lower part is highly acidic with pH 1.5—4.0 and intestine with pH 7—8.5. The physicochemical stability of compounds is substantially altered by pH variations and degraded into other chemical entities. In this study, GA was stable at pH 2.0 and 7.4 but quickly degraded at pH 9.2. GA is trihydroxybenzoic acid where the adjacent hydroxyl group at positions 3, 4, and 5 is responsible for the UV spectral shifting during the treatment at pH 3–11 (Friedman, & Jürgens, 2000), with major degradation product in alkaline conditions is purpurogallin-8-carboxylic acid (Honda *et al*., 2019). This compound can be found in fermented black tea and exhibits high antioxidant (Honda *et al*., 2019) and anti-inflammatory activities (Sang *et al*., 2004).

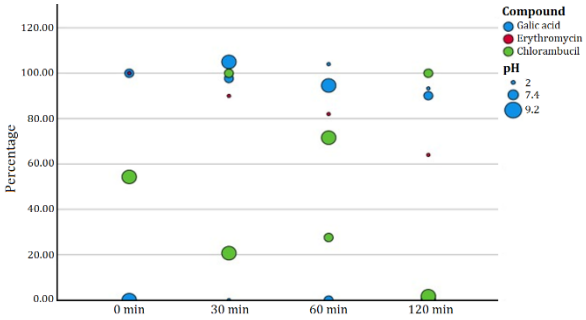

Figure 2. Percent parent remained of gallic acid, erythromycin, and chlorambucil at various pH

Permeability of GA across Caco-2 cells

Drug distribution is influenced not only by differences in pH but also by the intestinal epithelial barrier that separates the external and internal environments, acting as the first limiting step for the ingestion of drugs. The intestinal epithelium composed of delicate monolayer cells serves two distinctive functions: (1) as a natural barrier that prevents the entry of hazardous substances from the gut lumen, such as foreign antigens, toxins, and microorganisms into the circulation (Williams *et al*., 2015), and (2) as a selective filter that facilitates the absorption of dietary nutrients, electrolytes, water, and various beneficial substances from the intestinal lumen (Groschwitz, & Hogan, 2009). Selective permeability is mediated via two major routes, namely, paracellular or transcellular (Ménard, Cerf-Bensussan, & Heyman, 2010).

Caco-2 cell monolayer culture is a well-recognized and widely used model for the intestinal permeability study of new drugs. Intestinal absorption of polyphenolic compounds, such as corilagin, ellagic acid, apigenin, orientin, and (-) epicatechin-3-gallate, was hardly absorbed across the Caco-2 monolayer and could be mediated by the

paracellular pathway with the involvement of carrier proteins, such as P-glycoprotein and multidrug resistance proteins (Mao *et al*., 2016). Similarly, in our test, GA has a low permeability with Papp value $<2 \times 10^{-6}$ cm/s for the apical and basolateral directions. Konishi *et al*. discovered that GA has an absorption rate of 100 times slower than p-coumaric acid, suggesting that its transepithelial transport is through paracellular pathways (Konishi, Kobayashi, & Shimizu, 2003). In our oral administration in rats, GA was detected in serum blood as early as 15 min, indicating that GA was absorbed through the intestine by paracellular pathways. To overcome this limitation, structure modifications (Awang *et al*., 2016) and encapsulation approaches in various carriers, including mesoporous silica nanoparticles (Rashidi *et al*., 2014), cellulose acetate (Phiriyawirut, & Phaechamud, 2012), xanthan polysaccharide nanofibers (Faralli *et al*., 2019), and cyclodextrin (Aytac *et al*., 2016), could improve the absorption rate. In addition, GA loaded into the transdermal patches delivery poly(L-lactic acid) fiber mat retained the GA stability conveyed by the constant radical scavenging activity of GA (Chuysinuan *et al*., 2009).

Stability and binding of GA in mouse, rat, dog, and human blood plasma

Unstable compounds have rapid clearance and short half-life, resulting in poor pharmacological efficiency. GA was subjected to mouse, rat, dog, and human blood plasma for 120 min, and the percentage parent remained were calculated from LC-MS/MS data. From the concentration-time profile of plasma stability (Fig. 3A), linear regression and time-dependent GA clearance were observed in all plasma types. At 120 min, significant differences ($p < 0.05$) was measured in the concentration of GA in the plasma of human (62%) vs. mouse (9%), human (62%) vs. rat (11%), and human (62%) vs. dog (15%). Compared with the reference compound, enalapril clearance showed significant differences for all plasma with the highest stability of approximately 102% in dog, 84% in human, 35% in mouse, and the least stable in rat (0%) (Fig. 3B). Comparison between the in vitro half-lives (t1/2) showed that GA displayed longer stability in human plasma with t1/2 of 185 min followed by mouse (60 min), dog (56 min), and rat (53 min). Different species have different plasma stability due to different mechanisms and enzymes. Testing against all of these species may provide the possible information on toxicity, metabolism and clearance routes which is crucial for developing an appropriate dosage protocol (Toutain *et al*., 2010). In addition to the plasma stability test, 10 μM of GA was further assessed for its ability to bind to human plasma in vitro for 5 h. Results showed that only 5.6% of GA was bound to the plasma. In addition, 29% of GA was stable at 5 h of treatment.

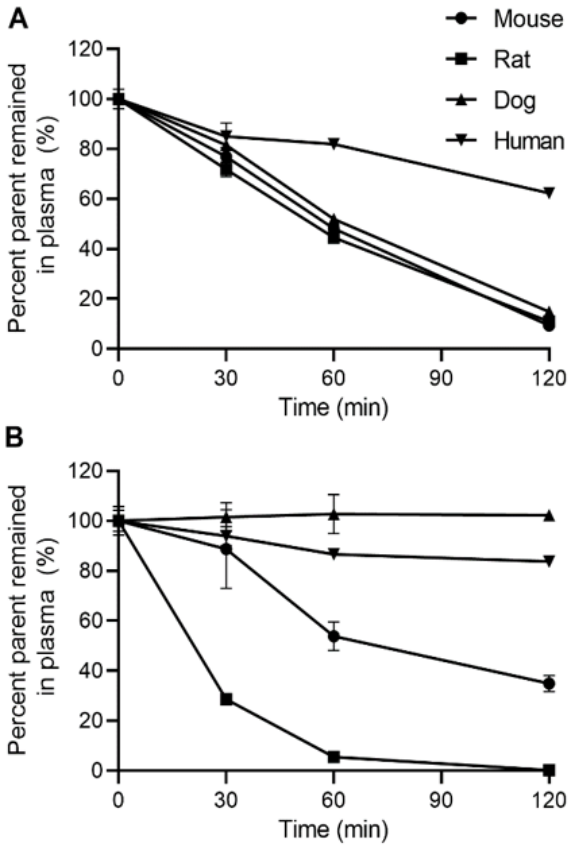

Figure 3. Percent parent remained in mouse, rat, dog, and human plasma. A) GA and B) positive control (enalapril).

Once the drug is absorbed into the bloodstream, it is transported to the target cells by (1) binding to the blood plasma components (serum albumin, lipoprotein) or (2) acting as a free drug. We discovered that GA was unstable in the plasma of mice, rats, and dogs but was moderately stable to human plasma. The reaction between the enzyme–drug complex significantly influences the bioavailability of drugs in blood plasma. Enzymatic reactions, such as hydrolysis and esterase, cause drugs to be molecular arranged or bound irreversibly to proteins, leading to rapid clearance, short half-life, and poor in vivo performance. Drugs

with specific functional groups, such as amide, ester, and sulfonamides, are hydrolyzed by plasma enzymes (Konsoula, & Jung, 2008). In addition, the binding of GA to human plasma proteins was capacity-limited, especially on the serum albumin, the most abundant component in blood plasma. GA binding to plasma proteins is relatively low. Thus, the free-drug theory would be the main event for GA pharmacological activity. Theoretically, the pharmacological activity of drugs at the site of action is exhibited by the active unbound fractions at steady-state conditions (Chen *et al*., 2020), and this fraction will be further metabolized in the liver and excreted in the urine.

In vitro metabolic stability of GA in mouse, rat, dog, and human liver microsomes

Liver microsomes are derived from the endoplasmic reticulum of hepatocytes. Liver provides subcellular fractions containing microsomal drug-metabolizing enzymes responsible for the metabolism of drugs, transforming them into more easily excretable compounds. The susceptibility of drugs to biotransformation provides the rate of disappearance over time. These data are usually reported as intrinsic intestinal clearance, which is extremely important as an early estimation of metabolic clearance in vivo in selecting and designing drugs with favorable pharmacokinetic properties. For example, if the drug is rapidly metabolized, more daily doses may be required to equilibrate blood and tissue concentrations to achieve an ideal therapeutic effect. By contrast, for slow-metabolized drugs, the dose needs to be adjusted, and more preclinical toxicity tests, such as sub-chronic and chronic toxicity tests, should be conducted as long-time exposure may cause toxic build-up. In our test, GA was exposed to four species of liver microsomes to determine the interspecies variation microsomal stability.

GA (1.0 μM) was incubated in mouse, rat, dog, and human microsomes for 30 min to assess the interspecies differences of metabolic stability. Results showed (Fig. 4) a slow rate of microsomal oxidation of GA in all species. GA was most stable in dog with a T1/2 of 374 min, which translated to low in vitro microsomal intrinsic clearance (mCl_{int}) of 6 μL/min/mg, in vivo intrinsic liver clearance (Cl_{int}) of 10 mL/min/kg, and hepatic clearance (CL_H) of 8 mL/min/kg. GA was also considered highly stable in human microsomes with a t1/2 of 107 min, a mCl_{int} of 22 μL/min/mg, a Cl_{int} of 29 mL/min/kg, and a CLH of 12 mL/min/kg. Compared with dog and human microsomes, rodent microsomes showed rapid clearance of GA. Mice has a t1/2 of 34 min, a mCl_{int} of 68 μL/min/mg, a Cl_{int} of 143 mL/min/kg, and a CL_H of 40 mL/min/kg. Rat microsomes showed the most rapid clearance of GA with a t1/2 of 32 min, a mCl_{int} of 72 μL/min/mg, a Cl_{int} of 329 mL/min/kg, and a CL_H of 71 mL/min/kg.

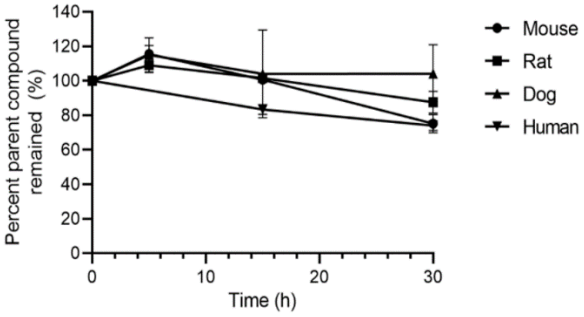

Figure 4. Percent parent remained of GA in mouse, rat, dog, and human microsomes.

In vitro cytochrome P450 inhibition study of GAf

GA is excreted in its original form in the urine. In orally administered (50 mg) GA of healthy female volunteers, about 12% of GA and 25% of GA metabolite (4-O-methyl GA) was detected in urine (Shahrzad *et al*., 2001). In another study, more than half of GA was metabolized into 4-O-methyl GA in rat urine, with a 4-O-methyl GA ratio of 0.55–0.76 total GA, indicating that a significant amount of GA was excreted in its original form (Zong *et al*., 1999). CYP450 isomers play prominent responsibilities in drug metabolism and abundantly present in hepatocyte microsomes. The presence of certain drugs could influence the metabolic activity of these enzymes, subsequently altering the bioavailability of other coexisting drugs. This phenomenon is also known as drug–drug interaction. Inhibition of drug-metabolizing enzymes is a key determinant of drug–drug interactions, resulting in adverse drug reactions in patients receiving multiple drugs.

In our test (Fig. 5), GA at 1 and 10 μM showed stimulation of activity of CYP 2C8 (1.5%/30%), CYP 2C19 (7.4%), CYP 2D6 (12%/14%), and CYP 3A4 (midazolam) (19%/26%). The percentage inhibition by positive inhibitors for each CYP isoform activity in human liver microsomes was within the in-house acceptable range, suggesting the assay system suitability. Mild inhibition can be observed in CYP 1A2 (9.2%/14.5%), CYP 2C9 (10%/21%), CYP 2B6 (52%/52%), and CYP 3A4

(Testo) (33%/45%). Hence, GA is not recommended to be consumed with drugs metabolized by these enzymes, such as artemisinin, bupropion, cyclophosphamide, efavirenz, ifosfamide, methadone, and testosterone (Zanger, & Klein, 2013). Conversely, GA also increased the activity of CYP2C8, CYP2D6, and CYP3A4 (MDZ) on metabolizing certain drugs, including thiazolidinediones, meglitinides, 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, chemotherapeutic agents, antimalarials, antiarrhythmics, and retinoid derivatives (Daily, & Aquilante, 2009).

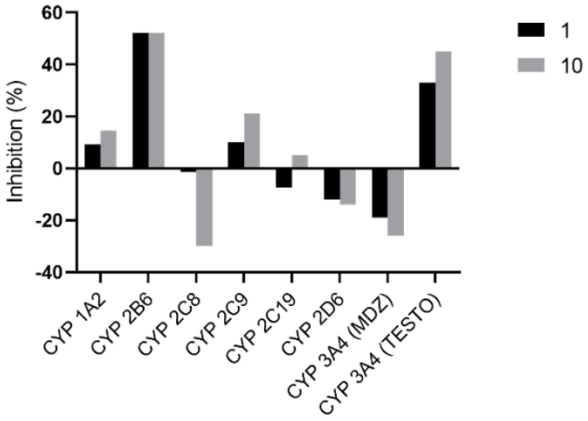

Figure 5. CYP450 isomers activity during the treatment of 1 and 10 μM of GA.

***In vivo* pharmacokinetic study of GA**

Preclinical pharmacokinetic studies in animal models are required to improve understanding of ADME patterns of a particular compound. Our study compared the bioavailability of GA in male Wistar rats' plasma upon oral (50 mg/kg) and intravenous (5 mg/kg) administration. The mean plasma concentration versus time profile of GA is presented in Fig. 6. A time-dependent clearance of GA in both administration routes was observed where 5 mg/kg intravenous showed faster clearance of GA (last measured clearance at hour 2) than 50 mg/kg oral administration (last measured clearance at hour 4). The peak plasma concentration (C_{max}) of GA for IV administration (5 mg/kg) was 1603 ng/mL detected at 7.5 min (T_{max}) post-dose with a total exposure of GA to last measured ($AUC_{0\text{-}last}$) of 926 ng*h/mL, and total exposure of GA to infinity ($AUC_{0\text{-}\infty}$) of 930 ng*h/mL. The GA residence time to last measure ($MRT_{0\text{-}last}$) was 24 min, whereas the residence time to infinity ($MRT_{0\text{-}\infty}$) was 34 min. GA showed a high clearance (CL) of 90.1 mL/min/kg and a high volume of distribution (Vss) (3062 mL/kg) with a short elimination half-life (t1/2) of 37 min. For oral administration, higher C_{max} was detected at 1743 ng/mL after 60 min of administration with a larger $AUC_{0\text{-}last}$ of 5077 ng*h/mL and a AUC0-∞ of 6964 ng*h/mL. Oral $MRT_{0\text{-}last}$ was 102 min, whereas $MRT_{0\text{-}\infty}$ was 187 min. The CL was 120 mL/min/kg with a longer t1/2 of 121 min.

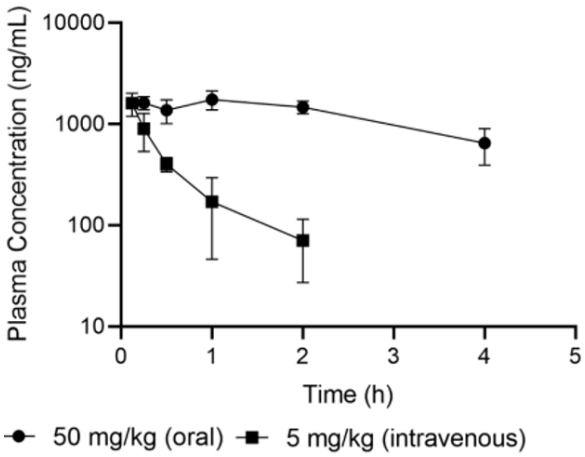

Figure 6. Mean plasma concentration vs. time profiles of gallic acid at 50 mg/kg oral and 5 mg/kg intravenous dose in male Wistar rats.

The Tmax in IV administration was eightfold faster than that in oral administration with 60 min. This result might be due to the limited GA absorption demonstrated via the in vitro Caco-2 absorption test, resulting in poor intestinal absorption. Moreover, the t1/2 of GA in the elimination phase of oral administration was twice longer than that of IV, suggesting that intestinal absorption is the first limiting step that influences GA bioavailability in rats. In addition, the t1/2 of oral and IV administration was relatively short because of the low plasma and tissue binding that allows this compound to be eliminated faster from the bloodstream in the form of unbound free molecules. The bioavailability of GA is 54%, which is relatively low. This result can be explained by the low permeability trans-membrane across the intestinal epithelium, instability under the basic condition of the duodenum and pancreatic juice, enzymatic reaction in blood plasma, and low solubility in body fluid. This study observed total degradation of GA in basic pH (9.2) but stable in body pH and acidic conditions. Theoretically, the pharmacological activity of drugs is exhibited by the active unbound fraction, where it will be diffused and distributed in the targeted tissue. Each

drug is uniquely distributed in our bodies. Very few drugs that are highly bound to tissue remain in the circulation, the plasma concentration is low, and the volume of distribution is high. Meanwhile, drugs that are highly bound to plasma protein remain in the circulation and have a low volume of distribution (Kok-Yong, & Lawrence, 2015). GA has a relatively high VSS (3064 mL/kg), which agrees with the low blood plasma protein-binding capacity.

CONCLUSION

GA was stable in acidic and neutral pH but degraded in basic condition. In addition, it showed poor intestinal absorption permeability, reaching maximum concentration in blood eight times slower in oral versus IV administration. GA was unstable in mouse, rat, and dog but moderately stable in human plasma serum with low binding ability in human plasma. Despite showing high metabolic stability in the microsomal extract of mouse, rat, dog, and human, GA mildly inhibited the activity of CYP 1A2, CYP 2C9, CYP 2B6, and CYP 3A4 (Testosterone) but stimulated the activity of CYP 2C8, CYP 2C19, CYP 2D6, and CYP 3A4 (Midazolam). The oral bioavailability of GA was 54%, with short elimination half-life and a high volume of distribution. The present data might be useful for future research on developing GA-based products from natural resources.

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