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Identification of gallic acid as a active ingredient of *Syzygium aromaticum* against tacrolimus-induced damage in renal epithelial LLC-PK1 cells and rat kidney

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ABSTRACT

Tacrolimus (FK506), a calcineurin inhibitor, is an effective immunosuppressive agent mainly used to lower the risk of organ rejection after allogeneic organ transplant. However, FK506-associated adverse effects, such as nephrotoxicity, may limit its therapeutic use. In this study, we confirmed that epigallocatechin-3-gallate (EGCG), sanguiin H-6, and gallic acid increased cell survival following FK506-induced cytotoxicity in renal epithelial LLC-PK1. Among these compounds, gallic acid exerted the strongest protective effect, further confirmed in the FK506-induced nephrotoxicity rat model. Additionally, we identified supporting evidence for the nephroprotective function of gallic acid using molecular docking and bioavailability investigations.

Calcineurin inhibitors are effective immunosuppressive agents; however, these agents present adverse effects such as nephrotoxicity, limiting their applicability.[1] Tacrolimus (FK506) is one such immunosuppressive drug used primarily to lower the risk of organ rejection after allogeneic organ transplant.[2–4] Adverse effects of FK506 can result in treatment cessation despite observed efficacy. Notably, FK506 treatment demonstrates nephrotoxicity in 17% to 44% of renal transplant recipients and in 18% to 42% of liver transplant recipients.[5]

The mechanisms of FK506-induced nephrotoxicity remain unclear. Mechanisms of adverse effects include functional glomerular and tubular changes, increasing apoptosis and inflammatory responses in proximal tubular cells.[2,6] Moreover, a previous study has suggested that oxidative stress could underlie the observed calcineurin nephrotoxicity, producing reactive oxygen species (ROS) through the NADPH oxidase pathway.[7] The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure and volume homeostasis.[8,9] Reportedly, treatment with tacrolimus leads to RAS impairment. Several studies have suggested that tacrolimus treatment increases the plasma renin activity[10,11], as well as the renin mRNA levels in the renal cortex in tacrolimus-treated rats.[12] Previously, several investigations have suggested that tacrolimus interferes with RAS and is associated with increased oxidative stress. Additionally, it is well known that the FK506 induces the increased expression of transforming growth factor-beta 1 (TGF-ß1).[13] FK506 induced TGF-ß1 is known to act on TGF-ß/SMAD signaling, causing renal fibrosis. [14,15]

Gallic acid, a 3, 4, 5- trihydroxybenzoic acid, is a member of the hydroxybenzoic acids and is found to be present in several plants, including tea leaves, grapes, apple peels, and wine.[16] Additionally, this chemical has shown beneficial effects such as antimicrobial and anticancer activities, as well as protective effects in various diseases, including gastrointestinal, cardiovascular, metabolic, and neuropsychological diseases.[17] Recent studies have suggested that gallic acid ameliorates inflammatory activity,[18–20] with the protective function attributed to its ability to inhibit ROS induced cellular damage, upregulate glutathione peroxidase expression, and mitigate the presence of free radicals[21].

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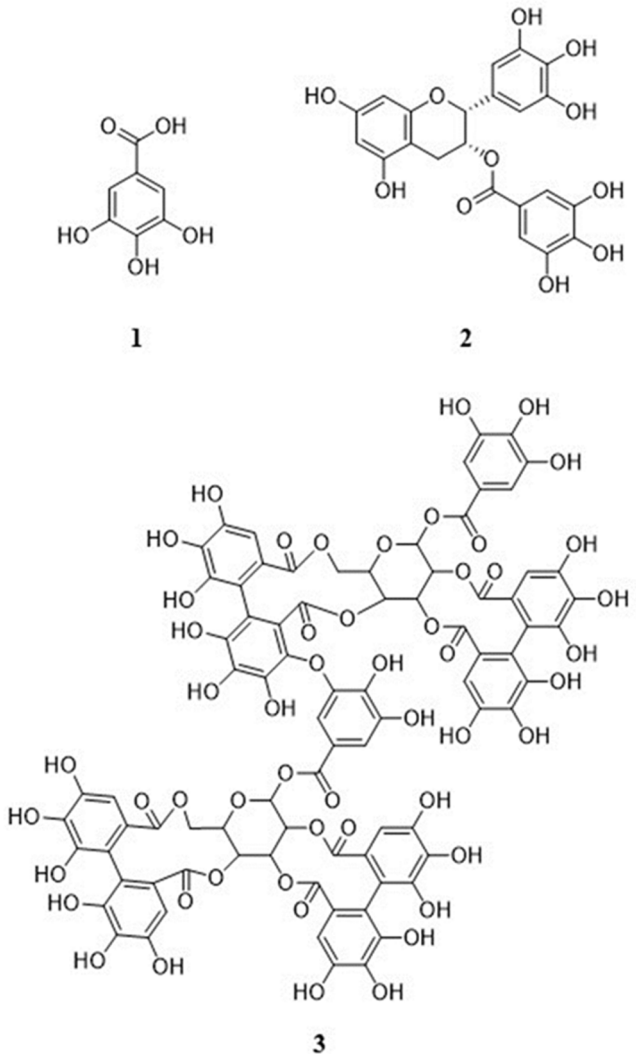


Fig. 1. Chemical structure of gallic acid (1), epigallocatechin gallate (EGCG) (2), and sanguiin H-6 (3).

In the present study, we examined the effect of some select compounds containing the gallic acid structure (gallic acid, epigallocatechin gallate (EGCG), and sanguiin H-6) on FK506-induced cytotoxicity and nephrotoxicity in a mouse model. Additionally, we identified evidence demonstrating the nephroprotective function of gallic acid using molecular docking and bioavailability studies.

Calcineurin inhibitors have immunosuppressive properties owing to the inhibition of calcineurin, a calcium and calmodulin-dependent phosphatase. Intracellularly, tacrolimus competitively binds FKBP12, inhibiting the phosphatase activity of calcineurin.[1] FK506 is one of the principal immunosuppressive drugs used for solid-organ transplantations, including the liver, kidney, and heart.[2–4] However, in clinical settings, FK506 treatment may demonstrate adverse effects such as nephrotoxicity, which could reduce the overall benefits in transplant donors. Previous studies have suggested that FK506 generates ROS, thus disturbing the antioxidant system in proximal tubules.[7,11,21]

To determine the effects of FK506-induced cytotoxicity of pig kidney proximal tubule LLC-PK1 cell viability, the 3-[4, 5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) method was used. As shown in Fig. 2., gallic acid, EGCG, and sanguiin H-6 exhibited a protective effect on LLC-PK1cells in a concentration-dependent manner, from 12.5 μM to 100 μM. The differences observed in the protective effect of each

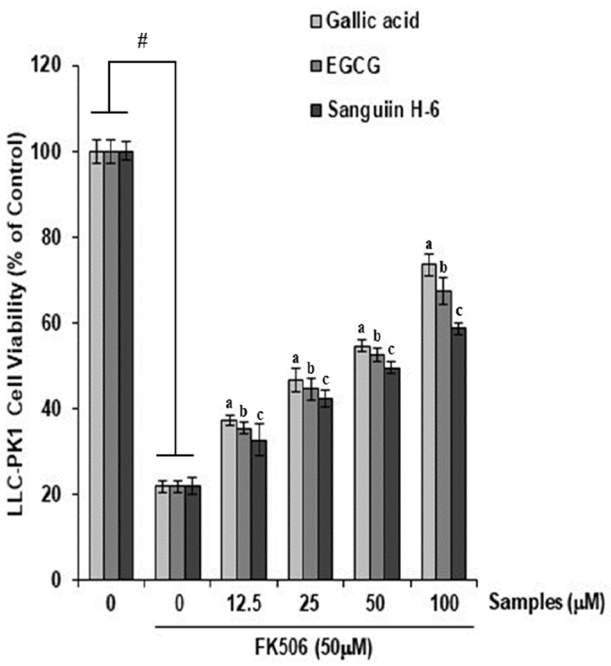


Fig. 2. Comparison in the protective effect of gallic acid, EGCG, and sanguiin H-6 against FK506-induced cytotoxicity in LLC-PK1 cells. The cells were pretreated with indicated concentrations of gallic acid, EGCG, and sanguiin H-6 for 2 h, and then FK506 was added to the culture medium to reach a final concentration of 50 μM, followed by incubation for 24 h. The cell viability was measured using the MTT assay. The results are expressed as the percentile of absorbance of treated samples compared to that of the control. All values are expressed as the mean SD. Statistic different is analyzed as one-way ANOVA followed by tukey post hoc tests. $^{a}p < 0.001$ vs FK506 50 μM (gallic acid), $^{b}p < 0.001$ vs FK506 50 μM (EGCG), $^{c}p < 0.001$ vs FK506 50 μM (Sanguiin H-6), $^{\#}p < 0.001$ vs control.

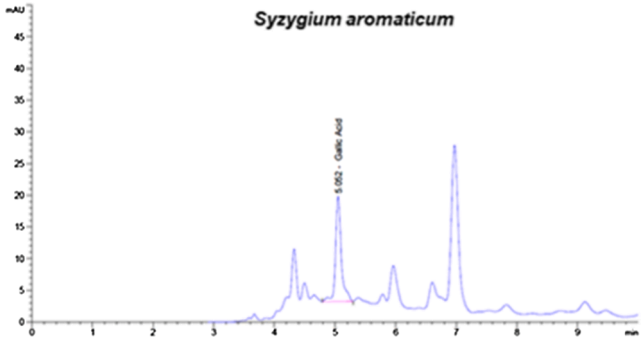


Fig. 3. HPLC chromatogram for the analysis of gallic acid in S. aromaticum extract.

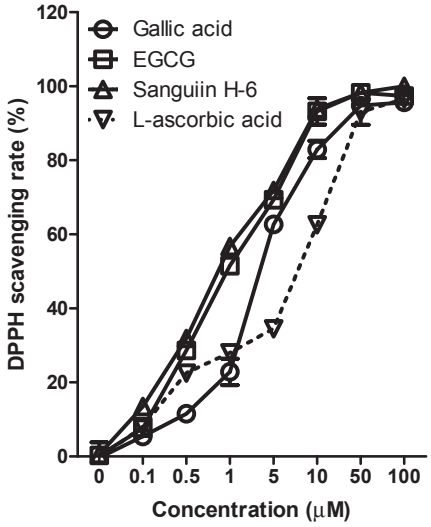


Fig. 4. Comparison of antioxidant effects of gallic acid, EGCG, and sanguiin H-6. The antioxidant capacity of compounds was evaluated using the DPPH scavenging assay. L-ascorbic acid used as a positive control to evaluate activity.

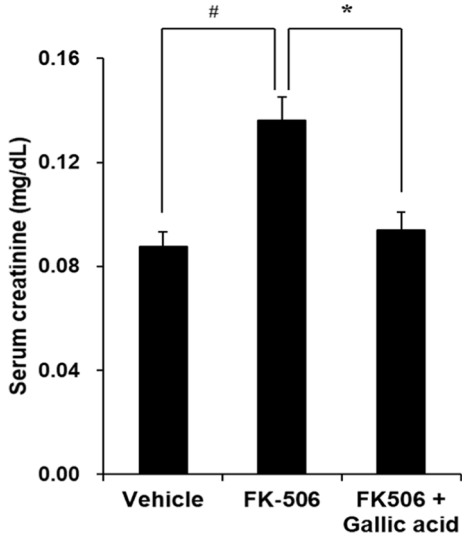


Fig. 5. Effect of gallic acid against FK506-induced kidney dysfunction in rats. After the experimental period, blood samples were obtained from the inferior vena cava. Serum creatinine levels were measured using a creatinine parameter assay kit. All values are expressed as the mean SD. Statistic different is analyzed as one-way ANOVA followed by tukey post hoc tests. *$p < 0.05$ vs FK506, $^{\#}p < 0.05$ vs. vehicle.

compound on cell viability can be related to the chemical structures. As shown in Figs. 1 and 2, the simpler chemical structure provided greater cell survival. The protective effect of these polyphenols was related to the size of the chemical structure rather than the number of gallic acid structures contained in these chemical structures.

Previously, studies have suggested that FK506 generates ROS through NADPH oxidase and disturbs the antioxidant system in the

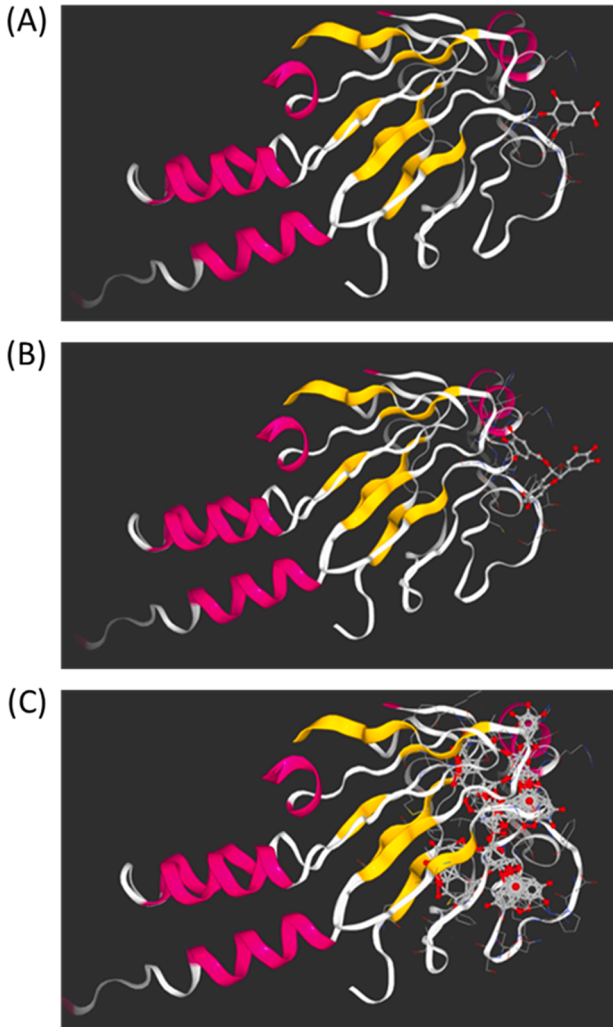


Fig. 6. Results of molecular docking between SMAD3 and three compounds. The binding results between the crystal structure of human SMAD3 MH2 domain and gallic acid (A), EGCG (B), and sanguiin H-6 (C) are presented. Docking was calculated and shown using Kdeep. SMAD3, Mother nuclei against decapentaplegic homolog 3; MH2, Mothers against decapentaplegic homology 2; EGCG, epigallocatechin-3-gallate.

proximal tubules.[7,11,21] These effects can induce apoptosis and inflammation in proximal tubular cells. We demonstrated that gallic acid, EGCG, and sanguiin H-6 protected LLC-PK1 cells in FK506 induced cytotoxicity. In several studies, gallic acid[22,23], EGCG[24,25], and sanguiin H-6[26,27] have reported potent antioxidant activity. In this study, we further confirmed the antioxidant efficacy of these compounds using the DPPH scavenging assay (Fig. 3). Compared with the positive control (L-ascorbic acid), gallic acid, EGCG, and sanguiin H-6 exerted superior antioxidant activity in a concentration dependent manner. Ascorbic acid is one of the well-known antioxidants, and previous studies suggest that has protective effect in contrast-induced nephropathy [28,29] which is generate oxidative stress. In our experiment, it was used as positive

Table 1
The binding affinity results of molecular docking; K_d, dissociation constant; pKd, −log (K_d); ΔG, Gibbs free energy; EGCG, epigallocatechin-3-gallate.

Molecule Name	Molecular Weight	pKd	ΔG (kcal/mol)	Ligand Efficiency (ΔG/[number of heavy atoms])
Gallic acid	170.02	4.35	−5.86	−0.49
EGCG	458.08	4.59	−6.19	−0.19
Sanguiin H-6	1870.16	11.14	−15.04	−0.11

control for the DPPH assay. Thus, our experiment indicates that the protective mechanism affording cell viability may be related to the antioxidative activity against FK506-induced ROS.

Phenolic compounds are abundant substances in aqueous and alcoholic extracts of Syzygii flos. Syzygii flos, commonly known as clove, is a dried flower bud in the pre-flowering stage of *Syzygium aromaticum* Merrill et Perry and belongs to the Myrtaceae family. *S. aromaticum* is not only used in flavor for foods but has also been used for stomach disorders and as analgesic in traditional remedies. *S. aromaticum* has a lot of effectiveness, such as antioxidant, antimicrobial, antinociceptive, and antiviral activities reported[30]. There is a correlation between polyphenol content and antioxidant activity, and gallic acid is one of the major compounds of phenolic molecules found in clove[31,32]. According to the literature, aqueous and ethanolic extract powder of dried *S. aromaticum* buds contains a lot of gallic acid equivalent[33–36]. To find out the renal protective effect of gallic acid in *S. aromaticum* extract, the gallic acid was extracted by 70% aqueous ethanol from dried Korea *S. aromaticum* buds and identified. And 8.02 mg/g gallic acid was found in that dried herb. (Fig. 4)

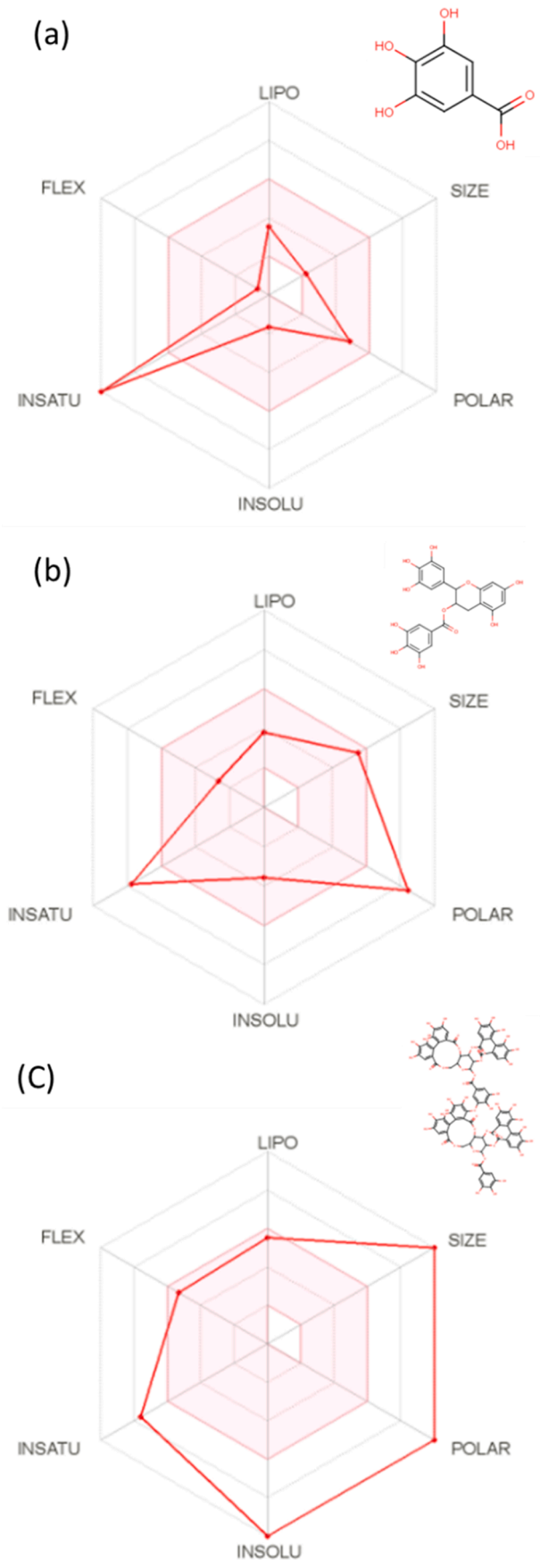
To examine the protective effects of gallic acid on FK506-induced kidney dysfunction in rats, serum creatinine levels were assessed using an assay kit to determine the kidney dysfunction status. Creatinine levels are closely related to damaged kidney function. In this study, we used 8-week-old male Sprague Dawley rats, randomly divided into three groups: group 1, control group receiving normal saline for 14 days; group 2, kidney toxic group receiving FK506 for 14 days; group 3, treatment group first receiving FK506 with the same schedule as group 2, followed by gallic for 14 days. As shown in Fig. 5., serum creatinine levels increased in FK506 alone treated group by approximately 1.8-fold when compared to the control group. Previous studies have reported that FK506-induced nephropathy is related to the production of oxidative stress, induced by the abnormal production of ROS.[7,11] Moreover, ROS production in the glomerulus[37] and mesangial cells[38] has been experimentally demonstrated. Gallic acid treatment reduced the increased serum creatinine levels to nearly those observed in the vehicle group (Fig. 5). Asci et al.[22] have suggested that, in rats, gallic acid demonstrates nephroprotective abilities against methotrexate-induced kidney damage. Additionally, in another study evaluating gallic acid against $HgCl_2$-induced liver damage, gallic acid reported hepatoprotective abilities, with increased antioxidant enzyme activity.[23] Thus, by increasing the activation of the antioxidant enzyme, gallic acid decreases the oxidative damage caused by FK506 induced ROS. Furthermore, in most cases using FK506, increased blood pressure reportedly caused adverse effects on renal function.[39] Current studies have reported that FK506 impacts blood pressure.[40,41] Reportedly, the angiotensin 2 receptor blocker telmisartan decreases FK506 induced nephrotoxicity and inflammation. Jin et al.[42] have reported that gallic acid controls hypertension in an animal model for essential hypertension. Furthermore, another paper has suggested that gallic acid regulates blood pressure and attenuates cardiac fibrosis in rat primary cardiac fibroblasts.[43] Thus, our findings indicated that gallic acid prevents kidney damage attributed to oxidative stress due to abnormal ROS production, and attenuates the blood pressure increase induced by FK506.

Additionally, we identified the mechanism underlying the nephroprotective function of gallic acid via *in-silico* analysis. FK-506 leads to increased TGF-ß1 expression, which is known to inhibit interleukin-2-dependent T cell proliferation.[44] TGF-ß1 plays an important role in suppression of the immune system; however, it also demonstrates side effects of fiberizing cells by activating the SMAD pathway.[1] Among the many proteins that constitute the SMAD pathway, SMAD3 is known to play an especially important role in renal fibrosis.[45] Furthermore, it has been established that all three test compounds bind to the SMAD3 protein to suppress the SMAD signaling pathway.[46–48] Therefore, to confirm the result of the in vitro experiment, we attempted molecular dockings between the three components and the SMAD3 MH2 domain, which are TGF-ß1 receptors.

Based on the molecular docking calculation, it was observed that gallic acid, EGCG, and sanguiin H-6 demonstrated binding potential with Mother nuclei against decapentaplegic homolog 3 (SMAD3) protein (Fig. 6). The bonds between SMAD3 and gallic acid, EGCG, and sanguiin H-6 were estimated using molecular docking. The crystal structure of the human SMAD3 MH2 domain was obtained from the Protein Data Bank (PDB ID: 1MJS)[49], and the compound structures were extracted from the PubChem database (Pubchem CID: 370 (Gallic acid), 65,064 (EGCG), 16,130,897 (Sanguiin-H6)).[50] Binding affinity was calculated using Kdeep[51], a 3D-convolutional neural network-based framework. On determining the dissociation constant and Gibbs energy, the binding efficiency of sanguiin H-6 was the highest. However, the binding power of gallic acid appeared to be the best based on ligand efficiency, which considers the number of non-hydrogen atoms (Table 1). This finding was consistent with the result demonstrated by protected LLC-PK1 cells with the same concentration of test compounds.

Using the swissADME platform[52], we further predicted the drug-likeness of the three test compounds. The platform evaluates drug-like properties such as lipophilicity, size, polarity, solubility, saturation, and flexibility. Drug-likeness properties of gallic acid, EGCG, and sanguiin H-6 were shown by radar chart (Fig. 7). Gallic acid demonstrated suitability as a drug in five of the six evaluated areas (lipophilicity, size, polarity, solubility, and flexibility), while EGCG and sanguiin H-6 showed suitable results in four (lipophilicity, size, solubility, and flexibility), and two (lipophilicity and flexibility) of the six evaluated areas, respectively. Based on these findings, it could be postulated that gallic acid has greater potential as a drug than the two other compounds. Collectively, our *in-silico* analyses suggested that gallic acid demonstrates better renal protection than EGCG and sanguiin H-6. However, in order to administer these natural products to human, many variables must be considered such as microbial metabolism in the gut or first-pass metabolism in the liver. For example, ginsenoside, the main pharmacological component of ginseng, has a low ADME property score[53,54], but compound K which is decomposed through gut microbial metabolism has a high drug similarity score. Pharmacokinetic researches will be needed to administer gallic acid to people with FK506-induced nephrotoxicity in the further studies.

In summary, gallic acid, EGCG, and sanguiin H-6 decreased oxidative stress and protected LLC-PK1 cells from FK506-induced cytotoxicity.



(caption on next column)

Fig. 7. The bioavailability radar graph of three compounds. The radar graphs were constructed using the swissADME website tool. The pink area represents the optimal range for each physicochemical properties (lipophilicity: the partition coefficient between *n*-octanol and water, size: molecular weight between 150 and 500 g/mol, polarity: topological polar surface area solubility: the molar solubility in water. saturation: fraction of carbons in the sp3 hybridizationand flexibility: no more than 9 rotatable bonds) and the suitable space for oral bioavailability is represented by a regular hexagon radar chart with pink shade. ADME properties of the compound are presented by the distorted red hexagon radar chart. (A) ADME properties of gallic acid. (B) ADME properties of EGCG. (C) ADME properties of sanguiin H-6. LIPO, lipophilicity; POLAR, polarity; INSOLU, insolubility; INSATU, insaturation; FLEX, flexibility. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The chemical structure of EGCG, and sanguiin H-6 contains a gallic acid structure, and gallic acid exhibited the strongest antioxidant effect, so an animal experiment using gallic acid was conducted. We confirmed the protective effect of gallic acid on FK506-induced renal dysfunction in rats. In-silico analyses also suggest that gallic acid possesses a better nephroprotective effect against FK506 nephropathy than EGCG and sanguiin H-6 through deactivation of the SMAD pathway. Therefore, gallic acid structure is important in that the compounds containing this structure, such as EGCG or sanguiin H-6, exert renal protective activity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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