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In silico evaluation of coconut milk phenolic antioxidants and their inhibition of oxidative stress in intestinal *Lactobacillus* spp. in vitro

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Abstract

Coconut milk is widely used in culinary applications in many Asian countries. The beneficial effect of coconut milk on gut health is limited to traditional knowledge and the beneficial effect of phenolic compounds present in coconut milk on gut microorganisms is not known. The aim of this study is to evaluate the effect of the phenolic compounds present in coconut milk on improving gut health and providing protection to gut microorganisms against inflammatory and oxidative stress conditions using *Lactobacillus* spp. The in silico analysis of the present study predicted the formation of 41 metabolites from 7 parent phenolic compounds present in coconut milk. According to in silico findings, phenolic compounds present in coconut milk and their metabolites display antioxidant and intestinal anti-inflammatory activities. The protective effect of the phenolic extracts of coconut milk against oxidative stress was evaluated in vitro by treating human intestinal *Lactobacillus* spp. with phenolic extracts of coconut milk followed by exposing them to oxidative stress and monitoring the levels of protein carbonyls and lipid peroxides under oxidative stress conditions. The results indicated that the phenolic extracts of coconut milk provide protection to *Lactobacillus* spp. against oxidation under H_2O_2-induced and aerobic oxidative stress conditions suggesting that coconut milk may mitigate the harmful influences of oxidative stress on gut microbiota hence enhancing gut health. Present findings also encourage the development of coconut milk as a vegan alternative to cow's milk.

Keywords *Cocos nucifera* · Coconut milk · Oxidative stress · Lipid peroxidation · Protein carbonylation · Gut microbial transformation

Introduction

There is a lot of interest recently in the effect of antioxidants on gut health. Gut microbiota plays a vital role in human health by maintaining the integrity of the gut mucous layer, involving in nutrient and drug metabolism, and protecting the host against pathogens. For example, *Lactobacillus* spp. that associate with the host symbiotically determine the oxidative stress level in the host intestine and assist in the digestion of complex nutrients (Reverón et al. 2012). Antioxidants present in diets may support gut health through direct involvement of inhibiting oxidative stress in the gut environment and through providing protection to microbiota against

challenging environmental conditions. Antioxidants present in green tea and botanical decoctions have been reported to protect intestinal epithelial cells from oxidation-induced apoptosis (Miller et al. 2001). The beneficial effects of phenolic antioxidants on gut microbiota have been attributed to the interaction of phenolic antioxidants in several metabolic pathways that supports the growth of useful bacteria (Rajoka et al. 2021). Even though the antioxidant and anti-inflammatory properties of phenolic compounds are known to be beneficial in promoting gut health, the impact of dietary phenolic compounds and their metabolites on the gut microbiota has been less explored (Valdés et al. 2015). The beneficial effects of coconut milk phenolic compounds on gut health have not been explored up to date.

Coconut milk is the aqueous emulsion of the solid endosperm of coconut (*Cocos nucifera*) that is used in culinary applications when preparing both vegetarian and non-vegetarian dishes in many Asian countries. Phenolic antioxidants of coconut milk are known to inhibit the oxidative damage to lipids and proteins of yeast cells under

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induced oxidative stress conditions (Karunasiri et al. 2020a). Phenolic extracts of coconut oil meal also inhibit protein carbonylation, lipid peroxidation, mitochondrial DNA damage and improve the GSH/GSSG ratio in oxidative stress-induced Hep-2 cells (Karunasiri et al. 2020b). The beneficial properties of coconut-based phenolic antioxidants suggest that phenolic-rich coconut milk could also render beneficial effects on gut health. Studies done with coconut endosperm indicate that fermentation reactions caused by yeast and other fungal species improve the nutritional status of the coconut endosperm (Wong et al. 2020, 2021). Reports also indicate that some host-beneficial bacteria and phenolic antioxidants have mutually beneficial roles in the human gastrointestinal tract (Piekarska-Radzik and Klewicka 2021). Among microbiota, *Lactobacillus* spp. present in the gut has the highest tolerance of phenolic compounds in the environment (Reverón et al. 2012).

In the present study, the effect of dietary phenolic compounds in coconut milk on gut health was evaluated by in silico and in vitro studies. Even though coconut milk is traditionally known to be beneficial for gut health, systematic evidence for such claims is extremely limited (Tolulope et al. 2019). Therefore, the present study was conducted to evaluate the effect of phenolic antioxidants of coconut milk on the gut microbes using *Lactobacillus* spp as a model.

Phenolic antioxidants undergo metabolism during absorption and after absorption (Feng et al. 2021). Human intestinal gut microbiota also plays a vital role in metabolizing dietary components that enter the intestinal tract (Ramakrishna 2013). In addition to the reported phenolic compounds present in coconut milk, the metabolic products of the phenolic compounds of coconut milk may also contribute to the health status of gut microorganisms. These metabolites may possess molecular characteristics, biological activities, pharmacokinetic activities, and toxicity properties that are different from their parent compounds (Rowland et al. 2018). Due to the lack of experimental data to examine the gut microbial metabolism of phenolic compounds present in coconut milk, the phenolic extract of coconut milk (PECM) was prepared by removing proteins and lipids of coconut milk as described by Karunasiri et al (2020a, b). A computational study was designed to anticipate the phenolic metabolites of PECM. In silico studies were conducted to predict the antioxidant activity, anti-inflammatory activity, behavior in the human body, and antibacterial activities of the phenolic compounds and their metabolites. The safety of the phenolic compounds of coconut milk and their metabolites were also evaluated based on the endocrine-disrupting effect and the probability of interaction with multiple human receptors. Protein carbonyls and lipid peroxides were quantified in vitro as oxidative stress markers in *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum, Lactobacillus delbrueckii* ssp. *lactis, Lacticaseibacillus casei* and *Limosilactobacillus*

fermentum to evaluate the protective effect of PECM against oxidative damage.

Results and discussion

In silico metabolite prediction

The beneficial effects of coconut endosperm materials on the gut microbiome are limited (Wong et al. 2020). The phenolic compounds in coconut milk are derived from the white coconut kernel and brown coconut testa. The phenolic compounds reported to be present in coconut milk (gallic acid, chlorogenic acid, *p*-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid and ferulic acid) are thermally stable under cooking conditions (Karunasiri et al. 2020a; Seneviratne et al. 2009; Senanayake et al. 2019). Human gut microbial biotransformation predicted the formation of 41 metabolites from 7 parent phenolic acids (Table S1). Based on the computational study, gut microbiota metabolized parent gallic acid, chlorogenic acid, para-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid and ferulic acid into 6 (G_{M1}–G_{M6}), 12 (C_{M1}–C_{M12}), 3 (P_{M1}–P_{M3}), 8 (CA_{M1}–CA_{M8}), 3 (V_{M1}–V_{M3}), 3 (S_{M1}–S_{M3}) and 6 (F_{M1}–F_{M6}) metabolites, respectively. Interestingly, some of the predicted metabolites namely G_{M1} (pyrogallol), CA_{M1} (dihydrocaffeic acid), CA_{M2} (4-vinylcatechol) and F_{M2} (4-vinylguaiacol) have been experimentally confirmed using in vivo or in vitro studies (Landete et al. 2021; García-Villalba et al. 2020).

The structures of parent phenolic acids and the respective metabolites predicted by the reactions of human gut microbial biotransformation are given in Fig. S1. Most of the predicted metabolites (29/41) were formed through the reactions of aromatic OH-glucuronidation (10/41), 4′-dehydroxylation of substituted benzene (5/41), O-glucuronidation of aromatic acid (4/41), decarboxylation of phenolic acid (4/41), O-glucuronidation of aliphatic acid (3/41) and alkyl-OH-glucuronidation (3/41). Out of all reactions of human gut microbial biotransformation, all parent phenolic acids were predicted to undergo aromatic OH-glucuronidation to form their respective metabolites. The metabolic transformations of a compound can significantly alter its molecular characteristics, biological activities, pharmacokinetic properties, and toxicity. Therefore, parent phenolic acids and their metabolites were subjected to further analysis of in silico biological activity, endocrine disruption potential and absorption, distribution, metabolism, and excretion/toxicity (ADME/T) predictions.

In silico biological activity prediction

The antioxidant, intestinal anti-inflammatory and antibacterial activities of parent compounds and their metabolites

were investigated using prediction of activity spectra for substances (PASS) webserver which is a useful tool for rapid identification of active compounds for the biological activity of interest. Table 1 shows that all parent phenolic acids and the metabolites have higher Pa (Probability of a compound being active) values than Pi (Probability of a compound being inactive) values, indicating that their antioxidant, intestinal anti-inflammatory, and antibacterial activities are possible.

All compounds demonstrated adequate antioxidant, intestinal anti-inflammatory, and antibacterial potential predictions, with Pa ranging from 0.199 to 0.878, 0.197 to 0.775 and 0.186 to 0.701, respectively. Many of the predicted metabolites were shown to be more antioxidant, intestinal anti-inflammatory and antibacterial than their parent forms. Among the seven phenolic acids present in coconut milk, chlorogenic acid (Pa − 0.83) exhibited the highest antioxidant activity, while p-hydroxybenzoic acid (Pa − 0.743) and vanillic acid (Pa − 0.72) showed the highest intestinal anti-inflammatory potential. Both metabolites, CA_{M3} and F_{M3} are formic acid. Because of the low number of carbon atoms in the primary chemical structure, the activity spectra were not predicted for formic acid in PASS server. Moreover, the PASS server did not identify the intestinal anti-inflammatory potential of C_{M4}, C_{M6} and C_{M7}. Out of 41 predicted metabolites, 44% had Pa > 0.7, suggesting a high probability of being active as antioxidant molecules, whereas 29% had 0.5 < Pa < 0.7, indicating a moderate potential to function as antioxidants. Only 22% of the metabolites showed a relatively low tendency for antioxidant activity with Pa < 0.5. According to the in silico results, the majority of compounds (34/48) demonstrated moderate to high antioxidant activity, indicating their potential to alleviate the harmful effects of oxidative stress on gut microbiota via direct antioxidant effects.

Among the tested metabolites, 12% indicated significant intestinal anti-inflammatory potential with Pa > 0.7, while 17% demonstrated moderate intestinal anti-inflammatory potential with Pa 0.5–0.7. However, a considerable proportion of the metabolites (59%) were found to have low intestinal anti-inflammatory activity whereas another 12% was not detected for intestinal anti-inflammatory potential. Except for the metabolites C_{M3}, C_{M5}, C_{M8}, C_{M11}, and C_{M12}, all compounds in the low anti-inflammatory intestinal potential range (Pa < 0.5) had higher Pa values than their corresponding Pi values and the Pi values were close to zero, suggesting that these compounds are likely to be active. Gut microbes exposed to phenolic compounds are known to up-regulate protein-controlled defense mechanisms of cells thereby protecting cells (Cardona et al. 2013). Phenolic compounds and their metabolites are also responsible for the reduction of C-reactive protein, which is an inflammatory marker. According to a human intervention study,

phenolic compounds promote the growth of *Lactobacillus* spp in the gut (Tzounis et al. 2011). Present-day diets are rich in fats and sugars and such imbalanced diets cause intestinal inflammation (Lobionda et al. 2019). The findings of the present study imply that the parent phenolic acids of coconut milk, as well as their anticipated metabolites, have the potential to act synergistically as intestinal anti-inflammatory agents to minimize the impact of intestinal inflammation on gut microbiota caused by diets rich in fat and sugar. These findings are consistent with the reported effect of coconut milk on ameliorating heat-stress-induced gastrointestinal tract dysmotility by reducing oxidative stress and inflammatory responses (Ajeigbe et al. 2022). However, more experimental research is required to prove the intestinal anti-inflammatory effect of these parent phenolic acids and their metabolites.

Only the metabolite C_{M4} exhibited a strong potential to act as an antibacterial agent, with Pa value of 0.701. Overall, 56% of metabolites displayed moderate antibacterial activity, whereas 37% had Pa < 0.5, suggesting a relatively weak potential to operate as antibacterial agents. Notably, excluding CA_{M1} and F_{M1} metabolites, all compounds in the low antibacterial potential range (Pa < 0.5) reported greater Pa values than their corresponding Pi values and Pi values were nearly zero, indicating that these compounds are likely to be active. Previous in vitro and in vivo studies on ferulic acid, gallic acid, chlorogenic acid, caffeic acid, vanillic acid, and plant extracts enriched in them have demonstrated their ability to inhibit the growth of pathogenic bacteria while promoting the growth of commensal microbes such as *Lactobacillus* spp. and *Bacteroides* spp. (Piekarska-Radzik and Klewicka 2021). More studies are needed to understand how these parent phenolic acids and their metabolites selectively support the growth of commensal microbes while inhibiting harmful bacteria in the human gut.

In silico endocrine disruption potential prediction

To ensure the safety of human ingestion, all 48 compounds were analyzed for endocrine-disrupting effects. The predictions of endocrine disruption potential between 48 compounds and human nuclear receptors are illustrated in Fig. 1.

The androgen receptor antagonist (AR an.) was the most susceptible conformation followed by the glucocorticoid receptor (GR) among 18 human nuclear receptor conformations studied. Out of 48 compounds, 10%, 77%, and 13% of compounds exhibited high probability, medium probability, and low probability of binding toward AR an. conformation, respectively. For GR, 6%, 46% and 48% compounds showed high, medium, and low binding probability. Interestingly, all compounds showed a low probability of binding for estrogen receptor βantagonist (ER β an.), liver X receptor α (LXR α), liver X receptor β (LXR β), peroxisome

Table 1 In silico biological activities of 7 phenolic acids and their metabolites

No.	Description	Antioxidant		Intestinal anti-inflammatory		Antibacterial	
		Pa	Pi	Pa	Pi	Pa	Pi
1	Gallic acid (GA)	0.52	0.006	0.64	0.003	0.418	0.026
2	G_{M1}	0.655	0.004	0.537	0.005	0.332	0.048
3	G_{M2}	0.401	0.012	0.707	0.003	0.394	0.032
4	G_{M3}	0.423	0.01	0.64	0.003	0.403	0.029
5	G_{M4}	0.792	0.003	0.297	0.073	0.627	0.007
6	G_{M5}	0.594	0.009	0.317	0.059	0.594	0.009
7	G_{M6}	0.821	0.003	0.28	0.087	0.615	0.008
8	Chlorogenic acid (CA)	0.785	0.004	0.387	0.026	0.537	0.013
9	C_{M1}	0.83	0.003	0.386	0.027	0.478	0.019
10	C_{M2}	0.603	0.005	0.648	0.003	0.358	0.041
11	C_{M3}	0.837	0.003	0.197	0.193	0.684	0.005
12	C_{M4}	0.87	0.003	N.D.	N.D.	0.701	0.004
13	C_{M5}	0.724	0.004	0.214	0.166	0.673	0.005
14	C_{M6}	0.878	0.003	N.D.	N.D.	0.676	0.005
15	C_{M7}	0.878	0.003	N.D.	N.D.	0.676	0.005
16	C_{M8}	0.856	0.003	0.197	0.193	0.642	0.007
17	C_{M9}	0.727	0.004	0.395	0.024	0.523	0.014
18	C_{M10}	0.727	0.004	0.395	0.024	0.523	0.014
19	C_{M11}	0.819	0.003	0.233	0.14	0.589	0.009
20	C_{M12}	0.711	0.004	0.233	0.14	0.599	0.009
21	Para-hydroxybenzoic acid (PA)	0.32	0.02	0.743	0.002	0.384	0.034
22	P_{M1}	0.199	0.055	0.717	0.002	0.362	0.04
23	P_{M2}	0.648	0.004	0.364	0.035	0.58	0.01
24	P_{M3}	0.704	0.004	0.336	0.047	0.586	0.01
25	Caffeic acid (CAA)	0.603	0.005	0.648	0.003	0.358	0.041
26	CA_{M1}	0.339	0.018	0.515	0.006	0.206	0.111
27	CA_{M2}	0.522	0.006	0.488	0.008	0.375	0.037
28	CA_{M3}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
29	CA_{M4}	0.544	0.005	0.653	0.003	0.33	0.049
30	CA_{M5}	0.553	0.005	0.684	0.003	0.343	0.045
31	CA_{M6}	0.78	0.004	0.332	0.049	0.608	0.008
32	CA_{M7}	0.78	0.004	0.332	0.049	0.608	0.008
33	CA_{M8}	0.75	0.004	0.349	0.041	0.611	0.008
34	Vanillic acid (VA)	0.374	0.014	0.72	0.002	0.376	0.036
35	V_{M1}	0.238	0.04	0.775	0.002	0.324	0.052
36	V_{M2}	0.604	0.005	0.35	0.04	0.579	0.01
37	V_{M3}	0.678	0.004	0.323	0.055	0.58	0.01
38	Syringic acid (SA)	0.403	0.012	0.688	0.003	0.395	0.031
39	S_{M1}	0.295	0.024	0.704	0.003	0.364	0.04
40	S_{M2}	0.606	0.004	0.332	0.049	0.567	0.011
41	S_{M3}	0.717	0.004	0.306	0.066	0.594	0.009
42	Ferulic acid (FA)	0.54	0.005	0.661	0.003	0.333	0.048
43	F_{M1}	0.33	0.019	0.524	0.005	0.186	0.131
44	F_{M2}	0.459	0.008	0.497	0.007	0.352	0.043
45	F_{M3}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
46	F_{M4}	0.426	0.01	0.717	0.002	0.274	0.07
47	F_{M5}	0.68	0.004	0.348	0.041	0.58	0.01
48	F_{M6}	0.69	0.004	0.356	0.038	0.597	0.009

Pa - Probability of a compound being active

Pi - Probability of a compound being inactive

Italics - Experimentally confirmed using in vivo or in vitro studies
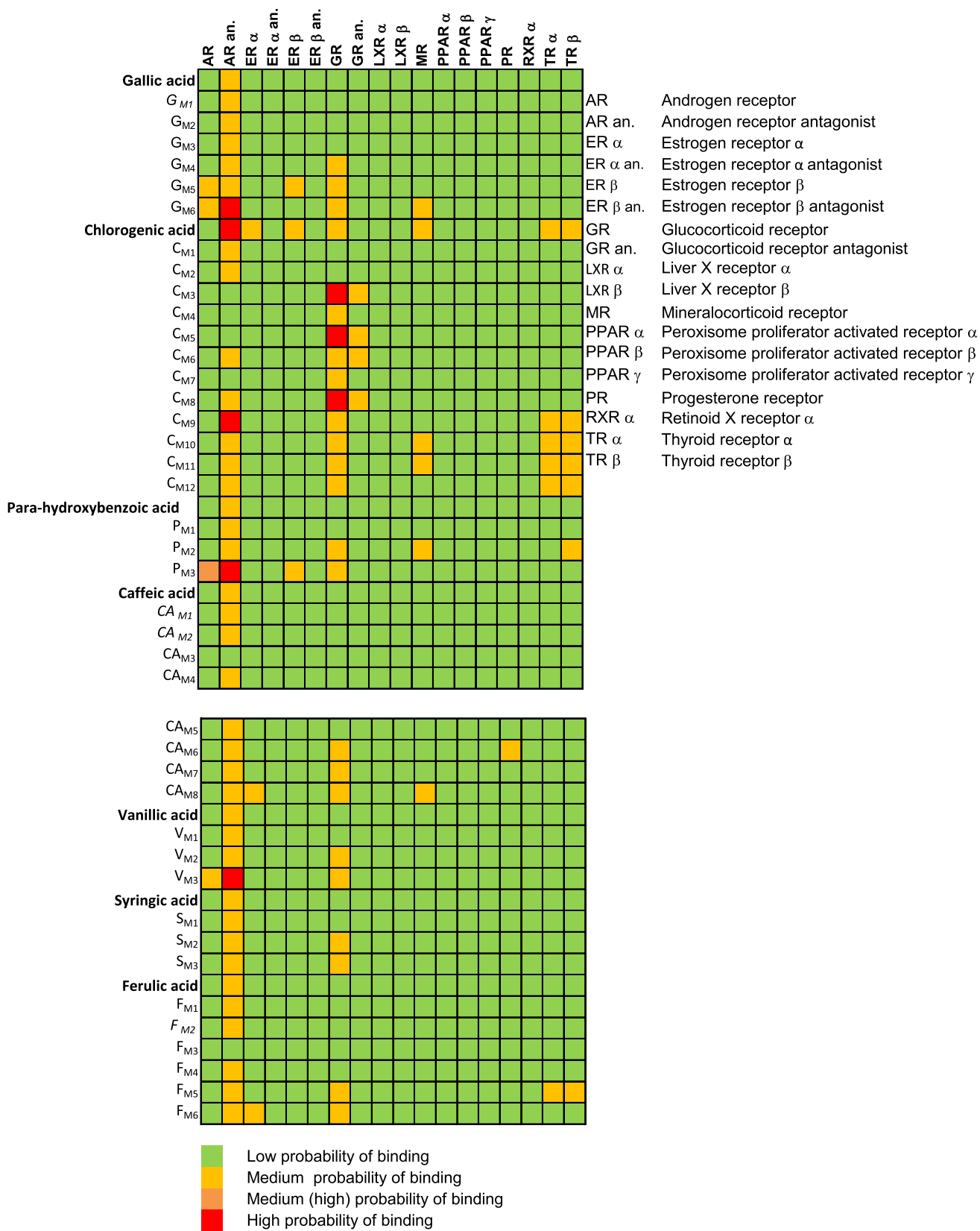
N.D., Not detected

Fig. 1 Endocrine disruption potential of 7 phenolic acids and their derivatives as obtained from Endocrine Disruptome webserver

proliferator-activated receptor α (PPAR α), peroxisome proliferator-activated receptor β (PPAR β), peroxisome proliferator-activated receptor γ (PPAR γ), and retinoid X receptor α (RXR α). The interactions (high and medium probability) between 87% compounds and AR an. conformation suggest the potential anti-androgenic activity of the parent phenolic acids and their metabolites used in the study. Moreover, 52% of compounds binding to GR (high and medium probability) indicate exerting glucocorticoid-like activity.

A chemical showing a high probability of interactions with multiple human nuclear receptors can lead to serious health consequences due to endocrine disruption (Kolsek et al. 2014). Chlorogenic acid was the only compound in the current investigation that interacted with the highest number of human nuclear receptors (7/18) via one high probability and six medium probabilities of binding. However, chlorogenic acid is poorly absorbed in the human gut unless the absorption is supported by other food ingredients (Weerakoon et al. 2021). The metabolites CA_{M3} and F_{M3} ($CA_{M3} = F_{M3}$ = formic acid), demonstrated a low probability of binding with all 18 confirmations of human nuclear receptors. The remaining compounds (47/48) showed a medium to high probability of binding to less than six human nuclear receptors. It was observed that most of the compounds demonstrated a low probability of binding with human nuclear receptors and bind to fewer human nuclear receptors. Hence, the parent compounds and the metabolites derived from them are unlikely to be hazardous to human health. In fact, a study conducted on the antioxidant potential of ethanolic extract of *Psidium guineense* Sw. leaves, which includes all seven parent compounds present in coconut milk, revealed that these phenolic extracts significantly reduced the TBARS and protein carbonyl formation in serum macromolecules of Wistar rats while not imparting any toxic effect (Senanayake et al. 2018).

In silico ADME/T prediction

The ADME/T study was carried out to better understand the behavior of each chemical compound inside the human body and whether they may have any adverse effects on humans. Prediction of the absorption was determined based on three descriptors such as human intestinal absorption, water solubility, and whether the compounds may serve as substrates for P-glycoprotein. The SwissADME online web server was used to generate the brain or intestinal estimated permeation predictive model (BOILED-Egg), which provides a graphical representation of human intestinal absorption and brain penetration of the 48 compounds as a function of lipophilic nature (WLOGP) and polarity of the molecules (TPSA). The BOILED-Egg graph for parent phenolic acids and their predicted metabolites is presented in Fig. 2.

In the BOILED-Egg graph, compounds occupying the white region (egg white) exhibit a high probability of

Fig. 2 BOILED-Egg graph for parent phenolic acids and their predicted metabolites
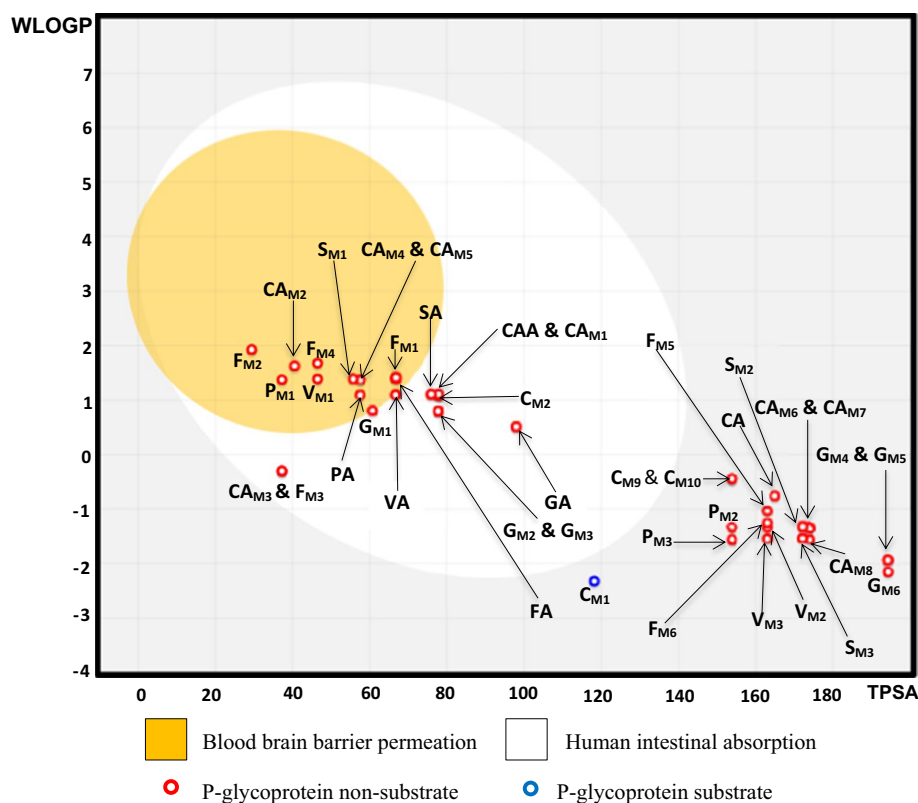
human intestinal absorption while the compounds occupying the yellow region (egg yolk) show a high probability of blood–brain–barrier permeation. Moreover, the red color indicator of the compound denotes the non-substrate of P-glycoprotein whereas the blue color indicator represents the substrates of P-glycoprotein. The compounds that occupy the gray color region have a low probability of human intestinal absorption as well as blood–brain–barrier permeation.

High intestinal absorption was observed for 46% of the compounds whereas 54% showed low intestinal absorption. Except for the metabolite C_{M1} derived from chlorogenic acid, none of the other 47 compounds are substrates for P-glycoprotein. P-glycoprotein is an ATP-binding cassette transporter that can pump xenobiotics out of cells to protect organisms against toxic substances. Hence, substrates for P-glycoprotein are easily pumped out of the cells. The water solubility scores of most compounds (73%) vary between − 4 to − 2 range. Therefore, they are soluble in water at 25 °C, while 4% are in the highly soluble range (0 <) followed by 23% compounds in the very soluble range (− 2–0).

The distribution of compounds was predicted using the steady-state volume of distribution (VDss) and blood–brain–barrier permeability (BBBp). In general, compounds with a higher VDss value are more likely to be distributed into body tissue rather than plasma (Pires et al. 2015). Overall, 79% of compounds showed low VDss values than the lower limit (− 0.15). Thus, they are more likely to be distributed in plasma rather than in the body tissue. Only 4% of compounds exhibited high VDss values than its upper limit (0.45). Hence, they are more likely to be distributed in body tissue rather than in the plasma. Another 17% of compounds showed VDss values ranging from − 0.141 to 0.374. Three-quarters of the compounds (75%) were very weakly permeable to the brain, whereas 25% passed across the blood–brain–barrier.

Metabolism was anticipated using cytochrome P450 models (CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4) for serving as substrates for cytochrome P450 or inhibitors of cytochrome P450. The F_{M2} metabolite was shown to be an inhibitor of CYP1A2. In terms of metabolism, the remaining 47 compounds were neither a substrate nor an inhibitor of the CYP isoforms studied.

The higher the total clearance score of a compound, the faster it is eliminated from the human body, and vice versa. The total clearance score of 19% of the compounds was less than 0.301 indicating that they are very slowly eliminated from the human body. In comparison to the higher cutoff level (1.176), 81% of the substances were found to have modest total clearance ranging from 0.307 to 0.817, demonstrating moderate removal from the human body.

Table 2 summarizes the in silico ADME/T predictions of the 48 compounds. Based on the ProTox-II findings, 65% of compounds were classified as the least toxic classes 5

(2000 < LD50 ≤ 5000) and 6 (LD50 > 5000). Therefore, they can be considered safer for oral consumption. Furthermore, 27% of substances were anticipated to have moderate toxicity (300 < LD50 ≤ 2000), while 8% were toxic if swallowed (50 < LD50 ≤ 300).

According to the in silico predictions, in terms of the various toxicity endpoints tested, only 17% (8/48: GA = gallic acid, G_{M1} = pyrogallol, G_{M2} = protocatechuic acid, C_{M2} = CAA = caffeic acid, CA_{M2} = 4-vinylcatechol, CA_{M4} = dihydro-3-coumaric acid, and CA_{M5} = p-coumaric acid) of the compounds were shown to be carcinogenic. Among them, GA, G_{M1}, CA_{M4} and CA_{M5} were weakly carcinogenic. However, several in vitro and in vivo investigations have reported anticancer activity for GA, G_{M1}, G_{M2}, C_{M2} = CAA, and CA_{M5} (Zhang et al. 2019; Revathi et al 2019; Tanaka et al. 2011; Alam et al. 2022; Kong et al. 2013). To the best of our knowledge, the remaining two carcinogenic substances (CA_{M4} and CA_{M2}) have not been experimentally investigated for their carcinogenicity. Therefore, further evidence is needed to arrive at any firm conclusion regarding their carcinogenicity.

Since the compounds investigated have almost no effect on CYP450 isoforms, the risks of liver damage are minimal. Only three metabolites (V_{M1}, F_{M4}, and P_{M1}) were weakly hepatotoxic. Interestingly, based on the *in silico* toxicity data, all 48 compounds were free from cytotoxicity in humans. Furthermore, only the metabolite G_{M1} was found weakly positive for mutagenicity. Notably, 77% of compounds obtained negative results for all toxicity descriptors, implying no severe human side effects.

Inhibition of protein carbonyl formation and lipid peroxidation in *Lactobacillus* spp. by coconut milk antioxidants

Proteins and lipids are major nutrient components of food and major structural components in the body. Protein carbonylation occurred under oxidative stress conditions causes irreversible protein modifications which are related to cellular damage and several age-related disorders. The level of oxidative stress can be quantified by the quantification of aldehydes and ketones resultant from the protein carbonylation based on the formation of hydrozones from DNPH (Suzuki et al. 2010). Compared with the control sample with no added antioxidants, the protein carbonyl concentrations decreased gradually with the increasing concentration of coconut milk antioxidants (Fig. 3).

The carbonyl production in oxidative stress-induced *Lactobacillus* spp. cultured under anaerobic conditions showed a significant, negative correlation with the increasing concentration of PECM (*L. acidophilus*: $r = − 0.84$, $P = 0.02$; *L. plantarum*: $r = − 0.82$, $P = 0.02$; *L. delbrueckii* ssp. *lactis*: $r = − 0.81$, $P = 0.03$; *L. casei*: $r = − 0.80$, $P = 0.03$; *L.*

Table 2 In silico pharmacokinetics and toxicity properties of 7 phenolic acids and their metabolites

No.	Description	Absorption	Distribution	Excretion	Toxicity					
		WS[a]	VDss[b]	TC[c]	LD50[d]	CLASS[e]	HEP[f]	CAR[g]	MUT[h]	CYT[i]
1	Gallic acid	− 2.56	− 1.855	0.518	2000	4	No	**Yes**	No	No
2	*GM1*	− 1.452	− 0.078	0.152	300	3	No	**Yes**	**Yes**	No
3	GM2	− 2.071	− 1.527	0.555	2000	4	No	*Yes*	No	No
4	GM3	− 2.248	− 1.947	0.603	2000	4	No	No	No	No
5	GM4	− 2.89	− 1.422	0.568	2190	5	No	No	No	No
6	GM5	− 2.89	− 1.446	0.569	3750	5	No	No	No	No
7	GM6	− 2.879	− 1.105	0.568	2260	5	No	No	No	No
8	Chlorogenic acid	− 2.449	0.581	0.307	5000	5	No	No	No	No
9	CM1	− 1.119	− 0.217	0.639	9800	6	No	No	No	No
10	CM2	− 1.565	− 0.559	0.582	2980	5	No	*Yes*	No	No
11	CM3	− 2.812	0.374	0.531	5000	5	No	No	No	No
12	CM4	− 2.714	− 0.004	0.544	5000	5	No	No	No	No
13	CM5	− 2.842	0.532	0.783	5000	5	No	No	No	No
14	CM6	− 2.903	− 1.203	0.564	5000	5	No	No	No	No
15	CM7	− 2.906	− 1.2	0.556	4000	5	No	No	No	No
16	CM8	− 2.897	− 0.922	0.817	5000	5	No	No	No	No
17	CM9	− 2.028	− 0.135	0.398	5000	5	No	No	No	No
18	CM10	− 1.999	− 0.255	0.394	9600	6	No	No	No	No
19	CM11	− 2.843	0.189	0.363	5000	5	No	No	No	No
20	CM12	− 2.756	− 0.141	0.375	5000	5	No	No	No	No
21	Para- hydroxybenzoic acid	− 1.877	− 1.557	0.593	2200	5	No	No	No	No
22	PM1	− 1.84	− 1.691	0.707	290	3	**Yes**	No	No	No
23	PM2	− 2.838	− 1.823	0.641	3750	5	No	No	No	No
24	PM3	− 2.763	− 1.326	0.66	2260	5	No	No	No	No
25	Caffeic acid	− 2.33	− 1.098	0.508	2980	5	No	*Yes*	No	No
26	*CAM1*	− 1.764	− 0.519	0.295	2000	4	No	No	No	No
27	*CAM2*	− 1.526	0.068	0.221	1560	4	No	*Yes*	No	No
28	CAM3	1.005	− 0.701	0.494	162	3	No	No	No	No
29	CAM4	− 2.408	− 1.255	0.663	2980	5	No	**Yes**	No	No
30	CAM5	− 2.438	− 1.2	0.662	2850	5	No	**Yes**	No	No
31	CAM6	− 2.744	− 1.721	0.202	5000	5	No	No	No	No
32	CAM7	− 2.868	− 1.635	0.182	4000	5	No	No	No	No
33	CAM8	− 2.782	− 1.653	0.13	5000	5	No	No	No	No
34	Vanillic acid	− 1.838	− 1.739	0.628	2000	4	No	No	No	No
35	VM1	− 1.934	− 1.385	0.678	1800	4	**Yes**	No	No	No
36	VM2	− 2.825	− 1.677	0.664	5000	5	No	No	No	No
37	VM3	− 2.807	− 1.299	0.673	2260	5	No	No	No	No
38	Syringic acid	− 2.223	− 1.443	0.646	1700	4	No	No	No	No
39	SM1	− 1.967	− 1.55	0.728	2000	4	No	No	No	No
40	SM2	− 2.863	− 1.654	0.706	5000	5	No	No	No	No
41	SM3	− 2.826	− 1.509	0.694	2260	5	No	No	No	No
42	Ferulic acid	− 2.817	− 1.367	0.623	1772	4	No	No	No	No
43	FM1	− 2.4	− 0.585	0.372	2000	4	No	No	No	No

Table 2 (continued)

No.	Description	Absorption	Distribution	Excretion	Toxicity					
		WS[a]	VDss[b]	TC[c]	LD50[d]	CLASS[e]	HEP[f]	CAR[g]	MUT[h]	CYT[i]
44	F_{M2}#	− 2.36	0.172	0.267	1560	4	No	No	No	No
45	FM3	1.005	− 0.701	0.494	162	3	No	No	No	No
46	FM4	− 2.256	− 1.16	0.755	1772	4	**Yes**	No	No	No
47	FM5	− 2.792	− 1.738	0.28	4000	5	No	No	No	No
48	FM6	− 2.74	− 1.821	0.225	5000	5	No	No	No	No

Italics—Experimentally confirmed using in vivo or in vitro studies

Bold – weakly active (probability < 0.7), Bold italics – active (probability > 0.7)

Metabolism: FM2# is an inhibitor for CYP1A2 isoform. Remaining phenolic acids and their metabolites are non-substrates and non-inhibitors for CYP (2D6, 3A4) and CYP (1A2, 2C19, 2C9, 2D6, 3A4) isoforms, respectively

[a]Water solubility (WS) [log (mol/L)], [b]Steady-state volume of distribution (VDss) [log (L/kg)], [c]Total clearance (TC) [log (ml/min/kg)], [d]Predicted median lethal dose (LD50) (mg/ kg), [e]Predicted toxicity class (CLASS), [f]Hepatoxicity (HEP), [g]Carcinogenicity(CAR), [h]Mutagenicity(MUT) and [i]Cytotoxicity(CYT)

Accepted range of values as defined by pkCSM webserver: Water solubility [log (mol/L)]—insoluble < − 10 < poorly soluble < − 6 < moderately soluble < − 4 < soluble < − 2 < very soluble < 0 < highly soluble/VDss [log (L/kg)]—high > 0.45, low < − 0.15/Total Clearance [log (ml/min/kg)]—high > 1.176, low < 0.301

Six toxicity classifications are given in ProTox− II, based on the worldwide harmonized method of chemical labeling categorization. Class I: death if swallowed (LD50 ≤ 5); Class 2: fatal if swallowed (5 < LD50 ≤ 50); Class 3: toxic if swallowed (50 < LD50 ≤ 300); Class 4: harmful if swallowed (300 < LD50 ≤ 2000); Class 5: may be harmful if swallowed (2000 < LD50 ≤ 5000); Class 6: non-toxic (LD50 > 5000)
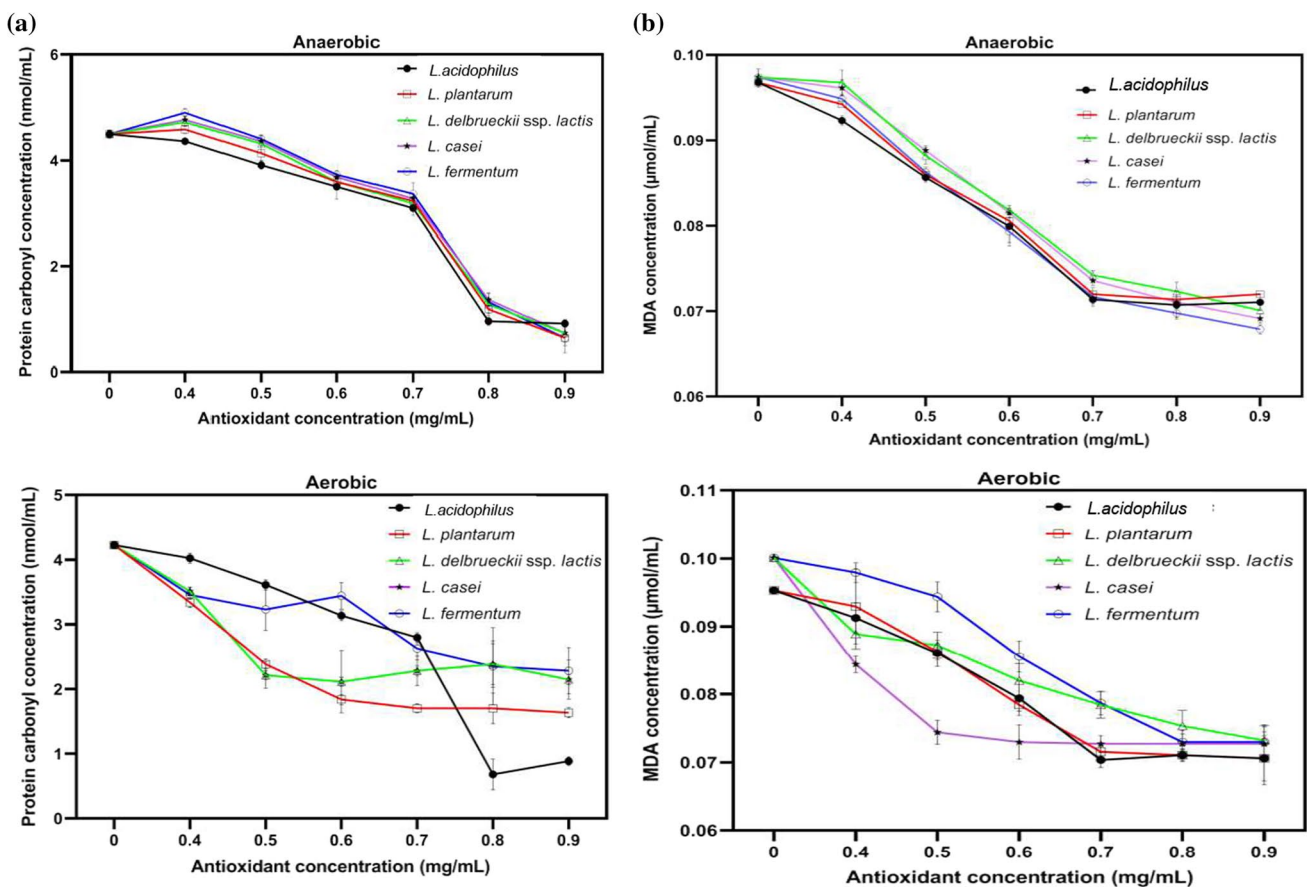

Fig. 3 Concentration dependent antioxidant activity of PECM against protein carbonylation (**a**) and lipid peroxidation (**b**) in oxidative stress-induced *Lactobacillus* spp. under aerobic (bottom) and anaerobic (top) conditions. Each data point represents mean ± standard deviation (n = 3)

fermentum: $r = -0.79$, $P = 0.03$). Furthermore, *Lactobacillus* spp. cultured under aerobic conditions also showed a significant, strong negative correlation between the carbonyl production and the increasing concentration of PECM (*L. acidophilus*: $r = -0.85$, $P = 0.02$; *L. plantarum*: $r = -0.95$, $P = 0.00$; *L. delbrueckii* ssp. *lactis*: $r = -0.87$, $P = 0.01$; *L. casei*: $r = -0.87$, $P = 0.01$; *L. fermentum*: $r = -0.95$, $P = < 0.001$).

Interestingly, the thiobarbituric acid reactive species (TBARS) concentrations of *Lactobacillus* spp. demonstrated a significant and very strong negative correlation with the increasing levels of PECM in both aerobic (*L. acidophilus*: $r = -0.93$, $P = 0.00$; *L. plantarum*: $r = -0.92$, $P = 0.00$; *L. delbrueckii* ssp. *lactis*: $r = -0.99$, $P = < 0.001$; *L. casei*: $r = -0.92$, $P = 0.00$; *L. fermentum*: $r = -0.91$, $P = 0.00$) and oxidative stress-induced anaerobic (*L. acidophilus*: $r = -0.94$, $P = 0.00$; *L. plantarum*: $r = -0.92$, $P = 0.00$; *L. delbrueckii* ssp. *lactis*: $r = -0.92$, $P = 0.00$; *L. casei*: $r = -0.92$, $P = 0.00$; *L. fermentum*: $r = -0.93$, $P = 0.00$) conditions suggesting strong inhibition of lipid peroxidation.

The general trend observed in the present experiment is that both protein carbonyl and lipid peroxide levels are significantly higher ($P \leq 0.05$) in *Lactobacillus* spp. grown under aerobic conditions compared to those grown under anaerobic conditions prior to induction of oxidative stress with hydrogen peroxide. *Lactobacillus* species are considered to be anaerobic. However, they are oxygen tolerant, and some strains are capable of using oxygen as a substrate (Pedersen et al. 2012). Hydrogen peroxide levels that are 2–3 times higher have been observed in some aerated *Lactobacillus* cultures compared to non-aerated cultures (Marty-Teysset et al. 2000). Therefore, higher oxidative stress levels may promote higher protein carbonyls and lipid peroxides under aerobic conditions. Overall results of protein carbonylation and lipid peroxidation under both H_2O_2-induced oxidative stress conditions and aerobic conditions show that coconut milk antioxidants provide protection against protein carbonyl formation and lipid peroxidation suggesting that coconut milk phenolics promote the tolerance of *Lactobacillus* spp. toward oxidative damage.

The predicted effects of the phenolic compounds and their metabolites by *in silico* studies do not clearly reflect the doses required for predicted activities. Lactic acid bacteria have been shown to tolerate up to 22.7 mmol/L concentrations of *p*-coumaric acid, caffeic acid, syringic acid and gallic acid in vitro (Filannino et al. 2016). The present study indicates that the tested *Lactobacillus* species maintained over 80% survival up to 0.8 mg/mL concentration of the mixture of seven phenolic compounds in vitro. Considering the total phenol content of coconut milk (~ 8 mg/L), assuming 50 mL coconut milk is consumed and dilution of food due to gastric juices, phenolic concentration in the intestine or any segment of the digestive tract never reaches 0.8 mg/

mL concentration of phenolic compounds. Therefore, consumption of coconut milk will not cause a phenolic overload in the intestine that might affect the gut microorganisms or the humans negatively.

Conclusion

Even though coconut milk is a common culinary ingredient in Asian diets, the nutritional information about coconut milk is mainly limited to macronutrient contents. In addition, even though antioxidant and anti-inflammatory effects of coconut milk in the intestine and other model systems were reported, the active ingredients responsible for these activities have not been studied. Further, the beneficial effects of dietary phenolic compounds present in coconut milk on gut health and the gut microbiome have never been explored. Most of the parent phenolic acids and the predicted metabolites of coconut milk were shown to have moderate to high antioxidant and intestinal anti-inflammatory activity with Pa > Pi values suggesting that the coconut milk-based phenolic compounds may promote gut health by inhibiting oxidative damage and inflammations in the gut environment. Most of the metabolites had a low probability of binding to human nuclear receptors, causing small risks to the endocrine system and posing minimal risk to human health. The obtained results revealed that only a few of the compounds have a weak mutagenic and hepatotoxic potential, while all compounds were devoid of cytotoxicity.

Improved antioxidant status upon exposure to phenolic antioxidants evident by the lower levels of protein carbonyls and lipid peroxides in *Lactobacillus* spp. under oxidative stress conditions supports some of the findings of the *in silico* studies. However, further in vitro and in vivo testing is required to confirm the preferentially beneficial effects of coconut milk on symbiotic bacteria over pathogenic bacteria in the gut. Further research on the impact of these parent phenolic acids and their metabolites on diverse gut microbes and human health is vital in promoting coconut milk as a functional food as well as a vegan replacement for cow's milk.

Materials and methods

The study includes an in silico prediction of metabolites of reported phenolic antioxidants of coconut milk and the evaluation of the biological activity, endocrine disruption potential, and ADME/T properties of the phenolic antioxidants and their predicted metabolites. The in vitro study evaluates the protective effect of phenolic antioxidants in

Fig. 4 Flow chart of the methodology
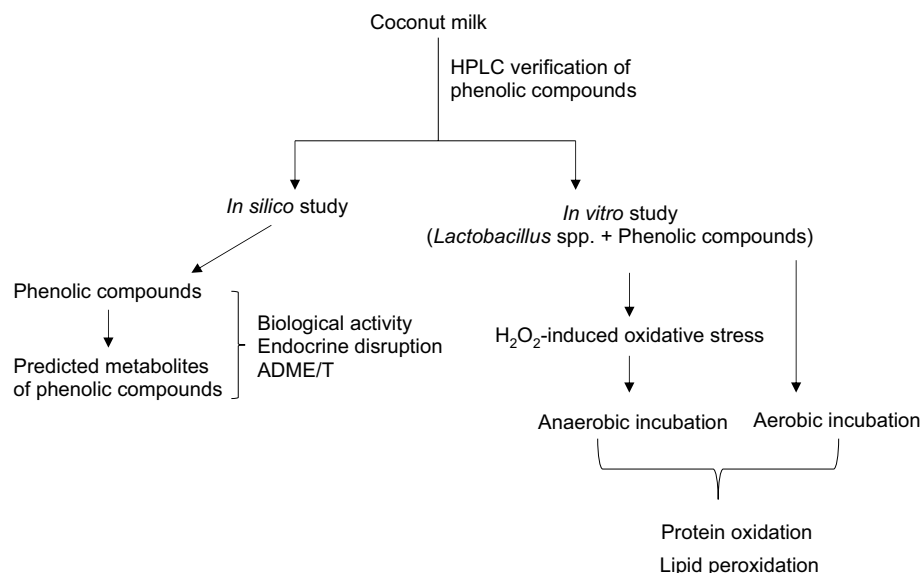
coconut milk against protein carbonylation and lipid peroxidation in *Lactobacillus* spp. under oxidative stress conditions. The overall methodology is summarized in Fig. 4.

Sampling and preparation of coconut milk

Mature coconuts (12–14 months old) were collected from ordinary tall coconut trees (*Cocos nucifera L.*). The coconut trees were selected based on the reported morphological characteristics to determine the nutritional composition of coconut milk. To prepare coconut milk, scraped coconut kernel (100 g) was mixed with distilled water (100 mL) using a kitchen blender (3 min) and the resultant slurry was squeezed through a cheesecloth to separate the milk portion.

In silico studies

In silico human gut microbial transformation prediction

Phenolic compounds reported to be present in the PECM were verified by the same high-performance liquid chromatography (HPLC) experiments as reported (Karunasiri et al. 2020a) in the coconut milk extract. BioTransformer 3.0 web-based tool (https://biotransformer.ca/) (Djoumbou-Feunang et al. 2019) was used to predict metabolic transformations of seven phenolic acids by human gut bacteria. The objective of metabolic transformation was selected as human gut microbial transformation to cover biotransformation that occurs only by the human gut microbiota and the number of reaction steps was set to one.

In silico biological activity prediction

The selected biological activities of 7 phenolic acids and their metabolites were evaluated using the Prediction of Activity Spectra for Substances (PASS) Server (http://www.way2drug.com/passonline/) (Lagunin et al. 2000). The online tool predicts the biological activity of a compound as probable activity (Pa) and probable inactivity (Pi) based on the analysis of structure–activity relationships for more than 250,000 compounds showing more than 3500 kinds of biological activities. The Pa and Pi values vary from 0.000 to 1.000. The activities showed $Pa > Pi$ were considered possible for a given compound with $Pa > Pi$. The $Pa + Pi < 1$, since these probabilities are calculated independently. The probability of experimental biological activity for a compound increases with higher Pa values and lower Pi values. If a compound has $Pa > 0.7$, the probability of experimental biological activity is high while $0.5 < Pa < 0.7$ indicates a moderate probability. If Pa is less than 0.5, the probability of detecting biological activity experimentally is quite low (Filimonov et al. 2014).

In silico endocrine disruption potential prediction

Endocrine Disruptome web server (http://endocrinedisruptome.ki.si/) (Kolšek et al. 2014) was employed for the prediction of the endocrine disruption potential of phenolic acids and their metabolites via a molecular docking approach against 14 distinct human nuclear receptors namely androgen receptor (AR), estrogen receptor α (ER α), estrogen receptor β (ER β), glucocorticoid receptor (GR), Liver X receptor α (LXR α), Liver X receptor β (LXR β), mineralocorticoid receptor (MR), peroxisome proliferator-activated receptor α (PPAR α), peroxisome proliferator-activated receptor β

(PPAR β), peroxisome proliferator-activated receptor γ (PPAR γ), progesterone receptor (PR), retinoid X receptor α (RXR α), thyroid receptor α (TR α) and thyroid receptor β (TR β). AR, ER α, ER β and GR were predicted for both agonistic (a.) and antagonistic (an.) actions.

In silico ADME/T prediction

The pharmacokinetics properties such as absorption, distribution, metabolism and excretion (ADME) of all compounds (phenolic acids and their metabolites) were studied using pkCSM web server (http://biosig.unimelb.edu.au/pkcsm/) (Pires et al. 2015) and SwissADME web server (http://www.swissadme.ch/) (Daina et al. 2017) while a variety of toxicities were evaluated using ProTox-II web tool (https://tox-new.charite.de/) (Banerjee et al. 2018). The pkCSM web tool was used to predict different ADME attributes such as water solubility (WS), steady-state volume of distribution (VDss), substrate of cytochrome P450 isoenzymes (S), inhibitor of cytochrome P450 isoenzymes (I) and total clearance (TC) whereas SwissADME tool predicted human intestinal absorption (HIA) and blood–brain–barrier permeability (BBBp).The ProTox-II web tool was employed to predict different toxicity parameters such as median lethal dose (LD50), toxicity class (CLASS), hepatoxicity (HEP), carcinogenicity (CAR), mutagenicity (MUT) and cytotoxicity (CYT).

In vitro studies

Bacterial strains and cultivation

Lactobacillus spp. (*L. acidophilus* (ATCC4356), *Lactiplantibacillus plantarum* (DMBUK113080), *L. delbrueckii* ssp. *lactis* (ATCC12315), *Lacticaseibacillus casei* (ATCC393) and *Limosilactobacillus fermentum* (ATCC14931)) cultures were obtained from the Department of Microbiology, the University of Kelaniya and each culture was stored in a glycerol stock (15–20%) at − 80 °C. *Lactobacillus* species were grown in MRS broth at 37 °C under anaerobic conditions.

The toxicity level of phenolic extracts and H_2O_2 on *Lactobacillus* spp.

PECM was freeze-dried until a powder was achieved. Then the powder sample was re-dissolved in distilled water and filter sterilized using a 0.45 μm filter. The resultant solution was used to make a concentration series of phenolic extract at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.2, 2.5, 3, 3.5 and 4 mg/mL. Sterile MRS broth (1 mL) was inoculated with *Lactobacillus* from the glycerol stock and incubated overnight at 37 °C in an anaerobic chamber. Then sterile MRS broth (20 mL) was inoculated

with the above overnight *Lactobacillus* culture (100 μL) and incubated at 37 °C in an anaerobic chamber until an OD 0.5 was achieved. Then 1 mL cultures were treated with each coconut milk extract (10 μL) while the control was treated with distilled water (10 μL) instead of the coconut milk extracts. Then all the samples were incubated overnight at 37 °C in an anaerobic chamber.

The concentration of H_2O_2 that does not affect the cell viability was determined by treating the control samples of *Lactobacillus* spp. with a concentration gradient up to 3 mM final solution in the cultures (Do et al. 1996). Briefly, after the overnight incubation period, the broth cultures of the *Lactobacillus* spp. were centrifuged (100 g, 3 min) to obtain cell pellets and the supernatants were removed. Then, the pellets were re-suspended in 1X PBS (1 mL). Samples were centrifuged (100 g, 3 min) again and the supernatants were removed. The pellets were re-suspended in 1X PBS (1 mL) and 15 mM $FeCl_2$ was added to reach a final concentration of 150 μM. The mixture was allowed to stand for 30 min at room temperature under anaerobic conditions. Then H_2O_2 was added at a concentration gradient. The samples were allowed to stand for 1 h at room temperature under anaerobic conditions. The control sample contained the same composition except for water in the place of H_2O_2.

The tetrazolium compound, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay was conducted to determine the cell viability. The MTT assay was carried out to determine the cell viability by using 5 mg/mL MTT in distilled water. The solution was filtered through the 0.22 μm filter into a sterile container. The MTT solution (100 μL) was added to the 1 mL of *Lactobacillus* culture and incubated for 20 min at 37 °C in an anaerobic chamber under dark conditions. Then the mixture was centrifuged for 3 min at 4000 g and the supernatant was decanted. The pellet was re-dissolved in 50% ethanol (300 μL) and the absorbance was measured at 480 nm. The viability percentages were calculated by using the following formula,

The percentage survival of *Lactobacillus* spp.

$$= \frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}} \times 100\%$$

Inhibition of protein carbonylation and lipid peroxidation by coconut milk antioxidants in oxidative stress-induced *Lactobacillus* spp. under anaerobic condition

Induction of oxidative stress on *Lactobacillus* spp.

Cells were treated with PECM up to 0.9 mg/mL as mentioned above and they were maintained in MRS broth at

37 °C in an anaerobic chamber. After the overnight incubation period, the broth cultures of the *Lactobacillus* spp. were centrifuged (100 g, 3 min) to obtain cell pellets and the supernatants were removed. Then, the pellets were re-suspended in 1X PBS (1 mL). Samples were centrifuged (100 g, 3 min) again and the supernatants were removed. Then and the pellets were re-suspended in 1X PBS (1 mL) and 15 mM $FeCl_2$ was added to reach a final concentration of 150 μM. The mixture was allowed to stand for 30 min at room temperature under anaerobic conditions. Then H_2O_2 was added to reach 2 mM (maximum concentration of H_2O_2 that does not significantly affect the viability of the *Lactobacillus* spp.) final H_2O_2 concentration. The samples were allowed to stand for 1 h at room temperature under anaerobic conditions. The control sample contained the same composition except for water in the place of H_2O_2. The experiment was repeated under aerobic conditions following the same procedure except for the incubation under aerobic conditions for 1 h instead of H_2O_2 treatment. Inhibition of protein carbonylation and lipid peroxidation by coconut milk antioxidants in *Lactobacillus* spp. were quantified according to the reported method by Karunasiri et al. (2020a).

Statistical analysis

All experiments were run in triplicate, and biological replicates were carried out unless otherwise indicated. A two-sample *t* test or one-way ANOVA was used for the determination of significant differences between the means. Differences were considered significant when $P \leq 0.05$. Data were analyzed using the GraphPad Prism v.9.4.0 software. The correlation between the formation of products of oxidation and the concentration of PECM is determined based on the Pearson correlation coefficient analysis.

Declarations

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