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Review Paper

In Silico Profiling, Acute Toxicity Study, and Haematological Analysis of Chlorogenic Acid in Swiss Mice

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ABSTRACT

Background: Chlorogenic acid (CGA) is a phytonutrient, widely distributed in diet and taken as a food supplement, and has been shown to possess various health benefits. However, *in silico* investigation and the effect on haematological parameters, especially in the light of acute ingestion, is scanty. The present study aimed to unravel, through predictive modeling, the effects of CGA (chlorogenic acid) in biological systems and assess its toxic effects in the context of acute exposure. This was achieved by conducting observations and analyzing haematological parameters as a preliminary approach.
Methods: Chemical absorption, distribution, metabolism, excretion, and toxicity (ADMET) was employed for the *in silico* profiling using admetSAR2.0. Acute toxicity study was conducted by weight-matching ninety-six male Swiss mice into eight groups (n=12) and exposed to distilled water (control), 1% ethanol (vehicle), and six doses of CGA (30, 60, 120, 240, 480, and 1,000 mg/kg body weight). After 24 h, the animals were observed for mortality; six of the animals were bled, and blood samples were analyzed for haematological parameters using an Autohaematology analyzer. The animals that survived were observed for 14 days post-treatment to monitor for mortality and signs of toxicity.
Results: The ADMET profile predicted that CGA had a tendency towards hepatotoxicity and toxicity in the respiratory and reproductive systems but no effect on the haematological investigation, except at the 1000 mg/kg dose. It is noteworthy that one animal died at the 60 mg/kg dose.
Conclusion: The CGA may be considered tolerable when its target organs are not the reproductive and respiratory systems or the liver, while being deemed safe concerning haematological parameters.

Keywords: ADMET profile, Acute toxicity study, Chlorogenic acid (CGA), Food supplement, Haematological parameters

Introduction

Food supplements are foodstuffs that support the regular diet. They are compounded sources of nutrients or other substances with a physiological or nutritional benefit, which are made available as capsules, tablets, or pills or dispensed as liquids [1]. Chlorogenic acid (CGA) is a polyphenol vastly distributed in foods, vegetables, fruits, spices, and herbs, including tomatoes, apples, eggplant, carrot, and green coffee beans, and is also administered as a food supplement and for therapeutic purposes [2-4]. Its medicinal attributes, including antidiabetic, antimicrobial, antioxidant, antihypertensive, anti-inflammatory, anti-obesity, anti-cancer, anti-neurodegenerative, and antilipidaemic properties, are well established [2,4-12]. However, studies investigating the safety assessment of CGA are limited, and predictive *in silico* investigations for target organs and biological systems are scarce. Therefore, the present study aimed to predict the possible somatic and toxic effects of CGA on various targets within the human system, as well as to investigate its effects on physiological features and haematological parameters following acute exposure in Swiss mice.

Materials and Methods

TEST MATERIAL: Chlorogenic acid was procured from AK Scientific (U.S.A.).

Chemical Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) Profile

The probability for human intestinal absorption, Caco-2, and blood-brain barrier permeability, reproductive toxicity, hepatotoxicity, human oral bioavailability, P-glycoprotein inhibitor, CYP1A2, CYP3A4, CYP2C9, CYP2C19 and CYP2D6 inhibition, eye irritation, renal

toxicity, Ames mutagenesis, eye corrosion, human ether-à-go-go inhibition, eye sensitization, respiratory toxicity, and carcinogenicity of CGA compound was predicted using admetSAR2.0 (http://lmmd.ecust.edu.cn/admetsar2/) [13].

Acute Toxicity Study

Ethical consideration and approval: The study was approved by the University of Ibadan Animal Care (Nigeria) and Use Research Ethics Committee. The identification number UI-ACUREC/19/095 was assigned, and there was compliance with the institutional and national guidelines for the care and use of animals.

Experimental Design: A total of 96 healthy male Swiss mice, weighing between 22-24 g, were weight-matched into eight groups, with each group consisting of 12 animals. The mice were obtained from the Central Animal House at the College of Medicine, University of Ibadan, Nigeria. Each group was housed in the experimental section of the facility in a standard laboratory cage, where they had free access to feed (Topfeed, Nigeria) and water. A 12-hour light/dark cycle was maintained, and they were allowed to acclimate for a week. After an overnight fast with free access to water, Group I received distilled water (control), Group II received 1% ethanol (vehicle), while Groups III-VIII received 30, 60, 120, 240, 480, and 1,000 mg/kg body weight doses of CGA, respectively. All administrations were carried out once orally since the route of administration in practice is via ingestion. At 24 h after administration, the animals were observed for signs of toxicity (redness of eye/blindness, restlessness, locomotive disability, halted reflexes, loss of hair, and sleeping pattern) and mortality. Six of the animals that survived in each group after 24 h of administration were bled via retro-orbital puncture using heparinized capillary tubes into K_2-EDTA specimen bottles for haematological analysis (packed cell volume, haemoglobin concentration, platelets count, total white blood cell count, red blood cell count, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, and differential leukocytes count [neutrophils, lymphocytes, monocytes, eosinophils, and basophils]). The remaining six animals in each group were observed for signs of toxicity (as enumerated before) and mortality for 14 days (delayed toxicity) [14,15]. The health status of the animals was verified daily by animal research staff or veterinary doctors. The blood samples were assayed using the Mindray BC 3000 Autohaematology analyzer (China) to determine the haematological parameters. It is noteworthy that this experiment was conducted three times.

Statistical Analysis

Where appropriate, data are expressed as mean±standard error of the mean and were subjected to one-way analysis of variance (ANOVA), with a P-value<0.05 considered significant. The analysis was performed using the GraphPad Prism 9.0 statistical package (GraphPad Software, La Jolla, CA, U.S.A.). A post-hoc assessment was conducted using Bartlett's test.

Results

ADMET Profile

The result for the ADMET profile is summarized in Table 1. The CGA was predicted to have positive human intestinal absorption and was not a blood barrier permeant. The phytonutrient was predicted to be a non-inhibitor of P-glycoprotein, human ether-à-go-go, CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2D6. The phytonutrient was predicted to not tend to eye irritation, skin sensitization, eye corrosion, or nephrotoxicity, and also has a negative bioavailability score. The phytonutrient was predicted to be a respiratory toxicant, toxic to reproduction, and does not affect Caco-2 cells. It also shows a tendency towards hepatotoxicity but is non-mutagenic and non-carcinogenic.

A. Admet Profiling

Table 1. Result for admet profile of chlorogenic acid

	Parameters	Result
1.	Human Intestinal Absorption	+
2.	Caco-2	-
3.	Blood-Brain Barrier	-
4.	Reproductive Toxicity	+
5.	Hepatotoxicity	+
6.	Human Oral Bioavailability	-
7.	P-glycoprotein Inhibitor	-
8.	CYP1A2 Inhibition	-
9.	CYP3A4 Inhibition	-
10.	CYP2C9 Inhibition	-
11.	CYP2C19 Inhibition	-
12.	CYP2D6 Inhibition	-
13.	Eye Irritation	-
14.	Renal Toxicity	-
15.	Ames Mutagenesis	-
16.	Eye Corrosion	-
17.	Human Ether-à-go-go-Related Gene Inhibition	-
18.	Skin Sensitization	-
19.	Respiratory Toxicity	+
20.	Carcinogenicity	-

Acute Toxicity Study

Table 2 reveals that on day 3, one of the animals in the 60 mg/kg CGA group developed blindness in the left eye on day 3 and died on day 5. Other signs of toxicity (restlessness, locomotive disability, loss of hair, halted reflexes, and change in sleeping pattern) were not observed, and mortality was not observed at the higher doses of CGA (Table 2). Table 3 indicates that CGA did not have any effect on packed cell volume, haemoglobin concentration, red blood cell count, white blood cell count, and platelets when compared to the control; however, when compared to 1% ethanol (vehicle), the haemoglobin concentration and red blood cell count increased significantly ($P<0.05$) in the group treated with 1,000 mg/kg body weight of CGA (Table 3). Tables 4 and 5 demonstrate that CGA did not affect the blood indices and differential leukocyte count when compared with the control and the group treated with the vehicle (Tables 4 and 5).

B. Acute Toxicity Study

Table 2. Behavioural changes and signs of toxicity

DAY	Redness of Eye/Blindness Group (Number of Animals)	Mortality Group (Number of Animals)
1 (24 h)	-	-
2	-	-
3	60 CGA (1)	-
4		-
5	-	60 (CGA) (1)
6	-	-
7	-	-
8	-	-
9	-	-
10	-	-
11	-	-
12	-	-
13	-	-
14	-	-

Note: n=6; **Abbreviations:** CGA- chlorogenic acid. The dose affected is stated with the number of animals in the bracket. Restlessness, locomotive disability, loss of hair, halted reflexes, and change in sleeping patterns were not observed throughout observation in the treatment groups. This result is the average of three replicates.

Table 3. Influence of chlorogenic acid on certain haematological parameters

DOSE (mg/kg)	PCV (%)	Hb (g/ 100mL)	Total WBC (x 10^3)	Platelets (x 10^5)	RBC (x 10^{12}/ L)
CONTROL	41.67±3.24	14.42±0.95	6.17±0.36	4.93±0.36	7.77±0.45
1% ETHANOL	38.83±2.24	13.60±0.83	7.65±1.54	6.91±0.27	7.68±0.45
30 CGA	40.67±0.92	14.07±0.33	6.85±0.69	5.82±1.30	8.17±0.23
60 CGA	44.17±1.42	15.13±0.29	5.77±0.85	6.00±0.35	8.17±0.15
120 CGA	41.67±2.44	14.55±0.82	5.97±0.47	6.22±0.65	8.42±0.27
240 CGA	39.00±2.97	13.65±0.98	5.38±1.33	6.18±0.61	7.77±0.51
480 CGA	44.17±0.95	15.98±0.67	7.62±0.80	4.69±0.40	8.72±0.10
1,000 CGA	46.17±1.58	16.17±0.43*	7.28±0.65	5.27±0.26	9.00±0.22*

Note: n=6; *- significantly different from 1% ethanol-treated group at $P<0.05$; **Abbreviations:** PCV- packed cell volume, Hb – haemoglobin concentration, WBC- white blood cell count, RBC- red blood cell count, CGA- chlorogenic acid

Table 4. Effect of chlorogenic acid on blood indices

DOSE (mg/kg)	MCV (fL)	MCH (pg)	MCHC (g/ 100mL)
CONTROL	51.67±0.61	18.17±0.17	35.50±0.34
1% ETHANOL	52.00±0.68	18.17±0.17	7.680±0.45
30 CGA	49.83±0.60	17.00±0.26	34.50±0.22
60 CGA	52.33±1.15	18.60±0.37	35.50±0.43
120 CGA	48.83±1.28	17.00±0.45	35.00±0.26
240 CGA	50.00±1.37	17.67±0.33	35.17±0.48
480 CGA	51.33±1.02	18.17±0.48	34.67±0.21
1000 CGA	51.00±0.37	18.33±0.33	35.83±0.31

Note: n=6; **Abbreviations:** MCV- mean cell volume, MCH- mean cell haemoglobin, MCHC- mean cell haemoglobin concentration, CGA- chlorogenic acid

Table 5. Effect of chlorogenic acid on differential leukocyte count

Dose (mg/kg)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Basophils (%)
CONTROL	38.67±6.88	59.33±6.84	0.00±0.00	1.33±0.61	0.00±0.00
1% ETHANOL	48.67±10.39	48.33±10.67	0 00±0.00	2.17±0.31	1.33±0.21
30 CGA	57.50±5.83	38.83±5.92	0.50±0.22	2.50±0.34	1.67±0.33
60 CGA	42.50±6.71	55.00±6.65	0.50±0.34	2.33±0.33	0.00±0.00
120 CGA	39.17±4.66	57.00±3.61	1.17±0.98	2.40±0.80	0.00±0.00
240 CGA	41.67±5.97	52.17±6.22	0.50±0.34	3.00±0.73	0.40±0.22
480 CGA	35.67±6.64	57.67±7.02	1.33±0.61	3.50±0.67	0.17±0.17
1000 CGA	46.00±7.54	49.67±8.30	0.33±0.21	3.00±0.86	1.00±0.45

Note: n=6; **Abbreviations:** CGA- Chlorogenic acid

Discussion

The ADMET analysis predicted that CGA had varying activities in various organs of the body and biological systems. In the acute toxicity study, the lethal dose was not observed, even at 1,000 mg/kg dose. However, mortality was observed at 60 mg/kg dose. In addition, CGA had no deleterious effect on haematological parameters.

The ADMET predicted that CGA can easily be absorbed by the human intestine, which is essential for efficacy. Oral bioavailability indicates the efficiency of drug delivery to the systemic circulation [16,17]. However, CGA (a phytonutrient) was predicted to be negative, thus implying that it is not effectively delivered to the circulatory system. Caco-2 permeation assesses the ability of the drug molecule to pass through intestinal cell membranes using passive diffusion [18,19]. As a result of CGA being a negative Caco-2 permeant, this suggests that it may find it difficult to pass through human intestine mucosa when used orally even though it is predicted to be easily absorbed by the human intestine.

P-glycoprotein inhibition and the blood-brain barrier give insight into the ability of CGA to be distributed around the peripheral nervous system and central nervous system. There is an understanding that drugs that can pass through the blood-brain barrier are likely to have neurological effects, especially if the target is in the brain and spine [20-22]. Since CGA has no blood-brain barrier permeability, it may have no therapeutic value if targeting a protein within the central nervous system. Drug molecules that interact with P-glycoprotein may affect the distribution of the drug and may lead to the accumulation of the molecules, which results in an adverse effect [23]. Accumulation of the drug molecule can also prevent the molecule from reaching the drug target. According to the results, CGA is predicted not to interact with P-glycoprotein.

In terms of metabolism, the outcome suggests that CGA is predicted to be metabolized efficiently. Clearly, CGA does not inhibit any CYP enzyme isoform, which means that the chances of interaction with the drug are low [24]. In other words, it is unlikely to result in adverse effects due to poor clearance and accumulation. While there is no direct parameter for excretion, such as renal clearance and half-time, the distribution suggests that CGA can be released from the nephrons without difficulties.

The possible deleterious effect of the phytonutrient on the reproductive system, respiratory system, and liver are predicted, while the innocuous effect on the skin, eye, and human ether-à-go-go gene is also demonstrated. In terms of genomic toxicity, CGA is predicted to be non-carcinogenic and non-mutagenic. In other words, CGA could be safe to use if the drug target is not situated within the liver, respiratory system, and reproductive system.

Previous *in silico* investigations have indicated the positive effects of CGA through different modes where it binds to enzymatic systems like glucose-6-phosphatase via translocase-1 to reduce blood sugar levels [25] to α-amylase and α-glucosidase thereby being able to reduce diabetes-related pathophysiology and attendant complications [11], and improved the vital biological properties of lactoferrin (a glycoprotein of mammals known to have antioxidant property) [26]. Moreover, conversely, Abedpour *et al*. [27] reported that CGA could possess the capacity to prevent polycystic ovary syndrome *in vivo*, which underscores the importance of *in vivo* investigations.

The animal experiment was repeated three times. Although the dose at 50% mortality (LD_{50}) could not be determined, upon conclusion of the 14 days of observation, one of the animals exposed to the dose of 60 mg/kg body weight died on day 5, and prior to this, blindness was observed in this animal on day 3. This does not support the ADMET prediction, thus indicating the importance of *in vivo* studies. This also suggests that a safe dose would be below 60 mg/kg. However, mortality was not observed at the higher doses (120, 240, 480, and 1,000 mg/kg). Similarly, in our study with *Drosophila melanogaster*, at 60 mg/kg diet of CGA, the flies showed 2.5 to 3 times the death observed at lower doses (under review). This may be because CGAs are esters of a trans-cinnamic acid residue and quinic acid of which are caffeoylquinic acid (CQA) and 5-O-caffeoylquinic acid (5-CQA) (which is an isomer of CQA) has been indicated to be absorbed through two significant pathways which are (a) unhindered absorption in the stomach and/or the upper part of the gastrointestinal tract or (b) slow absorption throughout the small intestine [28]. Therefore, we suggest that at 30 mg/kg, the dose is not sufficient to cause damage, while at 60 mg/kg, the dose is the adequate amount that needs to be absorbed by the stomach to cause damage, and at the high doses, CGA is not rapidly absorbed because of the increased concentration.

The haematological analysis reflected that when compared with the control, CGA did not have any effect on the haematological parameters. However, when compared with the group treated with the vehicle (1% ethanol), it significantly elevated the red blood cell count and haemoglobin concentration at the 1,000 mg/kg dose. The red blood cells function in the transportation of oxygen in the blood via the haemoglobin, from the lungs to other parts of the body and in the transport of nutrients [29]. This suggests that CGA may have an anti-anaemic effect at a high dose. Although CGA showed signs of toxicity at the 60 mg/kg dose, it enhanced haematological parameters at the highest dose (1000 mg/kg). The limitation of the *in silico* study is that investigations *in vivo* will be necessary to support the findings. The results of the acute toxicity study will serve as a basis for information

on the determination of dosage regimens in the practical administration of CGA for therapeutic purposes and food supplementation, as well as for further investigations in the reproductive system and the liver, amongst others.

Conclusions

In conclusion, CGA may be innocuous when the target is not in the reproductive and respiratory systems and the liver. Moreover, it does not exhibit any deleterious effect on haematological parameters. Instead, it enhances red blood cell formation and haemoglobin concentration. Moreover, there is a need to conduct *in vivo* studies to confirm *in silico* investigations.

Conflict of Interests

The authors have no conflict of interest to disclose.

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None.

Authors' Contributions

O. A-S. conceptualized the data, verified the methods, made substantial contributions to funding acquisition, supervised the article, wrote the original draft, and edited the manuscript. F. A. conceptualized the data, performed formal analysis, and reviewed the manuscript. O. O., J. A., A. A., and D. A. investigated the manuscript and performed a formal analysis. All authors approved the final version for publication.

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