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Integrating *In Vivo* Model, Molecular Docking and Network Pharmacology to Determine the Mechanism of *Theobroma cacao* Seed in Treatment of Diarrheal

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Abstract: *Theobroma cacao* is an economically important tropical-fruit tree where chocolate is obtained, and it is used as traditional medicine worldwide against several diseases. In the present study, *in vivo* model and computational biology approaches were used to elucidate the potential mechanisms of *T. cacao* in the treatment of diarrhea. The antidiarrheal and intestinal motility activity was conducted using an animal model induced diarrhea with MgSO4. In addition, an OECD acute oral toxicity test was carried out. Prediction analysis of the bioactive effects of *T. cacao* against diarrhea symptoms were carried out applying functional enrichment analysis, protein-protein interaction, ADME and drug-likeness analysis, and molecular docking. The analysis of the compound-target- pathway-antidiarrheal mechanism relationships was performed in Cytoscape. *T. cacao* (200 mg/kg) effectively inhibited diarrhea in mice, significantly lowering the diarrheal stools and intestinal motility, without toxicity signs. Gene set enrichment, molecular docking, and network pharmacology revealed 13 *T. cacao* compounds targeting 12 proteins that regulate 11 signaling pathways related to diarrhea. According to our research results, the *T. cacao* antidiarrheal effect could be due to the therapeutic action of quercetin, luteolin, and deoxyclovamide compounds on the ABCB1, ABCG2, CYP3A4, EGFR, ERBB2, IL6, SI, and SLC10A2 genes, related to Carbohydrate digestion and absorption, Bladder cancer, Bile secretion and Graft-versus-host disease as the most significant signaling pathways.

Keywords: Antidiarrheal Effect, Mechanism, Signaling Pathways, *Theobroma cacao*

1. Introduction

According to the World Health Organization (WHO), diarrhea is defined as the passage of three or more loose or watery stools in 24 hours and is the second leading cause of death in children under five years old [1]. Currently diarrheal diseases still remain among the top 10 causes of death worldwide. More worrying is that for common bacterial infections, including some forms of diarrhea, high rates of antimicrobial resistance have been observed [2]. Although mortality from diarrheal disease is decreasing globally, morbidity is not, and its incidence does not differ substantially between regions, but incidence and case morbidity rates are much higher in some countries [3].

In health, all the water and electrolytes ingested or secreted into the intestine are absorbed as part of the digestive process.

Reducing absorption efficiency by as little as 1% may be enough to cause diarrhea [4]. Such alteration may be due to altered rates of mucosal absorption or secretion in response to damage to the absorbent mucosa, bacterial toxins, enteric nerve function, or hormones [5].

Antidiarrheal medications are those that minimize the symptoms of diarrhea by improving consistency, reducing the frequency, or reducing the weight of stools [6]. The most used therapies in the treatment of diarrhea are opioid antidiarrheal drugs. These medications are effective for a wide variety of diarrheal conditions and can generally be used safely if they are closely monitored [4]. However, several side effects are described for its use, such as dependency, tolerance, constipation, sedation, dizziness, vomiting, and respiratory depression. Although constipation and nausea are considered the two main complications of opioids. Additionally, other side effects including delayed gastric emptying, hyperalgesia, immunological effects, and hormonal dysfunction have been observed [7].

The side effects of current antidiarrheal drugs, as well as the appearance of resistance to antimicrobials used for treatment of infectious diarrhea, justify the search for drugs based on natural products for treatment of diarrhea. Historically, medicinal plants have been used for treatment of various communicable and non-communicable diseases, and even today represent a rich source of important therapeutic agents as well as new lead compounds [8]. In recent years, several technological and scientific developments including improved analytical tools, genome mining and engineering strategies, and microbial culturing advances are addressing such challenges and opening new opportunities. Consequently, interest in natural products as drug leads is being revitalized [9].

One of the most recent research techniques is network pharmacology which considers the essence of disease are genes or encoded proteins, represented by one or more nodes in the network, are in an unbalanced state [10]. Herbal active ingredients have long been viewed as a rich source of therapeutic leads in drug discovery. Network pharmacology is expected to be a new strategy and powerful tool to find the bioactive compounds as well as their potential molecular targets from numerous herbs or herbal formulae [11]. The aim of constructing a network is to achieve the interaction between the bioactive compounds and targets and the interaction between various targets, and then find out and validate the key nodes via network analysis and network verification [12].

Several scientific reports indicate that medicinal plants are widely used for the treatment of diarrhea in different regions of the world, and it has also been described their anti-diarrheal effects is through their action as antisecretory agents, anti-peristaltic effects, anti-microbial and anti-spasmodic effects [13].

Theobroma cacao, present in South and Central America, is associated with cardiovascular health, lowering of blood pressure, and platelet activities. Its anti-malarial, antioxidant, Antitussive, anti-influenza, anti-diabetic, and Antihypertensive activities have also been reported [14]. In a preliminary study, the antidiarrheal effect of T. cacao seeds in an animal model has already been described [15].

T. cacao contains a higher concentration of carbohydrates, saponins, and phlorotannins, whereas tannins, glycosides, resins, and alkaloids were found in lower concentrations [16]. Alkaloid compounds such as theobromine and phenolic compounds (gallic acid, catechin, and epicatechin enantiomers) have been found as the majority in this species. [17] Likewise, other compounds such as quercetin, ferulic acid, and luteolin present in the seed [18, 19] have already been reported for their antidiarrheal activity [20].

Therefore, in the present study, the antidiarrheal activity of Theobroma cacao seed was determined in an animal model, to then identify the potential therapeutic targets and molecular signaling pathways regulated by T. cacao compounds in the treatment of diarrhea using molecular docking and network pharmacology.

2. Materials and Methods

2.1. In Vivo Tests

2.1.1. Extract Preparation

The extract was made from ground *T. cacao* beans. 83.6 g of beans were placed in a 500 mL round-bottom flask and the necessary amount of 90% ethyl alcohol was added, leaving it to macerate for 24 hours. A rotary evaporation in a Heidolph/Laborota 4000 equipment was made. The residue obtained was dried using calcium sulfate for 72 hours, obtaining a brown semi-solid substance. An extra defatting step was carried out using n-hexane. Subsequently, the mixture obtained was left for three days in drying. A solid and brown color product was obtained.

2.1.2. Experimental Design and Laboratory Animals

Male Swiss albino mice (25 g weight average) were used. They were kept in a controlled environment (22 ± 2°C), in 12/12 h light and dark cycles, fed with a commercial diet and water *ad libitum*, and received intragastric treatment. Americans Guide for the Care and Use of Laboratory Animals was followed [21]. Each animal was used once and then was euthanized. The minimum number necessary and the shortest observation time required to obtain consistent data were used for each trial. For antidiarrheal tests, two sets of animals were starved for 12 hours before the experiments and were randomly distributed into five groups (n = 10). Group 1 (negative control) received water; group 2 (positive control) received 0.075 mg of loperamide in 0.25 ml of water; groups 3, 4, and 5 received 100, 200, and 400 mg/kg body weight of the ethanolic extract of *T. cacao*. 10 mL/kg of substances were administered.

2.1.3. Antidiarrheal Effect

Thirty minutes after the treatment of that substance, each animal was administered magnesium sulfate (MgSO4) orally (0.25 ml, 0.2% w/v, vo) [22]. All animals were placed in individual cages bedded with filter paper. This paper was replaced every hour after noting its weight. The number and

type of stools excreted were recorded: hard (healthy) and diarrheal (stools with a liquid or semi-liquid appearance). The total number of diarrheal stools in group 1 was considered as 100%. The results were expressed as a percentage of diarrhea inhibition.

2.1.4. Intestinal Motility

The method described by Besra *et al*. [23] was used. Thirty minutes after substances treatment, each animal was administered $MgSO_4$ orally (0.25 ml, 0.2% w/v) and 0.1 ml activated charcoal as a food tracer prepared at 10% suspension in 5% arabic gum. After an observation period of one hour, each mouse was sacrificed and dissected. The small intestine was removed and its total length was measured (cm). The movement of charcoal from the pylorus was measured (cm). Intestinal transit was expressed as a percentage of the length moved by charcoal to the total length of the small intestine.

2.1.5. Acute Oral Toxicity

The test was performed according to the OECD method [24]. Eight-week-old Swiss male mice were randomly selected and housed in polycarbonate mice cages. A total of 10 animals were distributed into two groups of 5 animals each. Group 1 (saline control) did not receive plant extract while group 2 animals received 400 mg/kg of the ethanolic extract. Mice were observed for mortality and behavioral changes for 14 days after treatment. At the end of the test, they were euthanized for the macroscopic evaluation of the target organs.

2.1.6. Statistical Analysis

Data were analyzed using a normality test (Shapiro-Wilk, $P<0.05$). Antidiarrheal tests were analyzed by one-way analysis of variance (ANOVA), followed by a Dunnett's test for multiple comparisons. For toxicity test, an Independent Sample t-Test was performed. The results were expressed as mean ± standard deviation. A p-value of <0.05 was considered to have a significant difference, and a P value <0.01 indicated an extremely significant difference.

2.2. Network Pharmacology

2.2.1. Candidate Components Collection

Through the application of different electronic databases, scientific articles related to the chemical of the T. cacao seed were retrieved, assessed and evaluated [17, 18, 25]. A list of chemical compounds was obtained. The SMILES format of chemical structures was collected from the PubChem database. (https://pubchem.ncbi.nlm.nih.gov/).

2.2.2. Potential Target Prediction

The chemical compounds of *T. cacao* were uploaded to the SwissTargetPrediction platform [26] to predict their potential target proteins for Homo sapiens species. These proteins were then uploaded to the Enricher web server to obtain gene-disease associations (GDA) from DisGeNET [27]. Only genes related to diarrhea were selected. In addition, diarrhea-related genes were collected using the Online Mendelian Inheritance in Man database. (OMIM, http://omim.org/) [28]. The targets common to both lists (DisGeNET/OMIM) were selected and processed by String (https://string-db.org/) to obtain protein-protein interactions (PPI) and their respective network diagram using the Cytoscape 3.6.9 program [29]. This list of genes was considered therapeutic targets for *T. cacao* (PTT-Tc) in the treatment of diarrhea.

2.2.3. Gene Ontology (GO) and Pathway Enrichment

ShinyGO (http://bioinformatics.sdstate.edu/go/) [30] was used to perform the enrichment analysis for the ontological source GO [31]. Likewise, the analysis of the KEGG (Kyoto Encyclopedia of Genes and Genomes) routes was conducted [32], set to FDR < 0.05, a minimum pathway size of 2 and a maximum of 2000. To further capture relationships between terms and their significance, the twenty most significant KEGG pathways were selected and represented as a hierarchical clustering tree.

2.2.4. Absorption, Distribution, Metabolism, and Excretion (ADME) and Drug-Likeness (DL) Analysis

The ADME properties are important indicators of the effectiveness of herbs and play key roles in drug discovery and reducing costs and time. The chemical compounds of *T. cacao* were uploaded to swissadme [33] which was developed as a comprehensive source and open-source tool for the prediction of chemical ADME and DL properties.

2.2.5. Molecular Docking and Validation

Molecular docking was performed only between targets of the PTT-Tc gene list and their chemical components associated with *T. cacao* seed. The 3D protein structures of genes were downloaded from RCSB Protein Data Bank (PDB, http://www.rcsb.org) and saved in PDB format. Protein preparation (removal of ligands, water molecules, and cofactors that are resolved in the crystallized structures) was performed with the UCSF Chimera 1.16 program [34]. In addition, non-polar hydrogens and Kollman charges were added. The treatment of ligands was based on the minimization of their energies with the MMFF94 method [35], using the OpenBabel program [36]. Molecular docking was performed with the PyRx program, under the parameter of free rotation of all ligand bonds with conformational freedom and rigid receptor. The protein coordinates for docking were defined according to the active sites described by the authors responsible for the 3D annotation of the proteins in the PDB. Validation of the method was performed by Redocking on CYP3A4 protein (PDB: 1W0E) and its cocrystallized ligand protoporphyrin IX. The re-coupled complex was then superimposed on the reference co-crystallized complex using the UCSF Chimera 1.16 program and the root mean square deviation (RMSD) was calculated.

2.2.6. Analysis of Potential Mechanisms

Based on the results of the molecular Docking, ADME, and Drug Likeness analysis, a "multi compound - multi targets - metabolic pathways" network was created with the ClueGO [37] and CluePedia [38] Cytoscape tools. Two-sided hypergeometric (Enrichment/Depletion) tests, mid-P-values

corrected by Bonferroni step down method, min/ max GO level = 3 and 8, number of genes = 2, min percentage = 4.0, GO Fusion / Group = true/true, and Kappa Score Threshold = 0.4 were adjusted in ClueGO analysis. The network was built using only those BT-Tc genes associated with the signaling pathways obtained from the ClueGO analysis, and which are also related to chemical compounds of T. cacao with the best protein-ligand interaction energies and the best ADME and Drug Likeness results. Finally, the network was connected to previously reported antidiarrheal mechanisms for plant species [13].

3. Results

3.1. In Vivo Tests

The oral acute toxicity test shows ethanolic extract of *T. cacao* did not cause signs of toxicity in the animal model. A normal increase in animal weights was observed after 14 days (Table 1). No changes in appearance or weight of the internal organs are observed (Table 2). The extract of *T. cacao* seeds shows a significant reduction of effects induced by $MgSO_4$, as well as a reduction in the intestinal transit of the experimental animals (Table 3).

Table 1. *Body weight in oral toxicity test of the ethanolic extract of Theobroma cacao seeds at 400 mg/kg.*

Group	Day 0	Day 14	%	P value
Control	22.68 ± 3.279	26.98 ± 0.511	11.90 ± 2.217	
Treatment	21.30 ± 2.774	26.72 ± 0.698	23.69 ± 13.881	0.131

Values are expressed as the Mean ± SEM (standard error of the mean); %: weight gain. * $p < 0.05$.

Table 2. *Organ weight in oral toxicity test of the ethanolic extract of Theobroma cacao seeds at 400 mg/kg.*

Organ	Group	Mean ± SEM	p value
Liver	Control	1.646 ± 0.143	
	Treatment	1.376 ± 0.063	0.005*
Heart	Control	0.148 ± 0.015	
	Treatment	0.143 ± 0.015	0.599
Lung	Control	0.176 ± 0.022	
	Treatment	0.156 ± 0.023	0.197
Right kidney	Control	0.238 ± 0.024	
	Treatment	0.214 ± 0.024	0.186
Left kidney	Control	0.240 ± 0.021	
	Treatment	0.226 ± 0.011	0.23
Stomach	Control	0.504 ± 0.128	
	Treatment	0.396 ± 0.125	0.214
Spleen	Control	0.104 ± 0.025	
	Treatment	0.122 ± 0.030	0.329
Small intestine	Control	1.902 ± 0.234	
	Treatment	1.620 ± 0.175	0.063
Large intestine	Control	1.116 ± 0.120	
	Treatment	0.914 ± 0.203	0.092

* $p < 0.05$; SEM: standard error of the mean.

Table 3. *Effect of ethanolic extract from Theobroma cacao seeds on diarrhea induced with $MGSO_4$ and intestinal transit in animal models.*

Group	Diarrhea stool		Intestinal Motility Test (distance traveled)	
	Mean ± SEM (p value)	%	Mean ± SEM (p value)	%
H_2Od	13.50 ± 1.21	-	32.04 ± 2.60	-
Loperamide	0.63 ± 0.62 (< 0.001) **	95%	12.38 ± 3.36 (< 0.001) **	61.36%
EETc 100 mg/kg	4.00 ± 0.82 (< 0.001) **	70%	19.81 ± 1.79 (0.027) *	38.17%
EETc 200 mg/kg	2.38 ± 0.75 (< 0.001) **	82%	17.90 ± 2.63 (0.004) *	44.13%
EETc 400 mg/kg	2.13 ± 0.69 (< 0.001) **	84%	35.50 ± 2.86 -0.9	-10.80%

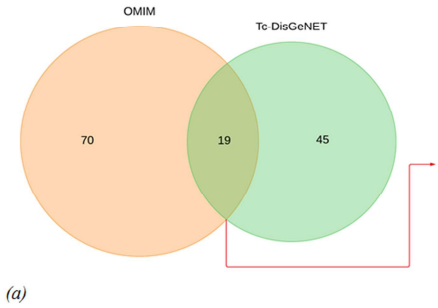
EETc: Ethanolic Extract of *T. cacao*. * $p < 0.05$; ** $p < 0.001$; SEM: standard error of the mean; %: diarrhea inhibition.

3.2. In Silico Analysis

3.2.1. Therapeutics Targets

Through literature review, 39 chemical constituents of the seed of *T. cacao* were collected [18, 19, 25, 39-42]. SwissTargetPrediction calculated 746 potential targets related to *T. cacao* compounds, 64 diarrhea-related genes were obtained according to Gene-Disease Associations (GDA) from the DisGeNET database. A total of 89 diarrhea-related targets were obtained from the OMIM database, and 19 overlapping genes were identified as therapeutic targets (Figure 1a, b). A Protein-Protein Interaction (PPI) analysis was carried out. The gene list was loaded into String (https://string-db.org/) and the resulting network was edited with Cytoscape 3.9.1 software. The network shows the

physical interactions in STRING [43] with a score > 0.132 on the members' list (Figure 2).



(a)

Gen	Protein	PDB ID
ABCB1	Multidrug resistance protein 1	6COV
ABCG2	ATP-binding cassette sub-family G member 2	5NJ3
CFTR	Cystic fibrosis transmembrane conductance	1XMI
CYP3A4	Cytochrome p450 family 3 subfamily a	1W0E
DGAT1	Diacylglycerol O-acyltransferase 1	6VP0
EGFR	Epidermal growth factor receptor	1IVO
ERBB2	Receptor tyrosine-protein kinase erbB-2	1S78
HTR3A	5-hydroxytryptamine receptor 3A	6HIO
IL2	Interleukin-2	1M49
IL6	Interleukin-6	5FUC
MME	Membrane metalloendopeptidase;	1DMT
MPO	Myeloperoxidase	1CXP
SI	Sucrase-isomaltase, intestinal	3LPO
SLC10A2	Ileal sodium/bile acid cotransporter	3EM0
SLC5A1	Sodium/glucose cotransporter 1	7SL8
SLC6A4	Sodium-dependent serotonin transporter	5I6X
TMPRSS15	Transmembrane serine protease 15	6ZOV
TNF	Tumor necrosis factor	1TNF
TTR	Transthyretin	1BM7

(b)

Figure 1. *Venn diagram showing the intersection between the Tc-DisGeNET and OMIM gene lists (a); description of common genes (b).*

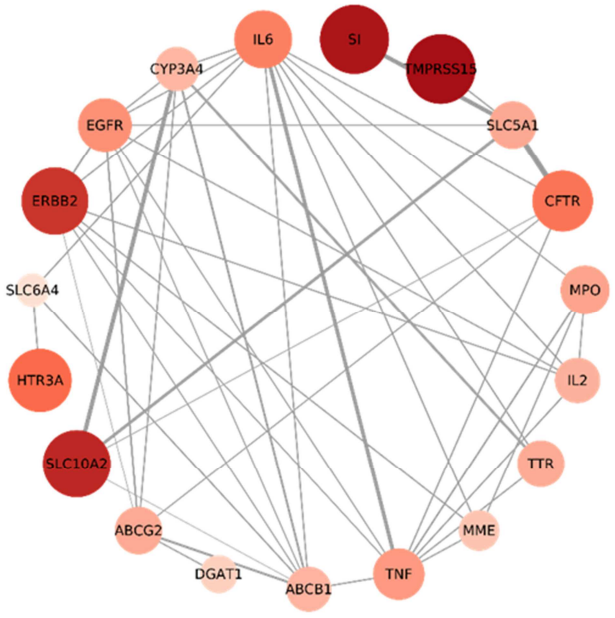


Figure 2. *Result of the PPI analysis of common genes in DisGeNET – OMIM, considered potential therapeutic targets for T. cacao (PTT-Tc). Larger, darker nodes indicate genes with higher significant P-values. Darker edges represent more related genes.*

3.2.2. GO and KEGG Analysis

The results in the ShinyGO PTT-Tc gene list consist of 3 parts: biological process, molecular function, and cellular component are listed in Figure 3. In the KEGG enrichment analysis [32], Bile secretion, ABC transporter, Inflammatory bowel disease, and Graf-versus-host disease are listed among the most significant signaling pathways.

3.2.3. ADME, DL Analysis, and Molecular Docking

From ADME and DL analysis, only compounds with high gastrointestinal absorption, a bioavailability scores equal to or greater than 0.55 [44], and no more than one violation of the Lipinski rules was selected (Table 4). According to literature reports, binding energy less than -5.0 kcal/mol is the standard for stable binding of ligands and receptors during molecular docking, and the lower binding energy means the molecular structure is more stable [45]. The 2D analysis of ligand-protein interactions was performed for those pairs with interaction energies equal to or less than -7 kcal/mol and is shown in Figure 4. The chemical compounds with the best ADME and DL values and best interaction energies were considered potentially responsible compounds for the antidiarrheal activity of *T. cacao* observed in vivo tests. Regarding the validation of the method (Figure 5), the superposition of the re-docked complex CYP3A4-protoporphyrin IX on the co-crystallized complex (PDB ID: 1W0E), fits properly and is consistent with the RMSD calculated between both ligands (1.038 angstroms), which validates the method used.

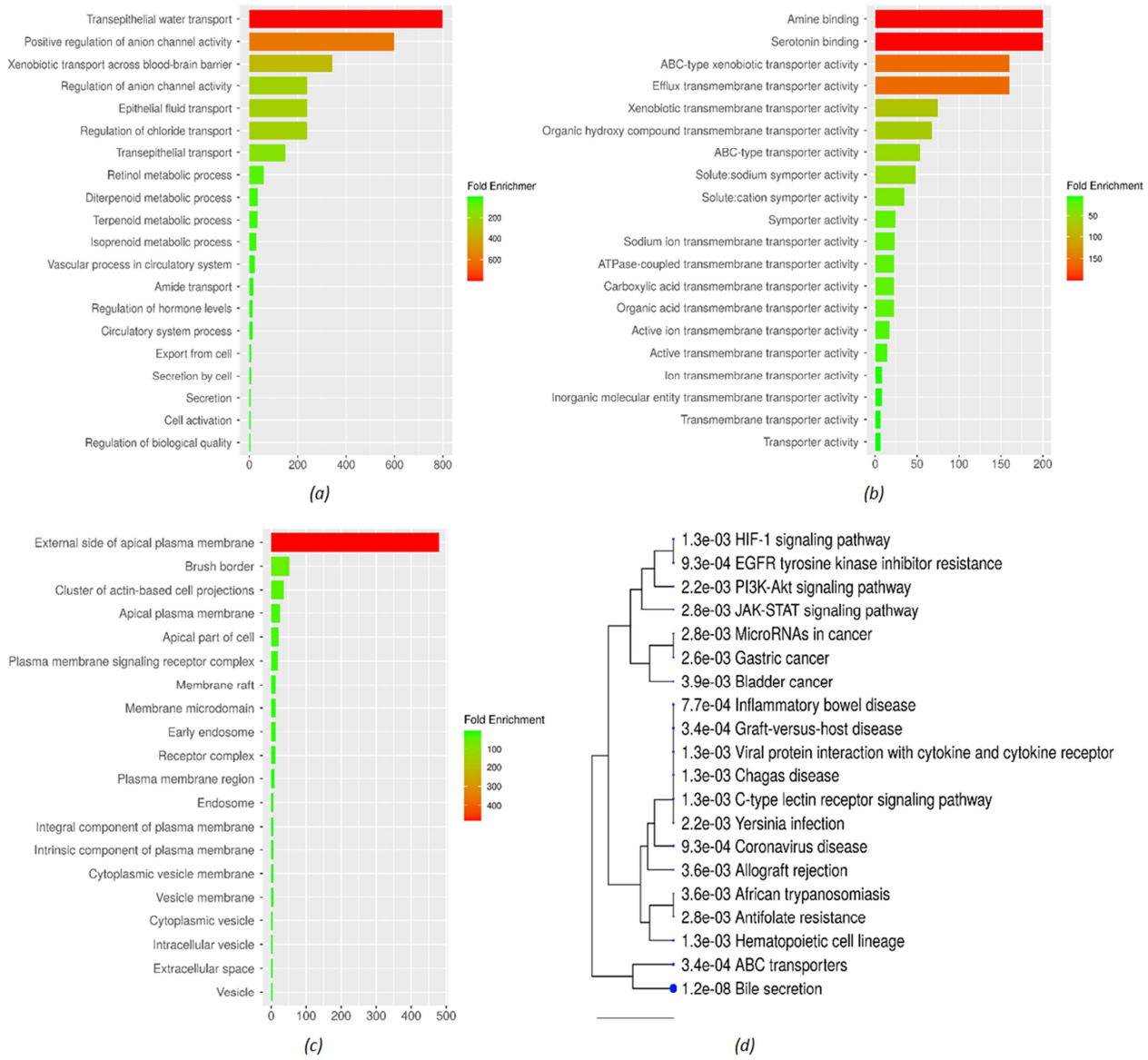


Figure 3. *GO enrichment analysis. Biological processes (a); Molecular function (b) and Cellular components (c). KEGG functional enrichment analysis in hierarchical clustering tree. Pathways with many shared genes are clustered together. Bigger dots indicate more significant P- values (d).*

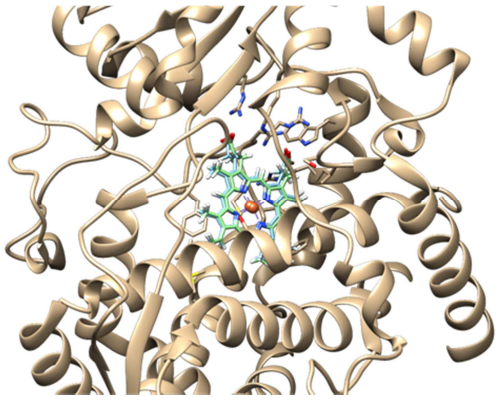


Figure 4. *Superposition of the re-coupled complex (green color, -13.3 kcal/mol) on co-crystallized complex of CYP3A4 protein (PDB: 1W0E) - protoporphyrin IX (light blue color), using UCSF Chimera 1.16 (RMSD=1.098).*

3.2.4. Analysis of the Potential Mechanisms of the Antidiarrheal Effect of T. cacao

From ADME and DL analysis, 13 from 28 *T. cacao* compounds collected from the literature, were identified as potentially responsible for antidiarrheal activity. The ClueGO tool allowed identified 11 signaling pathways, grouped into 4 functional groups represented by the signaling pathways Carbohydrate digestion and absorption, Bladder cancer, Bile secretion, and Graft-versus-host disease, as the most significant. ClueGO further revealed that only 12 of the 19 PTT-Tc genes appear associated with these signaling pathways. The molecular docking analysis shows only ABCB1, ABCG2, CYP3A4, EGFR, ERBB2, IL6, SI, and SLC10A2, have binding energies lower than -7 Kcal/mol with quercetin, luteolin, and deoxyclovamide, bioactives that showed to be *T. cacao* compounds with the best possibilities

of binding to most therapeutic targets (table 5). The functional groups represented by their most significant term and the relationships between compound - target, and targets – pathway, are visualized in the network (Figure 6).

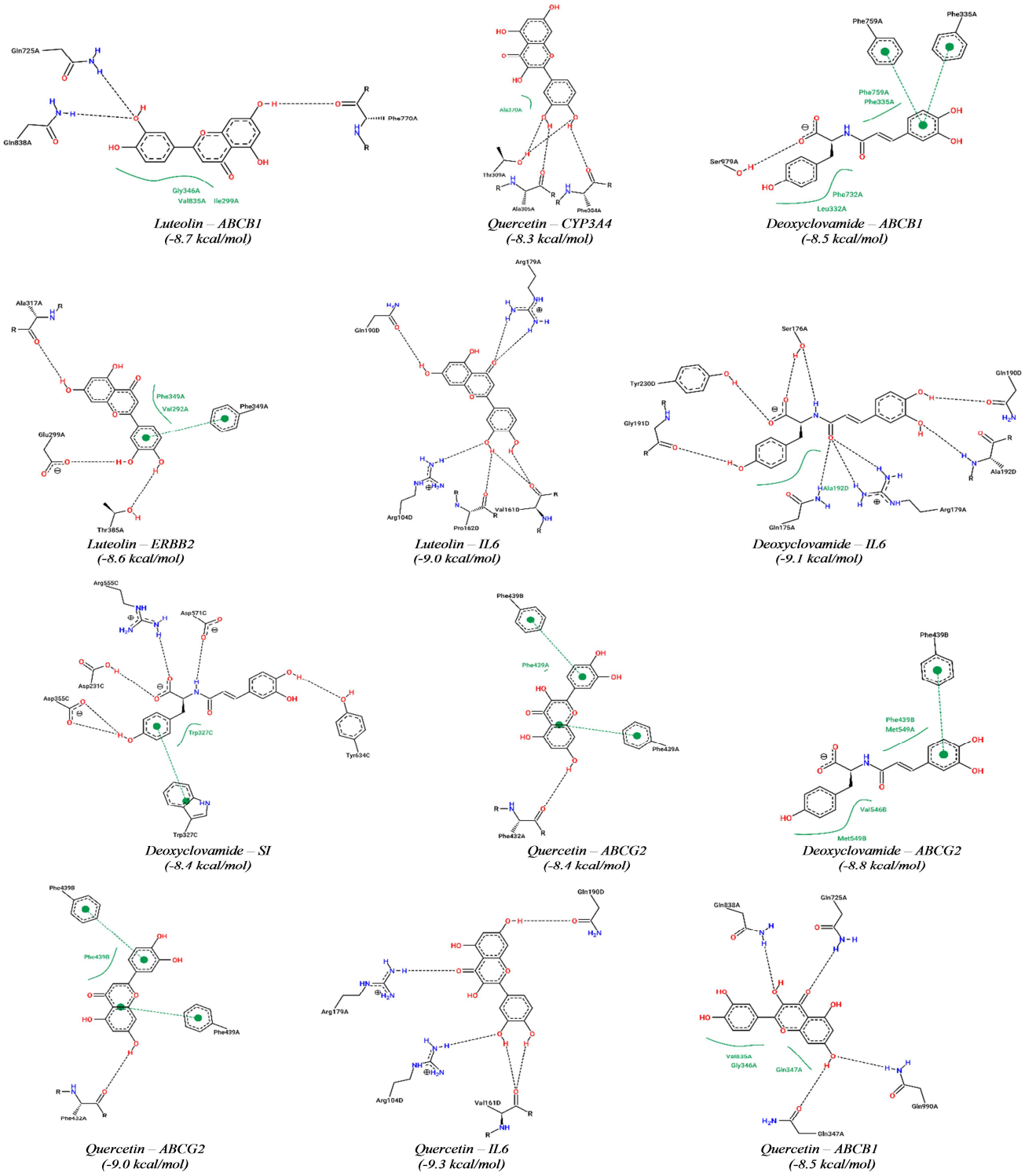


Figure 5. *2D analysis of the most relevant interactions between the bioactives Luteolin, Quercitin and Deoxyclovamide and the central therapeutic targets for T. cacao.*

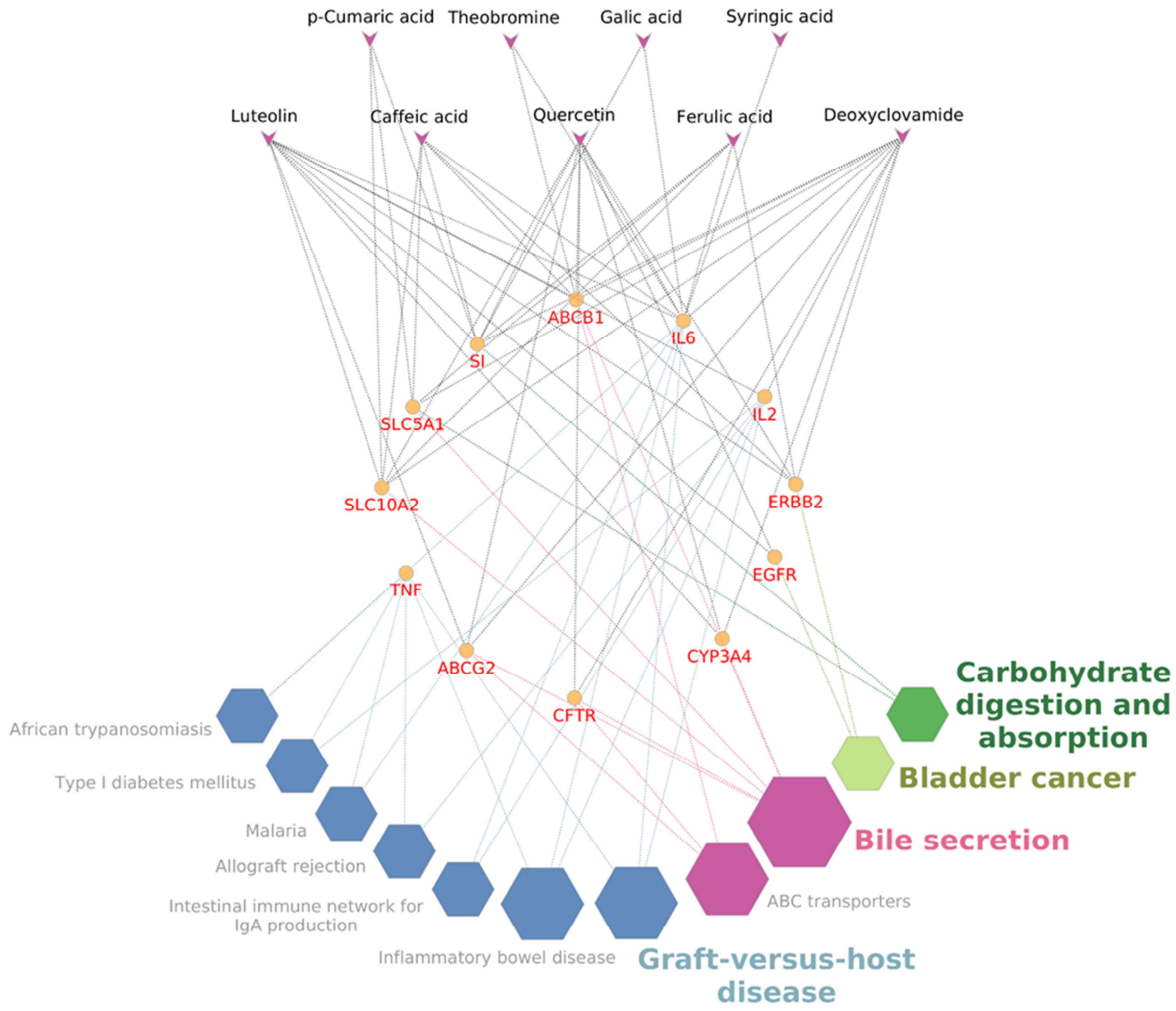


Figure 6. *Compound - target - pathway Network of T. cacao treating diarrhea. Purple V nodes: compounds of T. cacao with favorable human OB, IA, DL and binding energy ≤ -6.5 kcal/mol. orange ellipses: central therapeutic targets for T. cacao. Hexagons: signaling pathways inferred by ClueGO in Cytoscape. Larger hexagons and bold labels represent the most significant signaling pathways. The different colors in the hexagons represent the different functional groups.*

Table 4. *ADME and drug likeness analysis: Intestinal Absorption (IA), Oral Bioavailability (OB), drug likeness (DL).*

Compound	PubChem ID	IA	OB Score	DL
Orientin	5281675	Low	0.17	NO
Luteolin*	5280445	High	0.55	YES
Stearic acid*	*5281*	High	0.85	YES
Chlorogenic acid	1794427	Low	0.11	YES
Cyanidin 3 O galactoside	*441699*	Low	0.17	NO
Palmitic acid*	*985*	High	0.85	YES
Ferulic acid*	445858	High	0.85	YES
Procyanidin B1	11250133	Low	0.17	NO
Isoorientin	114776	Low	0.17	NO
Procyanidin B2	122738	Low	0.17	NO
Isovitexin	162350	Low	0.55	YES
Procyanidin C1	*169853*	Low	0.17	NO
Prunin	92794	Low	0.55	YES
Quercetin*	5280343	High	0.55	YES
Theobromine*	*5429*	High	0.55	YES
Caffeic acid*	689043	High	0.56	YES
Clovamide	6443790	Low	0.56	YES
Deoxyclovamide*	*14352555*	High	0.56	YES
Galic acid*	*370*	High	0.56	YES
Isoquercetin	*5280804*	Low	0.17	NO
p-Cumaric acid*	637542	High	0.85	YES
p-Hydroxybenzoic acid*	135	High	0.85	YES

Compound	PubChem ID	IA	OB Score	DL
Protocatechuic acid*	72	High	0.56	YES
Syringic acid*	10742	High	0.56	YES
Hyperoside	5281643	Low	0.17	NO
Linoleic acid	*5280450*	Low	0.17	NO
b-Sitosterol	222284	Low	0.55	YES
Campestanol	119394	Low	0.55	YES

IA: Intestinal Absorption. OB: Oral Bioavailability. "YES" when there is no more than 1 violation of the Lipinski Rules. Marked with * are the compounds potentially responsible for the antidiarrheal activity.

Table 5. *Binding energy of protein-ligand interaction (kcal/mol).*

Gen	PDB ID	Docking box *	Bioactives of *T. cacao* (Pubchem ID)						
			Luteolin (5280445)	Stearic acid (5281)	Palmitic acid (985)	Ferulic acid (445858)	Quercetin (5280343)	Theobromine (5429)	Caffeic acid (689043)
ABCB1	6COV	A	-8.7	-6	-6.4	-6.4	-8.5	-6.8	-6.6
EGFR	1IVO	B	-6.5	-4	-4	-5.2	-6.1	-4.7	-5.5
EGFR	1IVO	C	-7.3	-4.9	-5	-6	-7.2	-5.5	-6.1
EGFR	1IVO	D	-6.4	-4.1	-4.2	-4.9	-6.6	-4.7	-4.8
ERBB2	1S78	E	-8.6	-5.6	-5.8	-6.8	-7.9	-6.4	-6.8
IL2	1M49	F	-6.9	-4.1	-4.2	-5.7	-6.2	-5.1	-5.6
IL6	5FUC	G	-9.0	-5.4	-5.4	-6.8	-9.3	-6.5	-6.9
TNF	1TNF	H	-6.1	-4.9	-4.3	-5.1	-6.1	-5.3	-5.3
SLC5A1	7SL8	I	-4.5	-6	-5.6	-6.6	-4.7	-5.6	-6.8
SI	3LPO	J	-8.2	-5.6	-5.4	-6.6	-8.2	-4.8	-6.8
CYP3A4	1W0E	K	-8.3	-5.6	-5.6	-6.4	-8.3	-5.9	-6.4
CFTR	1XMI	L	-6.9	-4.2	-4.4	-5.3	-6.7	-5.8	-5.1
SLC10A2	3EM0	LL	-8	-5.9	-5.9	-6.8	-8.2	-5.9	-6.8
ABCG2	5NJ3	N	-9	-6.1	-6	-6.1	-8.4	-5.6	-6

Table 5. *Continued.*

Gen	PDB ID	Docking box *	Bioactives of *T. cacao* (Pubchem ID)					
			Deoxyclovamide (14352555)	Galic acid (370)	p-Cumaric acid (637542)	p-Hydroxybenzoic acid (135)	Protocatechuic acid (72)	Syringic acid (10742)
ABCB1	6COV	A	-8.5	-6.3	-6.4	-5.8	-5.9	-6.3
EGFR	1IVO	B	-5.9	-5.1	-5.1	-5	-5.3	-4.7
EGFR	1IVO	C	-7	-5.4	-5.8	-5.5	-5.5	-5.3
EGFR	1IVO	D	-5.7	-4.9	-4.9	-4.4	-4.8	-4.8
ERBB2	1S78	E	-8	-5.9	-6.3	-5.7	-6.2	-6.3
IL2	1M49	F	-6.8	-4.9	-5.7	-4.7	-5	-4.9
IL6	5FUC	G	-9.1	-6.5	-6.4	-6.3	-6.4	-6.5
TNF	1TNF	H	-6.4	-5.6	-4.9	-4.9	-5.4	-5.3
SLC5A1	7SL8	I	-6.5	-6.3	-7.1	-6.2	-6.4	-5.3
SI	3LPO	J	-8.4	-6.5	-6.5	-5.7	-6.1	-4.8
CYP3A4	1W0E	K	-8.4	-5.9	-6.3	-5.8	-5.8	-6.1
CFTR	1XMI	L	-7.1	-5.4	-4.9	-4.5	-5.2	-5.1
SLC10A2	3EM0	LL	-7.8	-5.8	-6.5	-6.2	-6.3	-5.6
ABCG2	5NJ3	N	-8.8	-5.5	-5.8	-5.2	-5.5	-4.9

*Related amino acids: A. Phe303, Ile306, Tyr307, Tyr310, Phe728, Phe336, Met69, Gln725, Phe983, Leu339, Phe343, Gln990, Ser344, Tyr953, Met68, Met949, Leu65, Ala871, Gln946, Glu875, Gln347, Trp232, Ile340, Met986, Ala987; B. Glu90, Leu98, Leu69, Gln16, Tyr45, Leu14; C. Phe357, Asp355, Val350; D. Gln384, Leu382, Phe412, Ile438; E. Thr290, Phe257, Val286, Pro294; F. Lys5, Arg38, Lys43, Tyr45, Phe42, Glu62, Leu72, Met39, Lys76; G. Phe78, Phe74, Arg179, Ar182, Arg30, Arg168; H. Gly40, Leu157, Ser52, Asn46; I. Gln457, Glu102, Trp291, Lys321, Asn78, Thr460, His83; J. His600, Ala576, Thr205, Thr204, Asp542, Asp443, Lys480, Phe450, Trp406; K. Tyr307, Leu211, Phe215; L. Gln493, Ser459, Phe650; LL. Tyr97, Leu90, Val74, Arg125, Trp49, Thr101, Ile92, Val83, Tyr14, Gly31, Tyr53, Gln51, Gln99; N. Phe432, Phe439; Met549, Val546, Ile543, Leu539.

4. Discussion

$MgSO_4$, an osmotic-acting laxative, has been reported to induce diarrhea by increasing the volume of intestinal content through the prevention of reabsorption of water [46]. In our study, $MgSO_4$ was used to establish an acute diarrhea model in mice, and the extract of *T. cacao* was able to reduce diarrheal stools. Intestinal motility also showed a tendency to decrease in groups receiving *T. cacao*, both comparable with loperamide as a reference drug, indicating its antidiarrheal activity. This agrees with the antidiarrheal effect reported in mice induced to diarrhea with castor oil [15]. Aquaporins (AQPs) 2 and 3 overexpression in both the apical and lateral mucosal epithelial cells in the colons of $MgSO_4$-induced diarrhea mice were reported.

Also, has been demonstrated that $MgSO_4$ as a laxative may increase the AQP3 expression level which caused the

transport of a large amount of water to the luminal side [47, 48]. AQPs have been involved in Bile Secretion signal pathway, one of the most important in our results and related to SLC5A1, CYP3A4, SLC10A2, ABCG2, and ABCB1 targets. Bile is a vital secretion, essential for digestion and absorption of fats and fat-soluble vitamins in the small intestine. Diarrhea is accompanied by malabsorption of sugars, nitrogen, fats, and micronutrients; and in a bile acid malabsorption (BAM), an excess of bile acids in the colon results in diarrhea [49]. Also, significantly altered levels of AQP3, 7, and 8, suggest that these AQPs may be involved in the pathogenesis of bile acid-induced diarrhea [50]. Therefore, the inhibition of transport via AQPs could be explain the observed antidiarrheal effect in this study via antisecretory activity, preventing the transport of water back into the lumen of the intestine.

It has been demonstrated that $MgSO_4$ promotes the release of cholecystokinin (CCK) from the duodenal mucosa, which increases the secretion and motility of the small intestine and thereby prevents the reabsorption of sodium, chloride, and water [51, 52]. The higher level of CCK was positively correlated with abdominal pain, therefore might be involved in the pathogenesis of diarrhea-predominant irritable bowel syndrome (IBS-D) [53]. Although CCK and its receptor CCKAR do not appear in our analysis as therapeutic targets for *T. cacao*, they should be considered as a potential pathway of antidiarrheal activity.

Our results show that Solute Carrier Family 5 Member 1 (SLC5A1) and Sucrase-Isomaltase (SI) are two of the main therapeutic targets in the treatment of diarrhea with *T. cacao*. These genes are key in the Carbohydrate digestion and absorption signaling pathway. Carbohydrate malabsorption is known to cause diarrhea [54] and is related to SLC5A1 inhibition causing a reduction in the Na+ gradient combined with Cl- secretion triggered by Ca+ accumulation [55]. Likewise, SI deficiency causes persistent bloating and diarrhea [56, 57]. The activation of SI and SLC5A1 by some bioactives of *T. cacao* (luteolin, quercetin, Deoxyclovamide, ferulic acid, and p-Cumaric acid) could then be related to an antispasmodic activity, and be related to reducing of diarrhea, through a significant increase in plasma levels of glucagon-like peptide-1 (GLP-1) [58], which, is involved in maintaining glucose, and has a beneficial role in the gastrointestinal tract [59].

ABC transporter signal pathway is another metabolic pathway that explains the antidiarrheal activity observed and associated with the activity of ABCB1, CFTR, and ABCG2 genes, all members of the ATP-binding cassette (ABC) transporter family and targeted by several *T. cacao* bioactives. In the case of P-glycoprotein (P-gp) transporter, encoded by the ABCB1 gene, it is expressed in intestinal cells, renal proximal tubule cells, and endothelial cells of brain capillaries normal tissues where it functions as a defense mechanism against potentially toxic substances ingested with the diet and protection of intestinal epithelium against pathogens [60]. A lower P-gp expression decreases the protection from the accumulation of toxic materials within the intestine [61], so the activation of this membrane transporter by compounds Luteolin, Stearic acid, Palmitic acid, Ferulic acid, and Quercetin, whose molecular docking results reveal its potential as a substrate for this protein, could be related to the therapeutic effect via enhanced intestinal absorption.

Graft-versus-host disease (GVHD) and Inflammatory Bowel Disease (IBD) were two of the most significant signaling pathways in the KEGG enrichment analysis. Intestinal GVHD involves the activity of inflammatory cytokines (IL) and Tumor Necrosis Factor (TNF) [62] and its clinical manifestations are nausea, anorexia, vomiting, high volume of watery diarrhea, intestinal bleeding, or abdominal pain [63]. Regarding IBD, imbalances in proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interferon-γ, (IFN-γ), interleukin (IL)-1, IL-6, and IL-12 and anti-inflammatory cytokines including IL-4, IL-10, and IL-11 are involved [64].

Therefore, the inhibition of IL2 and IL6 by luteolin, quercetin, and Deoxyclovamide whose ADME, Drug Likeness, and molecular docking analysis suggest that this compound could be related to the inhibition of the adverse effects of GVHD and IBD, may explain the antidiarrheal effect of *T. cacao*. Likewise, the inhibition of inflammatory cytokines by Deoxyclovamide would result in inhibition of Nitric Oxide (NO) activity with the consequent reduction of the laxative effect observed in the animal model [65, 66]. This agrees with the anti-IBD effects of phytochemicals that has been associated with modulating the levels of tumor necrosis factor α (TNF-α), interleukin IL-6, and inducible nitric oxide synthase [64].

Our results show that the signaling pathways mediated by the EGFR and ERBB2 receptors turn out to be among the most regulated in the treatment of diarrhea with *T. cacao*, as they are therapeutic targets for Luteolin, Quercetin, and Deoxyclovamide. EGFR and ERBB2 are receptors for an epidermal growth factor (EGF), an endogenous substance for tissue repair and cell protection secreted by the salivary glands, liver, pancreas, kidney, and intestines. EGF shows stimulation of electrolyte and nutrient absorption and the protective effects of gastrointestinal mucosa [67], via binding to its receptors (EGFR, ERBB2), whose activation depends on the dimerization of these and the consequent activation of signaling pathways related to gastrointestinal disorders (Figure 3d), such as PI3K-Akt whose inhibition causes diarrhea, nausea, etc. [68], JAK-STAT whose inhibition of the key non-membrane enzyme spanning protein tyrosine kinase activity (JAK) causes diarrhea [69], and MAPK which is a key regulator of inflammatory bowel disease, especially necrotic enteritis [70]. Another pathway regulated by EGFR and ERBB2 is the Bladder Cancer signaling pathway with a statistically significant weight in our results [71]. In patients with Crohn's Disease (CD) which is included in Inflammatory Bowel Disease (IBD), a trend toward an increased risk of Bladder Cancer was evident.

Our results show several compounds present in *T. cacao* have a potential antidiarrheal effect and whose activity has already been reported, and those that we can highlight are

Quercetin, Luteolin, and Ferulic acid. The antidiarrheal activity of Quercetin via inhibition of the activity of cyclooxygenases and lipoxygenases, as well as a calcium channel blocker activity is already known [72]. In recent work, quercetin has been reported for its probable ability to increase the microbial activity of the intestinal mucosa of mice and reduce the microbial activity of the intestinal contents through multiple targets, thereby achieving the effect of treating diarrhea [73]. Regarding, Luteolin it was reported that could increase the concentration of Na+ and K+ by upregulating the activity and gene level of Creatine Kinase (CK) and Na+/K+-ATPase, and could also decrease the volume and weight of small intestinal contents to exert antidiarrheal activity [74]. In a recent study, it's reported that quercetin, luteolin, and ferulic acid could be used to treat disorders such as diarrhea by regulating the contractile response via the L voltage-gated calcium ion channel, M3 muscarinic receptor, or other proteins, such as phospholipase C (PLC), to repolarize the membrane action potential [20]. This raises the hypothesis that the antidiarrheal activity of *T. cacao* is probably associated with an antisecretory and antimotility action.

Most herbal extracts are known to exhibit anti-diarrheal activity through one or more of the following mechanisms: anti-secretory activity, enhanced intestinal absorption, anti-motility or anti-peristaltic effect, anti-microbial effect, and anti-spasmodic action. These effects have been well demonstrated in several *in vivo* studies [13] and are related to our results.

5. Conclusion

The current study used an *in vivo* approach followed by an *in silico* evaluation to investigate the antidiarrheal activity of the ethanolic extract of *Theobroma cacao* seed. We reported according to *in vivo* tests, *T. cacao* seed reduces the diarrheal effects of $MgSO_4$ and no adverse effects were reported in the toxicological study. *In silico* analysis allowed us to identify the Carbohydrate digestion and absorption, Bladder cancer, Bile secretion, and Graft-versus-host disease signaling pathways, as the most significant, regulated by the therapeutic action of quercetin, luteolin, and deoxyclovamide on the ABCB1, ABCG2, CYP3A4, EGFR, ERBB2, IL6, SI, and SLC10A2 proteins, considered as the central therapeutic targets of the antidiarrheal activity of *T. cacao*. This study provides a preliminary hypothesis for mechanisms of the antidiarrheal effect of *T. cacao* seed and should guide future experiments, to elucidate the therapeutic mechanisms of *T. cacao*, and also guide drug discovery to treat diarrhea.

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