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Evaluation Of Anti-Cholelithiatic Activity Of Phenolic Acids: In-Silico And In-Vitro Approach

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Abstract
Worldwide, cholelithiasis, or gallstone disease, is a prevalent gastrointestinal disease. It is marked by biliary colic in the bile ducts and is linked to a high rate of morbidity and death. Gallstone disease has a complicated pathogenesis.Cholecystectomy(removal of gall bladder) is the only treatment for cholelithiasis. The aim of this study is to evaluate the potential of phenolic acid (vanillic acid) and safe alternative for gallstone dissolution. In this research, we employed computational modeling techniques (in-silico) using ligand -protein approach to predict the interactions between phenolic acids and key biomolecular targets implicated in cholelithiasis formation. The *In-silico* and *In-vitro* methods are used to evaluate phenolic acids for anti-cholelithiatic activity. Docking were performed with several receptors such as Vascular endothelial growth factor receptor 2 (VEGFR-2),Tumour Necrosis Factor alpha(TNFα), Peroxisome proliferator-activated receptor gamma (PPARγ), Interleukin-6 (IL-6), and Hydroxymethy lglutaryl-CoA (HMG CoA) and phenolic acids such as chlorogenic acid,coumaric acid,Caffeic acid ,Ferulic acid and Vanillic acid .Based on high binding affinity from *In-silico* method,*In-vitro* study was performed.*For In-vitro* anti-cholelithiatic method,cholesterol human gall stone were collected along with human bile from the hospital.The treatment was performed between standard drug Ursodeoxycholic acid (UDCA) 2mg/ml and test drug (Vanillic acid-2mg/ml,7mg/ml) which was incubated for 4 weeks. Weight of gall stone and the amount of cholesterol release in the bile before and after treatment were evaluated.The results of the *In-silico* show that vanillic acid has high binding affinity when compared to other phenolic acids .From *In vitro* study , the vanillic acid exert decrease in the weight of cholesterol gall stone and increase in the amount of cholesterol released from gall stone.Vanillic acid also shows morphological alterations in gallstone. The present study provides an overview of the pharmacological and clinical potential of the phenolic acids (Vanillic acid).Further ,In-vivo studies should be carried for understanding the mechanism or pathway behind vanillic acid over anti-cholelithiatic activity.

Introduction
Çerçi C, et al,showcased that the gallbladder is a pear shaped organ. It connects to the biliary tract, which is sometimes referred to as the biliary tree or biliary system at times. The network of ducts that enters the small intestine from the liver, gallbladder, and pancreas is known as the biliary system [1].The components of liver which is classified as intrahepatic that are found inside the liver, while the components classified as extrahepatic are found outside the liver .The gallbladder, which is part of the extrahepatic biliary system, is where concentrated and stored bile is kept. The liver secretes bile, a fluid necessary for the digestion of fat, the excretion of cholesterol, and possibly even antibacterial activity [2,3].
The gallbladder is located in the upper right quadrant of the belly and is joined to the liver's underside at the gallbladder fossa. Through the cystic duct, it is connected to the remainder of the extrahepatic biliary system.The gallbladder receives and holds the bile that the liver produces until it is needed for digestion [4,5].

Gallstones:
Bile deposit that has solidified is called a gallstone and the process is known as Cholelithiasis, the flow of bile is interrupted, and imbalances (biliary stasis) in the components of bile can lead to the formation of stones. Which is formed in gallbladder and it is composed of bile, bilirubin and cholesterol, resulting from substantially elevated bilirubin or cholesterol levels, it is asymptomatic in most cases, gallstone disease is solely linked with cholesterol gallstones that cause symptoms or even complication. [6,7].

Types of Gallstones:
Brown pigment stone:[8]
Location: Infected intrahepatic or extrahepatic ducts.
Chemical composition: Unconjugated calcium bilirubin crystals, calcium palmitate, monomeric calcium Bilirubinate, Stearate and mucin (glycoprotein).
Morphology: Brown stones tend to be single or discrete sets and to have a soft, soap-like or greasy consistency owing to the presence of fatty acid salts liberated from biliary lecithin's (phosphatidylcholine) by bacterial phospholipases.
% Radiodensity (radio- opaque/radio lucent): Lucent-100%

Black pigment stones:[8]
Location: Sterile gallbladder bile.
chemical composition: Calcium bilirubinate polymer (bilirubin pigment), Calcium phosphate, mucin and cholesterol.
Morphology: Generally black stones are small (sphere shaped), abundant, and fragile to the contact.
% Radiodensity (radio- opaque/radio lucent): Radio-opaque: 60%-85%.

Mixed cholesterol stones:[8]
Location: common bile ducts.
Chemical composition: Cholesterol, calcium phosphate, calcium carbonate, calcium salts and bile pigment.
Morphology: varying hues, irregular surface, round to oval shaped calculi and multifaceted.
% Radiodensity (radio- opaque/radio lucent): Radio-opaque : 10%-20% similar to cholesterol stones.

Pure cholesterol stones:[8]
Location: Gall bladder, bile ducts.
Chemical composition: 80%-100% cholesterol (cholesterol monohydrate).
Morphology: Pure cholesterol stones are pale yellow in color and they are ovoid and solid which has hard and granular surface.
% Radiodensity (radio- opaque/radio lucent): Radio-opaque: 10%-20%.

Pathophysiology:
The pathogenesis of cholelithiasis involves a complex interplay of genetic, environmental, and metabolic factors, including bile composition, gallbladder motility, nucleation, stone growth, and genetic and environmental influences. Understanding these mechanisms is crucial for the prevention, diagnosis, and management of gallstone disease.
Cholelithiasis , or the formation of gallstones, is primarily related to an imbalance in the components of bile. The pathophysiology involves several keyfactor:[9]

1. Bile Composition:
Gallstones form when there is an imbalance in the component of bile, which includes cholesterol , bile salts and bilirubin.
2. Gallbladder Motility:
Proper function of the gallbladder is essential for bile concentration and stone prevention. If the gallbladder doesn't empty completely or efficiently (e.g., due to reduced motility), bile can become concentrated, which increases the risk of stone formation.
3. Biliary Stasis:
Prolonged stasis or inactivity of the bile in the gallbladder can lead to the formation of stones. Conditions that lead to decreased gallbladder contraction or altered bile flow can contribute to stasis.

4. Inflammation:
Chronic inflammation of the gallbladder lining (cholecystitis) can alter bile composition and gallbladder motility, promoting stone formation.
Phenolic acids are found in a wide variety of naturally occurring materials, such as fruits, vegetables, whole grains, nuts, and seeds. Phenolic rings are defined by the presence of one or more hydroxyl groups connected to them. Phenolic acids can be categorized into two primary classes based on their structural makeup: hydroxybenzoic acids and hydroxycinnamic acids.
The body can be protected from harm caused by oxidative stress by phenolic acids' strong antioxidant capabilities, which allow them to scavenge free radicals. Furthermore, by immune response modulation and pro-inflammatory mediator production suppression, they demonstrate anti-inflammatory actions. Moreover, phenolic acids exhibit antibacterial activity against a range of harmful microorganisms, which suggests that they could find use in food preservation and pharmaceutical projects. [10,11].

Materials & Methods
Using a ligand-protein approach, receptors such as Vascular endothelial growth factor receptor 2 (VEGFR-2),Tumour Necrosis Factor alpha(TNFα), Peroxisome proliferator-activated receptor gamma (PPARγ), Interleukin-6 (IL-6), and Hydroxymethylglutaryl-CoA (HMG CoA) were docked with several phenolic acids such as Caffeic acid,Cholorogenic acid,Coumaric acid,Ferulic acid,Vanillic acid [12,13].

Insilico screening:
Docking:
The RCSB Protein Data Bank (PDB) was used to download the target protein in ".pdb" format for analysis.

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Ligand Selection :
Compound Library: Compile a library of compounds known or predicted to have anti- cholelithiatic properties. This may include phenolic acids, as indicated by your research interest, or other natural or synthetic compounds.

Preparation of protein:
Clean the protein structure by removing water molecues and any bound ligands or cofactors not involved in the docking. Add hydrogen atoms and assign appropriate charges.

Preparation of ligand:
Prepare the ligands for docking by optimizing their structures, predicting their charge states, and generating 3D conformations. The structures were downloaded from Protein Data Bank website (https://www.rcsb.org/?ref=nav_home) and saved in PDB format keeping a note on the parameters like mutation, resolution and method.

Visual screening and docking analysis:
Docking software was used to analyze and screen the chosen ligand with its optimized structure on various target proteins, including PPARγ(2ATH), IL-6(1-P9M), TNFα(2AZ5), HMG CoA(1R31), and VEGFR-2 (3V2A). The binding affinity results were obtained after docking.

In-vitro anti-cholelithiatic activity:
Human bile and gallstones (cholesterol stones) were taken from the hospital (SRM Medical College and Hospital, Kattankulathur , Ethics Clearance Number:8777/IEC/2024) for use in *In vitro* experiments. Every gall bladder stone was dried in an oven at 45 degrees Celsius, and its dry weight was recorded using an airtight electronic balance. Following that, each gallstone was cultured in human bile and treated independently for four weeks at 37 degrees Celsius using a combination of standard drug (ursodiol, 2 mg/ml) and vanillic acid at varying dosages (2 mg/ml, 7 mg/ml). The gallstones were removed during incubation, dried, and then their dry weights were recorded once a week. Weekly measurements of the quantity of cholesterol released by stones were also made using the autoanalyzer. The effects of the test drugs (vanillic acid) and the standard drug on gallstones were observed by comparing the weight of the gallstones and the amount of cholesterol produced before and after treatment.

Results
As the Table *1* demonstrate the docking data which shows possible interactions between a variety of receptors involved in inflammation, angiogenesis, metabolic control,cholesterol metabolism and five phenolic acids: Caffeic acid,chlorogenic acid, vanillic acid, coumaric acid, and ferulic acid.

Ligand	PPARγ(2ATH)	IL-6(1-P9M)	TNFα(2AZ5)	HMGCoA(1R31)	VEGFR- 2(3V2A)
Caffeic acid	-2.1	-4.4	-7.2	-6.8	-7.0
Cholorogenic acid	-1.4	-5.1	-8.6	-8.6	-7.3
Coumaric acid	-0.8	-4.1	-7.3	-6.7	-7.0
Ferulic acid	-1.5	-4.6	-7.3	-6.4	-7.3
Vanillic acid	-2.4	-5.3	-9.4	-8.8	-8.4

Table 1: Molecular docking of Phenolic acids

Vanillic acid displayed promising affinity towards PPARγ(-2.4), TNFα (-9.4) and HMG CoA (-8.8), indicating its potential dual roles in anti-inflammatory activity and cholesterol biosynthesis modulation. Furthermore, all ligands showed affinity towards VEGFR-2, with Chlorogenic acid, Coumaric acid, and vanillic acid demonstrating similar docking scores (-7.3), implicating their involvement in angiogenesis regulation. Since vanillic acid shows promising binding affinity, it was selected as drug for in-vitro activity. These findings underscore the therapeutic potential of these phenolic acids across a spectrum of disease conditions, although further experimental validation is warranted to elucidate their precise mechanisms of action and clinical relevance.

ANTI-CHOLELITHIATIC INVITRO ACTIVITY
Effectiveness in Dissolving Cholesterol Gallstones(Figure *01*):
In an *In-vitro* model, the study demonstrates that vanillic acid significantly dissolved cholesterol gallstones within 21 days of treatment. The weight of cholesterol gall stone was decreased in comparison with standard drug(ursodiol) and test drug(Vanillic acid).Morphological changes were observed such as colour change of cholesterol gall stone from yellow to white colour.

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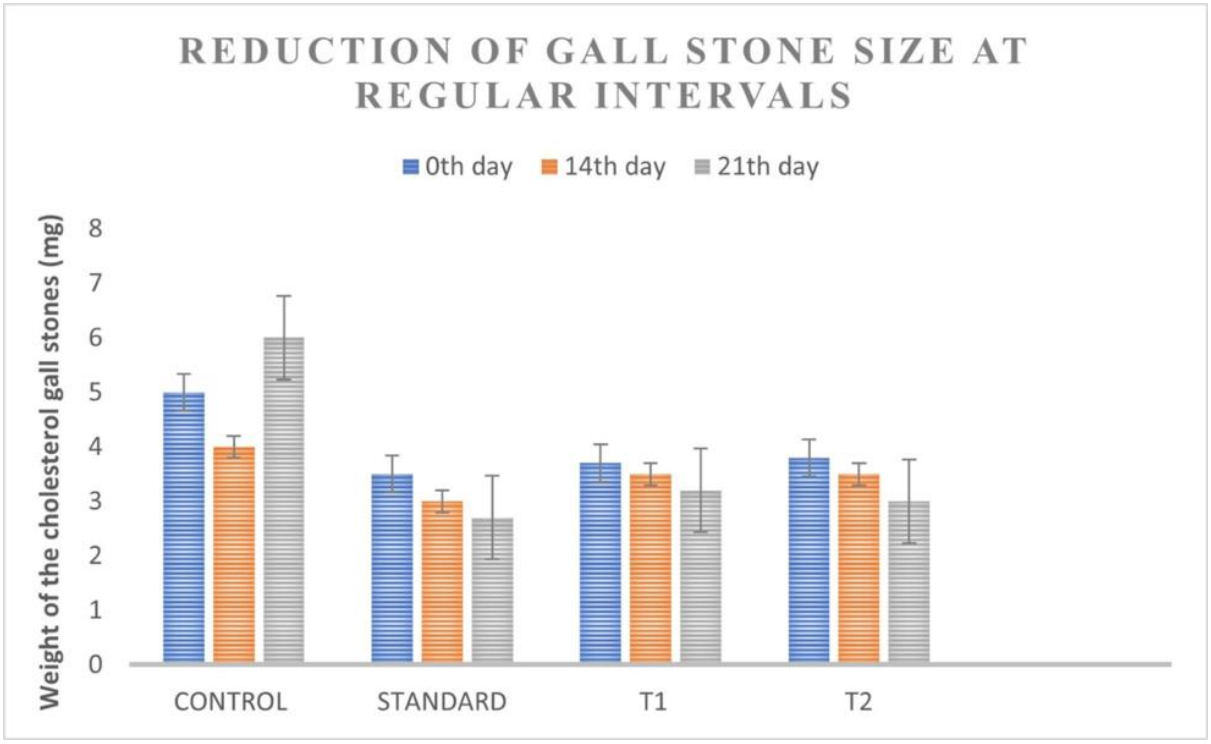

Figure 1: Reduction of Gall stone size at regular intervals

Changes in Morphology and Cholesterol Release(Figure *02*):
Vanillic acid treatment results in increase in cholesterol release from cholesterol gallstones, which are significant markers of anti-cholelithiatic activity. It is possible that prior studies on anti-cholelithiatic medicines evaluated the effectiveness of various agents in dissolving gallstones by measuring cholesterol release, morphological alterations.
In treatment of test drug ,7mg/ml shows significant reduction of gall stone size and increase in the cholesterol release which was comparison with standard drug ursodiol(2mg/ml).

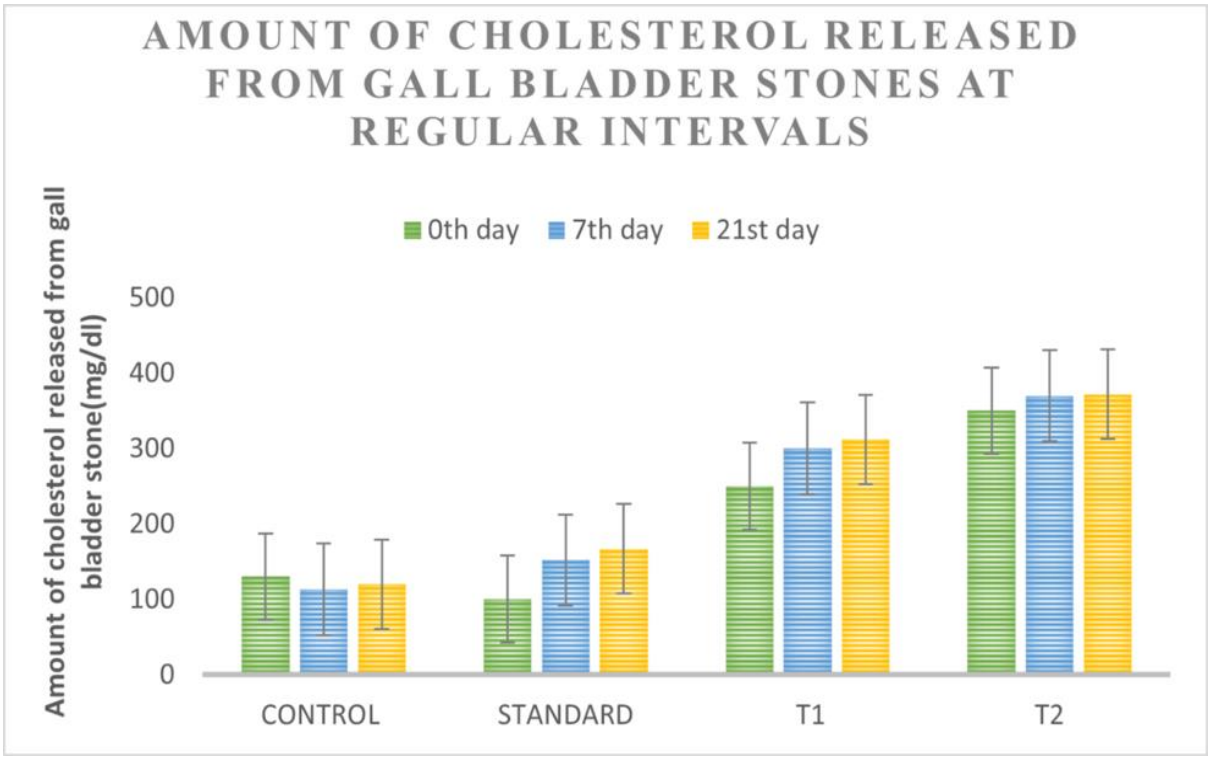

Figure 2: The quantity of cholesterol that is periodically released from gallstones

Discussion

Cholesterol gallstone is emerging worldwide, but there are no valuable therapeutic sources.The current treatment for systematic cholesterol gall stone is either oral litholysis (Ursodeoxycholic acid (UDCA) -When the stone is below 5 mm) or laproscopic surgery(cholecystectomy will be performed). The phenolic acid is already proven to have various activities such as anti-oxidant, anti-inflammatory, anti-microbial, cardiovascular protection, anti-cancer, neuroprotective, and anti-diabetic activity. The current study was initiated with *In-silico* molecular docking of phenolic acids such as caffeic acid,Cholorogenic acid,Coumaric acid,Ferulic acid and Vanillic acid with the PPARγ(2ATH) ,IL-6(1-P9M),TNFα(2AZ5) HMGCoA(1R31) and VEGFR- 2(3V2A) as receptors using ligand-protein approach.While HMG-CoA reductase directly influences cholesterol levels, VEGFR-2 may have an impact on liver function and bile production, and PPARγ, IL-6, and TNFα can influence cholesterol gallstone formation through their effects on inflammation and cholesterol metabolism.

Vanillic acid shows better binding energy PPARγ(-2.4),IL-6(-5.3),TNFα(-9.4), HMG-CoA(-8.8),VEGFR- 2(-8.4) among different types of phenolic acids. HMG-CoA plays a important role in anti-cholelithiatic activity by regulating cholesterol levels in the body . HMG-CoA reductase is responsible for controlling the formation of cholesterol, an essential component of gallstones, by facilitating the conversion of HMG-CoA to mevalonate. By blocking this enzyme, cholesterol production is decreased, which lowers bile cholesterol levels and lowers the chance of cholesterol gallstone development. HMG-CoA also has an impact on the formation of bile acid, which preserves the solubility of cholesterol in bile. As a result, inhibiting HMG-CoA can stop gallstones from forming by lowering cholesterol and changing the makeup of bile.Vanillic acid has the ability to affect HMG-CoA and TNFα. Because of its anti-inflammatory properties, illnesses linked to chronic inflammation may benefit from lowered TNF α levels. Its effects on lipid metabolism may also include modulating the activity of HMG-CoA , which lowers cholesterol and lowers the risk of cholesterol gallstones. Hence ,Vanillic acid was performed for the anti-cholelithiatic dissolution activity by an *In vitro* study. Human gallstones were used in this study and treated with the vanillic acid for 4 weeks. During the study ,cholesterol gall stone was completely dissolved after 21 days of treatment .The morphological changes were observed. The selection of vanillic acid for *In-vitro* testing is justified by its promising potential in cholesterol-modulating activities. However, while *In-silico* findings are promising, they serve as a preliminary step. Experimental validation through *In-vitro* assays and subsequent *In-vivo* studies is crucial to confirm these interactions and to better understand the mechanisms underlying the observed effects. The efficacy and safety of vanillic acid in clinical settings must be evaluated before it can be considered a viable therapeutic option.

Conclusions:

Vanillic acid shows anticholelithiatic properties against gall bladder stones, especially cholesterol gallstones. The study suggests that the vanillic acid may facilitate the dissolution of gallstones by increasing cholesterol release and causing morphological changes that enhance their fragility. However.,previously anti-cholelithiatic activity of vanillic activity were not attempted.While these *In silico* and *In-vitro* findings are promising, they serve as a preliminary step.
Experimental validation through *In-vitro* assays and subsequent *In-vivo* studies is crucial to confirm these interactions and to better understand the mechanisms underlying the observed effects. The efficacy and safety of vanillic acid in clinical settings must be evaluated before it can be considered a viable therapeutic option.The results show that vanillic acid has potential as a safe and natural option for gallstone dissolving; nevertheless, more studies are required to confirm its effectiveness and safety in human patients.

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