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Ethanol extract of Celery (Apium graviolense) dissolved bilirubin gallstones *in vitro*

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ABSTRACT

Background: Oral litholysis is the medical alternative to the surgical treatment of cholelithiasis. While it is modestly effective against the cholesterol type, it is completely of no effect against the bilirubin (or pigment) type. Whole celery plant is used in ethnomedicine for gallstone-related diseases and could be a source of new gallstone dissolving agents with possible bilirubin stone-dissolving capabilities.

Objectives: This work was aimed at investigating the potential dissolution capacity of an ethanol extract of whole celery plant on bilirubin gallstones *in vitro.*

Methods: Gallstones obtained from a female sickle cell patient were collected from the department of surgery, Lagos University Teaching Hospital (LUTH) Lagos, Nigeria, and confirmed bilirubin gallstones by morphological and UV spectrophotometric analyses. The dissolution capacity of an ethanol extract of celery on the gallstones was evaluated in vitro by stirring three separate Pre-weighed gall stones in 1 % w/v aqueous solution of the crude extract over 72 hours, checking the gall stones for weight reduction every 10 minutes for 2 hours; hourly up to 5 hours and then 24 hourly from 24 to 72 hours. Average weight reduction % was calculated at each point of measurement and compared, using t-test, with that of the control experiment in which an equal volume of distilled water was used in place of the extract suspension.

Results: The ethanol celery extract was found to cause a weight reduction % that was significantly higher than that of the negative control at each point of measurement, $p < 0.001$.

Conclusion: This experiment has shown that ethanol extract of celery has bilirubin gallstone dissolving capacity and could therefore be further explored for the discovery of new gallstone dissolving agents with possible bilirubin gallstone-dissolving capacity.

Key words: Gallstone disease, Bilirubin cholelithiasis, Celery, Gallstone Oral litholysis

Extrait d'éthanol de céleri *(Apiumgraviolense)* de calculs biliaires de bilirubine bilirubines dissous *in vitro*

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RESUME

Contexte: La litholyse orale est l'alternative médicale au traitement chirurgical de la lithiase biliaire (cholélithiase). Bien qu'elle soit modérément efficace contre le type de cholestérol, elle est complètement sans effet contre le type de bilirubine (ou pigment). LaPlante entière decéleri est utilisée en ethnomédecine pour les maladies liées aux calculs biliaires et pourrait être une source de nouveaux agents de dissolution des calculs biliaires avec des capacités possibles de dissolution des calculs de bilirubine.

Objectifs: Ce travail vise l'étude de la capacité de dissolution potentielle d'un extrait d'éthanol d'une plante entière de céleri sur des calculs biliaires de bilirubine *in vitro*.

Méthodes: Des calculs biliaires obtenus auprès d'une patiente drépanocytaire ont été collectés au département de chirurgie du LagosUniversityTeachingHospital (centre hospitalier universitaire, LUTH) de Lagos, au Nigéria, et des calculs biliaires de bilirubine confirmés par des analyses morphologiques et spectrophotométriques UV. La capacité de dissolution d'un extrait éthanoïque de céleri sur les calculs biliaires a été évaluée *in vitro* en agitant trois calculs biliaires pré-pesés séparés dans une solution aqueuse à 11 % w/v de l'extrait brut sur 72 heures, en vérifiant les calculs biliaires pour la réduction de poids tous les 10 minutes pendant 2 heures ; toutes les heures jusqu'à 5 heures, puis toutes les 24 heures de 24 à 72 heures. Le pourcentage moyen de réduction de poids a été calculé à chaque point de mesure et comparé, en utilisant le test t, à celui de l'expérience témoin dans laquelle un volume égal d'eau distillée a été utilisé à la place de la suspension de l''extrait.

Résultats: L'extrait d'éthanol de céleri s'est révélé provoquer une réduction de poids en% qui était significativement plus élevée que celle du témoin négatif à chaque point de mesure, $p < 0,001$.

Conclusion: Cette expérience a montré que l'extrait d'éthanol de céleri a une capacité de dissolution de calculs biliaires de bilirubine et pourrait donc être exploré davantage pour la découverte de nouveaux agents de dissolution de calculs biliaires avec une capacité possible de dissolution de calculs biliaires de bilirubine.

Mots-clés: maladie biliaire, lithiase biliaire de la bilirubine, céleri, litholyse orale des calculs biliaires

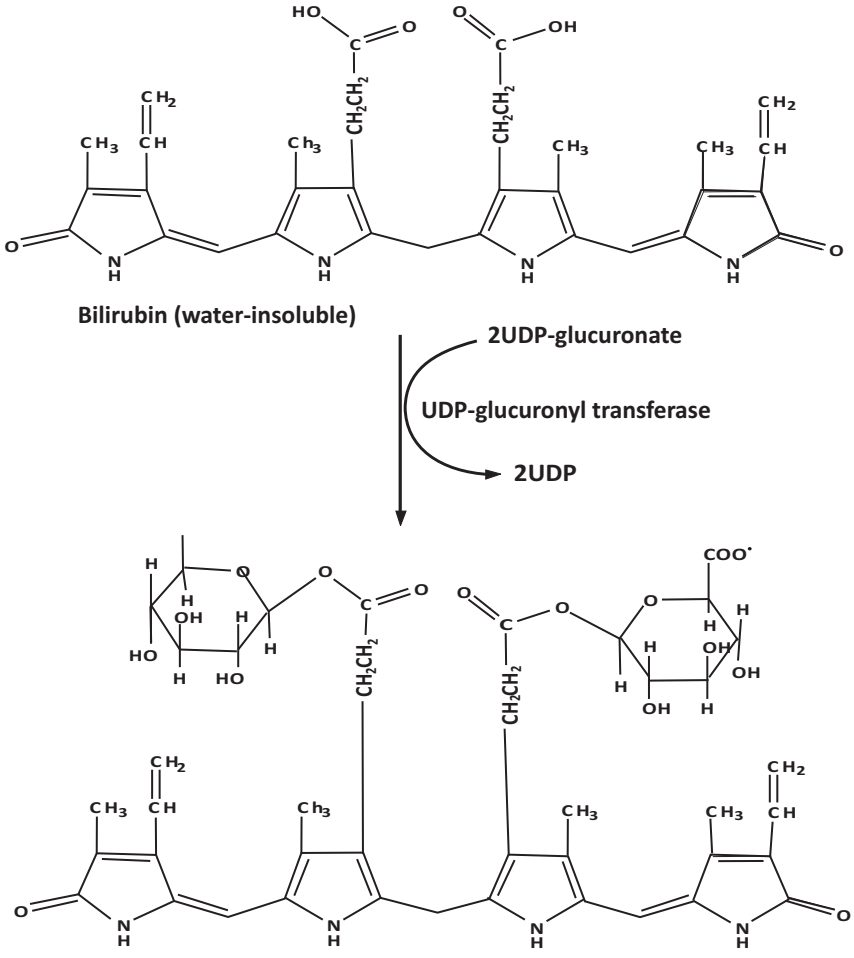
INTRODUCTION

Cholelithiasis or gallstone disease occurs as a result of precipitation of cholesterol and/or bilirubin components of bile, forming stones that could, at the very least, block the orifice of the bile duct to cause right upper abdominal pains or lead to much more severe conditions including acute biliary pancreatitis, choledocholithiasis, cholangitis, gallstone ileus and gallbladder cancer.[1-4]

Relative cholesterol/bilirubin concentrations have been the basis of classification of gallstones and, hence, of the gallstone disease by various authorities, the most widely accepted classification being the one by the National Institute of Health (NIH) of the United States of America, which primarily classifies gallstones into cholesterol and pigment stones, and further sub-classifies pigment stones into black and brown stones. By this system, gallstone is cholesterol stone if it contains greater than 70% w/w cholesterol; pigment stone if it contains less than 30% w/w cholesterol and mixed stone if it contains between 30% w/w and 70% w/w cholesterol.[5-6]

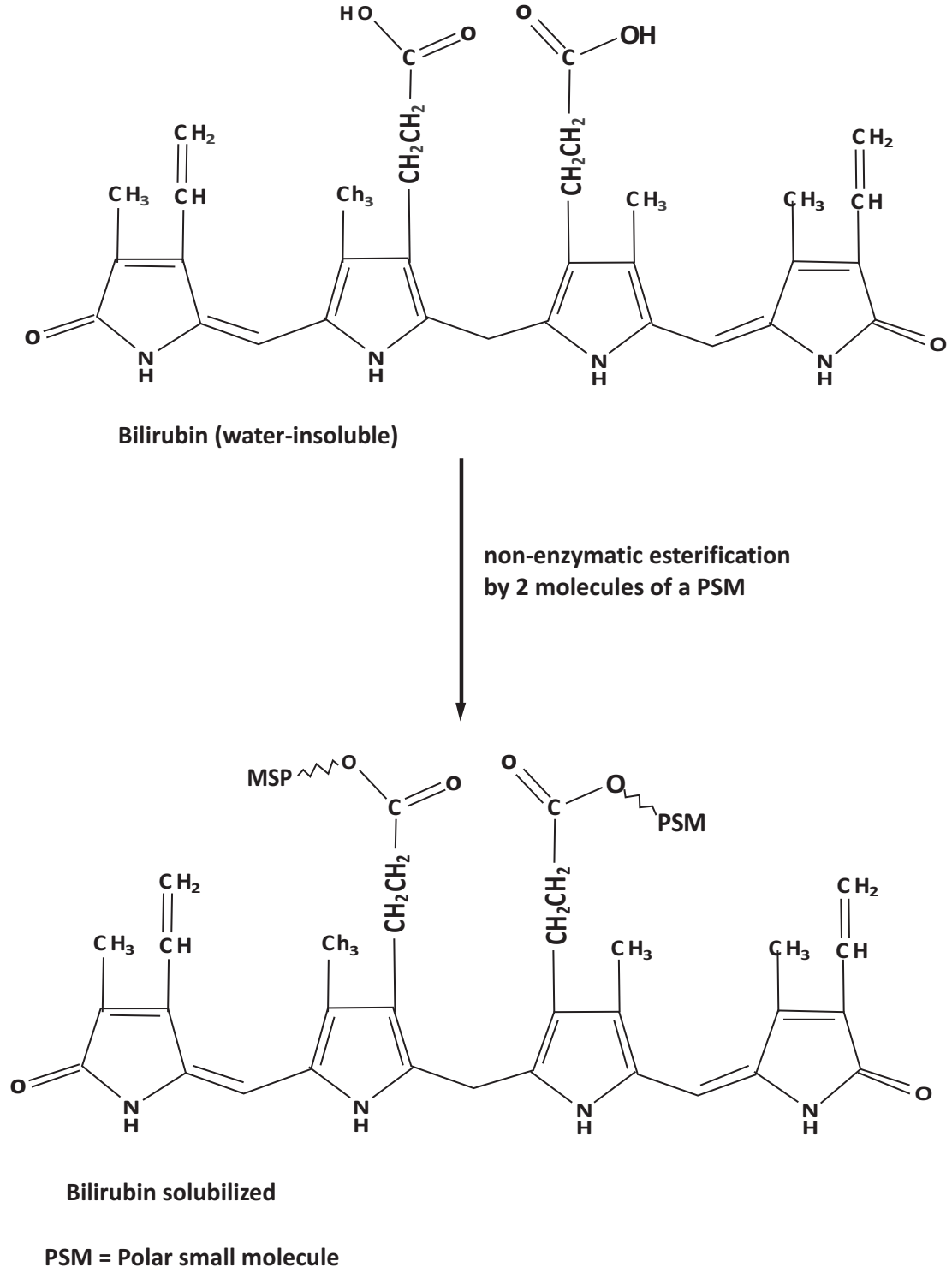
Cholecystectomy (open or laparoscopic) is the current treatment of choice for cholelithiasis. It nevertheless has the demerits of long recuperation time, post-surgical complications and high surgery cost.[7-8] Medical treatment by oral litholysis using bile acids is a very limited alternative in that it has the disadvantages of gastrointestinal disturbances, high rate of recurrence, and requirements for long duration of drug use. Furthermore, the available two bile acids (chenodeoxycholic and ursodeoxycholic acids) used as oral litholytic agents cannot dissolve bilirubin (or pigment) stones at all, making cholecystectomy the only available treatment for this kind of gallstone disease.[8] There is therefore a high need for the discovery of not only new gallstone dissolving agents but more especially those with bilirubin gallstone dissolving capability.

Bilirubin, an otherwise lipophilic molecule, is made water-soluble in the liver by glucuronidation (Scheme 1) before storage in the gall bladder. It is when this system is overwhelmed that bilirubin deposition, along with cholesterol, as gallstones is inevitable.



Scheme 1: Solubilization of bilirubin in the hepatocytes by enzymatic glucuronidation.

Non-enzymatic esterification of bilirubin by extremely polar small molecules could therefore be conjectured as a possible gall stone oral litholysis mechanism. (**Scheme 2**).



Scheme 2: Possible solubilization of bilirubin by non-enzymatic esterification of the two propionyl side chains of bilirubin by 2 polar small molecules (PSM)

Internalization into the hydrophobic cores of some form of complex polymers could be another mechanism. Yet another possible mechanism of solubilization of bilirubin is micellation by some form of biosurfactants.[9] It is quite notable that nature is rich in materials that could be useful in effecting any of the aforementioned non-enzymatic solubilization conjectures for bilirubin. Exploring plants like celery, with gall stone dissolution ethnomedicinal claims, is therefore a step in the right direction in the search for bilirubin stone dissolving agents.

Celery *(Apiumgraveolens L.),* belonging to plant family Apiaceae (formerly known as Umbelliferae) is a biennial, herbaceous plant, commonly cultivated in different parts of the world including U.S.A, India and Africa. Various parts of celery as well as the whole plant have been applied traditionally for various medicinal purposes including anti-bacterial, anti-fungal, cholesterol/lipid-lowering, cardio-protective, anti-diabetic, anti-hypertensive, hepato-protective, anti-inflammatory and gallstone disease treatment purposes.[10-11]

On one hand, some of the aforementioned ethnomedicinal uses of celery have been scientifically confirmed and a number of phytochemical components responsible for the activities isolated: For instance, the antihypertensive and vasodilatation activities of methanol, hexane and dichloromethane extracts of celery whole plant were reported by Moghadam et al., (2013).[12] Glycosides isolated from celery seeds including sedanolide, senkyunolide-N and senkyunolide-J have been shown to have prostaglandin H endoperoxide synthase-I (COX-I) and prostaglandin endoperoxide synthase-II (COX-II) inhibitory activities and thus demonstrated to be wholly or partly responsible for the anti-inflammatory activities of celery plant.[13] On another hand, a number of celery therapeutic properties are only rationalized by its high contents of antioxidant principles. For instance, the hypoglycemic effect of the n-butanol extract of its seed has been largely assumed to its high flavonoids contents believed to mop up free radicals produced in diabetes.[14] This antioxidation conjecture is also the main suggested rationale for the multiple pharmacological activities reported for the apiin and apigenin flavonoid constituents found in virtually all the parts of the celery plant.[11]

However, there are no information yet on the scientific basis for the ethnomedicinal use of celery in the treatment of gallstone disease. In our research efforts tailored towards finding effective medical alternative to surgery in the treatment of gallstone disease, we evaluated the gallstone dissolving potentials of an ethanol extract of a dried sample of whole celery in vitro.

METHODOLOGY

Plant collection and extraction

Fresh celery whole plant, weighing 220 grams, was purchased from a herbal market at Mushin local Government Area of Lagos State, Nigeria. It was identified at the botany department of the University of Lagos, Lagos, Nigeria, and oven-dried at 50°C for two days. The dried plant (25grams) was pulverized and extracted by cold maceration in ethanol (500 ml) for seven days. The extract was filtered and concentrated to dryness in vacuo at 45°C.

Gallstones collection and morphological examination

Gallstones were obtained from the Surgery department of the Lagos University Teaching Hospital (LUTH), Idiaraba, Lagos, Nigeria after a laparoscopic cholecystectomy on a middle age female sickle cell patient. The stones were observed for their external morphological features such as colour, shape and texture before and after cross sectioning. They were afterwards stoned in normal saline.

UV/Visible analysis of gallstones

Gallstones were characterized by analyzing them for their cholesterol contents in a UV-Visible spectrophotometric analysis using the calibration curve method as follows: Graded concentrations (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml) of cholesterol standard (Aldrich, Germany) were prepared in ethanol. UV-Visible absorbance readings were taken for each of these solutions, using ethanol as blank, to obtain a calibration curve. A stock solution of the gallstone sample of a nominal cholesterol concentration of 100 µg/ml was prepared and diluted 1 in 2. The absorbance of the resulting 50 µg/ml solution was taken on the Cary 50 UV-Vis spectrophotometer and its corresponding actual cholesterol concentration obtained from the regression equation of the calibration curve and used to calculate the % of cholesterol by weight in the gallstone sample as follows:

$$\text{Cholesterol \% (w/w)} \frac{\text{Actual Concentration}}{\text{Nominal Concentration}} \times 100$$

Evaluation of gallstone dissolution capability *In-vitro*

A 100 ml of a 1% w/v solution of the dried ethanolic extract of *Apiumgraveolens* was prepared in distilled water and put in a beaker. Another beaker containing 100 ml of distilled water was set as control. One pre-weighed gallstone sample was put in each of the two beakers and subjected to stirring at 500 rpm at room temperature using a magnetic stirrer. The weight reduction of stones was measured every 10 minutes for 2 hours; then hourly for 5 hours and afterwards daily from 24-72 hours. The procedure was carried out in triplicates. For reweighing in the course of the experiment, the stones were lifted out of the solutions using a spatula or by filtration, in case crumbling had occurred as a result of stirring. Stones were blotted dry before weighing. The solvents were not replaced during the study. Dissolution was defined as a reduction in the stone mass. % weight reduction, W_R, was calculated as follows:

$$\%W_R = (W_0 - W_t)/W_0 \text{ X } 100$$

Where W_0 is the initial weight of gallstone, W_t is the weight at time t

Statistical analysis was done with t-test at each point of measurement.

RESULTS

The gallstone samples were observed to be black in color and amorphous in texture on cross-sectioning. Fig. 1 shows the colour of a sample of the gallstones against a white aluminium foil background.

Fig.1: Visual black colour of gallstone sample against a white aluminum foil background

UV/Visible analysis of gallstone cholesterol content

The absorbance of the nominal 50 µg/ml solution (with respect to cholesterol) of the sample gallstone was 0.0167. The calibration curve obtained by measuring the absorbances of five graded concentrations (20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml, 100µg/ml) of a cholesterol standard is shown in fig. 2. Calculating the concentration using the regression equation *y=0.0092x - 0.0087* (from the calibration curve, fig. 2) gave the actual concentration of the test sample as 2.76 µg/ml. The % by weight of cholesterol in the gallstone sample was thus calculated as 5.52 % w/w.

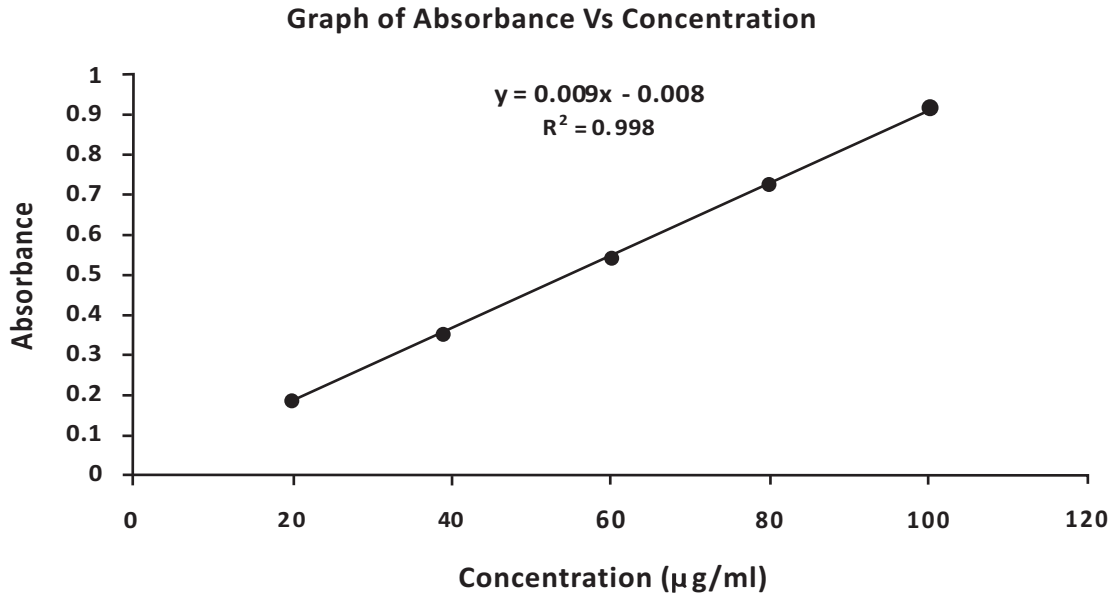


Fig. 2: Calibration curve obtained by plotting absorbance against concentration of five different concentrations of cholesterol standard

Gallstone dissolving capacity of celery plant

There were statistically significant differences in the Average Weight Reduction % produced by the ethanol extract of celery compared to that produced by distilled water at each of the points of measurements, p values ranging from < 0.05 to < 0.0001 as shown in Table 1 below.

Table 1: A time course of mean % weight reduction (% W_R) of gallstones by celery ethanol extract and distilled water (negative control) over a period of 72 hours.

Time (Mins)	% Mean W_R ± S.E.M	
	Distilled water	*Apiumgraveolens*
10	0.30±0.02	0.37±0.02*
20	0.90±0.01	1.38±0.01****
30	1.61±0.08	2.31±0.02***
40	2.20±0.05	3.51±0.02****
50	2.38±0.02	5.62±0.01****
60	2.51±0.02	7.37±0.02****
70	2.92±0.31	7.80±0.08****
80	3.03±0.02	8.07±0.04****
90	3.08±0.04	8.25±0.03****
100	3.40±0.05	8.48±0.02****
110	4.06±0.03	8.81±0.07****
120	4.21±0.05	9.70±0.17****
180	4.42±0.05	10.58±0.01****
240	4.48±0.04	11.19±0.12****
300	4.61±0.06	11.38±0.06****
360	5.08±0.11	11.76±0.03****
1440	5.39±0.04	12.50±0.04****
2880	5.56±0.08	13.49±0.10****
4320	6.44±0.08	27.42±0.32****

Data are expressed as mean ± S.E.M (n=3); *p < 0.05, ***p < 0.001, & ****p < 0.0001 are statistically significant compared with control (distilled water) (Student's t-test).

Figs. 3 and 4 below show a graphical comparison of the time-course gallstone weight reduction (or dissolution capacity) by the ethanol celery plant extract and distilled water.

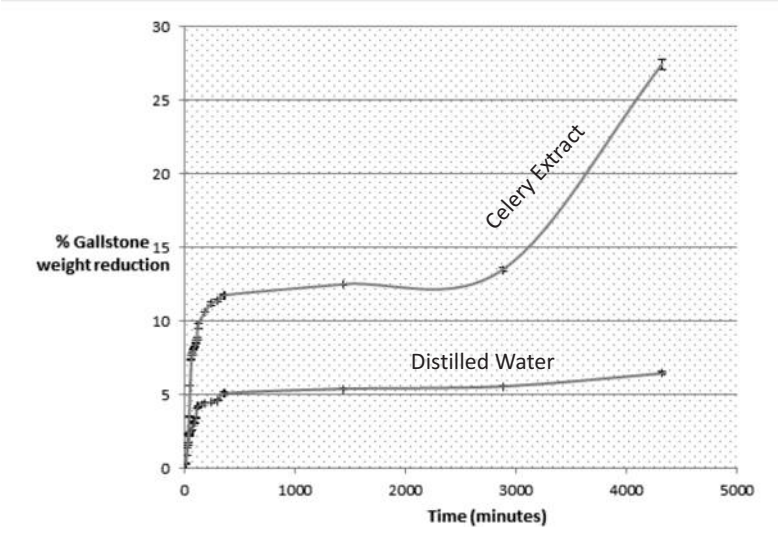


Figure 3: Graphical time-course comparison of % weight reduction of gallstone in ethanol celery (*Apiumgraveolens)* extract and distilled water from 10 to 4320 minutes.

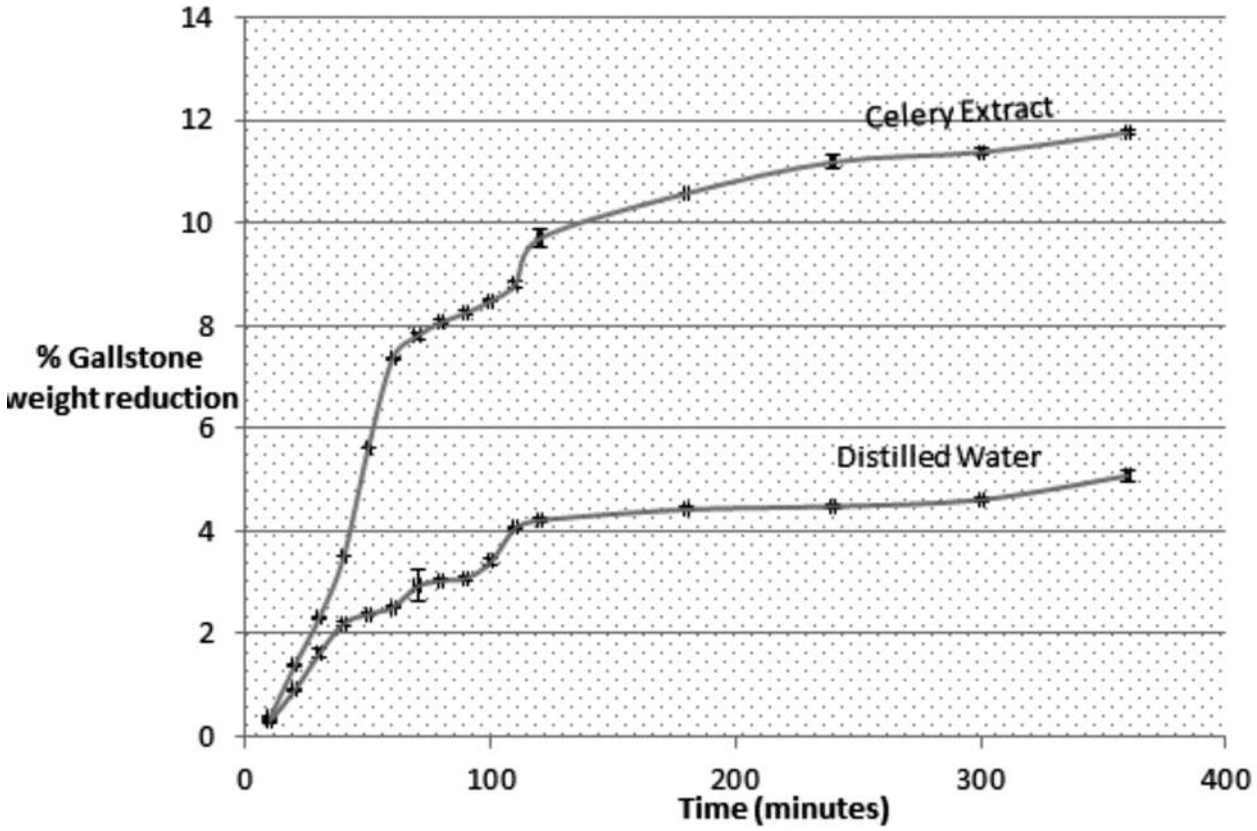


Figure 4: Graphical time-course comparison of % weight reduction of gallstone in ethanol celery (Apiumgraveolens) extract and distilled water from 10 to 360 minutes.

DISCUSSION

The prevalence of bilirubin-rich gallstones in haemolytic disorders is very high[15-16] and, hence, our preference for gallstones from sickle cell anaemia patients. The UV/Visible spectrophotometric analysis of the gallstone samples actually confirmed this correlation as it showed them to be extremely low in cholesterol contents (5.52%) by weight, a value much lower than the 30% maximum cholesterol threshold required for bilirubin gallstones. The morphological observations of the gallstone samples showing them to be black in colour (Fig 1) and amorphous on cross-sectioning were also in tandem with these results, as bilirubin or pigment stones are usually not only amorphous but also black or brown, depending on the level of oxidation that has taken place on the otherwise green bilirubin.[5]

Celery has been confirmed to be hypocholesterolaemic.[17] Lowering cholesterol concentration in the general circulation may tilt the precipitation/dissolution equilibrium of cholesterol in the gall bladder in favour of dissolution. This may therefore be one of the underlining mechanisms for the traditional use of the plant in the management of gallstone disease, though not verifiable by the in vitro evaluation employed in this study. The *in vitro* technique used was designed to explore gallstone dissolving capacities that boarder directly on dissolution and not on any biochemical process. The gallstone dissolving capacity of the celery extract evaluated therefore centered on the evaluation of the ability of the plant extract to solubilize cholesterol and/or bilirubin at room temperature using weight reduction % of the gallstone samples as dissolution extent yardstick.

Generally, the comparison of the weight reduction % and hence dissolution of the gallstone produced by the extract and the control at each of the designated times of measurement shows that the extract statistically significantly dissolved the gallstones compared to the control, $p < 0.001$ at virtually all points of measurement (Table 1). This result portends that the celery extract showed significant gallstone dissolution capability, the pattern of which is depicted in the % Weight reduction vs time graphs and deciphered as follows.

The dissolution pattern exhibited by both the celery extract and the control can be easily gleaned from the graphs presented in Figs. 3 of the results section. The two curves (control and celery plant extract data) show similar patterns, though significantly different: Both have two

steep parts separated by a plateau. Each of the steep parts is ascribable to active dissolution of one of the two major components of the gallstone mixture, i.e., cholesterol or bilirubin.

Because bilirubin is largely adjudged to be more lipophilic than cholesterol, the former is expected to be less soluble in the experimental medium than the latter, though their relative solubilities are yet to be quantitatively determined.[18] The first steep phase of the curve was thus ascribed to the dissolution of cholesterol and the second to that of bilirubin, the intervening plateau being suggestive of saturation of the dissolution medium with dissolved cholesterol molecules as dissolution continued. This intervening saturation in the dissolution pattern would not occur in vivo as the gallstones under this situation would intermittently find themselves in a fresh dissolution medium (i.e., bile), because new bile is continually produced by the liver as old one is secreted via the bile duct into the small intestine in response to food stimuli for participation in the digestive process.[19] The rather larger sharp rise observed in the gallstone weight reduction % at the onset of the second dissolution phase is a further allusion to the fact that the second phase is associated with bilirubin dissolution on account of the fact that since the stones were largely bilirubin, the onset of its dissolution would be expected to have a profound effect on the weight reduction and hence of the dissolution extent of the entire stone.

The second dissolution phase of the gallstone was very marginal for the control and started at about 4000 min. For the celery extract, however, this phase is very conspicuous and started much earlier at about 3000 min into the experiment. This shows that the celery extract certainly possesses some principles significantly aiding the onset and extent of dissolution of bilirubin in aqueous medium.

This work has only explored the potentials of celery as a possible source of oral litholytic agents for gallstones, especially of the bilirubin type. It has not proven celery safe for use in the treatment of gallstone disease and associated symptoms.

CONCLUSION

This experiment has shown that ethanol extract of celery has bilirubin gallstone dissolving capacity, positioning it for further investigation ultimately aimed at the discovery of drugs effective for oral litholysis of gallstones, particularly the pigment stones, the removal of which as at present has no alternative to surgery.

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