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In-vitro Dissolution Study of Gallstone with Medicinal Plant Extracts

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Abstract

Background: *Gallstone disease poses a substantial economic burden on healthcare systems globally, necessitating safer alternatives to current treatments like dissolution therapy and cholecystectomy. Natural compounds from plants offer a potential solution, but research on their cholelitholytic activity is limited. In vitro dissolution studies are crucial for identifying effective plant-based therapies.* ***Objective:*** *This study aims to investigate the in vitro cholelitholytic activity of six plants and Ayurvedic medicines, selected based on ethnopharmacological knowledge and folk medicinal practices.* ***Methods:*** *Gallstone samples were categorized as combined cholesterol gallstones (CCGS) or black pigment gallstones based on external morphology and cross-sectional analysis. In vitro dissolution studies were conducted using extracts from Bergenia ciliata, Berberis asiatica, Cuscuta europaea, Kalanchoe pinnata, Teraxacum officinale, Macrotyloma uniflorum, and Ayurvedic medicines (Cystone®, Gokshuradi, and Calcury). The samples were immersed in the extracts and controls separately and incubated in a shaking water bath. The gallstone dissolution capacity was assessed by recording the dry weight of the samples at multiple time points.* ***Results:*** *T. officinale was highly effective in dissolving black pigment gallstones, while B. asiatica exhibited superior efficacy for CCGS. M. uniflorum and C. europaea also demonstrated significant dissolution activity against black pigment gallstones. However, K. pinnata was less effective for both gallstone types. B. ciliata and C. europaea exhibited equal effectiveness against both types. Ayurvedic medicine extracts were less effective compared to plant extracts.* ***Conclusion:*** *This in vitro study showed the plants can dissolve GS effectively. However, the effectiveness of the plant to dissolve GS depends on the type of the stone. The findings from this study serve as a basis for further in vivo research.*

Keywords
Gallstone, *in vitro* dissolution, plant extracts, combined cholesterol gallstones, black pigment gallstones.

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1 Introduction

Gallstones (GS), which are solid deposits formed inside the gallbladder, are mainly composed of cholesterol and bilirubin [1]. The formation of GS involves supersaturation, nucleation, crystallization, and aggregation of the insoluble components of the bile like cholesterol and bilirubin [2]. When the

concentration of these insoluble components exceeds their solubility or there is a decrease in bile acid concentration or the presence of foreign substances, gallstones form [3]. Gallstone disease is a prevalent health problem that affects 10-20% of the global population [4] and is a significant contributor to morbidity and mortality [5], with an estimated global cost of $6.5 billion annually [6]. Its prevalence in Nepal is 4.87% with females being more affected than males [7]. Therefore, gallstone disease imposes a significant economic burden on healthcare systems worldwide [8].

The treatment of gallstone disease can be done through surgery or non-surgical methods. Non-surgical methods include taking bile acids like chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) orally (oral dissolution therapy) [9] or installing litholytic solvent like methyl tert-butyl ether (MTBE) [10], 2-methoxy-6-methylpyridine (MMP) [11], or ethylenediaminetetraacetic acid (EDTA) [12], directly into the gallbladder through a percutaneous transhepatic catheter (contact dissolution therapy) [13]. However, these methods have limitations due to side effects, toxicity, low efficiency [14], incomplete dissolution [15], and gallstone reoccurrence is common [16]. Laparoscopic cholecystectomy, the surgical removal of the gallbladder, is considered the best option [17] but has postoperative complications such as bile leakage, bile duct injury [18], persistent pain [19], and fat intolerance [20]. Due to the limitations and potential risks of current treatment options, natural compounds from plants could be a safer alternative. It is believed that herbal medicines have lesser or no side effects. While extensive studies have been conducted on the cholelitholytic activity of organic solvents, research on plants is limited, and *in vitro* dissolution studies are necessary to identify potential plant-based therapies. Therefore, in this study, we investigated the *in vitro* cholelitholytic activity of six plants (selected based on ethnopharmacological knowledge and folk medicinal practices) and Ayurvedic medicines available in the market. The results of this *in vitro* study will provide a basis for further research *in vivo*.

2 Materials and Methods

2.1 Collection and Pre-treatment of Gallstones

Dr. Barun Kumar Shah, Norvic International Hospital, Thapathali, Kathmandu, Nepal has provided the gallstone samples. Cholecystectomy was performed to extract gallstones from patients. Altogether, 12 CCGS (from two patients) and 15 black PGS (from one patient) were collected. The collected gallstones were thrown away materials with no human tissues or genetic material. The gallstone samples were washed with deionized water and dried at 60 ℃ in an incubator until the constant weight [21].

2.2 Macroscopic Classification of Gallstones

Based on the external morphology and internal cross-sectional analysis, we categorized the gallstones into two groups: combined cholesterol gallstone (CCGS) and black pigment gallstone (black PGS). Photographs of the gallstones were taken, their morphological features like shape, size, color, and internal cross-section were studied. The outer dimensions of the stone were measured with a digital vernier caliper. One gallstone from each patient was cut into equal halves using Jeweler's saw and the internal cross-section was studied. The remaining gallstones were stored on a desiccator filled with calcium chloride in a dark cabinet, and later on, they were used for *in vitro* dissolution with different extracts of plant and herbal medicines.

2.3 Collection of Plant and Ayurvedic Medicines

The rhizomes of *Bergenia ciliata* were collected from Central Nepal, Parbat District, Panchase. The root of *Berberis asiatica* and tendrils of *Cuscuta europaea* were collected from Central Nepal, Parbat District, Modi rural municipality-7, Ranpu. The leaves of *Kalanchoe pinnata* were collected from Central Nepal, Kaski District, Pokhara-15, Nayagaun. Leaves of *Teraxacum officinale* were collected from Central Nepal, Kathmandu District, Kirtipur. The legumes of *Macrotyloma uniflorum* were purchased from the local market of Kirtipur, Nayabazar. Herbarium samples of each plant were prepared and the plants were identified from National Herbarium and Plant Laboratories, Godawari, Lalitpur. Ayurvedic medicines (Cystone®, Gokshuradi, and Calcury) were purchased from Arogyadham Homoeopathic Hospital, Pokhara with the suggestion of homeopathic physician Dr. Bishnu Prasad Chapagain. The plant materials were washed with tap water, cut down into small pieces, and shed dried for two weeks. The shed dried plant samples were crushed into powder with the help of a grinding mill at the Central Department of Chemistry. The powder samples were put into an airtight glass jar and stored at ambient temperature.

2.4 *In vitro* Dissolution Study of Gallstones

The dried and powdered plant samples were extracted with ethanol using Soxhlet apparatus. To

obtain crude extracts, 50 g sample powder was refluxed with 500 mL of ethanol at 40 °C until a clear solution was obtained in the thimble. The dilute extract was evaporated under reduced pressure using a rotatory evaporator at 40 °C. The semisolid crude extract obtained from rotavapor was dried at ambient temperature for several days to get the dry crude extract. The dried crude extracts were collected into 15 mL flat-bottom borosil culture tubes, labeled properly, and stored at 4 °C in a refrigerator. 50 mg/mL extract solution was prepared by dissolving 5 g of the solid crude extract in 100 mL of distilled water. The solution was slightly warmed and sonicated for half an hour, let to settle down to get a clear stock solution. The clear solution was collected into a 100 mL volumetric flask, the flask was stoppered, labeled, and stored in a refrigerator at 4 °C.

Two tablets of each ayurvedic medicine were powdered in a mortar and pestle and the powder was taken into the centrifuge tube with a screw cap. Distilled water (10 mL) was added and the content was kept in a water bath for a night at 37 °C, centrifuged at 25 °C in a high-speed centrifuge at 8000 rpm to get clear supernatant. The clear supernatant was collected into a 100 mL volumetric flask, the flask was labeled and stored at 4 °C in a refrigerator [22].

2.5 Dissolution of Gallstones with Plant and Medicine Extracts

For the *in vitro* dissolution study, the protocol used by Igimi et al. [23] was followed with slight modification. The gallstone samples that were oven-dried (at 60 ℃) and stored in a desiccator in a dark cabinet at least for 15 days were taken for dissolution. The gallstone samples were taken, weighed, and immersed *in vitro* into 10 mL of the extract, positive control, and negative control separately. Borosil culture tubes (15 mL volume) with round bottom and screw cap were used for the purpose. The tubes with content were put in a test tube rack and kept in a shaking water bath at 37 ℃ with gentle and constant shaking (60 rpm). The plant extracts were changed every day to minimize any effect due to saturation of the solution by dissolved components of the stone [23]. The stones were taken out of the extract by filtration through filter paper (Whatman No1), washed with distilled water, transferred into a pre-weighed crucible, dried at 60 °C in an incubator for two days to get constant weight, and the weight reduced was noted [11,24]. To determine the gallstone dissolution capacity of each extract and solvent, we recorded the dry weight of the stone samples at three different time points (4, 94, and 190 h) after they were directly immersed in the solution. Photographs of the stones were also taken after each time interval.

The stone samples with comparable weight and size were taken for dissolution study. The average weight of different CCGS samples was 14.48±0.21 mg and that of black PGS samples was 21.46±0.22 mg. In the present research, 2% EDTA solution maintained at pH 9.5 adjusted with HCl and 95% ethanol was used as a positive control whereas distilled water was used as a negative control. It was found that the higher the pH of the EDTA solution higher is its litholytic activity [25]. The pH of 8.5 is within the harmless range to use to the human body [25], but we maintained the pH 9.5 because studies have found that this is the most effective pH at which EDTA shows the maximum dissolution of GS [26].

First of all, the weight dissolved by negative control at every time point was subtracted from the weight reduction value recorded for each of the preparations at respective time points to calculate the actual weight dissolved by the preparations. Then the percentage dissolution was calculated by using the following formula [11, 24]:

$$\%\text{Dissolution(w/w)} = \frac{\text{Actual wt. dissolved}}{\text{Initial wt. of the stone}} \times 100 \quad (1)$$

3 Results

3.1 General Observation and Macroscopic Classification of Gallstones

The photographs showing the external morphology and internal cross-section of the gallstone samples are given in **Figure 1**. The gallstone sample, which is multifaceted, whitish-brown with a hard and smooth outer surface [27, 28], with a distinct inner pigmented yellow core and a white external shell **(Figure 1a, b)** [28, 29] was classified as a combination cholesterol gallstone (CCGS). The gallstone sample, which was irregular with a rough and thin yellowish surface [27, 28], amorphous in cross-section [28, 29] with a black inner part and outer thin yellowish layer **(Figure 1c, d)** was classified as black pigment gallstone (black PGS) [28]. Furthermore, we conducted a separate study to support this macroscopic classification via UV-vis and SEM-EDS analysis [28].

3.2 *In vitro* dissolution study of gallstones

The final data obtained for cumulative dissolution of gallstone samples in different extract and solvent preparations is presented in **Table 1**. The observation tables for recorded weight (initial, after 4 h, 94 h, and 190 h), dissolution and cumulative dissolution are included in Supplementary material

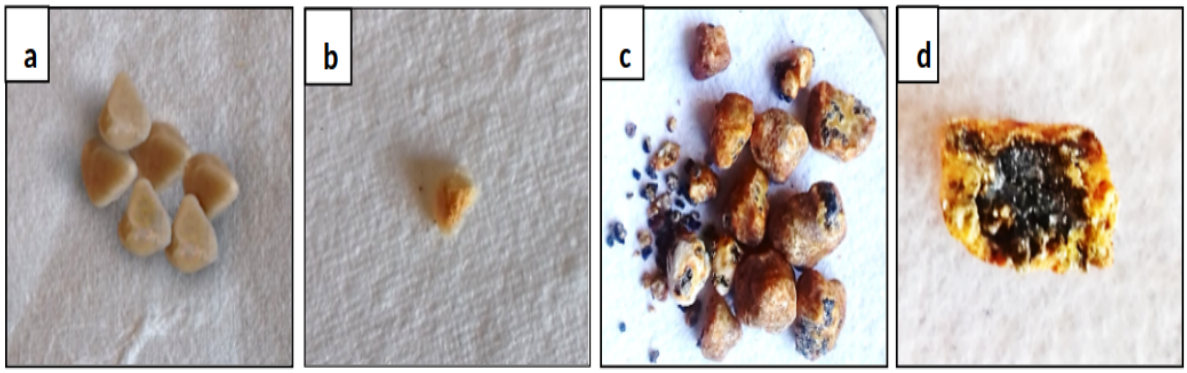

Figure 1: Photographs of the gallstone samples; (a) CCGS, (b) Internal cross-section of CCGS, (c) Black PGS, and (d) Internal cross-section of black PGS

(**Annexure 1**). Photographs of gallstones at different time are also given in Supplementary material (**Annexure 2**). After 190 hours, 14.3 mg of the CCGS and 8.6 mg of the black PGS was dissolved in EtOH, whereas in EDTA, 11.5 mg of the black PGS and 5.1 mg of CCGS was dissolved. Therefore, it can be inferred that the CCGS was more soluble in EtOH and black PGS was more soluble in EDTA (**Table 1**). **Figure 2** illustrates the comparative study of the final dissolution of gallstone samples in different extract and solvent preparations. It shows that E1 (*M. uniflorum*), E4 (*C. europaea*), E5 (*T. officinale*), and EDTA dissolved black PGS more effectively than CCGS, while the M2 (Calcury) showed almost similar dissolution for both the stones. In contrast, E2 (*B. asiatica*), E3 (*B. ciliata*), M1 (Cystone), M3 (Gokshuradi), and EtOH showed better dissolution for CCGS than for black PGS, while in E6 (*K. pinnata*), dissolution of both the gallstones was comparable.

Table 1: Comparative study of the final dissolution of CCGS and black PGS in different extract and solvent preparations.

Extract or Solvent	Weight dissolved after 190 hours (mg)	
	CCGS	Black PGS
E1 (*M. uniflorum*)	5.6	9.3
E2 (*B. asiatica*)	10.8	5.4
E3 (*B. ciliata*)	4.7	3.7
E4 (*C. europaea*)	7.3	8.5
E5 (*T. officinale*)	3.0	9.6
E6 (*K. pinnata*)	2.2	2.1
M1 (Cystone®)	3.1	1.8
M2 (Calcury)	3.9	4.0
M3 (Gokshuradi)	5.8	3.5
EDTA (+ve control)	5.1	11.5
EtOH (+ve control)	14.3	8.6

Note: EDTA and EtOH are used as positive controls and distilled water as a negative control. The data for each experiment is expressed after subtracting the data obtained for the negative control.

4 Discussion

Close interpretation of the graph in **Figure 2** shows that, in E1 (*M. uniflorum*), E4 (*C. europaea*) and E5 (*T. officinale*), black PGS was more soluble than CCGS. Among different extracts preparations (both plant and medicine), E5 (*T. officinale*) was the most effective for dissolving black PGS (9.6 mg). In comparison with the most effective positive control EDTA (11.5 mg), the efficacy of E5 (*T. officinale*) to dissolve black PGS can be considered as comparatively excellent. E5 (*T. officinale*) was more effective than another positive control EtOH (8.6 mg). The efficacy of E1 (*M. uniflorum*) to dissolve black PGS (9.3 mg) was almost equivalent to that of the most effective plant E5 (*T. officinale*). This shows that E5 (*T. officinale*) and E1 (*M. uniflorum*) can effectively dissolve pig-

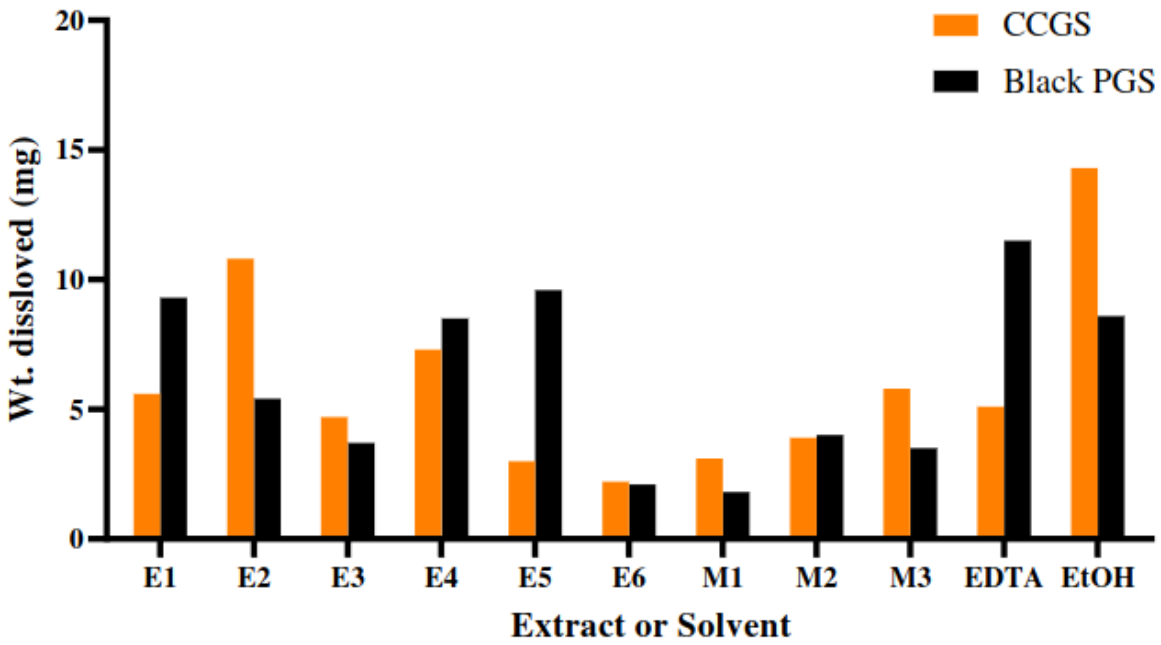

Figure 2: The comparative study of the final dissolution of CCGS and black PGS in different extract and solvent preparations

ment containing stones. Moreover, this fact was further supported by the result obtained for cholesterol predominant CCGS which shows that E5 (*T. officinale*) and E1 (*M. uniflorum*) dissolved significantly lesser amount of CCGS than black PGS (3.0 mg in E5 and 5.6 mg in E1). M2 showed almost similar efficacy to dissolve both the CCGS (3.9 mg) and GSB (4.0 mg) whereas M1 (Cystone®) and M3 (Gokshuradi) showed better dissolution for CCGS than black PGS. Similarly, E2 (*B. asiatica*) and E3 (*B. ciliata*) dissolved CCGS more effectively than black PGS. The efficacy of E6 (*K. pinnata*) was similar for both the stones (2.2 mg CCGS, 2.1 mg black PGS). A close inspection revealed that E6 (*K. pinnata*) was the least effective plant to dissolve both the CCGS and black PGS. Among different plant extracts, E2 (*B. asiatica*) was the most effective (10.8 mg) to dissolve CCGS. In comparison with the most effective positive control EtOH (14.3 mg), the efficacy of E2 (*B. asiatica*) to dissolve CCGS can also be considered significant. E2 (*B. asiatica*) was found to be more effective than another positive control EDTA (5.1 mg). Moreover, E2 (*B. asiatica*) dissolved only 5.4 mg of bilirubinate predominant black PGS which was almost half of the dissolution obtained for CCGS. Form all of these interpretations we can conclude that E2 (*B. asiatica*) is effective to dissolve cholesterol containing gallstones like CCGS.

E4 (*C. europaea*), although, was found to be more efficient to dissolve black PGS than CCGS, there is no significant difference between the values obtained (8.5 mg of black PGS vs 7.3 mg of CCGS). Dissolution of black PGS in E4 (*C. europaea*) is far close to the dissolution in E5 (*T. officinale*) with only 1.1 mg difference. On the other hand, a significant difference was obtained for dissolution of CCGS in E4 (*C. europaea*) and the most efficient plant E2 (*B. asiatica*) and positive control EtOH (difference of 3.5 mg with E2 and 7.0 mg with EtOH). Therefore, it can safely be inferred that E4 (*C. europaea*) was also effective in dissolving pigment stones like GSB.

Among different medicine extract, M3 (Gokshuradi) was the most effective to dissolve CCGS, it dissolved CCGS (5.8 mg) more efficiently than black GSB (3.5 mg, 16.20%). Even though, M3 (Gokshuradi) including other medicine extracts were far less effective than E2 (*B. asiatica*) (10.8 mg) and E4 (*C. europaea*) (7.3 mg) for dissolving CCGS. On the other hand, among different medicines, M2 (Calcury) showed the highest dissolution for black PGS (4.0 mg). But, M3 was still less effective than E1 (*M. uniflorum*), E2 (*B. asiatica*), E4 (*C. europaea*), and E5 (*T. officinale*). Therefore, it can be inferred that the medicine extracts were less effective than most of the plant extracts in dissolving gallstones. These ayurvedic medicines are recommended for oral dissolution therapy of kidney stones and not for gallstones. This fact could explain lesser efficacy of these commercial ayurvedic medicines over plant extracts in dissolving gallstones.

Chekroune and Benamara [30] reported dif-

ferent plant preparations to dissolve a significant amount of GS. About 209.37 mg, 97.42 mg, and 15.02 mg of the cholesterol-bilirubin containing GS were reduced in *Herniaria hirsuta* extract, lemon juice, and their mixture respectively after 312 hours of immersion whereas the olive oil/lemon juice emulsion dissolved the stone with 291.1 mg weight completely after 168 hours of immersion [30]. In the present study, among all plant extracts and gallstone samples, the maximum weight reduction after 190 h was 10.8 mg in E2 (*B. asiatica*) for CCGS. Due to the difference in immersion period of the stone, direct comparison of our report with the reported data seems to be inappropriate, however, it can be concluded that we also found *in vitro* cholelitholytic activity of different plant extracts.

The *in vitro* dissolution study showed that CCGS was more soluble in EtOH than in EDTA whereas black PGS is more soluble in EDTA than in EtOH. The insoluble residue of CCGS was left even after 190 hours of incubation in EDTA (5.1 mg dissolution) whereas CCGS was completely dissolved in EtOH after 94 hours (11.6 mg dissolution). The efficacy of EDTA to dissolve black PGS (11.5 mg) was almost double that for CCGS (5.1 mg); however, almost half of the black PGS was left undissolved even after 190 hours of incubation (**Annexure 1: Table 3**). This indicates that CCGS is easily dissolvable by using cholesterol solvent like ethanol whereas black PGS contains a greater proportion of insoluble components and it is difficult to dissolve completely both in cholesterol solvent like EtOH and in calcium chelating solvents like EDTA. This finding is consistent with the result reported by Lin et al. [24].

Lee and co-worker [31] reported that 1.1 mg, 9.1 mg, and 30.0 mg of the CGS stone sample with 69.3 mg initial weight was dissolved in absolute ethanol after 3, 6, and 9 hours of incubation, respectively and the stone was dissolved completely after 18 h. In the present study, we have recorded 2.7 mg and 14.3 mg dissolution of the CCGS (wt. 14.48±0.21 mg) after 4 and 94 hours respectively. The stone was dissolved completely after 94 hours. Based on this data, the dissolution we obtained in EtOH for CCGS was lower than that reported by Lee et al. The difference arises due to the lower concentration of EtOH we used (95% EtOH). In the case of black PGS (wt. 21.46±0.22 mg), EtOH was not found effective and only 8.6 mg of the stone was dissolved after 190 h. The low solubility of the black PGS in EtOH is due to the presence of a high concentration of insoluble bilirubinate as the main component which was confirmed by SEM and EDS in a separate study by our group [28].

5 Conclusion

From the morphological and cross-sectional study, gallstones were classified as combined cholesterol gallstone (CCGS) or black pigment gallstone (black PGS). *In vitro* dissolution studies were also conducted using plant and medicine extracts, revealing that *T. officinale* was the most effective in dissolving black PGS, whereas *B. asiatica* was the best for CCGS. *M. uniflorum* and *C. europaea* were also found to be effective in dissolving black PGS, while *K. pinnata* was the least effective for both types of gallstones. *B. ciliate* and *C. europaea* were equally effective in dissolving both types of GS. Medicine extracts were less effective than plant extracts. This *in vitro* study showed the plants can dissolve GS effectively. However, the effectiveness of the plant to dissolve GS depends on the type of the stone. The study suggests that further research is necessary to confirm the effectiveness of these plants in real biological systems through animal models. Furthermore, the potential compound in plant that show cholelitholytic or anti-cholelithogenic activity and mechanism of action is still unknown and requires further research. The present research is limited to *in vitro* study. Although normal human body temperature (37 °C) was maintained while preforming *in vitro* dissolution study, other factors like normal bile pH is not considered.

List of abbreviations

GS = Gallstone
CCGS = Combination cholesterol gallstone
Black PGS = Black pigment gallstone
CDCA: Chenodeoxycholic acid
UDCA: Ursodeoxycholic acid
MTBE: Methyl tert-butyl ether
MMP: 2-Methoxy-6-methylpyridine
EDTA: Ethylenediaminetetraacetic acid

Ethics approval and consent to participate

Not applicable.

Human and animals right

No animals/humans were used for studies that are basis of this research.

Availability of data and materials

All the data are provided in the Supplementary material which is available on the publisher's website along with the published article.

Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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Supplemetary materials

Supplementary material is available on the publisher's website along with the published article.

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