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SCOLICIDAL POTENCY OF BILE AGAINST *ECHINOCOCCUS GRANULOSUS*

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KEY WORDS

Hydatid cyst
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Cysto-biliary fistula

ABSTRACT

Cysto-biliary fistulas are commonly diagnosed, either before or after surgical intervention, in patients with enlarged or advanced-stage hydatid cysts (HCs). Analysis of cystic contents in these patients shows that diminished scolex vitality is more common in patients with cysto-biliary fistulas. This study aims to demonstrate the scolicidal effects of bile on *Echinococcus granulosus* (EG) scolices at various bile concentrations and over specific periods. The time- and concentration-based scolicidal potency was analyzed in EG scolex samples obtained from HC patients. The integrity of the cyst wall and the scolicidal effect of the contents were evaluated using a vital staining technique with 0.1% eosin. Bile samples were collected from patients with cholelithiasis who were scheduled for surgery. Scolicidal efficacy of 100% was observed in all samples at the 1/4 bile concentration after 10 min and at the 1/8 concentration after 60 min. At a 1/16 concentration, scolicidal efficacy was 66% at 1 min, 89% at 10 min, 93% at 30 min, and 98% at 60 min. At a 1/32 bile concentration, scolicidal efficacy was 59% at 1 min, 68% at 10 min, 89% at 30 min, and 95% t 60 min. At a 1/64 concentration, the scolicidal efficacy was 53% at 1 min, 58% at 10 min, 77% at 30 min, and 93% at 60 min. After 1 hr, reasonable scolicidal efficacy was determined up to a 1/64 concentration; however, significant decreases in scolicidal efficacy were observed at 1/128 and 1/256 bile concentrations. Theoretically, bile, which is an endogenous secretion, can be used as a suitable and potent scolicidal agent. This study lays the groundwork for future in vivo clinical trials utilizing synthetic bile acids for scolicidal purposes or for studies evaluating the scolicidal effects of bile in patients who develop cysto-biliary fistulas.

Studies of patients with liver hydatid cysts (HCs) show that the progression of the cystic stage decreases the vitality of the cysts (Kayaalp et al., 2002a). Research also indicates that the presence of biliary fistulas increases in patients with advanced-stage liver HCs (Dinakar Reddy and Thota, 2018; Kayaalp et al., 2002b, 2003). The theory that increased cyst size and intracystic pressure leads to the formation of cysto-biliary fistulas due to the pressure exerted on nearby biliary tracts is widely accepted. In advanced-stage liver HC patients, the diminished vitality of cyst contents, formation of cysto-biliary fistulas, and presence of pus-filled cysts are also noted (Ramia et al, 2012; Shi et al., 2016).

Surgical procedures are recognized as one of the most effective treatment options for HC disease, alongside puncture, aspiration, injection, and reaspiration (Goksoy et al., 2008). During these procedures, scolicidal agents are used to prevent secondary contamination. The ideal scolicidal agent must exhibit potent scolicidal activity on *Echinococcus granulosus* (EG) while causing minimal toxicity to the biliary tracts and having minimal side effects (Besim et al., 1998). Saline solutions are the most commonly used scolicidal agents, affecting intra- and extra-cystic osmotic pressure differences, which lead to the disintegration of the cuticular layer and subsequent death of the scolices (Kayaalp et al., 2001). Shi et al. (2016) also demonstrated that bile salts activate the caspase-3 pathway in scolices, leading to apoptosis. However, no study has yet explored the scolicidal activity of bile on EG.

The purpose of this study is to demonstrate the scolicidal effects of bile at various concentrations on EG scolices and over different periods and to determine whether the reduction in cyst contents vitality is due to bile contamination.

MATERIAL AND METHODS

Study design

The study was conducted at 2 centers. Patient evaluations and surgeries were completed at the Republic of Türkiye (TR) University of Health Sciences, Kartal Dr. Lütfi Kırdar Training and Research Hospital, General Surgery Department, and the scolicidal analysis was conducted at Istanbul University-Cerrahpaşa, Faculty of Medicine, Microbiology Department, Parasitology Laboratory.

HC patients were enrolled in the study until a sufficient number of viable EG scolices was obtained to account for the possibility of insufficient sample quantity or viability. For gallbladder patients, those with HCs suitable for sampling were included as they were identified. One cholelithiasis patient also was included in bile sampling. To ensure the homogeneity of the bile fluid to be obtained, only patients with symptomatic cholelithiasis scheduled for elective surgery were included. All patients undergoing either procedure were informed of the study, and written consents were obtained. Patients with HCs were specifically informed about the risk of contamination and the benefits of preoperative albendazole treatment. Only patients who refused preoperative albendazole treatment were included in the study. Unwilling patients were not included in the study.

HC patients

EG scolices were obtained from any intra-abdominal HC patient with viable cyst contents. For patients with suspected HC disease, the viability of the cyst contents was routinely confirmed preoperatively with abdominal ultrasonography (USG) and serologic tests (indirect hemagglutination assay [IHA]). Abdominal computerized tomography scans were performed when HC disease was clinically suspected or indicated by USG results, and magnetic resonance cholangiopancreatography (MRCP) for liver HC was requested in cases with suspected cysto-biliary fistula.

Patients with HCs from whom EG scolices were to be obtained were excluded when they met any of the following criteria: under 18 or over 80 yr of age, negative IHA test results, cysto-biliary fistula detected during preoperative imaging or preoperatively, HC type 4 or 5 according to World Health Organization (WHO) classification (Eckert et al., 2001), cyst size less than 6 cm, preoperative use of albendazole, known liver failure or chronic liver disease, or unwillingness to voluntarily participate in the study. Procedures for treatment of HCs included cystotomy, drainage, and omentopexy (CDO), pericystectomy, and splenectomy. During these procedures, to maintain the viability of the scolices and protect patients from allergic shock, the entire surgical area was initially covered with hypertonic saline and betadine (10% povidone-iodine) drapes.

For CDO procedures, cysts were aspirated, and the hydatid fluid, germinative membrane, and daughter vesicles were removed before administering scolicidal agents into the cyst cavity. For splenectomy or pericystectomy procedures, cyst contents were sampled after resection was completed. Obtained samples were preserved at 4 C in cold chain containers for transportation for further scolicidal analysis.

Gallbladder patients

For patients with cholelithiasis, preoperative routine tests included complete blood count, biochemical parameters, and USG. MRCP was requested based on abnormal blood test results, abnormal USG results, or clinical suspicion of bile duct disease. Patients with chronic liver disease, inflammatory biliary disease, or abnormal liver function test results were excluded from the study to ensure homogeneity of the collected bile samples.

In patients scheduled for laparoscopic cholecystectomy due to cholelithiasis and from whom bile fluid was to be obtained, exclusion criteria were under 18 or over 80 yr of age; history of inflammatory hepatobiliary diseases such as pancreatitis, cholecystitis, or hepatitis; history of emergency surgery; known liver failure or chronic liver disease; history of biliary stasis or endoscopic retrograde cholangiopancreatography in the preoperative period; abnormal liver function test results; or unwillingness to voluntarily participate in the study. After routine laparoscopic cholecystectomy, the gallbladder contents of the patients were preserved at 4 C in containers, each with at least 10 ml.

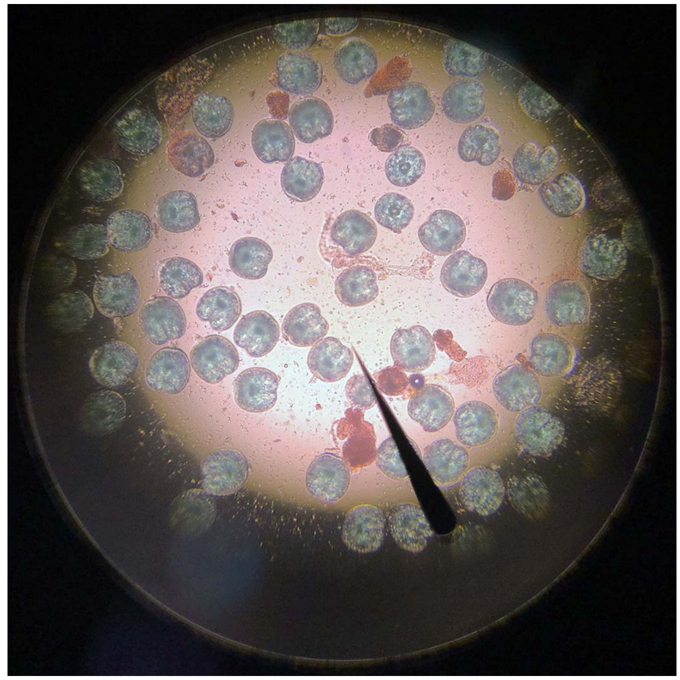

Figure 1. Checking bile fluid samples for scolex quantity and vitality. Live protoscolices are stained green, whereas dead protoscolices are stained red. Color version available online.

Scolicidal analysis and inclusion

Both bile and scolex samples were stored at 4 C in cold chain containers for infectious materials and transported to the Istanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Microbiology, Parasitology Laboratory within approximately 1 hr. At the laboratory, the samples were kept at room temperature to allow hydatid sand formation. Each slide was confirmed to contain at least 250 live scolices. The viability of the protoscolices was confirmed under a microscope by their lack of dye absorption (0.1% eosin stain) and observable motility. Samples with viable scolices, indicated by the absence of eosin staining, were included in the study.

Bile fluids were diluted using micropipettes with sterile distilled water at concentrations ranging from 1/1 to 1/256. Samples were prepared as 20 preparations in test tubes. From each preparation, 2 ml was placed on 4 slides. After counting the scolices, 1 ml of 0.1% eosin stain and 1 ml of bile concentration were added. The number of live scolices was recorded at 1, 10, 30, and 60 min for each slide. Additionally, to determine the viability with eosin stain alone, 2 ml of hydatid fluid and 1 ml of 0.1% eosin stain were added to 1 slide, and the viability of the scolices was monitored (Fig. 1).

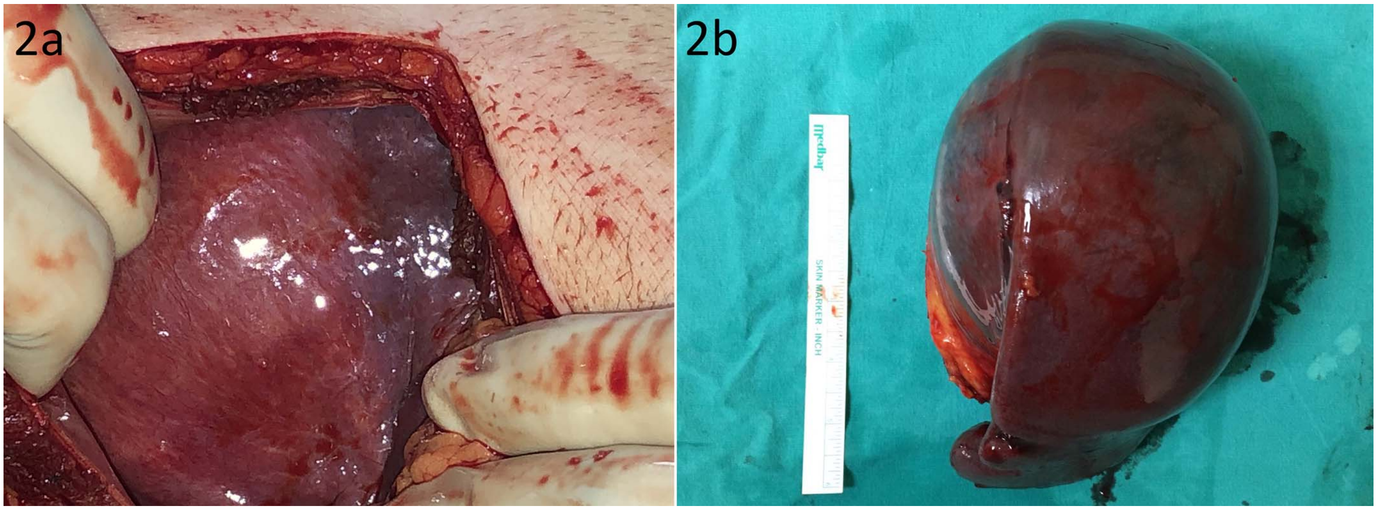

Figure 2. Hydatid cyst contents collection and transfer process. (**a**) Aspiration of hydatid cyst contents before cystotomy, drainage, and omentopexy procedure, right lobe of liver. (**b**) Splenectomy specimen with hydatid cyst. Color version available online.

Statistical analysis

The data were analyzed with SPSS 26.0 (IBM Co., Armonk, New York). Descriptive statistical measures were used (mean, standard deviation, median, frequency, and ratio). Because the sample size was less than 50, the Shapiro-Wilk test was employed to assess the normality of the data distribution. Given that some parameters did not conform to a normal distribution and due to the presence of repeated measures, the non-parametric Friedman test was utilized. When the Friedman test yielded significant results, the Wilcoxon signed-ranks test was applied to determine the significance of result differences between specific time intervals. Differences were considered significant at $P < 0.05$ for all analyses.

RESULTS

The study started at the General Surgery Clinic of TR University of Health Sciences Kartal Dr. Lütfi Kırdar Training and Research Hospital General Surgery Department with the approval of the ethics committee at the same center (26 June 2019, 2019/514/156/11). The study was carried out between July and August 2019 with suitable preoperative results for 4 HC patients and 1 cholelithiasis patient.

In our study, 4 patients underwent surgery for HC disease under the specified criteria. Two patients presented with recurrence at least 5 yr after the initial diagnosis, and all cases involved liver HCs. According to the WHO classification (Eckert et al., 2001), 2 liver HC patients had type 3 cysts and 1 had a type 2 cyst. Two patients underwent CDO procedures, 1 patient underwent a pericystectomy (Fig. 2a), and the remaining patient underwent a splenectomy (Fig. 2b).

The patient who underwent pericystectomy was a primary case. The remaining 2 CDO patients were excluded from the study due to the presence of infected HCs discovered through surgery and microscopic analysis. During the analysis, the cyst contents of the pericystectomy patient were found to lack a sufficient quantity of scolices, leading to the exclusion of this patient. Only the patient who underwent splenectomy met the necessary criteria for both the quantity and viability of scolices.

Bile at a 1/1 concentration showed 100% scolicidal efficacy at the 1 min measurement (Fig. 3a). As the bile concentration was diluted, a decrease in scolicidal efficacy was observed. Bile at a 1/2 concentration demonstrated 98% efficacy at 1 min, increasing to 100% scolicidal efficacy after 10 min. The 1/4 concentration bile showed 90% efficacy at 1 min and reached 100% efficacy in subsequent periods. Bile at a 1/8 concentration showed 70% efficacy at 1 min (Fig. 3b), 80% at

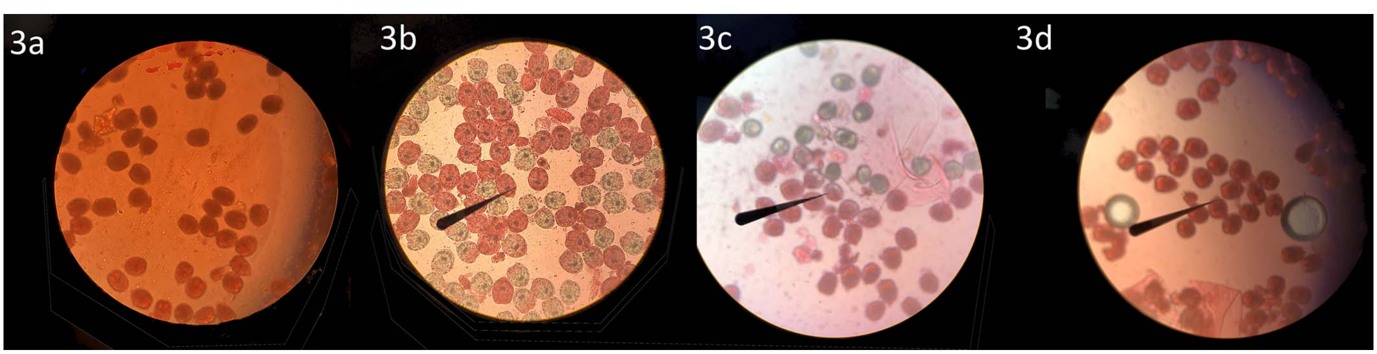

Figure 3. Evaluation of scolicidal potency of various bile concentrations: (**a**) 1 min in 1/1 bile solution, (**b**) 1 min in 1/8 bile solution, (**c**) 10 min in 1/64 bile solution, (**d**) 60 min in 1/64 bile solution. Color version available online.

Table I. Bile concentrations and resulting scolicidal activity over time.

Bile concentration	Dead scolices (%/min) at: 1 min	10 min	30 min	60 min
1/1	100	100	100	100
1/2	98	100	100	100
1/4	90	100	100	100
1/8	70	80	100	100
1/16	66	77	93	98
1/32	59	68	89	95
1/64	53	58	77	93
1/128	18	25	37	48
1/256	8	9	14	17
Control (0.1% eosin)	0	0	0	0

10 min, and 100% efficacy after 30 min. At a 1/16 concentration, bile showed 66% efficacy at 1 min, 77% at 10 min, 93% at 30 min, and 98% at 60 min. Bile at a 1/32 concentration exhibited 59% efficacy at 1 min, 68% at 10 min, 89% at 30 min, and 95% at 60 min. At a 1/64 concentration, bile demonstrated 53% efficacy at 1 min, 58% at 10 min (Fig. 3c), 77% at 30 min, and 93% at 60 min (Fig. 3d). Bile at a 1/128 concentration showed 18% efficacy at 1 min, 25% at 10 min, 37% at 30 min, and 48% at 60 min. Bile at a 1/256 concentration demonstrated 8% efficacy at 1 min, 9% at 10 min, 14% at 30 min, and 17% at 60 min. In the control group in which 0.1% eosin solution was used, no significant scolicidal activity was observed at 60 min, and the scolices remained viable throughout the study (Table I).

The Shapiro-Wilk test indicated that the data for 1 min ($P > 0.05$, skewness = 0.602, kurtosis = −0.611) and 10 min ($P > 0.05$, skewness = 0.909, kurtosis = −0.254) conformed to a normal distribution, whereas the data for 30 min ($P < 0.05$, skewness = 0.909, kurtosis = −1.328) and 60 min ($P < 0.05$, skewness = 1.105, kurtosis = −0.690) did not conform to a normal distribution. Given the non-normal distribution and the presence of repeated measures, the non-parametric Friedman test was applied to evaluate the differences in scolicidal efficacy across the time intervals. The Friedman test revealed differences in scolicidal activity between the time intervals ($\chi^2 = 13.056$, $P = 0.005$). The Wilcoxon signed-ranks test was then conducted to determine which specific time intervals differed. The Wilcoxon test indicated a difference between the 1 min and 10 min intervals ($Z = -2.524$, $P = 0.012$). However, no differences were found between the 10 min and 30 min intervals ($Z = -0.943$, $P = 0.345$) or between the 30 min and 60 min intervals ($Z = -0.674$, $P = 0.500$) (Table II).

Analysis of the effect of the bile dilutions on the scolicidal effect revealed that there were bile dilutions that did not conform to a normal distribution: 1/2 bile ($P = 0.001$, skewness = 2.000, kurtosis = 4.000) and 1/4 bile ($P = 0.001$, skewness = 2.000, kurtosis = 4.000). Again, the non-parametric Friedman test was applied this time to evaluate the differences in scolicidal efficacy across the bile dilutions. Results indicated that there were significant differences in scolicidal efficacy across the different bile dilutions ($\chi^2 = 31.368$, $P < 0.001$). To identify specific differences between consecutive bile dilutions, the Wilcoxon signed-ranks test was applied but indicated no differences between consecutive bile dilutions: 1/1 bile and 1/2 bile ($Z = -1.000$, $P = 0.317$), 1/2 bile and 1/4 bile ($Z = -1.000$, $P = 0.317$), 1/4 bile and 1/8 bile ($Z = -1.414$, $P = 0.157$), 1/8 bile and 1/16 bile ($Z = -1.826$, $P = 0.068$), 1/16 bile and 1/32 bile ($Z = -1.826$, $P = 0.068$), 1/32 bile and 1/64 bile ($Z = -1.826$, $P = 0.068$), 1/64 bile and 1/128 bile ($Z = -1.826$, $P = 0.068$), and 1/128 bile and 1/256 bile ($Z = -1.826$, $P = 0.068$) (Table II).

DISCUSSION

In our study, we demonstrated that the scolicidal impact of bile decreases with dilution ($P < 0.001$) and increases over time ($P = 0.005$). Bile concentrations up to 1/64 showed potent scolicidal activity over time, indicating that bile is a strong scolicidal agent. The bile, obtained strictly from the gallbladder, maintained its scolicidal potency down to a 1/32 concentration, which is close to physiological conditions. Following these analyses, 2 conclusions were reached by demonstrating the scolicidal effects of bile fluid on EG scolices. First, bile fluid exhibits scolicidal activity and could potentially be used as a scolicidal agent. Its main advantage in this aspect lies in being an endogenous secretion, which is less likely to have side effects such as cholangitis, anaphylactic shock, or several electrolyte abnormalities seen with other scolicidal agents.

Table II. Comparison of scolicidal efficacy across time intervals and bile concentrations.

Statistical test	Time interval (min)	Mean rank	χ^2	P value	Bile concentration	Mean rank	χ^2	P value
Friedman	1	3.61	13.056	0.005	1/1	2.00	31.368	<0.001
	10	2.50			1/2	2.25		
	30	2.11			1/4	2.50		
	60	1.78			1/8	3.25		
					1/16	5.00		
					1/32	6.00		
					1/64	7.00		
					1/128	8.00		
					1/256	9.00		
Wilcoxon signed-ranks		Z				Z		
	1–10	−2.524		0.012	1/1–1/2	−1.000		0.317
	10–30	−0.943		0.345	1/2–1/4	−1.000		0.317
	30–60	−0.674		0.500	1/4–1/8	−1.414		0.157
					1/8–1/16	−1.826		0.068
					1/16–1/32	−1.826		0.068
					1/32–1/64	−1.826		0.068
					1/64–1/128	−1.826		0.068
					1/128–1/256	−1.826		0.068

Although we have theoretically demonstrated the effects of bile in our study, its practical application will involve further research in many areas. The ratio of cyst size to gallbladder volume must be appropriately considered. Because cholecystectomy adds an extra surgical burden, it should be undertaken with care and only with patients with additional cholecystectomy indications. Although the inherent risks of cholecystectomy, such as bleeding, infection, or bile fistula, are low, the procedure should be limited to suitable patients, and performance of these procedures without meeting the mentioned conditions may lead to ethical concerns. The second conclusion that can be drawn from this study is the scolicidal efficacy in HC samples exposed to bile obtained by opening the bile ducts. This result may encourage physicians to delay surgical treatment in cases where the bile ducts have been opened but have not become complicated with conditions such as cholangitis. Instead of surgery, patients could be monitored and given and oral anthelmintic drugs to combat the disease.

Cyst enlargement and increased intracystic pressure can lead to the elevation of pressure in the nearby biliary tract, resulting in the rupture of the cyst and spillage of the contents into the biliary tract, which happens in cases of cysto-biliary fistulas. When these models are analyzed, diminished vitality of cyst contents is also observed. However, the main reason for this phenomenon has not yet been determined (Ramia et al., 2012).

Studies of bile solids indicate that they consist of bile salts, electrolytes (specifically potassium, calcium, sodium, and chloride), fatty acids, cholesterol, bilirubin, and lecithin (Chiang, 2004; Portincasa et al., 2008; Boyer, 2013). The primary scolicidal effect of bile is expected to be due to its high bile acid and electrolyte content. There are no comparative published studies on the scolicidal effect of these specific components.

Saline solutions, used widely due to their sodium concentration and osmotic pressure effects, induce scolex apoptosis by affecting the cuticular membrane. However, increased concentrations of saline both enhance scolicidal efficacy and elevate the risk of side effects (Kayaalp et al., 2001; Shi et al., 2016). Other scolicidal agents, such as cetrimide and chlorhexidine, offer strong efficacy but also carry risks. For example, cetrimide is effective but should be used cautiously due to adhesive effects on peritoneal surfaces (Besim et al., 1998; Sonisik et al., 1998). In our study, bile at 1/64 dilution exhibited potency similar to that of high concentrations of saline solutions, and 1/8 dilution exhibited potency similar to that of cetrimide solutions.

Phytotherapeutic agents, such as *Sambucus ebulus,* show promise; at high concentrations, *S. ebulus* had scolicidal efficacy similar to that of a 1/8 bile concentration (Gholami et al., 2013). In studies most relevant to bile's scolicidal properties, chenodeoxycholic acid was tested on scolices and had dose-dependent efficacy, though with slower scolicidal effects compared with those of bile rich in electrolytes (Shi et al., 2016). In our study, bile fluid from the gallbladder, which is rich in electrolytes, yielded more rapid results. Unlike other scolicidal agents with increased risk of short- and long-term adverse effects, bile does not exhibit such effects probably due to its endogenous nature. Moreover, bile is as effective as other reliable and potent scolicidal agents.

The main limitation of our study was the lack of detailed analysis of bile's base components. Bile samples were obtained from cholelithiasis patients after at least 8 hr of fasting, when bile concentrations are typically 5–30 times higher. We avoided bile from patients with external drainage or biliary diversion because these procedures can lead to abnormal bile composition due to altered liver and biliary function, affecting bile salt secretion and levels of secretin and cholecystokinin (Kayaalp et al., 2002b; Portincasa et al., 2008; Ramia et al., 2012).

Only patients with normal preoperative liver function and no history of liver failure or chronic liver disease were included for bile sampling. Osmotic pressure from electrolyte differences likely induced apoptosis, whereas bile salts impacted scolices through the caspase-3 pathway (Shi et al., 2016). Although we did not quantify bile salts, we examined dilutions down to 1/256. By excluding recurrent cases and verifying the viability and quantity of the scolices, we ensured reliable measurements. The Friedman test indicated significant differences across bile concentrations ($P < 0.001$) and time ($P = 0.005$), whereas the Wilcoxon test indicated differences only between results at 1 min and those at 10 min ($P = 0.012$). No significant differences appeared among subsequent dilutions, likely due to 100% efficacy by 10 min for the first 3 dilutions. Given this rapid scolicidal effect, we suggest evaluating shorter time intervals for greater accuracy.

This study has demonstrated the scolicidal efficacy of bile fluids in an in vivo environment and provided support for an observational understanding of the progression of cyst stages in patients who develop cysto-biliary fistulas during follow-up. These results lay the groundwork for future studies aimed at evaluating the scolicidal efficacy of synthetic bile acids combined with hypotonic solutions in the selection of the ideal scolicidal bile.

CONCLUSION

In this study, bile had potent scolicidal activity that increased with higher concentrations and over longer periods. Because it is an endogenous secretion, bile may be an ideal scolicidal agent due to its potency and fewer side effects. Further analysis of the scolicidal effects of synthetic bile acids can be evaluated in future studies. Additionally, this study supports the hypothesis of lower vitality of cyst contents in patients with cysto-biliary fistulas.

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