The anthelmintic effects of the ethanol extract of *Terminalia catappa* L. leaves against the ruminant gut parasite, *Fischoederius cobboldi*

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SUMMARY

Presently, no effective anthelmintic drugs have been used to treat and control paramphistomosis, a severe disease of ruminants. In this study, we have investigated the *in vitro* anthelmintic effect of the leaves of *Terminalia catappa* L. crude extract (TcCE) and albendazole (ABZ) on adult *Fischoederius cobboldi* after incubating the flukes in RPMI-1640 medium containing the TcCE at various doses and times. The TcCE-treated flukes at all dosages exhibited rapid decrease of motility, and the relative motility (RM) values were decreased sharply from start to 3 h. Worms were killed after 6 and 12 h of treatment with 1000, 1500 and 2000 μg mL^{-1} as well as 500 μg mL^{-1} of TcCE, respectively. By light microscopy examination, the flukes exhibited the earliest alteration in a limited area of the tegument. At scanning electron microscopy level, the flukes' tegument showed similar sequence of morphological alterations after treatment with ABZ and TcCE that consisted of swelling of ridges and folds, followed by blebbing and rupturing of the blebs, leading to the erosion, lesion and disruption of the tegument. Hence, *in vivo* studies should be performed to examine whether the TcCE may serve as a powerful anthelmintic drug for treatment of paramphistomosis.

Key words: *Fischoederius cobboldi*, *Terminalia catappa* L., anthelmintic drug, motility, survival, tegument, light microscopy, scanning electron microscopy.

INTRODUCTION

Paramphistome, also known as rumen fluke, is one of the most common parasites that reside in the rumen and reticulum of domestic and wild ruminants, i.e. cattle, goats, sheep and buffaloes. Rumen flukes belong to the superfamily Paramphistomoidea and are an important cause of paramphistomosis in many countries (Horak, 1971; Hanna *et al*. 1988; Wang *et al*. 2006; Sanabria and Romero, 2008; Anuracpreeda *et al*. 2008, 2012). Adult paramphistomes cause chronic ulcerative ruminitis and anaemia (Hanna *et al*. 1988; Rolfe *et al*. 1991; Anuracpreeda *et al*. 2013*b*). Large numbers of immature parasites cause severe acute gastroenteritis, dehydration, maldigestion and death, particularly in young animals (Ilha *et al*. 2005; Khan *et al*. 2008). In addition, paramphistomosis in domesticated animals causing economic losses with morbidity and mortality rates as high as 80–90% in livestock industry (Prasitirat *et al*. 1977*a*, *b*; Gupta *et al*. 1978; Hanna *et al*. 1988; Khan *et al*. 2008; Tariq *et al*. 2008; Anuracpreeda *et al*. 2015). It was reported that the outbreaks of clinical paramphistomosis caused by immature flukes in ruminants are not diagnosed and subclinical infection often passes undiagnosed. The prevalence of paramphistomosis is high and distributed in tropical and subtropical regions in Africa, Australia, Eastern Europe, Russia as well as South and Southeast Asia (Gupta *et al*. 1978; Nikitin, 1979; Hanna *et al*. 1988; Rolfe *et al*. 1991; Geurden *et al*. 2008; Tariq *et al*. 2008; Panyarachun *et al*. 2010). It has been revealed that paramphistomosis by *Gastrodiscoides hominis*

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Leiper, 1913 (Gastrodiscidae) was found in pigs, equines and occasionally reported in humans in Burma, China, India, Kazakhstan, the Philippines, Vietnam and Thailand (Ahluwalia, 1960; Dutt and Srivastava, 1972; Kumar, 1980; Harinasuta *et al*. 1987). In Thailand, the occurrence of paramphistomosis, mainly from *Fischoederius cobboldi*, has also been reported (Chethanon *et al*. 1985; Prasitirat *et al*. 1997*a*; Anuracpreeda *et al*. 2012). *Fischoederius cobboldi* are digenetic trematode parasites which are member of the family Gastrothylacidae. The disease causes a lower nutrition conversion, decrease in milk production, loss of weight and affects the productivity, resulting in considerable economic losses. Likewise, the prevalence of this disease ranged from 63·3 to 80·0% in beef cattle, while it was as high as 14·0–35·8% in dairy cattle (Chethanon *et al*. 1985; Prasitirat *et al*. 1997*a*). Bithionol sulfoxide has been reported to be highly effective and recommended for the treatment of paramphistomosis in cattle, sheep and goats (Rolfe and Boray, 1988). However, the resistance of rumen flukes to this drug has emerged and may pose a serious problem as no other effective drug is available (Prasitirat *et al*. 1997*b*). Thus, new anthelminthics are urgently needed.

Although several plant extracts have been tested for their anthelmintic efficacies against nematode, cestode and trematode parasites (Robinson *et al*. 1990; Tandon *et al*. 1997; Wongsawad *et al*. 2005; Athanasiadou *et al*. 2007; Magalhães *et al*. 2010; Hossain *et al*. 2012), no similar studies have been performed on *F. cobboldi*. It has been reported that *Terminalia catappa* L., Malabar or Indian almond, is a multipurpose medicinal herb of the family Combretaceae. This species is broadly distributed in countries with tropical and subtropical regions, particularly in beach areas (Thomson and Evans, 2006). The extracts of leaves, bark and fruit of *T. catappa* have been commonly used for the treatment of several symptoms and diseases: dermatitis, homeostasis, fever, diarrhoea and urinary infection (Lin *et al*. 1997; Chen *et al*. 2000; Germosén-Robineau, 2014), microbial and fungal infections (Fyhrquist *et al*. 2002), hepatoma (Lin *et al*. 1997; Chen *et al*. 2000; Tanaka *et al*. 2001; Tang *et al*. 2003), cancer metastasis (Chu *et al*. 2007; Yeh *et al*. 2012), diabetes mellitus (Nagappa *et al*. 2003) and gastric infection (Nunes *et al*. 2012; Kumar *et al*. 2014). In Thailand, Atjanasuppat *et al*. (2009) previously described the anthelmintic effect of the ethanol extract obtained from leaves of *T. catappa*, locally known as Hu-kwang in Thailand (Veesommai and Janjittikul, 2001). Up to now, very few drugs were used for the treatment of paraphistomosis in ruminants. In the present study, we have investigated the anthelmintic effects of the ethanol extract of *T. catappa* leaves on motility, survival and the tegumental surface of adult *F. cobboldi in vitro* using relative motility (RM) assay and observation by light microscopy (LM) and scanning electron microscopy (SEM).

MATERIALS AND METHODS

Collection of adult flukes

Collection of adult parasites was done according to the method described by Anuracpreeda *et al*. (2015) and Panyarachun *et al*. (2013). Briefly, adult *F. cobboldi* were collected from the rumens of infected cattle and water buffaloes killed for consumption at local abattoirs in Pathumthani province, Thailand. After washing flukes several times with 0·85% NaCl solution, the healthy ones with normal structure and active motility were selected and immediately used for the experiments.

Preparation of plant materials and crude extracts

The leaves of *T. catappa* L. were collected from a local area of Songkhla Province, Thailand. The plant materials were identified by Dr Panupong Puttarak and the voucher specimens (specimens no. SKP 049 20 03 10) were deposited at the herbarium of Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand, for future reference. The plant name has been checked and corresponded to the latest revision in 'The Plant List' (www.theplantlist.org). The leaves from *T. catappa* were rinsed thoroughly to remove any foreign matter with tap water and dried by hot air oven at 50 °C for 2 days. Then, the dried plants were powdered using a commercial electric grinder, weighed and stored in a desiccator at room temperature in the dark. Each plants powder (625 g) were macerated with 6000 mL of ethanol at room temperature for 2 days and filtered through a filtering paper (Whatman® No. 1). The maceration processes were performed three times. Thereafter, each filtrate was pooled and evaporated to dryness under reduced pressure at 45 °C by rotary evaporator. Finally, the resulting crude extracts (48·89 g) were kept in a refrigerator at −20 °C protected from light until use in later experiments.

Experimental designs

Drugs and media. The stock solution was made by dissolving 1 g powder of *T. catappa* crude extract (TcCE) in 2 mL of dimethyl sulphoxide (DMSO) (Sigma Co., St. Louis, MO). The sterile RPMI-1640 culture medium (Gibco) [pH 7·4 with HEPES 20 mM, supplemented with penicillin (50 IU mL^{-1}), streptomycin (50 μg mL^{-1}), gentamycin (50 μg mL^{-1}) and 20% fetal bovine serum (FBS)] was mixed with the drug stock solution to obtain the required concentrations at 500, 1000, 1500 and

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2000 μg mL^{-1} of the crude extract in the medium, and used for this experiment. A commercial anthelmintic albendazole (ABZ) (Sigma Co.) was used as the positive control, and the stock solution was prepared by dissolving 100 mg powder of ABZ in 1 mL of DMSO. The RPMI-1640 medium containing antibiotics and FBS was mixed with the drug stock solutions of ABZ to give a concentration of 100 μg mL^{-1}. Culture medium containing antibiotics and 0·1% (v/v) DMSO without TcCE was used as the negative control.

In vitro *incubation with the drugs*. Nine hundred adult flukes were separated into six groups (150 flukes per group): group 1 was the negative control; group 2 was treated with 100 μg mL^{-1} of ABZ as the positive control; groups 3–6 were treated with various doses of TcCE as mentioned in section of drugs and media. Three replicates were done for each group. The flukes in culture medium were incubated with 5% CO_2 for 24 h at 37 °C and were observed under a stereomicroscope for motility at 1, 3, 6, 12 and 24 h of incubation times (30 flukes per incubation time). The time required for complete inactiveness or paralysis and death of fluke was recorded, and the tegumental changes were examined under LM and SEM.

The motility of the parasites at each incubation period was scored using the following criteria (Kiuchi *et al*. 1987): score 3 = moving the whole body, score 2 = moving only some parts of the body, score 1 = immobile but not dead, unstained with 1% vital dye, and score 0 = immobile and dead, stained with vital dye [the vital dye was composed of 1% methylene blue diluted in 0·85% (w/v) NaCl]. The efficacies of the tested drugs against adult *F. cobboldi* were evaluated from the RM value (Kiuchi *et al*. 1987), and calculated as follows:

$$\text{RM value} = \frac{\text{MI test}}{\text{MI control}} \times 100$$

$$\text{Motility index (MI)} = \frac{\sum nN}{\sum N}$$

n = score, N = number of flukes with the score of n

LM analysis. Preparation of parasite specimens for LM examination was performed according to the method described by Anuracpreeda *et al*. (2013*a*, 2014). Briefly, flukes from each group were fixed in Bouin's fixative solution for 12 h, and stored in 70% ethanol. They were dehydrated with a series of ethanol, cleared with xylene and embedded in paraffin. Then, the serial-sections with 6 μm-thickness were cut by a Leica RM2125 microtome, and stained with haematoxylin and eosin. These specimens were observed for abnormalities and photographed under a light microscope (Olympus BX51).

SEM analysis. For SEM examination, parasite specimens were prepared as described by Anuracpreeda *et al*. (2015). Briefly, adult worms were fixed in 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer (Sigma-Aldrich, USA) containing calcium acetate, pH 7·2, at 4 °C for 2 h. After washing three times with the same buffer, the specimens were re-fixed in 1% osmium tetroxide (Sigma-Aldrich) with 0·1 M sodium cacodylate buffer (pH 7·2) at 4 °C for 1 h. Subsequently, they were washed in three changes of distilled water, and dehydrated with increasing concentrations of ethanol (from 50 to 100%). They were dried in a HCP-2 critical point drying apparatus (Hitachi, Japan) using liquid carbon dioxide as a transitional medium for 15 min. Thereafter, the specimens were mounted on aluminium stubs and coated with gold in an ion-sputtering apparatus (SPI-Model sputter coater; Structure Probe, USA) for 4 min. Finally, the specimens were examined with a JSM-5400 electron microscope (JEOL, USA) operating at 10–15 kV.

RESULTS

Evaluation of the motility of F. cobboldi

Negative control group. As shown in Table 1 and Fig. 1, all flukes incubated in RPMI-1640 medium containing 0·1% DMSO remained actively mobile with whole body movement (score 3) throughout the experimental period of 24 h (RM = 100).

Positive control group. At 6 h incubation, flukes treated with 100 μg mL^{-1} of ABZ started to show decreased motility. All flukes were still alive with active movement of whole (70%) and some parts (30%) of their bodies (RM = 90). At 12 h examination time point, only 30% of flukes showed no movement, but they were still alive and unstained with vital dye (RM = 73). After 24 h incubation, 30% of flukes were still active (score 3), 40% of them exhibited movement of only some parts of their bodies (score 2), and 30% of those showed no movement, but unstained with vital dye (score 1) (RM = 67) (Table 1 and Fig. 1).

TcCE group. When flukes were treated with TcCE at various concentrations (500–2000 μg mL^{-1}), the worm motility and the RM value decreased through the experimental period at a more rapid rate than the positive control group (flukes treated with ABZ). In 500 μg mL^{-1} of TcCE, the worms showed rapid reduction of the RM value from start to 3 h (RM = 23), then the RM value was stable between 3 and 6 h (RM = 20–23). Then the flukes became completely immobile and killed at 12 h (RM = 0). In 1000, 1500 and 2000 μg mL^{-1} of TcCE, the RM value dropped rapidly from start to 6 h (RM = 0). All of the worms ceased to be mobile at 6 h, and

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Table 1. RM values (%) of control and flukes treated with ABZ and TcCE at different hours after incubation

	Incubation time (h)					
Groups	0	1	3	6	12	24
Control	100	100	100	100	100	100
ABZ						
100 μg mL^{-1}	100	100	100	90	73	67
TcCE						
500 μg mL^{-1}	100	67	23	20	0	0
1000 μg mL^{-1}	100	67	13	0	0	0
1500 μg mL^{-1}	100	67	10	0	0	0
2000 μg mL^{-1}	100	67	5	0	0	0

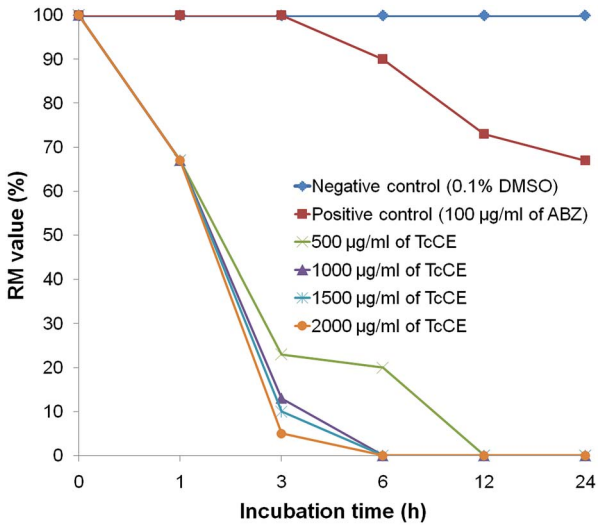

Fig. 1. RM values of adult *F. cobboldi* after *in vitro* incubations in ABZ and the crude extract of *T. catappa* L. (TcCE) at various doses and times. Each point in the graph represents the response from 30 flukes.

most of them took up the vital dye which indicated that they were killed and dead (Table 1 and Fig. 1).

Alterations of tegumental surface as observed by LM

Negative control group. After 24 h of incubation in the medium without drugs, all parasites showed normal appearance of the tegument with no damage of the tegumental folds and grooves, muscles and basement membrane (Fig. 2A).

Positive control group. At 24 h post incubation, all ABZ-treated flukes exhibited some vacuoles, small blebbing and disrupted blebs on the tegumental surface, while the underlying structures still exhibited normal appearance (Fig. 2B).

TcCE group. In all treated worms, TcCE exhibited more severe effects on the tegument and underlying structures than ABZ. For instance, TcCE at the concentration 500 μg mL^{-1} at 1 h incubation caused partial disruption of the tegument, numerous vacuoles and blebbings. These blebs were disrupted, resulted in the thinning of tegument at 3 h post incubation, while the underlying structures still showed normal finding (Fig. 2C). In TcCE at concentration 1000 μg mL^{-1} at 3 h incubation, a general disruption of the tegument was found. The components in the tegument were degenerated and partly sloughed off, but muscle underlying the tegument showed less severe morphological change (Fig. 2D). At 6 h incubation in 1500 and 2000 μg mL^{-1} of TcCE, the tegument was totally destroyed and sloughed off, but the underneath muscle layers and parenchyma were less damaged (Fig. 2E and F). These findings indicated that the tegument is the most affected target organ from the treatment.

Alterations of tegumental surface as examined by SEM

The body of the adult *F. cobboldi* can be divided into three parts based on the body shape and microtopography of the surface: (1) the anterior part covers an area from the oral sucker to the genital canal; (2) the middle part is the largest area of the body with most of the ventral pouch; and (3) the posterior part covers a large posterior sucker.

Negative control group. The adults *F. cobboldi* of control group which were incubated for 24 h in the medium containing 0·1% DMSO showed normal tegumental syncytium with no damage of the surface architecture. The key features of surface topography of the flukes were reported earlier by Anuracpreeda *et al.* (2012). Flukes possess conical-shaped body, concave ventrally and convex dorsally. The tegument exhibited transverse folds alternating with grooves and was spineless. The anterior sucker is positioned at the anterior part (Fig. 3A), and the genital pore is located ventrally at the anterior one-third of the body. The posterior sucker is positioned close to the posterior part of the body (Fig. 3D). There are two types of sensory papillae on the surface: type 1 is bulbous in shape with nipple-like tips (Fig. 3E), and type 2 is a similar shape and size and also has short cilia on tips (Fig. 3B). At high magnification, the surface of each fold is composed of a meshwork of ridges separated by

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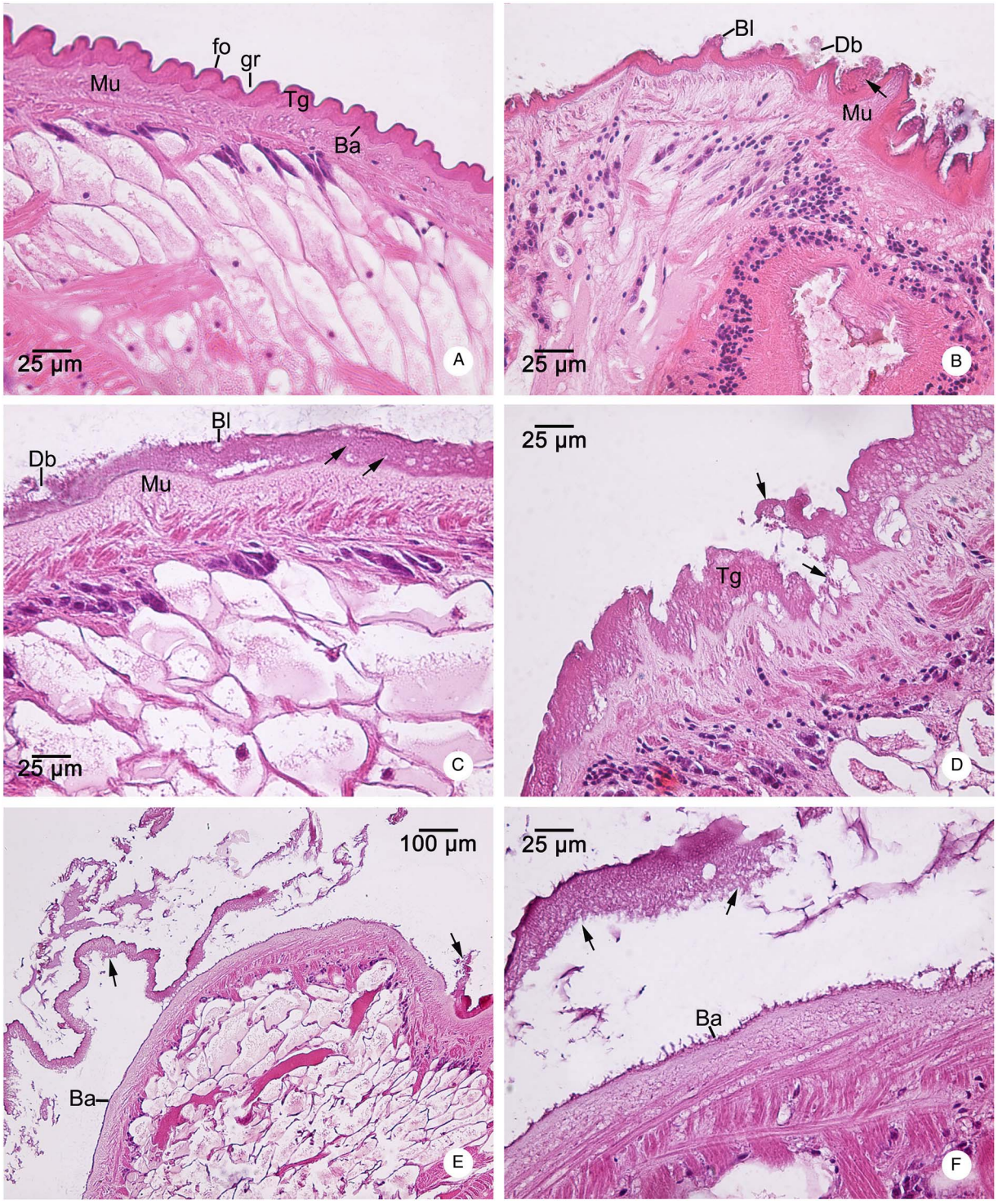

Fig. 2. LM micrographs of untreated (A), ABZ-treated (B) and TcCE-treated adult *F. cobboldi* (C–F). (A) The negative control fluke incubated in RPMI-1640 medium containing 0·1% DMSO for 24 h, showing normal features of tegument (Tg) without spines, folds (fo), grooves (gr), muscle (Mu) lying underneath the basement membrane (Ba). (B) The fluke treated with 100 μg mL^{-1} of ABZ for 24 h, showing some vacuoles (arrow), formation of small blebs (Bl) and disrupted blebs (Db) on the tegument, while the muscle (Mu) and underlying structures appear normal. (C) Fluke treated with 500 μg mL^{-1} of TcCE for 3 h, showing numerous vacuoles (arrows), blebs (Bl) and disrupted blebs (Db), while the muscle (Mu) and other structures underneath the basement membrane show normal. (D) Following 3 h incubation in the TcCE at the concentration 1000 μg mL^{-1}, the fluke shows disrupted tegument (Tg, arrows). (E and F) After 6 h incubation with 1500 μg mL^{-1} (E) and 2000 μg mL^{-1} (F) of TcCE, appearance of extreme damage of the tegumental surface with extensive degeneration and sloughing (arrows) from the basement membrane (Ba).

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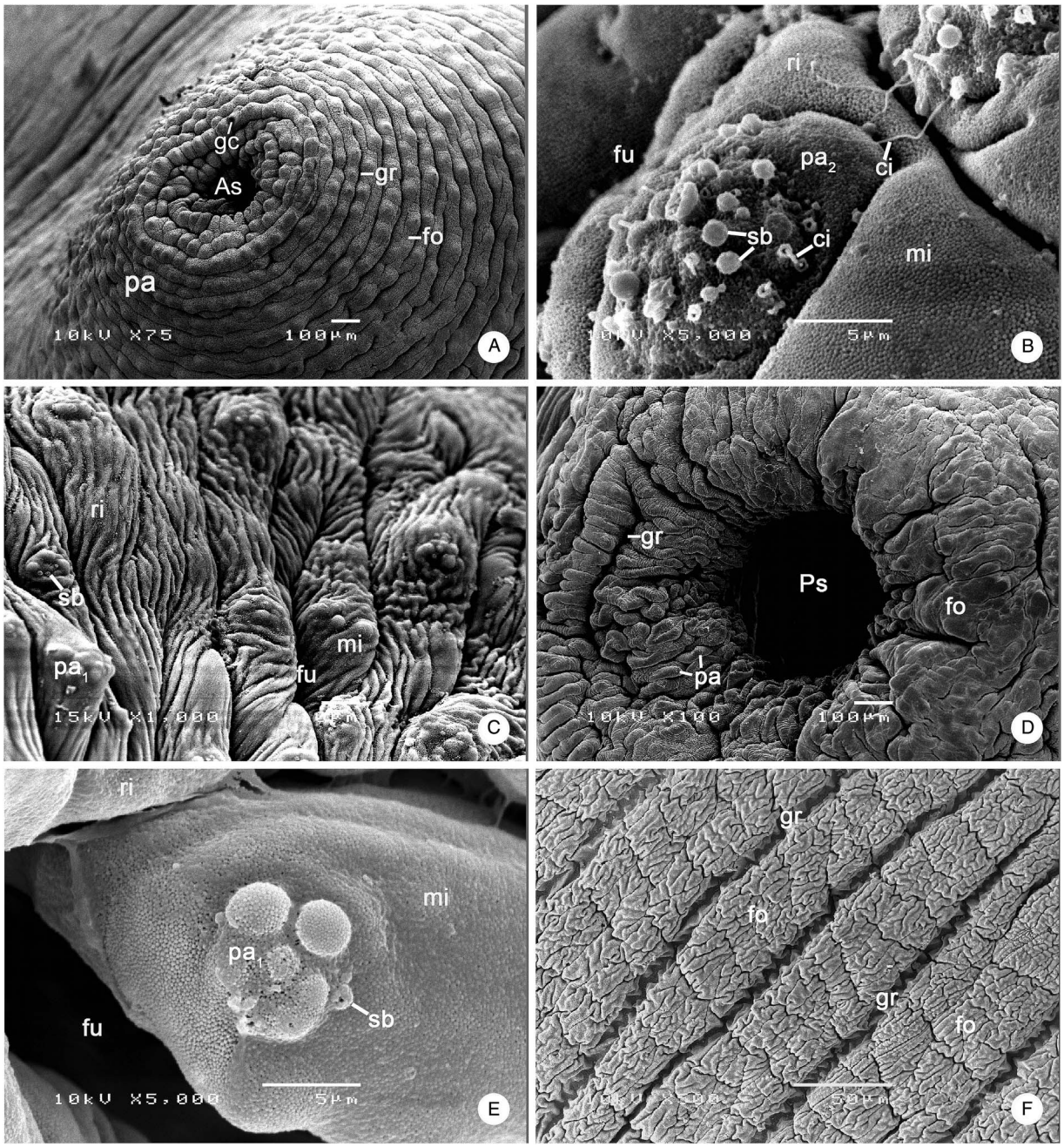

Fig. 3. SEM micrographs of the tegumental surface of the control adult *F. cobboldi* treated with 0·1% DMSO for 24 h. (A–E) Anterior, middle and posterior parts of the ventral surface of adult *F. cobboldi*. (A) The anterior sucker (As) surrounded by rows of papillae (pa), pores of glands (gc), folds (fo) as well as grooves (gr), while spines are absent. (B) At higher magnification, the surface shows numerous clusters of type 2 papillae (pa_2) with short cilia (ci) on the nipple-like tips surrounded by small bulbs (sb) and the surface also exhibits furrows (fu) and a series of ridges (ri) bearing numerous microvilli (mi). (C) The middle part appears highly corrugated with ridges (ri) bearing numerous microvilli (mi) separated by furrows (fu) and numerous type 1 papillae (pa_1) with nipple-like tips surrounded by small bulbs (sb). (D) The surface area of the posterior sucker (Ps) exhibits folds (fo) and grooves (gr) bearing rows of papillae (pa), while spines are absent. (E) An enlarged view of the surface area immediately posterior to the posterior sucker exhibits ridges (ri), furrows (fu) and type 1 papillae (pa_1) with nipple-like tips enclosed by small bulbs (sb) and numerous microvilli (mi). (F) The dorsal surface area appears as highly corrugated folds (fo) alternated with grooves (gr).

furrows whose surface bears numerous closely packed microvilli (Fig. 3B, C and E). The dorsal surface exhibits similar surface features as the ventral surface, but papillae have less numerous and are smaller than ventral surface (Fig. 3F).

Positive control group. Similarly to the control group, flukes treated with ABZ showed the general features of the tegument at 24 h post incubation. However, slight swelling of the tegument was observed on the anterior and posterior parts. Blebs

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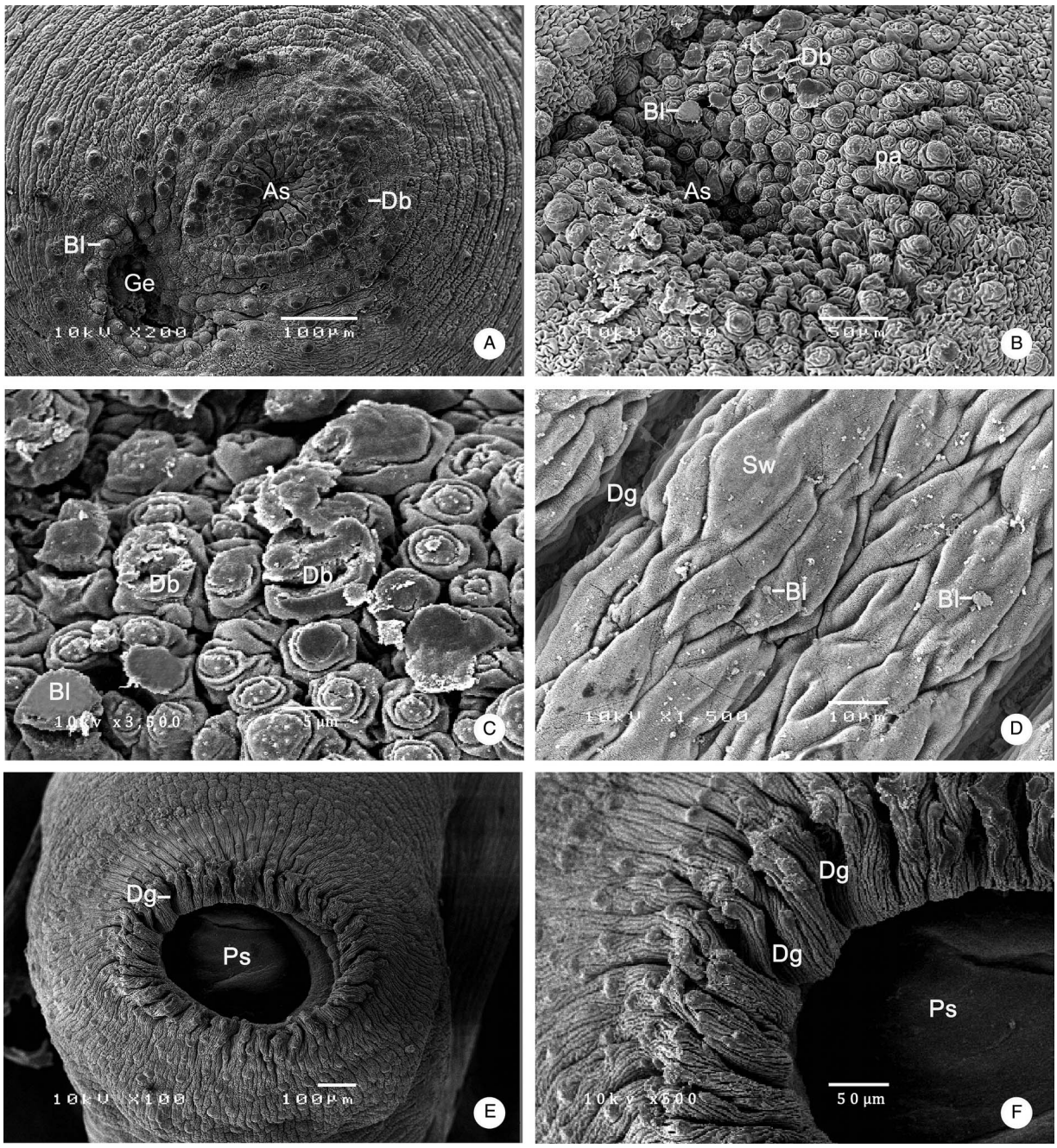

Fig. 4. SEM micrographs showing the tegumental surface alterations in adult *F. cobboldi* treated with ABZ at the concentration of 100 μg mL^{-1} for 24 h. (A–F) Anterior, middle and posterior parts of the ventral surface of adult *F. cobboldi*. (A, B) The anterior sucker (As) and genital canal (Ge) show mild swelling of the tegument, the papillae (pa) with blebs (Bl) and some disrupted blebs (Db). (C) A higher magnification shows blebs (Bl) and some disrupted blebs (Db) on the papillae. (D) The middle part appears as swollen tegument (Sw) with deep grooves (Dg) and blebs (Bl). (E and F) The posterior sucker (Ps) exhibits mild swelling of the tegument with wide and deep grooves (Dg).

on top of papillae were observed around the anterior sucker (Fig. 4A and B) and also some blebs were ruptured (Fig. 4C). In addition, wide and deep grooves were observed on the folds on the ventral and dorsal surfaces (Fig. 4D), particularly along the posterior sucker (Fig. 4E and F). These tegumental surface changes affected the anterior and ventral parts more drastically than the posterior and dorsal parts.

TcCE group. The sequences of changes and severity of tegumental damage of flukes after treatment with TcCE were composed of the following: (1) swelling of ridges and folds, (2) blebbing formation on the tegument, (3) disruption of blebs, (4) erosion and lesion of the tegument and (5) total disintegration of the tegument. Grading of the tegumental changes and disruption in the flukes during the course of incubation with TcCE is summarized in Table 2.

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Table 2. Summaries of the sequence of tegumental alterations and damages in adult *F. cobboldi* after *in vitro* treatments with ABZ and TcCE as examined by SEM

Drugs (μg mL^{-1})	Incubation time (h)	Ventral surface of tegument			Dorsal surface of tegument		
		Anterior	Middle	Posterior	Anterior	Middle	Posterior
Control	1	▬	▬	▬	▬	▬	▬
	3	▬	▬	▬	▬	▬	▬
	6	▬	▬	▬	▬	▬	▬
	12	▬	▬	▬	▬	▬	▬
	24	▬	▬	▬	▬	▬	▬
ABZ							
100	1	▬	▬	▬	▬	▬	▬
	3	▬	▬	▬	▬	▬	▬
	6	▲	▬	▬	▬	▬	▬
	12	▲	▲	▬	▬	▬	▬
	24	▲●■	▲	▲	▲	▲	▬
TcCE							
500	1	▲●■	▲	▲●	▲●	▲	▲
	3	▲●■	▲●■	▲●■	▲●	▲	▲●
	6	●■◆	●■◆	■◆	▲■◆	▲■◆	▲■◆
	12	‡	■◆	‡	‡	■◆	■◆
	24	‡	‡	‡	‡	‡	‡
1000	1	▲●■	▲●	▲●	▲●	▲●	▲●
	3	●■◆	▲●■	●■◆	●■◆	▲●■	●■◆
	6	‡	■◆	‡	‡	■◆	‡
	12	‡	‡	‡	‡	‡	‡
	24	‡	‡	‡	‡	‡	‡
1500	1	●■	●■	●■	●■	●■	●■
	3	●■◆	●■◆	■◆	■◆	●■	■◆
	6	‡	■◆	‡	‡	◆	‡
	12	‡	‡	‡	‡	‡	‡
	24	‡	‡	‡	‡	‡	‡
2000	1	■◆	■	■	■◆	●■	■
	3	◆	◆	◆	■◆	●■	■◆
	6	‡	◆	‡	‡	◆	‡
	12	‡	‡	‡	‡	‡	‡
	24	‡	‡	‡	‡	‡	‡

▬Normal appearance.
▲Swelling of the tegumental ridges and folds.
●Blebbing formation on the tegument.
■Rupturing of the blebs on the tegument.
◆Erosion and lesion of the tegument.
‡Total demolition of the tegument.

Three hour post incubation with 500 μg mL^{-1} of TcCE, the ventral surface of the tegument exhibited swelling and deep grooves at the anterior and posterior parts of the ventral surface (Fig. 5A and B). Several blebs were observed around the anterior and posterior suckers (Fig. 5C), and some blebs were ruptured. On the dorsal surface, the tegument showed moderated swelling of ridges and folds, and blebs were also observed on the anterior and posterior parts. Following 6 h treatment, numerous blebs covered the swollen tegument along the anterior, middle and posterior parts, especially around the posterior sucker, where blebs were ruptured, and erosion and lesions were observed in this region (Fig. 5D). On the dorsal surface, disrupted blebs and swollen tegument were observed on the anterior, middle and posterior parts of the body. At 12 h incubation, the treated flukes showed body deformity, most tegumental surfaces on the anterior and posterior parts of both ventral and dorsal surfaces were eroded and lesions were more pronounced throughout the ventral surface. In addition, all parts of the dorsal surface appeared to be highly disrupted and showed complete lesion.

TcCE at concentrations 1000, 1500 and 2000 μg mL^{-1} caused similar sequence of damages on the flukes' tegumental surface, with more severe destruction appearing at the earlier incubation time. At 3 h examination time point, tegumental damages were similar, but occurred more rapidly and severely than those seen at concentration 500 μg mL^{-1} of TcCE. The anterior and posterior parts of the ventral and dorsal surfaces exhibited a large area of the tegumental swelling, disruption, lesion and erosion (Figs 5E and F, 6A–E). Severe lesion was also observed on the anterior part of the ventral surface, whereas the dorsal surface showed severe

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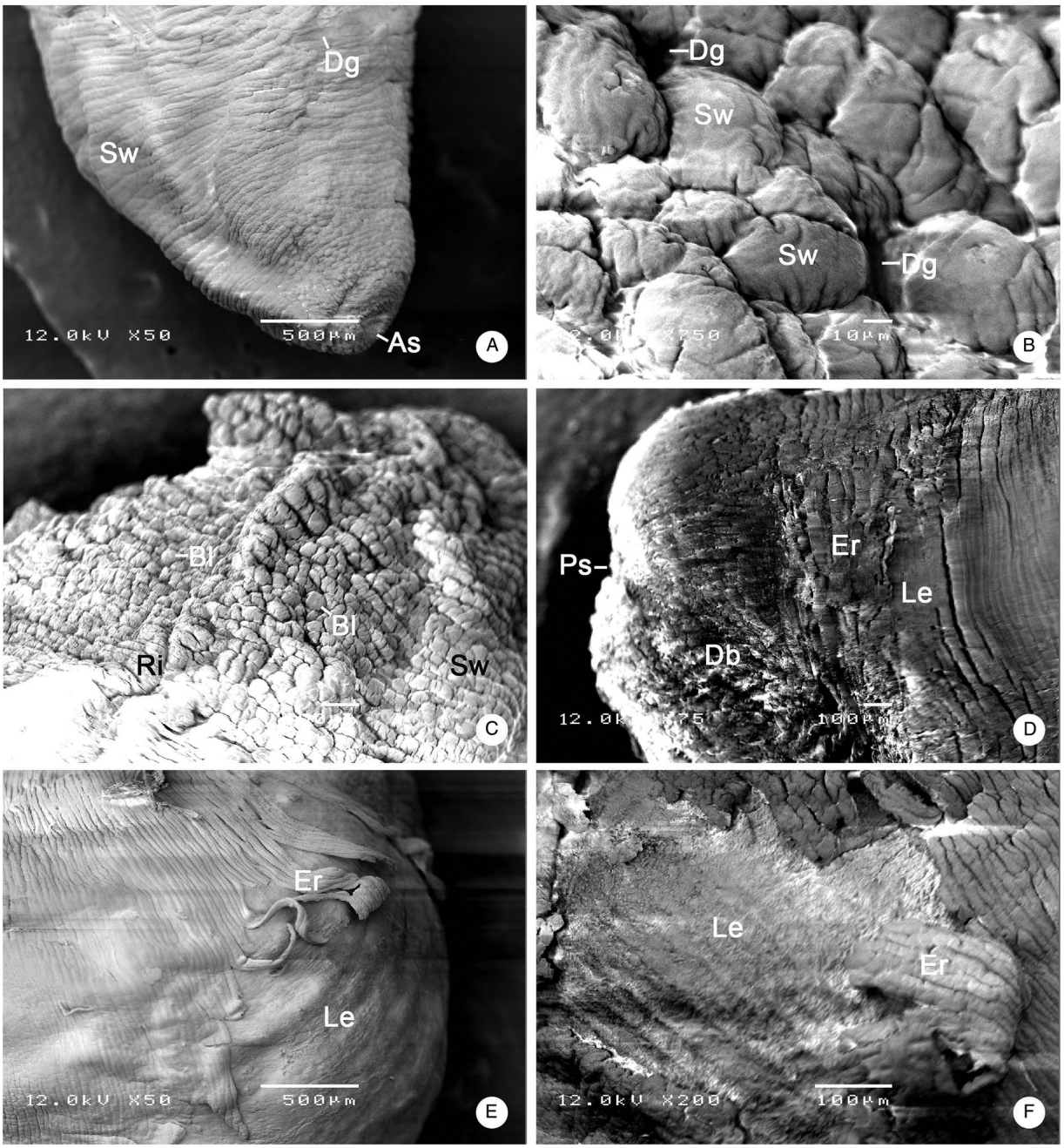

Fig. 5. SEM micrographs of the adult *F. cobboldi* treated with 500 μg mL^{-1} (A–D) and 1000 μg mL^{-1} (E, F) of TcCE *in vitro*. The sequence and severity of the tegumental alterations can be divided into five levels, (1) swelling, (2) blebbing, (3) disruption of blebs, (4) erosion and lesion as well as (5) basement membrane disruption and total destruction of the tegument. (A, B) At 3 h post incubation in 500 μg mL^{-1} of TcCE, the anterior part of the ventral surface of the anterior sucker (As) exhibits swollen surface (Sw) divided by deep grooves (Dg). (C) After 3 h incubation with 500 μg mL^{-1} of TcCE, severe swelling (Sw) and numerous blebs (Bl) of tegumental ridges (Ri) occur around the anterior part of the ventral surface. (D) At the 6 h examination time point, the posterior part of the ventral surface appears severe disrupted blebs (Db), erosion (Er) and lesion (Le) of the tegument especially around the posterior sucker (Ps). (E) Following 3 h incubation in TcCE at 1000 μg mL^{-1}, there is extensive erosion (Er) of the ventral surface of the anterior part which leads to severe lesion (Le) of the tegument. (F) At the 3 h examination time point, the dorsal surface of the middle part of the tegument exhibits severe erosion (Er), which eventually becomes lesion (Le).

disruption of basement membrane (Fig. 6C). At 6 h incubation, the flukes treated with these concentrations showed severe erosion, lesion and disruption of the basement membrane, and exhibited extreme deformity and the total destruction of the tegument on both ventral and dorsal surfaces (Fig. 6F).

DISCUSSION

Up to now, the natural products from plant extracts have been used as new alternative sources for development of potential anthelmintic drug (Geary *et al.* 2012; Hossain *et al.* 2012). Our work is the first study to demonstrate the *in vitro* anthelmintic

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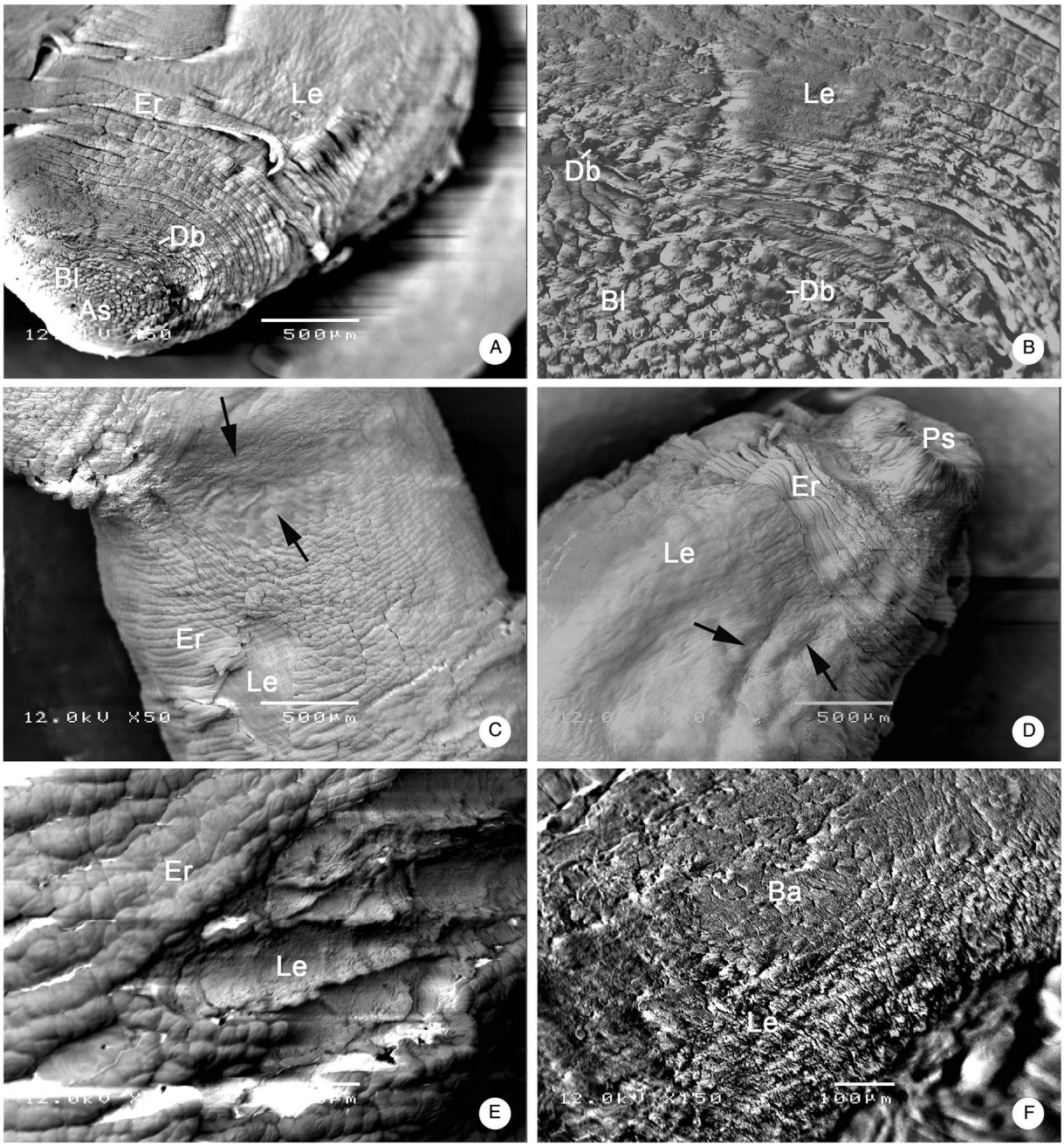

Fig. 6. SEM micrographs of the adult *F. cobboldi* treated with 1500 μg mL^{-1} (A–C) and 2000 μg mL^{-1} (D–F) of TcCE *in vitro*. (A, B) At 3 h post incubation in 1500 μg mL^{-1} of TcCE, the anterior part of the ventral surface of the anterior sucker (As) shows numerous blebs (Bl), severe disrupted blebs (Db), extensive erosion (Er) and lesion (Le) of the tegument. (C) The anterior part of the dorsal surface after treatment with 1500 μg mL^{-1} of TcCE for 3 h, showing erosion (Er) and lesion (Le) of the tegument, and depression of some areas (arrows). (D, E) The flukes treated with 2000 μg mL^{-1} of TcCE at 3 h, showing severe erosion (Er), large lesion (Le) and depression of some areas (arrows) through the ventral surface of the posterior part closed to the posterior sucker (Ps) (D) and of the middle part of the tegument (E). (F) Following 6 h incubation in 2000 μg mL^{-1} of TcCE, the anterior part of the ventral surface shows severe damage and extreme deformity over the whole surface of the tegument with vast lesions (Le) exposing a large area of the basement membrane (Ba) is completely lost.

effects of the ethanol extract of *T. catappa* L. (TcCE) leaves against adult *F. cobboldi* by estimating the RM, survival and tegumental surface changes in the treated parasites. Depending on dosages, TcCE can reduce motility and cause of death of adult flukes as evaluated by the RM values. The RM of flukes treated with TcCE significantly decreased from 1 to 6 h incubation, with the lowest RM (RM = 0) in the flukes treated with 500–2000 μg mL^{-1} for 12 h, indicating 100% mortality. These results indicate that the crude extract could kill *F. cobboldi* infection at proper dosages. In addition,

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these results are similar to those of other closely related trematode parasites. For instance, Saowakon *et al*. (2009) reported that a complete immobilization and death of *Fasciola gigantica* was observed after 12 h incubation with 750–1000 $\mu g\ mL^{-1}$ of *Artocarpus lakoocha* crude extract. In addition, Tandon *et al*. (1997) revealed that *Paramphistomum* sp., which was incubated in 500 $\mu g\ mL^{-1}$ of root-tuber extract of *Flemingia vestita* exhibited decreased motility and death within 12 h after incubation. In contrast to the test drug, ABZ that is a standard broad spectrum drug has been used to kill various trematode parasites in human and animals (Halton, 2004; Hossain *et al*. 2012). In the present study, we have used ABZ to treat the parasites and compare the results with TcCE. Our results revealed that the worms treated with ABZ showed slow reduction of the RM value. It is possible that ABZ-treated parasites required more time and higher dose for paralysis or death as previously reported by Hossain *et al*. (2012) who used 10 mg mL^{-1} of ABZ to test with *Paramphistomum explanatum* and only showed minor effect to the flukes'mobility.

In the present study, LM has been used to investigate the earliest alteration in a limited area of the tegument of *F. cobboldi*. Therefore, the tegument is the most affected target organ when the parasites were treated with TcCE. For anthelmintic action, SEM has been used to be a beneficial instrument for investigating the tegumental surface alteration of the parasites. The tegument is a vital structure of the parasites since it can synthesize and secrete a number of antigens that can affect the hosts. Moreover, it plays an important role in maintaining the parasites' homeostasis including protection against the host's digestive enzymes, immune responses, supporting internal organs, the absorption and exchange of nutritive and waste molecules, osmoregulation and perception of sensory stimuli (Sobhon and Upatham, 1990; Sobhon and Apinhasamit, 1996; Meaney *et al*. 2002, 2003, 2004; Anuracpreeda *et al*. 2015). From our results in this study, it is evident hereby that the tegument is shown to be a primary target of TcCE, which could be absorbed by the tegument of fluke. TcCE possess a rapid and drastic effect on *F. cobboldi*. The definite sequences of pathological alterations observed in the tegumental surface initially comprised the swelling of the tegumental folds and ridges that manifested first as small path which scattered in multiple areas. It is likely that the swelling which is the earliest sign of change could be elicited by the osmotic imbalance due to the disruption of ion pumps present on the apical plasma membrane (Skuce *et al*. 1987). Thereafter, the blebbing formation appeared on the surface which was then ruptured, as a result in the erosion and lesion of the tegument. Eventually, the surface exhibited the disruption of the basement membrane as indicated total destruction of the tegument over large areas. Following these alterations of the tegumental surface, the flukes exhibited immobility and death. The disruption of the tegument clearly visible to the naked eye, were observed in all specimens at the higher dosages. The flukes' surface exhibited dark and followed by the peeling of the tegument. In contrast, the flukes might also ingest TcCE through their anterior sucker because numerous blebs were frequently found at the anterior part of worm, particularly around the anterior sucker. In *F. cobboldi*, there are types 1 and 2 dome-shaped papillae which function as sensory receptors resemble those demonstrated in *Carmyerius spatiosus* (Anuracpreeda *et al*. 2015), *Paramphistomum cervi* (Panyarachun *et al*. 2010) and *F. gigantica* (Dangprasert *et al*. 2001). The sensory function is related to feeding at the oral aperture, pressure detection on the general body surface, and sexual reception around the genital pore (Bennett, 1975*a*, *b*). These papillae were also destroyed by TcCE which could cause the loss of sensory functions. Likewise, the damage of the posterior sucker might affect the anchoring onto the hosts' rumen and reticulum (Anuracpreeda *et al*. 2012, 2015).

Regional differences in response to the TcCE were also observed, the ventral surface being more drastically affected than the dorsal surface of the flukes, and the anterior, middle as well as posterior parts of the flukes were generally more destroyed than the lateral areas. The alterations of the swollen appearance and extensive blebs began along the lips of the anterior sucker and genital canal, and the posterior sucker also was damaged. These may depend on the thickness, variation of body structure, routes of drug uptake and metabolism of drug in different parts of the parasites' tegument. The severity of tegumental damage increased at higher drug concentration and longer incubation times. In ABZ treatment, the surface changes of flukes showed similar sequence as that of TcCE but with the least severity. The tegumental alterations observed in this work are similar to that investigated in adult *F. hepatica* treated with artremether (Keiser and Morson, 2008), as well as were also observed in the tegument of adult *F. gigantica* after incubation with crude extract of *A. lakoocha* (Saowakon *et al*. 2009) and with artesunate (Tansatit *et al*. 2012). Furthermore, similar tegumental changes were noted after incubation of adult *P. explanatum* in methanol extract of *Bombax malabaricum* (Hossain *et al*. 2012).

In conclusion, the results obtained in this study clearly indicated that the ethanol extract of *T. catappa* L. leaves (TcCE) possesses the anthelmintic activity against *F. cobboldi*. Hence, TcCE has a potential to be an efficacious anthelmintic drug for treatment of paramphistomosis.

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REFERENCES

Ahluwalia, S. S. (1960). *Gastrodiscoides hominis* (Lewis and McConnell, 1876) Leiper, 1913-the amphistome parasite of man and pig. *The Indian Journal of Medical Research* **48**, 315–325.

Anuracpreeda, P., Wanichanon, C. and Sobhon, P. (2008). *Paramphistomum cervi*: antigenic profile of adults as recognized by infected cattle sera. *Experimental Parasitology* **118**, 203–207.

Anuracpreeda, P., Panyarachun, B., Ngamniyom, A., Tinikul, Y., Chotwiwatthanakun, C., Poljaroen, J. and Sobhon, P. (2012). *Fischoederius cobboldi*: a scanning electron microscopy investigation of surface morphology of adult rumen fluke. *Experimental Parasitology* **130**, 400–407.

Anuracpreeda, P., Chawengkirtikul, R., Tinikul, Y., Poljaroen, J., Chotwiwatthanakun, C. and Sobhon, P. (2013*a*). Diagnosis of *Fasciola gigantica* infection using a monoclonal antibody-based sandwich ELISA for detection of circulating cathepsin B3 protease. *Acta Tropica* **127**, 38–45.

Anuracpreeda, P., Poljaroen, J., Chotwiwatthanakun, C., Tinikul, Y. and Sobhon, P. (2013*b*). Antigenic components, isolation and partial characterization of excretion–secretion fraction of *Paramphistomum cervi*. *Experimental Parasitology* **133**, 327–333.

Anuracpreeda, P., Srirakam, T., Pandonlan, S., Changklungmoa, N., Chotwiwatthanakun, C., Tinikul, Y., Poljaroen, J., Meemon, K. and Sobhon, P. (2014). Production and characterization of a monoclonal antibody against recombinant cathepsin L1 of *Fasciola gigantica*. *Acta Tropica* **135**, 1–9.

Anuracpreeda, P., Phutong, S., Ngamniyom, A., Panyarachun, B. and Sobhon, P. (2015). Surface topography and ultrastructural architecture of the tegument of adult *Carmyerius spatiosus* Brandes, 1898. *Acta Tropica* **143**, 18–28.

Athanasiadou, S., Githiori, J. and Kyriazakis, I. (2007). Medicinal plants for helminth parasite control: facts and fiction. *Animal* **1**, 1392–1400.

Atjanasuppat, K., Wongkham, W., Meepowpan, P., Kittakoop, P., Sobhon, P., Bartlett, A. and Whitfield, P. J. (2009). *In vitro* screening for anthelmintic and antitumour activity of ethnomedicinal plants from Thailand. *Journal of Ethnopharmacology* **123**, 475–482.

Bennett, C. E. (1975*a*). Surface features, sensory structures and movement of the newly excysted juvenile *Fasciola hepatica*. *Journal of Parasitology* **61**, 886–891.

Bennett, C. E. (1975*b*). Scanning electron microscopy of *Fasciola hepatica* during growth and maturation in the mouse. *Journal of Parasitology* **61**, 892–898.

Chen, P. S., Li, J. H., Liu, T. Y. and Lin, T. C. (2000). Folk medicine *Terminalia catappa* and its major tannin component, punicalagin, are effective against bleomycin-induced genotoxicity in Chinese hamster ovary cells. *Cancer Letters* **152**, 115–122.

Chethanon, U., Ausavamatha, P. and Piriyayon, S. (1985). Parasitological survey of cattle in the south of Thailand. *Proceedings of the 4th Annual Livestock Conference, 3–5 July 1985. Department Livestock Development*, pp. 281–290.

Chu, S. C., Yang, S. F., Liu, S. J., Kuo, W. H., Chang, Y. Z. and Hsieh, Y. S. (2007). *In vitro* and *in vivo* antimetastatic effects of *Terminalia catappa* L. Leaves on lung cancer cells. *Food and Chemical Toxicology* **45**, 1194–1201.

Dangprasert, T., Khawsuk, W., Meepol, A., Wanichanon, C., Viyanant, V., Upatham, E. S., Wongratanacheevin, S. and Sobhon, P. (2001). *Fasciola gigantica*: surface topography of the adult tegument. *Journal of Helminthology* **75**, 43–50.

Dutt, S. C. and Srivastava, H. D. (1972). The life history of *Gastrodiscoides hominis* (Lewis and McConnel, 1876) Leiper, 1913, the amphistome parasite of man and pig. *Journal of Helminthology* **46**, 35–46.

Fyhrquist, P., Mwasumbi, L., Haeggström, C. A., Vuorela, H., Hiltunen, R. and Vuorela, P. (2002). Ethnobotanical and antimicrobial investigation on some species of *Terminalia* and *Combretum* (Combretaceae) growing in Tanzania. *Journal of Ethnopharmacology* **79**, 169–177.

Geary, T. G., Chibale, K., Abegaz, B., Andrae-Marobela, K. and Ubalijoro, E. (2012). A new approach for anthelminthic discovery for human. *Trends in Parasitology* **28**, 176–181.

Geurden, T., Somers, R., Thanh, N. T., Vien, L. V., Nga, V. T., Giang, H. H., Dorny, P., Giao, H. K. and Vercruysse, J. (2008). Parasitic infections in dairy cattle around Hanoi, northern Vietnam. *Veterinary Parasitology* **153**, 384–388.

Germosén-Robineau, L. (2014). *Farmacopea Vegetal Carbeña*. CICY editorial, Yucatán, Mexico p. 360.

Gupta, P. P., Singh, B. and Dutt, S. C. (1978). A note on amphistomiasis in an adult buffalo. *The Indian Veterinary Journal* **55**, 491–492.

Hanna, R. E. B., Williamson, D. S., Mattison, R. G. and Nizami, W. A. (1988). Seasonal reproduction in *Paramphistomum epiclitum* and *Gastrothylax crumenifer*, rumen paramphistomes of the Indian water buffalo, and comparison with the biliary paramphistome *Gigantocotyle explanatum*. *International Journal for Parasitology* **18**, 513–521.

Harinasuta, T., Bunnag, D. and Radomyos, P. (1987). Intestinal fluke infections. *Baillieres Clinical Tropical Medicine and Communicable Diseases* **2**, 695–721.

Horak, I. G. (1971). Paramphistomiasis of domestic ruminants. *Advances in Parasitology* **9**, 33–71.

Halton, D. W. (2004). Review: microscopy and the helminth parasite. *Micron* **35**, 361–390.

Hossain, E., Chandra, G., Nandy, A. P., Mandal, S. C. and Gupta, J. K. (2012). Anthelmintic effect of a methanol extract of *Bombax malabaricum* leaves on *Paramphistomum explanatum*. *Parasitology Research* **110**, 1097–1102.

Ilha, M. R., Loretti, A. P. and Reis, A. C. (2005). Wasting and mortality in beef cattle parasitized by *Eurytrema coelamaticum* in the state of Parana, southern Brazil. *Veterinary Parasitology* **133**, 49–60.

Keiser, J. and Morson, G. (2008). *Fasciola hepatica*: tegumental alterations in adult flukes following *in vitro* and *in vivo* administration of artesunate and artemether. *Experimental Parasitology* **118**, 228–237.

Khan, U. J., Tanveer, A., Maqbool, A. and Masood, S. (2008). Epidemiological studies of paramphistomosis in cattle. *Veterinarski Arhiv* **78**, 243–251.

Kiuchi, F., Miyashita, N., Tsuda, Y., Kondo, K. and Yoshimura, H. (1987). Studies on crude drugs effective on visceral larva migrans. I. Identification of larvicidal principles in betel nuts. *Chemical and Pharmaceutical Bulletin* **35**, 2880–2886.

Kumar, V. (1980). The digenetic trematodes, *Fasciolopsis buski*, *Gastrodiscoides hominis* and *Artyfechinostomum malayanum*, aszoonotic infections in South Asian countries. *Annales de la Société belge de médecine tropicale* **60**, 331–339.

Kumar, B. G., Kumari, D., RAjeshwar, G., Umadevi, V. and Kotla, N. G. (2014). Antiulcer activity of ethanolic extract of *Terminalia catappa* leaves against gastric ulcers by pyrolic ligation induced model in rats. *International Journal of Pharmaceutical Sciences and Drug Research* **6**, 38–40.

Lin, C. C., Chen, Y. L., Lin, J. M. and Ujiie, T. (1997). Evaluation of the antioxidant and hepatoprotective activity of *Terminalia catappa*. *The American Journal of Chinese Medicine* **25**, 153–161.

Meaney, M., Fairweather, I., Bernnan, G. P., Ramasamy, P. and Subramanian, P. B. (2002). *Fasciola gigantica*: tegumental surface alterations following treatment *in vitro* with the sulphoxide metabolite of triclabendazole. *Parasitology Research* **88**, 315–325.

Meaney, M., Fairweather, I., Bernnan, G. P., McDowell, L. S. L. and Forbes, A. B. (2003). *Fasciola hepatica*: effects of the fasciolicide clorsulon *in vitro* and *in vivo* on the tegument surface, and a comparison of the effects on young- and old-mature flukes. *Parasitology Research* **91**, 238–250.

Meaney, M., Fairweather, I., Bernnan, G. P. and Forbes, A. B. (2004). Transmission electron microscopy study of the ultrastructural changes induced in the tegument and gut of *Fasciola hepatica* following *in vivo* drug treatment with closulon. *Parasitology Research* **92**, 232–241.

Magalhães, L. G., Kapadia, G. J., da Silva Tonuci, L. R., Caixeta, S. C., Parreira, N. A., Rodrigues, V. and Da Silva Filho, A. A. (2010). *In vitro* schistosomicidal effects of some phloroglucinol derivatives from *Dryopteris* species against *Schistosoma mansoni* adult worms. *Parasitology Research* **56**, 395–401.

Nagappa, A. N., Thakurdesai, P. A., Venkat Rao, N. and Singh, J. (2003). Antidiabetic activity of *Terminalia catappa* Linn fruits. *Journal of Ethnopharmacology* **88**, 45–50.

Nikitin, V. F. (1979). The course of acute enzootic amphistomiasis in claves in the lower Volga region. *Helminthological Abstract Series A* **48**, 463.

Nunes, A. F., Viana, V. S. L., Brito Junior, E. C., Rabelo, R. S., Nunes Filho, D. M., Nunes, P. H. M. and Martins, M. C. C. (2012). Antiulcerogenic activity of ethanol extract of the bark from *Terminalia catappa* in gastric ulcer model induced by ethanol in *Rattus norgegicus*. *Pharmacologyonline* **9**, 98–101.

Panyarachun, B., Sobhon, P., Yotsawan, T., Chotwiwatthanakun, C., Anupunpisit, V. and Anuracpreeda, P. (2010). *Paramphistomum cervi*: surface topography of the tegument of adult fluke. *Experimental Parasitology* **125**, 95–99.

Panyarachun, B., Ngamniyom, A., Sobhon, P. and Anuracpreeda, P. (2013). Morphology and histology of the adult *Paramphistomum gracile* Fischoeder, 1901. *Journal of Veterinary Science* **14**, 425–432.

Prasitirat, P., Chompoochan, T., Nithiuthai, S., Wongkasemjit, S., Punmamoamg, T., Pongrut, P., Chinone, S. and Itagaki, H. (1997*a*). Prevalence of amphistomes of cattle in Thailand. *Parasitology* (*Hungary*) **29–30**, 27–32.

Prasitirat, P., Nithiuthai, S., Ruengsuk, K., Kitwan, P., Bunmatid, C., Roopan, S. and Itagaki, H. (1997*b*). Efficacy of bithionol sulfoxide, niclosamide and fenbendazole against natural rumen fluke infection in cattle. *Helminthologia* **34**, 155–157.

Robinson, R. D., Williams, L. A. D., Linda, J. F., Terzy, S. I. and Mansingh, A. (1990). Inactivation of *Strongyloides stercoralis* larvae *in vitro* by six Jamaican plant extracts and three commercial anthelmintics. *The West Indian Medical Journal* **39**, 213–217.

Rolfe, P. F. and Boray, J. C. (1988). Chemotherapy of paramphistomosis in sheep. *Australian Veterinary Journal* **65**, 148–150.

Rolfe, P. E., Boray, J. C., Nichols, P. and Collins, G. H. (1991). Epidemiology of paramphistomosis in cattle. *Australian Veterinary Journal* **21**, 813–819.

Sanabria, R. E. F. and Romero, J. R. (2008). Review and update of paramphistomosis. *Helminthologia* **45**, 64–68.

Saowakon, N., Tansatit, T., Wanichanon, C., Chanakul, W., Reutrakul, V. and Sobhon, P. (2009). *Fasciola gigantica*: anthelmintic effect of the aqueous extract of *Artocarpus lakoocha*. *Experimental Parasitology* **122**, 289–298.

Skuce, P. J., Anderson, H. R. and Fairweather, I. (1987). The interaction between the deacetylated (amine) metabolite of diamphenethide (DAMD) and cytochemically demonstrable Na+/K+-ATPase activity in the tegument of *Fasciola hepatica*. *Parasitology Research* **74**, 161–167.

Sobhon, P. and Upatham, E. S. (1990). Snail hosts, life-cycle, and tegumental structure of oriental schistosomes. UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, pp. 57–88.

Sobhon, P. and Apinhasamit, W. (1996). *Opisthorchis viverrini*: the effect of colchicines and cytochalasin B on the adult tegument. *The Southeast Asian Journal of Tropical Medicine and Public Health* **27**, 312–317.

Tanaka, T., Nonaka, G. I., Ishimatsu, M., Nishioka, I. and Kouno, I. (2001). Revised structure of cercidinin A, a novel ellagitannin having (R)-hexahydroxydiphenoyl esters at the 3, 4-positions of glucopyranose. *Chemical and Pharmaceutical Bulletin* (*Tokyo*) **49**, 486–487.

Tandon, V., Pal, P., Roy, B., Rao, H. S. P. and Reddy, K. S. (1997). *In vitro* anthelmintic activity of root-tuber extract of *Flemingia vestita*, an indigenous plant in Shillong, India. *Parasitology Research* **83**, 492–498.

Tang, X. H., Gao, J., Wang, Y. P., Xu, L. Z., Zhao, X. N. and Xu, Q. (2003). Hepatoprotective effects of chloroform extract from leaf of *Terminalia catappa* in relation to the inhibition of liver IL-6 expression. *Zhongguo Zhong Yao Za Zhi* **28**, 1170–1174.

Tansatit, T., Sahaphong, S., Riengrojpitak, S., Viyanant, V. and Sobhon, P. (2012). *Fasciola gigantica*: the *in vitro* effects of artesunate as compared to triclabendazole on the 3-weeks-old juvenile. *Experimental Parasitology* **131**, 8–19.

Tariq, K. A., Chishti, M. Z., Ahmad, F. and Shawl, A. S. (2008). The epidemiology of paramphistomosis of sheep (*Ovis aries* L.) in the north west temperate Himalayan region of India. *Veterinary Research Communications* **32**, 383–391.

Thomson, L. A. J. and Evans, B. (2006). *Terminalia catappa* (tropical almond), 2·2. In *Species Profiles for Pacific Island Agroflorestry: Permanent Agriculture Resources (PAR)* (ed. Elevitch, C. R.). http://www.traditionaltree.org

Veesommai, U. and Janjittikul, T. (2001). *Plant Materials in Thailand*, 2nd Edn. Amarin Book Center Co., Ltd., Bangkok, Thailand, p. 637.

Wang, C. R., Qiu, J. H., Zhu, X. Q., Han, X. H., Ni, H. B., Zhao, J. P., Zhou, Q. M., Zhang, H. W. and Lun, Z. R. (2006). Survey of helminths in adult sheep in Heilongjiang Province, People's Republic of China. *Veterinary Parasitology* **140**, 378–382.

Wongsawad, C., Wongsawad, P., Luangphai, P. and Kumchoo, K. (2005). *In vitro* effects of aqueous extract from *Artocarpus takoocha* Roxb on tegumental surface of *Haplorchis taichui*. *The Southeast Asian Journal of Tropical Medicine and Public Health* **36**(Suppl. 4), 112–116.

Yeh, C. B., Hsieh, M. J., Hsieh, Y. S., Chien, M. H., Lin, P. Y., Chiou, H. L. and Yang, S. F. (2012). *Terminalia catappa* exerts antimetastatic effects on hepatocellular carcinoma through transcriptional inhibition of matrix metalloproteinase-9 by modulating NF-κB and AP-1 Activity. *Evidence-based Complementary and Alternative Medicine* **2012**, Article ID 595292. doi:10.1155/2012/595292.