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ORIGINAL ARTICLE

Aquaculture

High-resolution chemical profiling and antiparasitic potential of the tropical shrub *Dillenia suffruticosa*

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Abstract

The aquaculture industry is growing rapidly throughout the world, but due to intensification, fish hatcheries are often faced with infestations of parasites, which can lead to economic loss. Among these parasites, the leech *Zeylanicobdella arugamensis* (Hirudinea: Piscicolidae) has been reported to impact hybrid groupers and other hosts. The objective of this study was to test the antiparasitic potential of chromatographic fractions of a crude methanolic extract of the tropical shrub *Dillenia suffruticosa*. The phytochemical composition of the shrub was determined using high-resolution liquid chromatography (LC)–quadrupole time-of-flight (QTOF)–mass spectrometry (MS) to narrow down the metabolites responsible for its antiparasitic properties. Seven fractions of a methanolic extract of *D. suffruticosa* were obtained through flash column chromatography. Various concentrations of the fractions were prepared and tested against *Z. arugamensis*. In the bioassay conducted with fresh leeches, significant mortality was induced by fraction 6 at 31.66 ± 4.88 min, followed by fraction 5 (39.58 ± 2.94 min), fraction 3 (63.75 ± 6.61 min) and fraction 4 (65.25 ± 4.98 min). Chemical profiling using LC–QTOF–MS identified 17 secondary metabolites comprising triterpenoids, sterols, flavones, a glycoside, a non-flavone phenolid, a pyrrolizine, a fatty acid and a fatty amide. Thus, our study indicated that the *D. suffruticosa* fractions contained potent bioactive compounds with antiparasitic potential.

Keywords Aquaculture · Anti-parasitic activity · Hybrid groupers · *Zeylanicobdella arugamensis* · *Dillenia suffruticosa* · Liquid chromatography-quadrupole time-of-flight–mass spectrometry · Secondary metabolites

Introduction

Aquaculture plays a vital role in the supply of fish, which are an important source of protein in Malaysia (Othman et al. 2017). In 2016, the estimated production of fish for human consumption in Malaysia was 1.99 million tonnes, which was valued at USD 3.45 billion (RM 13.18 billion) (Department of Fisheries 2016; Othman et al. 2017). In Malaysia, the main fish cultured in open water cages are groupers [hybrid grouper *Epinephelus fuscoguttatus* × *Epinephelus*

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lanceolatus, orange-spotted grouper *Epinephelus coioides* (Hamilton 1822), tiger grouper *E. fuscoguttatus* (Forsskål, 1775) and Malabar grouper *Epinephelus malabaricus* (Bloch and Schneider, 1801)]; snappers [yellowstreaked snapper *Lutjanus lemniscatus* (Valenciennes, 1828), mangrove snapper *Lutjanus argentimaculatus* (Forsskål, 1775), John's snapper *Lutjanus johnii* (Bloch, 1792) and red snapper *Lutjanus erythropterus* (Bloch, 1790)]; milkfish *Chanos chanos* (Forsskål, 1775); fourfinger threadfin *Eleutheronema tetradactylum* (Shaw, 1804); Indian threadfin *Alectis indica* (Rüppel, 1830); pompano *Trachinotus blochii* (Lacepède, 1801); red tilapia *Oreochromis niloticus* (Linnaeus, 1758); and Asian seabass *Lates calcarifer* (Bloch, 1790) (Othman et al. 2017; Shapawi et al. 2019). The aquaculture industry is expected to play an important role in national food security, and as a potential catalyst for poverty alleviation (Fathi et al. 2018). It also generates opportunities for employment, business and investment (Othman et al. 2017). Unfortunately, though, the rapid development of the aquaculture industry is associated with many problems, and parasitic infestation is one that warrants immediate attention.

In Malaysia, parasitic infestations are problematic in marine aquaculture (Venmathi Maran et al. 2009). Particularly problematic is *Zeylanicobdella arugamensis* de Silva, 1963 (Annelida, Hirudinea, Rhynchobdellida, Piscicolidae), a parasitic marine leech that affects a large number of cultured groupers and other fish species (Kua et al. 2014; Ravi and Shariman Yahaya 2017). *Z. arugamensis* was first reported in grouper (*E. coioides*) in Malaysia. It was initially considered host-specific to grouper, but has since been frequently isolated from various other major species of marine fish, such as snappers [*L. johnii*, *L. argentimaculatus* and *L. stellatus* (Akazaki, 1983)] and sea bass (*L. calcarifer*) (Kua et al. 2010), and recently from humphead wrasse *Cheilinus undulatus* (Rüppel, 1835) (Venmathi Maran et al., in preparation). Infestations of this blood-sucking parasite result in mortality of the host fish in a very short time due to blood loss and secondary bacterial infection (Kua et al. 2010). The parasite is widely distributed in Southeast Asian countries, but its prevalence and intensity of infection are highest in Brunei Darussalam (Azmey et al. 2020). Controlling the prevalence of this parasitic leech is essential for aquaculture management (Azmey et al. 2020). Different chemicals, especially formalin, are used for the treatment of parasitic leeches in aquaculture facilities. These chemicals are extremely harmful to fish, handlers and consumers (Leal et al. 2018). In the course of our ongoing investigation to discover a natural remedy for *Zeylanicobdella arugamensis* (Shah et al. 2020), we reported significant antiparasitic activities of fractions of *Dillenia suffruticosa* (Griff ex Hook.f. and Thomson) Martelli (1887), a medicinal plant used for the treatment of various ailments such as stomach ache and fever, and post-childbirth (Borneo Post Online: https://www.theborneopost.com/2011/09/06/local-plants-trove-of-medicinal-and-health-supplements/ "Accessed 5 June 2020"). The main objective of this investigation was to demonstrate the potent antiparasitic potential of *D. suffruticosa* by performing polarity-based fractionation, identifying the most potent fraction, and identifying the secondary metabolites in the active fractions via liquid chromatography (LC)–quadrupole time-of-flight (QTOF)–mass spectroscopy (MS).

Materials and methods

Reagents

Silica (SiO_2) gel was purchased from Merck (Darmstadt, Germany). The column was prepared for the fractionation using a mixture of hexane and ethyl acetate (both from Merck). Normal phase thin layer chromatography (TLC) (SiO_2) plates were purchased from Merck, Germany, and the developed TLCs visualized using 5% molybdophosphoric acid in ethanol (Nacalai, Japan). Formalin (37% aqueous formaldehyde solution) was purchased from Sigma (Germany), and high-performance liquid chromatography grade (HPLC) grade methanol from Merck. LC–MS-grade acetonitrile was purchased from J. T. Baker (Philipsburg, NJ). Deionized water was produced using a Milli-Q system (Merck) at a resistivity of > 18.2 MΩ cm. The reference mass solution containing 5.0 mM purine and 2.5 mM of 1H,1H,3H-tetrafluoropropoxy phosphazene, was purchased from Agilent Technologies (Santa Clara, CA). LC–MS-grade formic acid was acquired from Fisher Scientific (Fair Lawn, NJ). Polyvinylidene fluoride syringe filters (0.22-µm pore size, 13-mm diameter) were purchased from Merck.

Plant collection

Mature and young leaves of the plant *D. suffruticosa* (6–10 years old) were obtained from the lowlands of Papar (5.7346°N, 115.9319°E), Sabah, East Malaysia. The plants were shrubby, 4–6 m tall and had yellow flowers. The leaves are alternate, oval, penniveined and serrated. The collected mature leaves were approximately 12–40 cm long and dark green in colour, while the young leaves were 5–10 cm long and reddish in colour (Fig. 1). A voucher specimen (MDS-002) was deposited after identification of the plant sample at the Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah, Kota Kinabalu, Malaysia.

Extraction

The leaves were cleaned with distilled water and oven-dried at 37 °C. The dried leaves were ground separately in a mechanical grinder then stored in an airtight container. Dry

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Fig. 1 The tropical shrub *Dillenia suffruticosa*, leaves of which were collected from Papar, Sabah, East Malaysia

leaf powder (60 g) was extracted with HPLC grade methanol (300 ml) using the Soxhlet method (50–60 °C for 72 h). The methanol residues were removed from the extract using a vacuum rotary evaporator. The samples were kept at − 80 °C for 24 h, then lyophilized by freeze drying. The freeze-dried samples were stored in a freezer for further studies (Srivastava and Srivastava 2018).

Flash column chromatography was performed using a 20 × 100-mm glass column with a 250-ml reservoir. Columns were prepared with SiO_2 gel impregnated in hexane. Once the column has settled overnight, 500 mg of the crude extract was dissolved in 2 ml hexane and spiked onto the column. Fractionation was performed using a solvent-gradient system with hexane (H)-ethyl acetate (E) [fraction (F)1 (H:E, 9:1); F2 (H:E, 8:2); F3 (H:E, 7:3); F4 (H:E, 6:4); F5 (H:E, 5:5); F6 (E, 100%)]. The final eluant, 100% ethyl acetate, was collected as two separate portions, fraction 6 and fraction 7, as two different coloured compounds were eluted. The total volume of each fraction was 250 ml. The fractions were collected, concentrated and their profiles determined via TLC to determine their separation. The fractions were also subjected to LC-QTOF–MS analysis to determine their secondary metabolites.

Anti-parasitic bioassay

Adult *Z. arugamensis* were obtained from infested hybrid groupers (*E. fuscoguttatus* × *E. lanceolatus*) (Fig. 2a, b) from the aquaculture facilities of University Malaysia Sabah, and identified based on their morphological features (Kua et al. 2010). The adult leeches used in the study were divided into nine groups (six leeches per group). Group 1 was the negative control, which was treated with seawater. Group 2, the positive control, was treated with 0.25% formalin solution. Groups 3, 4, 5, 6, 7, 8 and 9 were treated with *D. suffruticosa* fraction 1 (1.3 mg/ml), fraction 2 (20 mg/ml), fraction 3 (20 mg/ml), fraction 4 (20 mg/ml), fraction 5 (20 mg/ml), fractions 6 (20 mg/ml) and fraction 7 (20 mg/ml), respectively (Fig. 2c, d). The mortality time was recorded, and the remaining live leeches further observed for short intervals up to 720 min to make sure that all of them had died by the end of the experiment (Mann 1962; Gholami-Ahangaran et al. 2012).

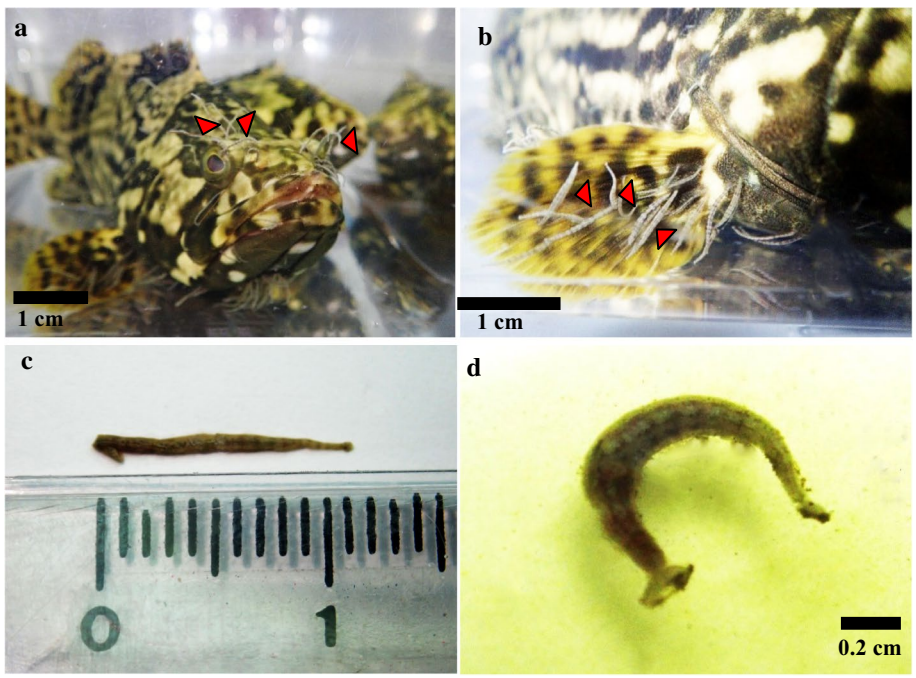

Fig. 2 **a**, **b** *Zeylanicobdella arugamensis* infestation of hybrid groupers (in a hatchery), **c** a normal untreated leech, **d** a fraction-treated leech

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Liquid chromatography-quadrupole time-of-flight–mass spectrometry

The fractions were analyzed using an Agilent 1290 Infinity LC system coupled with an Agilent 6520 QTOF–MS system (Agilent Technologies, Santa Clara, CA). A 2.0-µl sample was injected into a reverse phase column (narrow bore, 2.1 mm × 150 mm, 3.5 µm; Agilent Zorbax Eclipse XDB-C18; Agilent Technologies). The column was maintained at 25 °C at a flow rate of 500 µl/min during the analysis. The mobile phases were solvent A (H_2O–0.1% formic acid) and solvent B (acetonitrile–0.1% formic acid). The gradient elution program was initiated at 5% of solvent B for 5 min, then from 5 to 100% solvent B in 15 min, then maintained at 100% solvent B for 5 min. Later, the columns were conditioned as initial for 5 min before the next injection.

The data acquisition was set between a mass-to-charge ratio (*m/z*) of 100 and 1500. Positive heated electrospray ionization was deployed at 4 kV. The ion source conditions were set as follows: gas temperature of 300 °C, drying gas flow at 10 l/min, and nebulizer flow at 45 pounds per square inch gauge. The mass spectrometer was calibrated with Tuning Mix (Agilent Technologies) before each batch analysis. The internal mass calibration standards, betaine and hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazene, were introduced during the runs; in the positive ion mode, these internal mass calibration standards had *m/z* of 121.0508 and 922.0097, respectively.

Automated tandem mass spectrometry was employed for metabolite matching, and data acquisition was set between an *m/z* of 50 and 1500 with nitrogen using a collision energy of 20 eV. Acquired data were processed using Agilent MassHunter Qualitative Workflows software (version B.08.00) and matched against METLIN metabolites and lipid databases (Guijas et al. 2018) with a maximum mass tolerance of 2 p.p.m.

Statistical analysis

The statistical analyses were performed using IBM SPSS Statistics 25 Window package (IBM, Armonk, NY). Significant differences between groups were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test. All results are presented as mean ± SD, and *p*-values less than 0.05 are considered significant.

Results

Anti-parasitic activity of solvent fractions of *D. suffruticosa* methanolic extract

The effects of the *D. suffruticosa* fractions on the leech *Z. arugamensis* are indicated in Table 1. There was no mortality in the negative control group, while in the positive control group treated with formalin, all the leeches were killed within 3.90 ± 0.84 min. Mortality was also observed in the groups treated with fractions 3–6. Among all the fractions with anti-leech potential, the shortest time to mortality was seen for fraction 6, followed by fractions 5, 3 and 4, in that order. There was no indication that fractions 1, 2 and 7 were toxic to the leeches.

Table 1 Mortality time and percentage of leeches treated with different fractions of *Dillenia suffruticosa*

Fraction no.	Group	Mortality time (min; mean ± SD)	Mortality (%)
1	Negative control	720.0 ± 0.0	0
2	Positive control (formalin 0.25%) (v/v)	3.9 ± 0.8[a]	100
3	Fraction 1 (1.3 mg/ml)	720.0 ± 0.0[b]	0
4	Fraction 2 (20 mg/ml)	720.0 ± 0.0[b]	0
5	Fraction 3 (20 mg/ml)	63.7 ± 6.6[a,b,c,d]	100
6	Fraction 4 (20 mg/ml)	65.2 ± 4.9[a,b,c,d]	100
7	Fraction 5 (20 mg/ml)	39.5 ± 2.9[a,b,c,d,e,f]	100
8	Fraction 6 (20 mg/ml)	31.6 ± 4.8[a,b,c,d,e,f]	100
9	Fraction 7 (20 mg/ml)	720.0 ± 0.0[b,e,f,g,h]	0

Values are mean ± SD of six leeches per group

Fraction 1 [hexane:ethyl acetate (H:E) 9:1)]; *Fraction 2* (H:E, 8:2); *Fraction 3* (H:E, 7:3); *Fraction 4* (H:E, 6:4); *Fraction 5* (H:E, 5:5); *Fraction 6* (E, 100%)

[a]Significance at $p < 0.05$ compared with the negative control group

[b]Significance at $p < 0.05$ compared with the formalin [0.25% volume/volume (*v/v*)]-treated group

[c]Significance at $p < 0.05$ compared with fraction 1 (1.3 mg/ml)

[d]Significance at $p < 0.05$ compared with fraction 2 (20 mg/ml)

[e]Significance at $p < 0.05$ compared with fraction 3 (20 mg/ml)

[f]Significance at $p < 0.05$ compared with fraction 4 (20 mg/ml)

[g]Significance at $p < 0.05$ compared with fraction 5 (20 mg/ml)

[h]Significance at $p < 0.05$ compared with fraction 6 (20 mg/ml)

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Effects of *D. suffruticosa* fractions on the behaviour of *Z. arugamensis*

In the control group treated with seawater only, the leeches swam normally, using their posterior and anterior suckers for locomotion. Aggressive swimming activity was observed in the group treated with 0.25% of formalin. There was no change in the locomotion and swimming behaviour of the groups treated with *D. suffruticosa* fractions 1, 2 and 7. In contrast, the groups treated with fractions 3–6 showed strong swimming activity for the first 5–15 min, then moved less, became weak, and died.

LC-QTOF-MS analysis and metabolite identification

All seven fractions of the *D. suffruticosa* extract were subjected to LC-QTOF–MS analysis. A total of 17 secondary metabolites and two compounds produced from chlorophyll breakdown were detected. The secondary metabolites were bayogenin (1), crataegolic acid (2), propapyriogenin A2 (3), acetyl-11-keto-β-boswellic acid (4), allocholesterol (5), hongguanggenin (6), hecogenin acetate (7), 3,7,8,4′-tetrahydroxyflavone (8), luteolin-7-sulfate (9), scutevulin (10), heterodendrin (11), *p*-coumaroylquinic acid (12), tiliroside (13), isodomedin (14), phalaenopsine T (15), 7-hydroxy-10*E*,16-heptadecadien-8-ynoic acid (16) and *N*–*N*-(2, 2-dihydroxyethyl) arachidonoyl amine (17). These 17 metabolites comprised triterpenoids (1, 2, 3, 4), sterols (5, 6, 7), flavones (8, 9, 10, 13, 14), a glycoside (11), one other phenolic compound (12), a pyrrolizine (15), a fatty acid (16) and a fatty amide (17). There were no significant peaks for fractions 1 and 2. Fraction 3 contained two chlorophyll breakdown products, pheophytin a and pheophorbide a, in addition to isodomedin (14). Fraction 4 consisted of two triterpenoids and one sterol compound. Fraction 5 contained the highest number of compounds: two triterpenoids, two sterols, a glycoside, a pyrrolizine and a fatty amide. Fraction 6, on the other hand, consisted of four flavones, a phenolic compound and a fatty acid. The chemical structures of the secondary metabolites (1–17) in fractions 3–6 of *D. suffruticosa* are shown in Fig. 3; their retention times and molecular formulae as generated from the LC-QTOF-MS are given in Table 2.

Discussion

Medicinal plant extracts can be an excellent alternative treatments for leech infestations, and secondary infections, due to the different metabolites with potential pharmaceutical value that they contain (Bahmani and Rafieian-Kopaei 2014). There is currently very limited scientific information about the utilization of pharmaceutical plants as biocontrol agents against leeches (Bahmani and Rafieian-Kopaei 2014). We recently reported the antiparasitic potential and phytochemical composition of a methanolic extract of *D. suffruticosa* (Shah et al. 2020). In the present study, we further determined the phytochemical composition of solvent fractions of a crude methanolic extract of *D. suffruticosa* by high-resolution LC–QTOF-MS to further examine the specific compounds responsible for its antiparasitic properties.

The time of mortality of the leeches after exposure to different concentrations of the fractions is given in Table 1. *D. suffruticosa* fraction 6 (20 mg/ml) was the most effective, with 100% mortality at 31.66 ± 4.88 min, followed by fraction 5 (20 mg/ml) (39.58 ± 2.94), fraction 3 (20 mg/ml) (63.75 ± 6.61) and fraction 4 (20 mg/ml) (65.25 ± 4.98). In contrast, faction 1 (1.3 mg/ml), fraction 2 (20 mg/ml) and fraction 7 (20 mg/ml) of the methanolic extract showed no activity against the leeches. Our results are in agreement with those of previously published work. Gholami-Ahangaran et al. (2012) tested the activity of a methanolic extract of the common grape vine *Vitis vinifera* L. (Vitaceae) against the aquatic leech *Limnatis nilotica* (Savigny, 1822) (Hirudinidae), and found that doses of 300 and 600 mg methanolic extract killed immature *L. nilotica* in an average time of 260 ± 63 and 200 ± 50 min, respectively. Forouzan et al. (2012) evaluated the antiparasitic potential of ginger *Zingiber officinale* Roscoe (Zingiberaceae) methanolic extracts at 32×10^4 p.p.m. against the same leech, and mortality was observed within 24 ± 4.07 min. Different concentrations of the extracts of two medicinal plants, Baikal skullcap herb *Scutellaria baicalensis* Georgi (Lamiaceae) and noni *Morinda citrifolia* Linnaeus (Rubiaceae), tested for 96 h against the marine parasitic leech *Piscicola geometra* (Linnaeus, 1761) (Piscicolidae), resulted in 100% mortality in the group treated with the former, and 80% mortality in the group treated with the latter extract (Rizky et al. 2018). In another study, different concentrations of extracts of tobacco *Nicotiana* spp. (Solanaceae); an aromatic, deciduous, spiny shrub, the winged prickly ash *Zanthoxylum alatum* Roxb. (Rutaceae); and nightshade *Solanum khasianum* Linnaeus (Solanaceae), were tested against aquatic Asian buffalo leech *Hirudinaria manillensis* (Lesson, 1842) (Hirudinea), and were found to cause 100% mortality in an average time of 2.11 ± 0.11 min (*Nicotiana* spp.), 3.00 ± 0.33 min (*Z. alatum*) and 24.89 ± 2.34 min (*S. khasianum*) (Bam et al. 2016).

In the current study, formalin (0.25% volume/volume) was used as the positive control (Forouzan et al. 2012). Formalin, which is highly toxic to fish as well as to human beings, is used for parasitic disinfestation in the aquaculture industry (Morrison et al. 1993; Francis-Floyd 1996). Many other toxic chemicals such as tetracyclines, chloramphenicol, benzalkonium chloride, acriflavine, sulfonamides, nitrofurans, oxolinic acid and trichlorfon have also been used for this purpose in Malaysia and Singapore (Morrison et al.

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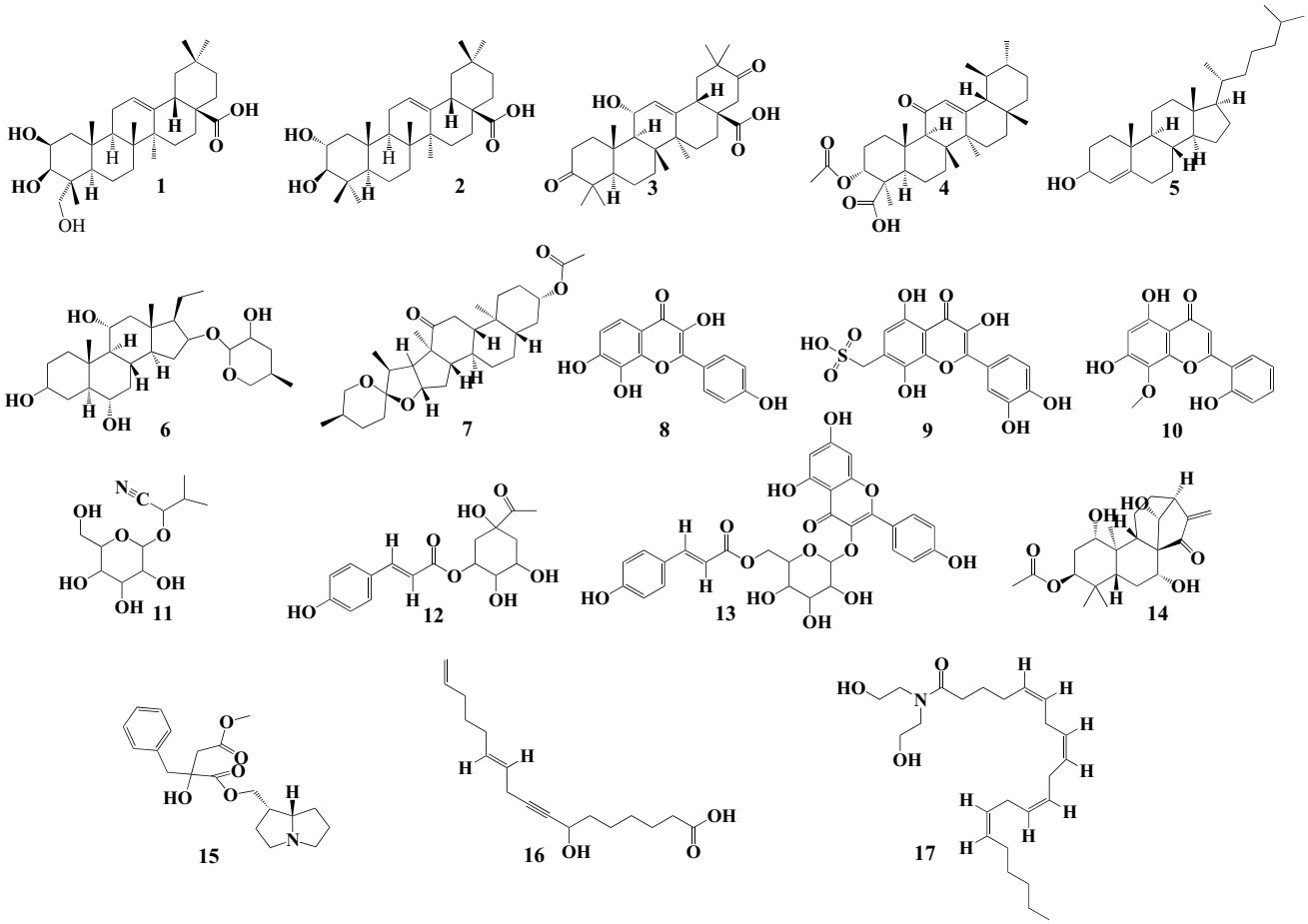

Fig. 3 Chemical structures of the 17 secondary metabolites detected in fractions 3–6 of *D. suffruticosa*. Bayogenin (*1*), crataegolic acid (*2*), propapyriogenin A2 (*3*), acetyl-11-keto-β-boswellic acid (*4*), allocholesterol (*5*), hongguanggenin (*6*), hecogenin acetate (*7*), 3,7,8,4′-tetrahydroxyflavone (*8*), luteolin-7-sulfate (*9*), scutevulin (*10*), heterodendrin (*11*), *p*-coumaroylquinic acid (*12*), tiliroside (*13*), isodomedin (*14*), phalaenopsine T (*15*), 7-hydroxy-10*E*,16-heptadecadien-8-ynoic acid (*16*) and *N*–*N*-(2, 2-dihydroxy-ethyl) arachidonoyl amine (*17*)

1993; Shariff et al. 2000). Thus, the development of safe and cost-effective biocontrol agents is not only highly desirable but also vital due to the current increase in fish production.

LC-QTOF was used in this study to produce profiles of the chromatographic fractions of the methanolic extract of *D. suffruticosa*; the detected metabolites were identified through automated tandem MS. The tandem mass spectra indicated the presence of terpenoids [acetyl-11-keto-β-boswellic acid (4), bayogenin (1), isodomedin (14), propapyriogenin A2 (3), crataegolic acid (2)], steroids [allocholesterol (5) and hongguanggenin (6)], flavonoids [3,7,8,4′-tetrahydroxyflavone (8), scutevulin (10) and tiliroside (13)], one other phenolic [p-coumaroyl quinic acid (12)], etc. Among these metabolites, bayogenin (1), isodomedin (14), crataegolic acid (2), tiliroside (13) and hecogenin acetate (7) have been reported to have various degrees of antiparasitic activity (Kubo et al. 1977; Xu et al. 1996; Wen et al. 2006; Argentieri et al. 2008; Hernández-Carlos et al. 2011; Lozano-Mena et al. 2014; Calzada et al. 2017; Santos et al. 2018).

It has been reported that a dose of 0.5 µg/µl bayogenin (1) can fully immobilize the larvae of a plant pest, the nematode *Meloidogyne javanica* (Trueb) Chitwood (Heteroteridae), and that the same dose of this pentacyclic triterpenoid also caused about 71% mortality in the ectoparasitic fan-leaf virus nematode *Xiphinema index* Thorne and Allen, 1950 (Longidoridae) after 48 h exposure (Hernández-Carlos et al. 2011). Isodomedin (14), a diterpene, was reported to exhibit antifeedant activity against the larvae of the African armyworm *Spodoptera exempta* (Walker, 1856) (Noctuidae) (Kubo et al. 1977). Tiliroside (13) showed 50% inhibition of the activity of the anaerobic parasitic amoebozoan *Entamoeba histolytica* Schaudinn, 1903 (Entamoebidae) and the flagellate protozoan *Giardia lamblia* Kofoid and Christiensen, 1915 (Hexamitidae) at concentrations of 17.5 µg/ml and 17.4 µg/ml, respectively (Calzada et al. 2017).

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Table 2 Secondary metabolite profiles of fractions of *D. suffruticosa* analyzed by liquid chromatography-quadrupole time-of-flight–mass spectrometry

Fractions	Retention time	Mass to charge ratio (*m*/*z*)	Formula	Mass error p.p.m.	Matched metabolites
1[a]	NA	NA	NA	NA	NA
2[a]	NA	NA	NA	NA	NA
3	3.99	415.2091	$C_{22}H_{32}O_6$	− 0.44	Isodomedin (**14**)
	19.59	871.5742	$C_{55}H_{74}N_4O_5$	− 0.82	Pheophytin a
	19.84	593.2772	$C_{35}H_{36}N_4O_5$	− 1.91	Pheophorbide a
4	15.35	506.3833	$C_{30}H_{48}O_5$	1.97	Bayogenin (**1**)
	17.58	473.3258	$C_{29}H_{44}O_5$	1.62	Hecogenin acetate (**7**)
	19.25	513.3574	$C_{32}H_{48}O_5$	0.27	Acetyl-11-keto-β-boswellic acid (**4**)
5	11.72	362.1959	$C_{20}H_{27}NO_5$	0.26	Phalaenopsine T (**15**)
	12.86	502.3527	$C_{30}H_{44}O_5$	− 0.31	Propapyriogenin A2 (**3**)
	15.59	473.3633	$C_{30}H_{48}O_4$	− 1.78	Crataegolic acid (**2**)
	16.70	279.1549	$C_{11}H_{19}NO_6$	0.75	Heterodendrin (**11**)
	17.53	487.3392	$C_{28}H_{48}O_5$	0.33	Hongguanggenin (**6**)
	17.97	409.3436	$C_{27}H_{46}O$	1.95	Allocholesterol (**5**)
	18.87	409.3428	$C_{24}H_{41}NO_3$	− 0.58	*N*,*N*-(2,2-dihydroxy-ethyl) arachidonoyl amine (**17**)
6	8.02	339.1071	$C_{16}H_{18}O_8$	0.81	*p*-Coumaroylquinic acid (**12**)
	9.33	287.0547	$C_{15}H_{10}O_6$	0.63	3,7,8,4′-Tetrahydroxyflavone (**8**)
	9.35	367.0119	$C_{15}H_{10}O_9S$	− 0.04	Luteolin 7-sulfate (**9**)
	10.43	595.1432	$C_{30}H_{26}O_{13}$	1.71	Tiliroside (**13**)
	10.51	301.0701	$C_{16}H_{12}O_6$	1.78	Scutevulin (**10**)
	14.80	296.2216	$C_{17}H_{26}O_3$	0.17	7-Hydroxy-10E,16-heptadecadien-8-ynoic acid (**16**)
7[a]	NA	NA	NA	NA	NA

NA Not applicable

[a]No notable peaks were observed in fractions 1, 2 and 7

Crataegolic acid (2), which is also known as maslinic acid, has been reported to have significant antiparasitic properties (De Pablos et al. 2010; Lozano-Mena et al. 2014) as well as anti-cancer (Reyes-Zurita et al. 2011), anti-tumour (Li et al. 2010), anti-HIV (Xu et al. 1996), antioxidant (Montilla et al. 2003) and anti-obesity activities (Liu et al. 2007). In the current study, significant antiparasitic properties were noted for multiple fractions of *D. suffruticosa*, especially fractions 6 and 5. Among all the fractions analyzed, most of the terpenoids, steroids and flavonoids were in fractions 6 and 5, which correlates with the cytotoxicity of these fractions as reported above.

During the experiment, we observed that the parasitic leeches *Z. arugamensis* used their anterior (small) and posterior (large) suckers for swimming. They swum freely in the water column and attached either to the fish or to the fish tank generally by using the larger posterior sucker, then extended their body to attach again to the fish or tank using the small anterior oral sucker (Kabata 1985; Nagasawa and Uyeno 2009). The swimming pattern was a zig-zag with rapid movement, and was well organized (Kabata 1985; Kua et al. 2014; Shah et al. 2020). When the leeches were exposed to the various concentrations of the plant extracts, they oscillated randomly and could not attach using their suckers to the bottom of the tank. They then gradually became fragile, weaker, and died (Bam et al. 2016; Shah et al. 2020). *Z. arugamensis* was previously considered host-specific to grouper; however, this leech has been frequently isolated from various other major species of cultured marine fish in Malaysia belonging to four different families: the Serranidae, Lutjanidae, Latidae and Labridae (Kua et al. 2014; Ravi and Shariman Yahaya 2017). This parasitic leech was found to infest these marine cultured fishes in large numbers on all the fins, the jaw, eyes and even inside the oral cavity, as also seen in previous studies (Cruz-Lacierda et al. 2000; Nagasawa and Uyeno 2009).

We demonstrated here the antiparasitic potential of fractions of a methanolic extract of *D. suffruticosa.* Seven fractions were obtained through high-pressure chromatography techniques. The highest mortality of the parasitic leeches (100%) was obtained with fraction 6 in an average time of 31.66 ± 4.88 min, followed by fraction 5 (39.58 ± 2.94 min), fraction 3 (63.75 ± 6.61 min) and fraction 4 (65.25 ± 4.98 min). Phytochemical analysis of the

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D. suffruticosa fractions indicated the presence of various metabolites such as terpenoids (acetyl-11-keto-β-boswellic acid, bayogenin, isodomedin, propapyriogenin A2, crataegolic acid), steroids (hongguanggenin, allocholesterol), flavonoids (3,7,8,4′-tetrahydroxyflavone, tiliroside and scutevulin) and one other phenolic compound (*p*-coumaroyl quinic acid). Thus, our study indicated that *D. suffruticosa* fractions are a rich source of bioactive compounds with antiparasitic potential. Further research is needed for the identification, purification and isolation of the bioactive compounds responsible for the antiparasitic activity of *D. suffruticosa*.

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