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Insecticidal activity of plant-derived extracts against different economically important pest insects

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Abstract With the aim of selecting potential botanical insecticides, seven plant extracts (*Daphne mucronata* (Family: Thymelaeaceae), *Tagetes minuta* (Asteraceae), *Calotropis procera* (Apocynaceae), *Boenninghausenia albiflora* (Rutaceae), *Eucalyptus sideroxylon* (Myrtaceae), *Cinnamomum camphora* (Lauraceae) and *Isodon rugosus* (Lamiaceae)) were screened for their toxic effects against four important agricultural pest insects, each representing a separate insect order; pea aphids of *Acyrthosiphon pisum* (Hemiptera), fruit flies of *Drosophila melanogaster* (Diptera), red flour beetles of *Tribolium castaneum* (Coleoptera), and armyworms of *Spodoptera exigua* (Lepidoptera). Aphids were the most susceptible insect with 100% mortality observed after 24 h for all the plant extracts tested. Further bioassays with lower concentrations of the plant extracts against aphids, revealed the extracts from *I. rugosus* (LC_{50} 36 ppm and LC_{90} 102 ppm) and *D. mucronata* (LC_{50} 126 ppm and LC_{90} 198 ppm) to be the most toxic to aphids. These most active plant extracts were further fractionated into different solvent fractions on polarity basis and their insecticidal activity evaluated. While all the fractions showed considerable mortality in aphids, the most active was the butanol fraction from *I. rugosus* with an LC_{50} of 18 ppm and LC_{90} of 48 ppm. Considering that high mortality was observed in aphids within

Highlights • Insecticide activity of botanical extracts against four important agricultural pests: *Acyrthosiphon pisum* (Hemiptera), *Drosophila melanogaster* (Diptera), *Tribolium castaneum* (Coleoptera) and *Spodoptera exigua* (Lepidoptera)
• *Acyrthosiphon pisum* aphids were the most susceptible with 100% mortality observed after 24 h for all the plant extracts tested
• First report that the butanol fraction of *Isodon rugosus* was identified to show the highest insecticidal activity at very low concentrations among all of the tested fractions against *A. pisum*.

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24 h of exposure to a very low concentration of the butanol fraction from *I. rugosus*, we believe this could be exploited and further developed as a potential plant-based insecticide against sucking insect pests, such as aphids.

Keywords *Acyrthosiphon pisum* · Botanical insecticides · Fractionation · *Daphne mucronata* · *Isodon rugosus*

Introduction

The management of insect pests has always been and will continue to be a constant challenge to agricultural researchers and producers alike. As insect resistance to commonly used pesticides builds and the removal of more toxic pesticides from the market continues, controlling insect pest infestations will become increasingly more difficult. Therefore, as farmers struggle to remain profitable in a highly competitive global economy, they will be constantly faced with the dilemma of producing high quality, pest-free crops within economical means, and without endangering the environment and the worker's safety. This struggle has resulted to increased research into alternative control methods, which are cost-effective, environmentally-friendly and capable of keeping insect pests at bay.

As plants constitute a rich source of bioactive chemical compounds, botanical insecticides may present attractive alternatives to currently used synthetic chemical insecticides for pest management (Pino et al. 2013; Miresmailli and Isman 2014; Pavela 2016). Besides their insecticidal potential, they have been reported to pose little threat to the environment or to human health as compared to synthetic pesticides (Pavela 2014, 2016). Several secondary metabolites present in plants serve as a defense mechanism against insect attacks and it has been demonstrated that the pesticidal properties of plant chemicals can be specific to particular target species, biodegradable to non-toxic products and potentially suitable for use in an integrated pest management (IPM) program (Markouk et al. 2000; Isman 2006; D'Incao et al. 2013). Despite the attractive insecticidal potential of phytochemicals and the abundant scientific literature documenting the bioactivity of plant derivatives against insect pests, there still remain few prospects for commercial development of new botanical products. This is partly due to regulatory barriers and the availability of competing products (newer synthetics, fermentation products, microbials), and principally due to lack of knowledge about the many other existing plants with insecticidal properties against insect pests. Over 6000 plant species are known to have insecticidal properties and many of these plants are used by farmers in developing countries (Walia and Koul 2008). Furthermore, only a small percentage of these plants has been screened for insecticidal activity, out of which many of the studies were either not complete or the bioassay procedures used were inappropriate or unsuitable (Isman 2013).

In this study, seven plant species, namely *Daphne mucronata* (Thymelaeaceae), *Tagetes minuta* (Asteraceae), *Calotropis procera* (Apocynaceae), *Boenninghausenia albiflora* (Rutaceae), *Eucalyptus sideroxylon* (Myrtaceae), *Cinnamomum camphora* (Lauraceae) and *Isodon rugosus* (Lamiaceae), were screened for their insecticidal potential against important agricultural pests (Table 1). These plants are unique in comparison to several other plant species grown in northern Pakistan. The folk or ethnobotanical uses of these particular plant species in the area as medicine and insect repellents are common and have been so for decades. With a variable degree of bioactivity, all of the seven plant species showed considerably higher repellent activities in comparison to other plants grown in the area. Exploring the scientific bases of this important trait and transforming local knowledge into commercial uses was the major and long term goal of this study.

This study presents the results of preliminary screening bioassays of the seven plants against four important agricultural pests, each representing a separate insect order, that is, *Drosophila melanogaster* (fruit fly; Diptera), *Acyrthosiphon pisum* (pea aphid; Hemiptera), *Tribolium castaneum* (red flour beetle; Coleoptera) and *Spodoptera exigua* (beet armyworm; Lepidoptera). The beet armyworm, *S. exigua*, is a polyphagous pest damaging major agriculture areas including field, flower and vegetable crops (Taylor and Riley 2008). Due to its polyphagous ability, vast distribution and strong migratory properties (Stewart et al. 2002), it has the ability to attack vast areas of both horticultural cultivations and field crops (Han et al. 2008). It has many host crops including sugar beet, tobacco, cotton, soy bean in Mediterranean and African countries (Mushtaq et al. 2008). Other hosts include lettuce, cabbage, egg-plant, potato, pea,

spinach, alfalfa and corn (Dingha et al. 2004; Rizwan-ul-Haq et al. 2009). The common fruit fly, *D. melanogaster*, is also a polyphagous pest like *S. exigua*. The larvae mostly damage pulpy fruits, such as banana and guava, by feeding on pulp, rendering the fruit soft and unmarketable. With a short life cycle of 10 days from egg to adult and the possibility of many generations in one season, *D. melanogaster*, if not controlled, can rise to significantly high numbers with potential economic impact on fruit production and storage (Yasmin et al. 1995). The red flour beetle, *T. castaneum*, is a worldwide pest that causes damage to stored products including grains, flour, pastas, beans and nuts in food warehouses, mills, urban homes and retail stores, and it can survive over 3 years (Via 1999; Weston and Rattlingourd 2000; Rees 2004). It can damage stored grains either through feeding or by reducing their quality severely through larval feces. Their long term presence on flour and cereal grains usually leads to mould development (Bennett 2003; Baldwin and Fasulo 2004). The pea aphid, *A. pisum*, is one of the most important groups of insect pests in the world. Aphids cause direct damage by sucking the phloem of plants and indirect damage by transmitting plant pathogenic viruses and promoting fungal growth on the excreted honey dew (Dixon 1998; Van Emden and Harrington 2007). *A. pisum* is responsible for crop damage worth hundreds of million dollars every year and is a vector of more than 30 viruses, including red clover vein mosaic virus, pea streak virus and bean yellow mosaic virus (Barnett and Diachun 1986; Jones and Proudlove 1991; Brault et al. 2010).

The objective of this study was to screen for plants which contain compounds that could be exploited as botanical insecticides. This objective was specifically achieved by screening methanolic extracts from seven selected plant species against four selected pest insect species of economic importance. Based on the degree of toxicity to the insect pests, the resulting methanolic extract of the most potent plants were further fractionated via liquid-liquid extractions on polarity basis, to identify the fraction with the active metabolites. The results obtained from this study provide first data for further chromatographic purification, spectrometric analysis, evaluation and exploitation of the active secondary metabolites present in the most active plant fractions for the control of selected insect pests.

Materials and methods

Plant material

Seven plant species from different families were field collected from the lower northern areas of Pakistan (34.1558° N, 73.2194° E) (Table 1).

Preparation of crude plant extracts

The plants were shade-dried for 3 months and then ground to powder using an electric grinder. Plant metabolites were then extracted by macerating the plant powder in methanol at room temperature. After 2 days, the solvent layer was filtered with Whatman filter paper No.1 and this process was repeated three times. The resulting filtrate was concentrated by using a rotary evaporator at 35 °C and the obtained crude methanolic extracts were stored at 4 °C until use for the bioassays. The yield for each plant crude methanolic extract is given in Table 2.

2.1.2. Preparation of fractions

The most active methanolic plant extracts were selected on the basis of their toxicity, following screening against all of the target insect species. These active methanolic plant extracts were further fractionated on polarity basis into four fractions, i.e., butanol, dichloromethane, hexane and ethyl acetate. The crude methanolic extract of each plant was suspended in 5 parts (*w*/*v*) of water and then extracted successively by *n*-hexane (4 × 150 mL), dichloromethane (4 × 150 mL), ethyl acetate (4 × 150 mL) and *n*-butanol (4 × 150 mL). The extracts were dried with Na_2SO_4, filtered and concentrated by evaporation under reduced pressure with a rotavapor at 40 °C. The resulting *n*-hexane residue, dichloromethane residue, ethyl acetate residue and *n*-butanol residues obtained from each of the plants were weighed and stored at 4 °C until use for the bioassays. The yield for each fraction is given in Table 2.

Insect cultures

All of the target insect cultures for this study were reared in the Laboratory of Agrozoology, Ghent University, Belgium.

Table 1 Details of plant species used to test for biopesticidal potential

Scientific Name	Common Name	Family	Part used	Ethnobotanical uses	References
Daphne mucronata	Kashmir Daphne (Kutti Lal)	Thymelaeaceae	Aerial parts	Use to treat bone, skin diseases, allergies, skeleto-muscular and rheumatism problems	Ali and Qaiser (2009); Afzal et al. (2009); Hussain et al. (2012); Hamayun (2007); Murad et al. (2011).
Tagetes minuta	Mexican Marigold (Gule Sad Barg)	Asteraceae	Aerial parts	Use to treat colds, stomach problems, respiratory, inflammation, chest infections, wounds, cuts and as antispasmodic, antiseptic and antiparasitic	Nikkon et al. (2011); Kamaraj et al. (2011).
Calotropis procera	Sodom Apple (Ak)	Apocynaceae	Aerial parts	Anticancer, antimicrobial, anti-inflammatory, analgesic, antipyretic, antiulcer and antifertility	Ahmed et al. (2006); Yoganarasimhan (2011).
Boenninghausenia albiflora	White Himalayan Rue (Pissu Mar Booti)	Rutaceae	Aerial parts	Treat cuts, wounds, malaria, scabies, headaches	Manandhar (2002); Duke and Ayensu (1985).
Eucalyptus sideroxylon	Red Ironbark (Gond or Safeda)	Myrtaceae	Leaves	Antifungal, antibacterial, anticancer, antioxidant	Ashour (2008); Miranda et al. (2016).
Cinnamomum camphora	Camphor Tree (Kafoor)	Lauraceae	Leaves	Relief chest congestion, rheumatism, bronchitis, sprains, asthma, muscle pain Antioxidant, anticancer, antimicrobial	Ho et al. 2009; Salman et al. (2012); Ghanta et al. (1990); Liu et al. (2002); Edris (2007); Lin et al. (2007).
Isodon rugosus	Wrinkled Leaf Isodon (Boi)	Lamiaceae	Aerial parts	Used to treat dysentery and cure generalized pain of the body, toothache, earache, abdominal pain, gastric problems, pyrexia, blood pressure, rheumatism and microbial infections, as bronchodilator, antidiarrheal and hypoglycaemic	Shuaib and Khan (2015); Akhtar et al. (2013); Sabeen and Ahmad (2009); Ahmad et al. (2014); Khan and Khatoon (2007); Adnan et al. (2012); Shuaib et al. (2014); Sher et al. (2011); Ajmal et al. (2012); Janbaz et al. (2014)

Table 2 Yield for plant crude methanolic extracts and *Daphne mucronata* and *Isodon rugosus* fractions

Plant crude extracts (Dry powder)/Fractions	Yields (g)
Daphne mucronata (3.5 kg)	180
Tagetes minuta (4 kg)	187
Calotropis procera (3.5 kg)	133
Boenninghausenia albiflora (300 g)	18.25
Eucalyptus sideroxylon (700 g)	49.33
Cinnamomum camphora (500 g)	32.53
Isodon rugosus (1 kg)	90
D. mucronata, hexane	41.84
D. mucronata, butanol	44.79
D. mucronata, ethyl acetate	4.95
D. mucronata, dichloromethane	2.84
I. rugosus, hexane	8.82
I. rugosus, butanol	6
I. rugosus, ethyl acetate	1.33
I. rugosus, dichloromethane	3.1

Acyrthosiphon pisum

A continuous colony of the *A. pisum* was maintained on young plants of *Vicia faba* L. (Fabales: Fabaceae) at 23 ± 2 °C and 65 ± 5% relative humidity (RH) under a 16:8 h light:dark photoperiod (Nachman et al. 2011). For the bioassays, adult aphids were transferred from fresh leaves into separate boxes from which neonates were collected after 24 h. The collected neonates were then used for the bioassays.

Drosophila melanogaster

Fruit flies were reared on agar-yeast-corn meal artificial diet that was prepared as described by Taning et al. (2016), at 25 °C, light regime of 16 h light and 8 h dark, and 65% RH. Adult flies were used for the bioassays.

Tribolium castaneum

T. castaneum beetles were reared on wheat flour, supplemented with 5% brewer's yeast in an incubator at 30 °C and 60% RH in darkness (Walski et al. 2016). Adult beetles were collected and used for the bioassays.

Spodoptera exigua

An established colony of beet armyworms (*S. exigua*) was reared in a growth chamber at 25 °C, 65% RH and a 16 h light: 8 h dark photoperiod. Pupae of *S. exigua* were placed in 40 × 25 × 25 cm Plexiglass cages, lined on the inner wall with white A4 paper as a substrate for oviposition. The emerging adults were fed with a 10% solution of honey in water. Eggs laid on the paper were transferred to a plastic container and the hatching larvae were fed on an agar-based artificial diet (Smagghe et al. 1998). Second-instar larvae were used for the bioassays.

Insect bioassays

Screening of the methanolic plant extracts at 2% against four pest insects

Economically important insect pests representing four insect orders; the pea aphid *A. pisum* (Hemiptera), the fruit fly *D. melanogaster* (Diptera), the red flour beetle *T. castaneum* (Coleoptera) and the beet armyworm *S. exigua* (Lepidoptera), were used for the first screening bioassays. For all these bioassays, the plant extracts were used at a 2% concentration. Two controls were used in the bioassays, that is methanol (for controlling for methanol in the treated groups) and water (untreated group).

For the bioassays with *A. pisum*, 8 mg of each plant extract was dissolved in 8 µl of methanol and 392 µl of liquid artificial diet, to a final concentration of 2%. 100 µL of the liquid diet mixture, was then pipetted onto the first layer of parafilm and sealed by stretching a second layer of parafilm on it (Nachman et al. 2011). Ten newborn (<24 h old) aphids in each treatment were placed on the second layer of the parafilm and then covered with a hollow plastic ring having a ventilated lid, to prevent the escape of aphids. These cages were placed in an inverted position in six well plates. Three replications were used for each plant extract. Mortality was assessed after 24 h by gentle probing of the aphids with a brush and observing post-mortem color change of the body (Sadeghi et al. 2009). For the bioassays with *D. melanogaster*, adult flies were selected. 200 µL of each plant extract solution (20 mg of the extract in 1 mL of methanol) was layered on the surface of the fruit fly diet in a 50 mL tube and after drying under a hood flow, 10 flies were placed into each tube. Three replications were used for each plant extract. Mortality was assessed

after 24, 48 and 72 h. For the bioassays with *S. exigua*, second instar larvae were used. In each treatment, 50 μL solution of each extract (20 mg of the extract in 1 mL of methanol) was layered on the diet in each well (Smagghe et al. 1998). After letting the methanol dry off the surface of the diet, one insect was added in each well. A total of 20 insects were used for each treatment. Mortality was analyzed at 24, 48 and 72 h. For the bioassays with *T. castaneum*, adult beetles were exposed to flour discs (Xie et al. 1996; Yu et al. 2013) containing the plant extracts in them. In brief, the flour discs were prepared by dissolving 8.4 mg of each plant extract in 420 μL of methanol and then mixed with 120 mg of corn flour. Aliquots (35 μL) of the mixture containing each of the plant extracts were placed in wells of a 96 well plate (Greiner CELLSTAR)), for making flour discs, and dried overnight under the flow hood to form solid flour diet discs for the beetles. Two control flour discs were prepared (methanol and water). Five flour discs were placed together with 10 beetles into each falcon tube per treatment. A total of 10 flour discs were used in two replications for each treatment. Mortality was analyzed at 24, 48 and 72 h after exposure to the treated flour discs.

Selection of the most susceptible target insect and bioassays with seven crude methanolic plant extracts at different concentrations against the selected insect (A. pisum)

After screening the plant extracts against the target insects at a 2% plant extract concentration (2.3.1.), the most susceptible insect to all of the plant extracts, which was the pea aphid, *A. pisum*, was selected for further bioassays and analyzed at lower concentrations than 2%. In these bioassays, artificial diet test cages were prepared as described by Sadeghi et al. (2009). In brief, the experimental setup was the same as in the screening phase, with the only difference being the different concentrations tested in the bioassays. Five concentrations were used for each of the plant crude extracts against the aphids. A stock solution of 1% was prepared by adding 1 mg of each plant crude extract in 100 μL of methanol. Five concentrations of 1000, 500, 200, 100 and 50 ppm were then prepared from the stock solution by diluting the stock solution with the artificial diet of aphids. A final volume of 300 μL for three replications of each treatment (100 μL for each replication) was prepared. Two controls (untreated artificial diet and methanol-treated artificial diet) and three replications were used for each treatment in the bioassays. Mortality was analyzed after 24 h of treatment. Out of the seven plant species, the two most active plant species were further fractionated and analyzed against *A. pisum*.

Bioassays with fractions against pea aphids

Four fractions (hexane, dichloromethane, ethyl acetate and butanol fractions) from each of the most active plants were tested in these bioassays. A 1% stock solution for each of the fractions was prepared by dissolving 1 mg of each fraction in 100 μL of their respective solvents. Then from 1% stock solution, five concentrations (500, 200, 100, 50 and 25 ppm) for each fraction were prepared by diluting in the artificial diet of aphids. Then, 100 μL of each solution was layered between two parafilm layers and cages were prepared as described by Sadeghi et al. (2009). Ten newborn (<24 h old) aphids were exposed to the treated diet in each cage. Three replications were used for each treatment. Five controls were used, i.e., hexane, dichloromethane, ethyl acetate, butanol treated artificial diet, and untreated artificial diet.

Data analysis

Probit analysis of mortality vs concentration was conducted to estimate lethal concentrations (LC_{50} and LC_{90}) with their corresponding 95% confidence intervals (95% CI) by POLO Plus V 2.0 (LeOra Software, Berkeley, CA). LC values were considered to be significantly different when their respective 95% CI did not overlap.

Results

Screening of the methanolic plant extracts at 2% against four different insects

In the preliminary screening bioassays, all of the plant extracts caused 100% mortality in *A. pisum*, while moderate mortality was observed in *S. exigua* and *D. melanogaster*. No mortality was observed in *T. castaneum* for all of the plant extracts (Table 3). Based on these results *A. pisum* was chosen for further bioassays.

Table 3 Screening all seven crude methanolic plant extracts at a concentration of 2%. Data are expressed as mean mortality percent of *Acyrthosiphon pisum* (pea aphid), *Drosophila melanogaster* (fruit fly), *Spodoptera exigua* (beet armyworm) and *Tribolium castaneum* (red flour beetle) at 24, 48 and 72 h post-treatment. Results are based on 3 repeats of 10 insects each per extract in case of *A. pisum* and *D. melanogaster*, 2 repeats of 10 insects in case of *S. exigua* and *T. castaneum*

Treatments (2% methanolic extracts)	*Acyrthosiphon pisum*[1]	*Drosophila melanogaster*			*Spodoptera exigua*			*Tribolium castaneum*[2]
	24 h	24 h	48 h	72 h	24 h	48 h	72 h	72 h
Tagetes minuta	100	0	0	0	6	6	6	0
Cinnamomum camphora	100	27	34	45	0	0	6	0
Eucalyptus sideroxylon	100	0	0	0	0	6	6	0
Boenninghausenia albiflora	100	0	0	0	0	0	0	0
Calotropis procera	100	0	7	7	11	18	22	0
Isodon rugosus	100	0	0	0	0	6	6	0
Daphne mucronata	100	0	0	0	0	6	6	0

Data are corrected for mortality in the treated groups with Abbott's formula. Control mortality was always <20%

[1] With pea aphids of *Acyrthosiphon pisum*, there was 100% mortality already at 24 h after treatment and the experiment was stopped. In contrast all control aphids survived

[2] With red flour beetles of *Tribolium castaneum*, there was 0% mortality with the insects treated at 24, 48 and 72 h post-treatment

Toxicity of the methanolic plant extracts at different concentrations against *A. pisum*

Except for *T. minuta* and *C. procera* with negligible toxic effects (no LC_{50} and LC_{90} could be calculated with the concentrations tested), all of the other plants analyzed showed strong toxic effects against *A. pisum* after 24 h of exposure to their respective methanolic extracts (Table 4). The crude extract from *I. rugosus* caused the highest mortality (LC_{50} 36 ppm and LC_{90} 102 ppm) as compared to the other plant extracts tested. Although the crude extract from *D. mucronata* caused the second highest mortality (LC_{50} 126 ppm and LC_{90} 198 ppm), this was not significantly different from *E. sideroxylon* (LC_{50} 136 ppm and LC_{90} 374 ppm) and *B. albiflora* (LC_{50} 160 ppm and LC_{90} 380 ppm) since their 95% confidence limit values overlap with each other (Table 4). *I. rugosus* and *D. mucronata* were selected for further fractionation and evaluation against aphids.

Bioassay with fractions of *Isodon rugosus*

The crude methanolic extract of *I. rugosus* with the highest bioactivity against aphids was further partitioned successively into hexane, dichloromethane,

Table 4 Toxicity of crude methanolic extracts against newborn (24 h old) nymphs of pea aphids (*Acyrthosiphon pisum*) after exposure for 24 h on artificial diet supplemented with different concentrations of crude extracts

Crude Extracts	LC_{50} (95% CI) ppm	Ratio	LC_{90} (95% CI) ppm	Ratio	Slope ± SE	Chi-Square	HF
Isodon rugosus	36 (18–49) a	1.0	102 (80–156) a	1.0	2.8 ± 0.8	8.1	0.6
Daphne mucronata	126 (113–142) b	3.5	198 (171–248) b	1.9	6.6 ± 1.2	3.9	0.3
Cinnamomum camphora	624 (505–813) c	17	2002 (1380–3703) c	20	2.5 ± 0.4	4.1	0.3
Eucalyptus sideroxylon	136 (113–163) b	3.8	374 (294–528) d	3.7	2.9 ± 0.4	4.5	0.3
Boenninghausenia albiflora	160 (136–190) b	4.4	380 (302–531) d	3.7	3.4 ± 0.5	8.2	0.6
Tagetes minuta	–	–	–	–	0.7 ± 0.6	10.1	0.8
Calotropis procera	–	–	–	–	2.4 ± 1.0	2.2	0.2

Data is presented as 50% (LC_{50}) and 90% (LC_{90}) lethal concentration values (both in ppm) together with their respective 95% confidence interval (95% CI), the slope ± SE of the toxicity vs concentration curve, and the Chi-Square and heterogeneity factor HF as accuracy of data fitting to probit analysis in POLO-PlusV2. Different letters in the same column indicate significant differences due to non-overlapping of 95% CI. Ratio, LC_x, crude extract/LC_x, *Isodon rugosus*

ethyl acetate, and butanol fractions. The butanol fraction caused the highest mortality in aphids at a low concentration (LC_{50} 18 ppm and LC_{90} 48 ppm) after 24 h of exposure (Table 5). The ethyl acetate fraction (LC_{50} 18 ppm and LC_{90} 96 ppm) and dichloromethane fraction (LC_{50} 55 ppm and LC_{90} 146 ppm) caused comparable mortality at their LC_{90} and not LC_{50} (Table 5). The lowest mortality was obtained for the hexane fraction (LC_{50} 165 ppm and LC_{90} 532 ppm) after 24 h of treatment.

Bioassay with fractions of *Daphne mucronata*

Daphne mucronata crude methanolic extract was also partitioned into hexane, dichloromethane, ethyl acetate and butanol fractions. The highest mortality in the bioassays was found for the ethyl acetate (LC_{50} 68 ppm and LC_{90} 157 ppm) and dichloromethane fractions (LC_{50} 63 ppm and LC_{90} 158 ppm) (Table 6). The second most active fraction was the butanol fraction (LC_{50} 450 ppm and LC_{90} 1472 ppm), while the lowest mortality was observed with the hexane fraction (LC_{50} 685 ppm and LC_{90} 3557 ppm).

Discussion

Plants have evolved to produce many secondary chemical compounds to protect themselves against herbivores and pathogens and some have been used historically for pest management (Pino et al. 2013; Miresmailli and Isman 2014; Pavela 2016). With the aim of selecting potential botanical insecticides, in this study, we screened seven plant extracts for their toxic effects against *A. pisum*, *D. melanogaster*, *S. exigua* and *T. castaneum*. While these insects were randomly selected to analyze the effect of the botanical extracts on a broader context, the plants were particular selected for their ethnobotanical (phytoinsecticides) uses in the northern region of Pakistan, as insect repellents. To the best of our knowledge, this is the first report on the insecticidal activity of all of the selected plant extracts against all of the target insects.

Screening bioassays for all the plant extracts (at 2% concentration) against the target insects, revealed *A. pisum* as the most susceptible insect, showing the highest mortality. Compared to the controls, a 45% increase in mortality was observed in *D. melanogaster* treated with *Cinnamomum camphora* and a 22% increase in *S. exigua* treated with *Calotropis procera*. The observed mortality was not considered to be high enough in these insect species for the plant extracts tested, hence, they were excluded from further evaluation in this study. Another possible explanation for the low mortality observed, besides the presence of insecticidal compounds in the plant extracts, could be the delivery method of the plant extracts to the insects in the bioassays. While the aphids sucked up the plant extract mixed with liquid diet, *D. melanogaster* and *S. exigua* were exposed to solid diet layered with the plant extract. However, the observation of increase mortality in *D. melanogaster* and *S. exigua* compared to the controls for some plant extracts, indicated that the delivery method did not significantly influence the bioassays. No mortality was observed for *T. castaneum*, even after complete consumption of diet flour disc containing the plant extracts. A 100% mortality was observed after just 24 h for all the plant extracts tested against *A. pisum*. As such, *A. pisum* was selected as the most susceptible insect for further bioassays, in which lower concentrations (1000, 500, 200, 100 and 50 ppm) as

Table 5 Toxicity of *Isodon rugosus* solvent fractions against newborn (24 h old) nymphs of pea aphids (*Acyrthosiphon pisum*) after exposure for 24 h on artificial diet supplemented with different concentrations of solvent fractions

Fractions	LC_{50} (95% CI) ppm	Ratio	LC_{90} (95% CI) ppm	Ratio	Slope ± SE	Chi-Square	HF
Hexane	165 (126–231) a	10	533 (348–1151) a	11	2.5 ± 0.4	22.5	1.7
Butanol	18 (9–24) b	1.0	48 (38–74) b	1.0	2.9 ± 0.9	4.7	0.4
Ethyl acetate	18 (7–26) b	1.0	96 (69–164) bc	2.0	1.7 ± 0.4	5.2	0.4
Dichloromethane	55 (45–64) c	3.2	146 (115–209) c	3.0	3.0 ± 0.5	5.4	0.4

Data is presented as 50% (LC_{50}) and 90% (LC_{90}) lethal concentration values (both in ppm) together with their respective 95% confidence interval (95%CI), the slope ± SE of the toxicity vs concentration curve, and the Chi-Square and heterogeneity factor HF as accuracy of data fitting to probit analysis in POLO-PlusV2. Different letters in the same column indicate significant differences due to non-overlapping of 95%CI. Ratio, LC_x, fraction/LC_x, butanol or ethyl acetate

Table 6 Toxicity of *Daphne mucronata* solvent fractions against newborn (24 h old) nymphs of the pea aphid (*Acyrthosiphon pisum*) after exposure for 24 h on artificial diet supplemented with different concentrations of solvent fractions

Fractions	LC_{50} (95% CI) ppm	Ratio	LC_{90} (95% CI) ppm	Ratio	Slope ± SE	Chi-Square	HF
Hexane	685 (451–1541) a	11	3557 (1571–21,021) a	23	1.8 ± 0.4	6.6	0.5
Butanol	450 (348–668) a	7	1472 (910–3624) a	9	2.5 ± 0.5	3.4	0.3
Ethyl acetate	68 (58–79) b	1.1	157 (127–215) b	1.0	3.5 ± 0.5	8.1	0.6
Dichloromethane	63 (53–74) b	1.0	158 (126–221) b	1.0	3.2 ± 0.5	8.8	0.7

Data is presented as 50% (LC_{50}) and 90% (LC_{90}) lethal concentration values (both in ppm) together with their respective 95% confidence interval (95%CI), the slope ± SE of the toxicity vs concentration curve, and the Chi-Square and heterogeneity factor HF as accuracy of data fitting to probit analysis in POLO-PlusV2. Different letters in the same column indicate significant differences due to non-overlapping of 95%CI. Ratio, LC_x, fraction/LC_x, dichloromethane

compared to the initial screening concentration of 2% were tested, for all the plant extracts. Based on the highest mortality caused to the treated aphids by the lowest treatment concentration (50 ppm), *I. rugosus* and *D. mucronata* were selected for further fractionation and further evaluation. The liquid-liquid extractions were performed to determine the solvent fractions containing a higher concentration of the active metabolites against aphids. The highest bioactivity against aphids for the fractions obtained from *D. mucronata* was observed with the ethyl acetate and dichloromethane fractions, which are moderately polar solvents. This observation is similar to the findings of Zewdu (2010), where toxic effects of different solvent extracts of Birbira, *Millettia ferruginea*, were evaluated against *A. pisum*. It was reported that deionised water extract was more toxic causing 98% mortality, followed by acetic acid extract causing 89% mortality, whereas, the chloroform, toluene and hexane extracts were the least toxic. Compared to the fractions obtained from *D. mucronata*, the fractions from *I. rugosus* were more toxic to the aphids, even at lower concentrations. The highest bioactivity for the fractions obtained from *I. rugosus* was observed with the butanol and ethyl acetate fractions, with the smallest LC_{90} values obtained for the butanol fraction. It can be said that the butanol fraction is more active at a lower concentration when compared to the ethyl acetate fraction. In a similar study, Baek et al. (2013) found that the butanol fraction showed more activity compared to other tested fractions, when dried seeds of *Macleaya cordata* extracted with methanol and successively partitioned into hexane, butanol and water fractions, were analyzed against the cotton aphid, *Aphis gossypii*. The presence of alkaloids, saponins, tannins, flavonoids, phenols, terpenes, glycosides and steroids has been reported in the butanol fraction for some plant species such as *Sapindus saponaria* (Gangula et al. 2013). Since some of these compounds are known to have insecticidal properties and all of them have been reported to be present in extracts of *Isodon rugosus* (Zeb et al. 2014a, b, 2016), this could explain the high toxicity against aphids observed with the butanol fraction. Baek et al. (2013) reported the presence of alkaloids, 8-hydroxydihydrochelerythrine and 8-methoxydihydro sanguinarine in the butanol fraction of *Macleaya cordata* seeds, which were very active against *Aphis gossypii*. Considering that the active metabolite in the butanol fraction of *I. rugosus* was not isolated and characterized in this study, strong discussions and conclusions could not be made concerning which metabolite is the principle causative agent of mortality in treated aphids. Nevertheless, the high mortality observed in aphids after a short exposure of 24 h to the butanol fraction of *I. rugosus* motivates the need for further isolation, identification and characterisation of the active compound.

Based on the results of this study, *I. rugosus* and *D. mucronata* extracts could serve as a source of potential insecticides against *A. pisum*. The butanol fraction from *I. rugosus* with the lowest LC_{90} value of 48.2 ppm, could further be subjected to different chromatographic techniques and analyzed to identify the toxic compounds and subsequently exploited as a potential pesticide against *A. pisum*. Since for various pharmacological activities these plants are used as a source of natural compounds, the extracts obtained are expected to be of low or no hazard to human beings or other animals. The genus *Daphne* has been known for the production of valuable bioactive natural products including flavonoids, coumarins, lignan, coumarinolignans and triterpenoids (Rasool et al. 2009). *D. mucronata* is considered as an important medicinal plant in northern areas

of Pakistan as well as in several regions of Iran. It has high ethnopharmacological value and has been used in conventional medication to treat many diseases (Afzal et al. 2009; Mosaddegh et al. 2012; Hussain et al. 2012; Ghasemi et al. 2013). *I. rugosus* on the other hand is known to contain some important bioactive compounds including flavonoids, terpenoids, saponins, tannins, cardiac glycosides, coumarins and steroids (Janbaz et al. 2014). It is used in traditional medicine in Pakistan to treat many diseases (Khan and Khatoon 2007; Ajmal et al. 2012; Sher et al. 2011; Sabeen and Ahmad 2009; Akhtar et al. 2013). Extracts and different solvent fractions of this plant are known to exhibit antifungal (Rauf et al. 2012a), antibacterial, phytotoxic (Rauf et al. 2012b) and antioxidant effects (Rauf et al. 2013). As both of these plants are already known to have considerable medical importance, it is of interest that these plants were found to be the best sources of potential pesticides against *A. pisum* in our study.

Conclusions

In conclusion, the extracts from *I. rugosus*, especially the butanol fraction, and *D. mucronata* could be exploited to develop potent aphicides, because of the high mortality caused at low concentrations to aphids. These potential botanical insecticides may fit well in IPM programs designed to control aphids. Considering that these plants are already used for medicinal purposes, they will be safer compared to the current conventional pesticides used to control aphids. Nevertheless, more research into their toxicity will be warranted. Most importantly, this study provides the basis for further exploration on the isolation and identification of bioactive compounds from the butanol fraction of *I. rugosus*, to be used for the development of a formulation for effective application.

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Compliance with ethical standards

Conflict of interest All the authors declare no conflict of interest.

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