Asian Pacific Journal of Tropical Disease

journal homepage: http://www.apjtcm.com

Original article https://doi.org/10.12980/apjtd.7.2017D7-175

Larvicidal efficacy of *Scabiosa arenaria* Forssk. (Dipsacaceae) organs extracts against *Culex pipiens* L.

Malek Besbes Hlila[1*], Ali Lamari[2], Amel Omri Hichri[1], Hichem Ben Jannet[3], Maha Mastouri[1], Mahjoub Aouni[1], Boulbaba Selmi[4]

[1]*Laboratory of Transmissible Diseases and Biological Active Substances, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Avenue Avicenne, 5000 Monastir, University of Monastir, Monastir, Tunisia*

[2]*Laboratory of Histology and Cytogenetic (Research Unit of Genetic, Genotoxicity and Childhood Illness UR12ES10), Faculty of Medicine, University of Monastir, Street Avicenne, Monastir 5019, Tunisia*

[3]*Laboratory of Heterocyclic Chemistry, Natural Products and Reactivity, Team: Medicinal Chemistry and Natural Products, Department of Chemistry, Faculty of Sciences of Monastir, University of Monastir, Monastir, Tunisia*

[4]*Laboratory of Bioresources: Integrative Biology and Exploiting, Higher Institute of Biotechnology of Monastir, Department of Molecular Biology, Cellular and Biotechnology, University of Monastir, Monastir, Tunisia*

ARTICLE INFO

Article history:
Received 20 Jul 2017
Received in revised form 24 Aug 2017
Accepted 10 Sep 2017
Available online 29 Nov 2017

Keywords:
Scabiosa arenaria Forssk
Culex pipiens L
Larvicidal activity
Mosquito

ABSTRACT

Objective: To determine the larvicidal activity of sixteen extracts of *Scabiosa arenaria* Forssk. (*S. arenaria*) against the third instar larvae of *Culex pipiens* L. (*C. pipiens*).
Methods: Larvicidal activity of *S. arenaria* extracts was determined according to World Health Organization. Twenty third instars of the *C. pipiens* were exposed to different concentrations (15 µg/mL to 60 µg/mL) of extracts. Mortality was recorded after 24 h of exposure.
Results: The roots ethyl acetate fraction of *S. arenaria* had a significant toxic effect on larvae of *C. pipiens*, with high mortality percentage (100.0% ± 2.2%) at 60 µg/mL, and 50% lethal concentration value of LC_{50} = (15.00 ± 0.09) µg/mL.
Conclusions: Results show that roots ethyl acetate could be used for the production of natural bio-pesticides which could decrease our dependence on chemical pesticides.

1. Introduction

Insect vectors, particularly mosquitoes (Diptera: Culicidae) are among the most dangerous and serious insect pests of medical significance. There are about 2 700 species of mosquito worldwide; the three more important genera are the *Culex*, *Anopheles* and *Aedes*. They are well-known vector borne illness, a few of which result in millions of fatal, epidemic diseases such as yellow fever, dengue fever, West Nile virus, Japanese encephalitis, Louis encephalitis, malaria, Zika virus, chikungunya and filariasis causing deaths in animals and humans in the world each year[1-4]. The species *Culex pipiens* L. (*C. pipiens*) has a large geographic distribution and may be found through the world in all urban and sub-urban tropical and temperate zones. The principal larval developmental or breeding sites of this mosquito are artificial containers, septic tanks, water irrigation channels, animal watering basins and temporary pools[5], where they play significant roles in the transmission of parasite and many illnesses that infect human, like the West Nile virus, Louis encephalitis virus, bird malaria (*Plasmodium* spp) and filarial worms[6,7]. We found many preparations and apparatuses designed to prevent or decrease such vectors, comprising microbial formulations and pesticides[8] such as, the conventional insecticides, pyrethroids and organophosphates. The pyrethroids are nowadays the most extensive insecticides for the indoor fight against mosquito adults

*Corresponding author: Malek Besbes Hlila, Laboratory of Transmissible Diseases and Biological Active Substances, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Avenue Avicenne, 5000 Monastir, University of Monastir, Monastir, Tunisia.
Tel: 216 97663356)
E-mail: angelkm2007@yahoo.fr
Foundation Project: Supported by the Ministry of High Education and Scientific Research of Tunisia (Grant No. 11/TM06).
The journal implements double-blind peer review practiced by specially invited international editorial board members.

while organophosphate insecticides are commonly utilized in the mosquito reproduction sites as larvicides[9,10]. Nevertheless, these conventional insecticides have not been absolutely successful due to augmentation resistance developed by numerous mosquito species[11], and also side effects on the environment and public health. This represents a major obstacle encountered in effective control programs[12]. Therefore, in recent years, researches are increasingly directed their attention on the development of biodegradable phytopesticides of plant origin extracts, essential oils and plant derived bioactive compounds. The plant natural products are effective, environment-friendly and mitigate the long term environmental effects of pesticide use. In addition, pests rarely develop resistance against pesticide of plant origin[13-16]. Many species of the genus *Scabiosa* (Dipsacaceae) have been pharmacologically and chemically used for their potential new drugs and medicinal use[17-19]. The genus species were supposed to relieve itching of scabies and other skin diseases, including wounds caused by the bubonic plague[20]. *Scabiosa succisa* was utilized to heal bites of poisonous insects and in certain dermatoses like scabies, herpes, ulcer and ringworm[21]. In our previous works, several biological activities of *Scabiosa arenaria* Forssk. (*S. arenaria*) extracts were studied such as their antioxidant, antimicrobial, anti-glucosidase and anti-acetylcholinesterase[22-25]. However, to the best of our knowledge, no species of the family Dipsacaceae has been examined as a potential source of mosquitocides. In this scenario, this paper sheds light on the larvicidal activity of stems + leaves, flowers, fruits and roots extracts of *S. arenaria*.

2. Materials and methods

2.1. Plant collection

The botanical identification of the plant *S. arenaria* of this study was identified by the professor Fethia Harzallah (Laboratory of Genetic, Biodiversity and Valorisation of Bioresources, LR11ES41, Higher Institute of Biotechnology of Monastir, University of Monastir, Tunisia). This species was collected at the flowering stage. The deposition of voucher species (Sa 110) was done in the Laboratory of Heterocyclic Chemistry, Natural Products and Reactivity, Team: Medicinal Chemistry and Natural Products, Faculty of Sciences of Monastir, University of Monastir, Monastir, Tunisia.

2.2. Preparation of S. arenaria extracts

Plant samples (the flowers, the fruits, the roots and the stems + leaves) were air-dried at room temperature and in darkness for two weeks. Then, 100 g of dried and powdered samples were extracted three times with methanol-water 80:20 (v/v) for three days at ambient temperature. After filtration, the crude extracts were then dried under a vacuum in a rotary evaporator. A portion of these extracts were dried completely for the larvicidal activity and the residual aqueous layer was after that exhaustively extracted by liquid/liquid partition with ethyl acetate (EtOAc) and butanol (*n*-BuOH) to yield dried fractions. The crude extracts and its fractions (sixteen samples) were maintained at 4 ℃ before analysis.

2.3. Mosquito culture

A local strain of *C. pipiens* larvae was collected from Oued Smaile (Monastir, Tunisia). The species was reared in a plastic tray (24 cm × 35 cm × 5 cm) at 12:12 light/dark photoperiod, 60% ± 10% relative humidity at (26 ± 2) ℃, in an insectary in the Laboratory of Histology Cytology and Genetics, Faculty of Medicine, University of Monastir, Tunisia. The third instar larvae were used for bioassays.

2.4. Evaluation of larvicidal activity

The larvicidal activity test was carried out following the method used by the World Health Organization[26]. The stems + leaves, flowers, fruits and roots extracts of *S. arenaria* were dissolved in 96% ethanol and afterwards diluted in distilled water to donate a range of concentrations that were utilized for test (60 μg/mL, 30 μg/mL, 15 μg/mL and 7.5 μg/mL). 1 mL of every solution was added to 99 mL of faucet water in plastic goblet. Groups of 20 larvae were taken on a strainer with fine mesh and transferred gently to the test medium by tapping.

The *in vitro* bioassays were applied three times: three replicates are determined for every dose as well as the witness. The latter represents the treatments without the samples which were performed in the same way. Treated and control larvae were held in the same conditions used for colony rearing. No food has been given to the larvae during the treatment. The larvae were considered dead if they were unable to move and to reach the water surface[27]. The mortality response of larvae was carried out for each experiment, 24 h after incubation, and the mortality percentage was reported from the average of three repetitions.

2.5. Determination of lethal concentrations

The lethal concentrations (LC_{50} and LC_{90}), are defined as the concentrations that cause 50% and 90% of mortality in the population of larvae studied, respectively, for a given time and for a single dose[28]. These two concentrations were calculated.

3. Results

Results showing the effect of *S. arenaria* extracts on larvae of

the mosquito *C. pipiens* taken at the third larval stage (L3), showed a direct relation between the rate of larvae mortality and samples concentrations. The results are collected and presented in Figures 1–4. Most of the extracts tested, and after 24 h of exposure, showed low mortality at 7.5 µg/mL, between 3.33% and 35.00%. The best one was recorded for the roots EtOAc fraction (35.0% ± 0.9%). The mortality increased with concentrations. At 60 µg/mL concentration, the mortality of *C. pipiens* larvae was going from 10% (stems + leaves crude extract, the EtOAc and water fractions of the flowers and the fruits) to 100.0% ± 2.2% (EtOAc fraction of the roots). For the second concentration (30 µg/mL), mortality was ranged between 5% (stems + leaves crude and water extracts and the fruits crude extract) to 90.0% ± 2.1% (roots EtOAc fraction). The four extracts of *S. arenaria* roots screened for larvicidal activity were found more effective as compared to stems + leaves, fruits and flowers extracts. Analyses showed that the roots EtOAc fraction was the most effective against *C. pipiens* larvae and exhibited the lowest LC_{50} and LC_{90} values [(15.00 ± 0.09) µg/mL and (30.00 ± 0.08) µg/mL], respectively. The stems + leaves EtOAc fraction indicated also an interesting LC_{50} value [(40.0 ± 0.2) µg/mL], with mortality percentage of the order of 70.0% ± 1.5%. The LC_{50} values were considered effective (less than 100 mg/L) according to the classification of Thangam and Kathiresan[29].

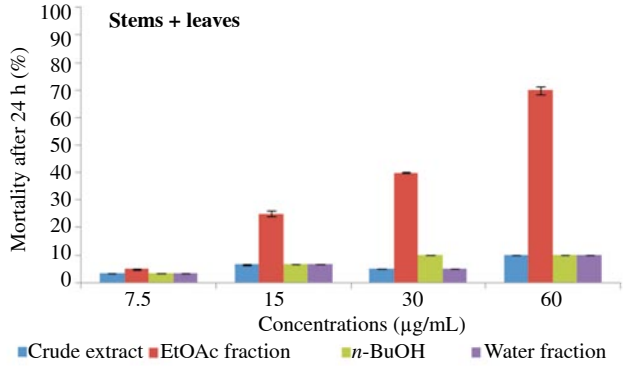

Figure 1. Evolution of *C. pipiens* mortality after 24 h of exposure to stems + leaves extracts from Tunisian *S. arenaria*.

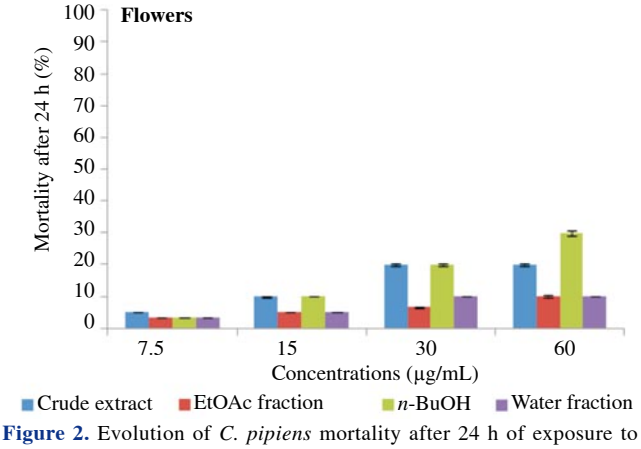

Figure 2. Evolution of *C. pipiens* mortality after 24 h of exposure to flowers extracts from Tunisian *S. arenaria*.

Thus, these results showed that the roots and the stems + leaves EtOAc fractions certainly contained many active substances such as the secondary metabolites, which were responsible for this interesting activity.

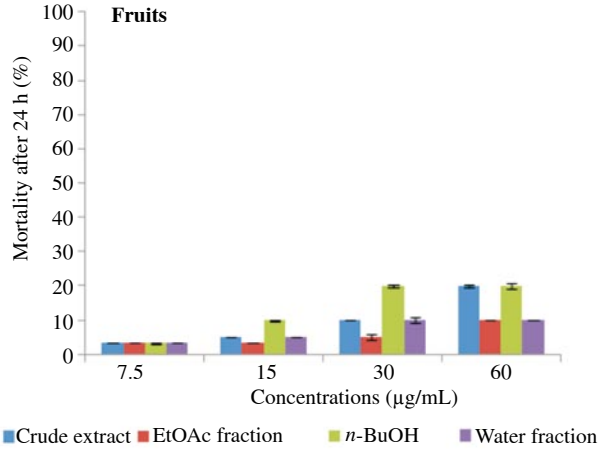

Figure 3. Evolution of *C. pipiens* mortality after 24 h of exposure to fruits extracts from Tunisian *S. arenaria*.

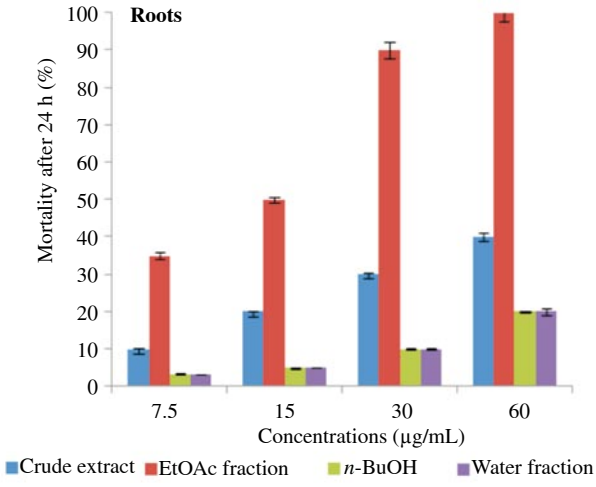

Figure 4. Evolution of *C. pipiens* mortality after 24 h of exposure to roots extracts from Tunisian *S. arenaria*.

4. Discussion

Several plants have represented a source of efficient and natural mosquitocidal agents[30,31]. Our paper represents the first work treating the larvicidal activity of species belonging to the Dipsacaceae family. In addition, few works touched the subject of sensibility of the mosquito *C. pipiens* by Tunisian plant extracts. Wafa *et al.*[32] have tested the larvicidal activity of aqueous extracts of leaves and seeds of *Ricinus communis* L. (Euphorbiaceae) against *C. pipiens* larvae. Toxicity showed a mortality of 100% after 24 h of exposure. This activity is due to its phenolic compounds. The same applies to Fatnassi *et al.*[33] who have worked also on a species belonging to the family Euphorbiaceae (*Jatropha curcas* L.) and found that the larval activity after 24 h of exposure, 100% mortality for aqueous seed extract. While in our work the organic extracts especially the EtOAc fractions exhibited the best larvicidal activity. While other

researchers from Tunisia, tested the toxicity of essential oils of plants like *Salvia officinalis* (Lamiaceae), *Hypericum* species (Hypericaceae) and *Pituranthos tortuosus* (Apiaceae) on the *C. pipiens*, such as Rouis *et al.*[34], Lamari *et al.*[35], Krifa *et al.*[36]. If we compare our results with other recent works around the world, several differences were found. Al-Mekhlafi *et al.*[37] and Al-Mekhlafi[38] showed that the EtOAc fractions of *Xanthium strumarium* (Asteraceae) seeds and *Carum copticum* (Apiaceae) fruits appear to have a weak larvicidal activity. On the contrary, the methanol extracts showed the best results (LD_{50} = 502.32 μg/mL and 122.26 μg/mL, respectively).

The interesting larvicidal activity of roots EtOAc fraction of our study plant *S. arenaria* may be due to its compounds, thereby, the chemical composition of this fraction was studied in the previous works of our research team, thus the phytochemical screening of roots extracts was analyzed and indicated the presence of tannins, flavonoids, coumarins, steroids and saponins[23]. The same team has studied the chemical study of the roots EtOAc fraction and shown its richness in flavonoids and phenolic acid such as rutin, taxifolin, *p*-coumaric acid, catechin[39].

In the literature, many studies have confirmed the strong toxicity of saponic extracts on *C. pipiens*[40-42]. As well as the larvicidal activity of flavonoids was also proved[43,44]. Overall, this research demonstrated larvicidal activity of sixteen extracts obtained from flowers, fruits, stems + leaves and roots of *S. arenaria* against the mosquito *C. pipiens*. The roots EtOAc displayed highest larvicidal activity among the tested extracts. This mosquitocidal activity encouraged the use of plant extract as alternative mosquito control agents. The plant extracts have many advantages like biodegradability, low toxicity to mammals. In addition, they may decrease the application of conventional insecticides which retard the development of resistance and diminish environmental pollution. Even so, more researches are required to examine the efficacy of different phenolic compounds found in the active extract.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This research was supported by the Ministry of High Education and Scientific Research of Tunisia (Grant No. 11/TM06).

References

[1] Aziz AT, Al-Shami SA, Mahyoub JA, Hattabi M, Ahmad AH, Rawi CS. An update on the incidence of dengue gaining strength in Saudi Arabia and current control approaches for its vector mosquito. *Parasite Vectors* 2014; **7**: 258.

[2] Bakr RFA, Al-Ghramh HA. Toxicological studies on the effect of some agricultural waste and plant extract as insecticidal agent on the mosquito, *Culex pipiens*. *Egypt Acad J Biol Sci* 2014; **7**(1): 11-21.

[3] Benelli G, Mehlhorn H. Declining malaria, rising dengue and Zika virus: insights for mosquito vector control. *Parasitol Res* 2016; **115**: 1747-54.

[4] Mehlhorn H, Al-Rasheid KA, Al-Quraishy S, Abdel-Ghaffar F. Research and increase of expertise in arachno-entomology are urgently needed. *Parasitol Res* 2012; **110**: 259-65.

[5] Martínez-de la Puente J, Ferraguti M, Ruiz S, Roiz D, Soriguer RC, Figuerola J. *Culex pipiens* forms and urbanization: effects on blood feeding sources and transmission of avian *Plasmodium*. *Malar J* 2016; **15**: 589.

[6] Turell MJ. Members of the *Culex pipiens* complex as vectors of viruses. *J Am Mosq Control Assoc* 2012; **28**: 123-6.

[7] Ward M, Benelli G. Avian and simian malaria: do they have a cancer connection? *Parasitol Res* 2017; **116**: 839-45.

[8] Benelli G. Research in mosquito control: current challenges for a brighter future. *Parasitol Res* 2015; **114**: 2801-5.

[9] World Health Organization. Pesticides and their application, for the control of vectors and pests of public health importance, sixth edition. Geneva: World Health Organization; 2006. [Online] Available from: http://www.who.int/whopes/resources/who_cds_ntd_whopes_gcdpp_2006.1/en/ [Accessed on 20th August, 2017]

[10] Liu ZL, Liu QZ, Du SS, Deng ZW. Mosquito larvicidal activity of alkaloids and limonoids derived from *Evodia rutaecarpa* unripe fruits against *Aedes albopictus* (Diptera: Culicidae). *Parasitol Res* 2012; **111**: 991-6.

[11] Karunamoorthi K, Sabesan S. Insecticide resistance in insect vectors of disease with special reference to mosquitoes: a potential threat to global public health. *Health Scope* 2013; **2**(1): 4-18.

[12] Benelli G, Pavela R, Canale A, Cianfaglione K, Ciaschetti G, Conti F, et al. Acute larvicidal toxicity of five essential oils (*Pinus nigra*, *Hyssopus officinalis*, *Satureja montana*, *Aloysia citrodora* and *Pelargonium graveolens*) against the filariasis vector *Culex quinquefasciatus*: synergistic and antagonistic effects. *Parasitol Int* 2017; **66**: 166-71.

[13] Govindarajan M, Benelli G. α-Humulene and β-elemene from *Syzygium zeylanicum* (Myrtaceae) essential oil: highly effective and eco-friendly larvicides against *Anopheles subpictus*, *Aedes albopictus* and *Culex tritaeniorhynchus* (Diptera: Culicidae). *Parasitol Res* 2016; **115**: 2771-8.

[14] Govindarajan M, Benelli G. Artemisia absinthium-borne compounds as novel larvicides: effectiveness against six mosquito vectors and acute toxicity on non-target aquatic organisms. *Parasitol Res* 2016; **115**: 4649-61.

[15] Govindarajan M, Benelli G. Eco-friendly larvicides from Indian plants: effectiveness of lavandulyl acetate and bicyclogermacrene on malaria, dengue and Japanese encephalitis mosquito vectors.

Ecotoxicol Environ Saf 2016; **133**: 395-402.

[16] Maurya P, Sharma P, Mohan L, Verma MM, Srivastava CN. Larvicidal efficacy of *Ocimum basilicum* extracts and its synergistic effect with neonicotinoid in the management of *Anopheles stephensi*. *Asian Pac J Trop Dis* 2012; **2**(2): 110-6.

[17] Bussmann RW, Glenn A, Meyer A, Kuhlman A, Townesmith A. Herbal mixtures in traditional medicine in Northern Peru. *J Ethnobiol Ethnomed* 2010; **6**: 10.

[18] Bussmann RW, Glenn A. Medicinal plants used in Northern Peru for reproductive problems and female health. *J Ethnobiol Ethnomed* 2010; **6**: 30.

[19] Bonet MA, Vallès J. Ethnobotany of Montseny biosphere reserve (Catalonia, Iberian Peninsula): plants used in veterinary medicine. *J Ethnopharmacol* 2007; **110**: 130-47.

[20] Culpeper N. *Culpeper's complete herbal: a book of natural remedies for ancient ills*. Hertfordshire: Wordsworth Editions Ltd; 1998.

[21] Girre L. [*Know and recognize medicinal plants*]. France: Bulletin des Bibliothèques de France; 1980. French.

[22] Besbes Hlila M, Omri A, Ben Jannet H, Lamari A, Aouni M, Selmi B. Phenolic composition, antioxidant and anti-acetylcholinesterase activities of the Tunisian *Scabiosa arenaria*. *J Pharm Biol* 2013; **51**(5): 525-32.

[23] Besbes Hlila M, Mosbah H, Msaada K, Ben Jannet H, Aouni M, Selmi B. Acetylcholinesterase inhibitory and antioxidant properties of roots extracts from the Tunisian *Scabiosa arenaria* Forssk. *Ind Crops Prod* 2015; **67**: 62-9.

[24] Besbes Hlila M, Mosbah H, Majouli K, Msaada K, Ben Jannet H, Aouni M, et al. α-Glucosidase inhibition by Tunisian *Scabiosa arenaria* Forssk. extracts. *Int J Biol Macromol* 2015; **77**: 383-9.

[25] Besbes Hlila M, Mosbah H, Majouli K, Ben Nejma A, Ben Jannet H, Mastouri M, et al. Antimicrobial activity of *Scabiosa arenaria* Forssk. extracts and pure compounds using bioguided fractionation. *Chem Biodivers* 2016; **13**: 1262-72.

[26] World Health Organization. Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides. Geneva: World Health Organization; 1981. [Online] Available from: http://apps.who.int/iris/bitstream/10665/69615/1/WHO_VBC_81.807_eng.pdf [Accessed on 20th August, 2017]

[27] Macedo ME, Consoli RA, Grandi TS, dos Anjos AM, de Olivira AB, Mendes NM, et al. Screening of Asteraceae (Compositae) plant extracts for larvicidal activity against *Aedes fluviatilis* (Diptera: Culicidae). *Mem Inst Oswaldo Cruz* 1997; **92**: 565-70.

[28] Benelli G. Commentary: data analysis in bionanoscience-issues to watch for. *J Cluster Sci* 2017; 28: 11-4.

[29] Thangam TS, Kathiresan K. Marine plants for mosquito control. In: Proceedings of the Second International Conference on Urban Pests. 1996; p. 431-5.

[30] Benelli G. Plant-mediated biosynthesis of nanoparticles as an emerging tool against mosquitoes of medical and veterinary importance: a review. *Parasitol Res* 2016; **115**: 23-34.

[31] Benelli G. Natural products as effective weapons against mosquito-borne diseases. *Curr Org Chem* 2016; **20**(25): 2647-8.

[32] Wafa G, Amadou D, Larbi KM, Hela EFO. Larvicidal activity, phytochemical composition, and antioxidant properties of different parts of five populations of *Ricinus communis* L. *Ind Crops Prod* 2014; **56**: 43-51.

[33] Fatnassi B, Khouja ML, El Ferchichi OH. Larvicidal efficacy of *Jatropha curcas* L. (Euphorbiaceae) leaf and seed aqueous extracts against *Culex pipiens* L. *Afr J Biotechnol* 2014; **13**(26): 2641-7.

[34] Rouis Z, Laamari A, Abid N, Elaissi A, Cioni PL, Flamini G, et al. Chemical composition and larvicidal activity of several essential oils from *Hypericum* species from Tunisia. *Parasitol Res* 2013; **112**: 699-705.

[35] Lamari A, Teyeb H, Ben Cheikh H, Douki W, Neffati M. Chemical composition and insecticidal activity of essential oil of *Salvia officinalis* L. cultivated in Tunisia. *J Essent Oil Bearing Plants* 2014; **17**(3): 506-12.

[36] Krifa M, Gharad T, Haouala R. Biological activities of essential oil, aqueous and organic extracts of *Pituranthos tortuosus* (Coss.) Maire. *Sci Hortic* 2011; **128**: 61-7.

[37] Al-Mekhlafi FA, Abutaha N, Mashaly AMA, Nasr FA, Ibrahim KE, Wadaan MA. Biological activity of *Xanthium strumarium* seed extracts on different cancer cell lines and *Aedes caspius*, *Culex pipiens* (Diptera: Culicidae). *Saudi J Biol Sci* 2017; **24**: 817-21.

[38] Al-Mekhlafi FA. Larvicidal, ovicidal activities and histopathological alterations induced by *Carum copticum* (Apiaceae) extract against *Culex pipiens* (Diptera: Culicidae). *Saudi J Biol Sci* 2017; doi: 10.1016/j.sjbs.2017.02.010.

[39] Besbes Hlila M, Mosbah H, Majouli K, Msaada K, Ben Salem Abid N, Ben Jannet H, et al. Characterization of anti-Coxsackie virus B3 constituents of *Scabiosa arenaria* Forssk by RP-HPLC-UV method. In: Berhardt LV, editor. *Advances in medicine and biology*. New York: Nova Science Publishers; 2016.

[40] Chaieb I, Ben Hamouda A, Trabelsi M, Ben Halima M, Ben Hamouda MH. Investigation of *Cestrum parqui* saponins to *Culex pipiens* larvae. *Pest Technol* 2009; **3**(1): 73-5.

[41] Bagavan A, Rahuman A, Kamaraj C, Kannappan G. Larvicidal activity of saponin from *Achyranthes aspera* against *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae). *Parasitol Res* 2008; **103**: 223-9.

[42] Weisman Z, Chapagain BP. Larvicidal activity of saponin containing extracts and fractions of fruit mesocarp of *Balanites aegyptiaca*. *Fitoterapia* 2006; **77**: 420-4.

[43] Perumalsamy H, Jang MJ, Kim JR, Kadarkarai M, Ahn YJ. Larvicidal activity and possible mode of action of four flavonoid and two fatty acids identified in *Millettia pinnata* seed toward three mosquito species. *Parasit Vectors* 2015; **8**: 237.

[44] Sun R, Sacalis JN, Chin CK, Still CC. Bioactive aromatic compounds from leaves and stems of *Vanilla fragrans*. *J Agric Food Chem* 2001; **49**: 5161-4.