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Leaves and seeds extracts of *Vitex agnus castus* L., an ecological and effective alternative to conventional insecticides against fruit flies (Diptera: Tephritidae)

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ABSTRACT

The objective of this work is to investigate the insecticidal activity of *Vitex agnus castus* (VAC; Lamiales: Lamiaceae) essential oils and organic extracts against the species *Ceratitis capitata* L. adults (Diptera: Tephritidae). Extraction of the essential oils from the leaves and seeds of the plant was carried out using hydro-distillation while organic extracts were obtained using the Soxhlet apparatus. The principal compounds of essential oils were 1, 8-cineol, α-pinene, β-farnesene and β-caryophyllène for leaves and 1, 8-cineole, sabinene, α-pinene, and β-farnesene for seeds. The insecticidal activity was determined by evaluating the adulticidal effect using direct contact application methods. Our extracts from leaves and seeds of VAC presented toxicity in *Ceratitis capitata* adults. Responses varied based on the type of extract, plant part, and exposure time. The good insecticidal activity was achieved by essential oils from leaves and seeds. The lethal concentration values (LC_{50}) were 0.660 mg mL^{-1} and 6.170 mg mL^{-1} after 24 h of incubation respectively. Thus, the essential oils from The VAC leaves and seeds exhibited insecticidal activity in *Ceratitis capitata* and can be used as an alternative to the control of these dipterans. Organic extracts from The VAC leaves and seeds showed moderate insecticidal activity against *Ceratitis capitata* after a long time of incubation. Prospective studies of these oils are needed on cytotoxicity, mutagenicity, and genotoxicity in human cells, in addition to the *in vitro* safety of these essential oils in human cells for safe use.

Keywords: Insecticide, Vitex agnus castus, Fruit flies, Organic extracts, Essential oils.
Article type: Research Article.

INTRODUCTION

Vitex agnus castus was formerly classified in the family of *Verbenaceae*, but now the phylogenetic classification locates this species in the *Lamiaceae* family (Aissaoui Zitoun & Hamama 2014). It is also known as chaste tree, chaste berry, and monk's pepper. It is also a shrub widely distributed in the middle east and southern Europe, joint Circum Mediterranean species. Common in Morocco, along the rivers (Bellakhdar 1997). VAC contains iridoids (Hajdú *et al*. 2007), flavonoids (Küçükboyaci & Şener 2010), diterpenoids (Hoberg *et al*. 1999), essential oils (Sørensen & Katsiotis 2000), and ketosteroids (Šaden-Krehula *et al.* 1990). Traditionally, VAC fruit extracts have been used in the treatment of many female conditions, including menstrual disorders (amenorrhoea, dysmenorrhoea), premenstrual syndrome (PMS), corpus luteum insufficiency, infertility, acne, menopause, and

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disrupted lactation (Daniele *et al.* 2005; Prilepskaya *et al*. 2006). Because of its dopaminergic effect, VAC could be considered as an efficient alternative phytotherapeutic drug in the treatment of slight hyperprolactinemia (Meier *et al*. 2000). Some data suggest that VAC flowers contain components that inhibit proliferation and induce apoptosis in human prostate epithelial cell lines. The extract may be useful for the prevention and/or treatment not only of benign prostatic hyperplasia, but also of human prostate cancer (Weisskopf *et al*. 2005). In the Oasis of Tata in the southeast of Morocco, different parts of VAC are used against the cold, back pains, dysmenorrhea, and sterility and also for tanning (Abouri *et al.* 2012). In other regions of Morocco VAC is used as a sedative, antispasmodic, and anaphrodisiac as an infusion of fruits and flowering tops (Sijel massi 1991). The Mediterranean fruit fly *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae), is one of the most devastating fruit pests worldwide which is a multivoltine and highly polyphagous species capable of utilizing at least 250 different hosts including fruits, nuts, and vegetables (Levinson 2003). Recently interesting results were obtained by studying the effect of natural substances like vegetable extracts and essential oils on control insect pests and disease vectors (Rodríguez-López *et al.* 2007; Benelli *et al.* 2012; Erdoğan & Yıldırım 2016; Ghabbari *et al.* 2018; Benelli *et al.* 2019; Pavela *et al.* 2020; Benelli *et al.* 2021; Rima *et al.* 2021). Chemical control of pests raises several concerns related to the environment and human health including acute and chronic poisoning of applicators and consumers, destruction of wildlife, disruption of natural biological control and pollination, extensive groundwater contamination, and evolution of resistance to pesticides in the pest population (Picollo *et al.* 2005; Nerio *et al.* 2010). Natural pesticides represent an attractive alternative to synthetic insecticides as they can be efficacious and less environmentally disruptive (Isman 2006; Oyarzún *et al.* 2008; Meissle, Mouron *et al.* 2010; Olivero-Verbel *et al*. 2010, Khater 2012; Marcombe *et al.* 2018; Porusia & Septiyana 2021; Alwan 2022; Shareif *et al.* 2022; Salih *et al.* 2022). Medicinal and aromatic plants are considered nowadays by numerous countries as one of the priority programs for the promotion of traditional products. As part of this program in Morocco, for instance, the valuation of the essential oils of VAC leaves and seeds also of its organic extracts could encourage the development of medicinal and aromatic plants sector and sustainable development in the marginalized regions of South-eastern Morocco. In this context, our main objective is to evaluate the potential insecticidal activity against Mediterranean fruit fly *Ceratitis capitata* of essential oil and organic extracts of The VAC leaves and seeds growing in the oasis of Tata localized in the south-east of Morocco.

MATERIALS AND METHODS

Sample collection

The study was carried out on essential oils; Methanol and Petroleum ether extract from The VAC leaves and seeds growing wild in the oasis of Tata, a marginalized oasis of the southeast of Morocco during June 5, 6, and 7 2014. Taxonomic identification of the species was confirmed and deposited in the laboratory of biotechnology, University of Ibn Zohr, Agadir, Morocco.

Isolation of the essential oils

In this study, 100 g of the leaves and seeds were submitted separately for 3 h to water-distillation, using a Clevenger-type apparatus according to the method of Demirci *et al.* (2008) with minor modification. The resulting essential oils were dried over anhydrous sodium sulfate and, stored at +4 °C for further experiment protocols. The percentage of essential oil yield was calculated as the weight of essential oils divided by the weight of dried botanical material.

Organic extracts obtaining or preparation

Preparation of the plant samples was conducted using the Soxhlet apparatus method. 20 g of plant powder was weighed in a thimble and subjected to 4-h Soxhlet extraction with 100 mL petroleum ether as a solvent. The solvent of extraction was removed by evaporation to dryness under reduced pressure at 40 °C until the sample weight was constant (Gharby *et al.* 2020). The remains of the plant material were extracted with methanol in a similar manner and yield was determined for each extraction.

Detection of alkaloids, terpenes, tannins, and saponins

Tests of absence and presence of alkaloids, terpenes, tannins, and saponins in the VAC methanol and petroleum ether extracts were carried out according to different protocols. The methanol and petroleum ether extracts were submitted to thin-layer chromatography (TLC) plates (GF 254 60; Merck). Plates were developed separately with

Ethyl acetate/Methanol/Water (100/13.5/10, v/v/v) for alkaloids and with benzene for terpenes. The components were visualized under visible and UV light (365 nm) followed by spraying with the following reagents to reveal spots of different groups: Dragendorff's and iodoplatinate's reagents for alkaloids and antimony chloride for terpenes. For the detection of tannins; to 2 mL of the methanol and petroleum ether extracts were added a few drops of an aqueous solution of $FeCl_3$. To detect the saponins, the calculation of the index of foam was used.

Total polyphenols and total flavonoids content

The dried extracts were dissolved again in methanol to obtain a mother solution with a known concentration. Total polyphenols were determined by the Folin-Ciocalteu method of Aguilar-Garcia *et al*. (2007). 50 µL of mother solutions brought to appropriate dilutions were mixed with 1.25 mL of 10 fold diluted Folin-Ciocalteu reagent. After 2 min of incubation at room temperature, 1 mL of 7.5% sodium carbonate was added and the mixture was incubated for 15 min at 50°C. A calibration curve of Gallic acid was established and the measurement was done at 760 nm. Total polyphenols values were expressed as milligram Gallic acid equivalent per gram of dried extract (mg GAE/g DE). Quantitative determination of total flavonoids was carried out using a simple spectrometric assay developed by Andary (1999) using 2-aminoethyl-diphenyl borate (NEU; 1% in Methanol). A volume of 1 mL of dissolved extract in methanol extract brought to the suitable dilution was mixed with 50 mL of the using NEU reagent. The absorption was determined at 409 nm by visible UV spectrophotometer and compared with quercetin standard (0.05 mg mL^{-1}) treated with the same reagent and under the same conditions as the extract. The percentage of total flavonoids quercetin equivalent is calculated using the following formula:

$$TFC\% = \frac{\text{Aext} * 0.05 * 100}{\text{Aq} * \text{Cext}}$$

where A_{ext} is the absorption of the studied extract, A_q is the absorption of quercitin and C_{ext} is the concentration of the extract in mg mL^{-1}.

Insecticidal activity

Insect cultures

The founding insect culture of *Ceratitis capitata* was collected from infested fruits of *Argania spinosa* that were rolling out on a layer of sterilized sand inside a mosquito cage. Adults of *Ceratitis capitata* were reared at 27 ± 2 °C, 50–60% relative humidity, and 16 h light: 8 h dark photoperiod. Adult flies were provided with water and a diet consisting of protein yeast autolysate and sucrose in a 1:4 ratio.

Contact toxicity bioassay

A contact toxicity bioassay (Kim *et al.* 2004) was used to evaluate the toxicity of essential oils and organic extracts of the VAC leaves and seeds in *Ceratitis Capitata* adults. The bioassays were conducted using groups of 10 *Ceratitis capitata* adults, 3-5 days old, kept in plastic containers (15cm × 10 cm × 5 cm) with transparent cover and fed with a formulation containing known concentrations of each essential oil and organic extracts. Every essential oil and every organic extract were prepared in a solution containing Tween 80%, water, yeast autolysate, and sucrose to obtain different concentrations ranging from 0 ppm to 50 000 ppm depending on every type of extract. 3 mL of each concentration solution was applied to a commercial sponge cylinder (h = 1 cm, r = 2 cm). The sponge cylinder was introduced in an open cylinder (h = 1.5 cm, r = 2.5 cm). Treated and control adults were held under the same conditions used for colony maintenance. Each trial was replicated three times and mortalities were determined at 24, 48, and 72 h post-treatment. Adults were considered to be dead if their legs did not move when they were prodded with a fine pin. All treatments were repeated three times.

Statistical Analysis

To determine whether there is a statistically significant difference among the obtained results for phytochemical analyses, variance analyses were carried out using SPSS 10.0 software package. Values of $p < 0.05$ were considered to be significantly different using Newman & Keuls tests.

For the study of the insecticidal activity of our extracts: essential oils and organic extracts; lethal activity was classified as follows:

Strong: mortality > 80%; moderate: mortality 80–61%; weak: mortality 60–40%; little or no activity: mortality <40%. PROBIT analysis was conducted to estimate lethal doses LC_{50} with their 95% confidence interval using SPSS 12.0 Statistical Software; LC values were considered significantly different when their respective 95% confidence interval did not overlap.

RESULTS AND DISCUSSIONS

Yields of different extracts from the VAC leaves and seeds

The hydro-distillation of the VAC leaves and the seeds growing in the oasis of Tata exhibited red-orange oils with 0.76 % ± 0.02 (w/w) of yield for leaves, while 0.71 % ± 0.03 (w/w) for seeds, based on the dry weight of plant material. These essential oils concentrations were higher than those reported in earlier literature (Stojković *et al.* 2011, Asdadi *et al.* 2015; Zahid *et al.* 2016; Rezaei *et al.* 2019). Methanol was the best solvent, extracting a larger quantity of material compared with petroleum ether for the VAC leaves in contrary with the seeds, where petroleum ether solvent displayed more material than methanol.

Table 1. The yield of essential oils, petroleum ether, and methanol extract from the VAC leaves and seeds.

Type of extract	Yields % (w/w)	
	Leaves	Seeds
Essential oil	0.76 ± 0.02	0.71 ± 0.03
Methanol extract	13.91 ± 0.10	2.78 ± 0.03
Petroleum ether extract	3.4 ± 0.06	4.74 ± 0.18

Phytochemical screening of leaves and seeds of the VAC leaves and seeds

The results of the phytochemical screening of petroleum ether extracts are listed in Table 2. The petroleum ether extract of the VAC leaves and seeds contain terpenes. Methanol extracts of both VAC leaves and seeds contain terpenes, alkaloids, and condensed tannins. Saponins are present only in the methanol extract of leaves and absents in the other extracts.

Table 2. Phytochemical screening of Methanol and Petroleum ether extracts from leaves and seeds of VAC.

	Leaves		Seeds	
Component	Petroleum ether	Methanol	Petroleum ether	Methanol
Terpenes	+++	+	+++	+
Saponins	ND	+++	ND	ND
Alkaloids	ND	+++	ND	+
CoNDensed tannins	ND	+++	ND	+++
Hydrolysable tannins	ND	ND	ND	ND

* ND: not detected

Total polyphenols and flavonoids content

The amount of total polyphenols varied widely in petroleum ether and methanol extracts of the VAC leaves and seeds. The highest amounts were found in methanol extract of leaves (82.63 ± 1.94 mg GAE/g), the difference was significant between this amount and the amount found in methanol extract of seeds (15.98 ± 0.38 mg GAE/g DP; $p < 0.05$). In the petroleum ether extracts of the VAC leaves and seeds, the contents of total polyphenols were medium and the differences were not significant between them. Content of total flavonoids was considerable in the methanol extract of leaves and seeds, while the petroleum ether extract of leaves and seeds exhibited low content of total flavonoids: 0.761 ± 0.026 mg GAE/g DP and 1.86 ± 0.22 mg GAE/g DP respectively (Table 3). Previous works highlighted the contents of total polyphenols and total flavonoids in the VAC leaves and seeds using different solvents. Sarikurkcu *et al.* (2009) found that the methanol extract of the VAC seeds contains 46.50 ± 1.39 µg Gallic acid equivalent per mg of extract of total polyphenols and 10.80 ± 0.26 µg quercetin equivalent per mg of extract of total flavonoids. The VAC leaves contain total polyphenols and total flavonoids in different concentrations including 46.85±1.42 of caffeic acid equivalent per gram of dry biomass of total polyphenols and 42.29 ± 1.86 mg catechin equivalent per gram of dry biomass of total flavonoids respectively (Latoui *et al.* 2012). The VAC leaves are rich in total polyphenols and total flavonoids than seeds.

Table 3. Total polyphenols and total flavonoid contents from the VAC leaves and seeds methanol and petroleum ether extracts.

	Leaves		Seeds	
Component	**Petroleum ether**	**Methanol**	**Petroleum ether**	**Methanol**
Total polyphenols content (mg GAE/g dried plant)	14.45 ± 0.77 [b]	82.63 ±1.49 [a]	13.52 ± 0.18 [b]	15.98± 0.25 [b]
Total flavonoids content (mg QE/g dried plant)	0.78 ± 0.22 [C]	37.27 ± 8.25 [A]	1.86 ± 0.22 [C]	13.25 ± 1.55 [B]

Each value is presented as mean ± standard deviation (n = 3). Different letters are significantly different according to Newman & Keuls tests at $p < 0.05$.

Insecticidal activity

The toxicity of organic extracts and essential oils from The VAC leaves and seeds to *Ceratitis capitata* adults is depicted in Table 4. The mortality of insects increased significantly depending on the elevation in the extract concentration. A significant difference was observed between the mortality rate (%) caused by essential oils from The VAC leaves and seeds essential oils caused strong mortality rate [(mortality > 80% at a low dose (500 ppm)] after 48 h of incubation, while weak mortality (60–40%) at 600 ppm after 24 h of incubation. The VAC seeds essential oil exhibited no activity (mortality <20 %) after 24 h. Gradually, as the insects were incubated for a long period with essential oils, the mortality rate upraised. Methanol extracts of leaves caused strong mortality after 72 h from 40 000 ppm. After 24 and 48 h of incubation, these extracts caused little or no mortality (mortality <40%). A methanol extract of seeds as well as the petroleum ether extract of leaves and seeds caused little or no mortality at the examined concentrations.

Table 4. Mortality rate (%) of *Ceratitis capitata* adults fed for 3 days with a formulation containing extracts from the VAC leaves and seeds.

Plant part	Extract type	Concentration of tested extract	Mean mortality rate (%) at different times		
			24 h	**48 h**	**72 h**
Leaves	Control	0	0.00	0.00	3.33
	Essential oil	50	0.00	33.33	70.00
		200	26.66	73.33	93.33
		600	53.33	93.33	100.00
	Methanol	5000	3.33	3.33	6.66
		20000	3.33	26.66	50
		50000	23.33	73.33	100
	Petroleum ether	5000	0.00	6.66	6.66
		20000	10.00	16.66	30.00
		50000	10.83	30.00	40.00
Seeds	Essential oil	50	0.00	20.00	40.00
		200	0.00	26.66	43.33
		600	10.00	30.00	50.00
	Methanol	5000	0.00	0.00	0.00
		20000	10.00	23.33	53.33
		50000	19.16	22.5	46.66
	Petroleum ether	5000	0.00	0.00	0.00
		20000	0.00	20.00	46.66
		50000	11.66	20.00	26.66

Note: Tree concentrations only are presented in the Table.

The LC_{50} values of essential oils, methanol, and petroleum ether extracts of the VAC leaves contact toxicity test are presented in Table 5. Essential oils of leaves and seeds were effective against adults of *Ceratitis capitata* in low doses (dose < 10 mg mL^{-1}). Methanol extracts of leaves and seeds showed LC_{50} values very high in comparison with essential oils of leaves and seeds.

Table 5. Contact Toxicity against *Ceratitis capitata* adults [LC_{50} values (mg mL^{-1}) and 95% confidence interval] of the essential oils, methanol and petroleum ether extracts of VAC leaves and seeds.

Incubation period	Extract type	Leaves				Seeds			
		LC_{50} (95%)	Slope ± SE	χ (df)	Sig.	LC_{50} (95%)	Slope ± SE	χ (df)	Sig.
24 h	Essential oils	0.660 (0.650-0.781)	28.168	19	0.080	6.170 (4.418-13.658)	42.621	25	0.015
	Methanol	82.739 (54.177-65.337)	23.653	16	0.097	75.815 (56.996-128.258)	29.237	16	0.022
	Petroleum ether	96.113 (63.910-447.092)	16.699	16	0.405	94.281 (75.927-141.819)	23.853	16	0.093
48 h	Essential oils	0.188 (0.084-0.275)	48.252	19	0.000	6.804 (3.928-12.429)	21.839	25	0.645
	Methanol	34.126 (25.909-50.830)	44.971	15	0.000	69.464 (52.101-128.258)	17.397	16	0.360
	Petroleum ether	57.051 (42.058-115.878)	27.154	16	0.040	79.496 (63.276-120.437)	25.454	16	0.062
72 h	Essential oils	0.044 (0.0003-0.12)	158.978	19	0.000	1.968 (0.835-6.945)	49.970	25	0.002
	Methanol	20.040 (16.757-24.075)	36.049	15	0.002	42.827 (32.057-73.541)	36.266	16	0.003
	Petroleum ether	46.012 (34.902-77.503)	31.756	16	0.011	63.544 (47.517-110.697)	49.970	25	0.002

The insecticidal activity varied based on extract type, part of the plant, concentrations of the essential oils and the organic extract, and the exposure time. Moreover, our results indicated that the higher concentrations of essentials oil and organic extracts for a relatively short period are much more effective than lower concentrations for a long period; 600 ppm of the VAC leaves essential oil exhibited 50 % mortality rate after 24 h of exposure, the same rate of mortality was achieved by 100 ppm after 48 h. Two reports on the VAC leaves and seeds essential oils were already published by Asdadi *et al*. (2015) which revealed that seeds contain monoterpenes hydrocarbons (35.68%) higherthan leaves, while the oxygenated monoterpenes and sesquiterpene hydrocarbons were mainly present in leaves. Obeng-ofari (1997) found that 1, 8-Cineol was highly repellent and toxic against some stored product beetles. Therefore, the higher toxicity of the VAC leaves essential oils could be attributed to their higher amount of the 1, 8-Cineol (19, 64 %). Jacobson (1989) pointed out that the most promising botanical insect-control agents are in the families: *Annonaceae*, *Asteraceae*, *Canellaceae*, *Lamiaceae* (formerly *Labiatae*), *Meliaceae*, and *Rutaceae.* Mediterranean plant VAC (monk's pepper) can be used as a spray to keep away especially Ixodesricinus and Rhipice phalussanguineus ticks from animals and humans for at least 6 h. Mosquitoes, biting flies, and fleas are also repelled for about 6 h (Mehlhorn *et al.* 2005).

CONCLUSION

The methanol extract of VAC leaves contained high levels of phenolic and flavonoid compounds higher than the methanol and petroleum ether extracts of seeds and also the petroleum ether extracts of leaves. The results obtained showed that the essential oil of VAC leaves is very effective against *Ceratitis capitata*. A significant difference was observed between the insecticidal activity of the VAC leaves and seeds methanol and petroleum extracts. In addition, the petroleum ether extracts were always more effective than the methanol extracts against *Ceratitis capitata.*

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Conflicts of Interest

There are no conflicts of interest**.**

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