Original Research Article

Biologically-guided isolation of acaricidal phytosterols: An *in vitro* study against *Rhipicephalus (B.) annulatus* ticks infesting cattle in Egypt

ABSTRACT

Aim: To find new and effective natural products to control *Rhipicephalus (Boophilus) annulatus* infesting cattle in Egypt through biologically-guided study.

Place and Duration of Study: Plant extraction and phytochemical study: Department of Pharmacognosy (2014-2016), Adult and larval immersion tests: Department of Parasitology, Faculty of Veterinary Medicine (2016).

Methodology: Acaricidal activity was evaluated using adult and larval immersion tests of the total alcohol extract of *Mesembryanthemum forsskaolii* Hochst. Ex. Boiss herb in addition to successive solvent extracts of different polarities (*n*-hexane, chloroform ($CHCl_3$), and methanol (MeOH). Acaricidal activities were measured by mean number of ticks died and antiparasitic efficacy (%) relative to the negative control. After specifying the most active fraction, chromatographic isolation of the secondary metabolites of the active *n*-hexane fraction was performed using silica gel columns and SephadexLH-20. Structure elucidation of the isolated compounds was done by extensive 1D/2D NMR study and mass spectroscopy. Re-evaulation of the acaricidal activity of the major isolated compounds was performed to determine the active natural products.

Results: At 10% concentration; *n*-hexane, $CHCl_3$ and the total alcohol extracts showed 100.00±0.00%, 100.00±0.00% and 86.66±11.15% adulticidal activity respectively compared to 80±10.00% of the acaricide chemical deltamethrin, and 100.00±0.00%, 93.33±2.88% and 96.66±2.88% larvicidal activity respectively compared to 95.00±0.00% of deltamethrin. Only *n*-hexane fraction retained its 100.00±0.00% mortality when its concentration was reduced to 5%. The polar methanol fraction of *M. forsskaolii* didn't show any degree of adulticidal or larvicidal activity against the tested tick species. Phytochemical investigation of the *n*-hexane extract led to the isolation of tricontanol (**1**), β-amyrin (**2**), β-sitosterol (**3**), Sitost-5-ene-3β,7α-diol (**4**) and β-sitosterol-3-*O*-glucosid (**5**). The isolated compounds are being reported for the first time from *M. forsskaolii*. The major compounds isolated from the most active fraction (*n*-hexane fraction) were retested for their acaricidal activity. In a concentration of 25 mg/ml; β-sitosterol showed 86.6±5.57% and 91.6±2.88% adulticidal and larvicidal activity respectively while β-sitosterol-3-*O*-glucosid recorded 76.66±5.57% and 98.33±2.88% respectively.

Conclusion: The *n*-hexane extract of *M. forsskaolii*, β-sitosterol and β-sitosterol-3-*O*-glucoside may be potentially used as natural alternatives in the control of *R. annulatus* infesting cattle in Egypt. Further studies including field efficacy, persistence and stability need to be done to provide a pharmaceutical preparation for tick control.

Keywords: Mesembryanthemum forsskaolii, Rhipicephalus (Boophilus) annulatus, β-sitosterol-3-*O*-glucoside, Tick, Egypt

1. INTRODUCTION

Ticks are the most predominant ecto-parasite of cattle all over the world especially tropical and subtropical areas. In Egypt*;* *Rhipicephalus (Boophilus) annulatus* is the most common tick species infesting cattle. Due to blood feeding habits of tick and its activity as vector of diseases; tick represents threats for the livestock industry and make great loss of economy in Africa (FAO, 1984). Babesiosis and anaplasmosis are examples of tick-borne diseases that seriously reduced productivity of cattle and their crosses. Significant losses in livestock cattle had been attributed to *R. annulatus* infestation that

developed increasing resistance to the chemical acaricides (Valente et al., 2014). The developed resistance occurs due to extensive use of these chemicals and there is a bad need for new, more effective and safer acaricides. There are many factors that accelerate development of acaricide resistance such as incorrect dilution, application methods and extensive acaricide pressure (Aguilar-Tipacamu et al., 2011; Abbas et al., 2014) and many cases of synthetic pyrethroid resistance in *R. microplus*, a close species to *R. annulatus*, from different regions worldwide were recorded (Mendes et al., 2011; Rodriguez-Vivas et al., 2012; Sharma et al., 2012; Shyma et al., 2012). The secondary metabolites from plants especially volatile oil isolates are active acaricides and are good potential alternative for the control of ticks that are susceptible or resistant to commercial acaricides (Rosado-Aguilar et al., 2017). Botanical acaricides have the advantages of low or no toxicity to mammals compared to chemical acaricides, rapid degradation in the environment and less chances of development of resistance (Ravindran et al., 2012; Shyma et al.,2014). Several plant extracts belonging to family Aizoaceae including *Mesembryanthemum forsskaolii* have been tested for their antimicrobial activities against several human pathogens and the non-polar *n*-hexane and $CHCl_3$ extracts of *M. forsskaolii* have shown moderate antimicrobial activities (Mohammed et al. 2012). In our previous communication on polar fraction of *M. forsskaolii* (Moawad et al. 2016), flavonoids were isolated.Six crude extract of wild plants including *M. forsskaolii* and their fractions were tested for acaricidal activity against the larvae of *Hyalomma dromedarii* Koch (Camel tick) and hexane extracts revealed high mortality rates (Abdel-Shafy et al. 2006). In this report, we are presenting biologically-guided isolation of non-polar acaricidal natural products from *M. forsskaolii* herb against *Rhipicephalus (Boophilus) annulatus* infesting cattle in Egypt.

2. MATERIAL AND METHODS

2.1. General Experimental Procedures

Column chromatography was performed with Sephadex LH-20 and silica gel for column and for TLC (Pharmacia Biotech AB, Uppsala). Identification of the isolated compounds was performed using one and two dimensional NMR spectra recorded using a Bruker Avance III 400 MHz (Bruker AG, Switzerland) with AEON Nitrogen-Free Magnet and BBFO Smart Probe. Data acquisition and processing was performed using Topspin 3.1 Software. $CDCl_3$ was purchased from Cambridge Isotope Laboratories, Inc., (Andover, MA, USA) to be used as NMR solvents. The plates were visualized by spraying with *p*-anisaldehyde's reagent, followed by warming with heat gun. Deltamethrin 50µg/ml (Butox ® 50 Intervet International, The Netherlands) was used as chemical acaricides (positive control).

2.2. Plant Material

Collection and identification of *M. forsskaolii* herb was done as previously described (Mohammed et al. 2012; Moawad et al. 2016).

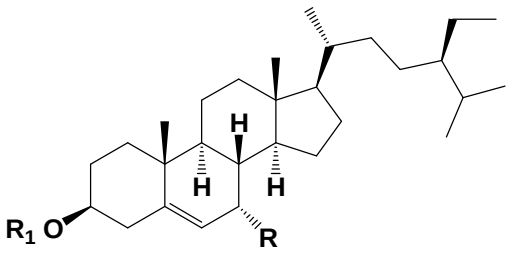
2.3. Preparation of extracts for the acaricidal activity and phytochemical study

The air-dried herb of M. forsskaolii (100 g) was successively extracted with *n*-hexane, $CHCl_3$ and MeOH. Another 50 g was extracted with EtOH 70 % to prepare the total alcohol extract. Four concentrations were prepared for the four different extracts (1.25, 2.5, 5 and 10%) in 50% DMSO-EtOH. The prepared concentrations were applied on adult ticks and unfed larvae.

The air-dried herb of *M. forsskaolii* (1 kg) was extracted and fractionated as described in our previous communication (Moawad et al. 2016). Ten grams of the *n*-hexane fraction (HX) were fractionated on VLC using silica gel (90 g, 15 x 3.7 cm) eluted with petroleum ether and increasing the polarity by adding 5% increments of ethyl acetate and collecting 100 ml fractions. TLC of the fractions was done and similar ones were combined to get five fractions. Fractions were screened by TLC using *n*-hexane–EtOAc (8:2) and sprayed with *p*-anisaldehyde's reagent followed by heating.

Fraction (HX-1, 1.250 g) eluted with petroleum ether-EtOAc (90:10-80:20) was chromatographed on silica column (30g, 28x2 cm) eluted in the same way and collecting 10 ml fractions to get 40 fractions. Fractions (10-14) were combined and chromatographed on silica column (10 g, 12x1 cm) eluted with pet. ether - EtOAc (99:1) isocratically to yield compound **1** (3 mg) and compound **2** (4 mg). Fractions (20-25) were combined and crystallized from MeOH to yield compound **3** (870 mg). Fraction (HX-2, 170 mg)

eluted with petroleum ether -EtOAc (65:35) was chromatographed on Sephadex LH-20 eluted with DCM - MeOH (9:1) and collecting one ml fractions. The fractions were TLC monitored using $CHCl_3$-MeOH (9.5:0.5) and sprayed with *p*-anisaldehyde's reagent to get a major blue spot. This fraction was rechromatographed on Sephadex LH- 20 using $CHCl_3$-MeOH (1:1) then silica gel and isocratically eluted with 1% $CHCl_3$-MeOH (99:1) to get compound **4** (20 mg).



	R	R_1
3	H	H
4	OH	H
5	H	β-D-glucose

Fig. 1: Structure of steroids isolated from *M. forsskaolii* herb.

Fraction (HX-3, 1g) eluted with EtOAc (100%) was chromatographed on silica gel for column (30g, 28x2 cm) eluted with $CHCl_3$ with 2% increments of MeOH. The fraction eluted with 5% MeOH in $CHCl_3$ (300 mg) is rechromatographed on Sephadex LH-20 eluted with DCM-MeOH (1:1) and collecting 1 ml fractions. The fractions were TLC monitored using $CHCl_3$-MeOH (9:1) and sprayed with *p*-anisaldehyde's reagent to get compound **5** (100 g). The isolated compounds are presented in Figure 1.

2.4. Ticks collection

Collection of fully engorged female *R. annulatus* ticks was performed according to Rodriguez-vivas et al. 2012) from naturally infested cattle making sure that none of which had received any tick treatments for at least 20 days. The ticks were kept in clean plastic bottles with lids containing small holes. The collected ticks were transported to the Parasitology Laboratory, Faculty of Veterinary Medicine, Beni-Suef University for identification and experimental application.

2. 5. Tick preparation and study design

The freshly collected females were separated, carefully washed, and then dried on absorbent paper. Engorged females weighing not less than 140 mg, with no signs of injury were used in the study. Ticks were divided into eight groups (10 ticks each) to evaluate the activity of *M. forsskaolii* herb and its major compounds. The groups were negative control group (50% DMSO-EtOH), total alcohol extract, *n*-hexane fraction, $CHCl_3$ fraction, MeOH fraction, β-sitosterol, β-sitosterol-3-*O*-glucosid and finally the positive control deltamethrin (50µg/ml). All experiments were done in triplicates in clean labeled petri dishes.

2. 6. Adult Immersion Test (AIT)

AIT was performed as described by Sharma et al., (2012) with little modifications. The ticks were weighed and divided into eight groups taking into consideration to have three replicates for each concentration. The different groups of ticks were immersed in 10 ml of each treatment by placing them directly into Petri

dish and stirred with glass rod. After 2 min, the liquid was poured off through a sieve and the ticks were transferred to a filter paper for drying and then kept separately in clean Petri dishes. Simultaneously, the ticks in the control group were treated with 50% DMSO-EtOH. The treated ticks were kept in biochemical oxygen demand (B.O.D.) incubator at a temperature of 27±2 ℃ and relative humidity of 80 ± 10 %. The mortality was recorded after 24 to 72 hours post treatment. Mortality % = (Number of dead tick in treated groups - Number of dead tick in control groups) × 100 / Total number of treated tick

2.7. Larval immersion Test (LIT)

The different concentrations of the products were screened against the unfed (15 days old) larvae. One ml of each solution was transferred to1.5 mL micro centrifuge tubes and then approximately 100 larvae were added to each one. Control solutions were prepared adding one ml 50% DMSO-EtOH. Immediately after addition of larvae, tubes were closed and shaken vigorously for 30 seconds and then gently for 10 min (Klafke et al., 2006). The tubes were then opened and the larvae transferred with a paint brush to a filter paper. After drying, paper was folded and closed with clips forming a packet. The packets were incubated at 27–28ºC and 80–90% relative humidity for 24 h then the mortality was determined.

2.8. Statistics

Statistical analysis of data was performed using Statistical Package for Social Science (SPSS for Windows (IBM), version 22, Chicago, USA) to determine if variables differed between treatments. ANOVA tests and subsequent Duncan's multiple range tests were applied to determine the differences between means. Results were presented as means±SD. Probability values of less than 0.05 ($P<0.05$) was considered significant.

3. RESULTS AND DISCUSSION

The use of natural products from botanical sources as acaricides has been the focus of research in many countries, principally to withstand the noticeable increasing frequency of acaricides resistant tick strains. The use of botanicals in veterinary medicine is sustainable and ecologically sound (Habeeb, 2010). Low toxicity of the purified chemicals of natural source, cheaper costs, and availability are attracting factors that promote the use of natural products in medicine (Yeh et al., 2003). The n-hexane extracts of *M. forsskaolii* have a previously reported acaricidal activity against camel tick larvae *(Hyalomma dromedarii* Koch) (Abdel-Shafy et al. 2006). We are presenting a biologically-guided isolation of acaricidal natural products from *M. forsskaolii* herb against the most common tick species infesting cattle (R. annulatus). The total alcohol extract plus different fractions of different polarities were screened *in vitro* to detect the most active extract, Table 1, Figure 2. The *n*-hexane extract was found to be the most active at concentration of 5-10% in 50% DMSO-EtOH. Phytochemical investigation of the *n*-hexane extract led to the isolation of five compounds; tricontanol (Amin et al., 2016), β-amyrin (Mahato and Kundu 1994) (Vázquez et al., 2011), β-sitosterol (Amin et al., 2016; Zhang et al., 2005), Sitost-5-ene-3β,7α-diol (Tasyriq et al., 2012) and β-sitosterol-3-*O*-glucosid (Amin et al., 2016); Figure 1. The structures of these compounds were elucidated by extensive 1D and 2D NMR spectroscopy and confirmed by comparison of their ^{13}C NMR data with those reported in the literature. The two major compounds β-sitosterol and its 3-*O*-glucosid- were tested for adulticidal and larvicidal activity and both compounds showed potent activity and may be responsible for the acaricidal activity of the *n*-hexane extract *of M. forsskaolii* herb with or without other minor compounds.

With adult immersion test, *M. forsskaolii* total alcohol extract showed variable degrees of adulticidal and larvicidal activities against *R. annulatus* ticks post 24 hours. It showed significant mortality percent ($P\leq0.05$) of 20.00±.00%, 66.66±11.15%, and 86.66±11.15% at concentration of 2.5%, 5% and 10% in a comparison with control non-treated group (50% DMSO-EtOH). Dead tick showed black coloration of the cuticle and complete immobility. Furthermore, it recorded significant larvicidal activity ($P\leq0.05$) of 20.00±.00%, 50.00±.00 at 1.25 and 2.5% concentrations respectively, and 96.66±2.88% at both 5 and 10% concentrations with larval immersion test. So the successive fractions of increasing polarities were prepared and the acaricidal activity was repeated for these fractions.

The *n*-hexane extract showed significant degrees of adulticidal and larvicidal activity against *R. annulatus* ticks post 24 hours. It showed significant mortality percent ($P \leq 0.05$) of 76.66±5.57%, and 93.33±11.15% at concentration of 1.25%, 2.5% respectively, and 100.00±.00% mortality at both 5% and 10% concentrations. Furthermore, it recorded significant larvicidal activity ($P \leq 0.05$) of 20.00±.00%, 70±.00% at concentrations 1.25% and 2.5% respectively, and 100±.00% mortality at both 5% and 10% concentrations which were significantly higher than deltamethrin.

The $CHCl_3$ fraction showed variable degrees of adulticidal and larvicidal activity against *R. annulatus* ticks post 24 hours. It showed significant mortality percent ($P \leq 0.05$) of 26.66±5.57%, 26.66±5.57%, and 100.00±.00 % at concentration of 2.5%, 5% and 10%. Furthermore, it recorded significant larvicidal activity ($P \leq 0.05$) of 10.00±.00%, 20.00±.00% at 2.5% and 5% concentrations respectively, and 93.33±2.88% at 10% concentration. The polar MeOH fraction didn't show any degree of adulticidal or larvicidal activity against *R. annulatus* ticks.

Among the tested extracts, *n*-hexane extract was the only extract that retained its 100.00% mortality at 5% concentration thus considered to be the most active. Chromatographic isolation of the non-polar constituents of the *n*-hexane extract afforded two major pure compounds that were identified as β-sitosterol and β-sitosterol-3-*O*-glucosid. In a concentration of 25 mg/ml; β-sitosterol showed 86.66±5.57% and 91.66±2.88% adulticidal and larvicidal activity respectively. While β-sitosterol-3-*O*-glucosid recorded 76.66±5.57% and 98.33±2.88%.

Table 1: Mean values of adult and larval mortality of different extracts of *M. forsskaolii* herb and major compounds against *Rhipicephalus (B.) annulatus* ticks.

Treatment	Concentration (%)	Mortality (adult) %[1]	Dead larvae %[2]
Control 50% DMSO- ETOH	----	0.00±0.00^{a}	0.00±0.00^{a}
Total alcohol extract	10%	86.66±11.15^{d}	96.66±2.88^{d}
	5%	66.66±11.15^{c}	96.66±2.88^{d}
	2.5%	20.00±.00^{b}	50.00±.00^{c}
	1.25%	13.33±11.15^{a}	20.00±.00^{b}
n-hexane fraction	10%	100.00±.00^{e}	100.00±.00^{e}
	5%	100.00±.00^{e}	100.00±.00^{e}
	2.5%	93.33±11.15^{e}	70.00±.00^{c}
	1.25%	76.66±5.57^{d}	20.00±.00^{b}
DCM fraction	10%	100.00±.00^{e}	93.33±2.88^{d}
	5%	26.66±5.57^{b}	20.00±.00^{c}
	2.5%	26.66±5.57^{b}	10.00±.00^{b}
	1.25%	6.66±5.57^{a}	5.00±.00^{a}
MeOH fraction	10%	0.00±.00^{a}	0.00±.00^{a}
	5%	0.00±.00^{a}	0.00±.00^{a}
	2.5%	0.00±.00^{a}	0.00±.00^{a}
	1.25%	0.00±.00^{a}	0.00±.00^{a}
β-sitosterol	25 mg/ml	86.66±5.57^{d}	91.66±2.88^{d}
β-sitosterol-3-*O*-glucosid	25 mg/ml	76.66±5.57^{d}	98.33±2.88^{e}
Deltamethrin	50µg/ml	80.00±10.00^{d}	95.00±0.00^{d}

[1] values are presented in Mean ± SD post 24-72 hours post treatment
[2] values are presented in Mean ± SD post 24 hours post treatment
Superscripts of same letters in the same column are non-significant.
Superscripts of different letters in the same column are significant at $P \leq 0.05$.

Deltamethrin 50µg/ml showed 80.00±10.00% and 95.00±0.00% adulticidal and larvicidal activity. There was a significant increase ($P \leq 0.05$) in adulticidal activity of 10% total alcohol extract and 5-10% *n*-hexane extract compared to deltamethrin. Meanwhile there was a significant increase ($P \leq 0.05$) in larvicidal activity of 5-10% *n*-hexane extract and β-sitosterol-3-*O*-glucosid in comparison with deltamethrin. TLC

screening of the $CHCl_3$ and *n*-hexane fractions revealed the presence of trace amounts of β-sitosterol-3-*O*-glucosid in the $CHCl_3$ fraction which may attribute its higher activity at 10% concentration, while the polar fraction contains flavonoids (Moawad et al. 2016) and its negative results indicates that flavonoids are secondary metabolites of no acaricidal activity.

Tick, like many insects, are unable to synthesis cholesterol de novo. Large amounts of cholesterol and its esters are present in the cuticular lipids of adult female cattle ticks and their eggs (Cherry, 1976). So, engorged tick absorb cholesterol from blood meals and transfer it to their eggs. β-sitosterol is ethyl derivative of cholesterol and this structure similarity allows the incorporation of β-sitosterol and related phytosterols in many pharmaceutical food supplements for cholesterol lowering. The proposed mechanism of cholesterol lowering involve interference with intestinal solubility, interaction with digestive enzymes, protein–mediated absorption and gene regulation (Cowles et al., 2002). So, the phytosterols present in *M. forsskaolii* may interfere with cholesterol absorption in tick by the same mechanism. The reported phytosterols are present in almost all plants and consumed by human beings in fruits and vegetables every day and no toxicity was reported so far. There is a topical preparation for treatment of burns (Ang et al.,2000) contains β-sitosterol as main component (MEBO® ointment, Gulf Pharmaceutical Industries, URES). Since the systemic and topical use of β-sitosterol is already known for humans, so its use as acaricides will be mostly safe for cattle.

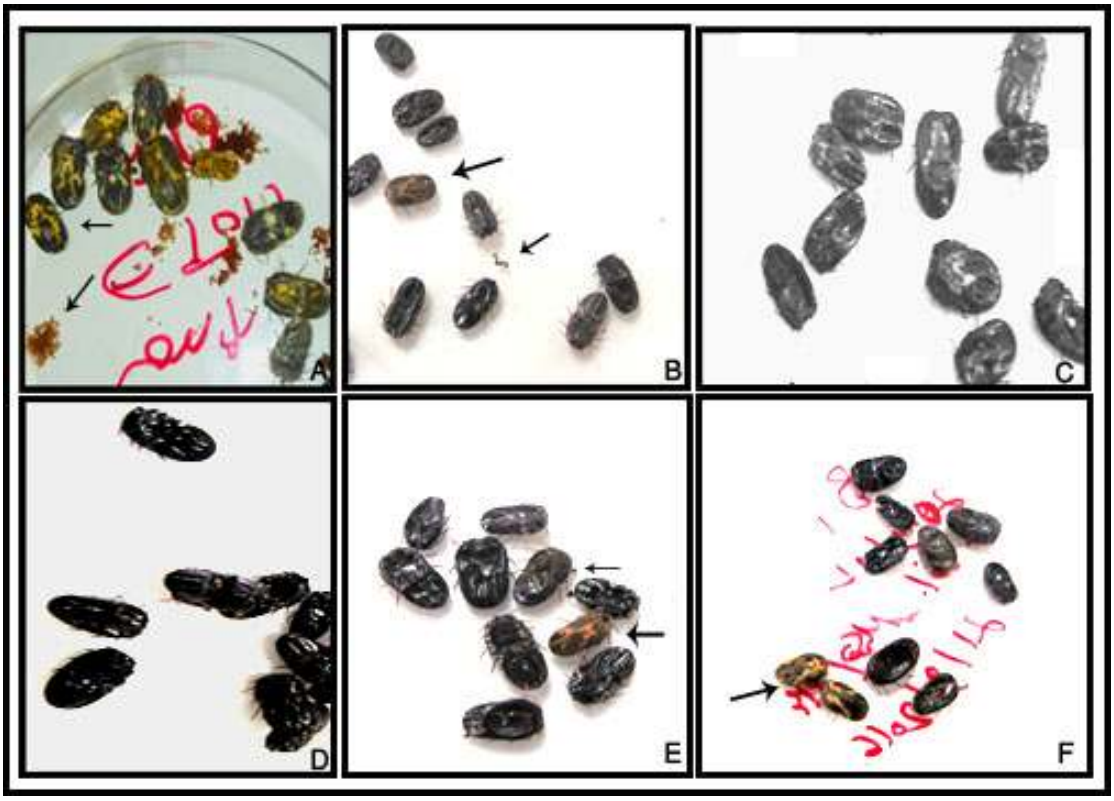


Fig 2: Acaricidal activity of different extracts of *M. forsskaolii* herb and major compounds 72 hours post treatment: negative control group (A), deltamethrin (B), *n*-hexane extract (C), $CHCl_3$ fraction (D), β-sitosterol (E), β-sitosterol-3-*O*-glucosid (F). Arrows refer to deposited egg mass or life tick (yellow colored cuticle). Dead tick showed black coloration of the cuticle and complete immobility.

Structure elucidation of compound 4

The ^{13}C NMR spectrum of compound (**4**) indicated the presence of 29 carbon signals including one double bond; consisting of an olefinic quaternary carbon signal at δc 146.3 (C-5) and one olefinic methine signal at δc 123.9 (C-6), two oxygenated methine carbon signals [δc 71.3 (C-3) and 65.4 (C-7)], and six

methyl carbon signals [δc 19.8 (C-27), 19.0 (C-26),18.8 (C-21), 18.2 (C-19), 12.0 (C- 29) and 11.6 (C-18)]. This information led us to conclude that compound (**4**) was a stigmastane-type steroid with two hydroxyl groups and one double bond. It gives blue color with *p*-anisaldehyde's reagent which is a characteristic reaction of 7-hydroxy-Δ^5-sterols (Deshmane and Dev 1970). Determination of the final structure of compound (**4**) was accomplished by 2D NMR experiments, gradient heteronuclear single quantum correlation (gHSQC), and gradient heteronuclear multiple bonding connectivity (gHMBC) and was consistent with reported data for Sitost-5-ene-3β,7α-diol which is a rare sterol (Tasyriq et al. 2012).

4. CONCLUSION

Bioactive plant natural products such as extracts, fractions, and isolated compounds that may constitute prototypes for the exploitation of acaricides against *R. annulatus* are highly promising alternatives. Steroids in *M. forsskaolii* Hochst. Ex. Boiss which are located in the n-hexane extract are responsible for the acaricidal activity of the test plant while flavonoids located in the polar fraction have no effect. The *n*-hexane extract of *M. forsskaolii*, β-sitosterol and β-sitosterol-3-*O*-glucoside may be potentially used as safer alternatives in the control of tick populations. Further studies including field efficacy, persistence, stability and toxicology need to be done to provide a pharmaceutical preparation for tick control.

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CONSENT

not applicable

ETHICAL APPROVAL

not applicable

REFERENCES

Abbas RZ, Zaman MA, Colwell DD, Gilleard J, Iqbal Z. Acaricide resistance in cattle ticks and approaches to its management: the state of play. Vet Parasitol 2014; 203: 6–20.

Abdel-Shafy S, Soliman M, Habeeb S. *In vitro* acaricidal effect of some crude extracts and essential oils of wild plants against certain tick species. Acarologia 2006; XLVII:33–42.

Aguilar-Tipacamu G, Rosario-Cruz R, Miller RJ, Guerrero FD, Rodriguez-Vivas, RI, Garcia-Vazquez Z. Phenotype changes inherited by crossing pyrethroid susceptible and resistant genotypes from the cattle tick *Riphicephalus (Boophilus) microplus*. Exp Appl Acarol. 2011; 54:301-311.

Amin E, Moawad A, Hassan H. Biologically-guided isolation of leishmanicidal secondary metabolites from *Euphorbia peplus* L. Saudi Pharm J,2016;25(2): 236-240. doi: 10.1016/j.jsps.2016.06.003

Ang ES, Lee ST, Gan CS, See PI, Chan YH. The role of alternative therapy in the management of partial-thickness burns of the face; experience with use of moist exposed burn ointment (MEBO) compared with silver sulphadiazine. Ann Acad Med Singapore, 2000; 29: 7-10.

Cherry LM. Utilization of cholesterol by the cattle tick *Boophilus microplus*: Cholesterol economy in the engorged female adult. Insect Biochem, 1976; 6(6): 587-594. https://doi.org/10.1016/0020-1790(76)90005-6

Cowles RL, Lee J, Gallaher DD, Stuefer-Powell CL, Carr TP. Dietary stearic acid alters gallbladder bile acid composition in hamsters fed cereal-based diets. J Nutr, 2002;132: 3119-3122.

Deshmane SS, Dev S. Higher Isoprenoids-II triterpenoids and steroids of *Scaccharum officinarum.* Tetrahedron 1970; 27:1109–1118.

FAO. Ticks and tickborne disease control: a practical field manual. Vol. 1. Food and Agriculture Organization of the United Nations. Rome. 1984; pp. 299.

Habeeb SM. Ethno-veterinary and medical knowledge of crude plant extracts and its method of application (traditional and modern) for tick control. World Appl Sci J. 2010;11(9):1047-1054.

Klafke GM, Sabatini GA, Albuquerque TA, Martins J R, Miller RJ, Schumaker TS. Larval immersion tests with ivermectin in populations of the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) from State of Sao Paulo, Brazil. Vet Parasitol 2006; 142:386–390. doi: 10.1016/j.vetpar.2006.07.001

Mahato SB, Kundu AP. ^{13}C NMR Spectra of pentacyclic triterpenoids—a compilation and some salient features. Phytochemistry 1994; 37:1517–1575. doi: 10.1016/S0031-9422(00)89569-2

Mendes MC, Lima CKP, Nogueira AHC, Yoshihara E, Chiebao DP, Gabriel FHL, Ueno THE, Namindome A, Klafke GM. Resistance to cypermethrin, deltamethrin and chlorpyriphos in populations of *Rhipicephalus* (*Boophilus) microplus* (Acari: Ixodidae) from small farms of the state of SãoPaulo, Brazil Vet Parasitol 2011; 178: 383–388

Moawad A, Amin E, Mohammed R. Flavonoids and 2D-DOSY NMR of flavonol mixture from *Mesembryanthemum forsskaolii* (Aizoaceae). European J Med Plants 2016; 16: 1–8. doi: 10.9734/EJMP/2016/27794

Mohammed R, Abo-youssef A, El-Hawary SS. Biological investigation of some wild Aizoaceae and Chenopediaceae species growing in Egypt. J Nat Prod, INDIA 2012; 5:193-206

Ravindran R, Juliet S, Sunil AR, Ajith Kumar KG, Nair SN, Amithamol KK, Bandyopadhyay A, Rawat AK, Ghosh S. Acaricidal activity of *Cassia alata* against *Rhipicephalus (Boophilus) annulatus*. Exp Appl Acarol 2012; 56:69–74. doi: 10.1007/s10493-011-9489-6

Rodriguez-vivas RI, Hodgkinson JE, Rosado-aguilar JA, Villegas-perez SL. The prevalence of pyrethroid resistance phenotype and genotype in *Rhipicephalus (Boophilus) microplus* in Yucatan, Mexico. Vet Parasitol 2012; 184: 221–229. doi: 10.1016/j.vetpar.2011.09.017

Rosado-Aguilar JA, Arjona-Cambranes K, Torres-Acosta JFJ, Rodríguez-Vivas R, Bolio-González ME, Ortega-Pacheco A, Alzina-López A, Gutiérrez-Ruiz EJ, Gutiérrez-Blanco E, Aguilar-Caballero AJ. Plant products and secondary metabolites with acaricide activity against ticks. Vet Parasitol 2017; In Press, Accepted Manuscript

Sharma AK, Kumar R, Kumar S, Nagar G, Singh NK, Rawat SS, Dhakad ML, Rawat AK, Ray DD, Ghosh S. Deltamethrin and cypermethrin resistance status of *Rhipicephalus (Boophilus) microplus* collected from six agro-climatic regions of India. Vet Parasitol 2012; 88: 337–345. doi: 10.1016/j.vetpar.2012.03.050.

Shyma KP, Kumar S, Sharma AK, Ray DD, and Ghosh S. Acaricide resistance status in Indian isolates of Hyalomma anatolicum anatolicum. Exp Appl Acarol 2012; 58(4): 471-81.

Shyma KP, Gupta JP, Ghosh S, Patel KK and Veer Singh. Acaricidal effect of herbal extracts against cattle tick *Rhipicephalus (Boophilus) microplus* using in vitro studies. Parasitol Res 2014;113(5): 1919-26.

293 Tasyriq M, Najmuldeen I A, In LL, Mohamad K, Awang K, Hasima N. 7α-Hydroxy-β-Sitosterol from
294 *Chisocheton tomentosus* induces apoptosis via dysregulation of cellular Bax/Bcl-2 ratio and cell cycle
295 arrest by downregulating ERK1/2 activation. Evid Based Complement Alternat Med 2012; 2012: 765316.
296 doi: 10.1155/2012/765316

297 Valente PP, Amorim JM, Castilho RO, Leite RC, Ribeiro MF. In vitro acaricidal efficacy of plant extracts
298 from Brazilian flora and isolated substances against *Rhipicephalus microplus* (Acari: Ixodidae). Parasitol
299 Res. 2014; 113:417–423. doi: 10.1007/s00436-013-3670-2

300 Vázquez LH, Palazon J, Navarro-ocaña A. The Pentacyclic Triterpenes α, β-amyrins: A Review of
301 Sources and Biological Activities. In: Rao V (ed) Phytochemicals – A Global Perspective of Their Role in
302 Nutrition and Health. In Tech 2011; pp 478–502.

303 Yeh GY, Eisenberg DM, Kaptchuk TJ. Systematic review of herbs and dietary supplements used in
304 glycemic control in diabetes. Diabetes Care, 2003; 26: 1277-94.

305 Zhang X, Geoffroy P, Miesch M, Julien-David D, Raul F, Aoudé-Werner D, Marchioni E. Gram-scale
306 chromatographic purification of beta-sitosterol. Synthesis and characterization of beta-sitosterol oxides.
307 Steroids 2005; 70:886–95. doi: 10.1016/j.steroids.2005.06.003

308

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