

Original Research Article

β -sitosterol and its 3-O-glucosid as novel acaricides against *Rhipicephalus (B.) annulatus* ticks

ABSTRACT

Aims: to find new and effective natural products to control *Rhipicephalus (Boophilus) annulatus* infesting cattle in Egypt

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, CHCl_3 , and MeOH). 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.

Results: At 10% concentration; *n*-hexane, CHCl_3 and the total alcohol extracts showed $100.00\pm 0.00\%$, $100.00\pm 0.00\%$ and $86.66\pm 11.15\%$ adulticidal activity respectively compared to $80\pm 10.00\%$ of the acaricide chemical deltamethrin, and $100.00\pm 0.00\%$, $93.33\pm 2.88\%$ and $96.66\pm 2.88\%$ larvicidal activity respectively compared to $95.00\pm 0.00\%$ of deltamethrin. Only *n*-hexane fraction retained its $100.00\pm 0.00\%$ 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- $3\beta,7\alpha$ -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 $86.6\pm 5.57\%$ and $91.6\pm 2.88\%$ adulticidal and larvicidal activity respectively while β -sitosterol-3-*O*-glucosid recorded $76.66\pm 5.57\%$ and $98.33\pm 2.88\%$.

Conclusion: the *n*-hexane extract of *M. forsskaolii*, β -sitosterol and β -sitosterol-3-*O*-glucoside should be incorporated in pharmaceutical preparations for tick control as potent and safe alternatives.

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

1. INTRODUCTION

Ticks are the most predominant ecto-parasite of cattle all over the world especially tropical and subtropical area. In Egypt; *R. (B.) 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. Chemical acaricides are usually used for tick control but extensive use of these chemicals developed resistance and there is a bad need for new, more effective and saver 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 India, Mexico, Brazil, North America and Australia were recorded (Nolan et al., 1989; Miller et al., 2007; Mendes et al., 2011; Rodriguez-Vivas et al., 2012; Sharma et al., 2012). Significant losses in livestock cattle had been attributed to *R. annulatus* infestation that developed increasing resistance to the primary acaricides (Valente et al. 2014). The active secondary metabolites produced by plants showing acaricidal and insecticidal properties are a promising alternative for the control of arthropods infesting animals. Botanical

acaricides have the advantages of low or no toxicity to mammals compared to chemical acaricides, also rapid degradation in the environment and less chances of development of resistance (Ravindran et al. 2012). The use of therapeutic plants in veterinary medicine has developed increasingly during the last ten years. Six crude wild plant extracts 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). Several plant extracts belonging to Aizoaceae family including *M. 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. In this report, we are presenting biologically-guided isolation of non-polar acaricidal natural products from *M. forsskaolii* herb.

2. MATERIAL AND METHODS

2.1. General Experimental Procedures

One and two dimensional NMR spectra were 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 and Pyridine- d_5 were purchased from Cambridge Isotope Laboratories, Inc., (Andover, MA, USA) to be used as NMR solvents. Column chromatography was performed with Sephadex LH-20 and silica gel for column and for TLC (Pharmacia Biotech AB, Uppsala). The plates were visualized by spraying with *p*-anisaldehyde's reagent, followed by warming with heat gun. Deltamethrin was used as chemical acaricide (Intervet International, The Netherlands).

2.2. Plant Material

Collection and identification of *M. forsskaolii* herb of 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 of the four different extracts were prepared (1.25, 2.5, 5 and 10%) in 50% DMSO-EtOH. The ready 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 pet. 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).

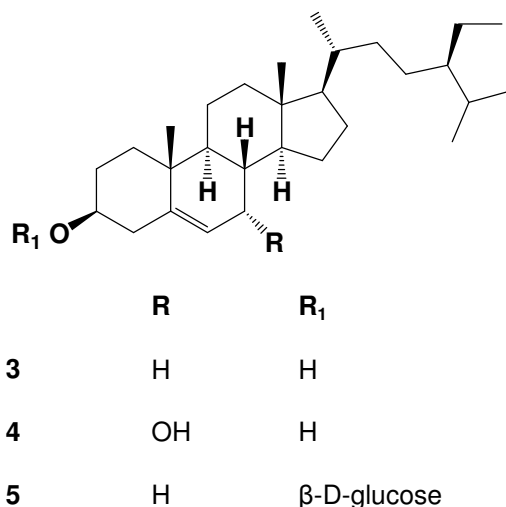


Fig. 1: Structure of steroids isolated from *M. forsskaolii* herb.

Fraction (HX-3, 1g) eluted with EtOAc (100%) was chromatographed on silica gel for column (30g, 28x2 cm) eluted with CHCl₃ with 2% increments of MeOH. The fraction eluted with 5% MeOH in CHCl₃ (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 CHCl₃-MeOH (9:1) and sprayed with *p*-anisaldehyde's reagent to get compound **5** (100 g). 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, total alcohol extract, *n*-hexane fraction, CHCl₃ fraction, MeOH fraction, β-sitosterol, β-sitosterol-3-*O*-glucosid and finally the positive control deltamethrin. 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 between the eight groups taking into consideration to have three replicates for each concentration. The different groups of ticks were immersed in 10 ml of the different concentrations by placing them directly into Petri dish and stirred with glass rod. After 2 min, the acaricide 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 °C

and relative humidity of 80 ± 10 %. The mortality was recorded after 24 to 72 hours of treatment (PT). The ticks which did not oviposit even after 7 days will be considered as dead (Sharma et al. 2012).

Mortality % = (Number of dead tick in treated groups - Number of dead tick in control groups) \times 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 to 1.5 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 27–28°C 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 \pm SD. Probability values of less than 0.05 ($P < 0.05$) was considered significant.

3. RESULTS AND DISCUSSION

Biologically-guided isolation of acaricidal natural products from natural source was done using *M. forsskaolii* herb. The total alcohol extract plus different fractions of different polarities were screened 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 (Zhang et al. 2005), Sitost-5-ene-3 β ,7 α -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 ($P \leq 0.05$) of 20.00 \pm 0.00%, 66.66 \pm 11.15%, and 86.66 \pm 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 ($P \leq 0.05$) of 20.00 \pm 0.00%, 50.00 \pm 0.00 at 1.25 and 2.5% concentrations respectively, and 96.66 \pm 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 \pm 5.57%, and 93.33 \pm 11.15% at concentration of 1.25%, 2.5% respectively, and 100.00 \pm 0.00% mortality at both 5% and 10% concentrations. Furthermore, it recorded significant larvicidal activity ($P \leq 0.05$) of 20.00 \pm 0.00%, 70 \pm 0.00% at concentrations 1.25% and 2.5% respectively, and 100 \pm 0.00% 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 \pm 5.57%, 26.66 \pm 5.57%, and

100.00±0.00 % at concentration of 2.5%, 5% and 10%. Furthermore, it recorded significant larvicidal activity ($P \leq 0.05$) of 10.00±0.00%, 20.00±0.00% 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%.

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.

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±0.00 ^b	50.00±0.00 ^c
	1.25%	13.33±11.15 ^a	20.00±0.00 ^b
<i>n</i> -hexane fraction	10%	100.00±0.00 ^e	100.00±0.00 ^e
	5%	100.00±0.00 ^e	100.00±0.00 ^e
	2.5%	93.33±11.15 ^e	70.00±0.00 ^c
	1.25%	76.66±5.57 ^d	20.00±0.00 ^b
DCM fraction	10%	100.00±0.00 ^e	93.33±2.88 ^d
	5%	26.66±5.57 ^b	20.00±0.00 ^c
	2.5%	26.66±5.57 ^b	10.00±0.00 ^b
	1.25%	6.66±5.57 ^a	5.00±0.00 ^a
MeOH fraction	10%	0.00±0.00 ^a	0.00±0.00 ^a
	5%	0.00±0.00 ^a	0.00±0.00 ^a
	2.5%	0.00±0.00 ^a	0.00±0.00 ^a
	1.25%	0.00±0.00 ^a	0.00±0.00 ^a
β -sitosterol	25 mg/ml	86.66±5.57 ^d	91.66±2.88 ^d
β -sitosterol-3- <i>O</i> -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 $P \leq 0.05$.

^b is significant with ^a at $P \leq 0.05$

^c is significant with ^{a, b} at $P \leq 0.05$.

^d is significant with ^{a, b, c} at $P \leq 0.05$

^e is significant with ^{a, b, c, d} at $P \leq 0.05$.

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 δ_c 146.3 (C-5) and one olefinic methine signal at δ_c 123.9 (C-6), two oxygenated methine carbon signals [δ_c 71.3 (C-3) and 65.4 (C-7)], and six methyl carbon signals [δ_c 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-3 β ,7 α -diol which is a rare sterol (Tasyriq et al. 2012).

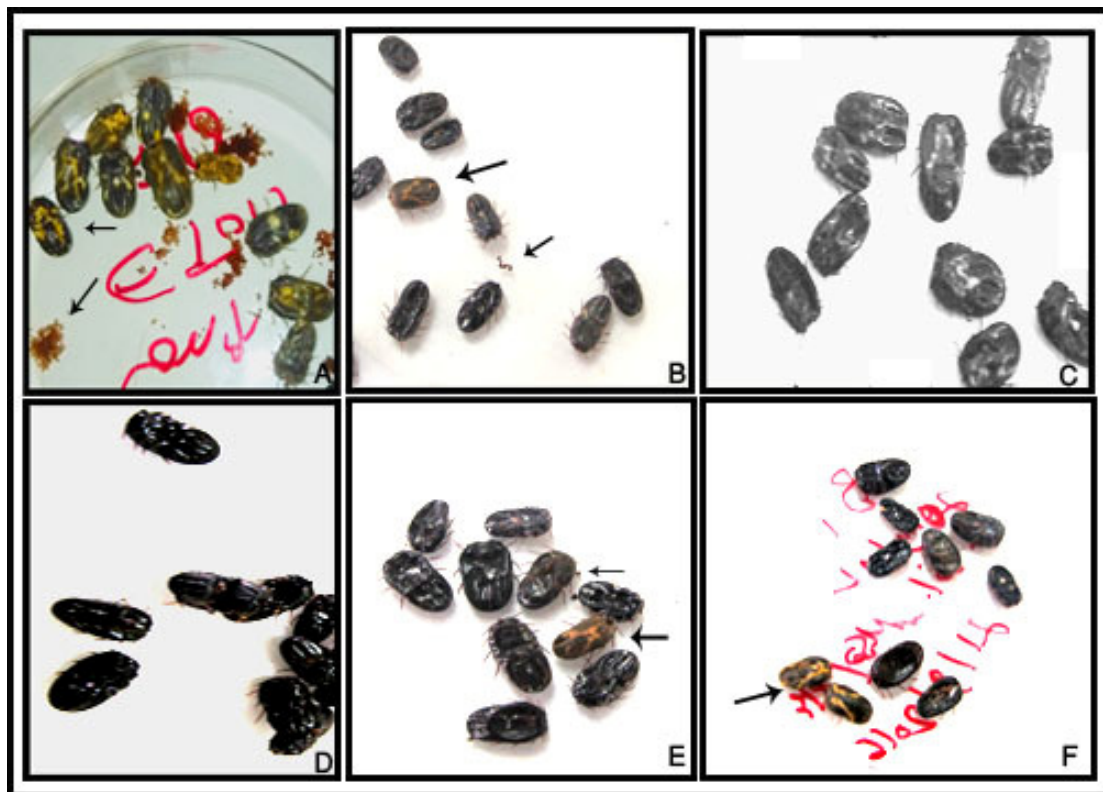


Fig 2: Acaricidal activity of different extracts of *M. forsskaolii* herb and major compounds 72 hours post treatment: negative control group (A), deltamethrin (B), *n*-hexane extract (C), CHCl_3 fraction (D), β -sitosterol (E), β -sitosterol-3-*O*-glucosid (F). Arrow refers to deposited egg mass or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

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.

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 *Rhipicephalus (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; in press, corrected proof. doi: 10.1016/j.jsps.2016.06.003

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.

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. ¹³C NMR Spectra of pentacyclic triterpenoids—a compilation and some salient features. *Phytochemistry* 1994; 37:1517–1575. doi: 10.1016/S0031-9422(00)89569-2

Miller RJ, Davey RB, George JE. First report of permethrin-resistant *Boophilus microplus (Acari: Ixodidae)* collected within the United States. *J Med Entomol* 2007; 44, 308–315.

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

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

Mohammed R, Abo-youssef A, El-Hawary SS. Biological investigation of some wild *Aizoaceae* and *Chenopodiaceae* species growing in Egypt. *Journal of Natural Products, INDIA* 2012; 5:193-206

Nolan J, Wilson JT, Green PE, Bird PE. Synthetic pyrethroid resistance in field samples in the cattle tick (*Boophilus microplus*). *Aust Vet J* 1989; 66, 179- 182.

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

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;188:337–345. doi: 10.1016/j.vetpar.2012.03.050

Tasyriq M, Najmuldeen I A, In LL, Mohamad K, Awang K, Hasima N. 7 α -Hydroxy- β -Sitosterol from *Chisocheton tomentosus* Induces Apoptosis via Dysregulation of Cellular Bax/Bcl-2 Ratio and Cell Cycle Arrest by Downregulating ERK1/2 Activation. *Evid Based Complement Alternat Med* 2012; 2012: 765316. doi: 10.1155/2012/765316

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

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

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