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Evaluation of acaricidal effect of terpinolene and γ-terpinene on *Hyalomma anatolicum* and *in silico* screening of herbs

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ABSTRACT

Importance: Terpenoids of a plant origin are a class of alternatives to chemical acaricides for tick control. Despite this, few studies have evaluated the efficacy of terpenoid-containing herbal extracts or individual terpenoids in *Hyalomma anatolicum*.
Objective: This study evaluated the killing efficacy of terpinolene and γ-terpinene against *H. anatolicum* to identify herbs rich in these compounds as potential candidates for future materials in the control of *H. anatolicum*.
Methods: Terpinolene and γ-terpinene were subjected to adult tick immersion experiments to evaluate their killing effect on *H. anatolicum*, following their identification post-collection. The herbs were screened for the two terpenoids using the HERB database, and the resulting data were categorized and analyzed.
Results: The acaricidal effect of terpinolene and γ-terpinene against *H. anatolicum* showed a clear dose-response relationship, with effective doses LC_{50} of 6.60 mg/mL and 4.86 mg/mL, respectively. *In silico* analysis revealed the presence of terpinolene and γ-terpinene in 52 and 36 herbs, respectively, with 16 herbs containing both compounds. These herbs could be grouped into 35 plant families. In particular, Lamiaceae and Apiaceae emerged as the most represented families, each accounting for 9.23% of the total herbs identified, followed by Zingiberaceae (7.69%) and Asteraceae and Rutaceae (6.15% each).
Conclusions and Relevance: Terpinolene and γ-terpinolene have good killing effects against *H. anatolicum*. Herbs rich in the two terpenoids are promising candidates as plant-derived materials for managing *H. anatolicum*.

Keywords: Tick identification; *Hyalomma anatolicum*; terpenes; herbs

INTRODUCTION

Ticks are medically important blood-feeding ectoparasites that can infest humans and animals. Ticks are the primary vectors of several pathogens, including viruses, bacteria, helminths, and protozoa. The global economic losses caused by tick infestations amount to $22–30 billion annually [1]. The Ixodidae family, which includes *Hyalomma anatolium*, has

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Conflict of Interest
The authors declare no conflicts of interest.

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a broad distribution across India, Nepal, Pakistan, Central Asia, North Africa, and Eastern Europe [2]. *H. anatolium* is distributed in Xinjiang and Gansu provinces of China [3]. The species has been reported to transmit several pathogens, such as *Theileria lestoquardi*, *T. equi*, *Babesia caballi*, and the Crimean–Congo hemorrhagic fever virus [4].

Chemical acaricides are the primary method used to manage ticks in animal farms. Organophosphates and pyrethroids are the most preferred acaricides for controlling tick infestations in large animals [5]. On the other hand, chemical acaricides are plagued by issues such as environmental contamination and the development of resistance in ticks. Recently, the interest in botanical acaricides has increased because of their minimal toxicity and the lack of drug resistance to ticks [6]. Plants contain a wide range of compounds such as alkaloids, steroids, and terpenoids [7]. These compounds, derived primarily from extracts, essential oils, and plant-derived natural compounds, are potential botanical acaricides and repellents [8-10]. Natural compounds possess acaricidal or repellent properties or both against several ticks [6]. Terpenoids are the major natural products used for tick control among this group of natural substances. Previous research has established that terpinolene, a monoterpene compound, showed considerably higher efficacy in repelling *Dermacentor variabilis*, which is comparable to DEET [11]. Carvacrol and thymol have acaricidal efficacy against *Rhipicephalus microplus* and *R. sanguineus* [12,13], while both have larvicidal and repellent properties against *Ixodes Ricinus* [14]. γ-terpinene has significant acaricidal activity against *H. marginatum* [15].

Thus far, plant essential oils or extracts have been used as acaricides or repellents for *Hyalomma*, namely *H. dromedarii* [16,17], *H. anatolicum* [18], and *H. scupense* [19]. In China, several kinds of Chinese medicinal herbs and their compounds have been used to manage ticks [20-22]. Considering the lack of studies on managing *H. anatolicum*, this study evaluated the killing effects of terpinolene and γ-terpinene against *H. anatolicum*. In addition, Chinese herbs containing these compounds were screened from the HERB database [23], which includes information on the relationship between Chinese herbs and their corresponding ingredients. This study provides new insights into screening Chinese medicinal herbs for managing *H. anatolicum*.

METHODS

Chemical reagents

The terpinolene, γ-terpinene, and amitraz were purchased through Shanghai Yi'en Chemical Technology Co., Ltd., Beijing J&K Scientific Technology Co., Ltd. and Fengcheng Animal Drugs Co., Ltd., respectively. Amitraz was used as the positive control.

Collection and identification of ticks

In the middle of May, ~1,800 ticks in different growth stages (unfed, half-engorged, and engorged adult ticks) were collected from the body surface of cattle in the town of Aydin Lake. The morphological identification of unfed adult ticks was performed by referring to the method described by Perveen et al. [24]. *H. anatolicum* morphologically identified as unfed adult ticks were cultured under laboratory conditions (27 ± 2°C, relative humidity 80 ± 5%, length: width: (16:8) with reference to the method of Agwunobi et al. [25]. Similarly, these ticks were used for subsequent immersion experiments. Semi- or fully engorged female ticks were placed under the same laboratory conditions as the material for passaging cultures.

Ticks genomic DNA extraction and polymerase chain reaction

Eight randomly selected *H. anatolicum* were cleaned with PBS solution and underwent molecular biology identification. Tick genomic DNA extraction was performed using a commercial tissue genomic DNA extraction kit provided by TIANGEN BIOTECH(BEIJING) Co., LTD., and the extracted genomic DNA was placed at −20°C for subsequent experiments. Eight representative samples of *H. anatolicum* were amplified by ixodid tick-specific 16S rDNA primers: F: 5'-CTGCTCAATGAATATTTAAATTGC-3', R: -CGGTCTAAACTCAGATCATGTAGG-3' [26]. Sangon Biotech (Shanghai) Co., Ltd. finished the sequencing at the end.

Molecular identification of ticks

Based on the BLAST results of the representative sequences in this study, *H. anatolicum* from China, Egypt, India, Iraq, Kazakhstan, Pakistan, Spain, Tajikistan, Turkey, and *Demodex canis* [27] were selected as the outgroups for homologous clustering analysis in this study. Specifically, L-INS-i in MAFFT v7.487 was used for multiple sequence alignment, and the 'automated1' parameter in trimAl v1.2 was used for sequence trimming. Finally, a maximum likelihood phylogenetic tree was constructed in the iqtree v2.2.2.6 program, using HKY+F+G4 as the best model, bootstrap = 5,000.

Adult immersion test

A tick adult immersion test was carried out as described by Agwunobi et al. [28] with a slight modification. Briefly, the working concentrations of terpinolene and γ-terpinene were 40 mg/mL, 30 mg/mL, 20 mg/mL, 15 mg/mL, 10 mg/mL, 5 mg/mL, and 2.5 mg/mL. The negative control was a 50% ethanol solution. Amitraz is a common chemical acaricide used locally, and terpenoids can act on the same receptors as amitraz [29,30]. Therefore, a 12.5% amitraz solution was used as a positive control.

Screening of herbal medicines containing γ-terpinene and terpinolene

Herbs containing terpinolene and γ-terpinene components were retrieved from the HERB database (http://herb.ac.cn/) [23]. The herbs that contained both compounds were analyzed by Venny 2.0. The herb family categories corresponding to different herbs were counted through the iPlant database (https://www.iplant.cn/). The herb names and plant families corresponding to the herbs were corrected through the Species Bank of the Chinese Natural Herbarium (https://www.cfh.ac.cn/Spdb/spsearch.aspx). These results were visualized using Cytoscape 3.7.2.

Statistical analysis

One-way ANOVA was used to assess the significance of the data from the adult tick immersion experiment, and post hoc analyses were conducted using a Tukey's test. The half-lethal concentration (LC_{50}) and their respective 95% confidence intervals were analyzed by probit analysis. Statistical analyses of the data were performed by SPSS v.19.0 (IBM Corp., USA), and the data were visualized using GraphPad Prism v8 software (GraphPad Software Inc., USA).

RESULTS

Tick's identification

Morphological identification showed that the unfed adult ticks exhibited an elliptical shape. Specifically, the females and males were approximately 5 mm and 4 mm in length, respectively. The males and females have cervical and lateral grooves, with the medial spur

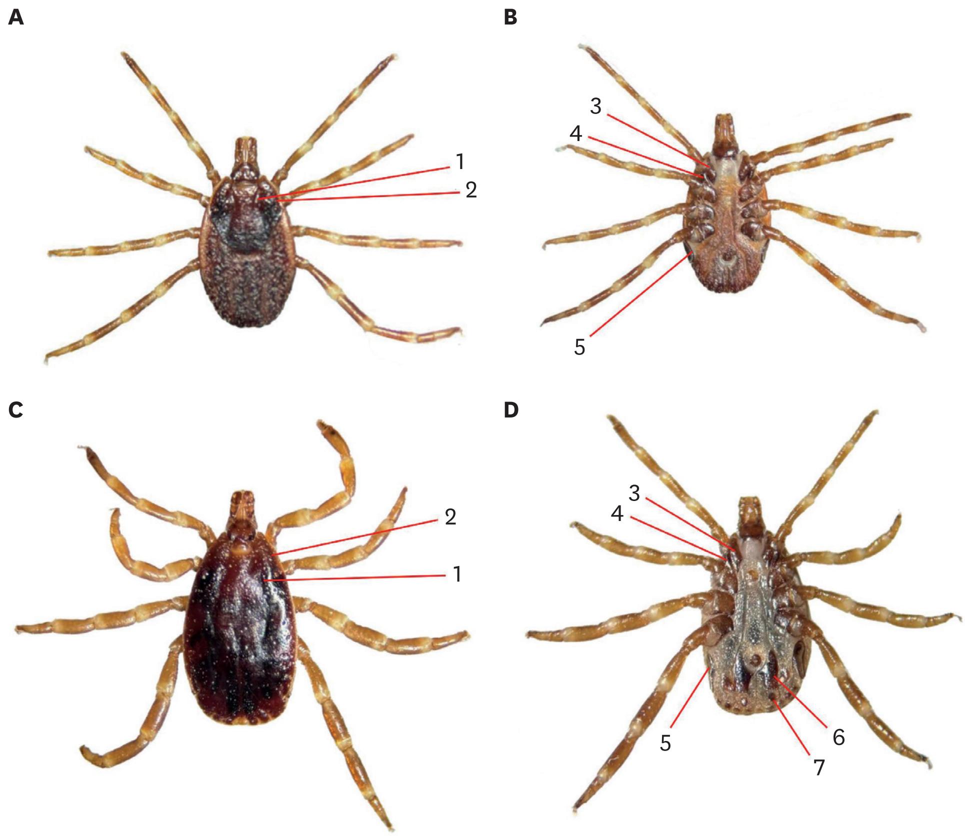

Fig. 1. Morphological characteristics of *H. anatolicum*.
(A) Female back, (B) female ventral surface, (C) male back, (D) male ventral surface. 1: cervical grooves, 2: lateral grooves, 3: coxae I spurs, 4: coxae I lateral spur, 5: spiracle plate, 6: adanal plate, 7: subanal adanal plate.

of Coxa I being closely aligned with the lateral spur. The medial spurs were wider than the lateral spurs. They had a pair of symmetrical spiracle plates. Males had clear adanal and subanal plates located just below, which were distinguishable from females (**Fig. 1**).

Phylogenetic analyses showed that the 16S rDNA sequences of the eight representative ticks for this study had a high homology with *H. anatolicum* originating from China, India, and Iraq (**Fig. 2**). The morphological identification and phylogenetic analysis confirmed that the ticks collected were *H. anatolicum*.

Acaricidal activity of two monoterpenes

The adult immersion test findings for terpinolene and γ-terpinene against *H. anatolicum* showed that both compounds had acaricidal activity. The positive control amitraz solution exhibited 100% lethality, whereas the 50% ethanol solution showed low toxicity to unfed adult *H. anatolicum* because it briefly incapacitated the ticks, but the recovery was rapid (**Fig. 3**). The acaricidal effects on *H. anatolicum* varied at different concentrations (**Fig. 3**). The impact of both substances on the mortality rate of *H. anatolicum* increased as the concentrations increased. Significance analysis revealed significant differences between 2.5 mg/mL and 5 mg/mL terpinolene and γ-terpinene solutions, other solutions of different concentrations, and the positive control ($p < 0.01$). On the other hand, there was no significance between the other working concentrations of the two terpenoids and the positive

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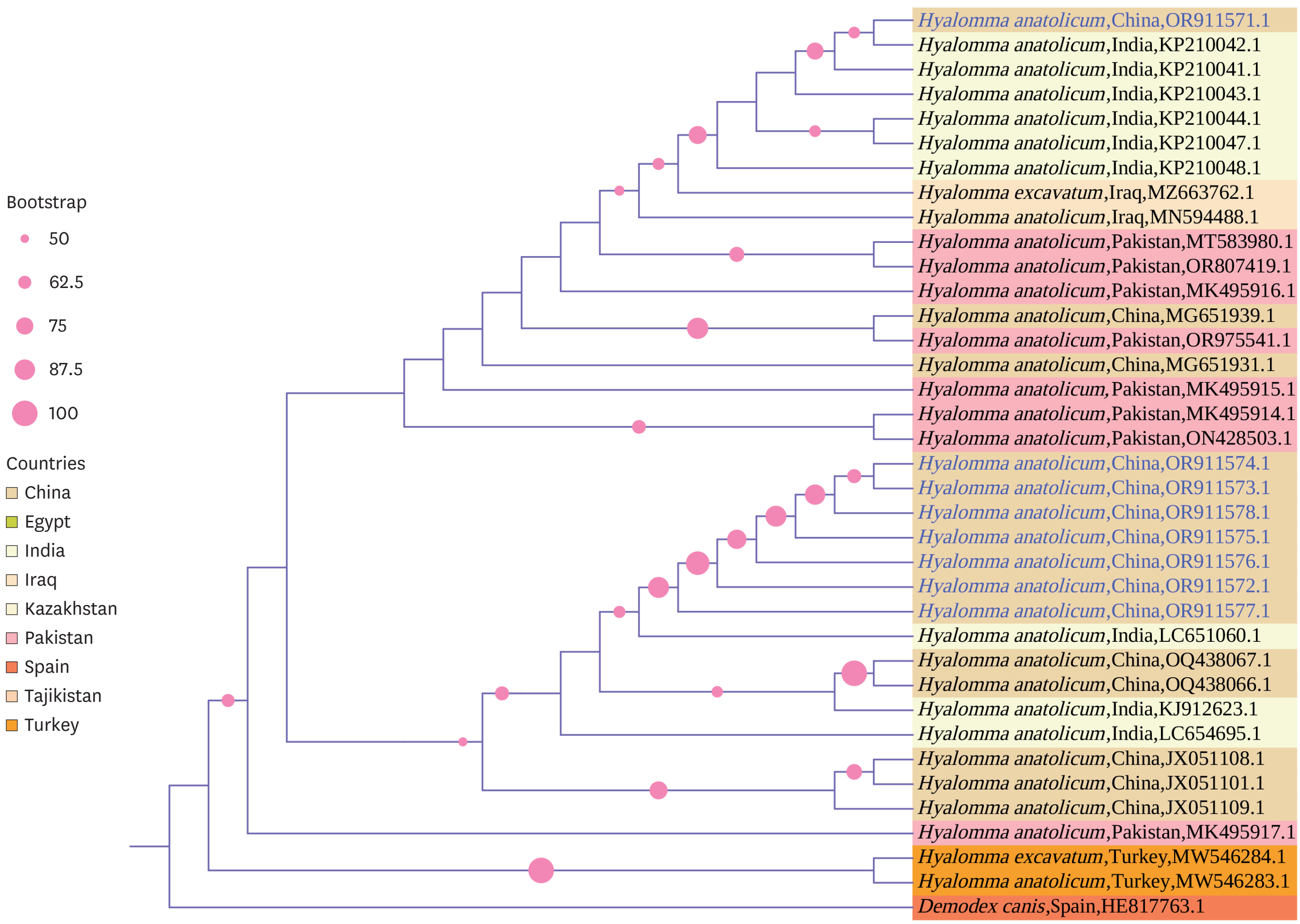

Fig. 2. Phylogenetic analysis of *H. anatolicum* and other tick species in different countries and regions in this study.

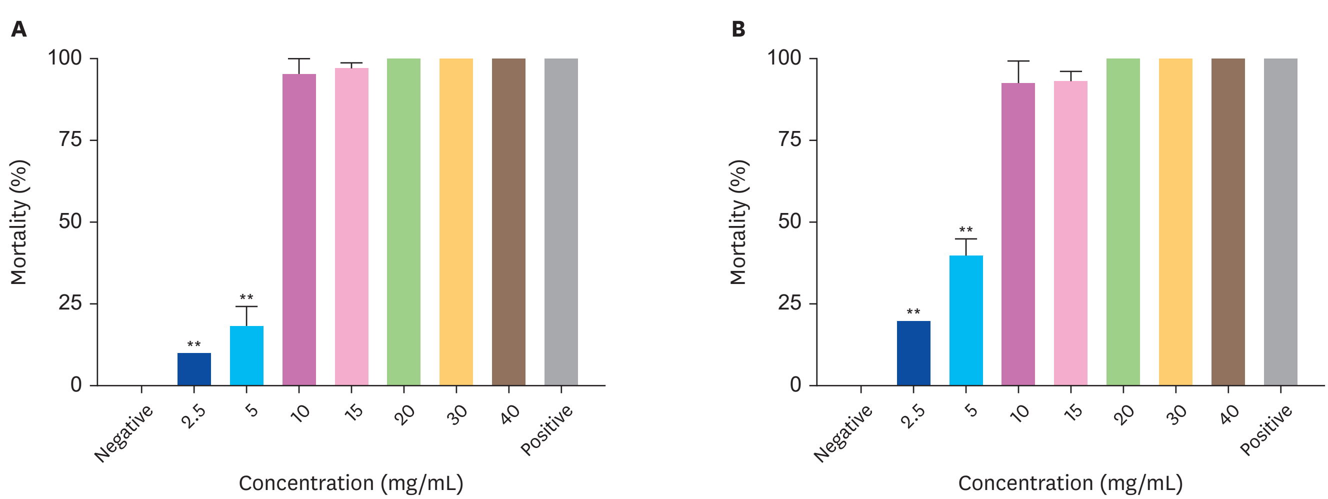

Fig. 3. Acaricidal effects of terpinolene and γ-terpinene on *H. anatolicum* at different working concentrations.
**$p < 0.01$.

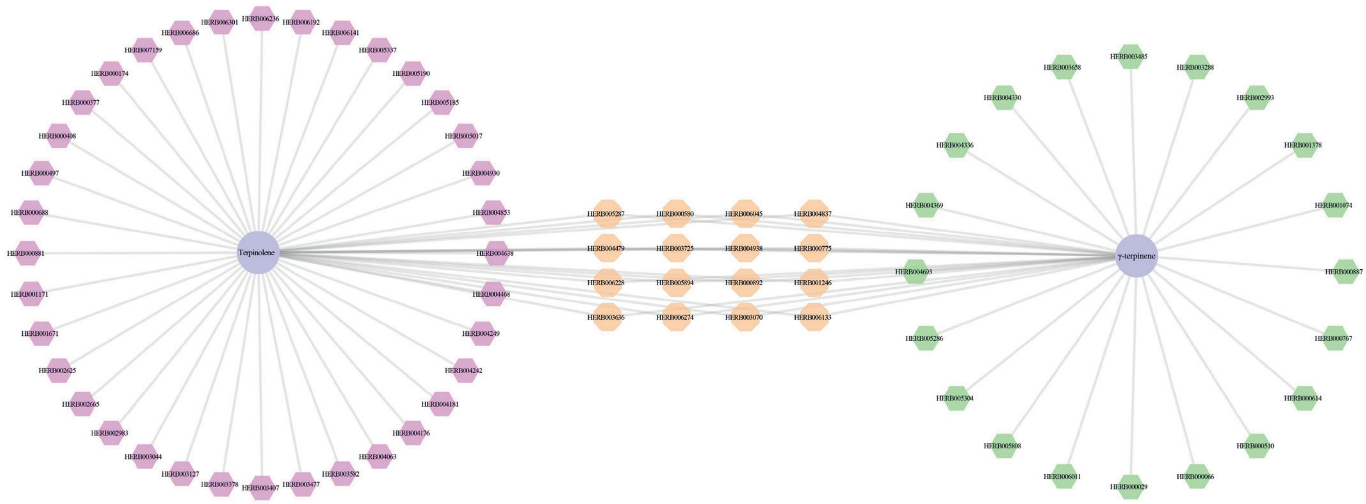

Fig. 4. Statistics of Chinese herbs containing terpinolene and γ-terpinene.
Number of herb species containing terpinolene (A, n = 36), γ-terpinene (B, n = 20), and both terpinolene and γ-terpinene (C, n = 16).

Table 1. Calculated LC_{50} of terpinolene and γ-terpinene on adult ticks of *Hyalomma anatolicum*

Compound	LC_{50} (mg/mL)	95% CI (mg/mL)	R^2	y = ax + b
Terpinolene	6.60	5.41–8.00	0.925	y = 0.367x − 2.424
γ-Terpinene	4.86	4.25–5.49	0.931	y = 3.637x − 2.498

CI, confidence interval.

control solution ($p > 0.05$). (**Fig. 4**). The specific results of the acaricidal activity (**Fig. 4**) and γ-terpinene against *H. anatolicum* were 6.60 mg/mL and 4.86 mg/mL, respectively (**Table 1**).

Analysis of herbs containing terpinolene and γ-terpinene

Fifty-two and 36 herbal medicines containing terpinolene and γ-terpinene, respectively, were retrieved from the HERB database. *Sedum sarmentosum*, *Amomum testaceum*, and *Verbena officinalis* are 16 herbs that contain terpinolene and γ-terpinene components (**Fig. 4**). The herbs originated from 36 plant families, including Lamiaceae, Apiaceae, and Zingiberaceae. Lamiaceae and Apiaceae had the highest representation (9.23% each), followed by Zingiberaceae (7.69%) and Asteraceae and Rutaceae (6.15% each) (**Fig. 5**).

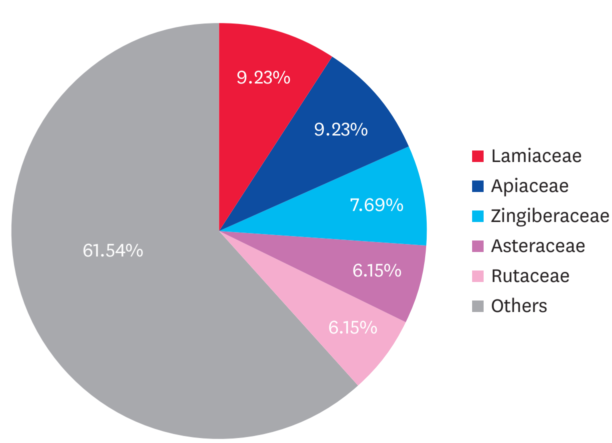

Fig. 5. Distribution of plant families corresponding to herbs containing terpinolene and γ-terpinene.

DISCUSSION

Forty-nine species of ticks have been documented, with *H. anatolicum* being the most dominant in Xinjiang region [31]. Locally, it contains a variety of pathogens, including *Theileria equi*, *Babesia caballi*, *T. ovis*, *Borrelia burgdorferi s.s.*, and *Anaplasma bovis* [3,32]. Precise identification of the tick species allows a deeper comprehension of the ecology, life cycle, and epidemiology of tick-borne diseases associated with different tick species [24]. In this study, morphological identification and molecular characterization were used to ensure that the ticks used for the drug treatments were *H. anatolicum*, which are endemic to the area and of public health and veterinary importance. These results findings provide support for the identification of *H. anatoicum*.

This study examined for the first time the killing effect of terpinolene and γ-terpinene on the *H. anatolicum* under semi-field conditions. Two terpenoids were effective against unfed adult *H. anatolicum* ticks. Both showed dose-effect relationships, but the LC_{50} values of γ-terpinene were lower than those of terpinolene. Previous studies reported that the botanical component terpinolene mainly acts as a repellent against ticks [33,34], whereas the present study showed that terpinolene had a killing effect on *H. anatolicum*. These findings suggest terpinolene may be a natural product with acaricidal and repellent properties. γ-Terpinene is a component of several plant essential oils used in tick control [15,35-37]. γ-Terpinene has also been used to control unfed adult *H. marginatum*. More than 90% of *H. marginatum* were dead after being treated with 10 μL/L of γ-terpinene for a few hours, and 87% of adult ticks were dead after 24 h [15]. Therefore, γ-terpinene might be a potential natural product that could be applied to reduce the number of *Hyalomma* ticks.

The application of plant essential oils on hard ticks is a viable tick control method [38], and many herbs have been developed for this purpose in China [20,22,25]. Terpenoid is a major class of compounds in essential oils [38]. This study searched the HERB database for Chinese herbal medicine containing terpinolene and γ-terpinene through the "ingredient"-"herb" correspondence. Fifty-two and 36 herbs contained terpinolene and γ-terpinene, respectively. Among them, several herbs have been used for tick control, such as *Foeniculum vulgare*, *Citrus reticulata*, *Cedrus atlantica*, *Zingiber officinale*, and *Citrus aurantium* for *Rhipicephalus microplus* [11,21,39]. *Nelumbo nucifera* is used to control *Haemaphysalis bispinosa* [40]. *Zingiber officinale* has been used to manage *H. anatolicum* and *Rhipicephalus bursa* [41,42]. What is worth mentioning is that there are currently 16 herbs that possess the compounds terpinolene and γ-terpinene. They are not currently used for tick control.

Despite the lack of direct evidence linking plant families to their effectiveness in tick-killing, statistical reviews suggest that the plant families with the highest tick control efficacy have shifted from Fabaceae and Euphorbiaceae to Lamiaceae [9,43]. In addition to these criteria, this study thoroughly investigated the plant families that are home to herbs rich in terpinolene and γ-terpinene. The primary objective was identifying the specific plant families in which these herbs are commonly found. The majority of these herbs are primarily composed of two families: Lamiaceae and Apiaceae, each accounting for 9.23% of the total, followed by Zingiberaceae (7.69%) and Asteraceae and Rutaceae (6.15% each) (**Fig. 5**). The focus was unconsciously drawn to the herbs belonging to the Lamiaceae family that contain terpinolene and γ-terpinene. These herbs included *Mentha canadensis*, *Mosla chinensis*, *Ocimum kilimandscharicum*, *Perilla frutescens*, *Schizonepeta tenuifolia*, and *Vitex rotundifolia*. *Vitex rotundifolia* and *Mosla chinensis* contain terpinolene and γ-terpinene. A search of the iplant database revealed the distribution ranges of *Mentha canadensis*, *Perilla frutescens*, and *Schizonepeta tenuifolia*. These herbs are primarily found in the Xinjiang region of China. In particular, Perilla frutescens [44], which has acaricidal properties, is a potential candidate for controlling *H. anatolicum*. In the future, extracts or essential oils derived from these herbs may be capable of providing effective management against *H. anatolicum*.

The present study used an *in silico* method to screen herbs containing two terpenoids with a good killing effect against the unfed adult *H. anatolicum*. Future studies are needed to determine the effectiveness of these herbal extracts in managing *H. anatolicum*. Nevertheless, the same natural products or plant essential oils are not equally effective against different species of ticks [45,46]. Moreover, it is also necessary to consider how various factors, such as the growth, development, physiological activity, and climate of medicinal plants, influence the effectiveness of tick control [47]. Compared to existing chemical acaricides, plant-derived insecticides still need to address sustainability issues, standardization of plant extracts, and development of formulations [38,48,49]. In addition, uncertainty in the dosage of acaricides of a plant origin and the concentration of active ingredients is one factor limiting the large-scale use of plant-derived acaricides [47]. More importantly, safety evaluations (e.g., cytotoxicity and acute/chronic toxicity in animals) are needed before plant-derived acaricides can be used. Overall, solving the above problems during research on plant-derived acaricides will help accelerate their development and allow early applications in the market.

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