Veterinary Parasitology 329 (2024) 110210

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

# Attractant and repellent properties of *Senna didymobotrya* plant extracts to *Amblyomma variegatum* and *Rhipicephalus appendiculatus*

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ARTICLE INFO

*Keywords:*
Attractant
Repellent
*Senna didymobotrya* extract
*Amblyomma variegatum*
*Rhipicephalus appendiculatus*
Sustainable tick control

ABSTRACT

The growing challenge of acaricide resistance and geographical range expansion of invasive tick species demands other interventions, like plant-based alternatives, for sustainable tick control. Leaves, flowers, seedpods, and twig branch extracts of *Senna didymobotrya* were analyzed using coupled gas chromatography mass spectrometry (GC-MS). Response of adult *Amblyomma variegatum* and *Rhipicephalus appendiculatus* to extracts was evaluated. The most attractive plant extract was fractionated and ticks' responses to its fractions assessed. Potential tick attractants in the attractive plant part extract and its fractions were identified by GC-MS analysis. Non- significant qualitative and quantitative differences were observed in the plant parts' extract composition ($R = 0.6178$). Flower extracts attracted both species, with a 0.1-fold higher attraction in *A. variegatum* compared to the standard attraction aggregation attachment pheromone (AAAP). Leaf and seedpod extracts repelled ticks at various concentrations. Bioassays after fractionating flower extracts identified hexane and ethyl acetate fractions as most attractive to *A. variegatum* ($P < 0.001$) and *R. appendiculatus* ($P < 0.001$), respectively. Chemical analysis of the most attractive extracts and fractions identified compounds, including documented acarine attractants, squalene and linoleic acid. A squalene and linoleic acid blend (1:1) at 1 mg/mL significantly attracted adult *A. variegatum* ($P < 0.01$) and *R. appendiculatus* ($P < 0.001$). The results of this study broaden comprehension of how ticks respond to plants in nature, and showcase the promising potential for integrating these insights into effective tick management programs.

## 1. Introduction

*Amblyomma variegatum* and *Rhipicephalus appendiculatus* are prominent tick species in sub-Saharan Africa (Jongejan and Uilenberg, 2004). With an extensive range across tropical sub-Saharan Africa and introduction in the Caribbean, *A. variegatum* is highly adaptable to domestic livestock (Jongejan and Uilenberg, 2004; Walker et al. 2003). It is a key vector for transmitting *Ehrlichia ruminantium*, the causative agent of heartwater, and significantly promotes the occurrence of dermatophilosis in cattle (Pavis et al., 1994; Walker, 1996). *Rhipicephalus appendiculatus* is of paramount importance in East and Southern Africa, where in its adult stage, it prefers to feed on the ears of various domestic and wild ruminants (Jongejan and Uilenberg, 2004; Walker et al. 2003). This tick species transmits *Theileria parva* and *Theileria taurotragi*, the causative agents of East Coast Fever (ECF) and benign bovine theileriosis, respectively, in cattle (Minjauw and Mcleod, 2003).

Acaricides have traditionally been central in tick control, but their overuse, often at suboptimal doses, has led to challenges, including resistance development, chemical residue persistence, limited effectiveness duration, non-target vertebrate toxicity, and high costs (de la Fuente, 2018; Kasaija et al., 2021; Nwanade et al., 2020). In response to these issues, there is a growing interest in eco-friendly alternatives, particularly plants as a sustainable and effective tick control solution.

Various plants, their extracts, and essential oils exhibit multifaceted behavioral effects on ticks, serving as attractants (Hassan et al., 1994; Nana et al., 2010; Zorloni et al., 2010), acaricides or repellents (Adenubi

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https://doi.org/10.1016/j.vetpar.2024.110210
Received 20 March 2024; Received in revised form 13 May 2024; Accepted 15 May 2024
Available online 17 May 2024

et al., 2018; Benelli and Pavela, 2018; Nwanade et al., 2020; Quadros et al., 2020). These plant-based solutions offer advantages, including low toxicity to mammals, minimal environmental contamination due to their short persistence, potential synergistic actions, limited development of resistance owing to their intricate chemical compositions, and relatively cost-effective acquisition (George et al., 2014).

*Senna didymobotrya* (Fresenius), commonly African senna, is an evergreen distinctive smelling shrub native to East and Central Africa (Witt and Luke, 2017). Traditionally, various communities in Kenya used the plant to manage livestock diseases and ticks (Njoroge and Bussmann, 2006; Wanzala et al., 2012). The ability of *S. didymobotrya* extracts to modify tick behavior has been documented (Opiro et al., 2013; Wanzala et al., 2014). Though plant secondary metabolites (PSMs) are known to differ between various parts of the same plant (Popović et al., 2020), there exists information paucity on tick behavioral responses to the different parts of *S. didymobotrya* and the chemical basis of these responses. Further, though *S. didymobotrya* is an extensively used medicinal plant, available literature focuses on its morphological identification (Jeruto et al., 2017). However, this approach may be hindered by the risk of incorrect identification, particularly due to its morphological resemblance to other species, such as *Senna italica*. Moreover, intraspecific morphological and chemotypic variations influenced by environmental and geographical factors further compound the challenge (de Sá Filho et al., 2022; Ho et al., 2021). Additionally, there is limited knowledge on the molecular diversity of *S. didymobotrya* across different climatic zones.

The objective of this study was to use plant morphological characteristics together with molecular markers to definitively identify *S. didymobotrya* samples, prior to evaluation. Secondly, it aimed to evaluate the behavioral responses of hard ticks (*A. variegatum and R. appendiculatus*) towards extracts derived from various parts of *S. didymobotrya*, including leaves, flowers, seedpods, and twig branches. Additionally, due to scarcity of information regarding tick attractive compounds in plants, the study sought to identify potential tick attractants within the most attractive extracts and fractions through chemical analysis and bioassay-guided experiments. The elucidation of this information holds promise for the development of environmentally friendly tick management strategies.

## 2. Materials and methods

### 2.1. Collection, morphological and molecular identification of Senna didymobotrya

Prior approval for plant sample collection was obtained from the National Commission of Science, Technology, and Innovations (NACOSTI), Kenya (License No.: NACOSTI/P/21/10632). The plants were harvested from open community fields, making it unfeasible to determine their developmental stage. Sampling was conducted during the plant's flowering season. On-site, *S. didymobotrya* samples were identified based on their morphological characteristics (Jeruto et al., 2017). The plant samples were randomly collected from four climatic zones in Kenya to assess environmental factors' influence on phylogenetic diversity. The samples were collected from Kirinyaga, Nairobi, Kajiado, Homabay, and Migori counties (Fig. 1). The environmental factors of the counties are listed in Table 1. Freshly collected plant parts (leaves, flowers, seedpods and twig-branches) were separately packed in well-labelled gunny bags and transported to the laboratory at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville Campus in Nairobi, on the same day.

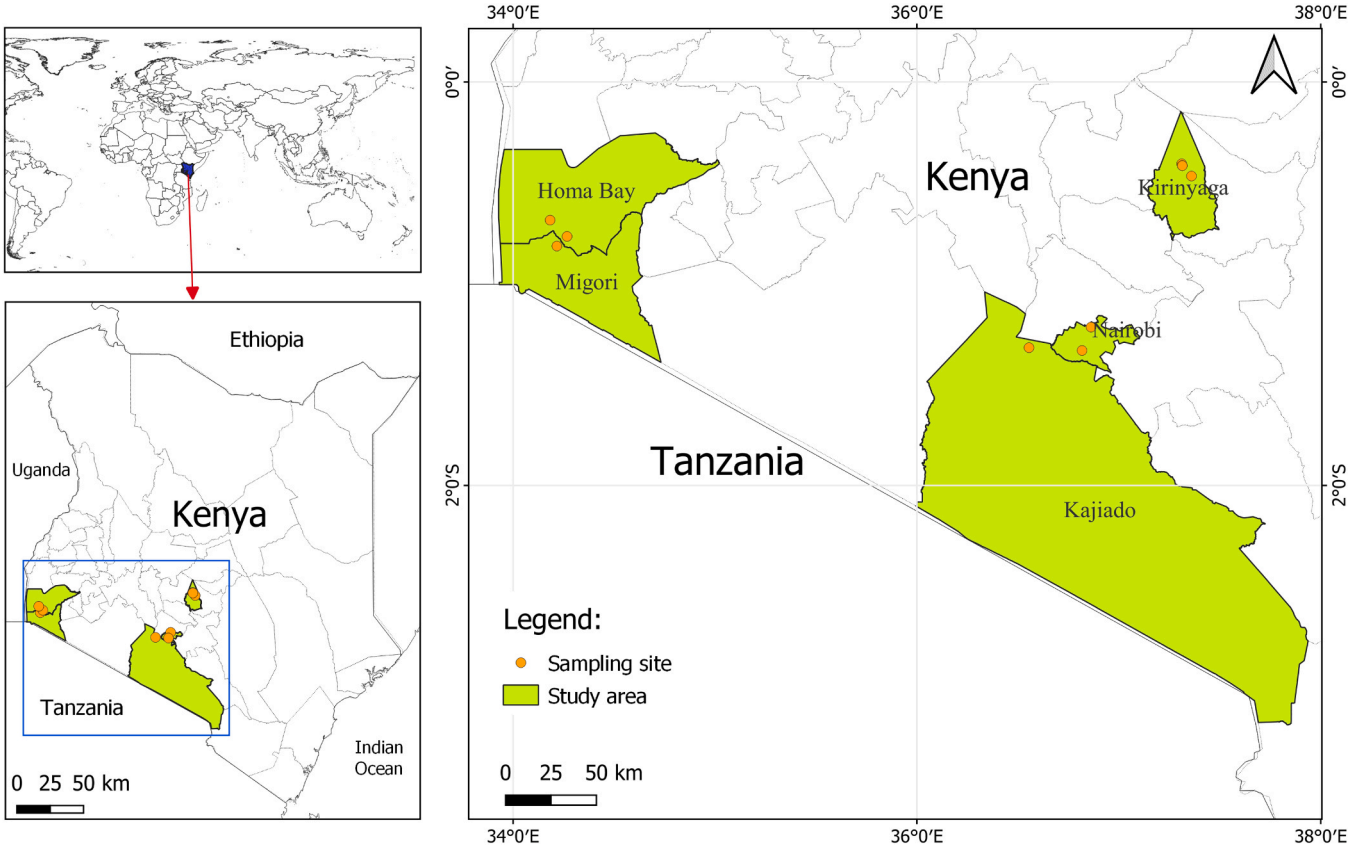


**Fig. 1.** Map of *Senna didymobotrya* sampling sites in Kenya created with QGIS v.3.22.3.
Shapefiles obtained from https://gadm.org/download_world.html (world map) and https://gadm.org/download_country.html (Kenya administrative boundaries) under CC-BY open access license.

**Table 1**
Geographic locations, altitude, and climatic characteristics of sampled counties.

| County | Latitude (°S) | Longitude (°E) | Altitude (m ASL) | Climate | Annual Rainfall (mm) | Annual Temperature Range (°C) |
|---|---|---|---|---|---|---|
| Kirinyaga | 0.6 | 37.33 | 1158 – 5199 | Tropical wet and dry or savanna | 1100 – 1250 | 14 – 25 |
| Nairobi | 1.3 | 36.82 | 1660 – 1800 | Cool, temperate | 610 | 14.78 – 24.9 |
| Kajiado | 2.1 | 36.78 | 1582 | Tropical wet and dry or savanna | 400 | 15.96 –23.89 |
| Homabay | 0.5 | 34.45 | 1193 | Tropical rainforest | 1200–2500 | 15.55 – 25.05 |
| Migori | 1.1 | 34.48 | 1394 | Tropical monsoon | 700 – 1800 | 15.55 – 25.05 |

ASL = above sea level. (Weather and Climate, 2023)

Total DNA was extracted from individual leaf samples from each sampling site using the ISOLATE II Plant DNA Kit (Bioline, London, UK) following the manufacturer's instructions. DNA samples were used for PCR amplifications targeting the ribulose-1,5-bisphosphate carboxylase large subunit (*rbcL*) gene fragment with the primers: rbcL1F (5'-ATGTCACCACAAACAGAGACTAAAGC-3') and rbcL724R (5'-TCGCATGTACCTGCAGTAGC-3').

PCR reactions were performed in 20 μL volumes with 4 μL 5× HOT FIREPOL Blend Master Mix (Solis BioDyne, Tartu, Estonia), 0.6 μL of 10 μM forward and reverse primers, 25 ng of DNA template, and PCR grade water in ProFlex PCR systems thermocycler (Applied Biosystems, Foster City, CA, USA). Cycling conditions for *rbcL* amplification included: initial denaturation at 95 °C for 15 min; 30 cycles of denaturation at 95 °C for 20 sec, annealing at 66 °C for 1 min, and extension at 72 °C for 2 min 30 sec; with a final extension at 72 °C for 7 min. PCR amplicons were resolved on 2% ethidium bromide-stained agarose gels at 80 V for 1 hour and visualized against a 100 bp DNA ladder (Solis BioDyne, Tartu, Estonia) under UV transillumination using a Kodak Gel Logic 200 Imaging System (SPW Industrial, Laguna Hills, CA, USA). Positive amplicons were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and sent to Macrogen Inc. (Seoul, South Korea) for Sanger sequencing. Nucleotide sequence data reported in this paper are available in the GenBank™ (accessions **OP524161-OP524173**).

### 2.2. Preparation of crude plant extracts and solvent fractionation of the crude flower extract

After DNA confirmation of sample identity, each plant part samples of *S. didymobotrya* were pooled with their counterparts from the various locations for chemical analysis. Samples were air-dried in a screen house (19–35 °C, 30 – 90% RH) to a constant weight, ground to a fine powder using an SM 100 cutting mill (Retsch GmbH, Haan, Germany), and then macerated three times in 100% analytical grade methanol (Sigma Aldrich, St. Lois, USA) (0.25 kg/L) with intermittent shaking for 72 hours. The filtered extracts were pooled, concentrated *in vacuo,* weighed, and stored at −20 °C until used.

The crude methanolic flower extract, identified as the most attractive, was fractionated by liquid-liquid partitioning using solvents of increasing polarity. Briefly, 15 g of the flower extract was solubilized in 200 mL distilled water and partitioned in a separating funnel using hexane (4 × 100 mL) and ethyl acetate (4 × 100 mL). The resulting water fraction was lyophilized to acquire the aqueous fraction. The hexane and ethyl acetate fractions were concentrated *in vacuo* at 40 °C and, along with the aqueous fraction, weighed and stored at −20 °C prior to bioassays.

The crude extracts and fractions were reconstituted in their respective extracting solvents to prepare different concentrations (100, 50, 25, and 1 mg/mL) for use in behavioral bioassays. These concentrations were selected based on established physiologically relevant doses of plant extracts (Nana et al., 2010), and the concentration-enhancing effect of fractionation.

### 2.3. GC-MS analysis

The chemical composition of the crude (methanol) extracts of *S. didymobotrya*'s flowers, leaves, seedpods, twig branches and solvent fractions (hexane and ethyl acetate) of the flower extract were analyzed by coupled gas chromatography-mass spectrometry (GC-MS).

Sample preparation involved adding 1 mL of GC-grade dichloromethane (DCM) (Sigma Aldrich, St. Lois, USA) to every 5 mg methanolic crude extract and ethyl acetate fraction samples. The samples were vortexed for 1 min, ultra-sonicated for 10 min, and centrifuged at 14,000 rotations per minute for 5 min. The resulting supernatant was dried over anhydrous sodium sulphate, filtered, diluted to a final concentration of 100 ng/μL in DCM, and then analyzed in the GC-MS. Hexane fraction samples were prepared similarly, replacing DCM with GC-grade hexane (Sigma Aldrich, St. Lois, USA). Solvent blanks of DCM and hexane were analyzed to remove solvent and column contaminant peaks.

The GC-MS system featured a 7890 A gas chromatograph and a 5975 C mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA), with sample injections carried out by a 7683B autosampler (Agilent Technologies, Inc., Beijing, China), all controlled by a Hewlett-Packard workstation (HP Z220 SFF intel Xeon) running MSD Chemstation software B.02.02. The GC was fitted with a mid-polar (5%-phenyl)-methylpolysiloxane (HP5 MS) low bleed, fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (J&W, Folsom, CA, USA). Helium carrier gas was used at a 1.2 mL/min flow rate. Aliquots (1 μL) of the samples were injected into the GC-MS in splitless mode. The inlet temperature was set at 270 °C. The oven temperature was kept at 35 °C for 5 min, programmed to increase at a rate of 10 °C/min to 280 °C for 10.5 min, then programmed to 285 °C at a rate of 50 °C/min for 9.9 min. The ion source and quadrupole temperatures of the mass selective detector were maintained at 230 °C and 180 °C, respectively. The mass spectrometer was operated in full scan mode over a 38 – 550 $m/z$ mass range with electron impact (EI) acquired at acceleration energy of 70 eV. Filament delay time was set at 3.3 min.

Compounds were identified by comparing their gas chromatographic retention times and mass fragmentation spectra with reference spectra in Adams2, Chemecol, and the National Institute of Standards and Technology (NIST) 11 databases. Serial dilutions of squalene (≥ 98% purity) (Sigma-Aldrich, St. Louis, MO) were analyzed in full scan mode by GC-MS to establish a linear calibration curve (peak areas versus concentration) with the regression Eq. (1):

$$y = 1E+07x - 4E+08 \quad (1)$$

and determination coefficient of $R^2 = 0.9923$. This regression equation was used for relative external quantification of detected compounds within the analyzed samples.

### 2.4. Tested chemicals

The standard tick attractant, attraction-aggregation-attachment pheromone (AAAP) used in the study was prepared by combining nonanoic acid (0.8 mg), methyl salicylate (0.1 mg) and *o*-nitrophenol (0.2 mg) (Nana et al., 2010), all sourced from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Bioassays were conducted using a 0.02 mg/mL concentration, which significantly attracts *A. variegatum* (Nchu et al., 2009) and *R. appendiculatus* (Nana et al., 2010) adult ticks.

The standard repellent, N, N-diethyl-3-methylbenzamide (DEET) (≥

97% purity) from Sigma-Aldrich, was diluted in ethanol to 7.2%, matching some commercial DEET formulations was also included.

Behavioral response of ticks to squalene (≥ 98% purity) and linoleic acid (≥ 99% purity) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was evaluated. Concentrations (10, 1.0, and 0.1 mg/mL) for these compounds and a squalene: linoleic acid (1:1) blend were prepared in hexane. The 1.0 mg/mL concentration is within physiologically relevant levels for ticks (Yoder et al., 1999), while lower doses were used to determine the minimum effective concentration.

### 2.5. Ethics statement

Tick rearing on New Zealand white rabbits followed approved protocols at *icipe*'s Animal Rearing and Quarantine Unit (ARQU) under the authority of NACOSTI, Kenya (License No: NACOSTI/P/20/4253).

### 2.6. Experimental ticks

The hard ticks, *Amblyomma variegatum* and *Rhipicephalus appendiculatus*, were obtained from colonies raised at *icipe*'s ARQU according to Levin and Schumacher (2016). All life stages were fed on New Zealand white rabbits. Ticks, identified morphologically (Okello-Onen et al., 1999), were maintained in glass vials (30 per vial) with nylon mesh and stoppers over saturated sodium chloride solution to ensure 85 ± 5% relative humidity in aluminum tins. The tins were kept in a Sanyo MIR-153 incubator at 25 ± 1 °C with a 16:8 L: D hr photoperiod. Male and female unfed adults (2 – 3 months post-molting) were used in the bioassays due to their large size and ease of visibility.

### 2.7. Tick behavioral responses based on their host-seeking strategies

#### 2.7.1. Tick climbing bioassay

A two-choice tick climbing assay based on *R. appendiculatus*' ambush host-seeking strategy (Wanzala et al., 2014) was employed with slight modifications from Nana et al. (2010). The setup (Fig. 2) included an aluminum base (14.5 × 14.5 × 1.7 cm) with two aluminum rods (26 cm long × 0.7 cm external diameter) placed 7 cm apart, situated inside a water-filled tray (32 × 25 × 3.5 cm) to prevent tick escape. Aliquots (0.5 mL) of treatment (either 100, 50, 25 or 1 mg/mL, one at a time) and control were applied to individual autoclaved cotton wool plugs (0.2 g). After a 5-minute solvent evaporation period, the plugs were fitted onto the aluminum rods, one with treatment and the other with control. A test

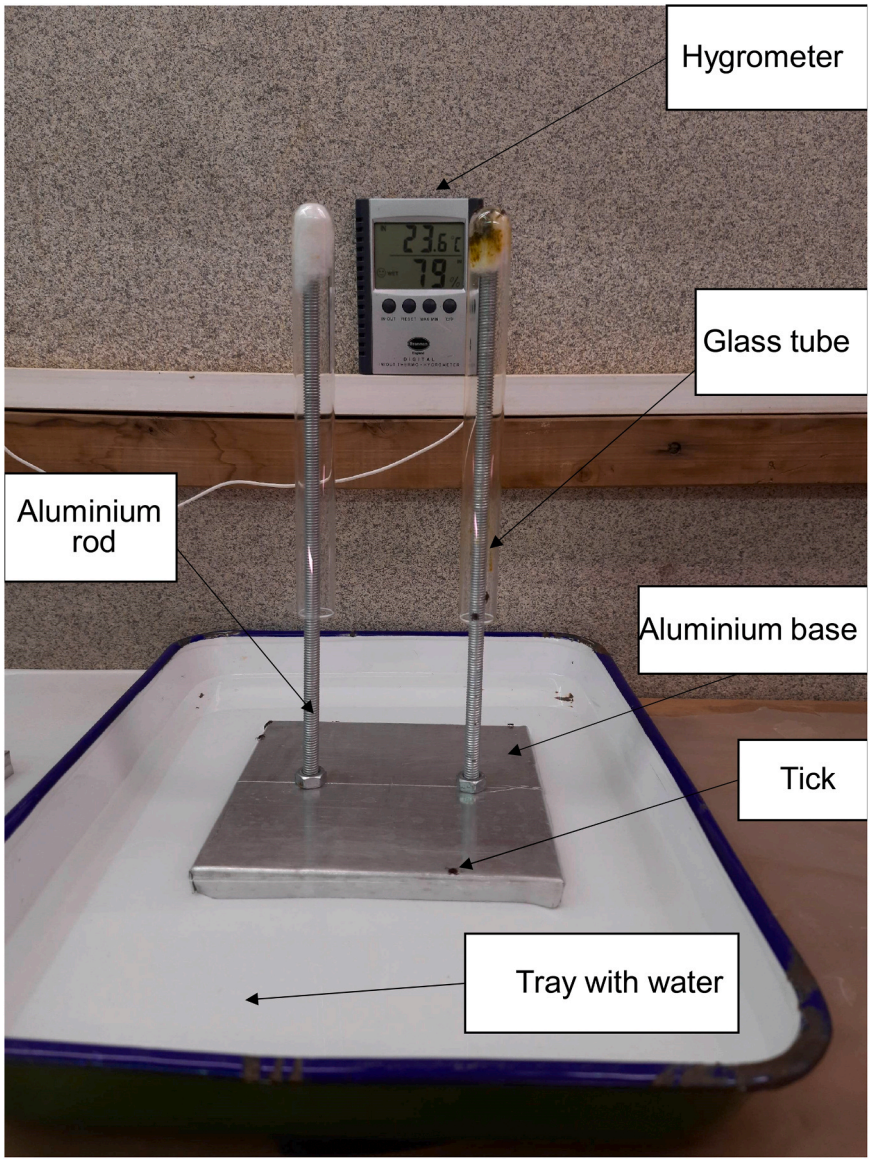


**Fig. 2.** Tick climbing bioassay setup.

tube (20 cm long and 2.4 cm internal diameter) was mounted over each cotton wool.

Ticks were assessed for host seeking activity by exposing them to human breath (Kimps et al., 2011), and only responsive ticks were used in the assays. Ten same-sex *R. appendiculatus* ticks were placed between the rods on the aluminum base. Experiments ran for 30 minutes at 23–26 °C and 70 ± 10% RH in a bioassay room. The final count included ticks that climbed (above 6 cm – space left between the bottom of the test tube and the aluminum base) the test and control rods, while non-climbing ticks were recorded but excluded from the analysis (Nana et al., 2010). The apparatus was cleaned after every test run with soap under running tap water, rinsed with distilled water then 70% ethanol and dried at 40 °C. Treatment and control rods were alternated between assays to avoid positional bias. Treatments were randomized, and each assay was conducted eight times, with a total of 80 adult ticks (40 male and 40 female) tested per treatment.

### 2.7.2. Olfactometer bioassay

An olfactometer was used to evaluate *A. variegatum* responses based on its hunting host location strategy (Nchu et al., 2009). The olfactometer (Fig. 3) comprised a clear, cuboid Perspex tube (31.5 × 5.7 × 4 cm) with a central hole. Two removable 50 mL Falcon tubes, with their bottoms removed, were attached to each end of the tube, one on the right and the other on the left, housed the test substances. The Falcon tubes had holes in their corks, connected to a portable field air delivery and vacuum pressure pump (Sigma Scientific, Micanopy, FL, United States) via Teflon tubes. The olfactometer tube featured three sections: two response zones (10 cm zones on each side of the tube); and a no-response zone in the middle (10 cm). The hole in the middle served as both the release point for ticks and the attachment point for the vacuum. Air flowed into the olfactometer through the ports in the Falcon tube lids at the ends of the tube at a 5 mL/s flow rate (Nana et al., 2010; Nchu et al., 2009), and the vacuum removed air at the same flow rate.

Aliquots (0.5 mL) of each treatment and the control were applied to separate autoclaved cotton wool plugs (0.2 g), air-dried for 5 min, and introduced into the olfactometer, with the treatment in one arm and the control in the other. Host seeking tick activity before each assay was determined by blowing human breath against them (Kimps et al., 2011); and only ticks that responded were used. Five ticks of the same sex were released into the olfactometer at a time and allowed a maximum of 5 min to choose an arm. The final count included ticks that chose the test and control rods, while ticks that did not choose an arm were considered unresponsive and were excluded from the analysis. After each experiment, the olfactometer was cleaned with soap under running tap water, rinsed with distilled water then 70% ethanol, and dried at 40 °C. Treatment and control arms were swapped between assay replicates to prevent positional bias. Eighty adult ticks (40 males and 40 females) were tested for each treatment.

### 2.8. Data analysis

Forward and reverse *rbcL* chromatograms were loaded into Geneious Prime 2020.2.2 software (created by Biomatters, Auckland, New Zealand) (Kearse et al., 2012), inspected, trimmed, edited and aligned to generate consensus sequences. The consensus sequences were then compared to known sequences in the GenBank database using BLASTn (Altschul et al., 1990).

Study sequences were aligned with previously deposited *Senna* species' *rbcL* sequences obtained from GenBank using the MAFFT plugin in Geneious Prime 2022.2. The alignment file was exported to MEGA 7 (Kumar et al., 2016), where maximum-likelihood phylogenies were constructed with automatic model selection based on the Akaike Information Criterion under 1000 bootstrap iterations. The final phylogenetic trees were visualized using Fig Tree 1.4.4 (Rambaut, 2014). *Calpurnia aurea* (family Fabaceae: GenBank accession: OP524173) was included as an outgroup.

Chi-Square goodness of fit test was used to assess responses between male and female ticks that preferred treatments over controls. Given the lack of significant differences, data from both sexes were pooled. Non-responsive ticks to either the treatment or control were excluded from

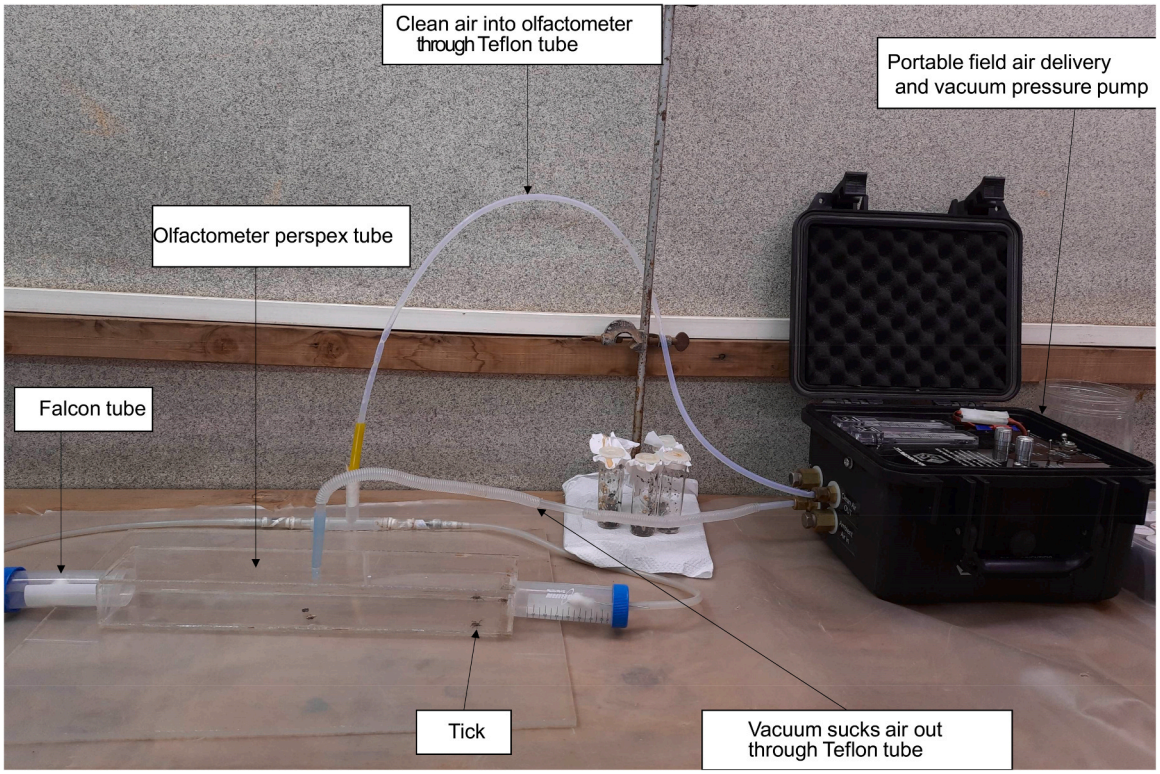


**Fig. 3.** Olfactometer setup.

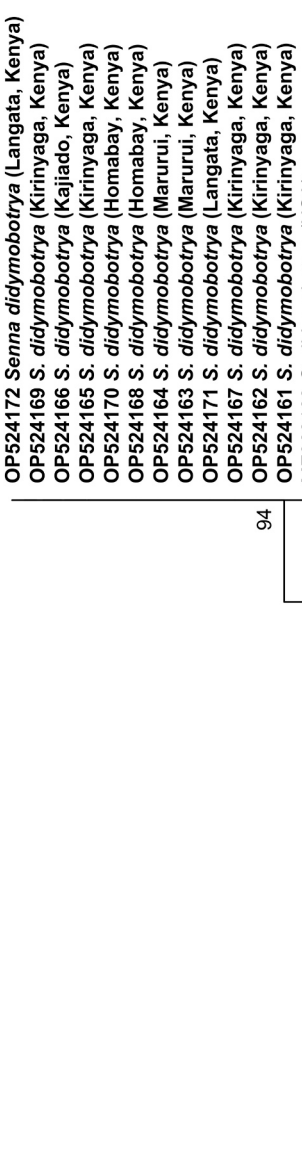


**Fig. 4.** Maximum likelihood phylogenetic tree of *rbcL* gene sequences from Kenyan plant samples. Thirteen sequences from the study are bolded. Bootstrap values at major nodes represent percentage agreement from 1000 replicates. Branch length scale indicates substitutions per site. *Calpurnia aurea* OP524173 (Fabaceae) serves as an outgroup (in brackets, bottom of tree).

the analysis. Counts of ticks responding to the treatment and the control were pooled across replicates for each test substance. Relative attraction index (RAI) was calculated as described by Nana et al. (2010) using the Eq. (2) below:

$$RAI = (Nt - Nc)/(Nt + Nc) \times 100 \quad (2)$$

Where:

RAI is relative attraction index

Nt is the total number of ticks in test and

Nc is the total number of ticks in control.

Chi-Square goodness of fit test was used to analyze the percentage of ticks responding to the treatments versus the controls.

Compound variations across replicates of *S. didymobotrya*'s leaves, flowers, seedpods, and twig-branches were illustrated through heatmap clustering. One-way analysis of similarity (ANOSIM) test based on Bray-Curtis measure dissimilarity matrix was used to compare the chemical composition of the plant parts. In complement, a non-metric multidimensional scaling (NMDS) test was employed to visualize these differences. Similarity percentage (SIMPER) analysis was conducted on peak areas to relatively determine compounds contributing to the dissimilarity. All statistical analyses were performed in R statistical software (version 1.4.1717) (R Core Team, 2021) and PAST software (version 4.06) (Hammer et al., 2001).

## 3. Results

### 3.1. Molecular identification and phylogenetic analysis of plant samples

Query *rbcL* sequences from the study's representative samples from various locations were identified to species level using BLASTn analysis, showing 99–100% identity with GenBank nr database reference sequences (Supplementary Figure S1). BLAST homology search of the 13 *rbcL* query sequences returned identical results, confirming the plant samples as *Senna didymobotrya*. Maximum Likelihood analysis showed that the 13 *rbcL* accessions formed a monophyletic clade (Fig. 4) matching a reference *S. didymobotrya* sequence.

### 3.2. Chemical composition of extracts of Senna didymobotrya plant parts

Qualitative and quantitative variations in *S. didymobotrya* extract composition were observed (Fig. 5). The flower, leaf, seedpod, and twig branch extracts contained 34, 40, 36, and 32 compounds, respectively (Table 2). Hexane and ethyl acetate fractions contained 22 and 19 compounds, respectively (Table 2). One-way ANOSIM using Bray-Curtis dissimilarity revealed no significance in the chemical composition of the plant parts (R = 0.6178, $p = 0.0014$) (Fig. 6). The NMDS clustered the four plant parts by their chemical profiles (Fig. 6A). The Shepard plot had a stress value below 0.2, indicating excellent NMDS ordination (Fig. 6B). SIMPER analysis, combining all plant parts, showed a 65.48% dissimilarity, with (3$\beta$, 5$\beta$)-stigmast-7-en-3-ol (11.15%), phytol (9.63%), hexadecanoic acid (7.32%), stigmasterol (4.42%), and methyl lineolate (4.32%) contributing preeminently (Fig. 6C). Flower and twig branch extracts had the lowest average dissimilarity (62.8%), followed by flower and seedpod (64.86%), and flower and leaf extracts (69.04%). Phytol (17.7%), (3$\beta$, 5$\beta$)-stigmast-7-en-3-ol (11.77%), and hexadecanoic acid (8.85%) played a significant role in the dissimilarity between flower and leaf extract (Fig. 5).

### 3.3. Amblyomma variegatum and Rhipicephalus appendiculatus responses to Senna didymobotrya's crude methanol extracts

No significant difference in response was observed between adult male and female ticks of both species ($p > 0.05$) (Supplementary Table S1 and S2). Adult ticks exhibited varied responses to different plant parts and tested concentrations (Fig. 7 and Supplementary Table S3).

Adult *A. variegatum* were significantly attracted to the flower extract compared to the solvent (control) at 100 mg/mL ($\chi 2 = 33.15$, df = 1, $P < 0.001$), 50 mg/mL ($\chi 2 = 6.93$, df = 1, $P < 0.01$), and 25 mg/mL ($\chi 2 = 5.54$, df = 1, $P = 0.02$) (Fig. 7A). The ticks were also attracted to twig

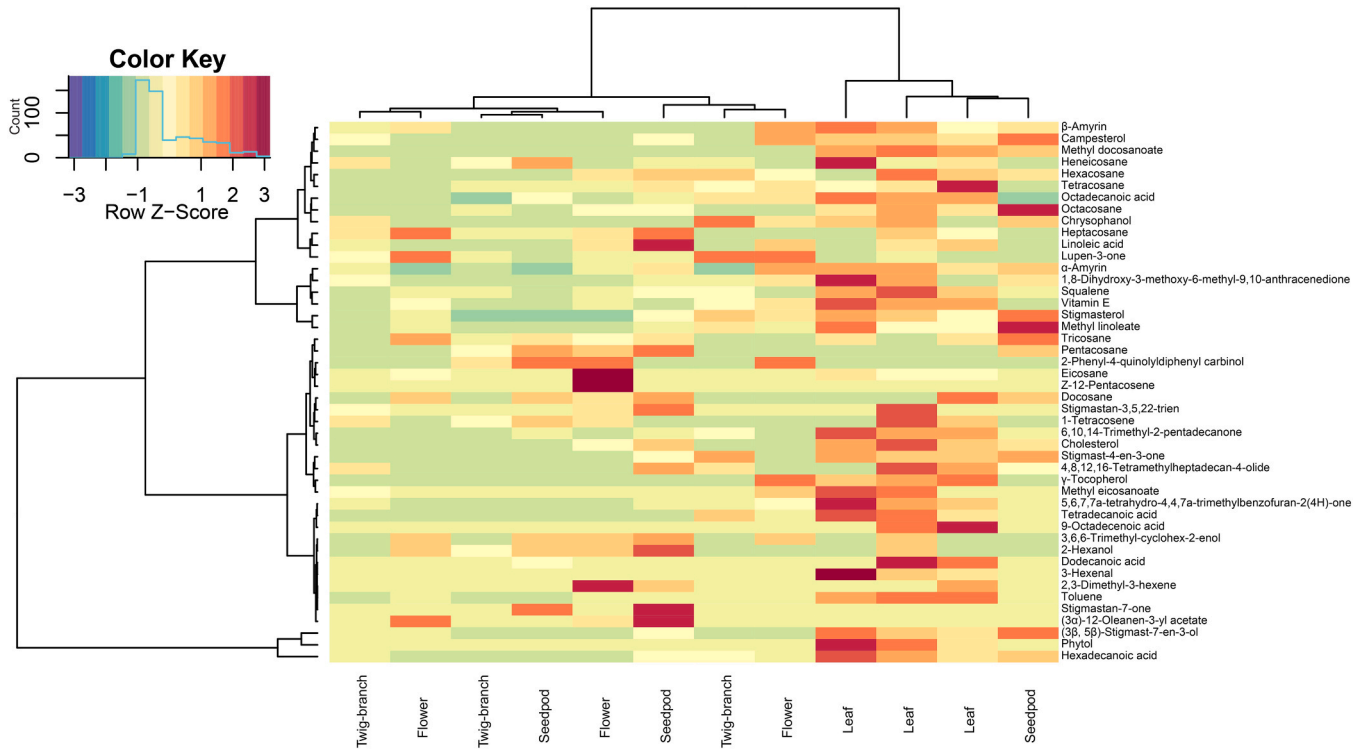


**Fig. 5.** Heatmap clustering showing chemical composition variations in *Senna didymobotrya*'s leaf, flower, seedpod and twig branch extracts. Intensity of colors represents compound abundance across replicates of the plant parts.

**Table 2**
Mean compound amounts in *Senna didymobotrya* extracts (n=3) presented as μg/g for plant parts (flower, leaf, seedpod, twig branch) and ng/mg for hexane and ethyl acetate fractions.

| RT (min) | Compound Identity | Chemical Class | Flower | Leaf | Seedpod | Twig branch | Hexane Fraction | Ethyl acetate Fraction |
|---|---|---|---|---|---|---|---|---|
| 5.39 | Toluene | Benzene | 8.0 ± 0.00 | 8.7 ± 0.09 | 8.1 ± 0.04 | 8.0 ± 0.00 | 458.1 ± 31.02 | |
| 6.46 | 3-hexenal | Aldehyde | | 10.0 ± 0.78 | | | | |
| 6.63 | 2-hexanol | Alcohol | 10.9 ± 0.07 | 10.9 ± 0.00 | 11.9 ± 1.02 | 9.7 ± 0.00 | | |
| 8.60 | 2,3-dimethyl-3-hexene | Alkene | 8.6 ± 0.59 | 8.3 ± 0.26 | 8.3 ± 0.26 | | | |
| 9.43 | 3,6,6-trimethyl-cyclohex-2-enol | Alcohol | 11.7 ± 1 | 9.5 ± 1.38 | 11.0 ± 1.47 | 8.1 ± 0.01 | | |
| 18.51 | 2,4-bis(1,1-dimethylethyl)-phenol | Phenol | | | | | | 1027.9 ± 135.99 |
| 19.24 | 5,6,7,7a-tetrahydro-4,4,7a-trimethylbenzofuran-2(4 H)-one | Ketone | 11.9 ± 0.00 | 19.1 ± 2.58 | 9.3 ± 0.43 | 9.7 ± 0.32 | | |
| 19.50 | Dodecanoic acid | Fatty acid | | 11.4 ± 0.69 | 8.8 ± 0.00 | | | |
| 21.67 | Tetradecanoic acid | Fatty acid | | 16.9 ± 2.24 | 10.2 ± 0.00 | 15.5 ± 0.00 | 726.9 ± 72.74 | |
| 22.53 | 6,10,14-trimethyl-2-pentadecanone | Sesquiterpene | | 40.3 ± 3.91 | 11.8 ± 1.21 | 18.8 ± 0.00 | 505.9 ± 465.64 | |
| 23.33 | 7,9-dimethyl-hexadecane | Alkane | | | | | | 408.8 ± 292.41 |
| 23.42 | Nonadecane | Alkane | | | | | | 429.5 ± 388.77 |
| 23.49 | 9-octadecenoic acid | Fatty acid | | 19.2 ± 2.10 | | | | |
| 23.64 | Ethyl hexadecanoate | Ester | | | | | | 2657.2 ± 1316.99 |
| 23.72 | Hexadecanoic acid | Fatty acid | 46.9 ± 22.86 | 277.3 ± 59.82 | 148.3 ± 61.42 | 82.1 ± 38.52 | | |
| 24.02 | Eicosane | Alkane | 40.4 ± 22.60 | 19.6 ± 1.57 | 11.1 ± 0.00 | 9.9 ± 0.55 | | 2153.9 ± 1506.51 |
| 24.60 | Methyl octadeca-9,12,15-trienoate | Ester | | | | | | 2775.5 ± 0 |
| 24.96 | Methyl linoleate | Ester | 21.9 ± 3.61 | 111.8 ± 45.21 | 106.1 ± 79.43 | 35.2 ± 27.11 | | 45.2 ± 0 |
| 25.12 | Phytol | Alcohol | | 479.2 ± 124.25 | 16.4 ± 4.81 | 19.4 ± 0.00 | | |
| 25.14 | Ethyl linoleate | Ester | | | | | | 2940.6 ± 0 |
| 25.28 | Methyl octadecanoate | Ester | | | | | 878.6 ± 60.63 | |
| 25.55 | Linoleic acid | Fatty acid | 50.5 ± 23.05 | 46.6 ± 3.96 | 56.5 ± 45.37 | 14.7 ± 4.24 | 4323.0 ± 712.58 | 2791.8 ± 2749.44 |
| 25.55 | Octadecanoic acid | Fatty acid | 37.3 ± 14.11 | 103.6 ± 4.68 | 35.8 ± 13.23 | 47.6 ± 25.57 | | |
| 25.86 | Heneicosane | Alkane | 14.7 ± 0.00 | 58.1 ± 23.65 | 51.3 ± 22.97 | 37.0 ± 4.74 | 3391.6 ± 100.88 | |
| 25.90 | Docosane | Alkane | 22.6 ± 2.82 | | 27.1 ± 1.43 | | 5050.3 ± 528.64 | |
| 26.72 | Tricosane | Alkane | 27.2 ± 12.36 | 34.8 ± 1.65 | 41.4 ± 8.30 | 15.6 ± 0.00 | 1726.2 ± 1677.10 | 1888.0 ± 958.55 |
| 26.97 | Methyl eicosanoate | Ester | 18.9 ± 10.87 | 45.7 ± 19.04 | 8.1 ± 0.05 | 14.1 ± 6.03 | | |
| 27.25 | 4,8,12,16-tetramethylheptadecan-4-olide | Isoprenoid γ-lactone | 8.0 ± 0.00 | 41.8 ± 17.27 | 37.5 ± 11.51 | 31.2 ± 1.01 | 3773.8 ± 425.01 | |
| 27.34 | Chrysophanol | Anthraquinone | 71.7 ± 0.00 | 93.8 ± 7.92 | 96.8 ± 0.00 | 103.6 ± 33.72 | | |
| 27.62 | 1-tetracosene | Alkene | | 48.3 ± 9.71 | 37.4 ± 0.00 | 24.1 ± 1.97 | | |
| 27.83 | Tetracosane | Alkane | 27.6 ± 12.47 | 77.0 ± 31.08 | 37.6 ± 13.99 | 32.5 ± 12.23 | 10509.2 ± 1424.65 | 4205.9 ± 4162.50 |
| 28.59 | Methyl docosanoate | Ester | 8.1 ± 0.01 | 86.6 ± 6.80 | 26.4 ± 18.27 | 8.1 ± 0.02 | | |
| 28.62 | Pentacosane | Alkane | 19.7 ± 11.64 | 8.1 ± 0.01 | 52.8 ± 5.95 | 14.0 ± 5.94 | 68.1 ± 3.09 | 10199.9 ± 5108.83 |
| 29.25 | 2-phenyl-4-quinolyldiphenyl carbinol | Alcohol | 42.5 ± 0.69 | | 40.6 ± 0.00 | 25.6 ± 0.00 | | |
| 29.36 | Hexacosane | Alkane | 48.0 ± 3.38 | 81.5 ± 15.49 | 61.3 ± 7.72 | 62.4 ± 0.00 | 6652.3 ± 1334.47 | |
| 29.69 | Z-12-pentacosene | Alkene | 34.2 ± 25.91 | | | | | |
| 29.76 | 1,8-dihydroxy-3-methoxy-6-methyl-9,10-anthracenedione | Anthraquinone | 47.5 ± 36 | 174.9 ± 38.51 | 61.7 ± 19.73 | 40.0 ± 10.12 | | |
| 29.86 | Heptacosane | Alkane | 66.8 ± 33.42 | 72.3 ± 17.12 | 59.8 ± 39.76 | 36.2 ± 19.88 | | 44.3 ± 2.59 |
| 30.70 | Octacosane | Alkane | 28.1 ± 20.04 | 71.3 ± 9.34 | 98.3 ± 46.49 | 17.0 ± 8.94 | | |
| 31.04 | Squalene | Triterpene | 27.1 ± 6.62 | 157.2 ± 23.93 | 46.2 ± 11.87 | 43.5 ± 13.37 | 7525.0 ± 292.56 | 5179.2 ± 2490.63 |
| 32.99 | β-tocopherol | Tocopherol | | | | | | 4429.7 ± 0.00 |
| 33.74 | γ-tocopherol | Tocopherol | 25.7 ± 17.32 | 51.4 ± 4.66 | 8.2 ± 0.17 | 8.1 ± 0.04 | 2521.5 ± 1050.64 | |

(*continued on next page*)

**Table 2** (*continued*)

| RT (min) | Compound Identity | Chemical Class | Flower | Leaf | Seedpod | Twig branch | Hexane Fraction | Ethyl acetate Fraction |
|---|---|---|---|---|---|---|---|---|
| 34.21 | Stigmastan-3,5,22-trien | Sterol | 12.1 ± 3.92 | 19.6 ± 11.03 | 18.1 ± 9.70 | 10.7 ± 2.30 | | |
| 34.88 | Cholesterol | Sterol | 10.5 ± 2.40 | 33.3 ± 3.40 | 18.6 ± 5.35 | 8.5 ± 0.34 | | |
| 35.00 | Vitamin E | Tocopherol | 56.2 ± 16.55 | 136.5 ± 16.52 | 16.3 ± 3.40 | 24.6 ± 12.05 | 19223.1 ± 1746.47 | 3139.0 ± 3095.56 |
| 36.79 | Campesterol | Sterol | 30.8 ± 22.60 | 56.8 ± 4.27 | 41.9 ± 20.21 | 16.9 ± 7.45 | 5108.3 ± 103.77 | |
| 37.43 | Stigmasterol | Sterol | 73.8 ± 27.15 | 133.7 ± 23.33 | 100.3 ± 54.99 | 68.8 ± 36.66 | 9904.3 ± 395.62 | |
| 37.44 | Stigmastan-3,5-diene | Steroid | | | | | | 1229.8 ± 1189.04 |
| 38.69 | (3$\beta$, 5$\beta$,24 *S*)-stigmast-7-en-3-ol | Sterol | 56.9 ± 28.76 | 366.8 ± 77.54 | 231.8 ± 135.64 | 42.5 ± 22.21 | 2245.8 ± 171.15 | |
| 38.94 | $\gamma$-sitosterol | Sterol | | | | | 18759.1 ± 1602.13 | |
| 39.50 | $\beta$-amyrin | Triterpene | 48.1 ± 19.97 | 69.5 ± 14.87 | 26.7 ± 12.84 | 15.2 ± 6.63 | 2617.5 ± 192.95 | |
| 39.51 | (3$\alpha$)-12-oleanen-3-yl acetate | Triterpene | 17.5 ± 3.43 | | 25.7 ± 0.00 | | | |
| 39.92 | Stigmastan-7-one | Triterpene | | | 10.0 ± 0.41 | | | |
| 40.05 | Lupen-3-one | Triterpene | 95.0 ± 29.51 | 16.1 ± 7.97 | 19.4 ± 11.30 | 65.5 ± 30.24 | | |
| 40.54 | α-amyrin | Triterpene | 68.4 ± 43.48 | 126.9 ± 15.12 | 75.2 ± 32.74 | 30.8 ± 13.64 | 3982.3 ± 1952.37 | |
| 41.87 | Stigmast-4-en-3-one | Terpene | | 47.8 ± 2.14 | 29.7 ± 11.51 | 58.4 ± 0.00 | 3227.0 ± 491.72 | |

RT (min) = retention time in minutes

branch crude extracts over the control at 100 mg/mL ($\chi2 = 5.10$, df = 1, $P = 0.02$) (Fig. 7A). Additionally, *A. variegatum* adults were attracted to various plant parts at the highest concentration (100 mg/mL) over the control: twig branches ($\chi2 = 5.10$, df = 1, $P = 0.02$), seedpods ($\chi2 = 4.00$, df = 1, $P = 0.05$) (Fig. 7A). However, the leaf at 1 mg/mL ($\chi2 = 13.80$, df = 1, $P < 0.001$) and the flower at 1 mg/mL ($\chi2 = 7.11$, df = 1, $P < 0.01$) (Fig. 7A) significantly repelled *A. variegatum* adults, with moderate comparability to DEET ($\chi2 = 33.52$, df = 1, $P < 0.001$). The relative attraction index of *A. variegatum* adults to the flower at 100 mg/mL (57.58%) exceeded that of the AAAP (51.52) (Supplementary Table S3).

Similarly, *R. appendiculatus* adults preferred the crude flower methanol extract over the control at all concentrations tested, except at 1 mg/mL (Fig. 7B). Adult *R. appendiculatus* significantly preferred the control over seedpods at 25 mg/mL ($\chi2 = 11.85$, df = 1, $P < 0.001$) and twig branches at 1 mg/mL ($\chi2 = 8.01$, df = 1, $P < 0.01$), with moderate comparability to DEET ($\chi2 = 37.70$, df = 1, $P < 0.001$) (Fig. 7B). The twig branches extract significantly attracted *R. appendiculatus* adults at 100 mg/mL ($\chi^2 = 5.98$, df = 1, $P = 0.01$) (Fig. 7B).

### 3.4. Responses of Amblyomma variegatum and Rhipicephalus appendiculatus to solvent fractions of the crude flower extract

There was no significant difference in response between adult male and female ticks of both species ($p > 0.05$) (Supplementary Table S4 and S5). The two species responded differently to hexane, ethyl acetate, and water residual fractions of the crude methanol flower extract tested against the control (Fig. 8 and Supplementary Table S6).

Adult *A. variegatum* were significantly attracted to the hexane fraction at 50 mg/mL ($\chi2 = 15.52$, df = 1, $P < 0.001$) (Fig. 8A). These ticks significantly preferred the solvent (control) over the water residual fraction at 1 mg/mL ($\chi2 = 4.94$, df = 1, $P = 0.03$) (Fig. 8A).

Adult *R. appendiculatus* were attracted to the hexane fraction at 25 mg/mL ($\chi2 = 4.34$, df = 1, $P = 0.04$) (Fig. 8B). These ticks were significantly attracted by the ethyl acetate fraction at 1 mg/mL ($\chi^2 = 17.90$, df = 1, $P < 0.001$) (Fig. 8B) over the control. At 50 mg/mL ($\chi2 = 8.65$, df = 1, $P = 0.003$) (Fig. 8B), the ethyl acetate fraction was the most repellent to *R. appendiculatus* adults.

### 3.5. Responses of Amblyomma variegatum and Rhipicephalus appendiculatus to synthetic compounds

Squalene and linoleic acid were evaluated as potential tick attractants, informed by prior studies identifying them as acarine attractants (Dunn et al., 2019; Yoder et al., 1998). Their inclusion was also informed by their ranking among the top 15 compounds contributing to the differentiation between plant parts, as illustrated by the SIMPER analysis (Fig. 6C). No significant difference in response was observed between adult male and female ticks of both species ($p > 0.05$) (Supplementary Table S7 and S8). Adult ticks exhibited different responses to the tested compound concentrations but responded similarly to the blend of synthetic compounds (Fig. 9 and Supplementary Table S9).

Adult *A. variegatum* significantly preferred squalene ($\chi2 = 22.15$, df = 1, $P < 0.001$), linoleic acid ($\chi2 = 4.50$, df = 1, $P = 0.03$), and the squalene: linoleic acid (1:1) blend ($\chi2 = 13.57$, df = 1, $P < 0.01$) at 1 mg/mL (Fig. 9A). Nevertheless, *A. variegatum* adults significantly avoided the blend at 10 mg/mL ($\chi2 = 8.65$, df = 1, $P < 0.01$) (Fig. 9A).

Adult *R. appendiculatus* were significantly attracted by the squalene: linoleic acid (1:1) blend ($\chi2 = 23.85$, df = 1, $P < 0.001$) at 1 mg/mL but significantly avoided it at 10 mg/mL ($\chi2 = 6.72$, df = 1, $P < 0.01$) (Fig. 9B). The relative attraction index of *R. appendiculatus* adults to the squalene: linoleic acid (1:1) blend (48.84) exceeded their relative attraction to the positive control, AAAP (45.45) (Fig. 9B and Supplementary Table S9).

## 4. Discussion

Molecular identification targeting the *rbcL* locus confirmed the identity of *Senna didymobotrya*. A prerequisite for employing DNA markers in species identification is that species are monophyletic (Hebert et al., 2003). In this study, the *rbcL* locus proved valuable for deducing evolutionary linkages, with the samples of *S. didymobotrya* from various locations in Kenya forming a monophyletic clade (94% bootstrap support).

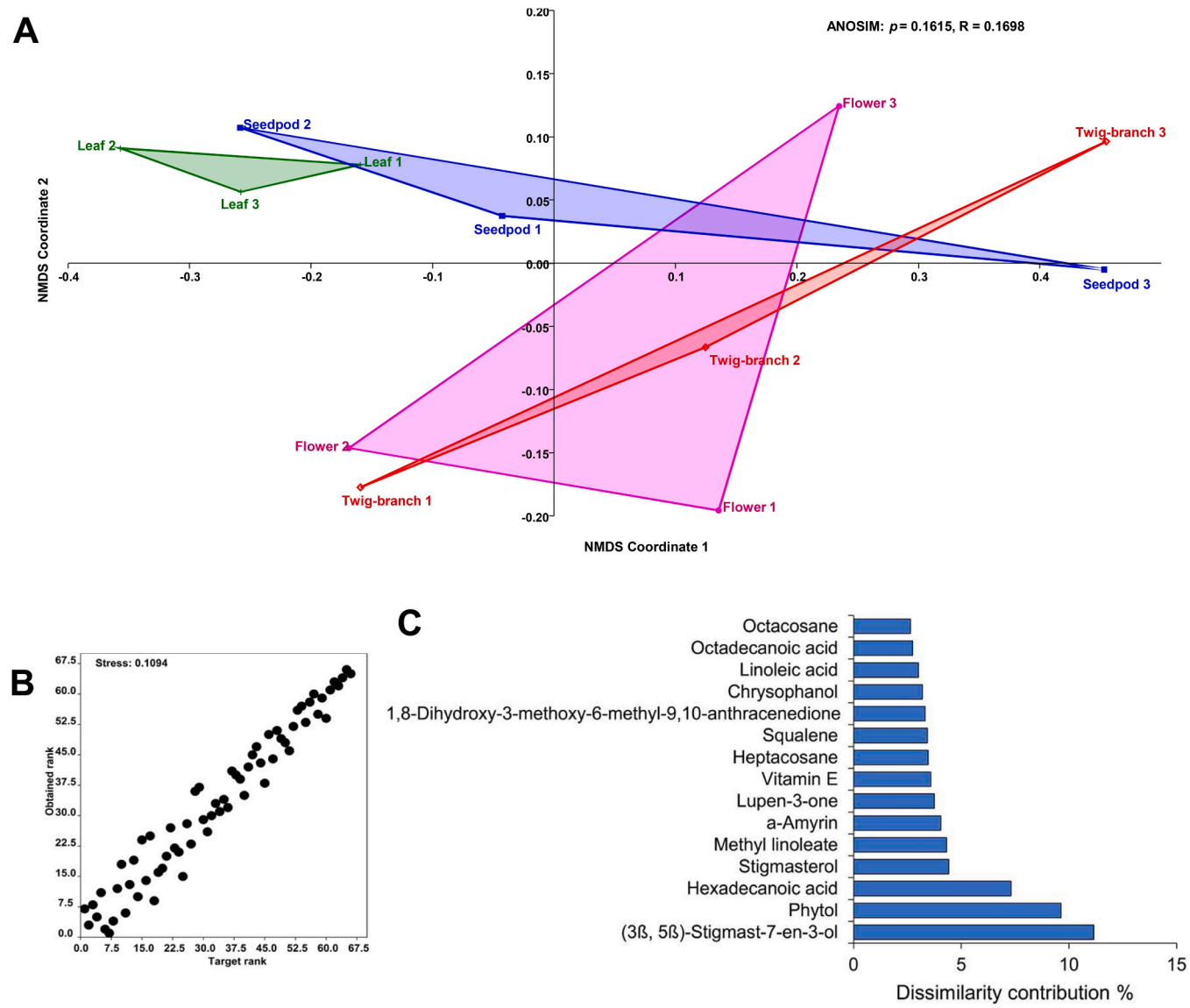


**Fig. 6.** Non-metric multidimensional scaling (NMDS) and similarity percentages (SIMPER) analyses for *Senna didymobotrya*'s chemical composition. (A) NMDS plot using Bray-Curtis dissimilarity measure in flower, leaf, seedpod, and twig branch extracts of *Senna didymobotrya*. (B) Shepard plot with a stress value < 0.2, depicting excellent NMDS ordination. (C) Histogram from SIMPER analysis displaying the top 15 volatiles contributing to differentiation between the plant parts.

While *S. didymobotrya*'s ability to influence the behavioral responses of ticks has been documented (Opiro et al., 2013; Wanzala et al., 2014), the chemical basis behind these responses has remained insufficiently characterized and elucidated. Plant secondary metabolites (PSMs) often exhibit specific phylogenetic patterns, with certain compounds limited to particular plant lineages like genera or families (Piasecka et al., 2015). Within monophyletic clades, PSMs tend to show similarity, resulting in plants within a taxon sharing common metabolite content and bioactive properties (Liu et al., 2017; Zhang et al., 2021). This clarifies why the plant part samples from various collection locations were pooled for analysis.

Plants have previously demonstrated the ability to either repel (Adenubi et al., 2018; Benelli et al., 2016; Nwanade et al., 2020) or attract ticks (Hassan et al., 1994; Nana et al., 2010; Zorloni et al., 2010), which has led to their consideration in integrated tick management. Utilizing plants and their extracts in tick management programs offers advantages, as they are friendly to non-target organisms in the environment, are biodegradable, and consist of a repertoire of chemical compounds that act synchronously on both behavioral and physiological processes, reducing the chances of selection for resistance by target arthropods (George et al., 2014).

Behavioral assays in this study revealed distinct responses from *A. variegatum* and *R. appendiculatus* adults to the different plant part extracts of *S. didymobotrya* and concentrations, regardless of their sex. This differential response may be due to ticks' ability to perceive subtle differences in the extract compositions. Such perceptiveness is crucial to avoid the formation of interspecific aggregations in areas where the species are sympatric (Sonenshine, 2004, 2008) or when they share the same host (Mulenga, 2013). Wanzala et al. (2004) reinforces ticks' sensitivity to different chemical profiles by demonstrating that host-derived attractant and repellent odors guide sympatric *R. appendiculatus* and *R. evertsi* to the inner ears and anal regions, their respective feeding sites on bovids.

Both *A. variegatum* and *R. appendiculatus* adults exhibited a preference for the crude methanol flower extract over the control. This suggests that these ticks likely detected specific behavior-altering chemical signals, prompting them to move in an oriented manner towards the odors emitted by the flower extract. Interestingly, the study's findings align with the discovery that *Anopheles gambiae*, a hematophagous arthropod and malaria vector, also demonstrates an olfactory preference

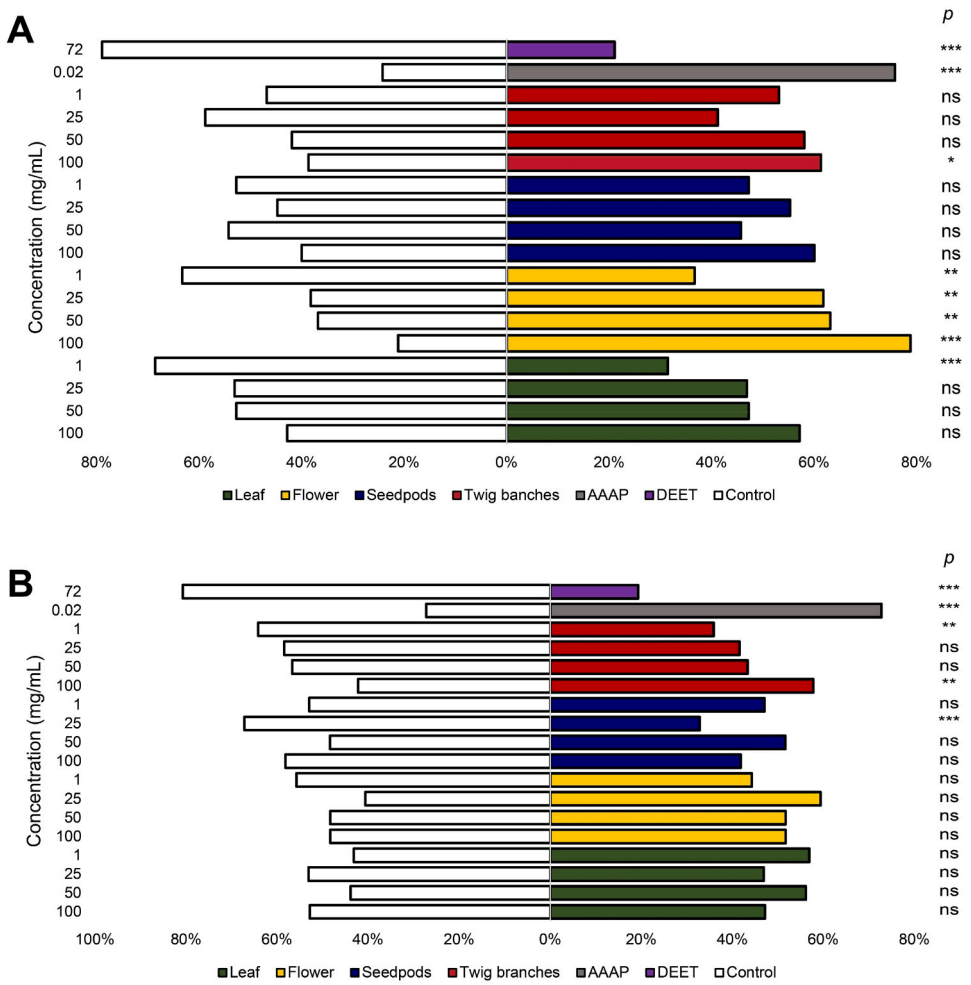


**Fig. 7.** Responses (%) of (A) *Amblyomma variegatum* and (B) *Rhipicephalus appendiculatus* adults to methanol extracts of *Senna didymobotrya*, AAAP and DEET compared to solvent control in olfactometer and tick climbing bioassays, respectively. *p* denotes statistical significance levels: ns (no significant difference, $P > 0.05$), *, **, *** (significant differences at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from $\chi^2$ test at $\alpha = 0.05$.

for *S. didymobotrya* flowers (Nikbakhtzadeh et al., 2016). However, it's essential to consider that despite the shared preference for *S. didymobotrya* flowers by the two tick species and the mosquito *A. gambiae*, their differing biology may influence the reasons behind their preference for the flower.

This study found that at high doses, the flower extract attracted *A. variegatum* while at low doses, it repelled these ticks. However, these findings diverge from previous research by Logan et al. (2008), Xu et al. (2015) and Mogwambo (2013), which observed similar responses in mosquitoes and ticks, respectively, but with opposite dose-dependent effects. The different responses in our study could be a function of the concentration of active compounds and the presence of other compounds in the extracts, which may cause distinct activation of tick chemoreceptors, resulting in different odor codes and hence different behaviors. Further studies are therefore recommended to develop a better understanding of these findings.

The dual-choice assay results also demonstrated significant attraction of *A. variegatum* adults to the twig branch extracts of *S. didymobotrya*. This attraction may be attributed to the similarity in chemical composition between the flower and twig branch extracts, as evidenced by their ordination in the NMDS and their overall average dissimilarity in the SIMPER analyses.

The leaves and seedpod extracts repelled both *A. variegatum* and *R. appendiculatus* ticks at varying concentrations, moderately similar to DEET. Similarly, Opiro et al. (2013) reported avoidance of the methanol extracts (87.7%) of *S. didymobotrya* by *R. appendiculatus* larvae in fingertip bioassays. Essential oil from *S. didymobotrya*'s aerial parts exhibited *in vitro* repellence to *R. appendiculatus*, with approximately 35% and 90% repellence at 0.1 mg and 50 mg dose, respectively (Wanzala et al., 2014). Differences in repellence percentages between this study and prior research may be due to differences in tick developmental stages, extraction methods and solvents used. Repellence of ticks by the leaf and seedpod extracts could be attributed to the presence of repellent compounds within these plant parts. Chemical analysis of the crude methanol extracts of the leaves and seedpods of *S. didymobotrya* showed the presence of known repellents, such as phytol, which is effective against *R. appendiculatus* (Ndungu et al., 1999). Other known repellents of ticks within the leaf and seedpod plants extracts include dodecanoic acid (Bissinger and Roe, 2013) and octacosane (Ndungu et al., 1999). The findings of Faraone et al. (2020) support this study's results that in the presence of antagonistic compounds (both attractants and repellents), as was the case in the leaf and seedpod extracts, the attractant stimuli declines, which deters the ticks' responses.

Adults of *A. variegatum* preferred the hexane fraction of the flower extract, suggesting that the attractant compounds were non-polar (Abubakar and Haque, 2020). Adults of *R. appendiculatus* were significantly attracted by the ethyl acetate fraction, implying that the active compounds were polar/mid-polar (Abubakar and Haque, 2020). Chemical analysis of the ethyl acetate fraction detected 2, 4-bis (1, 1-dimethylethyl) phenol, which probably triggered the attraction of the *R. appendiculatus* adults. Ticks of the genus *Rhipicephalus*, for instance

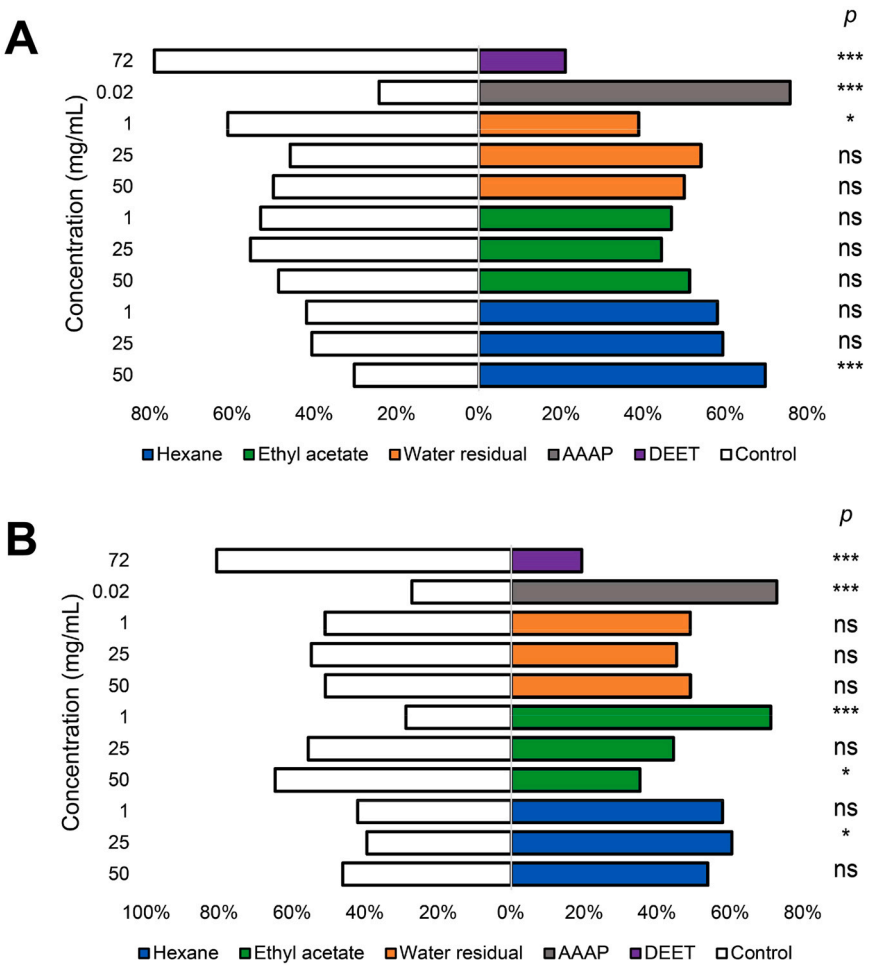


**Fig. 8.** Responses (%) of (A) *Amblyomma variegatum* and (B) *Rhipicephalus appendiculatus* adults to solvent fractions of crude methanol extract of *Senna didymobotrya* flowers, AAAP and DEET against solvent control in olfactometer and tick climbing bioassays, respectively. *p* denotes statistical significance levels: ns (no significant difference, $P > 0.05$), *, **, *** (significant differences at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from $\chi 2$ test at $\alpha = 0.05$.

the cattle tick, *R. microplus*, are known to exhibit a generic attraction response to phenolic compounds (Yoder et al., 2008). However, the response to this compound should be ascertained using other coupled chemical-biological detectors such as coupled gas chromatography-single sensillum recording (GC-SSR), among others.

Squalene, a naturally occurring triterpenoid lipid commonly found in mammalian skin and blood, significantly attracted *A. variegatum* adults in this study. At 1 mg/mL, squalene was highly attractive, but at 10 mg/mL, its attractiveness reduced. This is consistent with reports by Allan (2010) and Bissinger and Roe (2013). Similar concentration-dependent responses were also noted with *A. americanum*, where low squalene concentrations (1%) attracted 72% of ticks, while higher concentrations (10% and 100%) exhibited reduced attraction (62% and 21%, respectively) (Yoder et al., 1999).

Conversely, *R. appendiculatus* adults only preferred squalene over the control, though not significantly, at the lowest dose tested, and there was no observation of a behavioral dose-response. This may be attributed to the fact that squalene potentially acted as an arrestant rather than an attractant, consistent with other *Rhipicephalus* ticks (Carnohan et al., 2016; Yoder et al., 2008). In the current study, the positioning of squalene-treated cotton plugs on top of aluminum rods, along with the release of *R. appendiculatus* adults on the aluminum base, likely prevented direct contact of many *R. appendiculatus* ticks with the plugs, explaining the absence of aggregation. The differing responses of *A. variegatum* and *R. appendiculatus* adults to squalene may be hypothesized to be due to their host searching strategy, with it acting as an attractant to hunters and as an arrestant to ambushers (McMahon and Guerin, 2002).

Linoleic acid, present on the skin surface of humans, cattle, and other mammals infested by ticks (Pappas, 2009; Poon et al., 1978), exhibited dose-dependent behavioral effects on both *A. variegatum* and *R. appendiculatus* adults. *Amblyomma variegatum* displayed non-significant attraction to a lower linoleic acid dose (1 mg/mL), while *R. appendiculatus* exhibited significant attraction to the highest tested dose. This suggests that linoleic acid might function as an attractant for ticks, pending further validation, akin to its role in attracting sheep scab mites (Dunn et al., 2019). A synthetic squalene: linoleic acid blend (1:1) attracted both *A. variegatum* and *R. appendiculatus* ticks at varying concentrations. This observation aligns with Sonenshine's (2008) highlight that specific compound blends in the right ratio can maximize behavioral responses of ticks.

The potential of *S. didymobotrya* extracts to attract ticks presents an opportunity for tick control using the attract and kill strategy, which provides an eco-friendly alternative to traditional acaricides. This strategy involves luring ticks into slow-release formulations or devices containing infect/kill agents, such as entomopathogens or acaricides, respectively, thereby minimizing the quantity of infect/kill agent used and environmental harm (Carr and Roe, 2016). Botanicals such as *S. didymobotrya* emerge as promising attractants for this strategy due to their low toxicity, short persistence, and potential synergistic effects (George et al., 2014). While current research on attractants is constrained, the combination of botanical attractants with

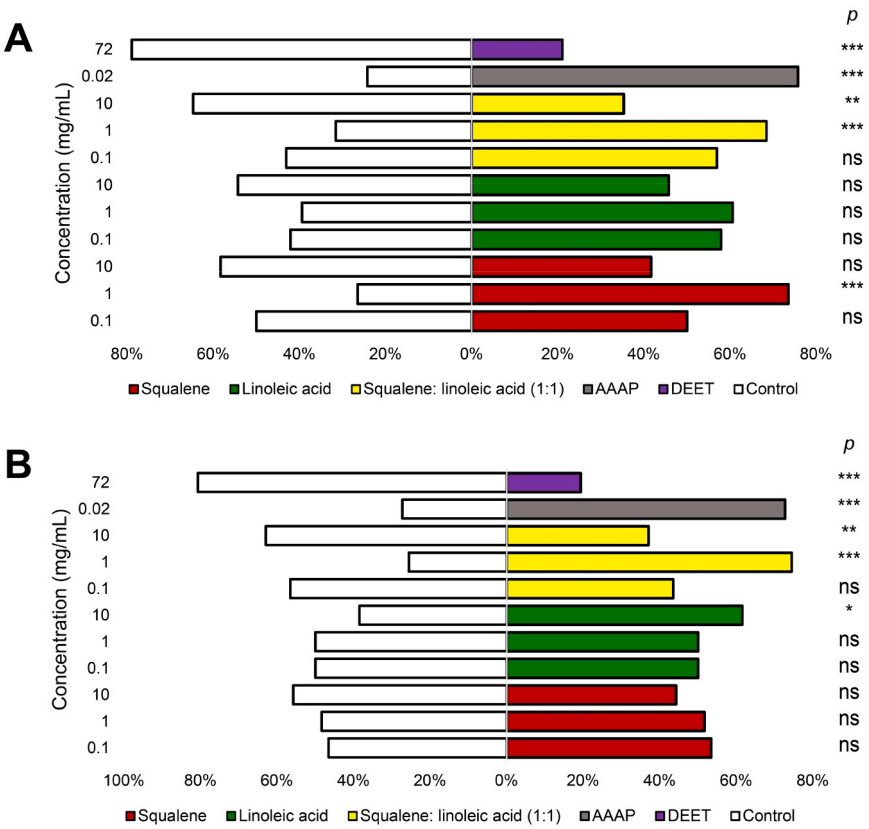


**Fig. 9.** Responses (%) of adult (A) *Amblyomma variegatum* and (B) *Rhipicephalus appendiculatus* to squalene, linoleic acid, squalene: linoleic acid blend (1:1), AAAP and DEET against solvent control in olfactometer and tick climbing bioassays. *p* denotes statistical significance levels: ns (no significant difference, $P > 0.05$), *, **, *** (significant differences at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively) from $\chi^2$ test at $\alpha = 0.05$.

entomopathogenic fungi has been previously investigated (Nana et al., 2010) and appears to offer a sustainable option.

In conclusion, this study used *rbcL* molecular markers to successfully confirm the identities of plant samples as *S. didymobotrya*. Additionally, it documented the attraction of hard ticks (*A. variegatum* and *R. appendiculatus*) to *S. didymobotrya* flower and twig branch extracts, while observing the repellence of leaf and seedpod extracts to these ticks. Through behavioral assays and chemical analysis, potential tick attractants, namely squalene and linoleic acid, were identified within the most attractive extracts and their solvent fractions. To harness these findings for practical tick control, further field experiments are crucial to validate the responses of ticks to these extracts and compounds. This groundwork could facilitate their integration with infect/kill agents, such as entomopathogenic fungi or acaricides, in an off-host attract-and-kill strategy, or in off-host push-pull traps, offering a sustainable approach to tick control.

## Funding

The authors gratefully acknowledge the financial support for this research by the following organizations and agencies: the German Ministry for Economic Cooperation and Development (BMZ) through the Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ) ICTDL grant number: 18.7860.2-001.00; the Swedish International Development Cooperation Agency (Sida); the Swiss Agency for Development and Cooperation (SDC); the Australian Centre for International Agricultural Research (ACIAR); the Norwegian Agency for Development Cooperation (Norad); the German Federal Ministry for Economic Cooperation and Development (BMZ); and the Government of the Republic of Kenya. The views expressed herein do not necessarily reflect the official opinion of the donors.

## CRediT authorship contribution statement

**Joel Ltilitan Bargul:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Xavier Cheseto:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis. **Komivi Senyo Akutse:** Writing – review & editing, Validation, Supervision. **Meshack Amos Obonyo:** Writing – review & editing, Validation, Supervision. **Daniel Masiga:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization. **Diana Wairimu Kinyua:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

The authors appreciate Dr Merid Getahun and Basilio Njiru for providing the olfactory assay equipment. The authors also thank Dennis Getange, Emmanuel Kiarie, Joseck Esikuri, Newton Nyagah and Sospeter Wafula for technical support. An anonymous reviewer improved an earlier version of this manuscript.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at doi:10.1016/j.vetpar.2024.110210.

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