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Antiparasitic properties of leaf extracts derived from selected *Nicotiana* species and *Nicotiana tabacum* varieties

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ABSTRACT

Within the traditional pharmacopeia, tobacco (*Nicotiana* spp.) is often cited as an efficient pesticide. This activity is generally attributed to nicotine, but tobacco plants contain other alkaloids that could potentially contribute to this effect. In this study, we tested methanolic extracts of *N. glutinosa, N. glauca, N. debneyi*, and *N. tabacum* (putrescine N-methyltransferase line, burley TN90 and Stella, Virginia ITB 683 and K326), selected according to alkaloid content. Their antiparasitic activity was evaluated in bioassays against adult fleas (*Ctenocephalides felis*), blowfly (*Lucilia cuprina*) larvae, nematodes (*Caenorhabditis elegans*), and ticks (*Rhipicephalus sanguineus* larvae and adults, *Ixodes ricinus* nymphs). None of the extracts killed fleas and blowfly larvae effectively at the concentrations tested. Only *N. tabacum* K326 and *N. glutinosa* exhibited moderate anthelmintic activity. All extracts significantly repelled *R. sanguineus* ticks, but not *I. ricinus,* and the nicotine-rich extracts rapidly knocked down all tick species and stages at high concentrations. The link between nicotine and tick knockdown was confirmed by successfully testing the pure alkaloid at concentrations found in the tobacco extracts. In contrast, repellent activity could not be correlated to the individually tested alkaloids (nicotine, nornicotine, anabasine, anatabine), although anatabine and nornicotine were active in the tick bioassay at high concentrations.

1. Introduction

Tobacco (*Nicotiana spp.*) leaves, powder, extracts, or fumigants have been used for centuries to control agricultural pests or parasites of medical and veterinary importance (McIndoo, 1943). However, because of safety concerns regarding tobacco's major alkaloid, nicotine, and the discovery of more specific and potent synthetic pesticides, no nicotine-based products are currently commercially available (Bradbury, 2008). Synthetic neonicotinoids are structurally related to nicotine and widely used as agricultural and veterinary pesticides; however, unlike tobacco-related alkaloids, synthetic neonicotinoids have a higher selectivity for the insect nicotinic acetylcholine receptor (nAChR) and reduced binding to vertebrate nicotinic receptors (Tomisawa and Casida, 2005). Their unique physicochemical features (photostability, non-volatility, and hydrophilicity) explain their success as pesticides but also their excessive use, which led to extensive contamination of the environment. Neonicotinoids have now become a major concern for the survival of ecosystems (Hladik et al., 2018). The proven impact on pollinators and aquatic and soil communities (Chagnon et al., 2015), and a more problematic toxicity profile than once perceived (Wang et al., 2018), pushes forward initiatives to limit or totally ban their use in agriculture and move away from the worldwide use of synthetic pesticides.

This desire to find alternatives to the use of synthetic pesticides has rekindled interest in the plant pharmacopeia and identification of more environmentally friendly solutions for pest control. Moreover, limited access to expensive chemicals by less-affluent populations has prompted many groups to review and/or rediscover ethnobotanical drugs of medicinal, veterinary, and crop protection relevance (Adenubi et al., 2018; Benelli et al., 2016; Mvine et al., 2011; Niroumand et al.,

Abbreviations: DEET, N,N-diethyl-m-toluamide; DMSO, dimethyl sulfoxide; nAChR, nicotinic acetylcholine receptor; PMT, putrescine N-methyltransferase; UHPLC-MS, ultra-high-performance liquid chromatography coupled with mass spectrometry

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2016; Rahuman, 2011). As part of the traditional pharmacopeia, tobacco is often cited as an effective pesticide and is also used as an insect or tick repellent to prevent vector-borne diseases (Jufri et al., 2016; Pavela et al., 2016). Nicotine may not be the sole bioactive molecule in tobacco; for example, the anabasine-rich *N. glauca* has been listed as an arthropod repellent (dos Santos Silva et al., 2014). Anti-trypanosomal activity attributed to anabasine has also been observed in parasitized bumble bees feeding on floral nectar containing this alkaloid (Richardson et al., 2018).

Tobacco leaves contain more than 4,000 components, and crude leaf methanolic extracts are very complex mixtures (Eich, 2008; Leffingwell, 1999; Rodgman and Perfetti, 2013). As the activity of mixtures may be quite different from the simple additive effect of individual constituents' activities, due to the likely chemical multiple interactions between molecules (synergy, potentiation, inhibition, or coalition), investigating both mixtures and single major components of the mixtures may further help understanding of the observed biological effects (Hayes et al., 2019).

In the present study, we investigated the bioactivity of extracts of tobacco leaves of varying alkaloid contents and individual alkaloids, including minor entities, for which data on activity against parasites are non-existent. Eight tobacco varieties and species were selected based on their previously known major alkaloid content (Saitoh et al., 1985): four *N. tabacum* nicotine-rich varieties, two grown in the field (burley Stella and Virginia ITB 683) and two grown in the greenhouse (burley TN90 and Virginia K326); one burley transgenic strain from the putrescine N-methyltransferase (PMT) lines, used to obtain an anatabine-enriched extract; and three wild tobacco species containing high levels of anabasine (*N. glauca* and *N. debneyi*) or nornicotine (*N. glutinosa*). Methanolic extracts were prepared from mature tobacco leaves and evaluated for their efficacy against ectoparasites and nematodes in a panel of bioassays. The selection of organisms was based on their representativeness of parasites of medical and veterinary importance, the type of activity (systemic or contact insecticides/acaricides, insect growth regulators, arthropod repellents, anthelmintics) generally searched for in anti-parasitic drug discovery programs (Buckingham et al., 2014; Schorderet Weber et al., 2017), and the availability of bioassays in multi-well plate formats with those organisms. The systemic and contact insecticidal activity was evaluated against adult cat fleas (*Ctenocephalides felis*), insect larvicidal or growth-regulating activity was tested against blowfly (*Lucilia cuprina)* larvae, the anthelmintic potential of the extracts was evaluated against nematodes (*Caenorhabditis elegans*), and repellence/deterrence and contact acaricidal effects were measured against ticks of different species and stages (*Rhipicephalus sanguineus* larvae and adults and *Ixodes ricinus* nymphs). The study aimed at determining: i) whether leaf crude extracts of different tobacco varieties containing complex mixtures of alkaloids and non-alkaloid components had any effect in the bioassays, ii) whether the potential effect observed with the tobacco leaf extract mixtures could be attributed solely to the alkaloids, and iii) and whether a particular alkaloid could be responsible for the observed effect. For this purpose, tobacco alkaloids were quantified in each extract, and full dose-response curves were generated with pure nicotine, nornicotine, anabasine, and anatabine in a concentration range corresponding to the one found in the tobacco leaf crude extracts (Fig. 1).

2. Materials and methods

2.1. Organisms

2.1.1. Tobacco plants

Description of the *Nicotiana* species and varieties are given in Table 1. Selection was based on their alkaloid content, previously known from internal data and literature (Saitoh et al., 1985), and on plant availability at the facility.

2.1.2. Insects, nematodes, and ticks for bioassays

Adult *C. felis* fleas, *L. sericata* blowflies, and *C. elegans* (wild-type) nematodes were sourced from the in-house colonies and cultures of INVENesis. *R. sanguineus* ticks were purchased as fully engorged females or adult unfed ticks from Ecto Services Inc. (Henderson, NC, USA). For egg-laying and larval development, females were maintained at 28 °C and 80% relative humidity. Hatched larvae and adult ticks were kept under the same environmental conditions until use. *I. ricinus* nymphs were purchased at Insect Service GmbH (Berlin, Germany) and kept at 28 °C and 80% relative humidity until use.

2.2. Plant cultivation

Seeds of *N. tabacum* TN90 and K326 cultivars as well as *N. glauca*, *N. glutinosa*, and *N. debneyi* were obtained from internal seed storage. The transgenic TN90 PMT line (06TN2048) was obtained from Altria Client Services LLC (Richmond, VA, USA). PMT lines were produced using *Agrobacterium*-mediated transformation, as described in patent WO2015157359A1 (Kudithipudi et al., 2015). Seeds were sown into soil-containing floating trays. Well-developed plantlets were transferred to 5 L pots and cultivated under a 16/8-h artificial light/dark photoperiod until fully grown.

At the time of flowering, all plants were topped. Two weeks after topping, representative, fully grown leaves were sampled from each plant. All samples were immediately used for crude extract preparation in methanol.

For field cultivation, seeds of *N. tabacum* Stella (burley tobacco) and *N. tabacum* ITB 683 (Virginia tobacco) were obtained from a local source and sown into soil-containing floating trays, grown in the greenhouse, and transplanted to the field according to tobacco agricultural practices. Plantlets were planted at a density of 24,000 per hectare in western Switzerland (canton de Vaud). Leaf samples were collected from fully mature plants two weeks after topping and immediately transported to the laboratory for crude methanol extract preparation.

2.3. Crude extract preparation

All plant leaves were oven-dried at 60 °C for 24 h and disrupted by shaking with glass beads at 400 rpm for 8 h. For each selected tobacco variety/species, 2 g of ground leaf powder were placed in 50 mL glass bottles. Twenty mL of methanol (high-performance liquid chromatography-grade, ≥ 99.9% purity, Sigma-Aldrich, St. Louis, MO, USA) were added to the ground leaves. The mixture was then sonicated (Branson 3510-DTH Ultrasonic Cleaner; Danbury, CT, USA) for 30 min and decanted into a filter column holding a Whatman® filter paper (125 mm Ø, cellulose paper; Maidstone, UK). The filtrate was sonicated with 20 mL of methanol and filtered again. The resulting filtrate was then placed into a rotary evaporator to remove the solvent, and the remaining extract was further lyophilized (Labconco cat. no. 7934030; Kansas City, MO, USA) for 16 h until all water was removed. Methanolic leaf extracts were prepared at either AnalytiCon Discovery GmbH (Potsdam, Germany) or internally, following the same extraction procedure.

2.4. Alkaloid quantification in tobacco crude extracts

Samples for pyridine alkaloid (nicotine, nornicotine, anatabine, anabasine, cotinine, and myosmine) analysis by ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS) were prepared by dissolving approximately 25 mg of crude extract in water/methanol (3:7, with 500 ng/mL quinoline as internal standard; 5 mL), filtering (Fisherbrand™ Sterile PES Syringe Filter with pore size of 0.2 µm; Thermo Fisher Scientific, Waltham, MA, USA), and diluting 1:200 with the extraction mixture. A simultaneous determination of all six alkaloids was performed on an Ultimate 3000 UHPLC system

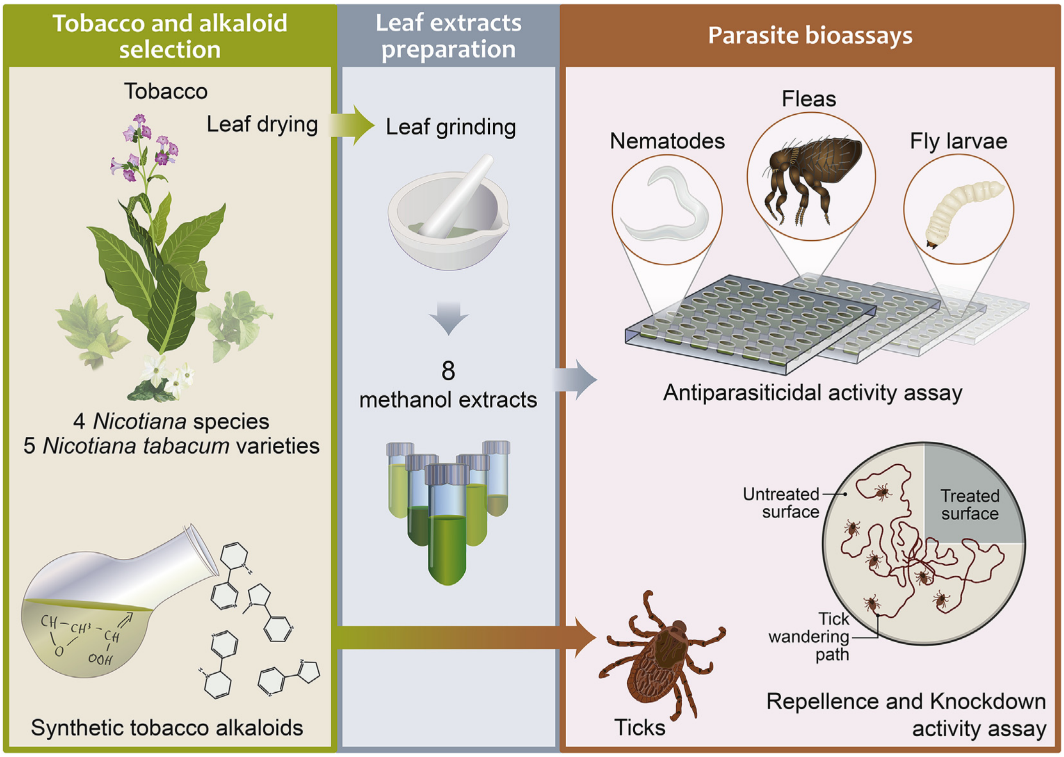


Fig. 1. Tobacco leaf extract and synthetic alkaloid testing in parasite bioassays. In a first step, tobacco species and varieties were selected according to their tobacco alkaloid content. In a second step, methanolic extracts were prepared and evaluated against cat flea, blowfly larva, nematode, and tick bioassays. In a third step, pure synthetic tobacco alkaloids were tested against ticks at similar concentrations than those found in the tobacco leaf extracts to determine whether a particular alkaloid could be solely responsible for the observed effect.

coupled to a Q Exactive™ mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was performed on an Acquity HSS T3 column (1.7 µm, 100 × 2.1 mm; Waters, Milford, MA, USA); the column temperature was set to 45 °C. Eluents were ammonium acetate in water (10 mM; pH 8.9; eluent A) and ammonium acetate in methanol (10 mM; eluent B) applied as a gradient (0 min 10% B; 0.25 min 10% B; 4.25 min 98% B; 5.25 min 98% B; flow: 0.5 mL/min). The injection volume was 5 µL. Nicotine, nornicotine, anabasine, anatabine, cotinine, and myosmine were eluted for 3.89 min, 2.76 min, 3.27 min, 3.36 min, 2.62 min, and 3.47 min, respectively, and detected as [M+H]+ pseudomolecular ions after positive electrospray ionization. No further analysis was conducted to separate and quantify S and R enantiomers of nicotine, nornicotine, anabasine, anatabine, and cotinine in the extracts. The concentration of each target alkaloid was expressed in mg per gram of dry weight. The molar concentration of the target alkaloids in the crude extract was calculated for the concentrations of the crude extracts used in the tick repellent test and was expressed in µmol/m^2 (see section 2.5.6), according to the following formula:

Concentration of target alkaloid (µmol/m^2) = (concentration extract [mg/m^2] × concentration of target alkaloid in extract [µg target alkaloid/mg extract])/molecular weight of target alkaloid [µg/µmol]

2.5. Compounds

2.5.1. Tobacco alkaloids

Tobacco pure alkaloids were used in the tick repellent/knockdown contact test to characterize the activity observed with the tobacco leaf extracts. (S)-Nicotine, (S)-nornicotine, (S)-anabasine, (S)-cotinine, and myosmine standards were purchased from Sigma-Aldrich. (S)-anatabine and (R)-anatabine enantiomers were synthesized by WuXi AppTec Co., Ltd. (Shanghai, China) (Fig. 2).

Racemic anatabine was obtained according to a modified procedure (Deo and Crooks, 1996). The (S)-anatabine and (R)-anatabine enantiomers were separated by chiral supercritical fluid chromatography and converted into hydrochloride salts for improved alkaloid stability.

Stock solutions of the alkaloids were prepared as 20 mM solutions in ethanol immediately prior to use. Stock solutions were kept at 22 °C in the dark for longer-term storage.

2.5.2. Bioassay positive controls

Positive controls (thiamethoxam, dicyclanil, ivermectin, and N,N-

Table 1
Plant characteristics and related crude extract denomination.

Plant species	Strain	Origin	Major alkaloid	Tobacco leaf extract ID
Nicotiana tabacum	PMT	Greenhouse	Anatabine	PMT
N. tabacum	Stella (burley)	Field	Nicotine	Stella
N. tabacum	K326 (Virginia)	Greenhouse	Nicotine	K326
N. tabacum	TN90 (burley)	Greenhouse	Nicotine	TN90
N. tabacum	ITB 683 (Virginia)	Field	Nicotine	ITB 683
N. glutinosa	Wild species	Greenhouse	Nornicotine	Glutinosa
N. glauca	Wild species	Greenhouse	Anabasine	Glauca
N. debneyi	Wild species	Greenhouse	Anabasine	Debneyi

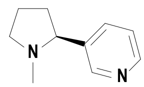
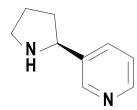
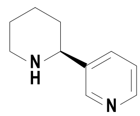
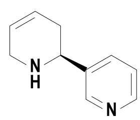
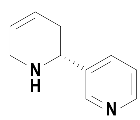
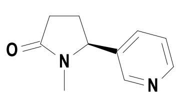
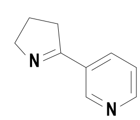
	chemical structure	name	MW
1		(S)-nicotine	162.23
2		(S)-nornicotine	148.21
3		(S)-anabasine	162.23
4		(S)-anatabine dihydrochloride	233.14
5		(R)-anatabine dihydrochloride	233.14
6		(S)-cotinine	176.22
7		myosmine	146.19

Fig. 2. Selected tobacco alkaloids. MW, molecular weight.

diethyl-m-toluamide [DEET]) used in the bioassays were obtained from Merck (Kenilworth, NJ, USA).

2.6. Bioassays

All bioassays were performed by INVENesis Sàrl, Neuchâtel, Switzerland. Concentrations of the extracts in the bioassays were selected according to the organisms' upper limit of dimethyl sulfoxide (DMSO) tolerance or specific bioassay constraints. Positive controls were tested at their minimum effective concentration.

2.6.1. *C. elegans development test (egg to larval stage 4)*

This assay was used as a primary screen to evaluate the anthelminthic potential of a test compound or extract. Plant extracts diluted in DMSO were mixed with the *C. elegans* culture medium at concentrations of 150 μg/mL, 47.5 μg/mL, 15 μg/mL, 4.8 μg/mL, 1.5 μg/mL, and 0.48 μg/mL and distributed into 384-well plates. Approximately 100 eggs of *C. elegans* were deposited in each well of a 384-well plate together with a bacterial suspension of *Escherichia coli* as a food supply. Plates were sealed and incubated at 25 °C for 48 h to allow the development of the nematodes up to the L4 stage. Positive (ivermectin, commercial anthelmintic) and negative (DMSO) controls were evaluated in parallel. The minimum effective concentration of ivermectin was determined using a 12 dilutions range based on data previously obtained in the laboratory. Concentrations of 41.5 μg/mL, 13.1 μg/mL, 4.1 μg/mL, 1.3 μg/mL, 0.41 μg/mL, 0.13 μg/mL, 0.041 μg/mL, 0.013 μg/mL, 0.0041 μg/mL, 0.0013 μg/mL, 0.00041 μg/mL, and 0.00013 μg/mL were used. In the negative control, DMSO alone was mixed with the *C. elegans* culture medium at the same concentration found in the highest plant extract concentration (0.75%). For all plant extracts, positive and negative controls, each concentration tested was run in triplicate. The effect of the treatment was measured by monitoring nematode motility with an automated data acquisition system. Efficacy was expressed as the percent motility reduction compared with motility in the negative controls. An 80% reduction in nematode motility was considered significant.

2.6.2. *L. sericata development test (egg to L3 stage)*

The assay was used as a primary screen to evaluate the insect growth regulator potential of a test compound or extract, targeting insects that are pests or parasites during their larval stage.

A protein-rich artificial medium treated with the plant extracts diluted in DMSO was distributed into 96-well plates at concentrations of 65 μg/mL, 20.6 μg/mL, 6.5 μg/mL, 2.1 μg/mL, 0.65 μg/mL, and 0.21 μg/mL. Approximately 10 freshly laid *L. sericata* eggs were deposited in each well on top of the treated food and left to develop for three days under conditions optimal for larval development. Positive (dicyclanil, commercial insect growth regulator) and negative (DMSO) controls were evaluated in parallel. The minimum effective concentration for dicyclanil was determined using a 6 dilutions range based on data previously obtained in the laboratory. Concentrations of 3.96 μg/mL, 1.25 μg/mL, 0.40 μg/mL, 0.13 μg/mL, 0.04 μg/mL, and 0.013 μg/mL were used. In the negative control, DMSO alone was mixed with the medium at the same concentration found in the highest plant extract concentration (0.33%). For all plant extracts, positive and negative controls, each concentration tested was run in triplicate. The effect of the treatment on development and survival was automatically assessed by motility quantification. Efficacy was expressed as the categorical percent motility reduction compared with motility in the negative controls, with values of 0%, 60%, 80%, and 100%. An 80% reduction in motility was considered significant.

2.6.3. *C. felis adult oral test*

The assay was used as a primary screen to evaluate the systemic insecticidal potential of a test compound or extract. Approximately 10 *C. felis* of a mixed sex adult population are placed per well in a suitably formatted 96-well plate, allowing the fleas to access and feed on blood treated with the plant extracts diluted in DMSO at concentrations of 200 μg/mL, 63.3 μg/mL, 20 μg/mL, 6.33 μg/mL, 2 μg/mL, and 0.63 μg/mL via an artificial feeding system. Fleas were fed on treated blood for 24 h. Positive (thiamethoxam, a commercial neonicotinoid insecticide with systemic and contact activity) and negative (DMSO) controls were evaluated in parallel. The minimum effective concentration for thiamethoxam was determined using a 6 dilutions range based on data previously obtained in the laboratory. Concentrations of 1.85 μg/mL, 0.58 μg/mL, 0.18 μg/mL, 0.058 μg/mL, 0.018 μg/mL, and 0.0058 μg/mL were used. In the negative control, DMSO alone was mixed with the blood at the same concentration found in the highest plant extract concentration (1%). For all plant extracts, positive and negative controls, each concentration tested was run in triplicate. The effect of the treatment was assessed by monitoring flea motility by machine vision. Efficacy was expressed as the percent motility reduction compared with motility in the negative controls, with 100% meaning no movement recorded. An 80% reduction in motility was considered significant.

2.6.4. *C. felis adult contact test*

The assay was used as a primary screen to evaluate the contact insecticidal potential of a test compound or extract. Plant extracts diluted in methanol were deposited onto the bottoms of wells in a 96-well plate and the solvent left to evaporate. The final concentration of the test compound was, therefore, expressed in mg/m^2 and set at 565 mg/m^2, 179 mg/m^2, 56.6 mg/m^2, 17.9 mg/m^2, 5.65 mg/m^2, and 1.79 mg/m^2. Approximately five fleas of a mixed-sex adult population are placed in each well and left in contact with the treated surface for 24 h. Positive (thiamethoxam) and negative (DMSO) controls are evaluated in parallel. The minimum effective concentration for thiamethoxam was determined using a 6 dilutions range based on data previously obtained in the laboratory. Concentrations of 3.84 mg/m^2, 1.21 mg/m^2, 0.39 mg/m^2, 0.122 mg/m^2, 0.038 mg/m^2, and 0.012 mg/m^2 were used. For all plant extracts, positive and negative controls, each concentration tested was run in triplicate. The effect of the treatment was assessed by machine vision and monitoring flea motility after 24 h of exposure. Efficacy was expressed as the percent motility reduction compared with motility in the negative controls, with 100% meaning no movement recorded. An 80% reduction in motility was considered significant.

2.6.5. Tick repellent/knockdown contact test

The assay is used to evaluate the repellent/deterrent potential of a test compound or extract. The test includes a preliminary assessment of knockdown efficacy as a secondary endpoint. Knockdown can be defined as a state of intoxication and paralysis that usually precedes death (Wickham et al., 1974). The test relies on the questing behavior of ticks that explore their habitat to find a suitable host hunting site and their propensity to avoid areas treated with repellent or irritating substances. Tick knockdown is expressed as a reduction in total motility over a defined period of time and not by collecting and counting the number of dead and live ticks at the end of the exposure period.

Plant extracts or synthetic pure alkaloids diluted in methanol (extracts) or ethanol (alkaloids) were deposited on the bottom of wells in a six-well plate. Only one quadrant (2.54 cm^2) of the bottom surface of each well was treated. The solvent was left to evaporate. The final concentration of the plant extracts was, therefore, expressed in mg/m^2, or in μmol/m^2 for the synthetic alkaloids. Final concentrations applied for plant extracts were 893 mg/m^2 (*R. sanguineus* adults only), 446 mg/m^2, 141 mg/m^2, 44.6 mg/m^2, 14.1 mg/m^2, and 4.46 mg/m^2, and 300 μmol/m^2, 100 μmol/m^2, 30 μmol/m^2, 10 μmol/m^2, and 3 μmol/m^2 for pure alkaloids. Approximately 50 *R. sanguineus* tick larvae and exactly five *R. sanguineus* adults or five *I. ricinus* nymphs were added to the untreated area of each well. Positive (DEET, commercial tick repellent) and negative (100% methanol (leaf extracts) or ethanol (pure alkaloids)) controls were evaluated in parallel. In the study with plant extracts, DEET was only tested at the concentration of 8.5 mg/m^2. From the laboratory history, this concentration always resulted in significant repellence greater than the 50% threshold, and is used here as bench mark. In the bioassay with the pure alkaloids, DEET was tested as the same concentrations as used for the alkaloids, 300 μmol/m^2, 100 μmol/m^2, 30 μmol/m^2, 10 μmol/m^2, and 3 μmol/m^2 (respectively 57.4 mg/m^2, 19.1 mg/m^2, 5.7 mg/m^2, 1.9 mg/m^2, and 0.57 mg/m^2). For all plant extracts, pure alkaloids, positive and negative controls, each concentration tested was run in three or more test replicates. After a 1-min incubation on a heating plate, the frequency of tick movement in the treated quadrant and in each of the untreated quadrants was recorded by machine vision. Tick movement on the untreated surface was computed as the median of movement on the three untreated quadrants. To estimate deterrence, recordings were performed over a period of 2 min for *R. sanguineus* larvae and for 3 min for *R. sanguineus* adults and *I. ricinus* nymphs after the 1-min pre-exposure period. To assess knockdown effect, recording was extended to 9 min (starting at the 1-min pre-exposure period and lasting an additional 8 min).

Deterrence activity was calculated as follows:

Percent deterrence = 1 − (movements on treated area / movements on untreated area) × 100

A difference of 50% between total movements recorded in the treated area versus movements recorded in the untreated area was considered significant.

The tick knockdown effect was calculated similarly:

Percent knockdown = 1 − (movements at the end of the imaging period / movements at the beginning of the imaging period) × 100

A difference of 50% between total movements recorded during the 1-min pre-exposure and total movements measured at 9 min of recording was considered significant.

2.7. Statistical analysis

The median of replicate measurements of the parasiticidal activity observed with the tobacco crude extracts was used in the statistical analysis (SAS 9.2, SAS Institute Inc., NC, USA). As a first step, a multicollinearity analysis was performed and led to the exclusion of cotinine in the further statistical analysis. Linear models were then used with a stepwise inclusion of predictors, as well as their second-order interaction, to predict the role of the alkaloid in the observed efficacy.

3. Results

3.1. Parasite bioassays with tobacco leaf crude extracts

None of the extracts showed any efficacy against insects (adult cat fleas, oral and contact bioassays, and blowfly larvae) tested in the bioassays. No systemic or contact activity was observed, even at the highest concentration tested (Tables 2–4). The respective positive controls, dicyclanil and thiamethoxam, were both active at concentrations at least 1,000 times lower than the highest extract concentration tested (minimum effective concentrations of positive controls: < 0.013 μg/mL for dicyclanil; 0.058 μg/mL (oral flea), and 0.038 μg/mL (contact flea) for thiamethoxam).

When the tobacco extracts were tested on *C. elegans* nematodes, two extracts, Glutinosa and K326, were active at the first (150 μg/mL) and second (47.5 μg/mL) dilutions tested, respectively (Table 5). These activities remained low compared with the activities of ivermectin, which was still positive at 0.0013 μg/mL. However, crude extracts are complex mixtures, and the activity could be attributable to other compounds present in low quantities, which could exert strong activity against nematodes at higher concentrations. No further investigation was conducted with those two extracts.

All eight tobacco leaf extracts showed significant repellent activity against *R. sanguineus* larvae over several dilutions (Fig. 3A). Five

Table 2
Percentage of activity and minimum effective concentrations of tobacco extracts in the primary screen against *L. sericata* larvae.

Test Item	Concentration [μg/mL]	Percentage of activity [%]	Minimum effective concentration [μg/mL]
PMT	65	5	> 65
Stella	65	2	> 65
K326	65	2	> 65
TN90	65	0	> 65
IBT 683	65	0	> 65
Glutinosa	65	4	> 65
Glauca	65	4	> 65
Debneyi	65	2	> 65
Dicyclanil	3.96	100	< 0.013
	1.25	100	
	0.40	100	
	0.13	100	
	0.04	100	
	0.013	100	
DMSO (0.33%)		1	–

The symbol > indicates that the minimum effective concentration exceeded the highest concentration tested. The minimum effective concentration is the smaller concentration for which the calculated median of activity is greater than the limit of activity significance (80%). The median of activity was calculated from three test replicates.

Table 3
Percentage of oral activity and minimum effective concentrations of tobacco extracts in the primary screen against adult *C. felis*.

Test Item	Concentration	Percentage of activity	Minimum effective concentration
	[μg/mL]	[%]	[μg/mL]
PMT	200	2	> 200
Stella	200	0	> 200
K326	200	0	> 200
TN90	200	0	> 200
IBT 683	200	2	> 200
Glutinosa	200	2	> 200
Glauca	200	0	> 200
Debneyi	200	2	> 200
Thiamethoxam	1.85	100	0.058
	0.58	100	
	0.18	100	
	0.058	100	
	0.018	72	
	0.0058	58	
DMSO (1%)		0	–

The symbol > indicates that the minimum effective concentration exceeded the highest concentration tested. The minimum effective concentration is the smaller concentration for which the calculated median of activity is greater than the limit of activity significance (80%). The median of activity was calculated from three test replicates.

Table 4
Percentage of contact activity and minimum effective concentrations of tobacco extracts in the primary screen against adult *C. felis*.

Test Item	Concentration	Percentage of activity	Minimum effective concentration
	[mg/m^2]	[%]	[mg/m^2]
PMT	565	21	> 565
Stella	565	12	> 565
K326	565	6	> 565
TN90	565	34	> 565
IBT 683	565	56	> 565
Glutinosa	565	3	> 565
Glauca	565	6	> 565
Debneyi	565	3	> 565
Thiamethoxam	3.84	100	0.038
	1.21	100	
	0.39	100	
	0.12	99	
	0.038	95	
	0.012	2	
DMSO		0	–

The symbol > indicates that the minimum effective concentration exceeded the highest concentration tested. The minimum effective concentration is the smaller concentration for which the calculated median of activity is greater than the limit of activity significance (80%). The median of activity was calculated from three test replicates.

extracts (Stella, K326, TN90, ITB 683, and Glauca) had minimum effective concentrations similar to or lower than those of the positive control, DEET. Moreover, Stella and TN90 rapidly knocked down 74% and 77% of tick larvae, respectively, at a concentration of 446 mg/m^2. At same concentration, two other extracts, K326 and ITB 683, knocked down 42% and 48% of the ticks, respectively.

Concentration curve responses for all extracts were bell shaped, and the highest concentration (446 mg/m^2) was not the most effective. Although the knockdown activity exhibited by half of the extracts could have influenced tick motility during repellence recording and biased the evaluation of repellence, this effect was also observed in extracts without knockdown activity. In the bioassay, ticks were restricted to a closed area without possibility to escape. At high concentrations, a

Table 5
Percentages of activity and minimum effective concentrations of tobacco extracts in the primary screen against nematodes.

Test Item	Concentration	Percentage of activity	Minimum effective concentration
	[μg/mL]	[%]	[μg/mL]
PMT	150	0	> 150
Stella	150	0	> 150
K326	150	97	47.5
	47.5	95	
	15	0	
TN90	150	0	> 150
IBT 683	150	0	> 150
Glutinosa	150	98	150
	47.5	38	
Glauca	565	0	> 150
Debneyi	565	0	> 150
Ivermectin	41.5	98.5	0.0013
	13.1	99	
	4.10	99	
	1.3	99	
	0.41	99	
	0.13	99	
	0.041	98.5	
	0.013	98	
	0.0041	97	
	0.0013	90	
	0.00041	33	
	0.00013	19	
DMSO (0.75%)		0	N/A

The symbol > indicates that the minimum effective concentration exceeded the highest concentration tested. The minimum effective concentration is the smaller concentration for which the calculated median of activity is greater than the limit of activity significance (80%). The median of activity was calculated from three test replicates.

tobacco extract deposited on one quadrant of the test well could influence bigger parts of the well and, depending of the vapor pressure of its multiple constituents, could saturate the total atmosphere of the well. Exposed ticks would try to escape this irritant environment without finding an exit, hence generating more movements in the well, including in the treated surface.

Based on these positive results, the eight tobacco leaf extracts were further tested against adult *R. sanguineus* (Fig. 3B) and nymphs of *I. ricinus* (Fig. 3C) to evaluate their spectrum of activity on other tick stages and species.

In *R. sanguineus* adult ticks (Fig. 3B), the repellent activity of the extracts was generally lower than that observed with larvae (Fig. 3A). Six extracts (K326, TN90, ITB 683, Glutinosa, Glauca, and Debneyi) exhibited significant repellence at the highest concentration of 893 mg/m^2. The minimum effective concentrations based on the median efficacy was 141 mg/m^2 for TN90, 44.6 mg/m^2 for K326 and ITB 683, and 14 mg/m^2 for Glauca. Tick knockdown was observed with Stella extract over three concentrations (893 mg/m^2 to 44.6 mg/m^2) and with TN90 at 893 mg/m^2 and 446 mg/m^2. Tick knockdown also occurred with Glauca and Debneyi extracts at 14 mg/m^2 and 44.6 mg/m^2, respectively.

In the *I. ricinus* nymph tick bioassay (Fig. 3C), few extracts exerted significant tick repellence. Stella and K326 were active at 446 mg/m^2, and TN90 was active at 44.6 mg/m^2. In addition, Glutinosa showed borderline activity over two concentrations (446 mg/m^2 and 141 mg/m^2). Tick knockdown was, on the contrary, very high (between 89% and 99.8%) in Stella, K326, TN90, and ITB 683 at the highest tested concentration of 446 mg/m^2, and 70% of the ticks were still affected by 141 mg/m^2 TN90 leaf extract.

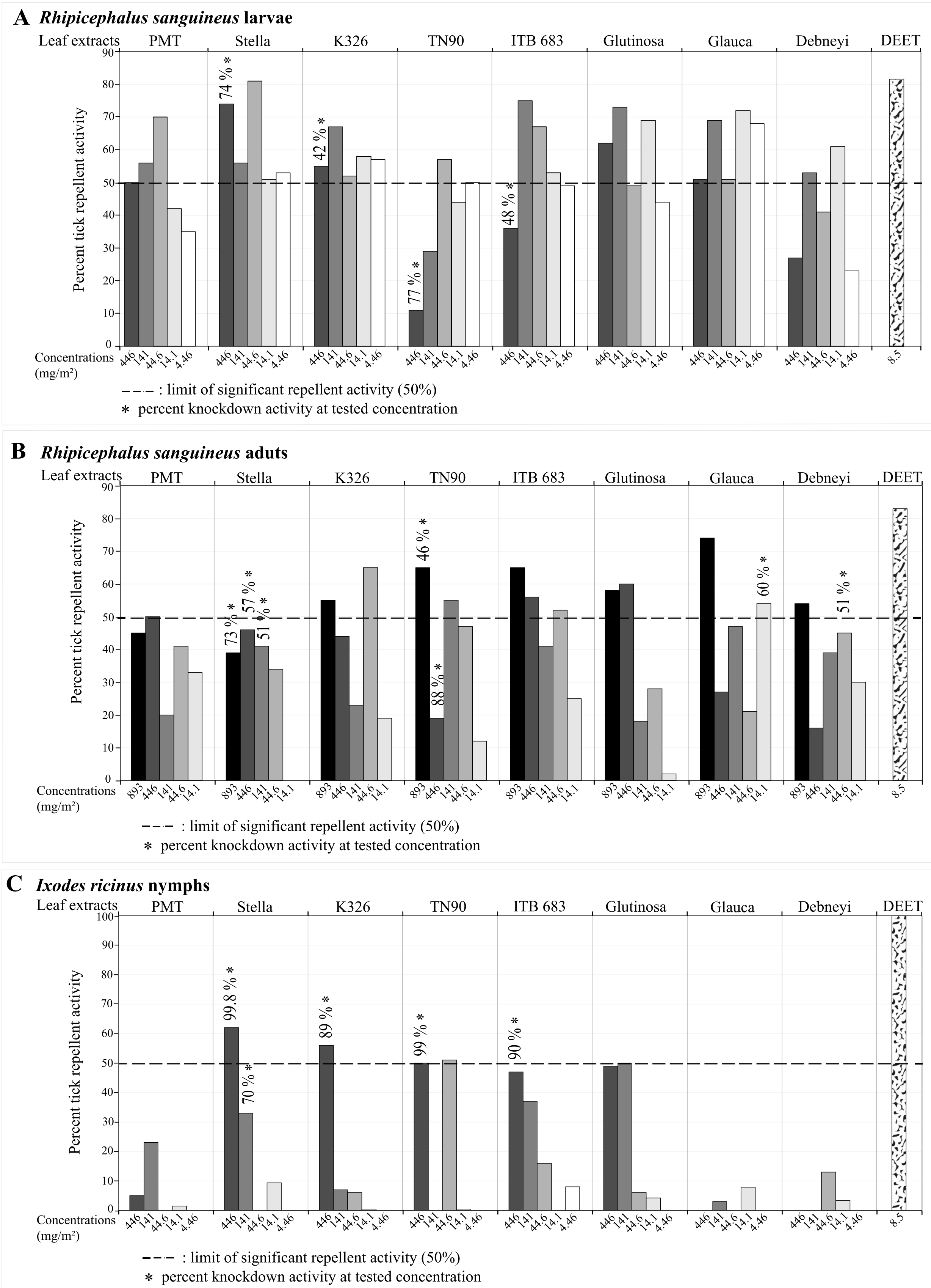


Fig. 3. Repellent activity of eight tobacco leaf extracts against (A) *R. sanguineus* tick larvae, (B) *R. sanguineus* adult ticks, and (C) *I. ricinus* nymphs. For each concentration, median efficacies of three test replicates are shown. DEET was used as a positive control and tested only at 8.5 μg/m^2 . *, percent of tick knockdown activity observed at the tested concentration in addition to repellence. Repellence is monitored for 2 min, starting 1 min after ticks are released into the treated well. Knockdown is determined as the difference in motility between the first minute and the ninth minute after tick release.

Table 6
Pyridine alkaloid concentrations in tobacco extracts expressed as mg per gram of crude extract weight.

Extract	Alkaloid					
	Nicotine	Nornicotine	Anabasine	Anatabine	Cotinine	Myosmine
PMT	3.95 (18.12)	0.3 (1.37)	0.78 (3.56)	**16.68 (76.55)**	0.04 (0.2)	0.04 (0.2)
Stella	**108.38 (93.47)**	1.88 (1.62)	1.01 (0.87)	4.55 (3.92)	0.1 (0.09)	0.03 (0.02)
K326	**48.93 (92.87)**	0.96 (1.82)	0.55 (1.04)	2.16 (4.09)	0.07 (0.14)	0.02 (0.04)
TN90	**90.9 (92.67)**	1.45 (1.48)	1.1 (1.12)	4.48 (4.57)	0.11 (0.11)	0.06 (0.06)
ITB 683	**38.82 (87.8)**	0.47 (1.07)	1.65 (3.73)	3.18 (7.19)	0.06 (0.13)	0.04 (0.08)
Glutinosa	11.02 (65.24)	**4.78 (28.32)**	0.27 (1.6)	0.55 (3.23)	0.22 (1.31)	0.05 (0.31)
Glauca	4.67 (46.76)	0.15 (1.47)	**5.02 (50.28)**	0.09 (0.95)	0.03 (0.26)	0.03 (0.28)
Debneyi	6.57 (81.25)	0.37 (4.59)	**0.88 (10.93)**	0.07 (0.92)	0.1 (1.19)	0.09 (1.11)

Values in parentheses denote the percentage composition of all measured alkaloids. The value of the major pyridine alkaloid in each plant extract is in bold.

3.2. Quantification of selected alkaloids in tobacco extracts

The species in genus *Nicotiana*, including *N. tabacum* varieties, accumulate alkaloids to various extents (Saitoh et al., 1985; Sisson and Severson, 1990). Among the multitude of tobacco constituents (carbohydrates, amino acids, pyridine alkaloids, pigments, isoprenoids, terpenoids, carboxylic acids, polyphenols, sterols, and inorganic compounds (Leffingwell, 1999)), alkaloids trigger particular interest due to the antiparasitic history of nicotine. To understand if alkaloids would play a role in the biological activity observed against ticks and which alkaloid may be responsible for this efficacy, we quantified the main pyridine alkaloids in the selected tobacco plants (nicotine, nornicotine, anabasine, anatabine, cotinine, and myosmine) using UHPLC-MS (Table 6).

Nicotine was the major alkaloid in crude extracts of *N. tabacum* field-grown Stella and ITB 683 varieties and greenhouse-grown TN90 and K326 varieties. Anatabine was the major alkaloid in the crude extract of the transgenic *N. tabacum* TN90 PMT line, but nicotine also constituted a sizable portion of the alkaloid pool in this variety (18.1%). The highest concentration of nornicotine (4.78 mg/g) was measured in the *N. glutinosa* extract (Glutinosa), but nicotine (11.02 mg/g) was the major alkaloid in this extract. The highest concentration of anabasine was found in the *N. glauca* extract (Glauca, 5.02 mg/g), but nicotine was present in this extract at a similar quantity and proportion. Anabasine was also relatively highly concentrated as a portion of total measured alkaloids in the *N. debneyi* extract (Debneyi, 0.88 mg/g), although nicotine was the major compound (6.57 mg/g).

To understand the potential role of the major alkaloids in the tobacco crude extracts tested for anti-tick activities, we analyzed full dose-response curves with the pure alkaloids (S)-nicotine, (S)-nornicotine, (S)-anabasine, (S)-anatabine, and (R)-anatabine in the tick repellent/knockdown bioassay. To establish an appropriate concentration range for this assay, the quantity of each alkaloid measured in each extract dilution (4.46–893 mg/m^2) was transformed into molar concentrations. Alkaloid concentration ranges in the crude extracts were 1–596 μmol/m^2 nicotine, 0.14–28.8 μmol/m^2 nornicotine, 0.024–27.6 μmol/m^2 anabasine, and 0.5–93 μmol/m^2 anatabine.

3.3. Tick repellent/knockdown efficacy of selected pure alkaloids against R. sanguineus larvae

(S)-nicotine, (S)-nornicotine, (S)-anabasine, (S)-anatabine, and (R)-anatabine were tested at 300 μmol/m^2, 100 μmol/m^2, 30 μmol/m^2, 10 μmol/m^2, and 3 μmol/m^2, covering most of the concentrations measured in the eight tobacco leaf extracts. Cotinine and myosmine were not considered for testing, as they constituted no more than 1.3% of the total alkaloid content of the extracts (see Table 6). Three independent test sets with triplicates were generated (Table 7). All pure alkaloids, with the exception of (S)-anabasine, exhibited repellent activity against *R. sanguineus* larvae at 300 μmol/m^2. Interestingly, (S)-anabasine was more active at 100 μmol/m^2, with a median of 58% activity. (S)-nornicotine was only active at the highest tested concentration of 300 μmol/m^2 (81% activity). The best tick repellent effects were observed with (R)-anatabine (62% at 30 μmol/m^2, 84% at 100 μmol/m^2) and (S)-anatabine (54% at 30 μmol/m^2, 86% at 100 μmol/m^2). At 30 μmol/m^2, (S)-nicotine and (R)-anatabine still exerted 55% and 62% repellence, respectively. These results were comparable with the effects of the positive control, DEET, at same concentration, the median percentage of activity for DEET being 64% at 30 μmol/m^2. DEET, however, remained more active at 10 μmol/m^2 than the two isomers of anatabine, with a median of 57% (versus medians of 34% and 38% for (R)- anatabine and (S)-anatabine, respectively).

(S)-nicotine and (R)-anatabine effectively knocked down *R. sanguineus* larvae at 300 μmol/m^2 (medians of 95% and 53%, respectively) (Table 7). At 100 μmol/m^2, the median activity of (R)-anatabine was still 55% but dropped down below the limit of significance for nicotine, with a median of 37%. No other significant tick knockdown effect could be observed with the other pure alkaloids.

3.4. Contribution of the tobacco major alkaloids in tick repellence/knockdown activity of the leaf extracts, statistical analysis

Tobacco plant alkaloid content is influenced by many factors, such as plant variety, batch, growing conditions in greenhouse, location, air pollution and weather conditions for field varieties, and storage and drying conditions. Crude leaf extracts derived from those plants are very complex mixtures with unknown interactions between constituents. All contribute to the variability observed in the bioassays, making an assessment of the role of individual constituents of the leaf extracts very difficult. Keeping in mind those limitations, we investigated the hypothetical contribution of the major alkaloids quantified in the eight different tobacco leaf extracts in the activity observed in the tick repellent/knockdown bioassay. Bioassay data from *R. sanguineus* larvae and adults and from *I. ricinus* nymph ticks were combined and analyzed using linear statistical models. Tick species and stage influence on the activity of the leaf extracts was also evaluated. Due to variability within the three replicates, the median was used in the statistical analysis for tick repellence and knockdown.

In the linear model used to predict knockdown activity, nicotine explained 35% of the variance of the model and was significantly correlated with the knockdown activity of the leaf extracts ($p < 0.0001$). Tick species and stage modestly influenced the model, explaining only 2.3% of the variance ($p < 0.05$). In the linear model used to predict repellence, no alkaloid could be identified as predominantly influencing the activity observed with the extracts tested. However, tick species and stage significantly impacted the repellent activity of the extracts, being responsible for 51% of the variance of the model ($p < 0.0001$).

For nicotine, the outcome of the model was in accordance with the results obtained with the pure alkaloid, although only tested against *R. sanguineus* tick larvae (see Table 6). Pure (S)-nicotine knocked down 95% of the tick larvae at 300 μmol/m^2 (48.7 mg/m^2) and 37% at

Table 7
Repellent and knockdown activity of pure tobacco alkaloids against *R. sanguineus* tick larvae.

Compound	Concentration [µmol/m²]	Repellence			Knockdown		
		Median (Q1–Q3) [%]	N	Minimum effective concentration [µmol/m²]	Median (Q1–Q3) [%]	N	Minimum effective concentration [µmol/m²]
(S)-nicotine	300	**56.2 (36.5–81.9)**	9	300–30	**94.5 (80.3–100)**	9	300
	100	27.8 (0–67.9)	6		37.1 (10.2–73.9)	6	
	30	**55.0 (13.6–85.0)**	9		26.4 (2.1–42.4)	9	
	10	41.8 (36.7–53.0)	6		1.3 (0.2–18.1)	6	
	3	18.1 (3.2–27.3)	6		3.4 (0–9.9)	6	
(S)-nornicotine	300	**81.1 (70.1–93.1)**	9	300	18.7 (8.8–48.6)	9	> 300
	100	42.2 (20.4–68.3)	6		9 (6.45–18.7)	6	
	30	27.9 (4.5–81.9)	9		9.9 (6.4–13.3)	9	
	10	35.3 (27.3–48.5)	6		0.4 (0–17.2)	6	
	3	12.3 (2.2–18.4)	6		0.7 (0.1–7.8)	6	
(S)-anabasine	300	40.5 (11.1–65.1)	9	> 300–100	9.5 (6.7–14.4)	9	> 300
	100	**58.0 (55.2–63.1)**	6		6.0 (0.9–9.0)	6	
	30	46.9 (30.4–92.6)	9		10.4 (7.5–24.4)	9	
	10	42.4 (28.8–60.8)	6		6.9 (0.9–13.2)	6	
	3	6.6 (0.7–18.0)	6		0 (0–0)	6	
(S)-anatabine dihydrochloride	300	**79.8 (72.6–83.8)**	9	30	14.2 (0–24.0)	9	> 300
	100	**86.2 (66.5–99.3)**	6		23.8 (18.6–32.8)	6	
	30	**54.4 (34.7–58.5)**	9		14.7 (3.2–21.2)	9	
	10	38.1 (28.1–49.4)	6		4.2 (2.4–12.6)	6	
	3	38.4 (26.3–46.9)	6		0 (0–10.1)	6	
(R)-anatabine dihydrochloride	300	**88.3 (79.6–94)**	9	30	**52.7 (32.8–60.1)**	9	100
	100	**84.3 (77.5–87.8)**	6		**54.5 (35.2–61.2)**	6	
	30	**62.0 (37.8–69.1)**	9		11.1 (1.3–16.8)	9	
	10	34.4 (24.3–49.2)	6		7.5 (0–26.0)	6	
	3	20.8 (3.5–32.8)	6		0 (0–0)	6	
DEET	300	**100 (100–100)**	9	10	11.1 (3.1–17.7)	9	> 300
	100	**75.7 (67.5–86.8)**	6		5.2 (0.8–17.3)	6	
	30	**63.9 (55.3–94.5)**	9		2.4 (0–6.4)	9	
	10	**57.3 (42.6–65.1)**	6		10.0 (4.4–15.2)	6	
	3	31.1 (28.1–32.2)	6		0.7 (0–17.2)	6	
Ethanol	Solvent control	9.7 (0–24.5)	27	nd	13.4 (5.4–26.6)	28	nd

Q1, first quartile (25%) of the dataset. Q3, third quartile (75%) of the dataset. N, number of test replicates. nd, not determined. The minimum effective concentration is the smaller concentration for which the calculated median of activity is greater than the limit of activity significance (50%). The percentage values exceeding the limit of activity significance are bolded.

100 µmol/m² (16.2 mg/m²), as was also observed in the crude extracts exerting lethal effects at 446 mg/m² against the same tick species and stage (TN90: 77%, 40.5 mg/m² nicotine; Stella: 74%, 48.3 mg/m² nicotine; K326: 42%, 21.8 mg/m² nicotine; ITB 683: 48%, 17.3 mg/m² nicotine). The knockdown effect of the leaf extracts containing high levels of nicotine was also observed at 446 mg/m² against *R. sanguineus* adults (Stella and TN90) and *I. ricinus* nymphs (Stella, K326, TN90, and ITB 683).

Although (R)-anatabine as a pure compound displayed significant knockdown activity at 300 µmol/m² and 100 µmol/m², anatabine was not identified in the model to have an influence on the effect observed with the leaf extracts. Anatabine was not analyzed in the statistical model as two separate enantiomers but rather as a racemate; this alkaloid only accounted for 13.1 µmol/m² in Stella, 11.1 µmol/m² in TN90, 7.9 µmol/m² in ITB 683, and 5.35 µmol/m² in K326 at a leaf extract concentration of 893 mg/m², 10 times below the minimum effective concentration limit of the pure alkaloid. In addition, no particular knockdown effect could be observed in the anatabine-rich PMT leaf extract for the same reasons.

In terms of repellence, the minimum effective concentrations of the extracts ranged from under 4.7 mg/m² to 44.6 mg/m² for *R. sanguineus* larvae. Alkaloids tested as pure compounds were generally not active at concentrations equivalent to those measured in the crude extracts (molarity of the corresponding alkaloid at crude extract minimum inhibitory concentration: 3 µmol/m² nicotine, 0.45 µmol/m² nornicotine, 0.14 µmol/m² anabasine, and 4.7 µmol/m² anatabine). As repellence exerted by the leaf extracts was lower by a factor of 3–10 against *R. sanguineus* adults and almost never reached the bioassay significance limit against *I. ricinus* nymphs, no comparison of activity could be drawn between leaf extracts and pure alkaloids. This difference in sensitivity between tick species and stages was also strongly pointed out by the statistical model.

4. Discussion

In *N. tabacum* burley (TN90 and Stella) and Virginia (K326, ITB 683) varieties, nicotine is the most predominant alkaloid, as frequently reported in agricultural cultivation. In this study, the transgenic *N. tabacum* TN90 PMT line accumulated anatabine as the major alkaloid, confirming previously reported effects of transformation (Kudithipudi et al., 2015). The concentrations of alkaloids in the crude extracts of wild species were similar to those reported earlier in the literature (Eich, 2008; Saitoh et al., 1985; Sisson and Severson, 1990). Anabasine is rarely the major alkaloid in species of the genus *Nicotiana*, and particularly not in leaves (Kaminski, personnal communication). Apart from genetic factors, growing environment may also affect alkaloid accumulation and composition (Saitoh et al., 1985; Sisson and Severson, 1990), and studies on these factors are lacking. Targeting nornicotine in wild species is difficult, as it rarely occurs in leaves as a major alkaloid. From previous studies, *N. glutinosa* was selected as representing a good alkaloid mixture, with nornicotine accounting for 5% of the total alkaloid content. *N. tabacum* converter plants could be used in future experiments, because almost all nicotine is converted to nornicotine during leaf senescence.

Although tobacco extracts have been used for centuries to control insect pests (Bradbury, 2008), in this study, all of the tobacco leaf extracts were inactive against cat fleas and blowflies at the concentrations tested. Claims for the efficacy of nicotine against Diptera and Siphonaptera often mention highly concentrated nicotine or tobacco leaf extract preparations (McIndoo, 1943). It is likely that the concentrations used in this study were not sufficient to exert effects. Nonetheless, significant activity of aqueous tobacco extract against *Musca domestica* fly larvae within 48 h has been reported at 3 μg/mL (Ogbalu et al., 2014). Different test species, bioassay setups, and extraction methods may account for this discrepancy in results. In addition, nicotine is known to be fairly selective, and not all insect species are affected by this alkaloid at low concentrations (El-Wakeil, 2013).

Nicotiana spp. have been reported to exhibit anthelmintic activity (Iqbal et al., 2006; Sastya et al., 2017). Aqueous or methanolic extracts of powdered leaves were successfully tested off-host and *in vivo* against gastrointestinal nematodes in sheep. Similarly, two of our leaf extracts, Glutinosa and K326, were active against *C. elegans* at 150 μg/mL and 47.5 μg/mL, respectively. *C. elegans* and parasitic nematodes both express nAChRs in their central nervous systems (Holden-Dye et al., 2013), suggesting that the observed anthelmintic activity may be attributable to nicotine and related tobacco alkaloids. However, in our study, the two extracts effective against *C. elegans* differed in their nicotine and nornicotine contents, while other extracts with higher nicotine yields were completely inactive at the concentrations tested (e.g., Stella and TN90). Non-alkaloid tobacco constituents, such as tannins, saponins, reducing sugars, flavonoids, and triterpenes, were not investigated in our study and may be responsible for the observed activity (Sastya et al., 2017).

The most striking findings of this study were obtained when tobacco crude extracts were tested against *R. sanguineus* tick larvae. In the scope of revisiting traditional pharmacopeia and identifying alternatives to synthetic acaricides, *N. tabacum* and *N. glauca* have been investigated with success against the cattle ticks *R. (Boophilus) microplus* (Castelblanco Sepulveda et al., 2013; dos Santos Silva et al., 2014; Mansingh and Williams, 1998) and *R. appendiculatus* (Dipeolu and Ndungu, 1991). Our results confirmed these observations and highlighted nicotine as the alkaloid predominantly responsible for rapid tick knockdown. The nicotine-rich Stella, K326, TN90, and ITB 683 extracts knocked down ticks efficiently within minutes at a concentration of 446 μg/m^2. This effect was also observed when these leaf extracts were tested against *R. sanguineus* adults and *I. ricinus* nymph ticks. Although the major nicotine-derived synthetic neonicotinoids targeting insect nAChRs are inactive against ticks (Meinke, 2001), nicotine appears to be less specific and, in our case, more active against ticks than against insects. Although less investigated, ticks do possess nAChRs in their synganglion that appear to differ from insect nAChRs, hence their insensitivity to neonicotinoids (Erdmanis et al., 2012). A functional nAChR has now been characterized in the *R. sanguineus* synganglion, opening perspectives for new targets in the tick central nervous system (Lees et al., 2014). The knockdown effect obtained with the two anabasine-enriched extracts Glauca and Debneyi at 14 mg/m^2 and 44.6 mg/m^2 against *R.sanguineus* adults are more difficult to interpret and may only be false positives. The two extracts previously had no knockdown activity on the more sensitive *R. sanguineus* larvae, and the effect was not observed at higher concentrations. Additional testing would be necessary to verify those results.

All tobacco extracts were repellent against *R. sanguineus* tick larvae, sometimes at concentrations equivalent to or lower than the positive control, DEET (Stella, K326, TN90, and Glauca). However, the repellent effect exerted by leaf extracts was 3–10 times lower in *R. sanguineus* adults and almost never reached the bioassay significance limit against *I. ricinus*. Repellent efficacy on other tick species and stages has been reported previously, with a 40% ethyl acetate extract of *N. tabacum* being active against *Hyalomma marginatum rufipes* adult ticks (Magano et al., 2011). The minimum evaluation time was 10 min, and the mg concentration of the extract was not specified. In our study, repellence was measured only for 2 min, and we do not know if the concentrations used were equivalent to those used by Magano et al. or lower. Additional assays, with higher concentrations and/or designed for longer exposure, may be worth conducting with some of the eight tobacco extracts, in particular the nornicotine-containing Glutinosa, to understand why they did not respond more positively in our bioassay against *I. ricinus* nymphs, as this tick species and stage are the principal vector of Lyme disease to humans in Europe (Földvari, 2016).

Unlike nicotine and tick knockdown, repellence could not be correlated to any of the major tobacco alkaloids. When tested as pure alkaloids against *R. sanguineus* larvae in the tick bioassay, significant repellence was observed for all compounds, but at much higher concentrations than measured in the extracts at their minimal effective concentrations. Molecular interactions resulting in synergy or potentiation between some or all alkaloids, together with other non-alkaloid tobacco plant constituents, could be responsible for the repellent activity recorded with the extracts. However, the same leaf extracts were clearly less effective on *R. sanguineus* adults and *I. ricinus* nymphs, and molecular potentiation could become debatable in this case. As the pure alkaloids were not tested against *R. sanguineus* adults and *I. ricinus* nymphs, attempting further comparison with the results obtained with the tobacco leaf extracts was considered to be unsound.

Assessing repellence is related to the questing behavior of ticks and to their host specificity. For example, all instars of *R. sanguineus* prominently feed on dogs and occasionally on other mammals, including humans (Dantas-Torres, 2010). *I. ricinus* nymphs are more ubiquitous and feed on a wide variety of hosts, such as birds, reptiles, and mammals, including humans (Földvari, 2016). It may be envisaged that both species of ticks do not react similarly to the same olfactory stimuli. In addition, a same species and stage may react differently to various molecules, as observed in a study testing commercial repellents and natural products against *I. ricinus* nymphs (Kröber et al., 2013). The physicochemical properties of single repellent molecules and their biological effect on ticks and other blood-feeding arthropods are still subject to various controversial hypotheses (Del Fabbro and Nazzi, 2013). Assessing very complex extract mixtures and not individual molecules complicates the picture to its maximum. Nonetheless, a very low vapor pressure seems to be an important feature for triggering repellence, and tobacco alkaloids would fulfill this requirement. Moreover, the molecular structure itself and the steric and chemical interactions with the tick olfactory receptors could be the most important parameter (Del Fabbro and Nazzi, 2013). Nothing has been yet investigated on most alkaloids or complex leaf extract mixtures.

Among the tested pure alkaloids, (S)-anatabine and (R)-anatabine exhibited the lowest minimum effective concentration against *R. sanguineus* larvae, close to that of DEET. (R)-anatabine also significantly knocked down ticks at 300 μmol/m^{2}and 100 μmol/m^2. However, the PMT extract containing the highest levels of anatabine was also the one with the highest minimum effective concentration for repellence, and no knockdown activity was recorded with this leaf extract. Although enriched in the PMT tobacco variety, the quantity of anatabine contained in the corresponding extract remained 10 times below the minimum effective concentration of the pure alkaloid. (R)-anatabine only accounted for about 15% of total anatabine, as previously measured in burley tobacco leaves (Armstrong et al., 1999). Therefore, it is not surprising that no knockdown activity could be observed with the PMT leaf extract. Nonetheless, our study is the first report of a significant activity of anatabine on arthropods of medicinal and veterinary importance. Its mode of action, likely to be related to nAChRs, remains to be confirmed in ticks. In addition, preliminary results with *Aedes aegypti* mosquitoes confirm and broaden the repellent potential of anatabine (results not shown). However, additional testing on other arthropod species and stages is still required to further characterize its spectrum of action.

Anatabine is known to exert beneficial effects on the mammalian central nervous system, improving memory and cognition (Ferguson et al., 2017), and displays anti-inflammatory properties by regulating the nuclear factor-kappa B pathway and pro-inflammatory cytokine production (Paris et al., 2013). In a recent study performed on human α7 and α4β2 nAChRs (Alijevic et al., submitted), (S)-anatabine and (R)-anatabine showed a different receptor affinity profile than nicotine. (R)-anatabine (EC_{50} 3.4 μM, I_{max} 21.6%) was a more potent α4β2 nAChR partial agonist than (S)-anatabine (EC_{50} 7.7 μM, I_{max} 8.3%). Unlike nicotine, however, none of the enantiomers activated the α7 nAChR. Moreover, ticks utilize monoamine oxidase as an important metabolic pathway for biogenic amines (Kaufman and Sloley, 1996). Substantial monoamine oxidase inhibition by anatabine and nornicotine (Williams et al., 1999) might contribute into the observed tick repellent effect. A better safety profile than nicotine due to differentiated mode of action could promote this minor tobacco alkaloid as a candidate for vector-borne disease prevention by repelling ticks and mosquitoes away from their potential hosts.

In summary, the antiparasitic activity of tobacco leaf extracts was confirmed against nematodes and ticks, but surprisingly not against the insect species tested. All tobacco extracts containing high levels of nicotine efficiently and rapidly knocked down ticks in a similar way to when pure nicotine was tested. All our extracts, including field varieties and wild species, showed strong repellence against ticks, justifying their ethnobotanical use in many countries where synthetic commercial acaricides and repellents are too expensive (Pavela et al., 2016). As the effect could not be solely attributed to the presence of one particular alkaloid and was stage- and species-specific, further studies are needed to understand the mechanism of repellence against ticks effected by the tobacco leaf extracts and to unravel the difference in sensitivity between stages and species. The pure alkaloids, particularly anatabine, were also active in the tick bioassay, but attributing complex mixture effects to individual chemical entities was not possible with the subset of alkaloids and tobacco leaf extracts tested at the experimental stage. Nevertheless, new repellents of natural origin that are safer than the current standard DEET remain a topic of great interest, especially in the scope of vector-borne disease prevention (Bissinger and Roe, 2010; Schorderet Weber et al., 2017).

Declaration of interest

All authors, except J.-L. Perret, are employees of Philip Morris International. Philip Morris International is the sole source of funding and sponsor of this study.

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References

Adenubi, O.B., Ahmed, A.S., F.O, F., McGaw, L.J., Eloff, J.N., Naidoo, V., 2018. Pesticidal plants as a possible alternative to synthetic acaricides in tick control: a systematic review and meta-analysis. Ind. Crops Prod. 123, 779–806. https://doi.org/10.1016/j.indcrop.2018.06.075.

Armstrong, D.W., Wang, X., Lee, J.-T., Lui, Y.-S., 1999. Enantiomeric composition of nornicotine, anatabine, and anabasine in tobacco. Chirality 11, 82–84. https://doi.org/10.1002/(SICI)1520-636X(1999)11:1%3C82::AID-CHIR14%3E3.0.CO;2-C.

Benelli, G., Canale, A., Mehlhorn, H., 2016. Tick repellents and acaricides of botanical origin: a green roadmap to control tick-borne diseases? Parasitol. Res. 115, 2545–2560. https://doi.org/10.1007/s00436-016-5095-1.

Bissinger, B.K., Roe, R.M., 2010. Tick repellents: past, present, and future. Pestic. Biochem. Physiol. 96, 63–79. https://doi.org/10.1016/j.pestbp.2009.09.010.

Bradbury, S., 2008. Reregistration eligibility decision (RED) document for nicotine. In: U.S.E.P.A. (EPA).

Buckingham, S.D., Partridge, F.A., Satelle, D.B., 2014. Automated, high-throughput, motility analysis in *Caenorhabditis elegans* and parasitic nematodes: applications in the search for new anthelmintics. Int. J. Parasitol.: Drug and Drug Resistance 4, 226–232. https://doi.org/10.1016/j.ijpddr.2014.10.004.

Castelblanco Sepulveda, L., Sanabria Rodriguez, O.J., Cruz Carillo, A., Rodriguez Molano, C.E., 2013. Preliminary report of the ixodicidal effect of some plant extracts on ticks *Boophilus microplus*. Rev. Cubana Plantas Med. 18, 118–130.

Chagnon, M., Kreuzweiser, D., Mitchell, E.A.D., Morrissey, C.A., Noome, D.A., Van der Sluijs, J.P., 2015. Risks of large-scale use of systemic insecticides to ecosystem functioning and services. Environ. Sci. Pollut. Res. 22, 119–134. https://doi.org/10.1007/s11356-014-3277-x.

Dantas-Torres, F., 2010. Biology and ecology of the brown dog tick, *Rhipicephalus sanguineus*. Parasites Vectors 3, 26. https://doi.org/10.1186/1756-3305-3-26.

Del Fabbro, S., Nazzi, F., 2013. From chemistry to behavior. Molecular structure and bioactivity of repellents against *Ixodes ricinus* ticks. PLoS One 8. https://doi.org/10.1371/journal.pone.0067832.

Deo, N.M., Crooks, P.A., 1996. Regioselective alkylation of N-(diphenylmethylidine)-3-(aminomethyl)pyridine: a simple route to minor tobacco alkaloids and related compounds. Tetrahedron Lett. 37, 1137–1140.

Dipeolu, O.O., Ndungu, J.N., 1991. Acaricidal activity of kupetaba, a ground mixture of natural products, against *Rhipicephalus appendiculatus*. Vet. Parasitol. 38, 327–338. https://doi.org/10.1016/0304-4017(91)90144-K.

dos Santos Silva, F., Albuquerque, U.P., Costa Junior, L.M., da Silva Lima, A., Borba di Nascimento, A.L., Monteiro, J.M., 2014. An ethnopharmacological assessment of the use of plants against parasitic diseases in humans and animals. J. Ethnopharmacol. 155, 1332–1341. https://doi.org/10.1016/j.jep.2014.07.036.

Eich, E., 2008. Solanaceae and Convolvulaceae: Secondary Metabolites. Biosynthesis, Chemotaxonomy, Biological and Economic Significance. Springer-Verlag, Berlin.

El-Wakeil, N., 2013. Botanical pesticides and their mode of action. Gesunde Pflanz. 65, 125–149. https://doi.org/10.1007/s10343-013-0308-3.

Erdmanis, L., O'Reilly, A.O., Williamson, M.S., Field, L.M., Turberg, A., Wallace, B.A., 2012. Association of neonicotinoid insensitivity with a conserved residue in the loop D binding region of the tick nicotinic acetylcholine receptor. Biochemistry 51, 4627–4629. https://doi.org/10.1021/bi300522s.

Ferguson, S., Mouzon, B., Paris, D., Aponte, D., Abdullah, L., Stewart, W., Mullan, M., Crawford, F., 2017. Acute or delayed treatment with anatabine improves spatial memory and reduces pathological sequelae at late time-points after repetitive mild traumatic brain injury. J. Neurotrauma 34, 1676–1691.

Földvari, G., 2016. Life cycle and ecology of *Ixodes ricinus:* the roots of public health importance. In: Braks, M.A.H., van Wieren, S.E., Takken, W., Sprong, H. (Eds.), Ecology and Prevention of Lyme Borreliosis. Wageningen Academic. https://doi.org/10.3920/978-90-8686-838-4.

Hayes, A.W., Li, R., Hoeng, J., Iskandar, A., Peitsch, M.C., Dourson, M.L., 2019. New approaches to risk assessment of chemical mixtures. Toxicol Res Applic 3, 1–10. https://doi.org/10.1177/2397847318820768.

Hladik, M.L., Main, A.R., Goulson, D., 2018. Environmental risks and challenges associated with neonicotinoid insecticides. Environ. Sci. Technol. 52, 3329–3335. https://doi.org/10.1021/acs.est.7b06388.

Holden-Dye, L., Joyner, M., O'Connor, V., Walker, R.J., 2013. Nicotinic acetylcholine receptors: a comparison of the nAChRs of *Caenorhabditis elegans* and parasitic nematodes. Parasitol. Int. 62, 606–615. https://doi.org/10.1016/j.parint.2013.03.004.

Iqbal, Z., Lateef, M., Jabbar, A., Ghayur, M.N., Gilani, A.H., 2006. *In vitro* and *in vivo* anthelmintic activity of *Nicotiana tabacum* L. leaves against gastrointestinal nematodes of sheep. Phytother Res. 20, 46–48. https://doi.org/10.1002/ptr.1800.

Jufri, M., Irmayanti, E., Gozan, M., 2016. Formulation of tobacco based mosquito repellent to avoid dengue fever. Int J PharmTech Res 9, 140–145.

Kaufman, R., Sloley, D., 1996. Catabolism of dopamine and 5-hydroxytryptamine by monoamine oxidase in the ixodid tick, *Amblyomma hebraeum*. Insect Biochem. Mol. Biol. 26, 101–106. https://doi.org/10.1016/0965-1748(95)00069-0.

Kröber, T., Bourquin, M., Guerin, P.M., 2013. A standardised *in vivo* and *in vitro* test method for evaluating tick repellents. Pestic. Biochem. Physiol. 107, 160–168. https://doi.org/10.1016/j.pestbp.2013.06.008.

Kudithipudi, C., Hayes, A.J., Lusso, M.F.G., Morris, J.W., 2015. Tobacco Having Altered Leaf Properties and Methods of Making and Using.

Lees, K., Jones, A.K., Matsuda, K., Akamatsu, M., Sattelle, D.B., Woods, D.J., Bowman, A.S., 2014. Functional characterisation of a nicotinic acetylcholine receptor α subunit from the brown dog tick, *Rhipicephalus sanguineus*. Int. J. Parasitol. 44, 75–81. https://doi.org/10.1016/j.ijpara.2013.11.002.

Leffingwell, J.C., 1999. Basic chemical constituents of tobacco leaf and differences among tobacco types. In: Davis, D.L., Nielson, M.T. (Eds.), Tobacco: Production, Chemistry, and Technology. Blackwell Science, pp. 265–284.

Magano, S.R., Mkolo, M.N., Shai, L.J., 2011. Repellent properties of *Nicotiana tabacum* and *Eucalyptus globoidea* against adults of *Hyalomma marginatum rufipes*. Afr. J. Microbiol. Res. 5, 4508–4512. https://doi.org/10.5897/AJMR11.334.

Mansingh, A., Williams, L.A.D., 1998. Pesticidal potential of tropical plants - II. Acaricidal activity of crude extracts of several Jamaican plants. Int. J. Trop. Insect Sci. 18, 149–155. https://doi.org/10.1017/S1742758400007797.

McIndoo, N.E., 1943. Insecticidal Use of Nicotine and Tobacco. A Condensed Summary of the Literature, 1690-1934. Agricultural Research Administration. Bureau of

Entomology and Plant Quarantine, pp. 1–16. https://doi.org/10.5962/bhl.title.122351.

Meinke, P., 2001. Perspectives in animal health: old targets and new opportunities. J. Med. Chem. 44, 641–659. https://doi.org/10.1021/jm990564h.

Mvine, J., Van Damme, P., Kamoga, G., Kudamba, Nasuuna, M., Jumba, F., 2011. Ethnobotanical survey of pesticidal plants used in South Uganda: case study of Masaka district. J. Med. Plants Res. 5, 1155–1163.

Niroumand, M.C., Farzaei, M.H., Karimpour-Razkenari, E.E., Amin, G., Khanavi, M., Akbarzadeh, T., Shams-Ardekani, M.R., 2016. An evidence-based review on medicinal plants used as insecticide and insect repellent in traditional Iranian medicine. Iran. Red Crescent Med. J. 18, e22361. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4867175/pdf/ircmj-18-02-22361.pdf.

Ogbalu, O.K., Bobmanuel, R.B., Membere, O., 2014. Larvicidal effect of aqueous leaf extract of tobacco (*Nicotiana tabacum*) on the third instar larvae of *Musca domestica*. IOSR-JAVS 7, 35–40. https://doi.org/10.9790/2380-071243540.

Paris, D., Beaulieu-Abdelaahad, D., Abdullah, L., Bachmeier, C., Ait-Ghezala, G., Reed, J., Verma, M., Crawford, F., Mullan, M., 2013. Anti-inflammatory activity of anatabine via inhibition of STAT3 phosphorylation. Eur. J. Pharmacol. 698, 145–153. https://doi.org/10.1016/j.ejphar.2012.11.017.

Pavela, R., Canale, A., Mehlhorn, H., Benelli, G., 2016. Application of ethnobotanical repellents and acaricides in prevention,control and management of livestock ticks: a review. Res. Vet. Sci. 109, 1–9. https://doi.org/10.1016/j.rvsc.2016.09.001.

Rahuman, A.A., 2011. Efficacies of medicinal plant extracts against blood-sucking parasites. In: Mehlhorn, H. (Ed.), Nature Helps. Springer-Verlag, Berlin Heidelberg, pp. 19–53. https://doi.org/10.1007/978-3-642-19382-8_2.

Richardson, L.L., Adler, L.S., Leonard, A.S., Andicoechea, J., Regan, K.H., Anthony, W.E., Manson, J.S., Irwin, R.E., 2018. Secondary metabolites in floral nectar reduce parasite infections in bumblebees. Proc R Soc B 282. https://doi.org/10.1098/rspb.2014.2471.

Rodgman, A., Perfetti, T.A., 2013. The Chemical Components of Tobacco and Tobacco Smoke, second ed. CRC Press, Boca Raton.

Saitoh, F., Noma, M., Kawashima, N., 1985. The alkaloid contents of sixty *Nicotiana* species. Phytochemistry 24. https://doi.org/10.1016/S0031-9422(00)80751-7.

Sastya, S., Kumar, R.R., Vatsya, S., 2017. Evaluation of anthelmintic efficacy of *Nicotiana tabacum* against gastrointestinal nematodes of goats. Int J Curr Microbiol App Sci 6, 780–789. https://doi.org/10.20546/ijcmas.2017.610.094.

Schorderet Weber, S., Noack, S., Selzer, P.M., Kaminsky, R., 2017. Blocking transmission of vector-borne diseases. Int J Parasitol Drug Drug Resist 7, 90–109. https://doi.org/10.1016/j.ijpddr.2017.01.004.

Sisson, V., Severson, R., 1990. Alkaloid composition of the *Nicotiana* species. Beitr. Tabakforsch. Int. 14, 327. https://doi.org/10.2478/cttr-2013-0610.

Tomisawa, M., Casida, J.E., 2005. Neonicotiniois indsecticide toxicology : mechanisms of selective action. Annu. Rev. Pharmacol. Toxicol. 45, 247–268. https://doi.org/10.1146/annurev.pharmtox.45.120403.095930.

Wang, X., Anadon, A., Wu, Q., Qiaoo, F., Ares, I., Martinez, M.A., 2018. Mechanisms of neonicotiinoid toxicity: impact on oxidative stress and metabolism. Annu. Rev. Pharmacol. Toxicol. 58, 471–507. https://doi.org/10.1146/annurev-pharmtox-010617-052429.

Wickham, J.C., Chadwick, P.R., Steward, D.C., 1974. Factors which influence the knockdown effect of insecticide products. Pest Manag. Sci. 5, 657–664. https://doi.org/10.1002/ps.2780050518.

Williams, J.R., Delorenzo, R.J., Burton, H.R., 1999. Monoamine oxidase (MAO) inhibitors and uses thereof. US Patent: US6929811B2.