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Invasive brown algae (*Sargassum* spp.) as a potential source of biocontrol against *Aedes aegypti*

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Influxes of sargassos are responsible for economic and environmental disasters in areas where they bloom, especially in regions whose main income relies on tourism and with limited capacity for sanitation and public health response. A promising way of valorization would be to convert this incredible biomass into tools to fight the deadly vector mosquito *Aedes aegypti*. In the present study, we generated hydrolates and aqueous extracts from three main *Sargassum* morphotypes identified in Guadeloupe (French West Indies): *Sargassum natans VIII*, *Sargassum natans I* and *Sargassum fluitans*. We conducted a chemical characterization and a holistic evaluation of their potential to induce toxic and behavioral effects in *Ae. aegypti*. Despite the low insecticidal potential observed for all the extracts, we found that *S. natans VIII* and *S. fluitans* hydrolates deterred oviposition, induced contact irritancy and stimulated blood feeding behavior in host seeking *Ae. aegypti* females, while aqueous extracts from *S. natans I* and *S. fluitans* deterred both blood feeding behavior and oviposition. Chemical characterization evidenced the presence of phenylpropanoid, polyphenols, amino acids and esters. Thus, *Sargassum* spp. aqueous extracts and hydrolates could be used to manipulate *Ae. aegypti* behavior and be valorized as control tools against this mosquito.

Keywords *Sargassum*, *Aedes aegypti*, Control, Behavior, Deterrent, Toxic, Green extraction, Chemical identification

Sargassos are pelagic drifting brown algae belonging to the genus *Sargassum*. These last 10 years, mass stranding of millions of tons of these brown algae have been starting to be washed-up along the Coasts of the Caribbean. This unprecedented blooming, which was never documented before, is causing anomalous quantities of sargassos to be regularly washed ashore along the eastern coastline of the Caribbean Sea to the coast of western Africa[1,2], where it was given the name of Great Atlantic *Sargassum* belt[3]. In 2018, its size was estimated of 8850 kms, carrying more than 20 million metric tons of biomass, constituted of three distinct morphotypes: *Sargassum fluitans*, *Sargassum natans I* and *Sargassum natans VIII*[3–6]. Sargasso influxes are responsible for environmental and economic disaster in regions already debilitated by sanitary issues with low response capacities of public health programs, and whose main income rely on tourism[7]. Exposure to decomposing *Sargassum* has also been shown to induce toxicological syndrome associated with acute exposure to hydrogen sulphide (H_2S), resulting in neurological, digestive and respiratory disorders[8]. In Guadeloupe (French West Indies), during the massive stranding that occurred from January to August 2018, 3341 cases of acute exposure to noxious gases emitted by sargassos were reported[9]. Due to the global threat they cause and their devastating consequences, sargassos have recently been declared an international public health concern[9]. So far, the main way to evacuate sargassos relies in their collection and burning, which represents a huge economic cost that governments cannot always assume. In this context, an alternative route for reducing sargasso burden would be to valorise this free resource. These last years, research and industries have been focusing in developing innovative applications from sargassos in various sectors such as bioenergy, bioplastics, agriculture and cosmetics (for review see[10,11]). In the context of a growing need for alternative sources of biocontrol against mosquitoes, sargassos would represent a logical candidate in the Caribbean due to their characteristic of free and abundant resources. Indeed, *Aedes aegypti* mosquitoes constitute a major health threat considering their large geographic distribution around the globe and the numerous arboviruses they transmit, the most prevalent being dengue fever, chikungunya virus, Zika virus

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and yellow fever[12]. These last decades, the global incidence of *Aedes*-borne diseases dramatically increased, from 505.430 cases in 2000 to 5.2 million in 2019[13]. A recent study projected that dengue fever will impose a burden of 306 billion international dollars on worldwide economy between 2020 and 2050, with tropical and subtropical areas being the most affected, and with lower and middle-income countries bearing the higher health and economic impacts[14]. Recently, the Caribbean has experienced an unprecedented crisis of co-occurring dengue, chikungunya, and Zika viruses, where 186,050 cases of dengue, 911,842 cases of chikungunya, and 143,127 cases of Zika were reported between 2013 and 2019[15–17]. Guadeloupe has been classified in the top 3 regions with the most cases for all these diseases [16]. In all the endemic areas for mosquito-borne diseases, insecticide resistance has dramatically spread among vector populations, threatening the efforts made to control pathogen circulation. In Guadeloupe, multiple resistance to the major classes of insecticide used in vector control have been reported[18,19] highlighting the crucial need for alternative control strategies. In this context, sargassos would be logical candidates to be tested for their effect against mosquitoes, as brown algae are known to be rich in secondary metabolites such as phenols, polyphenols and terpenoids[10,20,21], compounds with documented insecticidal properties[22]. The use of sargassos in integrated vector management would then be an excellent valorisation pathway, as it would contribute to mitigate the burden caused by two major health issues in this part of the world. In the context of upcycling sargassos, several *Sargassum* species have already been tested for their toxicological properties against mosquitoes and have demonstrated promising results. Yet, extracts of *Sargassum polycystum*, *Sargassum wightii* and *Sargassum swartzii* have been effective to induce mortality in larvae of several mosquito species such as *Ae. aegypti*, *Culex quinquefasciatus, Anopheles sundaicus* and *Anopheles stephensi*[21,23–25]. Some of these extracts also showed potential against adult vectors in inducing toxic and repellent effects[23,24]. By contrast, the potential of the sargasso species responsible for the blooming in the Caribbean has only been tested once, where *S. natans* extract showed larvicidal activities against *Ae. aegypti* both when tested as a solvent extract and as silver nanoparticles[26]. Besides, to the best of our knowledge, the characterization of chemicals emitted by sargassos that can induce behavioural or physiological responses in mosquitoes has never been assessed. In the context of the development of long-lasting and cost-effective control tools against *Ae. aegypti* in the Caribbean context, there is a need for understanding the toxicological patterns of the three main morphotypes circulating in the Caribbean, *S. fluitans*, *S. natans I*, *S. natans VIII*, against *Ae. aegypti*, as well as to characterize bioactive chemicals within these extracts.

The present study aims at evaluating the potential of *Sargassum* spp. to be used as a tool to fight the vector mosquito *Ae. aegypti*. To achieve this goal, we generated aqueous and hydrolate extracts of *S. fluitans*, *S. natans* I *and S. natans VIII* and characterized their chemical composition. We also investigated their potential for inducing repellent and toxic effects on mosquito immature stages and adults, as well as in mediating key behaviours such as oviposition and blood feeding.

Results

For all the assays except oviposition experiments, extracts were tested both as hydrolates dosed at 10% and 90% and as lyophilized (powder) aqueous extracts at 1000 ppm. Experimental groups were *S. natans I* hydrolate 10% (N1H10), 90% (N1H90), powder extract (N1P); *S. natans VIII* hydrolate 10% (N8H10), 90% (N8H90), powder (N8P), *S. fluitans* hydrolate 10% (FH10), 90% (FH90), and powder (FP).

Chemical identification

Based on their nature, hydrolates and powder extracts were respectively analyzed by GC–MS and ^{1}H-NMR spectroscopy. Total Polyphenol Content (TPC) analysis was also conducted to quantify phenolic compounds in powder extracts.

Hydrolate extracts: GC–MS analysis

Hydrolate extracts obtained from the three *Sargassum* morphotypes were characterized by two phenylpropanoid compounds: chavicol ($C_9H_{10}O$, RT = 20.93) and *m*-eugenol (chavibetol) ($C_{10}H_{12}O_2$, RT = 23.03). Their concentration varied according to the *Sargassum* morphotype, with N8H and FH displaying the higher values when compared to N1H (Fig. 1, Supplementary file S1).

Powder extracts: NMR spectroscopy

RMN analysis of powder extracts revealed a total of 36 compounds, with chemical composition being dominated by amino acids (33%) and esters (16.6%) (Fig. 2). Among all, 18 were common to the three *Sargassum* morphotypes and belonged to molecular families such as amino acids, esters, carboxylic acids and amines (Fig. 2, Supplementary file S1). Five additional compounds are also shared between *S. natans I* and *S. fluitans* powders. Four compounds are common between *S. natans I* and *S. natans VIII* but absent from *S. fluitans,* and only one additional compound is shared between *S. fluitans* and *S. natans VIII*. Some compounds are unique in a given morphotype, allowing their differentiation: *S. natans I* is characterized by ethanol, 1-methyl-L-histidine and proline; *S. natans VIII* is characterized by anserine, dimethyl sulfoxide (DMSO) and malonate and *S. fluitans* by dimethylamine, mannose, methylamine, pyroglutamic acid and trehalose.

Total phenolic compounds (TPC)

S. natans VIII hydrolates showed lowest phenolic content with 0.2 mg GAE/g, followed by *S. natans I* (0.3 mg GAE/g) and *S. fluitans* (0.4 mg GAE/g).

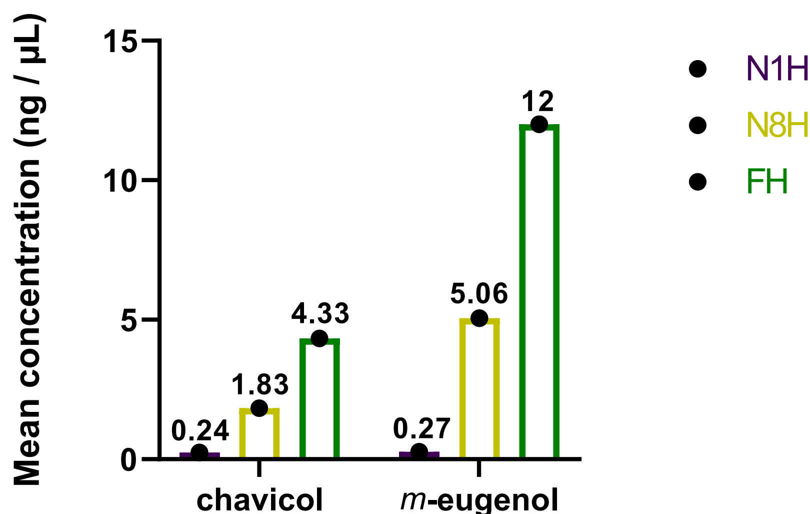

Fig. 1. Concentration of phenylpropanoids (chavicol and *m*-eugenol) in hydrolates extracts of *S. natans I* (N1H), *S. natans VIII* (N8H) and *S. fluitans* (FH). Numbers above bars indicate exact concentrations.

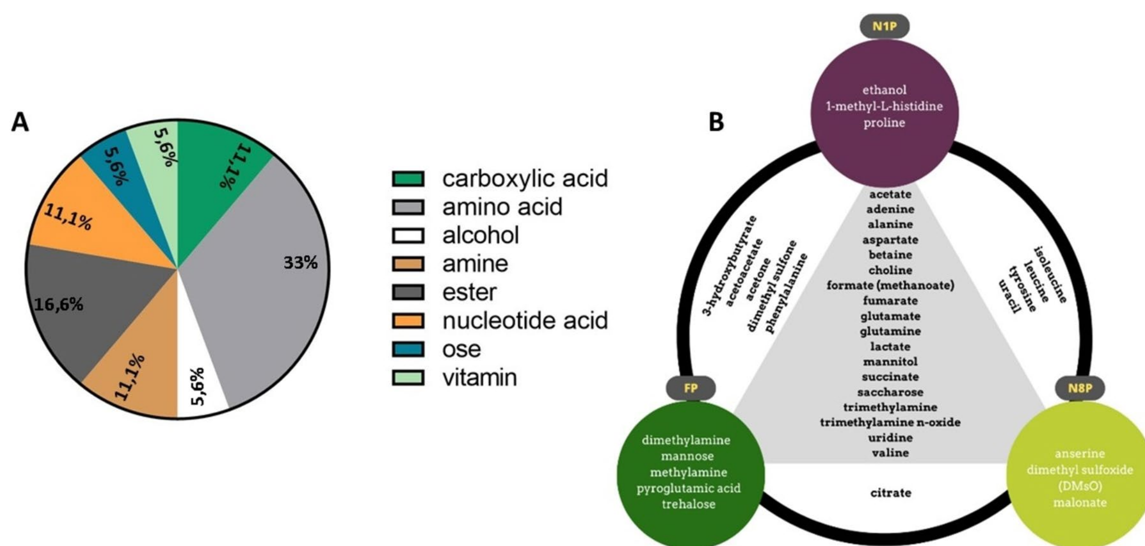

Fig. 2. Relative abundance of chemical families (**A**) and list of compounds (**B**) found by ^{1}H-NMR in powder extracts obtained from the three *Sargassum* morphotypes: *S. natans I* (N1P), *S. natans VIII* (N8P) and *S. fluitans* (FP). Percentages were calculated as the number of compounds found in a chemical family divided by the total number of compounds observed in the extracts. Compounds listed in the grey triangle are common between the three morphotypes. Compounds listed between two color circles are common between two morphotypes, and compounds listed inside of the color circles are only present in one morphotype.

Toxicity assays

The potential of extracts for inducing toxic effects was tested both against larval and adult stages.

Larvae

Larvicide assays were performed based on the WHO protocol[27]. Preliminary assays showed less than 10% mortality associated with exposure to *Sargassum* spp. extracts. Hence, larvae were kept in the cups until emergence and mortality was recorded every day for assessing the potential of the extracts to induce delayed mortality.

Survival rate was measured until 10 days of exposure, after which adults started to emerge in the solution. At that time, survival rate in the control group was above 90%, and ranged from 70 to 95% across tested groups. Only one compound, *S. fluitans* powder (FP), induced a significant decrease in the survival rate compared to control, with 70% females surviving after 10 days of exposure (Z value = – 3.03, P = 0.037) (Fig. 3A).

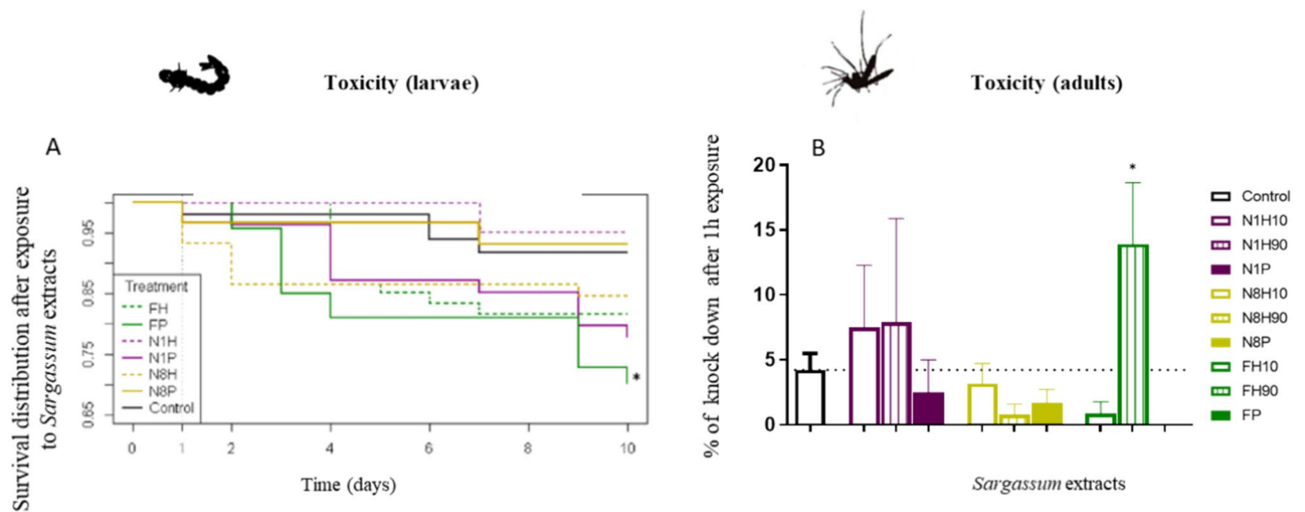

Fig. 3. Survival distributions of *Ae. aegypti* larvae exposed to *Sargassum* spp. extracts (**A**) and knockdown rate of female mosquitoes after 1 h exposure to *Sargassum* spp. extracts (**B**). (**A**) Asterisk shows significant differences (*** P < 0.001; ** P < 0.01; * P < 0.05). FH: *S. fluitans* hydrolate; FP: *S. fluitans* powder; N1H: *S. natans I* hydrolate; N1P: *S. natans I* powder; N8H: *S. natans VIII* hydrolate; N8P: *S. natans VIII* powder. N = 420 larvae tested by groups of 10 across 6 replicates per compound and control. (**B**) Values show mean ± SEM. Dotted line shows the percentage of Kds in the control group. Asterisk shows significant differences (* P < 0.05). FH: *S. fluitans* hydrolate; FP: *S. fluitans* powder; N1H: *S. natans I* hydrolate; N1P: *S. natans I* powder; N8H: *S. natans VIII* hydrolate; N8P: *S. natans VIII* powder. N = 2025 mosquitoes tested by groups of 25 across 6 replicates for *Sargassum*—treated papers and 27 replicates for the control. Control (untreated paper) was tested each day of the experiments to account for the effect of the day and the experimenter on the variability, which explain the higher number of replicates in the control group.

Adults

Toxicity assays were performed by using WHO recommendations for insecticide testing[28], where groups of females were exposed by tarsal contact for 1 h exposure to papers impregnated with either the control solution (acetone and silicone oil) or *Sargassum* spp. extracts (diluted in acetone and silicone oil). After 1 h exposure, knockdown rate was 4.2% (± 5.86) among the control group. Only one compound, FH90, induced 14% (± 4.73) knockdown after 1 h exposure, this effect being significant compared to the control (Z-value 3.08, P = 0.04) (Fig. 3B). At 24 h post-exposure, a trend for a higher mortality rate was observed only in the group exposed to N8H90 extract (7.3% (± 3.84) compared to the control 3.5% (± 1.43)), but the difference was not statistically significant ($X^2 = 2.5$, Df = 9, P = 0.98).

Behavioural assays

Behavioural assays were performed to assess the potential of *Sargassum* spp. extracts to induce behavioural effects against females during key steps of their lifecycle: host seeking, blood feeding and oviposition.

Feeding assays

Dual choice feeding assays were conducted for assessing the relative preferences of female mosquitoes when provided the opportunity to feed upon a feeder treated with either ultrapure water (control) or *Sargassum* spp. extracts (diluted in ultrapure water). Experiments were performed by using a blood pouch. Preliminary experiments showed no significant difference with fresh human blood when considering the blood feeding rate (Kruskal–Wallis test, P = 0.5) and the relative preference between *Sargassum*-treated membranes and control-treated membranes (Kruskal–Wallis test, P = 0.13).

Relative preference: first choice. When presented together with water, *S. natans I* N1H10 induced significant repellency compared to control, this extract being chosen in first choice in only 25% (± 16) of the tests ($X^2 = 4.19$, Df = 1, P = 0.04). Feeders treated with N1H90 or with N1P were chosen first in 38% (± 18) and 50% (± 17) of the tests respectively, with no significant behavioural effect compared to water ($X^2 = 1.01$, Df = 1, P = 0.32 and $X^2 = 0$, Df = 1, P = 1). *S. natans VIII* N8H10 was selected as first choice in 44% (± 18) of the assays, with no significant difference recorded with control feeder ($X^2 = 0.23$, Df = 1, P = 0.64). However N8H90 induced significant attractiveness compared to control feeder, as it was chosen as first choice in 80% (± 13) of the assays ($X^2 = 7.71$, Df = 1, P = 0.006). N8P was chosen as first choice in 40% (± 16) of the assays, which was not significantly different from the control ($X^2 = 0.81$, Df = 1, P = 0.37). *S. fluitans* FH10 was visited first in 67% (± 17) of the tests, although the difference with control was not significant ($X^2 = 2.04$, Df = 1, P = 0.15). In the same way, feeders treated with FH90 or FP were visited first in 33% (± 17) and 50% (± 17) of the assays, with no significant difference compared to control ($X^2 = 2.04$, Df = 1, P = 0.15 and $X^2 = 0$, Df = 1, P = 1) (Fig. 4.A).

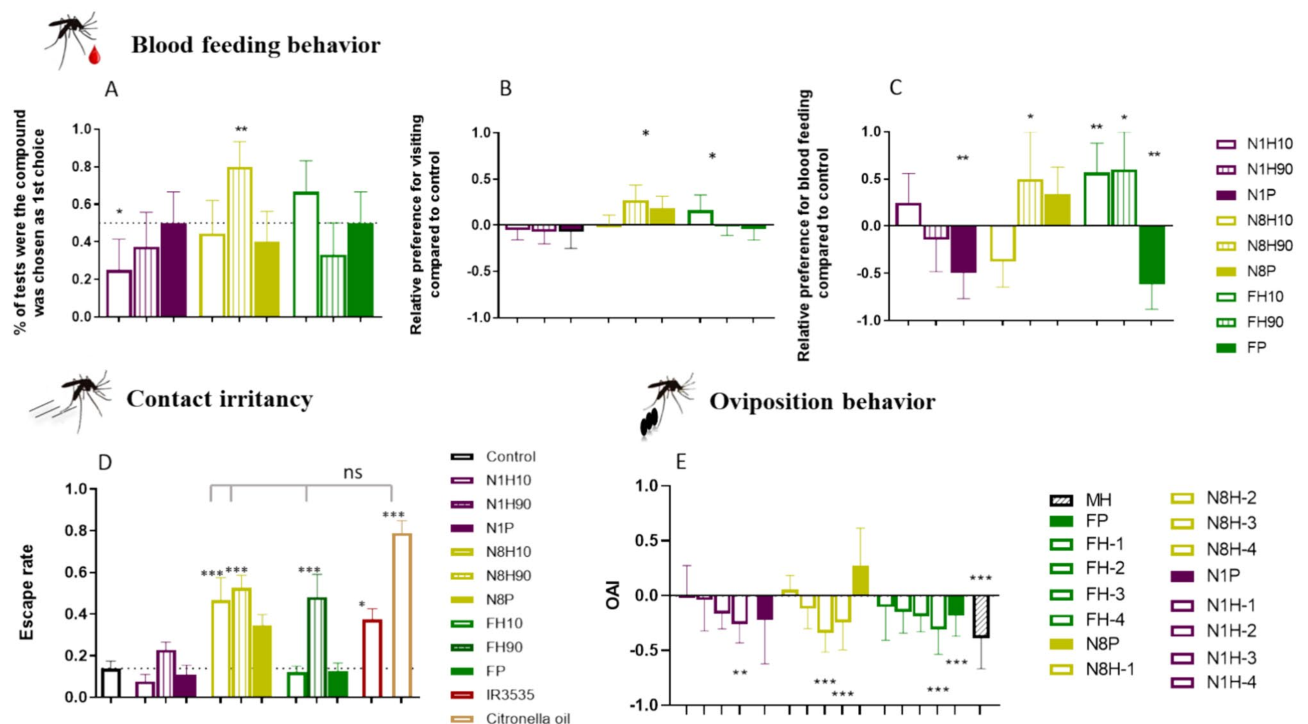

Fig. 4. Effect of the exposure to *Sargassum* spp. extracts on mosquito behavior during blood feeding (**A–C**), contact irritancy (**D**) and oviposition (**E**) assays. (**A**) Percentage of tests where the tested compound was selected as a first choice. Dotted line on shows equal preference (50%). (**B**) Relative preference for visiting the treated feeder compared to the control; (**C**) relative preference for blood-feeding on the treated feeder compared to the control. N = 900 mosquitoes tested by groups of 10 across 10 replicates per compound for (**A–C**). (**D**) Escape rate of female mosquitoes exposed to *Sargassum* spp. extracts in contact-irritancy HITSS assays. Dotted line shows the escape rate in the control group. Grey line shows the comparison with citronella oil. N = 1490 mosquitoes tested by groups of 10 across 10 replicates for *Sargassum*—treated papers and 38 replicates for the control. Control (untreated paper) was tested each day of the experiments to account for the effect of the day and the experimenter on the variability, which explain the higher number of replicates in the control group. E: Oviposition index of females exposed to powder and to hydrolates diluted at 10^{-4} to 10^{-1}. MH = mix of the three hydrolates diluted at 10^{-2}. N = 1600 mosquitoes tested by groups of 20 across 5 replicates per compound. Values show mean ± SEM. FH: *S. fluitans* hydrolate; FP: *S. fluitans* powder; N1H: *S. natans I* hydrolate; N1P: *S. natans I* powder; N8H: *S. natans VIII* hydrolate; N8P: *S. natans VIII* powder. Asterisk shows significant differences (***P < 0.001; **P < 0.01; *P < 0.05).

Relative preference: number of females visiting the treated feeder. Index preference was calculated to estimate the relative preference of responding females for visiting the treated feeder compared to the control, with positive values showing stimulation and negative values showing deterrence. *S. natans I* extracts induced no significant behavioural response compared to control, with relative preference ranging from − 0.05 (± 0.10) for N1H10 to − 0.07 for both N1H90 (± 0.13) and N1P (± 0.18) (X^2 tests, P > 0.05). In the same way, *S. natans VIII* N8H10 induced no significant behavioural effect, with an index value of 0.01 (± 0.10). However, N8H90 induced significant attraction, with index value of 0.27 (± 0.16) (X^2 = 5.05, Df = 1, P = 0.025). N8P showed a positive index value of 0.19 (± 0.13) but the difference with control was not significant (X^2 = 3.20, Df = 1, P = 0.07) likely due to inter-replicate variability. *S. fluitans* FH10 showed significant attraction with index of 0.16 (± 0.17) (X^2 = 5.05, Df = 1, P = 0.02). FH90 and FP did not induce significant behaviour, with index values of − 0.02 (± 0.09) and −0.04 (± 0.12) (X^2 tests, P > 0.05) (Fig. 4B).

Blood feeding among responding females. Index preference was calculated to estimate the relative preference of responding females for blood feeding on the treated feeder compared to the control. Positive values show stimulation and negative values show deterrence. *S. natans I* N1H10 and N1H90 elicited no significant behavioural effect (X^2-tests, P-value > 0.05), with index values of 0.25 (± 0.31) and − 0.14 (± 0.34), respectively. N1P however induced significant deterrent effect on blood feeding, with index value of − 0.5 (± 0.27) (X^2 = 9.2, Df = 1, P = 0.002). *S. natans VIII* N8H10 showed negative index (− 0.38 (± 0.27)), indicating deterrence, although the effect was not significant (X^2 = 0.44, Df = 1, P = 0.5). However, females displayed significant preferences for blood feeding towards N8H90 compared to water, with index value of 0.5 (± 0.5) (X^2 = 5.5, Df = 1, P = 0.02). N8P also showed positive values (0.34 (± 0.28)), indicating stimulation, but this was not significant (X^2 = 0, Df = 1, P = 1). Females also exhibited significant preference for blood feeding upon membrane treated with *S. fluitans* hydrolate extracts, with index values of 0.57 (± 0.31) for FH10 (X^2 = 6.69, Df = 1, P = 0.01) and 0.6 (± 0.4) for FH90

($X^2 = 4.32$, Df = 1, P = 0.04). Finally, FP showed significant deterrent effect, with index value of − 0.61 (± 0.27) ($X^2 = 10.20$, Df = 1, P = 0.001) (Fig. 4.C).

Contact irritancy and repellency assays
Repellency and contact irritancy assays were conducted by using a high-throughput screening system (HITSS) previously described[29]. HITSS allows to observe both irritancy by contact with a treated paper (contact irritancy) and avoidance of a treated paper without contact (spatial repellency).

Contact irritancy
Mean escape rate (ER) of females exposed to control papers (*i.e.* treated with solvent) was 14% (± 3.5). Positive controls, citronella oil and synthetic repellent IR3535, elicited significant escape rate compared to control with a mean escaping females of 79% (± 5.86) for citronella oil (Z value − 6.91, P < 0.001) and of 37% (± 5.11) for IR3535 (Z value − 3.29, P = 0.04). *S. natans I* elicited low behavioural response, with no significant differences between control for N1H10 (ER: 8% (± 3.26); Z value 1.05, P = 0.99), N1H90 (ER 23% (± 3.58); Z value − 0.89, P = 0.99) and N1P (ER 11% (± 4.51); Z value 0.51, P = 1). *S. natans VIII* hydrolates elicited significant escape rate compared to control, with 47% (± 10.71) mosquitoes escaping in presence of N8H10 (Z value − 4.39, P < 0.01) and 53% (± 6.20) with N8H90 (Z value − 4.83, P < 0.01). These were as effective as citronella oil for inducing escape, as difference with this positive control were not significant for both N8H10 (Z value − 2.65, P = 0.12) and N8H90 (Z value − 2.49, P = 0.33). N8P induced an escape rate of 35% (± 5.04), but this was not significant compared to the control (Z value − 2.91, P = 0.13). *S. fluitans* hydrolate FH10 as well as FP induced no significant escape rate (ER 12% (± 2.90); Z value 0.31, P = 1 and ER 13% (± 3.64); Z value 0.18, P = 1). However, FH90 induced a significant irritant effect, with escape rate of 48% (± 10.84) (Z value − 4.82, P < 0.01). Such effect was as effective as citronella oil, as no differences were observed between these compounds (Z-value − 2.36, P = 0.42) (Fig. 4.D, Table S2).

Repellency
Repellency index was calculated, where positive values show repellency and negative values show attraction. All tested extracts induced repellent effect, with positive repellency index (RI). Citronella oil elicited a RI of 0.49 (± 0.067) and IR3535 a RI of 0.35 (± 0.076). *S. natans I* N1H10 and N1H90 elicited a RI of 0.08 (± 0.045) and 0.14 (± 0.043), respectively. N1P elicited RI of 0.12 (± 0.026). These effects were significantly lower than the RI of citronella oil (Z value − 5.68 for N1H10; − 5.68 for N1H90 and − 5.26 for N1P, P < 0.001 for both). *S. natans VIII* N8H10 and N8H90 elicited RI of 0.06 (± 0.030) and 0.15 (± 0.082) respectively. RI of − 0.02 (± 0.041) was recorded N8P. These values were significantly lower than those of the positive control citronella oil (Z value − 5.64 for N8H10, − 5.64 for N8H90 and − 7.14 for N8P, P < 0.001 for both). *S. fluitans* RI values were 0.06 (± 0.040) for FH10, 0.03 (± 0.033) for FH90 and 0.12 (± 0.025) for FP. These effects were significantly lower than the RI of citronella oil (Z value − 6.57, − 6.57 and − 5.30, P < 0.001 for both) (Table S3).

Oviposition assays
Dual choice oviposition assays were carried out in order to evaluate the preference of females for ovipositing in a substrate containing either *Sargassum* spp. extract or ultrapure water. Oviposition Activity Index (OAI) was calculated, where positive values show stimulation and negative values show deterrence.

A dose–response effect was observed for all the extracts, with a deterrent effect increasing with the dose (Fig. 4.E). For *S. natans I* hydrolate, the percentage of eggs collected in the bowls were 51% (± 5.87) for 10^{-4}, 48% (± 6.24) for 10^{-3}, 42% (± 3.04) for 10^{-2}, and 37% (± 3.76) for 10^{-1}, the latter inducing significant deterrence (Z value − 3.75, P = 0.004). Powder extract received less eggs than did water, with 39% (± 8.87) of eggs, although the difference was not significant (X^2-test, P-value > 0.05) likely due to inter-replicate variability. In the same way, *S. natans VIII* extracts collected 53% (± 2.88) eggs for 10^{-4}, 44% (± 4.06) for 10^{-3}, as well as 33% (± 3.84) and 38% (± 5.57) of eggs for 10^{-2} and 10^{-1} concentrations, these two being significantly deterrent (Z value − 6.18 and − 4.45, P < 0.001 for both). Powder extract received 64% (± 7.68) of eggs, although this difference was not significant (X^2-test, P > 0.05). The percentage of eggs collected in bowls containing *S. fluitans* were 45% (± 6.75) for 10^{-4}, 43% (± 4.28) for 10^{-3}, 40% (± 3.10) for 10^{-2} and 35% (± 5.08) for 10^{-1}, the latter being significantly deterrent for oviposition (Z value − 4.55, P < 0.01). Powder extract at 100 ppm was also deterrent, with 41% (± 4.18) of eggs being deposited in bowls containing this extract ($X^2 = 35.17$, Df = 1, P < 0.001). Finally, the mix of the three hydrolates at doses of 10^{-2} was the most effective at inducing deterrence, with 30% (± 6.10) of eggs being deposited in bowls containing the substance ($X^2 = 28.67$, Df = 1, P < 0.001) (Fig. 4.E).

Discussion
In this study, we obtained by steam distillation *Sargassum* spp. extracts (hydrolates and lyophilized aqueous extracts) from three different morphotypes and characterized their chemical composition through complementary methods. Furthermore, we conducted a holistic evaluation to assess their potential to induce deleterious effects on the vector mosquito *Ae. aegypti*. A recent study highlighted a lack of literature and knowledge on *S. natans* and *S. fluitans*. As a consequence, valorisation pathways proposed so far are implemented by using different *Sargassum* species than those present in the Caribbean[11]. To the best of our knowledge, this is the first complete overview of the potential of *Sargassum* species affecting the Caribbean, *S. fluitans*, *S. natans I* and *S. natans VIII*, to induce toxic, irritant and repellent effects on a vector mosquito, as well as to impact two critical behaviours, blood feeding and oviposition.

Chemical signature is dominated by phenylpropanoids, amino acids and esters

Chemical characterization by GC–MS showed high similarities in the profile between the three *Sargassum* morphotypes. Hydrolates are characterized by two phenylpropanoid compounds (chavicol and *m*-eugenol). Studies performing GC–MS characterization on other *Sargassum* species found a profile either dominated by hydrocarbons, alcohols and esters[30] (in our study these families of natural products were identified by ^{1}H-NMR), or a dominance of 1, 2-benzoldicarbonsaeure and cyclopropanepentanoic acid[31]. For these studies, sample was dried and resuspended in solvent, in opposition with the extraction methods used in our study, which could thus explain the differences observed. Interestingly, a recent study evidenced genetic divergence between *S. natans I*, *S. natans VIII* and *S. fluitans*, these three morphotypes actually belonging to distinct taxonomic clades[32]. Different concentrations of chavicol and *m*-eugenol were observed between the three *Sargassum* morphotypes: *S. fluitans* had the highest concentration of both molecules (4 ng/µL and 12 ng/µL, respectively), followed by *S. natans VIII* (2 ng/µL and 5 ng/µL, respectively) and by *S. natans,* where the lowest concentrations were found (≤ 0.3 ng/µL for both molecules). These differences could be considered as a potential chemical marker of the clades.

Powder extracts show a profile dominated by amino acids and esters, with half of compounds being present in the three morphotypes, and a quarter being specific to each morphotype, allowing their differentiation. Amino acids such as valine, phenylalanine and leucine have also been found in other *Sargassum* species such as *S. cinctum*[33]. Our data also evidenced the presence of phenolic compounds in the three morphotypes at low concentrations, ranging from 0.2 to 0.4 mg/g. This is concomitant with a previous study showing a mean of 0.8 mg/g of such compounds in *S. fluitans* and *S. natans* extracts[34]. Other study however observed higher contents, with values ranging from 1.20 to 3.11 mg/g[35].

Interestingly, a recent study characterizing several *Sargassum* species using NMR technique also reported high presence of amino acids, as well as alcohol and sugars, with similar compounds as those observed here, such as mannitol, glutamine and glutamate[36]. The authors observed a differential metabolic profile of *S. natans VIII* compared to *S. natans I* and *S. fluitans*, with lactate being the main chemical responsible for this discrimination. In our samples, lactate was present in the three morphotypes, but the concentration was indeed higher in *S. natans VIII* extract (0.0034 mMol) than in *S. natans I* and *S. fluitans* (0.0021 and 0.0012 mMol respectively). The authors also reported glutamine as a discriminant chemical between *S. natans* and *S. fluitans*, which is concomitant with our data showing concentration of around 0.003 mMol in both *S. natans* morphotypes, and of 0.007 mMol in *S. fluitans*. As characterization of the genus *Sargassum* is known to be arduous due to the low genetic variability and similar morphological features between species[37], the use of techniques such as NMR or GC/MS would help describe chemical profiles, find chemical markers and facilitate chemotypes differentiation.

Sargassum spp. extracts show potential for inducing irritant and deterrent effects in mosquitoes

Results of the experiments are summarized in Table 1.

Despite similarities in the chemical profile observed in GC–MS between the hydrolates belonging to the three *Sargassum* morphotypes, they displayed different effects against mosquitoes (Table 1). One possible explanation would be differences in the quantity of phenylpropanoid and phenolic compounds within the extracts. *S. fluitans* extracts, for which the most significant effects were recorded on mosquitoes, showed the highest concentrations of chavicol (4.33 ng/µl), *m*-eugenol (12 ng/µl) and phenolic content (0.4 mg/g). By contrast, S. *natans I* extracts, which induced the less significant effects on mosquitoes, was the one with the lowest concentration of chavicol (0.24 ng/µl) and *m*-eugenol (0.70 ng/µl). These chemicals have high biological activity, with documented insecticidal and fungicidal properties[38–40]. They are also from plant origin and can therefore induce behavioral input, serving as cues for sugar-feeding. In the same way, the presence of esters in powder extracts could explain the deterrent effects observed, these chemicals being documented for having deterrent and toxic properties against insects[41–44].

Both hydrolate and powder extracts showed low potential for inducing insecticidal effects against *Ae. aegypti*. When tested against adults, only FH90 induced 17% knockdown effect after 1 h exposure but with no associated mortality. Against larvae, only FP showed reduced survival rate but with a low effect, as 75% larvae still survived

	Toxicity		*Behavior*		
	Larvicide	**Adulticide**	**Irritancy–contact**	**Blood feeding**	**Oviposition**
N1H10	0	0	0	Deterrent	Deterrent
N1H90	0	0	0	0	NT
N1P	0	0	0	Deterrent	0
N8H10	0	0	Irritant	0	Deterrent
N8H90	0	0	Irritant	Stimulant	NT
N8P	0	0	0	0	0
FH10	0	0	0	Stimulant	Deterrent
FH90	0	Yes but low	Irritant	Stimulant	NT
FP	Yes but low	0	0	Deterrent	Deterrent

Table 1. Summary of the observed insecticidal and behavioral effects induced by *Sargassum* spp. extracts on *Ae. aegypti*. 0 = no effect; NT = not tested.

after 10 days of exposure. These observations contrast with previous studies showing high potential of *Sargassum* species for inducing larvicidal effects. Yet, a study with *S. natans* observed 89% mortality at 900 ppm[26]. In the same way, another *Sargassum* species, *S. wightii* induced larval mortality rate of 90% at 350 ppm when using methanol extract[21]. Our results however are concomitant with other study observing no toxic effect of *S. tenerrimum* extract even at 100 mg/L[45]. Several factors could explain the observed discrepancies. First, two of these studies used other *Sargassum* species. Then, sample preparation was performed differently, as study on *S. natans* and *S. tenerrimum* used ground extract that was resuspended for the bioassay, and study using *S. wightii* used solvent extract. In our study, the process of Clevenger distillation used to obtain hydrolate extracts is likely to extract selectively Volatile Organic Compounds (VOCs), retaining compounds with high melting point and potentially responsible for insecticidal properties, such as phenolic compounds and esters. Such compounds are present in powder aqueous extracts, but the low concentration of polyphenols in these could explain the low toxic effect observed. Further investigations are then needed to characterize the bioactive compounds responsible for larval mortality, and to assess whether the extraction process and solvent used in our study retain or dilute certain compounds such as polyphenols compared to other techniques[46].

Our data show strong potential of *Sargassum* extracts, especially hydrolates, for inducing deterrence in contact-irritancy as well as in oviposition assays. In the first one, extracts were as efficient as citronella oil and IR3535 for inducing females to escape. Our observations are concomitant with arm-in-cage assays showing repellent effect of another *Sargassum* species, *S. polycystum*, where a maximum repellency of 93% when using 300 µL of extract was reported[23]. In our oviposition assays, a dose response pattern was observed for hydrolates, with an increased deterrent effect alongside the increased dose, the strongest deterrence being observed at a dose of 10^{-1} for the three extracts. Interestingly, the mixing of the three hydrolates at a dose of 10^{-2} increased the deterrent effect and outreached the effects of hydrolates tested individually at 10^{-1}. Powder extract of *S. fluitans* also elicited strong oviposition deterrence to females. To the best of our knowledge, this is the first study reporting irritant and ovideterrent effect of *Sargassum* hydrolates and aqueous extracts against a vector mosquito.

Then, contrasted observations resulted from blood feeding assays. Whereas *S. natans I* hydrolate dosed at 10% and powder extracts of *S. natans I* and *S. fluitans* showed deterrent effect, *S. natans VIII* and *S. fluitans* hydrolates elicited attraction and stimulation of blood feeding. Interestingly, *S. natans VIII* hydrolate was chosen preferentially as first choice, as well as receiving most visits and most blood fed females compared to the control. Mosquito rely on plant fluids to obtain energy-rich meals[47]. Chavicol and *m*-eugenol, found in hydrolates, are known to be present in plant extracts[48,49]. As they are present in higher quantities in *S. fluitans* and *S. natans VIII* hydrolates than in *S. natans I*, one possible explanation could be that the presence of these compounds in higher quantities in the feeders containing *S. fluitans* and *S. natans VIII* would have lured females and attract them towards cues that they interpreted as energy rich meal. As observed in many plant–insect systems, a same compound can either be toxic or lure depending on the dose[50].

Selection of the best candidates to be tested in further assays

The present study allows to screen the extracts with potential to be kept in further studies. FP, N8H10 and N1H10 seem to be the best candidates. Yet, FP induces an effect on larval survival (even if low), as well as being deterrent both against host seeking and gravid females. Then, N8H10 shows irritant effect against adults as well as deterrent effect in oviposition assays, and N1H10 induces deterrence both against host seeking and gravid females.

Then, FH90 and N8H90 showed irritant effect in forced contact but attractancy during blood feeding assays. In the same way, FH10 induced deterrence in oviposition assays but stimulation in blood feeding assays. These extracts should not be considered as candidates for implementation of repellents because of these contrasted effects. However, their effect should be further evaluated together with other attractive compounds to evidence a potential synergistic effect in the development of attractive lures against host seeking females. The attractant effect of N8H90 being the most important, this extract could be considered as an attractant candidate for behavioral assays in laboratory such as olfactometer and stimulant for experimental infections. It is worth noting that females tested during blood feeding assays were gravid, in contrast with females tested during contact irritancy assays. Such physiological changes might induce differences in the chemical perception of the tested compound and therefore eliciting contrasted behaviour[51]. Additional studies performing contact-irritancy assays of these candidates against gravid females would allow to decipher the role of physiological state in the observed behaviour.

Finally, N1P showed deterrent effect in blood feeding assays only, and N8P and N1H90 did not show any effect in any of the tests conducted. Therefore, these extracts do not fill the first step of validation and should not be considered in priority to be tested further.

Next steps for the validation of the best candidates

In order to bring the best candidates (FP, N8H10, N1H10) towards the next step of validation, additional assays are needed. First, at the behavioural level, the effect should be confirmed at larger distance by using validated protocols for testing the efficacy of control tools. Hence, the first step includes the testing of the three candidates in oviposition assays in semi-field conditions. If the ovideterrent effect is confirmed, a mix of FP, N8H10 and N1H10 should be tested to evidence potential synergistic effects. Oviposition deterrents are great tools in vector control management programs, as they can be used both in push–pull methods by redirecting females towards an attractant, as well as used in sites where water cannot be removed[52–54]. To confirm the irritant and deterrent effect against host seeking and blood feeding females, the extracts should be tested together with host volatiles, such as in arm-in-cage assays and in tunnel tests following the WHO protocol[55]. At the chemical level, testing FP, N8H10 and N1H10 extracts in electrophysiological studies such as single sensillum recording (SSR) would allow to assess whether the extract acts by contact or at distance. Characterization of organic volatile compounds

through headspace techniques would allow to identify volatiles acting as long-distance. If the results are conclusive, in the future, in-depth chemical studies could be performed with the final goal of obtaining formulations that can be deployed for vector control in the field (i.e. lures for traps, repellents). For instance flash chromatography technique would allow a fractionation of extracts and a bioguided-purification of the natural products, even from complex blend, favoring the identification of bioactive compounds within the extracts. Then, metabolomics studies would provide data on potential biomarkers that might help explaining the behavioural effects observed.

It is worth noting that Sargassos have been shown to be biosorbent for heavy metals[56,57]. Pre-treatment of the macroalga to eliminate these contaminants is feasible but would increase the cost. Yet, further assays including toxicological assays are compulsory to implement the best formulation in terms of cost, efficiency, and which complies with health and environmental regulations. Interestingly, *S. natans VIII* seems to accumulate lower quantities of metal than *S. natans I* and *S. fluitans*[35], which could be a parameter to take into account for the selection of candidates.

Conclusion

In the present study, we propose first evidence of mosquitocidal activity of pelagic sargassos affecting the Caribbean and we offer clues for understanding the chemicals involved. Our results showed great potential of *S. natans VIII* and *S. fluitans* hydrolates in deterring oviposition and inducing contact irritancy in *Ae. aegypti*. Also, aqueous extracts from *S. natans I* and *S. fluitans* were able to deter both blood feeding behavior and oviposition, which confirm the potential of sargassos to be incorporated in the toolbox to fight dengue vectors. Chemical characterization evidenced the presence of phenylpropanoid, polyphenols, amino acids and esters, and highlighted a possible role on the behavioural effects observed. Given the international public health concern of sargassos and the urgent need for new tools for integrated vector management, this complete overview suggests an avenue for *Sargassum* spp. valorisation that would not only reduce the burden of *Sargassum* spp. nuisance, but also use this abundant resource to combat a deadly vector.

Methods

Study site and *Sargassum* collection and preparation

Fresh specimens from *Sargassum* genus were collected on shores of Guadeloupe during February 2020 to June 2021 in Saint-François (16.278826106426443, − 61.247932039347425) Sainte-Anne (16.222340645449364, − 61.393059508536915) and Saint Felix, Le Gosier (16.20162363406598, − 61.45887205992317). Separation of the three main morphotypes (*i.e. S. natans I*, *S. natans VIII*, *S. fluitans*) was immediately performed in laboratory by visual identification of the morphological features of fresh specimens as previously described[4–6] and by using both Sargassum identification guide[58] and Sargazoom project[59]. Material was kept at − 20 °C until processing.

Clevenger distillation of *Sargassum* spp.

The extractive work was developed with the three different *Sargassum* morphotypes separately. The extraction method employed was a Clevenger distillation. For each sample, 1 kg of fresh material was extracted in 1L of water at 100 °C for 1 h. After each Clevenger extraction two types of samples were obtained. The first were hydrolate extracts, being the hydrodistilation products of Clevenger extraction and composed mainly of Volatile Organic Compounds. Then, water contained in the extraction vessel was filtrated, the *sargassum* were discarded and the aqueous extract was frozen at − 20 °C and lyophilized at 0.15 Bar and − 50 °C for 1 week. Both types of extracts, *i.e.* hydrolates and lyophilized extracts were kept at − 20 °C until use. Lyophilized extracts were resuspended in either solvent or ultrapure water for the assays.

The doses of extracts used for each experiment are given in Table 2.

For all assays except oviposition experiments, experimental groups were then *S. natans I* hydrolate 10% (N1H10), 90% (N1H90), powder extract (N1P); *S. natans VIII* hydrolate 10% (N8H10), 90% (N8H90), powder (N8P), *S. fluitans* hydrolate 10% (FH10), 90% (FH90), and powder (FP).

Chemical identification

GC–MS analyses

GC–MS was performed to identify the volatiles present in hydrolate extracts after steam distillation. Similarly to behavioural experiments, samples were diluted in acetone for a final concentration of 90%. Analyses were conducted on a Trace 1300 GC equipped with a DB-1-14092018 capillary column (DB-1 Agilent Technology®

Experiments	Dose tested : hydrolates	Dose tested: lyophilized extracts	Solvent used (control = solvent only)
Toxicity (larvae)	Pure	1000 ppm	Ultrapure water
Toxicity (adults)	10% and 90%	1000 ppm	Acetone (2/3) + silicone oil (1/3)
Irritancy -repellency	10% and 90%	1000 ppm	Acetone (2/3) + silicone oil (1/3)
Blood-feeding assays	10% and 90%	1000 ppm	Ultrapure water
Oviposition assays	0.01–10%	100 ppm*	Ultrapure water

Table 2. Detail of the doses of *Sargassum* spp. extracts and of the solvents used for the bioassays. *A lower dose was used for oviposition experiments to avoid any bias linked to change in water color.

length 60 m, diameter 0.250 mm, internal diameter 0.50 µm). This type of column has been proven efficient to screen chemicals and particularly volatile compounds from brown algae material[60–63].

Helium was used as the carrier gas and applied at a constant flow rate of 1 mL/min. Two µL of sample were injected in splitless mode. The GC injector (CombiPal TriPlus RSH, ThermoFisher Scientific®) and MS transfer line temperatures were set at 250 and 275 °C respectively. The ion source temperature was constantly maintained at 250 °C. The oven was programmed 3 min isothermal at 45 °C, then 45 °C to 280 °C at 8 °C min^{-1}, with 2 min hold at 280 °C. Mass spectra were recorded on a TSQ 9000 MS in electronic impact (EI) at 70 eV. Spectra of each sample was compared with the respective blank (acetone) to subtract possible contaminants. Retention times of a series of *n*-alkanes (C7–C40 alkanes standard solution, 49452-U, Sigma-Aldrich®) were used to convert retention times into retention indices. Compounds were identified based on their retention indices and mass spectra, which were compared with data of the NIST MS-Search 2.3 software library as well as those recorded in databases (The Pherobase[64]). Peak areas were measured on the GC-FID samples using the Chromeleon software (ThermoFisher, version 7.2.9).

NMR spectroscopy

NMR spectroscopy was used to characterize the compounds present in lyophilized extracts. Study samples were spiked with 600µL of D_2O in 5 mm NMR tubes. Measures were then performed on a Bruker Avance Neo 600 MHz spectrophotometer (Bruker BioSpin, France), equipped with a 5 mm BBI probe and a SampleJet autosampler. Temperature was set at 300 K. The spectra were obtained using a classical 1D 1H experiment in D_2O. All data processing was performed with TopSpin 4.0.7 software. The identification of the compounds present in the extracts was done with the ChenomX NMR Suite 8.6 Professional software.

Total phenolic compounds (TPC) analysis

Quantification of total phenolic compounds, assessed by the Folin Ciocalteau method[65] was performed on the powder extracts using the Polyphenol Quantification Assay Kit KB-03-006TPC (Bioquochem, Spain) following manufacturer instructions. Briefly, standard curve was built by using serial dilutions of gallic acid at concentrations ranging from 0 to 300 µg/ml. Absorbance at 700 nm was assessed using a iMark™ Microplate Absorbance Reader (Bio-rad, California) spectrophotometer. Absorbance of *Sargassum* spp. extracts was then assessed using a mixture containing 20 µl of each sample, 100 µl of Folin's reagent (dilution 1/10) and 80 µl of sodium carbonate (Na2CO3). The polyphenol content of each sample was estimated by plotting the sample absorbance on the calibration curve. Results are reported as mg GAE/g.

Toxicological and behavioural assays

Ethic statement

The use of fresh human blood from healthy volunteers to feed mosquitoes was approved by the internal ethics committee of the Pasteur Institute of Guadeloupe, established since September 2015 (no agreement number for internal ethics board), after receipt of written informed consent from the participants. For feeding assays, due to the high volume needed, blood patches constituted of blood > 40 days-old for non-therapeutic use were provided by the French Blood Agency (EFS) under a cession agreement N° PLER-01/2020/EFS-IPGUAD. All methods were performed in accordance with the relevant guidelines and regulations.

Mosquito colony

A metapopulation of *Ae. aegypti* was established by sampling larvae from July to August 2019 in 5 different localities of Guadeloupe: Les Abymes, Pointe-à-Pitre, Deshaies, Saint-François, and Anse Bertrand. Mosquitoes rearing took place under laboratory conditions of 26 ± 1 °C and 40–60% RH with a light: dark photoperiod of 12:12 h. Larvae were reared in groups of about 200 in 1L of dechlorinated tap water and were fed with rabbit pellets. Adults were given ad libitum access to a 10% sucrose solution. Experiments were performed on the 8th to 13th generation of this colony in the same environmental conditions as for the rearing. All experiments were performed on females 7–10 days post-emergence.

Toxicity assays

Larvae. Larvicide assays were performed based on the WHO protocol[27] with slight modifications for taking into account the relatively small amount of *Sargassum* extract available. Briefly, batches of 10 L2–L3 larvae were rinsed and placed in disposable cups with 50 ml of either *Sargassum* extract or control (ultrapure water). As preliminary assays showed low 24 h mortality associated with exposure to *Sargassum* spp. extracts (< 10%), larvae were kept in the cups until emergence (for up to 10 days) for assessing the potential of the extracts to induce delayed mortality. At the beginning of the study, each cup was provided with 80 mg of rabbit pellets. This amount of food, selected during preliminary assays, allowed to keep the larvae alive without inducing undesired bacterial development or biofilms in the water (data not shown). Cups were observed every day, where the recorded parameters were (i) the number of dead larvae and (ii) the developmental stage of the larvae present in the cup. A total of 6 replicates was performed for each condition and controls.

Adults. Toxicity assays were performed following WHO recommendations[28] slightly adapted to our study. Groups of 25 females were exposed by tarsal contact to papers impregnated with either the control solution (acetone and silicone oil) or *Sargassum* spp. extracts (diluted in acetone and silicone oil). After 1 h exposure, females were transferred to tubes containing non-impregnated papers and provided with 10% sugar solution. Knockdown and mortality rates were respectively recorded at 1 h and 24 h post exposure. A total of 6 replicates

was performed for each condition and control. Paper impregnation was performed by applying 2 ml of the solution (control or treatment) to 12 × 15 cm Whatman® Grade 1 Qualitative Filtration Paper (Sigma- Aldrich, Inc. Missouri, USA). After impregnation, all papers were allowed to air dry overnight for solvent evaporation and kept at + 4 °C until use. Each paper was used only once.

Behavioural assays

Feeding assays. Dual choice feeding assays were performed in order to assess the relative preferences of female mosquitoes when provided the opportunity to feed upon a feeder treated with either ultrapure water (control) or *Sargassum* spp. extracts. To do this, groups of 10 females (starved for 24 h) were placed in 30 × 30 × 30 cm laboratory cages (MegaView Science Education Services Co., Taiwan). Two Hemotek feeders (Hemotek Ltd, Great Harwood, England) were prepared with 2 ml of blood and were closed with pork intestine membrane, after which each membrane was impregnated with 50 µl of either ultrapure water (control) or *Sargassum* spp. extracts using a micropipette. Feeders were then placed on top of the cage, at opposite sides. Mosquito behaviour was observed for 10 min using the quantitative analysis of behaviour software JWatcher[66], where the following parameters were instantaneously recorded: (i) number of mosquitoes on each feeder and (ii) number of blood fed mosquitoes. A total of 10 replicates was performed for each condition.

Due to the high volume of blood needed, the blood used for these experiments was a pouch > 40 days-old provided by the French Blood Agency. Hence, preliminary experiments were performed to confirm that this type of blood would not influence female blood feeding behaviour. Blood feeding behaviour between fresh blood from a human volunteer and blood from the pouch was compared across 5 replicates of *S. natans I* and *S. natans VIII* hydrolates by using the same protocol as described above.

Contact irritancy and repellency assays. Repellency and contact irritancy assays were performed using a high-throughput screening system (HITSS) previously described[29] and adopted by the WHO guidelines for efficacy testing of spatial repellents[67]. Briefly, HITSS device is constituted of one clear Plexiglas chamber and two metal cylinders. The metal cylinders contain metal drums, where treated and control papers can be affixed. This dual-choice chamber system allows to observe directional flight behaviour by testing for two different modes of action: repellency without contact with the treated paper (i.e. spatial repellency) and irritancy by contact with the treated paper (i.e. contact irritancy). To do this, 2.8 ml of the solution were applied to 10.7 × 32 cm filter papers. After impregnation, all papers were allowed to air dry for 20 min for solvent evaporation and kept at + 4 °C until use. Each paper was used 4 times.

Before and after use, the device was soaked in alkaline detergent (RBS T105; Chemical products R. Borghgraef, Brussels, Belgium), then rinsed with ethanol and with clear water. During the tests, an opaque cloth was placed above the device so that mosquito behaviour was not influenced by luminosity. The position of the chamber containing the treated paper was alternated between replicates to avoid any bias. For these assays, 1 g IR3535 dosed at 35% (m/m) and 3% citronella oil were used as positive controls based on previous literature[68–70]. Females used for the assays have had the possibility to mate and have never had access to blood meal.

Contact irritancy. This assay used one metal cylinder (holding the treated paper) and the clear chamber, connected together with a butterfly valve. Groups of 10 females were placed in the metal cylinder through an opening in the end cap for 30 s acclimation. Then, the butterfly valve was opened and mosquitoes were given the opportunity to escape in the clear chamber. After 10 min, the butterfly valve was closed again and mosquitoes in each branch were counted. Contact irritancy was measured as the number of mosquitoes that have moved to the clear chamber relative to the total number of mosquitoes. A total of 10 replicates was done for each condition.

Spatial repellency. For these assays, the two metal cylinders were connected at opposite sides of the clear chamber and connected to it through butterfly valves. One of the cylinders held the treated paper whereas the second held the control paper. Groups of 10 mosquitoes were transferred into the clear chamber for a 30-s acclimation. Then, the butterfly valves connecting to the two cylinders were simultaneously opened. After 10 min, the valves were simultaneously closed and the number of mosquitoes in each cylinder as well as in the clear branch was counted. Spatial activity index (SAI) was calculated and weighted as previously described[29,71] as $[(Nc - Nt)/(Nc + Nt)] * [(Nc + Nt)]/N]$ where and Nc = number of mosquitoes in the control chamber, Nt = number of mosquitoes in the treated chamber and N = total number of mosquitoes in the replicate. SAI values range from − 1 to 1, where 1 indicates the strongest repellency (movement of all mosquitoes away from the treated paper), 0 indicates no response, and − 1 indicates attractiveness (movement of all mosquitoes towards the treated paper).

Oviposition assays. Blood from a healthy human volunteer was provided to females, after which groups of 20 fully engorged individuals were kept in 30 × 30 × 30 cm cages. Dual choice oviposition assays were carried out three days after blood feeding. To do this, two ceramic bowls (Ø: 8 cm) were filled with 100 ml of solution (treatment or control) and placed at opposite corners of the cage. A strip of filter paper (Whatman TM, no. 2300 916) was partially immersed into each bowl to serve as an oviposition substrate. After 24 h, bowls and papers were removed from the cage and eggs were visually counted under a binocular magnifier to assess female preferences. A total of 5 replicates were performed for each condition, for which the position of the bowls into the cage was randomly attributed. Before each trial, bowls were soaked overnight in RBS T 105 alkaline detergent, rinsed and sterilized at 100 ˚C for 1 h.

Statistical analyses

All statistical analyses were performed using the software R 3.3.2[72]. For larvicide assays, survival was evaluated with a mixed effects Cox proportional hazards regression model (packages survival, coxme[73,74]) after which Kaplan Meier curves were obtained. All percentage data (adulticide assays, contact-irritancy assays, first choice in blood feeding assays and oviposition assays) were analysed by using binomial generalized linear mixed model (GLMM, lme4 package) with replicate coded as a random factor.

In blood feeding assays, preference indexes were calculated for assessing the relative preference for both visiting the feeder and for blood feeding compared to the control, calculated and weighted as follows: ((NT − NC)/(NT + NC)) * ((NT + NC)/T). Regarding the preference for visiting the feeder, NT indicates the number of females visiting the treated feeder, NC indicates the number of females visiting the control feeder and T indicates the total number of responding females. For calculating the preference for blood feeding, NT indicates the number of blood fed females in the treated feeder, NC indicates the number of blood fed females in the control feeder and T indicates the total number of blood fed females in the cage. For oviposition assays, the Oviposition Activity Index (OAI) was calculated as previously described[75]. For these assays, a positive value indicates a preference toward the treated solution, whereas a negative value indicates aversion. Calculated indexes (spatial repellency assays, preference for visiting and blood feeding in blood feeding assays) were processed by using a linear mixed-effects model (lmer function, lme4 package) with replicate coded as random factors. Model selection was performed using AIC and analysis of the residuals (plotresid, RVAideMemoire package[76]).

Data availability

All data are available upon reasonable request.

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