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Evaluation of Phytochemicals, Antibacterial, Antioxidant and Larvicidal potential of the brown algae *Spatoglossum asperum* against mosquito vectors

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Abstract

The purpose of the present investigation is to find out whether *S. asperum's* ethyl acetate and chloroform extracts has larvicidal capacity towards *Aedes aegypti, Culex quinquefasciatus*, and *Anopheles stephensi*. Various quantities of ethyl acetate and chloroform seaweed extracts were given to chosen larvae in the initial 4th instar. Each trial was conducted three times, with a control group present throughout the study. The fatality rate was constantly monitored every 24 hrs. Utilizing FTIR and GC-MS examinations, the functional groups and phytochemical substances in the extracts were determined. Clinically obtained bacteria were used to test antibacterial properties employing the well plate process, and the results suggest that both extracts have the ability to inhibit the bacterial growth. The FTIR signals validated the carbonyl groups, amino acids, hydroxyl groups, and alkynes. The GC-MS studies found the presence of anticonvulsant, insecticidal, antibacterial, antioxidant, cytotoxic, and hepatoprotective active compounds. Ethyl acetate extract had a LC_{50} rate of 114.73 mg/L, 81.60 mg/L, 150.71 mg/L against *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi*. Whereas the chloroform extract had a LC_{50} rate of 73.27 mg/L, 74.11 mg/L, 66.38 mg/L against *Ae. aegypti, Cx. quinquefasciatus* and *An. stephensi* respectively. According to these investigations, *S. asperum* ethyl acetate and chloroform extracts have a mediocre chance of working as larvicidal agents against mosquito vectors.

Keywords: Antioxidant; Histopathology; Larvicidal; Phytochemical; Vector; Spatoglossum Asperum

1. Introduction

The mosquito is the primary vector for many of the globes most pressing public health issues, including dengue fever, malaria, yellow fever, filariasis, and others [1]. Multiple problems, with emissions, pesticide resistance, and toxic hazards to humans, have arisen from the careless application of chemical-based insecticides. To manage the rising number of parasites chemical insecticides including organochlorine, organophosphorus, carbamates, pyrethrins, and pyrethroids are routinely utilized. The excessive application of these chemical pesticides is not safer due to environmental hazards and the emergence of resistance in non-target organisms [2]. As a result, other strategies free of such issues are required in current times, including the creation of ecologically sound, recyclable, affordable indigenous methods comprised of lawful scientific and technological weapons with more powerful compatible capabilities towards such vectors [3].

Aside from localized cutaneous reactions, mosquito bites can also result in systemic allergic symptoms such as rashes and swelling of the vessels [4]. Especially in nations with tropical and subtropical climates, illnesses transmitted by

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mosquitoes have a financial impact, including deficits in commercial and labor productivity; nonetheless, no region of the globe is immune to diseases transmitted via vectors. Treatment for this illness Since mosquitoes have gained susceptibility to insect vectors employing artificial pesticides has been ineffective [5].

Marine algae have been described to possess primary and secondary metabolites with wide range of distinctive biological properties. Consequently, the application of marine algae has grown more popular recently because of their sustainable mosquito control strategies [6]. Due to their antiviral, antimicrobial, antifungal, and cancer-fighting abilities, chemical substances found in aquatic environments are a vital resource for numerous medicinal purposes. Numerous macroalgae found in the oceans contain a variety of secondary substances including polyphenols, terpenes, sterols, lectins, proteinase inhibitors, fatty acids, enzymes, and polysaccharides, which may have bioactive properties that are useful for medicinal uses [7]. Seaweeds have been shown to have mosquitocidal capabilities an addition to possessing distinct bioactive secondary compounds with therapeutic benefits [8].

Spatoglossum asperum is a brown seaweed, it has been reported for its antibacterial [9, 10], antioxidant [11, 12], antidiabetic [13, 14], wound healing [15, 16] properties. Seaweed has been shown in numerous tests to possess potent mosquitocidal properties. The discoveries of *S. asperum* mosquitocidal capabilities will help researchers of healthcare, insecticide, and mosquito control campaigns. When contrasted with the use of seaweeds for nutritional and pharmaceutical applications, the few reported studies of mosquitocidal properties of seaweeds reveal limited information on the bio-efficacy of seaweed-based insecticide. *Turbinaria conoides*, *Caulerpa scalpelliformis, Gracilaria corticata, Turbinaria decurrens, Sargassum tenerrimum* are described for their anti-mosquitocidal action.

The results of the present study would be useful in promoting research aiming at the development of new agent for mosquito control based on bioactive chemical compounds from indigenous plant source. In view of the recently increased interest in developing plant origin insecticides as an alternative to chemical insecticide, this study was under taken to assess the adulticidal, repellent, and larvicidal potential of the extracts from the medicinal plant against the medically important mosquito vector, *Cx. quinquefasciatus, Ae. aegypti and An. stephensi.*

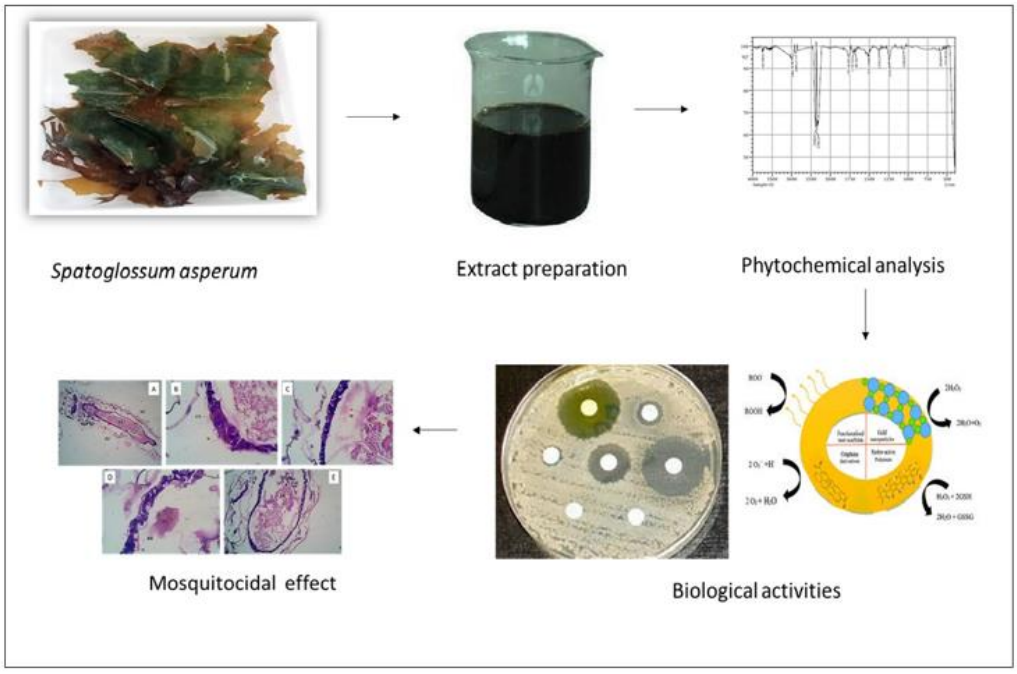


Figure 1 Graphical abstract of the present work

2. Materials and methods

2.1. Sample collection and extraction

The brown seaweed *Spatoglossum asperum* has been gathered from the beaches of Mandapam, Ramanathapuram District, TamilNadu. Dr. Palanisamy, a scientist at the Botanical Survey of India in West Bengal, verified the seaweed species that were collected. After being cleaned with sterile water, the confirmed sample was placed in a shaded area

for a duration of three weeks. The dried materials have been crushed in an electric grinder and sieved. Chloroform and ethyl acetate, both polar solvents, were utilized in the current investigation for obtaining bioactive chemicals from the algae materials. A variety of polar substances, including sugar- or protein-attached polyphenols, tannins, salts, saponins, mucus, glycosides, and organic acids, can be effectively extracted using polar solvents. 100 ml of each extract were macerated with 10 gms of the resulting material for 48 hrs with a constant stir. Following that, the samples were passed through using a Whatman No. 1 filter paper.

2.2. Phytochemical analysis

The Ethyl acetate and chloroform extracts of *S. asperum* has undergone initial secondary metabolites evaluation to detect a variety of different natural elements [17].

2.3. FTIR study

In order to understand more about the functional categories found in the seaweed, the FTIR signal of a *S. asperum* algae extract that had been dissolved in ethyl acetate and chloroform were estimated. Fluorescence can be beneficial to figure out the structures of newly identified materials that occur in plants. The raw methanolic extract was analyzed using FTIR, therefore it was fed into an FTIR spectroscope (FTIR-8400S, Shimadzu) with a 500–4000 cm^{-1} analytical region.

2.4. GC-MS study

On the GCMS-QP 2010 Plus Shimadzu system, GC-MS research of ethyl acetate and chloroform extract of *S. asperum* was done. Mass spectral analysis of phytocompounds: detection and analysis Employing the NIST and WILEY8 Records, GC-MS was carried out. They were comparing the unknown substances spectra of the substance that is unknown to those of known importance in the NIST database. Names, chemical formulas, and structural descriptions were given to the features that were discovered.

2.5. Antibacterial activity

The obtained ethyl acetate and chloroform extracts were subjected to antibacterial efficacy. Gram-positive *Bacillus subtilis, Streptococcus pneumonia*, and *Staphylococcus aureus* and the Gram-negative *Escherichia coli, Salmonella typhimurium,* and *Vibrio parahaemolyticus* were gathered from Christian Medical College (CMC), Vellore. Well plate procedure is used in this investigation. Different dosages of (25, 50, 75 and 100 mg/mL) were poured into the well. The positive reference was streptomycin. Compounds were allowed to diffuse for 30 mins at ambient temperature in the loaded discs. The discs were kept at 37°C for 24 hrs. The average values for each organism were determined, and their pure zone diameters were measured [18]. There were three separate runs of the experiment.

2.6. Antioxidant activity

2.6.1. DPPH activity

Using a UV spectrophotometer, the antioxidant capacity of methanolic extracts towards 2,2-diphenyl-1-picrylhydrazyl radical was examined. For this activity, the approach of Prieto [19] was utilized. In methanol, a 0.1mM solution of DPPH was made. 1mL of the made-up solution was added with 1mL of extract at 100 to 1000 µg/mL. The mixture was left at ambient temperature for 30 mins. At 517 nm, the reflectance was determined. Ascorbic acid was used as a reference point. Fewer absorbance values suggest a greater degree of action.

2.6.2. Larvicidal activity

The experimental specimens of *Ae. aegypti, An. stephensi*, and *Cx. quinquefasciatus* were obtained from ICMR, Vector Control Research Centre (VCRC) in Madurai. Every step of the larvicidal measurement adheres to the standard WHO protocol. In an experimental environment, the larvae were nurtured on plastic containers and fed a 2:1 mixture of yeast and dog cookies. 25 young 4^{th} instar mosquito larvae were placed in 100 mL disposable jars stuffed with water. The mortality rate of mosquitoes after 24 hrs of treatment was noted. All tests were undertaken in three separate batches at precise temperatures and relative humidity. Deceased larvae have been detected by their inability to migrate.

2.6.3. Histopathological study

Following the treatment, alterations in the gastrointestinal tract cell layer were noticed. The early 4^{th} instars of *Cx. quinquefasciatus, Ae. aegypti*, and *An. Stephensi* mosquito larvae were treated and stored in 10% formalin. The materials were anchored, then dried and fixed in paraffin. The already assembled slides were sliced with a microtome. The staining was carried out using hematoxylin and eosin. The slides were sterilized with xylene, the sections' abnormalities

were viewed under a light microscope, and the alterations were captured with images. The outcomes were contrasted with those of the untreated sample [20].

2.6.4. Statistical analysis

To figure out LC_{50}, the average data on larval mortality was examined using probit analysis. Standard deviations, or means, were used to present the data. We checked for statistical significance using the paired-sample t-test. To assess comparisons between time points, we utilized analysis of variance (ANOVA) with repeated measures. SPSS® version 20 used for the statistical test The P value of ≤ 0.05 was regarded as relevant.

3. Results and discussion

3.1. Phytochemical screening

Secondary metabolites present in the ethyl acetate and chloroform extracts of *S. asperum* were tannin, flavonoid, protein, carbohydrate and terpenoids. The phytochemicals present in *D. dichotoma* they used six solvents for extraction among them ethyl acetate extract displayed the extreme amount of the metabolites. Diana *S. vulgare* extract shows the presence of many secondary metabolites [21]. Singkoh [22] investigated the presence of secondary metabolites in the brown algae *P. australis* and it shows the presence of alkaloids, flavonoids, tannins, phenols, terpenoids, steroids and saponins. Ragunath [23] found the secondary metabolites in the brown algae *P. boergesenii* and *C. racemose* contains phenolic compound, terpenoids, phlobatannins and amino acids. The chloroform extracts from the specified brown *seaweeds D. dumosa, D. dichotoma, D. indica, P. boergesenii, and P. tetrastomatica* comprise a large proportion of the bioactive factors, according to Rachel's phytochemical examines of the marine algae.

3.2. FTIR analysis

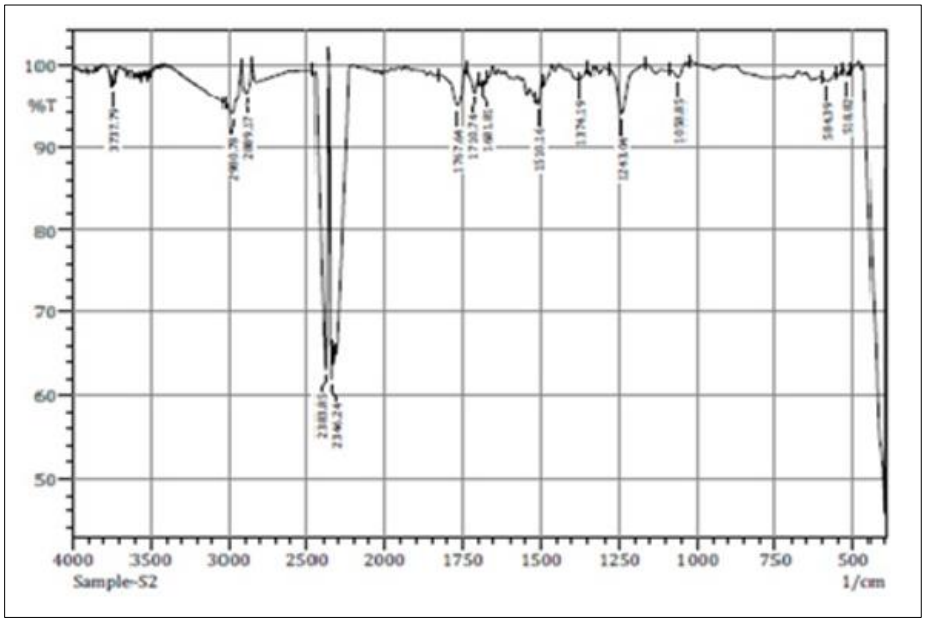


Figure 2 FTIR analysis of ethyl acetate extracts of *S. asperum*

The results of FTIR studies have shown that the ethyl acetate extract of *S. asperum* contains a number of different chemical components. The FTIR reports of ethyl acetate and chloroform extracts of *S. asperum* was shown in figure 2 & 3. The FTIR analysis of ethyl acetate shown the peak at 3737.79 cm^{-1} assigned to O-H alcohol, N-H amine salt were assigned the peak at 2980.78 cm^{-1}, the peak at 2383.85 cm^{-1} allocated to O=C=O carbon-dioxide, 1710.74 cm^{-1} was assigned to C=O aliphatic ketone, peak at 1681.81 cm^{-1} was assigned to C=O conjugated aldehyde, peak at 1510.16 cm^{-1} assigned to N-O nitro compounds, peak at 1243.04 cm^{-1} assigned to C-N amine, the peak at 1058.85 cm^{-1} assigned to C=O primary alcohol. Whereas the chloroform extract of *S. asperum* contains The FTIR analysis of chloroform extract of *S. asperum* 3874.72 cm^{-1} assigned to O-H stretching of alcohol, 2924.85 cm^{-1} assigned to O-H stretching of carboxylic acid, 2300.92 cm^{-1} allotted to N=C=O isocyanide, 1710.74 cm^{-1} assigned to C=O aliphatic ketone, 1621.06 cm^{-1} assigned to C=C conjugated alkene, 1544.88 cm^{-1} assigned to N-O nitro compound, 1460.98 cm^{-1} assigned to C-H alkane, 1280.65 cm^{-1} allotted to C-O aromatic ester, 1164.92 cm^{-1} assigned to C-O ester, 1057.88 cm^{-1} assigned to S=O sulfoxide, 971.09

cm^{-1} assigned to C=C alkene, 838.01 cm^{-1} assigned to C-Cl halo compounds, 722.29 cm^{-1} assigned to C=C alkene, 668.29 cm^{-1} assigned to C-Br halo compounds. As per the findings of *S. tenerrimum*. The findings from the FTIR inquiry confirmed the existence of several different functional groups, such alcohol, phenol, aliphatic, anhydrides, aromatics, and aliphatic amines. The outcomes of the FTIR study conducted on algae isolates indicated the presence of harmful interaction sites on the surfaces of the algae. These sites were identified as carboxyl, amino acid, and hydroxyl groups associated with the algae [24].

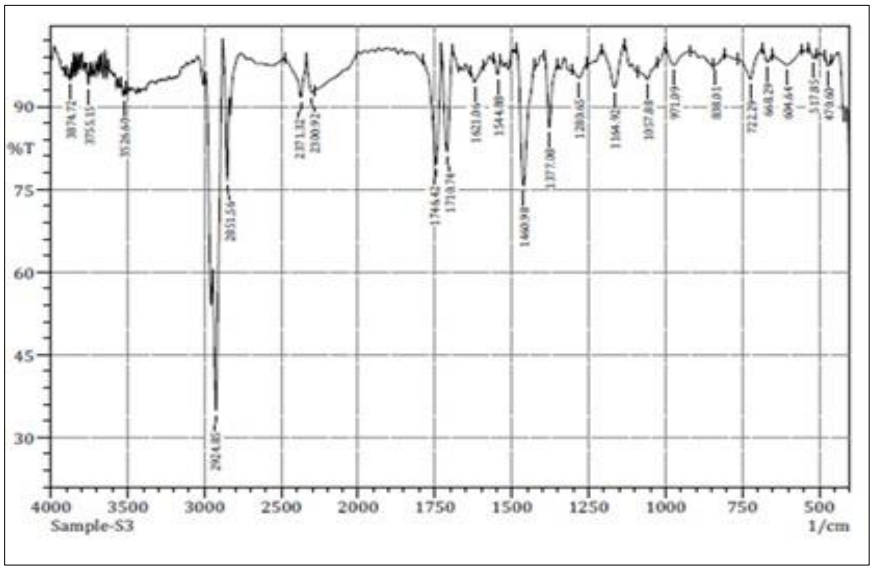


Figure 3 FTIR analysis of chloroform extracts of *S. asperum*

3.3. GC-MS study

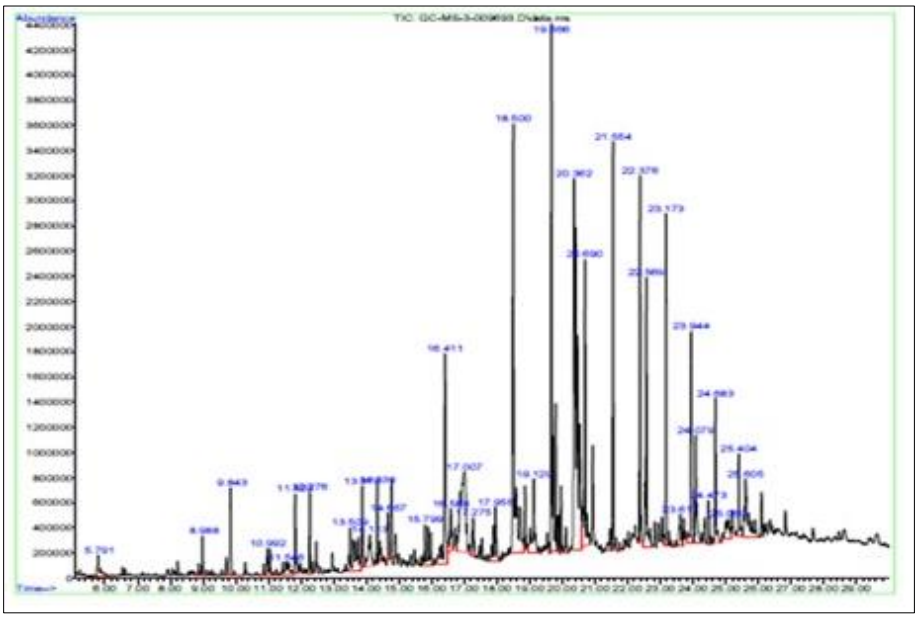


Figure 4 GC-MS analysis of ethyl acetate extracts of *S. asperum*

Using GC-MS analysis, 35 bioactive compounds from the ethyl acetate extract of *S. asperum* exhibiting various phytochemicals were discovered. Figure 4 & 5 shows the retention time, peak area, biological activities and percentage peak area of various bioactive compounds. The GC-MS analysis confirmed the presence of bioactive compounds in the extracts. Among them 13 compounds were reported for its activity. The prevailing compounds were Tridecane, 1-iodo (0.90), Benzene (2.10), Eicosane (4.28), Heneicosane (4.06), Pentadecane (9.35), Hexadecane (0.90), Phenol, 2,4-bis(1,1-dimethylethyl)- (1.13), Dodecanoic acid (0.58), Tetradecanoic acid (8.29), cis-Vaccenic acid (16.22). Whereas the chloroform extract contains Dibutyl phthalate (3.34), cis-vaccenic acid (16.22), capparatriene (0.36%), henicosane

(0.62), cis-bete-farnesene (7.75), 5-Androsten-17. alpha-ethynyl-3 (14.53). All the identified compounds have Anti-fungal, antipyretic, Antioxidant, antibacterial, cytotoxic, anticancer, antidiabetic and anti-inflammatory activities. The presence of bioactive elements in the brown algae *Padina boergesenii* it contains many compounds that contains Insectifuge, nematicide, Antibacterial, antiasthma, diuretic, hepatoprotective, hypocholesterolemic, antispasmodic activity and anti-eczematic activities [25].

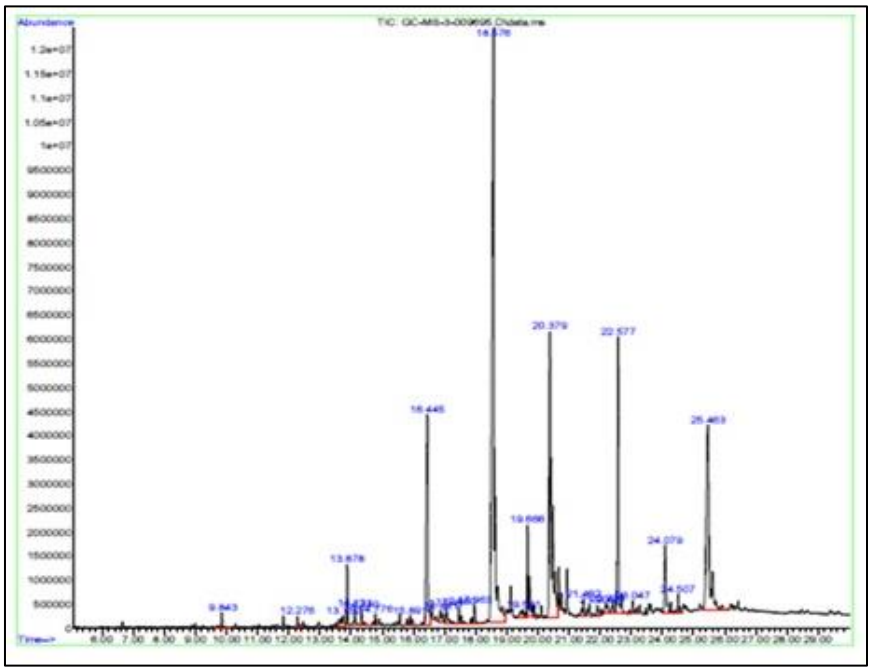


Figure 5 GC-MS analysis of chloroform extract of *S. asperum*

3.4. Antibacterial activity

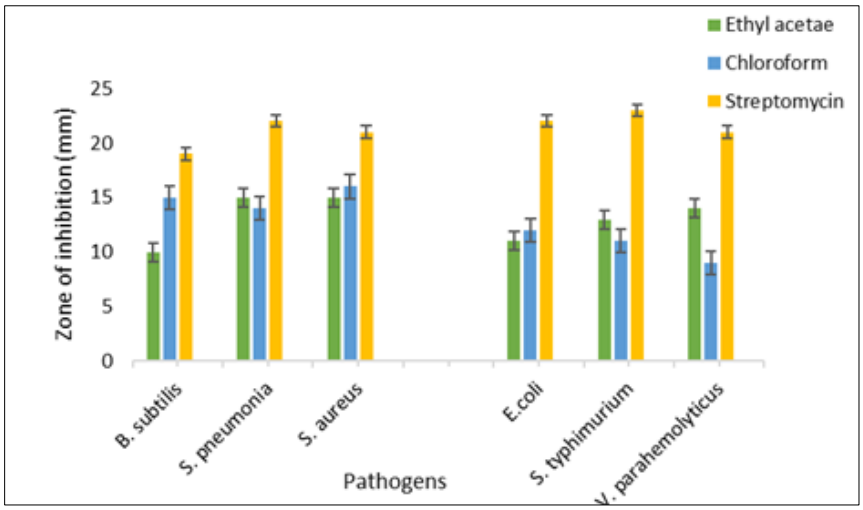


Figure 6 Antibacterial activity of ethyl acetate and chloroform extract of *S. asperum*

The antibacterial activity of the extracts was shown in figure 6. The ethyl acetate extract shows maximum activity against Gram^{-ve} *V. parahaemolyticus* 14±0.5, *E. coli* 14±2.0 and the Gram^{+ve} *S. pneumoniae* 15±1.50, *S. aureus* 15±1.7. The maximum activity of ethyl acetate extract against the Gram^{-ve} *S. typhimurium* 13±1.5, *E. coli* (14±1mm) and the Gram^{+ve} *B. subtilis* (13±2.6mm), *K. pneumonia* (12±2.6mm). The chloroform extract shows maximum activity towards Gram^{+ve} *S. aureus (*15±1.7mm) and *S. pneumoniae (*15±1.50mm). The main chemical components of algal cells are believed to be botanical compounds including phenol, flavonoids, and tannin substances, which, relying on their concentration and setting, may either activate or inhibit the growth of bacteria. The human microbes *S. aureus, S. mutans*, and *E. coli* are prevented from growing by the brown algae *P. australis* [22]. The brown algae *P. boergesenii* and *C. racemosa* has the

ability to inhibit the growth of both gram positive and negative microbes and it shows the maximum activity towards. the maximum activity of *C. racemose* against *Ps. aeruginosa* 23.6 ± 0.3, *S. aureus* 22.0 ± 0.8 and *P. boergesenii* shows *K. pneumoniae* 18.2± 0.3, *B. subtilis* 19.7 ± 0.2 [23]. According to earlier studies, algal extracts were typically more successful towards Gram^{+ve} than Gram^{-ve} bacteria. This is presumably due Gram^{-ve} bacteria possess more intricate cell membranes.

3.5. Antioxidant activity

3.5.1. DPPH activity

Based on our findings, the raw extract of *S. asperum* has a powerful anti-oxidant ability. The DPPH activity of ethyl acetate and chloroform extracts of *S. asperum* was shown in figure 7. The DPPH-reducing test found that a crude extract of *S. asperum* was exceptionally effective at a dose of 200 µg/ml. The DPPH value was extremely similar to the reference value, indicating that the plant-mediated antioxidant capacity was likely to benefit future investigations into biology [26]. It is believed that radicals' capability to scavenge DPPH radicals stems from their propensity to provide hydrogen. By accepting an electron or hydrogen radical, DPPH transforms into a dielectric molecule that is both rigid and free-radical. The brown seaweed *C. trinodis* has been studied for its DPPH activity and it shows 70.44% [24]. The DPPH analysis of *S. wightii* shown 79.1±0.23% inhibition additionally, the investigation shows that brown algae crude extract had stronger peroxyl radical scavenging activity than red seaweed raw extract [27].

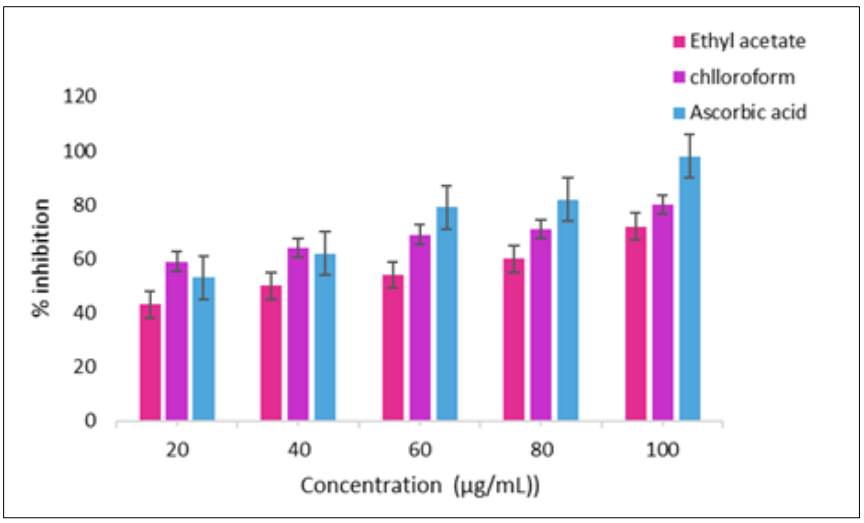


Figure 7 DPPH activity of ethyl acetate and chloroform extract of *S. asperum*

3.6. Larvicidal activity

The mosquito larvicidal activity of the brown seaweed against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* were given in table 1 & 2. Among all the three tested species ethyl acetate extract has virtuous activity against the malarial parasite *Cx. quinquefasciatus*. After contact to the seaweed extract, larvae, and female adults showed signals of drunkenness, such as frantic motion tremors, seizures, and final fatality [28]. the seaweed extract cause nerve poisons to the tested mosquito. *Gracilaria corticate, S. microystum T. decurrens and T. conoides were analyzed towards the 4th instar larvae of Ae. aegypti, Cx. quinquefasciatus, An. stephensi and the outcome shows all the tested extracts were effective against the tested mosquito larvae[1]. It has been proven that seaweed modifies multiple phases of the lifespan of mosquitoes in a sublethal manner. A hormonal defect following treatment is truly what is most likely the reason for the treated larvae's prolonged larval transformation [*29]. *Bryopsis pennata, Sargassum binderi* and *Padina australis* were tested against the 4th instar larvae of the dengue vector *Aedes aegypti and the outcome reveled that B. pinnata was highly active against Ae. aegypti and the LC_{50} value was* 82.55 µg/mL, followed by *S. binderi* and *P. australis* [30]. The methanol extract of *S. asperum* exhibits significant mosquitocidal action against *Ae. aegypti* larvae [31]. A variety of pharmacological effects have been associated with bioactive substances derived from Sargassum species, notably steroids, quinones, tannins, phlorotannin, fucoidans, sterols, and glycolipids. The chemicals that makeup seaweed is an important aspect of its bioactivity.

Table 1 Larvicidal activity of chloroform extracts of *S. asperum* against selected larvae

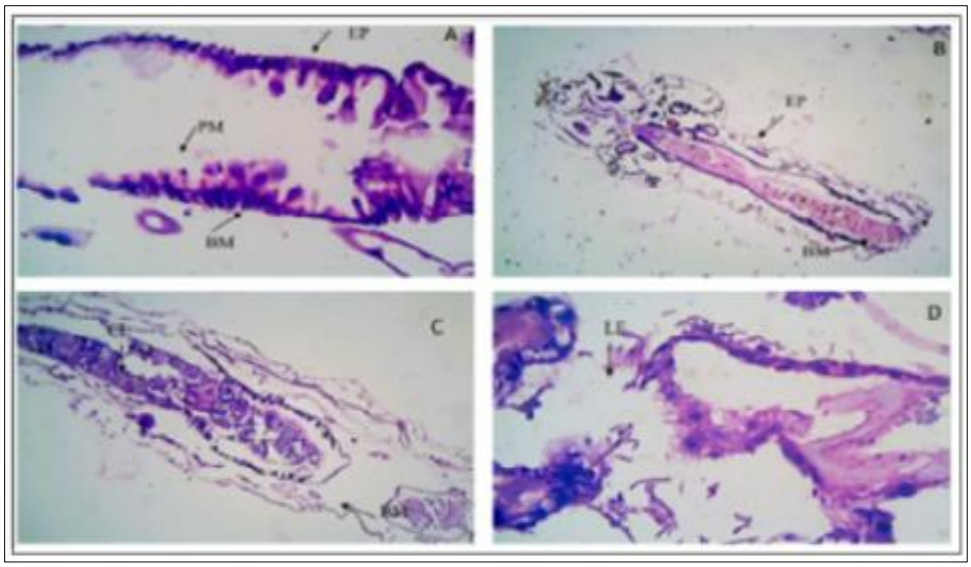
Mosquito species	Treatment period	LC_{50} (mg/L)	LC_{90} (mg/L)	R^2	Regression equation
Ae. aegypti	24hrs	210.99	5285.66	0.9964	y=0.561x+3.02
	48hrs	51.76631	114.4722	0.9492	y = 3.3212x-1.1939
	72 hrs	73.27544	177.9508	0.9241	y = 3.7132x-1.3646
Cx. quinquefasciatus	24hrs	374.19	11753.71	0.983	y = 1.0553x+1.9725
	48hrs	208.6698	1558.834	0.9191	y = 1.4656x+1.6006
	72 hrs	74.11228	206.063	0.9857	y = 2.8768x-0.3793
An. stephensi	24hrs	239.6934	953.4546	0.9283	y = 2.1343x-0.0789
	48hrs	211.2117	1687.718	0.7784	y=1.4181x+1.7033
	72 hrs	66.38127	225.9436	0.8404	y = 2.4023x+0.6229

Table 2 Larvicidal activity of ethyl acetate extracts of *S. asperum* against selected larvae

Mosquito species	Treatment period	LC_{50} (mg/L)	LC_{90} (mg/L)	R^2	Regression equation
Ae. aegypti	24hrs	449.007	862.78	0.4307	y =4.5119x-6.9667
	48hrs	176.06	962.98	0.8911	y=1.7356x+1.1024
	72 hrs	114.73	844.695	0.9713	y = 1.4763x+1.9593
Cx. quinquefasciatus	24hrs	296.949	1677.26	0.9892	y = 1.0775x+0.1089
	48hrs	158.279	837.336	0.9213	y = 1.4741x-1.0317
	72 hrs	81.6098	251.131	0.8994	y = 0.33x+2.965
An. stephensi	24hrs	521.022	4346.1	0.997	y = 0.21x+2.9667
	48hrs	203.006	1836.12	0.9863	y=1.5098x-1.7085
	72 hrs	150.716	1217.31	0.9714	y = 1.6511x-1.7085

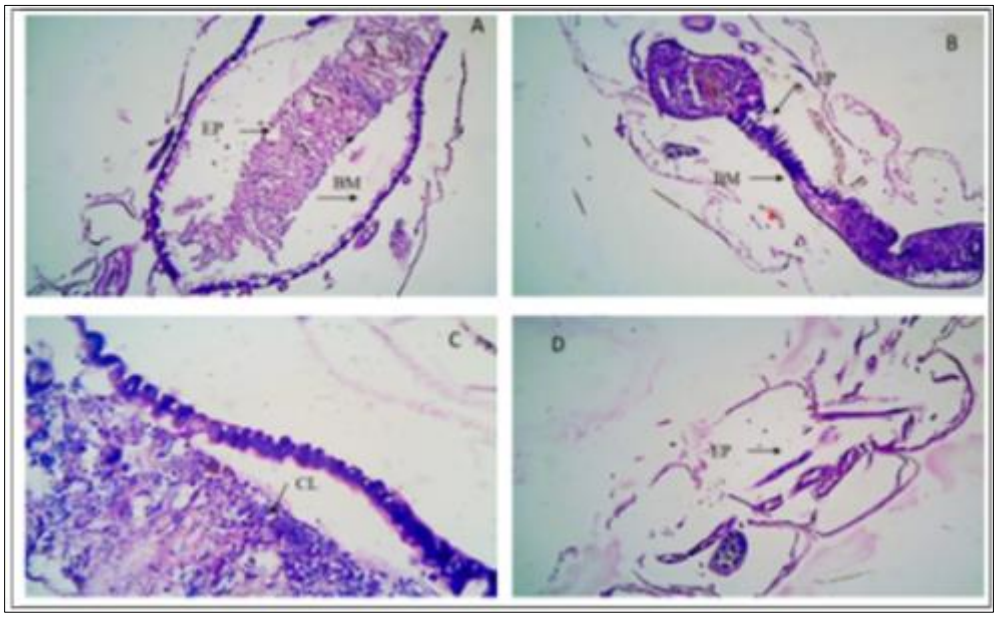
3.7. Histopathological changes

The histopathological changes in the tested organisms were given in the figures 8, 9 & 10. The seaweed extract alters the histopathological activities of the selected mosquitoes when compared to the control. The 4th instar of *Ae. aegypti* was treated with the brown seaweeds *B. pennata, S. binderi* and *P. australis*, the histological examination proved that the middle-gut epithelium of larvae subjected to algae extracts was altered cyto-pathologically [28]. *S. wightii* extract was tested against the 1-4th instar stages of *An. sundaicus* and it showed the repellency of 89%. among all the larval stages tested the 2nd instar stage was very susceptible to the extract and it caused the damage to the gut system [30]. *Canistrocarpus cervicornis*, *Laurencia dendroidea* were very active against the 4th instar stage of *Ae. aegypti* at the 300 ppm. A halogenated sesquiterpene molecule, known as elatol, was obtained using a series of fractionation steps applied to the n-hexane extract derived from the aforementioned seaweed species. The isolated chemical had significant larvicidal activity, as seen by its LC_{50} value of 10.7 ppm. The potential extraction of elatol from seaweed presents a promising opportunity for the development of a new drug for treatment targeting dengue [32].



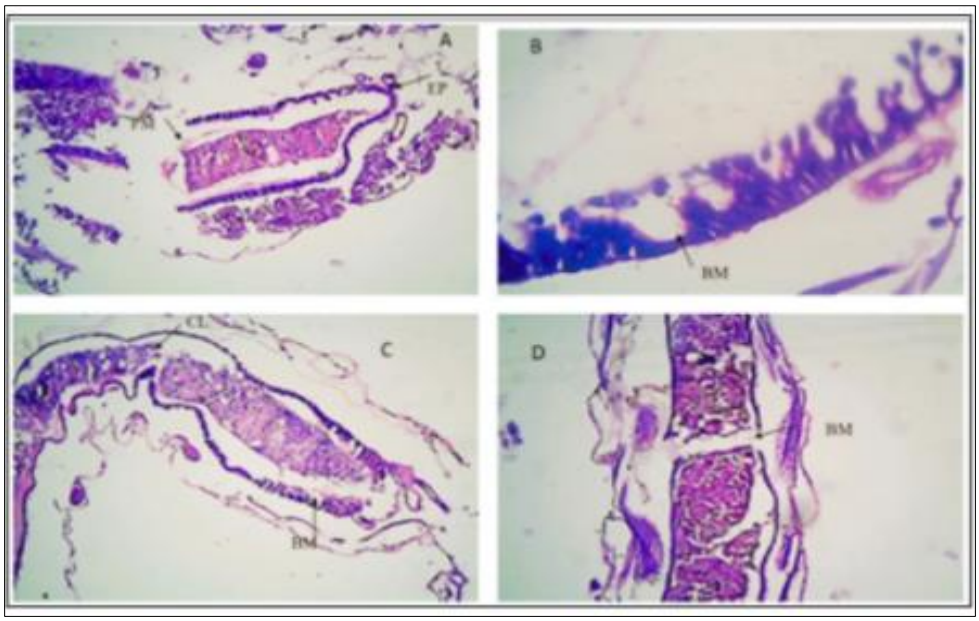
[A]- Control, [B]-Treated with Chloroform extract, [C, D]- Treated with ethyl acetate extract

Figure 8 Histopathological analysis of *Ae. aegypti* treated with chloroform and ethyl acetate extract of *S. asperum*



EP (Epithelial cells), BM (Basal membrane), PM (Peritrophic membrane), LC (cell lysis), A- control, B & C-treated with ethyl acetate, and D-treated with chloroform

Figure 9 Histopathological analysis of *Cx. quinquefasciatus* treated with chloroform and ethyl acetate extract of *S. asperum*



EP (Epithelial cells), BM (Basal membrane), PM (Peritrophic membrane), LC (cell lysis), A- control, B & C-treated with ethyl acetate, and D-treated with chloroform

Figure 10 Histopathological analysis of *An. stephensi* treated with chloroform and ethyl acetate extract of *S. asperum*

4. Conclusion

Marine brown algae are an invaluable source of biologically active substances with diverse medicinal effects. The present investigation examined the possible antibacterial, antioxidant, and mosquito larvicidal properties of chloroform and ethyl acetate extracts of *S*. *asperum* against *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus*. In addition, several risk factors and the emergence of insect resistance to traditional pesticides have led to a preference for larvicides produced from natural sources over synthetic ones. Biological pesticides, particularly those derived from plants and marine algae with higher selectivity and rapid degradability, hold incredible promise. GC-MS analysis confirms the presence of biologically active compounds. The presence of various functional groups in both chloroform and ethyl acetate extracts determined FTIR analysis. The antioxidant assay results from this investigation are consistent with the extracts' antioxidant activity demonstrated in earlier studies. After 72 hrs of treatment, the chloroform extract was extremely active against *Cx. quinquefasciatus*, while the ethyl acetate extract had modest activity against *An. stephensi*. Nevertheless, the precise mechanism and the specific chemical accountable for the larvicidal effects remain uncertain. Hence, additional research is warranted to investigate the active chemicals responsible for the observed effects and elucidate their mechanisms of action in field trials. These findings will be crucial in establishing *S. asperum* as a viable anti-mosquito product for implementation in mosquito control programs to combat and safeguard against mosquito-borne diseases.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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