Phytochemicals from the algal species *Chara braunii*

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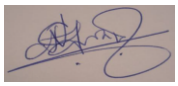

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Abstract

The algal species *Chara braunii* contains complex compounds that can be exploited for biosynthesis of agro-chemicals for the management of insect pests and phytopathogens. A phytochemical screening of various extracts from the plant reveals the presence of steroids, glycosides, tannins and saponins. A pure compound of steroids was isolated and identified using semi-qualitative approach. The crude extracts from this plant are of great importance to the project. They will be assayed to determine their bioactivities against plant-parasitic nematodes, insect pest and fungal/bacterial pathogens.

Keywords: Phytochemistry *Chara braunii*, plant-parasitic nematodes, steroids

Introduction

Plants are sources of botanical compounds capable of controlling pathogens and insect pests of agricultural importance. These botanicals are safe to the users and the environment. Currently, studies have focussed on the extraction and profiling of the phytochemical compounds from different plant species. Algal species have been shown to contain complex chemical profiles. The chemical constituents from these plants have been used to control various plant-parasitic nematodes (Ghareeb et al., 2019).

For instance, extracts from the brown marine algae *Ascophyllum nodosum* have been shown to suppress the reproduction of the root-knot nematodes *Meloidogyne javanica* and *M. incognita* in plants (Wu et al., 1997; Ghareeb et al., 2019). Treatment of sunflower plants with extracts from the marine algae *Botryocladia cabillaceae* have been shown to reduce formation of galls and egg masses (Ibrahim et al., 2007). Further, extracts from *Ulva fasciata* contain polyphenolic and diterpenoids compounds that are toxic to pathogenic bacteria (Silva et al., 2013. Other studies have shown that compounds from marine plants can increase plant growth parameters and therefore used to manufacture bio-fertilizer (Ghareeb et al., 2019). However, results of extracts from 32 seaweeds have shown that their activities are species dependent (Khan et al., 2015).

This study aimed to analyse the bioactivities of extracts from fresh water algae plant species specifically the study aimed to (i) isolate and characterize active compounds from the fresh water algae species *Chara braunii* and analyse the bioactivities of its compounds against plant pathogens and insect pests.

The central hypothesis of this study is that fresh water algae plant species contain biologically active compounds for disease and insect suppression.

Material and methods

Sample collection, drying and grinding of samples

The algal species used in this study was collected from Kesses dam, Eldoret (N 00°17.268` E 035°19.886; elevation 2,200 m) and identified as *Chara braunii*. Algal samples were cleaned of any necrotic parts. The whole algae plant was used in the extraction of phytochemicals. The algae was dried under a shade and ground into fine powder using an electric grinder at the Centre for Traditional Medicine and Drug Resistance (CTMDR), Kenya Medical and Research Institute (KEMRI) in Nairobi. The powder was stored in khaki brown paper bags at room temperature until further use.

Fig. 1: Collection of algae species. A: Dr. Njira in a boat ride during the collection of the samples at Kesses Dam in Eldoret, B: In-situ pictures of algae in water and C: fresh collected samples of the algae. Photo by the author.

Extraction of phytochemicals

Organic extraction was done using four organic solvents: methanol, ethyl acetate, hexane and DCM + methanol. For methanol, ethyl acetate and hexane extraction procedures, 500 ml of the solvent were added separately into 50 g of the algal powder in a conical flask. A ratio of 1:1 of DCM and methanol were applied for the DCM + methanol extraction procedure where 500 ml of a mixture of the two solvents was added into 50 g of the algal powder in a flask. The flasks were covered with aluminium foil and left to stand overnight at room temperature. Later, the extracts were filtered through Whatman filter papers (no 2) and the filtrate rota-evaporated (Rotavopor R-300) to remove the solvents. Afterwards, the dry compounds were reconstituted using the same solvents and stored at room temperature until further use.

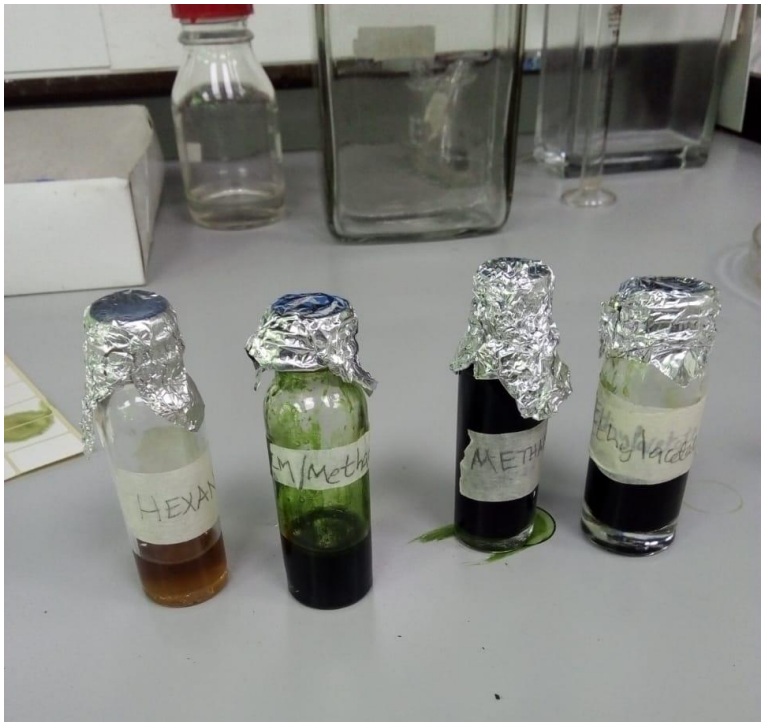

Fig. 2: Crude extracts of algae based on the different solvents: Hexane, DCM + methanol, methanol and ethyl acetate. Photo by the authors.

The total yields of the extracts were calculated using the formula:

$$Yield = \frac{Weight\ of\ the\ extract}{Total\ weight\ of\ the\ plant\ materials} x100$$

Fig. 3: Drying of the extracts on a rato-vaporator (Rotavopor R-300). Photo by author.

Water extracts were obtained after 100 g of dried sample powder was put in a flask into which was added 1 liter distilled water and placed in a water bath at 70˚C for 2 hours. This was then filtered through Whatman filter papers (no 2) into a round bottomed flask and the filtrate placed in a freeze drier for 72 hours at -104˚C and 800mbar. The flasks were then placed in a bowl with dry ice and acetone added to the bowl. These extracts were then dried using a vacuum drier. Both organic and water extracts were stored at 4°C until use.

Fig. 4: Drying of water extracts. A: freeze-drying of the extracts on dry ice and acetone and B: drying of the water extract on vacuum machine. Photo by the authors.

Phytochemical screening

This test was performed to determine the different classes of compounds present in the extracts. The targeted classes of compounds were phenols, steroids, glycosides, tannins, saponins and flavonoids. The methodology applied for determination of the different compounds is summarised in Table 1. These phytochemicals were confirmed by colour changes.

Table 1: Methods applied in analysis the different classes of compounds present in algal extracts.

Class of compounds	Methodology	Indicator	Reference
Steroids	A volume of 10 ml of chloroform was added to 2 ml of each extract (methanol, ethyl acetate, hexane and DCM + methanol) and gently	A colour change from blue to green indicates the presence of steroids	Thilagavathi et al. 2015

	shaken. Later 1 ml of acetic hydrant and 2ml of sulphuric acids were added successively.		
Glycosides	3-4 drops of ferric acid, acetic acid and sulphuric acids were successively added to 1 ml of each extract (methanol, ethyl acetate, hexane and DCM + methanol) while gently shaking.	A colour change from blue to green indicates the presence of glycosides.	Rao et al., 2016
Tannins	5 ml of each extract (methanol, ethyl acetate, hexane and DCM + methanol) was added to 2 ml of 5% $FeCl_3$ solution and gently shaken until a change of colour was detected.	A change of colour from green to black indicates the presence of tannins.	Rao et al., 2016
Saponins	A volume of 1 ml of each extract (methanol, ethyl acetate, hexane and DCM + methanol) was added to 9 ml of distilled water and shaken vigorously for 15 minutes. The extract was allowed to stand for another 10 minutes before analysing the results.	Formation of a 1-cm form indicates the presence of saponins.	Thilagavathi et al. 2015
Flavonoids	1 to 5 drops of concentrated hydrochloric acid (HCl) were added into 1 ml of each extract (methanol, ethyl acetate, hexane and DCM + methanol) while gently shaking.	Formation of red colour indicate the presence of flavonoids.	Rao et al., 2016

Thin Layer Chromatography (TLC)

TLCs were run to give a general overview of the different compounds present in the algal extracts. To do this, drops of the extracts were spotted on aluminium silka gel coated TLC (TLC Silica gel 60 F_{254}) and placed in solvent tanks containing petroleum ether (PET ether) and acetone in the ratio 9:1. After running up to the solvent front, the TLCs were dried in the oven and sprayed with a solution of 1 g vallinin in 100 ml sulphuric acid followed by heating at 100°C for 5 minutes. The TLCs were then observed under UV^{+} light. Vallinin aids in the visualization of the organic compounds.

Preparatory Thin Layer Chromatography

To have a wider overview of the separation of the compounds, 12 cm by 12 cm glasses were coated with silka gel and 1-ml volume of the extracts spotted. Later, the glasses were placed in tanks containing PET ether and acetone in the ratio of 9:1 and left to run until the solvent

reached the front line. The glasses were removed from the oven, spayed at the margins with a solution of vallinin in 100 ml sulphuric acid followed by heating at 100˚C for 5 minutes. Clear band were scooped, dissolved in hexane and filtered through Whatman filter paper.

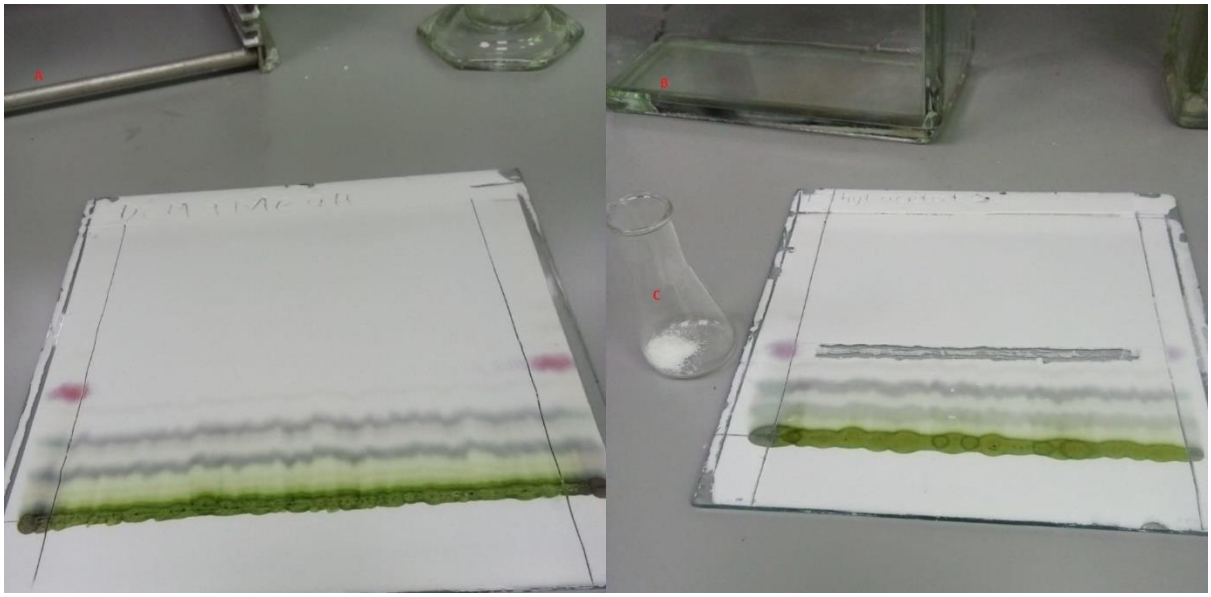

Fig. 5: Preparatory Thin Layer Chromatography. A: a picture of the glass plate sprayed at the margin with vallinin, B: scooped silka gel containing the purified compound of the extract and C: silka gel in flask ready for reconstitution. Photo by the authors.

Identification of the compounds

A semi-qualitative approach was applied to identify the compound from algal extracts. Filtrates from the clear bands of the preparative thin layer chromatography were spotted on aluminium silka gel coated TLCs. A well-characterised steroid compound was used a control. The TLCs were placed in tanks containing PET ether and acetone in the ratio of 9:1 and left to run until the solvent reached the front line. Later, they were removed from the tanks, spayed with a solution of vallinin in 100 ml of sulphuric acid followed by heating at 100°C for 5 minutes.

Column chromatography

This was performed to fractionate the compounds in the algal extracts. A stationary phase of silka gel was prepared by dissolving 10 g of silka gel in water at the ratio of 1:10 (water: silka gel). A 1-g of the extract (DCM + methanol) was weighed and added on top of the stationary phase. To maintain a uniform layer of the compound while adding the solvents, a 1-cm layer of cotton wool was placed on top of the compound. A 50 ml of 100% hexane was added and eluted. This was followed by another 50 ml of 10% acetone. The concentration of acetone was gradually increased from 20%, 30%, 40%, 50%, 75% up to 100% (acetone). Eight fractions were obtained. The composition of these compounds was visualised under TLCs as described above. These fractions will be assayed for bioactivity against plant-parasitic nematodes, insect pests, fungal and bacterial pathogens under laboratory conditions. The best performing fraction will be further fractionated until a pure compound is obtained.

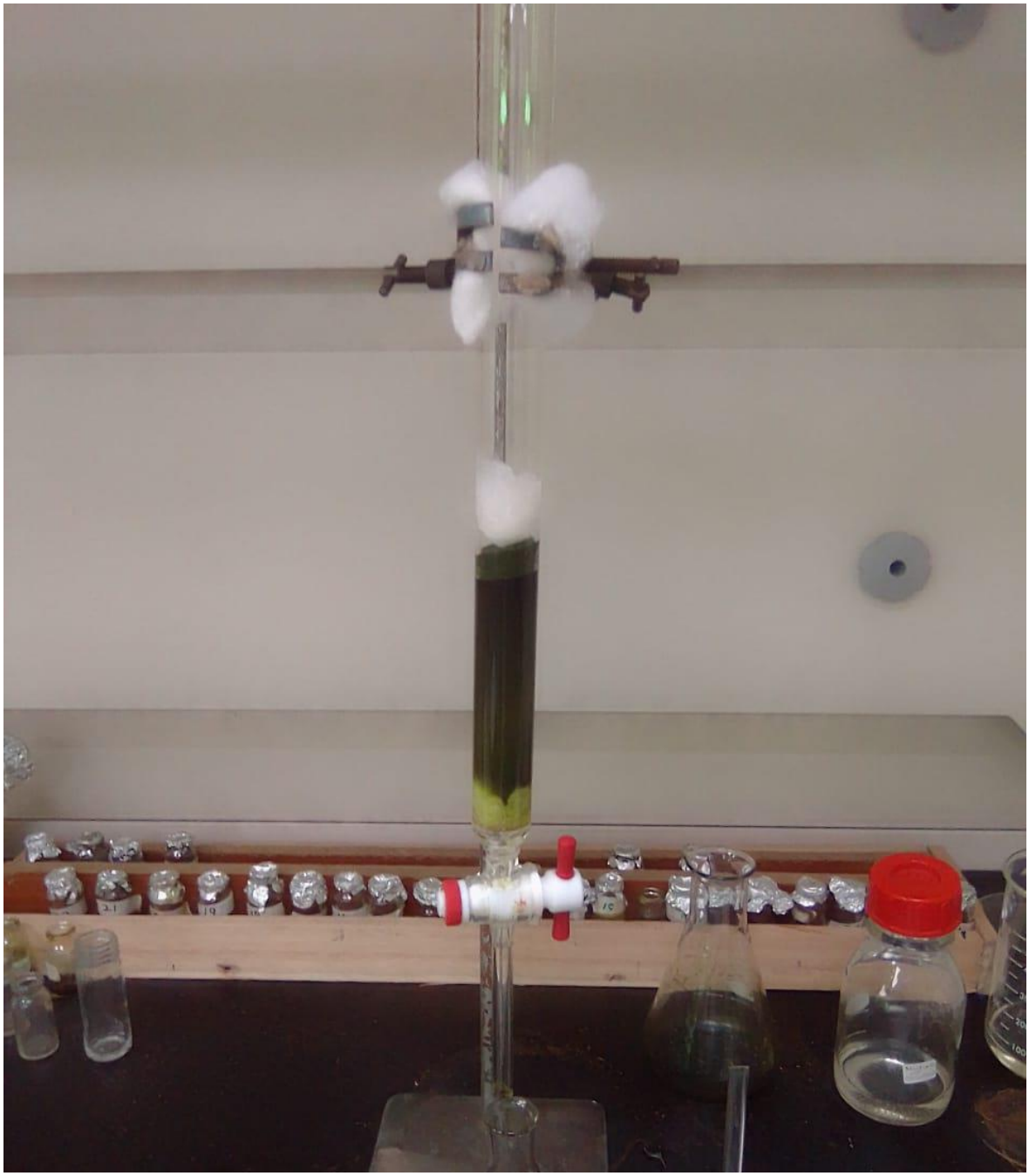

Fig. 6: Column chromatography to fractionate the compounds in the algal extracts. Photo by authors.

Separation of compounds using a separation funnel

A complementary test was performed to separate the compounds. Ten grams (10 g) of the crude extract was dissolved in 250 ml of ethyl acetate in a separating funnel. Another 250 ml of water was added to the solution. After gently shaking for 5 minutes, the experiment was left to stand for 24 hours or until when clear phases were visible. The bottom phase (water) was eluted first followed by the top phase (ethyl acetate). Compounds from both the phases were recovered after evaporating the solvents. The compounds present were visualised under TLCs as described above.

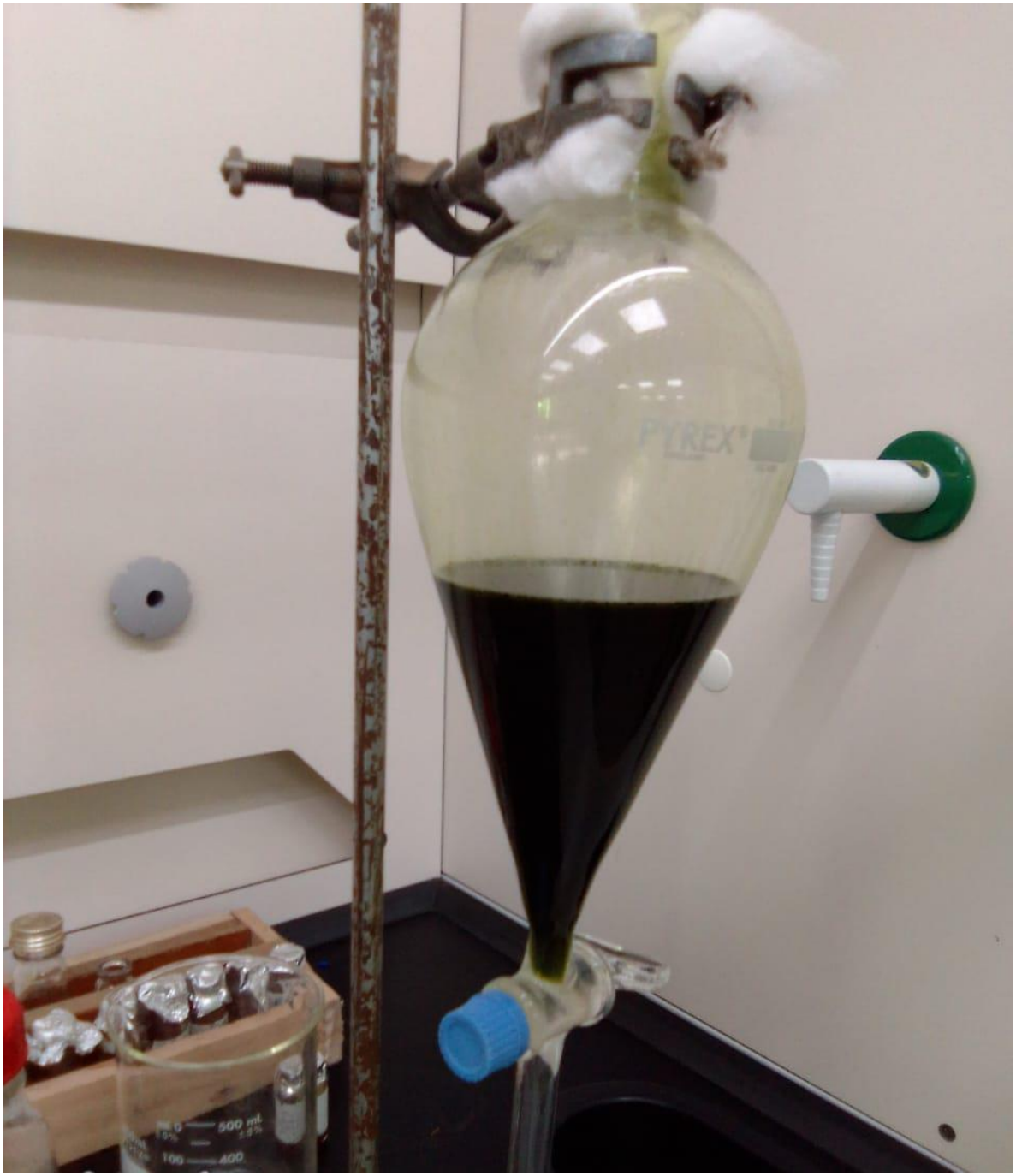

Fig. 7: Separation funnel containing the extracts. Photo by authors.

Soxhlet extraction

This extraction method was intended to extract essential oils. One-hundred grams (100 g) of the algal powder was dissolved in 5 litres of water and placed on a heating element connected to the Soxhlet apparatus. The experiment was left to stand overnight. Essential oils were drained from the Soxhlet apparatus and analysed.

Fig. 8: Soxhlet extraction for essential oils. Photo by the authors.

Results

Phytochemicals

Table 2 shows the percentage yield of the methanol, ethyl acetate, hexane and DCM + methanol extracts. Hexane extracted the highest content of the compounds, followed by ethyl acetate and methanol/DCM + methanol, respectively.

Table 2: Percentage yield of the extract

Extract	Weight of the plant material (g)	Weight of the extract (g)	Yield of the extract (%)
Methanol	50	1.00	0.2
Ethyl acetate	50	2.00	0.4
Hexane	50	3.00	0.6
DCM + methanol	50	2.00	0.2

Phytochemical screening of the extracts showed a complex composition of the compounds. Methanol, ethyl acetate and DCM + methanol extracted steroids, glycosides and tannins while hexane solvent was able to extract the glycosides and saponins compounds (Table 3).

Table 3: Phytochemical screening of the extracts to determine the composition of the compounds

	Solvents				
Phytochemicals	Compound	Methanol	Ethyl acetate	Hexane	DCM + methanol
	Steroids	+	+	-	+
	Glycosides	+	+	+	+
	Tannins	+	+	-	+
	Saponins	-	-	+	-
	Flavonoids	-	-	-	-

(+)Present, (-) Absent

Water extracts

About 3 g of the water extract were obtained after freeze-drying under the vacuum pump. The extract was stored under room temperature until further use.

Thin Layer Chromatography (TLC)

Investigation by TLC of the crude extracts from the algal species revealed a complex chemical profile rich in several compounds. Hexane and DCM + methanol solvents extracted most compounds. Ethyl acetate was able to extract just few compounds while methanol solvent extracted the least compounds (Fig. 9).

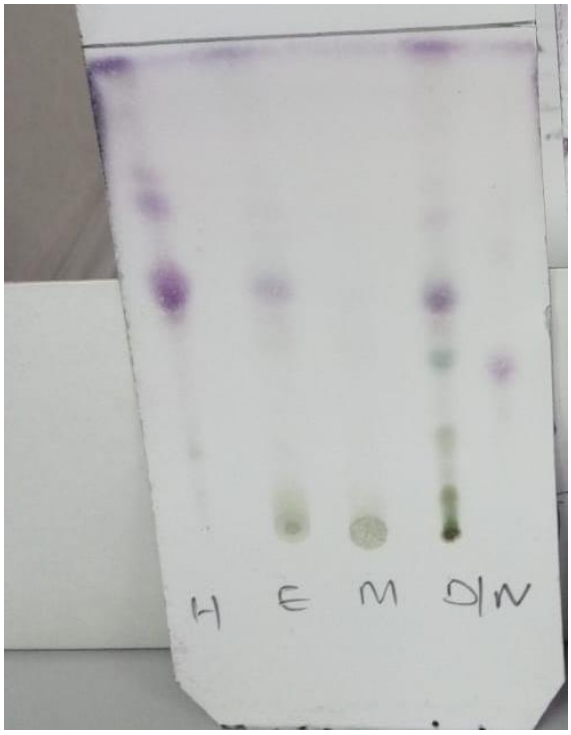

Fig. 9: TLC of the four algal extracts. H: Hexane; E: Ethyl acetate; M: Methanol; D/M: DCM + methanol. Photo by authors

Preparatory Thin Layer Chromatography and identification of the compounds

Analysis of the bands formed on the glass coated silka gels revealed a complex composition of different compounds present in the algal extracts (Fig. 10). After scooping, dissolving and filtration of the compounds, one major compound was isolated and purified. Preliminary analysis showed that the compound belonged to the class of steroids. A TCL run with a standard steroid compound confirmed that the isolated compound shared similar characteristics with the standard steroid compound (Fig. 11).

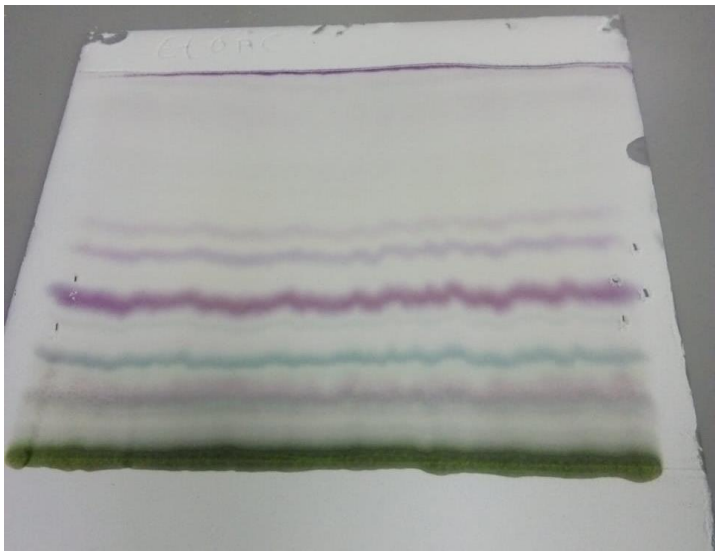

Fig. 10: Results of preparative thin layer chromatography. Photo by author.

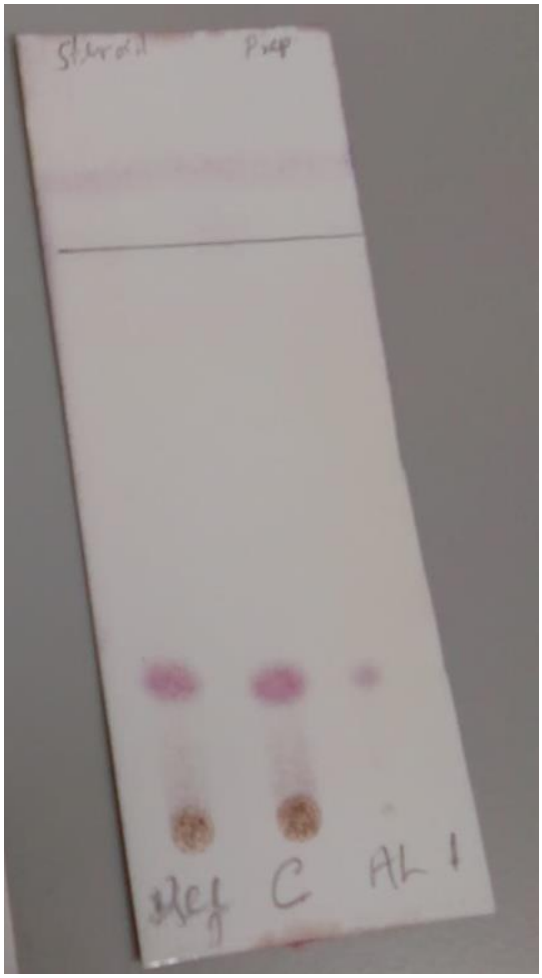

Fig 11: Identification of compounds present in the algal compounds. Ref: is a commercial steroid compound, C: is a well-characterised steroid from the CTMDR lab and AL is the purified steroid compound from the algal extracts. Photo by authors.

Column chromatography

Eight fractions were obtained. A TLC analysis to visualise the compound present showed that as concentration of the solvent is gradually changed, the content of the extracted compounds decreases. More compounds are extracted when 100% hexane is used. This observation was similar for 10%, 20%, 40% and 50% acetone. Pure acetone extracted lest compounds (Fig. 12).

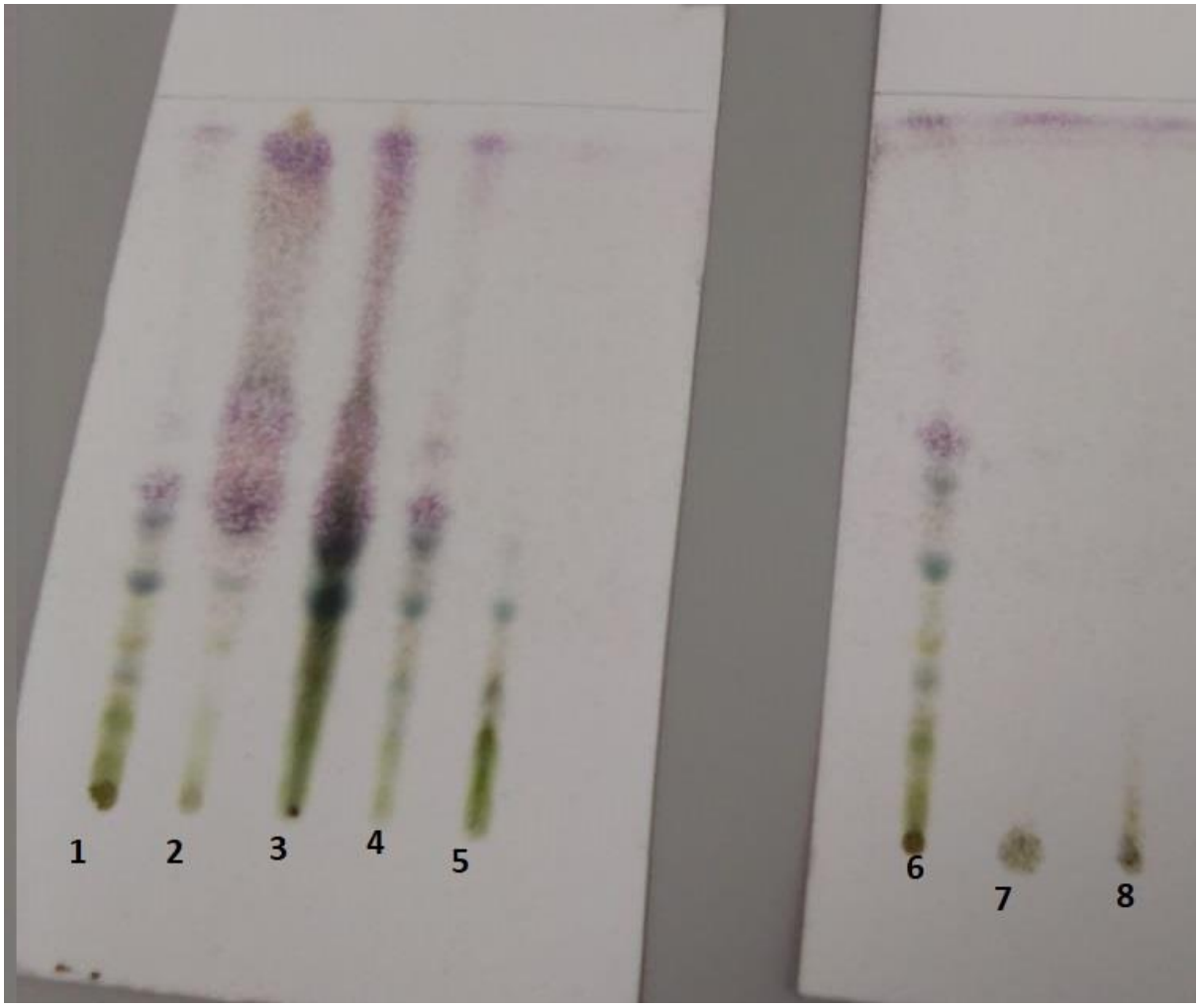

Fig. 12: Fractions of algal compounds. 1: a fraction obtained when 100% hexane was used, 2: a fraction obtained at 10% acetone, 3: a fraction obtained at 20% acetone, 4: a fraction obtained at 30% acetone, 5: a fraction obtained at 40% acetone, 6: a fraction obtained at 50% acetone, 7: a fraction obtained at 75% acetone and 8: a fraction obtained at 100% acetone. Photo by authors.

Separation of compounds using a separation funnel

The results of the TLCs for the compounds extracted through the separation funnels were similar those obtained when hexane was used as solvent (Fig. 9).

Soxhlet extraction

This experiment was for demonstration purposes hence the results obtained were not scientifically validated.

Discussion

The main objective of this staff exchange programme was to isolate and characterise phytochemical from the algal species *Chara braunii*. Results have shown that this plant species contains complex compounds. Hexane was the most effective solvent in the extraction process compared to ethyl acetate, methanol and DCM + methanol. Previous studies have shown that hexane is an excellent solvent for extracting phytochemicals (Thilagavathi et al., 2015; Rao et al. 2016). Steroids, glycosides, tannins and saponins were present in algal extracts. These compounds play various roles in plants.

Key among them is the induction of defence responses against plant pathogens and insect pests. For instance, saponins from Medicago species have been demonstrated to suppress the infection and reproduction of the root-knot nematode *Meloidogyne incognita* (D'Addabbo, et al., 2020). Saponins are large group of chemicals that consist of triterpene or steroids aglycone (D'Addabbo, et al., 2020). In animals, saponins have been shown to be effective against the gastrointestinal nematodes from donkey (Maestrini et al., 2019) and goats (Maestrini et al., 2020). Plant steroids are important in promoting growth. They are good candidate for synthesis of bio-fertilizers.

The results from this study are interesting as they inform new thematic areas of research. For instance, the bioassay of the compound to determine their activities against pathogens and insect pest is an important aspect. Further, synthesis of bio-fertilizers using these compounds can greatly assist farmers who incur huge burden of procuring chemical fertilizers that are hazardous to them and their environment.

Conclusions

Phytochemical screening of extracts from the algae, *Chara braunii*, indicated that they contain steroids, glycosides, tannins and saponins that could possibly be purified for use in agriculture. The proposal to use plant-based bio-pesticides is due to the high cost of production of synthetic pesticides, their high levels of toxicity, persistence in soils and the environment and, non-selectivity of their action. Plant-based pesticides have the advantage of being biodegradable and less expensive. Pesticidal-activity has been established in several plant families.

Time did not allow us to test the bio-activity of our algal extract on plant nematodes as intended. However, we have dried extracts from the algae and unprocessed dried samples. We aim at carrying out this phase of our research at the School of Sciences and Aerospace Studies, Department of Biological Sciences laboratories.

Acknowledgements

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Supplementary materials

Fig. 13: Dr. Njira Pili performing an extraction procedure of the algal extracts. Photo by authors.

Fig. 14: Dr. Dorcas Lusweti spotting the algal extracts on a TLC plate. Photo by authors.

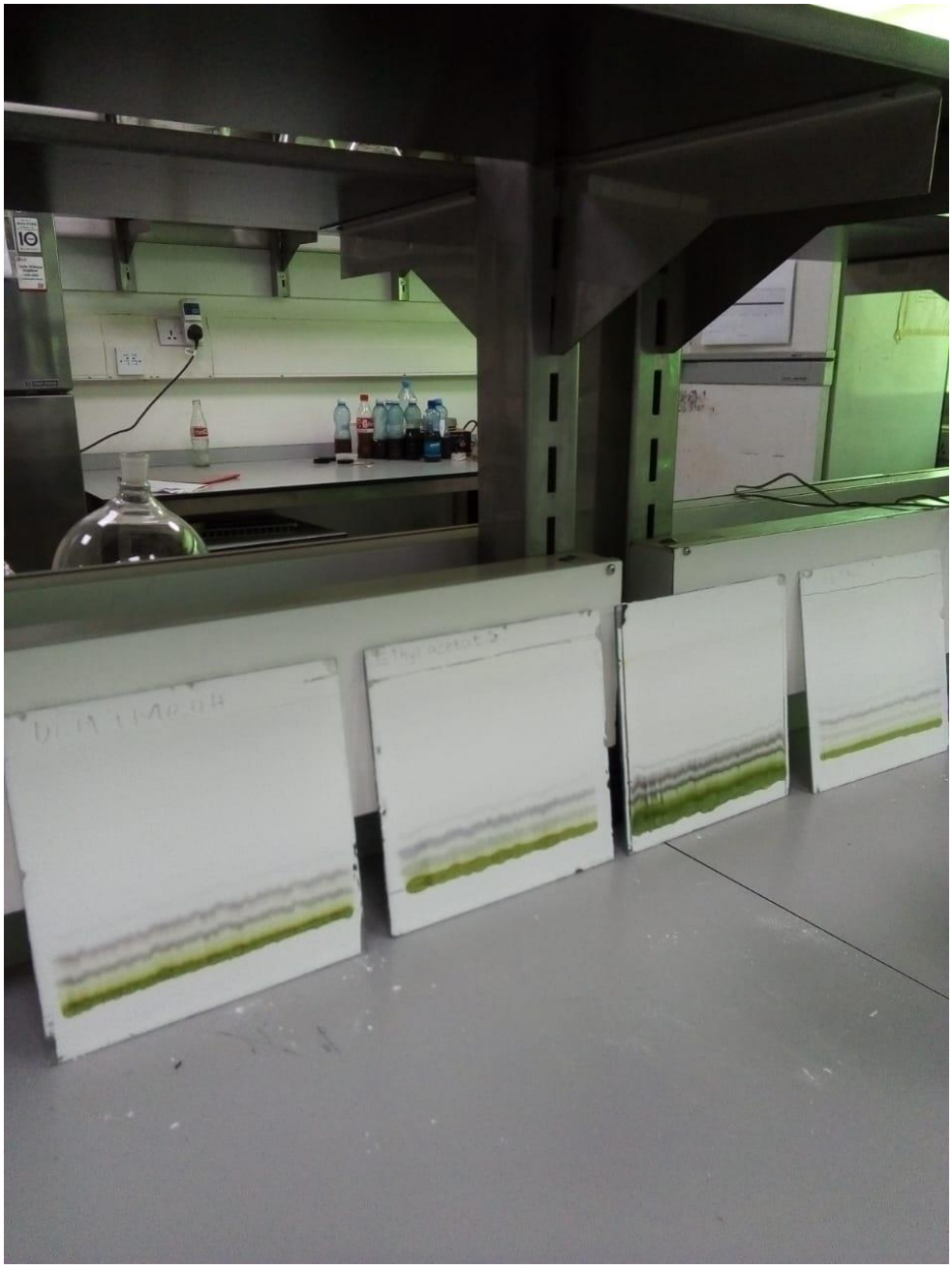

Fig. 15: Results of various preparative thin layer chromatography. Photo by authors.

WORK PLAN

S/N	Activity	**DAY**									
		1	2	3	4	5	6	7	8	9	10
1	Sorting and grinding of the materials										
2	Extraction of the compounds, phytochemical screening, and performance of the										

	TLCs, column chromatograph etc.								
3	Characterisation and identification of the compounds								
4*	Publish the results	After the exchange period							

NB.

Activity 4 will continue beyond the exchanged period.