Preliminary Phytochemical Screening, Proximate Analysis, Antioxidant and Antibacterial Activities of an Algal Species of *Hydrodictyon Reticulatum*

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Recommended Citation

Munir, M., Khan, A. M., Qureshi, R., Murtaza, S., & Munazir, M. (2020). Preliminary Phytochemical Screening, Proximate Analysis, Antioxidant and Antibacterial Activities of an Algal Species of *Hydrodictyon Reticulatum*, *Journal of Bioresource Management*, 7 (4).

DOI: <https://doi.org/10.35691/JBM.0202.0147>

ISSN: 2309-3854 online

(Received: Nov 29, 2020; Accepted: Dec 19, 2020; Published: Dec 31, 2020)

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PRELIMINARY PHYTOCHEMICAL SCREENING, PROXIMATE ANALYSIS, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF AN ALGAL SPECIES OF *HYDRODICTYON RETICULATUM*

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ABSTRACT

The freshwater algae grow in a wide range of aquatic habitats across the globe and are rich in secondary metabolites. The present study was designed to conduct the phytochemical screening, proximate analysis, antioxidant and antibacterial activities of water net (*Hydrodictyon reticulatum*), an algal weed from Pakistan. The pure and dried algal mass was crushed into a fine powder and four solvent-based extracts were used for phytochemical screening. The results of preliminary qualitative screening indicated the presence of flavonoids, phenols, quinones, steroids, resins, anthraquinones, glycosides, lignin, proteins, saponins, tannins, reducing sugars, alkaloids, terpenoids, fats, and oils, whereas the quantification results revealed the high quantities of total ash, crude proteins, crude fiber, alkaloids, carotenoids, flavonoids, and chlorophyll a. The studied methanolic extracts with maximum concentration (150 µg/ml) showed significantly (p-value ≤ 0.05) higher antioxidant and antibacterial activities. The IC₅₀ values of the crude methanolic extract of water net were found very close to streptomycin (positive control) against both bacterial pathogenic species of *Klebsiella pneumoniae* and *Staphylococcus aureus*. This study concluded that the crude extracts of water net possessed antibacterial activity at par to streptomycin drug, and requires further detailed study for the separation, identification, and testing of the pure phytochemical compound to be used by the nutraceutical and or pharmaceutical industry.

Keywords: Reducing power assay; phosphomolybdenum assay; proximate analysis; principal component analysis; water net.

INTRODUCTION

For centuries, algae have been used in various countries as food and to cure various diseases. Like plants, algae have many phytochemical compounds required for maintenance and improvement of human health (Selvi and Selvaraj, 2000; Rasala and Mayfield, 2015; El-Baz et al., 2020; Korzeniowska et al., 2020; Sahu et al., 2020). Algae are also rich in minerals, proteins, vitamins, carotenoids, phenolics and polyunsaturated fatty acids (Batista et

al., 2017). Therefore, many finished products obtained from the different algal species are commonly used as food or in the feed sector in Europe (Enzing et al., 2014) especially for aquacultures (Ahmad et al., 2020) and honey bees (Ricigliano, 2020). Different companies including Terravia USA and Dulcesol Spain commercialized algal protein, culinary oil and baked products of *Chlorella* respectively. According to an estimate, algal products market may reach to US \$ 44.7 billion by the year 2023 (Batista et

al., 2017). The phytochemical screening of the freshwater algae and seaweeds is on the rise to search valuable bioactive compounds with profitable properties (Ali et al., 1999; Rajauria et al., 2016). The proportion of trace elements is higher in the marine algae than the terrestrial plants and their constituent phytochemicals are mostly used in textile, pharmaceutical, food, confectionary and dairy industries for stabilizing and thickening of products (Seenivasan et al., 2012; Scieszka and Klewicka, 2019). Natural products are mostly obtained from the plants and seaweeds (Iliopoulou et al., 2002; Soobrattee et al., 2005), and now the trend to exploit freshwater algae for diversity of metabolites is increasing as well (Cardozo et al., 2007; Gupta et al., 2013).

The discovery of antioxidant and antiproliferative compounds from algae made them important organisms for food and pharmaceutical industry (Kelman et al., 2012; Neethu et al., 2017; Bottone et al., 2019) and various algal species say *Chlorella vulgaris* (Wang et al., 2010), *C. pyrenoidosa* (Hu et al., 2007) *C. ellipsoidea* (Ko et al., 2012) were investigated. The polysaccharides, proteins, polyphenols, sterols, carotenoids, chlorophylls and vitamins isolated from algal biomass have shown protective effect against free radical induced oxidative stress (Sheih et al., 2009; Ko et al., 2012; Dore et al., 2013; Machu et al., 2015; Chen et al., 2016; Khan et al., 2020; Sansone and Brunet, 2020). Although, algae have high chemo-diversity, rapid growth, photosynthetic activity, and least requirement of space and nutrients, not been explored well to reduce the product cost and to increase the industrial interest (Sansone and Brunet, 2020).

The anti-infectious agents of algae can also serve as good alternative source but till now majority of traditional and modern medicine are exploited from the terrestrial resources (Zbakh et al., 2020). Algae has broad spectrum activities against pathogenic bacteria, fungi, viruses,

and trypanosomes (de Felício et al., 2015; Lee, 2019; Seddek et al., 2019; Vehapi et al., 2019). A series of bromophenols from red algae strongly inhibited the growth of different pathogens including *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus*, *Proteus vulgaris* and *Salmonella typhimurium* bacteria and *Candida albicans*, *Aspergillus fumigatus*, *Trichophyton rubrum* and *T. mentagrophytes* fungal species (Oh et al., 2008). The marine algae are explored extensively when compared to freshwater algae. Therefore, the freshwater ecosystem also needed to be investigated for the bioactive compounds.

The freshwater macro-alga like *Hydrodictyon reticulatum* belongs to family Hydrodictyaceae, and commonly known as water net. The cells of the thallus are joined to form reticulate or net like structure that provides a strong adaptive capacity. Due to its fast rate of reproduction over a wide range of temperatures, it is globally distributed in lakes, ponds and rivers habitat. Additionally, its biomass is easy to harvest (Halder, 2015; Wehr et al., 2015; Fu et al., 2016). It contains a variety of bioactive compounds (Yokota et al., 1987; Ghazala and Shameel, 2005; Sahu et al., 2020). *H. reticulatum* could also treat polluted water by biosorption of trace elements (Fu et al., 2016; Sahu et al., 2020). It possesses antispasmodic (Pérez Gutiérrez and Solís, 2007; Gutierrez et al., 2012), antioxidant (Mridha et al., 2017), antifungal (Kamble et al., 2012) and strong antibacterial activities against gram positive bacterial strains (Gräf and Baier, 1981) owing to its unsaturated fatty acids (Olfers-Weber and Mihm, 1979), Brassinosteroids (Yokota et al., 1987), and sterols (Sahu et al., 2020).

The *H. reticulatum* contained a variety of bioactive compounds but a small work related to its phytochemistry and bioactivities particularly antioxidant and antimicrobial activities was conducted. Therefore, the objective of this study is to investigate the phytochemical screening,

proximate analysis, antioxidant and antibacterial activities of *H. reticulatum*.

MATERIALS AND METHODS

Collection and Identification of Algal Material

The *Hydrodictyon reticulatum* L. Bory (Family: Hydrodictyaceae; Order: Sphaeropleales; Phylum: Chlorophyta) is commonly known as water net, and a free floating, common, invasive species in Pakistan. About 5 kg of water net was collected from the fresh water reservoir of Rawal Lake, Islamabad (33.7027° N, 73.1261° E) during July, 2015. The collected material was brought in plastic bags to the Laboratory of Phycology, Department of Botany, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan. The water net was thoroughly washed repeatedly by using the tap water to remove the accessories. Ten different random samples from the fresh material were further subjected to microscopy for confirmation of its purity. The identification was confirmed by matching the morphological characteristics of the species mentioned in the standard manual (Prescott, 1962). A small quantity of the identified water net was fixed with 3% formalin, numbered and deposited for future references and record. Finally, the algal material was air dried and grounded to powder and stored at room temperature for further usage (Lim and Darah, 2004).

Extraction and Concentrations

The powdered and dried algal material (100 g) was macerated in 1000 ml of methanol (99.9%) in a conical flask, properly covered by using aluminum foil, labelled and stored at room temperature for 24 hours. It was followed by centrifugation (2800 rpm) for 15 minutes and filtration of supernatant by using sterile filter paper (Whatman No 1). The solvent of supernatant was evaporated for dryness of the extract in the vacuum by

using the rotary evaporator (40°C). It was followed by preservation/keeping of crude extract in the air tight containers and storage in the refrigerator (5 °C) until further used. By using this crude extract, a total of four different concentrations (viz. 25, 50, 100 and 150 µg/ml) were prepared by using the methanol as solvent (Alanis et al., 2005). The same procedure above was also performed by using three other solvents named 2-butanol (99%), n-hexane (99%) and benzene (99%), and finally their antioxidant potential was determined. Similarly, the same four concentrations were prepared in di-methyl sulfoxide (DMSO) to seek their anti-bacterial capabilities.

Preliminary Phytochemical Tests

The following qualitative and quantitative phytochemicals tests were performed.

i. Test for Alkaloids

An amount of 0.5 gram of crude extract obtained from the each of the four solvents was stirred in 5 ml of 1% aqueous HCl on a steam bath for 10 minutes. One ml of this filtrate was further treated with few drops of Dragendorff's reagent. The formation of orange coloured precipitates showed the presence of alkaloids (Ekpo and Etim, 2009).

ii. Test for Phenols

An amount of 5 mg of each of extract was added in the 5ml of distilled water (dH₂O), followed by continuous shaking for few minutes and addition of 5-6 drops of 1% FeCl₃. Distilled water with aspirin (instead of extract) was used as control. The appearance of blue, red, green or violet coloration in the test samples was observed for total phenols presence (Pasto and Johnson, 1979).

iii. Test for Flavonoids

Alkaline reagent test was used for flavonoids detection. 2 ml of 2% NaOH solution was added in the 0.2 g of each of algal extracts, it gives intensive yellow color, which changed to colorless upon addition of 1 ml of diluted HCl (Jaradat et al., 2015).

iv. Test for Tannins

About 0.2 g of extracts were mixed with 2 ml of dH₂O and heated on water bath for few minutes. 2-3 drops of FeCl₃ were added and dark green color was observed which indicated the presence of tannins (Egwaikhide and Gimba, 2007).

v. Test for Resins

About 0.5 g of methanolic extracts were added in small amount of dH₂O (5 ml). The mixture was shaken for fifteen minutes and formation of foam layer/turbidity indicated the presence of resins (Kokate, 1999).

vi. Test for Anthraquinones (Borntrager's test)

The extracts (0.5 g each) were mixed with chloroform (5 ml) and shaken for five minutes, followed by filtration. The equal amount of ammonia solution (10%) was then added in the filtrate. Pink-violet color in the ammonical layer indicated the presence of anthraquinones (Dhanalakshmi and Angayarkanni, 2013).

vii. Test for Quinones

Aqueous algal extracts (1 ml) were mixed with 1 ml of concentrated H₂SO₄ solution. Red color indicated the presence of quinones (Ganatra et al., 2012).

viii. Test for Glycosides (Keller-Killianit test)

100 mg of extracts were mixed in 5 ml of 1% FeCl₃ solution in 5% glacial

acetic acid. Followed by the addition of 1-2 drops of concentrated sulphuric acid. A blue color was observed which confirms the presence of de-oxy sugars (Trease, 1989).

ix. Test for Lignin

Maule's test was used to check the presence of lignin. Few drops of aqueous potassium permanganate solution (1%) were added in algal extracts (0.5 ml). Then, 3 drops of ammonium chloride and one drop of ammonium hydroxide solution were added. Red color indicated the presence of lignin (Leelavathi and Prasad, 2015).

x. Test for Saponins

Algal extracts (0.5 g) were added in 5 ml of dH₂O and shaken well till froth appeared. This froth was mixed with olive oil and shaken well. Emulsion was formed which indicated the presence of saponins (Leelavathi and Prasad, 2015).

xi. Test for Steroids (Salkowski test)

About 10 ml each of chloroform and concentrated H₂SO₄ were mixed with algal extracts (0.5 g) and allowed to stand for few minutes. The upper chloroform layer was observed red whereas lower sulphuric acid layer with yellowish-green fluorescence (Ganatra et al., 2012).

xii. Test for Terpenoids

About 2 ml of chloroform was added in the 5 ml aqueous algal extracts. It was followed by evaporation on the water bath and boiling with 3 ml concentrated sulphuric acid. Appearance of grey color showed the presence of terpenoids (Gul et al., 2017).

xiii. Test for Reducing Sugars

About 0.5g of each extract was mixed with dH₂O (5 ml) and 4 drops of Fehling solution (A: CuSO₄.5H₂O and B: Sodium potassium tartrate) were added and shaken. The solution was heated for ten minutes in a water bath and dark red precipitates were observed (Sofowora, 1993).

xiv. Test for Proteins

Algal extracts (1mg each) were dissolved in dH₂O (1 ml) and filtered. Biuret test was used to check the presence of proteins. 1 ml of Biuret reagent (1.5 g of CuSO₄.5H₂O and 6 g of NaKC₄H₄O₆.4H₂O was added in 500 ml of dH₂O. It was followed by the addition of 300 ml of 10% NaOH and total volume was increased to 1 liter) was added in the 1 ml of test solutions. Violet color indicated the presence of proteins (Krish and Das, 2014).

xv. Test for Fats and Oils

About 3 mg of extract was added in 4 drops of alcoholic KOH solution (0.5 N). Few drops of phenolphthalein was added in the mixture and heated on water bath for two hours. Fats and oils presence was checked with the formation of soap (Kokate, 1999).

Quantitative Phytochemical Screening

i. Determination of Alkaloids

The quantitative phytochemical screening of alkaloids was carried out by using the method of Harborne (1998). Powdered samples (5 g) were added in 200 ml of ethanolic acetic acid (10%) and incubated for four hours. The filtrates were heated on water bath to reduce the volume. Three drops of dilute NH₄OH were added in the filtrate and precipitates were formed. The precipitates were washed, dried and

weighed. The following formula (Eq. 1) was used for the estimation of total alkaloid contents.

$$TAC(\%) = \frac{FWP}{IWE} \times 100 \quad (\text{Eq.1})$$

Here, TAC = total alkaloid contents (%); FWP = final weight of the precipitates and IWE = initial weight of the crude algal extracts.

ii. Determination of Flavonoid

Powdered samples (1g) were extracted with 10 ml of aqueous methanol (80%) and kept at room temperature for few hours and then filtered the extract by using Whatman filter paper number 42 (125 mm). The filtrate was evaporated on water bath for dryness, and the residue was weighed. Flavonoid contents were estimated by using the Eq.2 (Leelavathi and Prasad, 2015) as under;

$$FC(\%) = \frac{WFE}{WS} \times 100 \quad (\text{Eq.2})$$

Here, FC = flavonoids contents (%); WFE = weight of the flavonoids extracted and WS = weight of the sample.

iii. Determination of Protein

Biuret reagent was used for determination of the protein contents. The crude sample (5 mg) was added in 1 ml dH₂O with 4 ml of Biuret reagent. Mixture was incubated for 30 minutes at room temperature and centrifuged for ten minutes at 4000 rpm. The supernatant was collected and density was measured by using spectrophotometer at 540 nm. Similar amount of dH₂O and Biuret reagent was run as blank (Raymont et al., 1964).

iv. Determination of Chlorophylls and Carotenoids

About 1 g of fresh algal material was ground with 20 ml of acetone (80%) and the paste was centrifuged for 15 minutes at 3000 rpm. The supernatant was shifted to a 100 ml beaker. The residue was again grounded with 20 ml of acetone and repeated the procedure 5 times until the residue becomes colorless. The volume of the test solution was increased to 100 ml by adding more acetone. The absorbance of the extract in 80% acetone and blank (80% acetone only) solutions were read at 480, 645 and 663 nm. The spectrophotometer was calibrated to zero absorbance by using a blank of acetone first. Furthermore, the blank of acetone were included in each run and the absorbance of blanks were subtracted from the absorbance of the targeted solutions (Arnon, 1949; Kizhedath and Suneetha, 2011). For carotenoids estimation, the absorbance of the same algal extract was read at 480 nm (Kirk, 1965; Vimala and Poonghuzhali, 2015). The following formulae in the form of Eq.3 to Eq.6 were used for the chlorophylls and carotenoids estimation.

$$Chl.A - \left(\frac{mg}{g}\right) = \frac{12.7(A_{663}) - 2.69(A_{645})}{1000 \times W} \times V \quad (Eq.3)$$

$$Chl.B - \left(\frac{mg}{g}\right) = \frac{22.9(A_{645}) - 4.68(A_{663})}{1000 \times W} \times V \quad (Eq.4)$$

$$Chl.Total - \left(\frac{mg}{g}\right) = \frac{20.2(A_{645}) + 8.02(A_{663})}{1000 \times W} \times V \quad (Eq.5)$$

$$Carotenoids - \left(\frac{ug}{g}\right) = \frac{A_{480} + [0.114(A_{663})] - 0.638(A_{645})}{1000 \times W} \times V \quad (Eq.6)$$

Here, A = absorbance at respective wave

length; V= volume of the target solution in ml (which is 100 ml in this study) and W = weight of the dried algal material (which is 1 g in this study). Thus, $V / (1000 \times W) = 100 / (1000 \times 1) = 0.1$ in this study.

v. Determination of lipids

About 50 g of dried powder of water net was dissolved in 200 ml of chloroform-methanol (1:1) mixture for homogenization for 2 minutes followed by addition of 50 ml of dH₂O and homogenized for further 30 seconds. The mixture was added in 50 ml tubes and centrifuged (3000 rpm at room temperature) for 5 minutes. The supernatant was collected and the residue was treated again by adding 20 ml of chloroform-methanol (1:1) and the whole process was repeated 4 times. The supernatants were combined and transferred to separating funnel. The bottom chloroform layer (containing lipids) was separated and passed through 2.5 cm thick layer of anhydrous sodium sulfate and Whatman no 1 filter paper. The solvent was removed by using the rotary evaporator (40 °C). The extracted lipids were calculated (Folch et al., 1957) by using the equation 7-8 as under;

$$WL = (WC + LE) - (WC) \quad (Eq.7)$$

$$LC\% = \frac{[LE(g)]}{WOS} \times 100 \quad (Eq.8)$$

Here, WL = weight of lipids; WC = weight of container; LE = lipids extracted in grams and WOS = weight of the original sample.

Proximate Analysis

The proximate analysis of the water net for its moisture contents, total ash contents, and crude fibers was done by using the standard methods (Helrick, 1990).

Determination of Antioxidant Activity

Antioxidant activity was analyzed by DPPH, reducing power and phosphomolybdenum assay.

i. DPPH Assay

A total of four different concentrations (25, 50, 100 and 150 µg/ml) of each of four different algal crude extracts (obtained by using methanol, 2-butanol, n-hexane and benzene as initial extracting agents) were prepared in the methanol. The same concentrations were also prepared for ascorbic acid to use as standard/reference. 0.1 mM solution of DPPH in methanol was prepared and 1 ml of DPPH solution was added in the 3 ml solutions of all concentrations. It was followed by vigorous shaking, and allowed to stand for 30 minutes in dark at room temperature. The absorbance of all the mixtures was measured against the blank/control (DPPH) at 517 nm. The lower absorbance values of the mixtures represents higher free radical scavenging capabilities. Furthermore, the change of DPPH radical color from purple to yellow indicates the presence of electron/proton donor molecules (Gyamfi et al., 1999). Percentage scavenging activity (Inhibition %) was calculated by using the equation 9 formula as under;

$$\text{Inhibition (\%)} = \frac{A_{517}(\text{Control}) - A_{517}(\text{Sample})}{A_{517}(\text{Control})} \times 100$$

(Eq.9)

ii. Reducing Power Assay

About 25 µl of each of 16 (4 concentrations x 4 solvents) different algal extracts/targeted samples (250 µg/ml) were mixed with 50 µl of 50 µM phosphate buffer (pH 6.6) and 50 µl of 0.1% (weight/volume) potassium ferricyanide. It was followed by incubation for 20 minutes at 50°C. The test tubes were cooled and 100 µl of 1% (weight/volume) trichloro-acetic acid

solution was added. It was followed by centrifugation at 3000 rpm for 10 min. The upper layer was removed and mixed with 25 µl of 5 mM ferric chloride solution. The absorbance of the samples was measured at 700 nm. Ascorbic acid was used as standard/reference. Reducing power of the samples were presented as µg of ascorbate equivalent per dry weight of the test sample in mg (Selvakumar et al., 2011).

iii. Phosphomolybdenum Assay

Antioxidant activity of the 16 different targeted samples (as also mentioned in DPPH and reducing power assay) was also determined by using phosphomolybdenum assay as communicated by (Prieto et al., 1999). 0.1 ml of each sample solution was mixed with 1.0 ml of reagent (0.6 M sulfuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate) in an eppendorf tube. All the tubes were capped, and incubated at 95 °C (90 minutes). It was followed by cooling of tubes to room temperature. The absorbance of the targeted solutions, ascorbic acid (control) was measured at 695 nm against blank (reagent). Activity was estimated by using the equation 10 formula as under;

$$\text{Inhibition (\%)} = \frac{A_{695}(\text{Control}) - A_{695}(\text{Sample})}{A_{695}(\text{Control})} \times 100$$

(Eq.10)

Antibacterial Activity

The response of two bacterial strains i.e. *Klebsiella pneumoniae* (ATCC12537) and *Staphylococcus aureus* (ATCC118536) as test microbes was tested against the 16 (4 concentrations x 4 solvents) water net extracts (prepared in DMSO) by using agar well diffusion method (Amtul, 1997). The Muller Hinton Agar (MHA) medium was prepared by dissolving 38 g of MHA in the 1000 ml of distilled water, boiled for 1 min for

complete dissolution. It was then autoclaved at 15 lbs pressure at 121°C for 15 min and allowed to cool to room temperature. The pH value of the medium was 7.3. The medium was poured (25 ml) in sterilized petri-plates (100 mm). The petri-plates were inoculated by using existing colonies through streak plating method, with the help of a sterile metallic loops. The sterile borer was used to make 6 mm wells and filled with 20 µl of each extract. Petri-plates were sealed, labelled and kept in incubator for 24 hours at 37 °C. The antibacterial activity was determined by measuring the zone of inhibition (mm). Streptomycin was used as positive control.

Statistical Analyses

A total of 32 different treatments were tested in triplicates (n = 96) against the five dependent response variables (three antioxidant and two anti-bacterial). The independent variables includes drug type (1. standard, 2. algal crude extract), concentrations (1. 25 µg/ml, 2. 50 µg/ml, 3. 100 µg/ml, 4. 150 µg/ml) and solvent types (1. methanol, 2. 2-butanol, 3. n-hexane, 4. benzene). The normality and homogeneity of the collected data was tested by using Kolmogorov-Smirnov and Levene tests respectively. Student t-test was used to seek the influence of drug type towards the response of target variables whereas, two-way multivariate analysis of variance (MANOVA) was employed in SPSS (ver. 25) to seek the main effects and interactions of the two nominal predictors (concentrations and solvent types) on the continuous outcome variables (scavenging and antibacterial responses). Tukey's honest significant difference (HSD) was used as post hoc for all pairwise comparisons, when required (p-value ≤ 0.05). IC₅₀ values were calculated online (<https://www.aatbio.com/tools/ic50-calculator/>) by using the collected data of scavenging and antibacterial activities of the studied samples. Principal component

analysis (PCA) was performed, and three antioxidant and two antibacterial variables were included in the analysis as supplementary variables to seek the interrelation of these with the four different concentration and solvent types categories (Khan et al., 2019a; 2019b).

RESULTS

Preliminary Phytochemical Screening

The preliminary qualitative phytochemical testing results of water net depicted that maximum number of different phytochemical types/classes (13 out of 15) were detected in the crude extract when methanol was used as extracting agent (Table 1).

Table 1: Results of qualitative screening and distribution pattern of phytochemicals of *H. reticulatum*.

N o.	Phytochemicals	Benzene	2-Butanol	n-Hexane	Methanol
1.	Alkaloids	-	+	-	+
2.	Anthraquinones	-	-	-	+
3.	Fats and oils	+	+	+	-
4.	Flavonoids	+	+	+	+
5.	Glycosides	-	-	-	+
6.	Lignin	-	-	-	+
7.	Phenols	+	+	+	+
8.	Proteins	+	+	+	+
9.	Quinones	-	-	-	+
10.	Reducing sugar	+	+	+	+
11.	Resins	+	-	+	-
12.	Tannins	+	-	-	+
13.	Terpenoids	+	+	+	+
14.	Saponins	-	-	-	+
15.	Steroids	+	+	+	+

Legends: ("+" present; "-" absent)

Only, Fats/oils and resin types were non-detectable in the methanolic crude extract. Similarly, the crude extracts of water net obtained by using three other solvents (viz. 2-butanol, n-hexane and benzene) extracted eight different phytochemicals. Some phytochemicals including phenolic compounds, proteins,

reducing sugars, terpenoids and steroids were detected in the crude extracts of all solvents (Table 1).

Antioxidant Activity

The significance of the recorded scavenging activity (% inhibition) of the crude extracts of *H. reticulatum* was tested by using MANOVA, based on two independent categorical variables (concentrations and solvents). The results of three different antioxidant assays (viz. DPPH, reducing power and phosphomolybdenum) of water net are as follows;

Figure 1: Quantitative phytochemicals and proximate analysis results of *H. reticulatum*.

i. DPPH Assay

The results of this assay depicted that there was significant difference in the group means as suggested by the values of overall corrected model (F: 3.23, p-value: <0.05) (Table 2). The main effect of concentrations was also statistically significant (F: 5.67, p-value <0.05). The influence of the initial solvent (solvent type) was also significant (F: 10.024, p-value <0.05), whereas the joint interactions of both input variables (concentration and solvent) were non-significant (Table 2). Therefore, further pairwise comparison of the different levels (groups) was studied by using Tukey's (HSD) as post hoc test. The pairwise comparison of four concentration groups depicted that the influence of the higher concentrations (100 and 150 µg/ml) was significantly (p-value <0.05) high when

compared to 25 and 50 µg/ml concentrations. In other words, the mean DPPH values contributed by the lowest concentration (25 µg/ml) of the crude extract were significantly (p-value <0.05) lower than the mean values of both 100 and 150 µg/ml concentration groups (Fig. 2). The estimated marginal means graph depicted that the highest concentration (150 µg/ml) of the crude extract showed maximum inhibition (%) while also interacting with all solvents except n-hexane, and gave higher mean values than the grand mean (65.83%, Figure 2). Therefore, it was observed that increase in concentration continuously resulted in the increase of scavenging activity.

Figure 2: Pairwise comparison and statistical significance of the groups of DPPH antioxidant assay (The different alphabetical lettering in brackets along with the concentrations and solvents indicates significant (p < 0.05) difference of the group means by Tukey's HSD comparisons).

This indicated the dose-dependent activity of all the extracts. As far as the influence of the type of solvent was concerned, the results depicted that the crude extracts obtained by using the methanol followed by 2-butanol as initial solvents, the inhibition (%) or scavenging activity was significantly (p-value <0.05) higher across all the concentrations except for 25 µg/ml. Therefore, based on initial solvent, the order of antioxidant activity of the crude extracts was methanol > 2-butanol > benzene > n-hexane. The joint plot depicted that two higher concentrations (100 and 150 µg/ml) and two initial solvents (methanol and 2-

butanol) were important in this regard (Fig. 2). IC₅₀ values of the standard ascorbic acid and different extracts of *H. reticulatum* are shown in Table 3.

ii. Reducing Power Assay

The statistical analysis results of this assay were a bit different than the DPPH assay. There was a statistically significant difference in the group means, which was not depicted by the overall corrected model (F value: 1.178, p-value 0.306), but the main effects of concentrations were significant (F: 4.072, p-value: 0.01), and of solvents and joint interactions were non-significant (F: 1.77, p-value: 0.159 and F: 0.014, p-value: 1 respectively) (Table 2).

Pairwise comparison of the four concentration groups depicted that the influence of all the three higher concentration (50-150 µg/ml) groups towards this assay were non-significant and alike with respect to each other, and significantly higher than the 25 µg/ml concentration group (Figure 3).

Figure 3: Pairwise comparison and statistical significance of the groups of reducing power antioxidant assay. The different alphabetical lettering in brackets along with the concentrations and solvents indicates significant ($p < 0.05$) difference of the group means by Tukey's HSD comparisons.

Both, 100 and 150 µg/ml concentration groups were detected with high reducing power mean values than the grand mean (1.31) value, especially when methanol and benzene were also interacting/involved as initial solvents. As the main effects of solvents were non-

significant, but the crude extracts obtained by using the methanol gave reasonably higher values than the grand mean (1.31), especially for the last three higher concentrations (50 µg/ml-150 µg/ml). Contrastingly, unlike 2-butanol in DPPH, benzene was detected as the second most important solvent, and supported the higher scavenging activity in this assay in case of 100 µg/ml and 150 µg/ml concentrations. Therefore, the order of importance of initial solvents was recorded a bit different when compared to DPPH and it was methanol > benzene > 2-butanol > n-hexane (Fig. 3). IC₅₀ values of this assay for the standard ascorbic acid and different extracts of *H. reticulatum* are shown in Table 3.

iii. Phosphomolybdenum Assay

The results of phosphomolybdenum assay were also similar to reducing power assay, and the main effects of the four concentrations towards the response (phosphomolybdenum assay) variable were statistically significant (F value: 6.8, p-value: <0.001), therefore depicted a significant difference in the four group means with varied antioxidant potential (Table 2). The MANOVA results of overall corrected model for this assay were non-significant (F value: 1.56, p-value 0.103), and the similar results were acquired for the main effects of four solvents (F value: 0.96, p-value: 0.417) and interactions of concentrations and solvents (F value: 0.024, p-value: 1) (Table 2). The Tukey's post hoc test results of four concentrations depicted that the influence of two higher concentrations (100 µg/ml and 150 µg/ml) were significantly ($p < 0.05$) higher as compared to the first two lower concentrations, and also well above the grand mean value (75.1%) (Fig.5). In other words, the contribution of both the two lower concentrations (25 µg/ml and 50

µg/ml) towards the scavenging activities were non-significant with respect to each other, and was well below the grand mean value of the groups. However, it was also observed that the influence of the second concentration group (50 µg/ml) was a bit close to the third group (100 µg/ml), and hence both were non-significant with respect to each other (Fig. 4). Although the overall contribution of the solvent types was non-significant in this assay, it was noted that all the solvents performed well in case of last two higher concentrations as compared to reducing power assay (Figure 4).

Figure 4: Pairwise comparison and statistical significance of the groups of phosphomolybdenum antioxidant assay. The different alphabetical lettering in brackets along with the concentrations and solvents indicates significant ($p < 0.05$) difference of the group means by Tukey's HSD comparisons.

Although the main effect of solvent types was non-significant, while seeking the influence of solvent types towards this assay, the results showed that the crude extracts obtained by using the methanol and benzene behaved almost alike, and gave higher antioxidant activities as compared to 2-butanol and n-hexane. Although, the overall order of importance of the initial solvents towards this assay was recorded alike to reducing power assays, and it was methanol > benzene > 2-butanol > n-hexane, it was also observed that the extracts obtained by using the non-polar benzene solvents contributed good antioxidant activity (higher than the methanol) especially at 25 µg/ml and 100 µg/ml concentration groups

in case of phosphomolybdenum assay. IC_{50} values of this assay for the standard ascorbic acid and different extracts of *H. reticulatum* are shown in Table 2.

Antibacterial activity

Like antioxidant activity assays, both input variables (concentrations and solvent types) comprising of four groups each (25, 50, 100 and 150 µg/ml; methanol, 2-butanol, n-hexane and benzene) of crude algal extracts of water net were prepared by dissolving in DMSO to seek the antibacterial potential of *Hydrodictyon reticulatum*. The influence of these 16 different treatments were tested against the gram positive bacteria *Staphylococcus aureus* and gram negative bacteria *Klebsiella pneumoniae*. The response of each treatment in terms of zone of inhibition (in mm) was recorded for both standard (streptomycin) and algal extracts. The MANOVA results depicted that the overall corrected model was non-significant for both *S. aureus* (F-value: 1.291, p-value: 0.227) and *K. pneumoniae* (F-value: 0.877, p-value: 0.592), however, the influence of different concentrations towards the zone of inhibition of both test microbes were significant (F-value: 5.224, p-value: 0.002 & F-value: 2.927, p-value: 0.039 respectively). Whereas, the contribution of the solvent types and interactions (concentrations and solvent types) were recorded as non-significant (Table 2).

The pairwise comparison of the concentration groups (25, 50, 100 and 150 µg/ml) towards the zone of inhibition of *S. aureus* by using Tukey's post hoc depicted that the two higher concentrations (100 µg/ml and 150 µg/ml) were significantly (p -value < 0.05) higher as compared to the first two lower concentrations, and also well above the grand mean value (3.96 mm) (Fig. 5). In other words, the contribution of both the two lower concentrations (25 µg/ml and 50 µg/ml) towards the antibacterial activity was non-

significant with respect to each other, and was well below the grand mean value of the groups. However, it was also observed that the influence of the second concentration group (50 µg/ml) was a bit close to the third group (100 µg/ml), and hence both were non-significant with respect to each other (Figure 5).

Figure 5: Pairwise comparison and statistical significance of the groups of zone of inhibition (mm) of *Staphylococcus aureus*. The different alphabetical lettering in brackets along with the concentrations and solvents indicates significant ($p < 0.05$) difference of the group means by Tukey's HSD comparisons.

Therefore, these comparisons were highly alike the phosphomolybdenum assay results. The contribution of solvent types were non-significant, and yet their order of importance was methanol > benzene > 2-butanol > n-hexane. Similarly, the pairwise comparison of the concentration groups (25, 50, 100 and 150 µg/ml) towards the zone of inhibition of *K. pneumoniae* by using Tukey's post hoc depicted that the influence of the highest concentrations (150 µg/ml) was significantly (p -value < 0.05) higher as compared to least concentration group (25 µg/ml) only, and all other pairwise comparisons were non-significant (Figure 6).

Again, the contribution of the solvent types towards the antibacterial (*K. pneumoniae*) activity was non-significant, and yet their order of importance was methanol > benzene > 2-butanol > n-hexane. Thus, overall the crude extracts of water net showed higher antibacterial activity against gram positive

Staphylococcus aureus (grand mean: 3.96 mm; Fig. 5) than the gram negative *Klebsiella pneumoniae* (grand mean: 3.03 mm; Fig. 6).

Figure 6: Pairwise comparison and statistical significance of the groups of zone of inhibition (mm) of *Klebsiella pneumoniae*. The different alphabetical lettering in brackets along with the concentrations and solvents indicates significant ($p < 0.05$) difference of the group means by Tukey's HSD comparisons.

Overall, the IC₅₀ values of the alcoholic algal extracts were recorded lowest and closer to their respective standard values. An IC₅₀ value of 33.34 µg/ml of methanolic extracts was recorded which was very close to standard drug (32.87 µg/ml) against *Klebsiella pneumoniae*. Thus, these extracts can be targeted for further detailed search of responsible phytochemicals (future antibiotic) with highest antibacterial potential (Table 3).

The significance of the two groups (standard and algal crude extract) of drug type input variable towards the five target variables was assessed by using student t-test. The results depicted the significant (p -value < 0.001) mean difference in all the recorded response variables, which represents that standard drug performed significantly higher than the crude algal extracts in all the studied assays (Table 4). Principal component analysis (PCA) results depicted that total variations in the response data (5 dependent variables) were 300. A total of 97% variations were explained (adjusted) by the both (treatments and initial solvent types)

Table 2: Results of two-way multivariate analysis of variance (MANOVA) of antioxidant and antibacterial potential of water net.

Variables	Source	Type III SS	DF	MS	F	p-value	R ²	Adj. R ²
Corrected Model	DPPH assay (% Inhibition)	9354.904	15	623.660	3.233	0.000	0.377	0.261
	Reducing power assay	7.048	15	0.470	1.178	0.306	0.181	0.027
	Phosphomolybdenum assay (% Inhibition)	5165.326	15	344.355	1.565	0.103	0.227	0.082
	Zone of inhibition of <i>S. aureus</i> (mm)	68.431	15	4.562	1.291	0.227	0.195	0.044
	Zone of inhibition of <i>K. pneumoniae</i> (mm)	21.136	15	1.409	0.877	0.592	0.141	0.02
Concentrations	DPPH assay (% Inhibition)	3281.219	3	1093.740	5.670	0.001		
	Reducing power assay	4.873	3	1.624	4.072	0.010		
	Phosphomolybdenum assay (% Inhibition)	4485.857	3	1495.286	6.797	0.000		
	Zone of inhibition of <i>S. aureus</i> (mm)	55.367	3	18.456	5.224	0.002		
	Zone of inhibition of <i>K. pneumoniae</i> (mm)	14.114	3	4.705	2.927	0.039		
Solvents	DPPH assay (% Inhibition)	5801.262	3	1933.754	10.024	0.000		
	Reducing power assay	2.124	3	0.708	1.775	0.159		
	Phosphomolybdenum assay (% Inhibition)	631.947	3	210.649	0.958	0.417		
	Zone of inhibition of <i>S. aureus</i> (mm)	12.137	3	4.046	1.145	0.336		
	Zone of inhibition of <i>K. pneumoniae</i> (mm)	6.109	3	2.036	1.267	0.291		

Variables	Source	Type III SS	DF	MS	F	p-value	R ²	Adj. R ²
Concentrations * Solvents	DPPH assay (% Inhibition)	272.423	9	30.269	0.157	0.997		
	Reducing power assay	0.051	9	0.006	0.014	1.000		
	Phosphomolybdenum assay (% Inhibition)	47.522	9	5.280	0.024	1.000		
	Zone of inhibition of <i>S. aureus</i> (mm)	0.926	9	0.103	0.029	1.000		
	Zone of inhibition of <i>K. pneumoniae</i> (mm)	0.913	9	0.101	0.063	1.000		
Error	DPPH assay (% Inhibition)	15432.520	80	192.906				
	Reducing power assay	31.914	80	0.399				
	Phosphomolybdenum assay (% Inhibition)	17598.604	80	219.983				
	Zone of inhibition of <i>S. aureus</i> (mm)	282.612	80	3.533				
	Zone of inhibition of <i>K. pneumoniae</i> (mm)	128.572	80	1.607				
Total	DPPH assay (% Inhibition)	440838.290	96					
	Reducing power assay	203.996	96					
	Phosphomolybdenum assay (% Inhibition)	564261.967	96					
	Zone of inhibition of <i>S. aureus</i> (mm)	1853.785	96					
	Zone of inhibition of <i>K. pneumoniae</i> (mm)	1030.226	96					

Table 3: IC₅₀ values ($\mu\text{g/ml}$) of the different solvent extracts of *H. reticulatum* showing its antioxidant and antibacterial potential.

Studied assays	Standard	Methanol	2-Butanol	n-Hexane	Benzene
DPPH assay	1.02	70.71	124.65	245.64	290.55
Reducing power assay	5.43	127.53	234.92	393.61	132.97
Phosphomolybdenum assay	4.76	96.54	219.73	175.99	91.65
Antibacterial (<i>S. aureus</i>)	28.05	44.56	155.76	288.53	78.13
Antibacterial (<i>K. pneumoniae</i>)	32.87	33.34	121.65	180.949	63.039

Table 4: Results of significance testing of drug type (standard vs. algal crude extract of *H. reticulatum*) towards the studied response variables.

Target variables	t-statistics	df	p-value	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
						Lower	Upper
DPPH assay (% Inhibition)	10.204	94	<0.001	23.29750	2.28323	18.76409	27.83091
Reducing power assay (% Inhibition)	18.563	94	<0.001	1.12938	0.06084	1.00858	1.25017
Phosphomolybdenum assay (% Inhibition)	13.602	94	<0.001	25.07875	1.84377	21.41789	28.73961
Zone of inhibition of <i>S. aureus</i> (mm)	18.179	94	<0.001	3.37458	0.18563	3.00602	3.74315
Zone of inhibition of <i>K. pneumoniae</i> (mm)	22.196	94	<0.001	2.28875	0.10311	2.08402	2.49348

independent variables, which were imported as supplementary variables including their levels during the analysis in Canoco software. PCA axis-1 explained majority of variations (0.93), and treatments (concentrations) were found highly correlated with the same axis. The solvent types were found more correlated with the PCA axis-2 (Figure 7).

This analysis also suggested that the results of reducing power and phosphomolybdenum assays are more correlated than the DPPH assay. The same way antibacterial response results against both test organisms viz. *Staphylococcus aureus* and *Klebsiella pneumoniae* are also highly correlated. The PCA biplot clearly showing the importance of 100 and 150 µg/ml concentrations, and methanol as initial solvent in this study as these are oriented closer to the origin of the biplot whereas all other treatments and solvents are placed much away from the response vectors (Figure 7).

DISCUSSION

Qualitative and Quantitative Analysis of Phytochemicals of Water Net

The polarity of extraction solvent affect the composition of phytochemicals in the crude extract (Abdel-Aal et al., 2015; Awotedu et al., 2020) and methanol is a good extraction solvent to isolate phytochemicals (Abdel-Aal et al., 2015; Rajkumar and Bhavan, 2017; Ramarajan and Janakiraman, 2019). The same way this study also observed that majority of phytochemicals were extracted by the methanol, and belong to alkaloids, anthraquinones, flavonoids, glycosides, lignin, phenols, proteins, quinones, reducing sugars, saponins, steroids, terpenoids and tannins classes (Table 1). Similar result was also reported by many workers (Yokota et al., 1987; Ghazala and Shameel, 2005; Nailwal et al., 2013; Stirk et al., 2013) that methanolic extract of *H. reticulatum* can efficiently extract various

phytochemicals including steroids, terpenoids, carbohydrates and fatty acids, and relatively less effective in case of lignin and resin contents (Kim et al., 2017). The earlier studies (Sunilson et al., 2009; Janarthanan and Kumar, 2013; Shiney et al., 2014; Leelavathi and Prasad, 2015) reported the presence of many secondary metabolites in green algae say in *Spirogyra longata* which possess ninety seven compounds. These include five alkaloids, six ketones, eight terpenoids, twenty hydrocarbons and four phenolic compounds (Abdel-Aal et al. 2015). In the same way detail of each phytochemical belonging to detected phytochemical classes of water net need further investigation. The quantitative results for *Spirogyra neglacta* by Yosboonruang et al. (2020) communicated that water extract gave phenolics (157.92mg GAE/g) and flavonoids (10.10mg GAE/g).

The quantitative phytochemical analysis depicted the order (based on quantity in mg/g) in *H. reticulatum* as alkaloids > carotenoids > flavonoids > proteins > total chlorophyll > lipids > chlorophyll a > chlorophyll b (Fig. 1). Therefore, the results revealed that different bioactive compounds are present in adequate quantity in *H. reticulatum*. (Sahu et al., 2020) reported that *Hydrodictyon* possessed carotenoids (1.8 mg/kg), sterols (35664 mg/kg), total phenols (2785 mg/kg), flavonoids (2305 mg/kg), oil (4.3% by weight), specimen collected from Kharun River. Another investigation revealed that it contains mono-unsaturated fatty acid (40.7%), di-unsaturated fatty acids (12.3%) and poly-unsaturated fatty acids (6.5%). The total unsaturated fatty acids of *Hydrodictyon* (59.5%) are greater than the *Spirogyra* (58.9%), *Pithophora* (55.1%), *Tolypothrix* (53.1%), *Rhizoclonium* (50.2%), and *Cladophora* (52.1%) (Kumar and Sharma, 2014). Due to its low protein contents, *H. reticulatum* was preferred as feed in combination with fish meal for *Tilapia*

zillii and *Oreochromis niloticus* fish (Appler, 1985). Green algae contain higher proportion of flavonoids, protein and chlorophyll contents (Muthuraman and Ranganathan, 2004; Janarthanan and Kumar, 2013; Leelavathi and Prasad, 2015). In the present study flavonoids (2.29 ± 0.01 mg/g) were found in the *H. reticulatum*, whereas according to another investigation (Cladophora and Trentepohia, 2013) flavonoids were not recorded in the water net where the authors used two-dimensional paper chromatography tool.

The proximate analysis results of this study revealed that water net has moisture content (80%), dry matter (5%), total ash (2.5%), crude fiber (1.8%) and crude fat (1.7%) (Fig. 2). The finding of moisture content of this study is similar to (Hennenberg et al., 2009) which also reported that aquatic biomass contain 80-90% of water. The quantities of ash and crude fiber contents of *H. reticulatum* of this study are inconsistent when compared with literature, and therefore need further investigation. (Appler, 1985) conveyed that ash content of *H. reticulatum* are (30.72%) which were grown at a nuclear power station for use in fish feed. Conversely, some other green algae were observed with least ash contents e.g. the ash content of four freshwater species (3 species of *Spirulina* and 1 species of *Chlorella*), and one marine water species of *Isochrysis* were 7.43%, 7.51%, 10.38%, 6.30% and 16.08% respectively cultured by using agar method (Tokuşoglu and Ünal, 2003). Similarly, the freshwater *Chlorella vulgaris* and *Arthrospira platensis* species also have low ash content compared to *Diacronema*, *Tisochrysis*, *Nanochloropsis*, *Odontella*, *Porphyridium* and *Tetraselmis* species (Bernaerts et al., 2018). The dried seaweeds had high ash content ranged from 12-45% (Gómez-Ordóñez et al., 2010; Lin et al., 2020), and insoluble cellulose fiber from 7.4-22.7% (Gómez-Ordóñez et al., 2010). Appler (1985) reported that *H. reticulatum*

collected from power station in Belgium has 14.08% of crude fiber compared to our results which is 1.8%. All these variations in the proximate analysis results might be reflection of varying environmental conditions of the specimen collection sites.

Antioxidant Activity of Water Net

The methanolic extract of *H. reticulatum* exhibited high IC_{50} value of 17.7 compared to other solvent and showed high antioxidant ability in case of DPPH assay (Table 2). Similar results in literature suggested that methanolic extract act as better antioxidant than others solvent extracts (Ismail and Hong, 2002; Razali et al., 2012; Dutta and Ray, 2020). In the present work absorbance decreased with the increase in concentration from 25 μ g/ml to 150 μ g/ml. Decreasing absorbance indicated increasing percentage scavenging activity (Krish and Das, 2014; Kumari, 2014; Renugadevi et al., 2018; Bursal et al., 2020). The observed antioxidant activity of *H. reticulatum* might be due to the presence of flavonoids and phenols (Table 1). These phytochemicals contain hydroxyl group and are well known to possess antioxidant activity (Abbas et al., 2015). The phenols trap free radicals and flavonoids scavenge free radicals and chelate metals (Brewer, 2011). The antioxidants donate hydrogen to DPPH and reduce it to diphenyl-picrylhydrazine (Bursal et al., 2020). The biomass of *H. reticulatum* contain total flavonoid and total phenols as 54.96mg QE/g and 23.95 mg QE/g respectively collected from Kolkata and Jharkhand, India. Moreover this methanolic extract of *H. reticulatum* showed moderate antioxidant potential (EC_{50} 40.3.9 μ g/ml) for nitric oxide scavenging assay in comparison with other algae viz., *Pithophora* and *Rhizoclonium* (Mridha et al., 2017). Based on solvent and concentrations groups, methanolic extracts and high dose showed significantly higher antioxidant values with DPPH assay (*p*

<0.05, Figure 3).

The reducing power assay does not require free radicals instead reduce the ferricyanide (Fe^{+3}) to ferrocyanide (Fe^{+2}) (Habashy et al., 2018). The Increasing absorbance in reducing power assay indicated the increasing reducing power (Jayshree et al., 2016). In this study ascorbic acid, methanol, butanol, benzene and hexane showed absorbance viz., 2.98 nm, 1.57 nm, 1.11 nm, 0.96 nm and 1.20 nm respectively at higher concentration (150 $\mu\text{g/ml}$) in the reducing power assay while at the lowest concentration (25 $\mu\text{g/ml}$) the same was recorded as 0.48 nm, 0.54 nm, 0.28 nm, 0.14 nm and 0.32 nm respectively. The reducing power increased with increase in concentration of sample. Similar findings have been reported in methanolic extracts of seaweeds (Kumaran and Karunakaran, 2007; Chandini et al., 2008; Vijayabaskar and Shiyamala, 2012; Damahe et al., 2014; Khalil et al., 2020). The antioxidant activity of majority the solvent extracts (treatments) were significantly different ($p < 0.05$) in case of reducing power assay as well (Figure 4). The seasonal changes impact the increase and decrease in phenolic contents in the algae of freshwater habitats along with the method of extraction and solvent type (Fabrowska et al., 2018). Therefore, this study also suggests seeking the impact of seasonal changes in the phenolic and flavonoids contents of *H. reticulatum*.

In the phosphomolybdate assay the antioxidants reduce Mo (VI) to Mo (V) forming a green phosphate/Mo complex with absorption maxima at 965 nm (Jayshree et al., 2016; Dutta and Ray, 2020). The percentage scavenging activity in phosphomolybdenum assay increased with increase in concentrations and maximum activity was recorded at the highest concentration (150 $\mu\text{g/ml}$) in this study and highest antioxidant activity was shown by the lowest IC_{50} value. Our results are in agreement with (Ganesan et al., 2008; Damahe et al., 2014; Jayshree et

al., 2016).

Figure 7: Principal component analysis triplot showing correlation of the studied assays and their relationship with the different groups of concentrations and solvent types.

Methanol and benzene extracts showed significantly higher antioxidant values than hexane and benzene with Phosphomolybdenum assay (Figure 4).

Antibacterial Activities of Water Net

In this study methanol extract of *H. reticulatum* showed the highest inhibition zone with *K. pneumoniae* while benzene and methanol extracts showed good inhibition with *S. aureus* as compared to other extracts. The antibacterial activity of methanol extract with both types of bacteria appeared to be because of its property of being good extraction solvent in this study. Like our results, the methanolic extract of twenty Moroccan species (Chlorophyta (9), Phaeophyta (3) and Rhodophyta (8)) were tested and six showed large zone of inhibition against three different bacterial species using disc diffusion method. Methanolic extract of the alga named *Enteromorpha compressa* showed maximum antibiosis against *E. coli* and *Caulerpa prolifera*, *Codium dichotomum*, *Cystoseira compressa*, *Cladostephus spongiosus* algal species were found effective against *S. aureus* with 24mm zone of inhibition, and *Hypnea musciformis* species was potent against three bacterial species including *S. aureus*, *E. coli* and *E. faecalis* (Zbakh et al., 2020).

The hot water extract of *H. reticulatum* showed antibacterial activity against *Staphylococcus albus* with 9 mm zone of inhibition and no activity was exhibited against *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* (Kamble and Chavan, 2010). The liquid pure cultures of *H. reticulatum* and *Aphanothece nidulans* under continuous illumination and ventilation manifested antibiosis against *S. aureus*, *Staphylococcus epidermis*, *K. aerogenes*, *P. aeruginosa*, *S. typhimurium* and *Candida albicans*, *E. coli*, *Streptococcus faecalis*, *Enterobacter aerogenes* and *Bacillus subtilis* (Gräf and Baier, 1981). The antimicrobial activities of various species depends on the extraction method, solvent type and sample collection time/season (ZAMIMI *et al.*, 2020). The antibacterial activity is due to presence of bioactive compounds in the extract viz., flavonoids, triterpenoids, saponins, sterols, carotenoids and chlorophylls (Bhagavathy *et al.*, 2011; Mouffouk *et al.*, 2020) . Both MANOVA and PCA results revealed that results of methanolic extracts and high dose (150 µg/ml) levels are more close to standard for all the 5 studied responses (3 antioxidant; 2 antimicrobial) in this study.

CONCLUSION

The flavonoids were detected and quantified for the first time in *H. reticulatum*. or water net. It is further concluded that different extracts of *H. reticulatum* possessed biologically active compounds (i.e. flavonoids, phenols, quinones, steroids, resins, anthraquinones, glycosides, lignin, proteins, saponins, tannins, reducing sugars, alkaloids, terpenoids, fats and oils). The IC₅₀ values of crude methanolic extract of water net was found very close to standard drug (Streptomycin) against both pathogenic *K. pneumoniae* and *S. aureus* species. Therefore, this study also concluded that if crude extract is so close to standard drug, the responsible pure phytochemical of

water net might have much stronger antimicrobial ability than the streptomycin against the studied pathogenic microbial species. Hence, future studies allied to separation, identification and testing of each individual pure phytochemical of water net are recommended, and needed to be conducted. This baseline study about the water net may provide some new avenues for further discovery of more effective phytochemicals in nutraceutical and/or pharmaceutical industry.

DECLARATION OF COMPETING INTERESTS AND FUNDING SOURCE

The author(s) declare that there is no known competing financial interest or personal relationships that could have appeared to influence the work reported in this manuscript. It is also declared that this study was not financially supported by any funding agency/institute.

AUTHOR(S) CONTRIBUTION

SM collected the field samples and did lab work; MM wrote the preliminary draft, and along with RQ, did overall supervision of the research work; AMK wrote methodology, results, did statistical analyses and interpretations; whereas MM did literature searches, wrote introduction and discussions. All author(s) read the manuscript and approved for submission.

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