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EVALUATION OF PHYTOCHEMICAL AND ANTIMICROBIAL POTENTIAL OF LEMNA MINOR FRACTIONS AGAINST PATHOGENIC ORGANISM ISOLATED FROM WATER SAMPLE

Samina Iqbal*[1], Zakia Khatoon[2], Saeeda Bano[1], Kanwal Abbasi[1], Kauser Siddiqui[1], and Nighat Sultana[1]

[1]PCSIR Laboratories Complex, Karachi; Karachi- 75280, Pakistan.

[2]Institute of Marine Science, University of Karachi, Karachi-75270, Pakistan.

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***Corresponding Author**
Samina Iqbal
PCSIR Laboratories Complex, Karachi; Karachi-75280, Pakistan.

ABSTRACT

Duckweeds are tiny, flowering aquatic plants found in lentic water. It has high nutritional, antimicrobial and plankton reduction potential. Active compounds of duckweed *(Lemna minor*) were alienated by four different solvents and tested for bacterial inhibition and antioxidant potential in order to evaluate its efficacy as an antibacterial agent against known pathogens. Results showed that ethyl acetate fraction exhibited antibacterial properties against all four tested organisms. However, the efficacy of the crude extracts against bacterial growth is dose-dependent. In the phytochemical study, the total phenolic content in *Lemna minor* was detected as 2.1%. Among different phenolic compounds, flavonoids in a concentration of 0.84% were found best in ethyl acetate fraction. In addition, Duckweed also showed 70.4 % antioxidant activity in ethanol extract. The screening of duckweed revealed the presence of a significant amount of protein content estimated as 0.17 g protein/g dry biomass and the total lipid content was 0.029 g /g dry biomass of *Lemna minor*.

KEYWORDS: Duckweed, Extract, Antibacterial activity, Antioxidant, Flavonoid.

INTRODUCTION

Duckweeds represent a small family of aquatic floating monocots consisting of 37 species distributed all over the world (Landolt, 1986; Sree et al., 2016). Duckweeds are fast growing angiosperms plants, when growth conditions are favorable they may cover ponds or lakes within a few days (Sree et al., 2015; Ziegler et al., 2015). Apart from this, it is a suitable plant

model for the toxicity evaluation of many substances due to its small size, rapid growth, and ease of culturing and handling (Tkalec et, al, 1998). Importantly, it is frequently observed that animals, such as ducks, swans, or geese, feed on duckweeds growing naturally in ponds or lakes. Of course, this is where the name, duckweed, comes from. Furthermore, these plants have also been used for a long time to feed domesticated animals, either by providing them access to duckweed grown ponds or by supplementing their diet with harvested duckweeds.

The different compound extracted from this plant has been exhibit the antioxidant activity. There is scientific information for 12 biologically active substances with antioxidant activity isolated from *Lemna Minor* (Petrova et al, 2020). Antioxidants are compounds that have ability to scavenge the free radicals and inhibit the oxidation of moieties and aging. Free radicals are reactive molecules or atoms that contain an unpaired electron and tend to react with other molecules, to gain electron and become stabilize but oxidize to another molecule. (Amin *et al.*, 2013). Many acute and chronic diseases caused by oxidative stress are mediated by reactive oxygen species.

Duckweeds are also very important due to a great source of nutrients. It has significant amount of protein in some species (40.0% protein per dry weight), which makes it valuable for human food. It contains 20-35% protein, 4-7% fat, 4-10% starch per dry weight. Apart from protein, these minute plants are floating factories of antioxidants like lutein, xanthin and tocopherols. Therefore, it can also be used as super food because duckweed protein has a better array of essential amino acids than most vegetable proteins. It closely resembles to animal protein and has ability to efficiently manage glucose levels and provide antioxidant and antibacterial compounds (Hillman and Culley, 1978). Indeed, harvested duckweed plants may be used without further processing as a complete feed for fish.

It should also be noted that resistance against commonly available antibiotics become increases, therefore it's the need of time to isolate new compounds that could be used as antimicrobial agent for the treatment of different disease (Desbois et al., 2009; Sieradzki, et al., 1999). Plants, herbs and spices are rich source of naturally occurring compounds and have been shown antimicrobial functions. Thus, could be serve as antimicrobial agent against infection causing bacteria in plants and human (Harborne J. B. 1984; Ferreiraet al, 1996). Therefore, the aim of current study was to estimate the total phenolic and flavonoid content of extracted compound along with antibacterial and antioxidant potential of *Lemna minor*.

OBJECTIVES

1. To evaluate the antimicrobial potential
2. To estimate the phytocompounds of duckeweed
3. Nutritional profiling of locally isolated strain of duckweed to use it as food supplement in future.

MATERIALS AND METHODS

All chemicals and solvents used in this study were analytical grade and purchased by Merck, Pakistan.

Plant Material: The plants of *Lemna minor* were collected from fresh water pond during the months of June and July. They washed thoroughly with distilled water several times then surface sterilized with a commercial bleach solution containing 5.0 % (v/v) sodium hypochlorite for 5 minutes. The excess bleach was washed off with sterile distilled water thrice. The material was then dried at room temperature in shade for 20 days. Afterward, ground into fine powder by grinder mixer.

Lipid extraction

The dried plant material was wrapped in filter paper, tied with string to keep it together, placed in a Soxhlet extractor with n-hexane in a ratio of 25 mL/g dry weight of extract for 6 hrs (Smyatskaya et al., 2018). Then solvent was removed from the lipid extract with a rotary evaporator. The yield of lipid was calculated by subtracting the dry weight of the final separated lipids to the dry weight of the initial biomass to obtain lipid yield data (g/g). The remaining biomass was used in the extraction of protein.

Protein extraction

The residual biomass after lipid extraction was dried in an oven at 50°C for 30 min to remove residual solvent, dissolved in 5.0 % NaCl (20 mL per g dry weight) and the pH of the resulting suspension was raised to 10 with 1 M NaOH (Zhang et al.,2014). The suspension was then stirred and heated on a hotplate for 30 min at 50°C, cooled to room temperature and left undisturbed for 6 hrs. The supernatant was collected, its pH adjusted to 4 with 1 M $C_6H_8O_7$ (citric acid) and stirred thoroughly while heating on a hotplate for 15 min at 50°C. The supernatant was allowed to cool to room temperature and left undisturbed for 12 hrs to precipitate the protein. The precipitated proteins were collected by centrifugation for 15 min

at 5,300 rpm. The dry weight of the precipitated protein was compared to the dry weight of the initial biomass to obtain protein yield data (g/g).

Extraction of Phytocompounds

To obtain the different fractions of *L. mino* 200 g of dry duckweed powder was transferred into four separate labeled round-bottom flasks and soaked into four different organic solvents hexane, ethyl acetate, acetone and ethanol for fractionation and leave at room temperature for 3 days. Afterward, the solvent was separated by rotary evaporator (Oreopoulou et al., 2019). A semisolid fraction was obtained after removal of solvent that farther dried under vacuumed pressure at 40°C. The extracts were stored hermetically in bottle at 2-8°C for further analysis.

Yield of plant extract = (crude extract weight/plant material weight) ×100

Phytochemical Analysis

To prepare the solution of plant extract for phytochemical analysis, crude extract was dissolved in methanol in a concentration of 5 mg/ml and all the four extracts were screened for total phenolic content, total flavonoid and anti-oxidant activity.

DPPH scavenging activity (%) = Ac – As/Ac × 100

Where,

Ac = Absorbance of Control

As = Absorbance of Sample

Total Phenolic Content

The total phenolic content was determined spectrophotometrically by using Folin-Ciocalteu's reagent with some modifications (Stankovic M.S. 2011). The extract (0.1ml; 5 mg/ml) was mixed with 0.1 ml of Folin-Ciocalteu's phenol reagent and kept for 5min. A volume of 1.0 ml of 7.0 % Na_2CO_3 solution was added to reaction mixture followed by addition of 1.30 ml of deionized water. The mixture was kept in the dark at room temperature for 90 min and then the absorbance was measured at 750 nm. A calibration graph was constructed using Gallic acid in the extract was expresses as milligram of Gallic acid equivalents in one gram of extract.

Total Flavonoids Content

Total flavonoid content was estimated by using aluminum chloride method. The extract (50 mg/ml) was prepared in dimethyl sulfoxide (DMSO). Extract/sample (0.6 ml) was transferred

to 10 ml test tube, then 0.6 ml aluminium chloride (2.0 %) was added and the mixture was incubated for 60 minutes at room temperature. The absorbance of the reaction mixtures was recorded at 420 nm. A calibration graph was constructed using Quercetin as standard and the total flavonoid content in the extract was expressed as milligram of Quercetin**.**

Bacterial Isolation and Inhibition Studies

Bacterial culture of *E.coli* and *Pseudomonas aerugenosa* were isolated from water. After purifying the isolates by classical method cultures were identify according to Bergey's manual of systemic bacteriology and *Staphylococcus aureus* and Bacillus *pumilis* cultures were obtained from culture bank of PCSIR Labs Complex, Karachi.

Bacterial Inhibition Studies

Four fractions of extract were screened for antibacterial potential against two gram-positive (*Staphylococcus aureus, Bacillus pumilis*) and two gram-negative bacteria (*Escherichia coli, Pseudomonas aerugenosa*) by agar well diffusion method (Ferreira et al, 1996; Ortega MG and Julian HR., 1996; Patra et al. 2009). For this purpose, 1.0% of standardized bacterial cell suspension (1.5×10^7 CFU/ mL) was incorporated with antibiotic agar no.1 and poured into pre-sterilized Petri dishes of 12×100 mm and left for solidification. After solidification, well of 8.0 mm were bored by sterilized cup borer under sterile conditions. Extracts solution of known concentration and standard antibiotics (gentamycin 0.3 %) were dispensed into respective wells. Plates were left for 30 minutes at room temperature to diffuse the test solution and then incubated at 35°C for 18-24 hours under aerobic conditions and zone of inhibition were measured in millimeter (mm) as described in the Kirby–Bauer method (1966).

Statistical Analysis

All experiments were performed in triplicate and reported results are mean ± standard deviation of three replicates. ANOVA test method is used for statistical analysis and results were considered as significant with $P<0.05$. Bacterial CFU was calculated by arithmetic mean of triplicate and transformed into log of base 10.

RESULTS AND DISCUSSION

Fronds of Duckweed are excellent source of protein and amino acids **(**Louiset al., 1980). So, the current study is focused on the phytochemical analysis of duckweed along with its antibacterial potential. The screening of duckweed extracts revealed the presence of

significant amount of protein content estimated as 0.17 g/g of dry bio mass and the total lipid content was 0.029 g /g of dry bio mass of *Lemna minor* (**Table 1**).

Table 1: Nutrient composition of duckweed harvested in Pakistan.

Lemna minor	**Nitrogen (g %)**	**Fat (g %)**	**Total Protein (g %)**	**Ash (g %)**
	5-15	2.9	17	13

Herbs with antimicrobial properties have been used both traditionally and commercially not only to increase the shelf-life and safety of foods but also as medicines (Dupont et al, 2006). Extracts of many edible and medicinal plants, herbs, and spices have been seen to possess antimicrobial functions and could serve as a source for antimicrobial agents against food spoilage and pathogens (Oussalah et al, 2006). Plants constituents are extracted on the basis of polarity from crude plant and they can be polar and nonpolar in nature. Therefore, compounds were extracted from duckweed by solvent extraction method and efficacy of different solvent extract of *Lemna minor* was evaluated against selected known pathogens. Results showed that the ethyl acetate and hexane fractions of duckweed are more potent antibacterial agent but ethanol extract didn't show any inhibition against any pathogen. It revealed that the active compounds are moderate to less polar in nature. They exhibited inhibition effect against all tested organism but *P. aerugenosa* and *S.aureus* were found more sensitive and inhibited by all three fraction except ethanol fraction (**Table 2**).

Table 2: Antimicrobial activity of *Lemna minor* extracts.

Fractions	**Bacterial Inhibition (%)**			
	E. coli	***S. aureus***	***P. aerugemosa***	***B. pumilis***
Ethanol[a]	A	A	A	A
Ethyl acetate[b]	17	11	10	15
Hexane[c]	15	10	9	15
Acetone[d]	A	10	11	A

a=50mgmL^{-1} in DMSO

b=125mgmL^{-1} in DMSO

c=100mgmL^{-1} in DNSO

d=125mgmL^{-1} in DMSO

A=absent

It has been reported earlier that the methanolic extract of duckweed are weak antibacterial in nature (Tan L. *et.al*. 2018) and it's also reflected by the use of high concentration of extracts for inhibition.

The duckweed extracts were also evaluated for in vitro antioxidant activity along with flavonoid and total phenolic content. For the estimation of free radical scavenging activity DPPH method has been widely used because DPPH remains unaffected by side reaction such as metal ion chelation and enzyme inhibition (Milan S.S, 2011). The ethanolic fraction of duckweed showed 70.4 % antioxidant activity while the least antioxidant activity was observed in acetone fraction that was only 21.0 % (Figure 1a&1b).

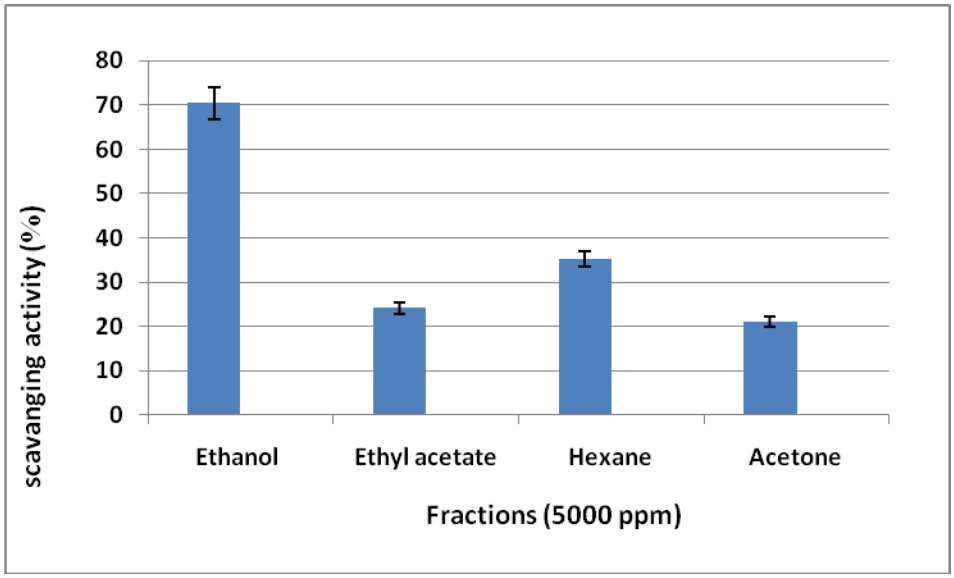

Fig. 1a: The DPPH radical scavenging activity of *Lemna minor* fractions.

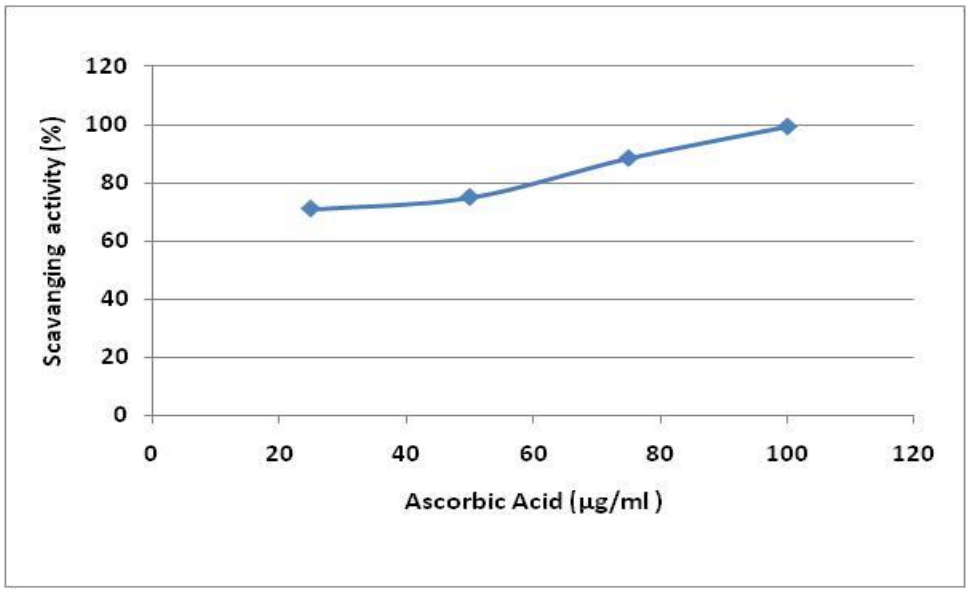

Fig. 1b: The DPPH radical scavenging activity of Ascorbic acid.

Indeed, antioxidant compounds have ability to remove or neutralize the free ROS radical from body before they oxidize the lipid, DNA and protein molecules of the body and in this

way help to protect from different diseases such as cardiovascular and cancer diseases. Free radicals of reactive oxygen species (ROS) are continuously produced during routine metabolic process and causes different human disease such as cancer, rheumatoid arthritis, Alzheimer's disease and aging by initiating oxidative events and as a result damage to the structure and functions of organ (Jacob andShenbagaraman,2011;Sen et al.,2010, Sensingh, 1989). For that reason, synthetic antioxidants are being extensively used in food industry but they are carcinogenic and have hepatic toxicity (Grice, 1986; Wichi, 1988). Therefore, plants having natural antioxidant constituent are good alternative of these synthetic compounds because they are biodegradable and non-toxic in nature. Among different phenolic compounds, flavonoids in a concentration of 0.84 % was found best in ethyl acetate fraction and the total phenolic content in *Lemna minor* was detected as 2.1 % in etrhanol fraction(Figure 2&3).

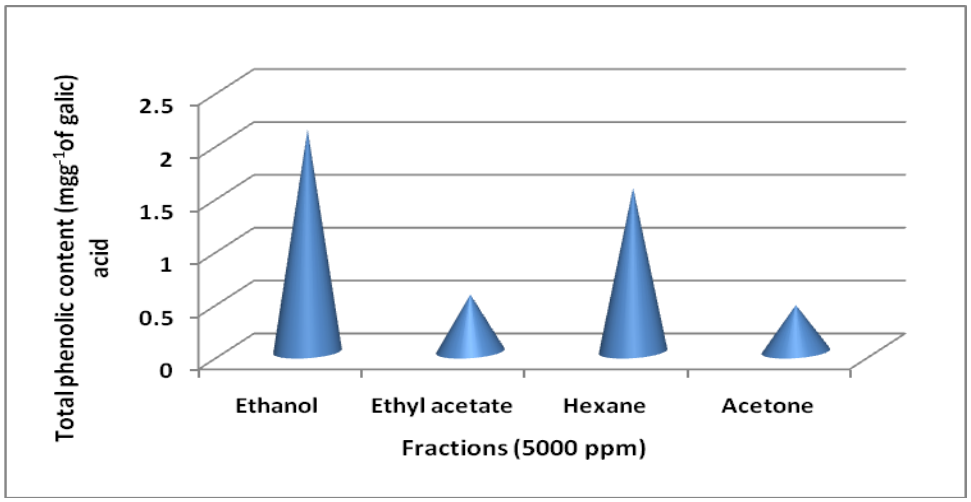

Fig. 2: Total Phenolic content of *Lemna minor.*

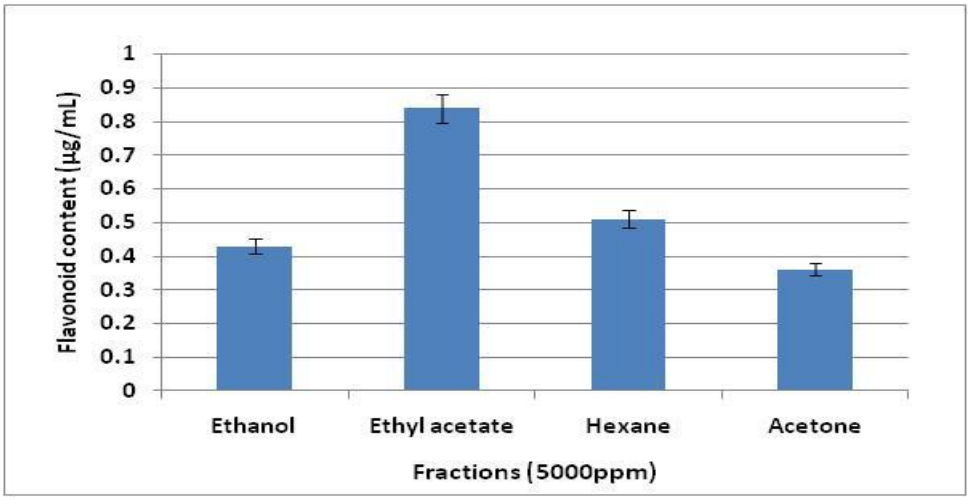

Fig. 3: Total flavonoid content of *Lemna minor.*

Phenolic compounds are highly soluble in polar organic solvents due to the presence of a hydroxyl group (Mohsen and Ammar, 2008). As a result, ethanolic extract of duckweed showed excellent antioxidant potential and high phenolic content. Phenolic and flavonoid molecules are important antioxidant components which are responsible for deactivating free radicals due to their ability to donate hydrogen atoms to free radicals. They also have ideal structural characteristics for free radical scavenging (Amarowicz et al, 2004). The significant linear correlation has been reported between total phenolic content and antioxidant activity. The presence of high concentration of phenolic components in the extract may effectively eliminate radicals which contribute directly to antioxidant effect of the system (Duh, 1994). Consequently, the high phenolic content of duckweed confirmed that the compound has good antioxidant activity (Milan S.S, 2011). It has also been reported that ethanolic and hexane fractions of natural products showed higher activity as compared to aqueous fraction due to high polarity (Majekodunmi et al, 1996; Martinez et al, 1996; Ahmed et al., 1998). Flavonoids are also secondary metabolites of plant and have antioxidant activity but its activity entirely depends on the structure of compound and hydroxyl group exchange pattern. Ethyl acetate and hexane fraction from duck weed exhibited high concentration of flavonoids. They protect the body from oxidative damage and also possess the anti-inflammatory effect.

CONCLUSION

Findings of this study showed that duckweed is also an excellent source of antioxidant compound along with protein. It also exhibited antibacterial effect against common water borne infectious organism but its weak antibacterial agent. Therefore, it could be incorporated into fish feeds formulas to serve as antibacterial agent against common gram positive and gram-negative pathogen. Moreover, it can also be used as food supplement for humans to combat the protein deficiency in mal nourished people. So, it would be beneficial for local fish farmers as protein supplement and antibacterial agent. The phytochemical screening of the *Lemna minor* extracts revealed the presence of flavonoid, antioxidants and phenolic content.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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