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DRUG STANDARDISATION AND *IN VITRO* ANTIINFLAMMATORY AND ANTIBACTERIAL ACTIVITY OF *Chlorella vulgaris*

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ABSTRACT

The methanolic crude extract of Chlorella vulgarisis was prepared and examined for invitro anti-inflammatory and antimicrobial activity. When compared to the positive standard, the extract showed potent anti-inflammatory activity by all the tested methods. The extract showed high inhibition in the growth of B.Subtilis when compared to the other tested strains. All microorganisms tested are susceptible to the treatment with methanolic extract of Chlorella vulgaris in a concentration dependent manner. Further, drug standardisation protocols are followed to evaluate and validate the quality, safety and bioefficacy of the extract.

KEY WORDS

Antibacterial, Anti-inflammatory, Ash content, Chlorella vulgaris, Moisture content

INTRODUCTION

Chlorella vulgaris is a single-celled fresh-water algae super-food and is thought to be one of the planet's earliest life-forms. Since the 1960's *Chlorella* has been popular as a multi-supplement taken to maintain health through optimal nutrition. *Chlorella* users report to experience more energy, improved physical appearance and protection from disease and illness. Infectious diseases are the leading cause of death world-wide and antibiotic resistance has become a global concern [1]. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens [2]. Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Unmatched availability of chemical diversity among natural products either as pure compounds or standardized plant extracts, provide unlimited opportunities for new drugs to lead in pharmaceutical industries. With an urgent need to discover new antimicrobial compounds, there is also need for the developing novel mechanisms of action for new and re-emerging infectious diseases [3]. In recent years, marine natural product search has yielded a considerable number of drug candidates [4]. Antibacterial substance, named 'chlorellin', was firstly isolated from *Chlorella*. The mixture of fatty acids was found to exhibit inhibitory activity against both Gram-positive and Gram-negative bacteria [5]. As microalgae where potentially explored only after 1950s, they were not considered previously for therapeutic purposes. Widespread research is presently undergoing to find the novel therapeutic useful agents to treat infectious diseases because it produces wide range of antibiotics [6,7]. Due to the emerging infectious diseases, viral infections and raise in antibiotic resistant bacteria, there is an urgent need for development of alternative treatment therapies against infectious diseases [8].

The chemical compounds responsible for the antibacterial activity in algae have been variously identified as bromophenols, carbonyls, halogenated aliphatic compounds, terpenes, isoprenylated and brominated hydroquinones, as well as phlobatannins

[9], unsaturated lactones, cyanogenicglycosides, sulphur containing compounds, phenols, phenolic glycosides, saponins and phytoalexins [10].

Inflammation is the reaction of living tissues to injury, infection or irritation. Lysosomal enzymes released during inflammation produce a variety of disorders which leads to the tissue injury by damaging the macromolecules and lipid peroxidation of membranes which are assumed to be responsible for certain pathological conditions as heart attacks, septic shocks and rheumatoid arthritis etc. The extra cellular activity of these enzymes is said to be related to acute or chronic inflammation. Stabilization of lysosomal membrane is important in limiting the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release or by stabilizing the lysosomal membrane [11].

HRBC or erythrocyte membrane is analogous to the lysosomal membrane [12] and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of human red blood cell membrane (HRBC) by hypo tonicity induced membrane lysis can be taken as an *in vitro* measure of anti-inflammatory activity of the drugs or plant extracts. Standardisation of drug means confirmation of its identity and determination of its quality and purity. In this regard, the present study focused on the anti-inflammatory and antibacterial potency of methanol extract of *Chlorella vulgaris* along with drug standardization.

MATERIALS AND METHODS

Preparation of methanolic extract of *Chlorella vulgaris*

1g of dried weight of *Chlorella vulgaris* was completely homogenized and soaked (w/v; 1:10) in 100 ml of methanol; and kept for 24 hours. This process was repeated until the extracted solvent became colourless except with that of water. The extracts were pooled together, filtered through Whatmann no. 1. The extract was dried at room temperature and stored in an air tight amber vial at 4˚C until further use.

DRUG STANDARDISATION

1. Pharmacognostic Study

Pharmacognostic study was carried on the basis of Morphological characters such as color, odor, taste, size, fracture, texture etc. using the sensory organs of our body [13].

2. Determination of foreign matter

Weigh 10 g to 50 g of the substance to be examined, or the minimum quantity prescribed in the monograph, and spread it out in a thin layer. Examine for foreign matter by inspection with the unaided eye or by use of a lens (6 ×). Separate foreign matter and weigh it and calculate the percentage present.

3. Physicochemical test

Solubility

The presence of adulterant in a drug could be indicated by solubility studies with various solvents [14].

i. Alcohol

5 gm of powdered material along with 100 ml of alcohol are shaken well occasionally for the first 6 hours and kept undisturbed for 18 hours. The liquefied extract thus obtained was concentrated in an vacuum oven and the percentage was calculated with the weight of the drug powder taken.

ii. Water

The procedure adopted for solubility percentage of alcohol is used with water instead of alcohol to get the water solubility.

Determination of moisture /Loss on drying procedure

Weigh about 1.5g of the powdered drug into a weighed flat and thin Porcelain dish. Dry it in the oven at 100°C or 105°C. Cool in desiccators and watch the loss in weight is usually recorded as moisture [15].

Determination of Ash

Total ash

About 2gm of powdered drug was weighed accurately into a tarred silica crucible. Incubated at 450°C in a muffle furnace until it is free from carbon. The crucible was cooled and weighed. Percentage of total ash was calculated with reference to air-dried substance [15].

Determination of total ash value formula:

Total ash value of the sample = 100 (z-x) % / y.

X= weight of empty dish.

Y= weight of the drug taken.

Z= weight of the dish + ash (after complete incineration).

Acid insoluble ash

Ash obtained from the total ash was boiled with 25ml of 2N HCl for a few minutes. Filtered through an ash less filter paper. The filter paper was transferred into a tarred silica crucible. Incinerated at 450°C in a muffle furnace until free from carbon. The crucible was cooled

and weighed. Percentage of acid insoluble ash was calculated with reference to air-dried substance.

Water soluble ash

Ash obtained from the total ash was boiled with 25 ml of distilled water for a few minutes and filtered through an ash less filter paper. The filter paper was transferred into a tarred silica crucible. Incinerated at 450°C in a muffle furnace until free from carbon. The crucible was cooled and weighed. Percentage of water-soluble ash was calculated with reference to air-dried substance.

Extractive value

Determination of alcohol soluble extractive value

About 5gms of air dried coarse powdered drug was weighed and macerated with 100ml of 90% alcohol in a closed flask for 24 hours, shaking frequently during the first 6 hrs and these allowed standing for 18 hrs. Thereafter it was filtered rapidly taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The %age of the alcohol soluble extractive values was calculated with reference to the air-dried drug.

Determination of water soluble extractive value

About 5gm of air-dried powdered drug was taken and macerated with 100 ml of chloroform water in a closed flask for 24 hrs shaking frequently during the first 6 hrs and then allowed to stand for 18 hrs. Thereafter, it was filtered rapidly taking precautions against loss of the solvent. 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of the water soluble extractive value was calculated with reference to the air-dried drug.

Determination of pH range

The powder sample was weighed to about 5g and immersed in 100 ml of water in a beaker. The beaker was closed with aluminum foil and left behind for 24 hours in room temperature. Later the supernatant solution was decanted into another beaker and the pH of the formulation was determined using a calibrated pH meter.

Anti-inflammatory Activity

Inhibition of albumin denaturation

A solution of 0.2% w/v of BSA was prepared in a Tris Buffer Saline and pH was adjusted to 6.8 using glacial acetic acid stock solutions of 10mg/ml of all the extracts were prepared by using methanol as a solvent. From these stock solutions 5 different concentrations of 100 - 500µg/ml were prepared. 50µl (0.05ml) of each extract was transferred to Eppendorf tubes using 1ml micropipette. 5ml of 0.2% w/v BSA was added to the entire above Eppendorf tubes. The control consists of 5ml of 0.2% w/v BSA solution with 50µl ethanol. The standard consists of 100µg/ml of diclofenac in methanol with 5ml 0.2% w/v BSA solution. The test tubes were heated at 72^0C for 5 minutes and then cooled for 10 minutes. The absorbance of these solutions was determined by using a UV-VIS Double beam spectrophotometer (ELICO SL 244) at a wavelength of 660nm [16]. Each experiment was carried out in triplicate and the mean absorbance was recorded. The percentage of inhibition of precipitation was determined on a percentage basis relative to control using the formula.

Percentage Inhibition

= [100 - (optical density of test solution – optical Density of product control) ÷ (optical density of test control) × 100

Proteinase inhibitory action

The test was performed using 2 ml of reaction mixture containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100 – 500 µg/ ml) were mixed together. The mixture was incubated at 37ºC for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 30 min. 2 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged, and the absorbance of the supernatant was read at 210 nm against buffer as blank [17]. Aspirin was taken as standard drug (100 – 500 µg/ ml). The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

% of proteinase inhibitory = (Abs control – Abs sample) X 100 / Abs control

HRBC Membrane Stabilization Method

The human red blood cell (HRBC) membrane stabilization method was used for the study of *in vitro* anti-inflammatory activity. The blood was collected from healthy human volunteer under aseptic conditions who was not taken any Non-Steroidal Anti-inflammatory Drugs for 2 weeks prior to the experiment and mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl). It was centrifuged at 3,000 rpm and the

packed cells were washed with isosaline (NaCl, pH 7.2) and a 10% suspension was made. To 0.5ml of test samples, 1ml of phosphate buffer (0.15 M, pH 7.4), 2 ml hypo saline (0.36% NaCl) and 0.5 ml of HRBC suspension were added. The solution was incubated at 37°C for 30 min and centrifuged at 3,000 rpm for 20 min. The content of the supernatant solution was absorbed spectrophotometrically at 560 nm [18]. Control was taken without the test sample. Diclofenac (100 - 500 µg/ml) was used as reference standard [19-21]. Percentage (%) of protection = [100 – OD of the drug treated sample/ OD of the control] × 100].

Membrane stabilization test

Preparation of Red Blood cells (RBCs) suspension

Fresh whole human blood (10 ml) was collected and transferred to the heparinized centrifuged tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline [22,23].

Heat induced hemolysis

The reaction mixture (2 ml) consisted of 1 ml of test solution and 1 ml of 10% RBCs suspension, instead of drug only saline was added to the control test tube. Aspirin was taken as a standard drug in the concentration ranges from 100 – 500 µg/ ml. All the centrifuge tubes containing reaction mixture were incubated in a water bath at 56°C for 30 min. At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates [24].

Percentage was calculated as follows:

% of inhibition = (100 – O.D of drug treated sample – O.D of control) X 100

Antibacterial Activity

Agar well-diffusion method was followed to determine the antimicrobial activity. Nutrient agar (NA) plates were swabbed (sterile cotton swabs) with 8 hour old - broth culture of *E.coli, S. aureus and B. subtilis*. Wells (10mm diameter and about 2 cm a part) were made in each of these plates using sterile cork borer. Stock solution of methanol extract of *Chlorella vulgaris* was prepared with a different concentration. About 100 µl of methanol solvent extracts were added sterile syringe into the wells and allowed to diffuse at room temperature for 2hrs. Control experiments comprising inoculums without methanol extract were set up. The plates were incubated at 37°C for 18-24 h for bacterial pathogens [25,26]. Methanol (100%) without *Chlorella vulgaris* extract was used as negative control and Streptomycin disc (30 µg) was used as the positive control. The diameter of the inhibition zone (mm) was measured. The experiment was repeated thrice, for each replicate the readings were taken in three different fixed directions and the average values were recorded [27].

RESULTS AND DISCUSSION

DRUG STANDARDISATION

Methods of standardization should take into consideration all aspects that contribute to the quality of the herbal drugs, namely correct identity of the sample, organoleptic evaluation, pharmacognostic evaluation, volatile matter, quantitative evaluation (ash values, extractive values), phytochemical evaluation, test for the presence of xenobiotics, microbial load testing, toxicity testing, and biological activity. The pharmacognostic study of *Chlorella vulgaris* showed green in color, produce musty odour which is found to be tasteless. The texture of *Chlorella vulgaris* was slightly rough, heavy fracture and size varies from 2-10 µm **(Table 1)**. Further, the physiochemical parameters of *Chlorella vulgaris* were studied.

DRUG STANDARDISATION

Table 1: Pharmacognostic Study

S.no	Properties	*Chlorella vulgaris*
1	**Colour**	Solid green
2	**Odour**	Musty
3	**Taste**	Tasteless
4	**Texture**	Slightly rough
5	**Fracture**	Heavy fracture
6	**Size**	2 -10µm

Table 2: Physicochemical Parameters of *Chlorella vulgaris*

Content	g/100g
Moisture content	**3.8**
Total Ash	**8.9**
Acid insoluble ash	**7.4**
Water soluble ash	**2.0**
Water soluble extractive	**3.8**
Alcohol soluble extractive	**7.9**
Solubility (water)	**3.2**
Solubility (alcohol)	**8.2**
PH	**8.5**

Table 3: Inhibition of albumin denaturation of methanol extract of *Chlorella vulgaris*

S.No	Concentration µg/ml	Standard % Mean ± SD	Methanol extract % Mean ± SD
1	100	26.78 ±0.007	28.85 ±0.005
2	200	36.78 ±0.015	35.07 ±0.014
3	300	43.64 ±0.006	42.83 ±0.017
4	400	52.29 ±0.007	54.82 ±0.006
5	500	71.50 ±0.123	72.67 ±0.025
IC 50 Values		336.227	329.351

The values are Mean±SD of triplicate values

Table 4: Protein inhibitory action of methanol extract of *Chlorella vulgaris*

S.No	Concentration µg/ml	Standard % Mean ± SD	Methanol extract % Mean ± SD
1	100	30.82 ±0.002	31.73 ±0.005
2	200	42.80 ±0.11	44.40 ±0.108
3	300	53.08 ±0.12	55.59 ±0.021
4	400	65.75 ±0.017	65.98 ±0.026
5	500	73.85 ±0.023	77.05 ±0.031
IC 50 Values		270.094	255.89

The values are Mean±SD of triplicate values

Table 5: HRBC membrane stabilization method of methanol extract of *Chlorella vulgaris*

S.No	Concentration µg/ml	Standard % Mean ± SD	Methanol extract % Mean ± SD
1	100	23.95 ±0.006	22.79 ±0.003
2	200	33.83 ±0.019	31.87 ±0.0213
3	300	49.33 ±0.005	51.36 ±0.008
4	400	57.79 ±0.015	61.53 ±0.021
5	500	73.55 ±0.023	79.16 ±0.023
IC 50 Values		318.756	304.621

The values are Mean±SD of triplicate values

Table 6: Membrane stabilization test: Heat induced haemolysis of methanol extract of *Chlorella vulgaris*

S.No	Concentration µg/ml	Standard % Mean ± SD	Methanol extract % Mean ± SD
1	100	40.63 ±0.025	40.94 ±o.009
2	200	49.11 ±0.006	49.48±0.012
3	300	55.48 ±0.021	55.98 ±0.032
4	400	62.34 ±.011	60.94 ±0.026
5	500	65.86 ±0.008	66.23 ±0.015
IC 50 Values		226.456	224.017

The values are Mean±SD of triplicate values

Table 7: Anti-bacterial Activity of Methanol Extract of *Chlorella vulgaris*

S.No	Concentration µg/ml	Zone of inhibition (mm ±SD)		
		E.coli	***S.aureus***	***B.subtilis***
1	25	4.6±0.04	4.3±0.01	7.5±0.03
2	50	6.9±0.02	6.4±0.12	8.7±0.10
3	75	11.6±0.28	10.9±0.8	12.5±0.21
4	100	14.9±0.21	12.8±016	15.8±0.26
Streptomycin 50 (+ Ve Control)		18.6±0.25	15.2±0.23	26.0±0.30
Methanol (- Ve Control)		-	-	-

The values are Mean±SD of triplicate values

Different Physicochemical parameters such as Total Ash (8.9 g/100g), Acid Insoluble Ash (7.4 g/100g), Water Soluble Ash (2.0 g/100g), Water soluble extractive (3.8 g/100g), Alcohol Soluble extractive (7.9 g/100g) and pH 8.5 was observed for the test sample *Chlorella vulgaris* **(Table 2)**. These values can be useful to detect adulteration.

The nutritive value of outdoor or indoor cultured *C. vulgaris* is of interest to the food industry, especially in countries where the weather conditions do not allow massive culture of higher plants. *Chlorella* has health benefits, such as assisting disorders such as gastric ulcers, wounds, constipation, anemia, hypertension, diabetes, infant malnutrition and neurosis. It has also been shown to have immune-modulating and anticancer properties [28-34]. Similar analysis has been reported with quality analysis of processed cheese enhanced with chlorella biomass [35].

***IN VITRO* ANTI-INFLAMMATORY ACTIVITY**

Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation, the mechanism of the anti-inflammation activity of different concentration of methanol extract of Chlorella vulgaris was tested for albumin denaturation. Methanol extract of Chlorella vulgaris was effective in inhibiting albumin denaturation. From the Table 3, Diclofenac sodium, a standard anti-inflammation drug showed the inhibition of 71.50% at the concentration 500 µg/ml. The maximum inhibition of 72.67% at the concentration 500 µg/ml was noted for the methanol extract of Chlorella vulgaris which was found to be slightly higher than the standard drug. Denaturation of proteins is a well-documented cause of inflammation. Phenylbutazone, salicylic acid, flufenamic acid (anti-inflammatory drugs) etc, have shown dose dependent ability to thermally induced protein denaturation [36]. Anti-inflammatory activity of Chlorella vulgaris was done by protein inhibitory action at different concentration in the range of 100 – 500 µg/ml which is noted in the Table 4. The methanol extract has showed highest proteinase inhibitory activity 77.05% compared to the standard drug aspirin which have showed the minimum proteinase inhibitory activity 73.85%.

In present investigation, the HRBC membrane stabilization method showed that the methanol extract of Chlorella vulgaris (79.16%) possess maximum activity at the concentration 500 µg/ml. while the standard showed decreased in activity which exhibit 73.55% **(Table 5).**

The mechanism of anti-inflammatory activity was studied through stabilization of RBCs membrane. The samples with different concentration were effectively inhibiting the heat induced haemolysis. Thus, the results showed that the samples inhibit the release of lysosomal content of neutrophils at the site of inflammation. The test samples inhibited the heat induced hemolysis of RBCs at varied concentration from 100 – 500µg/ml. At concentration 500µg/ml methanol extract showed the maximum inhibitions 66.23% which was slightly higher than the aspirin standard drug (65.86%) **(Table 6)**.

These provide evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect. This extract may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophil lysosomal constituents include bactericidal enzymes and protease, which upon extracellular release cause further tissue inflammation and damage [12]. The overall result of the present investigation showed that methanol extract of Chlorella vulgaris possessed high anti-inflammatory activities.

***IN VITRO* ANTI BACTERIAL ACTIVITY**

The methanolic extract of *Chlorella vulgaris* displayed different degrees of antimicrobial activities against

different bacteria. Antimicrobial activity of the methanol extracts of *Chlorella vulgaris* exhibited varying degree of antimicrobial activities against test microorganisms *E.coli, S. aureus and B. subtilis* **(Table 7)**. *S. aureus* showed low susceptibility i.e. 12.8 mm at concentration 100 µg/ml whereas, *E.Coli* produce moderate susceptibility i.e. 14.9 mm at concentration 100 µg/ml. Wide range of antimicrobial activity was exhibited between the range 25-100 µg/ml. *B .subtilis* was more potential when compared to *E Coli* and *S. aureus* which shows 15.8 mm at concentration 100 µg/ml. The negative control methanol alone doesn't exhibit any inhibitions which mean that in the methanol extract of *Chlorella vulgaris* the test sample alone produce inhibition against three different microorganisms. When compared with standard the test shows low susceptibility at all the tested concentration. The present results were similar to that reported on the antimicrobial activity of various solvent extracts of *chlorella vulgaris* [37]. In a similar study, *C. vulgaris* is reported to inhibit *B. subtilis* and *P. vulgaris* while *C. reinhardtii* are more efficiently inhibiting *E. coli, P. aeruginosa* and *S. aureus* [38].

CONCLUSION

The invitro anti-inflammatory and antimicrobial activity of methanolic extract of *chlorella vulgaris* is tested in the present study. The results proved the bioefficacy of the microalgae as anti-inflammatory and antimicrobial sources. The various polysaccharides, unsaturated fatty acids and pigments present in the microalgae is widely responsible for its bioactivity. The extract was also tested for various drug standardisation parameters. All studied standardization parameters like Pharmacognostic study and physicochemical parameters provide the knowledge in the identification, authentication and exploring *Chlorella vulgaris* as nutraceutical supplement in the combat of various diseases. Hence, further study is underway for the characterisation of bioactive compounds from *Chlorella vulgaris.*

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