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Quantitative Estimation of Chlorophyll and Carotene Content in *Triticum aestivum* Hexane Extract and Antimicrobial Effect against *Salmonella*

Anand Rajoria *, Archana Mehta, Pradeep Mehta, Laxmi Ahirwal
Laboratory of Plant Pathology and Microbiology, Department of Botany,
Dr. Hari Singh Gour University, Sagar 470003 MP, India

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Abstract: India is a rich and varied flora of medicinal plants since the Vedic period. Present study deals with the qualitative and quantitative analysis of hexane extract of *Triticum aestivum* grass. A qualitative analysis by thin layer chromatography (TLC) and a quantitative analysis by standard chemical protocol of secondary metabolites in the hexane extract of *Triticum aestivum* grass were studied. Using TLC, different components such as chlorophyll and carotene were analyzed. The R_f (0.28, 0.29, 0.37, 0.65, 0.74 and 0.93) values of the 6 developed spots in the hexane: ethyl acetate (16:4) solvent system were noted. In the quantitative analysis, chlorophyll and carotene content in *T. aestivum* was 0.54 ±0.016 g/l out of which chlorophyll-a, and chlorophyll-b were 0.288±0.05 and 0.305±0.05 g/l, respectively while carotene content was 0.42±0.066 g/l. The antimicrobial activity of hexane extract of *Triticum aestivum* grass against *Salmonella Enteritidis* showed potential effects with inhibitory zones in the range of 9-8 mm along with minimum inhibitory concentration (50 mg/ml) and reduction of cell count. These results may be helpful for rationale use of this plant in the modern system of health care.

Key words: *Triticum aestivum*, Hexane extract, Chlorophyll and Carotene content, Antimicrobial activity.

Introduction

Medicinal plants represent a rich source of secondary metabolites acting as natural drug agents. They are known to be biologically active compounds and therefore aid the pharmacological activities. It has been stated that the mechanism of the biological activity of the plant extracts/ bioactive compounds involves the inhibition of various cellular processes, increase in plasma membrane permeability and impairment of energy or synthesis of structural components in microbial cells [1].

Wheat is worldwide cultivated food crop and the second important staple cereal food. *Triticum aestivum* is commonly known as a bread wheat and grown in India in almost all the wheat-growing zones. Bread wheat was first introduced in India by Dr Borlaug of Mexico, and is frequently called as Mexican dwarf wheat, 86 % of the total wheat acreage grown in India. Wheat is one of the most important food crops in the world, providing 20 % of human dietary energy supply and serving as the main source of protein in developing nations [2].

Wheatgrass is a rich source of protein, carbohydrate, total dietary fiber, vitamin A, C, E, folic acid, niacin, riboflavin, iron, calcium, magnesium, selenium, chlorophyll and carotene. Each of which except carbohydrate is much more than a whole cereal grain of wheat and also contains 17 amino acids, 8 of which are essential [3]. The dry weight of wheatgrass contains 70% chlorophyll [4]. Wheatgrass is a safe alternative to treat chronic degenerative diseases such as cancer, diabetes, blood

*Corresponding author (Anand Rajoria)
E-mail: < anand.rajoria@gmail.com >

pressure and other diseases such as obesity, gastritis, ulcers, pancreas and liver problems, fatigue, anemia, asthma, eczema, hemorrhoids, skin problems, halitosis, body odor and constipation [5]. It is antimutagenic in nature [6] which improves hematological toxicity related to chemotherapy in breast cancer patients [7]. Wheatgrass juice is a good blood building natural source and is an effective alternative of blood transfusion in terminally ill cancer patients [4] and thalassemia patients [8].

In the present investigation, qualitative and quantitative analysis of phytochemicals and antimicrobial evaluation of *Triticum aestivum* was done. Importantly, we focused for the estimation of carotene and chlorophyll content in hexane extract of fresh grass and further evaluated thin layer chromatographic analysis for the color characteristics of bioactive compounds present in hexane extracts. Further, anti-pathogenic effects against *Salmonella Enteritidis* were also evaluated.

Materials and methods

Collection of plant material

The seeds of *Triticum aestivum* were collected from the Regional Agricultural Research Institute (RARI), Sagar (M.P.) and authenticated by Senior scientist of the institute. The seeds were sown during the month of September. Loamy soil (70 % sand + 30 % clay) was prepared and conditioned with compost. The pH of the soil was optimized from 6.9-7.2. The plants were harvested on the 10th day. The harvested plant materials were shade dried and ground to powder (Fig. 1). Dry powder was stored in airtight screw capped bottles at 4°C for further studies.

Chlorophyll and carotene estimation

1 g of leaf sample was ground in a pestle-mortar with 5 ml of distilled water to form a paste. The contents were transferred to a centrifuge tube and the total volume was made up to 10 ml with distilled water. 0.5 ml of supernatant from the tube was transferred to a tube containing 4.5 ml of 80 % acetone. The contents were centrifuged at 4000 rpm for 15 min. The absorbance of the supernatant was calculated to measure total chlorophyll, chlorophyll-a, chlorophyll-b and carotene at the following wavelengths of 645, 663, 490 and 638 nm, respectively [9].

Preparation of the hexane extract

Maceration technique [9] was used for the extraction. 10 g powder of *T. aestivum* was suspended in 100 ml of hexane in a 250 ml conical flask and kept on orbital shaker for 48 h at 37°C. After 48 h, the supernatant was filtered through Whatman filter paper No.1 and evaporated to dryness at room temperature. The viscous material was stored in sterile, air-tight bottles and kept in refrigerator for further studies.

Thin layer chromatographic analysis of hexane extract of *T. aestivum*

Thin layer chromatography (TLC) is an important analytical test for identifying unknown compounds, monitoring reactions, and testing chemical purity. It is a quick and simple method for determining the number of compounds in a mixture.

Fig. 1. (A) Plots of 1 m^2 of *T. aestivum* **(B)** Harvested Wheatgrass; **(C)** Dried and powdered *T. aestivum*

Hexane extract was to begin with, checked by TLC on analytical plates over silica gel. TLC was carried out to isolate the principle components that were present in most effective extract of the plant. The different solvent systems of different polarities were prepared and TLC studies were carried out to select the solvent system capable of showing better resolution.

The above prepared plant extract was applied on pre-coated TLC plates by using capillary tubes and developed in a TLC chamber using suitable mobile phase. The developed TLC plates were air dried and observed under ultraviolet light UV or with the aid of spraying reagents and some were placed in hot air oven for 1 min for the development of color in separated bands. The movement of the analyze was expressed by its retention factor (R_f). The R_f values of the visible spots can be determined by calculating the distance travelled by the colored product and total distance travelled by the solvent.

Ani-pathogenic activity by disc diffusion assay

A bacterial strain *Salmonella Enteritidis* was provided by the Institute of Microbial Technology (IMTECH), India. Anti-pathogenic activity of the extracts was performed by disc diffusion method [10]. Briefly, the hexane extract samples were dissolved in 5 % dimethyl sulphoxide (DMSO), and further diluted to obtain desired concentrations (10, 50, 100 and 200 mg/ml). The bacterial strain was sub-cultured by inoculating a single colony of the bacterial inoculum in the nutrient broth (30 ml) to get the viable count of 10^6 cells/ml. From this, an inoculum of bacterial strain (0.1 ml) was inoculated into the Molten-Muller Hinton agar medium. The discs (6 mm) were impregnated with 40 µl of test sample and allowed to dry for 5 min. Then, the disc containing the sample was placed on the seeded agar plates and allowed to stand for 1 h for pre-diffusion of the extract and incubated at 37°C/24 h. Antimicrobial evaluation was performed based on the diameters of the zones of inhibition around the disc and measured in mm. All experiments were done in triplicates. Streptomycin was used as a positive control, while DMSO (5 %) was used as negative control.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of the hexane extract of *T. aestivum* was tested by the two-fold dilution method [10]. A loopful of the bacterial culture of *Salmonella Enteritidis* was inoculated in the nutrient broth and incubated at 37°C for 24 h, and two-fold serial dilution method was followed as below. The crude hexane extract was first dissolved in 5 % dimethyl sulfoxide (DMSO). This solution was further diluted with 5 % DMSO and was added to NB to a final concentration of 10, 20, 40, 50, 60, 80, 100 and 200 mg/ml. The bacterial suspension of test pathogen was inoculated in NB medium in 25 ml of cap tube and incubated for 24 h at 37°C. The minimum concentration at which no visible growth was observed in the tube was defined as MIC, which was expressed in mg/ml. A set of tubes containing only seeded liquid medium was kept as control and 5 % DMSO control was also maintained. All the tests for MIC determinations were performed in triplicate.

Effect of *T. aestivum* hexane extract on viable counts

For viable counts, each of the tubes containing a bacterial suspension (approximately 10^7 CFU/ml) of *Salmonella Enteritidis* in NB broth medium was inoculated with the minimum inhibitory and its two-fold concentration of the *T. aestivum* hexane extract (50, and 100 mg/ml) in 10 ml LB broth, and kept at 37°C [11]. Samples for viable cell counts were taken out at 0, 20, 40, 60, 80, 100 and 140 min time intervals. Enumeration of viable counts on NB plates was monitored as followings: 0.1 ml sample of each treatment was diluted into buffer peptone water, thereby diluting it 10-fold and spread on the surface of LB agar. The colonies were counted after 24 h of incubation at 37°C. The controls were inoculated without extract for *S. Enteritidis* with the same experimental condition as mentioned above.

Result and discussion

Total chlorophyll and carotene content

In the present study, *T. aestivum* was subjected to estimate its chlorophyll and carotene contents. The total chlorophyll in *T. aestivum* was 0.54

±0.016 g/l out of which chlorophyll-a and chlorophyll-b were 0.288±0.05 and 0.305±0.05 g/l, respectively while carotene content was 0.42±0.066 g/l. (Table 1). The presence of pigments in plant tissues gives color to vegetables and fruits, which is different depending on variety and species. Pigments are substances with very different chemical structure, which are present in the form of porphyrin pigments, carotenoids, anthocyanins and flavones. The main porphyrin pigments found in green plants are chlorophyll-a, b and c. Chlorophyll a, the main pigment in plants, which convert light energy into chemical energy through photosynthesis process. The content of chlorophyll pigments varies by species [12]. Carotenoid pigments can be located in chromoplasts, contributing to the color of vegetables and fruits, or in chloroplasts, where, together with chlorophylls, are involved in the two phyto-systems. Among carotenoid pigments with 40 carbon atoms identified in vegetables and fruits, β-carotene is the most popular and widespread [12].

Thin layer chromatographic analysis of hexane extract of *T. aestivum*

Thin layer chromatography (TLC) was used to detect spots from different solvent extracts [13]. Hexane extract of *T. aestivum* was analyzed with following mobile phase, hexane: ethyl acetate (16:4). The hexane extract of *T. aestivum* revealed 6 different spots with R_f values 0.28, 0.29, 0.37, 0.65, 0.74 and 0.93, respectively, when hexane: ethyl acetate (16:4) solvent system was used (Table 2). Hexane extract of *T. aestivum* seems to have complex spots and this indicates that sequential purification and separation will be needed in all TLC plates. Prior to further purification there must be an improvement by increasing the ethyl acetate and then observing if there will be more separation, or may introduce another solvent which will improve the resolution of the spots [14].

TLC profiling of hexane extract of *T. aestivum* gives an impressive result, directing towards the presence of number of phytochemicals. Mixture of solvents with variable polarity in different ratio can be used for separation of pure compound from plant extract. The selection of appropriate solvent system for a particular plant extract can only be achieved by analyzing the R_f values of compounds in different solvent systems. Different R_f values of the compound also reflect an idea about their polarity. This information will help in the selection

Table 1. Total chlorophyll and carotene content in *T. aestivum*

Sample	Value in (g/l)
Total Chlorophyll	0.54±0.016
Chlorophyll-*a*	0.288±0.05
Chlorophyll-*b*	0.305±0.05
Carotene	0.42±0.066

The results obtained were expressed as Mean ± S.D. of triplicates

Table 2. R_f value of hexane extract of *T. aestivum*

Spot	R_f Value	Colour in UV light
1	0.28	Cream
2	0.29	Pink
3	0.37	Pink
4	0.65	Pink
5	0.74	Light Pink
6	0.93	Pink

Solvent system used: Hexane: Ethyl acetate (16:4)

of appropriate solvent system for further separation of compounds from plant extracts [15].

Anti-pathogenic activity

In the present study, hexane extract of *T. aestivum* showed most significant activity against *Salmonella Enteritidis* (zone of inhibition- 8 mm at 200 mg/ml concentration), 100 mg/ml showed the inhibitory zone of 7.3 mm, 50 mg/ml showed 7 mm inhibitory zones (Table 3). On the other hand, 10 mg/ml had no anti-pathogenic effect against *Salmonella Enteritidis*. The MIC value was in the range of 50-100 mg/ml. The blind control with 5 % DMSO used in this study did not inhibit any of the bacteria tested (Table 3).

Based on the sensitivity of the test pathogen *S. Enteritidis*, further studies to confirm the antibacterial mode of action of hexane extract of *T. aestivum,* MIC and 2xMIC concentration were chosen to analyze their effects on viable cells. The effect of hexane extract of *T. aestivum* on the growth of tested bacterial pathogen demonstrated reduced viability at the used concentrations (Fig. 1). The exposure of 0-80 min of hexane extract of *T. aestivum* (50 and 100 mg/ml) did not cause severe decline on the inhibition of cell viability of the tested pathogen, however, considerable amount of inhibitory effect was observed on the inhibition of the cell viability of *S. Enteritidis* at the exposure time of 120 min. Interestingly, the exposure of the hexane extract of *T. aestivum* for 160 min revealed complete inhibition of colony forming unit (CFU) numbers of *S. Enteritidis* (Fig. 2). On the other hand, complete inhibition of cell viable counts of *S. Enteritidis* was observed at 200 min exposure time of hexane extract of *T. aestivum* (50 and 100 mg/ml), and no CFU formation was observed (Fig. 1).

The current antimicrobials, which cover a wide array of targets, generally fall into two categories, one which kills the organisms and the other which merely inhibits the growth [1]. Antimicrobial action generally falls within one of the four mechanisms, three of which involve the inhibition or regulation of enzymes involved in cell wall biosynthesis, nucleic acid metabolism and repair, or protein synthesis, respectively. The fourth mechanism involves the disruption of membrane structure. The standard antibacterial drug target interactions are well studied and predominantly fall into three classes: inhibition of DNA replication and repair, inhibition of protein synthesis and inhibition of cell-wall turnover [1,16].

Basically, the antimicrobial activity of the medicinal plants is believed to be due to the presence of secondary metabolites which act either individually or in complex [17,18]. These secondary metabolites include the phytochemicals, whose antimicrobial potentials have already been reported by many researchers [17,18].

Similarly, chlorophyll is also reported to possess a great deal of antimicrobial potential. Jayavanth *et al.*[19] reported the antibacterial activity of chlorophyll in pure form against Gram-negative bacteria. Lilian *et al.*[20] analyzed the antimicrobial activity of a chlorophyll-based solution on isolates of *Candida albicans* and *Enterococcus faecalis* and concluded that the chlorophyll-based solution possessed potent antimicrobial activity.

Conclusion

In the present study, the antimicrobial efficacy of *T. aestivum* could be assigned to number of

Table 3. Anti-pathogenic effect of hexane extract of *T. aestivum* against *Salmonella Enteritidis*

Extract	Concentration (mg/ml)	Zone of Inhibition (mm)*	MIC
Hexane	10	-	-
	50	7.0	+
	100	7.3	++
	200	8.0	+++

Clearance zone including the diameter of the sterile disc (6 mm)

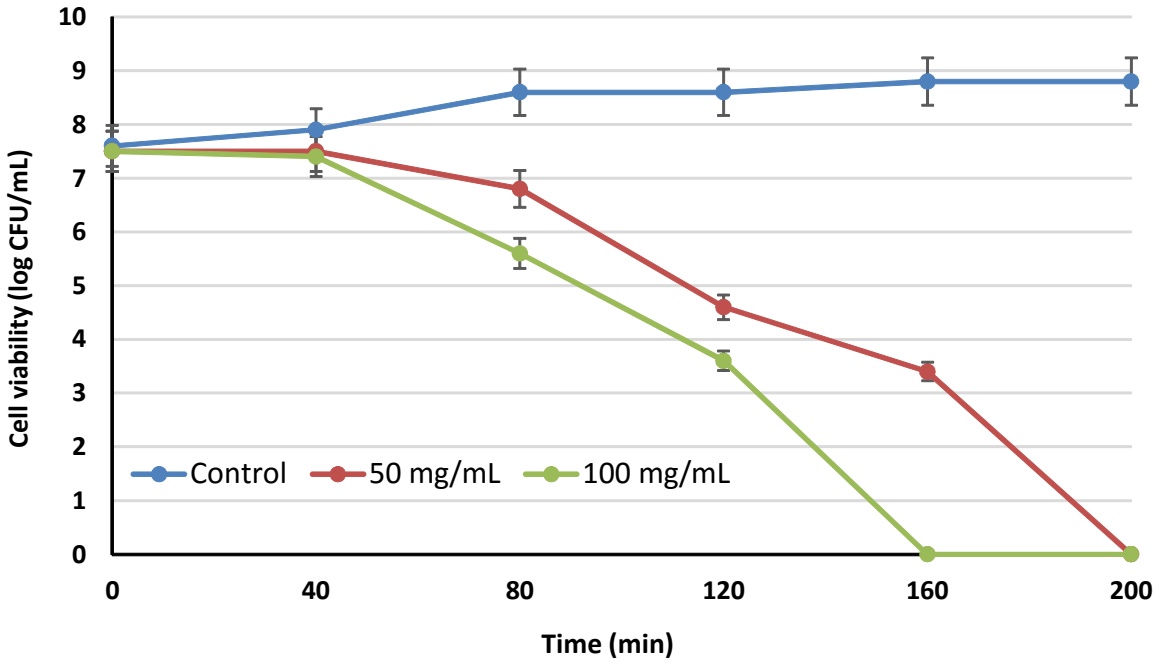

Fig. 2. Effect of hexane extract on cell viability of tested pathogen *Salmonella Enteritidis*

phytochemicals like flavonoids, alkaloids, tannins, terpenoids, glycosides and even chlorophyll seemed to have the potential to act as a source of useful drugs and also to improve the health status of the consumers. The antimicrobial data revealed that *T. aestivum* could be a potential source of natural antimicrobial having great importance as a therapeutic agent, which could be a useful substrate for the synthesis of new broad spectrum antibiotics for the treatment of infections caused by the pathogenic organisms. Further purification, identification and characterization of the active compounds would be our priority in future studies.

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