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Optimization of *Spirulina platensis* Biomass and Evaluation of its Protective Effect against Chromosomal Aberrations of Bone Marrow Cells

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Abstract

There are numerous studies investigating the effectiveness and potential clinical applications of *Spirulina* in treating several diseases. So, the present essay was designed to Cultivate *Spirulina sp.* in three different media; namely (modified commercial low cost Zarrouk medium (MS), Khul medium and Sea Water enriched medium), to compare Biomass concentrations (g/lDW) and optical density (OD) for 20 days, and phytochemical screening (Total phenolic, total flavonoids and Antioxidant activity) of the crude extract of all media with commercial *Spirulina* (dry product). In addition, examine possible potentials of *Spirulina* against hepatic intoxication induced by CCl_4 in albino male mice. Results revealed that, methanolic extract recorded the highest values in modified Zarrouk's medium (88.98 mg gallic acid equivalent/ml, 78.57 mg Rutin equivalent/ml and 82.04%) in Total phenolic, total flavonoids and Antioxidant activity respectively. Conclusively, *Spirulina* showed an ameliorating effect of CCl_4 induced chromosomal aberrations of bone marrow cells; which proves the protective role of it against the chromosomal damage.

Keywords: *Spirulina*; Cultivation; Phytochemical screening; Chromosomal aberrations; Bone Marrow Cells; CCl_4

Introduction

Water is very important to our existence in life. Potable water is the water that is free from Cyanobacteria is developed for marine algae belonging to the genus *Spirulina*, and previously grouped within the genus "Arthrospira". *Spirulina* is a planktonic photosynthetic filamentous cyanobacterium that forms massive populations in tropical and subtropical bodies of water which have high levels of carbonate and bicarbonate and alkaline pH values of up to 11 [1]. *Spirulina* has been introduced as food supplement, such as protein (60%-70% by weight), indispensable amino acid, vitamins, mineral substances, essential fatty acid, glycolipids and sulfolipids. Many authors investigated the potential use of seawater with some nutrients for commercial cultivation of *Spirulina platensis* at low cost [2]. The advantages of introducing natural sea water in *Spirulina* production medium are: 1) lower fertilizer cost; 2) saving farm land by using waste sea beach; 3) seawater culture is not easily polluted by heavy metals and contaminations [3]. The utilization of seawater media in the cultivation will reduce the production cost considerably [4].

Furthermore, *Spirulina* is cited in the published literature as having the potential to yield novel pharmaceutical; hence, *Spirulina's* primary and secondary metabolites have the potential to play a role as a source of powerful anti-viral agents [5], anti-bacterial agents [6], anti-HIV agents [7], anti-tumour agents [8], a-carotene and other phytopigments (carotenoids, chlorophyll and phycocyanin) that function as antioxidants [9], and mycosporine like amino acids (MAAs) and scytonemin as photo protectants [10] and sterols as antimicrobials [11].

Carbon tetrachloride (CCl_4) is one of the most common chemical agents used in the laboratory for the study of various liver disorders at acute and chronic condition [12]. Toxicity begins with the change in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structures [13]. The toxic metabolite CCl_4 radical is produced, which is further converted to trichloromethyl peroxy radical by cytochrome P450 2E1 enzyme. This radical binds covalently to the macromolecules and causes peroxidative degradation of cellular membrane leading to the necrosis of hepatocytes [14]. Interestingly, the oxidative DNA damage of CCl_4 was evaluated by means of the comet assay, which is widely used in genotoxicity testing in vitro and also becoming an important tool for evaluating the genotoxic potential and mutagenicity of many chemicals and natural compounds in vivo, whereas it play important roles in the determination of DNA damage level [15].

Treatment of *Spirulina platensis* (800 mg/kg/b.wt) to CCl4 challenged mice resulted in decreased liver marker enzymes activity, DNA damage and lipid peroxidation levels with increase in antioxidant status [16]. Chromosomal aberrations (CA) are the microscopically visible part of a wide spectrum of DNA changes generated by different repair mechanisms of DNA double strand breaks (DSB) and one of the important biological consequences of human exposure to ionizing radiation and other genotoxic agents. The chromosomal aberrations were appeared in the form of chromosomal fragment, chromatid, deletions, stickiness, ring chromosomes, Robertsonain centric fusion, centric fusion, chromatid gap and polyploidy [17,18].

The present investigation was designed to cultivate *Spirulina platensis* on different culture media (modified Zarrouk's medium, Khul medium and Sea Water enrichment medium) to compare the biomass yield and phytochemical screening of the crude extract of all media with commercial *Spirulina*, Selective highest value of *Spirulina* and evaluation of its protective effect against chromosomal aberrations of bone marrow cells.

Materials and Methods

Algal source

The cyanobacteria used in this study, *Spirulina platensis* strain which obtained from Algal Lab of Dr. Heba Saad El-Sayed presented in the marine hatchery in the National Institute of Oceanography and Fisheries, Alexandria Egypt. Zarrouk media was used for growing *Spirulina platensis* [19-21].

Culture collection and maintenance

Three types of media, namely commercial modified Zarrouk medium (used in mass production in *Spirulina* exporter in India, 2014) were prepared, in which pH was ranged between (9.0-9.5), Khul medium [20] (PH 6.5-7.0) and Sea Water enriched medium (PH 9.2) according to methods of Faucher et al., [22] with slight modification [23]. All the reagents used were of analytical grade (AR) and purchased from Sigma, except for sodium chloride (NaCl), which were substituted with Natural sea water, and also $NaHCO_3$ and urea, which are of commercial grades respectively, with concentrations equivalent to that found in Zarouk medium SM. The composition of the Reduced Cost media is shown in Table 1.

	Modified Zarrouk medium (commercial)	Kuhl's medium	Sea water enriched medium
$NaHCO_3$	8 .0 g/l	-	5.0 g/l
KNO_3	2.0 g/l	0.011 g/l	1.5 g/l
NaCl	5.0 g/l replaced with sea water%25	5.0 g/l	-
$NH_4PO_4.12H_2o$	0.08 g/l	-	-
$MgSO_4$. 7H20	0.16 g/l	-	-
Urea	0.015 g/l	-	-
$FeSO_4.7H_2O$	0.005 g/l	-	-
Complete fresh water to	1L	-	-
NaH_2PO_4	-	0.0399 g/l	-
Na_2HPO_4	-	0.0709 g/l	-
$MgSO_4.7H_2O$	-	0.0246 g/l	-
$CaCl_2.2H_2O$	-	0.0017 g/l	-
Fe complex stock (Mix of 0.695 gm $FeSO_4.7H_2O$ +0.93 gm EDTA)	-	1ml	-
Trace elements	-	g/l	-
H_3BO_3	-	0.0618	-
Mn $SO_4.H_2O$	-	0.151	-
Zn $SO_4.7H_2O$	-	0.2875	-
$CuSO_4.5H_2O$	-	0.0024	-
KH_2PO_4	-	-	0.1 g/l
Complete fresh water to 1L	-	-	Complete sea water to 1L

Table 1: Composition of media.

Growth and maintenance of the culture was done in an illuminated (4500 lux) growth room at 25°C ± 2°C under 24 hour continuous light illumination. Blower of 2H was used for continuous mixing of cultures. The growth of *Spirulina platensis* was determined by measuring the parameters, optical density and biomass concentrations in the cultures through the cell dry weight according to the method of Vonshak et al. [23]. The exhausted broth was collected for analyses. One portion was used for the determination of dry cell mass concentration (Biomass g/l), and optical density (OD) was measured by using a spectrophotometer at 560 nm. The dry weight was measured by 100 ml

of culture sampled and filtered through what man No.1 filter paper and dried for 1 hr at 105°C and weighed prior to filtration. The filtered wet biomass was then washed with two volumes of distilled water, dried as above and weighed.

Extracts preparation and preliminary phytochemical screening

Preparation of the Extract of *Spirulina platensis*: In raw materials that use absolute methanol to extract a wide range of phenolic and flavonoids compounds, which described by Sroka et al. [24].

Analysis of components of extracts

Determination of total phenolic content: Total phenolic compounds (TPC) of algal extracts were determined by Folin-Ciocalteu reagent according to the method of [24,25].

Determination of total flavonoid content: Flavonoid content of each extract was determined by the following colorimetric method [26].

Determination of antioxidant activity: DPPH method: The 2, 2 diphenyl-1-picrylhydrazyl (DPPH) test was carried out as described by Burits et al. [27].

Cytological study

Animals: Fifty Swiss Albino mice of weight between 29 gm to 33 gm were obtained from Institute of Graduate Studies and Research (IGSR), University of Alexandria. These animals were divided into five groups of ten animals each. Group I: This group received normal saline (0.9% NaCl) served as negative control. Group II: Animal in this group received subcutaneously olive oil (0.5 ml/kg body weight/day). Group III: Each animal received subcutaneously CCl_4 (1 ml/kg body weight/day) diluted with olive oil (1:1) as a solvent for the CCl_4. Group IV: Animals were pre-treated with *Spirulina* (800 mg/kg body weight/0.5 ml drinking water) orally 30 min after the single injection of CCl_4 (1 ml/kg body weight/day). Group V: *Spirulina platensis* group, which received *Spirulina* (800 mg/kg body weight/0.5 ml drinking water). The duration of the experiment was every other day for three successive weeks.

Chromosomal aberration assay: Animals were sacrificed 24 h. after the last treatment and chromosome smears of bone marrow cells were prepared according to Yosida et al. [28].

Statistical analysis: Data were subjected to one-way analysis of variance applying SAS program [29], by using general linear model GLM. Significant differences among treatment means were separated through using Duncan's multiple range procedure [30]. The values are expressed as means ± SE for 5 mice in each group. P-values <0.05 were considered significant according to Snedecor et al. [31].

Results and Discussion

Cultivation of *spirulina*

Spirulina is the most important commercial microalga for the production of biomass. It is mainly oriented towards the health food market, utilizing a chemically defined medium. Many authors achieved the fact that the convenient Zarrouk medium is not feasible for the commercial production due to its high production cost. Hence, these investigators tried to cultivate the *Spirulina* on cheap resources such as swine dung [32] Spent wash [33]. Dcow dung [34] etc., and also various supplementation have been made to achieve enhanced biomass yield and bio-products [34]. In this study, different cultivation media of *Spirulina* are formulated by using sea water, to reduce the production cost commercially, to compare the biomass yield, and evaluate of its protective effect against chromosomal aberrations of bone marrow cells. Growth of the organism was determined by measuring optical density and cell mass or Biomass. Optical densities against time for biomass concentration per day were represented in Figure 1, and the results obtained for growth biomass were shown in Figure 2.

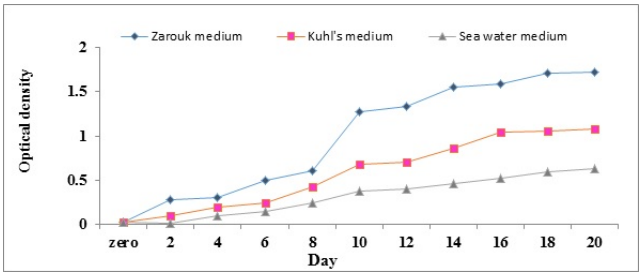

Figure 1: Growth measurements of *Spirulina platensis* by optical density (OD) cultured on different media for 20 days.

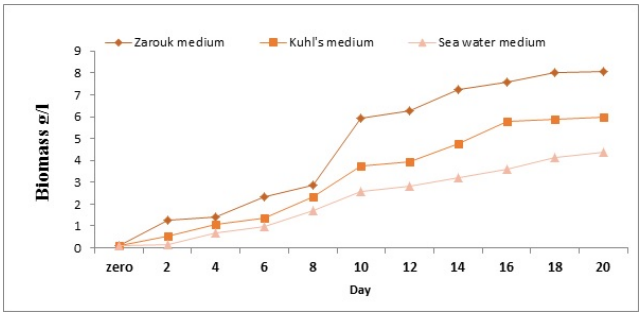

Figure 2: Growth of *Spirulina platensis* by Biomass concentrations (g/lDW) cultured on different media for 20 days.

It appeared from these data that the 10th day was the best of culturing the organism and reached the highest values of growth rate. Also, the data obtained cleared that Modified Zarrouk medium achieved the best biomass production (5.95 g/l) while cultivation of *Spirulina* in both kuhl and seawater enriched medium exhibited lower results than commercial Modified Zarrouk (3.75 g/l and 2.58 g/l respectively). These results are similar to the findings of [35,36], who found that Modified Zarrouk medium achieved the best biomass production and the highest values of growth rate. Also, the present study revealed that urea- supplemented Modified Zarrouk medium promoted *Spirulina* biomass production, mainly due to increasing protein production by the alga. These results are inconsistenent with the results obtained by Madkour et al. [37], who concluded that the biochemical composition of *Spirulina* grown on media containing urea (in another Modified Zarrouk) was changed significantly when compared with SM, giving lower production of protein and higher production of both carbohydrates and lipids; and explained that due to an adaptation mechanism to toxicity of high urea concentration

causing the accumulation of carbohydrates and lipids content in the alga at the expense of protein production. In our study, modified Zarrouk urea was in lower concentration that optimizes growth. Biomass and consequently protein production more than the other two media.

Extracts preparation and preliminary phytochemical screening

Total Phenolic (TP) and Total Flavonoid (TF) contents: In the present study, the total phenolics and flavonoids of four different algal methanolic extracts of *Spirulina* (Modified Zarrouk medium, Kuhl's medium, Sea Water medium and Commercial *Spirulina*) are present. Highly significant quantities of phenolic contents were found in crude extracts of all algal as of Gallic acid. The highest level of total phenolic content was shown in Modified Zarrouk medium (88.98 mg ± 0.12 mg Gallic equivalent/ml). Whereas, the lowest value was shown in Commercial *Spirulina* extract (48.93 mg ± 0.07 mg Gallic equivalent/ml), (Table 2).

The highest level of total flavonoid content was found in extract of Modified Zarrouk medium (78.57 mg ± 1.40 mg Rutin equivalent/ml).Whereas, the lowest value was found in extract of Commercial *Spirulina* (41.92 mg ± 0.45 mg Rutin equivalent/ml), (Table 2).

Evaluation of antioxidant activity: Total antioxidant levels (TAO) in the different media extracts were measured using Diphenyle-α-picrylhydrazyl (DPPH). Result showed an increase in (Modified Zarrouk medium, Kuhl's medium, Sea Water medium and Commercial *Spirulina*, respectively), the highest antioxidant activity was obtained from Modified Zarrouk medium and Kuhl's medium. Modified Zarrouk medium exhibited (82.04%) inhibition effect of DPPH. While, Kuhl's medium exhibited (66.33%) (Table 2). The results of the present study revealed that there are a strong correlation between antioxidant activity; and phenolic and flavonoids content. These results supported are by Ganesan et al. [38], who found that the capacity of phenolic and flavonoids are acting as antioxidants, depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities.

Interestingly, Plant Phenolic was considered in general as effective free radical scavengers and antioxidants. The reducing capacity of the extract may serve as a significant indicator of its potential antioxidant activity as reported by Meenakshi et al. [39]. However, some authors claimed that there is no correlation between the total phenolic content and the radical scavenging capacity [40]. Based on the results described above, we may conclude that phenolic flavonoid compounds increasing capacity are more informative for the determination of *Spirulina* antioxidative activity, so the cultivation of *Spirulina* in Modified Zarrouk medium represents an attractive option for the development of antioxidant supplementation, because of its high phenolic flavonoid content; thus the Modified Zarrouk medium could be considered as the best medium for further applications.

Algal extracts	Concentration of phenolic compounds (mg gallic acid equivalent/ml)	Concentration of Flavonoid compounds (mg Rutin equivalent/ml)	(Antioxidant activity %)
Modified Zarrouk medium	88.98 ± 0.12	78.57 ± 1.40	82.04 ± 0.79
Kuhl's medium	63.31 ± 0.14	62.50 ± 0.84	66.34 ± 3.42
Sea water medium	55.51 ± 1.99	50.97 ± 0.91	60.78 ± 2.78
Commercial *spirulina*	48.93 ± 0.07	41.92 ± 0.45	52.68 ± 1.56

Table 2: Total phenolic contents, total flavonoid contents and Antioxidant activity in the different algal extracts.

Cytological study

Analysis of chromosomal aberrations in mice bone marrow cells: Cytogenetic bio-monitoring, provides additional information on the DNA damage levels. Exposing to CCl_4 induces DNA damage, and *Spirulina* supplementation is able to modify the baseline levels of DNA damage. Our experimental findings indicated different types of structural chromosomal aberrations. A chromosomal fragment (F) is defined as a piece of chromatid without an evident centromere (Figure 3) and deletion (D) is defined as deleted material at the end of one chromatid (Figure 3b). Stickiness (S) (Figure 3c) is defined as adhering chromosomes and ring chromosome (R) is defined as a chromosome which is a result of telomeric deletions at both ends of the chromosome and the subsequent joining of the ends of the two chromosome arms (Figure 3), and centric fusion (C.F) as reverse of fusion, in which a two-armed chromosome is transformed into two acrocentric (Figure 3) and end to end association (E) (Figure 3), Eight shape (ES) (Figure 3) and polyploidy (P) (Figure 3) is defined as a cell in which the chromosome number is an even multiple of the haploid number or N, and is greater than 2N. Treatment with CCl_4 increase to 49.23% of the total chromosomal aberration. Interestingly, treatment with *Spirulina* ameliorated the chromosomal damages by 23.07%. These results confirmed the role of *Spirulina* antioxidant richness that could prevent or alleviate the oxidative damage of biomolecules such as DNA and lipids. So, studies including a combination of Chromosomal aberration analysis as well as comet assay give a clue about DNA damage and considered to be a good tool to detect the potential geno-toxicity of chemicals that lead to fixed mutations.

Different types of structural chromosomal aberrations were observed after the administration of CCl4 alone or in combination with *Spirulina* (Figure 3). The cytogenetically data presented in (Table 3) revealed that the percentage of chromosomal stickiness was 3% in Group (II, IV and V). On the other hand, the highest frequency of stickiness of 4.6% was recorded in animals treated with CCl_4. While, Centric fusion recorded the same percentage (1.53) in all groups. In Table 3, chromosomal fragment and chromatid deletions were scored as breaks in CCl_4 Group (21.5% and 12.3% respectively) compared to *Spirulina* treated group (6.15% and 7.69% respectively), while ring chromosomes and end to end fusion were scored as highest frequency in CCl4 group (3% and 6.15% respectively).

Groups	Percentage of aberration/50cells						Percentage of total chromosomal aberration
	S	F	D	CF	R	E	
Control	2.6	4	4.1	1.53	1.53	0	13.76
Olive Oil	3	4.6	4.6	1.53	1.53	0	15.38
CCl_4	4.6	21.5	12.3	1.53	3	6.15	49.23
Spirulina+CCl_4	3	6.15	7.69	1.53	1.53	3	23.07
Spirulina	3	4.6	1.53	1.53	1.53	0	12.3

*S: Stickiness; *F: Fragments; *D: Deletion; *CF: Centric fusion; *R: Ring chromosome; *E: Eight shape.

Table 3: Chromosomal aberration in bone marrow cells of mice.

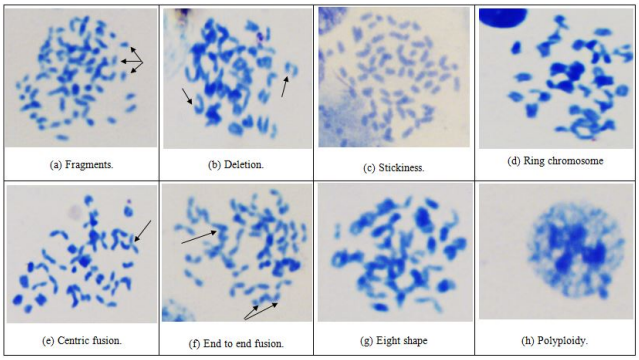

Figure 3: Chromosomal aberrations.

Many authors indicated that Double-strand breaks (DSBs) in DNA form as a result of exposure to exogenous agents such as radiation and certain chemicals, as well as through endogenous processes, including DNA replication and repair. In addition to these inadvertent occurrences, meiosis I entails the deliberate induction of DSBs, which triggers homologous recombination, thus helping to ensure normal chromosome segregation [41]. On the other hand, Most of the DSBs that can be attributed to endogenous processes are produced during DNA replication [42]. DSBs can also occur as a result of replication fork stalling due unusual DNA secondary structures, bulky lesions, polymerase blocking oxidative lesions, abasic sites, chemical or IR-generated inter-strand crosslinks, or as a result of collisions with transcription complexes and certain DNA binding proteins [43].

Table 3 shows the percentage of total chromosomal aberration in *Spirulina* treated groups as compared to CCl_4 group animals. Treatment with CCl_4 increase to 49.23% in the percentage of total chromosomal aberration and treatment with *Spirulina* improve the chromosomal damages caused by CCl_4, since the percentage of chromosomal aberration was found to be 23.07%. Moreover, results showed positive correlation between chromosome breaks and acentric fragments. These findings were supported by statistical analyses done by Snedecor et al. [31], who showed that the total number of chromatid breaks, chromosome breaks and acentric fragments were in a positive correlation with the total number of CA, as well as with the total percentage of aberrant cells. Oxidative stress induced lipid peroxidation may be one of the mechanisms causing DNA damage by CCl_4. Thus, the observed protective effect of *Spirulina* might be mediated through Reactive oxygen species ROS neutralizing capacity; whereby preventing damage to DNA and cellular components, leads to the attenuation of chromosomal aberration. It has been experimentally confirmed that ROS can have diverse effects on mammalian cell growth and, even small quantities, are capable of directing cells to undergo apoptosis or programmed cell death [44] and antioxidant supplements could prevent or alleviate the DNA damage. Finally, Our previous alkaline single-cell gel electrophoresis assay results showed that *Spirulina* supplementation can effectively alleviate DNA damage of blood cell caused by CCl4. This result was supported by Blasczyk et al. [45], who mentioned that Chromosomal aberration analysis of animals as well as comet assay are used as the most useful assays to detect the potential geno-toxicity of chemicals.

Conclusion

In conclusion, the present investigation revealed that the yield of *Spirulina* by using Modified Zarrouk's medium, which is cost wise very low expensive, is suitable for mass cultivation. Moreover, this study showed that *Spirulina* has hepatoprotective effect, powerful antioxidant action and free radicals scavenging activity. Thus, these marine algae and their bioactive compounds may be utilized for the development of natural antioxidants. Moreover, *Spirulina sp.* now is gaining more and more attention not only for the development of pharmaceutical industries but also as dietary supplement.

Conflict of Interest

We (authors) have declared that there is no conflict of interests in the study.

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