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Antioxidant Potential of *Zephyranthes citrina* Seed Extract in *Saccharomyces cerevisiae's* Oxidative Stress Response System

Sharangouda J. Patil[1*], Renuka Jyothi S[2]., Sadashiv S.O.,[3] Vishwantaha T[4]., Jamila Khatoon Adam[5], and Suresh Babu Naidu Krishna[5*]

[1]Department of Zoology, NMKRV College for Women (Autonomous), Bengaluru-560011, Karnataka, India
[2]Department of Life Sciences, Jain (Deemed to be) University, Bengaluru-560002, Karnataka, India
[3]Department of Food Technology, Shivagangothri, Davangere University, Davanagere-577 007, Karnataka, India
[4]Department of Microbiology, Maharani Science College for Women, Maharani Cluster University, Bengaluru-560001, Karnataka, India
[5]Department of Biomedical and Clinical Technology, Durban University of Technology, Durban-4000, South Africa
[*1]Corresponding author: shajapatil@gmail.com

Abstract

To investigate the in vitro and in vivo antioxidant and antidiabetic activity of *Zephyranthes citrina* seeds belong to the family Amaryllidaceae. Successive extraction was carried out using butanol and methanol as solvent system. Various phytochemicals were screened for alkaloids,, phenols, flavanoids, steroids etc. For in vitro antioxidant properties were evaluated using Diphenyl picryl hydrazyl (DPPH) radical scavenging method and reducing power assay and for in vivo antioxidant and antidiabetic testing done by using yeast cells. Methanolic extract of *Z. citrina* showed potent scavenging activity by DPPH method around 83.72% activity at a concentration of 5mg/ml. The reducing power activity of the extracts were evaluated using ferro cyanide as substrate, this shows how efficiently Fe^{3+} converts to Fe^{2+} in presence of extract. In vivo antioxidant capacity of methanol extract of *Z. citrina* was tested using to stress deficient antioxidant mutants which had high homology to humans such as superoxide dismutase (*sod1*Δ,), catalase (*cta1*Δ) in growth recovery assays. Methanol extract of *Z. citrina* seeds pretreatment exposed to hydrogen peroxide showed two-fold increase in the viability of antioxidant mutant strains *cta1*Δ (76.72%) and *sod1*Δ (78.27%). The glucose uptake by yeast (*S. cerevisiae*) was significantly more in all groups tested compared to control. The plant extract with 500µg concentration showed the maximum inhibition activity i.e., 54.54%.

Keywords: ROS, Antioxidant, *Zephyranthes citrina,,* Oxidative stress, *Saccharomyces cerevisiae*

Introduction

Oxidative stress results with an imbalance between prooxidant/antioxidant that leads to generation of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide and hydroxyl radicals etc. Ionizing radiation, redox-cycling chemicals in the environment or exposure to heavy metals are few alternate ways of ROS formation. Reactive oxygen species have been implicated in the induction and complications of diabetes mellitus, age related eye disease and neurodegenerative diseases such as Parkinson's disease (Cheeseman *et al.,* 1993). The effect of ROS in the cells is balanced by the antioxidants, which also play an important role in health maintenance and prevention of chronic and degenerative diseases (Lobo *et al.,* 2010; Pham-Huy *et al.,* 2008; Phaniendra *et al.,* 2015; Valko *et al.,* 2004; Valko *et al.,* 2007). The cellular

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components and the impact of oxidative stress on such chronic and degenerative diseases are being studied by researchers using in vitro and in vivo models.

Diabetes, is one such chronic disorder which is often referred to by doctors as Diabetes mellitus describing a group of metabolic disease in which the person has high blood glucose, either because insulin production is inadequate or because the body cells do not respond properly to high blood sugar levels, this may lead to frequent urination (polyuria), increased levels of thirst (polydipsia) and increased levels of hunger (polyphagia) (De Fronzo *et al.,* 2004; Velho *et al.,* 1997; Wang *et al.,* 2013). Other than insulin, other hormones that affect blood sugar levels are glucagon and epinephrine. Diabetes mellitus is caused because firstly the Beta cells of pancreas are destroyed due to autoimmune disorder. So no incident production is happening and no uptake of glucose from the bloodstream. Secondly Insulin is produced but the receptor mutated leading to know update of the glucose (Cheng *et al.,* 2005). The treatment for Diabetes usually involves hormone therapy and few plant-based drugs like metformin (Rao *et al.,* 1998; Xiu *et al.,* 2001). Usually insulin is taken for correcting the disorder, but insulin being expensive calls and finding for cheaper plant-based alternatives, this is a great motivator for evaluation of various medicinal plants for possible antidiabetic activity (Bhutani *et al.,* 2010; Heinrich *et al.,* 2004).

Various plants have been studied for their phytochemical properties In vitro for decades. Once was such species of plant is *Zephyranthes* which is mostly known for its ornamental and medicinal values. It belongs to the Family of Amaryllidaceae and is extensively studied for its chemical constituency. Although various phytochemical assays have been done on the species (Singh & Katoch 2015), managed to profile all the important phytochemicals observed from it. Numerous pharmacological studies have been reported from different species of *Zephyranthes.* Pharmacological activities of these species are majorly because of alkaloids present in these plants. Various activities reported by various research groups include antimicrobial, anticancer, antidiabetic, antimitotic, acetylcholinesterase as well as antineoplastic activities. Other than these properties the species exhibits cytotoxic effects. These physiochemical effects are attributed to the biological compounds present in the plant which include phenolic acids, terpenoids and flavonoids. The genus *Zephyranthes* has great potential for exploring the chemical compounds for identification and isolation for different pharmacological activity. Various species are known but only few of them are investigated for their phytochemical constituents. The information compiled in this review will help the researchers to use this genus for societal benefits.

In this study we have chosen *Zephyranthes citrina* seeds for evaluation of its various biological properties.

Materials and Methods

Collection of seeds

Zephyranthes citrina seeds were collected from a household garden near Gardens of Bengaluru, Karnataka, India. It was collected and stored prior to the commencement of the work as the flower blooms after the rain and are easily accessible at that stretch of the months of July to August. The seeds were dried and coarsely powdered.

Preparation of plant extract

The coarsely powdered seeds were subjected to successive Soxhlet extraction using solvent butanol and methanol.

Qualitative phytochemical screening

The phytochemical screening of the extracts of the plant was done out to know what are the class of organic compounds present in the different extracts of the seeds selected for the study, which will further facilitate for the

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identification of active constituents and their isolation (Koleva *et al.,* 2002).

In vitro antioxidant evaluation

Antioxidant properties were evaluated using Diphenyl picryl hydrazyl radical scavenging method and reducing power assay (Blois *et al.,* 1958; (Aruoma et al., 1997).

In vivo antioxidant testing

Chemicals, yeast strains, and growth conditions

Astaxanthin was purchased from Sigma (St. Louis, MO, USA). Culture media components and other chemicals were purchased from Himedia Laboratories (Mumbai, India). The Yeast, *S. cerevisiae* deletion strain (*sod1*Δ, *sod2*Δ, *tsa1*Δ, *cta1*Δ, *ctt1*Δ, *pep4*Δ and *fis1*Δ) collections constructed in the wild type, BY4741 (MATa*his3*Δ: *leu2*Δ*:met15*Δ*:ura3*Δ) was obtained from Thermofischer Scientific USA, Yeast strains were grown in YPD medium containing 2% (w/v) bacteriological peptone, 1% yeast extract, 2% Dextrose with or without supplemented with 200 µg/ml of Geneticin (G418 sulphate) for selection, for solid YPD agar media, 2% Bacto agar was used in addition to YPD liquid media) (Aruoma *et al.,* 1997)..

Optimization of Z. citrina seeds concentration

The exponentially grown wild type was treated or untreated with different concentrations (10-50µM) of astaxanthin. After incubating the cells overnight, the cells were 10 times serially diluted and spotted on to YPD plate or spread on to YPD plate for colony forming unit (CFU). After incubating the plates for 2 days, spot plates were imaged and CFU was counted and expressed as percent cell survival compared to control.

Antioxidant properties of Z. citrina seeds in S. cerevisiae

Exponentially grown yeast *S. cerevisiae* wild type (BY4741) and antioxidant mutants, superoxide dismutase (*sod1*Δ*)* catalase (*cta1*Δ*)* were treated or untreated with PE (500µg/ml) for two hours. Cells were then 10 times serially diluted for 5 times and 5µl were spotted onto YPD or YPD plate containing H_2O_2 (2mM). Culture plates were incubated at 30°C for 2 days and images were taken using gel doc (Syngene, Australia). For CFU assay, exponentially growing cells were pre-treated with or without of *Z. citrina* (500µg/ml)) for two hours followed by exposure to H_2O_2 (1mM) for one more hour in a shaker incubator at 30°C. Cell viability was determined by plating the cells with an appropriate dilution in triplicate on solidified YPD plate. After incubating the plates for 2 days, CFU was counted and expressed as percent cell survival compared to control (de Sá *et al.,* 2013; Dehshahri *et al.,* 2012; Pereira *et al.,* 2001).

Measurement of ROS level

The ROS scavenging ability of astaxanthin was determined by measuring the accumulation of ROS in the cells using fluorescence dye called 2,7-dichlorofluorescein-diacetate (H_2DCF-DA) (Ramamoorthy *et al.,* 2007).

Test for antidiabetics

Glucose uptake by yeast cells

This was achieved by a series of centrifugations and incubations, the percentage increase in glucose uptake by yeast cells was calculated using the following formula:

$$\%\ increase\ in\ Glu\ uptake = \frac{Abs\ control - Abs\ sample\ \text{x}\ 100}{Abs\ sample}$$

Result and Discussion

Extraction yield of the plant extract

Butanol and methanol are used for the extraction of the plant metabolites. Before starting with the assay, the yield of the plant extracted is calculated. The yield of the solvent extract is given in Table No. 1 & 2 & Graph 1.

Table 1. Yield, color and consistency of the extract of *Zephyranthes citrina* seeds

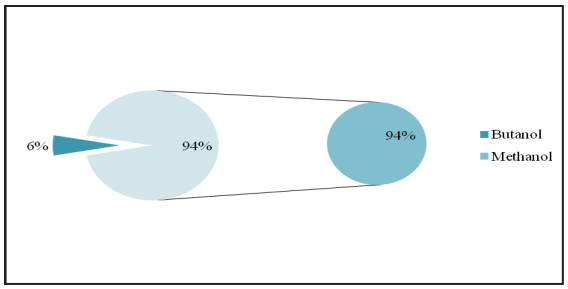
***Zephyranthes citrina* extracts**	**Yield (grams)**	**Color and consistency**
Butanol	00.65	Dark brown (gummy)
Methanol	10.37	Dark brown (gummy)

The phytochemical analysis of the seed extract (butanol) showed the presence of glycosides, carbohydrates and flavonoids. Whereas the seed extract (methanol) showed presence of alkaloids, flavonoids, steroids, glycosides, carbohydrates and phenols.. Phytochemical analysis helps to understand the components present in the plant extract. Thus, it is important to assay the extract for various metabolites and components (Bhat *et al.,* 2011). In this experiment we found that the steroid and flavanoid content in the plant extract was positive.

Table 2. Qualitative phytochemical analysis of *Zephyranthes citrina* seed extracts

Qualitative Tests		*Zephyranthes citrina* seed extracts	
		Butanol	Methanol
Alkaloids	Mayer's test Wagner's test	- -	+ +
Steroids	Libermann sterol test Solkowski's test	-	+ +
Glyco sides	Sulphuric acid test Molisch's test	- +	+ +
Carbo hydrates	Molisch's test Anthrone test	+ –	+ –
Saponins	Aqueous test	–	–
Flavones	Aqueous NaOH test Conc. H_2SO_4 test	+ -	+ +
Phenols	Lead acetate test	-	+
Tannins	Lead acetate test	-	+

Graph 1. Yield of butanol and methanol *Z. citrina* seed extracts (ZCSE)



This study showed that the presence of these phytochemicals in these plant extracts might be the reason for antioxidant and antidiabetic properties. The presence of these phytochemicals indicates that these may be responsible for enhancing the glucose uptake by the yeast cells, the reduction capability of DPPH radicals.

Determination of DPPH radicals Scavenging activity

DPPH is stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Blois *et al.,* 1958). The reduction capability of DPPH radical is determined by the decrease in its absorbance at 517nm, which is induced by antioxidants. The decrease in the absorbance is caused by the reaction between antioxidants molecules and radicals, which results in the scavenging of the radical by hydrogen donation (Kato *et al.,* 1998). It is visibly noticeable as a change in color from purple to yellow. From the results we infer that the methanol extract of *Zephyranthes citrina* showed more potent scavenging activity by DPPH method than the butanol extract.

This is an indication of antioxidant property. This result clearly suggested that the plant extract contain antioxidant properties which was further assayed in-vitro. In the in-vitro analysis, the most common method DPPH assay was performed which gives accurate results (Lai *et al.,* 2001). Earlier studies of the other species of the plant has shown antifungal,

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antimicrobial and anti-cancer properties (Funke *et al.,* 2006; Gorray *et al.,* 1986; Shanmugasundaram *et al.,* 1990). Further studies on the extract, like hydrogen peroxide assay and radical scavenging assay showed that the antioxidant property was present. There are various methods available to assess antioxidant activity of compounds. DPPH free radical scavenging assay is an easy, rapid and sensitive method for the antioxidant screening of plant extracts (Wang *et al.,* 2011; Jiju *et al.,* 2013). In presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases (Zhinshen *et al.,* 1999; Malick *et al.,* 1980). In this study, the scavenging activity of methanol extract was found to be dependent on the dose. Though the DPPH radical scavenging abilities of the extract was less than that of ascorbic acid (Aruoma *et al.,* 1998), the study showed that the extract has the proton-donating ability and could serve as free radical inhibitors or scavenger, acting possibly as primary antioxidant.

Table 3. Percentage of DPPH scavenging activity by *Zephyranthes citrina* seed extracts

Sample	O.D. at 517 nm	% of DPPH scavenging activity
1 mg/ml butanol extract	0.429	27.28
3 mg/ml butanol extract	0.364	38.30
5 mg/ml butanol extract	0.295	50.00
1 mg/ml methanol extract	0.389	35.59
3 mg/ml methanol extract	0.171	71.18
5 mg/ml methanol extract	0.096	83.72

***Reducing power activity* (Table 4)**

The result shows the reductive capability of the extract to potassium Ferro cyanide at different time interval. Here we investigate the Fe^{3+} to Fe^{2+} transformation in the presence of plant extract. The reducing capability of the extract can be monitored by the formation of blue color at 700nm. The maximum reducing power activity was found in methanol extract of plant 0.057 at 20 minutes and for the butanol plant extract maximum reducing power activity was found 0.037 at 30 minutes.

Table 4: Reducing power activity in terms of absorbance at 700nm

ZES (1mg/ml)	O.D at 700nm (10mins)	O.D at 700nm (20mins)	O.D at 700nm (30mins)
Butanol extract	0.028	0.033	0.037
Methanol extract	0.042	0.057	0.049

In vivo testing

Methanol extract of Z. citrina seeds protects antioxidant mutants under oxidative stress (Graph 2 & Figure 1)

The yeast seems to be a potentially useful eukaryotic model for studies of the effects of antioxidants at the cellular level, an alternative to mammalian cell lines and also yeast has provided significant information on oxidative stress and the mechanisms employed by cells in response to increased ROS. To analyze the antioxidant effect of methanol extract of *Z. citrina* seeds in *S. cerevisiae* cells against the hydrogen peroxide induced oxidation, first we carried out if methanol extract of *Z. citrina* seeds treatment would kill *S. cerevisiae* cells.

Graph 2: Antioxidant activity by CFU assay

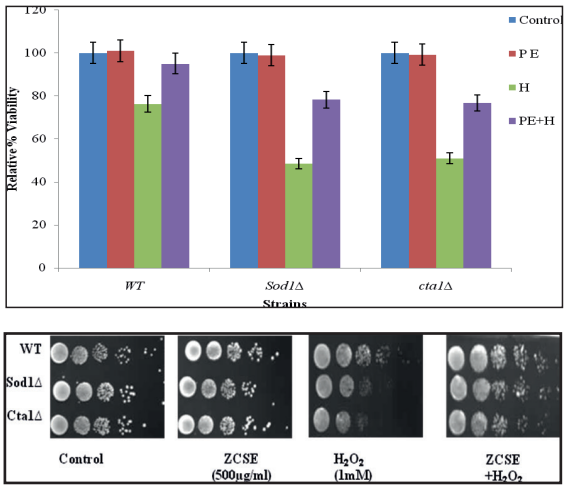

Figure 1. Spot assay of methanol extract of *Z. citrina* seeds protects antioxidant mutants under oxidative stress (ZCSE)

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The results showed that treatment with astaxanthin in the range of 10-50µM was not toxic to wild type strain and the cells continued to reach more than 100% growth compared to its untreated control. Since the cell growth was not affected by the astaxanthin, we decided to study its antioxidant, anti-apoptotic and antiaging capacity using the minimum concentration of astaxanthin (30µM) at which maximum percent growth (110%) was observed compared to untreated control. Thus, we directly exposed the wild type cells to methanol extract of *Z. citrina* seeds in different concentrations. Tolerance against methanol extract of *Z. citrina* seeds was measured after overnight exposition by CFU studies. The results showed that treatment with methanol extract of *Z. citrina* seeds in the range of 50-700µg/ml was not toxic for the wild type strain and cells continued to reach 100% tolerance in CFU. Since cells were not affected by the methanol extract of *Z. citrina* seeds we decided to study its antioxidant capacity using the minimum concentration of methanol extract of *Z. citrina* seeds (500 µg/ml) at which maximum percent growth (107%) was observed compared to untreated control.

The enzymatic machinery consisting of superoxide dismutases, reductases, catalases, peroxiredoxins, glutaredoxins, and glutathione transferases is utilized to maintain redox balance In addition to the enzymes, antioxidant small molecules are produced by the organism or taken up from the environment, which can act to delay or prevent oxidation of intracellular substrates such as lipids, DNA, or proteins. Such compounds have been at the center of an intense focus of research for their association with health-promoting properties (Bowers *et al.,* 1980; Wilson *et al.,* 1966; Sasaki *et al.,* 1966).

To evaluate the antioxidant capacity of methanol extract of *Z. citrina* seeds to protect yeast cells compromised antioxidant mechanism, we used 2 stress deficient antioxidant mutants which have high homology to humans such as superoxide dismutase (*sod1*∆,), catalase (*cta1*∆*)* in growth recovery assays (Sanchez- Moreno *et al.,* 2002; Daivies *et al.,* 2000). Hydrogen peroxide inside the cell after metabolic activation produces free radicals which can oxidize the organelles nearby and leads to oxidative stress. In the present study the antioxidant mutants *sod1*∆*, cta1*∆*, and* wild type were treated and untreated with methanol extract of *Z. citrina* seeds and subjected to hydrogen peroxide stress. The recovery of the surviving cells with astaxanthin was monitored for a period of 48-72 hrs. All the growth recovery assays were performed independently in triplicate. In the results there was a statistical difference, the antioxidant mutants *sod1*∆*, cta1*∆*,* were sensitive to 2mM Hydrogen peroxide. Whereas methanol extract of *Z. citrina* seeds treated cells showed the better survival growth in hydrogen peroxide induced stress plate indicated the rescued mechanism of methanol extract of *Z. citrina* seeds to antioxidant mutants from oxidative stress in spot assay studies. The hydrogen peroxide toxicity is associated with the production of the highly reactive hydroxyl radicals which are catalyzed by transition metals, such as iron and copper by fenton reaction in cells (Dasgupta *et al.,* 2004; Jain *et al.,* 2006). Methanol extract of *Z. citrina* seeds also protects antioxidant mutant *sod1*∆*, cta1*∆*,* strains from cell death induced by H_2O_2(1mM) in CFU study similar to the phenotype (spot assay) results. The cytosolic copper-zinc superoxide dismutase (sod1) and catalase A (cta1) gene which helps to breaks down hydrogen peroxide during fatty acid metabolism and scavenges O^{2-} radicals and the lack of these gene in respective strain showed more sensitivity such as 48.41% and 50.91% to hydrogen peroxide induced oxidative stress, but methanol extract of *Z. citrina* seeds pre-treatment exposed to hydrogen peroxide showed two fold increase in the viability of antioxidant mutant strains *cta1*∆(76.72%), and *sod1*∆(78.27%) indicating the lack of these gene function in respective strain was carried out by methanol extract of *Z. citrina* seeds in

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protecting the oxidative stress (Krishnaiah *et al.,* 2007). Our overall result indicated the methanol extract of *Z. citrina* seeds protects oxidative stress caused by hydrogen peroxide.

The extract was then used on yeast cell lines for *in vivo* test. The enzymatic machinery consisting of superoxide dismutases, reductases, catalases, peroxiredoxins, glutaredoxins, and glutathione transferases is utilized to maintain redox balance (Sadasivam *et al.,* 1987; Gacche *et al.,* 2004). In addition to the enzymes, antioxidant small molecules are produced by the organism or taken up from the environment, which can act to delay or prevent oxidation of intracellular substrates such as lipids, DNA, or proteins (Lowry *et al.,* 1951; David *et al.,* 2008; Hatano *et al.,* 1989; Chang *et al.,* 2002). Such compounds have been at the center of an intense focus of research for their association with health promoting properties. The *in vivo* tests helps to take the research further as the extract did not show high cytotoxicity, it can be used to test on animal cell lines for clinical research (Karthic *et al.,* 2008). The overall result indicated the methanol extract of *Z. citrina* seeds protects oxidative stress caused by hydrogen peroxide (Kobayashi *et al.,* 2003).

Methanol extract of Z. citrina seeds scavenges ROS by DCFDA

Biomarkers of oxidative stress are extremely useful in evaluating antioxidant and cytotoxic activity. Among them intracellular oxidation is one of the best characterized and explored biomarker to detect oxidative stress in cells. In this work we used the fluorescent probe 2,7dichlorofluorescein diacetate (H_2DCF-DA) to determine the levels of intracellular oxidation to wild type and antioxidant mutants during H_2O_2 stress (Fradovich *et al.,* 1986; Ohkawa *et al.,* 1979). H_2DCF-DA is a fluorogenic probe that can permeate inside the cell by passive diffusion and deacetylated by cytosolic esterases yielding H_2DCF which is more polar than the parent compound and becomes susceptible to the attack by ROS, yielding a high fluorescent product (Behera *et al.,* 2006; Harries *et al.,* 1935). The fluorescence of H_2DCF cells is proportional to the amount of ROS produced in the cells. In this experiment Oxidative mediated cell death in wild type and mutants are due to the generation of ROS by H_2O_2. To measure the level of oxidative stress cells were treated with the redox sensitive fluorochrome (H_2DCF-DA) as described in method. The levels of intracellular oxidation were measured in flurometer and in fluorescent microscope (Lee. *et al.,* 1992; Rose *et al.,* 1982). Direct exposure of H_2O_2 to cells produced an increase of H_2DCF fluorescence in the mutants compare to wild type strain indicating mutants are more susceptible to hydrogen peroxide oxidative stress but in PE treated a reduction of H_2DCF oxidation was observed in antioxidant mutants such as superoxide dismutase (*sod1Δ)* and catalase (*cta1Δ)*, indicating PE reduces oxidative stress caused by hydrogen peroxide and can act as ROS scavenger.

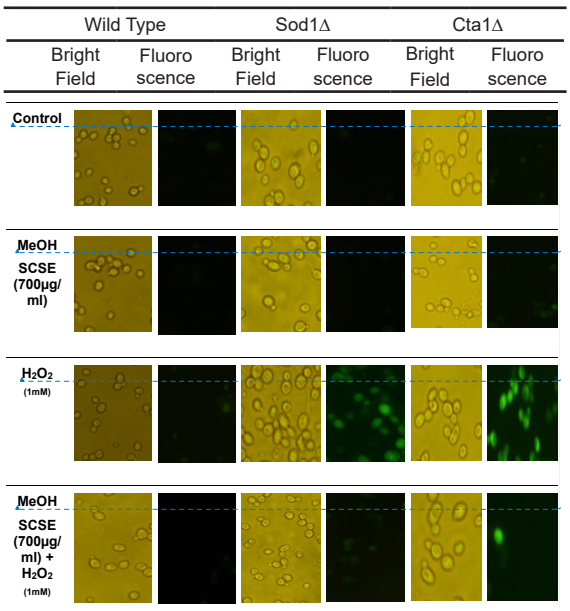

Figure 2: Methanol extract of *Z. citrina* seeds scavenges ROS by DCFDA (ZCSE)

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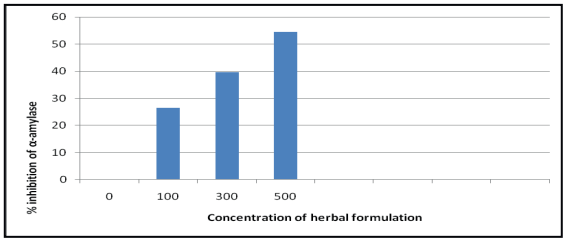
Graph 3: Relative % viability of cells vs concentration of astaxanthin



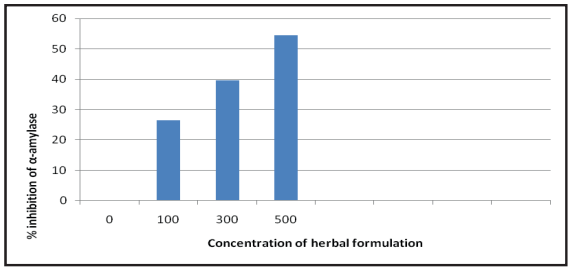
Uptake of glucose by yeast cells

In this assay, the glucose uptake rate increased with the increasing concentration of the extracts. The methanol extract with 500µg concentration showed the maximum inhibition activity i.e., 54.54%. It is specified that transport of glucose across yeast cell membrane occurs by facilitated diffusion down the concentration gradient. Hence the glucose transport occurs only if the intracellular glucose is effectively reduced or utilized (Kandra *et al.,* 2003; Dewi *et al.,* 2007; De Sales *et al.,* 2012; Kumar *et al.,* 2008).

Table 5. Glucose uptake by yeast cells of seed extract of *Zepharanthes citrina* seeds

Herbal formulation	Concentration of herbal formulation (µg/ml)	Absorbance at 540nm	Inhibition of α- amylase (%)
Control	0	1.21	0
1	100	0.89	26.44
2	300	0.73	39.66
3	500	0.55	54.54

Graph 4: Glucose uptake by yeast cells of Z. citrina seeds of methanol extract (ZCSE)



In this present study we have also evaluated *in vitro* alpha amylase and alpha glucosidase activity of crude methanol and butanol extract of *Z. citrina* seeds along with its Total antioxidant activity. The plant showed significant inhibition activity, so further the compound isolation, purification and characterization which is responsible for inhibiting activity, has to be done for the usage of antidiabetic agent.

Conclusion

Ethnomedicinal knowledge of Indian traditional medicinal plants may be beneficial to know the potentiality of different medicinal plants to yield antioxidant properties. It might have been brought by the acknowledgment of the significance of the medicinal plants as potential sources of new compounds of therapeutic interest and as sources of lead compounds in the drug development. In the present findings, we found that methanolic extract of *Z. citrina* seeds due to the presence of various secondary metabolite as per the phytochemical analysis and also it contains high amounts of phenolic and flavonoid compounds, which may be responsible for exhibiting high antioxidant activities. Thus based on a wide spectrum of activities exhibited by *Z. citrina*, the plant can be considered as an effective antioxidant resource for preventing oxidative stress mediated disorders and thus may serve as a good source for isolating new compounds for treatment against antitumor, antidiabetic or other neurodegenerative disorders.

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