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Original Article

Antioxidant and Cytoprotective Action of Aqueous Extract of *Citrus Sinensis* Fruit Peel against Endosulfan Induced Damage on *Saccharomyces Cerevisiae*

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ABSTRACT

In this study aqueous extract of *Citrus Sinensis* fruit peel was investigated with different antioxidant assays and cytoprotective action on xenobiotic induced oxidative stress in *Saccharomyces Cerevisiae* (yeast cells) was examined. The results showed that the aqueous extract showed protective effect in scavenging free radicals that were generated with different *in vitro* models with IC_{50} of 0.2 mg/ml on DPPH, 0.36 mg/ml on lipid peroxidation, 0.41 mg/ml in superoxide anion and also good activity in reducing power. Further the extract showed cytoprotective effect on xenobiotic induced (endosulfan) oxidative stress in yeast cells. The parameters that were studied in yeast cells were cell viability, reactive oxygen species (ROS), lipid peroxidation (TBARs), and lactate dehydrogenase leakage (LDH). Extract along with xenobiotic showed significant cytoprotective effect compared to that of xenobiotic induced cells alone this study shows that Citrus Sinensis fruit / peel can be consumed in our daily diet for boosting the immune system, which can minimize the free radical damage caused due to various oxidative stress.

Key word: *Citrus Sinensis* fruit peel extract, endosulfan, antioxidant, cytoprotective action.

1. INTRODUCTION

Several degenerative diseases such as arthritis, stroke, coronary heart diseases, diabetes and cancers are caused by endogenous and exogenous sources of free radicals.[1] Fruits and vegetables are known to lower the risk of degenerative diseases, since they are rich in

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phytochemicals which play a vital role in scavenging the free radical that are generated.[2]

Antioxidant are the molecules which protect the cells from the free radicals like superoxide, OH, NO by chelating and converting into detoxifying compounds. There is tremendous scope for the plant derived compounds since they have a broad-spectrum of activities in reducing the free radical implicated diseases.[3] It is been reported that plant extracts contain phenolic, flavanoids, tannins, saponins lignin and glycosides which reduce the level of oxidative stress and protect cell from undergoing damage and prevents the cell death.[4] Therefore there is a great deal of interest in edible plants that contain antioxidants and health – promoting phytochemicals, in view of their health implications.

Endosulfan is an organochlorine insecticide belonging to the cyclodiene group that is extensively applied in agriculture to protect crops. This chemical is used as toxicant in our experiments to induce oxidative stress in cell model study.

Citrus sinensis is commonly grown throughout the globe. It belongs to the family rutaceae, its fruit, commonly called orange is rich in vitamin C and other phytochemicals viz., polyphenols, flavones, anthocyanins hydroxycinnamicacid, tannins, saponins which are influenced in controlling several metabolic disorder associated problems like anticholesterol, anti-inflammatory, anticancer etc.[5] Swapna *et al* has reported the radical scavenging activity of citrus fruit peel extract on different solvent systems and has said that that the peel has a rich source of flavanones and polymethoxylated flavones.[6]

Cytoprotection using cell model against xenobiotics induced oxidative stress by phytochemicals has been reported.[7] *In vitro* models are very useful to understand the cytotoxicity of xenobiotic induced by free radicals and to examine the cytoprotective role of phtochemicals.[8] Cell injury is believed to be toxicological manifestation and pathobiology. Phytochemicals which reduces the level of oxidative stress is known to exhibit health promoting potential.[9] In this study, we have demonstrated the crude extracts of aqueous *citrus sinensis* fruit peel extract *in* various *in vitro* antioxidant assays systems, and amelioration of oxidative stress induced by endosulfan and cytoprotective action of the extract on yeast cells.

2. MATERIALS AND METHODS

2.1 Extraction

Citrus sinensis fruit peel separated from the fruit was washed thoroughly with tap water for two to three times and allowed to dry at room temperature. Further the dried peel was broken into small pieces and grounded into fine powder using grinder/mixer. Powdered material was extracted with Luke warm distill water in temperature controlled orbital shaker for 12-14 hr. The extractant was filtered using muslin cloth and further filtered in whatman No 1 paper, the extract obtained was concentrated using lyophilizer and stored at 4^0C till use.

2.2 Inhibition of Lipid Peroxidation

Microsomes were isolated from male wistar rats in 0.02mol/l tris buffer (pH 7.4) according to Kamat and Rubin.[10] Reaction was set to 100 µl of microsomes containing 1mmol/l Feso4 and ascorbic acid with or without extract in a total volume of 1 ml in 0.1ml/l phosphate buffer (pH 7.4) and incubated at 37^0C for 1hr. Reaction was stopped by adding 2 ml of 20% TCA and 1% TBA, boiled in water bath for 10 min, cooled and centrifuged. Absorbance was measured at 535 nm

2.3 DPPH radical scavenging assay

According to Guohua et al [11] DPPH assay was performed with slight modifications. 1 ml of DPPH solution (0.1 mmol/l, in 95% ethanol (v/v) was mixed with different concentration of extract and the reaction mixture incubated for 30 min at room temperature. The

optical density was measured spectrophotometrically at 517 nm against a blank. Depleting in the absorbance of DPPH indicates a higher radical scavenging activity.

2.4 Superoxide radical scavenging assay

Superoxide generated by the oxidation of Nitro Blue Tetrazolium chloride (NBT) coupled with NADH and Phenazine methosulphate (PMS) results in blue colour.[12] The reaction mixture contained NBT (0.lmM), NADH (l mM) with or without extracts in an total volume of 1 ml Tris buffer (0.02 M, pH 8.3). The reaction was measured spectrophotometrically at 560 nm each 30 sec for 1 min by including PMS (0.1mM) to the mixture.

2.5 Reducing power

The reducing power of the extracts was measured as described by the strategy depicted by Oyaizu.[13] To 1 ml of reaction mixture containing aqueous extracts *Citrus sinensis peel* in phosphate buffer (0.2 mol/l, pH 6.6) was incubated with 3 ml of 1% potassium ferricyanide at 50 °c for 20 min. After incubation, the reaction was ceased by adding 1 ml of 10% TCA solution and the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was mixed with distilled water (2.5 ml) and ferric chloride solution (0.1 g/ 100 ml), and the absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance indicated higher activity.

2.6 Phenol content

Phenol content in the extract was determined according to the method described by yamaguchi.[14] The extract was diluted 1: 50 times with distill water, to this 1 ml of the FC reagent was added and mixed well, and kept at room temperature for three minutes, further 3 ml of 2% Na_2Co_3 was added and incubated for 2 hr at 27-30^0c. Absorbance was measured at 760 nm. Standard graph was plotted using Gallic acid and total phenol was estimated from the standard graph.

2.7 Cytoprotection

Cell viability

Xenobiotics, endosulfan was chosen as toxicant, to check the cytoprotection of the extract. The concentration of endosulfan was used at 50 percent lethality (LC^{50}). Cytoprotection investigations were performed by incubating 1.0 ml of yeast cells (10 X 10^6) suspended in YEPD with xenobiotics (dissolved in DMSO) at LC^{50} concentration 0.01 mM with/without the extract for 1 hr in a shaking water shower at 37^0C. After the incubation period, an aliquot of cells was taken for viability test by the trypan blue exclusion method.[8]

Lactate dehydrogenase leakage

The supernatant obtained after incubation followed by centrifugation from the reaction mixture of yeast cells in the presence of xenobiotics with/without extract was assayed for LDH with sodium lactate as the substrate. [15]

Lipid peroxidation of yeast cell

The cells were centrifuged after incubation, and the cell pellet was washed in saline and the pellet was boiled in TCA (5.5%) and TBA (0.34%) for 15 min, cooled and centrifuged. The supernatant was measured in a spectrophotometer at wavelength of 535 nm.[16]

Reactive oxygen species (superoxide anion)

The cells (10 X 10^6) suspended in 1.0 ml YEPD were incubated with NBT (0.2 mM) with or without xenobiotics (in DMSO) and extracts in a shaking water bath at 37^0C. The generation of ROS by cells (respiratory burst) was measured by the formation of colored formazan due to reduction of NBT.[17]

2.8 Statistical analysis: Data are expressed as mean ± S.E. of three separate experiments

3. RESULTS AND DISCUSSION

3.1 DPPH

DPPH is a stable free radical which is measured at absorbance at 517nm. Basically it is used to screen the

samples for antioxidant activity. The principle involved in the reaction of DPPH is the capability of donating electron to the oxidized molecule. In reduced state it is purple in color and in oxidized state it is pale yellow. The decrease in absorbance or depletion of purple color is an index of strong free radical scavenging activity.[14] The results are shown in Table 1. Aqueous extract of *Citrus sinensis* fruit peel showed DPPH activity with an IC_{50} of 0.2 mg/ml. However, the activity was low compared to standard BHA. The potency of extract in scavenging the free radical may be attributable to donating hydrogen molecule.

3.2 Reactive oxygen species

The inhibition of reactive oxygen species (ROS) by aqueous extract with an IC_{50} of 0.41mg/ml is represented in Table1. Extract showed the highest activity compared to standard antioxidant molecule, BHA, which was not able to prevent the inhibition of ROS. The ROS which is produced under electron transport system of normal physiological process is harmful to the living system, though it is a minute oxidant but it leads to oxidation chain reaction producing more threatening free radicals such as hydroxyl radical and singlet oxygen which are unsafe, promotes to oxidative damage. [18] Since extract is showing good inhibition of ROS activity, consumption of citrus fruit in daily diet will definitely influence in scavenging the ROS that are generated also protects body from the endogenous radical sources.

3.3 Lipid peroxidation

Inhibition of lipid peroxidation by aqueous extract of *Citrus sinensis* fruit peel with an IC_{50} of 0.36 mg/ml is shown in Table.1. The theory behind the lipid peroxidation is cell is composed of cell membrane which is made up of lipid moiety (fatty acids) with polar and nonpolar group, this molecules is held by the hydrogen molecule and form chain like structure or mesh like appearance. If there is any damage to the cell, the outer layer *i.e.* cell membrane gets damaged by losing the hydrogen molecule and the protective cover breaks, which is an indication of cell damage. Normally this mediation of reaction is by the free radicals, which damages cells and leads to various degenerative diseases viz., arthritis, Parkinson and atherosclerosis.[19-20] In our result it was observed the inhibition was concentration dependant of the extract, from this result it is evident that the extract contains certain photochemical which prevent the cell damage by donating the hydrogen molecule and blocking the chain initiation of fatty acids which is present in the cell membrane.

Table 1: Antioxidant activity of aqueous extract of *Citrus sinensis* fruit peel

Aqueous extract	**Free radical scavenging activity IC_{50} mg/ml**				Phenol mg/g
	DPPH	LPO	ROS	Reducing activity	
	0.2	0.36	0.41	0.38	1.18 ± 0.02

3.4 Reducing Power

Reducing activity of aqueous extract of *Citrus sinensis* fruit peel is shown in Table 1. The activity was dependent with increase in concentration of extract. Reducing property of the extract is index of inhibitor potential by its ability to give the hydrogen molecule.[21]Mechanism of total inhibitor activity is concerned in varied mechanisms viz. binding of transition metal particle, prevention of chain initiation, inhibition of H abstraction, one such property in reducing activity is associated with the presence of reductones, that play a significant role in exerting the inhibitor activity in preventing the formation of peroxide by donating the atom and preventing the harm caused by atom. The result obtained suggests that crude extracts has potential biomolecules that neutralizes the free radicals by donating the atom.

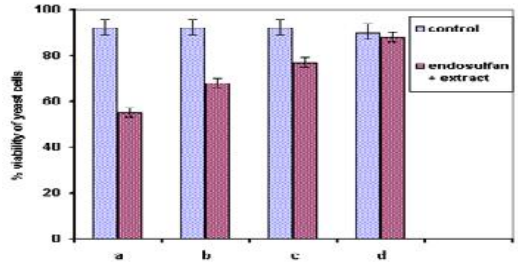

Fig 1: Cell viability of yeast cell cotreated with different concentration of extracts (a-100µg, b-200µg, c-300µg, d-400µg) and endosulfan (0.01 mM)

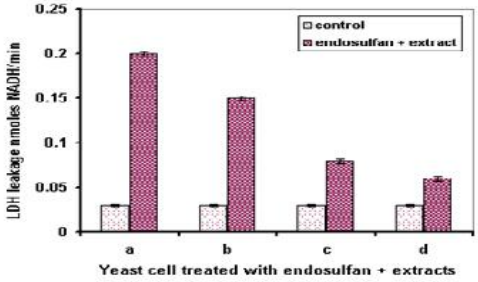

Fig 2: LDH leakage of yeast cell cotreated with different concentration of extracts (a-100µg, b-200µg, c-300µg, d-400µg) and endosulfan (0.01 mM)

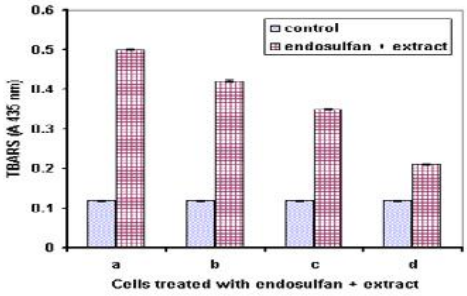

Fig 3: Lipid peroxidation of yeast cell cotreated with different concentration of extracts (a100µg, b-200µg, c-300µg, d-400µg) and endosulfan (0.01 mM)

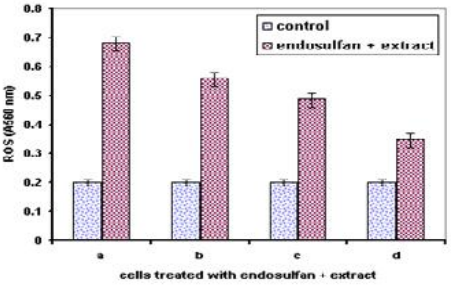

Fig 4: ROS of yeast cell cotreated with different concentration of extracts (a-100µg, b-200µg, c-300µg, d-400µg) and endosulfan (0.01 mM)

3.5 Cytoprotection of Citrus sinensis fruit peel on yeast cells against endosulfan induced damage

In vitro cell culture play a model framework in comprehension the role of the phytochemical in ameliorating the level of oxidative stress induced by xenobiotic in cells, which is measured by cell viability. A few studies on photochemical have indicated cytoprotective impact in both *in vitro* and *in vivo* models [8]. In this test we have demonstrated the *Citrus sinensis* fruit peel extract in anticipating xenobiotic induced cell death in yeast cells. endosulfan, organochlorine pesticide inducer of oxidative stress in cells is utilized as toxicant. Our outcomes indicated restraint of xenobiotic induced lipid peroxidation, inhibition of ROS by preventing cell death and reduction in level of LDL leakage with increase in concentration of extract. Cytoprotection observed as cell viability, was observed for cells co treatment with 100–500µg/ml of extract and 0.01mM endosulfan Fig.1. LDH leakage in the cells was altogether decreased when cells co treated with increasing concentration of extract, compared with endosulfan-treated Fig.2. Lipid peroxidation was depleted in the cells treated with high convergence of extracts, in which the development of Malondialdehyde was measured as marker record of lipid bilayer damage Fig 3. Reactive oxygen species (ROS), level increases when cells exposed to stress condition. The level of ROS was reduced when cells co treated with the extracts Fig 4. These outcomes demonstrate that the unrefined extract might contain cocktail of photochemicals, which improve the level of oxidative stress instigated by the endosulfan by protecting the cell from undergoing death. Further extract needs to investigate the photochemical in charge of keeping the cell alive.

4. CONCLUSION

The results of aqueous extract of *Citrus sinensis* fruit peel, suggest that it may contain various phytochemicals, which can reduce the level of free radicals that are generated in various *in vitro* conditions. The potency of extracts acts as reservoir of nutraceuticals in scavenging free radicals in the counteractive action and improvement of degenerative

maladies. Further the extracts have show the cytoprotective action on xenobiotic induced toxicity in yeast cells, which indicates that the extracts has molecules responsible in ameliorating level of oxidative stress caused by the toxicant and preventing the cell death. Though we have not isolated and characterized the antioxidant molecules responsible for antioxidant properties and cytoprotection it could be the phenolic compounds or peptides present in the extracts. Further studies in elucidating the antioxidant and cytoprotective molecules need to be analyzed.

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