academicJournals

Vol. 15(6), pp. 145-152, 10 February, 2016
DOI: 10.5897/AJB2015.15050
Article Number: 9EFD7D157196
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Possible oxidative effects of isotretinoin and modulatory effects of vitamins A and C in *Saccharomyces cerevisiae*

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Received 15 October, 2015; Accepted 17 December, 2015

Isotretinoin (ITN), chemically known as 13-*cis*-retinoic acid, is a part of the broad group of compounds related to vitamin A. It is particularly employed in the treatment of nodular cystic acne and as an inhibitor of proliferation of neoplastic cells, by exerting a regulatory effect on the cell differentiation. This study aimed at investigating the possible oxidative effects of ITN and modulatory effects of vitamins A and C in mutant and non-mutant *Saccharomyces cerevisiae* strains. In addition, to reconfirm the oxidative effects, five *in vitro* antioxidant assays were also prepared taking the alpha-tocopherol analogue, trolox as a standard. *In vivo* study conducted on *S. cerevisiae* cells was carried out with ITN 20 µg/ml taking hydrogen peroxide (H_2O_2) as stressor (STR), whereas ITN 5 to 50 µg/ml was considered for *in vitro* assays taking similar dose of trolox (TRO). Results show ITN to have oxidative effect in both *in vitro* and *in vivo* tests. In conclusion, ITN produced oxidative effects and there may be an hypervitaminosis effect with vitamins A and C, thus insinuation to genetic material.

Key words: Assay, isotretinoin, vitamin A, vitamin C, oxidative stress.

INTRODUCTION

Isotretinoin (ITN) is a chemical compound known as 13-*cis*-retinoic acid, marketed under the name Roaccutane; a synthetic isomer of tretinoin (TN) administered systemically. It is a drug under the class of retinoids (RTD), which according to Brito et al. (2010) is related to vitamin A (Vit A) having a similar chemical structure being

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considered the only natural RTD (first generation) undergoing clinical application through systemic therapy for acne treatment.

The RTD are involved in the proliferation and differentiation of various types of cells during fetal development and throughout the life as well as in the activation of retinoid-receptor complex. On the other hand, the activation of the complex can block the action of other transcription factors as AP1 whose expression appears to be exacerbated in various hyperproliferative and inflammatory conditions (Diniz et al., 2002).

The pharmacokinetic characteristics of ITN are analogous to Vit A, which after oral administration results in high plasma concentrations of ITN at the first 2 to 4 h. As a result, an oxidation process occurs and by cytochrome P450 enzymes in the stomach wall gives rise to 4-oxo-metabolites of ITN, TN and 4-oxo-tretinoin (OTN). OTN is evident to accumulate and increase the concentration in systemic circulation by consecutive administrations (Cajueiro et al., 2014).

Vitamins are organic substances essential to normal metabolism of living beings, they act as cofactors for enzymatic reactions, and their deficiency leads to malfunctioning of the organism (avitaminosis). However, the excess of vitamins also creates some hypervitaminosis related problems. Vit A, and more specifically, the retinoic acid (RA), is shown to maintain normal skin health by switching on genes and differentiation of keratinocytes (immature skin cells) in mature epidermal cells (Fuchs, 1981).

According to Pires (2008), ascorbic acid (AA), also known as vitamin C (Vit C), or ascorbate, is a lactone ($C_6H_{12}O_6$) whose molecular weight is 176.13 daltons, an essential ingredient in the metabolism of living cells with numerous physiological properties. The conversion of Vit C as dehydroascorbic acid normally occurs inside the body reversibly. This transformation capacity acts as an oxidorredutor system and is able to transport hydrogen in the breath processes at the cellular level. Azulay et al. (2003) claim that Vit C can acts as a pro-oxidant, promoting the formation of reactive oxygen species (ROS) such as H_2O_2, which compromise cell viability.

The main target of ROS include DNA, lipids, proteins and sugars, and their attacking order preferentially depends on numerous factors, such as the site of generation, types of macromolecules to be oxidized and the availability of metal ions metal associated to the biomolecules. However, the oxidized lipids, proteins and sugars can be removed via degradation which is not common phenomena for the DNA; since the molecule is responsible for all genetic information of all cells of a living organism (Berra et al., 2006), thus the oxidation to DNA may be a detrimental effect to all living cells.

Induction of oxidative damage to DNA bases occurs from its reaction with ROS. Lesions can occur due to direct oxidation of the nucleic acids or often, lead to the formation of breaks on one of the DNA strands (single breaks - SSB "single strand break") or single failures in approximately symmetrical positions on both strands of the DNA (double breaks - DSB "double strand break"). In addition, simple breaks can generate double breaks during cell replication (Valadares et al., 2012).

Taking into account that ITN as a drug with exaggerated doses of Vit A analogues may induce damage to the genetic material, thus the present study was undertaken to evaluate the net oxidative effects of ITN and the modulatory effects of Vit A and C in one proficient, three single mutants and two double mutants *Saccharomyces cerevisiae* strains. And to reconfirm the oxidative effect, some *in vitro,* namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) $ABTS^{\bullet+}$, hydroxyl radical ●OH, nitric oxide (NO) and reduction potential (RP) antioxidant assays were also prepared.

MATERIALS AND METHODS

Research type

To access the interaction of ITN with Vit A and C, this study was conducted in the Laboratory of Pharmacology and Genetics (LAPGENIC) at Federal University of Piaui (UFPI), Brazil.

Sources of reagents/chemicals and preparation for test concentrations

ITN was purchased from the Popular Pharmacies Teresina-PI, through a prescription given by a dermatologist. ITN at a concentration range of 5 to 50 µg/ml for *in vitro* while for *in vivo* antioxidant assay 20 µg/ml were used. Vit A and C were used at concentration of 17 µg/ml. All the other necessary reagents/ chemicals used in this study were collected from Sigma-Aldrich, St. Louis, MO; USA.

In vivo assay

Used S. cerevisiae strains

Table 1 contains the proficient (SodWT), single deficient (Sod1Δ, Sod2Δ and Cat 1Δ) and double deficient (Sod1ΔSod2Δ and Sod1Δ/Cat1Δ) of *S. cerevisiae* test strains.

Central disk test in yeast S. cerevisiae (in vivo)

This test was performed according to the aerobic metabolism pathway earlier described by Fragoso et al. (2008). Briefly, previously sub-cultured strains were linearly swabbed to the sterile Yeasts Extract Peptone Dextrose Broth (YEPD) media (0.5% yeast extract, 2% peptone, 2% dextrose and 2% bacteriological agar). Then 0.01 ml of test stressor (H_2O_2, STR)/STR/netagive control (NC) (specified concentrations) was applied on sterile paper disks and was treated accordingly, such as for alone (NC/ITN/STR) and co-treatment groups (ITN/Vit A/Vit C). The NC and STR groups were treated with sterile vehicle (0.9% NaCl) and 50 mM STR, respectively. Treatments were done immediately after swabbing the organisms in petri-dishes. Linearly organisms swabed are as shown in Figure 1. Dishes were then inverted (180°), kept into an incubator maintaining temperature 35±1°C for 72 h, followed by the

Table 1. Description of *Saccharomyces cerevisiae* strains used in the study (Oliveira et al., 2014).

Description	Genotypes	Source
EG103 (SodWT)	MAT α leu2 3, 112 his3 Δ 1,trp1 - 289 ura-3 52	Edith Gralla E, L Angeles
EG118 (Sod1Δ)	EG103 except sod1::URA3	Edith Gralla E, L Angeles
EG110 (Sod2Δ)	EG103, except sod2:: TRP1	Edith Gralla E, L Angeles
EG133(Sod1ΔSod2Δ)	EG103 except sod1::URA3 sod2::TRP1	Edith Gralla E, L Angeles
EG 223 (Cat1Δ)	EG103 except cat1::TRP1	Edith Gralla E, L Angeles
EG (Sod1ΔCat1Δ)	EG103 except sod1::URA3cat1::TRP1	Edith Gralla E, L Angeles

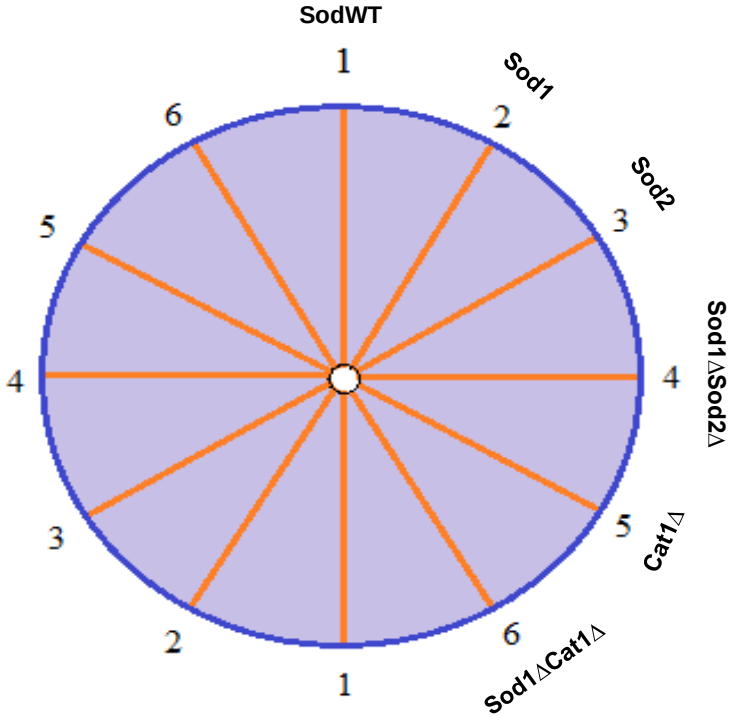

Figure 1. Test strains of *S. cerevisiae* positioning in the Petri dish.

measurement of inhibition zones in millimeters (mm) with a range from 0 mm (full growth) to 40 mm (no growth); these values being the size of the petri-dishes procured. All the treatments were performed in duplicate.

Percentage oxidative activity was calculated by using the following formula:

% oxidant = [(STR-test group) / STR] × 100.

In vitro reconfirmation assays

DPPH radical scavenging test

For the DPPH radical scavenging test, methodology described by Silva et al. (2005) with minor modifications was used. ITN and standard TRO were used at concentrations of 5, 10, 25 and 50 µg/ml. The antioxidant evaluation was performed calculating the percentage of scavenging of DPPH radicals by the following equation:

% scavenged DPPH radical = {($A_{control}$ - A_{sample}) × 100} / $A_{control}$)}

Where $A_{control}$ is the initial absorbance of DPPH in ethanol (absolute) solution, and A_{sample} is the absorbance of the reaction mixture containing ITN/TRO. Absorbance was taken at 517 nm. The half-minimal effective inhibitory concentration (EC_{50}) of the ITN/TRO was also determined. A NC was considered without sample.

ABTS radical scavenging test

This test was performed by the method earlier described by Re et al. (1999). Initially, the ABTS cationic radical is formed from the reaction of 5 ml of a 7 mM ABTS in 0.088 ml of a 2.45 mM solution of potassium persulfate ($K_2S_2O_8$), and incubated at room temperature in the absence of light for 16 h, then followed by dilution in ethanol to attain a solution of absorbance 0.70 ± 0.05 at 734 nm. Now, in a dark environment at room temperature, 0.040 ml of specified concentrations of ITN/TRO was transferred to vials with

Table 2. Effects of ITN, ITN+Vit C, ITN+Vit A+Vit C in *S. cerevisiae* strains compared to the NC and STR.

Group	Test strains					
	SodWT	Sod1Δ	Sod2Δ	Sod1Sod2Δ	Cat1Δ	Cat1Sod1Δ
NC	0.90±0.14	0.25±0.3	1.50±0.70	0.50±0.70	0.05±0.07	0.50±0.70
STR	11.0 ± 1.4	15.0±2.82	16.50±2.12	14.0±2.82	18.5±0.70	16.5±2.12
ITN (20 µg/ml)	3.05±0.2[a]	3.50±0.70[a]	3.75±0.35[a]	3.65±0.49[b]	3.70±0.14[a]	3.70±0.14
ITN (20 µg/ml) + Vit A (17 µg/ml)	3.05±0.21[a]	3.80±0.28[a]	3.95±0.07[a]	3.4 ± 0.28[a]	3.75±0.07[a]	3.20±0.28
ITN (20 µg/ml) + Vit C (17 µg/ml)	1.70±0.14[a]	1.05±1.06[a]	2.95±0.21[a]	1.55± 0.63[b]	3.45±0.21[a]	2.70±2.28
ITN (20 µg/ml) + Vit A (17 µg/ml) + Vit C (17 µg/ml)	1.15±0.21[a]	3.60±0.14[a]	3.55±0.35[a]	0.85±0.21[a]	4.00±0.0[a]	2.10±0.14

NC: Saline (0.9% NaCl); STR: stressor (H_2O_2); ITN: isotretinoin; Vit A: vitamin A; Vit C: vitamin C; one-way ANOVA followed by Tukey's multiple comparison test with significance [a]p<0.0001, and [b]p<0.001 compared with NC.

1.960 ml ABTS radical. Absorbance was taken after 6 min in a spectrophotometer at 734 nm and the results were expressed as percentage scavenged $ABTS^{\bullet+}$ radicals as DPPH test and EC_{50} was also calculated accordingly.

$^{\bullet}OH$ radical scavenging test

Fenton's reaction, described by Lopes et al. (1999) with minor modifications was used for the hydroxyl radical ($^{\bullet}OH$) scavenging test. Briefly, ITN/TRO (5, 10, 25 and 50 µg/ml) was added to the Fenton reaction mixture containing 6 mM phosphate buffer (pH 7.4). The reaction was carried out for 15 min at room temperature and stationed by the addition of phosphoric acid at 4% (v/v) and followed by addition of 1% thiobarbituric acid (TBA) (w/v in 50 mM NaOH). The reaction mixture was then heated for 1 h at 37°C and subsequently was cooled to room temperature. Finally, the absorbance was taken at 532 nm and percentage of radical scavenged as well as EC_{50} were calculated accordingly.

$NO^{\bullet}$ radical scavenging test

This test was produced from the spontaneous decomposition of sodium nitroprusside (SNP) in 20 mM phosphate buffer (pH 7.4). Once generated, nitric oxide ($NO^{\bullet}$) interacts with oxygen to produce nitrate ions, which were measured by the Griess reaction according to the method of Basu and Hazra (2006). The reaction mixture (1 ml) containing 10 mM SNP in phosphate buffer was interacted with specified concentrations of ITN/TRO and incubated at 37°C for 1 h. A 0.5 ml aliquot was removed and homogenized in 0.5 ml Griess reagent. Absorbance was taken immediately at 540 nm and percentage of radical scavenged along with EC_{50} was calculated accordingly.

RP test

The RP test was conducted by the modified method described by Oyaizu (1986). For this, 0.2 ml sample was added to 0.5 ml of 0.2 M phosphate buffer (pH 6.6) and 0.5 ml of $K_3Fe(CN)_6$ (1% w/v) and the reaction mixture was heated at 50°C for 20 min. Then 0.5 ml of trichloroacetic acid (10% w/v) was added with constant shaking, followed by addition of 1.175 ml distilled water and 0.125 ml of $FeCl_3$ (0.1%, w/v) after 5 min. Sample absorbance was taken at 700 nm against blank sample. To NC, only 0.2 ml vehicle was added and treated similarly. The activity as reducing power capacity is calculated as follows:

$$\text{Reducing potential (\%)} = [(A_{ts} - A_{bs})/A_{ts}] \times 100$$

where A_{ts} is the absorbance of test sample and A_{bs} is the absorbance of blank sample.

Statistical analysis

Values are mean±standard error of mean (SEM). Data was managed by using the program GraphPad Prism (version: 6.0). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests at 95% confidence intervals at p<0.05 significance.

RESULTS AND DISCUSSION

Oxidative effect of ITN in *S. cerevisiae*

In this *in vivo* study, ITN produced significant oxidative damage to all strains tested as compared to NC (Table 2). Since ITN is showing no statistical differences with STR group, thus suggesting it should be a ROS inducer to the *S. cerevisiae* strains.

ITN is a Vit A derivative that acts by binding to retinoid receptors, which participate in the growth and differentiation of cells and that is used in severe forms of acne (Brito et al., 2010). Besides this ITN is one of the most widely prescribed drugs which reduce the size of sebaceous glands and their secretion as well as the number of bacteria in both channels and the skin surface. This is thought to be not only a result of the reduction of sebum, the nutrient source for bacteria, but also a reduction of the inflammation by inhibiting chemotactic responses of neutrophils and monocytes (Nasser et al., 2011).

Lupulescu (1993) and Rutkowski (2012) emphasize that Vit A is a micronutrient, and due to its fat solubility, it is easily transformed into the human body in RA (the active form), the major forms may be mentioned as all *trans*-RA (ATRA, the most important) and 9-cis RA (9-*cis* RA); these are important for the cell growth and differentiation. However, ITN in large dose may produce hypervitaminosis which may develop trouble to the normal functions of the body due to over-production of free radicals (RLs). However, ITN is evidenced to design to initiate remodeling of the sebaceous glands; inducing changes in expression of genes that induce selective apoptosis (Duester, 2008).

According to Vellosa et al. (2013), oxidative stress is regarded as a metabolic condition where an imbalance occurs in pro- and antioxidant systems or body defense system; these conditions can mediate cell damage through oxidation of bio-macromolecules, such as, lipids, proteins and DNA.

The metabolisms of retinoids cause the rearrangement of three structural parts of their skeleton, justifying the existence of multiple analogues with potentially different biological effects (Gundersen and Blomhoff, 2001); otherwise, due to the oxidation process, the metabolism of ITN by cytochrome P450 in the stomach wall giving rise to 4-oxo-ITN metabolites, TN and 4-oxo-TN, where the main metabolite is 4-oxo-ITN, which eventually accumulates in the blood from consecutive administrations.

Co-treatment result of ITN with Vit A in *S. cerevisiae*

ITN at a dose of 20 µg/ml with 17 µg/ml of Vit A also showed significant oxidative damage (Table 2). There may be hypervitaminosis effect which is evident to induce oxidative stress in *S. cerevisiae* (Duester, 2008). According to Rodriguez et al. (2001), the wild proficient strain (SodWT) is produced for the defence of the cytoplasma and mitochondria. The enzyme responsible for the detoxification of STR is catalase (CAT); hence a decrease in the activity of this enzyme and an increase in the production of STR, can result in selective cytotoxicity of Vit C (Santos, 2012).

Ferrini et al. (2001) stated that the chemical tests for antioxidant activity are faster and simpler carry out. However, they are no representative cellular conditions in human. Microbial *in vivo* tests by using eukaryotic cells are most popular, and in this context, yeasts have been proven as suitable microorganisms for the determination of the antioxidant capacity of different compounds due to their rapidity, reproducibility and correlation with respect to humans.

Co-treatment results with Vit C in *S. cerevisiae*

A shown in Table 2, it is clear that ITN 20 µg/ml co-treated with 17 µg/ml of Vit C improved the survival of the tested *S. cerevisiae* strains in comparison to the Vit A co-treated ITN group. As Vit C is a well known antioxidant, there may be the effect of it. However, the damage was not significantly inhibited by this co-treatment. Vit C positively affects the immune system, minimizes the risk of inflammation, thereby the antioxidant activity of this vitamin has been intertwined with the protection of cell function in the appearance of free radicals by oxidative stress, thus the controlling of aging, inflammatory damage, and cancer (Nasser et al., 2010).

In addition, vitamins are the essential trace substances to the human body, but the recommended supplementation should be evaluated specifically for each case, as there are many organic and inorganic components in cells that can modulate the activity by them more specifically using Vit A and C (Lupulescu, 1993; Santos, 2012).

Co-treatment of ITN with Vit A and C in *S. cerevisiae*

In this occasion, again the oxidation effects come back. There may be a net effect due to the incorporation of Vit A and the induction hypervitamonisis. Going through data presentation (Table 3), it is clear that the oxidative effect was more prominent to the single mutants Sod1Δ, Sod2Δ and Cat1Δ. CAT is an antioxidant enzyme that is normally produced in all living organisms; it has an important role in protecting the body acting the defense against STR, protecting synergistically the cells. Furthermore, it has an action to retard or inhibit oxidation, thus acting at different levels of oxidative sequence by adapting mechanisms to oxidative stress (Silva et al., 2012).

However, Catania et al., (2009) reported that regular consumption of foods rich in Vit A and C can decrease the incidence of rectal and colon cancers. Carotene, which is the most important precursor of Vit A, is widely distributed in food and possess antioxidant capacity. It is absent in *S. cerevisiae* strains and is used in the concomitant therapy by ITN, Vit A and C; although eukaryotic cells are similar and have the same defenses similar to that of the cells in humans.

DPPH scavenging assay

DPPH radical scavenging assay is the most commonly performed test method in which antioxidant activity can be determined by monitoring the decrease in absorbance of the test sample. Anti-DPPH radical scavenging activity is tested here. Trolox, (+)-catechin, ethyl gallate, ascorbic acid and α-tocopherol are generally taken as standards (Antolovich et al., 2002). In our test, ITN did not show significant antioxidant activity in all tested concentrations as compared to the TRO (standard). The EC_{50} calculated for ITN was 21.23 ± 1.14 µg/ml, while for TRO it was

Table 3. *In vitro* reconfirmation assays adopting some antioxidant test methods.

Parameter	DPPH test (% radical scavenging)		ABTS test (% radical scavenging)		OH test (% radical scavenging)	
	ITN	TRO	ITN	TRO	ITN	TRO
5 µg/ml	6.04±0.01[a]	6.90±0.01[a]	6.21±0.01[a]	9.18±0.01[a]	7.38±0.01[a]	6.92±0.01[a]
10 µg/ml	1.37±0.01	7.88±0.01[a,e]	2.68±0.01	18.64±0.01[a,b,e]	5.77±0.01[a]	10.84±0.01[a,b,e]
25 µg/ml	4.73±0.01[a]	23.69±0.01[a,b,e]	2.97±0.01	31.36±0.01[a,b,c,e]	3.88±0.01	26.77±0.01[a,b,c,e]
50 µg/ml	3.97±0.01[a]	37.73±0.01[a,b,c,d,e]	1.41±0.01	56.78±0.01[a,b,c,d,e]	4.06±0.01[a]	35.21±0.02[a,b,c,d,e]
EC_{50} (µg/ml)	21.23±1.14	17.25±8.46	17.22±1.18	16.45±11.93	44.77±0.95	13.54±7.70
CI (µg/ml)	3.49-141.9	7.91-37.66	2.41-123.0	6.18-43.78	7.30-274.4	7.81-23.48
R^2	0.74	0.94	0.71	0.91	0.79	0.98
CTL	1.19±0.05		1.29±0.03		2.11±0.02	

	NO test (% radical scavenging)		RP test (% reduction capability)	
	ITN	TRO	ITN	TRO
5 µg/ml	9.48±0.01[a]	9.79±0.01[a]	6.16±0.01[a]	45.59±0.01[a,e]
10 µg/ml	6.14±0.01[a]	15.16±0.01[a,b,e]	5.82±0.01[a]	53.33±0.01[a,b,e]
25 µg/ml	6.68±0.01[a]	22.02±0.02[a,b,c,e]	2.99±0.01	65.33±0.01[a,b,c,e]
50 µg/ml	3.52±0.02	25.23±0.01[a,b,c,e]	2.63±0.01	74.54±0.01[a,b,c,d,e]
EC_{50} (µg/ml)	36.25±1.42	7.10±4.01	33.34±1.07	3.55±7.40
CI (µg/ml)	6.26-209.9	5.05-9.99	12.26-90.69	1.27-9.97
R^2	0.76	0.98	0.89	0.94
CTL	1.33±0.01		1.13±0.04	

ITN: Isotretinoin; TRO: torlox; CTL: control (chloroform); EC_{50}: half minimal effective concentration; CI: confidence interval; R^2: coefficient of determination; $p<0.05$ with 95% confidence interval where [a]compared to CTL, [b]for 5 µg/ml, [c]for 10 µg/ml, [d]for 25 µg/ml, [e]for ITN and TRO similar dose (one-way ANOVA Tucky test); values are the mean±SEM (n=5).

17.25 ± 8.46 µg/ml. The increase in doses also reduces the capacity of radical scavanged (Table 3), there may be an oxidative-like activity by large doses of ITN.

$ABTS^{\bullet+}$ scavenging assay

ABTS assay is the other familiar method for antioxidant test, where the scavenging activity of $ABTS^{\bullet+}$ radicals is done. It is a peroxidase substrate upon oxidation in the presence of H_2O_2 that generates a metastable radical cation (Antolovich et al., 2002). As shown in Table 3, it is clear that increased concentration reduced radical ($ABTS^{\bullet+}$) scavengeing activity by the ITN, which is ever opposite than the TRO. The EC_{50} calculated for ITN was 17.22 ± 1.18 µg/ml and for TRO was 16.45 ± 11.93 µg/ml, respectively.

$OH^{\bullet-}$ scavenging assay

Hydroxyl free radical ($^{-\bullet}OH$) generally reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes cellular destruction (Alam et al., 2013). Hydroxyl anionic radical measurement is done similarly to the DPPH and ABTS assays, where $^{-\bullet}OH$ is produced from the H_2O_2 and is scavenged by the sample tested. The ITN did not show antioxidant activity by reducing the $^{-\bullet}OH$ in all concentrations tested, compared to TRO. The EC_{50} calculated for ITN was 44.77 ± 0.95 µg/ml, while for the TRO it was 13.54 ± 7.70 µg/ml (Table 3).

NO● scavenging assay

Sodium nitroprusside in $NO^{\bullet}$ scavenging test is used as nitric NO radical source. In aerobic conditions, NO reacts with oxygen to produce stable nitrate ($NO_3^{\bullet-}$) and nitrite ($NO_2^{\bullet-}$) radicals, quantities of which can be determined using Griess reagent (Marcocci et al., 1994). In the NO kidnapping test, ITN by 5 µg/ml exhibited similiar activity like that of the TRO, but it was again discontinued from the 10 to 50 µg/ml as augmented doses caused reduction of inhibitory activity. The values of EC_{50} for ITN and TRO were calculated as 36.25 ± 1.42 and 7.10 ± 4.01 µg/ml, respectively (Table 3).

RP test

One of the rapid and sensitive antioxidant test method is

the RP assay, in which absorbance is increased by the formation of color complex with potassium ferricyanide, trichloro acetic acid and ferric chloride reaction. An increase in the absorbance indicates antioxidant activity of the test sample (Jayaprakash et al., 2001). There is also a similiar result like that of the $ABTS^{\bullet+}$ assay, as increased doses caused reduction of the antioxidant potential of ITN. However, TRO was found to have potential reducing capability in this occassion with an EC_{50} of 3.55±7.40 µg/ml and ITN of 33.34±1.07 µg/ml (Table 3).

Although the living system is co-existed with free radicals by developing diverse mechanisms for adapting them to advantageous physiological functions (Valko et al., 2007), but excessive production of ROS and NOS are implicated in various diseases (Carr et al., 2000; Valko et al., 2004; Kovacic et al., 2005; Neo et al., 2010). In our study, ITN should be considered as oxidative agent rather than antioxidant, otherwise high doses exhibiting less antioxidative potentials may be a marker for its oxidative damage by high doses.

Conclusion

ITN exhibited oxidative effect both in *in vivo* and *in vitro* studies. The oxidative effect of ITN was found to continue alone and with Vit A which was effectively modulated by Vit C (co-treatment). The modulatory effect of Vit C is due to its powerful antioxidant activity. Both co-treatment groups with Vit C notably reduced the oxidative effect to the tested yeasts, *S. Cerevisiae*; ITN alone and/or combination with Vit A opposed the effect of Vit C co-treated groups. In addition, ITN in the *in vitro* antioxidant tests exhibited antioxidant activity in a dose-reduced manners; this confirms ITN to have oxidative effects.

Conflict of interests

The authors have not declare any conflict of interest.

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