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## Original Article

### Comparative studies on the antioxidant potential of vanillin-producing *Saccharomyces boulardii* extracts

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Dichlorofluorescein diacetate;

Nitroblue tetrazolium; Total phenolic

content; Total flavonoid content

#### Abstract

Free radical-scavenging antioxidants have the potential as protective agents against various degenerative diseases caused by oxidative damage. Microorganisms are shown to be a promising new source of natural antioxidants. In the present study, supernatant (crude extract) and different solvent extracts (ethyl acetate, n-butanol, dichloromethane) of *Saccharomyces boulardii*'s growth were evaluated for the antioxidant activity using free radical scavenging (1,1-Diphenyl-2-picryl hydrazyl; DPPH) assay, superoxide radical scavenging (nitroblue tetrazolium; NBT) assay, and by estimating total phenolic and flavonoid contents (TPC and TFC). Antioxidative potential of *S.boulardii* extract was also tested in biological system, i.e. A549 lung cancer cell line using dichlorofluorescein diacetate (DCF-DA) assay. Organic extracts were further explored by HPLC and mass spectrometry techniques to identify active principles responsible for antioxidant activity. Comparative studies on antiradical ability (DPPH, NBT) revealed that crude extract has the highest activity than its other solvent counterparts as evident by its lower IC<sub>50</sub> values and higher TPC and TFC. *In vitro* studies also showed that *S.boulardii* extracts effectively quench intracellular reactive oxygen species. Vanillin was identified as one of the contributing antioxidant. It was found that *S.boulardii* extract is an efficient scavenger of free radicals and can be exploited as a potential new source of natural antioxidants.

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## INTRODUCTION

It is increasingly being realized that many of today's diseases are caused due to the 'oxidative stress' that results from an imbalance between formation and neutralisation of free radicals [1, 2]. All human cells protect themselves against free radical damage by enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH) or compounds such as ascorbic acid, tocopherol, etc. However, endogenous antioxidant supply is not unlimited as one antioxidant molecule can react with and neutralise only single free radical. These protective mechanisms are disrupted by various pathological processes. There is a constant need to replenish antioxidant resources, either endogenously or through supplementation to combat oxidative damage [3, 4]. Many synthetic drugs protect against oxidative damage but they have associated side effects.

An alternative solution to this problem is to consume antioxidants from natural sources, which may scavenge multiple reactive oxygen species (ROS) and can be used in various disease states to maintain a healthy status. The number of effective antioxidants that can be added to food products is limited and new compounds from new sources would be very useful. There is, therefore, a need to discover new and effective radical scavengers from natural sources.

Antioxidants obtained from plant sources suffer from few disadvantages viz extraction process involved is tedious and a lot of plant material has to be destroyed which is not environment-friendly. On the other hand, various microbial strains have been reported to possess antioxidant activity. Some of the bacterial probiotic (*Lactobacillus* sp) extracts have been evaluated pre-clinically for antioxidant activity [5, 6]. Researchers

have reported that metabolites produced by microorganisms (*Penicillium* and *Aspergillus*) may serve as antioxidants [7, 8]. These microorganisms can be cultured in higher amount with easier and environment-friendly ways of extraction for obtaining antioxidants. In addition, these microbes may provide easier set up for the production and purification of antioxidants as compared to plants. Hence, microbial strains can be exploited as a potential new source of natural antioxidants. Apart from the *Lactobacilli* and *Bifidobacteria*, *Saccharomyces boulardii* is a well-established anti-diarrheal probiotic strain owing to its anti-inflammatory and immune-modulatory properties [9, 10]. Reactive oxygen species (ROS) have prominent role in causing inflammation and antioxidants from natural and synthetic origin act as anti-inflammatory agents by scavenging them [11]. *S.boulardii* may contain certain antioxidant compounds possessing ROS scavenging activity which needs to be explored. Recent studies have also shown that *S.boulardii* ameliorates drug induced oxidative damage in rats and reduces DNA damage in the pancreatic acinar cells of acute necrotizing pancreatitis (ANP)-induced rats [12, 13]. The objectives of present work were to evaluate antioxidant potential of *S.boulardii* extracts and to identify the active compounds responsible for it.

## MATERIALS AND METHODS

### Chemicals

1,1-Diphenyl-2-picryl hydrazyl (DPPH), butylated hydroxy anisole (BHA), ascorbic acid, xanthine, xanthine oxidase (XO), nitroblue tetrazolium (NBT), Dulbecco's Modified Eagle's Medium (DMEM), Folin-Ciocalteu reagent (FCR), gallic acid, sodium carbonate, aluminium chloride and quercetin were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrogen peroxide ( $H_2O_2$ ) was procured from Calbiochem, and 2',7'-dichlorodihydrofluorescein-diacetate (DCF-DA), fetal bovine serum (FBS), and antibiotics were purchased from Invitrogen (Freiburg, Germany). Various organic solvents used were procured from Qualigens Chemicals (Mumbai, India), Central Drug House (New Delhi, India) and S.D. Fine-Chem (Mumbai, India). Media components were obtained from HiMedia (Mumbai, India).

### Microorganism and culture conditions

Microbial strain *Saccharomyces boulardii* ATCC 796 was procured from American Type Collection Culture (ATCC; Manassas, VA, USA). The stock culture was grown on agar plates of yeast extract-peptone-dextrose (YPD) medium (2% glucose, 2% peptone, 1% yeast extract, and 2% agar) at 30°C (pH 6.5 ± 0.2) for 48 h. From stock culture, two loopfuls of microorganisms were inoculated into 100 ml flask containing 20 ml medium and flasks were incubated at optimum growth

conditions on rotary shaker (200 rpm) to prepare starter culture.

### Cell culture

It has been reported that the lung is an organ sensitive to oxidative stress [14]. To study the effect of different polarity solvent extracts of *S.boulardii* on the oxidative stress induced by hydrogen peroxide, lung cancer (A549) cell line from ATCC was used. Cells were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotic at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

### Preparation of the solvent extracts of *S. boulardii*

Inoculum (2%, v/v) was transferred to 1000 ml production medium and incubated at 30°C for 48 h (200 rpm). Cell mass was centrifuged at 7,000g (15 min at 4°C) and supernatant was filtered through a Whatman No.1 filter paper under suction. Various fractions (250 ml) of *S.boulardii* broth filtrate (crude extract) were extracted using organic solvents (ethyl acetate, n-butanol and dichloromethane) (aqueous: organic: 1:5) with variable polarity. The process of extraction was repeated thrice with each sample using separating funnel. Organic extracts obtained were filtered over anhydrous sodium sulfate (1%, w/v). Both crude extract itself and organic solvent extracts were then concentrated to dryness *in vacuo* using rotavapor below 30°C and then reconstituted in distilled water (crude and n-butanol extract) or in dimethyl sulfoxide (DMSO) (ethyl acetate and dichloromethane [DCM] extracts) to get 10 mg/ml of test solution [15-17].

### Free radical scavenging activity (DPPH assay)

Free radical scavenging activity of the *S.boulardii* extracts was determined by DPPH assay according to the protocol given by Gautam *et al* with some modifications [18]. Seven dilutions of each extract were prepared (0.5-10 mg/ml). The reaction mixture comprised of 190 µl DPPH solution (24 µg/ml methanol) and 10 µl test extract. The total reaction volume was 200 µl having final concentrations of 25-500 µg/ml of test extracts. The control solution (expressed as 100% free radicals) contained 10 µl distilled water and DMSO instead of test extracts (crude, n-butanol and ethyl acetate, DCM, respectively) (only DPPH, no extract). BHA was used as standard. The reaction mixture was incubated in dark at room temperature for 30 min. Absorbance was read at 517 nm in a 96-well plate reader (Multiskan FC; Thermo Scientific, USA). All measurements were taken in triplicate. Radical scavenging activity of test extract was expressed as percentage reduction of absorbance. The IC<sub>50</sub> value (concentration of the extracts required to cause 50% inhibition) was determined by the linear regression analysis from the percentage inhibition values [15].

The ability to scavenge the DPPH radical was calculated using the following equation [19]:

$$\% \text{Free radical reduction} = [1 - (A_{\text{sample}} - A_{\text{sample blank}}) / A_{\text{control}}] \times 100$$

- $A_{\text{control}}$ : Absorbance of the control (DPPH solution without sample)

- $A_{\text{sample}}$ : Absorbance of the test sample (DPPH solution + test sample)

- $A_{\text{sample blank}}$ : Absorbance of the sample only (sample without DPPH solution)

#### Superoxide radical scavenging activity (NBT assay)

Superoxide radical scavenging ability of *S.boulardii* extracts was determined by NBT assay using xanthine-xanthine oxidase system according to the method of Liu *et al* with slight modifications [20]. The reaction mixture contained 20  $\mu$ l xanthine (100 mM), 20  $\mu$ l NBT (100  $\mu$ M) and 10  $\mu$ l of various concentrations of test extracts (25-500  $\mu$ g/ml). The final reaction volume was made up to 200  $\mu$ l with phosphate buffer (100 mM, pH 7.5). Reaction was initiated by the addition of 20  $\mu$ l xanthine oxidase (0.1 U/ml). The reaction mixture was incubated at room temperature for 5 min. Absorbance was measured at 560 nm. Test blank (only xanthine + XO + NBT + buffer, no extract) was expressed as 100% NBT-diformazan complex formation. The control solution contained 10  $\mu$ l distilled water and DMSO instead of test extracts (crude, n-butanol and ethyl acetate, DCM, respectively). Ascorbic acid was used as standard. All the measurements were taken in triplicate. Superoxide radical scavenging activity of test extract was expressed as percentage reduction of absorbance. The  $IC_{50}$  value was determined by the linear regression analysis from the percentage inhibition values.

The ability to scavenge the superoxide radical was calculated using the following equation [21]:

$$\% \text{Superoxide radical scavenging activity} = [1 - (A_{\text{sample}} - A_{\text{control}}) / A_{\text{test blank}}] \times 100$$

- $A_{\text{test blank}}$ : Absorbance of the blank (reaction mixture without test sample)

- $A_{\text{sample}}$ : Absorbance of the test sample (reaction mixture with test sample)

- $A_{\text{control}}$ : Absorbance of the sample only (reaction mixture without XO)

#### Determination of total phenolic contents (TPC)

The amount of total phenolics in the *S.boulardii* extract was determined using FCR with gallic acid as standard [22]. Extract (20  $\mu$ l, 1 mg/ml) was placed in a 2 ml cryovial, 1.58 ml distilled water and 100  $\mu$ l FCR were added to it and shaken thoroughly. After few minutes, 300  $\mu$ l sodium carbonate solution (20%, w/v) was added and the mixture was allowed to stand for 2 h at room temperature with intermittent shaking. Absorbance was measured at 765 nm. All the

measurements were taken in triplicate. TPC was calculated and expressed as milligrams of gallic acid equivalent (GAE) per gram of the dry test extract using gallic acid standard curve. The regression equation was  $y = 0.0005x + 0.0007$  ( $R^2 = 0.9981$ ) [23].

#### Determination of total flavonoid contents (TFC)

Concentration of total flavonoids was determined by the aluminium chloride ( $AlCl_3$ ) colorimetric assay (Dowd method) [24].  $AlCl_3$  in methanol (2%, 5 ml) was mixed with the same volume of extract solution (0.5 mg/ml). Absorption at 415 nm was taken after 15 min incubation at room temperature against a blank consisting of 5 ml test extract solution with 5 ml methanol without  $AlCl_3$ . All the measurements were taken in triplicate. Quercetin was used for preparing calibration curve (ranging from 0 to 100  $\mu$ g/ml) with the regression equation of;

$$y = 0.0064x + 0.034$$
 ( $R^2 = 0.9992$ )

where  $x$  is the concentration of quercetin in  $\mu$ g/ml and  $y$  is the absorbance reading at 415 nm. Total flavonoid contents were expressed as milligrams of quercetin equivalents (QE) per gram of dry extract [25].

#### Intracellular reactive oxygen species scavenging activity (DCF-DA assay)

Intracellular ROS scavenging properties of different *S.boulardii* extracts were analyzed using DCF-DA according to the protocol followed by Kang *et al* with some modifications [26]. A549 lung carcinoma cells were seeded at a concentration of  $1 \times 10^5$  cells/ml. Cells at 70% confluence were treated with varying concentrations of test extracts (100 and 200  $\mu$ g/ml) and incubated at 37°C for 30 min. Oxidative stress was induced by treatment of cells with  $H_2O_2$  (2 mM) for 30 min. Attached cells were washed with phosphate buffer saline (PBS) and incubated with DCF-DA for 10 min at 37°C. Cells were then washed with PBS and the fluorescence of oxidized DCF was observed in inverted microscope (Nikon Eclipse TE2000-S) at 10x magnification. Fluorescence intensity of the captured images was measured using ImageJ software.

#### Detection, extraction and analysis of vanillin from fermentation broth

Growing culture of *S.boulardii* gave characteristic aroma of vanillin, aromatic principle of vanilla pods that is widely used as flavouring agent. It is a simple molecule that contains aldehyde functional group in addition to hydroxyl group attached to aromatic benzene ring. In the presence of air vanillin is oxidized to vanillic acid. Fermentation broth of *S.boulardii* containing vanillin/vanillic acid in addition to medium components and other metabolites was subjected to extraction by various solvents (methanol, ethyl acetate, n-butanol and dichloromethane) to isolate vanillin. After work-up, samples were subjected to preparative

thin layer chromatography. Spots corresponding to vanillin standard were scratched and dissolved in methanol for further analysis. To confirm the presence of vanillin, HPLC analysis was performed using reversed phase column chromatography. Solvent system consisted of acetonitrile:water:formic acid in the ratio of 20:80:1. Chromatogram was developed at a flow rate of 0.9 ml/min and detection was done at 254 nm. Vanillin and vanillic acid standards were used as reference compounds. Sample purified by preparative TLC was dissolved in acetonitrile and subjected to isocratic elution using the system mentioned above. Samples were further analysed by mass spectroscopy [27].

### Statistical analysis

Experimental values were expressed as mean  $\pm$  SEM (standard error of the mean). Comparison of mean values between various groups was performed by one way analysis of variance (ANOVA) followed by multiple comparisons by Tukey's test using SigmaStat 3.5 software. P values less than 0.05 were considered to be significant. The IC<sub>50</sub> values were calculated from linear regression analysis.

## RESULTS

### Free radical scavenging activity

The crude extract of *S.boulardii* exhibited highest DPPH radical scavenging activity than other solvent extracts (*viz* ethyl acetate, n-butanol, DCM), but it was lower than that of the standard BHA (Fig.1). The crude extract showed comparable activity with BHA at maximum concentration tested (500  $\mu$ g/ml). IC<sub>50</sub> value is inversely proportional to antioxidant ability. IC<sub>50</sub> value of the crude extract (73.27  $\mu$ g/ml) was lower than other solvent extracts but higher than BHA (22.81  $\mu$ g/ml) (Table 1). Among the three different polarity fractions isolated from the crude extract by solvent partition, DCM extract revealed no or only moderate activity. The activity of *S.boulardii* crude extract was observed to be higher than that of the other three solvent extracts at all seven tested concentrations (25-500  $\mu$ g/ml). Thus, free radical scavenging activity of *S.boulardii* extracts was in the order as crude extract (CE) > n-butanol extract (BE) > ethyl acetate extract (EE) > dichloromethane extract (DE). These results indicate that *S.boulardii* crude extract possesses powerful antioxidant ability which may be due to the synergistic activity of mixtures of antioxidants.

### Superoxide radical scavenging activity

Superoxide radical scavenging activity of the crude extract of *S.boulardii* was higher than other solvent extracts, but was lower than standard ascorbic acid at lower concentrations (Fig.2). It showed activity

comparable with ascorbic acid at 300  $\mu$ g/ml. IC<sub>50</sub> value of the crude extract (99.93  $\mu$ g/ml) was lower than other solvent extracts but higher than ascorbic acid (45.18  $\mu$ g/ml) (Table 2). This indicates that the crude extract showed the most potent activity as compared to other solvents extracts. DCM extract revealed only moderate activity. Thus, superoxide radical scavenging activity of *S.boulardii* extracts followed the order: CE > BE > EE > DE, which is in agreement with the results of DPPH assay. These results indicate that *S.boulardii* crude extract is an effective scavenger of superoxide anions. This may be due to the presence of multiple antioxidants with relatively high superoxide scavenging activity.

### Total phenolic content as antioxidant agent

The concentration of phenolics in *S.boulardii* extracts was expressed as milligrams of gallic acid equivalent (GAE)/g of dry extract (Table 3). The amount of total phenols varied in different extracts with maximum value in CE (370.45  $\pm$  4.85) and minimum in DE (62.96  $\pm$  2.56). In addition, the amount of total phenolic compounds in CE was one and half fold greater than BE, two fold higher than EE and six fold more than that of DE (236.03  $\pm$  3.16, 177.12  $\pm$  3.65, 62.96  $\pm$  2.56, respectively). Total phenolic contents in *S.boulardii* extracts correlated with their free radical scavenging activity (*i.e.* correlation coefficient between data of DPPH assay and TPC was 0.9043).

**Table 1.** IC<sub>50</sub> values of *S.boulardii* extract by DPPH assay

S. No.	Test solution	IC <sub>50</sub> value ( $\mu$ g/ml)
1	Crude extract (CE)	73.27
2	Butanol extract (BE)	142.97
3	Ethyl acetate extract (EE)	231.67
4	DCM extract (DE)	450.87
5	BHA	22.81

BHA, butylated hydroxy anisole

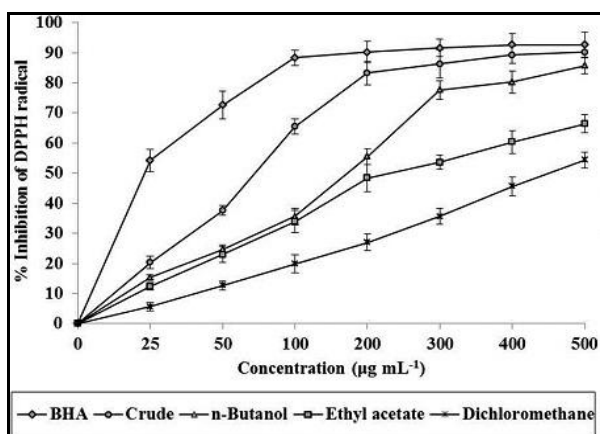
**Table 2.** IC<sub>50</sub> values of *S.boulardii* extract by NBT assay

S. No.	Test solution	IC <sub>50</sub> value ( $\mu$ g/ml)
1	Crude extract (CE)	99.93
2	Butanol extract (BE)	185.68
3	Ethyl acetate extract (EE)	261.85
4	DCM extract (DE)	422.89
5	Ascorbic acid	45.18

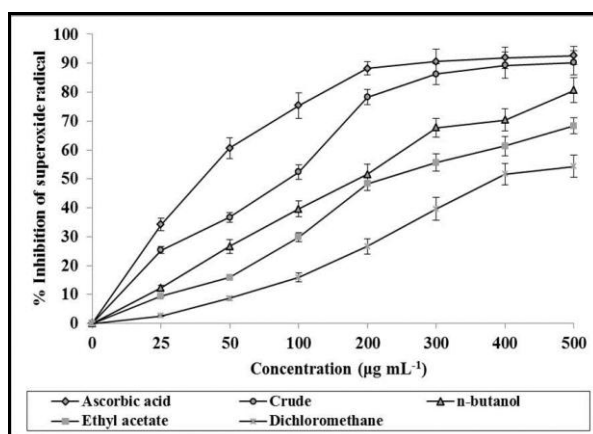
**Table 3.** Total phenolic and flavonoid content of the *S.boulardii* extract

S. No.	Extracts	TPC (mg/g) dry extract	TFC (mg/g) dry extract (in QE)
1	CE	370.45 $\pm$ 4.85	67.72 $\pm$ 2.44
2	BE	236.03 $\pm$ 3.16	35.36 $\pm$ 1.37
3	EE	177.12 $\pm$ 3.65	16.77 $\pm$ 0.86
4	DE	62.96 $\pm$ 2.56	5.79 $\pm$ 0.63

Data expressed as mean  $\pm$  SEM (n = 3)



**Figure 1.** DPPH radical scavenging effect of crude and solvent extracts of *S.bouldardii*. The radical scavenging ability of varying concentrations (25-500 µg/ml) of *S.bouldardii* extract was analysed by measuring their inhibitory effect on the absorbance of the DPPH radicals. Absorbance of the reaction was measured at 517 nm. The reaction was performed in triplicate and results were expressed as % inhibition of the absorbance of the DPPH radicals ± SEM (n=3).



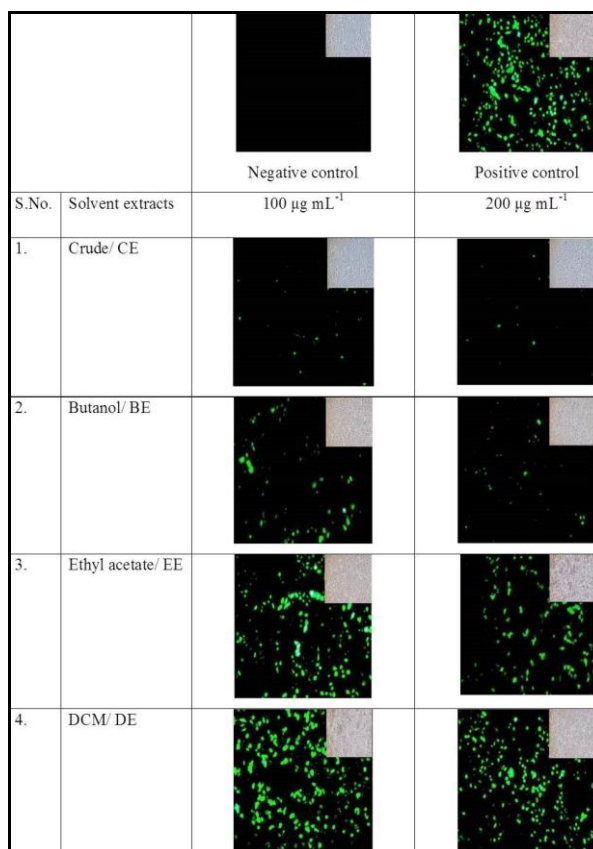
**Figure 2.** Superoxide radical scavenging effect of crude and different polarity solvent extracts of *S.bouldardii*. The superoxide scavenging ability of varying concentrations (25-500 µg/ml) of *S.bouldardii* extracts was analysed by measuring their inhibitory effects on the absorbance of the superoxide radicals. Absorbance of the reaction was measured at 570 nm. The reaction was performed in triplicate and results were expressed as % inhibition of the absorbance of the superoxide radicals ± SEM (n=3).

**Total flavonoid content as antioxidant agent**

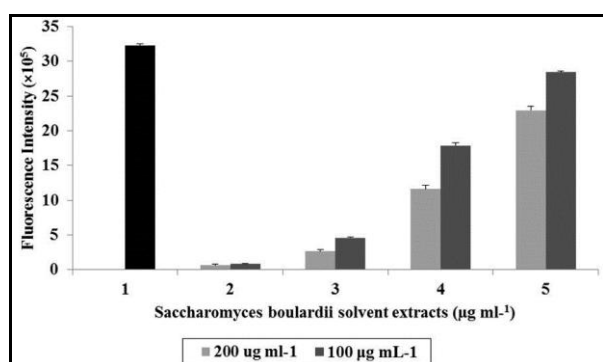
The concentration of flavonoids in *S.bouldardii* extracts was expressed as mg quercetin equivalent (QE)/g of dry extract (Table 3). *S.bouldardii* crude extract exhibited the highest total flavonoid contents at  $67.72 \pm 2.44$  mg QE/g, approximately two fold more than BE, four fold higher than EE and twelve fold more than DE ( $35.36 \pm 1.37$ ,  $16.77 \pm 0.86$ ,  $5.79 \pm 0.63$ , respectively). Like TPC, flavonoid contents also showed positive correlation with antiradical activity ( $R^2 = 0.7707$ ).

**Intracellular ROS scavenging activity**

In the present study, DCF-DA assay was performed to assess intracellular ROS scavenging activity of different *S.bouldardii* extracts using biological system, i.e. A549 lung cancer cell line and correlate it with the results of DPPH and superoxide scavenging activity assays. The fluorescent intensity of DCF-DA staining was enhanced in the 2 mM H<sub>2</sub>O<sub>2</sub>-treated A549 cells (positive control). However, *S.bouldardii* extracts reduced the intensity of the green fluorescence caused due to oxidative stress, reflecting a reduction in ROS generation (Fig.3). Decrease in the fluorescent intensity was found to be dose dependent and was higher in the case of *S.bouldardii* crude extract as compared to other solvent extracts at both concentrations of 100 and 200 µg/ml (Fig.4). In addition, we observed that, decrease in the fluorescence intensity was 37 and 50 fold than that of positive control in the case of CE for concentrations of 100 and 200 µg/ml, respectively. ROS scavenging activity was in the order as CE > BE > EE > DE. Thus, ROS scavenging activity of the *S.bouldardii* extracts was found to be consistent with the DPPH and superoxide scavenging activities of *S.bouldardii* extract.



**Figure 3.** Intracellular ROS scavenging effects of *S.bouldardii* extracts. Representative microscopy images illustrate the increase in green fluorescence intensity of DCF produced by ROS in 2 mM H<sub>2</sub>O<sub>2</sub>-treated A549 cells (positive control) against negative control and decrease in fluorescence on addition of different *S.bouldardii* extracts (100 and 200 µg/ml).



**Figure 4.** Intracellular ROS scavenging effect of *S. boulardii* extracts. 1, positive control; 2, crude extract; 3, butanol extract; 4, ethyl acetate extract; 5, DCM extract; data expressed as mean  $\pm$  SEM (n=3);  $P > 0.05$  for treated cells vs non-treated cells.

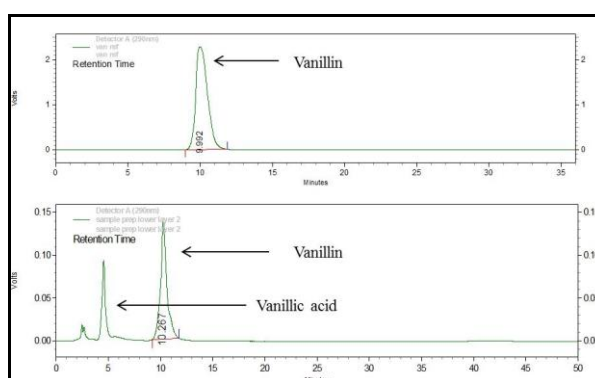
### Detection, extraction and analysis of vanillin from fermentation broth

Different organic solvents were tried to extract vanillin from culture supernatant. Extraction efficiency was much better when n-butanol was used. Extraction process was improved by initially acidifying the aqueous broth and repeating the process thrice. HPLC analysis showed two peaks corresponding to the retention time of 4 and 10 min. Standard vanillin also eluted after 10 min of run and the peak coincided with that of test sample. This confirmed the production of vanillin in yeast culture. Molecular weight of vanillin and vanillic acid is 152 and 167, respectively. Mass spectra analysis of sample showed peaks at 152 and 167 that corresponded to mass of vanillin and vanillic acid. Vanillin on exposure to air readily oxidizes to vanillic acid and it may be the reason of m/z-167 to be the base peak (Fig.5).

## DISCUSSION

### Antioxidant potential of *S. boulardii* extracts

A wide variety of microbes remain unexplored for structurally varied metabolites having valuable bioactivities including antioxidant activity [28]. Various scientific evidences are available regarding the metabolites derived from the genus *Saccharomyces*. The yeast *Saccharomyces cerevisiae* was found to produce (1 $\rightarrow$ 3)- $\beta$ -D-glucan that exhibits antioxidant and immunomodulating properties which justifies its use in anti-infective and antitumor therapy [29]. There is a strong evidence of production of complexes of nucleic acids and acidic nuclear proteins from *Saccharomyces cerevisiae* which neutralizes the oxidative stress generated by the ultraviolet irradiation or hydrogen peroxide [30]. Literature reports suggest that *Saccharomyces* produces certain metabolites (proteins, polysaccharides etc) responsible for its anti-inflammatory activity [31-33]. Such anti-inflammatory factors are hypothesized to show potential antioxidant activity.



**Figure 5.** HPLC analysis of vanillin (standard and from butanol extract).

The present study aimed to evaluate and compare antioxidant potential of *S. boulardii* ATCC 796 extracts using series of antioxidant activity assays and searching for active principles. Various antioxidants might be present in microbial extracts and nature of the antioxidants may be variable from polar to non-polar, therefore, crude filtrate of *S. boulardii* growth was extracted using different polarity solvents viz ethyl acetate (polar), n-butanol (intermediate polar), dichloromethane (non-polar) to partition antioxidants depending on their hydrophilic and lipophilic nature [34, 35].

### Antiradical properties of *S. boulardii* extracts

Antioxidant potential of *S. boulardii* extracts was tested using DPPH, which is a model free radical and shows a characteristic purple colour. It is reduced to non-radical form when it encounters the radical scavenger and its colour changes from violet to yellow based on the efficacy of antioxidants [36]. In NBT assay, superoxide anions, generated by XO mediated conversion of xanthine to uric acid and H<sub>2</sub>O<sub>2</sub>, convert NBT to blue coloured NBT-diformazan complex. SOD reduces the superoxide ion concentration and thereby lowers the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBT-diformazan complex is a measure of SOD activity/superoxide scavenging ability of the test extracts [37]. Quantitative evaluation of the *S. boulardii* extracts using DPPH and NBT assays revealed that, antioxidant activity of crude extract was significantly higher (lowest IC<sub>50</sub> value) in comparison to other three organic extracts. It exhibited a similar inhibition percentage to standard compounds at higher concentrations. This supports the fact that various microbial extracts or metabolites therein exhibit strong antiradical activity as reported in previous studies [7, 17, 38]. The order of antioxidant potential was: CE > BE > EE > DE. From this comparison, it may be concluded that *S. boulardii* crude extract contains potent antioxidant compounds with higher polarity, though this is not in agreement with some of

the previous studies reporting higher anti-oxidative potential of ethyl acetate extract as compared to other polarity solvent extracts from various microbial strains. That may be due to the lower polarity of antioxidative compounds [17, 34]. Reason for higher antioxidant activity of crude extract than other organic extracts may be breaking down of bonds between functional groups (*viz* hydroxyl, amine, *etc* responsible for antioxidant activity) during extraction with organic solvents.

#### **Total phenolic and flavonoid contents of *S.boulardii* extracts**

The phenolic compound and its derivatives, including simple phenols, flavonoids, phenylpropanoids, tannins, lignins, *etc.* contain aromatic rings and hydroxyl groups that determine the radical scavenging power of the compound [39]. In Folin-Ciocalteu assay, phenolate ion reduces molybdenum in the FCR to form blue coloured complex whose intensity is determined spectrophotometrically [40]. In Dowd method, flavonoids form (chelate type) internal complexes, with  $Al^{3+}$ . The intensity of the yellow colour of the chelate formed is spectrophotometrically determined [41, 42]. The findings from TPC and TFC clearly indicate that *S.boulardii* crude extract possesses a higher quantity of phenolics and flavonoids as compared to other different polarity extracts and results of TPC and TFC determination were in consonance with other antioxidant activity assays. Also, total phenolics and flavonoids showed positive correlation with free radical scavenging activity. Hence, it can be postulated that, phenolic and flavonoid compounds may be the contributing factors towards the antioxidant potential of *S.boulardii* extract. Furthermore, several studies have reported a significant correlation between the antioxidant activity present in various microbial extracts with their total phenolic and flavonoid contents suggesting microbial strains containing higher phenolic compounds can be a good source of antioxidants [17, 34, 43].

#### ***In vitro* evaluation of antioxidant potential of *S.boulardii* extracts**

DCF-DA assay was performed using A549 lung cancer cell model and  $H_2O_2$  as oxidative stress inducer. DCF-DA passively diffuses into the cells and is cleaved and oxidized by the intracellular ROS to the green fluorescence emitting compound DCF [44, 45]. Decrease in DCF fluorescence is a measure of ROS scavenging activity of the test extracts. Results of this particular work indicated that *S.boulardii* extract significantly reduced the susceptibility of A549 cells to ROS formation as measured by the inhibition of DCF fluorescence. It was realized that *S.boulardii* crude extract is an efficient scavenger of ROS in A549 cell model. These data supported the previous reports, showing protective effects of microbial metabolites

against hydrogen peroxide induced oxidative damage [46, 47].

#### **Vanillin as antioxidant in *S.boulardii* extract**

Vanillin is well-known flavoring agent used to provide sweet taste to various food items like biscuits, ice-cream, *etc.* Its antioxidant potential has also been identified in some of the previous studies [48-50]. Results of the HPLC analysis and mass spectrometry confirmed the formation of vanillin in *S.boulardii* growth supernatant. Being phenolic compounds, both vanillin and vanillic acid (oxidation product of vanillin) may be one of the active compounds contributing to the antioxidative potential of *S.boulardii* extract.

*S.boulardii* is a yeast probiotic strain and commonly used as anti-diarrheal and immuno-modulatory agent. To the best of our knowledge, apparently this is the first report on antioxidant activity of *S.boulardii* demonstrated by different assay procedures. *S.boulardii* extracts contain a mixture of antioxidants and exhibit multiple anti-oxidative activities which can be attributed to phenolic and flavonoid compounds such as vanillin. Thus, it can effectively diminish oxidative stress and help to ameliorate various disorders associated with it. Also, it can be perceived that, not only plants and mushrooms but some fungi like yeast may also be a good source of antioxidant compounds and *S.boulardii* is one such potential candidate offering a better scope for the production and easier downstream of such bioactive compounds. These findings will facilitate further studies to gain better understanding of the production of bioactive metabolites in yeast.

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#### **COMPETING INTERESTS**

The authors report no conflicts of interest for this work. The authors are solely responsible for the content and writing of the paper.

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