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Evaluation of antioxidant potential of anthocyanin-rich hydro ethanol extract of *Syzygium cumini* fruit pulp in various *in vitro* models and in food supplement enriched with omega-3 fatty acids

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Abstract

Omega-3 fatty acids have immense health benefits but are highly susceptible to oxidation due to high degree of unsaturation in their molecular structure. Therefore, development of omega-3 fatty acids fortified stable functional food is a challenging task. The aim of the study was to evaluate antioxidant potential of anthocyanin-rich hydro ethanol extract of *Syzygium cumini* fruit pulp extract in various *in vitro* models and in omega-3 fatty acids enriched food supplement in bulk oil and oil-in-water emulsions model systems along with chemical analysis of the fruit pulp extract and evaluation of its cytotoxic potential, if any. The *in vitro* antioxidant potential was evaluated by DPPH radical scavenging, $ABTS^{\circ+}$ decolorization and Fe^{2+} ion chelating methods. Antioxidant activity against oxidation of omega-3 fatty acids enriched food supplement in bulk oil and oil-in-water emulsions model systems was evaluated by monitoring primary oxidation, secondary oxidation and total oxidation of the omega-3 enriched oil using AOCS guidelines and iodometric method. Chemical analysis was performed by HR-LCMS analysis technique. Cytotoxic potential was evaluated by *Allium cepa* test. The test extract exhibited varying degrees of antioxidant activity in various *in vitro* models as well as against oxidation of omega-3 fatty acids enriched food supplement both in bulk oil and oil-in-water emulsions model systems. Chemical analysis revealed the presence of three major tentatively identified compounds malvidin-3,5-diglucoside, delphinidin-3,5-diglucoside and peonidin-3,5-diglucoside as major components of the extract. Test extract did not show any cytotoxic potential at recommended dosage level with LC_{50}> 1000 µg/ml. The results provide evidence that anthocyanin-rich hydro ethanol extract of *Syzygium cumini* fruit pulp may serve as a potential source of safe and effective, novel natural antioxidant for retarding oxidation of omega-3 fatty acids enriched food supplement which subsequently may help to develop omega-3 fortified stable functional foods in food industry.

Keywords: Oxidation of lipids; Omega-3 fatty acids; Auto-oxidation; *S. cumini* fruit pulp; Natural antioxidants; Omega-3 fortified functional food

1 Introduction

Functional foods provide important nutrients, protect against diseases and promote proper growth and development [1]. Omega-3 supplementation in food has been a significant recent trend in food fortification [2, 3]. In recent years, omega-3 fatty acids enriched oils are added to food to increase the nutritional value of food and reduce the risk of chronic disease (e.g. cardiovascular disease, arthritis, diabetes mellitus, Alzheimer's disease and cancer) [4, 5]. But, the main obstacle for developing omega-3 fatty acids fortified stable functional food is that they are highly susceptible to

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auto-oxidation due to high degree of unsaturation in their molecular structure [6]. The oxidation of double bonds in highly unsaturated omega-3 fatty acids reduces the nutritional benefits, and produced reactive oxygen species, as well as unhealthy volatile compounds with off-flavors and undesirable odors [7]. Relevant literature reveals that among the methods employed for controlling lipid oxidation, use of antioxidants is the most effective, convenient and economical means. Antioxidants are also used in health-related areas due to their ability to protect the body against oxidative damage (8). Synthetic antioxidants are generally used as preservatives to reduce the oxidation of unsaturated fatty acids in foods but they have accumulated evidence that they could be toxic and carcinogenic [8-10]. Therefore, innovation should continue to seek safe and effective, novel natural antioxidants as promising alternative to synthetic preservatives from other sources especially from plant origin for retarding oxidation of omega-3 fatty acids which subsequently may help to develop omega-3 fatty acids fortified stable functional foods [11, 12].

Relevant literature reveals that plant polyphenols especially flavonoids display a broad range of health-promoting bioactivities. Among these, their capacity to act as antioxidants has remained most prominent due to their redox potential and presence of a number of aromatic hydroxyl groups in their molecular structure [13-15]. Among the flavonoids compounds anthocyanins, a subgroup of flavonoids contains flavylium cation ($C_{15}H_{11}O^+$) in their molecular structure in addition to a number of aromatic hydroxyl groups [16-18]. This peculiar structure of anthocyanins makes them more potent antioxidant than other subgroup of flavonoids. *Syzygium cumini* fruit pulp (Family.*Myrtaceae*), commonly known as jamun or Indian black berryis rich in phenolic compounds especially anthocyanins and it is the only berry that contains all major anthocyanins (delphinidin, peonidin, petuinidin, malvidin, cyanidin and pelargonidin) in its fruit pulp [16]. Therefore, in the present investigation, an attempt has been made to evaluate antioxidant potential of anthocyanin-rich hydro ethanol extract of *Syzygium cumini* fruit pulp in various *in vitro* models and in food supplement enriched with omega-3 fatty acids with a view to shed some light on these important aspects.

2 Materials and methods

2.1. Collection and processing of plant materials

Fully ripe *Syzygium cumini* fruits were procured from local market (Baranagar Bazar Market, Kolkata, India). To remove any impurities adhered over the fruits surface, they were washed thoroughly under running tap water. The fruit pulp was manually peeled and dried in a hot air oven at 40 °C until it attains a constant weight. The dried pulps were ground using a grinder.

2.2. Preparation of anthocyanin-rich hydro ethanol extract

Anthocyanin-rich hydro ethanol extract of *S. cumini* fruit pulp powder was prepared following the method described elsewhere [19-21].Briefly 100 g of *S. cumini* fruit pulp powder was placed in a 500 ml conical flask, 300 ml of 70% hydro ethanol (acidified with 0.1% HCl)(85:15%, v/v) was added on it and mixed with constant agitation for 2h, centrifuged at 1000×g for 10 min and filtered. Residues were extracted two more times with 70% acidified hydro ethanol following the same procedure. The pooled filtrate was concentrated in a rotary evaporator (bath temp. 40°C) and finally dried under vacuum.

2.3. Estimation of total phenolics

Total phenolics content in test extract was determined by Folin-Ciocalteu method using gallic acid as standard [22]. Briefly 0.5 ml of test extract was mixed with 0.5 ml of 10% aqueous Folin-Ciocalteu reagent, stirred and left for 5 min in the dark. Then, 1 ml of aqueous 7.5% Na_2CO_3 solution was added and incubated in the dark at room temperature for 90 min. Reagent blank was also prepared using distilled water. The absorbance was measured against the prepared reagent blank at 765 nm. The total phenolic content in the extract was expressed as mg gallic acid equivalent (GAE) per g of dry extract. Each experiment was repeated thrice.

2.4. Estimation of total anthocyanin content

Total anthocyanin content of test hydro ethanol extract was estimated by pH differential method as described by Cheng and Breen [23] and expressed as cyanidin-3-glucoside equivalent using molar extinction coefficient of 26,900 $M^{-1}cm^{-1}$and molecular mass of 449.2 g mol^{-1}. Briefly, 0.2 ml of test extract was diluted in 0.8 ml of two different buffers (0.025 M potassium chloride *p*H = 1.0 and 0.4 M sodium acetate *p*H = 4.5), and incubated for 15 min at room temperature. After incubation, absorbance (A) was measured at 510 and 700nm and the obtained data were put in to the following formula.

$$A = [(A_{510} - A_{700})_{pH\ 1.0}] - [(A_{510} - A_{700})_{pH\ 4.5}]$$

Now, total anthocyanin content (TA) = (A × MW ×DF × 1000) / (ε × λ × m)

Where, DF is the dilution factor, λ is the cuvette optical path length (1 cm) and m is the weight of the test extract (g).

2.5. Evaluation of *in vitro* antioxidant activity

2.5.1. DPPH radical scavenging activity

Free radical scavenging activity of test extract of *S. cumini* fruit pulp was evaluated quantitatively using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as described by Wang et al. [24]. Briefly, 100µl of test extract in varying concentrations (3.125µg/ml – 100µg/ml) were taken in test tubes and 3.9 ml of 0.1mM solution of DPPH in methanol was added to these tubes and shaken vigorously. The tubes were then allowed to stand in dark at room temperature for 30 min. The control was prepared as above without test extract and methanol was used for zero adjustment. Absorbance of the samples was measured at 517 nm. Inhibition of the DPPH radical by the test extract was calculated according to the following formula.

$$(\%)\ \text{Free radical scavenging} = \frac{(\text{Ablank} - \text{Asample})}{\text{Ablank}} \times 100$$

Where A_{sample} is the absorbance of DPPH solution after reacting with a given concentration of anthocyanin-rich extract and A_{blank} is the absorbance of DPPH solution with methanol blank instead of test extract. The percentage of DPPH radical scavenging activity was plotted against the concentration of test extract and IC_{50} value of test extract against DPPH free radical scavenging activity was calculated. All tests were performed in triplicate.

2.5.2. ABTS radical cation ($ABTS^{\circ+}$) decolorization activity

ABTS radical cation ($ABTS^{\circ+}$) decolorization activity of test extract of *S. cumini* fruit pulp was determined by improved $ABTS^{\circ+}$ scavenging assay method [25]. Briefly, $ABTS^{\circ+}$ was prepared by mixing a 7mM ABTS (2,2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid) in water with 2.45mM potassium persulfate (1:1, v/v) and stored in the dark at room temperature for 12-16h until the reaction was complete and the absorbance was stable. $ABTS^{\circ+}$ solution was then diluted with methanol to obtain an absorbance of 0.700±0.05 at 734 nm. Now, 5µl of varying concentrations (3.125µg/ml - 100µg/ml) of test extract was added to tubes containing 3.995 ml of diluted $ABTS^{\circ+}$ solution, mixed thoroughly and kept for 30 min at room temperature. The absorbance of the solution was then measured at 734 nm. Methanol was used as negative control. All the measurements were carried out in triplicate. The $ABTS^{\circ+}$ scavenging activity was calculated using the following formula:

$$ABTS^{\circ+}\ \text{scavenging effect}\ (\%) = [(A_B - A_A/A_A)] \times 100$$

Where, A_B is the absorbance of $ABTS^{\circ+}$ + methanol and A_A is the absorbance of $ABTS^{\circ+}$ + test extract.

IC_{50} value of test extract against $ABTS^{\circ+}$scavenging activity was determined.

2.5.3. Ferrous (Fe^{2+}) ion chelating activity

Ferrous ion (Fe^{2+}) chelating activity of test extract of *S. cumini* fruit pulp was evaluated following the method of Dinis et al. [26]. Briefly, 50µl of 2mM $FeCl_2$ solution was added in tubes containing 100µl of varying concentrations (3.125µg/ml - 100µg/ml) of test extract. The reaction was initiated by the addition of 0.2ml of 5mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm using a spectrophotometer (Thermo Fisher Scientific, USA). Sample control was made without adding test extract. The percentage inhibition of ferrous-ferrozine complex formation was calculated using the formula:

% inhibition = $[1 - A_s/A_c] \times 100$.Where A_c is the absorbance of the negative control, A_s is the absorbance of the sample. Each experiment was repeated thrice. IC_{50} value of test extract against Fe^{2+} ion chelating activity was determined.

2.6. Evaluation of antioxidant efficacy against oxidation of omega-3 fatty acids in (i) bulk oil and (ii) oil-in-water emulsions model systems (Food models)

2.6.1. Preparation of oil-in-water emulsions

Before going to evaluate antioxidant efficacy of test extract against oxidation of omega-3 fatty acids enriched food supplement in bulk oil and oil-in-water emulsions model systems, at first an oil-in-water emulsions model of omega-3 fatty acids enriched food supplement was prepared following the method described by Yamamoto and Misawa

[27].Briefly for preparing oil-in-water emulsions model, 0.5 g of Tween 20 (as emulsifier) and 5 g of marine fish oil were weighed into a glass beaker and mixed together by a magnetic stirrer, then 44.5 ml deionized water was added and mixed thoroughly using a high-speed homogenizer.

Next, to evaluate antioxidant potential of test extract of *S. cumini* fruit pulp in bulk oil and oil-in-water emulsions model systems, the following three groups of samples were prepared.

2.6.2. Test extract treated group

- 1000µl of bulk oil + 50 µg/ml of test extract
- 1000 µl of oil-in-water emulsions +50µg/ml of test extract

2.6.3. Positive control treated group

- 1000µl of bulk oil + 50 µg/ml of BHT (maximum allowable limit in food industry)[28].
- 1000µl of oil-in-water emulsion+50µg/ml of BHT

2.6.4. Negative control treated group (without test extract or BHT)

- 1000µl of bulk oil
- 1000µl of oil-in-water emulsions

All samples were shaken, cooled, homogenized and kept in an oven at 60°C for 15 days. Aliquots were removed at different day intervals (0-, 3-, 6-, 9-, 12- and 15-day of incubation) for evaluation of antioxidant potential. The antioxidant potential was monitored by determining the peroxide value (PV) as indicator of primary oxidation [29], *p*-anisidine value (*p*-AV) as indicator of secondary oxidation [30] and TOTOX value [2PV+*p*-AV] as indicator of total oxidation [31].

2.7. Chemical Analysis

2.7.1. HR-LCMS analysis

The chemical composition of test extract was analyzed by HR-LCMS analysis technique. The instrument used is hrlcms-qtof-Agilent Technologies, USA. Data Acquisition Software is Agilent MAss Hunter and Data Processing Software is Agilent Mass Hunter Qualitative Analysis B.06. Column type is ZORBAX Eclipse Plus-C18 150x2.1 MM, 5 microns (Agilent).MS source conditions were as follows: capillary voltage 3500 V, Gas temperature 250°C, drying gas flow 13 L/min, sheath Gas temp 300, sheath Gas Flow 11, nebulizing gas pressure 35 (psig). Solvent used are, Solvent A:0.1% formic acid in Milli-Q water, and Solvent B: Acetonitrile. MS range m/z: 120-1200; MS scan rate: 1 spectra/sec. Injection Mode: 3.00 µl; Flow rate: 0.300 ml/min; Stop time: 40.00 min. The analysis was performed at SAIF, IIT Bombay, India.

2.8. Cytotoxicity Study

2.8.1. Allium cepa root growth inhibition assay

Cytotoxic potential of test extract of *S. cumini* fruit pulp was evaluated by *Allium cepa* root growth inhibition assay following the method described by Fiskesjo [32].Briefly healthy onion bulbs (20-25g) were procured from Baranagar Bazar Market, Kolkata, India, dried for 3 days at room temperature. Without destroying the root primordial, the outer scales of the dried bulbs were removed away. Washed thoroughly in running tap water to remove any impurities adhered over its surface and allowed to germinate at room temperature for 3-4 days in glass tubes containing tap water until the new roots were 3-4 cm in length. After the newly roots were emerged, a total of ten groups of bulbs containing six bulbs in each group were taken. The test extract at various concentrations (15.62, 31.25, 62.5, 125, 250, 250, 500, and 1000µg/ml) was given to eight test tubes. The negative control group received tap water only and positive control group received sodium azide (100µg/ml).The tubes were then incubated at room temperature for 96h in the dark. After incubation, the best developed 10 roots of *Allium cepa* bulb in each group were measured and mean root length (cm) was determined. Considering the average root length of the negative control group as 100% growth, decrease in root growth of test extract treated groups and positive control group were determined. A graph was plotted using various concentrations of extract vs. % inhibition in root length. LC_{50} value of test extract was determined from the graph. Moreover, at the end of the incubation period (96 h), visible morphological verifications, such as changes in root consistency and color as well as the presence of swelling, hooks or twists in the roots, if any, were recorded. Each experiment was repeated thrice.

2.9. Statistical Analysis

Data were expressed as mean ± S.D. of triplicate experiments. Data were statistically analyzed by one-way ANOVA and Tukey's post hoc test using R software with the level of significance set at $p < 0.05$.

3 Results

3.1. *In vitro* antioxidant activity

Figure 1 shows the results of antioxidant activity of test extract of *S. cumini*fruit pulp in various *in vitro* models (DPPH radical scavenging, ABTS radical cation decolorization and Fe^{2+} ion chelating). It was observed that test extract exhibited concentration- dependent varying degrees of antioxidant activity in various *in vitro* models. On the basis of IC_{50} values, the test extract was found to be effective in the following decreasing order: DPPH radical scavenging (IC_{50}: 35.30 µg/ml) > $ABTS^{\circ+}$ decolorization (IC_{50}: 39.38 µg/ml) > Fe^{2+} ion chelating (IC_{50}: 54.56 µg/ml) (Figure 1).

Assay	IC_{50} (µg/ml)
DPPH radical scavenging	35.30
$ABTS^{0+}$decolorization	39.38
Fe^{2+}ion chelating	54.56

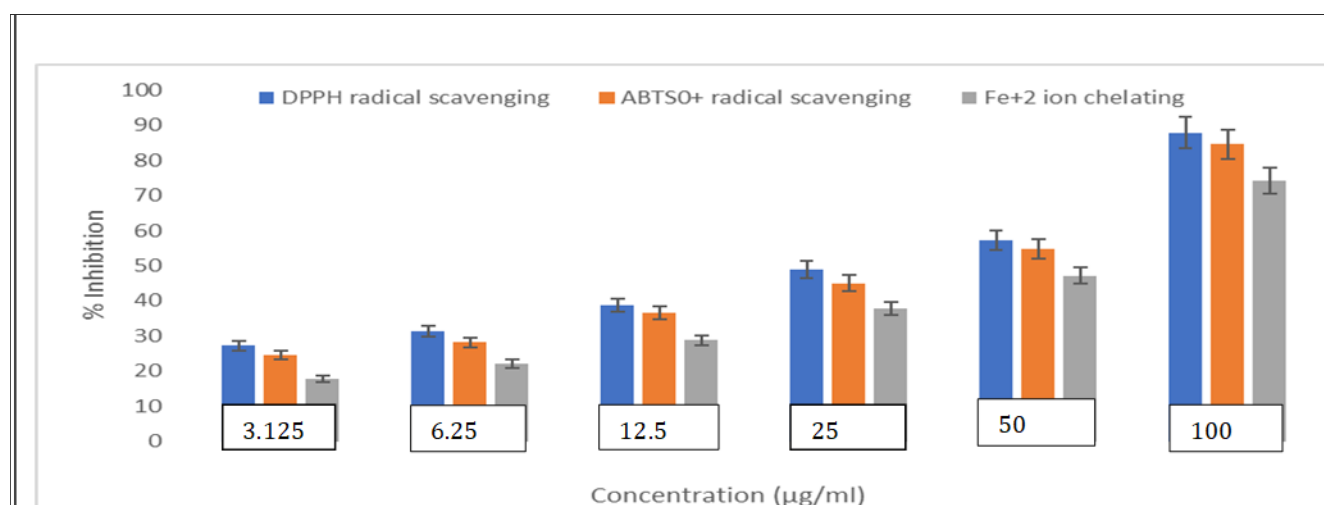

Figure 1 Antioxidant efficacy of anthocyanin-rich hydro ethanolextract of *S. cumini*fruit pulp in various *invitro* models (DPPH radical scavenging, $ABTS^{\bullet+}$ decolorization and Fe^{2+} ion chelating)

3.2. Antioxidant activity in bulk oil and oil-in-water emulsions model systems (Food models)

After evaluating antioxidant activity of test extract in various *in vitro* models, its effects against oxidation of omega-3 fatty acids enriched food supplement in two food model systems (bulk oil and oil-in water emulsions) were evaluated.

Table 1 shows the results of antioxidant potential of test extract against primary and secondary oxidation of omega-3 fatty acids enriched food supplement in bulk oil model system. It was observed that the test extract exhibited significant antioxidant activity ($P<0.05$) against both primary and secondary oxidation of omega-3 fatty acids in bulk oil model system from day 3 onwards whereas BHT was found to be significantly effective ($P<0.05$) from day 6 onwards compared to respective controls.

Table 1 Results on the effects of anthocyanin-rich hydro ethanol extract of *S. cumini*fruit pulp against primary and secondary oxidation of omega-3 fatty acids enriched food supplement in bulk oil model system

Treatment	Primary oxidation in bulk oil Peroxide value (PV) (meq/kg oil)						Secondary oxidation in bulk oil *p*-anisindine value (*p*-AV) (meq/kg oil)					
	Incubation period (days)						Incubation period (days)					
	0	**3**	**6**	**9**	**12**	**15**	**0**	**3**	**6**	**9**	**12**	**15**
Control	1.35 ± 0.07	7.84 ± 0.08	9.30 ± 0.24	12.34 ± 0.57	15.76 ± 0.57	19.91 ± 0.22	1.36 ± 0.06	11.90 ± 0.25	18.31 ± 0.13	26.27 ± 0.40	30.40 ± 0.49	33.78 ± 0.30
Anthocyanin Extract*	1.33 ± 0.09	5.02 ± 0.02	7.02 ± 0.18	7.99 ± 0.55	8.87 ± 0.50	9.40 ± 0.55	1.43 ± 0.06	9.61 ± 0.11	14.14 ± 0.46	18.46 ± 0.15	19.84 ± 0.47	21.22 ± 0.39
BHT#	1.32 ± 0.07	6.25 ± 0.60	6.27 ± 0.42	9.87 ± 0.43	11.48 ± 0.69	12.73 ± 0.32	1.41 ± 0.11	10.53 ± 0.40	15.40 ± 0.12	21.25 ± 0.24	23.50 ± 0.60	24.04 ± 0.52

Values are Mean ± S.D. of triplicate experiments. Mean values of *Anthocyanin-rich extract treated group against both primary and secondary oxidation of omega-3 fatty acids enriched food supplement in bulk oil model system were found to be significantly lower (P < 0.05) from day 3 onwards when compared with respective controls whereas for #BHT treated group it was found to be significantly lower (P<0.05) from day 6 onwards

Table 2 shows the results of antioxidant potential of test extract against primary and secondary oxidation of omega-3 fatty acids enriched food supplement in oil-in-water emulsions model system. A significant (P < 0.05) antioxidant activity of test extract and BHT against both primary and secondary oxidation of omega-3 fatty acids enriched food supplement in oil-in-water emulsions model system was observed from day 9 onwards compared to respective controls. Moreover, the test extract was found to be more effective in bulk oil (Table 1) than oil-in-water emulsions(Table 2) model systems against both primary and secondary oxidation of omega-3 fatty acids.

Table 2 Results on the effect of anthocyanin-rich hydro ethanol extract of *S. cumini* fruit pulp against primary and secondary oxidation of omega-3 fatty acids enriched food supplement in oil-in-water emulsions model system

Treatment	Primary oxidation in oil-in-water emulsions Peroxide value (PV) (meq/kg oil)						Secondary oxidation in oil-in-water emulsions *p*-anisindine value (*p*-AV)(meq/kg oil)					
	Incubation period (days)						Incubation period (days)					
	0	**3**	**6**	**9**	**12**	**15**	**0**	**3**	**6**	**9**	**12**	**15**
Control	1.39 ± 0.07	7.51 ± 0.33	10.65 ± 0.22	15.06 ± 0.66	16.87 ± 0.87	20.90 ± 0.48	1.82 ± 0.08	16.42 ± 0.11	21.00 ± 0.44	26.04 ± 0.38	32.94 ± 0.88	42.38 ± 0.30
Anthocyanin Extract*	1.35 ± 0.05	6.45 ± 0.19	8.36 ± 0.46	11.16 ± 0.09	12.03 ± 0.30	13.76 ± 0.32	1.79 ± 0.06	14.69 ± 0.19	18.08 ± 0.45	19.79 ± 0.61	23.89± 0.60	28.83 ± 0.56
BHT*	1.40 ± 0.02	6.90 ± 0.55	9.28 ± 0.10	12.24 ± 0.21	13.21 ± 0.68	15.09 ± 0.33	1.75 ± 0.07	15.25 ± 0.11	19.07 ± 0.14	22.27 ± 0.76	26.14 ± 0.60	32.29 ± 0.62

Values are Mean ± S.D. of triplicate experiments. *Mean values of both anthocyanin-rich extract and BHT treated groups in comparison to respective control values were found to be significantly lower (P < 0.05) from day 9 onwards.

The third important oxidative indicator of lipid oxidation is the total oxidation (TOTOX) which is the combined action of both primary and secondary oxidation of lipids. The results of antioxidant efficacy of test extract against total oxidation of omega-3 fatty acids in bulk oil and oil-in-water emulsions model systems are shown in Figure 2 and Figure 3 respectively. From both Figure 2 and Figure 3, it was observed that total oxidation of omega-3 fatty acids was reduced significantly ($P < 0.05$) in time-dependent manner both in bulk oil and oil-in-water emulsions model systems by the test extract compared to respective controls. The reduction in total oxidation by test extract in bulk oil model system (Figure 2) was found to be much greater than oil-in-water emulsions model system (Figure 3).

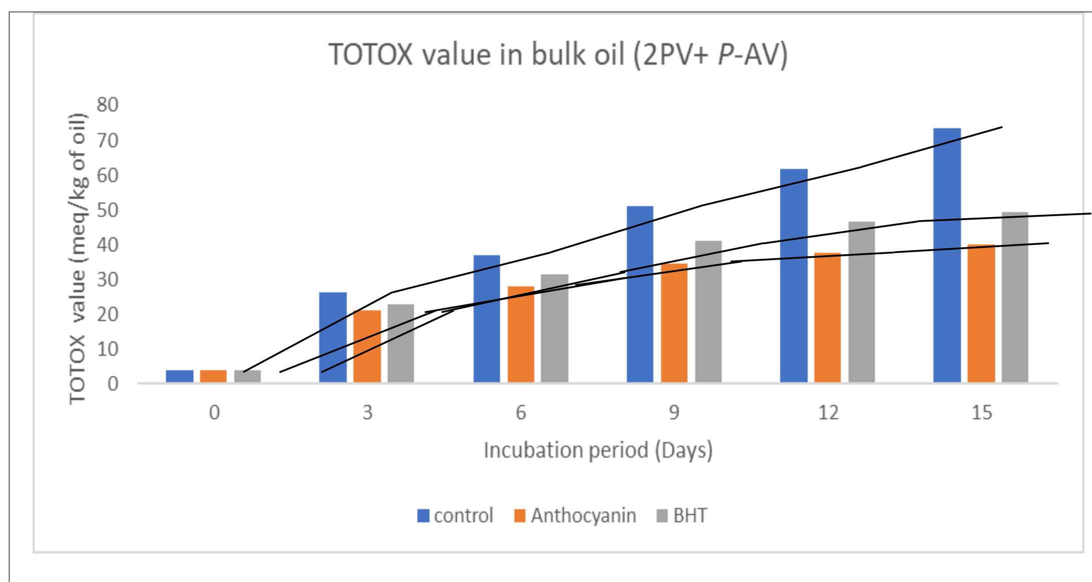

Figure 2 TOTOX value of anthocyanin-rich hydro ethanol extract of *S. cumini* fruit pulp against oxidation of omega-3 fatty acids in bulk oil model system

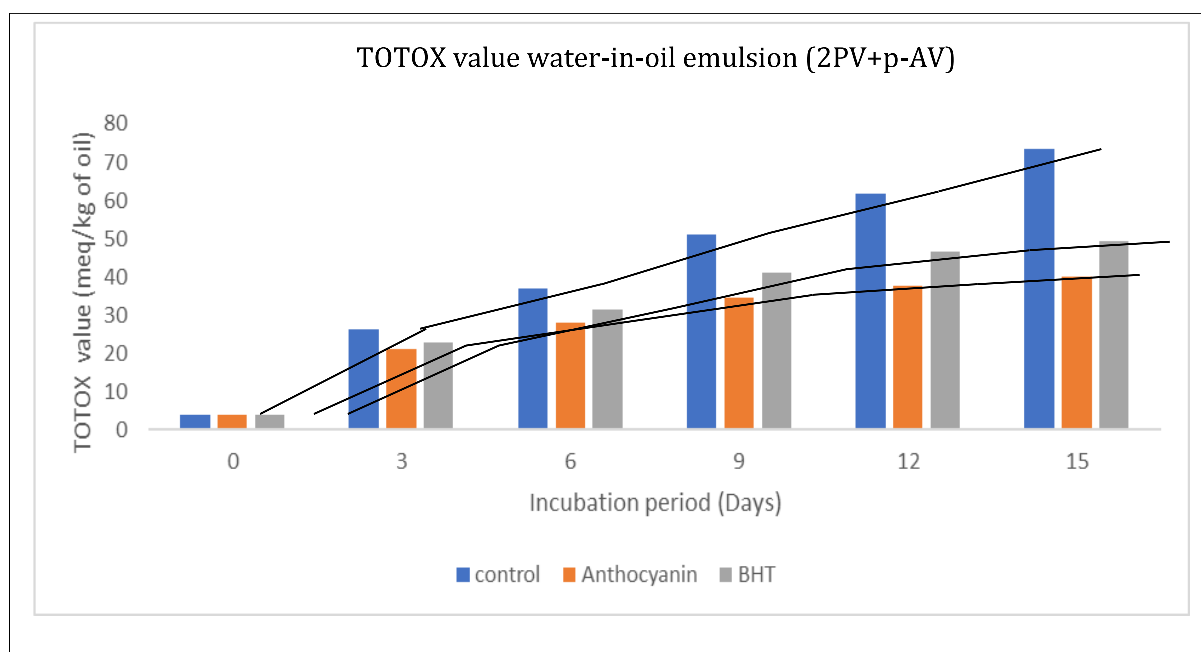

Figure 3 TOTOX value of anthocyanin-rich hydro ethanol extract of *S. cumini* fruit pulp against oxidation of omega-3 fatty acids in oil-in-water emulsions model system

3.3. Chemical analysis

HR-LCMS chromatograms of test extract (Figure 4) revealed the presence of twenty different chemical components of which only three components were present as major components of the extract. Based on retention time (R_T), mass and

available literature data, these three major components were tentatively identified as malvidin-3,5-diglucoside, delphinidin-3,5-diglucoside and peonidin-3,5-diglucoside (Figure 4).

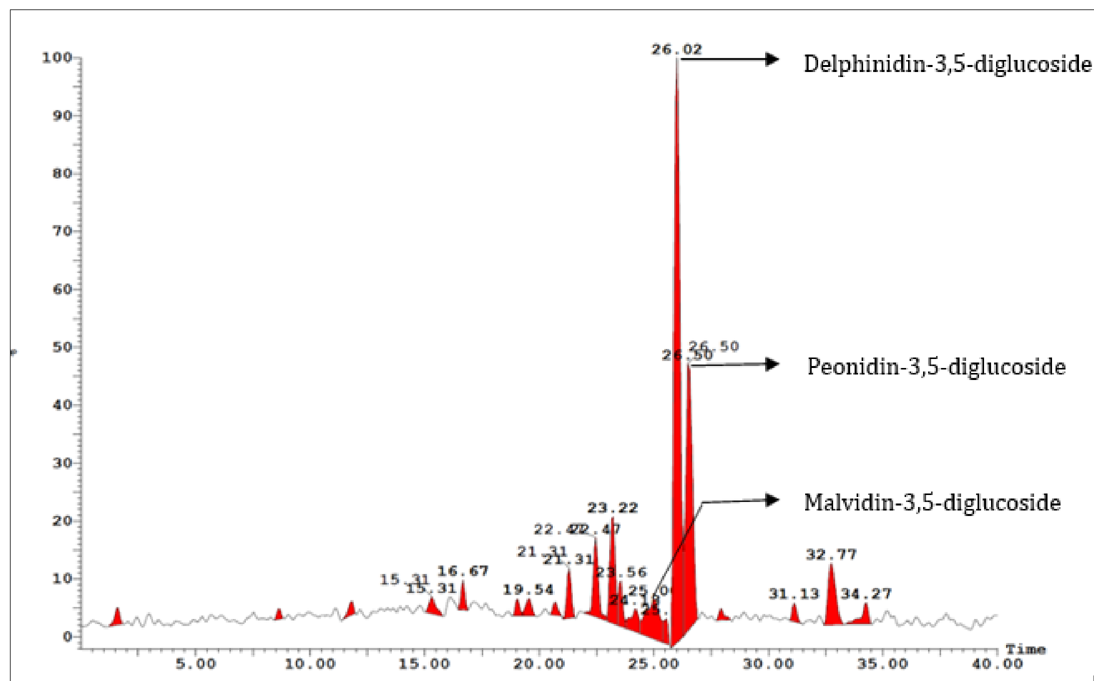

Figure 4 HR-LCMS chromatograms of anthocyanin-rich hydro ethanol extract of *S.cumini*fruit pulp

3.4. Cytotoxicity study

The results of cytotoxic potential of test extract in *Allium cepa* test are shown in Table 3. No noticeable cytotoxic potential of test extract was observed up to 1000 µg/ml concentration and LC_{50} of the test extract was found to be >1000 µg/ml(Table 3). Moreover, no visible morphological changes in root consistency and color as well as the presence of swelling, hooks or twists in the roots were observed.

Table 3 Root growth inhibitory activity of anthocyanin-rich hydro ethanol extract of *S. cumin* fruit pulp in *Allium cepa* test

Concentration of test extract (µg/ml)	Root Length (cm)	Root growth inhibition (%)	LC50 (µg/ml)
Negative Control (Tap water)	3.7±0.3	0	>1000
15.62	3.7±0.1	0	
31.25	3.7±0.2	0	
62.50	3.7±0.2	0	
125	3.7±0.1	0	
250	3.7±0.2	0	
500	3.6±0.1	2.70	
1000	3.4±0.2*	8.10	
Positive Control(Sodium azide;100µg/ml)	1.2±0.1	67.56	

*Not significantly different when compared with negative control but found to be significantly different (P<0.05) when compared with positive control

4 Discussion

Relevant literature reveals that both omega-6 and omega-3 polyunsaturated fatty acids are important components of cell membrane and human body needs them for many functions from building healthy cells to maintaining brain and nerve functions [33]. But they have opposite effects when it comes to cardiovascular system and inflammatory responses. High intake of omega-6 fatty acids enriched oils in our diet over time may cause certain diseases such as cardiovascular disease, arthritis, Alzheimer's disease, diabetes or even cancer due to pro-inflammatory property of eicosanoids (20-C signaling molecules) produced from metabolism of omega-6 fatty acids. Whereas, these diseases are suppressed by intake of omega-3 fatty acids enriched oils due to anti-inflammatory property of eicosanoids produced from metabolism of omega-3 fatty acids [34].Therefore, proper ratio between omega-6 and omega-3 fatty acids in our diet should be maintained. Western diets contain high level of omega-6 fatty acids but very low level of omega-3 fatty acids leading to omega-6/omega-3 fatty acids ratio of 15:1 to 20:1 or even higher. Ideally, this ratio should be 1:1 or to a maximum of 4: 1. Lower the ratio better is the chance to reduce chronic diseases [5, 35]. Therefore, in recent years, to increase the nutritional value of foods and reduce the risk of chronic diseases, omega-3 fatty acids enriched oils have been added to foods. But, developing omega-3 fortified stable functional foods is challenging, because the two principal omega-3 fatty acids components eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are highly susceptible to oxidation due to high degree of unsaturation in their molecular structure. This problem should be addressed to develop omega-3 fatty acids fortified stable functional foods. In the present investigation, we therefore evaluated antioxidant potential of anthocyanin-rich hydro ethanol extract of *S. cumini* fruit pulp in various *in vitro* models and in omega-3 fatty acids enriched food supplement in two food lipid model systems (bulk oil and oil-in-water emulsions) along with chemical analysis of the extract and assessment of its cytotoxic potential, if any, with a view to shed some light on these important aspects.

To evaluate antioxidant efficacy of test extract in various *in vitro* models, its effects in DPPH radical scavenging, $ABTS^{\circ+}$ decolorization and Fe^{2+} ion chelating activity were evaluated. DPPH radical scavenging method was used to know its effect on stable free radicals (antiradical), $ABTS^{\circ+}$ decolorization method was used to evaluate its effects on propagated free radicals (antioxidant) and Fe^{2+} ion chelating method was used to evaluate the effect of test extract on pro-oxidant metal ions (anti-prooxidant).The findings of these three *in vitro* methods may focus some light regarding possible mode of antioxidant action of test extract. From Figure 1, it was observed that test extract exhibited varying degrees of concentration-dependent antioxidant efficacy in various *in vitro* models. On the basis of IC_{50} values, the test extract was found to be more effective in DPPH radical scavenging (IC_{50}: 35.30 μg/ml) followed by $ABTS^{\circ+}$ decolorization (IC_{50}: 39.38μg/ml) and Fe^{2+} ion chelating (IC_{50}: 54.56μg/ml)(Figure 1) suggesting that the test extract is more effective for scavenging stable free radicals followed by propagated radicals and pro-oxidant metal ions. Our results are in consistent with the findings of other workers where varying degrees of antioxidant efficacy of different solvent extracts of *S. cumini* fruits in various *in vitro* models have been reported [16, 36].

Many *in vitro* methods are used to evaluate antioxidant efficacy of compounds by measuring free radical scavenging and metal ion chelating activities. These *in vitro* methods often correlate poorly with the ability of antioxidants to inhibit oxidative deterioration of foods. This is because the activity of antioxidants in food systems depends not only on free radical scavenging and metal ion chelating property but also on chemical, physical and environmental conditions (e.g. *p*H) of the food products. Thus, to accurately evaluate the potential of antioxidants in foods, model foods such as bulk oil and oil-in-water emulsions must be developed (37, 38).In the present investigation, we therefore, in addition to *in vitro* antioxidant activity study, evaluated antioxidant potential of test extract in two types of food lipid model systems: bulk oil and oil-in-water emulsions taking marine fish oil as omega-3 fatty acids enriched food supplement.

In food lipid model systems, the test extract showed varying degrees of antioxidant activity against oxidation of omega-3 fatty acids both in bulk oil and oil-in-water emulsions model systems suggesting that the test extract has the ability to reduce oxidation of omega-3 fatty acids enriched food supplement in both the food lipid model systems. The third important oxidative indicator of lipid oxidation is the total oxidation (TOTOX) which is considered to be the most important evaluation in determining oil's rancidity because combination of both primary oxidation (PV) and secondary oxidation (*p*-AV) give a good indication of the overall rancidity or quality of oil. The lower the TOTOX value, the better is the quality of oil (38,39). Therefore, after evaluating primary and secondary antioxidant efficacy of test hydro ethanol extract both in bulk oil and oil-in-water emulsions model systems, its effect on total oxidation (TOTOX) was also evaluated. It was observed that test extract has the ability to reduce total oxidation of omega-3 fatty acids both in bulk oil and oil-in-water model systems significantly ($P<0.05$) in time-dependent manner compared to respective controls (Figure 2 and Figure 3).These promising findings indicated that the test extract has the ability to protect omega-3 enriched food from rancidity as well as preserve its quality and may serve as an effective antioxidant against oxidation of omega-3 fatty acids enriched food. Moreover, test extract was found to be more effective in bulk oil (Table 1; Figure 2) than oil-in-water emulsions (Table 2; Figure 3).The difference in activity in oil-in-water emulsions model system

compared to bulk oil is not clear right now. It may be explained by their interfacial partitioning into the water phase of emulsion thus becoming less protective than in bulk oil system [39].

Now, before going to HR-LCMS analysis of test extract, its total phenolics and anthocyanin content were estimated. Total phenolics and total anthocyanin content of test extract were found to be 517 mg GAE $100g^{-1}$ and 289mg cyanidin-3-glucoside$100g^{-1}$ respectively. These findings suggest that *S. cumini* fruit pulp is a good source of bioactive compounds. Our results are in corroboration with the findings of other workers where total phenolics and total anthocyanin content of *S. cumini* fruit pulp found to be 182.42-2133mg GAE $100g^{-1}$ and 147.88-349 mg cyanidin-3-glucoside$100g^{-1}$ respectively[40-43]. Next, the test extract was subjected to HR-LCMS analysis to know the total number of components as well as major components of the test extract. Data from HR-LCMS chromatograms (Figure 4) revealed that the test extract contains twenty different components of which three as major components. Based on retention time (R_T), mass to charge ratio (m/z) and available literature data [16, 44, 45], the three major components were tentatively identified as malvidin-3,5-digluccoside, delphinidin-3,5-diglucoside and peonidin-3,5-diglucoside.

Next, cytotoxic potential of test extract, if any, was evaluated using *Allium cepa* root growth inhibition assay, because it is a useful step in determining the potential toxicity of a test substance including crude plant extracts and biologically active natural and synthetic compounds. *Allium cepa* test was used because this *in vitro* test is very useful as a first-tier analysis of cytotoxicity due to its simplicity, low relative cost, versatility and minimum laboratory facilities required for its performance [46]. It has been shown to have a correlation with tests in other living systems and serve as an indicator of toxicity of the tested material. Inhibition of root growth appears to be the most sensitive parameter because root growth inhibition is manifestation of an arrest of a cell division [47]. In the present investigation, test extract did not show noticeable cytotoxic potential up to 1000 μg/ml concentration when compared with negative control and LC_{50} of test extract was found to be >1000μg/ml (Table 3). According to Meyer et al. [48], crude plant extracts with LC_{50} > 1000 μg/ml can be considered as non-toxic. Our test extract having LC_{50}> 1000 μg/ml can therefore be considered as a safe substance.

5 Conclusion

The results provide evidence that anthocyanin-rich hydro ethanol extract of *S. cumini* fruit pulp exhibited strong antioxidant efficacy both in various *in vitro* models and in food lipid model systems and may serve as a potential source of safe and effective natural antioxidants for retarding oxidation of omega-3 fatty acids enriched food supplement both in bulk oil and oil-in-water emulsion model systems. These promising findings may help in developing omega-3 fatty acids fortified stable functional foods in food industry. This report may serve as a footstep on these important aspects.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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