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Impact of Aqueous/Ethanolic Goji Berry (*Lycium barbarum*) Fruit Extract Supplementation on Vitamin D Stability in Yoghurt

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A B S T R A C T

Deficiency of vitamin D is a multifaceted phenomenon. On one hand, it depends on sun exposure and consumption pattern while on the other hand, it depends onit's stability in target food matrix. It is assumed that the supplementation of bioactive phenolic compounds in target food matrix could result in higher stability of vitamin D. The current study was intended to evaluate the feasibility of supplementation of polyphenolic goji berry (*Lycium barbarum)* extract in yoghurt and to study its impact on vitamin D stability in yoghurt during shelf life. For that purpose yoghurt (fortified with vitamin D 600IU/100ml) was supplemented with 0.05, 0.10 and 0.15% (W/V) with both aqueous as well ethanolic extract. We have also evaluated consumer's acceptability of polyphenol extract supplemented yoghurt during its shelf life. The result indicates that supplementation of yoghurt with goji berry polyphenolic ethanolic extract (0.1% W/V) displayed greater ability to inhibit vitamin D degradation without affecting the consumer palatability during shelf life.

Introduction

Vitamin D refers to a group of secosteroid compounds, which are synthesized in plants and animals as the action of UV radiation. Vitamin D is susceptible to degradation and the extent depends on storage conditions such temperature, light and humidity, which is due to oxidation of the conjugate double bond (Grady and Thakker, 1980; LI and MIN, 1998). Vitamin D deficiency is prevailing even in countries with sufficient sunshine due to their socioeconomic constraints that prevent people to expose themselves to sunlight and to consume vitamin D rich food (Roy *et al.*, 2015; Wayse *et al.*, 2004). In that case, fortification of vitamin D in food becomes compulsion for healthy life. Fortification of vitamin D in food matrix also brings limitation within it, which is degradation of vitamin D in target food (Tangpricha *et al.*, 2003; Wagner *et al.*, 2008).

This creates need for supplementation of natural antioxidants within target food which offers antioxidant properties against the oxidation of vitamin D. Berry fruits like strawberry, blackberry, cranberry and goji berry are recognized for their diverse phenolic compounds which offer high antioxidative properties. Goji berry (*Lycium barbarum)*is

one of the well reported berry rich in diverse groups of phytochemicals (Benchennouf *et al.,* 2017; Chen *et al.,* 2014; Vulić *et al.,* 2016; Yang *et al.,* 2015; Zhang, 2013; Zhang *et al.,* 2016). Recent literature suggests that addition of natural fruits extract, rich in phenolic contents, offers high antioxidative properties as well as various other health benefits (Cossu *et al.,* 2009; El-Said *et al.,* 2014; Karaaslan *et al.,* 2011). Due high antioxidant activity goji berry extract have been applied in variety of food products such as sausages (Bulambaeva *et al.,* 2014), beverages (Ducruet *et al.,* 2017; Navarro *et al.,* 2011), dairy products (ROTAR *et al.,* 2014; Rotar *et al.,* 2015), bread (Yang, 2016) and chocolate (Morais Ferreira *et al.,* 2017).

To date milk is considered as the most suitable candidate for vitamin D fortification. But it brings several challenges with it one of them is the generation of reactive oxygen species (ROS) due presence of riboflavin which one of primary source for ROS (LI and MIN, 1998). There is scarcity of data on vitamin D fortification in foods other than milk. The stability of vitamin D has been reported in bread, processed dairy products (Wagner *et al.,* 2008), juice (Tangpricha *et al.,* 2003) and tea (Grant *et al.,* 2017). These reports suggest that vitamin D stability depends on matrix, composition, processing and local environments (pH, water activity and temperature of target food).Yoghurt is a fermented dairy product for lactose intolerant consumer. It is among the most common dairy products consumed across the globe, which also contains probiotic that makes it even healthier food. Yoghurt could be a potential vehicle for vitamin D delivery for various reasons: (i) the milk fat may offer high stability and bioavailability of vitamin D (ii) yoghurt is rich source of calcium and can consume by lactose intolerant consumers as it contains lactose in traces. Despite of various health benefits goji berry has been not exploited to supplement dairy product. There are very few studies present in current literature reporting supplementation of daily products with goji berry (ROTAR *et al.,* 2014; Rotar *et al.,* 2015). This brings immense opportunity to fortify yoghurt with polyphenolic aqueous as well ethanolic goji berry fruit extract. The present study was undertaken to evaluate the vitamin D stability in yoghurt supplemented with aqueous/ ethanolic polyphenolic goji berry extract during its shelf life.

Materials and Methods

Raw materials

Pasterurised toned milk (Motherdaiy, India), edible coconut oil and dried goji berry fruit (*Lycium barbarum)* were purchased from a local supermarket. Commercial yoghurt culture (yo-FAST-88) provided by Chr. Hansen, Hrsholm, Denmark.

Chemicals and reagents

HPLC grade chemicals (n-hexane, ethanol, and isopropyl alcohol), sodium chloride, Folin-Ciocalteu reagent, Dimethyl sulfoxide (DMSO) were procured from Merck chemicals Company, Germany. Vitamin D_3 and gallic acid were purchased from Sigma chemicals. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and Tween 80 was procured from SRL chemicals. While soya lecithin (Leciva 70S) was purchased from Vav Life Sciences, India.

Preparation of aqueous extract of goji berry polyphenol

Preparation of the gojiberry polyphenol aqueous extract was carried out by Cossu *et al.,* (2009) method with some modification (Cossu *et al.,* 2009). 5 g of lyophilized (Benchtop, Vir Tis, USA) gojiberrry fruit was

homogenized 20 ml milli Q water using tissue homogenizer (Polytron, Kinematic AG, Switterzerland) and subjected to boiling for 30 min and centrifuged at 6000g for 15 min. The super natent was collected frozed and concentrated using lyophilizer (Benchtop, VirTis, USA). The concentrated phenol extract was stored at -20^{0}C untile further use.

Preparation of ethanolic extract of goji berry polyphenol

Extraction of ethanolic polyphenol was carried out using Donno *et al.,* (2015)with some modification (Donno *et al.,* 2015). 5 g of lyophilized gojiberrry fruit (Benchtop, VirTis, USA) was homogenized 200 ml absolute ethanol and subjected to boiling for 30 min (Polytron, Kinematic AG, Switterzerland) and centrifuged at 6000g for 15 min.

The super natent was collected and concentrated using rotary evaporator at 55^{0}C for 5 min (Buchi, Switterzerland). The concentrated phenol extract was stored at -20^{0}C until further use.

Vitamin D nanoemulsion premix

Vitamin D_3 emulsion was prepared with four basic components oil phase, containing coconut oil, soy bean lecithin (Leciva), vitamin D and Tween 80, and aqueous phase containing mili Q water and 5% NaCl.

The emulsion was prepared according to Phase inversion temperature (PIT) process (Fathi and Varshosaz, 2013 Heurtault *et al.*, 2003; Kiani *et al.,* 2017). Preparation method includes two basic steps: (i) Formation of W/O emulsion by homogenizing all components (Coconut oil, Tween 80, Lecithin, NaCl, vitamin D and water) at 15000 rpm for 5 min (Polytron, Kinematic AG, Switzerland). Then solution was allowed to heat from room temperature to about 85^{0}C (above the PIT) and followed by cooling it to 65^{0}C (bellow the PIT) in shaker waterbath (Metrex Scientific Instruments, India). This resulted in formation of an O/W emulsion. In order to cross the phase inversion zone (PIZ) 3 temperature cycles were carried out from 85^{0}C to 65^{0}C. (ii) Sudden dilution with chilled water (0^{0}C) led to irreversible shock and breaking of the micro emulsion system and forming of stable nanoemulsion. Final vitamin D concentration in nanoemulsion premix was 100000IU/2.50mg/mL

Preparation of yoghurt

16g sucrose was added in vitamin D fortified toned milk and then heated in a waterbath at 85^{0}C for 30 min, cooled to approximately 43^{0}C, inoculated with commercial yoghurt culture and transferred to 100ml cups, incubated at 43^{0}C for 4h in BOD incubator (Metrex Scientific instrument, India)and stored at 4^{0}C overnight before further analysis.

Yoghurt fortification

To prepare fortified yoghurt with goji berry polyphenol extract and vitamin D premix, the coagulum was broken by gentle stirring using a hand blender and mixed with concentrated goji berry polyphenol aqueous/ethanolic extract (0.05, 0.10 and 0.15% W/V) and vitamin D 500IU/12.5µg/100mL as final concentration using vitamin D emulsion premix.

Resulting yoghurt samples were transferred to sterile plastic sample containers with air tight cap and stored at 4^{0}C in refrigerator. Yoghurt fortified with vitamin D only was considered as control. Total 7 formulations were made which were as such

Sensory evaluation

The sensory characteristics of yoghurt formulations fortified with goji berry polyphenolic aqueous/ethanolic extract as well as vitamin D were assessed for sensory characteristics by a panel of experienced 25 judges who were requested to record the score of liking or disliking quality parameters for color, flavor, body and texture on a 9-point hedonic scale and result was presented in terms of overall acceptability (Tunde-Akintunde and Souley, 2009).

Total phenolic content estimation

The total phenolic content of the yoghurt formulations supplemented with aqueous and ethanolic goji berry extract was determined by Folin-Ciocalteu method by measuring absorbance at 715nm in double beam spectrophotometer (Shimadzu UV-2600, Japan) (Zhang *et al.*, 2016).

Reactive oxygen scavenging activity (RSA) estimation

The RSA of the yoghurt formulations supplemented with aqueous and ethanolic gojiberry extract was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method by measuring absorbance at 517 nm in double beam spectrophotometer (Shimadzu UV-2600, Japan) (Yang *et al.*, 2015).

Extraction of vitamin D from yoghurt

Vitamin D extraction was carried out using (Dimartino, 2007; Pastore *et al.*, 1997) with some modification. 5 g of yoghurt sample containing vitamin $D_3$was taken in amber color bottle and 10 ml of DMSO was pipetted. The mixture was incubated at 45^0C in shaker water bath for 30 min (Metrex Scientific instrument, India). Mixture was allowed to cool down at room temperature. Further 100ml of n-hexane was added to bottle containing sample and mix thoroughly for 10 min on magnetic stir. Then mixture was allowed to stand for at least 10 min without agitation for phase separation. Then the supernatant was pipetted out and filtered with Whatman 1 paper through sodium sulphate to avoid the moisture. The filtrate was passed through a 0.45 µm nylon syringe filter and collected into a suitable HPLC vial and chromatographed.

Standard Preparation

Crystalline vitamin D_3 (cholecalciferol; 100 mg) was weighed into a 100.0 mL volumetric flask and dissolved and diluted to volume with n-hexane. This is the 40000 IU/mL stock solution. A 25.0 mL aliquot of stock solution was transferred using a pipet to a 50.0 mL volumetric flask and diluted to volume with n-hexane. This is the 4000 IU/mL working standard solution. Calibration was performed by using different dilution from 40, 400, 800, 1200, 1600 and 20000IU/mL.

Instrument conditions

Vitamin D was quantified using HPLC (UFLC, Shimadzu, Japan) equipped with auto injector using a silica column 250x4.6nm with particle size 5µm and pore size 10Å. The liquid chromatograph was operated in isocratic mode with a flow rate of 1.0 mL/min mobile phase (90% n-hexane and 10 % Isopropyl alcohol) and a wavelength of254 nm by photodiode array detector (AOAC, 979.24). Column temperature was 25^0C and a 10 μL injection volume was used. The retention time for vitamin D3 detection was found to be 4.72 min.

Statistical analysis

The data obtained in the present research was analyzed statistically using Graph prism pad

6.1 for analysis of variance (ANOVA). 95% confident interval ($p < 0.05$) was set throughout the data analysis to identify significant differences. Dunnett's multiple comparison tests and Tuckey's multiple compression tests were used to test the significant difference between different yoghurt samples.

Results and Discussion

pH of yoghurt supplemented with aqueous/ethanolic polyphenolic extract

The pH values of the goji berry polyphenolic aqueous and ethanolic extract supplemented yoghurt, fortified with vitamin D_3, was estimated on 1st day, 10th day and 20th day of storage and results are presented in figure 3. There was slight decrease in pH of all yoghurt formulations registered during their shelf life.

As result indicates on 1st day all yoghurt formulations significantly varied from control sample (Y) but on 10th day and 20th day pH of all formulations were not significantly different from control (1stday Y). Similar slight decrease in pH values was recorded in yoghurt when it was supplemented with grape and callus extract (Karaaslan *et al.,* 2011).

Total phenolic substance

Total phenolic content was estimated in goji berry polyphenolic aqueous and ethanolic extract supplemented yoghurt. The results obtained from yoghurt samples were displayed in figure 1. Among the assayed yoghurt samples highest phenolic content was observed in YE_3 (582.667±25 mg GAE/100g) formulation while it was least in control (50±5 mg GAE/100g) formulation within 1st day yoghurt formulations. It was also observed that total phenolic content of each formulation is significantly decreased during storage. Maximum loss in total phenolic content was recorded in YA_3formulation which varied from575±22 to 531±32 mg GAE/100g. While maximum retention of total phenolic content was noticed in YE_3 formulation which was recorded from 582±25 to 574±10 mg GAE/100g.

DPPH radical scavenging activity (RSA)

The free radical scavenging activity of yoghurt samples was measured by DPPH RSA method and results were displayed in figure 3. The results revealed that as the concentration of polyphenolic extract increases the antioxidant activity enhanced significantly for both aqueous and ethanolic extract (Fig. 2). Results clearly indicate that yoghurt supplemented with ethanolic goji berry extract have greater antioxidant activity than that of yoghurt supplemented with aqueous goji berry fruit extract. Our results were supported by another study where yoghurt was supplemented with polyphenolic extract (Cossu *et al.,* 2009; El-Said *et al.,* 2014; Karaaslan *et al.,* 2011) The least antioxidant activity was observed in control sample (10.97±2.9% in Y formulation) while maximum antioxidant activity was observed in YE_3 formulation (64.40±1.2%). It was observed that free radical scavenging activity (RSA) decreases during the storage. The maximum decrease in RSA was YA_1 (33.16 ±2.5 to 8±2.1 % form 1st day to 20th day respectively) yoghurt formulation while it was least decreased in YE_3 formulation (64.46 ±1.1 to 53.68±1.81 % form 1st day to 20th day respectively). The RSA in all formulations were significantly higher in goji berry extract supplemented yoghurt formulation but yoghurt supplemented with 0.15% (W/V) had maximum RSA values. The substantial quantity of TPC in yoghurt was well correlated with RSA. Similar kind of decrease in RSA during shelf life was registered when curd was fortified with strawberry polyphenolic extract (Singh *et al.,* 2013).

Fig.1 Total phenol content of yoghurt fortified with goji berry polyphenolic aqueous/ethanolic extract and vitamin D. Values are expressed in mean ± SD (n=3) ($p \leq 0.05$). a, b, c, d, e, and f represent significant differences between Y × YA_1, Y × YA_2, Y × YA_3, Y × YE_1, Y × YE_2, and Y × YE_3 within the 1st day, 10th day and 20th day yoghurt formulations where Y formulation is considered as control with each group (1st day, 10th day and 20th day). Further a*, b* and c* represent significance difference between 1st day x 10th day, 1st day x 20th and 10th day x 20th day within individual samples (Y, YA_1, YA_2, YA_3, YE_1, YE_2 and YE_3) at different time periods (1st day, 10th day and 20th day) where 1st day yoghurt were taken as control. Yoghurt (YA_1/YE_1, YA_2/YE_2 and YA_3/YE_3) formulations were fortified with 0, 0.05, 0.10, 0.15% (W/V) with goji berry polyphenolic aqueous/ethanolic fruit extract

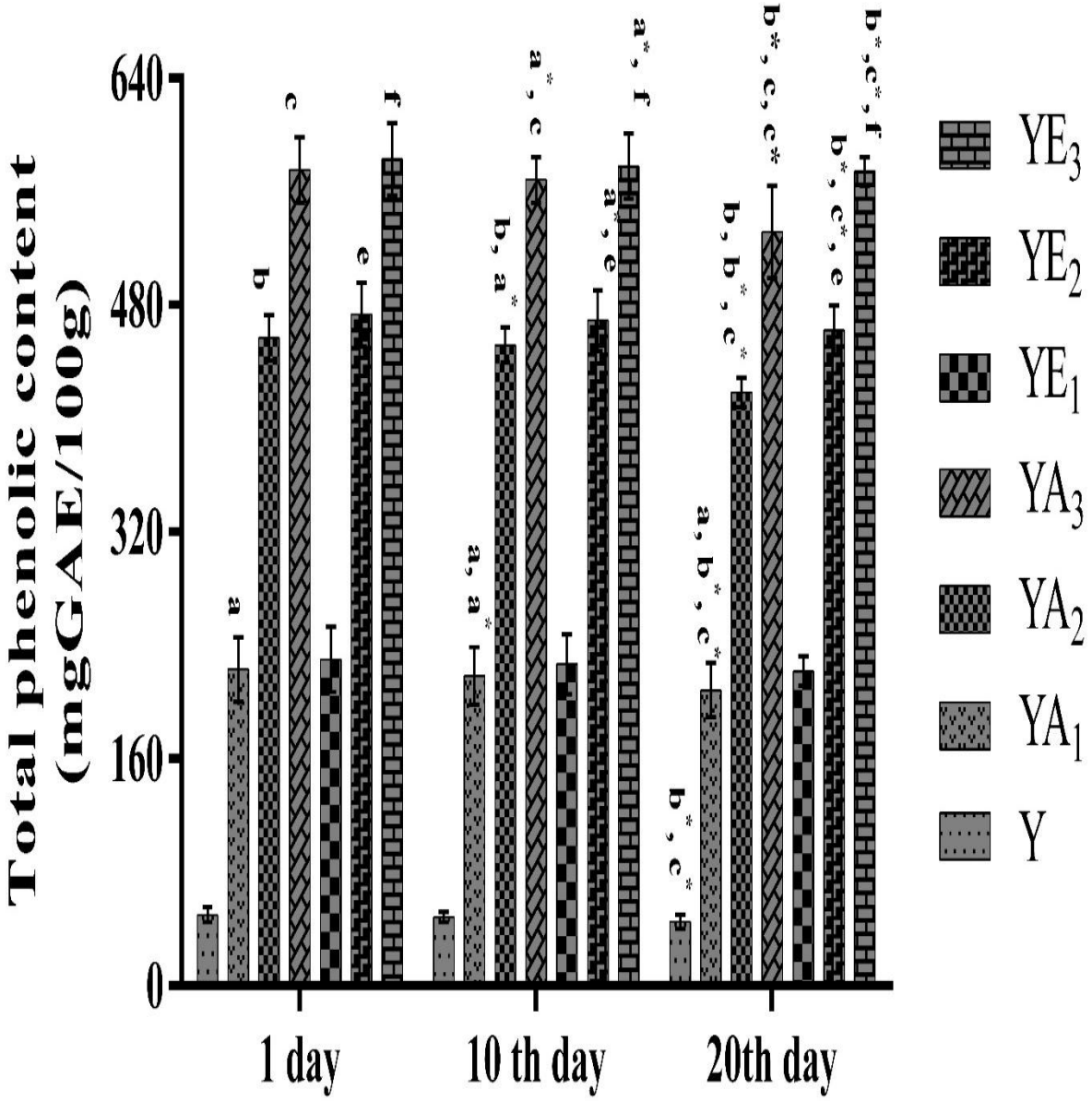


Fig.2 Antioxidant activity of yoghurt fortified with goji berry polyphenolic aqueous/ethanolic extract and vitamin D. Values are expressed in mean ± SD (n=3) ($p \leq 0.05$). a, b, c, d, e, and f represent significant differences between Y × YA_1, Y × YA_2, Y × YA_3, Y × YE_1, Y × YE_2, and Y × YE_3 within the 1st day, 10th day and 20th day yoghurt formulations where Y formulation is considered as control with each group (1st day, 10th day and 20th day). Further a^*, $b^*$and c^* represent significance difference between 1st day x 10th day, 1st day x 20th and 10th day x 20th day within individual samples (Y, YA_1, YA_2, YA_3, YE_1, YE_2 and YE_3) at different time periods (1st day, 10th day and 20th day) where 1st day yoghurt were taken as control. Yoghurt (YA_1/YE_1, YA_2/YE_2 and YA_3/YE_3) formulations were fortified with 0, 0.05, 0.10, 0.15% (W/V) with goji berry polyphenolic aqueous/ethanolic fruit extract

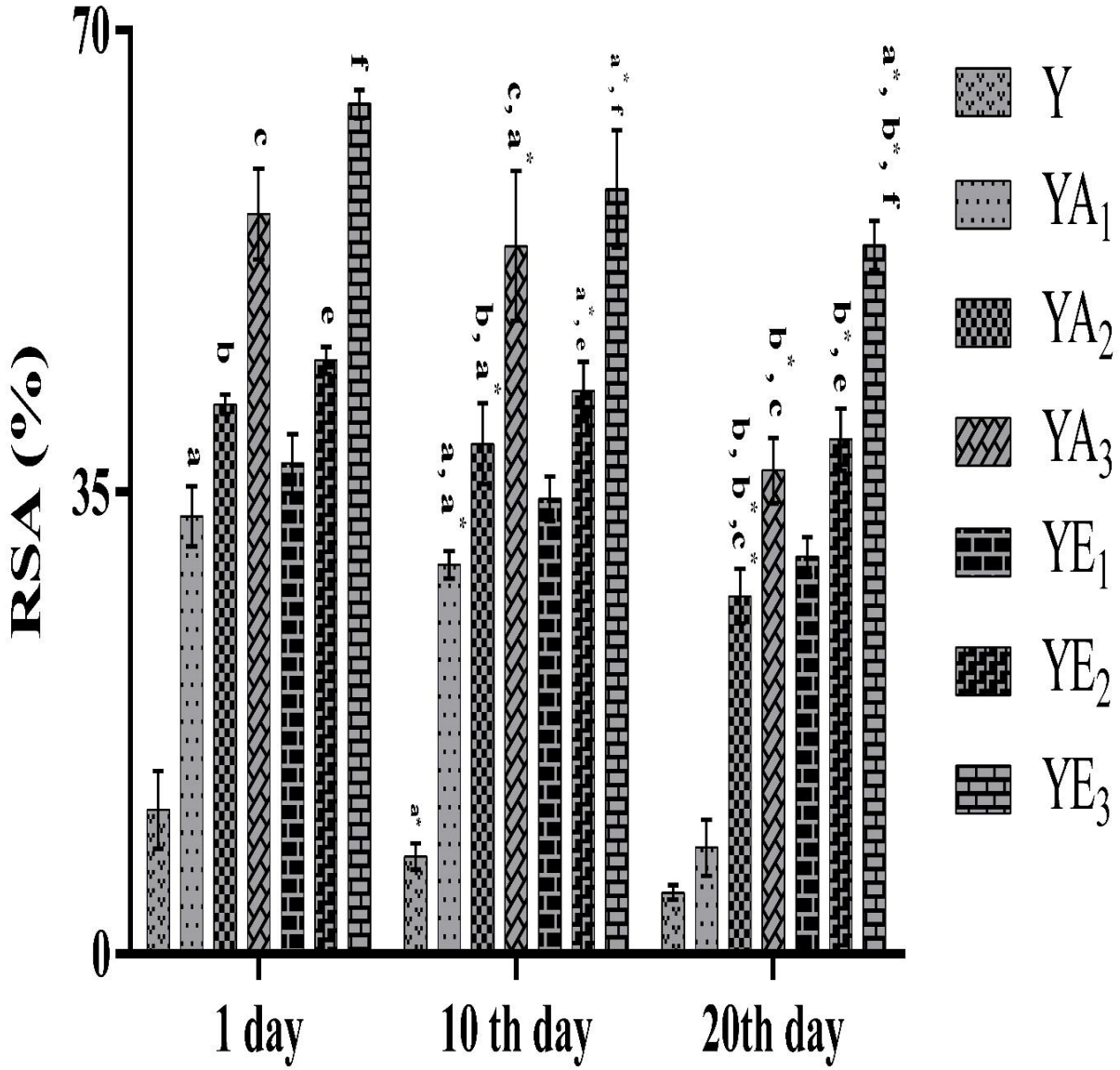


Fig.3 pH of yoghurt fortified with goji berry polyphenolic aqueous/ethanolic extract and vitamin D. Values are expressed in mean ± SD (n=3) ($p \leq 0.05$). a, b, c, d, e, and f represent significant differences between Y × YA_1, Y × YA_2, Y × YA_3, Y × YE_1, Y × YE_2, and Y × YE_3 within the 1st day, 10th day and 20th day yoghurt formulations where Y formulation is considered as control with each group (1st day, 10th day and 20th day). Further a^*, $b^*$and c^* represent significance difference between 1st day x 10th day, 1st day x 20th and 10th day x 20th day within individual samples (Y, YA_1, YA_2, YA_3, YE_1, YE_2 and YE_3) at different time periods (1st day, 10th day and 20th day) where 1st day yoghurt were taken as control. Yoghurt (YA_1/YE_1, YA_2/YE_2 and YA_3/YE_3) formulations were fortified with 0, 0.05, 0.10, 0.15% (W/V) with goji berry polyphenolic aqueous/ethanolic fruit extract

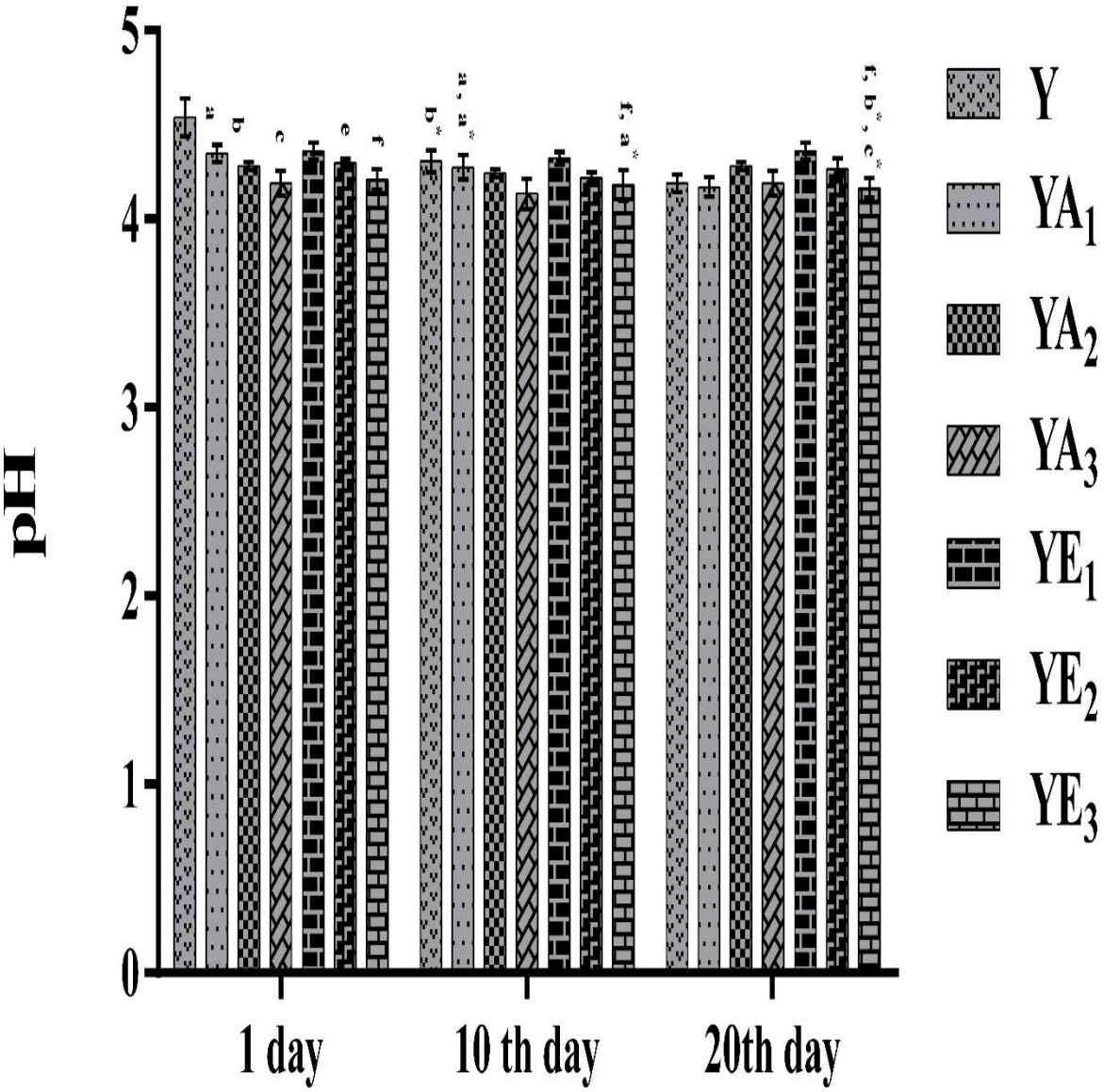


Fig.4 Vitamin D retention in yoghurt fortified with gojiberry polyphenolic aqueous/ethanolic extract and vitamin D. Values are expressed in mean ± SD (n=3) ($p \leq 0.05$). a, b, c, d, e, and f represent significant differences between Y × YA_1, Y × YA_2, Y × YA_3, Y × YE_1, Y × YE_2, and Y × YE_3 within the 1st day, 10th day and 20th day yoghurt formulations where Y sample is considered as control with each group (1st day, 10th day and 20th day). Further a^*, $b^*$and c^* represent significance difference between 1st day x 10th day, 1st day x 20th and 10th day x 20th day within individual samples (Y, YA_1, YA_2, YA_3, YE_1, YE_2 and YE_3) at different time periods (1st day, 10th day and 20th day) where 1st day yoghurt samples were taken as control. Where YA_1/YE_1, YA_2/YE_2 and YA_3/YE_3/ formulations were fortified with 0, 0.05, 0.10, 0.15% (W/V) with goji berry polyphenolic aqueous/ethanolic fruit extract

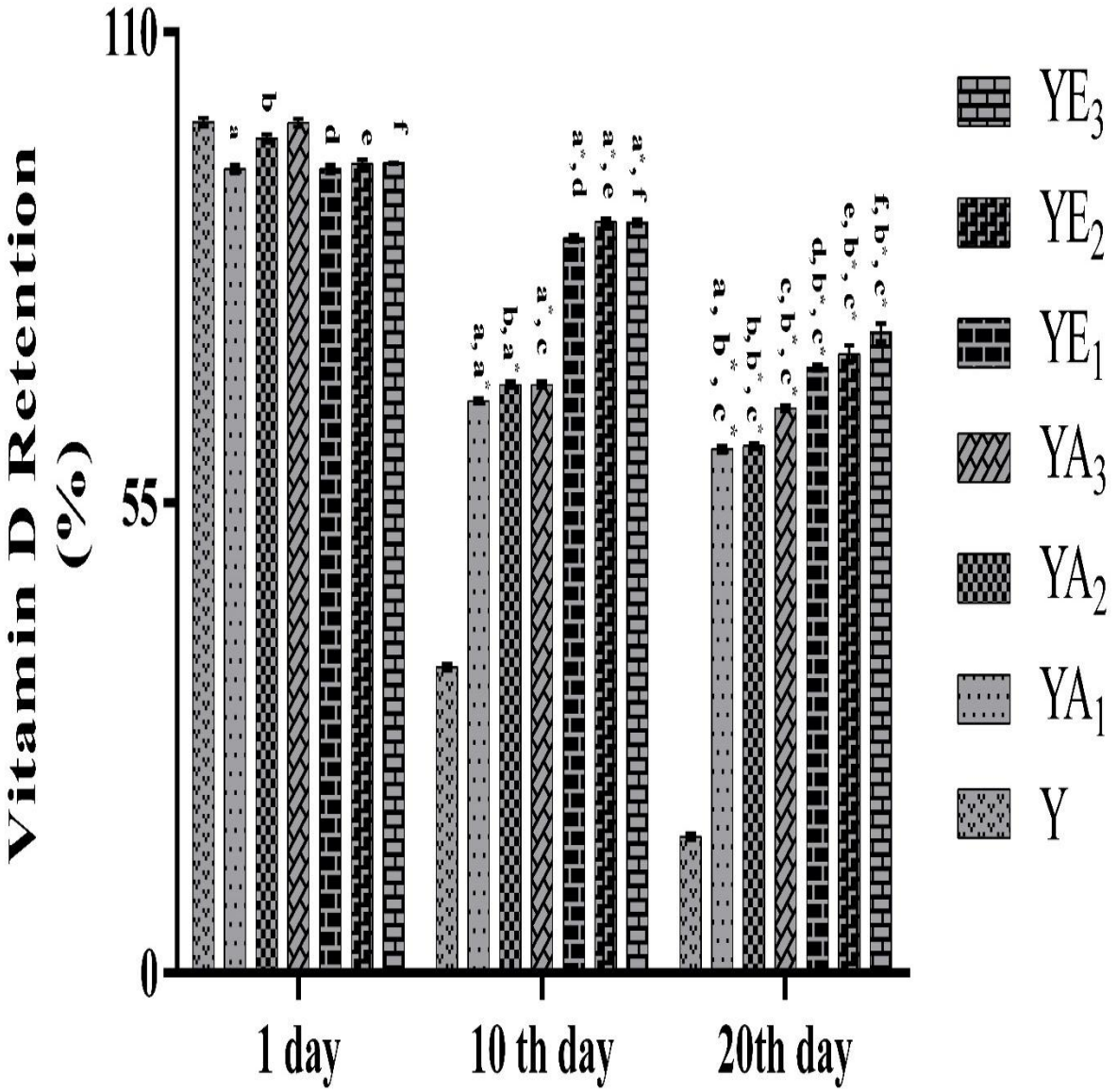
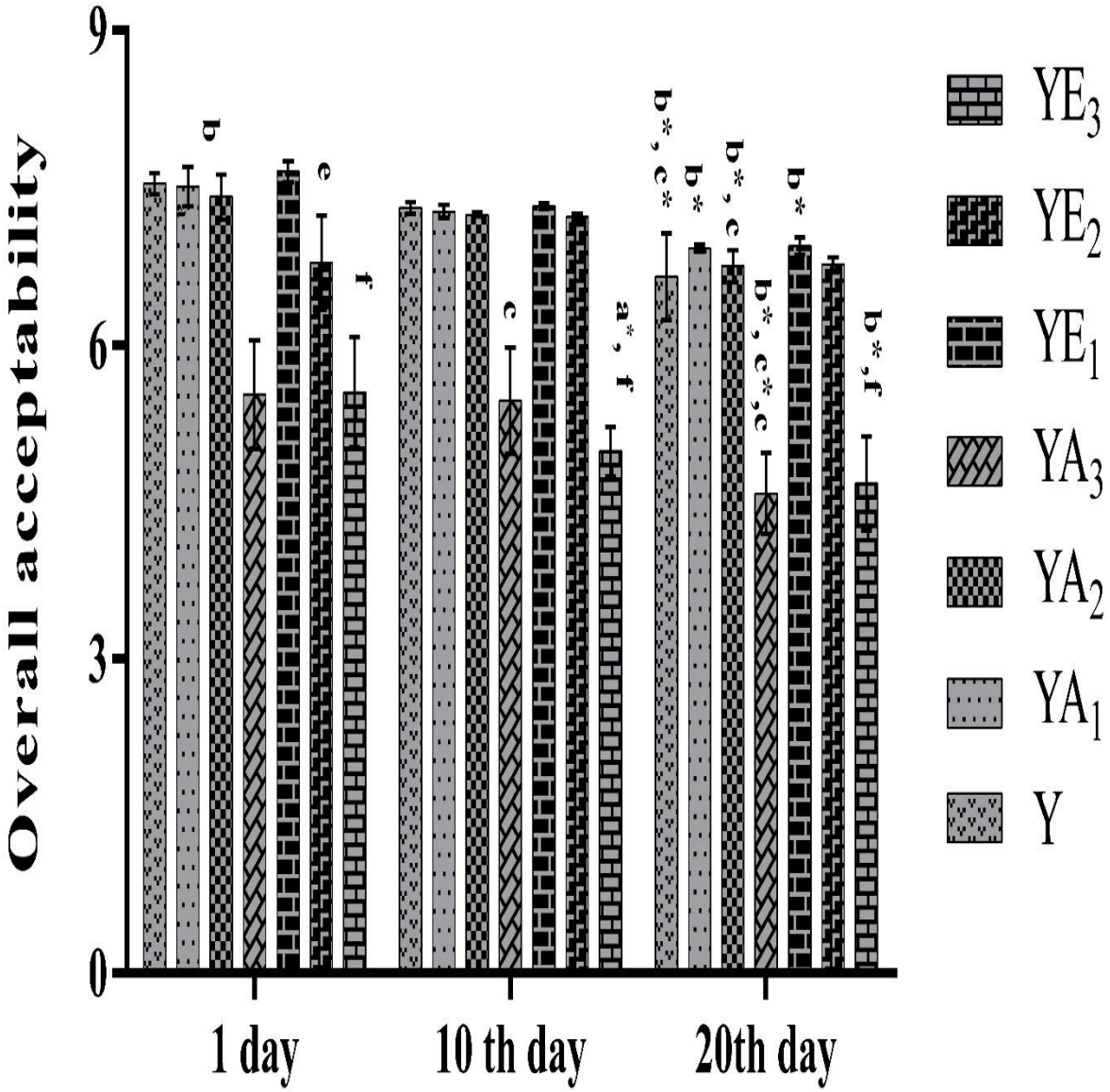


Fig.5 Overall consumer acceptability of yoghurt fortified with goji berry polyphenolic aqueous/ethanolic extract and vitamin D. Values are expressed in mean ± SD (n=3) ($p \leq 0.05$). a, b, c, d, e, and f represent significant differences between Y × YA_1, Y × YA_2, Y × YA_3, Y × YE_1, Y × YE_2, and Y × YE_3 within the 1st day, 10th day and 20th day yoghurt formulations where Y formulation is considered as control with each group (1st day, 10th day and 20th day). Further a*, b* and c* represent significance difference between 1st day x 10th day, 1st day x 20th and 10th day x 20th day within individual samples (Y, YA_1, YA_2, YA_3, YE_1, YE_2 and YE_3) at different time periods (1st day, 10th day and 20th day) where 1st day yoghurt were taken as control. Yoghurt (YA_1/YE_1, YA_2/YE_2 and YA_3/YE_3) formulations were fortified with 0, 0.05, 0.10, 0.15% (W/V) with goji berry polyphenolic aqueous/ethanolic fruit extract



Yoghurt fortification

Formulation code	Toned milk	Aqueous extract (g/100ml)	Ethanolic extract (g/100mLl)	Vitamin D µg/IU/100mL
Y	100	0	0	12.5/500
YA_1	100	0.05	0	12.5/500
YA_1	100	0.10	0	12.5/500
YA_3	100	0.15	0	12.5/500
YE_1	100	0	0.05	12.5/500
YE_2	100	0	0.10	12.5/500
YE_3	100	0	0.15	12.5/500

Vitamin D retention in polyphenolic extract supplemented yoghurt

Vitamin D was quantified in goji berry supplemented aqueous and ethanolic extract yoghurt and displayed in figure 4. The extraction efficiency of vitamin D from different yoghurt formulation lied between 90-95%. The results revealed that as the concentration of polyphenolic extract increases the vitamin D retention enhanced significantly for both aqueous and ethanolic extract. But yoghurt supplemented with ethanolic goji berry extract has greater vitamin D retention than that of aqueous extract. This can be correlated with high antioxidant activity and high total phenolic content in ethanolic extract than that of aqueous extract. Vitamin D is prone to oxidation which can be inhibited by addition of antioxidant. Figure 4 clearly shows that as the antioxidant activity decreases during storage vitamin D degradation also increases. The lowest vitamin D retention during storage of 20 days was recorded in control (Y) which varied from 99.55% on 1st day to 15.97% on 20th day) while the highest retention was registered in YE_3 formulation which changed from 94.7% on 1st day to 74.9% 20th day.

Sensory evaluation of soy blended yoghurt

Sensory evaluation is a key to potential consumer preferences. Sensory characteristics of goji berry extract supplement and vitamin D fortified yoghurt formulation are presented based on the mean score of and over all acceptability (mean of colour, flavour, body texture) of yoghurt prepared (Figure 5). It was observed that mean score for overall acceptability was in the range of 7.51-5.52 (Y and YA_3 within 1st day formulations). The highest acceptability was reported for control yoghurt while it was least for YA_3. Figure 5 also shows that the overall acceptability was decreased during the storage period of 20 days. Similar conclusion was made by several other researchers when they supplemented yoghurt with different types polyphenolic extracts (Cossu *et al.,* 2009 El-Said *et al.,* 2014; Karaaslan *et al.,* 2011). It is observable that the consumer acceptability of YE_2 formulation was next control formulation in overall acceptability. This indicates that yoghurt supplemented with 0.1% (W/V) ethanolic extract is the most suitable formulation for delivery of vitamin D as well as natural antioxidant without affecting the consumer palatability.

In conclusion, the present study validated that goji aqueous and ethanolic extracts can be effectively employed for production of polyphenol fortified yoghurt with high antioxidant properties. Ethanolic extract has displayed higher content of polyphenols and antioxidant activity followed than that of aqueous extract extracts, these extracts definitely affected the phenolic content in yoghurt during 20 days of refrigerated

storage. HPLC analysis revealed the absence of phenolic extract in yoghurts, vitamin D was susceptible to degradation. However, all yoghurts formulation showed high antioxidant activity compared to control yoghurt formulation (Y) during 20 days of storage. In addition, yoghurt formulations supplemented with ethanolic extract showed higher antioxidant activity among the other formulations. Further study on the consumer sensory evaluation reveals that as the concentration of aqueous/ethanolic goji berry fruit extract the overall consumer acceptability decreased. This study clearly suggests that yoghurt supplemented with 0.10% (W/V) ethanolic goji berry extract could be the most feasible option to ensure high antioxidant activity as well high vitamin D retention without affecting the acceptability and palatability of yoghurt.

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