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Stability and antioxidant activity of bacterial canthaxanthin in *aloe vera* model system.

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ABSTRACT

In this present study bacterial canthaxanthin (CX) (extracted from *Dietzia maris* NITD) was formulated with aloe vera (AV) to obtain a stable suspension. Antioxidant activity was determined through different radical scavenging methods. The formulation was then stored at 03 different temperature viz. $-4°C$, $4°C$ and $37°C$ for 05 weeks to check the stability of CX. Least degradation of CX was observed at $4°C$. Gibbs free energy (ΔG), the enthalpy of activation (ΔH) and entropy of activation (ΔS) was found to be ranging from $71.35 - 72.05\ kJ/mol$, $1.612 - 4.356\ kJ/mol$, and -242.86 to respectively at the stable temperature for all types of radical scavenging methods. Activation energy for the pigment (E_a) was found to be ranging from $4.020 - 6.765\ kJ/mol$. The thermal death time method (D-z model) was used to calculate the D value, Z value and half life $(t_{1/2})$ of the formulated pigment. FT-IR studies confirmed the presence of –OH stretching (carboxylic), C=C bending, -CH and -CH2 stretching (alkyl and alkane) etc. suggesting stable presence of CX in the formulation. The study suggests that CX may be formulated with AV for stable storage.

Keywords: Canthaxanthin, Aloe-vera, Stability, Activation energy, Storage temperature

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INTRODUCTION

Carotenoids possess various protective effects, such as their ability to absorb and reflect potentially damaging radiations, to quench singlet oxygen or as antioxidants by which sensitive tissues and reactive compounds are protected from oxidation (Goswami *et al.,* 2010). Theoretically, all carotenoids with similar conjugated double bond system should have chain breaking antioxidant capability (Burton, 1989). CX occurs normally in a wide variety of living organism and displays maturity in animals and protection of tissues (animals and plants) against oxidizing free radicals (Moller *et al.,* 2000). CX has remarkable possibilities for application as a nutraceutical owing to its anti-carcinogenic, tumour suppressing characteristics and antioxidant property (Goswami *et al.,* 2012). Degradation of carotenoids in food is complex in nature as various factors such as nature and composition of food processing treatment, packaging and storage conditions, activity of lipooxygenase and other enzymes, and coupled oxidation with lipids are considered to play a vital role (Dutta *et al.,* 2005). The polyene chain of CX is the cause of instability and is susceptible to oxidation and geometric isomerisation. Oxidation depends on the available oxygen and their physical conditions viz. heat, and is a highly deteriorative process leading to loss in nutritional value (Carvalho, 2015). It is because of various health benefits that consumers prefer the presence of antioxidants in food (Pizzale *et al.,* 2002; Koleva *et al.,* 2003). Due to the toxicological effect of synthetic antioxidants, antioxidants from natural products are preferred. Carotenoids are prone to degradation during thermal processing in industrial scale which results in decreased antioxidant efficacy (Razi Parjikolaei, 2015). Temperature and duration of storage are two important parameters responsible for the loss of pigment quality. Therefore it is essential to investigate the role of these two parameters (degradation and antioxidant efficacy) of the CX during storage.

Aloe vera (AV) or *Aloe vera* (L.) Burm.f. gel (family xanthorrhoeaceae) is a natural aqueous based model used widely in cosmetic and pharmaceutical industry due to its anti-inflammatory, anti-bacterial, antiseptic, ability to promote the wound healing, anti-diabetic, antioxidant properties (Reynolds & Dweck, 1999; Eshun & He, 2004; Pereira *et al.,* 2013; Kang *et al.,* 2014). The gel appearance is due to four different partially acetylated glucomannans present. However, AV gels are also rich in different enzymes, polyphenols, amino acids, vitamins etc. which may vary according to isolation and storage procedure (Eshun & He, 2004; Kiran & Rao, 2014). AV gel may be employed to effectively deliver poorly absorbable drugs through the oral route of drug administration (Hamman, 2008). In the present study, feasible temperature of storage of bacterial CX in AV gel formulation has been studied. Antioxidant efficacy of the formulated gel was studied. The thermodynamic parameter for CX degradation was calculated. The interacting functional groups of CX and AV were identified by FT-IR.

MATERIALS AND METHODS

Microorganism and carotenoid production

The experimental strain *Dietzia maris* NIT-D (accession number: HM151403) was previously isolated and established as efficient CX producer in our laboratory (Goswami *et al.,* 2012). Optimized medium supplemented with moderately aged ($6-8$ month) coconut water was used for optimal scale production of the pigment at initial broth $pH-6.7$, 120 rpm and $25°C$ (Bera *et al.,* 2015). Pigment extraction was done according to Goswami *et al.* (2012). Extracted pigment was concentrated using rotary evaporator (Yamato Rotary Evaporators, model RE301) followed by lyophilisation. Dried CX was stored at $4°C$ till further use. Pigment analysis was done using the UV–Vis spectrophotometer (U-2800, Hitachi), TLC (thin layer chromatography) in prepared silica gel plates (Merck) and High-performance liquid chromatography (HPLC) (Bera *et al.,* 2015) and compared with pure standards of CX (Sigma-Aldrich, USA).

Preparation of Aloe vera (AV) extract

Fresh and mature AV leaves were collected from Institute campus, washed with distilled water and stored in sterile plastic bags. AV gel was scrapped and collected in sterile glass tubes and dehydrated by lyophiliser. Desiccated AV gel was ground to a smooth powdered appearance and then extracted with methanol ($1:5$ w/v, overnight shaking at $25°C$ at 120 rpm). Methanolic extract was concentrated using rotary evaporator (Yamato, model RE301) followed by lyophilisation and stored at $4°C$ till further use.

Efficacy retention of CX in AV

To evaluate the efficacy and stability of canthaxanthin in AV model system, extracted pigment was mixed with prepared AV in equal concentration and the antioxidant efficacy was determined using different radical scavenging activity methods. DPPH• (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging ability (Aquino et al., 2001), ABTS (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) scavenging (Re *et al.,* 1999), hydroxyl radical scavenging activity (HRSA) (Yuan *et al.,* 2013) and nitrite scavenging activity (NSA) (Bera *et al.,* 2015) was determined of the extracted pigment, AV and formulated mixture. Vitamin-C was used as a positive control.

Stability and degradation of CX in AV

The IC_{50} value of CX, AV and CX-AV in different radical scavenging method was calculated. The formulated CX-AV mixture was stored at 03 different temperatures viz., $-4℃$, $4℃$ and $37℃$ for 05 weeks to check the stability of the pigment. The quality responses of most carotenoids either follow zero order or first order reaction (Dutta *et al.,* 2005). The following equation was used in this study:

$$\frac{dC}{dt} = -k(C)^n$$

Where k is the rate constant, n is the reaction order, C is the concentration of CX and t is the reaction time. The temperature dependence of the rate constant was determined by Arrhenius relationship:

$$k = k_0 \times \exp^{\left(\frac{-E_a}{RT}\right)}$$

Where k_0 is pre-exponential factor, E_a is activation energy (kJ/mol), R is the universal gas constant $(8.314\ J.mol.K)$ and T is absolute temperature (K).

The thermal death time method $(D-z$ model) was employed to determine decimal reduction time (D value), which is, the heating time needed to reduce the CX concentration by 90%, and the z value, that is, the temperature change required to alter the thermal death time by one log cycle with the relationships (Nayak *et al.,* 2011; Mitra *et al.,* 2016):

$$D = \frac{\ln 10}{k}$$

$$\log\left(\frac{D}{D_{ref}}\right) = -(T - T_{ref})/z$$

Where D_{ref} is the D value at the temperature T_{ref}. The half life $\left(t_{\frac{1}{2}}\right)$ of the CX was determined as

$$t_{\frac{1}{2}} = \frac{\ln 2}{k}$$

Thermodynamic parameters like the Gibbs free energy (ΔG) enthalpy of activation (ΔH) and entropy of activation (ΔS) was also calculated by Eyring-Polanyi equation.

$$\Delta G = \Delta H - T\Delta S$$

$$k = \frac{k_b}{h} T \times e^{-\Delta H - \frac{T\Delta S}{RT}}$$

Where T is absolute temperature(K), k_b is the Boltzmann constant$(1.381 \times 10^{-23}\ J/K)$, h is the Plank constant$(6.626 \times 10^{-34}\ Js)$.

Q_{10} values were determined using following formula

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}}$$

Where R_2 and R_1 were rate constant and T_2 and T_1were reference temperature in kelvin.

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FT-IR analysis of the formulation

For FTIR study, extracted pigment (i.e. CX), AV extract and formulated mixture was lyophilized in freeze dryer and subjected to KBr pellet formation. Spectrum was recorded in a range of $400 - 4000\ cm^{-1}$ (Thermo-Nicolet 6700, USA).

Statistical Analysis

All experiments were performed in triplicates. Data was presented as the mean ± standard deviation (SD) of their values. Analysis of variance (ANOVA) procedure was done followed by Duncan's test using SPSS 16 software (SPSS Inc., Chicago, USA). A probability value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Antioxidant efficacy of CX in AV model

Figure 1 shows the radical scavenging power by different assay method for the CX-AV gel formulation. The concentration of the extracted pigment was found to be 516.03 mg/l which was mixed with AV gel to obtain a final concentration of 1000 µg/ml. In case of DPPH assay, extracted CX and AV showed 22.97% and 13.29% inhibition respectively at 25 µg/ml concentration whereas the formulated mixture of CX-AV showed 23.56% DPPH inhibition at same concentration. At high concentration of 400 µg/ml, extracted CX and AV showed 82.87% and 53.83% inhibition respectively, whereas the formulated gel of CX-AV showed 83.81% DPPH inhibition. Significant difference ($p < 0.01$) in DPPH inhibition results was found between CX and CX-AV formulation. The scavenging ability in the DPPH assay was due to its chemical structure and its ability to donate hydrogen (Bera *et al.,* 2015) for both CX and AV.

ABTS assay showed less sensitivity of the test samples towards the substrate. At 250 µg/ml concentration, extracted CX and AV showed 31.78% and 31.91% inhibition respectively, whereas the formulated gel showed 42.65% ABTS inhibition at the same concentration. At high concentrations, AV scavenges ABTS radical more efficiently than CX. At 1250 µg/ml, extracted pigment and AV showed 58.96% and 74.37% inhibition respectively, whereas the formulated gel showed 80.91% ABTS inhibition at the same concentration. Probably the antiradical scavenging capacity of AV was acting synergistically with CX. In this method, ABTS scavenging results was not significant ($p > 0.05$) up to 750 µg/ml however beyond this concentration, the radical scavenging capacity of both CX and CX-AV formulation were significantly different ($p < 0.05$).

Nitrite is widely used in food processing industries as a preservative and colouring though nitrosamine is a reactive product of nitrite and amines (protein-rich staple vegetables and meat products) which has significant threat to animal health (Bera *et al.,* 2015). However NSA inhibition result with CX and CX-AV formulation was not significant ($p > 0.05$).

Extracted CX and AV showed 31.82% and 33.68% inhibition at a concentration of 100 µg/ml whereas the formulated gel of CX-AV showed 37.55% NSA inhibition at same concentration. At high concentration, extracted CX and AV showed 64.52% and 62.77% inhibition respectively at 600 µg/ml concentration whereas the formulated gel of CX-AV showed 61.13% NSA at same concentration. The hydroxyl groups of CX can usually inhibit the formation of nitrosamines by donating hydrogen (Bera *et al.,* 2015).

In case of HRSA, highly significant scavenging power ($p < 0.001$) was observed for CX and AV. Extracted CX and AV showed 33.65% and 26.48% hydroxyl ion scavenging power at 50 µg/ml concentration whereas the formulated gel of CX-AV showed 38.11% inhibition at same concentration. At high concentration, extracted CX and AV showed 91.11% and 84.79% inhibition respectively at 200 µg/ml concentration whereas the formulated mixture of CX-AV showed 95.67% hydroxyl ion inhibition at same concentration. However difference in HRSA results between CX and CX-AV formulation was not significant ($p > 0.05$). Hydroxyl radical generally reacts with aromatic group of other compounds to generate hydroxycyclo-hexadienyl radical which undergo further reactions to produce peroxyl radical, or decompose to phenoxyl-type radicals by water elimination. Reducing power of CX and hydrogen atom donating ability may be acting synergistically to facilitate the HRSA activity. Table 1 shows the obtained IC_{50} values with different radical

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scavenging method. Correlation coefficient (R^2) between CX and CX-AV formulation in HRSA system was found highest (R^2:0.9974) followed by DPPH (R^2:0.9964), NSA (R^2:0.9954) and ABTS (R^2:0.9903) assay.

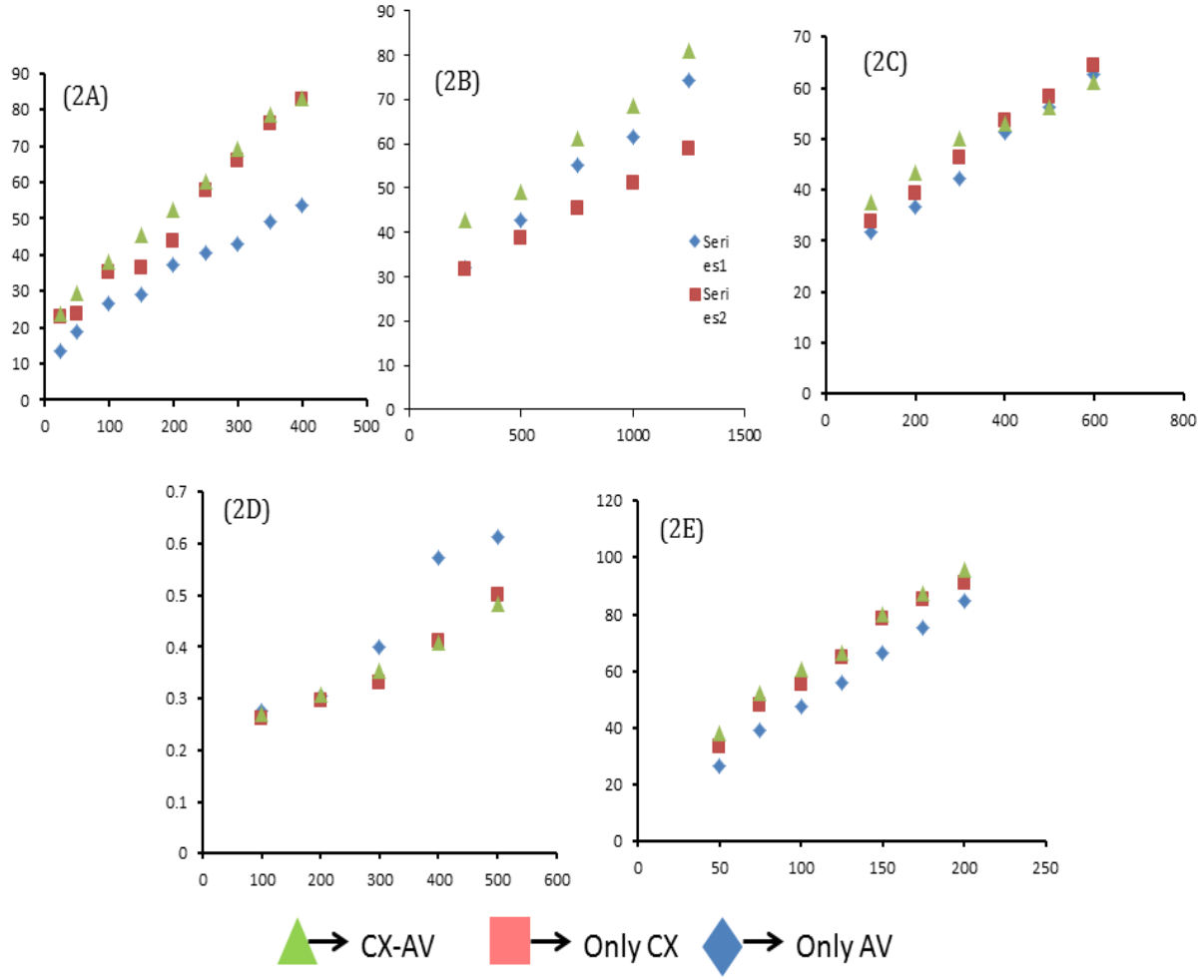

Figure 1. Different radical scavenging activity potentiality by different assay methods viz. (A) DPPH, (B) ABTS,(C) NSA, (D) RP and (E) HRSA. For Fig. A, B, C and E, X axis was taken as percentage of inhibition where Y axis was concentration (µg/ml) of the product i.e. CX/AV/ CX-AV. Only for Fig D, the X axis was the absorbance value at 700nm.

Table 1. IC_{50} **values** $(\mu g/ml)$ **of samples in different radical scavenging activity method**

Formulations	DPPH	ABTS	NSA	HRSA
CX	216.12	932.47	361.97	85.55
AV	354.29	674.49	398.99	107.69
$CX-AV$	181.53	477.21	343.65	75.53

Stability and degradation of CX in AV

It is essential to determine the stability of extracted canthaxanthin in order to apply it for industrial purpose. Four sets of CX-AV gel (with IC_{50} value concentration from 04 radical scavenging method) was kept at three different temperature of storage viz. $-4°C$, $4°C$ and $37°C$ for 05 weeks. The degradation was determined after every week. Figure 2 shows the degradation of CX at three different temperature of storage. It is observed that the highest degradation took place at $37°C$ $(58-72\%)$ and the least degradation took place at $4°C$ $(46-51\%)$.

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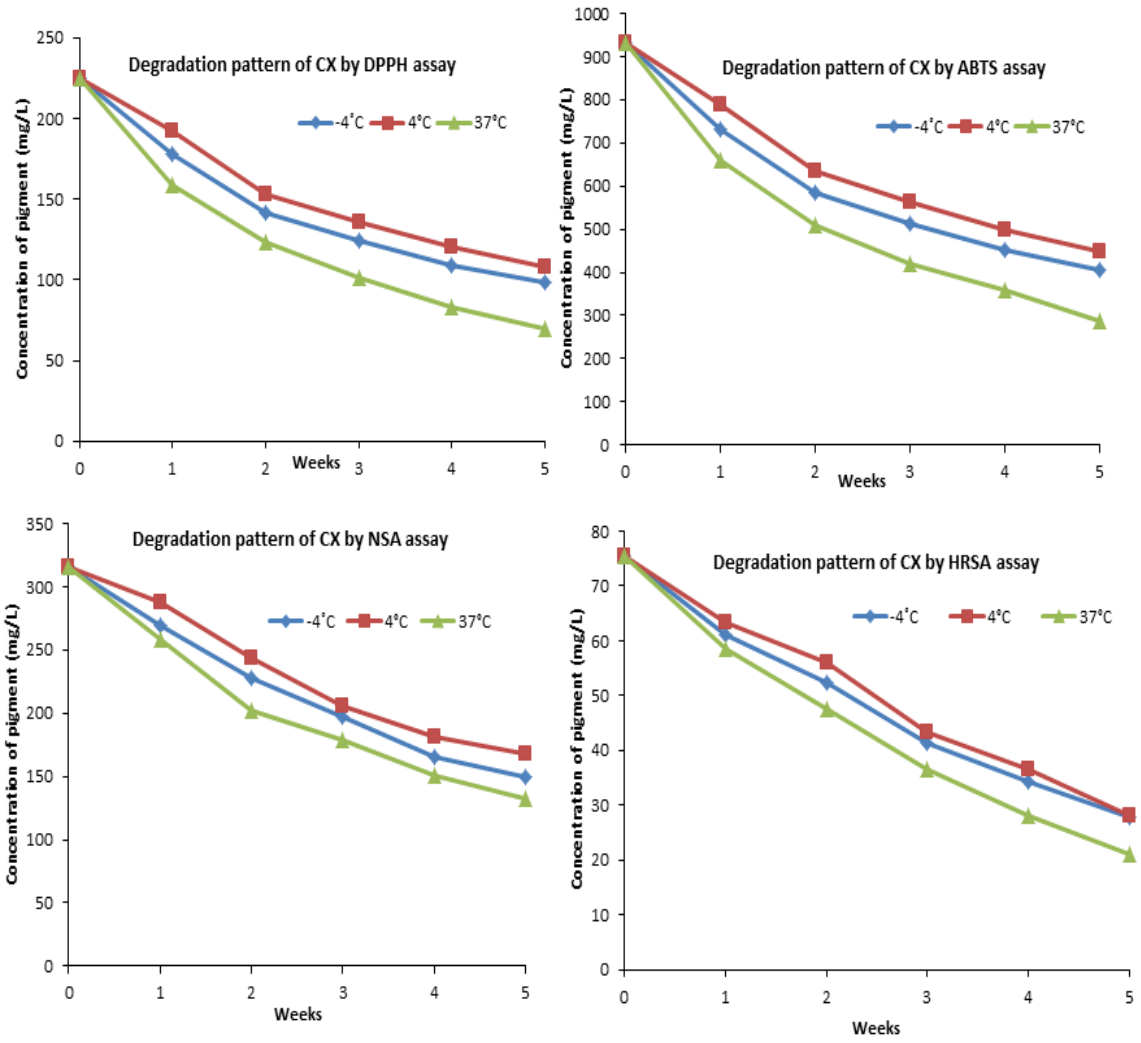

Figure 2. Degradation pattern of CX during 05weeks of storage at three different temperature.

In order to determine the rate of degradation, the values obtained from experimental results were fitted in different kinetic model systems. It was found that first order reaction fitted with the data. The degradation rate constants and correlation coefficients are given in Table 2. High k values at $37°C$ suggest that CX was least stable at $37°C$ and low k values at $4°C$ suggest least degradation at that temperature.

The thermodynamic parameters are given in Table 3. The $t_{\frac{1}{2}}$ and D values confirm that the CX in the formulated AV gel was most stable at $4°C$ and least stable at $37°C$. All the $t_{\frac{1}{2}}$ and D values for different scavenging method was not significantly different $(p > 0.05)$. However, at $4°C$ the $t_{\frac{1}{2}}$ and D value of NSA assay method was significantly higher than of HRSA assay $(p < 0.05)$. The activation energy varied from $4.02 - 6.75\ kJ/mol$ in the formulated AV gel mixture. E_a values obtained from DPPH and ABTS methods showed slightly higher than that of HRSA and NSA method. However, the values were not significantly different $(p = 0.9303)$ from E_a values obtained from HRSA and NSA method. Low activation energy signified less heat sensitivity suggesting that the canthaxanthin introduced in the AV gel system was heat stable and less susceptible for degradation. Results of Mitra *et al.* (2016) were in agreement with this study which reported an activation energy of $13.27\ kJ/mol$ for bacterial β-cryptoxanthin over a temperature range of $25 - 80°C$. In another study, Niamnuy *et al.* (2008) reported an activation energy of $10.6 - 12.17\ kJ/mol$ for astaxanthin obtained from dried shrimp over temperature range of $4 - 25°C$. The Q_{10}values ranged from 1.091 to 1.153 for all different radical scavenging methods $(p = 0.9656)$ For reactions such as enzymatically induced colour or flavour change in foods, degradation of natural pigments, non-enzymatic browning, and microbial growth rate, the Q_{10} is usually around 2 (Toledo RT, 2007).

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Table 2. Kinetic parameters of CX degradation during storage in AV model

Assay	Storage Temperature	k value	R^2
DPPH	−4°C	0.1561	0.9772
	4°C	0.1805	0.9536
	37°C	0.2502	0.9693
ABTS	−4°C	0.1826	0.9532
	4°C	0.1569	0.9756
	37°C	0.2488	0.9967
NSA	−4°C	0.1545	0.9955
	4°C	0.1315	0.9874
	37°C	0.1832	0.9844
HRSA	−4°C	0.1981	0.9983
	4°C	0.1869	0.9869
	37°C	0.2493	0.9976

Both Enthalpy (ΔH) and entropy change (ΔS) are the key factors that are considered in any process design in terms of transition state theory. Enthalpy measures the barrier for energy, which should overcome by reacting molecules, and is related to the strength of bonds that are broken and made in the formation of the transition state from the reactants. Negative entropy of activation indicates a smaller number of species in the transition state (Nayak *et al.,* 2011). In the present study, estimated ΔH of CX degradation varied from $1.612 - 4.356\ \mathrm{kJ/mol}$ when the formulated CX-AV gel was kept at different temperature of storage (Table 3). It suggested that CX was degraded to a less extent when the temperature of storage changed in the range of -4 to $37°\mathrm{C}$. The change in entropy ΔS was recorded as -242.86 to $-254.29\ \mathrm{J/mol.K}$ (Table 3). Negative ΔS values suggested declined randomness of the CX in AV gel thus inferring a stable system during long storage. Both ΔH and ΔS values calculated from four types of radical scavenging assay methods was not significantly different from each other. Gibbs free energy change (ΔG) for this experiment was recorded all positive and varied from 69.364 to $80.442\ \mathrm{kJ/mol}$ for all radical scavenging activity methods in a temperature range of -4 to $37°\mathrm{C}$ (Table 3) which are not significantly different $(p > 0.05)$ from each assay method. This suggested that degradation of CX in AV was non-spontaneous in nature. These results were in agreement with study done by Mitra *et al.* (2016). High temperature of storage may lead to fractional isomerization of CX from trans-to cis-configuration. Additionally, oxidation may reduce the pigment quality and concentration during long storage. The data obtained through kinetic study and thermodynamic calculation suggest that the extracted canthaxanthin was stable in the aleo-vera formulation, however the degradation was more at $37°\mathrm{C}$ and least at $4°\mathrm{C}$.

FT-IR analysis of the CX-AV formulation

FT-IR analysis of extracted AV gel (Figure 3A) indicated the presence of $-\mathrm{OH}$ (alcoholic group) or $-\mathrm{NH}$ (aliphatic group primary amine) for which a strong peak at $3250.13\ \mathrm{cm^{-1}}$ was obtained. A sharp peak at $1726.11\ \mathrm{cm^{-1}}$ specified the presence of $-\mathrm{C=O}$ group (acetyl stretching or aldehyde or aliphatic ketone or α-β unsaturated ester), $1623.13\ \mathrm{cm^{-1}}$ indicated $-\mathrm{C=C}$ (conjugated alkene) or $-\mathrm{NH}$ (amine bend), $1391.21\ \mathrm{cm^{-1}}$ indicated $-\mathrm{COO}$ asymmetric or $-\mathrm{S=O}$ group respectively. AV extract might contain sugar and glucan for which broad peaks obtained at $1061.72\ \mathrm{cm^{-1}}$ (Kiran & Rao, 2014). Extracted CX showed (Figure 3B) strong and sharp peaks at $2921.16\ \mathrm{cm^{-1}}$ and $1334.55\ \mathrm{cm^{-1}}$ due to $-\mathrm{CH2}$ (alkane stretching), $1653.26\ \mathrm{cm^{-1}}$ due to $-\mathrm{C=O}$ (conjugated ketone stretch) and $1560\ \mathrm{cm^{-1}}$ which indicated $-\mathrm{C=C}$ (unsaturated ketone)

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presence respectively. Most significantly, the presence of sharp peak at $971.53\ cm^{-1}$ may be attributed to di-substituted alkene (trans) which proved that the extracted pigment as trans-CX. CX-AV formulation showed slight modified peak pattern rather than its individual composition (Figure 3C). A broad peak at $3391.47\ cm^{-1}$ may be due to shifting of $-NH$ (amine) or $-OH$ (alcohol) group which might be due to the interaction of CX with AV. Another shifting was observed at $2930.1\ cm^{-1}$ which may be due to stretching of $-CH$ group (alkane). Other peaks at $1726.26\ cm^{-1}$, $1626.66\ cm^{-1}$, $1390.72\ cm^{-1}$ and $1062.58\ cm^{-1}$ which indicated less stretching or bending of chemical groups present in native CX or AV. Though AV is a natural aqueous based medium for cosmetics and nutraceutical industries, extract of different aged AV plant can show different antioxidant activity (Hu *et al.,* 2003; Eshun & He, 2004). It may be that the polyphenols, flavonoids present in AV act synergistically with the extracted CX and retain the stability and antioxidant efficacy during different temperature of storage.

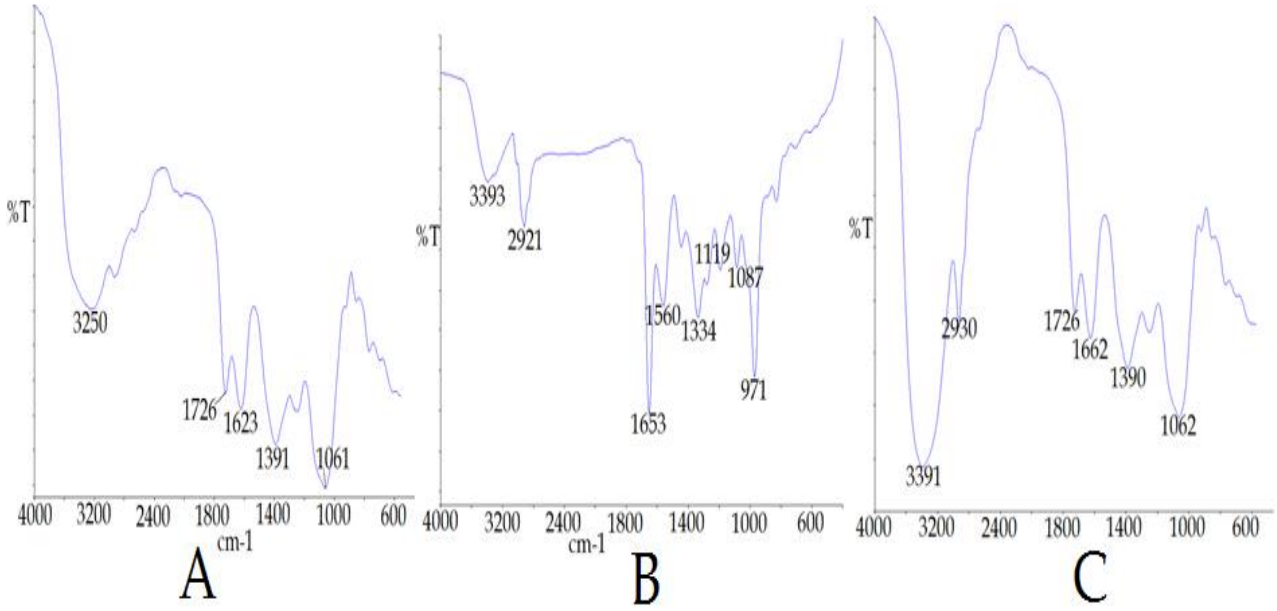

Figure 3. FT-IR pattern of AV (A), CX (B) and CX-AV (C) formulation

CONCLUSION

The present study investigates the stability of bacterial CX for long term preservation in natural model system to retain its maximum functional property. From the result we found Aloe vera (AV) solution may be considered to store bacterial CX at $4°C$ for up to 05 weeks in which least degradation of the carotenoid occurred. Different radical scavenging assays like DPPH, ABTS, NSA and HRSA may show different results due to different substrate sensitivity to CX. FTIR results revealed a possible interaction effect between CX and AV and alteration in $-OH, -NH$ and $-CH$ groups perhaps was involved to provide better stability of CX in AV model system. Altogether, carotenoids like CX and pharmaceutically important herbs like AV can be used together to prepare stable solutions with great antioxidant property. These results would be useful to formulate cosmeceutical solutions or nutritional supplements significant to human health related complications.

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Table 3. Thermodynamic and $D - Z$ model parameters of CX degradation during storage in AV model

Assay		Temperature (K)	$t_{\frac{1}{2}}$ (week)	D value(week)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol.K)	Q_{10}	E_a (kJ/mol)	Z value (°C)
DPPH		269K	3.840	12.756	69.686	4.356	-242.86	1.153	6.765	185.18
		277K	4.440	14.750	71.628					
		310K	2.770	9.203	79.643					
ABTS		269K	3.796	12.610	69.663	4.081	-243.80	1.149	6.489	188.68
		277K	4.417	14.675	71.614					
		310K	2.785	9.254	79.659					
NSA		269K	4.486	14.903	70.016	1.612	-254.29	1.105	4.020	277.78
		277K	5.271	17.510	72.050					
		310K	3.783	12.568	80.442					
HRSA		269K	3.498	11.623	69.364	2.101	-250.050	1.091	4.506	294.12
		277K	3.708	12.319	71.353					
		310K	2.780	9.236	79.759					

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