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E-mail: editorpjn@gmail.com

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Betalains from Red Pitaya Peel (*Hylocereus polyrhizus*): Extraction, Spectrophotometric and HPLC-DAD Identification, Bioactivity and Toxicity Screening

Anni Faridah¹, Rahmi Holinesti¹ and Daimon Syukri²

¹Faculty of Tourism and Hospitality, Padang State University, Indonesia

²Department of Agricultural Product Technology, Andalas University, Indonesia

Abstract: Betalains were extracted and analyzed from Red Pitaya Peel (*Hylocereus polyrhizus*). For this extraction, ethanol/water solvent systems were used. The observation such as UV-vis Spectrophotometric identification, total betalain content, HPLC-DAD analysis, antioxidant capacity, antibacterial activity and acute toxicity screening test were employed for the character of the main pigments presented in the extracts. Maximum pigment concentration (73 mg/100 g of fruit) was obtained. Four main compounds of betalain were detected by HPLC-DAD analysis with betanin as the first main compound and its concentration is 3081,97 ppm. The stability of the pigment was observed at temperature of 70°C. In addition to the antioxidant and antibacterial activities which were obtained from pigment, there was no mortality or any signs of behavioral changes or toxicity observed after oral administration of extract up to the dose level of 48500 mg/kg body weight in mice. This can be a benchmark that this pigment could be developed as a source of food colorant.

Key words: Betalains, red pitaya peel (*hylocereus polyrhizus*), extraction, spectro photometric, HPLC-DAD, bioactivity, toxicity screening

INTRODUCTION

Due to its unique taste, shape and the flesh colour, *hylocereus* species or better known as dragon fruit or pitaya from the Cactaceae family has become an interesting subject to many researchers (Mizrahi *et al.*, 1997). This fruit belongs to the family of Cactaceae and order of Caryophyllales. The peel and flesh of this species are red in colour.

According Saati (2011), red pitaya peel amounted to 30-35% of the weight of the fruit and it is often simply thrown away as garbage. Whereas the results shows that skin of pitaya also contains antioxidants and can lower cholesterol levels (Kanner *et al.*, 2001). The red pitaya fruit (*H. polyrhizus*) contains betalain that acts as antioxidants and natural dyes (Stafford, 1990; Wybraniec *et al.*, 2001; Wu *et al.*, 2006; Khalida, 2010). Red pitaya peel has a greater antioxidant potential than other fruits (Darmawi, 2011), in addition to its skin, pitaya is also reported to have antimicrobial activity (Sri Amalia *et al.*, 2014).

At present, most of red pitaya peels are normally treated as wastes and will be discarded during processing. Therefore, this study aims to evaluate bioactive compound profiles, total betalain contents, temperature stability test, anti-oxidant capacity, antibacterial and screening of toxicity of red pitaya peel extract. The findings obtained in this study could support the potential application of red pitaya peels as a natural food colorant.

MATERIALS AND METHODS

Materials: The fruits were collected from certain sources in Padang, West Sumatra, Indonesia and they were immediately transported to the laboratory. HPLC-grade water, methanol and formic acid were taken from Merck, Germany. Betanin standard was from Sigma Chemical Co. All other chemicals used in this study were analytical grade.

Instrumentation: a shimadzu UFLC series equipped with a photo diode array detector were utilized for chromatographic analysis and Shimadzu Spectrophotometer UV-1800 was used for spectrophotometric analysis.

Extraction and concentration of betalains pigment:

About 280 g of peel pitaya was mixed with 1 liter of ethanol 60% for 15 min at room temperature and shaken for 12 hours. This process was repeated for three times. The extract was filtered and concentrated under vacuum by a rotary vacuum evaporator at 40 degree Celsius.

Identification of betalains: The compound of betalains in the solution was identified by spectrophotometric at 200 to 800 nm with a UV-Vis Spectrophotometer.

Determination of total betalains (spectrophotometer):

The concentrated betalain extract was diluted with distilled water and the measurement was carried out at

wavelength of 530 nm. Meanwhile, the quantification was expressed as mg betalains/100 g using the following equation as determined by Castellar *et al.* (2003):

$$\text{Total betalains content (mg/100 g)} = \frac{A \times DF \times MW \times 1000}{\epsilon \times L}$$

where:

- A : Absorption value at 535 nm density
- DF : Dilution volume
- L : Path length of cuvette
- MW : Molecular weight of betalains (550 g/mol)
- ϵ : Extinction coefficient for betalains 60000 L/mol

Identification profile of bioactive compound by high performance liquid chromatography-photo diode array detector (HPLC-PDA): HPLC analyses were performed on HPLC (High pressure UFLC Shimadzu HPLC-System). The analytical column Shimpack 250 x 4 RP-18 (5 μ m) was operated at 30°C. Solvents were 0.4% (v/v) formic acid in water (A) and methanol (B), the gradient was from isocratic 10% B for 5 min, from 10 to 40% B for 5 min, from 40 to 90% B for 5 min, isocratic 90% B for 5 min, from 90 to 10% B for 3 min and isocratic 10% B for 2 min, at a flow rate of 1 mL/min, simultaneous monitoring was performed at 190-800 nm.

Determination of betalains compound (HPLC): Determination of total betalains was filtered with 0.45 μ m paper filter and injected to HPLC system then compared with standard betanin solution. Injection volumes were 10 μ L and the detection wavelength was 540 nm.

Temperature stability test: The measurement of temperature stability test which is based on the color intensity of betalains solution was performed on four temperature treatments (30, 50, 70 and 100°C). Each solution was measured with a maximum wavelength double beam spectrophotometer Shimadzu UV-1800 with an area measuring wavelengths between 200 -800 nm.

Antioxidant capacity test: Antioxidant capacity determined by DPPH-radical scavenging activity (DPPH assay). The ability to scavenge DPPH free radical was determined based on the method of Brand Williams *et al.* (1995) with minor modification. Briefly, reaction mixtures containing 0.025: 0.050: 0.125 and 0.175% extracts and 2 mL 40 mg/L DPPH solution were prepared, mixed and then reacted in the dark for 30 min. A control sample containing the same volume of solvent in place of extract was utilized to measure the maximum 2,2-diphenyl-1-picrylhydrazyl (DPPH) absorption. The absorbance at 517 nm was recorded to determine the concentration of the remaining DPPH.

Antibacterial test: For the purpose of evaluation of the antibacterial samples, Kirby-Bauet test method was used. The bacteria (*Staphylococcus aureus* dan *Escherichia coli*) are swabbed on the agar and the antibiotic discs are placed on top. The antibiotic diffuses from the disc into the agar in decreasing amounts and the further; it is away from the disc. If the organism is killed or inhibited by the concentration of the antibiotic, there will be no growth in the immediate area around the disc: This is called the zone of inhibition. The zone sizes are looked up on a standardized chart to give a result of sensitive, resistant, or intermediate.

Toxicity acute test: Acute oral toxicity test was performed as per OECD-423 guidelines. All the animals were randomly distributed into one control group and three treated groups. Each group contains five animals. Groups 1, 2, 3, 4 and 5 were orally administered 15, 150, 1500, 15000 and 48500 mg/kg body weight extract following the method of Lorke (Lorke *et al.*, 1983). The control group received vehicle alone. The animals were observed continuously for the first 24 h and 7 days for any signs of behavioral changes, toxicity, mortality and body weight.

RESULTS AND DISCUSSION

Betalains extraction: Extraction of betalains pigments from plants was typically done using solvent extraction processes. Betalains are polar molecules and consequently more soluble in polar solvents, however, the conditions of extraction process are also key factors in their overall solubility.

The extraction conditions such as solid-liquid ratio (solid loading), incubation temperature, incubation time, solvent type and solvent concentration are important in the stability and concentration of betalains that can be extracted from these particular crops.

Methanol is the most commonly used solvent for the extraction, but it is also considered more toxic and hazardous to handle than other alcohols. Water and ethanol are more environmentally friendly and can also recover anthocyanins with good quality.

The use of organic solvents mixture with water has become attention of the current research. The absorption of organic solvents such as ethanol into the pores of plants is better than water. This will cause the process of extraction by organic solvents would be more effective. However, because of the nature of betalains which tends to be stable at neutral pH, also requires the use of a mixture of water so that the pH is not low.

Pitaya peel of 280 g performed maceration using ethanol 60% solvent during 3 x 12 hours. The selection method was chosen in addition to being easy, simple and expected to reduce the risk of damage to the content of the compounds so it is a suitable method used in the study. The extract obtained was 91.4 g which means the rendement about 31.8%.

The use of 60% ethanol would make the water content in extract quite high; it is difficult in the process of solvent evaporation. But the absence of water use will also make compounds in the extracts were considered to contain betalains pigment. The pigment will be more easily damaged due to the low pH of ethanol.

Identification of betalains: Uv-Vis spectrum measurements on sample extract solution shows a maximum peak at a wavelength of 530 nm that is showed in Fig. 1. It is confirmed that the extract which is contained compounds of betalains, not as anthocyanin compound. The absence of peaks in the uv region shows the compound does not have a benzoyl group which is identical with the class of flavonoids. These are the characteristics of the anthocyanin.

Total betalains content: For peel components, the yield that was obtained using 60% ethanol as solvent was significantly higher than the one with water solvent. The concentration of betalains in water expressed as betanin was obtained in 7.72 mg/100 g. However, The concentration of betalains expressed as betanin equivalents per 100 g of peel was 73.24 mg, respectively when ethanol 60% was used as solvent.

High performance liquid chromatography-photo diode array detector (HPLC-PDA) analysis: Figure 2 shows the betalains profile of the extract using the HPLC-DAD chromatograms at 540 nm. A total of four betalains compounds were identified by their elution of the extract. The retention times of peaks in the HPLC chromatograph of the samples study were compared with that of the standards to identify the unknown. The qualitative and quantitative results of betalains detected in extract are presented in Table 1. The results indicated that betanin and isobetanin were found in the extract at 540 nm.

Flavonoid was also found in the extract in the UV observation area at 280, 360 nm (Fig. 3). There are several peaks that indicated as flavonoid compounds (peak 1, 2, 3, 4 and 5). To identify a group of flavonoid compounds in the extract still requires a further study such as LC-MS analysis.

HPLC (chromatographic) techniques with DAD and reversed-phased column are widely used and favorable for flavonoid betalains detection in the same time of the analysis. A group of flavonoids and betalains are compounds that differ from each other. A group of flavonoids provide maximum absorption in UV region, the group betalains provide maximum absorption at a wavelength of visible light. The use of DAD analysis can be done in one time analysis. The presence of flavonoids in the extract betalains is possible because the extraction process using 60% ethanol. Ethanol is also a good solvent in the extraction process of a group of flavonoids from the plant material.

Table 1: Qualitative and quantitative results of betalains for HPLC-DAD data

Standard					
Peak	RT	Area	Conc. (ppm)	Name	λ max
1	13.853	56598.8	1000	betanin	538
2	14.376	50767.2	1000	isobetanin	538
Extract					
1	13.855	174437.3	3081.97	betanin	538
2	14.362	4836.6	95.27	isobetanin	538

Table 2: Betalain degradation rates against the rise of temperature treatment

No	Temperature	Abs	Degradation (%)
1	30	0.799	0
2	50	0.780	0.28
3	70	0.781	0.28
4	100	-	100

Table 3: Degradation data of betalain compound from HPLC chromatogram data

No.	Temp.	Peak	Name	Area	Degradation (%)
1	30	1	Isobetanin	46580.5	-
		2	betanin	1400.6	-
2	100	1	Isobetanin	8569.1	81.60
		2	betanin	287.9	79.44

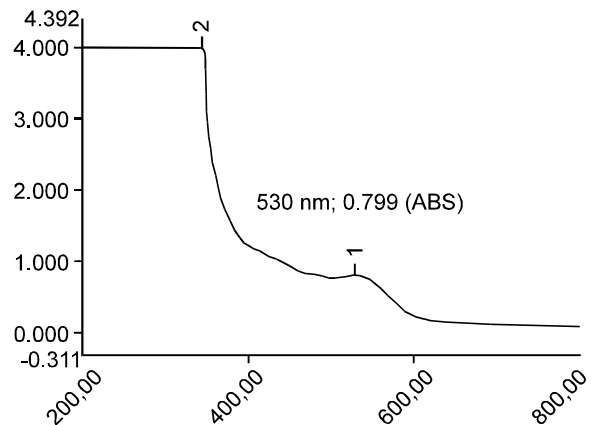


Fig. 1: UV-Vis Spectrophotometric data of red pitaya peel extract

Temperature stability test: Pitaya peel extract diluted with water solution (1:9 V/V) treated by heat at various temperature such as 30 (as a control), 50, 70 and 100°C. Heat treatment carried out for 15 min in the oven, a point to see the degradation or damage of betalains with the treatment given temperature. Reason took over the temperature variation to the application as a dye for food and beverage processing. In general, the food and beverage process used a water system that had 100°C boiling point of water (Santoni, 2013). Observation on the treatment that was given to the effect of temperature in the solution, has been diluted with a water solvent, where the water was the basis of a solvent. This process is commonly used in food processing. The spectrophotometric data for the treatment of temperature

against existing betalains compound in the extract was attached in the Fig. 4. At the maximum wavelength (530 nm), the absorbance of betalains was decreased against the rise of temperature (Table 2). From the data, it can be explained that the application of the betalains extract for red pitaya peel can only be used for applications that do not need the high temperature treatment, such as the production of ice cream.

The HPLC chromatogram results (Fig. 5) can give more information about the degradation of betalains

compound against the increase of temperature. HPLC chromatogram data give more detail about the absorbance of the compound that cannot be identified on spectrophotometric analysis. In spectrophotometric analysis the degradation on 100°C could not be determined, but on HPLC analysis, it can be determined. The result is on Table 3.

Antioxidant activity test: In order to evaluate antioxidant activity of chosen betalains extract, DPPH assay was

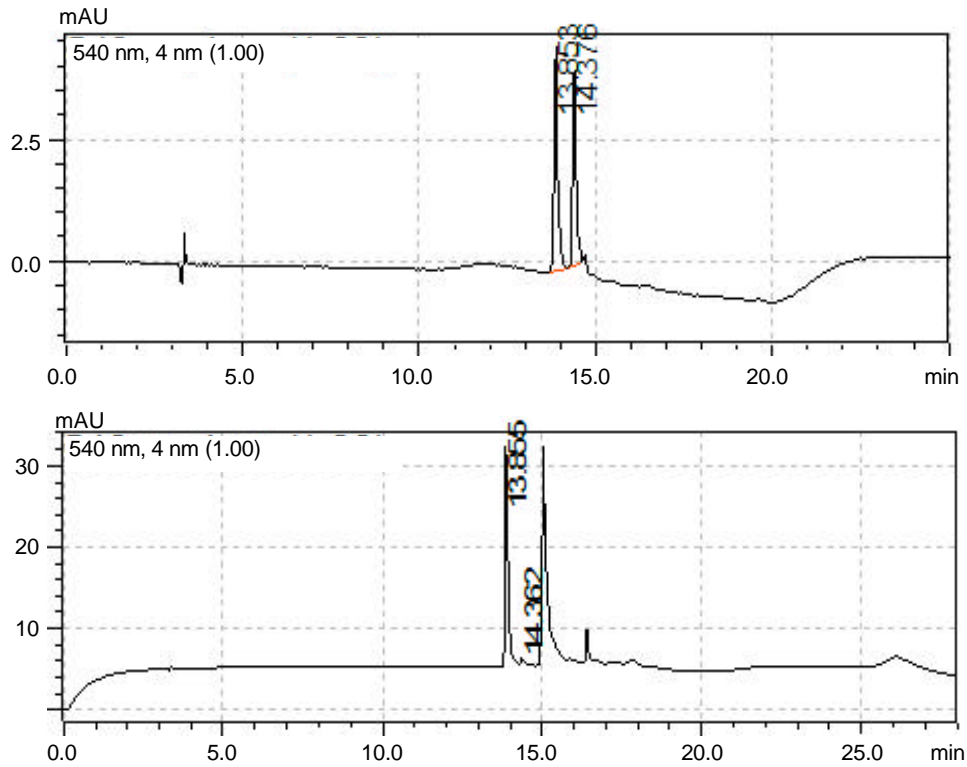


Fig. 2: HPLC chromatogram of betalains profile

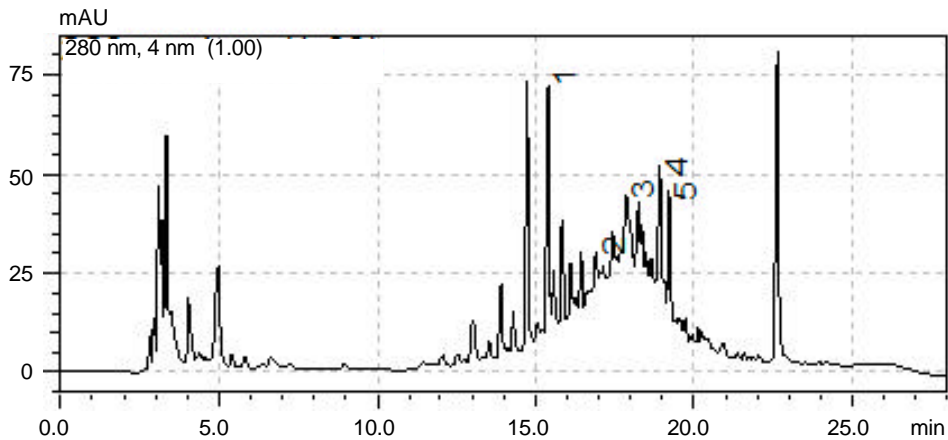


Fig. 3: Continue

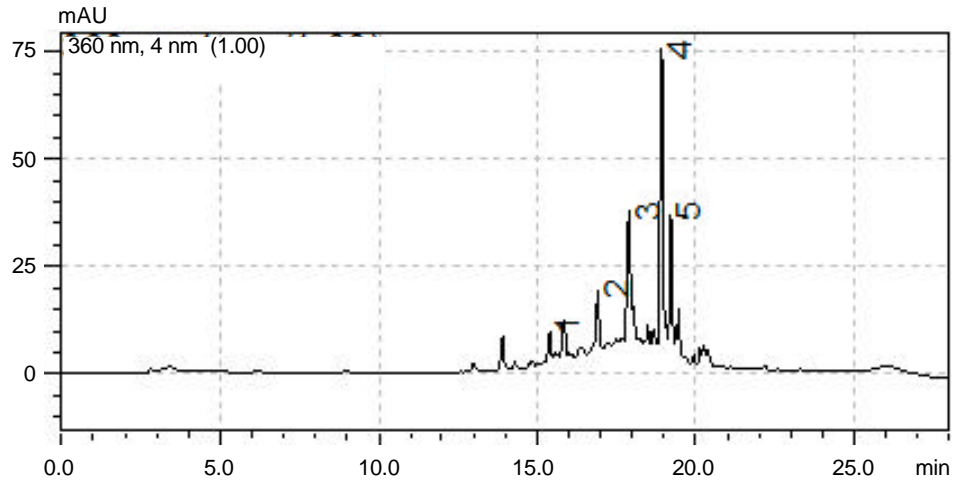


Fig. 3: HPLC-DAD chromatogram of flavonoid profile

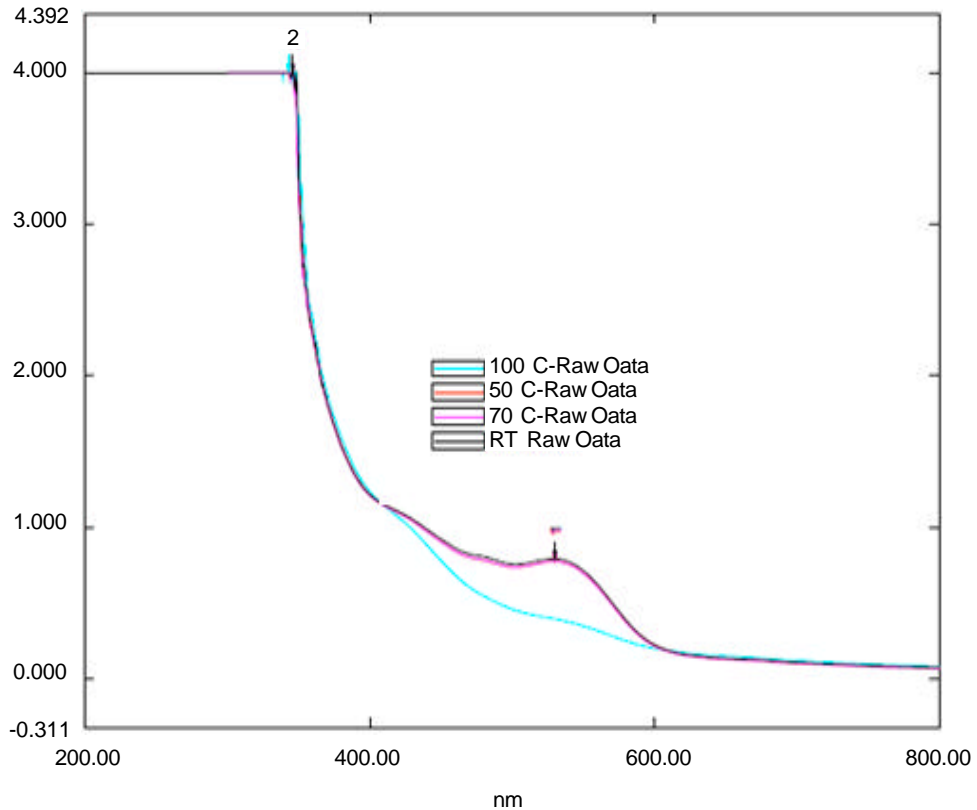


Fig. 4: Spectrophotometric data of existing betalains absorbance against temperature treatment of the red pitaya peel extract

applied and the result was 0.263%. The antioxidant activity of the extract samples gave IC₅₀ values of 0.263%. This indicates that the sample has a weak antioxidant activity. Betalains structure have no phenolic group, this may be the cause of their weak antioxidant activity.

Antibacterial activity: *In vitro* antibacterial activity of the extract is evaluated by disc diffusion method using selected Gram-positive and Gram negative bacteria. The inhibition zone on Gram-positive bacteria (*Staphylococcus aureus*) demonstrated higher susceptibility than Gram-negative (*Escherichia coli*). The

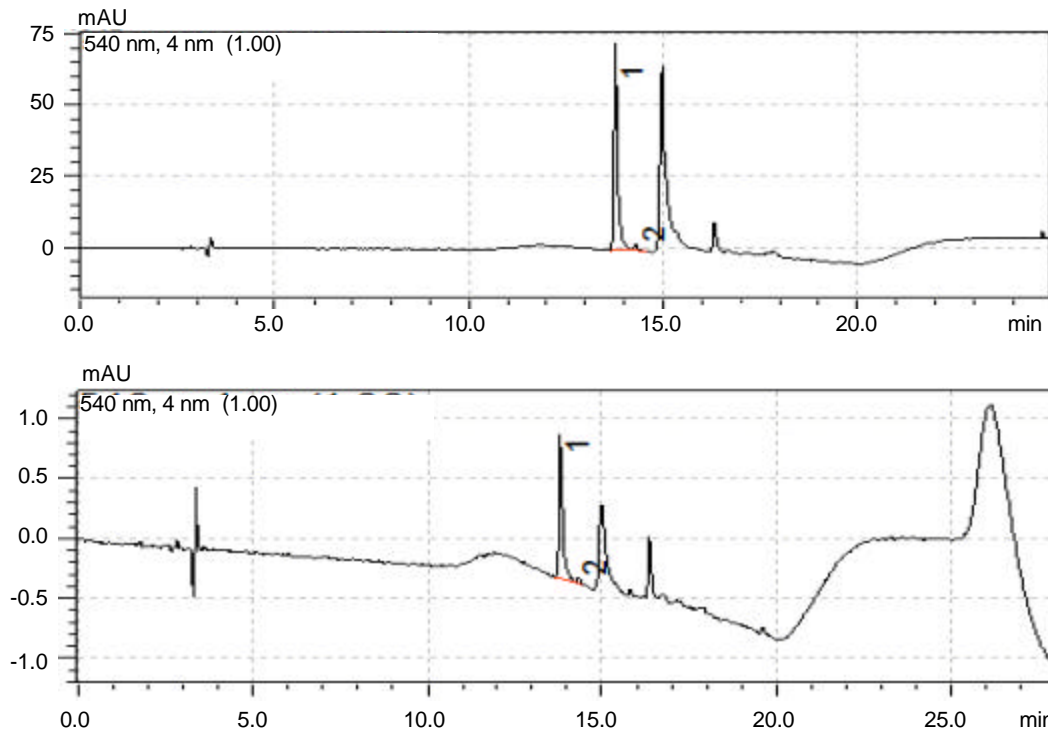


Fig. 5: HPLC chromatogram of betalains degradation against temperature treatment

data indicates that the exhibited extract activity is against the investigated food pathogens. The presence of betalains compounds which is a group of alkaloids is the most probable explanation of the resulting antibacterial capability.

Acute toxicity test: Experimental screening method is imperative in order to establish the safety and efficacy of traditional and herbal products and also to set up the active components of the herbal products (Mythilypriya *et al.*, 2007). In the acute toxicity test of the betalain extract, there was no mortality or any signs of behavioral changes or toxicity observed after oral administration of extract up to the dose level of 48500 mg/kg body weight in mice. There were no significant differences in the body weight between the control and treated groups.

Conclusion: Betalain were extracted and analyzed from Red Pitaya Peel (*Hylocereus polyrhizus*). Complementary data from the extraction process, spectrophotometric, HPLC, bioactivity and acute toxicity analysis provided data that strongly supported the development efforts of betalains pigment as the alternative food colorant.

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