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Research Article
Beetroot Nanoencapsulation in Flavored Beverage and its Effect as Hepatoprotective Agent in Rats

[1]Tarek N. Soliman, [2]Samera H. Shehata and [3]Safaa A. Salem

[1]Dairy Department, Food Industries and Nutrition Research Division, National Research Centre, El-Buhouth St., 12622 Dokki, Cairo, Egypt
[2]Department of Dairy Science and Technology, Menoufia University Shibin El kom, Cairo, Egypt
[3]Department Medicinal Food, National Organization for Drug Control and Research (NODCAR), Giza, 12553, Egypt

Abstract

Background and Objective: Beetroot juice is a biological antioxidant and acts as health-promoting minerals as well as soluble fibres and vitamins. This study aimed to encapsulate the Beetroot Juice Powder (BJP) by the conjugate sodium caseinate (NaCas) and Maltodextrin (MD) to protect it from environmental conditions. Produced flavoured acid beverage using BJP encapsulated using conjugates. **Materials and Methods:** Nano-encapsulation of BJP (20, 30, 40 mg g^{-1}) and determine the encapsulation efficiency, size and zeta potential. Rats were divided into 4 groups as follows, negative control, positive control and 2 test groups that received free BJP or encapsulated BJP. All rats except the negative control group were injected with CCl_4 twice a week. **Results:** The NaCas-MD conjugate has the advantage over the NaCas-MD complex of higher stability and BJP binding, also showing high encapsulation efficiency (>93.75%) of different levels of BJP. The flavoured beverage from the addition of BJP encapsulated by conjugate has better sensory and technological properties than fortified with BJP in the complex. Injection with CCl_4 leads to a decrease in body weight, serum parameters including, protein, albumin, GSH, CAT and SOD, also increase ALT, AST, ALP and liver weight. Moreover, a variable pathological alteration in liver tissue was found. At the end of the experiment receiving encapsulated beetroot juice led to improvement in all above body and liver weight, all biochemical parameters and histopathological elevation. **Conclusion:** Thus, it could be concluded that flavoured beverage containing BJP encapsulated by conjugate is of acceptable quality and high antioxidant activity. Also, it has a remarkable protective effect against acute hepatotoxicity.

Key words: Sodium caseinate, complex, conjugate, flavoured beverage, beetroot juice, carbon tetrachloride, liver function, oxidative stress, liver histopathology

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Corresponding Author: Tarek Nour Soliman, Dairy Department, Food Industries and Nutrition Research Division, National Research Centre El-Buhouth St., 12622 Dokki, Cairo, Egypt

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Beetroot juice is strong in available biologically antioxidants and a variety of other health-promoting minerals, include, iron, magnesium, calcium, potassium, phosphorus and sodium as well as soluble fibres and vitamins like B6, folic acid. Flavonoids, phenolic acids, carotenoids and ascorbic acid are one of the phytochemical components found in it[1]. Also, in one of the few vegetables that contain betalains, a group of highly bioactive pigments[2,3]. *In vitro* and *in vivo* animal models, betalain was found to have antioxidant and anti-inflammatory activity[3,4]. This has stimulated study in beetroot's potential role in oxidative stress and chronic inflammation-related diseases such as liver disease, arthritis and cancer. The roots and leaves of the beet have long been used as a folk treatment to cure several diseases, including immune system activation as well as liver and kidney diseases[5].

The nanostructures' trend has been paid attention to, because of its ability to improve solubility and bioavailability for bioactive ingredients without compromising other food properties[6]. Complex formation between proteins/polysaccharides presents protein stability versus aggregation or dissociation on a broad range of pH and temperature. Recently, researchers reported the bovine serum albumin/carrageenan complexes to give high binding and encapsulation efficiency for polyphenolic compounds compared to native proteins[7,8]. Moreover, complex protein/polysaccharides based on electrostatic reactions without noncovalent reactions are sensitive to environmental conditions. Maillard reaction is natural and proteins conjugate with polysaccharides such as whey proteins[9], casein and soybean protein, giving significantly increased functional properties such as solubility, foaming and emulsification.

Chemical-induced liver injury is mostly caused by oxidative stress in hepatic tissue and it is at the base of many diseases because there is still no effective therapeutics, antioxidants have been proposed as a treatment for injury prevention and/or attenuation[10]. Using carbon tetrachloride CCl_4 to induced liver injury is the better system of xenobiotic-induced hepatotoxicity and it's a powerful machine for evaluating natural compounds' hepatoprotective effects[11]. The liver swells and becomes tender and fat collects inside the organ also it may be damaged or destroyed in severe cases, leading to a reduction in liver function[12].

The objective of the present study investigates and characterizes the ability to encapsulate the Beetroot Juice Powder (BJP) by sodium caseinate-maltodextrin (NaCas-MD) in 2 forms, complex or conjugate. Also, produced and characterized strawberry flavored acid beverage using BJP encapsulated in the NaCas-MD conjugate. Then, studied the protective effect of free BJP or encapsulated against hepatotoxicity in rats.

MATERIALS AND METHODS

Study area: The study was carried out at the Dairy Department, National Research Centre, Department of Dairy Science and Technology, Menoufia University Shibin El Kom, Egypt and National Organization for Drug Control and Research from January-April, 2021.

Materials: Bovine sodium caseinate was obtained from Sigma/Aldrich (St. Louis, MO, USA). Maltodextrin (MD) was purchased from Alfasol Co., Turkey. Red beetroots (*Beta vulgaris* L.) was purchased from the local market, Cairo, Egypt. Beetroots are composed of 89.43 g water, 1.61 g protein, 0.17 g lipids and 8.56 g carbohydrates per 100 g. Of the carbohydrates, beets are composed of 29.3% fibre (2.8 g total dietary fibre/100 g beet) and 70.7% sugar (5.76 g sugar/100 g beets). All chemicals, kits and solvents used for spectral and HPLC analysis were of HPLC grade and all other solvents were of ACS grade.

Animals: Male rats weighing 140±10 g were obtained from the animal house of the National Organization for Drug Control and Research Giza, Egypt. Rats were kept 1 week under standard laboratory conditions and housed in stainless steel cages in an air-conditioned room with a temperature of 22±3°C and relative humidity of 30-70% for acclimatization. The investigation complies with the guide for the care and uses laboratory animals (NODCAR/II/29/2020). The experimental protocol was approved by the Institutional Ethics Committee of NODCAR, Giza.

Methods

Preparation of Beetroot Juice (BJ): Clean 1 kg beetroots and were cut, then frozen at -10°C for 24 hrs to destroy the cellular structure of beets. After 24 hrs the frozen up beets were brought out and kept at room temperature for thawing. The beetroots were minced by a blender and pressed to extract the juice. The juice was filtered first in organza and afterwards under vacuum on qualitative filter paper for the elimination of suspended solids. The weight of the extracted juice was measured by a digital balance of 350 mL. Juice extraction percent was calculated as:

$$\frac{\text{Weight of extracted juice}}{\text{Weight of fresh beet}} \times 100$$

of 35%. The moisture content of the juice was measured by an oven-dry method of 90%. Then freeze-dried the Beetroot Juice (BJ) to obtained Beetroot Juice Powder (BJP).

Preparation of NaCas-MD complex and NaCas-MD conjugates: Preparation of the NaCas (5%, w/v, protein) and MD (5%, w/v), were prepared in ultrapure water to obtain a 10% NaCas-MD solution, with low-speed magnetic stirring and allowed to solubilize for 2 hrs at 22°C. The solution was adjusted to pH 7.0 using 0.1 M HCl or 0.1 M NaOH, stored at 4°C for 12 hrs and readjusted to pH 7.0 as necessary. Then, the complex solutions were prepared by mixes the NaCas and MD (1:1). The NaCas-MD solution was frozen initially at -20°C for 12 hrs and then tempered at -80°C for 3 days. Freeze-drying was carried out under vacuum at a pressure of < 0.1 m bar for 72 hrs. The resulting material was milled and divided into 2 portions, the 1st portion NaCas-MD complex and the 2nd portion transferred to a plastic Petri dish (150×15 mm) and then placed on a perforated plate in a desiccator that contained a pre-equilibrated saturated potassium bromide solution generating an atmosphere with Relative Humidity (RH) of 80% at 80°C/24 hrs to prepared WPI-MD Conjugate[13].

Determination of available amino groups: The concentration of available amino groups in solutions, prepared from 0.1% dry heated NaCas and NaCas-MD was measured by the θ-phthalaldehyde (OPA) method as detailed by Nielsen *et al.*[14] with minor modifications as described by O'Regan and Mulvihill[15]. The concentration of available amino groups in the NaCas and NaCas-MD conjugate solutions at 80°C for 1 day, expressed as a percentage of available amino groups in the respective unheated solutions.

Determination of betalain content: The content of betalains (BC) in the samples was determined according to the method described by Skalicky *et al.*[16].

Total phenolic content: Total Phenolic Content (TPC) of samples was established using the Folin-Ciocalteu spectrophotometric method[17].

Determination of antioxidant activity DPPH: The Antioxidant Activity (AA) by DPPH was determine decreasing of DPPH solution using the spectrophotometric method described in Girones-Vilaplana *et al.*[18].

HPLC analysis: Separation and quantitative determination of polyphenols content of BJP were carried out using HPLC apparatus model 1100 (Agilent Technologies, CA, USA) system column: Agilent Eclipse XDB C18 (150×4.6 and 5 μm) according to Croci *et al.*[19].

Preparation of BJ-loaded NaCas-MD complex and NaCas-MD conjugate nanoparticles: For the preparation of beetroot juice-loaded NACas-MD complex and NaCas-MD conjugate, the powder was dissolved in distilled water with a concentration of 100 mg mL^{-1}. The BJ was added to the NACas-MD complex and NaCas-MD conjugate, solution at a concentration of 20, 30 and 40 mg mL^{-1} (curcumin to protein ratio of 20:100, 30:100 and 40:100). The solutions were agitated at room temperature for 1 hr in the beaker dark wrapped. Then homogenized by high-intensity ultrasound VCX800 (Vibra Cell, Sonics, Newtown, CT, USA) at an amplitude of 40% with a 13 mm diameter probe (high-grade titanium alloy) for 5 min in an ice bath. The resulted nanoparticles, a part of it are kept as the liquid to measure the particle size and zeta-potential, while the rest was freeze-dried.

Determination of encapsulation efficiency: Encapsulation Efficiency (EE) was presented as the ratio of encapsulated phenolic content to total phenolic content. The phenolic content in the core (CPC) and surface (SPC) of powder was determined by Robert *et al.*[20].

Preparation of beverage: The functional beverage was prepared using 600 mg BJP with 2 forms, the first form: 20 g (30 mg BJP encapsulated by NaCas-MD conjugated) and the second form is 600 mg BJP free with NaCas-MD complex with good agitation, avoiding entrapment of air. Then added 0.25% citric acid and added 3% strawberry flavour, then adjust to pH 3.2 using phosphoric acid with continuous mixing, sugar (2%) and vegetable oil (5%). Heating to 80-85°C for 15-30 sec may serve as a starting point for low pH beverages. Hot-fill containers and cool immediately. The beverage thus obtained was stocked undercooling. Chemical analysis (total solids, fat, proteins, carbohydrates and ash) and acceptability evaluations were performed for the fresh beverages. The sensory evaluations of beverages were judged by 30-panel members and asked to evaluate the beverages samples on a scale of 1-5 (representing excellent-5, very good-4, good-3, fair-2 and poor-1) for taste, texture, colour and overall acceptability[21].

Induction of hepatotoxic rats by CCl_4: Acute toxicity tests have been carried out following the Organization for Economic Cooperation and Development's (OECD)-423 guidelines. Hepatotoxicity was induced by an Intraperitoneal

injection of CCl_4 (2 mL kg^{-1} b.wt.) twice a week during the experimental feeding period according to Sundaresan and Subramanian[22].

Experimental design: Twenty four rats were randomly divided into 4 groups of 6 animals in each group negative control group (control$^-$), Hepatotoxicity group (control$^+$): Rats injected intraperitoneal CCl_4 (2 mL kg^{-1} b.wt.) suspended in olive oil (1:1) twice a week:

Group I : Rats received Beetroot Juice (BJP) 8 mL/kg/b.wt./day

Group II : Rats received Encapsulated Beetroot Juice (EBJP) 8 mL/kg/b.wt./day

All rats in tested groups I and II were Intraperitoneal injected with CCl_4 (2 mL kg^{-1} b.wt.) suspended in olive oil (1:1) twice a week and orally doses of BJ and EBJ were given in 3 doses/day for the 2 groups, respectively during the experimental period[23]. All rats were fed on a standard diet[24].

At the end of the experiment, body weight and liver weight was recorded and blood samples were taken from the orbital plexus of the eye of each rat[25]. Serum was separated by centrifugation at 3000×g for 10 min and used for serum biochemical analysis. Also, the liver was excised and used for histopathological examination.

Serum biochemical analysis: The activities of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined[26], protein and albumin[27] and alkaline phosphates were determined[28,29]. Reduced glutathione (GSH) was estimated by its reaction with dithio-bis-2-nitrobenzoic acid (DTNB)[30] and Catalase (CAT) activity was determined[31], Superoxide dismutase was determined according to SOD method[32].

Histopathological method: Specimens from the liver were fixed in 10% formalin then dehydrated and embedded in paraffin for light microscopic examination, sections of 5-micron thickness were cut and stained by Hematoxylin and Eosin (H and E) for general histological structure and counterstain which was done using Eosin stain.

Statistical analysis: The data presented as Mean Values±Standard Deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's test. The differences were considered significant at ($p \leq 0.05$). The IBM SPSS statistics 23 software program for statistical analyses [IBM Corp[33] IBM SPSS Statistics for Windows were used, Version 23.0. IBM Corp, Armonk, NY] with $p \leq 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Available amino groups: The Concentration of NaCas and MD and heating methods (such as wet heat or dry heat) can affect the degree of conjugation[13] found that the degree of conjugation NaCas 5% W/V and MD5 was 10.8% after 10 hrs wet heating at 90°C. However, the degree of conjugation in Fig. 1 showed the free amino groups absorbance at 340 nm were high in the wet heating for NaCas-MD at 80°C for 24 hrs, while the lowest in the native NaCas. The absorbance of native NaCas decreased from 100-95.97% in RH of 80% at 80°C/24 hrs. Nevertheless, the Available amino groups of NaCas-MD were decreased to 74.77% after a period of conjugation of 24 hrs, at RH of 80% at 80°C. The higher degree of conjugation found in the present study might be due to the period of conjugation 24 hrs may have led to more effective interactions between protein and polysaccharide.

Bioactive components: For characterizing beetroot juice powder the chosen exemplary index was total phenolics, total betaxanthins content and total betacyanins (spectrophotometric determined), individual phenolics and water-soluble vitamins (HPLC characterization) as well as free Radical Scavenging Activity on DPPH (RSA). The results are presented in Table 1 and 2.

Table 1 illustrates the total polyphenols value, determined by the Folin-Ciocalteu examination, which are the control

Table 1: Content of bioactive components and antioxidant activity of beetroot juice powder (BJP)

Bioactive components or activity	Content of BJP (mg/100 g)	
Total phenolic content	129.57±4.045	Gallic acid equivalents
Total betaxanthins content	85.45±2.09	Vulgaxanthin-I equivalents
Total betacyanins	86.29±2.21	Betanin equivalents
Radical scavenging activity	75.98±2.00	mol trolox equivalents

Table 2: HPLC analysis for polyphenols and soluble vitamins of BJP

Compounds	Area	Conc. (μg g^{-1})
Catechin	32.61	8.87
Caffeic acid	38.00	1.22
Ellagic acid	25.68	2.72
Naringenin	39.96	1.06
Propyl gallate	78.00	2.07
Quercetin	33.63	2.42
Cinnamic acid	346.53	3.17
Vitamin B 12	55.95	513.70
Vitamin B 2	113.63	241.50
Vitamin C	3.00	200.10
Folic acid	380.98	586.40

Table 3: Size (nm) and ζ potential (mV), polydispersity index (PDI) and encapsulation efficiency for NaCas-MD complex, NaCas-MD conjugated and BJP ratio encapsulated

Samples	Size (nm)	Calculated (PDI)	ζ potential (mV)	Encapsulation efficiency (%)
NaCas-MD conjugate	1291±833	0.387	20.40	-
20 mg BJP	436±195	0.396	17.40	96.78±2.12
30 mg BJP	469±184	0.445	16.80	95.55±2.40
40 mg BJP	488±207	0.489	16.50	93.75±1.95
NaCas-MD complexes	610±245	0.479	17.20	-
20 mg BJP	285±98	0.484	15.10	89.25±1.20
30 mg BJP	358±145	0.543	7.91	85.45±3.45
40 mg BJP	379±191	0.588	2.62	79.95±5.54

bioactive components in BJP of 129.57 mg Gallic Acid Equivalents (GAE)/100 g. Also, the total betaxanthins content and total betacyanins were similar without significant difference ($p<0.05$) it finds 85.45 mg Vulgaxanthin-I Equivalents (VE)/100 g and 86.29 mg Betanin Equivalents (BE)/100 g, respectively. The total phenolic content in beetroot varies from 4.02-15.50 mg of GAE g^{-1} of dried weight. Georgiev *et al.*[34], reported that the betaxanthin and betacyanin concentration proportion usually ranges from 1-3 and depends on beetroot varieties and method of juice or extraction production. The quantity of betalains in beetroot has been estimated as 1000 mg/100 g of total solids.

Furthermore, the characterization of individual phenolic components and watery vitamins was conducted by the HPLC method in Table 2. It was revealed that cinnamic acid, propyl gallate, naringenin, caffeic acid and catechin are found in large amounts in BJP and watery vitamins concentration ratio from high content to lowest, folic acid, B2, B12 and C. HPLC data agreed with total polyphenols content determined spectrophotometrically.

Characterization of BJP nano-encapsulated

Effect of types NaCas-MD on droplet size, zeta potential, PDI and encapsulation efficiency: The particle size and their polydispersity have an important role in colloidal stability, BJP encapsulation and bioavailability. Table 3 showed the results of the particle size, calculated PDI, ζ potential and encapsulation efficiency for BJP encapsulated by the NaCas-MD complex and NaCas-MD conjugate. As shown the effect of adding different ratios of BJP (20, 30 and 40 mg) particles on the size of the NaCas-MD complex were 436, 469 and 488 nm is higher than ($p<0.05$) the NaCas-MD conjugate were 285, 358 and 379 nm, respectively. However, the size particles of NaCas-MD conjugated 1291 nm are higher than NaCas-MD complex 610 nm. The size of the rest samples had to decrease by ultrasonic homogenizer processing. In addition, it was found that the particle size increased with the increasing addition of BJP. This may be to the presence of BJP adsorbed on or entrapped in caseins.

Also, the glycation of NaCas changes the distribution of protein surface charge, by decreasing the content of lysine on the protein surface, which is supported by the lower value of ζ potential of glycated NaCas. That indicates modification of protein structure significantly by glycation. The BJP encapsulated by NaCas-MD conjugate shows good stability and narrow size distribution. The ζ potential of all samples shows a negative charge, which causes more repulsive forces between particles doing more solution stability. Similar results with a decrease in ζ potential values after encapsulation by conjugation were shown[35]. As shown in Table 3 zeta potential of samples BJP encapsulated was decreased by increasing BJP addition, NaCas-MD conjugate charge of -20.40 mV decreased to -17.40, -16.80 and -16.50 mV with the addition of BJP (20, 30 and 40 mg g^{-1}), respectively. The same trend showed with BJP encapsulated by NaCas-MD complex. The PDI values of the NaCas-MD complex of 0.479 increased by increasing adding BJP different ratios (20, 30 and 40 mg g^{-1}) were 0.484, 0.543 and 0.588, respectively. Also, the same trend with The NaCas-MD conjugate shows a significant difference ($p < 0.05$). The Encapsulation Efficiency (EE) of BJP different ratios addition (20, 30, 40 mg) shows increasing with NaCas-MD conjugate shell of 96.78, 95.55 and 93.75% more than the shell of NaCas-MD complex of 89.25, 85.45 and 79.95%. However, the EE for BJP encapsulated by NaCas-MD conjugate or NaCas-MD complex shells was decreased with the increase of BJP addition.

From the results obtained, it was found that the best source for BJP encapsulation that can be incorporated in functional strawberry beverages was 30 mg BJP loaded to NaCas-MD conjugate, In terms of encapsulation efficiency, diffusion rate and stability.

Nutritional value of the beverage: Since NaCas-MD conjugate displayed great techno-functional stability in the acidic condition it motivated to insert it in acidic flavoured beverages this study cleared the effect of protein conjugation by Maillard reaction and application of it in dairy beverages. Table 4 shows no significant ($p<0.05$) in the nutritional value

Table 4: Nutritional value and radical scavenging activity of flavoured caseinate beverage

Parameters	Beverage NaCas-MD complex	Beverage NaCas-MD conjugate
Protein (%)	9.60±0.05	9.55±0.10
Carbohydrate (%)	12.24±0.24	12.02±0.19
Oil	5	5
Ash (%)	0.29±0.01	0.31±0.03
Nutritional value (Kcal)	132.38	131.28
Antioxidant activity (µg (GAE) g^{-1})		
Fresh	6.57±0.23	6.62±0.19
30 days	3.45±0.17	6.55±0.21

Table 5: Effect of free BJP and encapsulated BJP on body weight and liver weight in rats

Groups	Initial body weight (g)	Final body weight (g)	Body weight gain (%)	Relative weight liver (%)
$Control^{-}$	147.14±1.38^{a}	171.16±0.90^{a}	16.32	2.33±0.003^{a}
$control^{+}$	142.14±1.28^{a}	154.28±0.99^{b}	8.54	4.56±0.005^{b}
I	145.59±0.86^{a}	168.57±3.62^{c}	15.78	3.49±0.003^{c}
II	143.85±1.03^{a}	170.14±2.78^{c}	18.27	3.75±0.005^{c}

Negative control group: Rats received water, positive control group: Rats were intraperitoneally injected with CCl_4, group I: Rats were injected with CCl_4 + beetroot juice (BJP), group II: Rats were intraperitoneally injected with CCl_4 + EBJP: Encapsulated beetroot juice, mean values with different superscript letters in the same column are significantly different at the Duncan test ($p<0.05$) n = 6

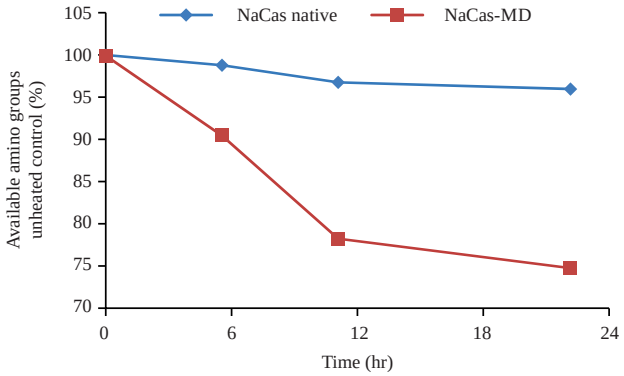

Fig. 1: Percentage of available amino groups in solutions prepared from NaCas and dry heated NaCas-MD
Conditions: pH: 7.0, Temp: 80°C, relative humidity: 80% for up to 24 hrs

of NaCas-MD complex and NaCas-MD conjugate beverages. Which the protein content of 9.60 and 9.55%, carbohydrates 12.24 and 12.02%, Ash of 0.29 and 0.31%, respectively. But the radical scavenging activity showed significantly ($p<0.05$) decreases with NaCas-MD complex beverage after 30 days storage from 6.57-3.45 µg (GAE) g^{-1}. While insignificant the change after storage period with NaCas-MD conjugate beverage, furthermore showed stability of NaCas-MD conjugate beverage and clearness from turbid protein and precipitate. Zhang *et al.*[13], reported that the Maillard reaction (casein with maltodextrin conjugated) affected a significant improvement in protein functionality (solubility, heat stability, emulsification, etc).

Sensory evaluation: Sensory attributes of BJP encapsulated by NaCas-MD complex or NaCas-MD conjugates beverage flavoured with strawberry are presented in Fig. 2. The results showed the fresh beverage prepared via NaCas-MD conjugate flavoured by strawberry takes a good acceptability score of 4.8 also no significant change in overall acceptability score of 4.7 on a 5 point scale after 30 days storage period, indicating that the staff members liked very well some attributes of the beverage via NaCas-MD conjugates. Because of the stability of colour and protein solubility under acid conditions. But, the fresh BJP encapsulated using NaCas-MD complex, beverage gained an overall acceptability score of 4.4 while decreasing to 2.8 after 30 days storage period.

Effect of BJP and encapsulated BJP on body and liver weight in rats: Table 5 showed increases in body weight of all groups at the end of the experiment. A significant decrease in the hepatotoxic positive control group which was intraperitoneally injected with CCl_4 twice a week compared to negative control was found, percentage of body weight gain at the end of the experiment was in values of 16.32 and 8.54% in the 2 groups, respectively. The reduction in body weight may be related to the toxicity because CCl_4 affects the activation and utilization of nutrients which due to maldigestion or malabsorption, that produced by gastrointestinal problems[36].

The tested groups I and II which were injected intraperitoneal CCl_4 twice a week and received (8 mL/kg/day) Beetroot Juice Powder (BJP) or Encapsulated Beetroot Juice Powder (EBJP) showed increases in body weight in both groups at the end of the experiment compared to the negative control group and hepatotoxic positive control group and non-significant differences between them were found, in percentage values of 15.78 and 18.27%, respectively.

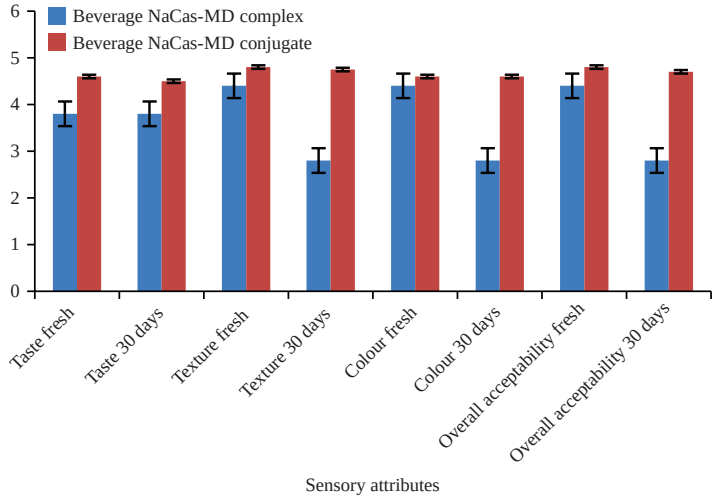

Fig. 2: Sensory attributes of the flavoured beverages

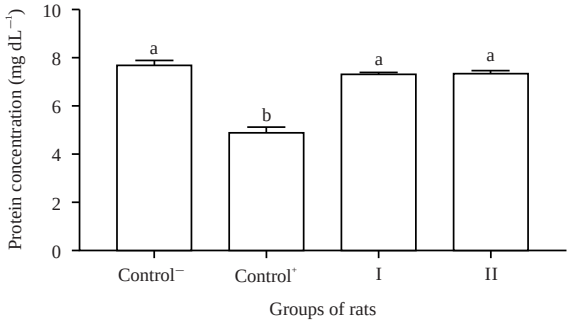

Fig. 3: Effect of BJ and encapsulated BJP on serum protein (n = 6 rats)

Also, Choi *et al.*[37] reported that *B. vulgaris* leaves extract resulted in a significant increase in body weight in diabetic rats through improved lipid and glucose metabolism.

A significant increase ($p<0.05$) in relative liver weight to body weight in the positive group compared to the negative control group was observed at the end of the experiment in percentage values of 4.56 and 2.33% in the 2 groups, respectively. The liver swells and becomes sensitive and fat stores inside the organ, also it may be damaged or destroyed in severe cases, due to a reduction in liver function[38].

On the other hand, there were non-significant differences in the relative weight of liver between tested groups I and II, in percentage values 3.49 and 3.75, respectively. Rats were given 250 and 500 mg of beetroot juice extract and 100 mg of silymarin to see if it could help with liver damage injury in rats[39].

Effect of BJP and encapsulated BJP on total protein: From results in Fig. 3 it could be observed that injection of CCl_4 in hepatotoxic positive control group caused a significant decrease ($p<0.05$) in total protein in serum compared to the negative control group in values of 4.29 and 7.73 mg dL^{-1} for the 2 groups, respectively at the end of the experiment. Levels of total protein are important indications of liver damage. Moreover, the tested groups I and II which were intraperitoneal injected CCl_4 twice a week and were given orally 8 mL/kg/b.wt./day BJ or EBJ showed a significant increase ($p<0.05$) in total protein compared to the positive control group. On the other hand, there was a non-significant difference between the 2 groups in values of 7.35 and 7.40 mg dL^{-1}, respectively at the end of the feeding period. Treatment with beetroot for 28 days improved the mean value of total protein significantly ($p<0.05$). Beetroot has a beneficial effect on liver function. The beetroot, juice and beetroot waste are high in antioxidants because they contain both total flavonoids and total phenols. As a result, it is recommended that liver disease patients consume it as part of their daily diet. Patients suffering from liver diseases can also drink beetroot juice to improve their liver function and increase antioxidant enzymes[23,40]. Encapsulated beet powders are promising natural pigments with a wide range of applications, furthermore because of their antioxidant activity beet powders may be beneficial to consumer health[41] because it has been used to protect bioactive[42].

Effect of BGP and encapsulated BJP on albumin: Figure 4 showed a significant reduction in serum albumin in the hepatotoxic positive control group compared to the negative control group in values of 2.83 and 3.92 mg dL^{-1}, respectively at the end of the experiment. Moreover, the tested groups I and II showed a significant increase ($p<0.05$) in albumin

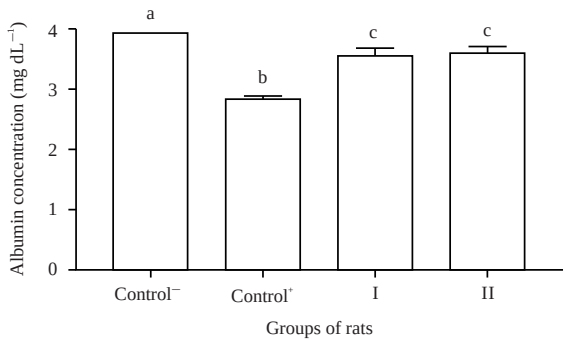

Fig. 4: Effect of BJ and encapsulated BJP on serum albumin (n = 6 rats)

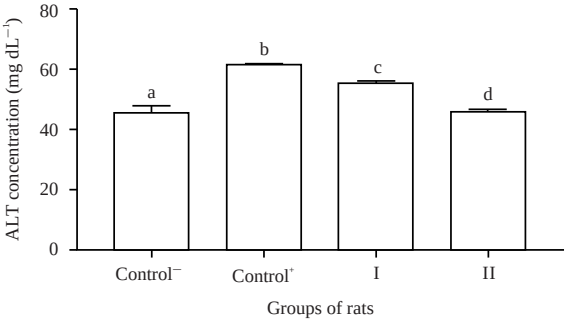

Fig. 5: Effect of BJ and encapsulated BJP on serum ALT (n = 6 rats)

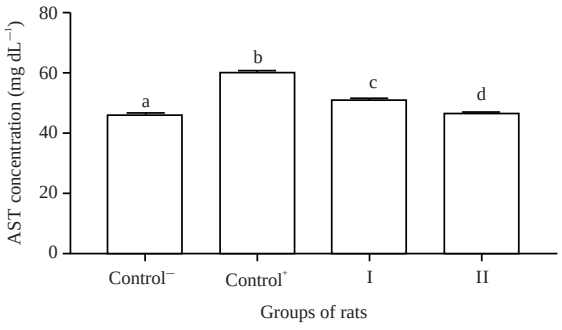

Fig. 6: Effect of BJ and encapsulated BJB on serum AST (n = 6 rats)

compared to the positive control group. On the other hand, there was a non-significant difference between the 2 groups in values of 3.55 and 3.59 mg dL^{-1}, respectively at the end of the feeding period with beetroot for 28 days which improved the mean value of total protein and albumin significantly ($p<0.05$). Phenolic components are classified into various types, the most important of which are flavonoids, which have strong antioxidant activity and are very effective at scavenging free radicals[43]. Antioxidant activity of protein-based encapsulation could be related to Maillard reactions between sugars from beetroot extract and whey proteins as well as bioactive components from beetroot extract[44].

Effect of BJ and encapsulated BJP liver enzymes: The effect of using free BJP and encapsulated beetroot juice powder on the activity of liver enzymes ALT, AST and ALP were found in Fig. 5 which showed a significant increase in ALT in the positive control group that was injected with CCl_4 (2 mL/kg/b.wt./day) twice a week for 4 weeks compared to the negative control group in values of 61.74 and 45.72 mg dL^{-1}, respectively. Results showed that hepatotoxicity was caused by a single dose of CCl_4, which was indicated by significant increases in the activity of liver enzymes ALT and AST. The leakage of cellular enzymes into plasma or serum can be used to detect hepatic damage[45].

The tested groups I and II showed a gradually significant decrease ($p<0.05$) in liver enzyme activity, ALT, compared to the hepatotoxic positive group the highest decrease was found in group II in value of 45.92 mg dL^{-1} followed by the group I in value of 55.50 mg dL^{-1}.

Beetroot ethanolic extracts have been shown to have hepatoprotective effects by improving liver function as seen by reduced blood ALT, AST and ALP levels. Also, it is an effect of dietary beetroot supplementation against oxidative stress induced by CCl_4 shown by reduced blood enzyme activity. In a rat model of hepatic injury caused by N-nitrosodiethylamine (NDAE), beetroot juice improved liver injury parameters, enzymes, DNA damage and other indicators after 28 days[41,46]. Microencapsulation beetroot could improve the sensitivity of natural compounds and for the betalains and antioxidant activity during storage[36].

A significant increase in AST was found in the positive control group which was injected with CCl_4 (2 mL/kg/b.wt./day) twice a week for 4 weeks compared to the negative control group in values of 60.20 and 46.09 mg dL^{-1}, respectively at the end of the experiment as seen in Fig. 6. CCl_4 is bio-transformed by the cytochrome P450 mediated reactions to produce trichloromethyl free radical (CCl_3)[45,46]. It can lead to produce lipid peroxide, which can damage cell membranes, change enzyme function and finally lead to liver injury[47]. The tested groups I and II were treated orally with 8 mL/kg/ b.wt., day beetroot juice and encapsulated beetroot juice, respectively, showed a gradually significant decrease in AST ($p<0.05$) compared to the hepatotoxic positive group, the highest decrease was found in group II in value of 46.70 mg dL^{-1} followed by the group I in value of 51.12 mg dL^{-1}.

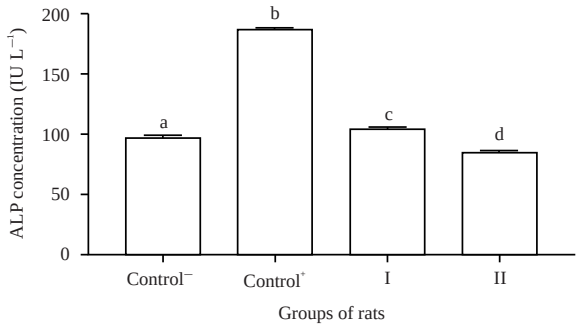

Fig. 7: Effect of BJ and encapsulated BJP on serum ALP (n = 6 rats)

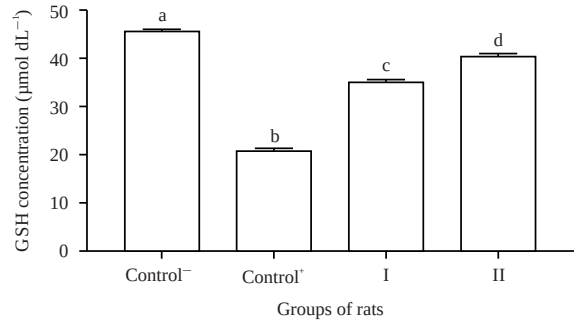

Fig. 8: Effect of BJP and encapsulated BJP on serum GSH (n = 6 rats)

Different litters assisted to maintain the plasma membrane and inhibited enzyme leakage through the cellular membrane, both of which are significant at $p<0.05$ antioxidant capability may play a role in this hepatoprotective action[48]. After 44 days of storage, the concentration of betalain, colour, antioxidant activity and redox potential of encapsulated beetroot juice in gum Arabic were all stable. This process requires encapsulating target molecules in capsules that release the content at controlled rates and under controlled conditions, preventing degradation[36,49].

Figure 7 demonstrated the effect of using free BJP and encapsulated BJP which showed a significant increase in ALP activity in the hepatotoxic positive group when compared with the negative control group in values of 187.13 and 97.02 mg dL^{-1}, respectively. The prototype for these enzymes indicates a pathogenic shift in biliary flow. The CCl_4-induced increase in this enzyme activity in serum, which could be due to stimulation of its production from sources other than the living, results in a high level of serum bilirubin[50,51].

The tested groups I and II have been treated orally 8 mL/kg/b.wt./day beetroot juice and encapsulated beetroot juice, respectively, showed a gradually significant decrease ($p<0.05$) in ALP activity compared to a hepatotoxic positive group, the highest decrease was found in group II in value of 85.01 mg dL^{-1} followed by the group I in value of 104.69 mg dL^{-1}, respectively.

Beetroot is high in phytochemicals such phenolic acids, ascorbic acid, carotenoids and flavonoids 1 as well as highly bioactive pigments like betalains, which have powerful antiradical and antioxidant properties as well as a variety of physiological effects[52], its effect of liver-protective by lower serum enzyme activity may play a role in hepatoprotective action[53].

Effect of BJ and encapsulated BJP on the level of enzymatic and non-enzymatic antioxidants: Figure 8 demonstrate the effect of beetroot juice and encapsulated BJP on the activity of non-enzymatic antioxidants Glutathione (GSH), its mean values in serum of positive control rats were significantly lower at $p<0.05$ in value of 20.68 µmol dL^{-1} compared to the negative control group in value of 45.49 µmol dL^{-1}. The GSH is an important endogenous antioxidant substance, the decrease of GSH content maybe lead to increased GSH consumption as it participates in the detoxification system for the metabolism of CCl_4 and enhanced susceptibly of hepatocytes to CCl_4 toxicity[54].

On the other hand, a significant increase in GSH activity was observed in the 2 tested groups I and II compared to the positive control group with the greatest value in group II which was given encapsulated BJP in value of 40.33 µmol followed by the group I which in value of 35.01 µmol, respectively. It was noticed that bioactive components such as vegetables and fruits prevent CCl_4-induced hepatotoxicity by increasing antioxidant levels such as SOD, CAT and GSH, fruits and vegetables have hepatoprotective effects mediated by antioxidant and anti-inflammatory mechanisms[55,56] encapsulation is one of the methods for protecting natural-source components while also increasing food product stability, shelf life and nutrient benefits as well as improving natural substance sensitivity[57,58].

Figure 9 showed a highly significant decrease in the hepatotoxic positive group compared to the negative control group at the end of the experimental period in values of 43.64 and 76.28 mmol dL^{-1}, respectively. The CCl_4 causes lower CAT and SOD activity in tissues[53,54].

On the other hand, a significant increase in CAT was observed in the 2 tested groups I and II compared to the positive control group with the greatest increase in group II which was given encapsulated BJP in value of 64.89 mmol dL^{-1} followed by the group I which was given beetroot juice in value of 56.10 mmol dL^{-1}.

Catalase is a hemeprotein that catalysis the reduction of H_2O_2 and can prevent the tissue from reactive free oxygen and hydroxyl radicals[47] antioxidants work in 2 ways: They either act as a "free radical scavenger" to neutralize free radicals directly or they improve the antioxidant system, which includes enzymatic antioxidants including catalase, superoxide dismutase and glutathione peroxidase as well as non-enzymatic antioxidants such as glutathione[57,58].

It was found that induction of hepatotoxic with CCl_4 injection induced significant decrease SOD in the positive control group compared to the negative control group as seen in Fig. 10 in values of 61.43 and 94.69 μ dL^{-1}. Superoxide dismutase (SOD) is a key defence enzyme and catalysis the dismutation of superoxide anions. The decrease in SOD activity can result in the removal of superoxide anions that may inactivate SOD, thereby causing the inactivation of H_2O_2 scavenging enzymes[47].

Significant increases in SOD activity were seen in both tested groups I and II when compared to positive control with the best decrease values in group II, which was given encapsulated BJP, at 89.02 μ dL^{-1} and group I, which was given beetroot juice, at 82.30 μ dL^{-1}. Bioactive components found in vegetables and fruits increased antioxidant levels such as SOD, CAT and GSH, indicating that vegetables and fruits have hepatoprotective effects mediated by antioxidant and anti-inflammatory mechanisms[51,52]. Also, the

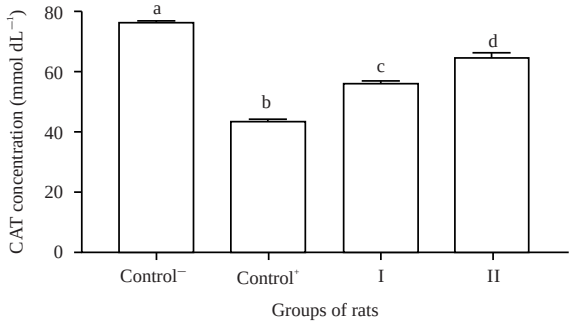

Fig. 9: Effect of BJ and encapsulated BJP on serum CAT (n = 6 rats)

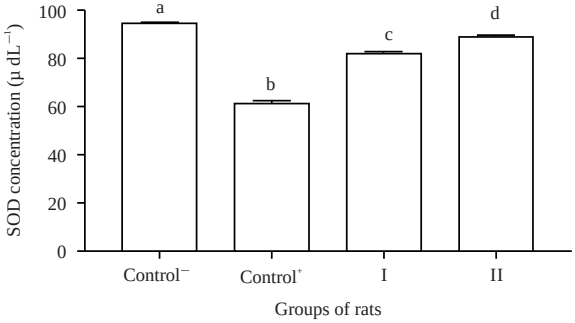

Fig. 10: Effect of BJP and encapsulated BJP on serum SOD (n = 6 rats)

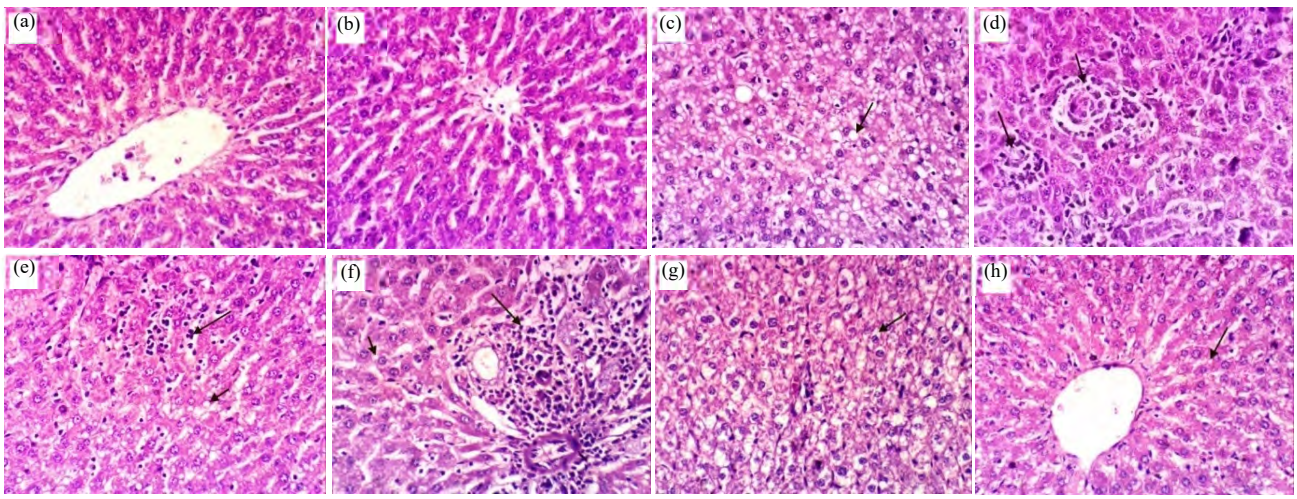

Fig. 11(a-h): Liver of rat from (a-b) Control negative group rats received instead water showing the normal histological structure of hepatic lobule, (c-d) Control positive group were intraperitoneally injected with CCl_4 showing hepatocellular steatosis and multiple focal hepatic necroses associated with mononuclear cells infiltration, (e-f) Group I were intraperitoneal injected with CCl_4^+ beetroot juice (BJP), showing hepatocellular steatosis and sinusoidal leukocytosis and hepatocellular steatosis and portal inflammatory cells infiltration and (g-h) Groupie were intraperitoneally injected with CCl_4 + encapsulated beetroot juice (EBJP) showing hydropic degeneration of hepatocytes and slight cytoplasmic vacuolization of hepatocytes (H and E X400)

release of polyphenolic compounds from encapsulating was more intense in intestinal fluid than in gastric fluid during the *in vitro* digestion[41].

Histopathological examination of the liver: Results of histopathological examination in rats are shown in Fig. 11. Liver in negative control group rats revealed the normal histological structure of hepatic lobule in Fig. 11a and b. Meanwhile, the liver of rats in the hepatotoxic positive group which was injected with CCl_4 showed hepatocellular steatosis focal hepatic necrosis and apoptosis associated with mononuclear cells infiltration in Fig. 11c and d. However, the liver of rats from group I was received orally beetroot juice revealed hepatocellular steatosis in Fig. 11e and f, sinusoidal leukocytosis (5), focal hepatic necrosis (6) on the other hand, group II which received orally encapsulated beetroot juice revealed improved picture, the liver showed hydropic degeneration of hepatocytes in Fig. 11g and slight cytoplasmic vacuolization of hepatocytes in Fig. 11h. There were no pathological changes or inflammatory indices in the hepatic tissues or the blood profile studied. The hepatic histology of beetroot juice-treated rats showed normal architecture, indicating the extract's safety. The efficacy of beetroot juice as a powerful antioxidant will be enhanced by the addition of these studies[59].

Beetroot helped to stabilize hepatocyte plasma membranes, lowering ROS levels. Flavonoids and polyphenols have been found in beta vulgaris in previous phytochemical also hepatoprotective effects may be due to antioxidant activity, flavonoids and polyphenols[60,61].

CONCLUSION

The NaCas-MD conjugate has the advantage over the NaCas-MD complex of better binding of BJP, exhibiting high encapsulation efficiency. Furthermore, the strawberry flavoured acidic beverage was selected to the stability of casein protein and delivery of encapsulated BJP in acidic conditions. Which shows to be stable under different processing conditions. Thus it could be concluded that flavoured beverage containing encapsulated BJP by conjugate is of acceptable quality and has high antioxidant activity. Also, it has a remarkable protective effect against acute hepatotoxicity, which may be due to its potential to increase antioxidant activity.

SIGNIFICANCE STATEMENT

This study discovers the BJP can bind with NaCas-MD conjugate. Also, the first time using caseins as the main source of protein in an acidic beverage without precipitation and clear beverage with stability and good functional properties during manufacture conditions. Thus a new theory on BJP can bind with NaCas-MD conjugate may be arrived at.

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