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Development of a Low Calorie Ready-to-Serve Beverage from *Hibiscus cannabinus* L

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Abstract

Hibiscus cannabinus, a leafy vegetable is known for rich in fibre, minerals with high acidity. It is also known to be one of the sources of an anti-obese factor (hydroxycitric acid). A low-calorie ready-to-serve (RTS) beverage was developed from *H. cannabinus* leaves using a natural sweetener (steviol glycoside). The beverage was prepared using the leaf aqueous extract, adjusting the acidity, and followed by the addition of a natural sweetener, steviol glycoside. The beverage was evaluated for changes in physicochemical and sensory parameters during a storage period of six months. The product was found to be acceptable for four months and scored very good (6.56) for overall quality after six months. A reduction in sweetness attribute was found when compared to control (8.0). The product was found to be shelf-stable with no microbial growth during storage. Significant decrease in steviol glycoside content (16.63%) and hydroxycitric acid content (54.4%) was observed after the end of storage. The RTS beverage from *Hibiscus* leaves with functional ingredients will ensure health and wellness of consumers.

Keywords: *Hibiscus cannabinus*; Low Calorie Ready To Serve Beverage; Steviol Glucoside; Hydroxycitric Acid

Abbreviations: RTS: Ready To Serve; ADI: Acceptable Daily Intake; CSP: Commercial Steviol Glucosides Powder; HCA: Hydroxycitric Acid; NEB: Non-Enzymatic Browning; PCA: Plate Count Agar; DRBC: Dichloran Rose Bengal Chloramphenicol Agar; RT: Room Temperature

Introduction

Ready-to-serve (RTS) beverages made up of fruit pulp have greater amount of water that is useful for body balancing by preventing dehydration. Fruit drinks contain high percentage of sugar and provide a few vitamins and minerals. The consumption of fruit-based beverages in the form of fruit blends and smoothies is increasing due to public awareness on the presence of various functional ingredients beneficial to health. The limited intake of free sugars below 10% of total energy intake constitutes a healthy diet and further reduction to < 5% of total energy intake will result in additional health benefits [1]. The usage of various synthetic/natural sweeteners in lieu of sugar is increasing day by day due to health concerns. Studies on sensory quality of synthetic sweeteners such as saccharin, acesulfame K, aspartame, and neotame revealed that aspartame solution resembles the sweetness of sucrose solution [2]. The RTS beverages from amla juice and ginger juice prepared with aspartame as a sweetener, scored maximum for all sensory quality attributes when compared to saccharine [3]. In another experiment, thaumatin/sucralose (1:1) blend had a closer sensory profile with 7% sucrose solution during the preparation of mango nectar [4].

Stevia (*Stevia rebaudiana*), a natural sweetener aqueous extract was compared for sweetness with sugar and applied in eleven beverage recipes namely, milk, coffee, tea, gajar halwa, milkshake, kheer, curd, lemon water, custard, halwa, and lapsi. The results indicated that 1.5% of stevia extract yielded sweetness comparable to 5% sugar in the products tested [5]. The procedure for the extraction of sweetener ingredients (steviol glycoside) from stevia leaves includes pre-treatment of leaves, hot water extraction, concentration, recrystalization, and separation. The steviol glycoside include substances such as Steviolbioside, Stevioside, Rebaudioside A, Rebaudioside B, Rebaudioside C (dulcoside B), Rubusoside, and dulcoside. The steviol glycoside contain a steviol backbone conjugated to several combinations of the sugar moieties such as glucose, rhamnose, xylose, fructose, and deoxyglucose [6]. The chemical properties, toxicity, legislation, extraction techniques and uses of stevia in the food industry were reviewed [7]. Steviol glycoside were permitted up to 200mg/kg in carbonated water, soft drink concentrates, yogurts, fruit nectars, dairy-based flavored drinks and non-carbonated water-based beverages and a higher quantity of 360mg/kg in jams, jellies and marmalades [8].

The steviol glucosides are 300 times sweeter than sugar and showed the negligible effect on blood glucose levels in humans and the EFSA (2010) prescribed an acceptable daily intake (ADI) limit of 4mg/kg body weight/day [9]. On the other hand, commercial steviol glucosides powder (CSP) exhibited good antioxidant activity when evaluated by DPPH, FRAP and ABTS assays. The CSP exhibited 47.64% inhibition in DPPH activity at 100µg/ml level [10]. *H. cannabinus* and *H. sabdariffa* (*Roselle*) belonging to family Malvaceae are found in the tropical and subtropical countries. They are termed as *gongura* in the local language in the southern Indian states. The physicochemical composition, uses, biological and pharmacological activities of *Hibiscus* was discussed thoroughly earlier [11]. *H. cannabinus*, red stemmed variety is generally preferred for its higher acidity for preparation of chutneys and pickles. *Gongura* based culinary preparations with pulses such as tur dhal, with non-vegetarian meats such as mutton, chicken and shrimp are popular in Southern India. It was indicated that *gongura* is a very rich source of iron, vitamins, folic acid and anti-oxidants essential for human health [12]. *H. cannabinus* leaves had a moisture content of 11.82%, ash 5.11%, lipids 2.33%, crude fibre (29.61%), protein (12.40%) and carbohydrates (37.67%) [13].

Seeds of *Hibiscus* are eaten roasted or ground in meals, while the leaves are consumed raw or cooked, or as a flavored vegetable. Literature is available on the preparation of beverages from *Rosella* (*Hibiscus safdariffa*), wherein calyces were used for the extraction of its bright red color. The plant extracts from *Hibiscus* varieties are known to possess an anti-obesity agent, L-hydroxycitric acid (HCA). HCA acts as a competitive inhibitor of the enzyme ATP-citrate lyase, which catalyzes the conversion of citrate and coenzyme A to oxaloacetate and acetyl coenzyme A (acetyl-CoA), primary building blocks of fatty acid and cholesterol synthesis [14]. Earlier workers [15] identified the principal acid (−)-hydroxycitric acid in the highly acidic fruits of *Garcinia species*, (+)-allo-hydroxycitric acid in the leaves of *H. cannabinus*, and its isomer in *H. sabdariffa*. The *Hibiscus* flowers (calyces) were used in the production of non-alcoholic beverages, wine, jam, jellies, and marmalades. Commercially, dried calyces from *H. sabdariffa* were extracted with hot water to yield an attractive red color, and then the °Brix was adjusted with sugar to 13 for a beverage production [16]. Further, the extracts were flavored with orange, pineapple, and apple fruit pulps to enhance vitamin C, mineral content and their acceptability [17]. So far utilization of leafy vegetables is not in practice to produce beverages. In the present study, an attempt has been made to utilize the leaves of *H. cannabinus* L. for producing a low calorie functional RTS beverage using steviol glucosides as a sweetener which will be an alternative way of consumption apart from regular traditional preparations.

Materials and Methods

Materials

Fresh *H. cannabinus* leaves were collected from the vegetable market, Uppal, Hyderabad. The chemicals were used in the study were procured from M/s. SD Fine Chem., Mumbai, India. The commercial stevia powder (steviol glycoside) was procured from M/s Stanpack Pharma Pvt Ltd, Mumbai, India. The analytical standard of hydroxycitric acid (HCA) was procured from Sigma Chemicals, USA. Microbiological media, plate count agar (HiMedia-M091A) and dichloran rose bengal chloramphenicol agar (HiMedia-M1881) were obtained from HiMedia, Mumbai, India.

Preparation of RTS Beverage

H. Cannabinus leaves were separated manually from stems and soaked for 5min in 2ppm chlorine (sodium hypochlorite) water for disinfection. The leaves were further washed with water to remove dust and adhering particles. Stems were separated manually to extract the red coloring pigments and used in the study. A portion of leaves were tray dried at 50±2 °C for proximate composition. Experiments were conducted for aqueous extraction of fresh leaves using ground leaves, chopped leaves with and without stem peel. The extraction of fresh leaves was carried out in boiling water at different ratios (1:5 to 1:30) for varying time periods ranging from 5 to 30min. After the treatments, the contents were filtered, and the filtrate was analyzed for pH, acidity, and color. The filtrate was diluted with sterile and potable water and adjusted the acidity. The required sweetness was obtained with steviol at 15-30mg/100mL by comparing with a control RTS beverage wherein a standard °Brix: acid ratio (15 °Brix: 0.15% acidity, test drink) was followed. The beverage was preserved by hot filling process and stored for six months at room temperature (29 ± 3 °C). A sweetener control (RTS beverage with sugar) was prepared for comparison during sensory evaluation.

Physicochemical and Sensory Analysis

The bottled RTS beverage kept for storage studies was drawn at regular intervals during the storage period as followed: 0, 2, 4 and 6 months. The beverage was analyzed for various physico-chemical parameters such as °Brix, acidity, pH, non-enzymatic browning (NEB), total polyphenols, anthocyanins using standards methods reported [18]. Brix was measured by using Hand refractometer (Erma, Japan). The acidity was measured by titrating the known amount of sample with standard NaOH solution using Phenolphthalein as an indicator. The pH of beverage was measured by using single electrode pH meter (Hanna, England). Non-enzymatic browning (NEB) was carried out by addition of ethanol, filtration and measuring the absorbance at 440nm. Total polyphenol content was determined by extracting with 80% ethanol, color development with Folin-Ciocalteu reagent and reading optical density at 675nm. Anthocyanins were analysed by extracting with acidified alcohol and reading absorbance at 535nm. The changes in color units for brightness, red and yellow (L*, a*, and b*) were recorded using a Hunter Lab colorimeter (UV-VIS1417, Hunter Lab, UK).

Steviol glucosides and HCA standards were prepared at 1mg/mL concentration, and 20µl was injected into HPLC system (LC 20AD Shimadzu, Japan). Steviol glycoside was identified using NH_2 column (250 x 4.6mm, particle size 5µ) and using a mobile phase consisting of acetonitrile: water (80:20, v/v). Hydroxycitric acid was quantified using a C_{18} column (250 x 4.6mm, particle size 5µ) employing 0.0025N H_2SO_4 as the mobile phase [19]. Both steviol

glucosides (Stevioside and Rebaudioside A) and HCA were detected at 210nm at 6.9,11.9 and 4.0min. when a flow rate of 1mL/min was optimized. The beverage samples were dissolved in respective mobile phases, filtered through 0.45µm membrane filter (Millipore, Merck, India) for determining the stevioside and HCA contents during the storage. Sensory analysis of the beverage products along with control was carried out by a well-trained panel of 8 judges. They were asked to score for sensory attributes like appearance, color, flavor, taste, overall acceptability using a 9 point Hedonic scale where score 1 is designated for dislike extremely and 9 for like extremely during initial and the storage period at RT [20].

Microbiological Analysis

The microbiological quality of the beverages was carried out by pour plate technique. The samples were prepared up to 10-2 dilution and plated on Plate Count Agar (PCA) for enumeration of viable mesophilic bacteria (Total Plate Count). Dichloran Rose Bengal Chloramphenicol agar (DRBC) was used for enumeration of yeast and molds. They were sampled in duplicate and incubated at 37 °C for two days, and 30 °C for five days respectively [21-23]. The samples were analyzed initially and after 2, 4, 6 months of storage at room temperature (RT).

Statistical Analysis

The changes in physicochemical parameters were analyzed in triplicate, and the mean values are presented. The values presented for sensory parameters are the mean of 8 panelists with standard deviation. The data were analysed statistically by Analysis of Variance (ANOVA) using SPSS 19.0 to evaluate the significance at $P<0.05$

Results and Discussion

Changes in Physicochemical Parameters

The fresh leaves possessed a moisture content of 86.51%. The composition of dried leaves indicated moisture content of 5.09%, total ash 5.44%, crude fat 9.03%, protein 19.03% and crude fibre 9.02% (Table 1). The results are comparable studies conducted with earlier [24]. The extraction of juice involved boiling of fresh leaves in water maintaining finely chopped leaves to water ratio at 1:10 for 15 min revealed the complete recovery of the pink color and total acidity. The juice was filtered through a four-layered muslin cloth to yield the clarified juice. The extract possessed pH 2.62, acidity 0.30% and exhibited a bright pink-red color with L*, a* and b* values of 30.27, 8.01, 3.78 respectively in Hunter Lab colorimeter. The acidic nature of extract might have resulted complete recovery of color. The HCA content was quantified about 275 mg/100g by HPLC. The water is known to be the optimal solvent for extraction of greatest quantity of functional compounds from *H. cannabinus* leaves with the highest levels of antioxidant activity [25]. A steviol glycoside content of 20mg/100mL was optimized to yield the sweetness comparable with the test beverage with sugar (15°Brix) and the acidity was adjusted to 0.18% .

Table 1: Composition of dried *H. cannabinus* leaves.

Parameter	Value
Moisture, %	5.09
Total ash, %	5.44
Crude fat, %	9.03
Total protein, %	19.03
Crude fibre, %	9.02
Iron, mg/100 g	41.50
Phosphorous, mg/100 g	171.00

Earlier, a process to produce a variety of low-calorie RTS fruit beverages from mango, jamun, pineapple, pomegranate and purple grapes was standardized using steviol glucosides at 20-35mg/100mL [26]. The physicochemical and microbiological quality of *H. cannabinus* RTS beverage samples are presented in Table 2. The changes in °Brix content of the beverage is negligible throughout the study. The variation in acidity was minor which maintained between 0.18 – 0.16 pH at 2.7. The NEB values increased from 0.067 to 0.071 during storage. The light pink color of the beverage is due the anthocyanin content of 1.6mg/100g. The Hunter color values of the RTS beverage were noted lower than extract as 32.58, 2.58, 4.52 for L*, a*, b* respectively which changed to 33.84, 0.57 and 5.23 respectively after six months of storage. Studies on effect of temperature showed that the anthocyanins reduced to 13% at 50 °C and 39% at 80 °C [27]. In the present study, the significant decrease was observed in red units is in accordance with a significant reduction in total anthocyanin content from 1.61 to 0.44mg/100mL after storage of six month at RT (Table 3).

Table 2: Quality changes in *H. cannabinus* RTS beverage during storage.

Parameter	Storage Period (Months)			
	0	2	4	6
°Brix	0.50±0.00	0.50±0.00	0.50±0.00	0.50±0.00
pH	2.76±0.01	2.62±0.01	2.54±0.02	2.73±0.02
Acidity, %	0.18±0.01	0.17±0.01	0.17±0.01	0.16±0.00
NEB	0.067±0.00	0.068±0.00	0.068±0.33	0.071±0.00
HunterLab values L*	32.58±0.03	33.00±0.2	33.76±0.05	33.84±0.04
a*	2.58±0.02	1.48±0.01[a]	0.87±0.02[a]	0.57±0.01[a]
b*	4.52±0.02	4.62±0.01[a]	4.71±0.02[a]	5.23±0.01[a]
Anthocyanins, mg/100g	1.61±0.03	1.24±0.0[a]	0.95±0.03[a]	0.44±0.02[a]
Total polyphenols, mg/100g	27.50±0.12	27.27±0.05	26.71±0.05	26.30±0.12

Steviol glycosides, mg/100g	17.68±0.18	16.52±0.13[a]	15.47±0.01[a]	14.74±0.05[a]
Hydroxycitric acid, mg/100g	275.27±4.26	204.25±3.91[a]	158.00±2.9[a]	125.50±9.58[a]
Total plate count, cfu/mL	<10.0	<10.0	<10.0	<10.0
Yeast and moulds, cfu/mL	<1.0	<1.0	<1.0	<1.0

Note: *N=3; a indicates significant difference at P<0.05 in a row.

Table 3: Quality changes in *H. cannabinus* RTS beverage during storage.

Parameter	Storage Period (Months)			
	0	2	4	6
Appearance	8.00±0.38	7.81±0.26	7.69±0.46	7.31±0.26[a]
Colour	7.50±0.53	7.25±0.27	7.13±0.35	6.56±0.32[a]
Flavour	8.00±0.38	7.63±0.23[a]	7.56±0.18[a]	6.88±0.23[a]
Taste	7.88±0.23	7.75±0.27	7.31±0.26[a]	6.50±0.46[a]
Overall Quality	7.88±0.35	7.75±0.27	7.56±0.18	6.56±0.50[a]

Note: *N=8; a indicates significant difference at P<0.05 in a row.

Changes in Steviol Glucosides and Hydroxycitric Acid

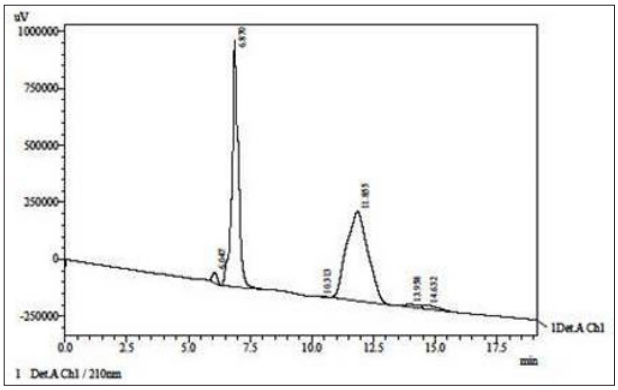

Figure 1: HPLC of commercial steviol glucoside.

HPLC analysis of steviol glycoside indicated the presence of two major peaks for Stevioside and Rebaudioside A along with other minor ingredients (Figure 1). Similarly, in the HPLC, standard HCA was detected at retention time of 4 min (Figure 2). Total steviol glycoside content of 17.68mg/100mL was noticed on the day of preparation by HPLC (Figure 3), which reduced to 14.74mg/100mL accounting for a loss of 16.63%. During the storage of two months, the decrease of steviol glucosides amounted to 6.56%. Reduction in steviol glucosides was also perceptible during sensory analysis as indicated by a lower score for sweetness. Hence, the addition of higher quantities of steviol glycoside (>25mg/100mL) can be recommended for retaining optimum sweetness in RTS beverages for six months storage. Other studies indicated the higher decrease in stevioside content (18%) in a low-calorie orange nectar prepared with reduced sugar content and high stevioside content (60 mg/100 mL) during storage at 25 °C for 2 months [28]. Some studies revealed that degradation of Rebaudioside A into six secondary compounds in carbonated beverages at pH 2.8-4.2 at various temperatures (5-40 °C) stored for six months [29]. Stevioside was found to be less stable than Rebaudioside A in different soft drinks after 24, 48, and 72 h storage at 80 °C [30]. A similar trend of decrease was observed when calculated for individual sweeteners such as Stevioside and Rebaudioside A in the present study from (Figure 3).

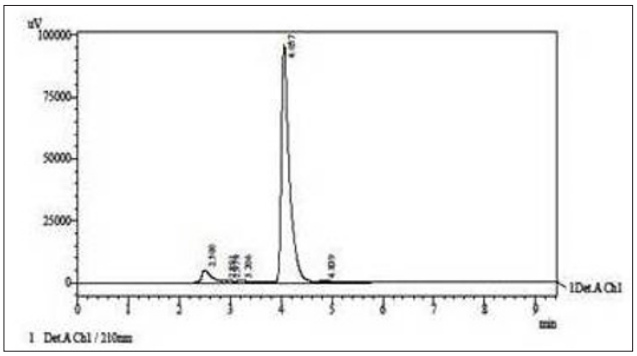

Figure 2: HPLC of standard Hydroxycitric acid.

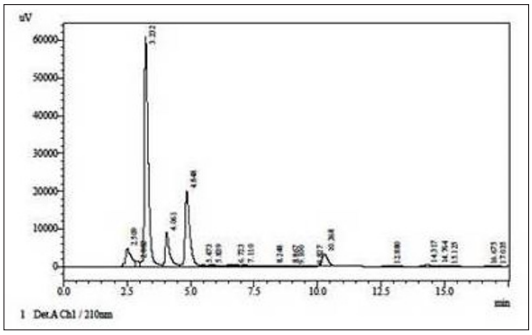

Figure 3: HPLC of Steviol glucosides in *H. cannabinus* RTS beverage.

HPLC analysis revealed the presence of HCA in the beverage at 275.27mg/100mL (Figure 4) the peak of which was confirmed by spiking with a standard on the day of preparation. Dilution of leaf extract and processing at higher temperature might have caused degradation of HCA content in the beverage. Significant reduction

was noticed in HCA content to an extent of 25.8, 42.6 and 54.4% respectively after storage for 2, 4, and six months. Earlier studies reported HCA to an extent of 1.01g/100mL in a formulation of Kokum RTS beverage [31]. Losses of HCA content to the extent of 17.96% and 15.57% were noticed in RTS beverage and fruit bar respectively at the end of two months storage. The losses in HCA content in kokum jam were two-fold when stored at RT compared to refrigerated conditions during storage for 90 days [32]. It was concluded that the shorter half-life of HCA to two months in water when prepared with or without heat treatment where beverage formulation contains 500mg Super Citrimax (HCA) and 150mg stevia [33].

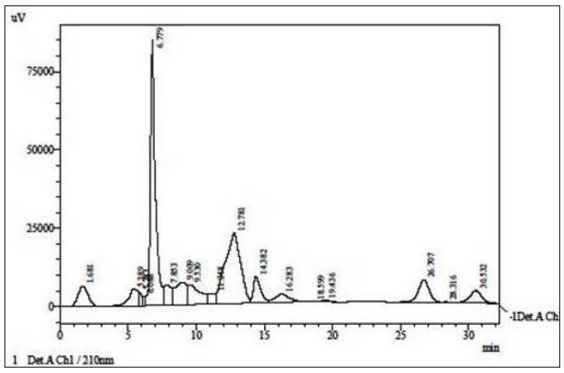

Figure 4: HPLC of hydroxycitric acid in *H. cannabinus* RTS beverage.

Changes in Sensory Quality

The sensory evaluation of the RTS beverage revealed its high acceptance by panelists on the day of preparation with an overall score of 7.88 which is similar to control (8.2). During the storage at RT, the low-calorie beverage maintained good score for overall quality (7.56) even though decrease in scores was observed for taste, and flavor after 4 months. After six months of storage, significant decrease was observed in scores of all sensory parameters (6.56) and the panel suggested for addition of more stevia/sweetness. The beverage showed reduced sweetness and increased acidity after six months. The control sample with sugar as a sweetener scored good (7.6) for overall quality compared to experimental beverage.

Microbiological Quality

The RTS beverage was found to be microbiologically safe as the growth of bacteria, yeast and molds were noted negligible on the day of preparation and no further growth after the hot-filling process and during storage for a period of six months. The hot-filling process is known as an effective technique in preserving beverages which are having pH below 4.6. The process not only help in removal of microorganisms during hot filling but also create anaerobic conditions for aerobic microbial growth by vacuum during cooling the bottles in addition to reducing oxidative deterioration of the beverage [34].

Conclusion

The RTS beverage was found to be shelf-stable and microbiologically safe during the period of 4 months. The acceptability of the beverage can be enhanced by the addition of higher steviol glycoside. The study further required the antioxidant activity and mineral content in the beverage for attention. The beverage from under-utilised *H. cannabinus* leaves with steviol glucosides can be popularized as a low-calorie functional drink useful for diabetic and obese populations.

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Highlights

a) *Hibiscus cannabinus*, a leafy vegetable is known for rich in fibre, minerals with high acidity.

b) They are generally consumed as chutney, chutney powder, spice mix and cooked dhal in India. The extract in acidic nature was consumed after sweetened for better palatability.

c) The *Hibiscus* extract was sweetened with a natural sweetener, steviol glucosides.

d) The presence of hydroxyl citric acid and addition of steviol glucosides brings the RTS beverage to functional food and widens the scope for commercialization.

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