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Original research

# Evaluation of functional properties in ready-to-drink beverage formulated with *Kalanchoe pinnata* (Akkapana) and Aloe vera

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A B S T R A C T

Medicinal plants have a good potency in preventing and curing diseases. This study was done to develop a functional Ready-to-drink beverage from *Kalanchoe pinnata* (Akkapana) leaf and *Aloe vera* gel with antiurolithiatic, antioxidant, antidiabetic, and anti-inflammatory properties. Sensory evaluation was conducted for different formulations with different volume ratios of *K. pinnata* and *Aloe vera*. Among those formulations, the highest sensory scores were obtained for flavor and overall acceptability in 70:30 (v/v %) of *K. pinnata* and *Aloe vera*. That formulation was selected as the final beverage for further analysis. Proximate composition and antinutritional factors were respectively analyzed quantitatively and qualitatively. Physiochemical properties in the final beverage were analyzed. Methanolic extracts of the above plant samples and final beverage were analyzed for evaluating the functional properties such as total phenolic (TPC), total flavonoid (TFC), carotene content (TC), antiurolithiatic, antioxidative, antidiabetic, and anti-inflammatory properties. Tannin was detectable in both plant materials while Saponin and Alkaloids were only present in *K. pinnata*. *K. pinnata* exhibited the highest TPC (1.3141 mg GAE/g dw), TFC (0.7364 mg RE/g dw) and TC (32.8049 mg/g dw) compared to *Aloe vera*. The highest DPPH inhibition (80.26%) and α-Amylase inhibition (23.33%) were shown by *K. pinnata* and the highest protein denaturation inhibition (71.43%) was exhibited in *Aloe vera* compared with other tested samples. Results of *in vitro* antiurolithiatic test in two plant materials and beverage exhibited inhibition action in both nucleation and aggregation assays. Results indicate *K. pinnata* leaf and *Aloe vera* gel can be effectively used for developing a functional beverage with antiurolithiatic, antioxidant, antidiabetic, and anti-inflammatory properties.

Keywords: Aloe vera; Antidiabetic; Anti-inflammatory; Antioxidant; Antiurolithiatic; *Kalanchoe pinnata*

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## 1. Introduction

Beyond the basic nutritional values, functional food has a positive effect on human health care as it contains bioactive components in its composition (Mudgil et al., 2016). In the functional food category, Functional beverages are the fastest-growing segment (Gruenwald, 2009). As natural substances are clearly preferred over chemically synthesized ones by people, functional botanical ingredients including medicinal plants are more popular than ever on the beverage market (Gruenwald, 2009).

About 80% of the world's community lives in less developed countries and relies on plants for their medical purposes (Waris et al., 2018). Medicinal plants are considered to be the backbone of traditional medicine. Sri Lanka is abundant with a huge number of medicinal plants. A long time ago our ancestors depended on traditional Ayurvedic treatments. Among those valued medicinal plants, *Kalanchoe pinnata* and Aloe vera are two plants that have high therapeutic and medicinal values.

*K. pinnata* (Bryophyllum) is a perennial plant belonging to the family *Crassulaceae*. The pharmacological studies so far have mostly been performed in-vitro and in-vivo and they proved that *K. pinnata* plant has anticancer, antimicrobial (Christiana et al.,2019), antileishmanial (Muzitano et al., 2006), antinociceptive, anti-inflammatory (Ojewole, 2005) antiurotilithiatic (Phatak & Hendre, 2015), immunomodulatory (Ibrahim et al., 2002), hepatoprotective (Pers, 2003), antioxidative (Phatak & Hendre, 2014), antidiabetic (George et al., 2018), antihypertensive (Bopda et al., 2014), etc.

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On the other hand, the Aloe vera plant has been commonly used for centuries due to its health, beauty, medicinal and skin care properties. The scientific name of Aloe vera is *Aloe barbadensis miller*. It belongs to *Asphodelaceae* (Liliaceae) family. More than 75 biologically active constituents have been discovered in the inner gel of Aloe vera (Kumara et al., 2019) and these herbs have properties such as antiulcer activity (Koo, 1994), antidiabetic (Bunyapraphatsara et al., 1996), anti-inflammation (Araújo et al., 2019), anti-hypercholestermic, anti-oxidative (Mohamed, 2011), immunomodulatory (Im et al., 2010), wound healing (Pers, 2012) and anti-urolithiasis (Kirdpon et al., 2006), etc.

Using those two valued medicinal plants together will give antidiabetics, antiurolithiatic, anti-inflammatory and antioxidative properties. Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from several pathogenic processes ranging from autoimmune destruction of the pancreatic b-cells with consequent insulin deficiency to abnormalities that result due to resistance to insulin action (American Diabetic Association, 2014). The global prevalence of diabetes mellitus (DM reached 463 million in 2019, and the World Health Organization (WHO) estimated that, worldwide, the number of people living with diabetes would increase to 700 million by 2045 (Ling et al., 2020). As uncontrolled diabetes can be a fatal situation, getting treatments and preventive agents is an essential fact.

Urolithiasis is the formation of stones (calculi) anywhere in the urinary and about 80% of stones are calcium based predominantly calcium oxalate (70%) or calcium phosphate (10%) (Dawson et al., 2012) and other types of stones are uric acid stones, struvite stones and Cystine stones (Chunningham et al., 2016). Preventing and treating kidney stones will be very important as it is the third most common urinary tract disease in humans.

Inflammation is an adaptative response involving multiple defense cell types and soluble mediators triggered in response to noxious stimuli by eliminating infectious agents and regulating the homeostasis of tissues (Araujo et al., 2019). The uncontrolled inflammatory response can cause a vast continuum of disorders including allergies, cardiovascular dysfunctions, metabolic syndrome, cancer, and autoimmune diseases. So, it is important to have natural anti-inflammatory treatments without any side effects (Ghasemian et al., 2016).

Although an organism is naturally equipped with antioxidant protection systems to cope with the harmful effects of ROS, the endogenous antioxidant defense system is not totally adequate to counteract oxidative stress (Gunathilake & Ranweera, 2016). So, using a product made with medicinal plants which have a high proportion of antioxidants is considered fundamental in the prevention of a variety of degenerative diseases (Waris et al., 2018).

This study is focused on developing a Ready-to-drink beverage by formulating *K. pinnata* leaf and Aloe vera gel with antiurolithiatic, antioxidant, antidiabetic, and anti-inflammatory properties by the addition of other proper ingredients. Also, in a situation where people have more attention on the traditional medicine system and are concerned about synthetic chemical drugs and their side effects, the natural herbal formulation will be a good solution to overcome them for maintaining a healthy life.

## 2. Material and Methods

### 2.1. Materials and reagents

Locally grown *Akkapana* leaves and Aloe vera leaves were collected from several areas in Galle district. Both leaves will be cleaned with distilled water. An edible proportion of *K. Pinnata* leaves were air dried at room temperature (30 ± 2 °C) for 2 hours. The Air-dried samples were dried at 45 °C to get constant weight by using a dehydrator. Those dried leaves were ground and obtained dried leaf powder was preserved at -18 °C in plastic sachets. For Aloe vera, the peels were removed and gel part was cut into several cubes. Then those gel cubes were dried by using a dehydrator and the dried gel was finely ground to get gel powder. The result was preserved in plastic sachets at -18 °C. All chemical reagents used in this study were of analytical grade.

### 2.2. Preparation of crude methanolic extract

For the preparation of methanolic extracts, two grams of dried samples were mixed with methanol (80% v/v) (20 mL) and vortexed at high speed for twenty minutes. Vortexed samples were centrifuged for 10 min at 1000 rpm and the supernatants were collected. Then the residue was re-extracted by following the same procedure mentioned above. The supernatant obtained was combined with the first extraction. The combined mixture was filtered (Whatman no 42) and the filtrate was evaporated in a rotary evaporator at 40 °C for concentrating the extract. Prepared extracts were stored at −18 °C until assayed within one week.

### 2.3. Proximate composition analysis

Proximate compositional values of moisture, carbohydrate, fat, protein, fiber and ash were determined using the method of the Association of Official Analytical Chemists.

### 2.4. Qualitative analysis of anti-nutrient factors in Kalanchoe pinnata leaves and Aloe vera gel

#### 2.4.1. Detection of alkaloids

About 5 g of the sample was measured into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol solution was added. The covered beaker was kept for 4 h. Then that solution was filtered and the filtrate was concentrated in a water bath to get one-quarter of the original volume. Then concentrated $NH_4OH$ was added in dropwise to the concentrated extract until the precipitation was completed. That residue is the alkaloid (Edeoga et al., 2005).

#### 2.4.2. Detection of saponins (foam test)

About 2.0 g of the dried powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. Then 5.0 ml of distilled water was added to 10 mL of filtrate and that mixture was shaken vigorously for having a stable persistent froth. Then obtained froth was mixed with 3 drops of olive oil and it was vigorously shaken. Resulted formed soluble emulsion indicates the presence of saponin in the extract (Edeoga et al., 2005).

#### 2.4.3. Detection of tannins

About 0.50 g of the dried powdered samples were boiled in 20 ml of water in a test tube. After filtrating the boiled mixture, a few drops of 0.1% ferric chloride were added. Brownish green or a

blue-black coloration indicates the presence of Tannin (Edeoga et al., 2005).

### 2.5. *Beverage formulation*

Beverage formulation was done in the steps described in Fig. 1.

### 2.6. *Sensory Analysis of beverage formulations*

Sensory evaluation was conducted for four different formulations which were prepared according to different *K. pinnata* juice volume and Aloe vera juice volume ratios as mentioned in Table 1. The panel consisting of 18 panelists was used for evaluating overall acceptability and other sensory parameters colour, flavor, aroma and after-mouthfeel (Ranaweera & Gunathilake, 2022). The scores were allocated for different quality characters according to a 9-point hedonic scale, indicating 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much and 1 = dislike extremely). The best out of the four formulations was selected as the final beverage formulation and that formulation was used for further analysis.

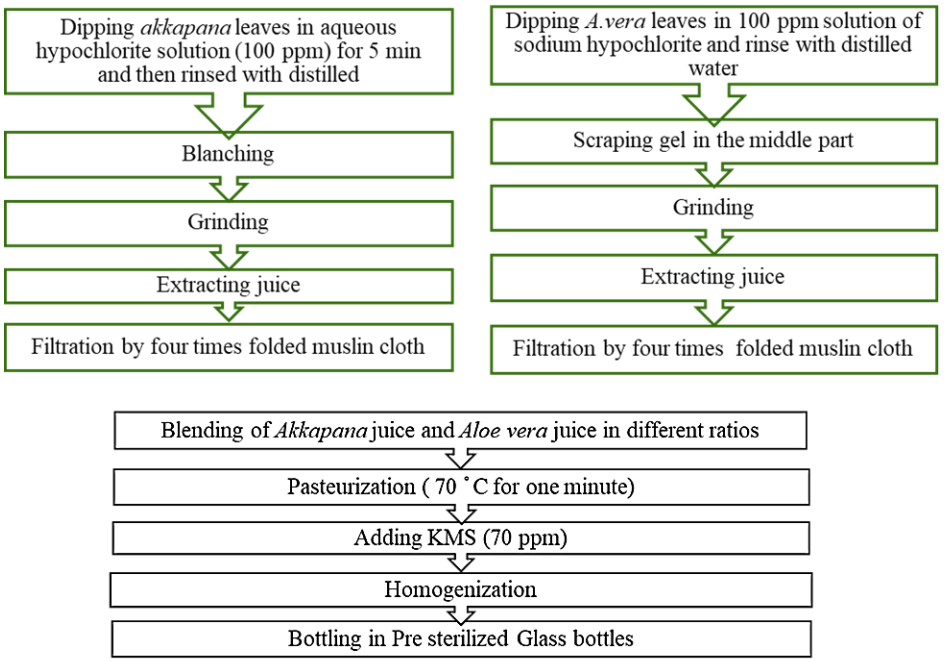


Fig. 1. Process flow chart of preparation of RTS beverage from *K. pinnata* leaf juice and *Aloe vera* juice.

Table 1. Four different beverage formulations.

| Formulation | *K. pinnata* juice volume (mL) | *Aloe vera* juice volume (mL) | KMS |
|---|---|---|---|
| 01 | 80 | 20 | 70.0 ppm |
| 02 | 70 | 30 | 70.0 ppm |
| 03 | 60 | 40 | 70.0 ppm |
| 04 | 50 | 50 | 70.0 ppm |

Table 2. Proximate composition of fresh *K. pinnata* leaves, fresh *Aloe vera* gel and formulated RTS beverage.

| | *K. pinnata* | *A. vera* | Beverage |
|---|---|---|---|
| Moisture | $93.41 \pm 0.65^{c}$ | $99.23 \pm 0.12^{b}$ | $99.47 \pm 0.12^{a}$ |
| Carbohydrate | $1.37 \pm 0.16^{a}$ | $0.62 \pm 0.12^{b}$ | $0.03 \pm 0.00^{c}$ |
| Crude protein | $1.21 \pm 0.12^{a}$ | Not detectable | $0.30 \pm 0.23^{b}$ |
| Crude fat | $1.90 \pm 1.51^{a}$ | $0.21 \pm 0.00^{b}$ | $0.03 \pm 0.11^{c}$ |
| Ash | $0.65 \pm 0.56^{a}$ | $0.12 \pm 0.00^{c}$ | $0.21 \pm 0.23^{b}$ |
| Crude fibre | $1.46 \pm 0.11^{a}$ | $0.38 \pm 0.12^{b}$ | $0.00 \pm 0.00^{c}$ |

Values are presented as means ± SD, n=3.

### 2.7. Product characterization in the final beverage

Total Soluble Solids (TSS) (Brix) and pH of RTS beverage were measured using a hand refractometer and pH meter, respectively. Total acidity (% citric acid) was determined using NaOH titration.

### 2.8. Product characterization in the final beverage

#### 2.8.1. Functional properties evaluation in the methanolic extracts of K. pinnata leaf, Aloe vera and final beverage

The total phenolic content was determined using Folin–Ciocalteu assay as described by Gunathilake et al. (2018) with slight modifications. Total phenols content was presented as mmol gallic acid equivalents (GAE) per g fresh weight of leaf (FW).

#### 2.8.2. Determination of total flavonoid content

The total flavonoid content was evaluated by using the colorimetric method at 510 nm described by Gunathilake et al. (2018) with slight modifications. Total flavonoid content in methanolic extracts of was expressed as mmol rutin equivalents (RE) per 1 g fresh weight.

#### 2.8.3. Total carotene contents

Carotene content was determined by following the strategy described by Gunathilake and Ranweera (2016). The absorbance of samples was read at 470, 653 and 666 nm on UV/VIS spectrometer (SP-3000). The concentration of carotene content was reported as g per g dry weight of the sample. Chla is chlorophyl and Chlb is chlorophyll b.

$$Chlorophyll\ a = 11.75(A662) - 2.350(A645) \quad (1)$$

$$Chlorophyll\ b = 18.61\ (A645) - 3.960\ (A662) \quad (2)$$

$$Carotene = 1000\ (A470) - 2.270\ (Chla) - 81.4Chlb /227 \quad (3)$$

#### 2.8.4. Antidiabetic activity-alpha amylase inhibitory assay

The assay was conducted by following the strategy described by Chiranthika et al. (2021) with slight modifications. By using the 3, 5-dinitrosalicylic acid (DNSA) method, the α-Amylase inhibition assay was performed. 100 µL of 2mM phosphate buffer (pH 6.9) solution was added to 100 µL of methanolic extract of the sample, and 200 µL of α-amylase enzyme solution was added. Then reacting mixture was incubated for 30 min at 37 °C. Thereafter 100 µL of the starch solution (1% in water (w/v)) was added to every tube and incubated for five min. The reaction was terminated by the addition of 200 µL DNSA reagent and the reaction mixture was boiled for five min during a water bath at 85–90 °C. The mixture was allowed to cool to room temperature and 5 ml of distilled $H_2O$ was added. Then the absorbance was measured at 540 nm employing a UV-Visible spectrophotometer. The control sample with 100% enzyme activity was prepared by replacing the plant extract with 100 µL of the buffer. For the control sample, the enzyme was replaced by phosphate buffer. The α-amylase inhibitory activity was expressed as percent inhibition by using the formula.

$$Inhibition\ (\%) = \frac{(absorbance\ of\ the\ control\ -\ absorbance\ of\ the\ sample)}{absorbance\ of\ the\ control} \times 100 \quad (4)$$

#### 2.8.5. Antioxidant activity- DPPH radical scavenging assay

Method was done by following the strategy described in Gunathilake et al. (2018). 3.9 mL of freshly prepared methanolic solution of DPPH (1 mM, 0.5 mL) was added to 100µL of extract. Then the mixture was vortexed for 15 seconds then allowed for 30 min within the dark at temperature. The absorbance of the resulting solution was read by using UV/VIS spectrometer at 517 nm. Control is the absorbance of the DPPH solution without adding extracts (control). The percentage inhibition of the radicals due to the antioxidant activity of extracts was calculated by using the formula.

$$Inhibition\ (\%) = 1 - \frac{Abs\ extract}{Abs\ control} \times 100 \quad (5)$$

#### 2.8.6. Antioxidant activity- DPPH radical scavenging assay

##### 2.8.6.1. Nucleation assay

The effect of methanolic extracts on calcium oxalate (CaOx) crystal formation was evaluated by means of the nucleation assay per the strategy described by Mosquera et al. (2020). Salt ($CaCl_2$) (5 mM) and sodium oxalate solution ($Na_2C_2O_4$) (7.5 mM) were prepared in Tris-HCl (0.5 M) and NaCl (0.15 M) buffer (pH 6.5). Several concentrations of the extract were prepared in distilled $H_2O$. One milliliter of the prepared extract concentrations was mixed with 3 mL of $CaCl_2$ and then three mL of $Na_2C_2O_4$ solution was added. The mixtures were incubated for 30 min at 37 °C in an oven and cooled down to room temperature. The absorbance of the mixtures was measured at 620 nm employing a spectrophotometer. The percentage of inhibition of nucleation was calculated by using the formula.

$$Inhibition\ (\%) = 1 - \frac{Abs\ extract}{Abs\ control} \times 100 \quad (6)$$

##### 2.8.6.2. Microscopic evaluation

In the absence or presence of extracts, the morphology of CaOx crystals and the number and size of crystals formed were determined using light microscope at 100 × magnification.

##### 2.8.6.3. Aggregation assay

The effect of extracts on CaOx crystal aggregation was determined by the assay described by Mosquera et al. (2020). CaCl2 and Na2C2O4 solutions (50 mM each) were mixed together, heated to 60 °C in a very water bath for 1 h and so incubated overnight at 37 °C. After drying, a CaOx crystal solution (0.8

mg/mL) was prepared in an exceedingly 0.05 M Tris-HCl and 0.5 M NaCl buffer (pH 6.5). One milliliter of every dilution extract was added to three mL CaOx solution, vortexed and incubated at 37 °C for 30 min. The absorbance of the mixtures was measured at 620 nm employing a spectrophotometer. Inhibition of aggregation was calculated by using the formula.

$$Inhibition\ (\%) = 1 - \frac{Abs\ extract}{Abs\ control} \times 100 \quad (7)$$

### 2.8.7. *Anti-inflammatory activity- effect on protein denaturation*

Anti-inflammatory activity was determined by Gunathilake et al. (2018). 0.2 mL of 1% egg albumin, 4.78 mL of phosphate-buffered saline (PBS, pH 6.4), and 0.02 mL of extract were mixed to form a reaction mixture with the final volume of 5 mL. The mixture was incubated in a water bath (37 °C) for 15 min. Then the reaction mixture was heated at 70 °C for 5 min. After cooling, the turbidity was measured using a UV/VIS spectrometer at 660 nm. Phosphate buffer solution is used as the control. Formula for calculating the percentage inhibition of protein denaturation is:

$$Inhibition\ of\ denaturation(\%) = 1 - \frac{Abs\ extract}{Abs\ control} \times 100 \quad (8)$$

## 3. Results and Discussion

### 3.1. *Proximate composition of Kalanchoe pinnata leaf, Aloe vera gel and final beverage*

*K. pinnata* fresh leaf, *Aloe vera* fresh gel and developed beverage were analyzed for moisture, protein, fat, fibre and total ash content. The results of these proximate composition values are summarized in Table 2. According to the results, except for the moisture content, all other analyzed proximate composition was significantly higher in *K. pinnata* leaf compared with Aloe vera gel. There was not any detectable protein content in the Aloe vera gel sample. Moisture, protein, fat, fiber and total ash content in formulated beverages were 99.47%, 0.03%, 0.30%, 0.03%, 0.21%, and 0.00%, respectively. *K. pinnata* leaf and *Aloe vera* gel both contained high moisture content when compared with other proximate composition values. That is because of being a succulent plant which contains high water concentration in their nature. Due to this nature especially in *Aloe vera* gel, externally added water that is required to add when developing the beverage can be reduced. Though during this study protein content in *Aloe vera* was not detectable, however according to the results of proximate analysis of Scala et al. (2012), protein content in *Aloe vera* gel is 0.12%.

### 3.2. *Qualitative antinutrient screening in Kalanchoe pinnata leaves and Aloe vera gel*

The qualitative analysis of several anti-nutrient factors for the *K. pinnata* leaf and *Aloe vera* gel was shown in Table 3. Under those three anti-nutrients, tannin, saponin and alkaloid were qualitatively analyzed. Tannin was present in *K. pinnata* at high concentrations while saponin and alkaloids were present in moderate concentrations. In *Aloe vera* gel only the tannin was present at low concentrations and saponin and alkaloids were not detected. According to Okunwu et al. (2020), the concentration of total alkaloids in the *K. pinnata* leaves was 9.63 g/100 g and the most concentrated alkaloid was caffeine while pyridine was the most abundant type of alkaloid. According to the results of Choudhary et al. (2012), other than the tannin, saponin was quantitatively found in *Aloe vera* gel powder and amounts of saponins and tannin content in gel powder were 0.01% and 0.01%, respectively.

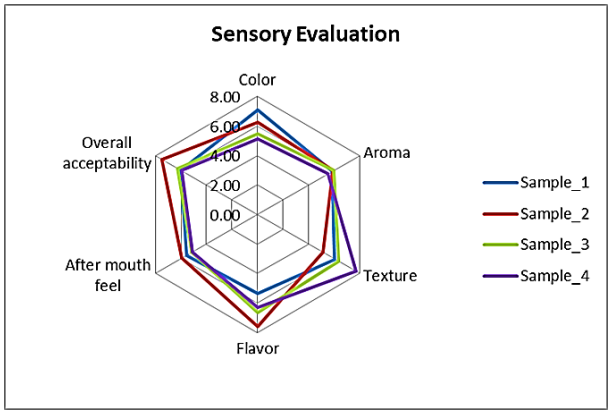


Fig. 2. Sensory analysis of different beverage formulations.

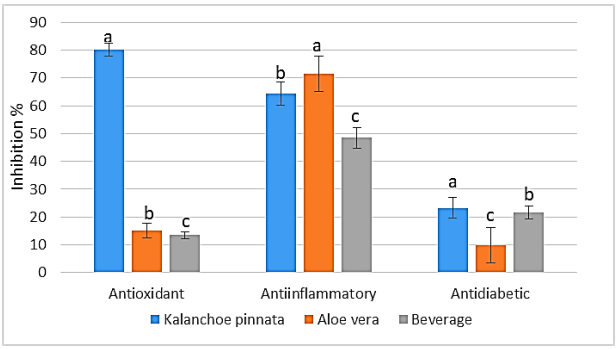


Fig. 3. DPPH radical scavenging activity (antioxidant properties), alpha amylase inhibition activity (antidiabetic property) and effect on protein denaturation (antiinflammatory activity) of methanolic extracts of fresh *K. pinnata* leaves, fresh *Aloe vera* gel and formulated RTS beverage. Values are presented as means ± SD, n = 3.

### 3.3. *Sensory analysis of beverages with different combinations of Kalanchoe pinnata leaf juice and Aloe vera gel juice*

The ultimate measure of product quality and success is the sensory quality of that food or beverage. Therefore, by following a sensory evaluation best one out of four juice blends were selected. Results of the sensory evaluation which was done by adopting a 9-point hedonic scale for the parameters of color, flavor, texture, after-mouth feel and overall acceptability were shown in Fig. 2.

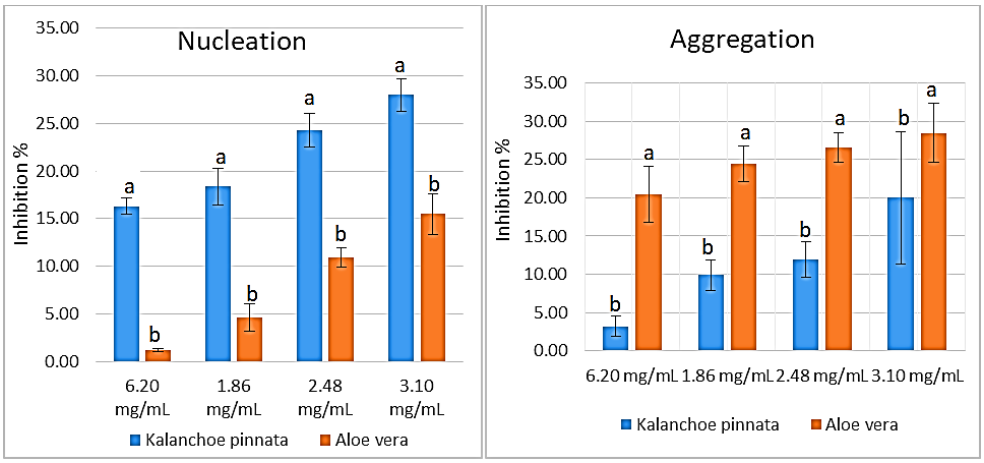


Fig. 4. Effect of the crude methanolic extracts of *K. pinnata* and *Aloe vera* on nucleation and aggregation of calcium oxalate crystals. Values are presented as means ± SD, n = 3.

Table 2. Qualitative analysis of several antinutrients for *K. pinnata* leaf and *Aloe vera* gel.

| Sample | Tannin | Saponin | Alkaloid |
|---|---|---|---|
| *K. pinnata* | +++ | ++ | ++ |
| *Aloe vera* | + | - | - |

+++ present at high concentration.
++ present at moderate concentration.
+ present at very low concentration.
−Not present.

Table 4. Total phenolic, total flavonoid and carotene content of crude methanolic extracts of *K. pinnata* leaves, fresh *Aloe vera* gel and formulated beverage.

| Sample | Total phenolic (mg GAE/g dw) | Total flavonoid (mg rutin /g dw) | Total carotene (mg/g dw) |
|---|---|---|---|
| *K. pinnata* | $1.31 \pm 0.46^{a}$ | $0.74 \pm 0.12^{a}$ | $32.80 \pm 3.23^{a}$ |
| *Aloe vera* | $0.76 \pm 0.07^{b}$ | $0.23 \pm 0.02^{b}$ | $4.19 \pm 0.14^{b}$ |

Values are presented as means ± SD, n = 3.

Sensory evaluation was done for four different formulations by 18 untrained panelists to find out consumer acceptability. Among the different combinations of *K. pinnata* juice and Aloe vera gel juice, the ratio of 70:30 (%v/v) (*K. pinnata*: Aloe vera beverage formulation had recorded the highest sensory scores for flavor and overall acceptability 7.60 and 7.47 scores, respectively). Hence that formulation was selected as the best beverage formulation to carry out further analysis. KMS was added for increasing the shelf life of the beverage as many commercial beverages are following this method for the same purpose mentioned. In this study, beverage formulation was not contained externally added sugar or flavors. It is for maintaining the natural quality of the product as most people are moving for natural healthy food and beverages.

### *3.4. Physicochemical analysis of final developed beverage*

The pH, Total soluble solids and titratable acidity values in formulated beverages were reported as 4.55, 4.00%, and 0.01% (citric acid), respectively. In regard to the pH and titratable acidity values, the beverage may have a sour taste. Having a very low TSS value provides evidence for very low sugar content in the formulation. The beverage has a lower pH value (pH 4) which lies within the acidic pH range. The natural sourness and bitterness of *K. pinnata* give the taste of the beverage as Aloe vera does not give a considerable impact on flavor. Also, the color of the beverage relies on the *K. pinnata* due to the transparency of Aloe vera juice.

During the process of obtaining Aloe vera gel, the method of gel filleting is very crucial since if the latex (containing aloin) residue was present in the removed gel, it makes a very bitter gel and imparts laxative properties (Javed & Atta, 2014). Therefore, careful removal of gel from the leaf without disturbing the latex portion present between the rind and the gel fillet is very important in beverage formulation. Aloe vera is usually a very viscous liquid. Hence enzymatic treatment can be applied to reduce the viscosity. Cellulase is a commonly used enzyme that is readily available commercially (Javed & Atta, 2014). When combining these two plant juices, unfiltered fibrous nature-like components in *K.*

*pinnata* juice become agglomerate and make green color cloudy-like substances. Those can be removed by filtration. So, there was no requirement to add stabilizers for preventing the sedimentation of developed beverages in this study.

### 3.5. Total Phenolics, Total Flavonoids and Carotene contents of the methanolic extracts of Kalanchoe pinnata leaf and Aloe vera gel

Total phenolics, total flavonoids and carotene contents of the methanolic extracts of *K. Pinnata* leaf, Aloe vera gel were analyzed and results are summarized in Table 4. Methanolic extract of *K. pinnata* showed the highest total phenolic content, flavonoid content and carotene content (1.3141 mg GAE/g dry weight, 0.7364 mg RE/g dry weight, 32.8049 mg/g dry weight respectively) compared with Aloe vera gel extract (0.7569 mg GAE/g dry weight, 0.2284 mg rutin /g dry weight, 4.1910 mg/g dry weight respectively). According to the phytochemical properties in *K. pinnata* described in Okunwu et al. (2020), the total flavonoid content was (5.348 g/100 g) and the most abundant class of flavonoids was flavones. In that same research related to *K. pinnata*, the concentration of phenolic compounds in the leaves was 7.437 g/100 g and cinnamic acid was the most concentrated phenolic while the least was singlic acid. Phenolic and polyphenolic compounds including phenolic, flavonoids, and carotenoids can be considered as several main contributors to the antioxidant activity of plants and those are responsible for important bioactivities (Nascimento et al., 2013; Gunathilake & Ranweera, 2016). Therefore, it can be suggested that there can be a positive correlation between the phenolic and polyphenolic profile and the antioxidant property as having higher antioxidant activity in *K. pinnata* than Aloe vera as mentioned in Fig. 3.

### 3.6. Anti-inflammatory activity of the methanolic extracts of Kalanchoe pinnata leaf, Aloe vera gel and developed beverage

Inhibition percentages of the antioxidant, anti-inflammatory and antidiabetic assays were graphically shown in Fig. 3. In the anti-inflammatory assay, methanolic extracts of *K. pinnata*, Aloe vera and beverage all were able to inhibit the protein denaturation. The inhibition percentage of protein denaturation of Aloe vera (71.43%) was the highest followed by *K. pinnata* (64.29%) significantly. According to the results, inhibition percentage of the anti-inflammatory assay in beverage was reported as 48.41%.

The excessive production of ROS may be a cause of tissue injury that may lead to an inflammatory process in the body. Flavonoids and other polyphenols exhibit significant antioxidant activity and the reduction of the concentration of ROS that can show an anti-inflammatory effect.

Therefore, the anti-inflammatory potential of plant species may have also resulted from the antioxidant activity which was provided by these mentioned compounds (Agarwal & Shanmugam, 2019). Aloe vera gel reduced inflammation that was induced by agents via the promotion of prostaglandin synthesis as well as increased infiltration of leucocytes. However, Aloe vera gel was less effective against inflammation caused by agents that produce allergic reactions (Kumar et al., 2019). Anti-inflammatory properties of phyto-compounds from methanol crude leaf extract of *K. pinnata* can be effectively used in novel drug designs to treat inflammatory disorders (Agarwal & Shanmugum, 2019). So, the beverage formulated with those two plants has a promising positive effect in treating inflammatory conditions.

### 3.7. Antioxidant activity of the methanolic extracts of K. pinnata leaf, Aloe vera gel and developed beverage

The antioxidant activity of methanolic extracts was evaluated by following DPPH radical scavenging assay. Among the methanolic extracts of two plant materials evaluated, *K. pinnata* (80.26%) showed significantly higher DPPH radical scavenging activity compared to Aloe vera (15.24%). According to the results, formulated beverage showed a value of 13.49% for the inhibition percentage in the DPPH Assay (Fig. 3).

Reactive oxygen species (ROS) such as free radicals are part of the normal human metabolism. They act against infectious agents that invade the body and, in several cells, -signaling pathways. However, the high concentrations, of these ROS and free radicals can cause oxidative stress which results in damaging lipids and DNA, etc. Therefore, it can cause for occurring many diseases, including inflammatory disorders, heart disease, stroke, atherosclerosis, diabetes and cancer. As a preventive action against these negative effects of ROS and free radicals, the human body produces enzymatic and non-enzymatic antioxidants (Nascimento et al., 2012). So, studying diets and natural plants which are rich in antioxidants is very important and it is an ongoing process still. The antioxidative potential of plant extracts can be measured using various in vitro assays such as total antioxidant capacity, reducing power, lipid peroxidation and DPPH radical scavenging assays, etc. Each of those assays is based on at least one feature of antioxidant activity. It is better to conduct two or more methods in evaluating antioxidant properties (Gunathilake & Ranweera, 2016). In the DPPH assay, DPPH produces stable free radicals whose odd electrons are paired off through electron capture from other electron donors like antioxidants (Isaac et al., 2012). The odd electron of a nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine and the deep violet color of the DPPH solution loses its' violet color and reduces the absorption. The presence of Glutathione Peroxidase (GPx), superoxide dismutase (SOD), vitamin E, carotenoids, vitamin C, flavonoids, and phenolic antioxidants in Aloe vera gel may be responsible for antioxidant effects (Kumar et al., 2019).

### 3.8. Anti-diabetic activity of the methanolic extracts of K. pinnata leaf, Aloe vera gel and developed beverage

In the antidiabetic assay, the alpha-amylase inhibition activity of *K. pinnata* (23.33%) was obtained as a higher inhibition activity compared to Aloe vera (9.85%) significantly. Formulated beverages resulted in 21.57% of inhibition in the alpha-amylase inhibitory assay (Fig. 3).

Alpha-amylase inhibition and alpha-glucosidase inhibition are two mechanisms that can be observed when anti-diabetic drugs are taken. Methanolic extracts of *K. pinnata* contain organic compounds which can bind to the alpha-amylase enzyme and inhibit its' hydrolyzing activity. It can reduce the glucose liberated in the bloodstream and control the blood glucose level (George et al., 2018). On the other hand, the alpha-glucosidase inhibitory activity of *K. pinnata* has also shown a significant inhibitory effect. The $IC_{50}$ value of the methanolic extracts of *K. pinnata* indicated that the extracts are more efficient in inhibiting alpha-glucosidase

activity than the regular standard anti-diabetic drugs such as Acarbose (George et al., 2018). Some findings suggested that *Aloe vera* gel and phytosterols derived from Aloe vera gel have a long-term blood glucose level control effect and it can be used as a treatment for type 2 diabetes mellitus (Tanaka et al., 2006). Clinical trials that have been done with diabetic Mellitus patients reveal that the treatment with aloe juice and glibenclamide together produced a significant decrease in blood sugar levels within two weeks (Bunyapraphatsara. et al., 1996). In addition to the gel, methanolic extract of whole *Aloe* leaf showed significant inhibition of 87%, compared to acarbose (5 mg/ml) which was used as a positive control and showed an inhibition percentage of 97% according to Ramirez et al. (2020).

Also relevant to *K. pinnata* the same thing was proven that a mixture of the extract and glibenclamide could be promising for treating diabetes effectively (Aransiola et al., 2014). *In-vitro* studies have suggested that flavonoids, alkaloids, phenolic acids, terpenoids, tannins and saponins play a crucial role in the management of diabetes and prevent the development of diabetic-associated complications through varied mechanisms (Waititu et al., 2018). Therefore, treating beverages with a mixture of these two plant materials may have good potential for treating diabetic conditions in people.

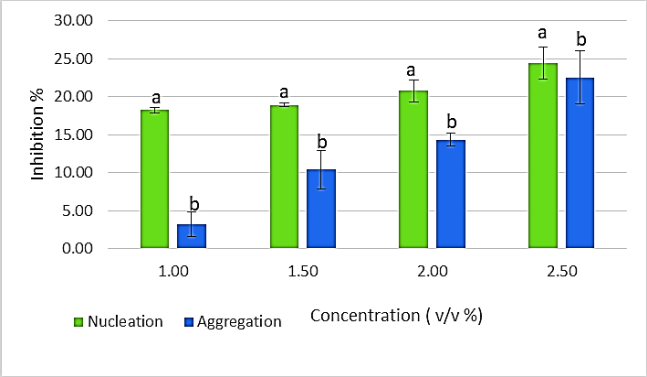


Fig. 5. Effect of the methanolic s extracts formulated beverage on nucleation and aggregation of calcium oxalate crystals. Values are presented as means ± SD, n = 3.

### 3.9. *Antiurolithiatic activity in methanolic extracts of K. pinnata leaf, Aloe vera gel and developed beverage*

Results of the aggregation assay of methanolic extracts of *K. pinnata* leaf, *Aloe vera* gel and developed beverage are presented in Fig. 4. All the methanolic extracts produced a significant reduction of nucleation and aggregation of preformed $CaO_X$ crystals. The highest inhibition of nucleation activity was obtained from *K. pinnata* extract compared with Aloe vera. However, in the aggregation assay, the inhibition activity of Aloe vera was significantly higher than that *K. pinnata*. All the extracts showed increasing trends in inhibition activities with the increase of the extract's concentration. Considering the methanolic extract of the developed beverage, it also exhibited inhibition action in both nucleation and aggregation assays (Fig. 5). Except for the methanolic extract of 2.5 ml of beverage, inhibition activity in the nucleation assay was significantly higher compared to the inhibition activity of the Aggregation assay. Microscopy analysis (Fig. 6) revealed an increase in the number and size of the crystals in the urolithiasis control group when compared with groups treated with the extract of *K. pinnata*, Aloe vera and formulated beverage. Also, according to the results of Elambarathi et al. (2018), there were fewer and smaller particles with increasing concentrations of *K. pinnata* extract as shown in various SEM micrographs and the main findings of that study was that the plant extract inhibited the nucleation and aggregation process of kidney stone formation.

In general, urolithiasis is a process of the removal or formation of stones in any part of the urinary system such that the kidney, the ureters, or the urinary bladder. The pathogenesis of calcium oxalate stone formation is a multistep process and includes nucleation, crystal growth and crystal aggregation. Nucleation basically is an event of phase change where dissolved substances in a supersaturated solution spontaneously become crystallize thermodynamically (Mosquera et al., 2020). The stone formation can also be influenced by the irregular urinary composition that proposed the unevenness between promoters and inhibitors in the kidneys (Rahim et al., 2021). As per the aggregation principle, all small crystals have aggregated to form larger crystals. The combination of aggregated crystals leads to the formation of solid mass called as kidney stones. Phytochemicals like saponins, phenolic, flavonoids, quercitrin, glycosides, carotenoids, saponin, kamferol and alkaloids, etc. inhibit the growth of $CaO_X$ crystals and inhibit $CaO_X$ crystal aggregation (Elambarathi et al., 2018). Changes in the chemical compositions of urine after *Aloe vera* gel consumption have shown potential for preventing kidney stone formation among children as well as in adults (Kridpon et al., 2006). The extract of *K. pinnata* demonstrated slightly better compared to cystone standard solution to inhibit $CaO_X$ aggregation (Kridpon et al., 2006). In this study, both plant extracts showed a positive inhibitory effect in both nucleation and aggregation assays. This result can also be observed in the methanolic extract of developed beverages too. With the increase in the concentration of extracts inhibitory effect gets increased.

## 4. Conclusion

In conclusion, results indicate that tested methanolic extracts of *K. pinnata* leaf and Aloe vera gel exhibit significant *in-vitro* inhibitory activities in alpha-amylase, protein denaturation, DPPH free radical scavenging assays as well as in the nucleation assay and aggregation assays. It can be claimed that those two plant materials have a positive effect in antioxidant, antidiabetic, anti-inflammatory and antiurolithiatic properties. *K. pinnata* and Aloe vera contain phytochemicals such as phenolic, carotene, flavonoids, saponin, and alkaloids which are responsible for these determined functional properties. According to the results of the functional property evaluation in formulated beverages, *K. pinnata* leaf juice and Aloe vera gel juice can be effectively used to produce a new functional Ready-to-drink beverage with antioxidant, antidiabetic, anti-inflammatory and antiurolithiatic properties which may be a good herbal remedial solution for the problem of rising non-communicable diseases. However, further investigation needs to be done related to *in vivo* studies for the above functional properties as well as to measure the effectiveness of the functional properties of beverages with storage life and the temperature treatments during processing by different *in vitro* and *in vivo* studies.

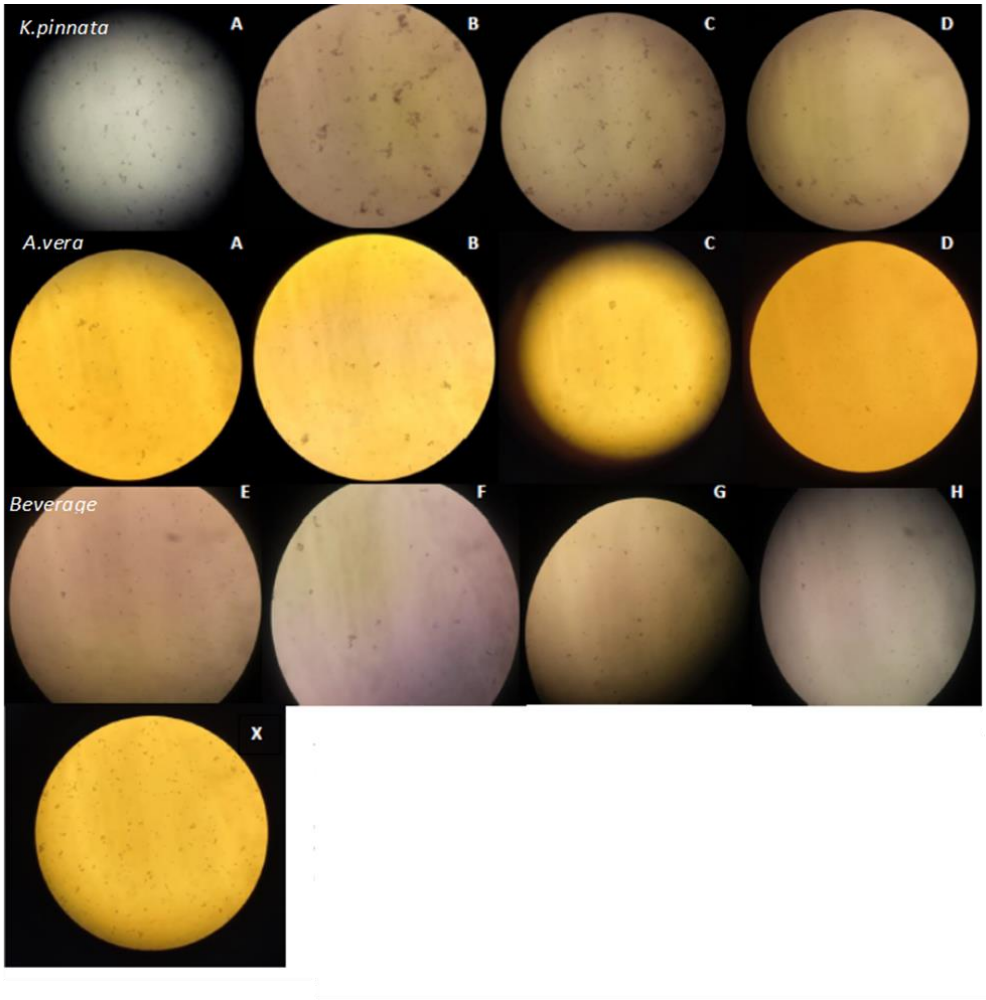


Fig. 6. Microscopic images of (X) control; (A) treated with extract (6.20 mg/mL), (B) treated with extract (1.86 mg/mL), (C) treated with extract (2.48 mg/mL), (D) treated with extract (3.10 mg/mL), (E) treated with beverage extract (1.00 mL), (F) treated with beverage extract (1.50 mL), (G) treated with beverage extract (2.00 mL), (H) treated with beverage extract (2.5 mL) (Magnification *100).

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## Conflict of interest

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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