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PRODUCTION OF NUTRABEVERAGE THROUGH FERMENTATION OF POMEGRANATE, STRAWBERRY AND PINEAPPLE JUICES WITH KEFIR GRAINS

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ABSTRACT: The term nutrabeverage was applied to the product of alcoholic fermentation made by using Kefir grains. In the present studies initially, the pH of the pomegranate juice sample was 3.7, 3.5 for strawberry and 3.8 pH for pineapple. The titrable acidity optimum was found in pineapple at 3.37 % while 16% TSS was found in pomegranate. The highest TSS content before fermentation was observed at 160brix, 90brix and 14.30 brix in the pomegranate, strawberry and pineapple juice respectively. After 15 days of fermentation at 25°C with pH 4.5, the lowest TSS content of 120brix, 8.30brix, and 100brix in the pomegranate, strawberry and pineapple nutrabeverage was observed respectively. The titrable acidity was found to increase after 15 days of fermentation at 25°C in all nutrabeverage while reducing sugar was found to the maximum before fermentation. The lowest reducing sugar content was 9.73%, 6.81% and 8.82% in the pomegranate, strawberry and pineapple nutrabeverage respectively recorded after fermentation. The maximum alcohol content 6.9%, 4.3% and 5.2% in the pomegranate, strawberry and pineapple nutrabeverage was observed at 25°C respectively at pH 4.5 after 15 days of fermentation. Vitamin C was found maximum before the fermentation of all juice. Additionally, this beverage has demonstrated strong antioxidant activity and sensory testing of the beverage produced positive findings.

INTRODUCTION: Various obstacles must be overcome by the food sector, such as rising consumer knowledge and demands for safer and more nutrient-dense food. The production of functional food products, or foods that can positively affect certain bodily functions in addition to their nutritional effects, is one of the innovations in the food industry.

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There are numerous fruit juices available for consumption such as apple juice, orange juice, and cranberry juice *etc*. Although different fruit juices have nutritious content with beneficial to health advantage. Using fruit juices as a substrate medium with kefir grains and baker's yeast for production of beverage product [22].

These innovations can improve people's health and well-being and/or lower their risk of contracting diseases. Prebiotics and probiotic foods are regarded as a significant category of functional foods in this context [8, 15, 28]. Fruit juices can also be utilised as a medium for fermentation or as a delivery system for probiotics because they have

high levels of sugars, dietary fibre, and other highly nutritious components. Antioxidant polyphenolics, which make it simple for consumers to accept them [9]. Recently, the pomegranate, pineapple (*Punica granatum* L.) has been cited as a pioneer in the healthy beverage sector. About 10% of the sugars in pomegranate fruit, sugars are mostly fructose and glucose, while 1.5% is pectin, organic acids such as ascorbic, citric, and malic, and bioactive substances like phenolics, flavonoids and anthocyanins make up the valuable to it. Pomegranate juice has at least 20% more antioxidant capacity than red wine and iced tea and is 2 to 8 times more potent than grape, caneberry, grapefruit, and orange juices [29]. The majority-consumed fruits are the strawberry (Fragaria x ananassa) and pineapple (*Ananas comosus*), both of which are rich in nutrients. These antioxidants help reduce oxidative damage brought on by chelating metals and free radicals [11]. Revealed that, pineapples have a favourable sugar ratio that makes them perfect for production of wine, and strawberries are a good source of vitamin C and manganese as well as folate (B9) and potassium. Because of their high antioxidant and plant component content, strawberries may be good for heart and blood sugar regulation.

In recent years, consumers have responded more favourably to "functional" foods or food ingredients that promise to have health benefits. Probiotics, which are foods containing live bacteria that can improve human or animal health by changing their intestinal microbiota, account up a sizeable portion of the food market [3]. The high nutritional benefit of lactic acid bacteria-containing fermented dairy products like yoghurt, sour milk, and kefir grains, Kefir grains, a naturally occurring dairy culture made up of a symbiotic group of yeasts and bacteria and utilised for the alcoholic and lactic acid fermentation of milk in the Caucasus region, include a wide variety of probiotic species. According to Chen *et al.* (2012) [4], the majority of them are lactic acid bacteria such *Lactobacillus plantarum, Lactobacillus acidophilus,* and *Lactobacillus kefiranofaciens*. Additionally, a number of studies have indicated that kefir grains contain beneficial components [14]. The problem is production and consumption designer food have recently become more popular with consequences for the environment, human health such as obesity, cancer, diabetes, ageing issues, and degenerative diseases are currently on the rise due to lifestyle choices. Due to their ability to stop or postpone the oxidation of easily oxidizable substrates, antioxidants are crucial in the management of various disorders. This has increased interest in naturally occurring, high-antioxidant liquids including tea, coffee, and wine. The purpose of this study was to make a fermented dairy-fruit juice beverage and investigate whether fruit juice may be used as a media for the growth of lactic acid bacteria, the consumption of sugar, and the creation of organic acids during the fermentation of kefir grains.

MATERIALS AND METHODS:

Collection of Fruit Samples and Kefir Grains: The fresh fruit samples of pomegranate, strawberry and pineapple were collected from the local area of Sinhgad College, Ambegaon bk. Pune for the production of nutrabeverage and stored in a polythene bag at room temperature until further process. The kefir grains were collected from the local market, in Pune. It contains *Lactobacillus acidophilus, Lactobacillus kefiranofaciens.* These kefir grains were kept in the refrigerator at 4°C until further processing.

POMEGRANATE **STRAWBERRY** **PINEAPPLE** **KEFIR GRAINS**

Sub-culturing and Maintenance of Kefir Grains: The Kefir grains were subcultured in fruit juices separately in a flask by inoculated with half loopful of pure culture of kefir grains under the aseptic

condition at pH in the range of 4.2- 4.6 and incubated at room temperature for 24 hours.

Preparation of Nutrabeverage:

Extraction of Sample from Pomegranate, Strawberry and Pineapple Fruits: Fruits of pomegranate, strawberry and pineapple were thoroughly washed and peeled. The extract juice from fruit with the help of grinder and mixer, extracted juice was mixed with water (1:1) and potassium metabisulphate (50mg/250ml) to prevent contamination and filtered through muslin cloth to obtain the clear juice. The T.S.S was adjusted to 22°Brix by the addition of 52 gm sugar in pomegranate, 53 gm in strawberry and 56 gm in pineapple.

Fermentation of Fruit Juice Extract with Kefir Grains: The fermentation was carried out by Lab scale Fermenter under anaerobic conditions. For fermentation conditions, the following treatment combinations were used: inoculums concentration of 12.5 ml of kefir subculture with an incubation period of 24hours at a temperature of 25^{o}C. The pH of juice extract was adjusted at 4.5. Then potassium metabisulphate (50mg/250ml) was added for the prevention of contamination.

Starter Culture Preparation: In the case of nutrabeverage production from the kefir grains mixed with pomegranate strawberry and pineapple fruit juice, an adjustment of 0 brix of the juice is generally required in order to have a sizable amount of alcohol in wine. Hence in the present study, TSS content of the known volume of juice was adjusted to the required 0 brix by the addition of sugar.

The pH was adjusted to 4.5; it was required for better fermentation and better quality of wine. After adjusting TSS, the juice was distributed in conical flasks, each containing 50 ml juice and pasteurized between 82^{o}C -85^{o}C for 20 minutes. Each 30 ml pasteurized juice after cooling was inoculated with a pure culture of kefir grains under aseptic conditions. The flasks were incubated at 30°C for 24 hrs. This 24 hrs old starter culture was used for nutrabeverage production by pouring 12.5 ml starter culture in 250 ml of juice.

Fermentation: A fermenter is a specially designed vessel, which is built to support and provide the optimum environmental conditions for optimum growth of microorganisms.

Clarification of the Nutrabeverage: Clarification of nutrabeverage was done to remove insoluble matter suspended in the nutrabeverage. This matter may include dead yeast cells, bacteria, tartrates, proteins, pectins, various tannins and other phenolic compounds, as well as pieces of pulp, stems and gums. The clarification was done using clarifying agent bentonite or gelatin (50mg/250ml)

Centrifugation of the Nutrabeverage: Centrifugation of nutrabeverage was carried out by using ultra centrifuge at 10000 rpm for 20 minutes for supernatant as nutrabeverage

Filtration, Storage and Aging of the Nutrabeverage: Nutrabeverage was filtered by using sterile muslin cloth after filtration nutrabeverage was stored in pre-sterilized glass bottles by appropriate labeling according to the different parameter and kept at room temperature for aging.

pH: The pH of fermented sample throughout the fermentation process was determined by using digital pH meter. The standard buffer solution was used to check proper working of electrode. The electrode was dipped into the standard solution and the pH of buffer solution was checked, which was indicated correctly on the display. Then glass electrode was aseptically immersed into the sample and the pH meter reading was recorded

Total Soluble Sugar Analysis by Hand Refractometer: Hand refractometer was used to measure sugar concentration in fermented sample (Ranganna 1979). The 1 or 2 drops of fermented sample was taken on the lens of the hand refractometer.

Titrable Acidity (TA): Titrable acidity was determined by using method of Association of Official Analytical Chemists (AOAC, 1970). For determination of titrable acidity 2ml of fermented sample was taken in test tube, and the burette was filled with 0.1N NaOH. Then two drops of phenolphthalein indicator was added and sample was titrated against 0.1 N NaOH with constant shaking. Titration was stopped when pink color was observed and the burette reading was noted.

The titrable acidity was calculated using following formula:

TA (%) = Normality (NaOH) X titre (ml) / Volume of Sample (ml) × 75

Reducing Sugar:

Standardization of the Fehling's Solution for Invert Sugar: Accurately weigh 4.75g of AR-grade sucrose. Transfer to 500 ml volumetric flask with 50 ml distilled water. Add 5 ml conc. HCl and allow standing for 24 h. Neutralize the solution with NaOH using phenolphthalein as an endpoint indicator and makeup to volume. Mix well and transfer 25 ml to a 100 ml volumetric flask and makeup to volume (1 ml = 2.5 mg of inverted sugar). Transfer to a burette having an off-set tip and titrate against Fehling's solution as described below for sample. (Practical manual of food chemistry and physiology, expt-4a).

Preparation of Sample: Weigh accurately 10-50 g sample as such (juices, beverages *etc*.) or homogenized sample (jams, preserves *etc*.) and transfer to 500 ml volumetric flask. Add about 100 ml water and neutralize with NaOH solution to phenolphthalein end point. Add 10 ml neutral lead acetate solution, shake and let stand for 10 min. Add potassium oxalate solution in small amounts until there is no further precipitation. Make up to volume, mix the solution well and filter through Whatman No. 1 filter paper. Transfer the filtrate to a 50 ml burette 4. having an off-set tip.

Preliminary Titration: Pipette out 5 ml each of Fehling A and B solutions into 250 ml conical flask. Mix and add about 10 ml water and a few pumice stone or glass beads. Dispense the sugar solution from the burette. Heat the solution up to boiling. Add 3 drops of methylene blue indicator. Continue the addition of the sugar solution drop wise until the blue colour disappears to a brick-red end point.

Final Titration: Pipette out 5 ml each of Fehling A and B solutions into a 250 ml conical flask. Add sample solution about 0.05 to 1.0 ml less than titre value of the preliminary titration. Heat the flask to boiling. Add 3 drops of methylene blue indicator. Complete the titration within 1 min by adding 2 to 3 drops of sugar solution at a time, until the indicator is decolourized. At the end point, the boiling liquid assumes the brick red colour. Note down the titre value. Perform the titration in duplicate and take the average.

Calculations: Based on the factor for Fehling's solution, V3 ml sample solution contains: 0.0025 V1 g reducing sugar (as invert sugar)

Therefore, % Reducing sugars in the sample = $0.0025 \times V_1 \times V_2 \times 100 / V_2 \times W$

$= 0.25 \times V_1 \times V_2 / V_3 \times W$

Specific Gravity: The specific gravity (osp.gr) was measured using the following equation, (refers to the USDA 0Brix measurement for the exact conversion numbers

Specific Gravity = 1 + (0.004× °Brix).

Alcohol Content: The total alcohol of the nutrabeverage samples were determined by the specific gravity method by using following formula (AOAC, 2000).

Ascorbic Acid (Vit-C): Ascorbic acid content was estimated by the method of Howard *et al*. (1999) [10].

Antioxidant Activity by ABTS Method: ABTS (2, 29-Azinobis 3-ethylene benzo thiazoline 6-sulphonic acid) Assay was used by the food industry and agricultural researchers to measure the antioxidant capacities of food and beverages [25]. In this assay, ABTS was converted to its radical cation by addition of potassium per-sulphate. This radical cation was blue in color and absorbs light at 734 nm. The ABTS radical cation was reactive towards most antioxidants including phenolics, thiols and ascorbic acid. During this reaction, the blue ABTS radical cation was converted back to its colorless neutral form (Raghavendra *et al*., 2017) [23].

RESULTS AND DISCUSSION: In this study, Kefir grain was collected from the local area, Pune. The Kefir grain was used for the production of nutrabeverage with pomegranate, strawberry, and pineapple juice. The quality of nutrabeverage can be characterized by its alcohol content which is the chief component found in all types of nutrabeverage [13]. Physicochemical characteristics of nutrabeverage after completion of fermentation under optimized environmental conditions were studied. Fruit juice is derived from the fruit's flesh

or from the fruit in its entirety. The process for creating fruit juice varies depending on the fruit, however many producers "press" or crush the fruit to extract the juice, pasteurize it, or add preservatives, and then package the finished product. Despite the fact that many fruit juices are healthy, drinking too much can have negative effects on one's health, such as weight gain or changes in blood sugar levels. *L. paracasei* SP3, which had been previously isolated from kefir grains and may have probiotic qualities [15], was tested in the fermentation of pomegranate juice to assess its technical potential, and the results were positive (Plessas et al., 2021). In contrast to dairy products, fruit juices like pomegranate juice have attracted a lot of attention in recent years as a substrate for probiotic administration [19]. Since fruit juices low pH values are their main flaw, more and more potential probiotics or specific probiotic strains are being used in these fermentations.

Fermentation Characteristics of Nutrabeverage: Physical characterstics of kefir grains and pomegranate, strawberry and pineapple juice.

TABLE 1: PHYSICAL CHARACTERISTICS OF SAMPLE

Characteristics	Kefir Grain	Pomegranate	Strawberry	Pineapple
Colour	White	Red	Red	Yellow
Shape	Globular	Globular	Globular	Oval
Weight	5gm	200gm	200gm	200gm

Physicochemical Analysis of Sample before Fermentation: The physicochemical characteristics of pomegranate, Strawberry and Pineapple fruit juice before fermentation. Initially, the pH of the pomegranate fruit juice sample was 3.7 whereas titrable acidity, TSS and reducing sugar were 2.6%, 16% and 15.78% respectively. In strawberry fruit juice sample the initial pH was 3.5 whereas titrable acidity, TSS and reducing sugar were 0.75%, 9%, 9.78% respectively. In the case of the pineapple juice sample the initial pH was 3.8 whereas titrable acidity, TSS and reducing sugar were found to be 3.37%, 14.3%, 14.06% respectively. These results are in agreement with the finding of Mena *et al.* (2015) and Dhumal *et al.* (1984) [5, 17].

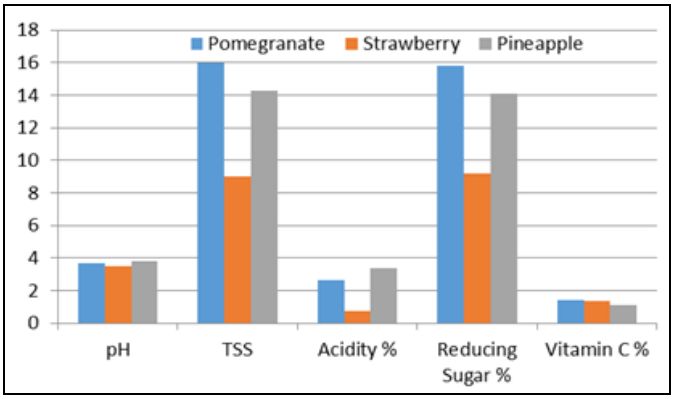

FIG. 2: PHYSICOCHEMICAL ANALYSIS OF SAMPLE BEFORE FERMENTION

Fermentation Process: The fermentation process was carried out in the fermenter. The 24 h old starter cultures were used for inoculation of samples for nutrabeverage production. The Kefir Grains required fifteen days to complete the fermentation process as it showed constant results with regard to reducing sugar. During the process of fermentation, the carbohydrates are degraded to ethyl alcohol and CO_2 due to the synthesis of amylases, and therefore, it might be due to their slow growth and poor utilization of carbon sources during the fermentation process [16].

Physicochemical Analysis of Sample After Fermentation: The present studies, focused on changes that occurred within compounds with reference to pH, TSS, titrable acidity, reducing sugars, alcohol content, and vit-c, after nutrabeverage production from Pomegranate, Strawberry and Pineapple juice samples by using Kefir grains. The pH of the fermented alcoholic beverage was decreased during the fermentation and again increased during the aging period. The pH before fermentation was observed 3.7, 3.5 and 3.8 for pomegranate, strawberry and pineapple juice respectively, during the fermentation at 25°C temperature and 4.5 pH. It was decreased gradually as observed 3.5, 3.2 and 3.6 in the pomegranate, strawberry and pineapple nutrabeverage respectively **Fig. 3**. The decrease in pH during fermentation indicates the increased titrable acidity due to fermentation 24. Given the significant buffering ability of the fermented juices, the initial pH value was specifically 3.2 and ranged between 3.2 and 0.2 for all of the time periods, as previous researchers have documented [18].

The pH values fluctuate as different levels of kefir grains are grown in the beverage. Depending on the kind of fermentation, the pH value reduced from an initial value of 4.23 to a lower value. The beverage fermented with 8% w/v kefir grains at a temperature of 25 ^{o}C had the lowest pH value of all the beverages tested, 3.40. Higher pH values 3.48-3.5 were obtained for beverages fermented at temperatures of 19 ^{o}C and 25 ^{o}C with 8%w/v and 5%w/v kefir grains, respectively. The beverage fermented with 5%w/v kefir grains had the highest pH value (Sabokbar and Khodaiyan 2008).

The total soluble solids (TSS) content of fermented alcoholic beverages decreased with the increase of the fermentation period. The highest TSS content before fermentation was observed at 160brix, 90brix and 14.30brix in the pomegranate, strawberry and pineapple fruit juice respectively. After 15 days of fermentation at 25^{0}C with pH 4.5, the lowest TSS content were120brix, 8.30brix and 100brix in the pomegranate, strawberry and pineapple nutrabeverage respectively observed. The decrease in TSS content of fruit alcoholic beverages indicates the utilization of sugar by kefir during fermentation.

The titrable acidity of fermented alcoholic beverages was increased till the end of fermentation. The titrable acidity was found minimum 2.62%, 0.75% and 3.37% in the pomegranate, strawberry and pineapple fruit juice respectively before fermentation. The titrable acidity was increased gradually with the increasing period of fermentation. After 15 days of fermentation at 25°C with pH 4.5, the highest titrable acidity were 4.12%, 1.87% and 4.5% in the pomegranate, strawberry and pineapple nutrabeverage respectively. Joshi (1998) [13], revealed that the titrable acidity of fruit wines vary between 0.5-1.0 percent. The reducing sugar content of fermented alcoholic beverages was decreased during the fermentation period. The reducing sugar was found maximum before fermentation were 15.78%, 9.78% and 14.06% in the pomegranate, strawberry and pineapple juice. The reduced sugar content decreased gradually with the increasing period of fermentation at 25°C with pH 4.5. The lowest reducing sugar content were 9.73%, 6.81% and 8.82% in the pomegranate, strawberry and pineapple nutrabeverage respectively. The alcohol content was increased with an increase in the fermentation period. The maximum alcohol content were found to be 6.9%, 4.3% and 5.2% in the pomegranate, strawberry and pineapple nutrabeverage respectively at 25°C at pH 4.5. Despite the low alcohol concentration (1% v/v), which complied with the guidelines for low or non-alcoholic beverages, respectable amounts of lactic acid were formed [7]. Low quantities of acetic acid were formed, likely as a result of the natural citric acid contained in pomegranate juice being enzymatically degraded, as other researchers have already noted in the lactic acid fermentation of fruit juices [26].

Vit-C content of fermented alcoholic beverages was decreased during the fermentation period. The vit-c was found maximum before fermentation were1.4%, 1.13% and 1.33%, in the pomegranate, strawberry and pineapple juice. After fermentation, it was decreased gradually with the increasing period of fermentation at 25°C and pH 4.5. Vit-c is a potential and effective scavenger of free radicals, decreased with an increase in the aging period. Microbes use the vit-c for their own defense system under high moisture content. Long-term storage decreases ascorbic acid content [10].

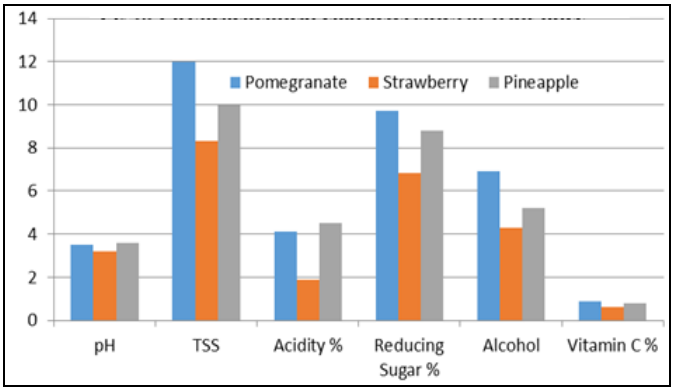

FIG. 3: PHYSICOCHEMICAL CHARACTERISTICS OF FRUIT JUICE AFTER FERMENTATION

Comparative Study of Antioxidant Activity of Pomegranate, Strawberry and Pineapple Fruit Juice before and after Fermentation: The average antioxidant activity of pomegranate, strawberry and pineapple fruit juice before fermentation were 0.137, 0.151, and 0.126 TEAC (µmol /mg) respectively. While the average antioxidant activity of pomegranate, strawberry, and pineapple nutrabeverage after fermentation was increased.

This is due to pomegranates and pineapple being rich sources of antioxidant compounds [12]. The highest antioxidant activity before fermentation was recorded for pineapple juice which was 0.151 TEAC (µmol/mg) and the lowest antioxidant activity was recorded for strawberries i.e. 0.126 TEAC (µmol/mg). The highest antioxidant activity after fermentation was recorded for pineapple beverage i.e. 0.315 TEAC (µmol/mg) and the lowest antioxidant activity were recorded for strawberry i.e.0.280 TEAC (µmol/mg).

TABLE 2: ANTIOXIDANT ACTIVITY OF JUICE BEFORE FERMENTATION

Fruit	R1	R2	R3	Mean TEAC (umol/mg)
Pomegranate	0.148	0.160	0.104	0.137
Strawberry	0.120	0.140	0.118	0.126
Pineapple	0.176	0.148	0.130	0.151

TABLE 3: ANTIOXIDANT ACTIVITY OF JUICE AFTER FERMENTATION

Fruit	R1	R2	R3	Mean TEAC (umol/mg)
Pomegranate	0.310	0.302	0.298	0.303
Strawberry	0.280	0.286	0.276	0.280
Pineapple	0.320	0.316	0.311	0.315

FIG. 4: LAB SCALE FERMENTER

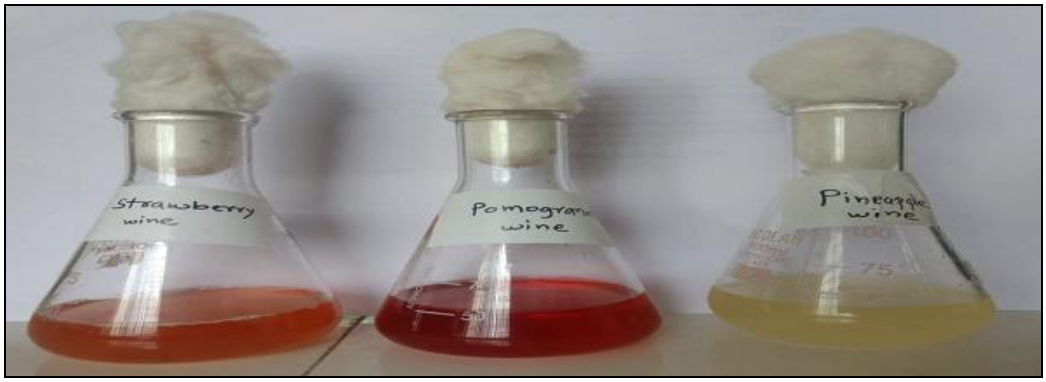

FIG. 5: NUTRABEVERAGE PRODUCTION OF FRUIT JUICES AFTER 15 DAYS

CONCLUSION: Studies including physicochemical parameters for the production of nutrabeverage were done in a simplified way. The various physical parameters studied while fermentation shows that Kefir grains were capable of fermentation of pomegranate, strawberry, and pineapple juice for nutrabeverage production. It was concluded that the production of nutrabeverage at pH 4.5, inoculums concentration 12.5 ml, temperature 25°C, and incubation periods 24 h was optimum for the nutrabeverage production by using the kefir grains. The value addition of pomegranate and pineapple must improve the antioxidant compound and overall physicochemical characteristics of the beverage. Currently, several beverages and food products are marketed based on their antioxidant capacity, and pomegranate and pineapple juice beverage has the potential to be presented in the lucrative market for their high content of antioxidant compound. These studies

needed facilitate the development of a value-added product that could serve as a new brand of potential cardio-protective kefir-based products.

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CONFLICTS OF INTEREST: Nil

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