FERMENTATION PROCESS, ANTIMICROBIAL ACTIVITY AND PHYSICOCHEMICAL ANALYSIS OF KEFIR GRAINS FERMENTED MILK, GRAPE (*VITIS VINIFERA* L.) AND APPLE (*MALUS PUMILA*) JUICES

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Abstract

The purpose of this study was to prepare low alcoholic fruit beverages through fermentation of grape juice and apple juice with kefir grains and optimum conditions for fermentation has been studied. The present study includes phytochemical investigation of selected fruits (grape and apple), antimicrobial activity and some physicochemical analysis of kefir fermented milk and fruits juice such as pH, titratable acidity, alcohol content, reducing sugar content and total soluble solids were determined. During the12 - 48 h of incubation, pH values of the fermented milk kefir and fruits-based beverages ranged from 5.8 to 3.0. These pH values were similar to those previously reported for kefir beverage. The antimicrobial activity of water kefir, apple juice kefir, grape juice kefir and milk kefir was screened with microorganisms such as three Gram positive bacteria - *Bacillus subtilis*, *Bacillus pumilus*, *Staphylococcus aureus*, two Gram negative bacteria - *Escherichia coli*, *Pseudomonas aeruginosa* and a fungus *Candida albicans* by agar well diffusion method. Milk kefir exhibited higher antimicrobial potency against the test organisms with inhibition zone diameters ranged between 20mm - 25 mm. Apple juice kefir and grape juice kefir also exhibited antimicrobial potency against the test organisms with inhibition zone diameters ranged between 18 mm - 23 mm.

Keywords : Kefir, kefir grains, probiotics, grape, apple, fermentation, antimicrobial activity

Introduction

Fermented foods and beverages play an important role in the human diet as they provide essential as well as contribute towards prevention of diseases. Lactic acid bacteria and yeasts are a major group of microorganism associated with fermented products. Some of the microorganisms, known as probiotics, confer health properties to human health. Thus, many different types of fermented foods and beverages containing probiotics are produced around the world to support wellness and health. (Athanasiadis *et al*., 2004) Probiotics are live microorganisms that have a beneficial effect on the host intestinal microbial balance. Advantages of probiotics are support body's ability to sbsorb nutrients and fight infection, support the immune system and help reduce chronic inflammation in digestive tract, treat high cholesterol and antibiotic associated diarrhoes. Lactic acid bacteria (LAB) and bifidobacteria are the most common types of probiotics.

Kefir is a unique fermented probiotic beverage produced by kefir grains (a mixture of lactic acid bacteria, acetic acid bacteria, and yeast). Kefir is nutrient-dense, with plenty of protein, B vitamins, potassium and calcium. It is incredibly beneficial for digestion and gut health. Kefir grains are multi-species natural starter culture, consisting of lactic acid bacteria (LAB), acetic acid bacteria and yeasts. Both the bacteria and yeast are surrounded by a polysaccharide matrix, called kefiran, which is a water-soluble branched glucogalactan, creating

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complex symbiotic community and widely used in fermented dairy products and beverages. It is very complex probiotic.

There are essentially two main types of kefir, and they differ in multiple ways. The two types of kefir are water kefir and milk kefir (Witthuhn *et al*., 2005).

Water kefir is a sour, alcoholic, and carbonated fermented beverage of which the fermentation is started with water kefir grains as inoculum. Water kefir grains consist of polysaccharide and contain microorganisms responsible for the water kefir fermentation. Its light and fizzy and can be flavored with any juice, tea or herbs. Fruit juice kefir is a delicious containing the probiotics benefits of kefir. The selected fruits for fermentation are grapes and apple fruits. Grapes are versatile fruits used in a wide range of popular fruits. It contains high levels of vitamins C, K and copper, good levels of vitamins B6, B2, B1, iron and potassium. The nutrients in grapes may help protect against cancer, eye problems, cardiovascular disease, and other health conditions. Grapes are suitable for people with diabetes, as long as they are accounted for in the diet plan. Apples are one of the most popular fruits in the world. They're also highly nutritious, high in vitamin C, fiber and several antioxidants which helps prevent absorption of dietary-LDL or bad cholesterol in the gut. Apples are low in calories; 100 g of fresh fruit slices provide just 50 calories. This fruit is good for diabetics, blood sugar regulation, protecting bones, boost brain power, aids weight loss (Joshi *et al.*, 2006).

Milk kefir is a fermented milk drink made with a yeast/bacterial fermentation starter of kefir grains. It is prepared by inoculating cow, goat, or sheep milk with kefir grains. Drinking of milk kefir is a healthy, fermented food with a consistency comparable to drinkable yogurt. It is a slightly sour, deliciously creamy and refreshing. Milk grains feed on lactose in milk (Guarner *et al*., 2005).

Various types of kefir beverages have been chosen for this research because fermented foods and beverages play an important role in the human diet as they provide essential nutrients as well as contribute towards prevention of diseases. In this research work, screening of phytochemical constituents, antimicrobial activity and physicochemical analysis of different kefir beverages were carried out.

Materials and Methods

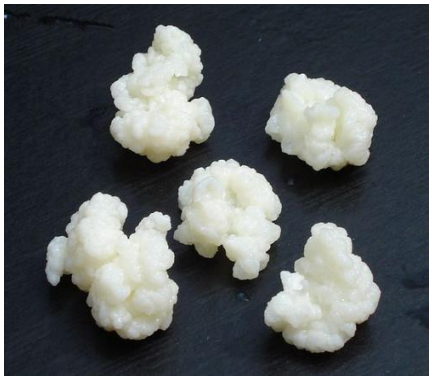
Sample Collection

Water kefir grains (Figure 1) and milk kefir grains (Figure 2) were purchased from the NIHON KEFIA Co., Ltd, Japan. Grape samples were collected from Thirimingalar Market, Yangon Region, originally from Yamethin Township, Mandalay Region and apple samples were also collected from Myitkyina Township, Kachin State.

Botanical Aspect of grape

Botanical name	:	*Vitis vinifera* L.
Family	:	Vitaceae
Genus	:	Vitis
Species	:	Vinifera

Figure 1 Water kefir grains



Botanical Aspect of apple

Botanical name	:	*Malus pumila* Mill.
Family	:	Rosaceae
Genus	:	Malus
Species	:	*M.*pumila

Figure 2 Milk kefir grains

Preliminary Phytochemical Investigation of Grape and Apple Juice

In order to find out the types of organic constituents present in the grape and apple fruits samples, preliminary phytochemical investigation was carried out according to the appropriate reported methods.

Investigation of the Development of Fermented Apple and Grape Juice Kefir Beverages Using Kefir Grains as a Starter Culture

Processing of water kefir

Water kefir grains (ca. 4 g) were added in a beaker or glass jar and then water and sugar (sucrose 4 g and distilled water 250 mL) were added. The gas jar was sealed with clean clothes and allowed to culture for 12 to 48 h at room temperature, then the kefir grains were removed to give the water kefir recipes (Figure 3).

Figure 3 Preparation of water kefir recipes

Preparation of fruit juice water kefir

The fermented water kefir (1F) was poured into a large glass jar or beaker and was added to each jar 60 mL of fruit juice or fresh fruit (apple or grape) and then allowed the kefir ferment for another 12 h (2 F). As the fruit was metabolized by kefir grains, the colour change was noticed in 24 h.

Processing of milk kefir

Milk kefir grains (ca. 4 g) were added in a jar and then fresh milk (250 mL) was added. The glass jar was sealed with clean clothes and allowed to culture for 12 to 48 h at room temperature, then the kefir grains were removed, resulting the milk kefir recipes (Figure 4).

Figure 4 Preparation of milk kefir recipes

Physicochemical Analysis of Kefir Beverages

Determination of pH

The pH values of kefir beverages were determined by pH meter.

Determination of total soluble solids content

Total soluble solid (ºBrix) content of kefir beverages was measured in units of degrees Brix by using ABBE refractometer.

Determination of acidity

200 mL of boiled and cooled distilled water was placed into a 50 mL conical flask and added 1mL phenolphthalein and titrated against 0.1 M sodium hydroxide until definite pink end point. Then 5 mL of grape juice sample was added and titrated against 0.1 M sodium hydroxide solution until same distinct end point. (Nummer, 2008). The volume of NaOH was noted and calculated with following equation:

$$\text{Titratable acidity A as tartaric acid } (\text{g}/100\ \text{mL}) = \frac{(\text{V})\ (\text{M})\ (75)\ (100)}{1000\ (\text{v})}$$

V = mL of sodium hydroxide solution used for titration
M = molarity of sodium hydroxide
v = sample volume (mL)

Determination of reducing sugar content

Reducing sugar content of kefir beverages was measured by Lane and Eynon method. (Leung et *al*., 1984).

Determination of alcohol content

Alcohol content of kefir beverages was measured by distillation method.

Screening of Antimicrobial Activities of Different Kefir Beverages by Agar Well Diffusion Method

Screening of antimicrobial activity

Antimicrobial activity of different kefir beverages (water kefir, milk kefir, apple and grape juice kefir) was tested against six pathogenic microorganisms by using agar well diffusion method. The extent of antimicrobial activity was measured from the diameter zone of inhibition. (Cruickshank et *al*., 1975)

Test organisms

Basic test organisms used in the research were kindly supplied by the Fermentation Department, Pharmaceutical Research Department, Ministry of Industry 1, and Yangon Region. These include *Bacillus subtilis, Bacillus pumilus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*.

Procedure

Nutrient agar was prepared according to the method described by Cruickshank et al., 1975. Nutrient agar was boiled and 20-25 mL of the medium was poured into the test tube and plugged with cotton wool and sterilized at 121 ºC for 15 min in the autoclave. After this, the tubes were cooled down to 30-35 ºC and poured into the sterilized petri dishes and 0.1-0.2 mL of the test organisms were added into the dishes. The agar was allowed to set for 2-3 h; then 10 mm agar wells were made by the help of sterilized agar well cutter. After that, about 0.2 mL of the sample was introduced into the agar well and incubated at 37 ºC for the 24 h. The inhibition zone which appeared around the agar well, indicated the presence of antimicrobial activity.

Results and Discussion

Preliminary Phytochemical Investigation of Apple and Grape Fruits

Preliminary Phytochemical analysis was performed in order to know different types of chemical constituent present in the fruit samples. The results were summarized in Table 1.

Both of the sample showed the presence of alkaloids, glycosides, flavonoids, carbohydrates, tannins, phenolic compounds, α-amino acids, reducing sugars, organic acid and saponins whereas steroid is absent in both grape and apple fruits. Terpenoids is present in apple fruits but absent in grape fruits.

Table 1 Results of Phytochemical Investigation of Grape and Apple Fruits

No.	Types of compounds	Extract	Test reagents	Observation	Remark	
					Grape	Apple
1	Alkaloids	1 % HCl	Mayer's Wagner's	red ppt yellow ppt	+ +	+ +
2	α-Amino acids	H_2O	Ninhydrin	violet colour spot	+	+
3	Carbohydrates	H_2O	10% α-naphthol and conc: H_2SO_4	red ring violet ring	+	+
4	Flavonoids	H_2O 70 % EtOH	dil:NH_3, conc: H_2SO_4 Mg turning and conc: HCl	yellow colour pink colour	+ +	+ +
5	Glycosides	H_2O	10% lead acetate	white ppt	+	+
6	Terpenoids	$CHCl_3$	Acetic anhydride & conc: H_2SO4	no pink colouration	-	+
7	Phenolic compounds	H_2O	1% $FeCl_3$	green colour deep blue colour	+	+
8	Reducing sugars	dil H_2SO_4	Benedict's solution	brick red ppt	+	+
9	Saponins	H_2O	Distilled water	frothing	+	+
10	Organic acids	H_2O	Bromocresol green	yellow colour	+	+
11	Steroids	PE	Acetic anhydride and conc H_2SO_4	no colouration	-	-
12	Tannins	H_2O	0.1 % $FeCl_3$	greenish yellow	+	+

(-) = absence, (+) = presence

Physicochemical Analysis of Different Kefir Beverages

Physicochemical analyses such as pH, reducing sugar content and acidity content, total soluble solids content and alcohol content of different kefir beverages were determined by reported methods and it was found that longer incubation period, higher acidity content and alcohol content but lower pH value, total soluble solid content and reducing sugar content.

pH

pH values of kefir beverages obtained from different fermentation times were measured by pH meter. The pH value of water kefir was ranged from 5.8 to 3.4.The pH value of milk kefir was ranged from 4.1 to 3.3. The pH value of grape juice kefir was ranged from 3.3 to 3.0. The pH value of apple juice kefir was ranged from 3.4 to 3.0 respectively. This suggests the oxidation of the alcohol to acid upon keeping longer incubation time. These results are reported in Table 2.

Table 2 pH Value of Water Kefir, Milk Kefir, Grape Juice Kefir and Apple Juice Kefir

No.	Samples	pH values			*Literature value
		12 h	24 h	48 h	
1	Water kefir	5.8	3.8	3.4	6.0-3.5
2	Milk kefir	4.1	3.7	3.3	
3	Grape juice kefir	3.3	3.0	3.0	
4	Apple juice kefir	3.4	3.3	3.0	

Acidity

Acidity content of kefir beverages obtained at different fermentation times were measured by titration method. The acid value of water kefir was ranged from 5.8 to 9.71.The acid value of milk kefir was ranged from 3.3 to 4.1. The acid value of grape juice kefir was ranged from 18.3 to 66.27. The acid value of apple juice kefir was ranged from 25.35 to 46.12 respectively. In this research, highest acidity content was observed in grape juice kefir. These results are reported in Table 3.

Table 3 Acidity of Water Kefir, Milk Kefir, Grape Juice Kefir and Apple Juice Kefir

No.	Samples	Acidity content (%)		
		12 h	24 h	48 h
1	Water kefir	5.8	6.8	9.71
2	Milk kefir	3.3	3.7	4.1
3	Grape juice kefir	18.3	39.57	66.27
4	Apple juice kefir	25.35	35.93	46.12

Alcohol content

Alcohol content of kefir beverages obtained from different fermentation times were measured by distillation method. The alcohol content of water kefir was ranged from 0.1 to 0.44.The alcohol content of milk kefir was ranged from 0.05 to 0.4. The alcohol content of grape juice kefir was ranged from 1.0 to 1.81. The alcohol content of apple juice kefir was ranged from 0.4 to 1.20 respectively. During the 12 h, 24 h and 48 h of incubation, alcohol content of fermented water kefir, milk kefir, apple juice kefir and grape juice kefir was increased. Highest alcohol content was observed in grape juice kefir. These results are reported in Table 4.

Table 4 Alcohol Content of Water Kefir, Milk Kefir, Grape Juice Kefir and Apple Juice Kefir

No.	Samples	Alcohol content (%)		
		12 h	24 h	48 h
1	Water kefir	0.1	0.2	0.44
2	Milk kefir	0.05	0.15	0.4
3	Grape juice kefir	1.0	1.48	1.81
4	Apple juice kefir	0.4	0.66	1.20

Total soluble solid content

Total soluble solid (ºBrix) content content of kefir beverages was measured in units of degrees Brix by using ABBE refractometer. The total soluble solid content of water kefir was ranged from 1.3 to 0.3. The total soluble solid content of milk kefir was ranged from 3.0 to 1.9. The total soluble solid content of grape juice kefir was ranged from 7.8 to 5.00. The total soluble solid content of apple juice kefir was ranged from 8.4 to 6.25 respectively. In this research, when longer incubation period were observed lower total soluble solid content. These results are reported in Table 5.

Table 5 Total Soluble Solid (ºBrix) Content of Water Kefir, Milk Kefir, Grape Juice Kefir and Apple Juice Kefir

No.	Samples	Total soluble solid (ºBrix)		
		12 h	24 h	48 h
1	Water kefir	1.3	0.85	0.3
2	Milk kefir	3.0	2.6	1.9
3	Grape juice kefir	7.8	6.25	5.00
4	Apple juice kefir	8.4	7.00	6.25

Reducing sugar content

Reducing sugar content of kefir beverages obtained different fermentation times were measured by Lane and Eynon method. The reducing sugar content of water kefir was ranged from 4.8 to 1.9.The reducing sugar content of milk kefir was ranged from 4.1 to 3.3. The reducing sugar content of grape juice kefir was ranged from 5.1 to 2.76. The reducing sugar content of apple juice kefir was ranged from 3.5 to 3.64 respectively. In this research, when longer incubation period were observed lower reducing sugar content. But highest reducing sugar content was observed in apple juice kefir .These results are reported in Table 6.

Table 6 Reducing Sugar Content of Water Kefir, Milk Kefir, Grape Juice Kefir and Apple Juice Kefir

No.	Samples	Reducing Sugar content (%)		
		12 h	24 h	48 h
1	Water kefir	4.8	3.0	1.9
2	Milk kefir	4.1	3.7	3.3
3	Grape juice kefir	5.1	4.05	2.76
4	Apple juice kefir	3.5	3.84	3.64

Antimicrobial Activity of Water Kefir, Apple Juice Kefir, Grape Juice Kefir and Milk Kefir against on Six Species of Microorganisms

Antimicrobial activity of water kefir , apple juice kefir, grape juice kefir and milk kefir were screened on six different strains of microorganisms such as *Bacillus subtilis*, *Bacillus pumilus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* by agar well diffusion method. It was found that milk kefir exhibited higher antimicrobial potency against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aerugino* and *Escherichia coli* with inhibition zone diameters ranged between 20 mm - 25 mm. But Milk kefir showed no activity on *Bacillus pumilus* and *Candida albicans.* Apple juice kefir and grape juice kefir also exhibited antimicrobial potency against the test organisms with inhibition zone diameters ranged between 18 mm - 23 mm. Therefore, prepared kefir beverages showed the antimicrobial activity and it may be used for the treatment of diseases infected by the microorganisms such as diarrhea, dysentery and urinary infections.The result of antimicrobial activities of different kefir beverages samples are reported in Table 7 and Figures 5 to 10.

Table 7 Antimicrobial Activity of Water Kefir , Apple Juice Kefir, Grape Juice Kefir and Milk Kefir against on Six Species of Microorganisms

No.	Sample	Inhibition zone diameters (mm) against different microorganisms					
		A	**B**	**C**	**D**	**E**	**F**
1	Water Kefir	12(+)	13(+)	23(+++)	14(+)	15(++)	13(+)
2	Milk Kefir	20(+++)	20(+++)	20(+++)	-	-	25(+++)
3	Apple Juice Kefir	23(+++)	22(+++)	23(+++)	23(+++)	20(+++)	23(+++)
4	Grape Juice Kefir	21(+++)	20(+++)	20(+++)	20(+++)	20(+++)	18(++)
5	(-) Control	-	-	-	-	-	-

Agar well - 10 mm
10mm ~ 14 mm (+)
15 mm ~ 19 mm (++)
> 20 mm above (+++)

A = *Bacillus subtillis*
B = *Staphylococcus aureus*
C = *Pseudomonas aeruginosa*
D = *Bacillus pumilus*
E = *Candida albicans*
F = *E.coli*

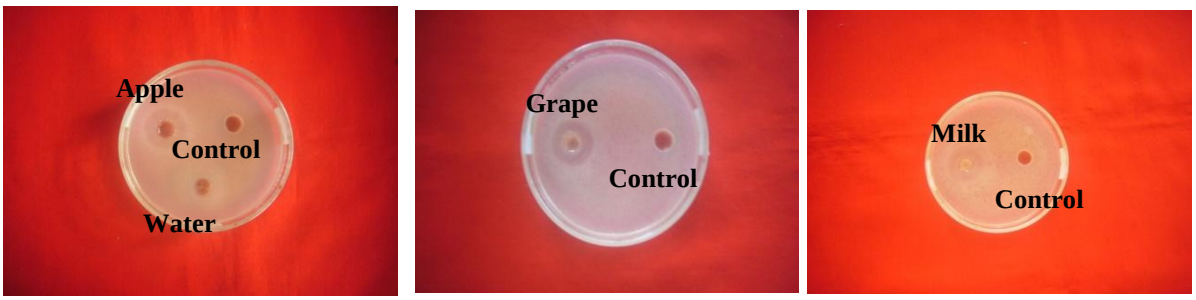


Figure 5 Antimicrobial screening of water kefir, apple juice kefir, grape juice kefir and milk kefir against *Bacillus subtillis* by agar well diffusion method

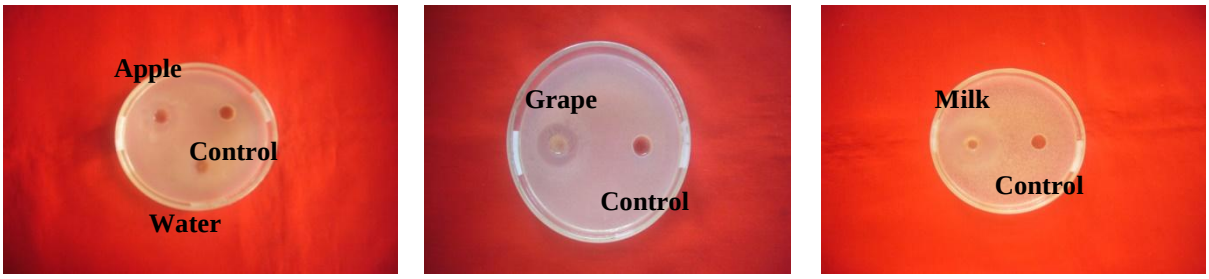


Figure 6 Antimicrobial screening of water kefir, apple juice kefir, grape juice kefir and milk kefir against *Staphylococcus aureus* by agar well diffusion method

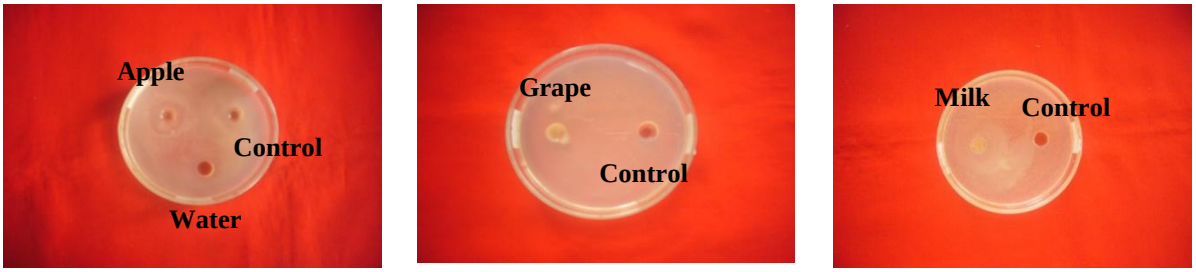


Figure 7 Antimicrobial screening of water kefir, apple juice kefir, grape juice kefir and milk kefir against *Pseudomonas aeruginosa* by agar well diffusion method

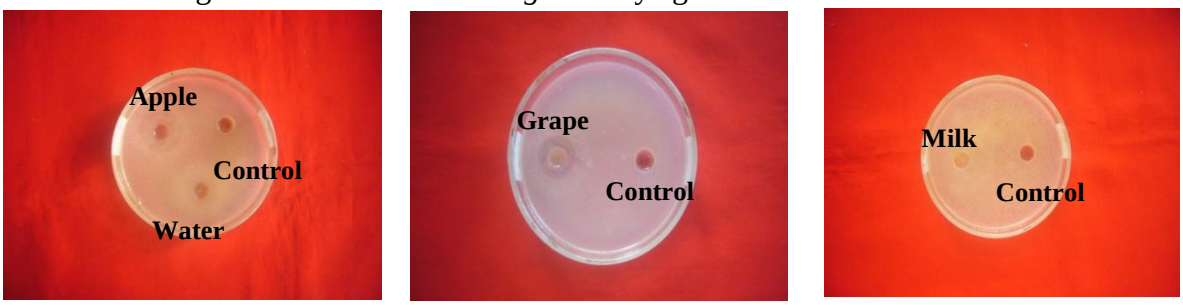


Figure 8 Antimicrobial screening of water kefir, apple juice kefir, grape juice kefir and milk kefi against *Bacillus pumilus* by agar well diffusion method

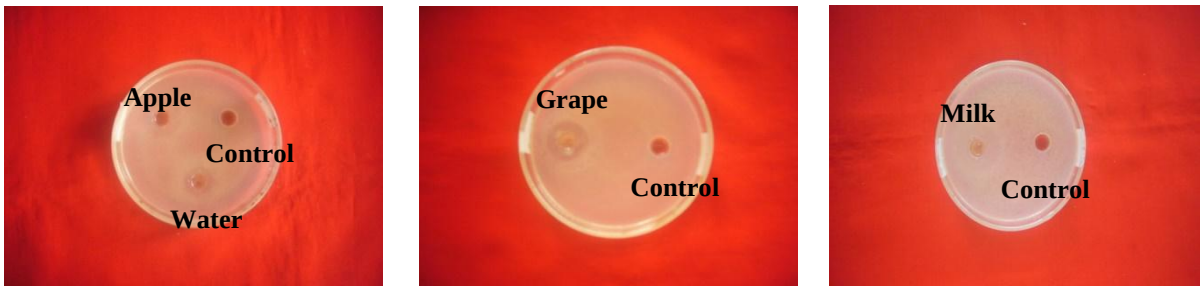


Figure 9 Antimicrobial screening of water kefir, apple juice kefir, grape juice kefir and milk kefir against *Candida albicans* by agar well diffusion method

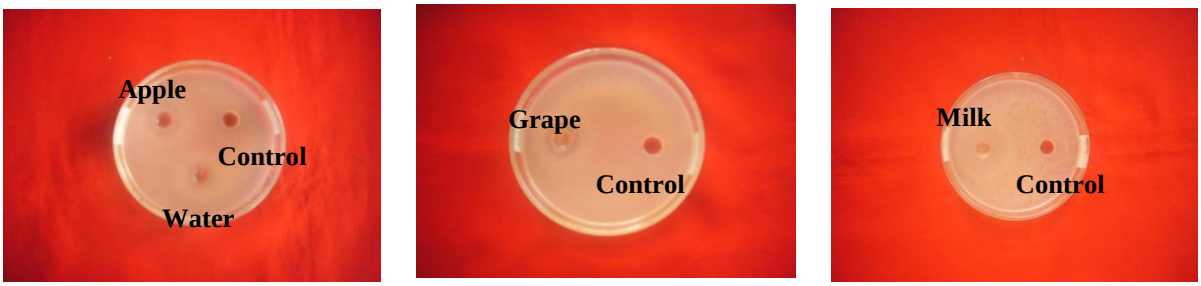


Figure 10 Antimicrobial screening of water kefir, apple juice kefir, grape juice kefir and milk kefir against *E.coli* by agar well diffusion method

Conclusion

The preliminary phytochemical investigation indicated the presence of alkaloids, glycosides, flavonoids, carbohydrates, tannins, phenolic compounds, α-amino acids, reducing sugars, organic acid and saponins in grape and apple fruits. Steroid was found to be absent in both grape and apple fruits. Terpenoids was found to be present in apple fruits but absent in grape fruits.

From the determination of the some physicochemical properties of kefir beverages, pH values of the fermented water kefir, milk kefir and fruits-based beverages ranging from 5.8 to 3.0 during the 12 h, 24 h and 48 h of incubation,. These pH values were similar to those previously reported for kefir beverage. During the 12 h, 24 h and 48 h of incubation, acidity content of different kefir beverages were increased from 3.3 % to 46.12 %, alcohol content was increased from 0.05 % to 1.81%, total soluble solid (TSS) content and reducing sugar content was reduced from 8.4 ºBrix to 0.3 ºBrix and 5.1 % to 1.9 %, respectively.

From antimicrobial screening tests, almost all of the kefir beverages (water kefir, milk kefir, grape Juice kefir and apple Juice kefir) exhibited antibacterial and antifungal activities.

Among them, milk kefir showed higher potency with inhibition zone diameter up to 25 mm on *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*. Apple juice kefir and grape juice kefir also exhibited antimicrobial potency with inhibition zone diameter up to 23 mm against on all test organisms. Thus kefir beverages may be useful in the treatment of bacterial and fungal infections owing to its effective antibacterial and antifungal actions.

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