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Anti-microbial, anti-fungal and anti-carcinogenic properties of coconut milk kefir

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Abstract
Fermented dairy products have been an important component of a nutritional diet. Kefir, a fermented yoghurt, is produced by the action of bacteria and yeasts that exist in symbiotic association in kefir grains. Traditionally, Kefir is manufactured by using cow, ewe, goat or buffalo milk. Coconut is classified as a highly nutritious "functional food" as it provides many health benefits beyond its nutritional content. The study focuses on studying the synergistic effect of coconut milk and kefir and to assess the antimicrobial, antifungal and anticancer activities of the newly formulated coconut milk kefir. The kefir grains were activated and then introduced into coconut milk to produce, coconut milk kefir. The antimicrobial activity was tested with two gram negative (Escherichia Coli, Salmonella typhi) and one gram positive bacteria (Staphylococcus aureus); and fungi (Saccharomyces cerevisiae and Aspergillus niger). Different concentrations of coconut milk kefir (20, 25 and 30 µl) were tested using Kirby-Bauer disk diffusion susceptibility test and using the Kirby-Bauer well diffusion method respectively. The Cytotoxic activity of coconut milk kefir was tested against breast cancer cell line (MCF-7) using Micro Culture Tetrazolium (MTT) assay, where, different concentrations (25, 50, 75,100 and125 µl) were tested at an optical density of 570 nm to calculate the percentage of viability. The newly formulated coconut milk kefir demonstrated the maximum zone of inhibition (39mm) against E.coli among the bacteria tested and against Saccharomyces cerevisiae (7mm) among the fungi tested. The anticancer study using MTT assay, revealed that coconut milk kefir had a cytotoxic effect of 24.73 to 57.79 percent on MCF-7 cells in a dose dependent manner. Thus, it can be inferred that coconut milk kefir has antimicrobial, antifungal and anticancer properties.

Keywords: Fermented products, Coconut milk kefir, Antimicrobial activity, Antifungal activity, Anticancer activity

1. Introduction
Fermented dairy products have long been associated with the ability to confer health benefits and longevity in those who regularly consume them. It is due to the beneficial effects of its microbiota on the gut. The kefir grains consist of a host of beneficial yeasts and bacteria, which confer health benefits in humans (Tamang JP *et al*., 2016 [29] and Tamime AY, 2002) [30]. Kefir is traditionally made with cow's milk but can also be made with milk from other sources such as goat, sheep, buffalo, or soy milk (Ismail AA *et al*., 1983 [11]; Liu JR, 2006 [18]; Motaghi M *et al*., 1997 [22]; Wszolek M, 2001) [33]. In addition to beneficial bacteria, kefir contains vitamins, minerals and essential amino acids that are beneficial in healing, energy, promoting longevity and homeostasis (Ot1es S and Cagindi O, 2003) [23]. Kefir contains vitamins B1, B2, B5 and C. The vitamin content of kefir is influenced by both type of milk and microbiological flora (Sarkar S, 2007) [28].
Food-borne diseases (FBD), cover illnesses acquired through consumption of contaminated food, and are also frequently referred to as food poisoning. Food borne diseases are an important cause of morbidity and mortality worldwide. The common types of FBD include botulism, *Salmonellosis*, Hepatitis A, Shigellosis, Camplylo bacteriosis and Norovirus infection. The organisms which are majorly responsible for FBD include: *E.coli*, *Campylobacter spp*., *Salmonella typhi*, Taenia solium, hepatitis A virus, aflatoxin and Shigella (WHO, 2015) [32]. Kefir acts against the pathogenic bacteria such as: Salmonella, Helicobacter, Shigella, Staphylococcus, Escherichia coli, Enterobacter aerogenes, Proteus vulgaris, Bacillus subtilis, Micrococcus luteus, Listeria monocytogenes, Streptococcus pyrogenes (Lopitz FO *et al*. 2006) [20], Streptococcus faecalis, Fusarium graminearum, and the fungus Candida albicans

(Ismaiel AA *et al*., 2011) [10]. Therefore, kefir appears to be a promising safe alternative natural food preservative offering protection against FBD.

Cancer is one of the ten leading causes of death in India. Breast cancer is the most common diagnosed malignancy in India. It is estimated that there are nearly 2 to 2.5 million cancer cases at any given point of time in India. Latest findings reveal that all conventional medical treatment for cancer is not helpful. Studies indicate it is found that survival in breast cancer is four times longer without conventional treatment (Jacob B *et al*., 2015) [12]. Cancer prevention is thus the optimal means of decreasing the cancer burden, and is particularly important given the severity of the disease. Several studies support the idea that some dietary components, such as fruits and vegetables, are protective factors against cancer (Key TJ *et al*., 2004) [14]. There is evidence that a diet rich in fermented foods such as fermented milk products may have beneficial properties in reducing risk of some cancers (De Moreno A *et al*., 2010 [5]; Larsson SC *et al*., 2008 [17]; Van't Veer P *et al*., 1989) [31].

Coconut (*Cocos nucifera* L.) is widely known as the tree of life, due to its significant contribution to human life. The lauric acid in coconut oil is used by the body to make the same disease fighting fatty acid derivative, monolaurin, which babies make from the lauric acid they can otherwise get only from their mother's milk. Lauric acid is known for its antimicrobial properties, and the monoglyceride derivative of lauric acid, monolaurin, is known to have more potent antimicrobial properties against lipid coated RNA and DNA viruses and numerous pathogenic protozoas (Kabara JJ, 2000) [13]. The aqueous extract of coconut is found to possess antineoplastic, antifungal and antimicrobial effects (Koschek PR, 2007) [15]. Enig, (2004) reported that the fatty acids in coconut like capric acid, lauric acid are found to be made of medium chain fatty acids, which allows them to be directly absorbed from the intestine and go straight to the liver to be rapidly metabolized and do not participate in the synthesis and transport of cholesterol.

Milk kefir has not been commonly used in India as it is not indigenous to India. Coconut milk and kefir have each been documented to have health benefits. Considering the health benefits of consuming fermented foods on a daily basis, this study was designed to observe the synergistic effect of coconut milk and kefir, when combined to produce a new product, coconut milk kefir; with special reference to anticancer and antimicrobial properties.

Materials and Methods

Preparation of coconut milk kefir

Fresh coconut milk was extracted using a standardized procedure. The inactive kefir grains were activated using fresh cold pasteurized cow's milk, by allowing the grains to ferment in the milk for 12-24 hours. The grains were then separated by filtering and added to another batch of fresh milk. The milk was changed every time until it reached a maximum of four cups and the kefir grains were then removed by filtering. The kefir grains thus activated were then introduced into the coconut milk and allowed to ferment for 12-24 hours, followed by the straining process to separate the kefir grains.

Antimicrobial studies

The antimicrobial effect of coconut milk kefir was tested with two gram negative (*Escherichia coli*, *Salmonella typhi*) and one gram positive (*Staphylococcus aureus*) bacteria; and fungi (*Saccharomyces cerevisiae and Aspergillus niger*) as test microorganisms. These organisms were chosen because they are common food borne pathogens. A sterile wire loop was used to place the test bacteria or fungi into a test tube with peptone water over an open flame.

Kirby Bauer disk diffusion susceptibility test procedure for bacteria

Antimicrobial susceptibility testing was done using the disk diffusion method to detect the antibacterial and antifungal activity of the samples (Perez C, 1990) [24]. The pathogenic organism was grown on Mueller Hinton Agar in the presence of various concentrations of the coconut milk kefir impregnated filter paper disks. The presence or absence of growth around the disks is an indirect measure of the ability of that compound to inhibit that organism. In a sterile petri plate, the autoclaved molten Mueller Hinton agar was poured and allowed to solidify. A sterile swab was dipped into the inoculum tube containing the bacterial organism. The test organism was inoculated by streaking the swab in a back- and-forth motion very close together, while moving across and down the plate. The plate was rotated at 60˚. The plate was rotated once more and the streaking action was repeated. This ensures an even distribution of inoculum that will result in a confluent lawn of growth. The plate was rimmed with the swab by running the swab around the edge of the entire plate to pick up any excessive inoculum that may have been splashed near the edge. Sterile filter paper disks were placed in the solidified agar with a help of tongs; using a micropipette. The samples were added in concentrations of 150 µl, 175µl and 200 µl onto the disks. The sample was allowed to diffuse through the agar and incubate the plates at 35˚C±2˚C. Following incubation, the diameter of the zone of inhibition was measured to the nearest millimetre using a ruler.

Kirby Bauer well diffusion susceptibility test procedure for fungi

The general media was prepared and was left for autoclaving. The culture of *Aspergillus niger* was inoculated in the nutrient broth and kept for incubation overnight in the shaker. The culture of *Saccharomyces cerevisiae* was made using 10 ml of sterile saline and 1 g of yeast. The general media plates were prepared. Sterile cotton swab was immersed in the culture suspension and then rotated and compressed against the wall of the test tube so as to express the excess fluid. The surface of the general media plates was inoculated with the swab. 150, 175 and 200 µl of the sample were added to the wells punctured in the plates. The plates were incubated for 48 hours at 37 °C. Following incubation, the diameter of the zone of inhibition was measured to the nearest millimetre using a ruler.

Anticancer Studies: The MTT Assay is based on the ability of live but not dead cells to reduce a yellow tetrazolium dye to a purple formazan product (Mossman T, 1983) [21].

Culturing of MCF-7 breast cancer cell line

MCF-7 Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10%foetal bovine serum, and grown to confluency at 37 ºC in humidified atmosphere with 5% CO_2 in a CO_2 incubator. The cells were plated in 96 well, flat bottom tissue culture plates at a density of approximately 1.2x 10^4 cells/ well. The cells were allowed to attach to the bottom of the well overnight, at 37ºC. The medium was then discarded and cells were incubated with different concentrations (25, 50, 75, 100 & 125 µL) of the sample for 24 hours. After the incubation, the medium was

discarded and 100µl fresh medium was added with 10µl of MTT (5mg/ml). After four hours, the medium was discarded and 100µl of Dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Then, the absorbance was read at 570nm in a micro titre plate reader. Cyclophosphamide was used as positive control.

Cell survival or viability was calculated by the formula:

Viability % = (Test OD/ Control OD) X 100

Cytotoxicity % = 100 – Viability%

Results and Discussion

The formulated product coconut milk kefir was off-white in colour. The taste of unflavoured kefir was "yeasty".

Antimicrobial and Antifungal studies

The antimicrobial activity of 20, 25 and 30 µl of coconut milk kefir against pathogenic microorganisms has been presented in table 1. The antifungal activity of 150,175 and 200 µl of coconut milk kefir against pathogenic microorganisms is presented in table 2.The results presented in tables 1, 2 and figure 1, indicate that coconut milk kefir showed antimicrobial and antifungal activity against both the gram negative bacteria (*Escherichia Coli*, *Salmonella typhi*) as well as the gram positive bacteria (*Staphylococcus aureus*) in addition to the two fungi (*Saccharomyces cerevisiae and Aspergillus niger*) tested. However, the coconut milk kefir was more potent against the gram-negative bacteria, with a zone of inhibition ranging from 8mm to 39 mm (Table 1); with a dose dependent increase in zone of inhibition. In comparison the anti-microbial effect on gram-positive bacteria, was lower with a zone of inhibition ranging from 5 mm to 10 mm (Table 1 and Table 2). The coconut milk kefir was most effective against *Escherichia Coli* at concentrations of 20 to 30 µl exhibiting 33-39 mm zone of inhibition. While there was anti-microbial activity against *Staphylococcus aureus* at 20 µl concentration there was a 10 mm zone of inhibition at 25 and 30 µl concentrations. The coconut milk kefir showed no anti-microbial activity against *Salmonella typhi* that causes typhoid or enteral fever at 20µl concentration but showed an inhibitory zone of 8 and 9mm respectively at 25 and 30 µl concentrations.

Studies by Santos *et al.* (2003) [26] and Chifiriuc *et al.* (2011) [3], revealed that milk kefir was found to be effective against pathogenic organisms such as *E. coli*, *Salmonella typhi*, *Salmonella enteritidis*, *Shigella flexneri*., *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia faecalis*. The antimicrobial activity of coconut milk kefir is attributed to the production of organic acids, peptides (bacteriocins), carbon dioxide, hydrogen peroxide, ethanol and diacetyl. These compounds helps in reducing the food borne pathogens and deterioration causing bacteria during beverage production and storage (Farnworth ER, 2005 [5]; Sarkar S, 2008) [27].

This is also similar to the findings by, Garrotte *et al*., (1997) [8] in which kefir caused zones of inhibition ranging between 11 to 20.50 mm against gram-positive and gram-negative species due to the undissociated forms of lactic and acetic acids that were produced during fermentation. The zones of inhibition of coconut milk kefir against *Escherichia coli, Salmonella typhi and Staphylococcus aureus* ranged from 10 to 39 mm, this enhanced effect could be because of the combined effect of coconut milk and kefir grains. Fresh kefir has been shown to have an intrinsic inhibitory power against *Staphylococcus aureus*, *Kluyvero myceslactis*, and *Escherichia coli*, but not against *Saccharomyces cerevisiae* or *Candida albicans* (Brialy C, 1995) [1]

Coconut milk kefir exhibited anti-fungal activity against *Saccharomyces cerevisiae and Aspergillus niger*. The zone of inhibition of coconut milk kefir against *Saccharomyces cerevisiae* was 7 mm for the concentration of 175 µl. The coconut milk kefir at concentration of 200 µl exhibited a zone of inhibition of 6 mm for *Aspergillus niger.* This is consistent with the findings of Rihakova *et al*., 2002 [25] who reported that coconut is found to be an effective antifungal agent as it can prevent spore germination and inhibit radial growth, this might be due to monolaurin the active component in coconut. Kefir also demonstrated antifungal activity against *Candida*, *Saccharomyces*, *Rhodotorula*, *Torulopsis*, *Microsporum* and *Trichophyton* species (Cevikbas A, 1994) [2].

Table 1: Anti-microbial activity of coconut milk kefir

Microorganism	Zone of Inhibition (mm)		
	20µl	25 µl	30 µl
Escherichia Coli	33	32	39
Staphylococcus aureus	-	10	10
Salmonella typhi	-	8	9

Table 2: Anti-fungal activity of coconut milk kefir

Fungal organism	Zone of Inhibition (mm)		
	150µl	175 µl	200 µl
Saccharomyces cerevisiae	6	7	3.5
Aspergillus niger	5	5	6

Staphylococcus aureus

Salmonella typhi

E. coli

Aspergillus niger

Saccharomyces cerevisiae

Fig 1: Zone of Inhibition exhibited by coconut milk kefir against the food borne disease pathogens

Anticancer study

The effect of coconut milk kefir on breast cancer cell lines was determined by measuring the cell viability by MTT assay. The

results of the MTT assay has been presented in tables 3, 4; figures 2, 3 and 4. From which it can be observed that the coconut milk kefir has shown significant cell death of 24.73 to 57.79 per cent in the MTT Assay. It was also noted that as the concentration of coconut milk kefir increased, the level of cytotoxicity also increased.

Table 3: Percentage of cell viability of sample and positive control

Concentration (µL)	Cell Viability %	
	Coconut Milk Kefir	Positive Control
25	75.27	24.88
50	68.45	19.34
75	61.21	16.60
100	55.26	12.02
125	42.21	6.32

PC – Positive control: Cyclophosphamide (1 mg/ mL)

Table 4: Percentage of cytotoxicity of sample and positive control

Concentration (µL)	Cytotoxicity %	
	Coconut Milk Kefir	Positive Control
25	24.73	75.12
50	31.55	80.66
75	38.79	83.40
100	44.74	87.98
125	57.79	93.68

PC – Positive control: Cyclophosphamide (1 mg/ mL)

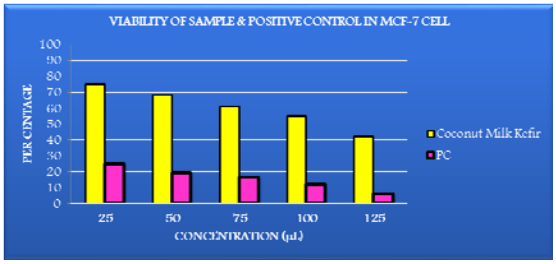


Fig 2: Viability of Sample and positive control in MCF7 cell

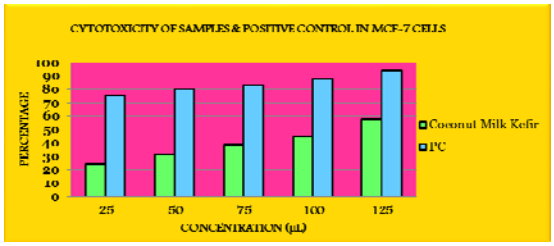
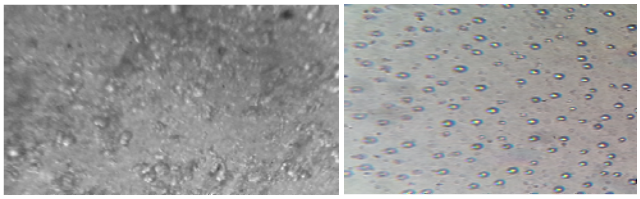
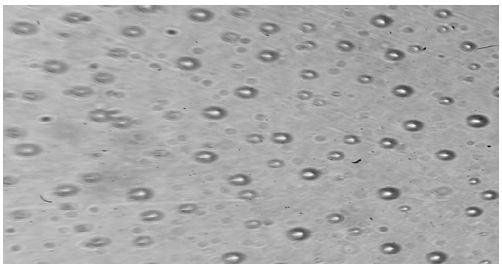


Fig 3: Cytotoxicity of Samples and positive control in MCF7 cells

The anticancer activity of fermented dairy products can be attributed, in general, to cancer prevention and the suppression of early-stage tumours, by the delaying of enzyme activities that convert pro-carcinogenic compounds to carcinogens, or by the activation of the immune system (Sarkar S, 2008) [27]. Kubo *et al.* (1992) [16] reported the inhibition of the proliferation of subcutaneously transplanted *Ehrlich ascites* tumours in mice. Several studies have reported that milk kefir helps in preventing growth of malignant cells by acting as antioxidants. They are found to be capable of binding with the tumour cells and thereby preventing metastasis. This function might be attributed to the non-microbial components released during milk fermentation (De Moreno a *et al*., 2006; Guven A and Gulmez M, 2003; Liu JR, 2002) [4, 3, 19]. It is evident that, coconut milk kefir prevented the growth and proliferation of breast cancer cell lines, when compared with the control cells lines. We can thus conclude that kefir can be considered a good probiotic and also may be used in the treatment of breast cancer.



Cell control Sample treated cells



Positive control treated cells

Fig 4: Coconut milk kefir exhibiting anticancer activity against MCF-7 cell lines

Conclusion

Coconut milk kefir, a novel alternative to the traditional beverages, is considered as an ideal probiotic due to the array of nutrients and functional components present in it. The anti-microbial activity of coconut milk kefir against certain common pathogenic organisms that cause food borne illness and gastrointestinal disorders indicates that it can be recommended as a food based remedy to prevent these illnesses. The results also suggest that coconut milk kefir may be considered among the more promising food components in terms of cancer prevention and treatment. Due to various nutritional and therapeutic attributes of kefir, it may occupy an important place in the daily diet.

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