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Effect of Biologically Active Compounds Recovered from Kefir, (A Probiotic Fermented Milk) On Thyroid Toxicity

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Abstract

Fermented milk is considered as a good source of nutrition for many people. One of the most important substances that could be used in milk fermentation is kefir grains. Kefir fermented milk is very important in many health conditions such as bacterial infections, high blood pressure and some hepatic conditions. The aim of this study was to determine the antibacterial activity of kefir fermented milk against some pathogenic microorganisms as Salmonella sp., Escherichia coli, Staphylococcus sp. and Candid albicans by using disc diffusion method. The efficacy of kefir fermented milk was the results revealed that the kefir fermented milk had a potent antibacterial activity against all tested pathogenic isolates and it also showed high significant protection in mice against CCl4 toxicity. In conclusion, kefir milk can be used as an antibacterial supplement and as a protective agent against thyroid cancer.

Keywords: *Kefier, Thyroid cancer, ccl4.*

Introduction

"Kefir is gotten from the Turkish word "keif" which signifies "nice feeling" (Adriana and Socaciu 2008) and the drink started in the Caucasian heaps of Russia [1-4]. Kefir is obtained from the fermenting activity of Kefir grains [5]. Traditionally, it is fermented in goatskin bags for 24 hours [6]. Kefir contains many ingredients that demonstrate biological activity, such as some probiotic bacteria and bioactive peptides [7] and onsets of activity varies according to the type of kefir and the time of fermentation [8]. It is self-carbonated fermented milk with a slightly acidic taste [1]. The kefir drink is produced from cow, goat, sheep [2], camel, buffalo or soy milk [9,10] that could be whole fat, low-fat, skimmed or fat-free milk [11]. This difference in the milk type and methods of fermentation affects the amount of grain produced, food composition and flavors of kefir [1]. Kefir grains are considered to be the most important component in the production of fermented kefir and can be reused again [12]. It contains many types of bacteria in addition to proteins and polysaccharides [13-15]. Although the kefir drink can be found in many countries, in Egypt the grains are not commercially available and are culturally donated from person to person. Partial sequencing of the gene encoding 16S rRNA was used for species identification [16,17]. Fermented milk produced by kefir grains contains yeast and lactobacilli [18-20]. Kefir has many applications in a variety of medical conditions such as; high blood pressure, allergy problems and coronary heart disease. Also, it strengthens the immune system and improves the digestive health.

Kefir antimicrobial activity is associated with the production of organic acids, peptides (bacteriocins), carbon dioxide, hydrogen peroxide, ethanol and diacetyl [17,21].

Review of Literatures Background

The history of Kefir is shrouded by legend, with the drink dating back hundreds of years. The word "kefir is derived from the Turkish word "keif" which means "Sense of comfort" [11] and the kefir drink its origins Returns to the Caucasian mountains of Russia, between the Black Sea and the Caspian Sea Today, it is still Made in Europe these days under many names, such as Kephir, Kiaphur, Kefer, Kepi and Kippi. [1-4]. The grains of Kefir, considered to be a gift from the prophet Mohammed [22], were passed from generation to generation among the Moslem tribes. These people considered the grains asource of family and tribal wealth, and keep the secret process of Kefir making [11]. Kefir is not product which a separate or cause to separate into curds or lumps. but is produced by the addition of Kefir grains to fresh milk [23]. The content was tied off in one corner of the leather bag where most of the grains were retained, and the Kefir separated from the grains by pouring the beverage off. This produced a foaming drink, creamy in consistency and texture, with an alcohol content of approximately 0.08 - 2.0%v/v) (Anfiteatros,2004). During the 24 h fermentation, the Kefir grains change the milk into a thick, astringent tasting beverage. During cold weather, the leather bag was placed in the sun during the day, or hung near a fireplace at night. It was also custom to hang the bag near a doorway,

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where by visitors would give the bag a gentle rock as they passed by (Koroleva, 1988) [22]. Kefir was also regularly subjected to a secondary fermentation, during which a mixture of fresh Kefir, fresh milk and the root of Snow Rose was poured into wooden barrels or clay crocks [24]. The container was then sealed airtight and the content fermented for some days. This produced a highly carbonated Beverage, with possibly a slightly higher alcohol content (Anfiteatros, 2004). Today, Kefir is still manufactured in [25].

It is also popular in Eastern European countries and is produced in small quantities in the former Czechoslovakia, Poland, Hungary, Finland, Sweden, Norway and Germany [24,26]. It is also available in the United States and is growing in popularity in Japan [1]. Kefir is refreshing, selfcarbonated fermented milk with a slightly acidic taste, yeasty. Favor and creamy consistency and when agitated, the beverage foams and fizzes. This led to kefir being named "the champagne of cultured dairy products". The history of Kefir is shrouded by legend, with the drink dating back many centuries. European countries and is produced in small quantities in Czekia, Poland, Hungary, Romania, Finland, Sweden and Germany. It is also available in the United States and is growing in popularity in Japan. The beverage is manufactured by fermenting milk with kefir grains, comprised microorganisms, polysaccharides and milk proteins. The microbial population of kefir grains primary includes lactic acid bacteria (LAB), namely lactococci and lactobacilli, yeasts, acetic bacteria and filamentous fungi. Kefir exhibits antimicrobial activity in vitro against some fungi and Gram-positive and Gram-negative bacteria. The exact cause of this inhibition is not known but now, is well known the ability of LAB to inhibit the growth of closely related bacteria. These inhibitions of pathogenic and spoilage microbes may be due to the production of organic acids, hydrogen peroxide, acetaldehyde, diacetyl, carbon dioxide or bacteriocins.

The microbiological and chemical composition of kefir indicates that it is a much more complex probiotic, as the large number of different bacteria and yeast found in it distinguishes it from other probiotic products. Since the yeasts and bacteria present in kefir grains have undergone a long association, the resultant microbial population exhibits many similar characteristics, making isolation and identification of individual species difficult. Many of these microorganisms are only now being identified by using advanced molecular, biological techniques. The study of kefir is made more difficult, because it appears that many different sources of kefir grains that are being used to produce kefir. The production of kefir depends on the synergistic interaction of the microflora in kefir grains. During the fermentation process, the yeasts and bacteria in kefir grains produce a variety of ingredients that give kefir its unique taste and texture. After fermentation, the finished kefir product contains many ingredients which proved to be bioactive. At least one exopolysaccharide-kefiran- has been identified in kefir, although others may be present. Many bacteria found in kefir have been shown to have proteinase activity, and a large number of bioactive peptides have been found in kefir [7]. KEFIR BEVERAGE Kefir is a refreshing, self-carbonated fermented milk with a slightly acidic taste [1], yeasty flavor and creamy consistency and when agitated, the beverage foams and fizzes [27,28]. This led to Kefir being named "the champagne of cultured dairy products" [11], The beverage is produced from cow, goat, sheep, camel, buffalo or soya milk [2,4], and the milk can be unpasteurized, pasteurized, whole fat, low-fat, skimmed or fat-free. The higher the fat content, the thicker and creamier the Kefir. Pasteurized milk is recommended since bacteria in raw milk may influence the microbial balance of the Kefir grains [11]. The nutritional composition and flavor of Kefir vary significantly and depend on a variety of factors, including the source and fat content of the milk composition of the grains, and fermentation conditions [1]. Kefir has a pH of [29], an ethanol content of 0.5 – 2.0% (v/v), a lactic acid content of 0.8 – 1.0% (m/v), a carbon dioxide content of 0.08 – 0.2% (v/v), and contains formic, succinic and propionic acids, as well as trace amounts of isoamyl alcohol, acetone and diacetyl [28]. Lactose is reduced [1] and β-galactosidase increases during fermentation [30]. There is also a small increase in proteolysis, leading to an increase in free amino acids [24].

Kefir Grains

Kefir grains play a natural starter culture role during the production of kefir and are recovered after the fermentation process by milk straining [17]. These grains are composed of microorganisms immobilized on a polysaccharide and protein matrix, where several species of bacteria and yeast coexist in symbiotic association [14,15]. In this ecosystem there is a relatively stable microorganism population, which interacts with and influences other members of the community. This population provides the synthesis of bioactive metabolites, which are essential for grain growth and microorganism inhibition, such as food pathogens and contaminants [15]. Kefir grains vary in size, from 0.3 to 3.0 cm in diameter (Figure 1), are characterized by an irregular, multilobular surface, united by a single central section, and their color varies from white to yellowish white. The grains are elastic and have a viscous and firm texture [14,19,20].

Fig 1: The physical appearance of Kefir grains. Although the kefir drink can be found in many countries, in Egypt the grains are not available commercially and are culturally donated from person to person. Kefir is obtained from the fermentative activity of Kefir grains [5]. The grains are insoluble in water and common solvents gelatinous, and irregular in size [23], varying from 0.3 – 3.5 cm in diameter [31]. They are white to yellow in colour and resemble small cauliflower florets.

Sheet-like structures and scroll-like forms of Kefir grains are easily distinguished from cauliflower-like forms. Kefir grains probably evolved through the curling of the sheet-like structures, with subsequent folding and refolding into a globular structure [26]. Kefir grains are comprised of a mass of actively growing and reproducing bacteria and yeasts [13], polysaccharides and other products of bacterial metabolism, together with milk protein [3]. When added to milk, the grains swell and form a jelly-like product. Microbial cells account for the major part of the grain together with autolysis products, curd proteins and carbohydrates such as Kefiran. Kefiran is water-soluble [32] and facilitates the formation of aggregates [4]. The grains are formed during

the process of Kefir making, and as far as is known, only from existing grains. The grains are initially very small but increase in size during fermentation as the microorganisms multiply and Kefiran accumulates [1]. Despite intensive research to produce Kefir grains from pure and mixed cultures, no successes have been reported [20]. This can be ascribed to the fact that very little is known about the mechanism of grain formation, and a combination of different factors may have an influence on the biomass increase of the Kefir grains. These factors include the renewal of the milk at regular intervals the cultivation temperature, grain washing, and the presence of essential nutrients in the correct concentration in the growth medium.

A variety of methods by which Kefir grains may be stored have been developed, and each method affects the activity of the grains differently. Grains stored in water can only be kept for 8-10 days, air-dried or lyophilized Kefir grains can be kept for 12-18 months, with no loss of activity (Marth & Yousef Garrote et al., 1997). Frozen grains stored at - 20 °C maintained microbial activity for 7 – 8 months, whereas refrigerated grains showed a decrease in activity after 10 days (Oberman & Libudzisz, 1998). Preservation of Kefir grains at-80°C has less of an effect on the microbial composition of the grains than preservation of the grains at -4° or -20 °C [31].

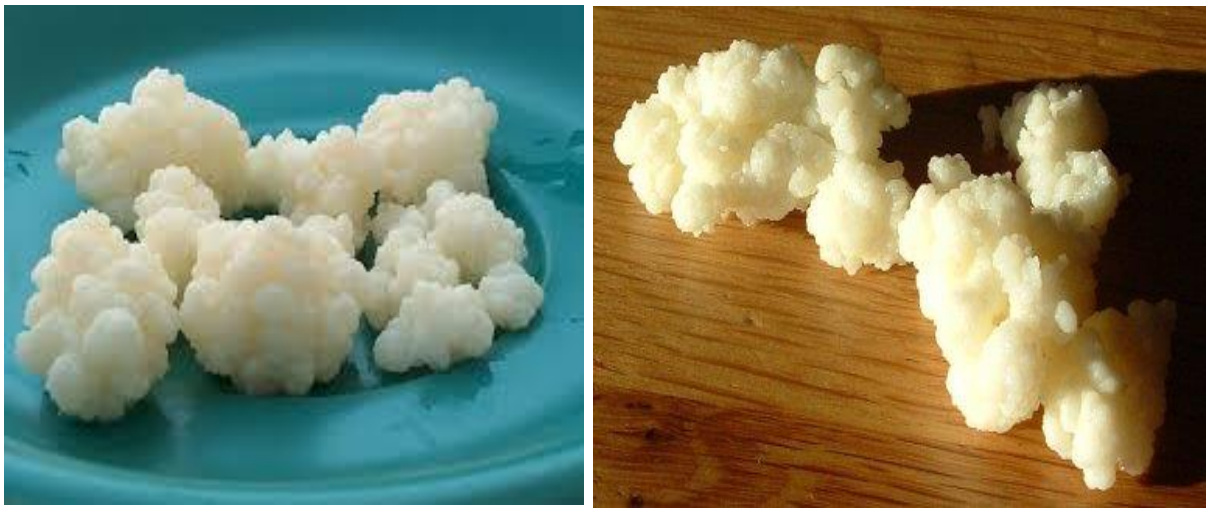


Figure 1: the kefir grains

Kefir has many applications in a variety of medical conditions such as high blood pressure, allergy problems and coronary heart disease. Also, it strengthens the immune system and improves the digestive health

The main objective of this study was to: -

1. investigate the antimicrobial activity of the fermented kefir in vitro against different pathogens.
2. Identification of microorganisms isolated from fermented kefir by partially sequencing of 16S rRNA gene
3. Evaluation of the protective ability of Kefir against carbon tetrachloride CCl4-induced thyroid toxicity in mice. evaluate its protective effect in mice.

Materials and Methods

Preparation and characterization of kefir

Kefir grains were collected from Cairo City, Egypt. They were: -

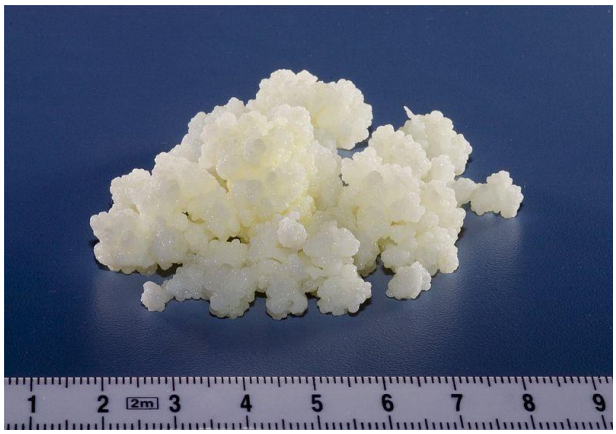
-varying in size (from 0.3 to 3.0 cm in diameter),

- irregular in shape

-and white to yellowish white in color.

-Also, the grains were flexible, softer in texture, viscous and insoluble in water and common solvents.

When milk was added, the grains swelled and produced a jellied called Kefiran.



Kefir has a pH of 4.2 – 4.6, an ethanol content of 0.5 – 2.0% (v/v), a lactic acid content of 0.8–1.0% (m/v), a carbon dioxide content of 0.08 – 0.2% (v/v). Kefiran was prepared by adding 100 g of kefir grains to 500 ml of purified milk at 25ºC in a dark place for 24 h - 48 h. Kefir grains were separated from the fermented milk by plastic sieve.

1-The antimicrobial activity of the fermented kefir:
Screening for antibacterial activity of the Kefir fermented milk was done using agar diffusion method against Gram-positive bacteria (*Staphylococcus aureus ATCC 44330* and *Bacillus subtilis)*, Gram-negative bacteria (*Escherichia coli* ATCC 5087, *Salmonella enteritidis* and *Pseudomonas aeruginosa)*, and yeast (*Candida albicans)*.

The Kefir fermented milk was filter sterilized using 0.45 um membrane filter and the indicator microorganisms were incubated overnight in brain heart infusion broth (Oxide) at 37°C. The antimicrobial activity was done based on seeding inoculation of each indicator microorganism in 20 ml Muller Hinton agar (Oxide), and then cups were prepared using Wassermann tubes with an external diameter of 5 mm. A fixed amount of 50 µl, 100 µl and 150 µl of tested kefir solution was distributed to each well. The plates were incubated for 24 h at 37°C. A positive control of antibiotic ampicillin (10 mg/ml) was also tested. Estimation of antimicrobial activities was done by measuring diameters of zone of inhibitions.

2-Evaluation of the protective ability of Kefir against carbon tetrachloride CCl4-induced thyroid toxicity in mice.

Animal grouping and treatment.

Three weeks old, clinically healthy, female Swiss albino mice (n=40) weighting 26–30 g was randomly divided into 4 groups (10 mice/group) after 7 days adaptation. They were housed in stainless-steel wire-mesh cages (four in a cage), at 24±2ºC temperature, 55% relative humidity and a 12 h light–dark cycle. The animals were provided a normal diet and tap water.

The groups were separately treated for as following:
Group I: animals were sham treated with 2 ml/kg distilled water through oral gavage, daily for 4 weeks; this group of animals served as the control.

Group II: animals were treated with 1.5 ml/kg body weight (b.w.) CCl4 dissolved in 1.5 ml corn oil through oral gavage, daily for 4 weeks.
Group III: animals were treated with 1.5 ml/kg b.w. CCl4+ 30 ml/kg b.w kefir through oral gavage, daily for 4 weeks.
Group IV: animals were treated with 30 ml/kg b.w. Kefir through oral gavage daily for 4 weeks.

Preparation of Fermented Kefir to feed animals.
The compound was prepared by washing the kefir grains with distilled water and raw milk, after that heated to 90 °C for 10 min in a water bath, then cooled to inoculation temperature (25 °C) and 10% active kefir grains added. The mixture was placed in a plastic container with screen cloth as a cover and incubated at room temperature for 24–48 hrs.

A plastic container is used because the acidity of fermented kefir may degrade metals such as aluminum and iron which could mix with the drink thereby causing harmful effects to the body.

After fermentation, kefir grains were sieved by filtration through a plastic sieve and washed for another process. Kefir drink was maintained at 4 ± 1ºC for 24 h and then used for microbiological and chemical analyses before feeding the animals in group III, kefir samples which were stored for more than 3 days were not used.

Animal treatment was continued for 4 weeks then the experiment was concluded, and animals were killed under anesthesia, blood samples were collected and thyroid.

Biochemical analysis:

Each blood sample was placed in dry clean centrifuge tube, and then centrifuged for 10 min at 3000 revolutions per minute (rpm) to separate the serum. Serum was carefully separated into clean dry Wasser man tubes by using a Pasteur pipette and used for determination of thyroid hormone tests; (T4) (biolab), (T3) (biolab) and (TSH) (biolab)] using standard techniques by manufactures.

Histopathological examination:
Thyroid:

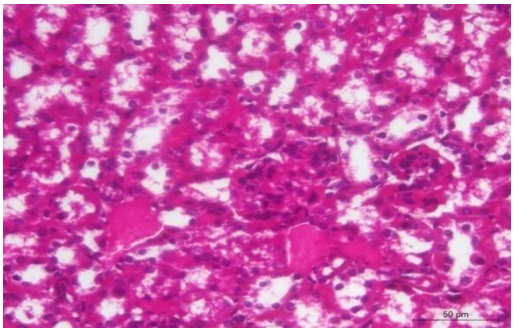
Thyroid nodules are lumps that occur in the thyroid gland. Thyroid nodules may be solid, cystic (fluid filled), or a combination of both and can develop in any location within the thyroid gland. At least 85% of thyroid nodules are benign thus thyroid cancer accounts for only a small percentage of all thyroid nodules. Thyroid nodules are generally not considered a serious condition and most often detected without producing any symptoms whatsoever. Thyroid nodules can produce symptoms and most commonly this is a lump or sensation of fullness in the neck. This page will tell you when we worry about thyroid nodules, and when we don't.

Thyroid nodules are most found when a doctor examines a patient's neck, feeling the thyroid gland. Sometimes thyroid nodules are found when a patient gets x-rays or scans of the neck for some other reason. Sometimes it is a screening x-ray or scan for carotid arteries or neck pain that shows nodules in the thyroid. Thyroid nodules that are large, develop in women with thin necks, or are present in the middle portion of the thyroid gland (called the isthmus) may be visible and discovered as a lump in the neck.

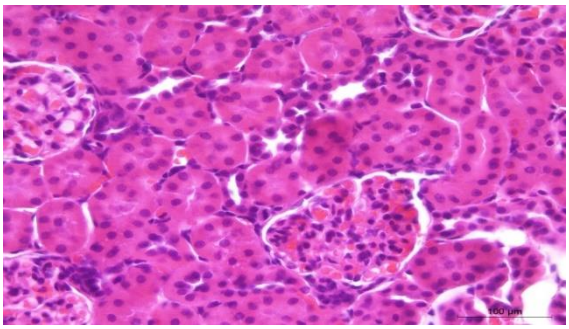
Histopathological examination methodology:
Tissue samples were collected from thyroid of all animals (Group I-IV). These samples were fixed in neutral formalin solution 10% for 72 hrs., after that fixed samples were processed and stained by Hematoxylin and Eosin.

Results

1-Control group:
In the control group, the thyroid was histologically normal without noticeable alterations. normal thyroid epithelium shows uniform nuclei and good and normal cytoplasm (Papanicolaou, ×100). (Fig. 1a)

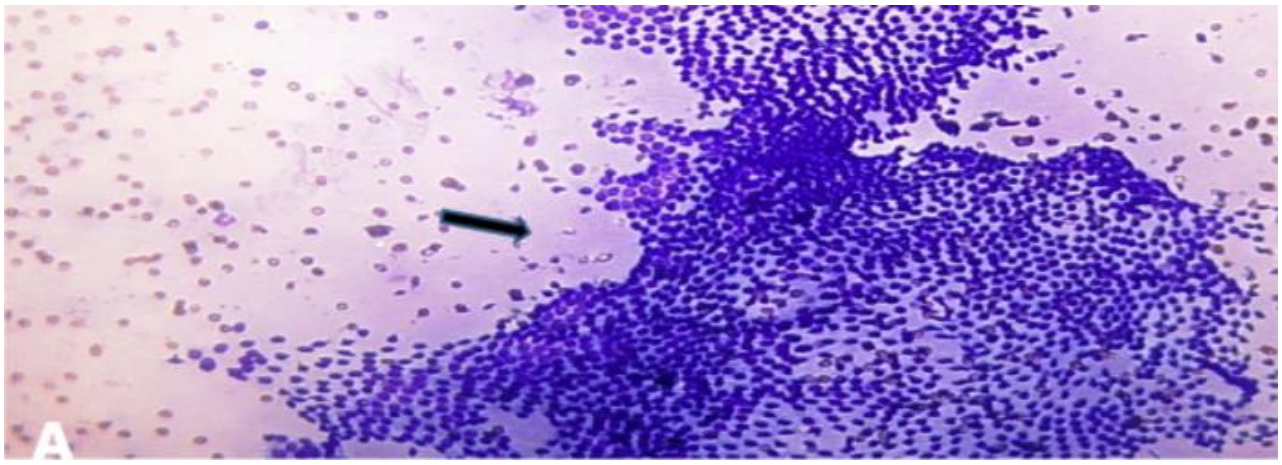


(A1)

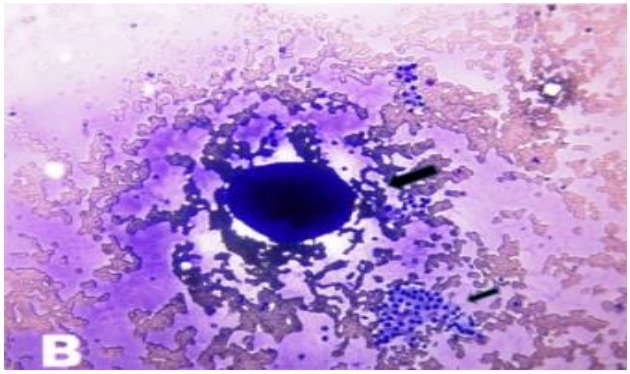


(B1)

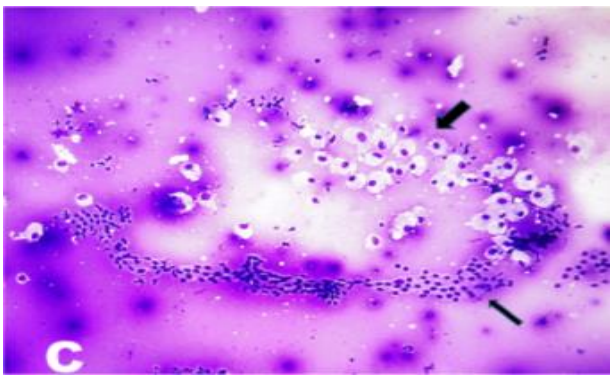
2-CCL4 group:



(A2)

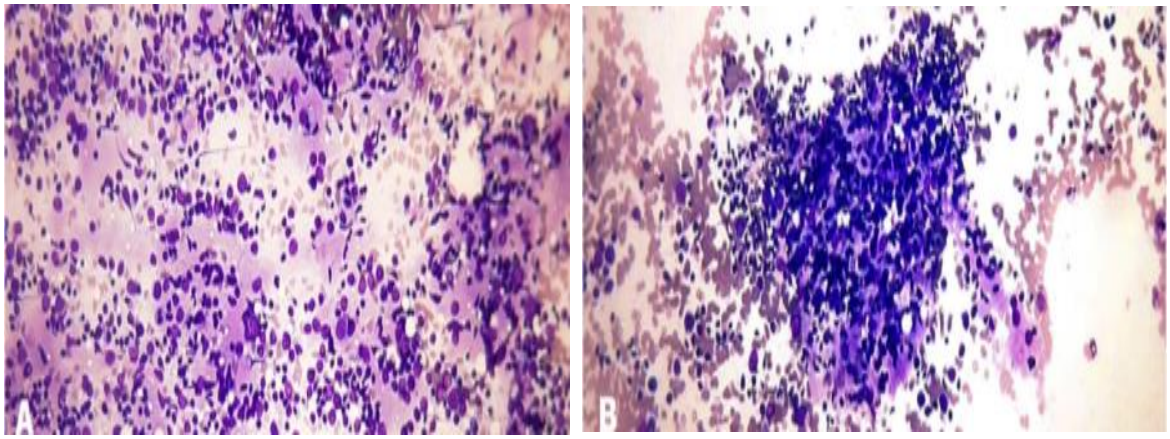


(B2)



(C3)

Figure 2. (a) malignant follicular nodule. Photomicrograph showing monolayer sheets of evenly spaced follicular cells having a honeycomb-like arrangement (arrow) (Smear, Giemsa, 400x magnification). (b) malignant follicular nodule. Photomicrograph showing globular mass of colloid with superimposed follicular cells (thick arrow) mixed with monolayer sheet of follicular cells (thin arrow) against the background of colloid and blood (Smear, Giemsa, 400x magnification). (c) malignant follicular nodule. Photomicrograph showing follicular cells arranged in sheets (honeycomb-like) (thin arrow) mixed with macrophages (thick arrow) against the background of colloid (Smear, Giemsa, 400x.



(A2.1) (B2.1)

Figure 2.1.(A) Lymphocytic (Hashimoto) thyroiditis. Photomicrograph showing polymorphous lymphoid population (Smear, Giemsa, 400x magnification). (b) Lymphocytic (Hashimoto) thyroiditis. Photomicrograph showing lymph histiocytic aggregates in lymphocytic (Hashimoto) thyroiditis (Smear, Giemsa, 400x magnification).

3-kifer group:

normal thyroids are referred to as colloid nodules and show loosely cohesive sheaths of follicular epithelium, colloid, blood, and rare macrophages. Colloid nodules are the most common cytology and contain an abundance of colloid with sparse follicular cells. There is considerable variation in the number of cells as well as the type and amount of colloid present (Fig.3).

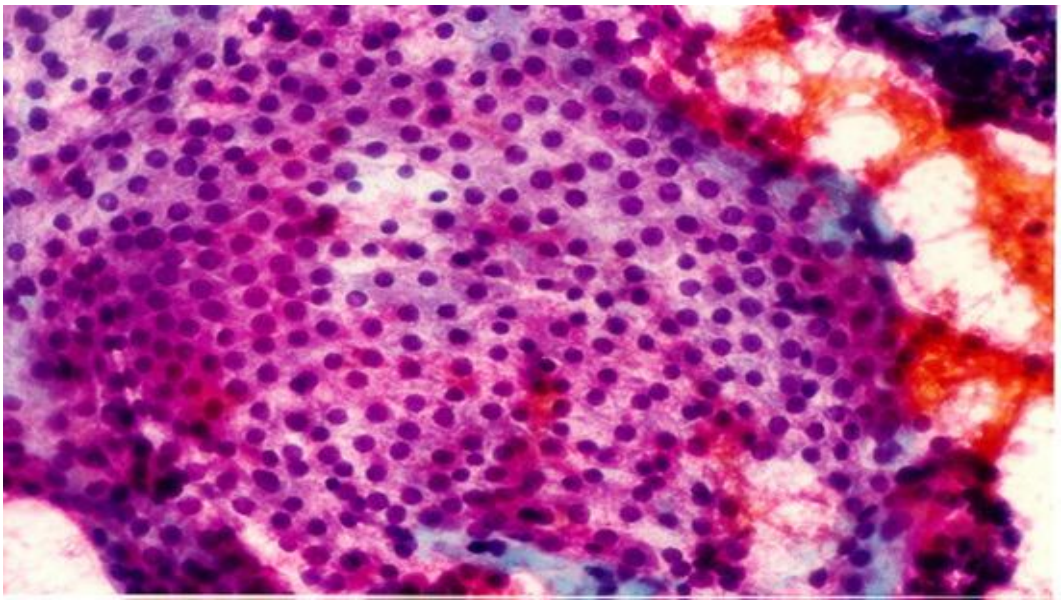


Figure 3: Colloid nodule. Sheath of normal thyroid epithelium shows uniform nuclei and pale cytoplasm (Papanicolaou, ×100).

Conclusion

In conclusion, our findings revealed that kefir has antimicrobial activity against pathogenic microorganisms and protective properties against CCl4-induced thyroid toxicity. These protective effects included anti-inflammatory effect and inhibition of CCl4 activity with improving of thyroid function. So, kefir may have the potential for clinical applications to the prevention and/or treatment of thyroid toxicity.

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