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The Potential Amoebicidal Effect of Some Milk forms as an Inhibitor for *Entamoeba histolytica* Viability Isolated from Diarrhea

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ABSTRACT

Milk includes numerous compounds such as lacto-peroxidase, lactoferrin, immunoglobulin G, secretory immunoglobulin A, and Lysozymes The invasive trophozoites of E. histolytica are capable to inhabit host tissues and provoke diseases such as intestinal colitis. The objective of the study was to compare different forms of milk's effect on E. histolytica trophopzoite by measuring protein concentration in vitro using a spectrophotometer and number of trophozoite by microscopy. For this purpose, E. histolytica trophozoites were exposed to several types of milk (human, sheep, cattle, goats, and formula) at concentrations of 6, 12, 18, and 24% for each type after 8, 16, and 24 hours of incubation. According to the findings, sheep and goat milk had lower total protein concentrations after 8 hours than other types of milk when compared to the control group. After 16 hours of incubation, raise the milk concentration to 24% leading to a reduction in the formula milk's total protein concentration to 5g/l. after 8 and 16 hours of incubation, sheep milk and cattle have significantly lower total protein concentrations (24% vs. 8%), respectively. The action of formula and human milk is increased by 24-hour incubation. Increase the trophozoite inhibition percentage compared to metronidazole in human milk, which was followed by sheep and cattle milk with respective values of 60.9%, 56.1%, and 46.3% respectively. Human milk, goat and formula milk had the greatest inhibitory activity after incubation 24hrs. at 24%.the extent of amoebicidal activity of milk was shown to depend on incubation period and concentration of milk.

Keywords- *Entamoebahistolytica*, human milk, oligosaccharides, Amoebicidal.

I. INTRODUCTION

The parasite *Entamoeba histolytica* is wide spread throughout the world. Amoebiasis, which is caused by parasite infection and accounts for more than 100,000 annual mortalities, is the third most common cause of death. Due to the parasite's cytotoxicity action, host cells are killed when it is present (1). The trophozoites and host cell make their initial contact at the membrane surface. The development of the disease depends heavily on the trophozoites' adhesion to the host cells, which is mediated by Gal/GalNAclectin. Cell adhesion molecules, which are also part of integral membrane proteins, also start cell-cell attachment(2). Human milk oligosaccharides (HMOs), a kind of complex carbohydrates found in large quantities in human milk, have been shown to recognized as essential, useful biomolecules for baby health. One of the most prevalent HMO components and the most predominate core structure is lacto-N-tetraose (LNT). Prebiotic property, antiadhesive antibacterial activity, antiviral action, and modification of intestinal epithelial cell response are only a few of the potential physiological effects of LNT that have been widely established (3,4). Both the amoebicidal effects of secretory immunoglobulin type A (sIgA) and the ability of human milk to kill amoebas have been documented. It is unclear,

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though, which milk ingredients are amoebicides (5). Since metronidazole is an efficient amoeba antibiotic, it is now the preferred medication for treating all types of amoebiasis. The amoeba parasite, however, has been linked to metronidazole medication resistance (6). Antiparasitic activity of milk from humans and different animals has been investigated by many authors. Bovine, goat and camel milks were the most investigated ones (7).The inhibitory activity of milk is related to the presence of several proteins such as lysozyme, lactoferrin and lactoperoxidase (8). It is necessary to obtain safe antiparasitic agents for human use from natural sources whether plant and animal sources.In spite of growing interest in the consumption of milk from various animals over the past years, there are limited comparative studies on the antimicrobial properties of the milk from different equine and ruminant animals. Therefore, this study aimed to compare the amoebicidal properties of milk from various source on *Entamoebahistolytica*viability in vitro.

II. MATERIALS AND METHOD

2.1 Isolation of human milk

Human milk was obtained from healthy volunteers recruited at the Pediatric Hospital and Azadi teaching hospital. after centrifugation, the lipid layer was removed and proteins were precipitated from the aqueous phase by the addition of ice-cold ethanol and subsequent centrifugation(9,10).

2.2 Source of animal milk

The animal milk was collected from different areas of Kirkuk city / Iraq. The hand milking method was used to collect the milk and transported directly by ice-cooled vials to the Hospital laboratory and kept in the refrigerator under 2-5 ℃ until use.Each type of milk was diluted at 6%,12%,18% and 24%.

2.3 Culture

Trophozoites of the E. histolyticaisolated from a humanpatient in Kirkuk City were cultured axenically in 8 ml of TYI-S-33 medium at 37 °C with a pH of 6.8 in 9 ml glass screw cap tubes then incubated and examined daily for amoeba growth for at least 5 days. In positive cultures the tubes were cooled for 5 minutes in an ice water bath, actively growing trophozoitesand sub cultured for amoeba maintains, after 48 hours (Log phase) trophozoites were obtained by cooling and centrifuging (300 g, 5 min) and then resuspending them in the suitable medium. 0.1 µl (2800 trophozoites) from this suspension was added to each treating experiment. Amoebas were treated with 6%,12%,18% and 24% of human milk, cattle milk, goat milk, sheep milk and formula milk. Milk proteins were detected using spectrophotometer. Direct microscopy was used to define the interaction of milk proteins (200µl) media and amoebas. Total Protein concentration was determined by biuret method and number of trophozoites after incubation 8hr.,16hr.,24hrs. was estimated.For compression controls wells were used (type of milks, trophozoite with media).The biuret method was used to determine the total protein concentration using this formula:-

A sample/A standard*C standard=C sample

In micro titer wells (2800) amoebaswere treated with doses of 6%, 12%, 14%, and 24% of formula milk, human milk, cattle milk, goat milk, and sheep milk and incubated for (8,16, 24 hours). The effect of different type of milk on amoebaes viability were detected by applying 0.4% trypan blue stain and counting the alive amoeba on hemocytometer using direct microscopy. For compression controls wells were used (trophozoite without treatment, trophozoite with metronidazole0.8 %). Each treatment was performed in three replicates. The effect of each milk type and inhibition rates were recorded using this formula (The effect percentage = No. of alive cells in each treatment /No.of alive cells in control x 100, Inhibitionrate =100- effective rate).

III. RESULTS

Comparing among different types of milk at different concentration in media contain Trophozoites of the E. histolytica after incubation at 37℃ for (8) hrs. The results appeared that all types of used milk had low total protein concentration compared to control group but sheep and goat milk were more significant compared to other types of milk as well as different concentrations were used in comparisons, and it had a noticeable impact compared to other kinds of milk. Compared to 6%, there was a 24% difference between sheep's milk and cow's milk, which was significant. This shows the maximum level of antimicrobial activity seen in goat and sheep milk, and when milk concentration rose to 12%, 18%, and 24%, total protein concentration declined and amebacidial activity was increased as table (1).

Table 1: Total protein concentrationin culture media treated with different types and concentrations of milk after 8 hrs. incubation

Milk form	Total protein concentration (g/l)						
	Culture media with troph.				Milk control mean	Culture media control mean	p-value
	6%	12%	18%	24%			
Human	19	17	16.5	14	28.75	30	0.042*
Sheep	36	24	12	10 *	103.75	30	0.001**

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Cattle	43	39	20	8 *	73.3	30	0.034*
Goat	78	70	52	45	120.5	30	0.008**
Formula	27	19	12	9	40.5	30	0.050 *

When elevated the time preceding incubation from 8-hours to 16-hour, the concentration of total protein showed significant decreased in all types of milk with exception Goat milk which recorded non-significant decrease compared to control group. Furthermore, the results demonstrated that the total protein concentration was lowered by formula milk (p 0.05), followed by cattle and sheep milk at 24 percent compared to 6% (p <0.05) as table (2).

Table 2: Total protein concentration in culture media treated with different types and concentrations of milk after 16 hrs. incubation

Milk form	Total protein concentration (g/l)						
	6%	12%	18%	24%	Milk control mean	Culture media control mean	p-value
Human	15	11	9	8	28.75	30	0.041 *
Sheep	34	27	14	10 *	103.75	30	0.021*
Cattle	37	33	17	9*	73.3	30	0.029*
Goat	62	58	33	25	120.5	30	0.064 ns
Formula	20	13	6	5**	40.5	30	0.013**

The results of table (3) revealed Incubation period for 24 hrs. significantly reduced total protein concentration at all concentration compared to control group. It's interesting to observe that the amoebicidal impact was noticeable in each case and referred to the incubation period's effect on raising the amoebicidal activity of all types of milk.

Table (3): Total protein concentration in culture media treated with different types and concentrations of milk after 24 hrs. incubation

Milk form	Total protein concentration (g/l)						
	6%	2%	18%	24%	Milk control mean	Culture media control mean	p-value
Human	10	7	5	1 *	28.75	30	0.021*
Sheep	31	19	13	5 *	103.75	30	0.004**
Cattle	29	15	9	7 *	73.3	30	0.013**
Goats	51	47	20	11 *	120.5	30	0.013**
Formula	14	9	4	3**	40.5	30	0.001**

The results of Table(4) revealed after incubation for (8)hrs. inhibitory effects of various concentration of milk in media contain trophozoite. The growth of Entamoeba histolytica trophozoites was significantly inhibited by human milk at 6%, with an inhibition percentage of 54.4% compared to metronidazole and other types of milk. Additionally, the number of trophozoites decreased as milk concentration increased. The milk from cattle at 24% had the highest amoebacdial activity it ,recorded inhibition percentage 74.7%. However, in the samples of goat milk, the number of trophozoites significantly decreased from 2405 to 1280.finally, formula milk had no significant inhibitory impact compared to control group (p>0.05).

Table 4: Effect of different types of milk on trophozoite viability after8hrs. incubation

Milk form	Number of trophozoites treated with different milk concentrations					
	6% (In %)	16%(In %)	18%(In %)	24%(In %)	p-value	p-value with control
Human	1270(54.3)	860 (69.1)	645(76.8)	412 (85.2)	0.14	0.044 *
Sheep	2630 (5.4)	1800(35.3)	1400(49.6)	1050(62.2)	0.075	0.051 *
Cattle	1630(41.4)	1000(64.0)	800 (71.2)	675 (75.7)	0.044*	0.048 *

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Goat	2405(13.5)	2000(28.1)	600 (78.4)	1280(53.9)	0.05*	0.026 *
Formula	2206(20.6)	1800(35.3)	1600(42.4)	1260(54.6)	0.08	0.077 ns
Negative control only trophozoite (No.2780) Positive control 0.8 % metronidazole (No.1800, In% 35.3)						

The result of table(5) revealed raising the incubation period to16 hours. Elevate the inhibition percentage of trophozoite compared to metronidazole in human milk followed by cattle and sheep milk which recorded 60.9%,56.1 %, and 46.3% moreover when increasing the concentration of types of milk to 24 %. the inhibition percentage elevated the inhibitory activity of cattle milk to 90.2%

Table 5: Effect of different types of milk on trophozoite viability after 16hrs.incubation

Milk form	Number of trophozoites treated with different milk concentrations				
	6% (In %)	16%(In %)	18%(In %)	24%(In %)	p-value
Human	800 (60.9)	600(70.7)	500(75.6)	300(85.3)	0.048*
Sheep	1100(46.3)	800(60.9)	600(70.7)	500(75.6)	0.027*
Cattle	900(56.1)	800(60.9)	400(80.4)	200(90.2)	0.04*
Goat	1500(26.8)	1200(41.5)	1100(46.3)	900(56.1)	0.088
Formula	1500(26.8)	1400(31.7)	1300(36.5)	1100(46.3)	0.07
Negative control only trophozoite (No. 2050).					
Positive control 0.8 % metronidazole (No. 700,In% 65.9)					

* Mean $p<0.05$

According to the results study , following a 24-hour incubation period, human milk showed the highest amoebicidal activity, with an inhibitory percentage of 92.0% at 24% of milk and a 71.9% inhibition percentage at 6% compared to other forms of milk. Further raising the goat milk to 24% results in a 1000 trophozoites reduction, indicating that the milk's amoebic action was concentration- and incubation-dependent as table(6).

Table 6: Effect of different types of milk on trophozoite viability after 24hrs.incubation

Milk form	Number of trophozoites treated different milk concentrations					
	6% (In %)	16% (In %)	18%(In %)	24%(In %)	p-value	p-value with control
Human	550 (71.9)	450 (77.0)	300(84.7)	150(92.3)	0.014*	0.019**
Sheep	800(56.2)	700 (64.3)	550(71.9)	450(77.0)	0.08	0.051 *
Cattle	750(61.7)	550 (71.9)	450(77.0)	350(82.1)	0.075	0.040 *
Goat	1500(23.5)	800 (56.2)	650(66.8)	500(74.5)	0.008**	0.038 *
Formula	700(64.3)	650 (66.8)	550(71.9)	500(74.4)	0.054*	0.002 **
Negative control only trophozoite (No. 1960).						
Positive control 0.8 % metronidazole (No. 400, In % 79.6)						

IV. DISCUSSION

An increasing amount of accumulated epidemiologic literature, using improved methodology and statistics, demonstrates the protective benefits of human milk for infants. An ever-increasing number of bioactive factors are being identified and measured in breast milk during the period of lactation. These factors can play multiple roles in the infant: nutritional, metabolic, modulatory and anti-inflammatory (11). The result of study when treated trophozoite with different types of milk at 6% following 8hrs. incubation, the lowest total protein conc. was observed for goat and sheep milk compared to control group and the highest the amoebicidal impact. Goat milk is known for anti-inflammatory properties and serves as an important source of angiotensin converting enzyme (ACE) which functions as an antihypertensive peptide and helps alleviate infections caused by pathogenic microbes (12). when using different concentrations of different types of milk on the growth on trophozite of E. histolytica in vitro. As it recorded each of cattles milk and sheep milk concentration of 6% therapeutic efficiency was (41.4%, 56.1%, 61.5%), respectively, after(8,16, 48hr) incubation

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Clarify(13). Increasing the in incubation period to 16hrs. elevate amoebicidal activity of cattle and human milk especially at 25% and observed non inhibitory effect of goat milk, thus reffered to impact of incubation milk on inhibitory effect. Another study recorded cattle milk showed a slightly lower amoebicidal activity than human milk (14).

The current finding showed among some milk forms, samples of cattle followed by human and sheep milk , the number of trophozoite was decreased significantly when increase the concentration from 6% to 24% for 16.hrs incubation. Also, the most affective animal milk after human milk was seen in cattle milk. That is incompatible with study who reported that at 10 mg/ml, the average Human oligosaccharide milk (HMO) concentration in mature human milk, HMO detached more than 80 % of the trophozoites within the first 30 min of exposure. Light microscopic observations showed that detached trophozoites rounded up and floated in clusters (15). While other study concluded that among few in literatures to determine oxidants and antioxidants content of breast milk and animal milk (Buffalo, cow, sheep, goat) and increased antioxidants with decreased oxidants levels in: sheep milk, goat milk, cow milk, buffalo milk and human milk respectively. On the other hand, the study showed decreased antioxidant and increased oxidants levels in human breast milk levels with increasing age of women and that might be decreased infants' health(16). Also, another study appeared mature human milk have significant lethal effect on *E. histolytica* (17).

The trophozoite after incubation at 37℃ for (24) hrs, all types of used milk appeaed significant decrease in total protein conc. and increase inhibition percent after incubatin24hrs.compared to 8hrs. and 16hrs. Furthermore, goat, human and formula milk after showed Higher amoebidal activity compared to metronidazole and other types of milk(18).

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