STUDY OF THE ANTIBACTERIAL ACTIVITY OF *Artemisia herba-alba* FOR USE AS A BIOCONSERVATIVE IN RAW COW'S MILK

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Abstract: The objective of our work is to highlight the antibacterial activity of the different extracts of *Artemisia herba-alba*: oil, ethanolic and aqueous extracts, this plant was harvested in the region of El Kantra wilaya of Biskra, and the use of their oil in the process of bioconservation of raw cow's milk. The highest extraction yield was found in ethanol extract with a value of 15.64%. Thus, the quantitative study showed a richness of polyphenol and flavonoid extracts, particularly ethanol extract. On the other hand, the aromatogram and liquid dilutions methods show a strong antibacterial activity of the various extracts against *S. aureus*, *E. coli* and *Salmonella* sp. bacteria, while *P. aeruginosa* has proved resistant. The results of microbiological analyses carried out on the milk reveal the effectiveness of the oil against psychrotrophic bacteria, which reduces the quality of the milk and leads to the appearance of serious defects, particularly flavors, which make the products unsafe to eat.

Keywords: *Artemisia herba-alba*; antibacterial activity; bioconservation; milk.

INTRODUCTION

It is recognized in the scientific world that products of natural origin are an important source of therapeutic agents for microbial diseases that cause a large number of deaths in terms of mortality rates, such that antimicrobial compounds excreted from plants can inhibit bacterial growth through different mechanisms [34]. The increase in bacterial resistance to antibiotics is a health problem that is mainly related to repeated and massive use of these products has led to a loss of effectiveness of antibiotics.

Medicinal plants are a numerically large group of important economic plants. They offer an alternative to drugs, they contain active components resulting from secondary metabolite produced from nutrient metabolism that are used by humans in their therapeutic arsenal [18]. These active components are distinguished by several categories such as alkaloids, flavonoids, tannins, essential oils and other compounds [14].

The food industries use substances of the food additive type to ensure the preservation of their products, these substances can be synthetic (chemical nature), which causes damage to the health of consumers with the character of accumulation and over time [30].

The current consumer trend towards a more natural diet has led to renewed scientific interest in these substances [13]. For two decades, studies have been conducted on the development of new applications and the exploitation of the natural properties of essential oils in the food field. The antimicrobial effects of different species of herbs and spices have long been known and used to increase the shelf life of foods [33]. For example, essential oils, currently used as food flavourings, are also known to have antimicrobial activities and could therefore be used as food preservatives [19], in this context, the present research work aims to investigate new natural substances of plant origin with antibacterial activity by evaluating the antibacterial effect of the various extracts of *Artemisia herba alba* (essential oils, ethanolic extract, aqueous extract) on pathogenic bacteria and aims at the possibility of using essential oils as a natural preservative agent in raw cow's milk. the choice of this plant is based on the traditional use of *A. herba alba* to keep the milk fresh, for this reason we tried in this study to scientifically prove the beneficial effect of this plant on a scientific basis

MATERIAL AND METHODS

Plant material

Harvesting the plant

The plant of *A herba alba* was harvested in January 2019 (flowering stage), the plant in its natural habitat located in the region of El Kantara (wilaya of Biskra).

Drying the plant.

The aerial part of the specie *A. herba alba* was air-dried, away from light, for 15 days and then stored in clean paper bags until the time of extraction.

Microbial equipment

The strains used to detect the antibacterial activity of the extracts are part of four genera of microorganisms, are referential strains (ATCC), these are: *Staphylococcus aureus* (ATCC 25293), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC27853) and *Salmonella* sp.

Extraction of essential oils

The extraction of essential oil from the aerial part of *Artemisia herba alba* was carried out by hydrodistillation in a clevenger-type [19].

After weighing 100 g of dry vegetable matter placed in a pyrex glass flask, with 1000 mL of distilled water the whole thing is brought to a boil in a flask heater for 3 hours. The essential oil of *A. herba alba* will be recovered in a clean and sterile bottle.

Conservation of essential oils

Essential oils are stored in a closed glass bottle in a refrigerator (4°C) away from light [2].

Essential oil yield

According to the Afnor standard (1986) [3], the yield of essential oil (Rd) is Rd= M'/M.100

- Rd : Essential oil yield expressed as a percentage (%);
- M': Mass of the essential oil obtained in grams (g);
- M: Mass of dry vegetable matter used in grams (g).

Preparation of *A. herba alba* extracts

Ethanolic crude extract

In this work we have tried to extract the total phenolic compounds. It is a solid-liquid extraction the principle consists in dissolving the active principle inside the solid and dragging it out using a solvent. Most authors suggest that solvent entry is through an osmotic mechanism and solute exit by dialysis or diffusion [6]. Ethanol has been used as a solvent.

Aqueous extract

Preparation of aqueous extracts according to the method cited by Ghedadba [16]. An aqueous maceration was also carried out on 100 g of powder with 1000 mL of distilled water and placed under agitation for 24 h after filtration the extract was lyophilized.

Quantitative study of the metabolites of *A. herba alba* extracts

Determination of total phenols

The determination of total polyphenols was performed with the Folin Ciocalteau colorimetric reagent using the Singleton and Rossi method [8]. The Folin Ciocalteau reagent is a yellow acid that is based on the alkaline reduction of the mixture of phosphotungestic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) of the folin reagent by the oxidizable groups of phenolic compounds, leading to the formation of blue colored reduction products absorbed in the visible at 765 nm [10].

Determination of flavonoids

The quantification of flavonoids in the extract was performed by an aluminum trichloride (AlCl3) colorimetric method adapted by Bahorun *et al.* (1996) [6]. In the presence of AlCl3, flavonoids, absorbance at 430nm, concentration (µg/mL). dihydroxylated on rings A or B and free hydroxyl groups in position C-3 and C-5 or ketone group in position C-4, are capable of forming a stable yellowish acid complex absorbed in the visible at 430 nm [4].

Evaluation of antibacterial activity

Two different methods are used to evaluate the antibacterial effect of essential oils and extracts: the method of disk diffusion in agar medium and the liquid micro-dilution method (MIC and MBC).

The essential oil, ethanolic and aqueous extract have been prepared using DMSO 10% which is inert on bacterial activity a concentration of 100mg/mL of each extract allows the detection of antibacterial activity.

The results of disk diffusion method are expressed by the diameter of the inhibition zone and can be symbolized by signs based on the sensitivity of the strains in mm [28]:

- not sensitive (-) or resistant: diameter < 8 mm;
- sensitive (+): diameter between 9 and 14 mm;
- very sensitive (++): diameter between 15 and 19mm;
- extremely sensitive (++++): diameter > 20 mm.

Performed in 96-well plates with a rounded bottom, the MIC corresponding to the minimum concentration of extract for which no observed visible growth to the naked eye was determined. In these wells, was estimated the MBC corresponding to the minimum bactericidal concentration.

Use of oils in the bio-conservation process

Sampling procedure. The cown's milk samples were collected in a sterile 1L vial with an aseptic manner and immediately subjected to a series of microbiological and analyses

The collected raw milk was distributed in sterile vials at the rate of 100 mL. Three different concentrations of *A. herba alba* essential oil: 12.5 mg/mL, 25 mg/mL and 6.25 mg/mL prepared using DMSO 10%. were added to the milk and homogenized. A negative control was prepared without the addition of essential oil. The individual samples are then stored in the refrigerator at 4°C for 13 days. The analyses were performed every 3 days (day 0, day 3, day 7, day 10 and day 13) [19].

Analysis of raw milk during storage

Microbiological analysis of raw milk during storage

The microbiological control was carried out according to the techniques described by Guiraud (2003) [19].

Preparation of decimal dilutions

After stirring the vials, 1 mL of the milk is taken from a propette with the sterile tip and added to 9 mL of the physiological water contained in a test tube. Then we shake on vortex and we proceed to successive dilutions up to10^{-5} [19].

Total mesophilic flora count (FTAM)

Principle. The enumeration was performed on Plate Count Agar (PCA). Its count reflects the general microbiological quality of a natural product and makes it possible to monitor its evolution. The number of "total" germs may give an indication of the freshness or decomposition state of the product [19].

Mode of operation. 1. Prepare the sterile petri dishes; 2. Seed cans per 1 mL of each dilution (10^{-1} to 10^{-5}); 3. Add 15 to 20 mL of PCA agar medium; 4. The mixture is homogenized by circular movements. After solidification, the boxes are turned over and incubated at 37°C for 24 hours, the operation is carried out twice. After this period, the lenticular colonies are counted [5].

Enumeration of psychotrophic microorganisms

Principle. In order to quantify this population, the germ count was performed on Plate Count Agar (PCA) with dilutions.

Mode of operation. 1. Prepare the sterile petri dishes; 2. Seeding the cans with 1 mL of each dilution (10^{-1} to 10^{-5}); 3. Add 15 to 20 mL of PCA agar medium; 4. The mixture is homogenized by circular movements. After solidification, the boxes are turned

over and incubated at 7°C for 13 days, the operation is carried out twice [27].

Determination of the UFC/mL number

Colonies were counted for each dilution to determine the number of UFC/mL using the following formula: UFC/mL= number of colonies ×1/Ve ×1/D (where: Ve - being the sowing volume; D -being the dilution taken into account).

Physico-chemical analyses

In order to get an idea on the quality of the collected milk, several physico-chemical parameters are evaluated. 1. *pH:* the pH is obtained using a pH meter. The value is read directly from the pH meter after the electrode is immersed in milk; 2. *Titratable acidity:* to 10 mL of milk, a drop of 1% phenol phthalein is added and then the acidity is monitored by adding the 0.1 N sodium hydroxide solution drop by drop using a tap burette until a persistent pink colour is obtained. The result is calculated according to the quantity of soda added. The change in colour determines the volume of soda [14]; 3. *The acidity of the milk* can be expressed by the following formula: AT=V x 10(D°) (where: AT - titratable acidity; V - the volume in mL corresponds to the drop of the burette).

The results of the analysis of the statistical data obtained in this work were carried out by the "Graph Pad Prism 7" software.

RESULTS

Extraction and Quantitative study

The essential oil of *A. herba alba* was obtained by hydrodistillation. It is a viscous, yellowish liquid with a strong odor. The ethanolic extract obtained is dark greenish in colour, unlike the aqueous extract, which is light brownish in colour. These yield of the *A. herba* alba extracts are represented in the following Table 1.

Determination of total polyphenols

Total polyphenols were determined by the Folin-Ciocalteu method. Gallic acid was used as the standard. The absorbance was read in a wavelength of 765 nm. The amount of polyphenols was reported in milligrams of gallic acid equivalent per milligram of dry weight of the extract (mg EAG/mg dw) (Fig. 1).

From the calibration curve, the concentrations of total polyphenols of *A. herba alba* extract are presented in the following table 2:

In this work the total polyphenol content shows that the ethanol extract represents the richest extract with (143.25 mg EAG/g dw) while the aqueous extract represents a low polyphenol content (6.58 mg EAG/g dw). The same result found by Abedalah (2015) [1].

Determination of flavonoids

Flavonoid determination was performed using the aluminum trichloride ($AlCl_3$) method, quercetin was used as a standard. The amount of flavonoids was reported in milligrams of quercetin equivalent per milligram of dry weight of the extract (mg EQ/mg dw) (Fig. 2).

From the calibration curve, the flavonoid concentrations of *A. herba alba* extract are as follows (Table3):

It was found in this study that the ethanolic extract is the richest extract in flavonoids with 16.77 mg EQ / mg dw and the aqueous extract represents a low content with 5.54 mg EQ / mg dw.

Study of the antimicrobial power of *A. herba alba* extracts

Aromatogram test

The antibacterial power of the extracts was estimated in terms of the diameter of the inhibition zone around the discs containing the extracts to be tested for *Escherichia coli* bacteria and *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella* sp [17]. The results obtained to determine the antibacterial activity of the extracts are presented in the following Table 4.

According to the results of Table 4, the studied bacteria are extremely sensitive for the positive control (gentamycin).

The results obtained show that white mugwort oil has an effect on *S. aureus* and *E. coli* bacteria, *Salmonella* sp. On the other hand, we have noticed that the ethanolic extract of *A. herba alba* is the most active extract on *S. aureus* and *E. coli* bacteria. Although the aqueous extract has an effect on *S. aureus* bacteria only. While the bacterium *P. aeruginosa* was not affected by the action of the three extracts.

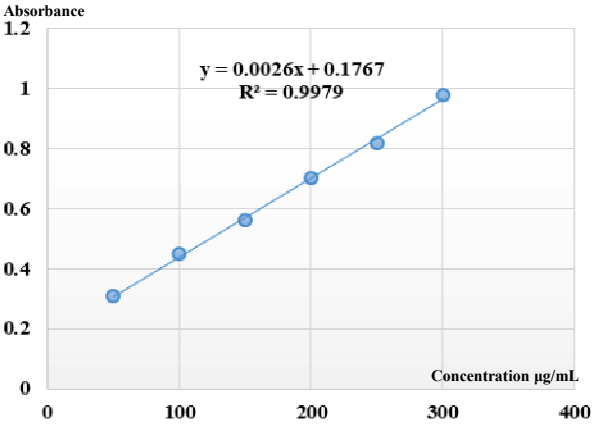

Figure 1. Calibration curve for gallic acid

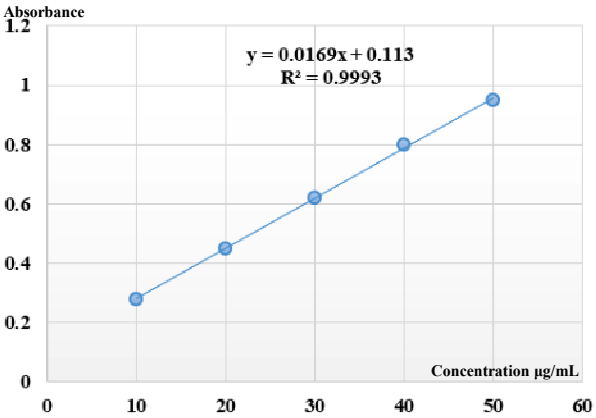

Figure 2. Quercetin calibration curves

Table 1. Yield of *A. herba alba* extracts

	yield in %		
The plant	**ethanolic extract**	**aqueous extract**	**essential oil**
A. herba alba	15.64%±0.01	12.54%±0.02	1.23%±0.002

Table 2. Determination of total polyphenols

Extracts	**Polyphenols**
Ethanolic	143.25±0.09
Aqueous	6.58±0.005

Table 3. Determination of total flavonoids

Extracts	**Flavonoides**
Ethanolic	16.77±0.02
Aqueous	5.54±0.11

Table 4. Diameters of the inhibition zones of the essential oil of *Artemisia herba alba*

	Zone d'inhibition (mm)			
Strain	**Essential oil**	**Ethanolic extract**	**Aqueous extract**	**Gentamycin**
S. aureus	16.48±0.11	20.31±0.49	14.47±0.002	33.37±0.59
E. coli	13.90±0.76	19.56±0.09	-	26.47±1.09
P. aeruginosa	-	-	-	32.53±0.22
***Salmonella* sp.**	13.94±0.51	-	-	27.66±2.22

Table 5. MIC of the raw extracts of *A. herba alba*

	Zone d'inhibition (mm)		
Strain	**Oil**	**Ethanolic extract**	**Aqueous extract**
S. aureus	25	50	100
E. coli	12.5	50	100
***Salmonella* sp.**	6.25	-	-

Table 6. MBC of the raw extracts *of A. herba alba*

	MBC (mg/mL)		
Strain	**Oil**	**Ethanolic extract**	**Aqueous extract**
S. aureus	50	50	-
E. coli	25	100	-
***Salmonella* sp.**	25	/-	-

The essential oil of *A. herba alba* has an important effect on *E. coli* and *Salmonella sp*., which are inhibited with diameters ranging from 13.90 mm and 13.94 mm, respectively. The *S. aureus* bacterium has an inhibition zone of 16.48 mm and is very sensitive to this oil.

The results obtained from aqueous extracts of *A. herba alba* showed that Gram-negative bacterial strains showed no zone of inhibition, which is reflected in the resistance of the strains to the action of the extracts. While the *S. aureus* Gram+ bacteria sensitive to this extract with a 14.47 mm diameter.

On the other hand, *S. aureus* and *E. coli* bacteria are sensitive to ethanol extracts from plants tested with a diameter of 20.31 mm and 19.56 mm respectively.

Liquid dilution method

Determination of the minimum inhibitory concentration (MIC)

After the incubation period, the appearance of a clear appearance in the microplate wells indicates inhibition of bacterial growth, and the other wells show a cloudy appearance indicates bacterial growth. The CMI results are shown in the following Table 5.

The extracts of *A. herba alba* are found to be active against the bacterial strains tested but with different degrees of activity, which resulted in the difference in MICs. There was a significant effect of *A. herba alba* oil on pathogenic strains studied, we enrolled the MIC 25 mg/mL 12.5 mg/mL, 6.25 mg/mL for *S. aureus* and *E. coli* and *salmonella*.sp, respectively. The two extracts (aqueous and ethanolic) have the same MIC for the strains *S. aureus, E. coli* with a value of 50 mg/mL and 100 mg/mL, respectively.

Determination of minimum bactericidal concentration

The results obtained on MBC of the raw extracts *of A. herba alba.*are presented in the following Table 6.

It was found in this study that the effective dose of the essential oil is 50 mg / mL against the bacterium *S. aureus* on the other hand 25 mg / mL against *E. coli*. On the contrary the ethanolic extract requires a dose of 100 mg / mL for inhibits the growth of the *E-coli* bacteria. The aqueous extract has no effect whatever the dose on any bacteria. The MBC and MIC values confirm the antibacterial activity of *A. herba alba* extracts observed with respect to these strains tested.

Bio-conservation results

The monitoring of the total flora, psychotropic bacteria, pH and titratable acidity of raw milk after storage at 4°C in the refrigerator, and after treatment with different oil concentrations showed the following results presented in figures 3, 4, 5 and 6:

Total flora

The results obtained are presented in Figure 3 and showed that in untreated milk (control), the number of total flora increased between days D0 and D3, then gradually decreased during the rest of the storage days. However, milk treated with the three concentrations of *A. herba alba* essential oil revealed that the number of total flora between days D0 and D3 decreased compared to the control. While the total flora has completely disappeared in D7 to D13 (Fig. 3).

The essential oil of *A. herba alba* has demonstrated its effectiveness in killing the population of the total flora (bactericidal effect) in raw milk preserved for 13 days. Our study confirms the possibility use of *A. herba alba* essential oil as a natural antibacterial agent in raw milk.

Psychrotrophic Bacteria

The results of monitoring the survival of psychotrophic bacteria over time are presented in Figure 4. While after treatment with oil, a slight increase in psychotrophs was observed during all storage days compared to the control (Fig. 4).

Based on the obtained results, raw milk stored at 4°C for 13 days demonstrated the efficacy of *A.herba alba* essential oil as a natural preservative that inhibited the growth of psychrotrophic microorganisms.

pH

The results of monitoring the pH evolution over time, showed that the pH of the treated raw milk gradually decreased over time (Figure 5). However, the pH of the treated milk with the different concentrations of the oil showed an increase from D0 to D3, then decreaseed in the rest of the storage days. The control pH was found to be more acidic than that of the treated milk (Fig. 5).

Acidity

Figure 6 shows the results of monitoring the evolution of titratable acidity over time. The control values represented a gradual increase during all days of storage. On the other hand, the treated milk with the oil showed stability on days D0 to D3, then an increase in the rest of the storage days. The acidity values of treated milk are lower than those of untreated milk (Fig. 6).

DISCUSSION

In our work the lowest yield is represented by the essential oil compared to the two extracts with a value of 1.23%, in comparison with other research studies, this rate is considered higher than that of essential oil extracted from the same species, harvested in the Matmata mountain range in Tunisia (0.65%) [5].

In fact, the best solvent for extracting phenolic compounds by the maceration method is ethanol with a yield of 15.64%, while distilled water is found to be low with a yield of 12.54%, in Morocco, correlatively to a previous study, our yield is equal to that obtained

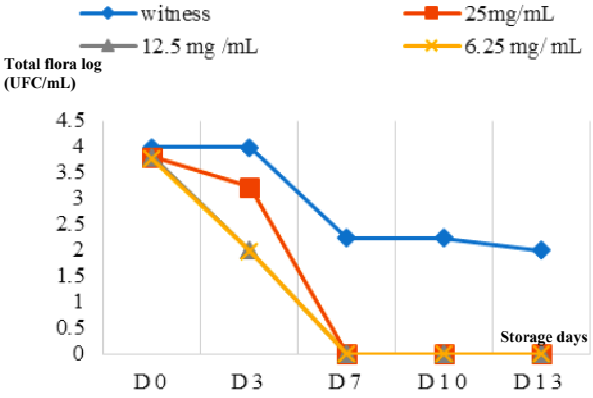

Figure 3. Monitoring of the survival of the total flora over time

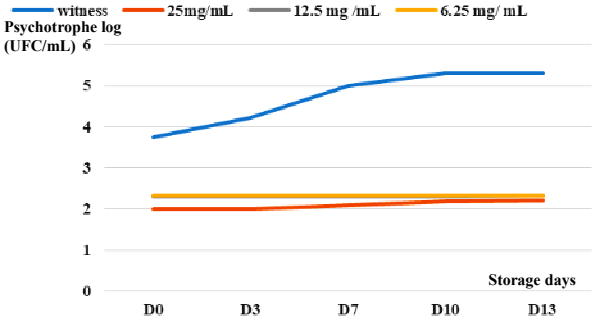

Figure 4. Monitoring the survival of psychotrophic bacteria over time

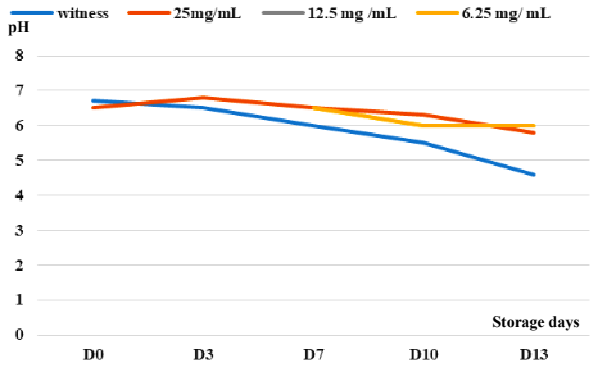

Figure 5. Monitoring of the pH evolution over time

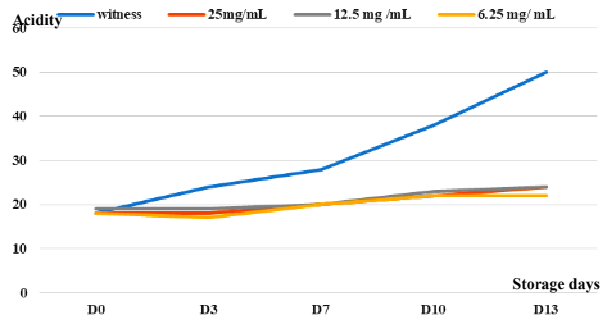

Figure 6. Monitoring of the evolution of acidity over time

in the Guerçif region in June (1.23%) [7] but higher than the yields obtained in September (0.56%) [12].

Generally speaking, the EO yields obtained are in the range 0.3%-1.6% cited by Lattaoui (1989) [23]. Work carried out by Benmensour (2001) [8] on the *A. herba alba* plant in different regions of Algeria has shown that EO yields vary from one station to another and from one harvest period to another. It increases from 0.16 to 0.32% for samples collected in June and from 0.47 to 0.97% for those collected in December. The high yields in December compared to those in June can be explained by the advanced stage of development and flowering of the plant and therefore an increased synthesis in EO the same result was found and explained by Mighri *et al.* (2011) [26]. Thus the difference in yield of essential oils from one station to another in the same country and from one country to another is based on two factors: climate change and degree of aridity [15].

Climate changes (rainfall and temperature) which are related to biomass density, and altitude in (m) different from one station to another so this parameter plays a positive role in better EO yield as reported by Rolet (1930) and Bellakhdar *et al*. (1984), and to their lower degree of aridity which implies a greater development of the plant and therefore a better yield.

These variations may be due to abiotic factors, such as the specific climate of the regions, the origin of the samples, geographical factors such as altitude, soil type and the harvesting season [11].

The content of phenolic extracts and total flavonoids of *A. herba-alba* in the extracts varies according to the complexity of these compounds, the variety of plants (differentchimiotypes), the difference in the harvest period and the region and the geographical conditions, the environmental factors, solvent extraction and the analytical method used [31].

Concerning the antibacterial activity of the essential oils of *A. herba alba*, our results are superior to Kahlouche (2014) [20] and Kheyar *et al* (2014) [22] study on the same plant studied with a diameter of 13.66 mm for *S. aureus* and 7.33 mm for *E. coli* on the other hand, the bacterium *Pseudomonas aeruginosa* has no inhibitory zones. This specific antibacterial activity of essential oils could be explained by the bioactive components of EO (synergistic effect between the components), the work carried out by Mighri *et al* (2009) [25] on essential oils of *A .herba alba* showed that the chemical composition was studied using both GC and GC / MS hair techniques. β-thujone, α-thujone, α-thujone / β-thujone and 1,8-cineole / camphor / α-thujone / β-thujone were respectively the main components of these types of oil. They act in particular at the membrane and cytoplasm level, and in some cases completely modify the morphology of the cells [9]. The difference in sensitivity to extracts can be attributed to the chemical nature of the crude extracts tested and to the bacterial strain [21].

Concerning the conservation of raw milk by the use of *A. herba alba* which is found effective in this study is explained by the antibacterial capacity developed by essential oils which results in the reduction of the zone of bacterial growth, this work is similar to previous work by Selim (2011) [32], who confirmed the antibacterial efficacy of *Thymus* essential oil against a population of foodborne pathogens.

At the end the titratable acidity and pH values of control and treated milk depend, depending on the content of casein, mineral salts and ions, the hygienic milking conditions, the total microbial flora and its metabolic activity [35]. Several research studies have focused on essential oils extracted from aromatic plants [24]. The various published results indicate that they have several biological properties.

The results obtained showed that the pH and acidity were influenced by the contribution of essential oils. Also, the presence of essential oils led to a significant decrease in microbial flora in raw cow's milk compared to the control [29]. Indeed, an inhibition of undesirable germs was observed in flavoured milk from the very first days.

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