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Isolation and Identification of Pathogenic Bacteria from Upper Respiratory Tract Infection in calves and Study the Antibacterial Activity of Propolis Extracts on these isolates

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ABSTRACT

This study aimed to isolation and diagnosis of most important bacteria related to upper respiratory tract infection in Calves, and propolis sample was collected for chemical quantification by HPLC technique and testing the antibacterial activity of alcohol and aqueous extract of Propolis sample against these isolates. The bacterial isolates were obtained by nasal and pharyngeal swabs, in laboratory were cultured on different media (differential and selective). The identification and diagnosis of these isolates were confirmed using the Vitek-2 system. The result revealed many isolates were *Staphylococcus aureus*, *Escherichia coli*, *Pasteurella multocida*, *Mannheimia haemolytica* and *Streptococcus agalactia*, all these clinical bacterial isolates were tested by propolis extracts and with different antibacterial drugs. The Iraqi Propolis samples obtained from Baghdad (2022 October) were used in this study. The concentrations of propolis extracts used in this experiment were 1, 2, 3, 4, and 5 mg/ ml. The bactericidal effects of the well and disc diffusion methods were investigated. The findings demonstrated that the efficacy of antibiotic against various bacterial isolates varied. The result of disk and wells diffusion agar (alcohol and aqueous extract) revealed that *Staph. aureus* was higher sensitive to all extracts of propolis than other type of bacteria followed by other type of bacteria. The results also showed that antibacterial effect of propolis extracts were increased when the concentration of propolis extract increased to all type of bacteria. There was no statistically significant difference between inhibitory ranges of Gram positive bacteria and Gram negative bacteria of Propolis samples.

Keywords: Upper Respiratory Tract, Propolis Extracts, bacteria , calves

Introduction:

Bovine respiratory disease (BRD) is considered the major cause of economic losses in dairy and beef cattle production due to its high morbidity and mortality rates (Hilton 2014), especially in less technology farms, such as family farms. BRD is the second major cause of losses in calf raising (Panciera & Confer 2010). Opportunistic bacteria are factors for the development of BRD (Holman *et al*. 2015). Pasteurella multocida, Mannheimia haemolytica and Mycoplasma bovis, are the major bacterial pathogens of BRD (Griffin *et al*. 2010).

Spreading and increasing antimicrobial resistance rates make physicins to search new approaches, Propolis is a non-toxic beehive product that is used for honeycomb construction and restoration (Olegario 2019). Propolis is derived from the Greek 'pro', which means 'in front,' and 'polis,' which means 'town' or 'city,' and bees use it to protect their hives from other insects. (Fawzy, *et al*., 2016) Honeybees use propolis, a resinous material, to seal fissures, smooth walls, and maintain constant moisture and temperature in the hive throughout the year. It's also effective against bacteria, fungi, and invading larvae (Al-Otaibi,2019). Propolis has been shown to have other biological actions, such as antibacterial properties. (Santos *et al.*, 2002), antifungal (Silici *et al*,. 2005), antiviral (Amoros *et al.,* 1992), antitumor (Akao *et al.*, 2003), immunomodulation and anti-inflammatory activities (Hu *et al.*,2005). Many studies have shown that propolis has antibacterial activity against bacteria such as Enterococcus spp., Escherichia coli, and Staphylococcus aureus. according to many reports, Propolis has been shown to be effective against Gram-positive bacteria but ineffective against Gram-negative bacteria, (Bueno-Silva, *et al.,* 2013). Propolis' widespread use in modern medicine has heightened interest in its chemical makeup. Many investigations have shown that the observed effects could be due to the complex ingredients' synergistic activity (Xuan, et al., 2011). Propolis has a wide range of pharmacological effects depending on where it was harvested. 50 percent plant resins, 30 percent waxes, 10% essential and aromatic oils, 5% pollens, and 5% other organic elements make up raw propolis. Propolis is made from the resins of poplars, conifers, birch, pine, alder, willow, and palm trees, according to reports. (Hu *et al.,*2005).

- The aims of this study were to isolate the most important bacteria related to respiratory disease in Upper Respiratory Tract Infection of healthy and dairy calves with clinical signs of BRD and chemical analysis of propolis by HPLC technique as well as assessment the bioactivity of alcoholic and aqueous extract of propolis against different type of bacteria in vitro by using two methods: disc and well diffusion agar.

Material and Methods.

- Clinical sample collection and microbiology identification.

Nasal and upper respiratory tract samples (n= 200) were collected after antisepsis from calves with signs of upper respiratory tract disease, and cultured to Brain Heart Infusion medium and stored at -4°C until further analysis. Plates were incubated in aerobiosis at 37°C for 24-48 days. After that, 10µL of this suspension were seeded on 5% sheep blood agar and incubated for 48h at 37°C. The obtained colonies were examined by gram stained and observed for hemolysis production. The colonies identification was performed for biochemical tests, The diagnosis was confirmed using the Vitek-2 system (Levinson, 2016)

and identified by clinical pathology Lab. at the University of Diyala, College of Veterinary Medicine, Diyala, Iraq.

Propolis collection:

Three samples of propolis were gathered from honeybee (Apis mellifera L.) colonies in Baghdad and diyala Province (Iraq), were collected at three different times during a year (January, July and September). Since preliminary HPLC analysis of the three samples revealed similar profiles, a single one (fifty grams) were selected for chemical quantification and biological testing. and stored in a dry area at 4°C until the process was complete.

- Ethanolic extract of propolis.

Propolis samples were gathered from honeybee colonies in Iraq's Baghdad Province. Propolis (fifteen grams) was collected for chemical quantification and biological testing. and stored in a dry area at 4°C until the process was complete. The propolis was broken into small pieces, blended to a fine powder, and extracted in a magnetic stirrer at room temperature for 48 hours with 70% ethanol (1:10 w/v). Whatman No.4 filter paper was used to filter the ethanolic extract solution. then concentrated in a rotary evaporator to yield the crude extract in paste form, which was then stored in a dry, dark location. The propolis extract was diluted in dimethyl sulfoxide (DMSO) to test for antibacterial activity. In this experiment, the doses employed were 1,2,3,4, and 5 mg/ml. (Mirzoeva *et al.*, 1997).

- Aqueous extraction of propolis:

In a dark brown container, 15 grams of propolis were mixed with 100 cc of double D.W. and left for 7 to 14 days at room temperature in a dark spot. The container was shaken twice or three times per day and placed in a warm, dark location. Whatman No.4 was used to filter the liquid. The extract was weighed after the water was evaporated in an oven at 45°C..and kept in a dark, clean container for later use. Distilled water was used to dissolve water or aqueous extract, which was then sterilized by filtering (using Millipore 0.45 filter paper) and diluted to 1,2,3,4, and 5 mg/ml. (Mirzoeva *et al.*, 1997).

Conditions analysis:

The investgation was conducted in the Departement of Environment and water laboratories of the ministry of science and technology .and according to the method presented by (Mradu, 2012) using a high- performance liguid chromatography device HPLC model (sykamn-) of german origin. Where the carrier phase was used : methanol distelled water: acetic acid (75:13:2) the separation column was(C18-ODS (25 cm×4.6mm) to separate the phenols and the ultraviolet detector was used: UV-360 nm, where the flow velocity of the carrier phase was :1ml/min, the compounds were used. The following standard caffeic acid, cinnamic acid, ferulic acid, p-coumaric acid and qurcetine, the concentration of the phenolic substances that were calculated according to the fpllowing equation:

$$\text{Compound concentration} = \frac{\text{Solution concentration Sample}}{\text{standad solution sapace}} x \frac{\text{Dilution factor}}{\text{sample weight}}$$

Determination of total phenolic compounds.

The total amount of phenolic compounds was determined in the ethanolic extract with a standard Folin -Ciocalteu reagent . The reaction mixture contained 100 µl of the extract, and

500 µl of the Folin-Ciocalteu reagent (Merck, Germany) and 1.5 ml of 20% sodium carbonate. The sample was then mixed on a vortex mixer and diluted with distilled water to the final volume of 10 ml. After 2 h reaction, the absorbance at 765 nm was determined and used to estimate the phenolic content using the calibration curve made with gallic acid (Sigma-Aldrich, Germany). The total amount of phenolic compounds was expressed in mg gallic acid equivalent (GAE) per g dry weight (Laouini and Ouahrani 2017).

Determination of total flavonoid content

The total flavonoid content of crude extract was determined by the aluminum chloride colorimetric method. In brief, 50 µL of crude extract (1 mg/mL ethanol) were made up to 1 mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% NaNO2 solution; 0.3 mL of 10% AlCl3 solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution were added, and the final volume of the mixture was brought to 10 mL with double-distilled water. The mixture was allowed to stand for 15 min, and absorbance was measured at 510 nm. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg rutin equivalent per g dry weight (habibatni *et al.,* 2017) .

-Antibacterial properties of extracts.

Well and disc diffusion methods were used to test the antibacterial effects (Bauer et al., 1966). The minimal inhibitory concentration was determined using the disc diffusion method. The bacteria were injected into Mueller Hinton broth and incubated at 37 C for 3-6 hours. These bacterial broths had 1.5 10^8 CFU/mL They were then soaked in sterile swabs. By streaking over the surface of the agar media, bacteria were injected on the surfaces of Petri dishes containing Muller Hinton Agar. Different concentrations of two extracts were impregnated into 5 mm diameter discs. The discs were then placed at well-spaced intervals on the surface of the agar plates in an aseptic manner The control was a blank disc impregnated with DMSO. The plates were incubated for 24 hours at 37 degrees Celsius. The existence of antibacterial action was indicated by clear inhibition zones around the discs. All antimicrobial activity data were averaged over three replicates, and the zones of inhibition were quantified in millimeters. (Karaman *et al*., 2003).

The agar well diffusion method was used to test the antimicrobial activity of propolis extract (Olurinola, 1996). Antimicrobial susceptibility was assessed on solid agar media in petri plates using the agar well diffusion method. Swabs were dipped in a bacterial suspension containing 1.5 10^8 CFU/mL and inoculated on Nutrient agar plate surfaces (NA Using sterile glass-made pipettes connected to a vacuum pump, wells (10 mm diameter and around 2 cm apart) were created in each of these plates. Propolis extract stock solutions were produced at concentrations of 1,2, 3,4, and 5 mg/ml. About 100 µl of propolis extract was injected into the wells using a sterile syringe and left to diffuse for 2 hours at room temperature. For 24 hours, the plates were kept at 37 degrees Celsius. The diameter of the inhibitory zone was calculated as the smallest distance (mm) between the sample's outside margin and the microbial growth's starting point. The experiment was repeated three times in triplicate, with readings obtained in three different fixed directions for each replication and the average values reported. (El-Deeb *et al*., 2020).

-Antibacterial sensitivity test.

The Kirby-Bauer disc diffusion method was used to test antibacterial sensitivity using Mueller-Hinton agar. The sensitivity of the bacterial isolates to each antimicrobial drug was assessed after incubation at 37 C° for 24 hours (Bauer *et al*., 1966) and the data were

interpreted using interpretive criteria published by National Committee for Clinical Laboratory Standards recommendations (NCCLS, 2020).

Statistical analysis:

The acquired data was subjected to statistical analysis using ANOVA utilizing the Sigma Sat for Windows application. In all analyses statistical significance was taken to be indicated by $p < 0.05$. Values are reported as mean and standard error (mean ± SE) (Joda, 2008).

Results:

- Bacterial isolation:

bacterial isolates were obtained 70 (35 %) isolates from about 200 samples. In which the gram-positive bacteria were the most prevalent isolated bacteria 47 (67.14 %). as *Staphylococcus aureus* 27 (57.44 %), *Streptococcus agalactia* 20 (42.55%), and In lower prevalence were 23 (32.8%) as Gram negative bacteria in which *E. coli* were 18 (78.26%), *Pasteurella multocida,* 3 (13%). *Mannheimia haemolytica* 2 (8.6%).

Total compound of propolis.

The results of the high-performance liquid chromatography examination of the propolis sample revealed numerous chemical materials with substantial concentrations, as shown in table-1.

Table (1) showing the results of the quantitative and qualitative analysis of the propolis sample.

No	Name	Con-
1	Total phenolic content (mg Gallic / gm)	27.5
2	Total flavonoid content (mg Rutin / gm)	10.5
3	Total glycoside content %	4.5
4	Total alkaloid content %	13.6
5	Total tannin content %	5.0
6	Total saponins content %	0.63
7	Caffeic acid (µg / gm)	52.6
8	p-Coumaric acid (µg / gm)	66.9
9	Ferulic acid (µg / gm)	18.9
10	Quercetin (µg / gm)	42.6
11	Cinnamic acid (µg / gm)	32.0

Our research revealed that the recommended HPLC technology successfully separated the standards components in the propolis samples (Figures 1). The HPLC fingerprints show the presence of cinnamic acid (2.50 min), ferulic acid (3.90 min), quinine (5.88 min), caffeic acid (6.90 min), and p-coumaric acid (8.05 min).

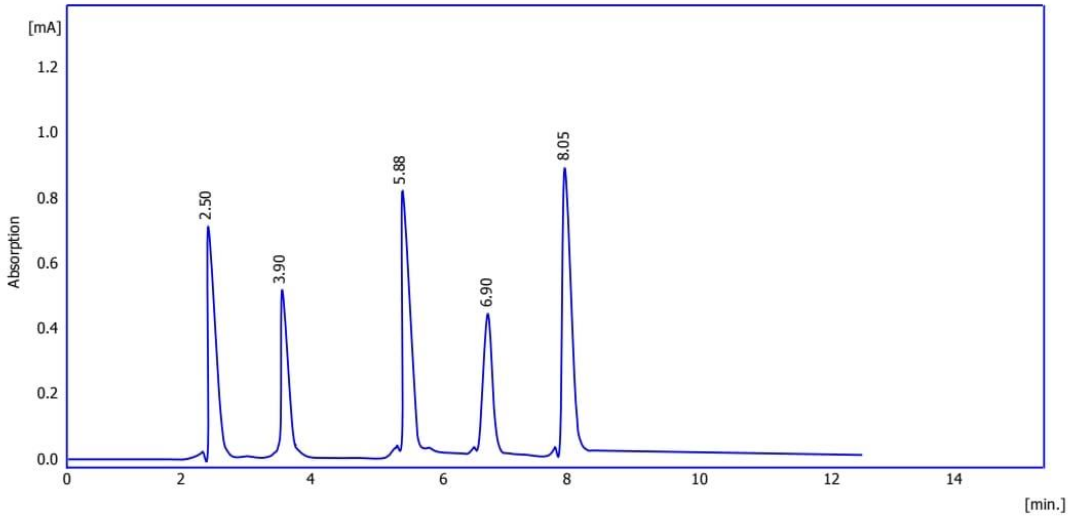
12/03/2023 01:40 م Chromatogram F:\ sample .PRM Page 1 of 1

Chromatography Laboratory
HPLC

Sample Info:

Sample ID	: sample	Amount	0
Sample	: sample	ISTD Amount	0
Inj. Volume [mL]	: 0.1	Dilution	1

Autostop	: 20.00 min	External Start	: Start - Restart, Down
Detector 1	: Detector 3	Range 1	: Bipolar, 2000 mAU, 10 Samp. per Sec.
Subtraction Chromatogram	: (None)	Matching	: No Change



Result chromatography Table (Uncal - F:\ sample))

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.50	12564.08	785.08	20.25	20.58	0.20	
2	3.90	10665.99	566.25	16.22	16.85	0.15	
3	5.88	14589.98	800.25	22.98	22.65	0.22	
4	6.90	9526.44	430.26	13.25	13.25	0.10	
5	8.05	18956.23	900.12	25.25	25.98	0.25	
	Total	66302.15	3481.59	100.00	100.00		

1- cinnamic acid. 2-Ferulic acid 3- qurcetine 4-caffeic acid 5- p-Coumaric acid.

Figure -1- showed the standards components in the propolis samples which separated by HPLC technology .

- Alcohol extracts of propolis:

The results revealed that alcohol extracts of propolis of disk diffusion agar have potent antimicrobial effect against all the isolated bacteria which were studied. The results showed that antibacterial effect was increased when the concentration of propolis extract increased to all type of bacteria. The *Staph. aureus* was higher sensitive to alcohol extracts of propolis (in which the zone of inhibition was 9.8 mm, 11.17 mm, 17.19 mm, 19.15 mm, and 21.09 mm) at concentration 1,2,3,4 and 5 mg/ml respectively, followed by *E. coli*, *Streptococcus agalactia*, *Mannheimia haemolytica* and *Pasteurella multocida* bacteria .

Alcohol extract of propolis exhibited significant difference ($P \leq 0.05$) between Staph. aureus, E. coli, and *Pasteurella multocida* bacteria at 3,4 and 5 mg/ml in contrast to other concentrations (1 and 2) mg/ml, according to the results of the disc method. There was a significant difference in inhibition between Streptococcus agalactia and *Mannheimia haemolytica* at 5 mg/ml and other concentrations (1,2,3, and 4) mg/ml. As in (table -2-).

Table -2- the diameter of the inhibitory zone (in mm) of Propolis alcohol extract against isolated pathogenic bacteria as determined by the disc technique

Concen. / Bacteria	1 mg/ml	2 mg/ml	3 mg/ml	4 mg/ml	5 mg/ml
Pasteurella multocida	7.12± 0.16 aA	9.15± 0.13 dA	9.19± 1.15 dAC	11.11± 1.12 bA	13.12± 1.15 cA
E. coli.	9.02± 1.13 aAB	10.17± 1.12 bA	15.19 ± 0.15 eB	17.15 ± 1.12 dB	19.19 ± 0.14 cB
Mannheimia haemolytica	8.13 ± 1.21 aB	9. 2 ± 0.12 abA	10.02 ± 0.15 bC	11.15± 1.31 cbA	14.01 ± 1.11 dA
Streptococcus agalactia	9.1 ± 1.21 aAB	9.9 ± 0.12 aA	10.07 ± 0.15 acC	11.18± 1.31 cA	13.05 ± 1.11 bB
Staph. aureus	9.8± 1.18 aB	11.17± 1.13 cA	17.19 ± 0.19 bB	19.15 ± 0.19 dC	21.09 ± 0.16 eC

Values are M ±MSE .Small letters show significance between concentration; Capital letters revealed the significance between bacteria, was at $P< 0.05$

The result of well diffusion agar of alcohol propolis extracts revealed that *Staph. aureus* was higher sensitive (10.05± 1.18, 11.17± 1.19, 12.6 ± 0.19, 17.15 ± 0.15, 22.07 ± 1.14 mm) at concentration 1,2,3,4 and 5 mg/ml respectively than other type of bacteria followed by *Streptococcus agalactia, Mannheimia haemolytica ., E. coli* and *Pasteurella multocida* bacteria, also the results of well method of these extracts show high significant difference ($P \leq 0.05$) between *Pasteurella multocida*, and other type of bacteria, at various concentration, (1,2,3,4 and 5) mg/ ml. Also The results of well method revealed that alcohol extract of propolis showed high significant difference ($P \leq 0.05$) between these concentration 3,4 and 5 mg / ml in comparison with other concentration (1 and 2) mg/ml. When the concentration of extract was increased to 4 and 5 mg, however, the effect on all types of bacteria was increased. (table -3-).

Table -3- The diameter of the inhibitory zone (in mm) of Propolis alcohol extract against isolated pathogenic bacteria as determined by the wells method.

Concenc. / Bacteria	1 mg/ml	2 mg/ml	3 mg/ml	4 mg/ml	5 mg/ml
Pasteurella multocida	5.12± 0.16 aA	6.15± 0.13 aA	9.18± 1.18 bA	10.12± 1.16 bA	13.12± 1.13 cA
Streptococcus agalactia	9.5± 1.17 aB	10.17± 1.15 aB	12.5 ± 0.15 bB	16.15 ± 1.19 cB	20.14 ± 0.13 dB
Mannheimia haemolytica .	9.2 ± 1.21 aB	12.02 ± 0.13 bB	12.5 ± 0.15 bB	15.7± 1.35 cB	16.4 ± 1.11 cC

E. coli.	9.16 ± 1.21 aB	11.0.2 ± 0.13 bB	11.5 ± 0.15 bB	15.4± 1.35 cB	16.11 ± 1.11 cC
Staph. aureus	10.05± 1.18 aB	11.17± 1.19 abB	12.6 ± 0.19 bB	17.15 ± 0.15 cB	22.07 ± 1.14 dD

Values are M ±MSE .Small letters show significance between concentration; Capital letters revealed the significance between bacteria, was at P< 0.05.

- Aqueous extract of propolis:

The current study showed that aqueous extracts of propolis of disk diffusion agar have antimicrobial effect against all the microorganisms studied. The result showed that antibacterial effect was increased to all type of selected bacteria, when the concentration of propolis extract increased. The *Staph. aureus* was higher sensitive to Aqueous extracts of propolis (in which the zone of inhibition was 9.05 mm, 10.17 mm, 14.19 mm, 14.15 mm, and 19.17 mm) at concentration 1,2,3,4 and 5 mg/ml respectively than other type of bacteria, followed by *Streptococcus agalactia, E. coli*, *Pasteurella multocida* and *Mannheimia haemolytica*.(Table - 4-)

Table-4- shows the diameter of the inhibitory zone (in mm) of Aqueous Propolis Extract against isolated pathogenic bacteria using the disc method.

Concent. / Bacteria	1 mg/ml	2 mg/ml	3 mg/ml	4 mg/ml	5 mg/ml
Bacillus Pasteurella multocida	6.11 ± 1.31 aAB	9. 1 ± 0.12 bB	10.2 ± 0.17 bB	11.18± 1.31 bAB	14.02 ± 1.21 cAB
E. coli.	7.3± 1.13 aAB	9.18± 1.17 aAB	9.19 ± 0.18 aAB	12.15 ± 1.12 bAB	15.18 ± 0.13 cAB
Proteus Mannheimia haemolytica	5.12± 0.15 aA	5.15± 0.12 aA	7.19± 1.15 aA	10.11± 1.14 bA	13.12± 1.15 cA
Streptococcus agalactia	8.1 ± 1.11 aB	9.4 ± 0.19 aB	10.09 ± 0.13 aB	13.18± 1.71 bB	16.15 ± 1.21 cB
Staph. aureus	9.05± 1.18 aB	10.17± 1.13 aB	14.19 ± 0.19 bC	14.15 ± 0.19 bB	19.17 ± 0.15 cD

Values are M ±MSE .Small letters show significance between concentration; Capital letters revealed the significance between bacteria, was at P< 0.05

The present study also revealed that aqueous extracts of propolis of wells diffusion agar have antimicrobial effect against all the bacterial studied. The *Streptococcus agalactia* showed higher sensitive to aqueous extracts of propolis (in which the zone of inhibition was 12.4 mm, 12.14mm, 17.17 mm, 20.15 mm, and 25.14 mm) at concentration 1,2,3,4 and 5 mg/ml respectively than other type of bacteria, followed by *Staph. aureus, Pasteurella multocida, E. coli*, and *Mannheimia haemolytica*..(Table - 5-).

Table -5- The diameter of inhibitory zone (in mm) of aqueous extract of Propolis against isolated pathogenic bacteria by wells method.

Concenc. / Bacteria	1 mg/ml	2 mg/ml	3 mg/ml	4 mg/ml	5 mg/ml
Bacillus Pasteurella multocida	11.16 ± 0.22 aB	13.0.2 ± 0.13 aB	13.7 ± 0.14 aC	17.6± 0.35 bC	20.12 ± 0.31 cC
Streptococcus agalactia	12.4± 1.6 aB	12.14± 1.13 aB	17.17 ± 0.12 bB	20.15 ± 1.11 cB	25.14 ± 1.13 dB
Proteus Mannheimia haemolytica.	6.16 ± 0.21 aA	9.0.2 ± 0.19 bC	11.8 ± 0.13 bC	15.8± 0.15 cC	18.6 ± 1.14 dC
E. coli.	5.33 ± 0.15 aA	5.42± 0.18 aA	8.18± 0.18 bA	10.18± 1.12 bA	14.17± 1.18 cA
Staph. aureus	12.06± 0.18 aB	13.27± 1.17 aB	16.8 ± 0.13 bB	18.15 ± 0.9 bD	23.09 ± 0.24 dB

Values are M ±MSE .Small letters show significance between concentration; Capital letters revealed the significance between bacteria, was at $P < 0.05$.

- Sensitivity test to antibacterial drugs.

The result of antimicrobial susceptibility test revealed that *Pasteurella multocida*, were susceptible to all antibacterial uses except amoxicillin (resist). *Streptococcus agalactia* and *Mannheimia haemolytica*. revealed susceptible to Cefotaxime, Erythromycin, Ciprofloxacin, but resist to Amoxicillin and Nitrofurantoin. The isolate *E. coli* were sensitive to Cefotaxime, Nitrofurantoin and Ciprofloxacin but resist to Erythromycin and Amoxicillin. The remaining isolates *(Staph. aureus)* were sensitive to Cefotaxime, Amoxicillin and Nitrofurantoin but resist to Erythromycin and Ciprofloxacin.

Discussion:

In the present investigation, a higher percentage of the samples were found positive for Gram positive bacteria in which *Staphylococcus aureus Streptococcus agalactia* in the group of diseased animals compared to the Gram negative bacteria in which *E.coli* , *Pasteurella multocida, , Mannheimia haemolytica*

In the study of Benesi *et al*. (2013) were isolated *Staphylococcus spp., Bacillus spp., Streptococcus spp., P. aeruginosa, and enterobacteria.* Oliveira, *et al*. (2016) was reported *P. aeruginosa, Bacillus spp., Staphylococcus spp., and E. coli*, but with less frequency. In several nations, Elshafee (2003) detected *Bacillus spp., Enterobacter spp., Staphylococcus spp,. Pseudomonas spp., Escherichia spp.* other Serratia species, several of the microorganisms mentioned were available in the environment of calves and is found in the lower as well as upper respiratory systems. Unknown species of the family Enterobacteriaceae family members retrieved from pure cultures were presented in the study of (Loneragan et al. 2001, Griffin et al. 2010 and Hammadi, 2023),. On the other hand, the *P. multocida* and *M. haemolytica* were important pathogens of current study, but in the study of Natalia, *et. al.* (2018), in which these microorganisms were not isolated. Similar results were obtained by

Benesi *et al*. (2013). However, these species were isolated in other studies (Angen *et al*. 2009, Oliveira *et al*. 2016).

Propolis also called as bee glue, is one of the few resinous substances collected from plant sources by honeybees (Castaldo and Capasso, 2002). Propolis is a chemical compound and the compound varies depending on the geographical area and due to the changes in the extraction procedure, it is known that propolis water extracts have a highly distinct chemical makeup from alcoholic extracts (Mello *et al.,* 2010). A broad class of phenolic chemicals known as flavonoids can be extracted more easily using solvents like methanol or alcoholic solutions with GL ranges of 70 to 96. According to HPLC fingerprint, similar findings were made by Mello et al. (2010) and Park and Ikegaki (1998). Both groups noted that propolis extract revealed the presence of caffeic and p-coumaric acids with more polar solvents (100% water, for example), whereas less polar solvents revealed substances that were more lipophilic (compounds discovered after 20 minutes of chromatographic running). Based on these findings, extractable solvents like ethanolic solutions are preferable to aqueous extracts, Although the quantitative amounts varied ($P \leq 0.05$), the HPLC analysis in the current study revealed that Aqueous Propolis Extract contained all the standard chemicals typically found in Propolis alcohol extract, The two extracts assessed in this study were from the same batch of Iraqi green propolis and through the same extraction method, however PWE was obtained following the hydrolysis of propolis extractable materials of PEE and water solubilization. Biological effects of phenolic chemicals, which are frequently present in both food and non-edible plants, have been documented to include antioxidant activity. A wide range of substances, primarily flavonoids, are present in propolis. It has been hypothesized that flavonoids and other phenolic chemicals can inhibit the onset of heart disease and cancer (Ahn, *et al*,. 2007). also, it is reported that the mode of action is Propolis attacks cytoplasm, cytoplasmic membrane, inhibit bacterial enzymes, cell division, and protein synthesis. The antibacterial mechanism also depends on the inhibition of bacterial RNA polymerase (Akao, 2003). This was in line with observations from multiple articles that each propolis sample included 80–100 chemical components of varying quantities. (Darwish *et al*., 2010 and Al-Otaibi,2019). This Studies evaluating in vitro activity of Propolis were performed by using clinical strains isolated from sheep diarrhea. all the tested bacterial isolates revealed higher sensitivity to prepared propolis extract. The reported data of current study reveals that the Propolis extract has a broad antibacterial activity against gram positive and negative strains (Bueno-Silva, *et al.,* 2013). according to Stepanovic *et al*. (2003), Propolis has a strong antibacterial action against gram positive bacteria, however it is less effective against gram negative bacteria. Scazzocchio *et al*., (2016) reported that Propolis is an active agent against various Staphylococcal clinical strains. Similar results were found by different authors (Bueno-Silva, 2013). Our data also supports these findings it is found that the iraqi Propolis were found to be active against clinical strains of gram positive and negative strains in concentration between 1 - 5 mg/mL. The current *S. aureus* findings were consistent with those of numerous investigators, who discovered that the inhibitory zones achieved by alcoholic propolis from various parts of the world were 18, 20, 24, 21.8, 24.3, and 21.8 mm, respectively (Stepanovi et al., 2003).Our result also agree with that of Mahmood and Abdul Hadi, (2012), where they found that the highest concentration of 200 mg/ml of alcoholic propolis extract gave the inhibition zone of 20 mm, while the lowest concentration of 6.2 mg/mL did not give any inhibition by the paper disc method against *E.coli* bacteria, and they found that the least effective inhibition was at a concentration of 125 mg/mL Also, agree with the findings of Al-Salmani and Hassan, (2011), who noticed that the highest concentration of 5 mg/ml of alcoholic propolis extract against *E.coli,* gave the highest inhibition activity of

7.20, 20 mm by wells and discs method, respectively, compared to the lowest concentration of 1 mg/ml, whose inhibition rate was 15.10, 6.83 mm by the method of wells and discs, respectively. The result of the alcoholic extract of current study was identical to the result of Pirog *et al*., (2018), which found an inhibitory effect of alcoholic propolis extract against *Mannheimia haemolytica* bacteria isolated from wounds. It also matches the result of the alfadam, (2005), which concluded that alcoholic propolis extract is effective in inhibiting of *Mannheimia haemolytica* by wells method. Aljanabi, (2021) found that the concentrations of 1000 and 800 mg/ml were able to inhibit these bacteria, whose diameters are 27.58 and 26.55 mm, respectively, on the remaining concentrations which are 600, 400, 200 mg/ml, whose diameters are 23.48, 24.13, 25.10 mm, respectively, in the same study the disk diffusion agar method, showed no significant differences between all concentrations of the aqueous extract in their effect on these bacteria. The inhibition of bacterial growth was proportional to increased propolis concentration due to increased concentration of active component of propolis in our investigation. This result agreed with Taylor *et al*., (1996) and Hernandez *et al*., (1994), who reported that propolis extract efficiency rose as propolis concentration increased. Propolis antibacterial action is related to a range of phenolic chemicals, mostly flavonoids, phenolic acids and their esters, and certain prenylated-coumaric acids, according to (Yaghoubi *et al*., 2007). Propolis and certain of its cinnamic acid derivatives and flavonoids were found to be responsible for uncoupling the energy transuding cytoplasmic membrane, which inhibited bacterial movement and may contribute to the antibiotic action (Bankova *et al.*, 2000).

As a conclusion, The antibacterial efficacy of alcohol and aqueous propolis extracts against pathogenic microorganisms is well established. As a result, it can be employed in the development of therapeutic agents, limiting the occurrence of antibiotic resistance among microorganisms that arise as a result of antibiotic misuse and overuse. The well method of extracting alcohol a nd aqueous extracts is more successful than the disk method.

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