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Parasitological, bacteriological and in-vitro studies on antibacterial activity of ethanolic extract of Calligonumcomosum in goats affected with fascioliasis in Taif, KSA.

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ABSTRACT

This study was carried out to detect the relationship between bacterial infections and liver fascioliasis. Grossly, post mortem examination of the livers infested with Fasciolagigantica in the present study, were hard, firm and tough in consistency with multiple, irregular pale brownish areas on the surface. Cut section of the liver revealed presence of large whitish areas of fibrosis. The affected ducts were enlarged, thickened, hard in consistency and in the form of cord like structures and protruded above the surface of the liver. In most cases, mature Fasciola worms were detected within the lumen of the affected bile ducts and bile stones. Several types of bacteria were isolated from liver surface, bile duct and Fasciola worms from goats suffering from chronic fascioliasis. Bacterial isolated from liver surface were Stenotrophomonasmalyophilia, Corynebacteriumpropinquum and Escherichia coli. The bacteriological bile culture, revealed isolation of AerococcusUrinae and Escherichia coli. While bacterial species isolated from Fasciolagigantica, were Leifsonia aquatic, Corynebacteriumrenale group, Corynebacterium species, Escherichia coli and Lactococcuslactis spp. cremoris. All the isolated microorganisms are pathogenic and can transmitted from animals to human causing very dangerous zoonotic diseases. The present investigation evaluated the in-vitro antibacterial activity of the crude ethanolic extracts of Calligonumcomosum 10% on some gram positive and negative bacteria. Further in vivo studies are required to validate our findings and improve our knowledge on the potential of the Calligonumcomosum extract as antimicrobial in relation to its chemical composition.
Keywords:*Fasciola ,Calligonumcomosum , goats, antibacterial.*

INTRODUCTION

Fasciola species plays an important role in the microbial invasion of the infected animals either by transportation or depressing the vital resistance of the host. Besides, infected liver constitutes a good media for bacterial multiplication, transportation of micro-organisms with the parasites occurs during the different stages of its life cycle either outside or inside the animal body. Anaerobic necrotic lesions of the liver produced by immature flukes occasionally provides a suitable environment for the germination of spores of Clostridium novyi type B bacteria in the liver. The bacteria will release toxins into the blood stream resulting in what is known as black disease in sheep and goats and sometimes cattle (1), (2) and (3).

Control of fascioliasis may be achieved by control of snail and by use of anthelmentic treatment of infected animals, (4). Snail control is difficult and expensive, and involves improved drainage, fencing and the possible use of copper compounds, which are lethal to snails and other non-target animals. Anthelmentic treatment is a regular

practice in enzootic areas, but fails to eradicate the parasite. Allopathic anthelmentics are not completely effective against common flukes (5) and have serious disadvantages in some developing countries in addition to cost, risk of misuse leading to drug resistance, environmental pollution and food drug residues (6). Moreover, almost all adversely affect milk and meat production of animals during the course of their treatment, and even for long after their use (5).

Infestation of goat herds results in major health issues which lead to poor animal performance and economic loss. Currently, chemical anthelmintics are used to control gastrointestinal parasites. However, the increased resistance of parasites to anthelmintics will eventually result in a major crisis (7).

Concerning the relationship between bacteria and Fasciola spp., (8) observed the presence of large numbers of bacteria in the bile ducts of rats infected with Fasciola hepatic and their absence from uninfected animals suggests that the helminth in some way alters the conditions in the bile duct to favour bacterial growth in previously unfavourable environment. Indeed, significant changes have been found in the chemical characteristics of bile fluid from rats and cattle after liver fluke infection.After fluke infection the phyrsico-chemical nature of the bile may have changed sufftcicntly to favour the multiplication of the particular bacteria. Kahan et al. 2008 (7)reported that, the bile duct is not normally inhabited by bacteria and the powerful detergent action of bile in disrupting certain types of bacteria is commonly used to differentiate the morphologically identical but harmless Streptococcus viridans from the virulent S. pneumoniae in the diagnosis of bacterial pneumonia.However bacterial invasion of both intestines and bile duct commonly accompanies biliary diseases and inflorescences of Escherichia coli and streptococcus faecalis and other bacteria are not uncommon in the bile ofpatients with obstructive biliary disease Erlandsen and Chase 1972 and(10) reported that, there are two possible methods of introduction of the bacteria; one is direct migration when the fluke moves from the small intestine through the liver, while the other is migration of bacteria normally present in the small intestine into the bite duct after the establishment of the liver fluke.

Cheema and Myhammed1980 (11) and (12) studied microorganisms associated with abscesses in sheep and goats. The bacteria isolated were Escherichia coli,Staphylococcus epidermidis, Pasteurella spp., Streptococcus spp., Corynebacteriumspp., Moraxella osloensis and Pseudomonasaeruginosa from the sheep abscesses and Staphylococcus epidermidisfrom, Peptostreptococcusanaerobius, Eubacteriumtortuosum,Corynebacteriumpyogenes, Pasteurellahaemolytica,Pasteurellamultocida and Corynebacterium pseudotuberculosis from the goat liver abscess.

Foster 1984(13) and (14) stated in the presence of fluke infection bacteria is thought to flourish due to changes in the biliary environment. This was confirmed in the early chronic phase, where a bacterial infection of the common bile duct was detected at 8 weeks post-infection in F. hepatica infected rats. They also stated, bile taken from rats infected with the liver fluke, Fasciola hepatica contained spiral bacteria, whereas bile from uninfected rats was free from spiral bacteria. The bacteria and its relationship to the bile duct epithelium and the liver fluke was studied with a combination of light microscopy, scanning and transmission electron microscopy. Its morphological characteristics suggest that the bacteria belong to the genus Spirillum. In contrast to many other co-infections of bacteria and helminths, the present one seems to be a fairly passive relationship so that neither the helminths nor the rat suffers from the presence of bacteria. Eguale and Abie 2003 (1), (15), (16) and (17) reported that, bacterial infection will often occur as a consequence of tissue damage, and the method of infection of many helminth parasites seems to be particular likely to lead to the introduction of a secondary infection.Infested animals with Fasciolaspp had suffered severe body weight loss and growth retardation. This economic loss can be due to anorexia caused in infected animals by restlessness. The bacteria will release toxins into the blood streamer resulting in what is known as black disease in sheep. Gonzalo-Orden et al. (18) reported introduction of bacteria into the biliary system during migration from the duodenum Escherichia Coli,Klebsiella pneumonia and Enterococcusfaecalisfrom bile of infected sheep. Mas-Coma et al. 2000 (19) stated in the rats, the bacterobilia was higher when the number of parasites in the common bile duct were increased. Concerning fascioliasis, there is considerable tissue reaction and calcification in the bile ducts due to the presence of even a small number of flukes. Sayed et al. 2008 (20) observed that, invasion of the liver by migrating immature liver fluke damages the tissue and results in reduction of the oxygen tension (anaerobic condition), that allows the germination and proliferation of closteridial spores with release of its toxins and induce hepatocellular necrosis. William et al. 2008 (21) studied the fascioliasis and bacterobilia in experimentally and naturally infected animals, E. Coli (50%), Klebsiella pneumonia (30%), Pseudomonasspp(8%),

Proteus spp(5%) were isolated. Tehrani et al. 1998 (22) recorded that, the following bacteria were isolated: Corynebacterium spp. (52.4%), Pasteurella spp. (11.3%), Escherichia coli (11.1%), Pseudomonas aeruginosa (7.2%), staphylococcus spp. (5.3%). Borai et al. 2013 (23) stated that, bacteriological examination revealed that Staphylococcus aureus, Corynebacteriumpyogenes and Escherichia coli were the most common aerobic bacteria while Clostridium perfringens and Fusobacteriumnecrophorum were the most common anaerobic bacteria isolated from the affected livers. Corynebacteriumovis was isolated from six cases of sheep.

This study was designed to: -

Study the pathological hepatic lesions induced by bacteria in liver infested with Fasciola and to detect the relationship between these bacterial infections and liver fascioliasis. Also study, in vitro, the effects of some medicinal Herbal drug extracts (Calligonumcomosum plant) as antimicrobial compound.

MATERIALIS AND METHODS:

Bacterial isolation and characterization:

A loopful from each affected livers, bile duct and surface of Fasciola streaked onto the nutrient agar, MacConkey agar and blood agar plates, then incubated aerobically at 37 °C for 24 hrs. Bacterial isolates were identified morphologically, according (24).

Identification of bacteria species.

Identification of bacteria species were done by Baltimore Biology Laboratory (BBL) Crystal panel viewer ofserial number 042611-007

Crystal Spec Nephelometer of serial number 11050002734

BBL Crystal mind software

BBL Crystal gram – positive & enteric / nonfermenter ID Kits, Cat number 245140 & 245000 respectively

BBL Oxidase Reagent Dropper Cat, number 261181 & BBL Indole Reagent. Dropper number 261185

Microbiological studies

Bile samples were simultaneous and examined according to (21), Fasciola worms were collected and counted under a dissecting microscope. First, the bile duct was examined for the presence of worms and stones, and then the rest of the organs were evaluated, the liver parenchyma, in particular (25).

Antimicrobial activity:

The antibacterial activity was evaluated by noting the zone of inhibition against the test organismsing to according (26) and (27).Determination of minimal inhibitory concentration (MIC) of the extract was done according to (28) and (26).

Minimal bactericidal concentration (MBC)

The Minimum bactericidal concentration (MBC) of the plant extract was determined using the method described by (29).

Pathological and bacteriological studies on livers affected with fascioliasis in goats.

The lesion specimens were collected from four affected livers,and placed in sterile plastic containers and shipped to the laboratory, on ice, for bacterial culturing. In laboratory and under complete sterile condition in laminar flow cabinet samples were taken from three places (liver surface, bile duct and surface of adult Fasciola).

Identificationof gram positive and negative bacterial isolates was done using standard procedures (24) and (30).

RESULTS AND DISCUSSION.

Types of bacteria isolated are shown in table (1, 2) and graph (1, 2, 3).

Table (1): Bacteria species isolated from different location of livers infected with *Fasciolagigantica.*

Location	Liver surface	Bile duct	Surface of *Fasciolagigantica*
Bacterial species isolated	*Stenotrophomonas Malyophilia*	*Aerococcus Urinae*	*Leifsonia aquatica*
	Corynebacteriumpropinquum	*Escherichia coli*	*Lactococcuslactis spp. cremoris*
	Escherichia coli		*Corynebacteriumrenale group*
			Corynebacterium species
			Escherichia coli

Table (2): Types and pathogeneses of the positive and negative gram microorganisms isolated from livers infested with fascioliasis.

Types of liver samples bacterial isolates	Pathogeneses
Gram –possitive *1- Leifsoniaaquatica*	Associated with the environment. Isolated occasionally in the clinical laboratory, but natural and pathogenic significances yet to be dfined.Associated with endophthalmitis,meningitis in a child,andepticemia as well as urinary tract infection in a child.*
2- Lactococcuslactis spp. cremoris	With few case reports of being an opportunistic pathogen.*
3- C.renale group	Pathogenic veterinary bacterium that causes cystitis and pyelonephritis in cattle.*
*4- C.*spp.	Diphtheria some others animal.*
5- C.propinquum	Respiratory pathogen.*
6 - Aerococcusurinae	Predisposed to urinary tract infection.*
Gram- negative *1- Stenotrophomonasmaltophilia*	Associated with human urinary tract and respiratory tract infections postoperative .*
2- Escherichia coli	Bacteremia and bacteria-related travelers diarrhea leading cause of neonatal meningitis and other infections including pneumonia.*

According to (31), (32).

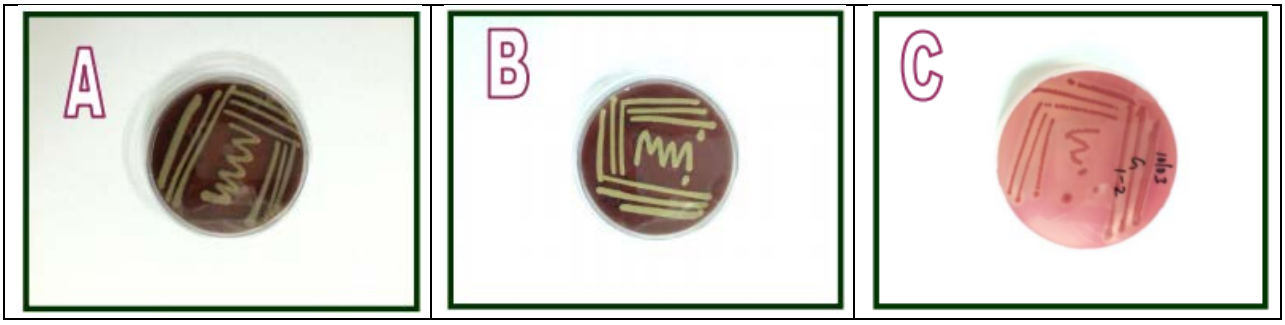

Fig (1) Bacteria isolated from liver surface infected with *Fasciolagigantica* and cultivated on blood agar (A, B) and manconkey (c).

A) *StenotrophomonasMalyophilia*

B) *Corynebacteriumpropinquum*

C) *Escherichia coli*

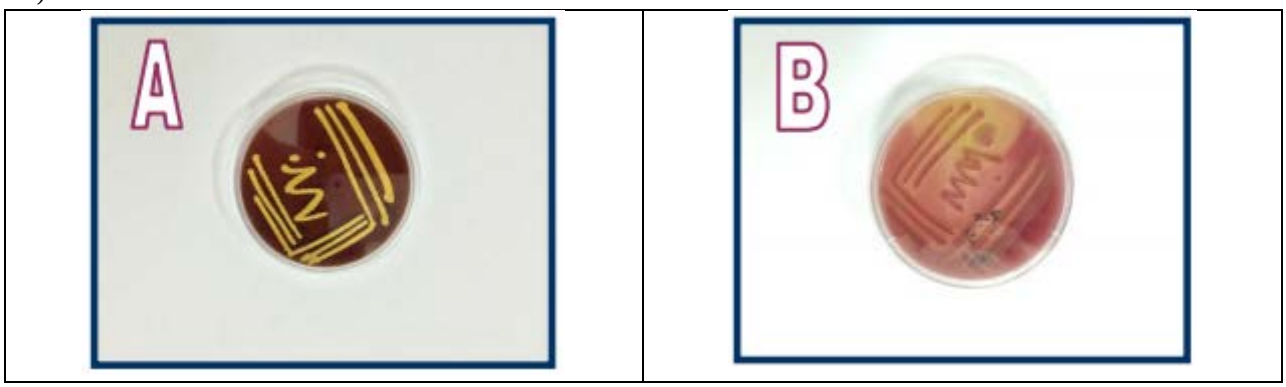

Fig (2) Bacteria isolated from bile duct infected with *Fasciolagigantica* and cultivated on blood agar(A) and manconkey(B).

A) *AerococcusUrinae*B) *Escherichia coli*

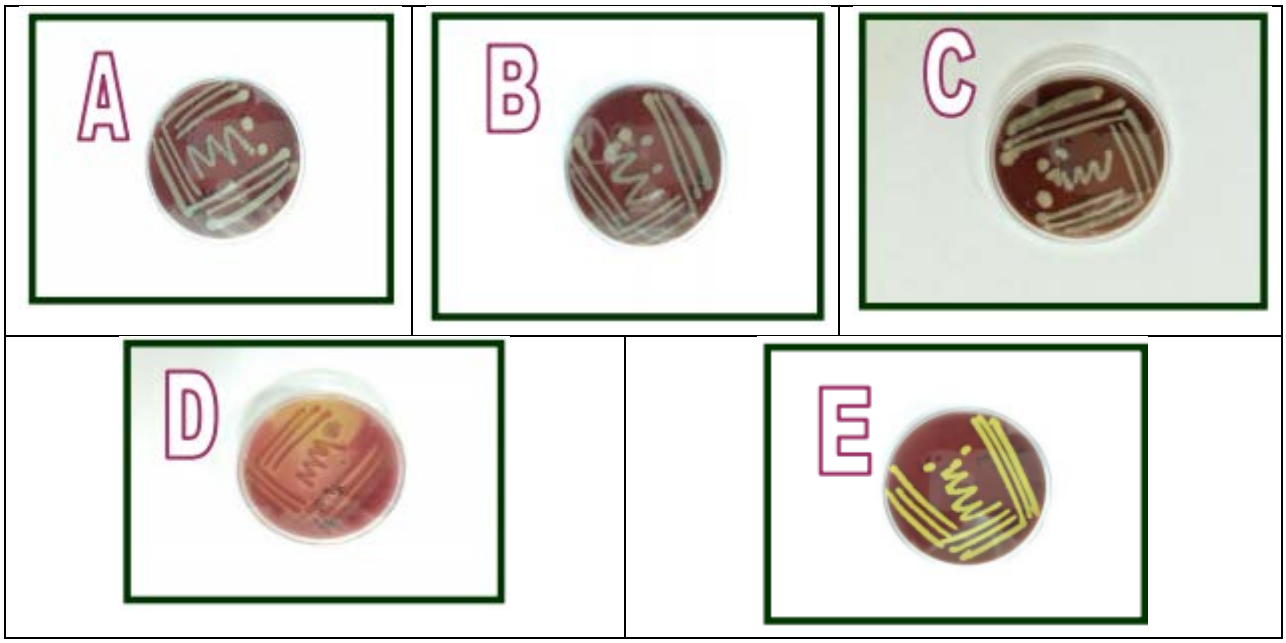

Fig (3) Bacteria isolated from surface of *Fasciolagigantica* and cultivated on blood agar (A, B, C, D) and maconkey (C)

A) *Lactococcuslactis spp.* (*cremoris*)B) *Leifsoniaaquatica* *C) Corynebacteriumrenale*group

D) *Corynebacterium*spp. E) *Eschericia coli*

DISCUSSION

Concerning pathological and bacteriological studies on livers affected with fascioliasis in goats.

Emerging evidence suggests a strong interaction between the gut microbiota and health and disease. The interactions of the gut microbiota and the liver have only recently been investigated in detail. The liver is receiving approximately 70% of its blood supply from the intestinal venous outflow, represents the first line of defense against gut-derived antigens and is equipped with a broad array of immune cells (macrophages, lymphocytes, natural killer cells, and dendritic cells) to accomplish this function. In the setting of tissue injury, whereby the liver is otherwise damaged (e.g., viral infection, toxin exposure, ischemic tissue damage, etc.), these same immune cell populations and their interactions with the infiltrating gut bacteria likely contribute to and promote these pathologies. Liver is

considered the most important organ for animal health production and reproduction. Many of the metabolic activities of the body occurred in the liver. Liver infection is an important disease that affects all kinds of meat producing animals, this lead to great losses to live-stock production and national income due to condemnation of great numbers of livers in the slaughter houses (33), (34) and (35).

Besides, infected liver constitutes a good media for bacterial multiplication, transportation of microorganisms with the parasites occurs during the different stages of its life cycle either outside or inside the animal body. Presence of both immature and mature flukes of Fasciola spp. cause acute and chronic hepatitis.

This study was carried out to detect the relationship between these bacterial infections and liver fascioliasis.The results of this study (Table 1) revealed that, the several types of bacteria which isolated from liver surface suffering from fascioliasis were Stenotrophomonasmaiyophilia,Corynebacteriumpropinquum and Eschericia coli.While Aerococcusurinae and Eschericia coli were isolated from the bile duct of the same infected liver. The results agreeed with those obtained by (9), (1), (2), (13), (20) and (36), who reported that, there are 2 possible methods of introduction of the bacteria; one is direct migration when the fluke moves from the small intestine through the liver, while the other is migration of bacteria normally present in the small intestine into the bile duct after the establishment of the liver fluke. They stated also, bile bacteria and its relationship to the bile duct, due to changes in the biliary environment produced as a result of the fluke infection, these changes subsequently allow a multiplication of bacteria normally present in the uninfected animal.

Table (2) showed the predominant incidence of the positive and negative gram microorganisms isolated from livers infested with fascioliasis which were Leifsoniaaquatica,Lactococcuslactissppcremoris,Corynebacteriumrenale group, Corynebacterium spp., Corynebacteriumpropinquum, Aerococcusurinae,Stenotrophomonasmalyophilia and Escherichia coli. Similar findings were described by (37),(38)and (39).Also, (40) who mentioned that, the most common bacteria isolated from the liver were A. pyogenes and anaerobic bacteria.

Bacteriological examination of samples with chronic fascioliasis showed the most frequently isolated aerobic or facultative organisms were E. coli and corynebacteriumpropinquum.These results agreed with the previous findings of (41), (42)and (43) who found that Fasciola infestation plays an important role in the damages which were attributed to toxic environment created by the organisms in liver tissue.The presence of some members of family Enterobacteriaceae in combination with other Clostridial bacteria such as C. perfringens may be explained by (44) that infection with pathogenic E. coli may cause altering the mucous membrane of the intestine, enable the clostridial microorganisms to vegetate and release its toxins which absorbed through the damaged gut and reached to the blood circulation and then to the liver. On the other hand, (45) considered E. coli as an incidental pathogen which contaminated the animal tissues during preparation of the carcasses from faecal material, skin and hides.These results nearly coincide with that observed by (41), (46) and (47).Similarly (48) isolated C.novyi and C. septicum in incidences of 6% and 2% respectively.

From the achieved results, it is concluded that livers of goats showed a very high proportion of gross lesions rather than different pathological disorders. Also Fasciola worms may be incriminated in aiding bacterial infections specially Stenotrophomonasmaiyophilia,Corynebacteriumpropinquum,Aerococcusurinae,Leifsoniaaquatica,Lactococcuslactis spp. cremoris,Corynebacteriumrenale group,Corynebacterium spp.and E. coliwhich lowering the hepatic viability.Generally, livers of slaughtered ruminant are considered as hazardous source of different mixed bacterial species. Moreover, their low value referred to high incidence of pathological lesions.During their migration phase through the abdominal cavity, liver parenchyma and bile ducts walls, young liver flukes may produce serious acute inflammatory tissue reaction, and blood loss owing to mechanical trauma and the young flukes then reach maturity in the bile ducts. These small liver flukes mainly produce chronic tissue reactions of the liver, such as fibrosis of small bile ducts, portal veins,and hepatic artery leading to biliary cirrhosis. Heavy infections may result in weight loss and emaciation and death of the host is mostly due to severe anemia and failure of liver function. Valero et al. 2006 (49) suggested an association between bactcriobilia and both duration and intensity of parasitic infection. They added that the obstruction caused by advanced chronic fascioliasis in the animals may be related to biliary sepsis.

Grossly, post mortem examination of the livers infested with Fasciola in the present study, were hard, firm and tough in con- sistency with multiple, irregular pale brownish areas on the surface.Cut section of the liver revealed presence of large whitish areas of fibrosis. The affected ducts were enlarged, thickened, hard in consistency and in the form of cord like structures and protruded above the surface of the liver. In most cases, mature Fasciola worms were detected within the lumen of the affected bile ducts and bile stones. There are strong association between time of infection with Fasciola, number of flukes, and the risk of developing pigment stones in the main bile duct.Gallstone presence increased with infection time. The relative risk of gallstone disease increased when the number of flukes per animals increased. The Fasciolacounte in this study revealed more than 300 flukes, post mortum findings were attributed to the effect of toxic products elaborated by Fasciola worms and presence of mature worms within the lumen of intra hepatic bile ducts. These findings were coincided with (2), (16), (25), (19), (20), (49) and (50).

Therapeutic efficacay of ethanolic extract C.comosum extracts on different types of bacteria.

Bile is usually sterile and the important factors in maintaining its sterility are the choledochalsphincter,the bile flow, and its bacteriostatic properties. When there is obstruction and stagnation,bacteria gain access tothe biliary system through either the papilla or the portal circulation.

The causes of biliary obstruction that predisposeto bacterial cholangitis are myriad. Biliary parasites cause necrosis, inflammation, fibrosis, strictures, and bile ducts cholangiectasis by several mechanisms as a direct result of the irritating chemical composition of the parasite, parasitic secretions, or eggs physical obstruction of the bile ducts induction of biliary stones formation; and introduction of bacteria into thebiliary system during migration from the duodenum.The main aim of this work is to investigate the relationship between presence of pathogenic bacteria in the bile versus fascioliasis using parasitological criteria: worm bacteriological bile culture and bacteriological fluke surface culture.

Concerning incidence of the positive and negative gram microorganisms isolated from livers infested with fascioliasisTable (1) showed that, the bacteriological bile culture revealed isolation of AerococcusUrinae and Escherichia coli. Bacterial species isolated from liver surface were Stenotrophomonasmalyophilia, Corynebacteriumpropinquum and Escherichia coli.While bacterial species isolated from Fasciolagigantica were Leifsonia aquatic, Corynebacteriumrenale group, Corynebacterium species, Escherichia coli and Lactococcuslactis spp. cremoris.All the isolated microorganisms are pathogenic and can transmitted from animals to human causing very dangerous zoonotic diseases.

The present investigation evaluated the in-vitro antibacterial activity of the crude ethanolic extracts of Calligonumcomosum 10% on some gram positive bacteria (Corynebacteriumpropinquum, Aerococcusurinae,Leifsoniaaquatica,Lactococcuslactis spp.) cremoris, Corynebacteriumrenale group and Corynebacteriumspp.and Gram negative bacteria(StenotrophomonasmaiyophiliaandEscherichia coli.)

Taking into consideration medicines from plant origin as an alternative form of health care is increasing because they are serving as promising sources of novel antibiotic prototypes (51). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found to have antimicrobial properties as anticancer agents, anti-diarrheal as well as antifungal activities (52) and (53).

In our current investigations we have found that the activity of ethanolic extract of Calligonumcomosum 10% against the gram positive and Gram negative bacteria showed the highest zone of inhibition for gram negative and positive bacteria .These findings might be in well agreement with (54) who observed that the significant level of antibacterial activity was in a similar range of inhibition zone for the growth of the bacterium , Listeria ivanovii (9 – 18 mm) when laboratory tested by the agar well diffusion method. The authors also demonstrated that the most active plant part causing inhibition is the extract from the leaves. Thus; the study ascertains the value of plant parts used, which could be of considerable interest towards the development of new drugs. The observed differences in sensitivity the Calligonumcomosum extract between gram positive and gram negative bacteria can probably be attributed to the structural and compositional variations in the nature of the cell wall between the two groups (55).

Results in current study were strongly agreed with results provided by (56), (57), (58) (59), (2), (3), (36) and (54).

Further in vivo studies are required to validate our findings and improve our knowledge on the potential of the Calligonumcomosum extract as antimicrobial in relation to its chemical composition.

Amazing and interesting to, isolated *Lactococcuslactis* spp.cremoris from the surface of Fasciolagigantica, collected from liver chronically suffered with Fasciolagigantica.Isolation of this microbe proved that the migration of bacteria can occur during the life cycle of Fasciola.However, it is a members of the genus Lactococcus and gram positive lactic acid bacteria.

These lactococci are found in various environments including plant surfaces (60) and (61), and recognized as pathogens in fish aquaculture (62). Lactococcuslactis is used worldwide in starter cultures for the manufacture of fermented dairy products, such as in the cheese-making process. Lactococcuslactis spp. can be found in various environments including animal sources, dairy products and silages (60), (61) and (63).

Lactococcuslactis has been observed at high numbers in the intestinal tract of the puffer fish Takifuguniphobles (marine fish). Additionally, the isolation of L. lactis strains from Amur Catfish Silurusasotus cultured in freshwater has been reported (64). Some strains of L. lactis have been isolated from intestinal tracts of several freshwater fish species. Mitchell 2002 (65) and (66) recorded that L. lactisspp.lactis may be isolated from the intestinal tracts of marine fish , and their excreta are continuously being introduced to the environment.

CONCLUSION

This study is of value in endemic areas, where possibility of bacteria co-infection with Fasciola is a common occurrence and highlights the importance of early chemotherapeutic intervention.

In the light of our present findings we can conclude that the ethanol leaf, root/bark extracts of Calligonumcomosum showed antibacterial effect in experimental models against Corynebacteriumpropinquum, Aerococcusurinae, Leifsoniaaquatica, Lactococcuslactis spp. cremoris, Corynebacteriumrenale group, Corynebacterium spp., and gram negative bacteria Stenotrophomonasmaiyophilia and Escherichia coliwhich therefore offer a scientific basis for using this plant as a good source of traditional microbiological and anthielmtic references. Further studies are required to validate our findings and improve our knowledge on the potential of the Calligonumcomosum extract as antimicrobial in relation to its chemical composition. Isolation of Lactococcuslactis spp. cremoris may therefore have commercial application such as probiotics for aquaculture and the development of functional foods and novel food additives.

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