

STUDIES ON ANTIBACTERIAL ACTIVITY OF LEAF EXTRACTS OF
***Limonia acidissima* Linn. WITH SPECIAL REFERENCE TO**
***Pasteurella multocida*.**

T H E S I S

Submitted
in partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE
IN
VETERINARY PHARMACOLOGY AND TOXICOLOGY

BY

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2018

DECLARATION OF STUDENT

I hereby declare that the experimental research work and Interpretation of the thesis entitled “**STUDIES ON ANTIBACTERIAL ACTIVITY OF LEAF EXTRACTS OF *Limonia acidissima* Linn. WITH SPECIAL REFERENCE TO *Pasteurella multocida***” or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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This is to certify that the thesis entitled, “**STUDIES ON ANTIBACTERIAL ACTIVITY OF LEAF EXTRACTS OF *Limonia acidissima* Linn. WITH SPECIAL REFERENCE TO *Pasteurella multocida*”** submitted by Mr. **SHIRANKAR SHRADDHYEY SHARAD** to the **Maharashtra Animal and Fishery Sciences University** in partial fulfillment of the requirement for the degree of **M.V.Sc** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

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“Research is to see what everybody else has seen and to think what nobody else has thought”

-Albert Szent-Gyorgyi

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ABBREVIATIONS

%	Percentage
µg	Micrograms
mg	Milligrams
µl	Micro liters
ml	Milliliters
MIC	Minimum Inhibitory Concentrations
MTCC	Microbial Type Culture Collection
EtOH	Ethanol
Me₂CO	Acetone
Aq.	Aqueous
P.E.	Petroleum ether
OD	Optical density
NA	Nutrient agar
NB	Nutrient broth
MHA	Mueller Hinton Agar
GC/MS	Gas chromatography/Mass spectrometry
CAS	Chemical Abstract Services
STEC	Shiga toxin producing <i>Escherichia coli</i>
VTEC	Vero cytotoxin producing <i>Escherichia coli</i>
ETEC	Enterohaemorrhagic <i>Escherichia coli</i>

API	Active Pharmaceutical Ingridient
o	Degree
C	Celsius
PSI	Pound force per square inch
Kg	Kilograms
Viz.	Namely
<i>et. al.</i>	And associates
NIST	National Institute of Standards and Technology
RT	Retention time

INTRODUCTION

Ayurveda deals with the plant medicines and comprises the information on the very new and old plant medicines. The origin of Ayurveda is attributed to Atharva Veda wherein several diseases are mentioned with their treatments. During the Samhita period (6th Century BC to 7th Century AD) there was systematic development of the science, when a number of classical works were produced by several authors and during this period there is evidence of organized veterinary and medical care. Ayurvedic therapies are typically based on complex herbal compounds. Ancient Ayurveda treatises also taught surgical techniques.

The *Kahun papyrus* (1900 BC) and Vedic literature of ancient India offers the first written records of veterinary medicine. First Buddhist Emperor of India Ashoka everywhere in his kingdom made provision for two kinds of medicines to take care of human and animal diseases. His efforts are worth appreciating to make plant medicines available everywhere. The Vedic Society in India was dominated by the 'cow culture' and Vedic people adored the cow and regarded it as the source of their good fortune, happiness and good health (Rigveda 6.28.1, 6). It is believed that the religious priests used their medical knowledge to keep the sacred cattle free from ailments.

Before the advent of modern allopathic system of medicine, it seems possible that the healing art was almost the same throughout the world including India. This system of medicine has given the term ethno-medicine (when implied to human treatment) and ethno-veterinary medicine (in the context of animal treatment). In India, ethno-veterinary practices were in vogue since time immemorial. In ancient India, the Vedic literature, particularly Atharvaveda is a repository of traditional medicine including prescriptions for treatment of animal diseases. Books written by Charaka, Sushruta, Palakapya (1000 BC) and Shalihotra (2350 BC) documented treatment of animal diseases using medicinal plants. Yajurveda cites importance of growth and development of medicinal plants and Atharvaveda mentions about the value of medicines in curing the diseases. Shalihotra undoubtedly appears to be the first veterinarian of pre-historic times. The ancient Indians were so equipped with the knowledge of

herbals, even Alexander acquired some of the skills used by Indians, particularly for treatment of snakebite (**Somvanshi, 2006**).

In current age, with the advent of science and development or emergence of new diseases the present day therapy is not sufficient to cure man and animal beings because of one or other reasons. This is especially true in case of antimicrobial therapy since many of the identified bacterial species are showing multidrug and cross drug resistance. There are ecological disturbances as well in the normal microbiota of an individual following antibiotic treatment. Study showed that even short-term administration of antibiotic can lead to stabilization of resistant bacterial populations in the human intestine that persist for years (**Jernberg et al., 2010**). Both medical and veterinary fields are facing prime problem of antimicrobial drug resistance against many commonly identified bacteria.

In India, the haemorrhagic septicaemia (HS) was reported as an endemic, highly fatal and economically important disease of large animals. About 46 - 55% mortality in bovines was attributed to haemorrhagic septicaemia during past four decades in India. Buffaloes and cattle were the most susceptible animal species with highest rate of incidence in buffaloes during wet season. The bacterial agent *Pasteurella multocida* serotype B: 2 was the etiology responsible for disease (**Benkirane and Alwis, 2002**). Many researchers found the incidence of haemorrhagic septicaemia with *Pasteurella multocida* serotype B: 2 in many states of India including buffaloes and cattle of Maharashtra state. Studies revealed high genetic diversity, broad host range, no geographical and host specificity among field isolates of *Pasteurella multocida* serotype B: 2 in India which may be regarded as probable cause of vaccine failure against this disease in India (**Saxena, et al. 2016**). The organism is no more an exception for the emergence of antimicrobial resistance. With the advent of modern chemotherapeutic agents, the resistance was also reported in *Pasteurella multocida* by many workers. Résistance to amoxicillin, augmentin and cotrimoxazole were found (**Jabeen, et al. 2013**), also high resistance were observed for erythromycin and sulfadiazine (**Naz, et al. 2012**).

The *Escherichia coli* is considered to be the most leading gram negative organism causing mastitis in India and World. The organism affects all age groups and species of animals with significant losses in domestic animals. Shiga

toxin or verocytotoxin-producing *Escherichia coli* (STEC or VTEC) was recognized as an important food borne pathogen, responsible for sporadic cases to serious outbreaks across the world and India also. The morbidity and mortality associated with several recent outbreaks due to shiga toxin producing-*Escherichia coli* have highlighted the threat due to this organism and made realize its zoonotic importance. The organism may cause serious human complications like bloody diarrhoea, haemolytic uraemic syndrome, haemorrhagic colitis and thrombocytopaenic purpura. Domestic animals, especially cattle and sheep are the main reservoirs and sources of infection **(Wani, et al. 2003)**. Recently, incidences of shiga toxin producing-*Escherichia coli* resistance were also detected by many researchers. Resistance for erythromycin, cephalothin, amikacin, kanamycin and gentamicin was found **(Mahanti, et al. 2013)** in India with ever changing genetic status of *Escherichia coli*.

The *Klebsiella pneumoniae* is well recognized environmental coliform responsible for respiratory tract infections and interstitial pneumonia in animals of India **(Amravathi, et al. 2016)**. The organism is also responsible for mastitis in dairy animals and considered as major gram negative bacteria causing mastitis and hence responsible for economic losses **(Hamadani, et al. 2013)**.

Nowadays, there are many problems arising out due to antimicrobial therapy around World including India. Surveys claim that in 2010, India was the world's largest antibiotic consumer. New Delhi metallo- β -lactamase (NDM) enzymes, which were first reported in 2008, were now detected worldwide. In India, *Escherichia coli* isolated from the community showed high resistance to ampicillin, nalidixic acid and co-trimoxazole. Apart from these, *Escherichia coli* showed resistance for aminoglycosides, third generation cephalosporins and fluoroquinolones. *Klebsiella pneumoniae* isolates showed resistance to fluoroquinolone and carbapenem **(Laxminarayan and Chaudhury, 2016)**. The above discussion regarding three gram negative bacteria namely *Pasteurella multocida*, *Escherichia coli* and *Klebsiella pneumoniae* visualize the depth of problem of antimicrobial resistance for common drugs of choice employed for treating infections in man and animals.

The plants are supposed to be helpful in fighting against many bacterial infections. It is known that India is blessed with very rich plant diversity and therefore the current study intended to validate the ethno medicinal plants for

their antibacterial activity. After going through the heavy literature on ethno medicinal plants, it was found that tribals and farmers of the Vidarbha region of Maharashtra, India use leaves of plant named *Limonia acidissima* to treat the infection of haemorrhagic septicaemia in livestock (Kulkarni, *et al.* 2014). Haemorrhagic septicemia is one of the fatal and economically important diseases of ruminants in India. In India, Haemorrhagic septicaemia (HS) took the heavy population of about 53,000 bovines every year and responsible for the highest mortality and second highest morbidity after foot and mouth disease, rinderpest and black quarter during the period of 1974-1986 (Sherikar, *et al.* 2004). Similar kind of findings were encountered by National Animal Diseases Referral Expert System (NADRES) showing haemorrhagic septicaemia as leading cause of mortality and second most observed disease during the period of 1991-2010 (Saminathan, *et al.* 2016). Hence, there is need for scientific validation and evaluation in order to explore antibacterial potential and ethnoveterinary use of such under explained plant which may prove as an alternate therapy to conventional one. Therefore, the plant *Limonia acidissima* is selected to screen its antibacterial properties with following objectives.

OBJECTIVES

1. Phytochemical analysis of the aqueous, acetone, ethanolic and petroleum ether leaf extracts for presence of active principles.
2. Evaluation of the *in-vitro* antibacterial activity of aqueous, acetone, ethanolic and petroleum ether leaf extracts of *Limonia acidissima* against standard strains *Pasteurella multocida*, *Escherichia coli* and *Klebsiella pneumoniae* by disc diffusion method.
3. Further confirmation of antibacterial activity of leaf extracts of *Limonia acidissima* against *Pasteurella multocida*, *Escherichia coli* and *Klebsiella pneumoniae* by tube dilution method.
4. Detailed analysis of phytoconstituents present in the most potent antibacterial leaf extracts of *Limonia acidissima* by GC/MS or LC/MS.

REVIEW OF LITERATURE

2.1 Diseases caused by gram negative organisms in domestic animals:-

Benkirane and Alwis (2002) reviewed the haemorrhagic septicaemia (HS) and stated it as an endemic threat of cattle and buffaloes of many tropical countries of Asia and Africa. Prevalence and severity of the disease was found to be more in buffaloes and young animals of both species compared to cattle and aged animals, with higher incidence of occurrence during wet moist season, humid conditions, greater population density, work stress and extensive free grazing. Author categorized hemorrhagic septicemia affected countries into three different classes on the basis of frequency of occurrence and economic losses attributed to hemorrhagic septicemia and put India in the prime class wherein disease was endemic problem with serious economic losses. The etiological agents found to be responsible were *Pasteurella multocida* serotype B:2 and E:2 in Asia and Africa respectively amongst many serotypes detected. Author suggested antimicrobial therapy for treatment but at the same time highlighted very serious problem of *Pasteurella multocida* resistance.

Author recommended herd vaccination immediately following an outbreak and prophylactic immunization prior to onset of monsoon in endemic areas. Economic drift due to haemorrhagic septicaemia was found to be significant in Asia compared to Africa because 146 million buffaloes and 432 million cattle became susceptible constituting 95% and 30% buffalo and cattle population of the world respectively in last few decades. Haemorrhagic septicaemia accounted for 46–55% of total bovine mortality during the last four decades. Author highlighted the fact that mortality caused by haemorrhagic septicaemia alone (46-55%) was almost equal to cumulative mortality (58.7%) caused by five leading endemic diseases like foot-and-mouth disease (FMD), rinderpest, blackquarter, anthrax and haemorrhagic septicaemia during the period of 1974 to 1986, indicating seriousness of this disease. India being leading milk producer of Asia with huge population of buffaloes contributing significantly in milk production, the disease eventually gains an economic importance.

Wani et al. (2003) studied Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *Escherichia coli* (EPEC) in calves and lambs below four

months age suffering from diarrhoea in India. Faecal samples of almost 391 calves and 101 lambs manifesting diarrhoea were screened and *Escherichia coli* from 249 bovine and 60 ovine samples were isolated. Bacterial genes like *stx1*, *stx2*, *eaeA* and EHEC *hlyA* were targeted by multiplex polymerase chain reaction (m-PCR) for screening and results revealed the presence of STEC and EPEC in diarrhoeic calves and lambs. One of the most important STEC serogroup O157, known for certain life-threatening infections in humans, was isolated from both bovine and ovine faecal samples. The most frequent serogroup among bovine and ovine EPEC was O26 (40%). There has been variability since the strains of STEC detected in the analysis did not resembled to those detected earlier from calves associated with diarrhoea and enteritis outside India. The study further revealed that, being associated with diarrhoeic diseases of humans in other part of globe STEC and EPEC in calves and lambs suffering diarrhoea in India may spread certain life-threatening diseases to humans and hence of zoonotic significance.

Sharma and Sindhu (2007) screened milk of 2057 buffaloes affected with clinical and subclinical mastitis from rural as well as urban dairy with the aim to study occurrence and etiology of mastitis in traditionally managed buffaloes of Haryana, India. Results of the study showed that prevalence of subclinical cases was more consisting 273 cases of *Escherichia coli* and 45 cases of *Klebsiella spp.* as compared to clinical cases of mastitis possessing 134 cases of *Escherichia coli* and 25 cases of *Klebsiella spp.* Out of 3447 isolates obtained from mastitic quarters, *Escherichia coli* and *Klebsiella spp.* contributes about 11.80% and 2.03% respectively.

Gani et al. (2008) conducted a study in fertile and sub fertile cows to detect the presence of bacteria in the uterus of normal and repeat breeder cows. The study includes uterine biopsy of 51 cows consisting of 14 normal and 37 repeat breeders. Bacterial infection was detected in majority of repeat breeders i.e. about 23 repeat breeder animals and 4 normal fertile animals which indicated a positive correlation between repeat breeding and uterine infections. Among the repeat breeders, *Escherichia coli* with prevalence rate of 29.7% (i.e. 11 biopsy samples) was one of leading infectious isolate investigated from repeat breeding cows. The *Escherichia coli*, was followed by some miscellaneous short minute rod shaped gram negative bacteria and *Pseudomonas sp.* with occurrence of

24.3% (9 biopsy samples) and 18.9% (7 biopsy samples) respectively from repeat breeder cows suffering with mucopurulent uterine discharges. The results of in-vitro antibiotic sensitivity testing showed high sensitivity of *Escherichia coli* isolates to Amoxicillin, Oxytetracycline, Streptomycin, Ciprofloxacin and Sulphamethoxazole.

Awandkar et al. (2009) conducted a study aimed at isolation of causative pathogen of mastitis followed by antibiotic sensitivity testing for determining current antibiogram status. Total 100 mastitic milk samples obtained from clinical mastitis cases of cattle and buffaloes in and around Udgir (Dist. Latur, Maharashtra) were processed and 63% showed bacterial growth, 7% showed fungal growth and remaining showed no growth. Amongst bacteria *Escherichia coli* with 28 samples (40%) was most prevailing bacteria followed by *Staphylococcus aureus* 24(34.29%) and *Candida* 7(10%). Apart from above mentioned major etiological agents some minor gram negative bacteria like *Pasteurella sp.1* (1.43%) and *Pseudomonas sp.1* (1.43%) were also detected. The antibiogram study clarified that ciprofloxacin was the most effective antibiotic followed by gentamicin, enrofloxacin, chloramphenicol and ceftiofur.

Radostit et al. (2009) mentioned that haemorrhagic septicaemia and pneumonic pasteurellosis were exclusively the diseases of cattle and water buffaloes caused by strain B and E in Asian countries. Author considered it an economically important disease in India. All age groups of animals were known to be susceptible, with exceptionally higher incidence in 6 months to 2 years age. The tonsils were supposed to be point of entry. The clinical condition characterized by acute fulminating septicaemia due to capsular antigen with high fever (106-107°F), profuse salivation, submucosal patechiation, severe depression and death within 24 hrs. Morbidity and case fatality rate vary between 50% and 100%. Author suggested sulfadimidine and oxytetracycline for therapeutic purpose and vaccination with alum precipitated, endotoxin free capsular antigen for prophylactic use in herds.

Author explained *Escherichia coli* (*E. coli*), a major cause of diarrhoea so called colibacillosis and an acute undifferentiated diarrhoea in all newborn farm animal species responsible for early economic losses. Some pathogenic serotypes found commonly in domestic animals were enterotoxigenic *E. coli*

(ETEC), enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) or shiga-toxin producing *E. coli* (STEC), necrotoxigenic *E. coli* (NTEC) and septicemic *E. coli*. Author further mentioned that, the enterohaemorrhagic *E. coli* O157:H7 being shared between humans and animals and proven as serious public concern across the world.

The coliforms *Escherichia coli* and *Klebsiella pneumoniae* were found to be associated with mastitis and histopathological changes in mammary glands in dairy cattle and sows. Both organisms were responsible for toxic agalactia. The wet saw dust bedding and moisture promotes growth of coliforms especially *Klebsiella* spp.

Sellyei et al. (2009) isolated *Pasteurella multocida* from clinically manifested poultry and swine and subjected those isolates for evaluation of antimicrobial susceptibility. Total 56 field isolates of *Pasteurella multocida* from poultry (20) and from swine (16 *Pasteurella multocida* toxin (PMT) positive and 20 PMT negative) were screened against 16 commonly used antibiotics. The rate of resistance was higher in *Pasteurella multocida* strains of poultry than swine. The *Pasteurella multocida* producing toxin (PMT) possessed resistance against only sulphonamides and apramycin. It is found that, the resistance to sulphonamides, tetracyclines, first-generation quinolones and aminoglycosides was remarkable. On the contrary, the antimicrobial activity of the classical penicillin, the newer macrolide (tulathromycin), the third-generation fluoroquinolone (enrofloxacin) and the fourth-generation cephalosporin (cefquinome) proved to be satisfactory against this bacterium.

Onat et al. (2010) screened clinically healthy Holstein cattle to know frequency and antibiotic susceptibility of *Pasteurella multocida* and *Mannheimia haemolytica* isolated from nasal cavity of cattle having at all no history of antimicrobial treatment prior to commencement of this experimental sampling. From 27 identified cases of *Pasteurella multocida*, 17 samples from cattle of 1 year or below and 10 samples from cattle of 2 year or above were found positive. All tested *Pasteurella multocida* isolates were found susceptible against florfenicol, also 27 *Pasteurella multocida* showed susceptibility against enrofloxacin, sulfamethoxazole/trimethoprim, erythromycin and ampicillin at the rates of 85.1, 92.6, 44.5 and 100 respectively.

Kumar et al. (2011) conducted a study in cattle and buffaloes of Uttar Pradesh, India with the aim to access status of haemorrhagic septicaemia (HS). Study comprised screening of total 400 samples obtained from apparently healthy (335), field cases of haemorrhagic septicaemia (55) and dead animals (10) suspected for haemorrhagic septicaemia from various sources. Most of the organisms were isolated from blood and edematous fluids of clinical cases. Almost 25 samples tested by examining the blood smear, impression smear of lung, liver, spleen and smear from heart blood of rabbit died in pathogenicity test showed presence of bipolar organism which clearly indicated *Pasteurella multocida*. On the basis of sugar fermentation ability and capsular typing by counter immuno eletrophoresis (CIE) test, isolates were categorized into 12 different biotypes which indicated presence of multiple biotypes with highest prevalence of *Pasteurella multocida* B:2 in the UP, India.

Ranjan et al. (2011) focused over isolation of bacterial pathogens from milk of bovines suffering from clinical cases of mastitis in Jharkhand, India. Almost 190 milk samples were collected from bovines and 138 samples yielded bacteria. From 138 isolates, *E. Coli* 17(8.95%) was most prevalent gram negative bacteria leading to mastitis. But, there were sporadic cases of *Klebsiella* spp. 3(1.57%).

Naz et al. (2012) mentioned that haemorrhagic septicaemia caused by *Pasteurella multocida* was an economically important disease of buffaloes in Pakistan. The study was comprised of samples of blood, bone marrow and tissues from liver, spleen and lung collected from carcasses of 20 haemorrhagic septicaemia suspected buffaloes around Lahore, Pakistan. Maximum number of isolates was recovered from samples of bone marrow, lungs and spleen. Sixteen isolates were identified as *Pasteurella multocida* and all isolates were serologically associated with Carter's sero group B. Antibigram study of all the field isolates depicted the highest sensitivity i.e. 87.5% for ciprofloxacin, ofloxacin, enrofloxacin and gentamicin, 81.25% for norfloxacin and amikacin, 75% for kanamycin and 56.25% for tetracycline. No resistance was observed for ciprofloxacin, enrofloxacin, ofloxacin and norfloxacin whereas maximum resistance was observed against erythromycin and sulfadiazine (50%). It was concluded that all *Pasteurella multocida* isolates belonged to Carter's group B and found sensitive to ciprofloxacin and ofloxacin.

Dhama et al. (2013) in his review discussed about major food borne pathogens from animals and their zoonotic importance. Such pathogens pose a serious public threat especially bacteria like *Escherichia coli* which elaborate toxins leading to intoxication. Further, it has been discussed that enterohaemorrhagic *Escherichia coli* (mostly strain *Escherichia coli* O157:H7) has worldwide occurrence and emerging as food borne pathogen. It was reported that enterohaemorrhagic verotoxic *Escherichia coli* (producing Shiga toxins or verotoxins i.e STX-1 and STX-2) known to be harboured by Cattle, shed intermittently in faeces and transmitted through undercooked beef and raw contaminated milk. Apart from Cattle other animals species viz. sheep, goat, dog, horse and deer too harbour this organism.

Hamadani et al. (2013) stated mastitis as an inflammatory disease of udder involving pathological changes in epithelial cells of udder leading to reduced functional capacity and losses extending to next generations. Author summarized and discussed mastitis with its epidemiology, predisposing factors, etiology, prevention, pathogenesis, types of mastitis, comparison with normal milk, economic losses due to mastitis, diagnosis and treatment. Author further classified pathogens causing mastitis into major and minor etiological agents. It is important to note that *Escherichia coli* and *Klebsiella pneumoniae* were categorized as major gram negative organisms leading to mastitis and *Pasteurella multocida* as uncommon pathogen responsible for mastitis. There were certain cases of environmental mastitis not exceeding 10% of the total mastitis, wherein *Escherichia coli* (environmental coliforms) was supposed to be the major etiology and unhygienic conditions or contact with contaminated environment to be predisposing factors.

Jabeen et al. (2013) analysed *Pasteurella multocida* B:2 strain isolated from cattle leading to haemorrhagic septicaemia. In respective study morphological/microbial, biochemical, molecular analysis along with the antibiotic sensitivity pattern of the *Pasteurella multocida* B:2 was carried out. Typical gray lustrous isolated colonies on Brain Heart Infusion and Blood Agar Base Medium were observed. Gram staining and microscopy showed gram negative and non-motile coccobacilli. Identification was conducted by conventional biochemical tests and percentage identification with Analytical Profile Index (API) kit was 96 %, which showed significant similarities with earlier studies. The results of

antibiotic sensitivity test revealed that organism was sensitive to only Ceftiofur and Gentamycin, susceptible to Amoxicillin and Aztreonam but found to be resistant to Augmentin and Cotrimoxazole. On molecular level its DNA was extracted and was run with marker having range from 0.5 to 10 kb. Its DNA was found heavier than 10 kb. From study it was concluded that accurate laboratory diagnosis of *Pasteurella multocida* depends upon isolation and identification of suspected bacterial colonies by microscopy and biochemical tests. Molecular analysis supposes to be a successful tool for differentiation of strains in a variety of bacterial infections.

Jeykumar et al. (2013) conducted study to recognize prevalence of mastitis causing pathogens and their drug sensitivity pattern in and around Namakkal, Tamilnadu. Total 72 samples were subjected to battery of test to undergo isolation, identification and antibiotic sensitivity testing. Almost 32 (44.44%) were positive for *Staphylococcus* sp., 4 (5.5%) for *Streptococci* sp., 30 (41.66%) for *Escherichia coli*, 6(8.33%) for *Klebsiella* sp. The study revealed sensitivity of organisms for enrofloxacin and ciprofloxacin but resistance for Penicillin-G.

Mahanti et al. (2013) studied Shiga toxin–producing *Escherichia coli* (STEC) in apparently healthy buffaloes of West Bengal, India which acted like reservoir for multidrug-resistant Shiga toxin–producing *Escherichia coli* for humans. Buffaloes from European and few Asian countries found to be reservoirs of STEC but no such prevalence was detected in Indian Buffaloes, even though India is home for majority of buffaloes.

Total 165 faecal samples were processed for bacterial isolation and 363 *Escherichia coli* isolates were obtained which were again subjected to biochemical and molecular characterization. The serogroup O120 was most frequent i.e. 11.7% out of eight serogroups detected in Indian buffaloes. The m-PCR and RAPD-PCR detected the genes responsible for Shiga toxin–producing *Escherichia coli* (STEC) with highest frequency of *stx1* gene (95.8%) followed by *stx2* gene (83.3%) from total STEC isolates. The isolated STEC strains were most frequently resistant to erythromycin (95.83%), cephalothin (62.5%), amikacin (54.17%), kanamycin (45.83%) and gentamicin (41.67%) but no ESBL-producing and quinolone resistance were detected in STEC. Nine strains of

STEC showed multidrug resistance which points towards developing resistance among organisms and post exposure threat to humans.

Mir et al. (2013) conducted a study aimed at isolation of various bacterial pathogens from 88 apparently healthy equines and 53 equines affected with respiratory tract diseases from various regions of Jammu and Kashmir, India. Total 141 nasal swabs were collected from both the nostrils of horses and processed for bacteriological isolation. It was interesting to found that every swab yielded at least one kind of bacterium and total 321 isolates were obtained out of which 51 (15.88%) were identified as gram negative. The bacterium *Klebsiella pneumoniae* was most predominant isolate recovered from mostly diseased equines (12.7%).Whereas, bacterium *E. coli* was second most prevalent isolate recovered from mostly apparently healthy equines (4.12%).

Nobrega et al. (2013) conducted a study to isolate *Klebsiella pneumoniae* on animals of three dairy herds with previous history of mastitis due to *Klebsiella pneumoniae*. The genetic similarities between different isolates were analyzed by pulsed field gel electrophoresis (PFGE) and studied status of extended spectrum β - lactamase (ESBLs) production. Milk samples from lactating cows and swabs performed at different locations, including milking parlors, waiting room, soil, animal's hind limbs and rectum were collected. *Klebsiella pneumoniae* was isolated from 27 cases of intramammary infections (IMI) and from 41 swabs. For farm A isolates from IMI and bulk tank were considered of the same PGFE subtype. One isolate from a bulk tank, three from IMI cases and four from environmental samples were positive in the DDST test. All eight DDST positive isolates harbored the bla_{shv} gene, one harbored the bla_{tem} gene, and three harbored the bla_{ct-m} gene, including the bulk tank isolate. Our study confirms that ESBL producing bacteria is present in different locations in dairy farms, and may be responsible for IMI. The detection of ESBLs on dairy herds could be a major concern for both public and animal health.

Kamran et al. (2014) isolated *Pasteurella multocida* from slaughtered healthy and dead or field cases of haemorrhagic septicaemia affected buffaloes from Sargodha division, Punjab. About eleven *Pasteurella multocida* isolates were subjected to identification and confirmation by various microbiological tests. All isolates were subjected to antibiotic susceptibility testing by disc diffusion

method and results of the study revealed that *Pasteurella multocida* were found highly sensitive to enrofloxacin (90.91 %) followed by gentamicin, chloramphenicol, ciprofloxacin and norfloxacin (72.73%). However, there was low level of susceptibility against ampicillin and amoxicillin (45.45%), amikacin (36.36%), sulfadiazine (18.18%) and erythromycin (18.18%). Finally, author concluded that resistance in *Pasteurella multocida* against the macrolides, sulfa drugs and beta lactam antibiotics has been increased and hence quinolones (enrofloxacin, ciprofloxacin and norfloxacin) and chloramphenicol were better choice for the treatment of hemorrhagic septicemia.

Podder et al. (2014) studied *Klebsiella* spp. from about 11 dairy farms in Newfoundland, Canada which was responsible for bovine clinical mastitis. Author discussed about inability and inadequacy of current methods, to distinguish between three gram negative organisms viz. *Klebsiella pneumoniae*, *Klebsiella variicola* and *Enterobacter cloacae*. Mass spectrometry and partial sequencing with *rpoB* gene, were done to distinguish between coliform organisms responsible for clinical mastitis. From total 61 collected samples detected as *Klebsiella* sp. again 45 samples were subjected to further analysis, wherein only 2 samples were reclassified as *Enterobacter cloacae* and complete samples from one farm were recognized as *Klebsiella variicola*. Finally author concluded that, *Klebsiella variicola* an environmental inhabitant which has not been previously isolated from mastitic milk may not be detected from milk samples in the past years due to the inability of standard tests to discriminate or distinguish it from other *Klebsiella* sp.

Qureshi and Saxena (2014) conducted a study over 100 cattle inclusive of both male-female and indigenous zebu-crossbreed cattle vaccinated with alum precipitated haemorrhagic septicaemia vaccine over a period of time for quantitative evaluation of antibody titer. The serum collected at 0, 42, 84 and 128 days post immunization were subjected to quantitation of antibody titer by Microtiter Agglutination Test (MAT), Indirect Haemagglutination Assay (IHA) and Monoclonal antibody based Indirect Enzyme Linked Immunosorbent Assay (ELISA). Author found that, ELISA mean titer (2.076) was higher than MAT (1.948) and IHA (2.020). Also, IHA was found sensitive tool compared to MAT, since mean IHA titer (2.020 ± 0.028) was higher than corresponding MAT titer (1.948 ± 0.030) on 42 day of post immunization.

Author concluded that, immune response to alum precipitated HS vaccine go on rising and attains the peak antibody titer at 42 day post immunization. Antibody titer showed slight reduction upto 84 day post immunization followed by significant decline over 128 day post immunization which confers very short term immunity and recurrence of the disease in vaccinated animals.

Rahal et al. (2014) reviewed the causes of respiratory affections in small ruminants. The author highlighted over the state of atmospheric air, climate, atmospheric pollution, oxidative stress along with bacterial etiological agents as determinants of respiratory disease in sheep and goats. Author mentioned *Pasteurella* sp. and *Klebsiella pneumoniae* were the commensal responsible for respiratory tract infection in sheep and goats. The ammonia and bacterial endotoxins were predisposing factors for respiratory tract infections by bacterial and viral pathogens. The broad spectrum antibiotics like fluoroquinolones were considered as effective in treating infection of sheep and goats. The author concluded that interaction between three sets of two-way communications between pathogen-environment, host-environment and host-pathogen leading to respiratory disease in small ruminants.

Tigga et al. (2014) studied swine pasteurellosis in outbraek suspected pigs of Ruwabandha (Bhilai), Anjora, Somni, Tedesara, Tirgajhola villages of Durg district in Chhattisgarh, India during August and September of 2011. The study was focused on isolation, characterization and antibiogram study followed by pathological changes of *Pasteurella multocida* infection in pigs. Nasal Swabs and blood samples from ailing pigs and heart blood and impression smears from morbid pigs were processed for detection and isolation of *Pasteurella multocida* by bacteriological methods. The test isolates were subjected to antimicrobial sensitivity profile by disc-diffusion method. The smears from heart blood, tissue impressions, Gram's staining and biochemical methods were employed for identification of the bacteria. Gross lesions were observed and histopathological examination was done. From antibiogram study conducted over swine isolates of *Pasteurella multocida*, it was found that they were 100% sensitive to Amoxicillin, Gentamicin, Enrofloxacin and showed 100% resistance to Ceftizoxim and Cloxacillin.

Kumar et al. (2015) conducted a study to isolate organism from bovine mastitic milk and to develop antibiogram for organisms prevalent in and around Ranchi, Jharkhand. About 150 cases of mastitis were processed for isolation of organisms followed by antibiotic sensitivity testing (AST). The organisms prevalent in the area were *Staphylococcus* spp., *Streptococcus* spp., *Escherichia coli*, *Pseudomonas* spp. and *Klebsiella* spp. The *Staphylococcus aureus* was most prevalent (37.25%) followed by *Escherichia coli* (11.76%), *Streptococcus* spp. (7.84%), *Pseudomonas* spp. (6.86%) and *Klebsiella* spp. (1.96%). The results of AST revealed that isolates showed maximum response to Ciprofloxacin (49.02%) followed by Ceftriaxone (42.16%), Enrofloxacin (37.25%), Gentamicin (34.31%), Amoxicillin+Sulbactam (33.34%), Ampicillin+Cloxacillin (18.63%) and Ceftiofur sodium (12.75%).

Langoni et al. (2015) conducted a study aimed at isolation of *Klebsiella pneumoniae* from ten dairy herds of Sao Paulo state of Brazil by applying routine microbiology cultures, biochemical tests and polymerase chain reaction. Under this study, milk samples from 1067 animals were screened from California mastitis test positive quarters. The environment in which dairy animals was kept were also targeted bulk tanks, whereas swab samples were collected from faeces, hind limbs of the animals, bedding and milking parlor.

Identification of *Klebsiella pneumoniae* was performed using conventional microbiology culture, biochemical assay and Polymerase Chain Reaction. Six cases of intramammary infection by *K. pneumoniae* were detected in six different cows in two farms. Moreover, *K. pneumoniae* was isolated in 77 swabs (34 from bedding in 9 farms, 7 from waiting rooms in 5 farms, 6 from milking parlors in 4 farms, 11 from rectums in six farms, and 19 from hindlimbs in 7 farms. Molecular analysis confirmed the agent was *K. pneumoniae*. At least one strain of the agent was identified in a certain site in all farms, showing the need of maintaining the hygiene in dairy farms. Mastitis caused by *K. pneumoniae* is even more severe due to its poor response to antibiotic therapy, rapid evolution to toxic shock and death of the animal. The organism was found to pose a serious public hazard.

Mahanti et al. (2015) conducted a study to detect Shiga toxin producing–*Escherichia coli* (STEC) from healthy goats of West Bengal, India. About 245 isolates from the 125 faecal samples were identified as *Escherichia coli*. The

isolates were screened for presence of *stx1*, *stx2*, *eaeA*, *ehxA* and *saa* genes by multiplex PCR (m-PCR). Out of 245 isolates almost 36 (14.7%) were detected possessing Shiga toxins genes i.e. *stx1* and *stx2*. It was found that *stx1*, *stx2*, *eaeA*, *ehxA* and *saa* were detected in 72.2%, 58.3%, 61.1%, 77.7% and 22.2% respectively from Shiga toxin producing-*Escherichia coli* (STEC) isolates. Antibiotic sensitivity testing of isolates revealed resistance to erythromycin (80.5%), amikacin (52.7%), cephalothin (50%), kanamycin (41.6%), neomycin (36.1%) and gentamycin (36.1%) and less frequently to norfloxacin (2.7%), enrofloxacin (2.7%), and ciprofloxacin (2.7%). Multidrug resistance was observed in eleven STEC isolates. Hence, STEC may pose serious health problems or occupational hazards to farmers living in close vicinity of such goat population.

Amaravathi et al. (2016) conducted a study on interstitial pneumonia of sheep. Out of 988 lung samples almost 187 suspected for pneumonia on the basis of gross lesions were histopathologically examined and subjected to bacterial isolations. Interstitial pneumonia was noticed in 5 cases. The results of histopathology revealed thickened alveolar septae with infiltration of mononuclear cells and macrophages. Bacterial isolation yields *Klebsiella spp.* from lungs affected with interstitial pneumonia along with *Edwardsiella spp.* and *Pseudomonas spp.*

Lakshmi and Jayavardhanan (2016) isolated and identified *Staphylococcus aureus* and *Escherichia coli* as a major bacteria causing mastitis in Herd of livestock farm, Mannuthy, Kerla. Milk samples collected from almost 50 mastitis affected animals were processed for isolation and identification of causative bacteria. From 50 samples, 28 contained gram positive and 19 contained gram negative organisms. Out of 28 gram positive about 18 (36%) were identified as *Staphylococcus aureus* and 13 (27%) out of 19 gram negative were confirmed as *Escherichia coli*.

Sahay et al. (2016) isolated the *Klebsiella pneumoniae* from pneumonic yak belonging to Shyro village of Arunachal Pradesh. The organism *Klebsiella pneumoniae* was detected in 18 hr old Brain Heart Infusion (BHI) enriched broth by multiplex PCR technique. On the basis of 16S rRNA sequence analysis and RNA secondary structure prediction, organism was found to be related to environmental *Klebsiella pneumonia* PB12 from River Mahananda from East

India and *Klebsiella pneumoniae* JPR 9 isolated from soil samples of Assam. The *in-vitro* antibiotic sensitivity testing revealed resistance to ampicillin.

Saminathan *et al.* (2016) in his review mentioned current status of some commonly occurring diseases of ruminants including haemorrhagic septicaemia (HS) with special reference to Indian scenario. Author claimed that haemorrhagic septicaemia found to be an endemic disease of cattle and buffaloes in India with relatively more severity in buffaloes than Cattle and young and young adults suppose to suffer more than older animals. Moreover, the disease is widely reported from bison, African buffalo, camels, elephants, horses, donkeys as well as from yak. The most prevalent serotypes found in India were B:2, A:1, A:1,3, A:3, A:4, A:3,4,12, F:3, D:1, D:3, F:1, F:4 and F:4, 12. In an Indian molecular study of MLST conducted by (Sarangi *et. al*, 2016), 10 different sequence types were identified as ST 122, ST 50, ST 9, ST 229, ST 71, ST 277, ST 129, ST 280, ST 281 and ST 282. The B:2 serotype in India in pigs causes sporadic septicaemic disease. Apart from buffaloes, sporadic occurrence of the disease with 62% outbreak rates in goats, 102% in sheep and 5% in pigs has been reported during 2007-2010.

Author emphasized on economic losses caused by haemorrhagic septicaemia. An estimated economic loss due to haemorrhagic septicaemia in India was Rs. 225 million per year. The economic loss due to haemorrhagic septicaemia has been calculated as Rs. 6816 per infected cattle and Rs. 10901 per infected buffalo. The total economic loss was Rs. 5255 crore estimated from throughout India. Among that, about 80.3% economic losses are due to direct effects of haemorrhagic septicaemia and 19.7% economic losses are due to indirect effects of haemorrhagic septicaemia. In haemorrhagic septicaemia, about 74.8% of the economic losses are due to affections in calves and 25.2% are due to losses in adults (Singh, 2014a). In India, haemorrhagic septicaemia is the leading cause of mortality and second most commonly encountered disease during 1991 to 2010 reported by National Animal Diseases Referral Expert System (NADRES).

Saxena *et. al.* (2016) conducted a study aimed at detection of *Pasteurella multocida* B:2 serotype in different animal species and exploration of genetic variations among different isolates of *Pasteurella multocida* sp. obtained from

different animal species compared to vaccine strain P52 of India. Total 42 isolates were analysed by Enterobacterial intergenic consensus sequence (ERIC)-PCR and organisms were categorized into 22 different profiles including some of Maharashtra state, specially isolated from buffaloes followed by cattle. *Pasteurella multocida* serotype B:2 was isolated from cattle, buffalo, sheep, goat, pig and wild animals (leopard and tiger).

At the end of study author concluded that there exist a genetic diversity, no geographic or host specificity among field isolates of *Pasteurella multocida* serotype B:2 isolated from various states of India. The highest percentage of variation was found in Cattle. But, there appears similarity in profiles between some wild and domestic animals indicating the transfer of bacteria amongst themselves. Vaccine strain P52 used for preparation of killed vaccine in India requires re-evaluation since it was found to be distantly related to field isolates and probably may be the cause of vaccination failure in India. Haemorrhagic septicaemia is considered to be the disease of mostly cattle and buffalo, however many isolates of serotype B: 2 were obtained from pigs, sheep and goats, which clearly indicated that host range of organism has become wide.

Zamri-saad and Annas (2016) highlighted the problem of vaccination failure of haemorrhagic septicaemia, vaccination coverage and vaccination regime. Being acute and highly fatal disease of animals, treatment with antibiotics only could not limit the disease and so researchers were focusing on vaccine development. Vaccination against hemorrhagic septicemia was started early in 1928. Both oil adjuvant and broth bacterin vaccine failed to gain field popularity and associated with post vaccination complications. The reports regarding vaccination failure due to improper annual vaccination, poor vaccination regime and inability to vaccinate large number of animals were well known. To overcome the difficulty to reduce large area under consideration and large number of animal population, OIE suggested implementation of zoning concept. It has been observed that vaccination coverage upto 70% leads to sporadic occurrence of disease and suggested as minimum criteria of population coverage.

2.2 Ethnoveterinary and ethnomedicinal plants used as an antibacterial:-

Das et al. (2009) evaluated antibacterial activity of aqueous and methanolic leaf extracts of *Spathodea campanulata* and *Tridax procumbens*

against bacteria isolated from clinical cases of bovine mastitis such as *Staphylococcus aureus* (coagulase positive and negative), *Escherichia coli*, *Streptococcus agalactiae*, *Streptococcus uberis* and *Klebsiella pneumoniae*. Both plants showed significant antibacterial activity. The methanolic extracts of *Spathodea campanulata* and *Tridax procumbens* showed significant activity however the aqueous extracts showed no observable effects.

Sharma et al. (2009) screened 15 medicinal plants used by tribals against urinary tract infection (UTI) causing organisms against human isolates. The antibacterial activity of aqueous, ethanol and acetone extracts of *Coriander sativum*, *Abutilon indicum*, *Boerhavia diffusa*, *Andrographis paniculata*, *Plantago ovata*, *Bacopa monnieri*, *Bauhinia variegata*, *Flacouratia ramontchi*, *Embelia tfergium*, *Euphorbia ligularia*, *Zinziber officinale*, *Terminalia chebula*, *Azadirachta indica*, *Ocimum sanctum* and *Cinnamomum cassia* was determined against 33 UTI isolates obtained from district hospital, Mandla, Madhya Pradesh, India. The isolation and identification of bacteria were done by standard protocol and found to be *Proteus mirabilis* (10), *Escherichia coli* (6), *Proteus vulgaris* (6), *Klebsiella pneumoniae* (5), *Enterobacter cloacae* (2), *Providencia pseudomallei* (2), *Pseudomonas aeruginosa* (1) and *Klebsiella oxytoca* (1) which then were screened by disc diffusion method.

Mubarack et al. (2011) studied the effects of extracts of three ethnoveterinary plants i.e *Asteracantha longifolia*, *Dactyloctenium indicum* and *Trichodesma indicum* collected from Coimbatore, Tamil Nadu. The aqueous and methanolic extracts of mentioned plants were tested against *S. aureus*, *E. coli*, *S. agalactiae* and *K. pneumoniae* isolated from bovine udder affected with mastitis. *Asteracantha longifolia* showed significant antibacterial activity against all the tested bacteria and remaining two plants showed the moderate type of activity. No extract were found to be effective against *Klebsiella pneumoniae*.

Raja et al. (2011) screened the antimicrobial potential of three ethnomedicinal plants *Chassalia curviflora* (*C. curviflora*) , *Cyclea peltata* (*C. peltata*) and *Euphorbia hirta* (*E. hirta*) used in folk medicines in Aarukani hills Kani tribe, Tamil Nadu, India against human bacterial pathogens. The author concluded that the *C. curviflora*, *C. peltata* and *E. hirta* are good source of antibacterial agents. Antibacterial efficacy was performed by disc diffusion

method against the selective standard pathogens viz., *Escherichia coli* (*Escherichia coli*) (ATCC 35218), *Staphylococcus aureus* (*S. aureus*) (ATCC 6538), *Salmonella typhi* (*S. typhi*) (MTCC 733), *Proteus vulgaris* (*P. vulgaris*), *Proteus mirabilis* (*P. mirabilis*) and *Streptococcus pyogenes* (*S. pyogenes*) and incubated for 24 hrs at 37°C. The plant *Cyclea peltata* showed highest degree of antibacterial activity against three organisms, which was followed by *Chaslia curviflora*. The plant *Euphorbia hirta* showed relatively low antibacterial activity with susceptibility of only one bacterium.

Doss et al. (2012) conducted an in-vitro antibacterial screening of some local wild ethnoveterinary plants used by tribals against pathogens isolated from clinical cases of bovine mastitis such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Streptococcus agalactiae*. The plants selected are *Dactyloctenium indicum* L., *Argemone mexicana* L., *Calotropis gigantea* L., *Trigonella foenum-graecum* L., *Acacia leucophloea*., *Acacia nilotica* L., *Achyranthes aspera* L., *Acalypha indica* L., *Coccinia grandis* L., *Asteracantha longifolia* L., *Abutilon indicum* L., *Cenchrus ciliaris* L., *Brachiaria* sp., *Trichodesma indicum* L., *Melinis repens*, *Barleria lupulina*., *Chloris inlata* L., *Medicago sativa* L., *Spermacoce hispida* L., *Rhynchosia capitata*, etc. collected from gardens and villages in and around Coimbatore, Tamil Nadu. Amongst all candidate plants three plants *Asteracantha longifolia*, *Rhynchosia capitata* and *Cenchrus ciliaris* possesses potential antibacterial activity. The most pronounced activity with inhibition zones of more than 24.0 mm was shown by methanol extract (inhibition zone of 25 mm against *S. aureus* at concentration 200mg /ml) of *A. longifolia* and aqueous extract (inhibition zone of 24 mm against *S. aureus* at concentration 200mg/ml) of *R. capitata*.

Kalayou et al. (2012) screened nine ethnoveterinary plants of Tigray region of Ethiopia for antibacterial activity. Some traditionally used plants *Achyranthes aspera*, *Ficus caria*, *Malva parviflora*, *Vernonia species.*, *Solanum hastifolium*, *Calpurinia aurea*, *Nicotiana tabacum*, *Ziziphus spina-christi* and *Croton macrostachys* were extracted with hydroalcoholic (70% methanol) solvent by maceration procedure. The extracts were tested against field isolates of bacteria obtained from equines. The antibacterial activity was tested by using disc diffusion method and broth dilution method at two different concentrations (100 & 200 mg/ml). Results of the study revealed that eight plants possess

antibacterial properties with dose dependent inhibition of bacteria. Plants *Calpurinia aurea*, *Croton macrostachys*, *Nicotiana tabacum*, *Achyranthes aspera* and *Vernonia sp.* were showing promising activity. Based on results of the study, author suggested use of these plants in wound infection, mastitis and gastrointestinal troubles.

Shinde and Mulay (2015) studied in-vitro antibacterial activity of leaf extract of some common Indian medicinal plants *Azadiracta indica* (Neem), *Cymbopogon citratus* (Lemon grass), *Mentha arvenis* (Pudina) and *Ocimum sanctum* (Tulsi) against four human pathogens *Escherichia coli*, *Salmonella typhi*, *Shigella sp.* and *Staphylococcus aureus* using agar well diffusion method. Growth of all test pathogens was inhibited by methanolic extracts of the plants, while chloroform extracts of the plants were found to be ineffective against all test pathogens. Amongst the four plants used, highest antibacterial activity was shown by *Azadiracta indica* (Neem). *Salmonella typhi* was found to be most sensitive while *Escherichia coli* was found to be least sensitive to different plant extracts.

2.3 *Limonia acidissima* as an antibacterial: -

Nadkarni (1954) in Indian Materia Medica mentioned that leaves of *Limonia acidissima* possess purgative and sudorific properties. Even the leaves can be used to treat snake bite. Dried fruits diminish intestinal fermentation.

Thomas and Ponnammal (2005) studied antibacterial activity of methanolic extract of various parts of *Limonia acidissima* against *Escherichia coli* and *Staphylococcus aureus*. The author discussed about utility of leaves in bowel infection of children, indigestion and carminative property. The extract of the leaves has shown almost 21 mm and 28 mm zone of inhibition against *Escherichia coli* and *Staphylococcus aureus* respectively, compared to pulp extract showing 40 mm and 43 mm zone size confirming highest activity. The methanolic extract of fruit pulp exhibited highest activity against both organisms followed by seeds, leaf, bark and rind. Hence, it seems clear that leaves possess poor antibacterial activity.

Deshpande (2006) has mentioned about botanical details, chemical constitution, biological activities and use of plant. The plant was described with

botanical name *Feronia limonia* or *Feronia elephantum* and with Marathi name Kavath or Kavith and categorized under family Rutaceae. The leaves were known to contain tannins, volatile oils, psoralen, bergapten, orientin, vitexin, saponarin, anisic oil, methylchavicol, trans-anethole, thymol, p-cymen, 7-ol, cis-anethol and estragole. Further, it was claimed that leaves bear an astringent, antidiarrhoeal, antiscorbutic, haematinic and stomachic activities. Looking towards general usage, leaves were used in diarrhoea, dysentery, intestinal flatulence, etc. indicating towards antibacterial activity.

Patil et al. (2012) in his experimental studies claimed that methanolic extract of fruit pulp of *Limonia acidissima* shows prominent antibacterial activity. Author mentioned that, flavonoids from plant parts (except bark) bear antimicrobial properties and coumarins from plant parts (except fruit pulp) possess antibacterial properties. However, author showed in his results that no bacteria being inhibited by methanolic leaf extracts, indicating poor antibacterial activity attributed to leaf.

Hossain (2013) evaluated the antibacterial and cytotoxic activity of fruit pulp petroleum ether and ethyl acetate extracts against ten pathogenic bacteria. The author claimed that extract possess moderate type of antibacterial activity. The maximum zone of inhibition was found against *Salmonella paratyphi B* and *Salmonella paratyphi* with 15 mm diameter at concentration of 500 ug/disc followed by *Staphylococcus aureus*, *Vibrio mimicus*, *Shigella butea* and *Shigella bordetella* with 14 mm zone of inhibition.

Jayashree and Londonkar (2014) performed investigation of antimicrobial potential of *Feronia limonia* Linn. against some gram negative bacteria like *Salmonella typhimurium* (MTCC 98), *Klebsiella pneumoniae* (MTCC 432), *Escherichia coli* (MTCC 45), *Pseudomonas aeruginosa* (MTCC 647). The petroleum ether, chloroform, methanolic and aqueous extracts were screened for their antibacterial activity. The results revealed that methanolic extract possess highest and chloroform extract possess lowest antibacterial activity.

Naidu et al. (2014) screened hexane, chloroform and methanol extracts of leaves of *Limonia acidissima* for *In-vitro* antibacterial activity against four Gram+ve and five Gram-ve bacteria. Methanol extract showed highest, chloroform extract showed mild to moderate and hexane extract showed lowest

zone of inhibition. A result of their screening confirmed their use against *E. coli*, *P. vulgaris* and *S. pneumoniae*. Based on observations of this study author further suggested that, antimicrobial activity of leaves of *Limonia acidissima* attributed to polar compounds since methanolic extract proved most effective in this study compared to other extracts.

Pandey et al. (2014) performed phytochemical analysis of pulp and rind of *Limonia acidissima* fruits for primary detection of phytoconstituents. The extract was found to contain alkaloids, phenols, flavonoids and saponins. Again the extract was subjected for detailed analysis by GC/MS technique.

Vijayvargia and Vijayvargia (2014) in their review on *Limonia acidissima* mentioned the antibacterial potential of plant and explained that the ethanolic extract of leaves of plant show broad spectrum activity against Gram+ve and Gram-ve bacteria.

Anebaracy et al. (2015) conducted study of antimicrobial activity of ethanolic extract of root of *Limonia acidissima*. The *Bacillus subtilis* and *Pseudomonas aeruginosa* were selected as test organisms and assayed by well diffusion method with each well containing 50 ul of extract. The zone of inhibition was found to be 5.98 $\pm$ 0.03 and 5.21 $\pm$ 0.01mm res. for *Bacillus subtilis* and *Pseudomonas aeruginosa*.

Lunkad et al. (2015) studied antibacterial activity of petroleum ether extract of leaves of *Limonia acidissima* against one Gram +ve (*Staphylococcus aureus*) and one Gram –ve (*Escherichia coli*) bacteria by well diffusion method. The finding showed significant activity against these bacteria and hence they claimed that leaves of plant *Limonia acidissima* can be used to treat infections.

Ponnuraj et al. (2015) studied antibacterial activity of methanolic extract of fruit pulp and also done spectroscopic analysis of extracts which leads to identification of phytoconstituents. The antibacterial activity showed dose dependent inhibition of clinical pathogens at varying concentrations i.e 100 to 500 mg/ml with highest zone of inhibition on 500 mg/ml. The methanolic extract showed highest zone of inhibition against *Salmonella typhi* with 27 mm, however it showed lowest zone of inhibition against *Bacillus cereus* with 19.3 mm.

MATERIALS & METHODS

The present study was carried out in the Department of Veterinary Pharmacology and Toxicology of Nagpur Veterinary College, Nagpur the constituent college of Maharashtra Animal and Fishery Sciences University, Nagpur. The facilities required for this work were provided by respective departments. Present study was designed to check the ethnoveterinary use of plant *Limonia acidissima* L. by exploring an antibacterial potential of this plant. To study above said objective various parameters like determination of zone of inhibition by disc-diffusion method, minimum inhibitory concentration by tube dilution and microdilution assay method, qualitative analysis of leaf extracts for presence of various phytochemicals and detailed analysis by Gas Chromatography-Mass Spectrometry technique.

3.1 Chemicals

Nutrient agar (NA), Nutrient Broth (NB), Mueller Hinton Agar (MHA), standard discs of Ciprofloxacin, Ceftriaxone, Gentamicin and Dimethylsulfoxide (DMSO) supplied by Hi-media and Merck were used. Active Pharmaceutical Ingredients (API) of standard drugs Ciprofloxacin, Gentamicin and Ceftriaxone provided by Zydus Pvt. Ltd. were used.

3.2 Bacterial strains

The lyophilized standard pathogenic strains of gram negative bacteria three namely *Pasteurella multocida* (MTCC-1148), *Escherichia coli* (MTCC-739) and *Klebsiella pneumoniae* (MTCC-109) were selected for the study and procured from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology, Chandigarh. The lyophilized bacterial cultures were revived as per the instructions given by Microbial Type Culture Collection (MTCC), Chandigarh.

3.3 Selection and Collection of Plant Material

The branches containing fresh green leaves were selected for experiment and procured from matured and naturally grown tree in the month of August. The selected tree was located in the premises of Department of Poultry Sciences, Nagpur Veterinary College, Nagpur.

3.4 Identification and authentication of plant

The collected plant material was identified by the Botany Department of Rashtrasant Tukdoji Maharaj University, Nagpur with the assistance of expert botanist. The plant was identified as *Limonia acidissima* and herbarium sheet with appropriate information regarding identified plant species were filled up by botanist and given authentication number **10056**. One herbarium sheet was deposited in the respective department for future reference and records. The picture of herbarium sheet of twinge of *Limonia acidissima* with identification details is shown in Plate1.

3.5 Preparation of extracts

The extraction was done as per the method suggested by **Rosenthaler (1930)** with little modifications adopted from methodologies employed by **Betoni et al., (2006)** and **Mahomoodally and Dilmohamed (2016)**. The extraction of leaves was done by cold exhaustive maceration method. The collected plant leaves were air dried under shed at normal room temperature. Dried leaves were subjected to grinding and converted into somewhat coarse powder form. Then 100 gm leaf powder was dispensed into stoppered round bottom long neck flask and added with 400 ml distilled water which is shown in Plate 2. The plant powder was allowed to macerate for 24 hrs at room temperature with vigorous intermittent shaking of the flasks for 10 - 15 minutes after every 2 - 3 hrs in order to facilitate proper dissolution and hence extraction. After completion of maceration process the contents present in flask were taken out and filtered through muslin cloth with care that complete filtrate was obtained. Filtrate collected in this manner was again subjected for further filtration by Whatman filter paper no. 1. The final filtrate was collected in a petridish (weighed already). The marc remained after filtration in above process was again added with 400 ml water and allowed to macerate for next 24 hrs. Similar process was repeated for third time till complete decolorization of marc occurred and filtrates obtained through second and third maceration process were combinely evaporated on hot water bath at 50°C with previously obtained filtrate. Finally solvent free, semi-solid aqueous extract was obtained.

The entire procedure was repeated in similar manner for different solvents like ethanol, acetone and petroleum ether. Finally, after completion of the

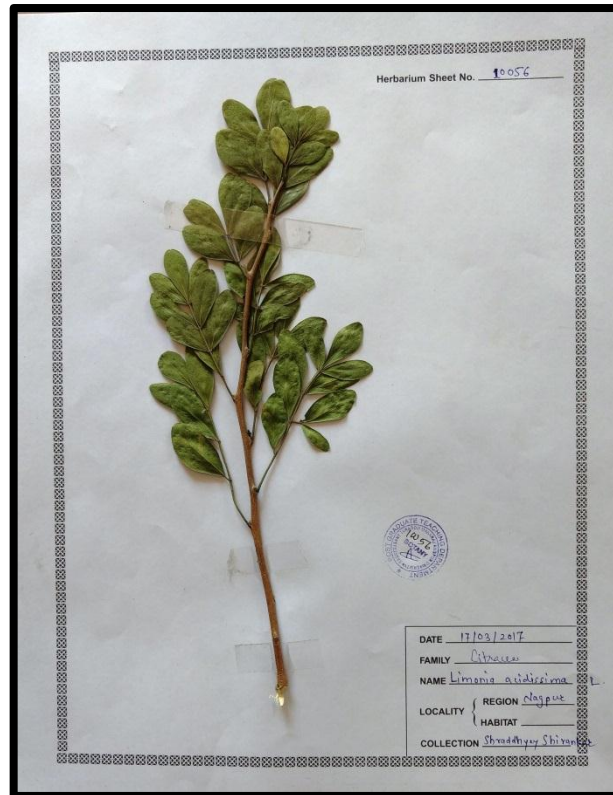


Plate 1: Showing herbarium sheet of *Limonia acidissima* with identification details.



Plate 2: Showing flasks containing powdered leaf plant material macerated with water, ethanol, acetone and petroleum ether.

procedure, cold macerated extracts of ethanol, acetone and petroleum ether were obtained. The extracts thus obtained were stored in dessicator for further use. The petriplates containing solvent free extracts obtained with an individual solvent are shown in Plate 3. All the solvents used for this extraction were analytical grade supplied by Hi-media. The percentage extractability has been calculated from above information for every individual extract.

3.6 Phytochemical study for qualitative analysis of certain active principles

The preliminary qualitative phytochemical analysis was done to detect presence or absence of various phytoconstituents in given leaf extracts of *Limonia acidissima* as per method described by **Rosenthaler (1930)** and **Raaman (2006)**.

1. Test for Alkaloids

About 50 milligrams of extract was stirred with dilute hydrochloric acid and filtered. The filtrate was then subjected to following tests.

(A) Mayer's test

Few millilitres of filtrate were taken in a test tube and few drops of Mayer's reagent were added along side wall of test tube. Formation of white or creamy precipitate indicates positive test.

Mayer's reagent: Mercuric chloride (1.358 grams) was dissolved in 60 millilitres of water and potassium iodide (5 grams) was dissolved in 10 millilitres of water. These two solutions were mixed and volume was made up to 100 millilitres with water.

(B) Wagner's test (Rosenthaler, 1930)

To the little amount of filtrate, Wagner's reagent was added. Appearance of brown flocculent precipitate indicates the presence of alkaloids.

Wagner's reagent: Iodine 1.27 grams and 2 grams of potassium iodide were dissolved in 5 millilitres distilled water and solution was further dissolved in water to make final volume 100 millilitres.

(C) Hager's test

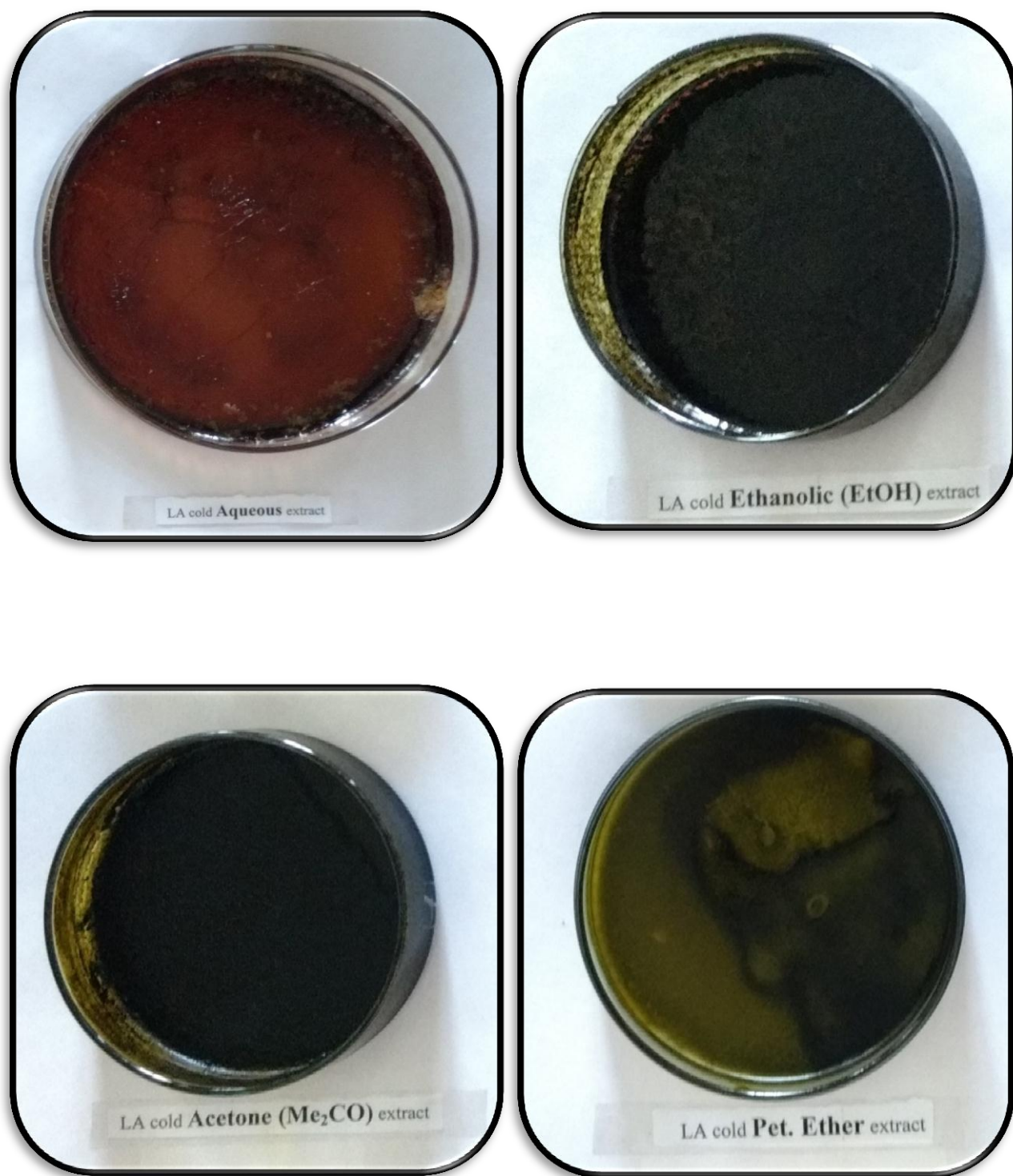


Plate 3: Showing petri plates of various types of leaf extracts of *Limonia acidissima* Linn. with aqueous, ethanolic, acetone and petroleum ether extracts from upper left to lower right figure res.

Few millilitres of filtrate were taken in a test tube and one or two millilitres of Hager's reagent were added. A prominent yellow color precipitate indicates positive test.

Hager's reagent: A saturated aqueous solution of picric acid was made.

(D) Dragendorff's test

To a few millilitres of filtrate, 1 or 2 millilitres of Dragendorff's reagent were added. A prominent yellow precipitate indicates positive test.

Dragendorff's reagent: It was prepared by mixing solution A (17 grams of Bismuth subnitrate + 200 grams of tartaric acid + 800 millilitres of distilled water) and solution B (160 grams of potassium iodide + 400 millilitres of distilled water) in 1:1 proportion (V/V).

2. Test for carbohydrates

Extracts (100 milligrams each) were dissolved individually in 5 millilitres of water and filtered. The filtrates were then subjected to following test.

(A) Fehling's test:

About 1 millilitre of filtrate was taken in a test tube and added with 1 millilitre of Fehling A and 1 millilitre of Fehling B solution and mixed well by shaking. The test tube was heated on water bath for 2 minutes. Appearance of red precipitate indicates positive test.

Fehling - A solution: Copper sulphate (34.66 grams) was dissolved in distilled water and volume made upto 500 millilitres.

Fehling - B solution: Potassium sodium tartarate (173 grams) and sodium hydroxide (50 grams) were dissolved in water and volume made upto 500 millilitres.

(B) Benedict's test

About 0.5 millilitre of filtrate was taken in a test tube and 0.5 millilitre of Benedict's reagent was added. The mixture was heated over boiling water bath for 2 minutes. A characteristic colored precipitate indicates test as positive.

Benedict's reagent: Sodium citrate (173 grams) and sodium carbonate (100 grams) were dissolved in 800 millilitres of distilled water and boiled to make

it clear. Copper sulphate (17.3 grams) dissolved in 100 millilitres distilled water was added to it.

3. Test for glycosides

The extract (50 milligrams) was dissolved in concentrated hydrochloric acid for 2 hrs on water bath, this hydrolysate was filtrated and filtrate was used for following test.

Legal's test

About 50 milligrams of extract was taken in a test tube and small amount of pyridine was added to it and mixed well. After that sodium nitroprusside followed by 10% sodium hydroxide was added. Development of pink color indicates positive test.

4. Test for saponins

The extract (50 milligrams) was taken in stoppered test tube and finally diluted upto 20 millilitres by adding distilled water. The tube was shaken for 15 minutes and observed for formation of foam. A two centimeter foam layer indicates positive test.

5. Test for proteins and amino acids

The extract (100 milligrams) was dissolved in 10 millilitres of distilled water and filtered through Whatman filter paper no. 1 and filtrate was again used for following test.

(A) Biuret test (Rosenthaler, 1930)

A few milligrams of residue were taken in water and 1 millilitre of 1% solution of sodium hydroxide was added followed by a drop of 1% solution of copper sulphate. Violet pink color development indicates positive test for proteins.

(B) Ninhydrin test

Two millilitre of filtrate was taken in test tube and few drops of ninhydrin solution were added. A characteristic purple color indicates positive test for presence of amino acids.

Ninhydrin solution: About 10 milligrams of ninhydrin dissolved in 200 millilitres of acetone

(C) Xanthoprotein test (Rosenthaler, 1930)

A little residue was taken in 2 millilitres of water and to it 0.5 millilitre concentrated nitric acid was added. Appearance of white or yellow precipitate indicates presence of proteins.

6. Test for phytosterols

Salkowski's test (Rosenthaler, 1930)

A small amount of extract was taken in 2 millilitres of chloroform and sulphuric acid was added alongside of test tube and test tube was shaken. Red color development in the chloroform layer and greenish yellow fluorescence in the lower layer indicates presence of sterols.

7. Test for phenolic compounds

(A) Ferric chloride test

About 50 milligrams of extract were dissolved in 5 millilitres of distilled water and transferred to test tube and to this 5% neutral ferric chloride solution was added. Development of dark green color indicates presence of phenolic compounds.

(B) Lead acetate test

About 50 milligrams extract was dissolved in distilled water and 3 millilitres of 10% lead acetate solution were added. A bulky white precipitate indicates presence of phenolic compounds.

8. Test for flavanoids (Rosenthaler, 1930)

A small quantity of residue was dissolved in 5 millilitres of ethanol (95%) and treated with a few drops of concentrated hydrochloric acid and 0.5 gram of magnesium metal turnings. Development of either pink or red color indicates presence of flavonoids.

9. Test for resins (Rosenthaler, 1930)

The alcoholic extract was dissolved in alcohol. To this, a few drops of water were added. The appearance of turbidity was considered as a positive test.

3.7 Determination of antibacterial activity

3.7.1 Preparation of media and plates

The agar was prepared as per the guidelines given by Hi-media. The dehydrated powdered media of Mueller Hinton Agar (MHA) was weighed accurately and taken in sterile conical flask and added with appropriate amount of distilled water. The flask was put for autoclaving at 15 psi pressure (Pound force per inch) for 15 minutes at 121°C. About 20-25 milliliters of autoclaved liquid agar media was poured into the sterile petri plates and allowed to solidify under laminar flow. After solidification of agar, the plates were kept in incubator at 37°C for 5 - 6 hrs to ensure sterility and avoid any contamination of plates. Plates with no visible growth were used for performing antibiotic sensitivity test.

3.7.2 Preparation of bacterial inoculums

Three sterile test tubes were taken and 2-3 milliliters of nutrient broth were added in each tube. A loop of single, isolated colony of *Pasteurella multocida* (MTCC-1148), *Escherichia coli* (MTCC -739) and *Klebsiella pneumoniae* (MTCC-109) were inoculated into three different tubes simultaneously and denoted with strain number on respective tubes. Inoculated tubes were incubated for 4 to 5 hours. After incubation, the tubes were spectrophotometrically matched at 620 nm by using nutrient broth to get final optical density of 0.08 which exactly yields starting number of $1 - 2 \times 10^8$ CFU/ml bacteria (Basri and Nor, 2014 and Balouiri, et al. 2016). The spectrophotometrically adjusted inoculum were used within 15 to 30 minutes of standardization in order to prevent change in number of bacteria.

3.7.3 Preparation of stock solution of extracts for various tests

Stock solutions required for various tests were prepared in following way. For well diffusion test 2, 4, 8, 16, 32 and 64 mgs of extracts were weighed accurately and dissolved in 280 µl of dimethyl sulfoxide (DMSO), stored in sterile vials and vortexed for 5 minutes for uniform mixing. With micropipette 70 µl was taken out from each vial which corresponds to 0.5, 1, 2, 4, 8 and 16 mgs respectively.

For microdilution assay 96 mg of extract was weighed accurately dissolved in 200 µl DMSO in sterile vial and vortexed for 5 minutes. From vial 50 µl extract was taken with the help of micropipette which exactly measures to 24mg initially and serially diluted while performing assay.

For tube dilution test exact 256 mg of extract was weighed and dissolved in 800 µl DMSO in sterile vial and vortexed for 5 minutes. About 200 µl dissolved extract was taken out from vial which contains 64mg extract initially and serially diluted further.

3.7.4 Well - diffusion method

The well-diffusion method proposed by **Ncube, et al. (2008)** was adopted with slight modifications. The Mueller Hinton Agar (MHA) was used to grow bacterial culture. About 100 microlitres (µl) of 5 to 6 hours old bacterial cultures or inoculums of each bacterial strain diluted with nutrient broth upto final optical density of 0.08 was seeded and spread evenly over the surface of agar by using L-loop spreader. The wells of 8 millimetres (mm) diameter size were punched into agar with sterile cork borer and after punching each well was sealed with 15 microlitres (µl) of MHA. Such seven similar wells were punched out with one in central position serving as negative control and six in series loaded with different concentrations of extracts. Then standard antibiotic sensitivity testing disc Ciprofloxacin (10µg), Gentamicin (10µg) and Ceftriaxone (10µg) were applied over inoculated agar surface serving as standard positive control. After this, central well was added with 70 microlitres (µl) of DMSO and other wells with 70 microlitres (µl) of increasing concentrations of extract i.e 0.5, 1, 2, 4, 8 and 16 mgs in respective wells. The plates prepared in this way were kept for incubation at 37°C for 18 - 24 hrs. After completion of incubation period the size of zone of inhibition was measured by zone scale reader provided by Hi-media.

3.7.5 Microdilution Assay

The microdilution assay was performed by method proposed by **Balouiri, et al. (2016)** and **Klancnik, et al. (2010)** with little modifications. Assay was performed in sterile flat bottom 96 well microtitre plate possessing 8 rows and 12 columns. Initially all the wells were added with 50 µl of nutrient broth except the wells of last column (12th) wherein 100 µl nutrient broth was added. The very first well of first column was added with 50 µl of extract containing 24 mg

concentration of drug, mixed properly with the help of pipette and 50 µl content of first well was transferred to second well. Similar serial double fold dilution was made across the column till eighth well was reached which yields 24mg, 12mg, 6mg, 3mg, 1.5mg, 0.75mg, 0.375mg and 0.1875 mg from first to last well and 50 µl was discarded from eighth well. This process was repeated in next two columns to produce triplicate results. Exact similar process was done for standard drug gentamicin, ciprofloxacin and ceftriaxone in duplicates with initial concentration of 0.25 mg in first well of 4th- 5th, 6th- 7th and 8th- 9th column respectively and diluted serially across respective columns. The 50 µl of spectrophotometrically adjusted inoculum to optical density of 0.08 at 620 nm consisting 1×10^8 CFU/ml organisms were added in all the wells except wells of last column. The last three columns served as controls. The tenth column contained 50 µl broth and 50 µl inoculum. The eleventh column contained 40 µl broth, 10 µl DMSO and 50 µl inoculum. The twelfth column contained only 100 µl nutrient broth without bacteria. The microtitre plate was covered with lid appropriately so that air does not get the chance to enter inside plate. The reactions set in above manner were incubated at 37°C for 18 hrs.

The plates were read over ELISA multiscan reader at 620 nm after the completion of incubation period. The well with practically zero absorbance were considered as minimum inhibitory concentration (MIC). After reading plates 15 µl resazurin dye were added in all the wells to check presence of growth. The plates were further incubated for next 6 to 8 hrs at 37°C. Plates were examined visually for any change in colour.

3.7.6 Tube dilution method

The tube dilution method explained by **Das et al. (2013)** was employed for the determination of minimum inhibitory concentration (MIC) of plant extracts with some necessary modifications. A series of ten sterilized test tubes were arranged in a series. All the test tubes were marked properly with name of bacteria and type of extract. In the first and last tube 1.6 millilitres of nutrient broth were taken and 0.9 millilitre in rest of all tubes. The first and last tube was added with 200 µl of 64 mg extract and 200 µl of DMSO making final volume upto 1.8 millilitres in first and last tube respectively and mixed well. About 0.9 millilitres from first tube were transferred to second tube and from second into third tube. This serial double fold dilution was made upto 8th tube which gives 32mg, 16mg,

8mg, 4mg, 2mg, 1mg, 0.5 mg and 0.25mg from first to last tube and 0.9 millilitres was discarded from eighth tube and also from tenth tube. The ninth tube contained only nutrient broth and therefore ninth and tenth tube served as positive control. At last 100 μ l of spectrophotometrically standardized inoculum at 620 nm to optical density of 0.08 was added in all ten tubes so as to make up total volume upto 1 millilitre. The whole set of test tubes were incubated at 37°C for 18 - 24 hrs.

After completion of incubation period the tubes were observed for presence of bacterial growth. The lowest concentration of the extracts that inhibits growth of the organisms, as detected by lack of visual turbidity, was designated as the minimum inhibitory concentration (MIC) of that extract. The 100 μ l content from first three to four tubes were sub-cultured on separately prepared Mueller Hinton Agar (MHA) plates and incubated at 37°C for next 18 - 24 hrs. The volume remained in tubes after sub-plating were again added with 20 μ l of resazurin dye as an indicator of bacterial growth. Resazurin dye added tubes were again incubated for next 6 - 8 hrs at 37°C.

3.8. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Preparation of the sample for gas chromatography:

The crude sample of *Limonia acidissima* aqueous leaf extract was mixed and dissolved with ethanol and filtered with 0.45 micron. The particle-free extracts were taken in a syringe and injected into GC-MS instrument. The injector volume of 2 μ l for analysis was fed into the system.

Gas chromatograph with a mass spectrometer:

Gas chromatograph with a mass spectrometry (GC-MS/MS) was performed with an instrument Make-Bruker Scion, Model-TQ MS System for the analysis of plant extract sample under following chromatographic conditions. The machine was equipped with the column DB-5MS Agilent (30m x 0.25mm1D), composed of 100% dimethyl polysiloxane. For GC-MS detection, electron ionization system with ionization energy of 70eV was used. Helium gas (99.99%) was used as the carrier gas at constant flow rate 1.0 ml/min with a split ratio of 10:1. The oven temperature was operated in following manner: 40°C held for 1 min, raising at the rate of 20°C min⁻¹ up to 150°C then, raising at the rate of 3°C

min⁻¹, hold for 0 min and raising at the rate of 20°C min⁻¹ up to 300°C with 10 min held, injector temperature and volume 250°C and 2µL, respectively. The total GC running time was about 36 min.

The mass spectrometer (MS) operating conditions were ionization voltage 70 eV, source temperature of 250°C, the inlet line temperature of 280°C, mass scan (m/z)-30-500, solvent delay: 3.0 min, with total MS running time for 34 min. The data was evaluated using total ion count (TIC) for compound identification and quantification. The mass spectra of compounds were identified by comparing the mass spectra obtained from their related chromatographic peaks with NIST mass spectral libraries. Further the identified compounds were searched over online sources and various literatures for detailed information.

RESULTS AND DISCUSSION

The current experimental study was conducted to ensure and validate traditional use of plant *Limonia acidissima* against common bacterial pathogens with special emphasis being given to bacteria *Pasteurella multocida*. Four different leaf extracts namely aqueous, ethanolic, acetone and petroleum ether were tested against three gram negative bacteria *Pasteurella multocida* (MTCC-1148), *Escherichia coli* (MTCC-739) and *Klebsiella pneumoniae* (MTCC-109). In order to confirm antibacterial potential of this plant various methods were employed with different concentrations of extracts under study and compared simultaneously with standard drug gentamicin, ciprofloxacin and ceftriaxone. Results obtained in the study are presented and discussed here under in this chapter.

4.1 Extractability percentage

The fresh procured leaves of matured plant *Limonia acidissima* were shed dried and subjected to cold exhaustive maceration with four different solvents i.e. distilled water, ethanol, acetone and petroleum ether. The percent extractability of *Limonia acidissima* leaves in water, ethanol, acetone and petroleum ether solvents were calculated as 24, 14, 2 and 1 percent respectively. The percent extractability, colour and consistency of individual extract are given in Table 1.

The percentage of extractability obtained through this extraction process is in accordance with the results obtained in the previous work done by various researchers. The highest percentage of extractability was obtained through aqueous extract i.e. 24%. However, percentage of extractability in water is not found in reviewed literature. The percentage of extractability with ethanol is recorded as 14% in present study which is almost double as compared to the extractability (7.89%) mentioned by **Panda et al., 2013**. The extractability with petroleum ether has been mentioned as 1% by **Panda et al., 2013** which is similar to our results. However, **Baneerjee et al., 2011** mentioned the total yield of 1.12 grams of petroleum ether extract; acetone extract 2.34 grams and alcohol extract 2.21 grams from 25 grams of leaves, which comes to 4.48, 9.36 and 8.84% respectively. As compared to this in present study petroleum ether and acetone extracts were less and alcoholic extract was yielded more (14%).

Table No. 1. The colour, consistency and percent extractability of aqueous, ethanolic, acetone and petroleum ether extract of leaves of plant *Limonia acidissima*.

Sr. No	Type of extract	Quantity used	Colour	Consistency	Percentage of extractability
1	Aqueous	100 gm	Brown or coffee colour	Semisolid and pasty	24
2	Ethanolic	100 gm	Dark olive green	Semisolid and pasty	14
3	Acetone	100 gm	Dark green	Semisolid	2
4	Petroleum ether	100 gm	Sea weed green	Semisolid	1

4.2 Phytochemical analysis

The detailed phytochemical investigation of plant extracts were done to detect the presence or absence of various phytoconstituents present in the extract. The qualitative phytochemical analysis of all four extracts was done. The results obtained in phytochemical analysis are presented in Table 2.

The aqueous extract showed presence of alkaloids, carbohydrates, proteins and amino acids, phenolic compounds and resins. As mentioned earlier phytochemicals in aqueous extract have not been mentioned in the literature it could not be compared with the present study. Ethanolic extract showed presence of glycoside and saponins in addition to those phytochemicals present in aqueous extract.

The ethanolic extract revealed presence of phytoconstituents, which is in mere agreement with previous study by **Panda et al., 2013** who detected all the phytochemicals in addition to phytosterols and flavanoids. **Singh and Vidyasagar, 2015** revealed the absence of steroids in leaf ethanolic extract which is in agreement with the present study. When the phytochemicals of the ethanolic extract obtained in this study compared with phytochemicals obtained in a study conducted by **Thilagavathi et al., 2015**, it is observed that all the phytochemicals matched, except presence of saponin and absence of flavonoid and saponins in two studies.

The petroleum ether extract in the present study revealed presence of alkaloids and resins. The results of phytochemical analysis with leaf petroleum ether extract seems almost match with previous results stated by **Attarde et al., 2011**, except for presence of steroids and absence of alkaloids. It is important to note that in the present study all the solvent extracts detected presence of alkaloids and resins. Many authors revealed presence of flavonoids and terpenoids/steroids in leaves, but not detected in this study from any of the tested extract. This variation could be due to the method of estimation of flavanoids which was followed in present study and alkaline reagent test followed by **Attarde et al., 2011**.

Table No. 2. Phytochemical analysis of aqueous, ethanolic, acetone and petroleum ether leaf extracts of plant *Limonia acidissima*.

Sr. No.	Active Principle	Test employed	Aqueous extract	Ethanolic extract	Acetone extract	Per. ether extract
1.	Alkaloids	Mayer's test	Absent	Absent	Absent	Absent
		Wagner's test	Present	Present	Present	Present
		Hager's test	Absent	Absent	Present	Present
		Dragendroff's test	Present	Present	Absent	Absent
2.	Carbohydrates	Fehling's test	Absent	Absent	Absent	Absent
		Benedict's test	Present	Absent	Absent	Absent
3.	Glycosides	Legal's test	Absent	Present	Absent	Absent
4.	Saponins	Foam test	Absent	Present	Absent	Absent
5.	Proteins and amino acids	Biuret test	Present	Absent	Absent	Absent
		Ninhydrin test	Present	Absent	Absent	Absent
		Xanthoprotein test	Absent	Present	Absent	Absent
6.	Phytosterols	Salkowski's test	Absent	Absent	Absent	Absent
7.	Phenolic compounds	Lead acetate test	Present	Present	Present	Absent
		Ferric chloride test	Present	Present	Absent	Absent
8.	Flavonoids	Test for flavonoids	Absent	Absent	Absent	Absent
9.	Resins	Test for resins	Present	Present	Present	Present

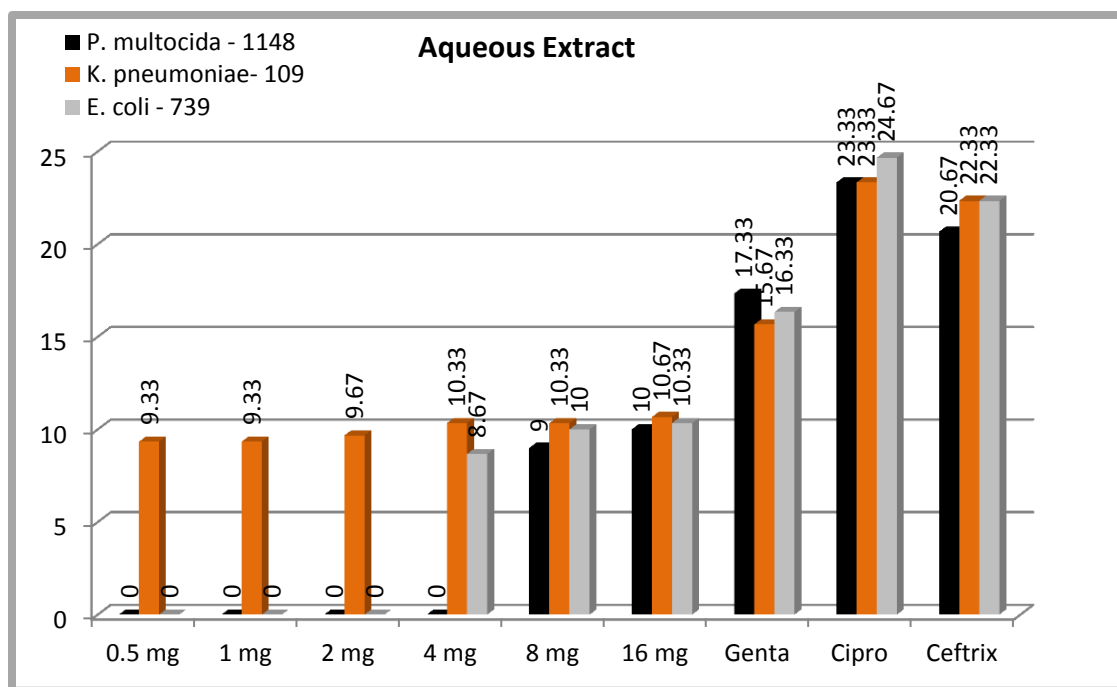
4.3 Antibacterial activity of *Limonia acidissima*

4.3.1 Well diffusion method

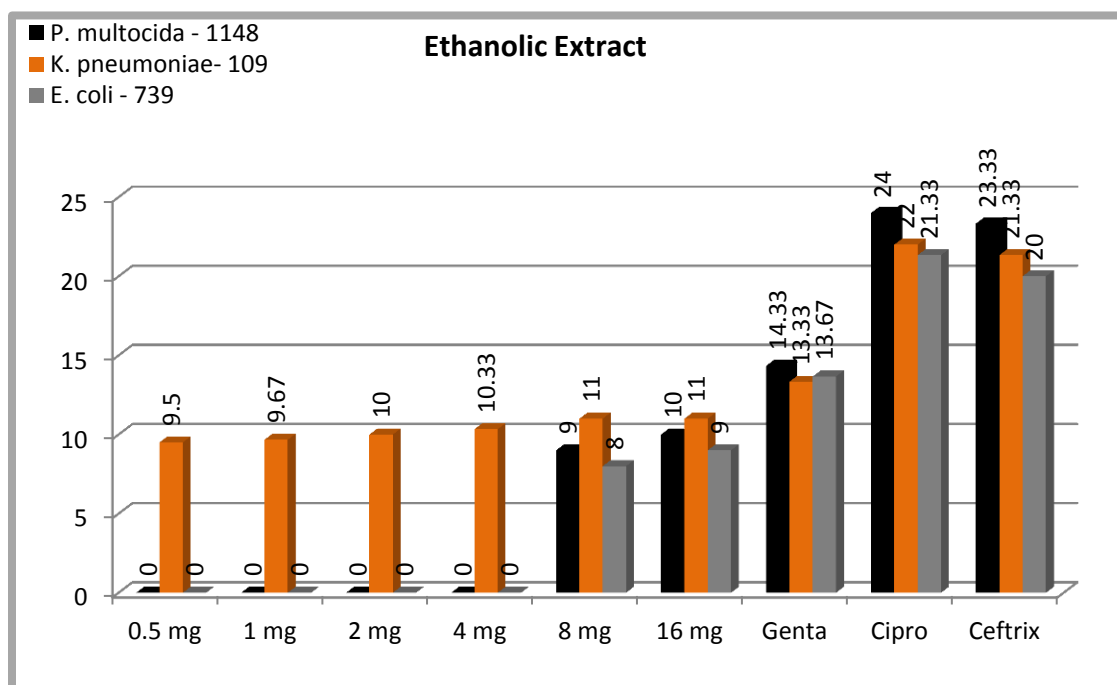
The agar well diffusion method is widely employed to test antimicrobial activity of plant extracts and supposed to be more suitable and sensitive method than disc diffusion method for evaluating natural products (Valgas *et al.*, 2007). All four extracts under investigation were tested individually against selected bacteria and readings were recorded as diameter of zone of inhibition in millimeters. All the extracts under study were diluted to get final concentration of 0.5, 1, 2, 4, 8 and 16 mg which were then used for the antibacterial activity. The zones of inhibition due to various concentrations of extracts were compared with zones of inhibition obtained due to standard drugs ciprofloxacin, ceftriaxone and gentamicin.

The **aqueous extract** was found to be active against all three bacteria under study. Antibacterial activity of aqueous extract was recorded at the dose of 8 mg onwards for *Pasteurella multocida* and 4 mg onwards for *Escherichia coli*, whereas it showed activity against *Klebsiella pneumoniae* right from initial doses indicating relatively greater susceptibility for aqueous extract. Further, from the results obtained it is evident that there was no appreciable dose dependent increase in size of zone of inhibition for *Pasteurella multocida*. However, antibacterial activity against *Escherichia coli* and *Klebsiella pneumoniae* was found to be slightly dose dependent. When compared with standard drugs, aqueous extract has non-significant effect against all the tested bacteria. Amongst standard drugs, the ciprofloxacin (23.33-24.67 mm) was found to be the best drug followed by ceftriaxone (20.67-22.33 mm) and gentamicin (15.67-17.33 mm) based upon their size of zones of inhibition. The zone size and sensitivity pattern for aqueous extract is depicted against each individual bacterium in Graph 1. It has not been mentioned in the literature reviewed about the antibacterial activity of cold aqueous extract against selected bacteria. The zones of inhibition of an aqueous extract against *Pasteurella multocida*, *Escherichia coli* and *Klebsiella pneumoniae* were shown in Plate 4.

Ethanollic extract (EtOH) was similarly tested against all selected bacteria. At higher concentrations i.e. 8 mg and 16 mg ethanolic extract produced observable zones against *Pasteurella multocida* and *Escherichia coli* but



Graph 1: Graphical representation of activity of an aqueous extract against *Pasteurella multocida*, *Klebsiella pneumoniae* and *Escherichia coli*.



Graph 2: Graphical representation of activity of an ethanollic extract against *Pasteurella multocida*, *Klebsiella pneumoniae* and *Escherichia coli*.

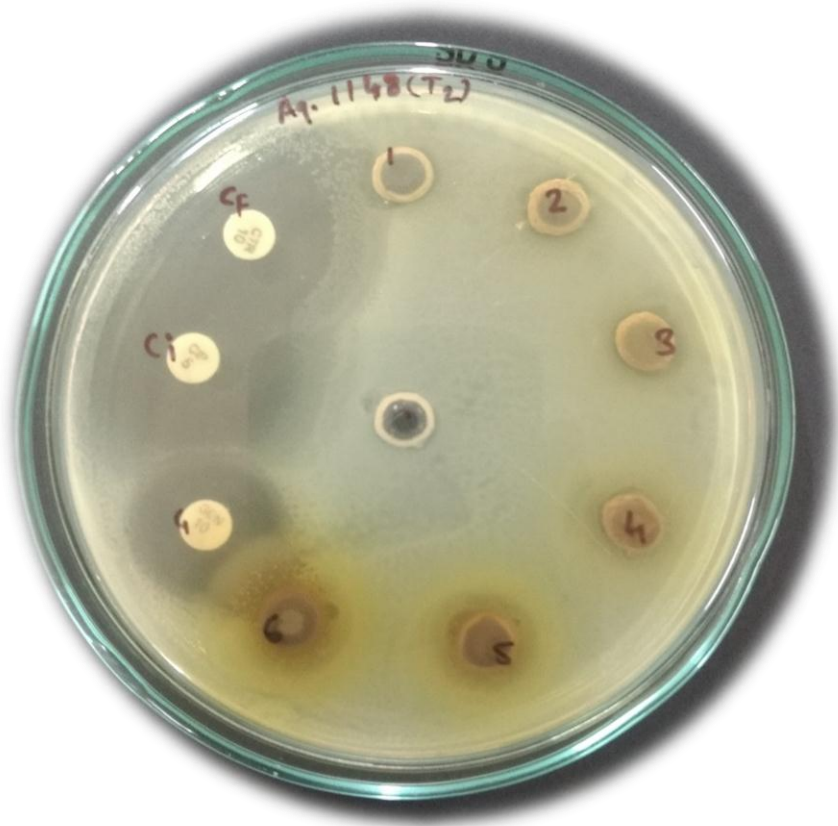


Plate 4: (a) Zones of inhibition of an aqueous extract against *P. multocida*.

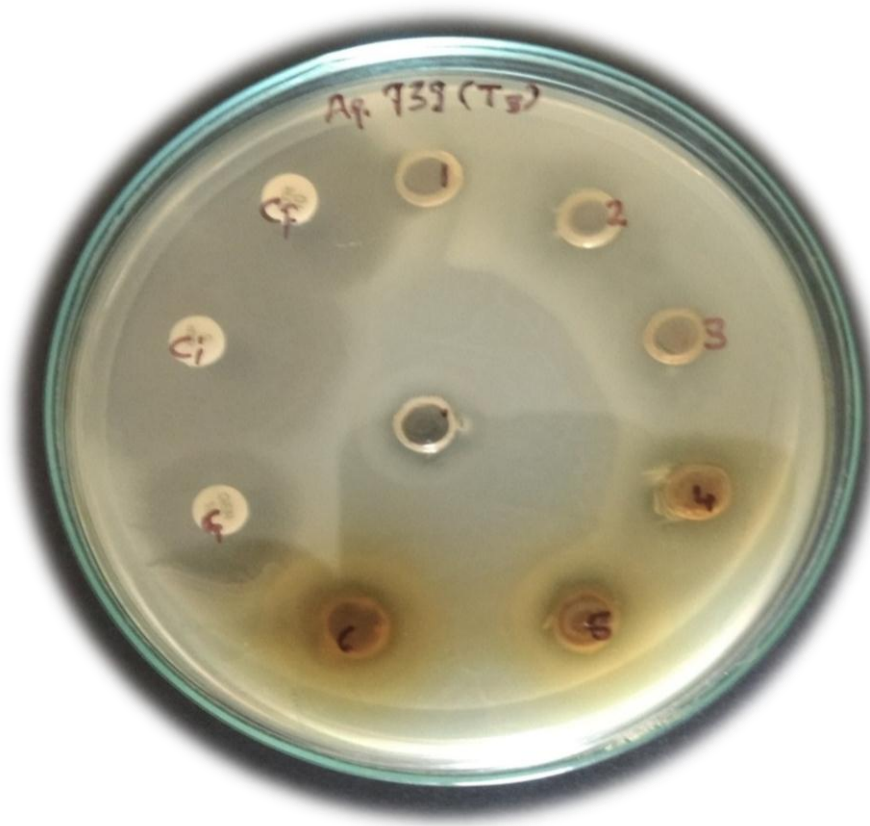


Plate 4: (b) Zones of inhibition of an aqueous extract against *E. coli*.

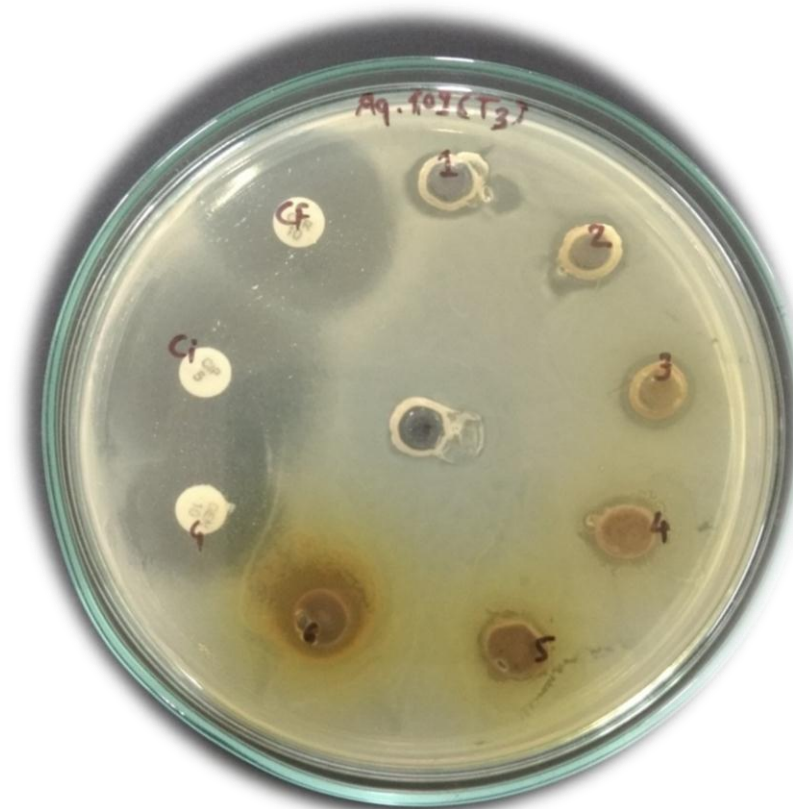


Plate 4: (c) Zones of inhibition of aqueous extract against *K. pneumoniae*.

Plate 4: Showing zones of inhibition of an aqueous extract against *P. multocida*, *E. coli* and *K. pneumoniae*. (Letters G, Ci, Cf indicates Gentamicin, Ciprofloxacin, Ceftriaxone and numbers 1, 2, 3, 4, 5, 6 indicates 0.5 mg, 1 mg, 2 mg, 4 mg, 8 mg, 16 mg concentration respectively of leaf extract).

ineffective at concentrations below 8 mg. The zone of inhibition was found to be dose dependent. The ethanolic extract produced slight dose dependent zones of inhibition against *Klebsiella pneumoniae* right from the lowest dose, which reflects relatively greater susceptibility of this bacterium to ethanolic extract. As compared to standard drugs the ethanolic extract showed non-significant effects against all tested bacteria. Amongst standard drugs, ciprofloxacin (21.33-24 mm) showed highest zone of inhibitions, ceftriaxone (20-23.33 mm) with moderate effect and gentamicin (13.33-14.33 mm) with lowest effect. The zone size and sensitivity pattern for ethanolic extract is depicted against each individual bacterium in Graph 2. Also, the zones of inhibition of an ethanolic extract against *Pasteurella multocida*, *Escherichia coli* and *Klebsiella pneumoniae* were shown in Plate 5.

Antibacterial screening with leaf ethanolic extract conducted by **Panda et al., 2013** on *Escherichia coli* strains, he observed that even after doubling the dose rates there was no appreciable increase in zone of inhibition. Similar observations are recorded in this study also. Another study with ethanolic leaf extract showed good antibacterial activity against some gram negative organisms like *Escherichia coli* and *Pseudomonas aeruginosa* with significant dose dependent increase in zone size and found very effective against *Escherichia coli* (**Singh and Vidyasagar, 2015**), which corresponds to some extent with this study. As ethanol and methanol are placed one after other based on polarity order and as stated by **Cowan, 1999** nearly all the identified components from plants which are active against microorganisms are most often obtained through initial ethanol or methanol extraction, antibacterial studies conducted on the methanolic extract of the leaf of *Limonia acidissima* are recorded here. Antibacterial studies by some authors (**Thomas and Ponnammal, 2005**) with methanolic leaf extract proved efficacy against *E. coli* with diameter of zone size 21mm. Similarly, **Naidu et al., 2014** observed highest activity in methanolic extract with largest zone of inhibition against *Escherichia coli* (24±0.13 mm) and relatively smaller zone against *Klebsiella pneumoniae* (17±0.45 mm). From the literature reviewed there is no evidence about study of ethanolic leaf extract against *Pasteurella multocida*.

The **acetone extract (Me₂CO)** was also tested against all organisms. The acetone extract has shown slight activity against *Klebsiella pneumoniae* and

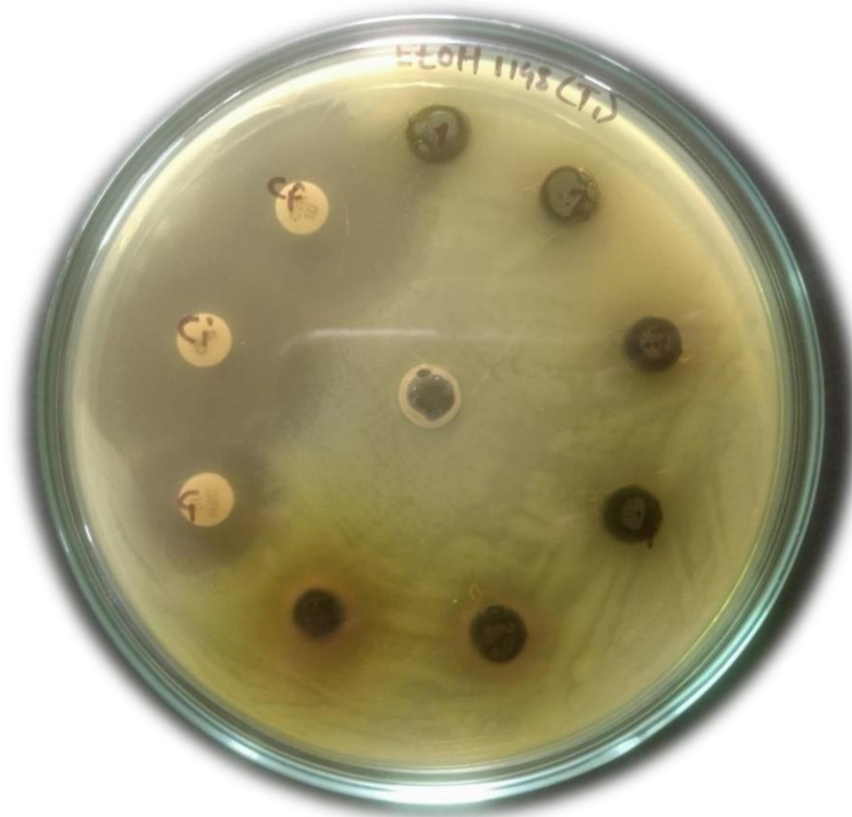


Plate 5: (a) Zones of inhibition of ethanolic extract against *P. multocida*.

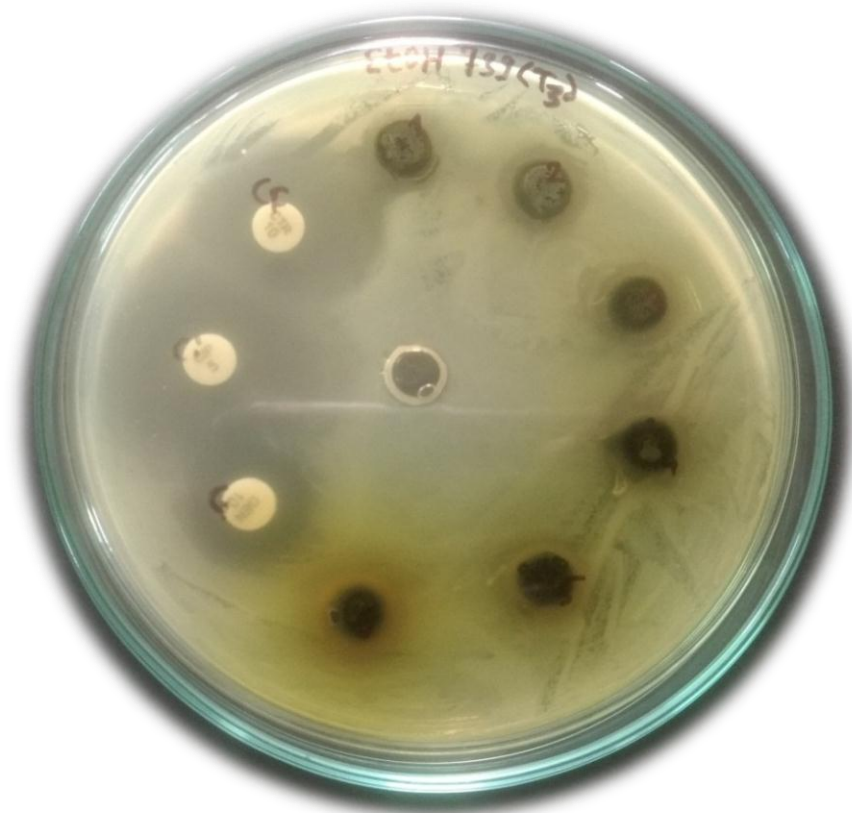


Plate 5: (b) Zones of inhibition of ethanolic extract against *E. coli*.

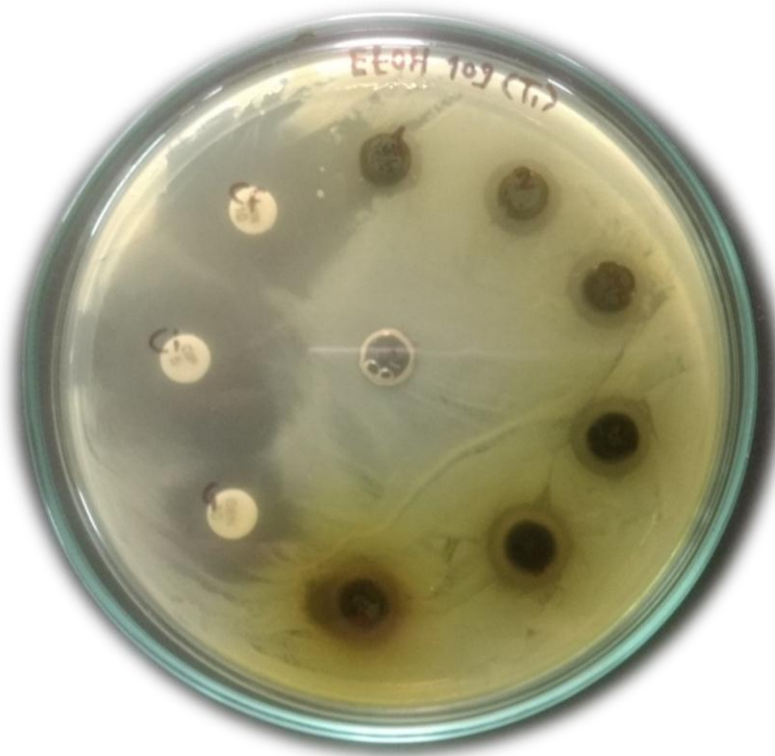


Plate 5: (c) Zones of inhibition of ethanolic extract against *K. pneumoniae*.

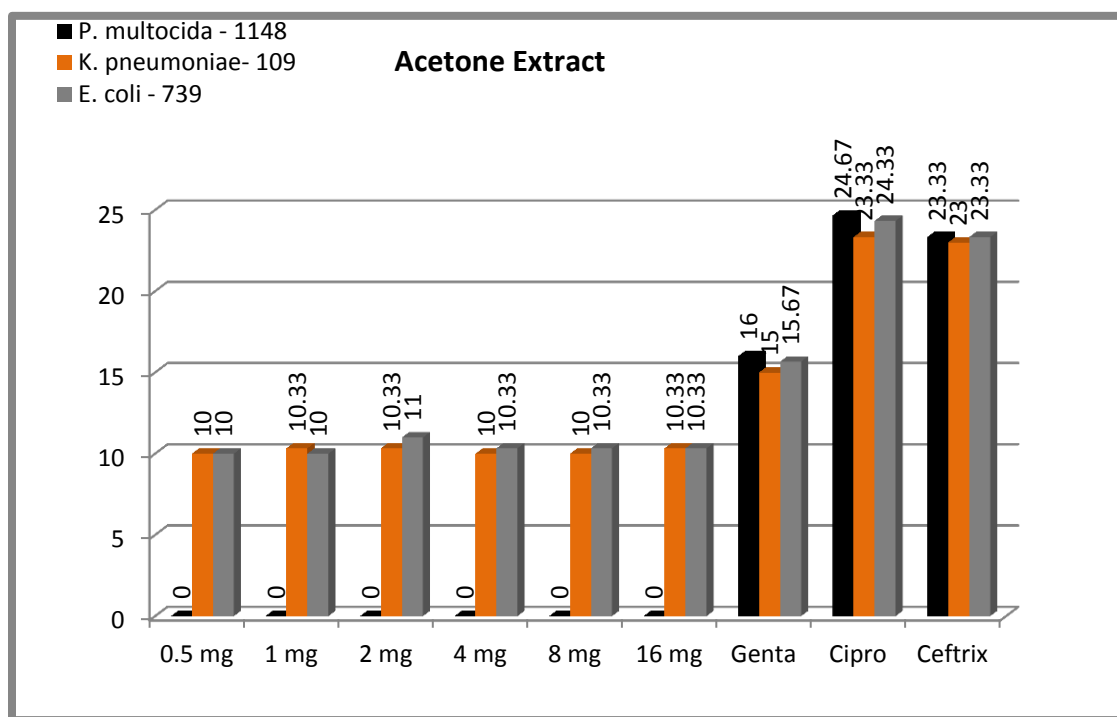
Plate 5: Showing zone of inhibition of an ethanolic extract against *P. multocida*, *E. coli* and *K. pneumoniae*. (Letters G, Ci, Cf indicates Gentamicin, Ciprofloxacin, Ceftriaxone and numbers 1, 2, 3, 4, 5, 6 indicates 0.5 mg, 1 mg, 2 mg, 4 mg, 8 mg, 16 mg concentration respectively of leaf extract).

Escherichia coli and no effect against *Pasteurella multocida* at any dose as there were no zones of inhibition. The concentration dependent increase in zone sizes was not observed in case of both the sensitive bacteria. The zone sizes for all the concentrations under study were found to be of almost similar size in case of both organisms. The ciprofloxacin (23.33-24.67 mm) was observed to produce highest zone of inhibitions, followed by ceftriaxone (23-23.33 mm) and gentamicin (15-16 mm) which again conferred highest potency of ciprofloxacin. The zone size and sensitivity pattern for acetone extract is depicted against each individual bacterium in Graph 3. The record of antibacterial activity of *Limonia acidissima* leaf acetone extract could not be available through literature reviewed.

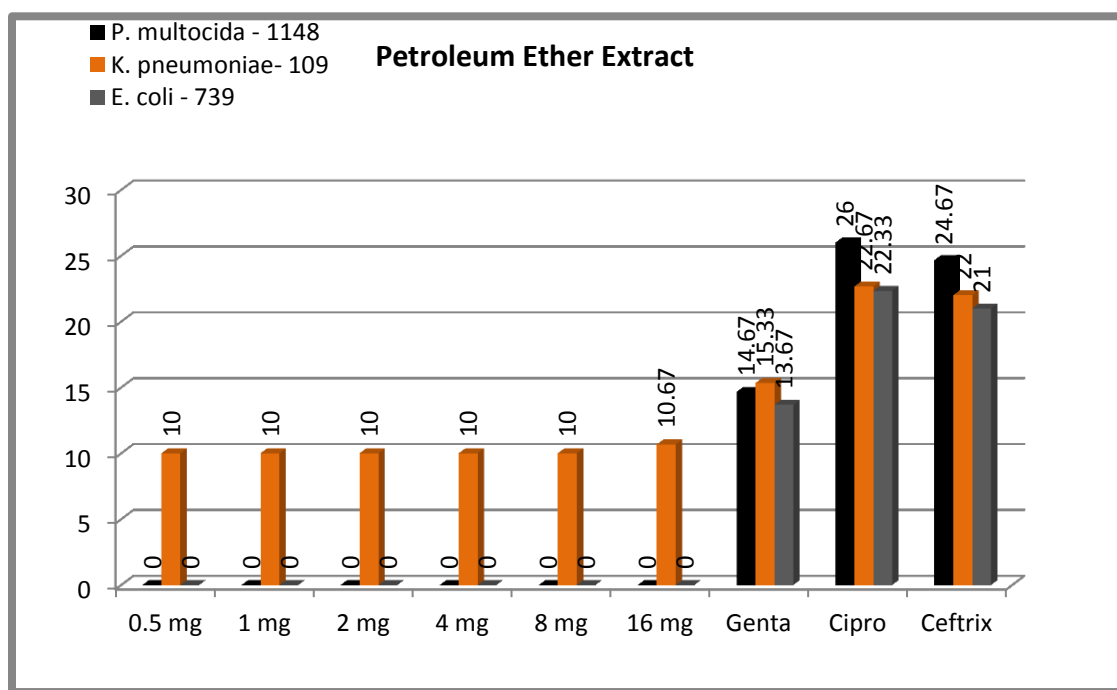
The **petroleum ether extract** was screened for antibacterial activity against similar bacteria in similar manner. This extract showed slight antibacterial activity against *Klebsiella pneumoniae* only and zone size was found to be remained almost similar for concentrations from 0.5 mg to 8 mg and showed slight increase at 16 mg concentration. It was observed that, petroleum ether showed no effect and zone against rest of the two bacteria namely *Pasteurella multocida* and *Escherichia coli*. The standard drug showed similar pattern of activity against all tested organisms with ciprofloxacin (22.33-26 mm) as a leading drug followed by ceftriaxone (21-24.67 mm) and gentamicin (13.67-15.33 mm). The zone size and sensitivity pattern for petroleum ether extract is depicted against each individual bacterium in Graph 4.

In a study, antibacterial activity evaluated by **Naidu et al., 2014**, the hexane extract produced some zone against *Escherichia coli* (12 ± 0.21 mm) and *Klebsiella pneumoniae* (10 ± 0.04). These results correlate with the present study with respect to antibacterial activity of *Klebsiella pneumoniae* and differ with *Escherichia coli*. The antibacterial potential of petroleum ether extract studied by **Lunkad et al., 2015** and showed some activity against *Escherichia coli* with maximum zone of inhibition upto 14.44 mm, which didn't correlates with current study wherein no activity was found against *Escherichia coli*. Activity of petroleum ether extract of leaf against *Klebsiella* sp. and *Pasteurella* sp. not discussed elsewhere in the literature reviewed.

The results of well diffusion test revealed sensitivity pattern against three organisms. The organism of main concern i.e. *Pasteurella multocida* was found to be inhibited with only aqueous and ethanolic extract at 8 and 16 mg with very



Graph 3: Graphical representation of activity of an acetone extract against *Pasteurella multocida*, *Klebsiella pneumoniae* and *Escherichia coli*.



Graph 4: Graphical representation of activity of petroleum ether extract against *Pasteurella multocida*, *Klebsiella pneumoniae* and *Escherichia coli*.

limited zone of inhibition i.e. 9-10 mm. It was noticed that acetone and petroleum ether extract did not inhibit *Pasteurella* sp. The bacterium *Escherichia coli* were inhibited by aqueous, ethanolic and acetone extracts, but not inhibited with petroleum ether extract. The acetone extract inhibited two bacteria *Klebsiella pneumoniae* and *Escherichia coli*. It was also observed that, petroleum ether could inhibit only one bacterium *Klebsiella pneumoniae*.

The sensitivity pattern for standard drugs ciprofloxacin, ceftriaxone and gentamicin were also analysed from this experiment. The zone size of ciprofloxacin ranges between 23.33 to 26 mm (Avg. **24.50** mm) for *Pasteurella multocida*, 22 to 23.33 mm (Avg. **22.83** mm) for *Klebsiella pneumoniae* and 21.33 to 24.67 mm (Avg. **23.16** mm) for *Escherichia coli*. The zone size of ceftriaxone against *Pasteurella multocida* was observed to be ranging from 20.67 to 24.67 mm (Avg. **23** mm), for *Klebsiella pneumoniae* ranging from 21.33 to 23 mm (Avg. **22.16** mm) and for *Escherichia coli* ranging from 20 to 23.33 mm (Avg. **21.66** mm). Similarly, the size of zone of inhibition produced by standard gentamicin against *Pasteurella multocida* was ranging from 14.33 to 17.33 mm (Avg. **15.58** mm), against *Klebsiella pneumoniae* it ranges between 13.33 to 15.67 mm (Avg. **14.83** mm) and against *Escherichia coli* it ranges from 13.67 to 16.33 mm (Avg. **14.83** mm). The ranges and average zone values confirmed ciprofloxacin as most effective drug against all tested bacteria followed by ceftriaxone and finally the gentamicin. It is important to notice that all standard drugs yielded maximum zone of inhibition against bacterium *Pasteurella multocida*. However, there were no significant differences in zones of standard drugs for remaining two bacteria *Klebsiella pneumoniae* and *Escherichia coli*. It is also observed that zone sizes of ciprofloxacin and ceftriaxone against *Klebsiella pneumoniae* are almost similar with no practical difference.

4.3.2 Tube dilution / Macrodilution test

The tube dilution method is more useful than disc or well diffusion method which provides quantitative data regarding concentration of drug necessary to inhibit bacterial growth. This method can further be used to know minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). For this each tube that showed no bacterial growth is further plated, the smallest concentration that produced no growth in the plate is the MBC. Accordingly tube dilution test was performed to assure the antibacterial potential of the plant leaf

extracts and taking into consideration the isolated colonies obtained it can be stated that there was an inhibition of bacteria at higher concentration. The series of tubes were incubated at 37°C for 18 hrs and examined for inhibition of growth and further 100 µl from tubes showing inhibition was sub cultured on Mueller Hinton Agar (MHA) plates and further incubated for 24 hrs and observed for growth. After incubation period plates shows complete lawn like growth or less than that or isolated colonies. Lesser the number of colonies higher is the inhibition of bacteria and vice-versa. For further confirmation of a bacterial growth inhibition resazurin dye (20 µl) was added as a growth indicator to tubes showing reduction of bacterial growth based on turbidity. The pink colour was indicative of presence of bacterial growth and blue colour showed inhibition of bacterial growth.

The **aqueous extract** of *Limonia acidissima* was tested against all bacterial strains to determine MIC of the extract. The aqueous extract showed slight reduction in turbidity at higher concentrations i.e. 32 mg, 16 mg and 8 mg for all bacterial strains and was sub plated on separate MHA plates. The aqueous extract yielded isolated colonies to some extent with little variation for *Escherichia coli* and *Klebsiella pneumoniae* bacteria. The *Pasteurella multocida* plates showed very little number of colonies at 32 mg and 16 mg and produced intermittent lawn over agar plates. Also, the colour of resazurin remained unchanged to some extent at 32 mg in all tubes of all three bacteria. This indicated that the aqueous extract inhibited the growth of *Escherichia coli* and *Klebsiella pneumoniae* to certain extent at higher concentrations. However, the inhibition of *Pasteurella multocida* was less as compared to other two bacteria. During review of literature it was noticed that aqueous extract was not evaluated for its antibacterial property. The results of tube dilution test of an aqueous extract against bacterium *Klebsiella pneumoniae* is shown in Plate 6 (a), (b) and (c).

Ethanollic extract (EtOH) was also processed in similar ways for estimating MIC of this extract. The ethanollic extract also showed reduction of turbidity but not the complete inhibition of bacterial growth. The tubes of higher concentration i.e. 32 mg, 16 mg and 8 mg were sub-plated to check inhibition of growth of all organisms. It was observed that not a single concentration could yield isolated colonies of any of the bacteria over the plates. The highest

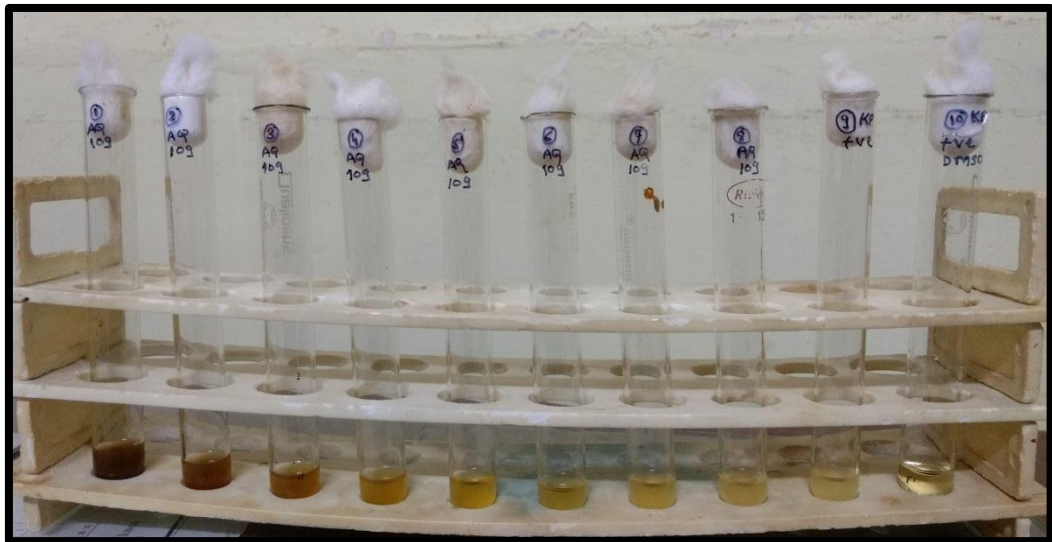


Plate 6: (a) Tube dilution test of an aqueous extract against *Klebsiella pneumoniae* showing turbidity in some tubes after 18 hrs of incubation.



Plate 6: (b) Tubes added with resazurin dye showing inhibition of growth in first tube i.e. 32 mg and presence of growth in rest of the tubes including positive control.

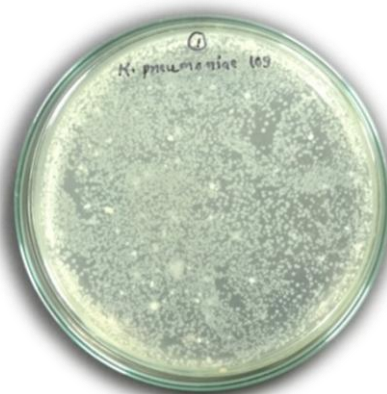


Plate 6: (c) MHA Plate showing isolated colonies of *Klebsiella pneumoniae* after subplating from first tube i.e. 32 mg.

concentration showed no change in colour of resazurin, but plating showed formation of lawn which was comparatively thin. This indicated either some bacteria might remain viable at highest concentration or temporarily growth might have inhibited. The results of tube dilution method are indicative of antibacterial activity of this extract against the all three bacteria under study, which is also supported by results obtained earlier in the well diffusion method wherein the zone of inhibition were recorded. Ethanolic extract have been evaluated for its MIC by **Panda et al., (1913)** who recorded it as 50 µg/ml. In study with leaf methanolic extract by **Naidu et al., 2014** the MIC was determined as 62.5 µg/ml and 500 µg/ml against *Escherichia coli* and *Klebsiella pneumoniae* respectively.

The **acetone extract (Me₂CO)** was also tested for determination of MIC of the extract. The observations were quite similar to that of ethanolic extract. The first three tubes with concentrations 32 mg, 16 mg and 8 mg were sub-plated on MHA plates. All the bacterial strains yielded growth on sub-plating, however intensity of growth was observed to be reducing with increasing concentration. The highest concentration of extract for all bacteria showed no change in colour after addition of resazurin. But, plates obtained after processing some volume from these tubes yielded lawn, which indicated that some bacteria were viable at highest concentration. Antibacterial property of this extract could not found from literature reviewed to selected bacteria.

The **petroleum ether extract** was processed for determination of MIC against each and individual bacteria. The results of this test for *Pasteurella multocida* and *Escherichia coli* were matching with well diffusion technique, wherein no zone of inhibition was observed for these two bacteria. Similarly, sub plates from first three tubes showed formation of lawn and showed change of colour from blue to pink even at the highest concentration indicating no effect over growth of these two bacteria. However, some sort of inhibition was expected based on well diffusion method in case of *Klebsiella pneumonia*, but after sub plating lawn was produced with comparatively reduced intensity.

Naidu et al., 2014 determined minimum inhibitory concentration with hexane extract against *Escherichia coli* and *Klebsiella pneumoniae*, which was found to be 250 µg/ml and > 1000 µg/ml respectively.

4.3.3 Microdilution assay

The microdilution assay was performed to determine minimum inhibitory concentration (MIC) of the various extracts against all test strains. The results were judged and discussed in terms of optical density (OD) and change of colour of wells after addition of resazurin dye. The results are presented and discussed below.

The *Limonia acidissima* **aqueous extract** showed inhibition of all three organisms. The growth of *Pasteurella multocida* was inhibited at 24 mg concentration in very first well showing blue colour and rest of the concentration showing pink well indicating bacterial growth. Based on visual inspection it is claimed that 24 mg would be the minimum inhibitory concentration (MIC) against *Pasteurella multocida*. Another two bacteria i.e. *Escherichia coli* and *Klebsiella pneumoniae* were also found to be inhibited at 24 mg suggesting the same concentration as MIC for both the organism under question.

The *Limonia acidissima* **ethanolic extract (EtOH)** showed inhibition of growth of *Pasteurella multocida* and *Escherichia coli* to some extent between concentrations 12 mg and 24 mg, as intensity of blue colour of indicator was relatively less indicating some bacteria may remain viable at that concentration. Hence, minimum inhibitory concentration for ethanolic extract could not be found out, however from the observations it can be elicited that it may be around 24 mg. At the same time this extract failed to inhibit the growth of *Klebsiella pneumoniae* as all the wells showed growth of organism, which does not correlate with the result of antibiotic sensitivity test with this extract, wherein it has shown concentration dependent antibacterial activity.

The *Limonia acidissima* **acetone extract (Me₂CO)** was tested against all bacteria under study. The initial concentrations of 12 mg and 24 mg were observed as inhibiting concentrations for *Klebsiella pneumoniae* and *Escherichia coli*. However, at 12 mg the intensity of blue colour was not so pronounced, which again pointed out towards higher minimum inhibitory concentration (MIC) i.e. 24 mg. The extract was not found to possess any activity against *Pasteurella multocida*, which is going hand in hand with results of well diffusion test. The results of microdilution assay showing minimum inhibitory concentration (MIC) of an acetone extract against *E. coli* bacterium were shown in Plate 7.

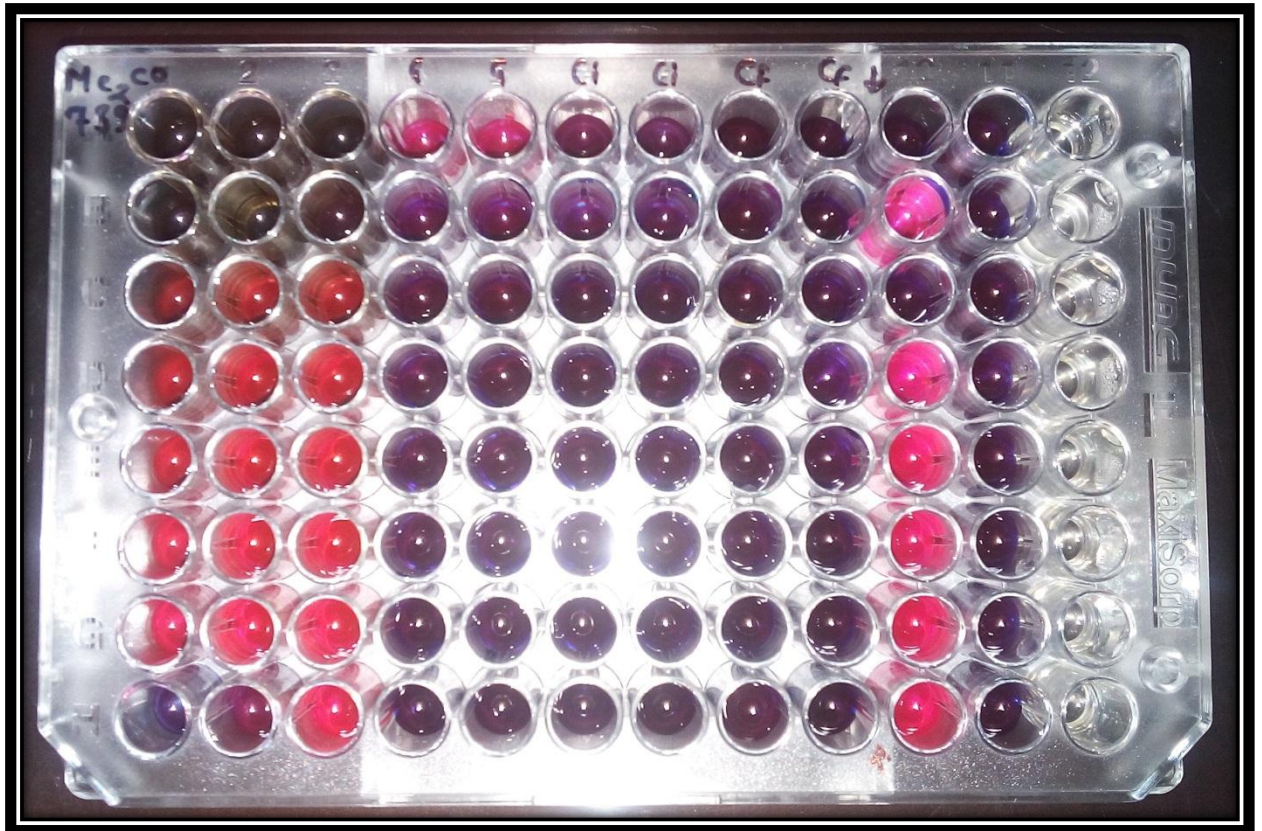


Plate 7: Microdilution assay showing minimum inhibitory concentration (MIC) of an acetone extract against *Escherichia coli* bacterium in first three wells at initial concentration 24 mg. (G-Gentamicin, Ci-Ciprofloxacin and Cf-Ceftriaxone).

The *Limonia acidissima* **petroleum ether extract** was tested against all bacteria under question. The extract was found to be useful against *Klebsiella pneumoniae* with inhibition of growth in first well indicating minimum inhibitory concentration at 24 mg. However, this extract was found ineffective against *Pasteurella multocida* which coincide with results of well diffusion test. However, the results for *Escherichia coli* are somewhat variable and not relating with results obtained by well diffusion method.

Based on results obtained in all the methods it can be eluted that aqueous leaf extract of *Limonia acidissima* possesses antibacterial property against *Pasteurella multocida*, *Escherichia coli* and *Klebsiella pneumoniae* wherein MIC was recorded around 24 mg. This was followed by ethanolic extract which showed activity against *Escherichia coli* and *Pasteurella multocida*. Acetone extract showed activity against *Escherichia coli* and *Klebsiella pneumoniae*. Whereas, petroleum ether extract showed activity against *Klebsiella pneumoniae* with some activity against other two bacteria. As aqueous extract was found to be most potent amongst the all extracts under study for selected bacteria it was presented for detailed analysis of phytoconstituents by Gas chromatography-Mass spectrometry (GC/MS).

4.4 Gas chromatography-Mass spectrometry (GC-MS) analysis

The aqueous leaf extract of *Limonia acidissima* was analysed by GC-MS to know the phytoconstituents present in an aqueous extract. The aqueous extract revealed eleven compounds which are DL-2,3-Butanediol, n-Undecanal, Ethyl hydroxyphenyl acetate, 3-Oxo-7,8-dihydro-a-ionol, Camphene, 4-Methoxyphenyl heptanoate, Lilac aldehyde A, 5-Eicosene, Bergaptan, Cycloicosane, Benzene,1-acetyl-3-ethyl-2-(2-ethenyl-6-ethylphenylazo). The chromatogram of an aqueous extract showing picks of identified compounds were shown in Plate 8. Also, the molecular structures of all revealed compounds were depicted in Plate 9. The four major entities found in aqueous extract are DL-2,3-Butanediol (24.07%), Ethyl hydroxyphenyl acetate (33.06%), 3-Oxo-7,8-dihydro-a-ionol (27.26%) and 4-Methoxyphenyl heptanoate (3.46%) with retention time 3.94, 12.28, 15.10 and 16.07 respectively. The compound bergaptan was mentioned earlier in the literature in slightly different form i.e. β -Bergaptan. All identified components of an aqueous extract are listed in Table 3.

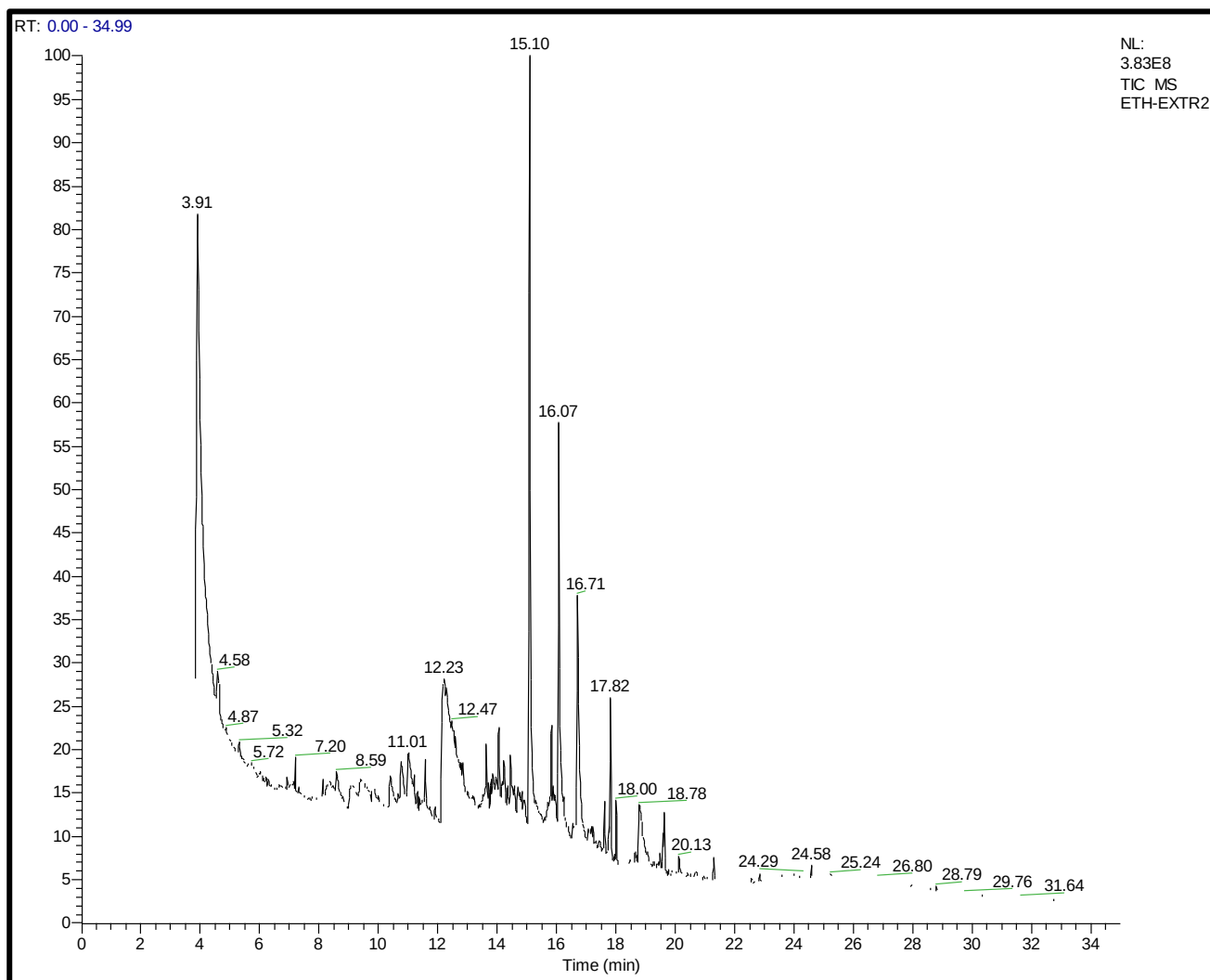
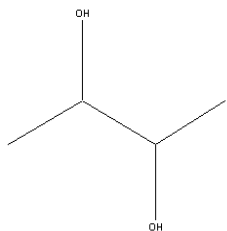


Plate 8: The chromatogram of an aqueous extract showing picks of identified compounds.

Formula C4H10O2, MW 90, CAS# 6982-25-8, Entry# 15293
2,3-Butanediol, (R*,R*)(S)



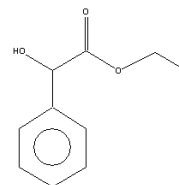
DL-2,3-Butanediol

Formula C11H22O, MW 170, CAS# 112-94-7, Entry# 2281
n-Undecanal



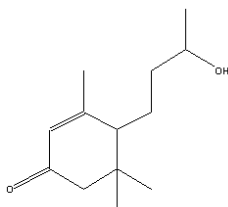
n-Undecanal

Formula C10H12O3, MW 180, CAS# 13704-09-1, Entry# 70074
Ethyl hydroxyphenylacetate #



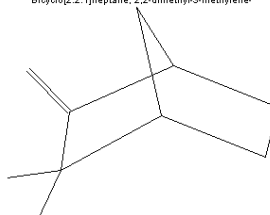
Ethyl hydroxyphenyl acetate

2-Cyclohexen-1-one, 4-(3-hydroxybutyl)-3,5,5-trimethyl-
Formula C13H22O2, MW 210, CAS# 36151-02-7, Entry# 5952



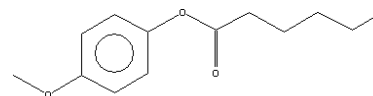
3-Oxo-7,8-dihydro-a-ionol

Camphene
Formula C10H16, MW 136, CAS# 79-92-5, Entry# 56938
Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-



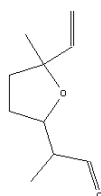
Camphene

heptanoic acid, 4-methoxyphenyl ester
Formula C14H20O3, MW 236, CAS# 69052-15-4, Entry# 87254
4-Methoxyphenyl heptanoate #



4-Methoxyphenyl
heptanoate

Lilac aldehyde A



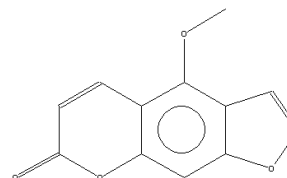
Lilac aldehyde A

5-Eicosene, (E)
Formula C20H40, MW 280, CAS# 74655-30-6, Entry# 18079
(5E)-5-Eicosene #



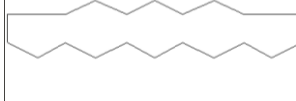
5-Eicosene

7-methoxy-2,9-glyoxybenzopyran-1-one, 4-methoxy-
Formula C12H8O4, MW 216, CAS# 484-20-8, Entry# 152841



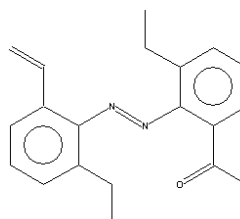
Bergapten

Formula C20H40, MW 280, CAS# 295-95-0, Entry# 18077
Cycloicosane #



Cycloicosane

Benzene, 1-acetyl-3-ethyl-2-(2-ethenyl-6-ethylphenylazo)-
Formula C20H22N2O, MW 306, CAS# NA, Entry# 176073



Benzene, 1-acetyl-3-ethyl-
2-(2-ethenyl-6-
ethylphenylazo)

Plate 9: The molecular structures of compounds identified in GC-MS analysis.

Table no 3. Compounds identified in the GC-MS analysis are mentioned in table with their names, retention time, peak area, percentage area and CAS identity number.

Sr No.	RT (min)	Name of compound	Peak area	% Area	CAS #
1	3.94	DL-2,3-Butanediol	11104197548	24.07	6982-25-8
2	11.02	n-Undecanal	29479547	0.06	112-447
3	12.28	Ethyl hydroxyphenyl acetate	15249312306	33.06	13704-09-1
4	15.10	3-Oxo-7,8-dihydro-a-ionol	12573632274	27.26	36151-02-7
5	15.83	Camphene	1251988832	2.71	79-92-5
6	16.07	4-Methoxyphenyl heptanoate	1596996119	3.46	56052-15-4
7	16.71	Lilac aldehyde A	1301492812	2.82	53447-45-3
8	17.82	5-Eicosene	1055209193	2.29	74685-30-6
9	18.82	Bergaptan	942381754	2.04	484-20-8
10	19.62	Cycloicosane	263705841	0.57	296-56-0
11	18.00	Benzene, 1-acetyl-3-ethyl-2-(2-ethenyl-6-ethylphenylazo)	761153701	1.65	611-14-3

SUMMARY AND CONCLUSION (S)

The present study was planned to test and validate the ethnoveterinary use of leaves of plant *Limonia acidissima* linn. against the diseases haemorrhagic septicaemia by tribals and farmers of Vidarbha region of Maharashtra, India. The current study was therefore conducted to explore an antibacterial potential of leaves of this plant with special emphasis being given on bacteria *Pasteurella multocida* along with some other gram negative organisms. The parameters studied under this topic included extraction of leaves of plant *Limonia acidissima* with four different solvents, phytochemical analysis of all the extracts, antibiotic sensitivity testing and determination of minimum inhibitory concentration (MIC) of extracts against bacteria under question and detailed analysis of phytoconstituents of effective leaf extract with gas chromatography-mass spectrometry (GC-MS) technique.

The extraction of leaves was done by using various solvents of different polarity like water, ethanol, acetone and petroleum ether. The highest percentage of extractability was obtained through aqueous extract which is 24 % followed by ethanol (14%), acetone (2%) and petroleum ether (1%) extract. The primary and qualitative analysis of all the four extracts was carried out for presence and absence of various active principles by phytochemical analysis. The aqueous and ethanolic extract revealed the presence of most of the active phytoconstituents like alkaloids, carbohydrates, glycosides, proteins, amino acids, phenolic compounds, saponins and resins. The petroleum ether extract revealed least number of active principles.

Antibacterial property of the extracts was analyzed against *Pasteurella multocida*, *Escherichia coli* and *Klebsiella pneumoniae* by using well diffusion test, tube dilution test and microdilution assay. The results of well diffusion test revealed sensitivity pattern against all three organisms. The organism of main concern i.e. *Pasteurella multocida* was found to be inhibited with only aqueous and ethanolic extract at 8 and 16 mg with very limited zone of inhibition i.e. 9-10 mm. It was noticed that acetone and petroleum ether extract did not inhibit *Pasteurella* sp. The *Escherichia coli* growth was inhibited by aqueous, ethanolic and acetone extracts, but not inhibited with petroleum ether extract. The acetone extract inhibited two bacteria *Klebsiella pneumoniae* and *Escherichia coli*. It was

also observed that, petroleum ether could inhibit only one bacterium i.e. *Klebsiella pneumoniae*. The aqueous and ethanolic extract showed very little or slight dose dependent increase in size of zone of inhibition. Whereas, acetone extract didn't follow dose dependent increase and zones diameters were in the same range. The petroleum ether showed steady zones against *Klebsiella pneumoniae*.

The standard drug ciprofloxacin was confirmed as most effective drug against all tested bacteria followed by ceftriaxone and gentamicin. The average zone diameters against *Pasteurella multocida*, *Klebsiella pneumoniae* and *Escherichia coli* for ciprofloxacin were 24.50 mm, 22.83 mm and 23.16 mm respectively. The average zone size for ceftriaxone was found to be 23 mm, 22.16 mm and 21.66 mm against *Pasteurella multocida*, *Klebsiella pneumoniae* and *Escherichia coli* respectively. Similarly, the average zone size against *Pasteurella multocida*, *Klebsiella pneumoniae* and *Escherichia coli* for gentamicin was 15.58 mm, 14.83 mm and 14.83 mm respectively. It is important to notice that all standard drugs yielded maximum zone of inhibition against bacterium *Pasteurella multocida*. However, there were no significant differences in zones of standard drugs for remaining two bacteria *Klebsiella pneumoniae* and *Escherichia coli*. It is also observed that zone sizes of ciprofloxacin and ceftriaxone against *Klebsiella pneumoniae* are almost similar with no practical difference.

The tube dilution test was performed to estimate minimum inhibitory concentration (MIC) of all the plant extracts against test bacteria. The aqueous extract performed slightly well in comparison with other three extracts. The aqueous extract produced some degree of intermittent lawn and some isolated colonies of *Pasteurella multocida*, whereas isolated colonies were observed against *Klebsiella pneumoniae* and *Escherichia coli* at higher concentrations. Similarly, other three extracts were also tested against all bacterial strains but none of them yielded isolated colonies, however reduced density of lawn or an intensity of growth.

The microdilution assay being more sensitive method was employed to further confirm minimum inhibitory concentration (MIC) of the extract. The highest concentration i.e. 24 mg observed as MIC of an aqueous extract against *Pasteurella multocida*, *Klebsiella pneumoniae* and *Escherichia coli*. The MIC of

ethanolic extract calculated against *Pasteurella multocida* and *Escherichia coli* was around 24 mg, however *Klebsiella* sp. was found to be inhibited. The acetone extract showed inhibition of *Klebsiella pneumoniae* and *Escherichia coli* at concentration around 24 mg which reflects MIC, but growth of *Pasteurella multocida* were not inhibited. The petroleum ether extract revealed MIC against *Klebsiella pneumoniae* which is 24 mg and found ineffective against *Pasteurella multocida*.

The aqueous extract being active against all three bacterial strains and found effective in all three antibacterial testing, it was subjected to detailed analysis by Gas chromatography and mass spectrometric (GC-MS) technique. The four major entities found in extract are DL-2,3-Butanediol (24.07%), Ethyl hydroxyphenyl acetate (33.06%), 3-Oxo-7,8-dihydro-a-ionol (27.26%) and 4-Methoxyphenyl heptanoate (3.46%) with retention time 3.94, 12.28, 15.10 and 16.07 respectively. From the results and observations of the present study it can be concluded that –

The *Limonia acidissima* leaves showed highest extractability in water followed by ethanol, acetone and petroleum ether. At the same time it revealed presence of maximum number of active principles indicating good ability of water to extract out phytoconstituents of *Limonia acidissima*.

The aqueous extract is having more antibacterial activity against all three tested bacteria as compared to other three extracts, with MIC of 24mg. It showed presence of eleven compounds amongst which four major compounds revealed are DL-2,3-Butanediol (24.07%), Ethyl hydroxyphenyl acetate (33.06%), 3-Oxo-7,8-dihydro-a-ionol (27.26%) and 4-Methoxyphenyl heptanoate (3.46%).

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VITA

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THESIS ABSTRACT

- a) Title of the thesis : **“STUDIES ON ANTIBACTERIAL ACTIVITY OF LEAF EXTRACTS OF *Limonia acidissima* Linn. WITH SPECIAL REFERENCE TO *Pasteurella multocida*”.**
- b) Full name of student : **SHIRANKAR SHRADDHYEY SHARAD**
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- d) Degree to be awarded : **Master of Veterinary Science**
- e) Year of award of degree : **2018**
- f) Major Subject : **Veterinary Pharmacology & Toxicology.**
- g) Total number of pages in the thesis : **50**
- h) Number of words in the abstract : **531**
- i) Signature of Student :
- j) Signature, Name and address of forwarding authority :

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ABSTRACT

The present study was conducted to validate ethnoveterinary use of leaves of plant *Limonia acidissima* Linn. against a well known animal pathogen named *Pasteurella multocida* responsible for haemorrhagic septicaemia in cattle and buffaloes.

The extraction of leaf with four different solvents viz. distilled water, ethanol, acetone and petroleum ether was performed by modified cold extraction involving exhaustive maceration. The percentage extractability of an individual extract was determined which revealed highest extractability with water followed by ethanol, acetone and petroleum ether. Same, extracts were subjected to phytochemical analysis which showed presence of many phytochemicals with maximum presence detected in an aqueous and ethanolic extract and least in petroleum ether.

The aqueous and ethanolic extracts found active against all three bacterial strains. However, both extracts were active against *Klebsiella pneumoniae* from lowest dose i.e. 0.5 mg to highest dose i.e. 16 mg. Very little dose dependent increase in zones of inhibition for these two extracts against all tested strains was recorded. Similarly, acetone extract yielded zones against *Klebsiella pneumoniae* and *Escherichia coli* but proved ineffective against *Pasteurella multocida*. Petroleum ether extract could inhibit only *Klebsiella pneumoniae* but ineffective against *Pasteurella multocida* and *Escherichia coli*. The standard drug ciprofloxacin was found most effective against all tested bacteria, which is followed by ceftriaxone and gentamicin. The *Pasteurella multocida* showed highest susceptibility for tested standard discs with largest zones of inhibition. However, susceptibility of *Klebsiella pneumoniae* for ciprofloxacin and ceftriaxone was almost similar.

The tube dilution method employed for determining minimum inhibitory concentration (MIC) of extracts revealed inhibition of growth and formation of isolated colonies at higher concentration. However, no such phenomenon was observed in case of any other extract but they could succeed in reducing density of lawn of bacterial growth. The microdilution assay was performed to counter evaluate the results of tube dilution method and for calculating minimum inhibitory concentration (MIC) of the extracts. The MIC of an aqueous extract against *Pasteurella multocida*, *Klebsiella pneumoniae* and *Escherichia coli* calculated is 24 mg. The MIC of ethanolic extract calculated against *Pasteurella multocida* and *Escherichia coli* must be around 24 mg. The MIC of acetone extract showed inhibition of *Klebsiella pneumoniae* and *Escherichia coli* at concentration around 24 mg. The petroleum ether extract revealed MIC against *Klebsiella pneumoniae* which is 24 mg.

The results of antibacterial testing leads to conclusion that an aqueous extract is most potent and being positive for most of the phytochemicals it was subjected to analysis by gas chromatography – mass spectrometry (GC/MS), which again identified eleven compounds from an aqueous extract with highest percentage of four compounds namely DL-2,3-Butanediol (24.07%), Ethyl hydroxyphenyl acetate (33.06%), 3-Oxo-7,8-dihydro-a-ionol (27.26%) and 4-Methoxyphenyl heptanoate (3.46%) with retention time 3.94, 12.28, 15.10 and 16.07 respectively. From the experiment performed it was concluded that an aqueous and ethanolic extract can be utilized for therapeutic purposes, however its use as an antimicrobial drug against animal pathogens cannot be assured but minor infections can treated. Since, very little activity was observed against *Pasteurella multocida*, one cannot justify the use of leaves in treatment of haemorrhagic septicaemia. However, isolation of active principles and in-vivo testing in animals should be done which will confirm and could highlight the future use of this plant.

प्रबंध सारांश

अ.	प्रबंधाचे शिर्षक	:	“लिमोनिआ अँसिडिसीमाच्या (कवठ) पानांच्या अर्काचा जिवाणू विरोधी कार्याचा मुख्यत्वे पाश्चुरेल्ला मल्टोसिडा विरोधी अभ्यास”
ब.	विद्यार्थ्याचे पुर्ण नांव	:	शिरणकर श्रद्धेय शरद
क.	मार्गदर्शकाचे नांव व पत्ता	:	डॉ. आ.पा. सोमकुवर प्राध्यापक तथा विभाग प्रमुख औषध शास्त्र व विषशास्त्र. विभाग, नागपूर पशुवैद्यक महाविद्यालय, नागपूर
ड.	प्रदान करण्यात येणारी पदवी	:	स्नातकोत्तर पदवी (एम.व्ही.एस.सी.)
इ.	पदवी प्रदान करण्याचे वर्ष	:	२०१८
फ.	मुख्य विषय	:	औषध शास्त्र व विषशास्त्र
ग.	प्रबंधातील एकूण पृष्ठे	:	५०
ह.	सारांशातील एकूण शब्द	:	४७९
ई.	विद्यार्थ्याची सही	:	
ज.	अग्रेषित करणाऱ्या अधिकाऱ्याची सही, नांव आणि पत्ता	:	(डॉ. आ.पा. सोमकुवर) सहयोगी अधिष्ठाता नागपूर पशुवैद्यक महाविद्यालय, नागपूर,

सारांश

सदर अभ्यास लिमोनिआ अँसिडिसीमा / कवठाच्या पानांच्या अर्काचा गायी व म्हशींमध्ये घटसर्पाची लागण करणाऱ्या पाश्चुरेल्ला मल्टोसिडा या जिवाणुविरोधी पारंपारिक उपचारापद्धती मधील वापर प्रमाणित करण्यासाठी केला गेला.

शुद्ध पाणी, इथॅनॉल, ॲसिटोन आणि पेट्रोलिअम इथर या चार द्रावणांमधील पानांचा अर्क हा थंड अर्क पद्धतीने काढण्यात आला. शेकडा उच्चतम अर्कता ही शुद्ध पाणी, इथॅनॉल, ॲसिटोन आणि पेट्रोलियम इथर या क्रमाने आढळली. अर्काच्या वनस्पती द्रव्यांच्या विश्लेषणामध्ये सर्वात जास्त वनस्पती द्रव्ये ही पाणी व इथॅनॉल च्या अर्कामध्ये आढळली.

पाणी व इथॅनॉल मधील अर्क हा तिनही प्रकाराच्या जिवाणुविरोधी सक्रिय आढळला. दोन्ही अर्क हे क्लेबसिएल्ला न्युमोनिए या जिवाणुविरोधी लघुत्तम मात्रा ०.५ मि.ग्रॅ पासून ते उच्चतम मात्रा १६ मिली ग्र. पर्यंत सक्रिय आढळले. दोन्ही अर्काची तिन्ही परिक्षित जिवाणुविरोधी मात्रावर निहाय कार्यक्षमता कमी असल्याचे दिसले. त्याचप्रमाणे ॲसिटोन मधील अर्क क्लेबसिएल्ला न्युमोनिए आणि ई. कोलाय या दोन जिवाणु विरोधी कार्यक्षम दिसला. परंतु पाश्चुरेल्ला मल्टोसिडा या जिवाणुविरोधी ते निष्क्रिय आढळले. पेट्रोलियम इथर मधील अर्क क्लेबसिएल्ला न्युमोनिए या जिवाणुला प्रतिबंध करू शकला मात्र पश्चुरेल्ला मल्टोसिडा आणि ई. कोलाय विरोधी निष्क्रिय असल्याचे दिसला. सिप्रोफ्लोक्सासिन हे मानक औषध तिनही परिक्षित जिवाणु विरोधी सर्वात प्रभावी आढळले, त्यानंतर सेफ्ट्रॅक्झोन आणि जेंटामायसिन हे क्रमशः परिणामकारक आढळले. पाश्चुरेल्ला मल्टोसिडा हा जिवाणु सर्व मानक औषधांना संवेदनशिल असल्याचे दिसले, परंतु क्लेबसिएल्ला न्युमोनिए या जिवाणुची संवेदनशिलता मात्र सिप्रोफ्लोक्सासिन आणि सेफ्ट्रॅक्झोन या औषधांसाठी जवळपास समान असल्याचे दिसली.

अर्काचे लघुत्तम प्रतिबंधित प्रमाण (MIC)निर्धारित करण्यासाठी ट्युब डायल्युशन पद्धतीचा अवलंब केला असता पाण्यांमधील अर्काची उच्चतम मात्रा जिवाणुच्या वाढीस प्रतिबंध आणि त्यांच्या विखुरलेल्या वसाहती साठी कार्यक्षम दिसली. ट्युब डायल्युशन पद्धतीने समोर आलेल्या परिणांमाचे पुनर्परिक्षण करण्यासाठी आणि अर्काची लघुत्तम प्रतिबंधित प्रमाण (MIC) पुन्हा निर्धारित करण्यासाठी मायक्रोडायल्युशन ऍसे या पद्धतीचा वापर करण्यात आला. पाण्यामधील अर्काचे लघुत्तम प्रतिबंधित प्रमाण हे पश्चुरेल्ला मल्टोसिडा, क्लेबसिएल्ला न्युमोनिए आणि ई. कोलाय यांच्या विरोधात २४ मि. ग्रॅ इतके आढळले. इथॅनॉल मधील अर्काचे लघुत्तम प्रतिबंधित प्रमाण हे पश्चुरेल्ला मल्टोसिडा, क्लेबसिएल्ला न्युमोनिए आणि ई. कोलाय यांच्या विरोधात जवळपास २४ मि.ग्रॅ आढळले. ॲसिटोनमधील

अर्काने क्लेबसिएल्ला न्युमोनिए आणि ई.कोलाय या जिवाणुविरोधी २४ मि.ग्रॅ एवढे लघुत्तम प्रतिबंधित प्रमाण दर्शविले. पेट्रोलियम इथर मधील अर्काने क्लेबसिएल्ला न्युमोनिए या जिवाणुविरोधी २४ मि. ग्रॅ एवढे लघुत्तम प्रतिबंधित प्रमाण दर्शविले.

प्रतिजैविक विश्लेषणाच्या परिणामावरून असे निष्कर्षित करण्यात आले की, पाण्यामधील अर्क हा सर्वात परिणामकारक असून त्यात जास्त वनस्पती द्रव्य आहेत. पाण्यामधील अर्काचे जि.सी.एम.एस. पद्धतीद्वारे विश्लेषण केले असता त्यात एकूण ११ घटक असल्याचे आढळले. त्या पैकी सर्वाधिक प्रमाण हे डी. एल—२,३—ब्युटानिडेऑल (२४.०७ टक्के), इथाईल हायड्रॉक्सीफिनिल ॲसिटेट (३३.०३ टक्के) ३—ऑक्सो, — ७,८ — डायहॅयड्रो—अ—आयनाॅल (२७.२६ टक्के) आणि ४ —मिथॉक्सीफिनिल हेप्टानोयेट (३.४६ टक्के) या चार घटकांचे आढळले व त्यांचा रिटेंशन टाईम हा अनुक्रमे ३.९४, १२.२८, १५.१० आणि १६.०७ असल्याचे दिसते. केलेल्या प्रयोगावरून असे निष्कर्षित करण्यात आले की, पाणी आणि इथॅनाॅल मधील अर्क हे वेगवेगळ्या उपचारांसाठी वापरले जाऊ शकतात तथापि त्याचा जनावरांमधील रोगकारक सुक्ष्मजिवविरोधी औषध म्हणून वापर खात्रीपूर्वक करू शकत नाही पण याचा उपयोग लघुसंक्रमांच्या उपचारासाठी होऊ शकतो. पश्चुरेल्ला मल्टोसिडा या जिवाणुविरोधी कार्यक्षमता हि फारच कमी आढळली म्हणून, घटसर्प या रोगाच्या उपचारासाठी पानांचा वापर समायोजित करू शकत नाही. तथापि, कार्यक्षम घटकांचे विलगीकरण आणि त्याची जनावरांमधील चाचणी करून पृष्टी करता येईल आणि या वनस्पतीचा भविष्यातील वापर अधोरेखित करता येईल.