Mechanistic Study on Antibacterial Efficacy of Babool Leaves Extract against *E coli* Isolated from Clinical Cases of Uterine Infections in Bovines

THESIS SUBMITTED FOR PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE

OF

MASTER OF VETERINARY SCIENCE

IN

VETERINARY PHARMACOLOGY & TOXICOLOGY

BY

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Enrolment No.: V-2181/20

COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY
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(2022)

CERTIFICATE

This is to certify that the thesis entitled, **"Mechanistic Study on Antibacterial Efficacy of Babool Leaves Extract against *E coli* Isolated from Clinical Cases of Uterine Infections in Bovines"** submitted by **Dr. Rajneesh Singh, Enrollment No. V-2181/20** in partial fulfilment of the requirements for the award of the **Master of Veterinary Science** in **Veterinary Pharmacology and Toxicology** of the **U.P. Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura (UP), India,** is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated...15.09.22...

(Soumen Choudhury)

Major Advisor and Chairman
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It is certified that the thesis entitled **"Mechanistic Study on Antibacterial Efficacy of Babool Leaves Extract against *E coli* Isolated from Clinical Cases of Uterine Infections in Bovines"** submitted by **Dr. Rajneesh Singh**, Enrollment No. **V-2181/20** in partial fulfillment of **Master of Veterinary Science** in **Veterinary Pharmacology and Toxicology** at **College of Veterinary Science & Animal Husbandry, U.P. Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura (UP),** India, embodies the original work done by the candidate himself. The candidate has carried out his work sincerely and methodically.

We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which being presented by him for the award of the degree of this University.

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%	:	Percent
<	:	Less than
>	:	More than
°C	:	Degree Celsius
µl	:	Micro litre
AI	:	Artificial insemination
AMR	:	Antimicrobial resistance
ANOVA	:	Analysis of variance
CFDA-AM	:	Carboxyfluorescein diacetate acetoxymethylester
cfu/mg	:	Colony forming units per milligram
CLSI	:	Clinical Laboratory and Standards Institute
DNA	:	Deoxy-ribonucleic acid
dNTPs	:	Deoxy-nucleotide triphosphates
DPPH	:	2,2 Diphenyl-1- picrylhydrazyl
DW	:	Distilled Water
EDTA	:	Ethylene diamine tetra-acetic acid
EtBr	:	Ethidium bromide
FIC	:	Fractional inhibitory concentration
gm	:	Gram
Mg	:	Microgram
MIC	:	Minimum inhibitory concentration
Min	:	Minute
mL	:	Millilitre
Mm	:	Millimetre
mM	:	Milli mole
mM/L	:	Millimole per litre
mV	:	Milli volt
PBS	:	Physiological buffer saline
PCR	:	Polymerase chain reaction
pg/ml	:	Picogram per millilitre
pH	:	Log hydrogen ion concentration
PI	:	Propidium iodide
PJ	:	*Prosopis juliflora*

RNA	:	Ribo-nucleic acid
RPM	:	Revolutions per minute
SEM	:	Standard error mean
TAE	:	Tris-Acetate-EDTA
TEM	:	Transmission electron microscope
Tris	:	Tris-hydroxy methyl aminoethane
TSB	:	Trypticase soy broth
µg/ml	:	Micro gram per millilitre

LIST OF TABLES

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Contents

ABSTRACT

Endometritis is one of the leading causes of reproductive inefficiency, infertility and decreased milk yield in high yielding dairy cows and buffaloes as well as in other species of animals and are associate with severe economic loss. Indiscriminate use of antimicrobial agents has resulted in development of resistance to the synthetic antimicrobials. Plant-derived active principles have promising potential to act as an alternative to conventional antimicrobial agents. In the present study, an attempt was made to investigate the mechanism of action of *Prosopis juliflora* leaves (PJ) extract against clinical isolates of *E. coli.*. Out of 42 clinical samples of uterine discharges and 23 isolates from cattle and buffaloes having history of uterine infection, we identified two isolates as virulent *E.* coli based on the cultural, biochemical and genotypic characterization. Ethanolic PJ extract showed presence of large quantity of total phenolic acid and flavonoid contents as well as promising *in vitro* antioxidant and radical scavenging activity. PJ extract exhibited a marked *in vitro* antibacterial effect against S4 isolate and reference strain (ATCC 25922) as evidenced by agar well diffusion test with MIC value of 0.39 mg/ml. This antibacterial action of PJ extract was shown to initiate at 6 h post-exposure while the complete bactericidal action was achieved at 12 h post-exposure. Additionally, the electron microscopy and fluorescent microscopy studies revealed that PJ extract produced damage to the bacterial cell wall and caused loss of cell membrane integrity leading to release of the intra-cytoplasmic contents and formation of vacuole resulting in cell death. Moreover, 12 h post-exposure to PJ extract significantly down-regulated mRNA expression of acrA and acrB efflux pump genes in clinical isolate of *E. coli* (S4). The cultural characteristic and crystal violet assays evidently suggested the strong biofilm producing ability of S21 isolate, and PJ extract was exhibited to cause 54.55 % inhibition of biofilm production by this isolate. Further, *in situ* visualization of biofilm production by scanning electron microscopy revealed that PJ extract decreased the multilayer growth of biofilms and free living cells by influencing the integrity of cell wall. Additionally, it was also observed that the disturbed cell wall of the bacterium led to failure in the emergence of cluster and incapable of maintaining their typical morphology in presence of the extract. Based on these above findings, it may be concluded that PJ extract have promising antimicrobial activity against clinical isolates of *E. coli* and the bactericidal action of the extract was possibly mediated by disruption of cell wall and cell membrane, attenuating the expression of efflux pump related genes as well as by inhibiting the bacterial biofilm production. Thus it may be inferred that PJ extract can be used as an alternative to conventional antimicrobials against uterine infection in large animals, however, *in vivo* efficacy testing warrants further investigation.

Introduction

CHAPTER-1

INTRODUCTION

Uterine disorders like metritis and endometritis are the major causes of reproductive inefficiency in farm animals and are associated with poor reproductive performance in high yielding dairy cows, buffaloes, mares, bitches and other species of animals resulting in decreased milk yield and infertility, and ultimately leading to heavy economic losses (Fourichon et al., 2000; LeBlanc et al., 2002; Gilbert et al., 2005; Chapwanya et al., 2009). Post-partum endometritis associated with microbial infection is one of the major etiological factors that cause infertility during subsequent breeding seasons (LeBlanc et al., 2002). Bacterial strains like *Escherichia coli* and *Trueperella pyogenes* along with anaerobic bacteria such as *Fusobacterium necrophorum* and *Prevotella melaninogenica* are commonly associated with clinical and subclinical endometritis in bovines (Gautam et al., 2010). Presence of pathogenic bacteria in uterus initiates inflammatory reaction in the uterus and interfere with the normal physiological process of uterine involution, ovulation and embryo survival (Sheldon et al., 2006). Besides pathogenic bacteria, dystocia, ketosis and parity also contribute to development of inflammation of uterus (Dubuc et al., 2010).

Endometritis is a localized inflammation of uterine wall and it can affect all layers of the uterus *viz*. endometrium, myometrium, and perimetrium. The uterus is typically aseptic, however, endometritis results from the travel of normal bacterial flora from the cervix and vagina and it is a major cause for bovine infertility. The causal organisms usually reach the uterus during coitus, insemination, parturition and/or after parturition. The retention of fetal membrane, abortion, dystocia, mounting by infected bull, unhygienic practices at insemination, hypocalcaemia, season and poor nutrition are the main factors associated with the development of endometritis. Endometritis also results from the rupture of membranes during childbirth (Taylor and Pillarisetty, 2020).

Incidence of clinical endometritis ranges between 18% and 37% (Etherington et al., 1984; Bartlett et al., 1986; Peeler et al., 1994; Drillich et al., 2002; Drillich et al., 2005); whereas subclinical endometritis ranges between 12% and 94% (Raab, 2004; Kasimanickam et al., 2004; Gilbert et al., 2005; Barlund et al., 2008). Prevalence of clinical and subclinical endometritis depends on occurrence of early

post-partum uterine diseases (Benzaquen et al., 2007), time of examination and diagnostic techniques employed (Drillich et al., 2002). Decreased fertility persists even after the clinical resolution of metritis and conception rate declines even up to 20% as compared to the healthy cow and around 3% cows remain infertile (LeBlanc et al., 2002). However, subclinical endometritis has been considered as the most common causes of conception failure in bovine in India (Raab, 2004). Intrauterine antimicrobial therapy is the most commonly adopted process in dairy animals even though results are variable (Drillich et al., 2005). Some of the commonly used antibacterial agents in veterinary practice for treatment of endometritis are oxytetracycline, penicillins, aminoglycosides, quinolones and cephalosporins etc. These antimicrobial therapies have varying degrees of success, inconsistent recovery rate, high cost of treatment, milk disposal problem after treatment, and emergence of microbial resistance (Santos et al., 2010). Moreover, the indiscriminate use of antimicrobial agents has resulted in development of resistance against these available antimicrobial agents. Therefore, management and treatment of clinical and subclinical endometritis remain a challenging task to the clinicians. Thus, there is an urgent and immediate need of an alternative and effective therapy for uterine infections, and use of some natural substances may strengthen the natural defense mechanism(s) in uterus. Conventional antimicrobials have a single and specific target site of action while plant-derived phyto-constituents exhibit multiple target sites due to presence of chemically and structurally diverse active principles and secondary metabolites. Thus, used of these compounds either alone or concurrently with existing antimicrobials against endometritis always open an effective alternative and complementary therapeutic strategy to fight against pathogens and to combat resistance.

Many plants have been reported to possess antimicrobial property e.g. *Moringa oleifera* (Vieira et al., 2010), *Eucalyptus citriodora* (Luqman et al., 2008), *Tegatus erecta* (Dixit et al., 2013), *Genera expansa* (McGaw et al., 2005), *Allium sativa* (Sarkar et al., 2006), *Ficus asperifolia* (Watcho et al., 2011), *Acacia nilotica* (Solomon et al., 2010), *Prosopis juliflora* (Ukande et al., 2019) etc. Babool plants are of different types and among them *Acacia spp* and *Prosopis spp.* are most frequently found in India. *Acacia spp* is commonly known as 'desi babool' whereas *Prosopis juliflora* is known as 'angaraji babul', 'vilayati babul', 'vilayati khejra' or 'vilayati kikar' in Hindi. Among different species of *Prosopis spp*, the mostly reported species

having medicinal values are *P. africana, P. alba, P. cineraria, P. farcta, P. glandulosa, P. juliflora, P. nigra, P. ruscifolia and P. spicigera.*

The *Prosopis* genus belongs to the Fabaceae or Leguminosae family, and includes about 45 species of spiny trees and shrubs. This plant is found in both subtropical and tropical areas of the world. *Prosopis* plants have been traditionally used for the treatment of asthma, birth/postpartum pains, callouses, conjunctivitis, diabetes, diarrhea, expectorant, fever, flu, liver infection, malaria, otitis, pediculosis, rheumatism, scabies, skin inflammations, spasm, stomach ache, removal of bladder and pancreas stones etc.(Ahmed et al., 2015; Umair et al., 2017; Younis et al., 2018). The paste, gum, leaves and pods smoke of *Prosopis* plants possess various bioactive properties, such as anticancer, antidiabetic, anti-inflammatory, antimicrobial and antioxidant effects (Almaraz-Abarca et al., 2007). Phytochemical analysis identified several bioactive compounds in different parts of this plant. Singh (2012) reported the presence of distinct classes of secondary metabolites namely, tannins, phenolics, flavonoids, alkaloids, terpenes and steroids in different parts of *P. juliflora* extracts. Though, the antibacterial efficacy of *Prosopis juliflora* (Babool) leaves was documented earlier, most of the studies reported its efficacy against food-borne pathogens of Gram-positive and/or Gram-negative in nature with no reports against bacteria of animal origins. Moreover, the mechanism of its antibacterial action is yet to be explored. Thus present study was designed with following objectives:

i) To isolate and characterise *Escherichia coli* from uterine infections of bovine origin, and

ii) To assess the mechanism of action of Babool leaves extract against *Escherichia coli* isolated from uterine infections in bovines.

Review of Literature

CHAPTER – 2

REVIEW OF LITERATURE

Endometritis, the inflammation of endometrium without any systemic illness, usually occurs at least 21 days after calving (Barlund et al., 2008). It is sub-divided into clinical and subclinical categories. Clinical endometritis is very well characterized by purulent or mucopurulent uterine discharges on 21 or 26 days postpartum, respectively. Subclinical endometritis is a condition characterised with more than 18 % polymorphonuclear (PMN) cells in uterine discharges. Although cows having subclinical endometritis do not show any uterine discharges but still they show poor reproductive performances. Due to the lack of noticeable clinical symptoms related to the reproductive system, subclinical endometritis is diagnosed late, usually when insemination becomes ineffective. Endometritis is common in post-partum dairy cows and is associated with impaired reproductive performance as reflected in reduced conception rate, reduced hazard of pregnancy over the breeding period and increased risk of reproductive culling (Gilbert, 2011). Inflammation of the uterus in cows causes pathological changes in the endometrium which disturbs fertilization, hampers implantation, and may even lead to early miscarriages (Lopez-Gatius et al., 1996). It also causes ovarian cycle disorders, including the formation of ovarian cysts, prolongation of the luteal phase and delay of first postpartum estrus (Herath et al., 2009).Thus, endometritis is also associated with lower conception rates, increased intervals from calving to first service or conception, and reduction in conception rate (Borsberry et al., 1989).

Despite numerous studies, implementation of new diagnostic methods and the use of different therapeutic methods, endometritis in dairy cows remains a serious economic problem all over the world. This is mainly due to the large economic losses caused by the low rate of artificial insemination and the necessity to cull animals in the herd (Galvao et al., 2012; LeBlanc, 2008). Parturition is a period of high risk for mother and offspring in all species, and cattle are no exception. Risk of physical damage during the birth process or failure to release the placenta after parturition (retention of placenta), also creates favourable environment for microbial infections in the cow. Some animals acquire infections of the uterus or mammary gland during

late gestation, which may lead to premature parturition, or may compromise fetal or calf health. However, the greatest impact on health and productivity is associated with microbial contamination of the uterine lumen after parturition. Amongst the mammals, *Bos taurus* and particularly dairy cattle farmed in intensive system, commonly acquire microbial contamination of the uterus. Indeed, 80–100 % of animals have bacteria in their uterine lumen within the first 2 weeks after calving. Although immune responses progressively eliminate the microbes, up to 40 % of animals still have a bacterial infection 3 weeks after calving.

2.1 Etiology of uterine infection

a. Bacterial agents

Escherichia coli and *Arcanobacterium pyogenes* are the most prevalent bacteria isolated from the uterine lumen of cattle with uterine disease, followed by a range of anaerobic bacteria such as *Prevotella* species, *Fusobacterium necrophorum*, and *Fusobacterium nucleatum* (Sheldon et al., 2002; Williams et al., 2005). The postpartum environment of the uterine lumen supports the growth of a variety of aerobic and anaerobic bacteria. Many of these bacteria are contaminants in the uterine lumen and are removed by a range of uterine defense mechanisms. Indeed, *A. pyogenes, F. necrophorum* and *Prevotella* species have been shown to act synergistically to enhance the likelihood of uterine disease and increase the risk and severity of clinical endometritis (Ruder et al., 1981; Olson et al., 1984). Numerically the most prevalent pathogens are *E. coli* (37 %) and *A. pyogenes* (49 %) (Williams et al., 2005). Furthermore, *E. coli* infections appear to precede and pave the way for the *A. pyogenes* infection (Williams et al., 2007).

b. Viral particles

Bovine herpesvirus 4 (BoHV-4) is the only virus consistently associated with uterine disease after parturition in cattle, and BoHV-4 infection is widespread in the European Union (Castrucci et al., 1986; Ackermann, 2006). Like other herpes viruses, BoHV-4 can establish persistent infections in its natural host, particularly in macrophages (Osorio and Reed, 1983), and enter many other cell types in different species (Donofrio et al., 2008).

c. Managemental factors:

This includes various factors like farm hygiene, cleanliness etc. that can lead to adverse health conditions leading to the establishment of post-partum bacterial infection.

d. Environmental factors:

Extreme weather conditions can be detrimental to the stressed cow after parturition which may alter the immune status, paving way for the pathogen entry. Also, various toxicants found in the environment like polycarbonated biphenyls were proven to cause infection to the uterus (Trabert et al., 2010).

2.2 Prevalence of uterine infections

The prevalence of uterine infections varies considerably (Lewis, 1997) and variations among these studies in prevalence rate is not surprising because many researchers (Curtis et al., 1985, Markusfeld, 1987) did not describe the diagnostic methods, the classification of the uterine infections, the postpartum period during which the infection were detected, the parity of the cows, the general characteristics of the cows, or the herd management practices (Lewis, 1997). For example, prevalence of endometritis in cows in Spain ranged from 2.6 to 4.5 % (Lopez-Gatius, 2003), in Denmark 6.25 % (Bruun et al., 2002), in Korea 47.6 % (Kim and Kang, 2003), while in Australia it varied from 5.6 % to 10.9 % (Moss et al., 2002) and in UK, a prevalence rate of 10.1 % was recorded (Borsbery and Dobson, 1989). Approximately one-third of post parturient cows develop metritis and 10 to 15 % have clinical endometritis (Borsberry and Dobson, 1989). However, subclinical cases of metritis often progress to endometritis, which is an important cause of infertility in cows and economic loss in the dairy industry (Gilbert et al., 2005). High prevalence of subclinical endometritis (53 %) was found among dairy herds in the United States using cytological methods for the diagnosis of uterine diseases (Gilbert et al., 2005). Following calving, the uterus of more than 90% of all cows becomes contaminated with bacteria, some of which are harmful leading to establishment of infection and uterine disease (Sheldon et al., 2002).

The prevalence rate of uterine infection in buffaloes was much higher than in cows, and 24.7 % prevalence rate was reported in India (Gupta et al., 1978; Rao and

Sreemannarayana, 1982). Raman and Bawa (1977) found a high prevalence rate of 38.54 % in buffaloes. While metritis and endometritis were recorded with its prevalence rate of 25 % and 20.68 % (Rao and Sreemannarayana, 1982), Rao (1982) recorded 30 % prevalence of endometritis among Indian buffaloes. In Pakistan, Usmani and co-workers (2001) recorded a prevalence of 24 % of uterine infection among buffalo. In Malaysia, the prevalence rate was the same as in India, with high prevalence of ovaro-bursal adhesions (Jainudeen, 1986). In Egypt, Serur and co-workers (1982) recorded a prevalence of 38.9 % and Ghanem and co-workers (2002) recorded 22.4 % of endometritis in Egyptian buffalos. In Iran, Moghami and co-workers (1996) recorded a prevalence of 33.2 % of endometritis in buffaloes. A prevalence rate of 29.4 % of infertility problems including endometritis and metritis were recorded in Iranian local breed buffaloes (Moghaddam and Mamoei, 2004)). In Iraq, it was recorded as 45.3 % of uterine infections (El-Dossokey and Juma, 1973). Al-Fahad and co-workers (2004) recorded 43.3 % prevalence of chronic endometritis in Basra buffaloes. While Alwan and co-workers (2001) recorded a prevalence of 47.9 % of endometritis in Baghdad buffaloes. Azawi (2006) documented higher prevalence of uterine infections in buffaloes than in cows which might be due to poor hygiene, vaginal stimulation for milk let down and possibly, wallowing. In contrast, the prevalence of uterine infections is low in Zebu cattle (Rao, 1982), and this may be related to the ease with which the Zebu expels the calf and placenta (Vale-Filho et al., 1986). In Brazil, the prevalence of endometritis in Zebu was recorded 3.3 % (Vale-Filho et al., 1986). In India, Saxena and coworkers (2006) detected 6.84 % incidence at Rajasthan in 2006. The latest available record from Punjab by Singh and co-workers (2017) reported the incidence of endometrtis in livestock is 17.65 %.

2.3 Pathogenesis of endometritis

The uterus of postpartum cows is usually contaminated with a range of bacteria, but clinical disease does not always develop. Infection and disease is dependent on adherence of pathogenic organisms to the mucosa followed by colonization or penetration of the epithelium and⁄or release of bacterial toxins that lead to establishment of uterine disease (Janeway et al., 2001). The development of endometritis also depends on the balance between the immune response of the cow, and the species and number of bacteria. This balance can be tipped in favour of

disease by risk factors such as retained placenta, dystocia, twins and stillbirth (Grohn and Rajala-Schultz, 2000; Kim and Kang, 2003). Unfortunately, these risk factors are not particularly amenable to intervention to reduce the incidence of disease and the factors that could be addressed, such as the cleanliness of the environment or the animals, are far less important (Noakes et al., 1991).

Uterine infection has been classified into mild, acute and chronic depending on histo-pathological changes observed in uterine tissues (Jubb et al., 1985; McEntee,1990). The histo-pathological changes in mild endometritis are not striking and characterized by light infiltration of inflammatory cells with slight desquamation of the superficial epithelium and no significant vascular changes. While in acute endometritis, there is a prominent leukocyte infiltration involving all mucosal elements including the glands, and massing at the surface, with suppurative and superficial necrosis. In chronic endometritis, there is a prominent leukocyte infiltration involving all mucosal elements, the uterine wall is thickened with suffused blood and edema, serosa is dull and finely granular with “paint brush” hemorrhages and thin deposition of fibrin or sub serosal vessels may be darkly congested (Dzhuroua and Gulubinov, 1981).

Uterine infections as well as cytological endometritis, which are not necessarily congruent, play a crucial role in subfertility in cows. It seems likely that underlying mechanisms for subfertility can be found on molecular level (Sheldon et al., 2014). Several studies found differences in endometrial gene expression of pro-inflammatory mediators, such as cytokines, antimicrobial peptides, acute phase proteins (APP) and prostaglandins between healthy cows and subfertile cows with subclinical or clinical endometritis (Fischer et al., 2010; Drillich et al., 2012; Hoelker et al., 2012; Peter et al., 2015; Ibrahim et al., 2016). Upregulated chemokine direct the polymorphonuclear (PMN) cells influx into the uterine lumen (Zerbe et al., 2003) and chemoattractive effect has been reported for Interleukin (IL) 8 and CXCL5 (Fischer et al., 2010; Galvão et al., 2011). Cytokines IL1A, IL1B, IL6 and TNFα can be regarded as mediators of nonspecific inflammatory processes but are also physiologically upregulated during the early puerperal period (Gabler et al., 2010). Highest cytokine and acute phase proteins was observed during the third week of postpartum regardless of their health status (Gabler et al., 2010; Chapwanya et al., 2012).

2.4 Efflux pumps: its role in bacterial virulence and drug resistance

Bacterial genome comprises of efflux pump (EP) genes, expressed under tight regulation of global/local transcription factors (e.g., BmrR: transcriptional regulator of efflux pump Bmr in *B. subtilis;* QacR: transcriptional repressor of QacA transporter in *S. aureus;* AcrR: transcription repressor of acrB efflux pump in *E. coli*) suggesting the important physiological roles of efflux pumps during cell development, stress adaptations and bacterial pathogenesis (Sun et al., 2014). Various substances that threaten the survival of the bacteria like antibiotics are extruded out from the cytoplasm via efflux pumps and bacteria may develop multiple drug resistance by overexpressing these efflux pumps (Wright, 2011) which are dependent on energy and transportation systems. The knowledge about these regulatory mechanisms may advance the understandings of physiologically originated antimicrobial resistance (Sun et al., 2014). The description of an efflux system able to pump tetracycline out of the cytoplasm of *E. coli* was first reported in early 1980s (McMurry et al., 1980) and since then many classes of efflux pumps have been characterized in both Gram-negative and Gram-positive pathogens. These efflux pumps may be substrate-specific (for a particular antibiotic such as *tet* determinants for tetracycline and *mef* genes for macrolides in *Pneumococci*) or have broad substrate specificity, which is usually found in multiple drug resistant bacteria (Poole, 2005). This mechanism of resistance affects a wide range of antimicrobial classes including protein synthesis inhibitors, fluoroquinolones, β-lactams, carbapenems, and polymyxins. The genes encoding efflux pumps can be located in mobile genetic elements (as initially described for the *tet* gene) or in the chromosome. Chromosomally encoded pumps can explain the inherent resistance of some bacterial species to a particular antibiotic (eg. *Enterococcus faecalis* intrinsic resistance to streptogramin A).

There are five major families of efflux pumps, namely i) Major facilitator superfamily (MFS), ii) Small multidrug resistance family (SMR), iii) Resistance-nodulation cell-division family (RND), iv) ATP-binding cassette family (ABC), and v) Multidrug and toxic compound extrusion family (MATE). These families differ in terms of structural conformation, energy source, range of substrates they are able to extrude, and the type of bacterial organisms in which they are distributed (Piddock,

2006). In addition to these carriers, another efflux protein named as Proteobacterial Antimicrobial Compound Efflux (PACE) superfamily has been described in *A. baumannii* and is structurally close to the SMR family (Hassan et al., 2015).

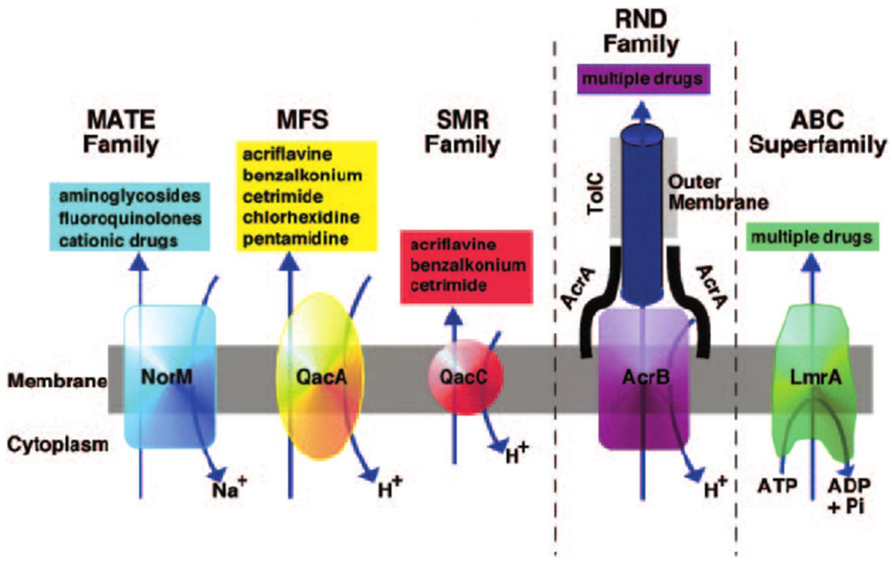


Fig.1: Major families of the transmembrane transporters which are responsible for development of resistance to diverse group of antimicrobials

The most clinically significant of these in Gram-negative bacteria are members of the RND family. These pumps exist as a tripartite system, spanning both the inner and outer membrane. The most well-characterized RND system in *Escherichia coli* is AcrAB-TolC system which, like other pumps in this family, is made up of three components: an inner membrane transporter (AcrB), an outer membrane protein channel (TolC) and a periplasmic adaptor protein (AcrA) (Du et al., 2014). Among different species, RND systems are highly conserved and comparative genomics shows that efflux pumps in *E. coli* (AcrB), *Pseudomonas aeruginosa* (MexB), *Campylobacter jejuni* (CmeB), *Acinetobacter baumannii* (AdeB) and *Neisseria gonorrhoeae* (MtrD) show high levels of homology (Piddock., 2006).

The three component system of RND pumps allows transport of compounds across both membranes. Efflux by the constitutively expressed RND system, MexAB-OprM in *P. aeruginosa* confers intrinsic levels of resistance to β-lactams, tetracycline, tigecycline, chloramphenicol, trimethoprim, sulfamethoxazole and norfloxacin (Li et

al., 1994; Srikumar et al., 1997; Poole, 2000). Intrinsic resistance to multiple antibiotics due to MDR efflux systems has also been described in *E.coli* (Ma et al.,1993), *Campylobacter jejunii* (Pumbwe and Piddock, 2002), *Haemophilus influenza* (Peric et al., 2003) and *Salmonella enteric serovar Typhimurium* (Miyamae et al., 2001). RND efflux pumps confer intrinsic levels of resistance and it is thought that the additional effect of non-RND transporters upon such resistance may be minimal. Nonetheless, MFS, SMR and MATE pumps can also play a role in MDR in Gram-negative bacteria.

MFS transporters tend to be substrate specific. For example, the CraA MFS pump in *A. baumannii*, which is homologous to MdfA pump in *E. coli,* only confers resistance to chloramphenicol (Roca et al., 2009). The *E. coli* MFS efflux pump EmrB has a wider substrate range than that of CraA, and confers resistance to a range of hydrophobic toxins, such as carbonyl cyanide m-chlorophenyl hydrazine (Lomovskaya and Lewis, 1992). Association of EmrB with the membrane fusion protein, EmrA and the outer membrane channel, TolC, forms a tripartite system and allows substrates to be transported across the inner and outer membranes (Tanabe et al., 2009).

SMR transporters are small, hydrophobic, bacterial multidrug-resistant efflux proteins (Paulsen et al., 1996) that have been shown to export various classes of antibiotics out of Gram-negative bacteria such as *P. aeruginosa* (Li et al., 2003), *Klebsiella pneumonia* (Srinivasan et al., 2013*), A. baumannii* (Srinivasan et al., 2009) and *E. coli* (Yerushalmi et al., 1995).

Several studies evidently suggest that bacterial strains lacking EPs exhibit reduced pathogenicity. For example *S. typhimurium* acrB or tolC deficient mutant poorly colonized in the avian gut, highlighting the requirement of complete AcrAB-TolC system for virulence (Buckley et al., 2006). The *S. typhimurium* strain lacking all the drug efflux assemblies became avirulent, when tested in a mouse model (Nishino et al., 2006). To confirm the role of EPs in bacterial pathogenesis, Hirakata et al. (2002) assessed the ability of EP (MexAB-OprM, MexCD-OprJ, MexEF-OprM, and MexXY-OprM) mutants of *P. aeruginosa* to invade Madin-Darby canine kidney cells. The findings revealed that except mexCDOprJ, all other systems evidenced decreased bacterial invasion abilities. EPs are also known to effect the bacterial cell

communication during the stress-responses, especially in the quorum-sensing (González and Keshavan, 2006). As transportation of autoinducers (chemical signals generated during quorum sensing) is a key-event during cell-cell interactions via quorum-sensing, drug EPs assist their transport across the membrane (Liang et al., 2016). Moore et al. (2014) confirmed a vital role played by MexAB-OprM efflux system in the secretion of a major auto-inducer N-acylated L-homoserine lactone by *P. aeruginosa* cells. The study also postulated this auto-inducer as a substrate for MexAB-OprM system (Moore et al., 2014). Further, Martinez and co-workers (2009) advocated the EP-mediated ceasing of quorum-sensing via augmented efflux of autoinducers, facilitating quick bacterial response to stress signals. One more physiological role attributed to EPs is in the biofilm formation. Recent studies confirm the involvement of many well-characterized efflux systems, AcrAB-TolC of *E. coli*, AcrD of *S. enterica*, AdeFGH of *A. baumannii* and MexAB-OpeM of *P. aeruginosa* in biofilm formation (Alav et al., 2018). Kvist and co-workers (2008) reported an up-regulation of 20 genes encoding EP-transporters in *E. coli* during the growth of biofilm. Similarly, the EP-mediated export of colanic acid for capsule-matrix formation was observed along with up-regulated TetA(C) in *E. coli,* facilitating the biofilm formation (May et al., 2009). Collectively, the physiological roles of EPs are vital for pathogenic stability and virulence maintenance in bacteria. The synthetic EPIs namely carbonylcyanidemchlorophenylhydrazone (CCCP), chlorpromazine and PAβN were reported to prevent biofilm formation in *E. coli, P. aeruginosa* and *S. aureus* (Baugh et al., 2014). However, investigations on evaluating phytochemicals for their antibiofilm potencies via inhibiting EPs are few. Fiamegos and associates (2011) isolated 4,5-O-dicaffeoylquinic acid from *Artemisia absinthium* which was proved to be a potent inhibitor of MFS pumps as well as anti-biofilm agent in *E. coli* and *E. faecalis* (Fiamegos et al., 2011). Recent reports advocate that nanomaterials in combination with phyto-EPIs can also be an effective therapy for containing drug-resistant infections (Gupta et al., 2017).

2.5 Conventional treatment strategies against uterine infection

Reproductive performance is one of the key components of dairy production management. The general principle of therapy of endometritis is to halt and reverse inflammatory changes that impair fertility (Gilbert, 1992). Practically, treatments aim

to reduce the load of pathogenic bacteria and enhance the processes of uterine defence and repair. A wide variety of therapies for endometritis have been reported, including systemic or local antibiotics, as well as systemic prostaglandin F2α (PGF2α) or estradiol. Treatment of postpartum endometritis has been reviewed from time to time (Gustafsson, 1984; Bretzlaff, 1987; Gilbert and Schwark, 1992; Olson, 1996). Well-designed, large-scale clinical trials with objective case definitions and economically meaningful outcomes are lacking. Many therapeutic trials suffer from a lack of negative controls, small numbers of animals resulting in little statistical power, or both. Most investigations have used diagnostic criteria for endometritis that were not validated as to their effect on reproductive performance, making it difficult or impossible to discern a true treatment effect. Some studies have used clinical or bacteriologic cure as the endpoint (Callahan and Horstman, 1993; Dohmen et al., 1995; Brooks, 2000), rather than economically relevant measures of reproductive performance, the probability and timing of pregnancy. Moreover, many studies have reported measures of reproductive performance that were biased or incorrectly analyzed statistically. There is little evidence for a benefit of any intrauterine (i.u.) antimicrobial on reproductive performance in cows with endometritis (Steffan et al., 1984; Thurmond et al., 1993), but some evidences show that routine postpartum use of PGF2α reduces time to pregnancy (Etherington et al; 1988; Risco et al., 1994). Most comparative clinical trials (Steffan et al., 1984; Sheldon and Noakes, 1998) and reviews (Gilbert and Schwark, 1992; Olson, 1996) have concluded that $PGF_{2\alpha}$ is at least as effective as or is preferable to intra-uterine infusion of antimicrobials for treatment of endometritis. However, there remains a lack of compelling results on this question, and intrauterine administration of antibiotics to dairy cows remains common.

Ideally, antimicrobial therapy for uterine infection should control pathogens from the uterus and should result in withdrawal periods for milk and meat that are as short as possible (Azawi, 2008). Although systemic or intrauterine antibiotic therapy is commonly used as the treatment for metritis (Azawi, 2008), it is recognized that antibiotic therapy cannot sterilize the uterus nor prevent recontamination that occurs during the early postpartum weeks (Sheldon and Dobson, 2004; Azawi, 2008). Furthermore, widespread usage of antimicrobials in food producing animal has

contributed to the emergence of antimicrobial resistance among pathogens, which complicates the treatment of infectious diseases (Tollefson et al., 1999).

The increasing level of resistance to frontline antimicrobial agents relevant to the treatment of human diseases is a significant public health concern (Tollefson and Miller, 2000) and has led to important changes in the perceptions and priorities of federal agencies with regard to usage of antimicrobials as growth promoters and prophylactic agents (Angulo et al., 2004). The FDA, USDA, and Center for Disease Control and Prevention (CDC) strongly promote the development of new classes of antimicrobials and other products able to eliminate or reduce risk of bacterial resistance. The use of pathogen-specific antimicrobials is expected to reduce the incidence of resistance development (Walsh, 2003).

Bacteriophage therapy, which uses virulent (lytic) bacteriophages to control bacterial growth, has shown to be an attractive alternative to conventional drugs (Viscardi et al., 2008) and has been successful in animal trials against a broad range of bacterial pathogens such as enteropathogenic *E. coli* (Callaway et al., 2008), *Pseudomonas aeruginosa* (Soothill, 1992), *Staphylococcus aureus* (Matsuzaki et al., 2003), *Acinetobacter baumannii* (Soothill, 1992) and vancomycin-resistant *Enterococcus faecium* (Biswas et al., 2002).

2.6 Medicinal plants as effective alternative to treat uterine infections

The importance of phytochemicals (natural products and plant-derived secondary metabolites) for the treatment of microbial infections increased in the late 1990's with the increased inefficacy of conventional antibiotics, due in part to their often extensive and inappropriate use in mammalian infections. Plants produce an enormous array of phytochemicals and it is commonly accepted that a significant part of this chemical diversity is related to defence/stress mechanisms including *in vitro* antimicrobial activity (Dixon, 2001).

Accumulating evidences suggest the therapeutic potentials of several medicinal plants, namely- *Saraca asoca, Aloe barbadensis, Gossypium herbaceum, Plumbago zeylanica, Azadirachta indica, Eucalyptus citridora, Moringa oleiofera, Tegatuseracta*. Garlic (*Allium sativum*), is being used since ancient times for its cytotoxic, antitumor, antifungal, antibacterial, antiviral, antiprotozoal properties

(Ledezma and Apitz, 1998). McGaw and co-workers (2005) reported the antibacterial activity of *Gunnera perpensa* against Gram-positive *Enterococcus faecalis* and *Staphylococcus aureus* and Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*. *Eucalyptus citriodora* (family Myrtaceae) oil showed anti-inflammatory (Grassmann et al., 2000), analgesic, antibacterial, antifungal (Hmamouchi et al., 1990), anti-tumor (Takasaki et al., 1995) and anti-diabetic effects (Swanston et.al., 1990). Sarkar and associates (2006) investigated the use of garlic extract against endometritis. About fifty per cent endometritic animals recovered from endometritis and became pregnant in garlic extract treated group. This could be due to significant reduction in bacterial load in garlic extract treated animals, as garlic extract is well known for its antimicrobial property against Gram-positive and Gram-negative bacteria (Yoshida et al., 1998; Chung et al., 2003). Garlic extract has been shown to stimulate release of cytokines such as IL-2, IFN-α, IFN- γ and increase the natural killer activity and enhances phagocytic activity of peritoneal macrophages (Kyo et al., 1998). Esparza and co-workers (1996) also evaluated the therapeutic efficacy of plant extracts in treatment of acute endometritis and uterine involution. Extracts of garlic (*Allium sativum* L), Eucalyptus (*Eucalyptus globulus* Labill), and Gordolobo (*Gnaphalium conoideum*) were applied as intrauterine infusions to 45 Holstein cows divided in 3 groups. Treatment was repeated every 48 hours until clinical response was observed. They have reported that garlic extract was most effective, although *Eucalyptus* extract was also effective, requiring more treatment. Huda and associates (2013) reported the comparative effect between *Cyperus esculentus* seeds extract and gentamicin on endometritis in mice. Earlier report from our laboratory also suggested the antibacterial and anti-inflammatory potential of *Eucalyptus robusta* leaves against *Staphylococcus aureus*- and *E. coli*-induced endometritis in rats (Tiwari et al., 2018).

2.7 Potential of natural compounds as efflux pump inhibitors:

Bacteria often minimize the accumulation of chemotherapeutic agents by expressing the efflux pumps (EP) resulting in development of drug resistance. Medicinal plants contain several phytochemicals which target bacterial efflux pump to combat multiple drug-resistance (Newman and Cragg, 2012; Prasch and Bucar, 2015). The anti-hypersensitive alkaloid reserpine which was isolated from *Rauwolfia vomitoria* is considered as one of the potent efflux pump inhibitors (EPI) (Stavri and

Piddock, 2007). Similarly, EP inhibitory activity of gallotannin (1,2,6- tri-O-galloyl-b-D-glucopyranose) isolated from hydro-alcoholic extracts of *Terminaliachebula fruits* was demonstrated against MDR uropathogenic *E. coli* (Bag and Chattopadhyay, 2014). Gallotannin induced 2 to 4 fold reduction in MIC value of test antibiotics via inhibiting the ethidium bromide (EtBr) pump. As EtBr is a known EP-substrate, inhibition of EtBr efflux backs the postulated EP inhibitory activity of gallotannin (Bag and Chattopadhyay, 2014). Methanolic extracts of *Acer saccharum* was also reported to exert drug efflux inhibitory potentials against *P. aeruginosa* (ATCC 15692 and UCBPPPA14), *E. coli* (ATCC 700928) and *P. mirabilis* (HI4320) (Maisuria et al., 2015). In another study, lysergol and its derivative, 17-O-3″,4″,5″-trimethoxybenzoyllysergol (a clavine alkaloid from *Ipomoea muricata*) was demonstrated to exhibit strong EP inhibitory potentials against resistant *E. coli* strains (MTCC1652 and KG4) (Maurya et al., 2013). Similarly, falcarindiol, isolated from *Levisticum officinale* exhibited EPI activities against the Gram-negative strains (Garvey et al., 2011). On the similar lines, Dwivedi and co-workers (2018) reported catharanthine potentiated the activity of tetracyclin and streptomycin against superbug *P. aeruginosa*. A pentacyclic triterpenoid (ursolic acid) isolated from leaves of *Eucalyptus tereticornis* and esterified derivatives of ursolic acid, 3-O-acetyl-urs-12-en-28-isopropyl ester and 3-O-acetyl-urs-12-en-28-n-butyl ester were also reported to show promising EP inhibitory action (Dwivedi et al., 2014). Molecular docking confirmed the targets of these compounds are AcrA/B, MacB, TolC (Dwivedi et al., 2014). Similarly, two alkaloids isolated from roots and rhizomes of *Berberis vulgaris*, the barberine and palmatine showed potent EP inhibitory efficacies against *P. aeruginosa* isolated from burn infections (Aghayan et al., 2017). Phenylpropanoids from the n-hexane and chloroform fractions of *Alpinia galanga* exhibited EP inhibitory activities against *Mycobacterium smegmatis* (Roy et al., 2012). A dose-dependent EP inhibition was observed with 1′ -S-1′ -acetoxyeugenol acetate (Roy et al., 2012). Naphthoquinonediospyrine isolated from *Diospyros montana* along with its derivatives were proved to be highly potent EPI and allowed bacterial cells to accumulate high concentrations of ciprofloxacin (Mukanganyama et al., 2015). The acylphloroglucinol (olympicin-A) isolated from n-hexane fractions of *Hypericum olympicum*, showed promising activities against *S. aureus* (Shiu et al., 2013). The radiometric accumulation assay of the strain overexpressing NorA pump

indicated the enhanced accumulation of (14)C-enoxacin, thus confirming efflux inhibition (Shiu et al., 2013). Traditionally, most of the investigations were aimed at identifying EPIs for Gram-positive strains for reversing their AMR characters with very few reports against Gram-negative members. This can be because Gram-negative bacteria are more difficult targets than their positive counterparts due to the presence of powerful efflux pumps and other effective membrane barriers (lipophilic layer) averting them from external impacts (Stavri and Piddock, 2007; Prasch and Bucar, 2015). Though, some approaches have emerged in recent years for improving antibiotic-penetration across the permeability membranes of Gram-negative bacteria such as the inhibition of new accessible target, identification of uptake pathways and the "Trojan Horse" approach (achieving fast or facilitated antibiotics uptake), establishing the rules of permeation (for predicting whether elevated uptake or reduced efflux would be the most efficient way for increasing the potency of specific antimicrobial class) and identifying potent EPIs (Zgurskaya et al., 2015). Bruns et al. (2017) successfully inhibited EmrD3 pump-mediated drug efflux from a Gram-negative bacterium *Vibrio cholerae* by garlic extract and its bioactive compound (allylsulfide). At relatively low concentrations, the extract seems to target the EmrD-3 pump, but at higher concentration garlic was shown to affect the respiratory chain. This example confirms targeting the energization of the efflux system by plant compounds as a potential strategy for drug efflux inhibition (Bruns et al., 2017). Further, the MFS conserved sequence motifs, present across the entire superfamily, provide vital information regarding alignments of MFS transporter sequences which may help in understanding the structural templates and actual binding events achieved via these transporters. Molecular dynamic simulation (MDS) studies of VMAT2 multidrug transporter (MFS family) revealed the presence of two domains of six trans-membrane helices (Yaffe et al., 2013). Recent advances in scientific and technological arena have added significant in-depth understandings of the structural and biochemical basis of drug efflux, substrate profiles, molecular regulation and inhibition of major EPs. Active EPs play a critical role in intrinsic and acquired drug resistance via overproduction or over-activation of pumps in Gram-negative bacteria, and the development of clinically useful EPIs or new antibiotics to bypass pump-effects continues to be a challenge in combating Gram-negative bacterial infections (Li et al., 2015). As practically all the antibiotics are susceptible to active drug efflux,

the potent EPIs can target these pumps antagonistically and can make old antibiotics effective again (the phenomenon also known as re-sensitization). Besides, considering the fact that several antimicrobial agents like lipophilic penicillin, many glycopeptides, oxazolidinones, macrolides and lipopeptide daptomycin are effective in treating only Gram-positive bacterial infections and their poor potencies against Gram-negative pathogens is at least partially due to their active drug efflux, novel and potent EPIs are needed to significantly broaden the range of these antimicrobial agents. All this clearly indicates that EPIs have tremendous potential in adjunctive therapies along with the known but otherwise ineffective antibiotics ultimately reducing the emergence of AMR and virulence (Opperman and Nguyen, 2015). But developing novel and potent EPI is difficult and needs to overcome several hurdles such as choice of antibiotics for potentiation and matching the pharmacological properties of EPI-antibiotics pair (Opperman and Nguyen, 2015; Zgurskaya et al., 2015). Considering the serious threats posed by the Gram-negative bacteria and their drug-resistance nature, more investigations aiming to target them with the novel, alternative and effective approaches including exploration of natural products are coming up. Though there are limited success stories, but they may lay the foundation for developing potent EPIs to avert the AMR phenotypes with the help of natural sources.

2.8 *Prosopis juliflora*: A promising plant with several medicinal value

Babool plants are of different types and among them *Acacia spp* and *Prosopis spp.* are most frequently found in India. *Acacia spp* is commonly known as desi babool whereas *Prosopis juliflora* is known as ‘angaraji babul’, ‘Kabuli kikar’, ‘vilayati babul’, ‘vilayati khejra’ or ‘vilayati kikar’ in Hindi. Among different species of *Prosopis spp*, the mostly reported species having medicinal values are *P. africana, P. alba, P. cineraria, P. farcta, P. glandulosa, P. juliflora, P. nigra, P. ruscifolia and P. spicigera.*

Prosopis juliflora belongs to the family Fabaceae and is also known as ‘mesquite’. It is a fast growing, thorny deciduous, drought-resistant plant and has a wide crown and deep-rooted. It is native to Central and South America - spreading from southern Mexico to Panama and from the Caribbean Islands to northern South America and an invader species in India that competes with the native species

(Pasiecznik et al., 2001; Harris et al., 2003; Lakshmi et al., 2010). It grows in all kinds of soil conditions, including wastelands at altitudes ranging from 0 to 1,500 m above sea level (Pasiecnik et al., 2001). This herb is well-known in the folkloric system of medicine because of its ethnobotanical importance. The plant has been reported to treat oral ailments like toothache (Hebbar et al., 2004). The leaves were used against asthma, bronchitis, conjunctivitis (Agra et al., 2008) as well as against skin diseases, blood and venereal diseases and act as an insecticide (Senthilkumar et al., 2009). The crude extracts of various parts and purified chemical components have been found to possess antimicrobial, insecticidal and different pharmacological activities (Malik and Kalidhar, 2005). This is also used traditionally for curing catarrh, colds, diarrhea, dysentery, excrescences, flu, hoarseness, inflammation, measles, sore throat, and for the healing of wounds (Hartwell, 1971).

Phytochemical analysis identified several bioactive compounds in different parts of this plant. Phytochemical analysis of different parts of *P. juliflora* extracts revealed distinct classes of secondary metabolites namely, tannins, phenolics, flavonoids, alkaloids, terpenes and steroids (Singh, 2012). In an another study the preliminary phytochemical screening of ethanolic leaves extract revealed the presence of tannins, phenolic acids, glycosides, flavonoids and alkaloids (Sathiya and Muthuchelian, 2008). Moreover, some compounds belonging to the alkaloid group of bioactive molecules were identified and characterized (Lakshmi et al., 2010). These include β-sitosterol (phytosterol), prosopidione (a terpenoid diketone) and three alkaloids namely secojuliprosopinal, 3'oxojuliprosopine and juliprosopine. Likewise, DART-MS analysis showed that *P. juliflora* leaves are a rich source of piperidine alkaloid and contain two diverse groups of alkaloids, one with an indolizidine ring in the centre of the molecule (viz., juliprosopine, juliprosine and juliprosinine) and other without indolizidine ring (*viz.* julifloridine, projuline and prosafrinine). Among them, juliprosopine and julifloridine were found to be present in highest concentrations (Singh et al., 2011). Similarly, several alkaloids such as juliflorine, julifloricine, julifloridine (Ahmad et al., 1978), juliprosine (Daetwyler et al., 1981), juliprosinene, juliflorinine (Ahmad et al., 1989), 3′-oxojuliprosopine, sceojuliprosopinol, 3-oxojuliprosine and 3′-oxo-juliprosine (Nakano et al., 2004) have been isolated from

leaves and have proven to be pharmacologically active (Ahmad et al., 1989; Aqeel et al., 1989)

2.9 Pharmacological activities of *Prosopis juliflora*

a. Antibacterial activity: Several alkaloid constituents present in the *Prosopis juliflora* were assessed for their antibacterial property using disc diffusion method on different Gram-negative and Gram-positive bacterial strains like *E.coli, Staphylococcus aureus*, etc. The maximum antibacterial effect is shown by leaf, pod and flower extract of the plant with minimum inhibitory concentration (MIC) value ranging between 25 µg/ml to 100 µg/ml. The extract of leaves showed the maximum activity among of all the other parts (Ukande et al., 2019). Juliflorine isolated from *Prosopis spp* is reported to provide protection against some human pathogenic bacteria *viz. Corynebacterium diphtheria var. mitis, Corynebacterium hofmanni, Bacillus subtilis, Staphylococcus aureus, and Streptococcus pyogenes* (Ahmad et al., 1989*).* Interestingly, juliflorine offered substantial antibiotic efficacy even against *Streptococcus faecalis,* which is resistant to most antibiotics (Criswel, 2004; Ventola, 2015). Compared with the antibiotics streptomycin and penicillin, ethanol extracts of *Prosopis spp.* provide antibacterial activity against various pathogenic bacteria (Sathiya and Muthuchelian, 2008). The crude methanolic extracts of *P. juliflora* showed antibacterial activity against *S. aureus* and *Escherichia coli* (Sukirtha and Growther, 2012). The compounds myo-inositol-4C-methyl and N-bchloropropionyltryptamine are particularly responsible for this activity (Vedak and Raut, 2014). In comparison to extracts of other parts of plant, the extract of *P. juliflora* pods shows higher activity against Gram-positive bacteria, such as *S. aureus* (Saheed et al., 2015). Furthermore, ethanolic leaf and root extracts of *P. juliflora* showed antibacterial activities against Gram-negative bacteria that were otherwise resistant to antibiotics such as minocycline, chloramphenicol and erythromycin (Odhiambo et al., 2015). It was also noted that alkaloids, saponins and tannins were likely candidates for this activity (Thakur et al., 2014).

b. Antifungal activity: Alkaloid-rich fractions of *P. juliflora* leave are reported to inhibit the growth of *Cryptococcus neoformans*, and the compounds zerumbone and cassine exert highest antifungal activity against this fungal species (Valli et al., 2014). The methanolic extract of leaves is thermostable and shows considerable antifungal

activity against seed-borne fungal pathogens, such as *Aspergillus candidus*, *Aspergillus columnaris*, *Aspergillus flavipes*, *Aspergillus flavus*, *Aspergillus fumigatus, Aspergillus niger, Aspergillus ochraceus* and *Aspergillus tamarii* (Satish et al., 2007; Napar et al., 2012; Bazie et al., 2014). In a recent report, *P. juliflora* water-soluble leaf ethanolic extract is shown to produce antifungal activity against food-poisoning fungal strains by alternating their hyphae and spores (Saleh and Abu‑Dieyeh, 2021).

c. Antiparasitic activity: The anthelmintic activity of aqueous leaf extracts of *P. juliflora* and roots of *Entada leptostachya* powder has been reported in livestock. This herbal drugs mixture contains several compounds like alkaloids, steroids, phenolic compounds, tannins, flavonoids and saponins (Mutembei et al., 2015). The encapsulated ethanolic extracts of *P. juliflora* leaves display ovicidal potential against *Haemonchus contortus*, a highly pathogenic nematode parasite of small ruminants (Kipyegon et al., 2015). Methanolic extracts of *P. juliflora* have high antiplasmodial activity against the malaria-causing *Plasmodium falciparum* and also antiflagellate activity against the vector of the Chagas disease, *Trypanosoma cruzi* (Al-Musayeib et al., 2012). Further, its activity against Leishmaniasis is also reported recently (Mutile et al., 2021).

d. Anti-inflammatory and anti-ulcerogenic activity: Methanolic extracts of *P. juliflora* bark was reported to exhibit anti-inflammatory activity by blocking prostaglandins in carrageenan- and histamine-induced paw edema model in rats (Sivakumar et al., 2009). Similarly, ethanolic extract of leaves of *P. juliflora* also demonstrated substantial anti-inflammatory action (Choudhary and Nagori, 2013). Compounds such as alkaloids, flavonoids, tannins, anthraquinones, and quinones from *P. juliflora* have been explored as therapeutic drugs against ulcer. These compound are shown to inhibit the growth of the ulcer-causing bacterium *Helicobacter pylori* through a mechanism where H^+-K^+-ATPase are inhibited in combination with antioxidant activity (Gopinath et al., 2013).

e. Miscellaneous activity: 24-methylencycloartan-3-one isolated from *P. juliflora* pods was found to be effective against diabetes (Alsaadi and Al-Maliki, 2015). Additionally, it is also reported to show potent hypoglycemic effect in alloxan induced diabetic rabbits. A new compound isolated from *P. juliflora* flowers was

reported to produce anticancer activity as a spindle inhibitor which can enhance the clastogenic effects in *A. cepa* cells. Further, this compound induced different mutations such as chromosomal aberrations, fragmentations and C-mitotic effects (Shachi, 2012). *P. juliflora* contains specific alkaloids that affect neurodegenerative diseases. The alkaloid juliflorine from *P. juliflora* can inhibit acetyl cholinesterase in a concentration-dependent manner possibly by targeting different amino acid residues (Tyr70, Asp72, Tyr121, Trp279, and Tyr334) belonging to the anionic and peripheral sites of acetyl cholinesterase (Choudhary et al., 2005). Juliflorine also showed dose-dependent spasmolytic and Ca^{2+}-channel-blocking activity in isolated preparations of jejunum from rabbits (Soreq and Seidman, 2001).

Materials and Methods

CHAPTER-3

MATERIAL AND METHODS

3.1 Plant Material:

Leaves of *Prosopis juliflora* plant were collected from Veterinary College Campus, DUVASU, Mathura during the month of June-July, 2021. Taxonomical identification of the plant was confirmed from CSRI-Central Institute of Medical and Aromatic Plants (CSRI-CIMAP), Lucknow with Accession No. 8360. Coarsely powdered leaves of *Prosopis juliflora* were then extracted by hot percolation method using Soxhlet apparatus. Three different solvents *viz.* methanol, ethanol and ethanol-water mixture (hydroalcoholic; 80:20) were used for extraction of phytoconstituents. Thereafter, the extract was concentrated under reduced pressure to obtain semi-solid mass using rotatory vacuum evaporator (Cole-Parmer, USA). The extract was transferred into air tight containers and stored at -20 °C until further use.

3.2 Chemicals/Reagents/Media Used

Table 1: List of the chemicals used

Chemicals	Make/Company
2,2- diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich
Agarose	Amresco
Aluminium chloride	SRL
Ammonium sulphate	Merck
Ascorbic acid	SRL
Carboxyfluoresceindiacetate acetoxymethylester (CFDA-AM)	Thermo Scientific
Casamino acid	Sigma-Aldrich
Cefotaxime	HiMedia
Chloramphenicol	HiMedia
Chloroform	Amresco

Chemicals	Make/Company
Di-potassium hydrogen phosphate	Merck
DNA Ladder	Invitrogen
EMB agar	HiMedia
Ethanol	Amresco
Ethidium bromide	SRL
Ethylenediaminetetraacetic acid (EDTA)	Merck
Ferrous sulphate	SRL
Ferrozine	Sigma-Aldrich
Folin-Ciocalteu reagent	Sigma-Aldrich
Gallic acid	Sigma-Aldrich
Gentamicin	HiMedia
Glacial acetic acid	Merck
Luria-Bertani Agar	HiMedia
MacConkey agar	HiMedia
Mac-farland standard	HiMedia
Magnesium sulphate	Merck
Methanol	SRL
Mueller Hinton Agar	HiMedia
Mueller Hinton Broth	Sigma-Aldrich
Peptone Water	HiMedia
Potassium dihydrogen phosphate	Merck
Propidium iodide	Sigma-Aldrich
Proteose peptone	Sigma-Aldrich
Quercetin	Sigma-Aldrich
Sodium hydroxide	Merck
Sodium nitrite	Sigma-Aldrich
Tryptic Soy Broth	Hi-Media

3.3 Isolation and identification of bacterial isolates*:*

3.3.1 Preparation of culture media:

Solid and liquid culture media were prepared by adding deionized triple distilled water to dehydrated culture media with or without agar, respectively. Media was sterilized by autoclaving at 121 °C for 15 min. Molten agar was cooled to 55 °C before preparation of culture plates and stored at 4 °C until further use.

3.3.2 Sample collection:

Uterine discharges were collected from 23 cows and 19 buffaloes, presented to Veterinary Clinical Complex, College of Veterinary Science & Animal Husbandry, DUVASU, Mathura, during September 2021-December 2021, with the history of uterine infection and clinical endometritis. Presence of the purulent uterine discharge on 21-26 days postpartum was considered as selection criteria for clinical endometritis while increase in polymorphonuclear cells (18%) in uterine discharge was considered for diagnosing subclinical endometritis. For collection of samples, the perineal area was washed with 0.01 % potassium permanganate solution prior to sample collection followed by swabbing with ethyl alcohol. The samples were collected by the double guarded method to prevent vaginal contamination and were processed in the laboratory for isolation of the bacterial organism. Collected samples were incubated overnight in buffered peptone (pre-enrichment) media.

3.3.3 Isolation of *Escherichia coli* from clinical samples:

Pre-enriched samples were inoculated in MacConkey agar plates in duplicate. For identification of *Escherichia coli (E. coli.)*, the lactose fermenter (pink colonies) were differentiated from non-lactose fermenter (colourless) and the pink colonies were then sub-cultured on MacConkey agar. The colonies were picked and stored as glycerol stock at -20 ºC for further use.

3.3.4 Identification of *Escherichia coli* bacterial strains:

3.3.4.1 Culture characteristic and Gram staining: The phenotypic characterization of selected bacterial colony was done by observing the colony characteristic, for example, pink colour colony on MacConkey agar while green metallic sheen on EMB agar. Further the bacterial colony was examined for Gram staining for confirmation of presence of Gm-ve rod shaped bacteria.

3.3.4.2 Biochemical identification: All the selected isolates were characterized by a panel biochemical tests *viz*. catalase, oxidase, ONPG, indole, methyl red, Voges-Prausker, citrate test and triple sugar fermentation test.

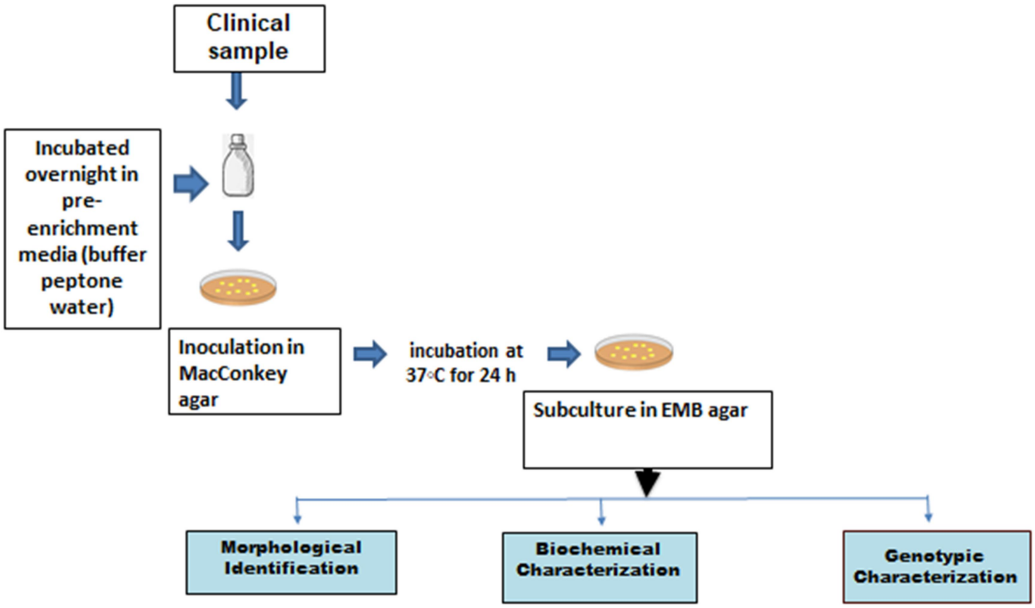


Fig 2: Schematic diagram showing the steps followed for isolation and identification of *Escherichia coli*.

3.3.4.3 Genotypic characterization: All the suspected samples were tested for the presence of *Pap, F41, Sta, csgA* and *csgD* genes for genotyping characterization of *Escherichia coli*.

3.3.4.3.1 Isolation of bacterial DNA: From all the suspected samples, bacterial DNA was isolated by snap chill method. Briefly, a loopfull of each isolate was mixed thoroughly with the nuclease-free water in microcentrifuge tube and the suspended isolates were denatured at 100 °C in boiling water for 10 minutes. After heat treatment the boiled cell lysate in microcentrifuge tubes were placed immediately on ice for 10 minutes followed by centrifugation at 12,000 rpm for 2 min. The supernatant (2µl) was used as DNA template.

3.3.4.3.2 Polymerase chain reaction: The PCR was carried out for detection of the genes (Table 2) in suspected bacterial samples. *Escherichia coli* (ATCC 25922) was used as positive reference. The primer sequences and thermal cycling conditions are summarized in Table 3 and 4, respectively.

Table 2: Description of primers

Gene	Sequence (5'-3')	Amplicon Size (bp)	Annealing Temp. (°C)	Reference
Pap	F-GCAACAGCAACGCTGGTTGCATCAT R-AGAGAGAGCCACTCTTATACGGACA	336	63	Yamamoto et al., 1995
F41	F-GCATCAGCGGCAGTATCT R-GTCCCTAGCTCAGTATTATCACCT	380	50	Shophia et al., 1998
Sta	F-GCTAATGTTGGCAATTTTTATTTCTGTA R-AGGATTACAACAAAGTTCACAGCAGTAA	190	50	Shophia et al., 1998
csgA	F-CAGCAATCGTATTCTCCGGTA R-CGTTGTTACCAAAGCCCAACC	408	58	Silva et al., 2009
cgsD	F-TTATCGCCTGAGGTTATCGTTT R-TAAATCTTCTTTGCAGGCGACA	601	58	Silva et al., 2009

Table 3: Composition of PCR mixture

Composition	Volume
PCR master mix (2X)	6.25 µl
Forward primer (10 pmol/µl)	1.0 µl
Reverse primer (10 pmol/µl)	1.0 µl
Template (1000 ng/µl)	2.0 µl
Nuclease free water	up to 12.50 µl

Table 4: Thermal cycling conditions

SN	Steps	Temperature and duration
1.	Initial denaturation	95 ºC for 5 min.
2.	Denaturation	95 ºC for 40 sec.
3.	Annealing	AT* for 1 min
4.	Extension	72 ºC for 1 min
5.	Step 2-4 repetition	40 cycles
6.	Final extension	72ºC for 5 min
7.	Held at	4ºC

*AT= annealing temperature for respective primers as described in Table 2.

3.3.4.3.3 Agarose gel electrophoresis: DNA amplified by PCR was subjected to agarose gel electrophoresis as described by Sambrook and Russel (2001). Four fifty gram (1.5%) agarose was dissolved in 30 ml 1X TAE buffer (Annexure-I) by heating and allowed to cool to 50 ºC. To this, 1.5 µl ethidium bromide (final concentration 0.5 µg/ml) was added and mixed thoroughly. The mixture was poured onto a gel-casting tray fitted with acrylic comb and allowed to solidify. Once the gel had formed, a few ml of TAE buffer was added near the comb, which was later removed carefully and the gel was immersed in an electrophoresis tank containing 1X TAE buffer. 1 µl of 6X DNA loading dye was mixed with 5 µl of DNA samples and loaded into the wells. Electrophoresis was carried out at 85 volts until the tracking dye reached the end of the gel. The DNA samples were visualized as bands under UV-illumination and documented using imaging system (iBright^{TM} CL 750, ThermoFischer Scientific, USA). The size of the amplified PCR products was calculated by comparing with 1 Kb plus DNA ladder (Invitrogen, USA).

3.3.5 Evaluation of *In vitro* efficacy of *Prosopis juliflora* leaves extracts (PJ) against *Escherichia coli* isolates

3.3.5.1 Preparation of *Prosopis juliflora* leaves extracts: The leaves of *Prosopis juliflora* were collected from the Veterinary College campus during the month of June-July, 2021 and these were shade dried and coarsely powdered before extraction. The extraction of the leaves was carried out by Soxhlet apparatus using three different solvents *viz.* methanol, ethanol and ethanol-water mixture (80:20). About 25-30 g coarsely a ground powder of leaves was taken in porous cellulose thimble and was placed in the extraction chamber above the flask containing the solvent. Extraction process was allowed to continue for several hours (approximately 48 h) involving 20-22 cycles till the solvent in siphon tube of the extractor became almost colourless. Thereafter, the extract obtained was concentrated in rotatory vacuum-evaporator under reduced pressure to obtain the semisolid mass extract. Percentage yield of the extract was determined and then extract was transferred into air tight containers and stored in -20 °C until further use.

3.3.5.2 Phytochemical examination of plant extract: The plant extract was reconstituted in deionized water and subjected to phytochemical analysis using gas chromatography (GC) to identify the major phytomolecules present in the extract.

The gas chromatography analysis of the extract was performed using flame ionization detector (FID) in gas chromatography (7890A GC) and mass spectrophotometry (MS; 5975CVLMSD) with triple axis detector system (Agilent Technologies, USA).

Programming of gas chromatography analysis conditions

For GC:

Column: HP-5 (30 m × 0.320 mm × 0.25 µm)

Temperature limits: -60° C to 325° C (350° C)

Carrier gas: Hydrogen

Flow rate: 3 ml/ min

Injection volume & mode: 2 µl (Split less)

Injector temperature: 260° C

Oven temperature: 100° C for 2 min then 200° C at 10 min and from 200-300° C

@15 °C/ min withhold time 5 and 22 min

GC- Run time: 34.983 min

For MS:

Electron ionization energy: 70 eV

Mass scan (m/z) fragments: 40 to 600 Da

3.3.5.3 Estimation of total phenolic content:

3.3.5.3.1 Preparation of standard: Total phenolic contents (TPC) in the test extract were determined by Folin–Ciocalteu colorimetric method as described by Singleton and co-workers (1965) with some modifications. The principle of the test is the Folin–Ciocalteu reagent (FCR) oxidizes the phenols present in plant extract resulting in dark blue colour mixture which is measured by UV-visible spectrophotometer. Gallic acid was used as standard and the total phenolic content in the test extract was expressed as gallic acid equivalents (mg GAE) per gram of test extract.

Stock solution of gallic acid was prepared in ethanol (1 mg/ml) and subsequently diluted to obtain different concentrations *viz.* 25, 50, 75, 100, 250 µg/ml. To each concentration, 5 ml of 10% Folin–Ciocalteu reagent (FCR) and 4 ml of 7% Na_2CO_3 were added to make the final volume to 10 ml. The mixture (blue in colour) was shaken well and incubated for 30 min at 40 °C in water bath. The

absorbance was measured at 760 nm against blank. All the experiments were carried out in triplicates, and the average absorbance values at different concentrations of gallic acid were used to plot the standard curve.

3.3.5.3.2 Preparation of Samples: Different concentrations of the extracts (500, 1000 and 2000 µg/ml) were prepared in ethanol and the reaction was carried out as described above for gallic acid standard. The absorbance for each concentration of the extracts was recorded. The average value of absorbance for each concentration of the extract was used to interpolate the value of gallic acid equivalent (GAE) phenolic acid from the standard curve.

3.3.5.4 Estimation of total flavonoid content:

3.3.5.4.1 Preparation of Standard: Total flavonoid contents in the extracts were determined by aluminum chloride colorimetric assay as described earlier (Zhishen et al., 1999; Yang et al., 2004). Quercetin was used as standard and total flavonoid in the test extract was expressed as quercetin equivalent (mg QE) per gram of test extract.

Stock solution of quercetin was prepared in ethanol (4 mg/ml) and subsequently diluted to obtain various concentrations *viz.* 0.25, 0.5, 0.75, 1.0 and 1.5 mg/ml. To 1 ml of each concentration of quercetin 4 ml of distilled water was added. At the same time, 0.3 ml of 5% $NaNO_2$ and 0.3 ml of 10% $AlCl_3$ after 5 min was added to the test tube. After 6 min, 1 M NaOH (2 ml) was added to the mixture and the final volume of the mixture was made to 10 ml with distilled water. The absorbance was measured at 510 nm using multimode plate reader (Molecular Devices, USA). Readings were taken in triplicate for each concentration of standards and the average value of absorbance was used to plot the standard curve.

3.3.5.4.2 Preparation of Samples: Different concentrations of the extracts (2500, 3000 and 4000 µg/ml) were prepared in ethanol and the reaction was carried out as described above for quercetin standard. The absorbance for each concentration of the extracts was recorded. The average value of absorbance for each concentration of the extract was used to interpolate the value of quercetin equivalent (QE) flavonoid content from the standard curve.

3.3.5.5. Evaluation of *in vitro* antioxidant activity:

3.3.5.5.1 Free radical scavenging activity:

The free radical scavenging activity of the test extract was assayed by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay as mentioned earlier (Karioti et al., 2004) with some modification. Briefly, 100 µl DPPH working solution (from 0.1 mM stock solution) was mixed with the 100 µl of extract or standard of different concentrations and incubated for 20, 40 and 60 min in dark before recording the absorbance at 517 nm. Extract of different concentrations (25, 50, 100, 250, 500, 1000, 1250 and 2500 µg/ml) was tested in triplicate for free-radical scavenging activity. The scavenging activity of the extract was compared with ascorbic acid (positive control) of different concentration (0.5, 2.5, 5, 6.25, 12.5, 25, 50 and 125 µg/ml). A blank containing vehicle instead of the test sample or ascorbic acid was included under the same condition. The free radical scavenging activity (RSA) as determined by the decolouration of the DPPH solution was calculated according to the formula:

$$\text{DPPH inhibition (\%)} = [(A_{blank} - A_{test})/A_{blank} \times 100]$$

Where, A_{test} is the absorbance of the reaction mixture containing the standard antioxidant or extract, and A_{blank} is the absorbance of the blank.

The IC_{50} value for extract and standard at different time interval (20, 40 and 60 min) was calculated. IC_{50} defines the concentration of extract or standard required to scavenge 50 % of DPPH free radicals.

3.3.5.5.2 Ferrous ion chelating assay:

The ferrous ion chelating activity assay was performed as described earlier by Singh and Rajini (2004). Briefly, 0.1 mM $FeSO_4$ (0.5 ml) was mixed with the extract (0.5 ml) of different concentrations (50, 100, 1000, 2500 and 5000 µg/ml) in triplicate, followed by adding 0.25 mM ferrozine (1 ml). The reaction mixtures were then incubated for 10 min and the absorbance was measured at 562 nm. Ethylenediaminetetraacetic acid (EDTA; 1000 µg/ml) was used as positive control. The ability of extracts to chelate ferrous ions was calculated using the following equation:

$$\text{Chelating ability (\%)} = [(A_{blank} - A_{test})/A_{blank}] \times 100$$

Where, A_{test} is the absorbance of the reaction mixture containing extract or EDTA, A_{blank} is the absorbance of the blank.

3.3.5.6 Agar well diffusion test: The *in vitro* antibacterial activity of PJ extract was evaluated by agar well diffusion method as described earlier (Perezet al., 1990). Briefly, sterilized Muller Hinton agar (MHA) media was transferred to sterile petri dishes (90 mm diameter) to a depth of 4 mm. Bacterial suspensions of *E. coli* (ATCC 25922), clinical isolate of *E. coli* (S4 and S21) having visually equivalent turbidity to 0.5 McFarland standards were used to inoculate on MHA plates. The surface was lightly and uniformly inoculated by cotton swab to ensure even distribution. With the help of well puncture device, wells (9 mm diameter) were punched on the agar plate. The dried PJ extract was freshly reconstituted either in sterile water or ethanol at two different concentrations *viz.* 20 mg/ml and 25 mg/ml each. The wells were filled with 100 µl of reconstituted extracts in different vehicles and the plates were incubated at 37ºC for overnight. Vehicle control (95% ethanol) was also plated in separate wells.

3.3.5.7 Determination of minimum inhibitory concentration (MIC): MIC of the PJ extract against reference strain of *E. coli* (ATCC 25922) and clinical isolate of *E. coli* (S4 and S21) was determined by broth microdilution method. Briefly, 50 µl of Muller Hinton broth (MHB) was taken into all the wells from column 2-10 except column 11 and 12 which were kept as growth control and sterility control wells, respectively. Working solutions of PJ extract (100 µl) was added to the first well of each row and the content was serially diluted in the subsequent well of each row to obtain two-fold dilution. From column 10, 50 µl of content was discarded. Bacterial suspension (50 µl) was added to each well except column 12 which served as growth control well. The plates were covered by sterile covers and incubated at 37ºC for 18h. Following incubation, the wells were examined for the formation of bacterial plaque formation. The lowest concentration of PJ extract failed to produce any visible bacterial growth was considered as MIC of the test extract. Cefotaxime was used as positive control.

3.3.5.8 Determination of fractional inhibitory concentration (FIC): FIC for PJ extracts against cefotaxime was calculated by checker board assay. PJ extract was serially diluted along the ordinate, while the standard antibiotic (cefotaxime) was serially diluted along the abscissa. Each well was inoculated with standardized

inoculum (100 µl of 10^5 CFU/ml) of clinical isolate of *E. coli* (S4) and the microtiter plates were incubated at 37 ºC for 18 h.

FIC was calculated using the following formula:

$$\Sigma FIC = FIC \text{ of Cefotaxime} + FIC \text{ of PJ}$$

$$\text{FIC Cefotaxime} = \frac{\text{MIC of Cefotaxime in combination}}{\text{MIC Cefotaxime alone}}$$

$$\text{FIC of PJ} = \frac{\text{MIC of PJ in combination}}{\text{MIC PJ alone}}$$

Synergy is defined as $\Sigma FIC \leq 0.5$

Indifference is defined as $0.5 \leq \Sigma FIC \leq 4$

Antagonism is defined as $\Sigma FIC > 4$

3.3.5.9 Time kill assay: The effectiveness of PJ extract against clinical isolate of *E. coli* (S4) was determined by time kill assay as mentioned earlier (Verma et al., 2007). Briefly, the freshly grown S4 isolate was adjusted to 1 X 10^8CFU/ml using 0.5 McFarland Standard and diluted with MHB to achieve 1:100 dilution. From this dilution, 1 ml each was transferred to two 50 ml sterile tubes containing 10 ml MHB. To one of the tubes, PJ extract (reconstituted in ethanol @ 2 MIC) was added and the other tube was taken as growth control. Immediately after mixing the bacterial suspension in both the tubes, 200 µl was aliquoted from each tube and 100 µl was serially diluted (10 fold) for 6 dilutions. 100 µl from first three dilutions (10^{-2}, 10^{-3}, 10^{-4}) from the treatment and control groups were platted on sterile MHA plates and the process was repeated after 2, 4, 6, 8, 12, 16,18 and 24 h. For 12, 16, 18 and 24 h lower dilutions (10^{-5}, 10^{-6}, 10^{-7}) were taken for ease in colony counting. The plates were incubated for 24 h and the colonies were counted using colony counter. The experiment was carried out in triplicate and in at least two independent experiments was averaged. The results were expressed as logarithms with corresponding standard errors (mean ± SEM).

3.3.5.10 Cell membrane integrity test: To evaluate the effect of PJ extract on bacterial cell membrane integrity, the uptake of fluorescent dye by the bacterial cell was evaluated in the absence and presence of PJ extract. Being the cell permeable fluorescent dye, 5-carboxyfluorescein diacetate, acetoxymethyl ester (CFDA-AM)

penetrate the live cell with intact cell membrane and emits green fluorescence while dead cells with disrupted cell membrane are stained red with propidium iodide.

Briefly, to 100 µl of the bacterial suspension (turbidity adjusted to 0.5 McFarland) in a microcentrifuge tube, PJ extract (@ 2 MIC) was added and incubated at 37 °C for 12 h. Tubes were then centrifuged @ 3000 rpm for 10 mins at 4 °C and the supernatant was discarded. The bacterial pellet was dissolved in 1.5 ml of sterile phosphate buffer saline (PBS) solution. The cells were then washed twice in PBS by centrifuging at 1000 rpm for 2 min. The final pellet was dissolved in 200-300 µl PBS depending on the pellet size and to this 1.5 µl 5-carboxyfluorescein diacetate, acetoxymethylester (CFDA-AM; 1 µM) per 200 µl bacterial pellet was added and incubated at 37 °C for 15 min. The bacterial suspension was further incubated with 3 µl propidium iodide (5 µM) per 200 µl of bacterial pellet and incubated at 37 °C for 5 min. Following washing, the supernatant was discarded and the pellet was resuspended in 200 µl PBS. From this suspension, 10 µl was taken and a thin smear was made on a clean glass slide. The smear was air dried and visualised under fluorescent microscope (EVOS-FL, Invitrogen, USA). The test was performed with both S4 clinical isolate and reference strain of E. coli. (ATCC 25922). Cefotaxime was used as positive reference control.

3.3.5.11 Transmission electron microscopy: Transmission electron microscopy (TEM) study was performed to unravel the target sites and possible mechanism of action of PJ extract against clinical isolate of *E. coli* (S4). Briefly, bacterial suspension having visually equivalent turbidity to 0.5 McFarland standards were used in this study and were subjected to following treatments:

i) Negative control (untreated bacterial culture)

ii) Positive control (bacterial culture incubated with cefotaxime (2 MIC)

iii) PJ extract low dose (bacterial culture incubated with 2 MIC of PJ extract)

iv) PJ extract high dose (bacterial culture incubated with 4 MIC of PJ extract)

v) PJ + Cefotaxime (bacterial culture incubated with 2 MIC PJ and 2 MIC cefotaxime).

After exposure to the test extract and/or cefotaxime for 12 h at 37 °C, the bacterial pellet was obtained by centrifugation @ 3000 rpm for 10 min. The pellet was further

washed thrice with sterile PBS (pH 7.4) by centrifugation at lower speed. The washed pellet was further suspended in fixative (2.5% glutaraldehyde + 2.5% paraformaldehyde in 0.1 M PBS). Further processing of the pellet and imaging was done from AIIMS, New Delhi.

3.3.5.12 mRNA Expression study: To evaluate the effect of PJ extract on bacterial efflux pump and outer membrane protein (OMP), genes expression of efflux pump and OMP related genes were studied in the clinical isolate (S4) following exposure to test extract.

3.3.5.12.1 Bacterial RNA isolation: Bacterial suspensions (S4) having visually equivalent turbidity to 0.5 McFarland standards were taken and divided into four groups *viz.*

i) S4 control

ii) S4 + PJ extract (@ 2 MIC for 6 h exposure)

iii) S4 + PJ extract (@ 2 MIC for 12 h exposure)

iv) S4 + PJ extract (@ 4 MIC for 6 h exposure)

Tube from each group was incubated at 37 °C for either 6 h or 12 h (as described above) and following incubation bacterial pellet was obtained by centrifuging @ 3000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was dissolved in 2 ml sterile PBS solution and stored in -80 °C until further use. Total RNA was isolated from the bacterial pellet from different groups using PureLink^{TM} RNA mini kit (Invitrogen, USA) as per the manufacturer's instructions. Approximately, 10^9 cells were homogenized with 300 µl lysis buffer (pre-warmed at 60 °C) in 1.5 ml microcentrifuge tube with the help of sterile micro-pestle. The homogenates were then sheared by passing the lysate through a 20 G needle syringe for at least 40-50 times. After incubating at room temperature for 5 min, the homogenates were centrifuged at 12,000 rpm for 2 minutes and clear supernatants were transferred to a fresh microcentrifuge tube and mixed properly with equal quantity of 70 % ethanol. The ethanol added mixture was then transferred to a filter column placed in 2 ml collection tube and spun at 12,000 rpm for 1 min. The follow through was discarded and 700 µl of wash buffer-I was added to the spin column and centrifuged at 12,000 rpm for 1 min. The spin column was then transferred to a fresh

collection tube and 500 µl of wash buffer-II was added to the spin column before spinning it at 12,000 rpm for 1 min. The follow through was discarded and washing with wash buffer-II was repeated once again before drying the spin column by centrifuging at 12,000 rpm for 2 min. Then the column was transferred to an RNase-free 1.5 ml microcentrifuge tube and 13 µl DNase/RNase-free water was added to the spin column and allowed to stand for 1 min at room temperature. The RNA was eluted by spinning at 12,000 rpm for 2 min. The samples were treated with RNase free-DNase and the DNase was subsequently inactivated by heating at 56 ºC for 10 min and immediately chilled to 4 ºC. The purity of the RNA was checked by Biophotometer (Eppendorff, Germany) and quantitated as follows:

$$1OD_{260} = 40\ \mu g/ml$$

The RNA samples showing A_{260}/A_{280} ratio 1.8-2.0 and A_{260}/A_{230} ratio>1.6 were used for cDNA synthesis.

3.3.5.12.2 cDNA synthesis: cDNA synthesis was carried out from the mRNA present in the total RNA using Revertaid® First strand cDNA synthesis kit (Thermo Scientific, USA) using moloney murine leukemia virus reverse transcriptase enzyme by following manufacturer's instructions. Briefly, 1000 ng RNA was mixed thoroughly with 1 µl of random hexamer primer (20 pmol/µl) and volume of each tube was adjusted to 12 µl using nuclease-free water (NFW). The solution was incubated at 65 °C for 5 min in thermocycler and immediately chilled on ice after incubation. To this, 2 µl dNTPs (10 mM), 4 µl reaction buffer (5x), 1 µl MMLV reverse transcriptase (200 U/µl) and 1µl RiboLock™ RNase inhibitor were added to make the total volume 20 µl. Afterwards, the tubes were incubated at 25 ºC for 5 min followed by 42 ºC for 60 min and 70 ºC for 5 min in a thermocycler (Biorad, USA).

3.3.5.12.3 Primer validation: Primer sequence was obtained from the previous publication and respective gene sequence was checked from NCBI nucleotide database. The cDNA was subjected to amplification at various annealing temperatures to optimize the PCR conditions. Reverse transcription real time (RT-qPCR) analysis was carried out after checking each primer for its specificity.

Table 5: Description of primers for qRT-PCR

Gene	Primer sequences	Amplicon size (bp)	Annealing Temp. (°C)	Reference
acrA	F5′- CTCTCAGGCAGCTTAGCCCTAA R5′- TGCAGAGGTTCAGTTTTGACTGTT	107	59.5	Swick et al., 2011
acrB	F5′- AGCTTCCTGATGGTTGTCGG R5′- ACGGCTGATGGCATCTTTCA	107	59.5	Swick et al., 2011
OmpF	F5′- AAGTAGTAGGTTGCGCCCAC R5′- AGTTCGATTTCGGTCTGCGT	118	61.0	Chetri et al., 2019
OmpC	F5′- ATTCTGGCAGTACGTCGGTC R5′- AAACAACTCCTGGACCCGTG	125	61.0	Chetri et al., 2019
RPS	F5′- GCAAAAACGTGGCGTATGTACTC R5′- TTCGAAACCGTTAGTCAGACGAA	104	59.5	Swick et al., 2011

3.3.5.12.4 Reverse transcriptase polymerase chain reaction (RT-PCR): The cDNA was checked for quality by performing PCR with standard RPS primer. The reaction was carried out at different annealing temperatures and the optimum annealing temperatures of the genes were determined as 59.5 °C and 61°C as mentioned in Table 5. The protocol used was as follows (Sambrook and Russel, 2001).

Table 6: Composition of RT-PCR mixture

Composition	Volume
PCR master mix (2X)	6.25 µl
Forward primer (10 pmol/µl)	0.5 µl
Reverse primer (10 pmol/µl)	0.5µl
Template (1000 ng/µl)	1.0 µl
Nuclease free water	up to 12.5 µl

Table 7: Thermal cycling conditions

SN	Steps	Temperature and duration
1.	Initial denaturation	95 ºC for 5 min.
2.	Denaturation	95 ºC for 30 sec.
3.	Annealing	AT* for 30 sec
4.	Extension	72 ºC for 1 min

5.	Step 2-4 repetition	40 cycles
6.	Final extension	72 ºC for 5 min
7.	Held at	4 ºC

*AT= annealing temperature for respective primer pairs as mentioned in Table 5.

3.3.5.12.5 Agarose gel electrophoresis: DNA amplified by PCR was subjected to agarose gel electrophoresis as described by Sambrook and Russel (2001). Four hundred fifty milligram (1.5%) was dissolved in 30 ml 1X TAE buffer (Annexure-I) by heating and allowed to cool to 50 ºC. To this, 1.5 µl ethidium bromide (final concentration 0.5 µg/ml) was added and mixed thoroughly. The mixture was poured onto a gel-casting tray fitted with acrylic comb and allowed to solidify. Once the gel had formed, a few ml of TAE was added near the comb, which was later removed carefully and the gel was immersed in an electrophoresis tank containing 1X TAE buffer. 1 µl of 6X DNA loading dye was mixed with 5 µl of DNA samples and loaded into the wells. Electrophoresis was carried out at 85 volts until the tracking dye reached the end of the gel. The DNA samples were visualized as bands under UV-illumination and documented. The size of the amplified PCR products was calculated by comparing with 1 Kb plus DNA ladder (Invitrogen, USA).

3.3.5.12.6 Reverse transcription real-time quantitative PCR (RT-qPCR):

RT-qPCR reactions were run on Real-Time PCR system (QuantStudio3, Applied Biosystem) and analysed by using QuantStudio™ Design and Analysis Softwares v1.2.x.

3.3.5.12.6.1 RT-qPCR reaction conditions: RT-qPCR was performed using SYBR Green master mix (PowerUp^{TM}SYBR^{TM} Green master mix [2X]; ThermoFischer Scientific, USA). Each sample was run in duplicate in 10 µl reaction. The 10 µl reaction mixture consisted of 5 µl SYBR Green master mix, 0.5 µl from 10 pmol/µl stock solution of each gene-specific forward and reverse primers, and 1µl of (1:4 dilution) of cDNA and volume was made up to 10 µl with nuclease free water (NFW). The real-time PCR reaction was started with UDG activation at 50 °C for 2 min, Taq polymerase activation at 95 ºC for 2 min followed by 40 cycles of amplification with

denaturation at 95 ºC for 15 sec, annealing at respective temperature (Table 5) for 30 sec and extension at 72 ºC for 1 min each. To assess the specificity of the amplified product, dissociation curve was generated at temperature of 60 °C through 95 °C. The results were expressed as threshold cycle values (C_T). This value is the cycle number when the fluorescence of the reporter dye is appreciably higher than the background fluorescence. The threshold, automatically adjusted by the instrument, was used for the generation of C_T values.

To study the relative change in gene expression, the $2^{-\Delta\Delta C}{}_T$ method was used as described previously by Livak and Schmittgen (2001). The formula used to calculate the fold change in gene expression was "fold change = $2^{-\Delta\Delta C}{}_T$," [where $\Delta\Delta C_T$ = ($C_{T,target\ gene}$ - $C_{T,RPS}$) treatment - ($C_{T,target\ gene}$ -$C_{T,RPS}$) control]. The gene-specific amplification was corrected for the difference in input of RNA by taking reference gene (RPS) in account. For treatment groups, evaluation of $2^{-\Delta\Delta C}{}_T$ indicates the fold change in gene expression relative to healthy control (i.e., fold change in healthy control = 1). The results were analyzed in comparison with the C_T (minimum threshold of amplification) value of the target gene and the reference gene (RPS).

Table 8: RT-qPCR thermal cycle conditions

Component	Volume (per well)	Step
SYBR green	5 µl	**Segment 1**
Master Mix (2X)		
Forward primer	0.5 µl	1 – 50 ºC : 2 min
(10 pmol/µl)		2– 95 ºC : 2min
		1 cycle
Reverse primer	0.5µl	**Segment 2**
(10 pmol/µl)		2 – 95 ºC :30 sec
cDNA Template	1.0 µl	3 – AT : 30 sec
(1:4 dilution from stock)		4 –72 ºC :30 sec
		40 cycles
Nuclease free water	up to 10 µl	**Segment 3**

	95 ºC: 15 sec
	60 ºC: 60 sec
	95 ºC: 15 sec
	1 cycle

*AT: respective annealing temperature

3.3.5.13 Biofilm inhibition assay:

The ability of biofilm formation by the bacterial isolates and the effect of test extract on bacterial biofilm formation were determined by three different methods as described below.

3.3.5.13.1 Congo red agar (CRA) method:

Congo red agar (CRA) method is a qualitative assay for detection of biofilm producer microorganism. The CRA medium was prepared by mixing 0.8 g of Congo red and 36 g of sucrose to 37 g/l of Brain heart infusion (BHI) agar. Bacterial suspensions of clinical isolate of *E. coli* (S4 and S21) were inoculated on CRA plates and incubated at 37 ℃ for 24 h. Appearance of black colonies with a dry crystalline consistency indicate biofilm producers, whereas colonies retained pink were considered as non-biofilm producers.

3.3.5.13.2 Microtiter plate method or Crystal violet assay method:

The detection and quantification of bacterial biofilm was performed by the microtiter plate method (O'Toole et al.1999) as described below.

3.3.5.13.2.1 Preparation of biofilm assay media:

The biofim assay media (Annexure-I) was prepared by supplementing Luria-Bertani broth with M63 minimal media to promote robust biofim production. The M63 minimal media was prepared as described earlier (Sturgill et al., 2004).

3.3.5.13.2.2 Preparation of bacteria inoculation

The bacterial isolates (S4 and S21) were grown in 5 ml LB broth. The overnight culture was diluted to 1:100 in fresh biofilm assay medium and 200 µl of the diluted culture was added per well in U-bottom 96 well microtiter plate. Each

isolates was used in triplicate for quantitative assay. Microtiter plate was incubated at 37 ℃ for 48 h without shaking.

3.3.5.13.2.3 Staining of biofilm

After incubation, all the liquid media was poured off by turning the plate upside down. Plate was submerged on a small tub of water. The water was shaken out and process was repeated for second time. 200 µl of 0.1% solution of crystal violet was added to each well of the microtiter plate. The plate was left undisturbed at room temperature for 20 min, rinsed four time with sterile distilled water followed by quick drying by shaking out the water and vigorously blotting on a stack of paper towel. The microtiter plate was turned upside down and allowed to dry overnight.

3.3.5.13.2.4 Quantification of bacterial biofilm:

To each well of microtitre plate, 200 µl of 30 % acetic acid was added to solubilise the crystal violet followed by incubation at room temperature for 20 min. The solubilised crystal violet was transferred to a fresh flat bottom microtiter plate. Quantification was done at 600 nm in microplate reader (Molecular Devices, USA). A background control with 30 % acetic acid in water was also included. Absorbance values were compared with the standard OD readings. The isolates were categories as mild, moderate and high biofilm producer as described in Table 9.

Table 9: Classification criteria for bacterial adherence and biofilm producers

Mean OD value	Adherence	Biofilm formation
≤0.12	Non-adherent	Mild
0.12 - 0.16	Moderately	Moderate
≥0.16	Strong	High

3.3.5.13.2.5 Biofilm inhibition assay:

The potential of test extract to prevent cell attachment and biofilm production was assessed by biofilm inhibition assay. As described above, the bacterial aliquot was added in U bottom 96 well microtiter plate and incubated at 37 °C for 48 h without shaking for establishment of bacterial biofilm. For evaluation of anti-biofilm activity of plant extract, bacterial aliquot were added along with the plant extract

(equivalent to MIC) and incubated for 37 °C for 48 h. Chloramphenicol (equivalent to MIC i.e. 10 µg/ml) was used as external positive control while ethanol was served as negative control. The final volume of each well was adjusted to 200 µl and the assay was conducted in triplicate. The biofilm mass was assayed using crystal violet staining assay as described previously. The inhibition of biofilm was quantified using following formula,

Percentage inhibition of Biofilm = [(OD (control)−OD(test)/OD(control)]×100

3.3.5.13.3 *In situ* Visualization of Biofilm:

Scanning electron microscopy (SEM) was performed for *in situ* visualization of the biofilm as described earlier (Vuotto and Donelli, 2014) with some modification. S21 clinical isolate of *E. coli* was showing highest biofilm production based on the culture characteristic and microtiter plate assay. Thus efficacy of test extract to inhibit biofilm production was further assessed using S21 isolate. Briefly, a loopful colony was inoculated to 5 ml broth and incubated overnight at 37 °C without shaking. Bacterial culture were then adjusted to the equivalent turbidity to 0.5 McFarland standard. The sterile coverslip (12 mm diameter) was placed in the bottom of each well of 6 well plate before filling the well with 7.5 ml of biofilm assay media and 1 ml of broth of bacterial culture and incubated at 37 °C for 48 h without shaking. The wells of the plate were divided in following groups:

i) Negative control (untreated bacterial culture)

ii) Positive control (bacterial culture incubated with chloramphenicol @ MIC)

iii) PJ extract low dose (bacterial culture incubated with PJ extract @ MIC)

iv) PJ extract high dose (bacterial culture incubated with PJ extract @2 MIC)

After exposure to the test extract and/or chloramphenicol, the culture media was removed from the wells by pipetting and washed three times gently with phosphate buffer solution (pH 7.4). The cover slips in the wells were then fixed in the fixative (2.5 % glutaraldehyde + 2.5 % paraformaldehyde in 0.1 M PBS) for overnight. Further processing of and imaging was done from AIIMS, New Delhi.

3.5. Statistical analysis:

Data were analysed using Graph Pad Prism version 4.00 software (San Diego, California, USA). Results are expressed as mean ± SEM with 'n' equal to number of observations. Difference in mean values was considered statistically significant at $p<0.05$ (Snedecor and Cochran, 1989).

Results

Present study was aimed to isolate and identify virulent *E. coli* isolates from uterine discharges of bovines with a clinical history of uterine infection. Further an attempt was also made to unravel the mechanism of antibacterial action of *Prosopis juliflora* leaves extract against these clinical isolates of *E. coli*. This study was conducted in two phases where in phase-I, isolation and identification of clinical isolates of *E. coli* was performed and in phase-II the *in vitro* efficacy of the plant extract against these clinical bacterial isolates along with its possible mechanism of action was delineated.

A. Phase I

4.1 Isolation and identification of clinical bacterial isolates

4.1.1 Morphological characterization:

Swab containing uterine discharge was inoculated for pre-enrichment in buffer peptone water and incubated overnight at 37 °C. The bacterial growth in buffer peptone water was ascertained by the presence of turbidity in enrichment media. Inoculums from buffer peptone water were streaked over Macconkey agar and incubated overnight at 37 °C. Lactose fermenter colonies were distinguished from non lactose fermenter by observing the pink colour colonies (Fig. 3). Pink colonies from the primary culture were picked and subcultured separately on eosin methylene blue (EMB) agar. All the *E. coli* positive isolates showed typical characteristics of 'metallic sheen' on EMB agar (Fig. 3). In total 23 isolates on MacConkey agar were isolated as *E. coli* from 42 clinical samples. Morphological examinations revealed the presence of Gram-negative rod shaped organisms which took pink colour after Gram's staining.

4.1.2 Biochemical characterization:

All these 23 bacterial isolates were characterized by panel of biochemical tests *viz.* catalase, oxidase, ONPG, indole, methyl red, Voges-Prausker, citrate test, triple sugar fermentation test. All the *E. coli* isolates (9 out of 23 isolates) showed typical results during their biochemical characterization, i.e. positive to indole, methyl red

catalase, ONPG and sugar fermentation tests whereas negative for VP and citrate utilization. Results were interpreted by observing the change in colour of respective growth media (Fig. 4).

4.1.3 Genotypic Characterization

Genotypic characterization of these selected bacterial isolates was carried out by amplifying the virulence genes (*Pap, F41, Sta, csgA* and *csgD*) from their genomic DNA. ATCC 25922 was used as reference strain for *E. coli*. As shown in Fig. 5 and summarized in Table 10, the PCR amplified products corresponding to *Pep1* (336 bp), *csgA* (408 bp) and *csgD* (601 bp) were amplified only from the genomic DNA from clinical isolate of S4 and S21. *Sta* gene was not amplified in any of the isolate whereas *F41* was amplified only in ATCC reference strain. Thus, further study was carried out using S4 and S21 clinical isolates.

Table 10: Presence of different virulent genes as detected by polymerase chain reaction using DNA isolated from selected bacterial isolates collected from uterine discharges of cattle and buffaloes.

E. coli Isolates	*Sta*	*F41*	*Pep1*	*CsgA*	*CsgD*
ATCC 25922	-	+	+	+	+
S4	-	-	+	+	+
S12	-	-	-	+	+
S13	-	-	-	+	+
S14	-	-	-	+	-
S15	-	-	-	+	+
S17	-	-	-	+	+
S19	-	-	-	-	+
S21	-	-	+	+	+
S22	-	-	-	+	+

'+' indicates presence while '-' indicates absence

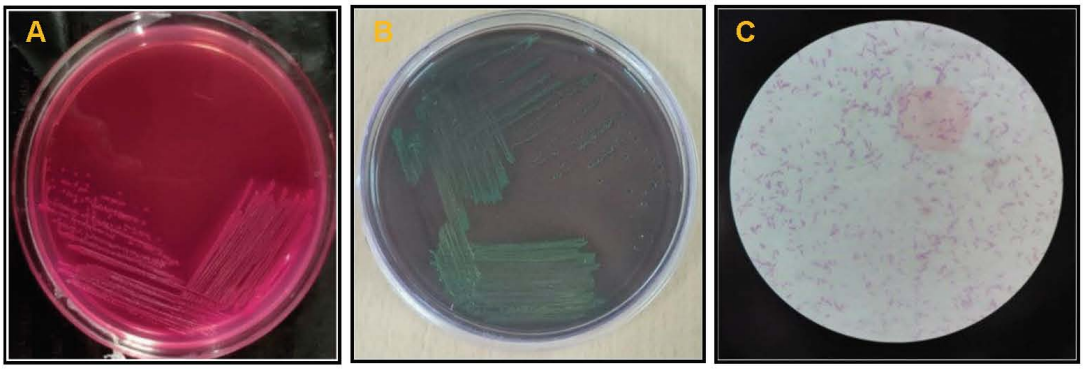


Fig. 3: Representative photograph showing the bacterial growth on Mac-Conkey agar (A), EMB agar (B) plates characterized by typical pink colour colonies and metallic sheen with dark centre colonies, respectively, of *E. coli* isolated from clinical cases. Gram staining of bacterial isolates showing Gram -ve rod shaped organism (C).

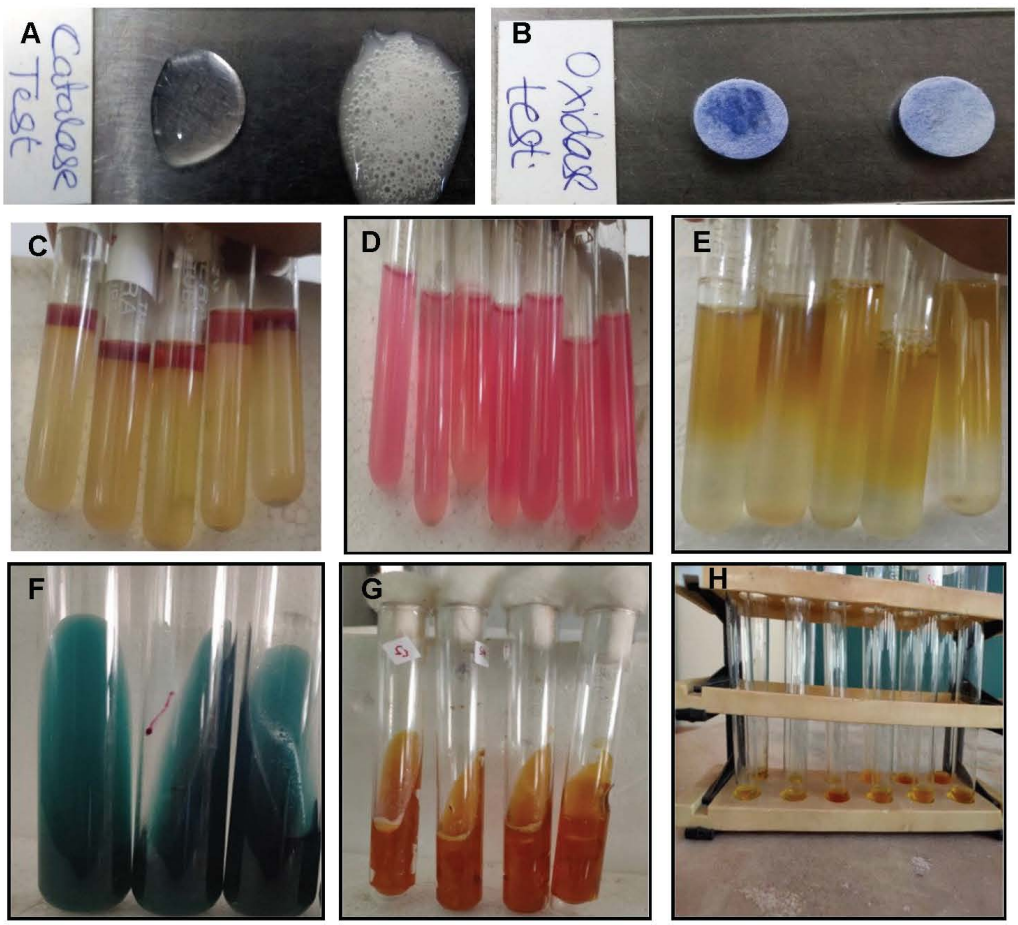
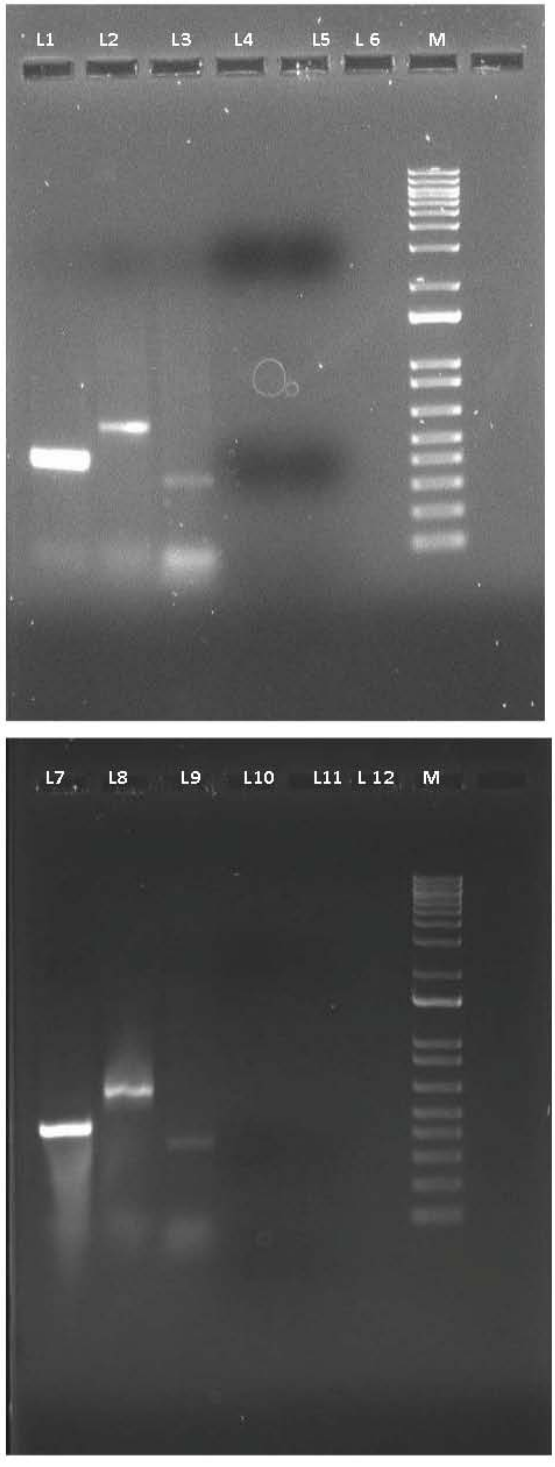


Fig. 4: Representative photograph showing the catalase +ve (A), oxidase –ve (B), indole test +ve (C), methyl red test +ve (D), Vogues-Proskauer test –ve (E), citrate utilization test –ve (F), sugar fermentation test +ve (G) and ONPG test +ve (H) for *E. coli* isolated from clinical samples.



Lane 1,7 : *csgA* gene for S4 and S21 isolates
Lane 2,8 : *csgD* gene for S4 and S21 isolates
Lane 3,9 : *Pap 1* gene for S4 and S21 isolate
Lane M : 100 bp DNA ladder

Fig 5: Representative agarose gel images showing the amplified products for *csgA* (408 bp), *csgD* (601 bp), *Pap1* (336 bp) genes in clinical isolates (S4 and S21) of *E. coli*.

B. Phase II

4.2 Phytochemical analysis of *P. juliflora* leaves extract

Following hot extraction of dried *P. juliflora* leaves, the solvent was completely evaporated with the help of rotary vacuum evaporator. The yields of methanolic, ethanolic and hydro-ethanolic extracts were calculated to be 36.79 %, 41.42% and 49.56 %, respectively. As the ethanolic extract dissolved in ethanol showed better antibacterial effect (as described hereunder), the phytochemical analysis of this extract was performed using gas chromatography-mass spectrophotometry (GC-MS) to delineate the nature of phytoconstituents present in this extract. Probable phytoconstituents identified in GC-MS analysis of PJ extract along with their retention time and peak areas are summarized in Table 11 and GC-MS chromatogram is depicted in Fig. 6. Perusal of the data revealed the presence of around 22 major compounds in the plant extract as listed in Table 11.

Table 11: Major phytoconstituents identified in crude hot ethanolic extract of *P. juliflora* leaves on gas chromatography-mass spectrometry (GC-MS) analysis

SN	Major phytoconstituents	Peak Area	Retention time
1.	Oxirane	1.97	0.271
2.	Diethyl Phthalate	11.00	7.899
3.	Bicyclo[3.1.1] heptane	1.23	10.017
4.	2-Hexadecene,	0.13	10.049
5.	9-Eicosene	0.13	10.049
6.	11-Tetradecyn-1-ol acetate	0.13	10.243
7.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.48	10.427
8.	Decanoic acid	2.26	11.832
9.	Pentadecanoic acid	2.26	11.832
10.	Phytol	0.87	12.826
11.	2-Bromononane	0.87	12.826
12.	Alpha.-D-Xylofuranoside	3.30	13.906
13.	9,12,15-Octadecatrienoic acid	1.52	13.976
14.	Isolongifolol	2.66	21.080
15.	Squalene	0.58	21.523
16.	Vitamin E	0.43	23.257
17.	Campesterol	0.45	23.694
18.	11,13-Dimethyl-12-tetradecen-1-ol acetate	1.36	23.808
19.	Stigmasterol	0.77	23.840
20.	beta.-Sitosterol	2.29	24.153
21.	Longifolenaldehyde	1.66	24.575
22.	13-Oxabicyclo[10.1.0]tridecane	0.33	25.671

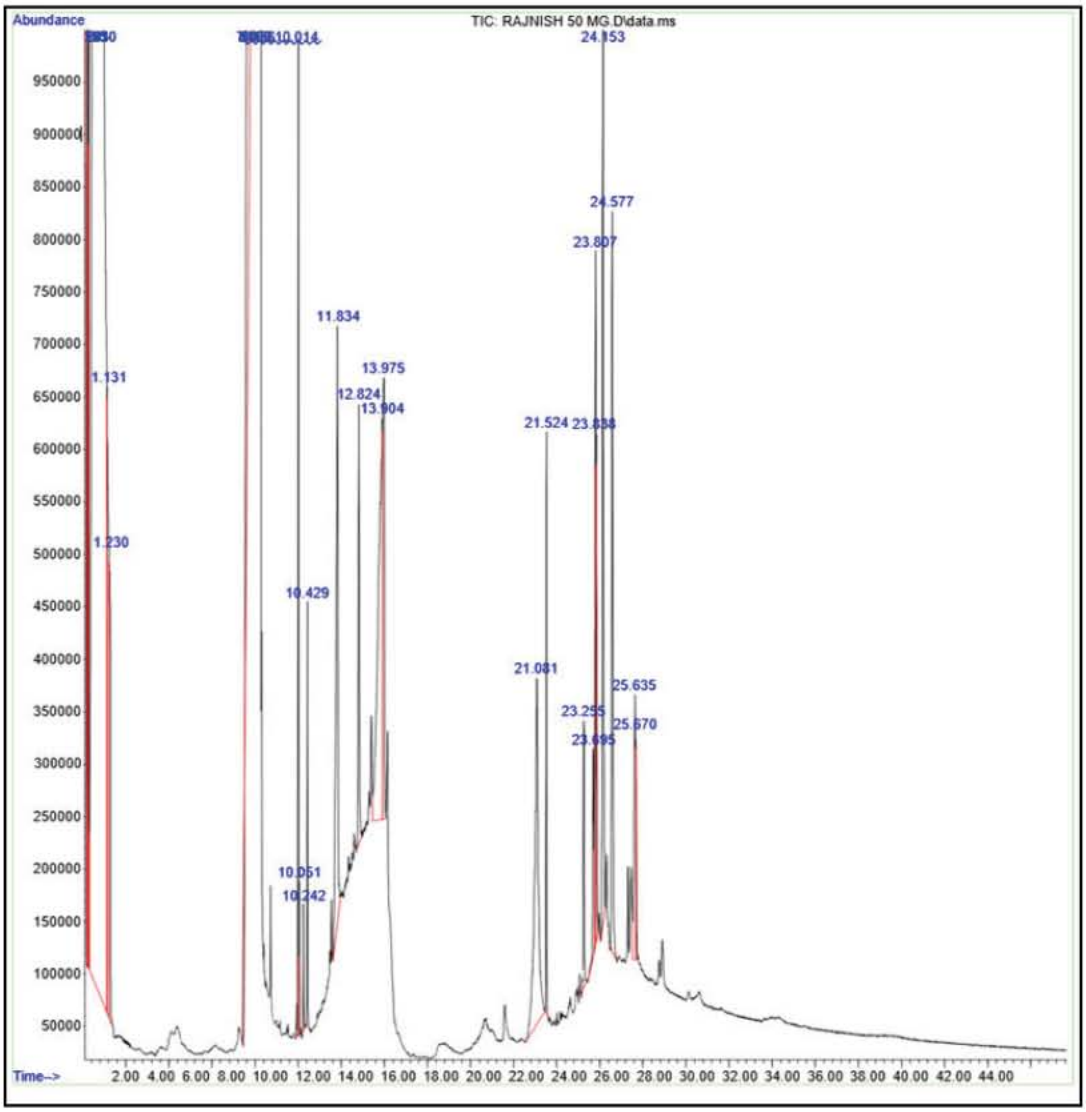


Fig. 6: Gas chromatography-mass spectrometry (GC-MS) chromatogram of the hot ethanolic extract of *Prosopis juliflora* leaves.

4.3 Estimation of phenolic contents:

Total phenolic contents in *P. juliflora* leaves (PJ) extract was estimated by Folin–Ciocalteu method using gallic acid as a standard. Fig. 7 depicts the standard curve of gallic acid at different concentrations. As summarized in Table 12, the average gallic acid equivalent (GAE) phenolic contents in PJ extract was calculated to be 120.68 ± 1.36 mg/g extract.

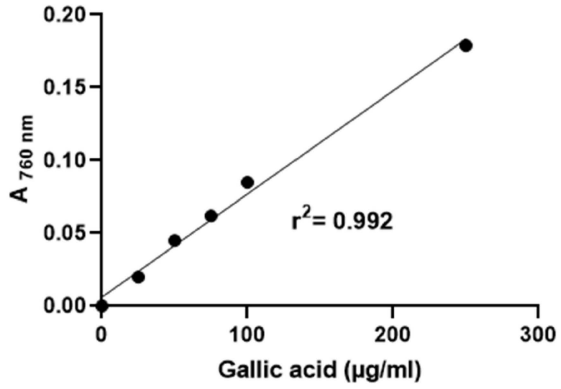


Fig. 7: Linear regression line showing the standard curve of gallic acid. Different concentrations (25, 50, 75, 100, 250 µg/ml) of gallic acid were prepared and following reaction with Folin–Ciocalteu reagent, their respective absorbance at 760 nm were recorded to plot the standard curve.

Table 12: Total Phenolic contents (TPC) in *P. juliflora* leave extract

***P. juliflora* leaves extract**	**Total Phenolic content (mg GAE/ g extract)**
500 µg/ml	118.21
1000 µg/ml	122.91
2000 µg/ml	122.91
Mean ± SEM	**120.68 ± 1.36**

4.5 Estimation of total flavonoid content:

Total flavonoid contents in *P. juliflora* leaves (PJ) extract was estimated using quercetin as a standard. Fig. 8 depicts the standard curve of quercetin at different concentrations. As summarized in Table 13, the average quercetin equivalent (QE) flavonoid contents in PJ extract was calculated to be 148.48 ± 3.27 mg/g extract.

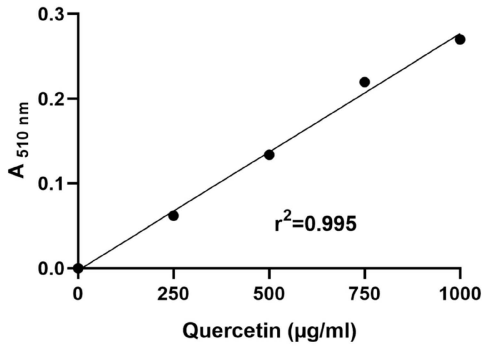


Fig. 8: Linear regression line showing the standard curve of quercetin. Different concentrations (0.25, 0.5, 0.75, 1.0 and 1.5 mg/ml) of quercetin were prepared and following reaction with aluminium chloride, their respective absorbance at 510 nm were recorded to plot the standard curve.

Table 13: Total flavonoids contents (TFC) in *P. juliflora* leave extract

***P. juliflora* leaves extract**	**Total Flavonoid content (mg QE/ g extract)**
2500 µg/ml	143.05
3000 µg/ml	148.07
4000 µg/ml	154.33
Mean ± SEM	**148.48 ± 3.27**

4.6 Estimation of *in vitro* antioxidant activity:

4.6.1 DPPH radical scavenging activity:

The antiradical activity of the extract was assessed by measuring the ability of the PJ extract to scavenge DPPH free radicals and was compared with the standard ascorbic acid. The radical scavenging activity (RSA) of ascorbic acid at 20, 40 and 60 min time intervals is depicted in Fig. 9 and summarized in Table 14. Perusal of the data revealed that the free radical scavenging activity of ascorbic acid increased with increasing the concentration (0.5 µg/ml to 125 µg/ml). Decrease in the IC_{50} value indicates the increase in the radical scavenging activity. However, no significant change in the IC_{50} value of ascorbic acid was found with increase in the time interval.

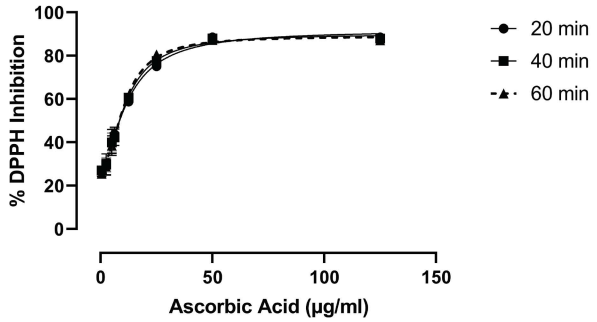


Fig. 9: Effect of ascorbic acid on per cent DPPH inhibition at different time interval (20, 40 and 60 min).

Table 14: Effect of ascorbic acid on per cent DPPH inhibition

SN	Ascorbic acid (µg/ml)	DPPH inhibition (%)		
		20 min	40 min	60 min
1.	0.5	26.62 ± 1.68	26.94 ± 0.64	25.48 ± 1.12
	2.5	29.81 ± 3.44	29.66 ± 2.36	28.55 ± 2.65
2.				
3.	5.0	40.39 ± 3.94	39.91 ± 3.18	38.46 ± 3.23
4.	6.25	43.61 ± 2.37	42.39 ± 1.20	42.32 ± 2.68
5.	12.5	58.85 ± 1.29	60.57 ± 1.01	60.61± 1.28
6.	25.0	75.16 ± 1.10	77.51 ± 1.40	80.57 ± 0.25
7.	50.0	88.47 ± 0.22	88.01 ± 0.08	87.26 ± 0.30
8.	125.0	88.46 ± 0.22	89.89 ± 0.05	87.13 ± 0.08
IC_{50} (µg/ml)		**12.03 ± 1.09**	**11.32 ± 0.67**	**10.63 ± 0.64**

Data are presented as Mean ± SEM of two observations. Data were analysed by one-way ANOVA followed by Tukey's *post-hoc* test.

The radical scavenging activity of PJ extract is illustrated in Fig.10 and summarized in Table 15. Perusal of the result revealed that PJ extract exhibited concentration–dependent increase in the per cent inhibition of DPPH activity. There was also increase in the radical scavenging activity of the extract with increase in the exposure time as evidenced by corresponding decrease in the IC_{50} values, though these changes in the IC_{50} values of PJ extract at different time interval was found to be statistically non-significant.

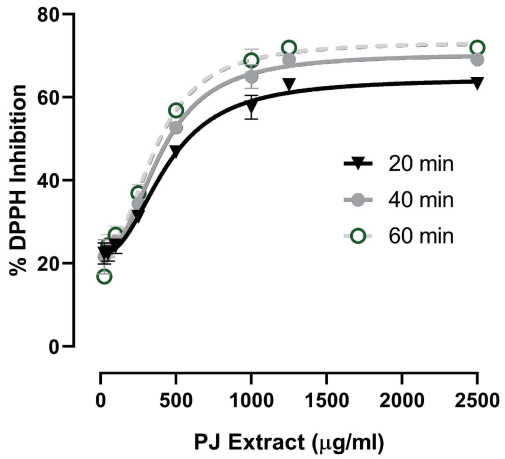


Fig.10: Effect of *P. juliflora* (PJ) leaves extract on per cent DPPH inhibition at different time interval (20, 40 and 60 min)

Table 15: Effect of *P. juliflora* (PJ) leaves extract on per cent DPPH inhibition

SN	PJ extract (µg/ml)	DPPH inhibition (%)		
		20 min	40 min	60 min
1.	25	22.38 ± 2.53	21.60 ± 4.16	16.84 ± 0.01
2.	50	22.40 ± 1.84	23.25 ± 1.78	24.40 ± 2.54
3.	100	24.10 ± 1.73	25.31 ± 1.32	26.91 ± 1.94
4.	250	31.19 ± 1.58	34.64 ± 1.45	36.91 ± 2.10
5.	500	46.81 ± 0.59	52.73 ± 0.57	56.83 ± 0.98
6.	1000	57.61 ± 2.87	64.99 ± 2.87	68.92 ± 2.74
7.	1250	62.98 ± 0.61	69.11 ± 0.32	71.97 ± 0.48
8.	2500	63.25 ± 0.51	69.11 ± 0.32	71.97 ± 0.48
IC_{50} (µg/ml)		**439.27 ± 33.46**	**401.26 ± 24.63**	**372. 16 ± 26.55**

Data are presented as Mean ± SEM of two observations. Data were analysed by one-way ANOVA followed by Tukey's *post-hoc* test.

4.6.2 Ferrous ion chelating ability

Ferrous ion chelating activity of ethanolic extract of *P. juliflora* leaves (PJ) extract exhibited a linear exponential relationship with extract concentration. The

absorbance of ferrous and ferrozine complex was decreased dose-dependently indicating that the chelating activity was increased following increase in the concentration of extract (50-5000 µg/ml). As shown in Table 16, the highest chelating activity (36.76%) of PJ extract was achieved at 5000 µg/ml concentration while EDTA at 1000 µg/ml concentration exhibited 92.86% chelating activity.

Table 16: Ferrous ion chelating ability (%) of *P. juliflora* leaves extract

***P. juliflora* leave extract**	**Ferrous ion chelating ability (%)**
50 µg/ml	16.32
100 µg/ml	18.31
1000 µg/ml	20.02
2500 µg/ml	25.51
5000 µg/ml	36.76
EDTA (1000 µg/ml)	92.86

4.7 *In vitro* antibacterial efficacy testing of *P. juliflora* leaves extract against *E. coli.*

The antibacterial activity of *P. juliflora* leaves extract was evaluated against clinical isolate of *E. coli* (S4 and S21) along with the reference strains (ATCC 25922) using agar well diffusion assay.

The vacuum dried methanolic, ethanolic and hydro-ethanolic PJ extracts were reconstituted either in ethanol or deionized water at two different concentrations *viz.* 20 mg/ml and 25 mg/ml. The antibacterial activity of these test extracts was initially evaluated against reference strain of *E. coli* (ATCC 25922) in terms of zones of inhibition. As summarized in Table 17, ethanolic PJ extract dissolved in ethanol and water showed comparatively higher (20.00 ± 1.54 mm and 17.00 ± 1.32 mm, n=5) zone of inhibition as compared to other combination of the extract and vehicles at 20 mg/ml concentration. Interestingly, increasing the concentration of the extract to 25 mg/ml did not show corresponding increase in the zone of inhibition (17.5 ± 0.93 mm and 16.75 ± 0.99 mm, n=5). Methanolic extract or hydro-ethanolic extract when

dissolved in either ethanol or deionized water did not produced better zone of inhibition as compared to ethanolic extract as shown in the Table 17. Thus in the future experiment, ethanolic extract was used by dissolving in either ethanol or water. Vehicles (methanol, ethanol, water) did not produce any marked antibacterial effect or zone of inhibition.

Table 17: Comparative zones of inhibition (mm) produced by *P. juliflora* leaves extract (PJ) reconstituted in different vehicles against reference strain (ATCC 25922).

Extract conc.	Zone of inhibition (mm), n=5					
	Methanolic extract (dissolved in water)	**Methanolic extract (dissolved in ethanol)**	**Ethanolic extract (dissolved in water)**	**Ethanolic extract (dissolved in ethanol)**	**Hydro-ethanolic extract (dissolved in water)**	**Hydro-ethanolic extract (dissolved in ethanol)**
20 mg/ml	$15.20^{b} \pm 0.20$	$17.00^{ab} \pm 0.45$	$17.00^{ab} \pm 1.32$	$20.00^{a} \pm 1.54$	$13.40^{b} \pm 0.51$	$14.80^{b} \pm 1.11$
25 mg/ml	$16.40^{a} \pm 0.40$	$17.00^{a} \pm 0.45$	$16.75^{a} \pm 0.99$	$17.5^{a} \pm 0.93$	$14.60^{a} \pm 0.51$	$15.40^{a} \pm 1.08$

Data are presented as Mean ± SEM of five observations. Mean values with different superscripts within rows are statistically significant.

Subsequently we evaluated the antibacterial efficacy of ethanolic extract of *P. juliflora* leaves against clinical isolates of *E. coli* (S4 and S21). As ethanolic PJ extract dissolved in ethanol or water showed better antibacterial action against reference strain of *E. coli*, we used this extract-vehicle combination in further study to evaluate the antibacterial efficacy against selected clinical isolates (S4 and S21). The respective antibacterial effect of PJ extract in terms of zone of inhibition is illustrated in Fig. 11 and summarized in Table 18. Perusal of the data revealed that ethanolic extract when dissolved in ethanol showed comparatively higher (though non-significant) zone of inhibition at both the concentrations (20 mg/ml and 25 mg/ml) of the plant extract against both S4 (13.78 ± 0.55 mm and 15.11 ± 0.61 mm, n=7, respectively) and S21 (12.50 ± 0.50 mm and 12.75 ± 0.48 mm, n=9, respectively) isolates.

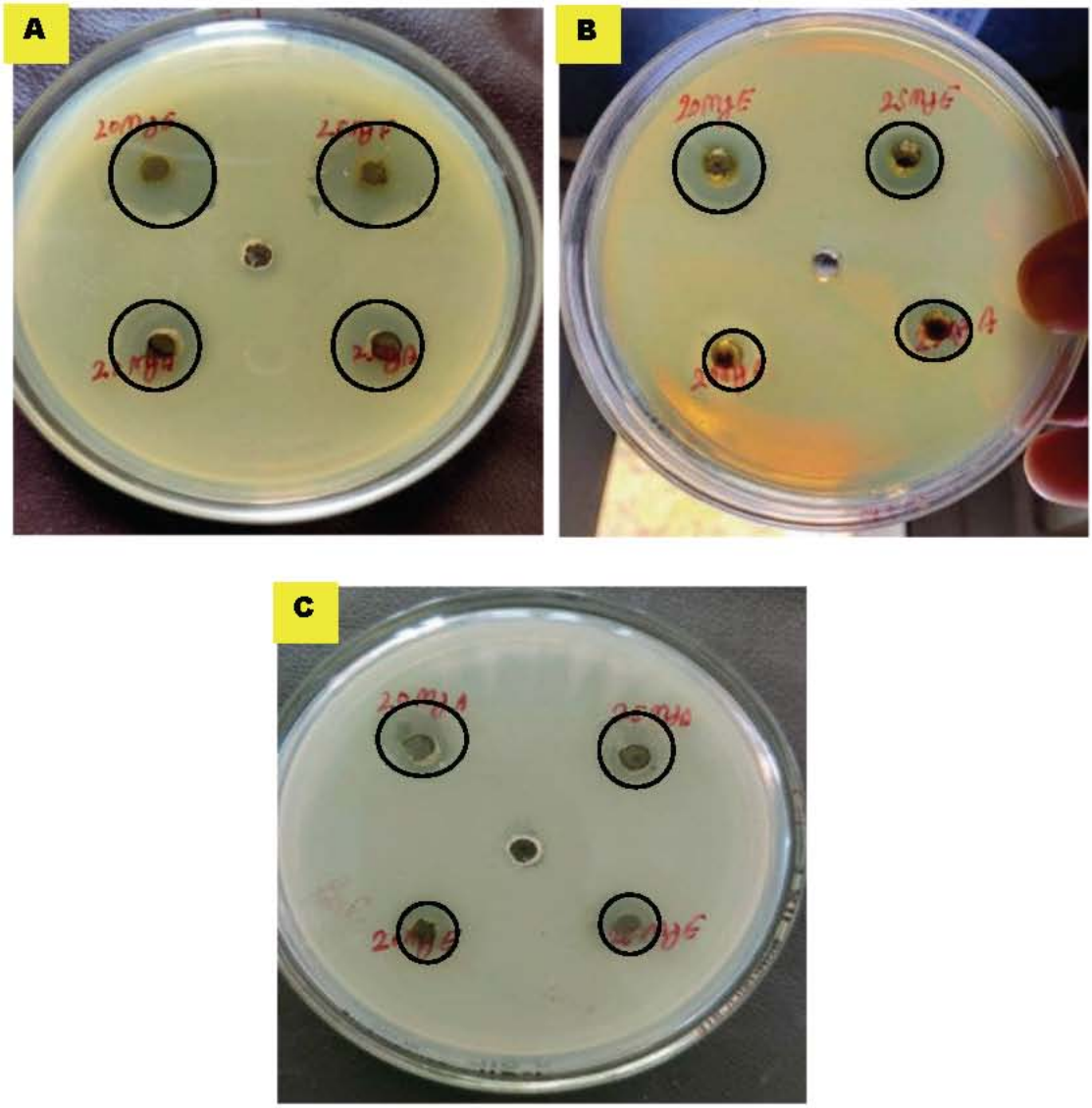


Fig. 11: Representative images showing the agar well diffusion test to evaluate the *in vitro* antibacterial activity of *Prosopis juliflora* leave (PJ) extract against (A) *Escherichia coli* (ATCC 25922), (B) clinical isolate S4 and (C) clinical isolate (S21). E: Ethanol solubilized, A: Aqueous solubilized extract.

Table 18: Comparative zones of inhibition (mm) produced by *P. juliflora* leaves extract (PJ) reconstituted in different vehicles against clinical isolates of *E. coli* (S4 and S21).

Organism tested	Zone of inhibition (mm)			
	PJ extract (ethanolic dissolved in water)		PJ extract (ethanolic dissolved in ethanol)	
	20 mg/ml	25 mg/ml	20 mg/ml	25 mg/ml
S4 isolate (n=7)	12.11 ± 0.31	12.44 ± 0.24	13.78 ± 0.55	15.11 ± 0.61
S21 isolate (n=9)	12.00 ± 0.41	12.50 ± 0.29	12.50 ± 0.50	12.75 ± 0.48

Data are presented as Mean ± SEM of seven to nine observations.

Further, to ascertain the antimicrobial activity of the PJ extract, the minimum inhibitory concentration (MIC) of the extract was determined by broth microdilution method against the clinical isolates (S4 and S21). As depicted in Fig. 12, the MIC values of PJ extracts against reference strain (ATCC 25922), clinical isolates of S4 and S21 were calculated to be 0.39 mg/ml, 0.39 mg/ml and 1.56 mg/ml, respectively. The MIC values of cefotaxime against these isolates were 0.04 µg/ml, 0.04 µg/ml and 32 µg/ml, respectively.

As illustrated in Fig. 13, the fractional inhibitory concentration (FIC) of cefotaxime for PJ extract was found to be 3.0 thus, indicating indifferent or no antagonistic effect.

4.8 Effect of *P. juliflora* leaves (PJ) extract on bacterial growth kinetics:

To assess the effect of PJ extract and/or cefotaxime on growth kinetics of S4 clinical isolate, time kill assay was performed. Fig. 14 illustrates the representative live bacterial counts at different exposure time (*viz.* 0, 2, 4, 6, 8, 12, 16, 18 and 24 h) to PJ extract and/or cefotaxime. As summarized in Table 19 and depicted in Fig. 15, significant antimicrobial action of PJ extract against S4 isolate was exhibited at 6 h post-exposure, while complete bactericidal action was observed at 12 h post-exposure. Cefotaxime produced its bactericidal effect at 6 h post-exposure.

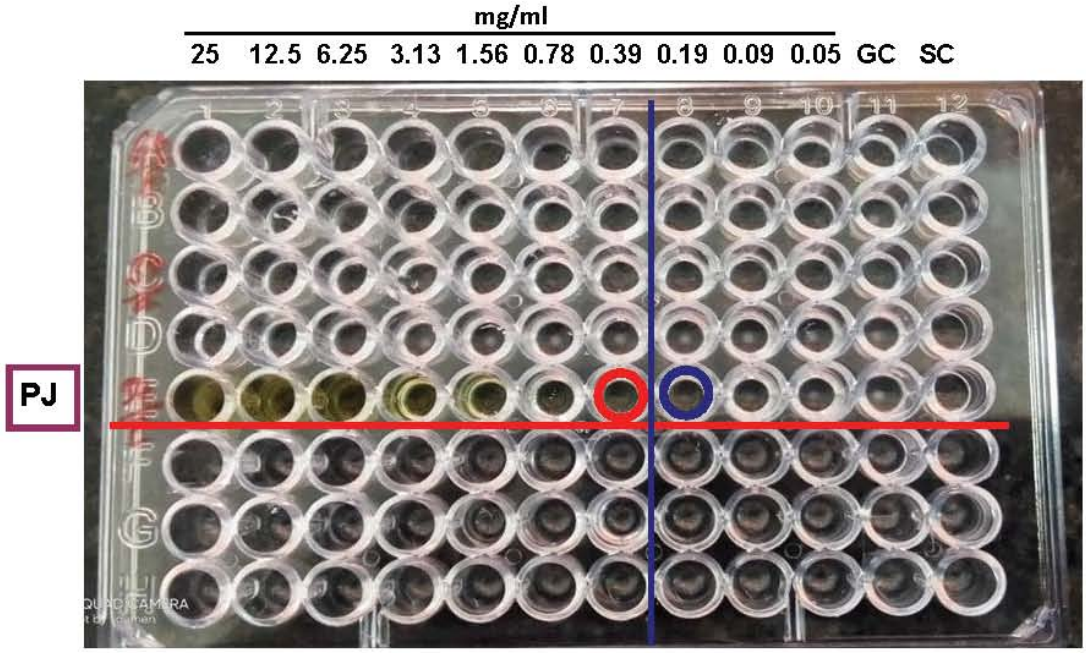


Fig. 12: Representative microtitre plate showing the MIC value of *Prosopis juliflora* leaves (PJ) extract as recorded by visual reading of bacterial plaque formation. PJ: *Prosopis juliflora* leaves extract; GC : growth control; SC : sterility control.

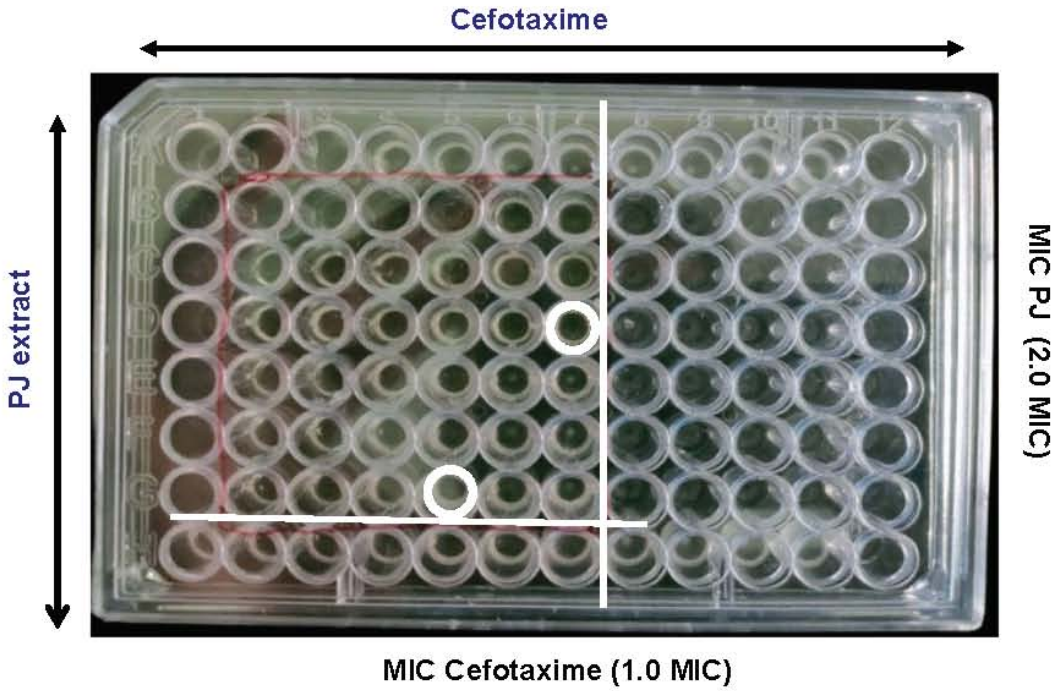


Fig. 13: Determination of FIC value by visual reading from representative microtitre plate. PJ: *Prosopis juliflora* leaves extract with concentration decreasing from left to right against cefotaxime.

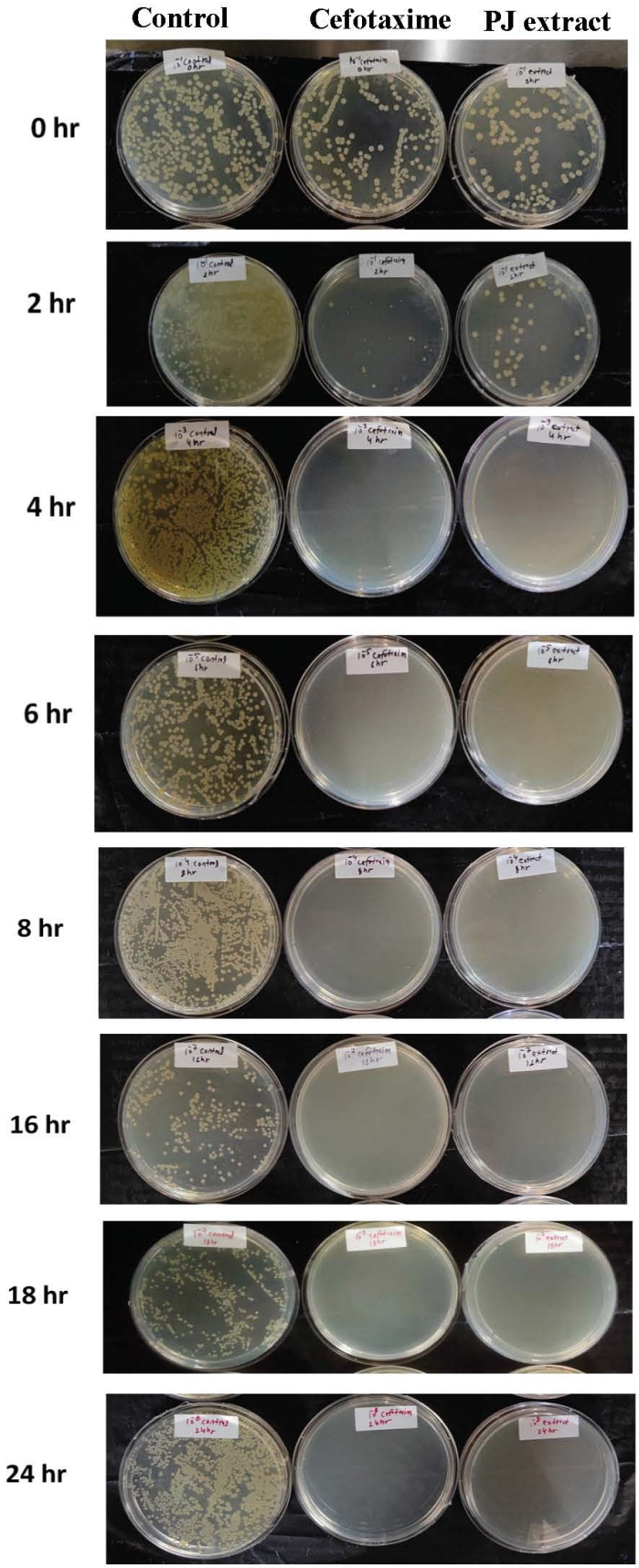


Fig. 14: Representative culture plates showing the effect of exposure to cefotaxime and *Prosopis juliflora* leaves (PJ) extract on bacterial (S4 isolate) growth at different time intervals.

Table 19: Effect on growth kinetics (at different time intervals) of clinical isolate of *E. coli* (S4) following exposure to *P. juliflora* leaves extract (PJ).

Time (h)	Bacterial count (Log CFU/ml)		
	Control (S4) n=3	Treatment (S4 + PJ) n=3	Treatment (S4 + Cefotaxime) n=3
0	4.66 ± 0.06	5.04 ± 0.28	4.63 ± 0.11
2	5.55 ± 0.24	4.55 ± 0.08	3.84 ± 0.09
4	7.97 ± 0.33	3.93 ± 0.19	1.33 ± 0.04*
6	7.81 ± 0.34	1.59 ± 0.05*	No visible growth
8	7.95 ± 0.33	1.49 ± 0.04*	No visible growth
12	10.38 ± 0.42	No visible growth	No visible growth
16	10.20 ± 0.46	No visible growth	No visible growth
18	10.03 ± 0.40	No visible growth	No visible growth
24	10.10 ± 0.40	No visible growth	No visible growth

Data are presented as Mean ± SEM of three observations. *p<0.05 *vs.* 0 h bacterial count of respective group.

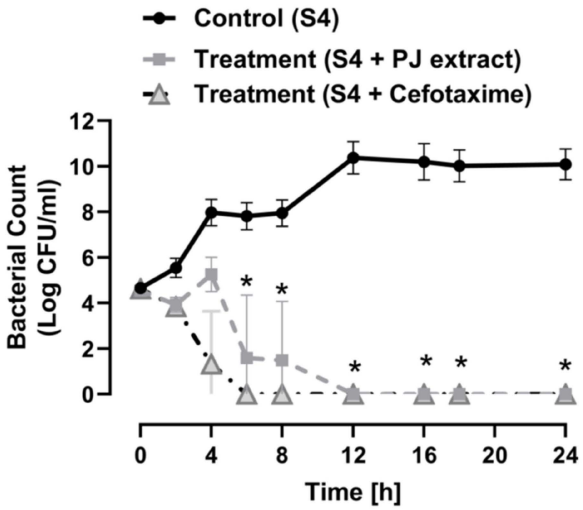


Fig.15: Effect of *P. juliflora* leaves extract (PJ) on growth kinetics (at different time intervals) of clinical isolate of *E. coli* (S4). Data are presented as Mean ± SEM of three observations. Vertical bars represent SEM. *p <0.05 *vs.* control (S4).

4.9 Effect on cell membrane integrity:

The CFDA dye is a membrane permeable dye which emits green fluorescence following breakage of ester bond by the esterase present in the cell membrane. Thus the bacterial cells showing the green fluorescence are indicative of live cells with intact cell membrane. On the other hand, dead cell with damaged membrane integrity emits red florescence with propidium iodide (PI). As shown in the Fig. 16, the smear from bacterial culture (S4) without treatment exhibited maximum green fluorescence. The potential of PJ extract to penetrate the bacterial cells (S4) and to damage the membrane integrity was evidenced from the higher number of the cell emitting red fluorescence (Fig. 16). Similarly, exposure to bacterial cells to cefotaxime (positive control), a known antimicrobial causing damage to cell wall also showed bacteria with red fluorescence.

Similarly, reference *E. coli* culture (ATCC 25922) without treatment showed higher green fluorescence (Fig. 16). Further, treatment with PJ extract produced more number of dead cells with loss of membrane integrity as shown in Fig.16. Cefotaxime (positive control) produced similar effect to that of PJ extract.

4.10 Effect on bacterial cellular ultrastructure:

Representative transmission electron microscopy (TEM) slides from different groups are summarized in Fig. 17-21. Gr-I showed normal bacterial structure with intact cell wall, cell membrane and cytopalsmic contents (Fig. 17a-d). The bacteria undergo normal binary fusion with intact cell membrane (Fig. 17a & b) and uniform distribution of cytoplasmic contents (Fig. 17c & d). Treatment of bacterial cells with cefotaxime (positive control, Gr-II) produced abnormal bacterial morphology with disrupted cell wall and loss of integrity of cell membrane (Fig.18a-d). Damage of the cell membrane integrity caused leakage of the cytoplasmic contents (Fig. 18a & d). The cell wall was also damaged during binary fusion with non-uniform distribution of cytoplasmic contents (Fig. 18b). Appearance of vacuolation along with detached cell membrane (Fig. 18c) was seen following cefotaxime treatment. Exposure to *P. juliflora* leaves extracts at lower dose (Gr-III) exhibited abnormal bacterial morphology with disrupted cell wall and loss of integrity of cell membrane (Fig. 19a). There was shrinking of cytoplasmic contents and translucent cytoplasm (Fig. 19b & c)

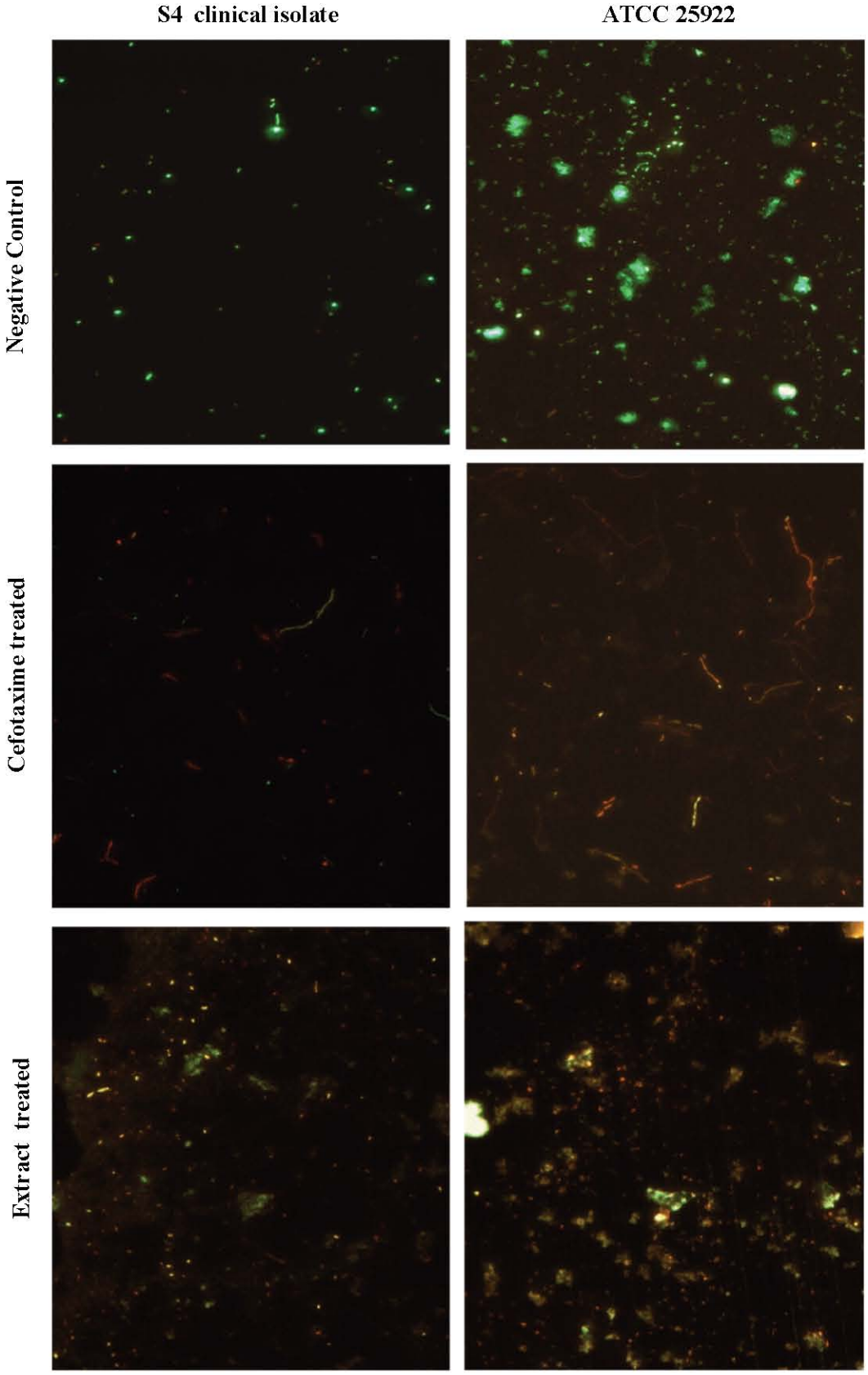


Fig. 16: Effect of *P. juliflora* leave (PJ) extract on cell membrane integrity of clinical isolate (S4) as well as reference strain (ATCC 25922) of *E. coli* as detected by fluorescent microscopy.

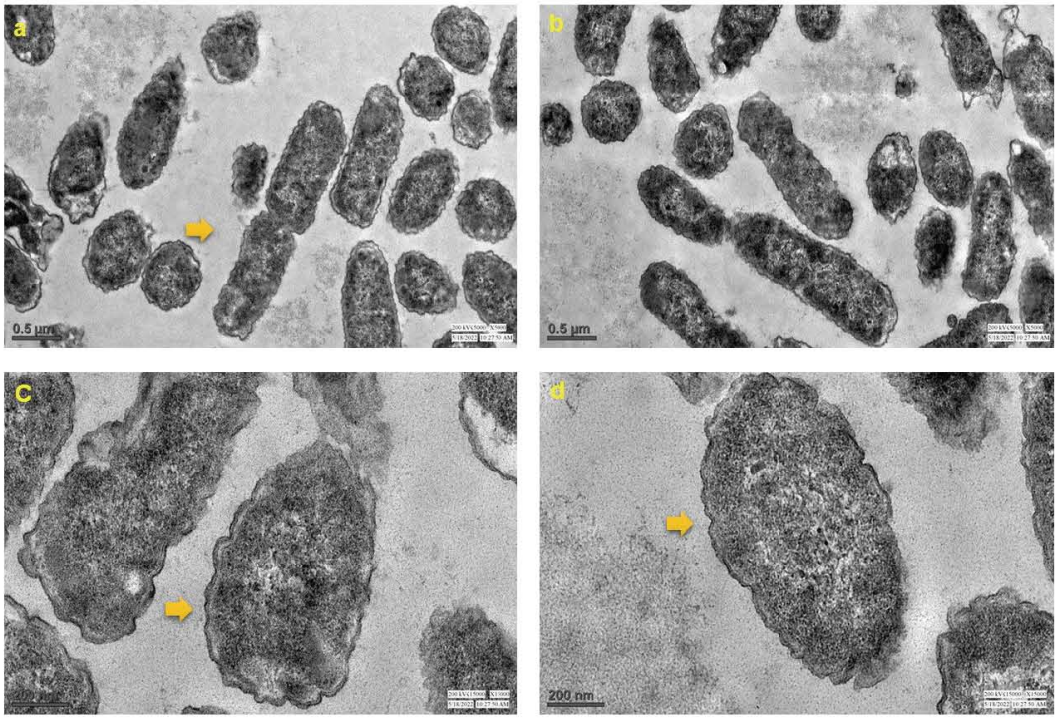


Fig. 17: Representative electron microscopy (TEM) slides from Gr-I showing normal bacterial structure with intact cell wall, cell membrane and cytoplasmic contents (a,b,c,d). The bacteria undergo normal binary fusion with intact cell membrane (a,b) and uniform distribution of cytoplasmic contents (c,d)

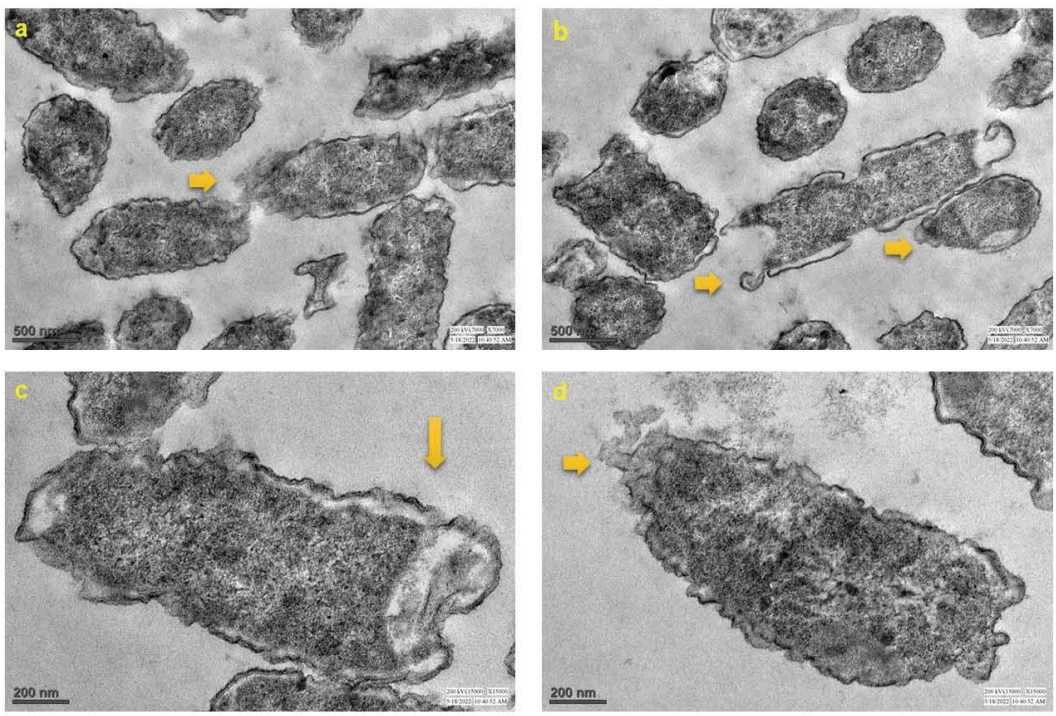


Fig. 18: Representative electron microscopy (TEM) slides from Gr-II showing abnormal bacterial morphology with disrupted cell wall and loss of integrity of cell membrane (a,b,c,d). Damage of the cell membrane integrity caused leakage of the cytoplasmic contents (a,d). The cell wall was also damaged during binary fusion with non-uniform distribution of cytoplasmic contents (b). Appearance of vacuolation along with detached cell membrane (c) were seen following cefotaxime treatment.

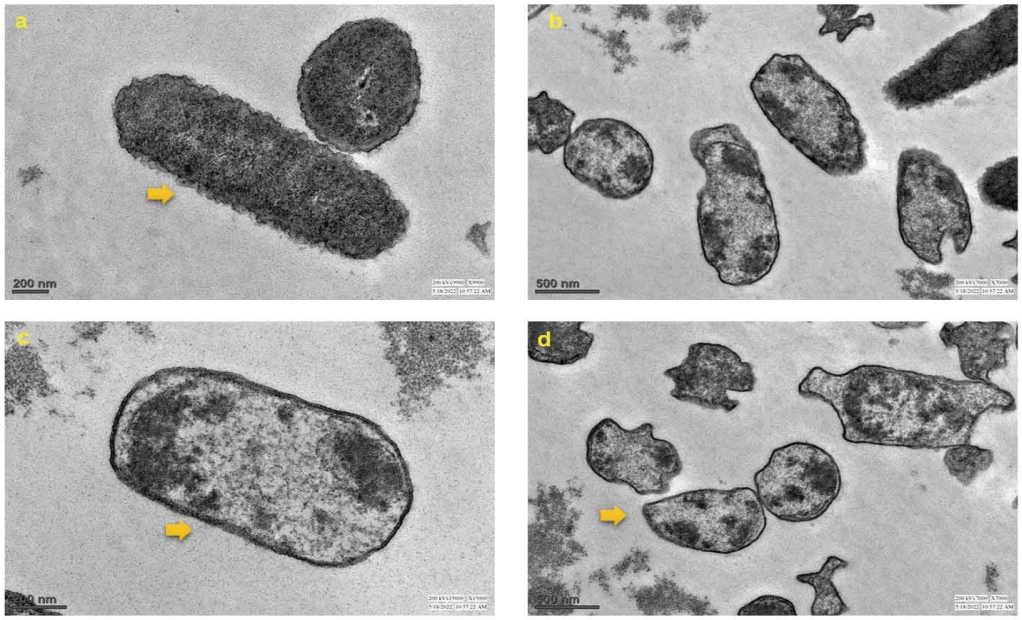


Fig. 19: Representative electron microscopy (TEM) slides from Gr-III showing abnormal bacterial morphology with disrupted cell wall and loss of integrity of cell membrane (a). There was shrinking of cytoplasmic contents and translucent cytoplasm (b,c) along with detachment of cell membrane (c). Appearance of misshapen cell (d) with cytoplasmic vacuoles were also prominent following exposure of bacterial cells to *P. juliflora* leave extract (2 MIC).

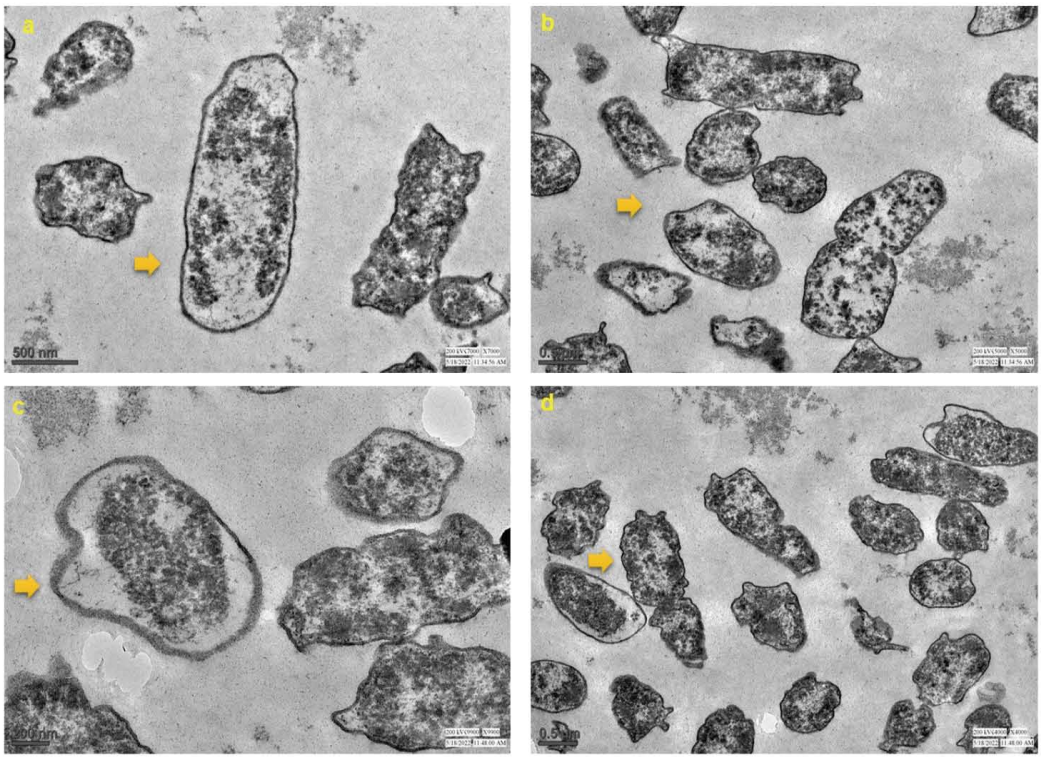


Fig. 20: Representative electron microscopy (TEM) slides from Gr-IV showing abnormal bacterial morphology with intensely disrupted cell wall and loss of integrity of cell membrane (a,b,c,d). There was clumping of cytoplasmic contents with vacuole formation (a,b) and loos of cell wall integrity (c). Detachment of cell membrane with misshapen cells (b,c) along with condensation and aggregation of chromatin (c,d). Improper distribution of cytoplasmic contents during binary fusion along with loss of cell wall structure (b,d).

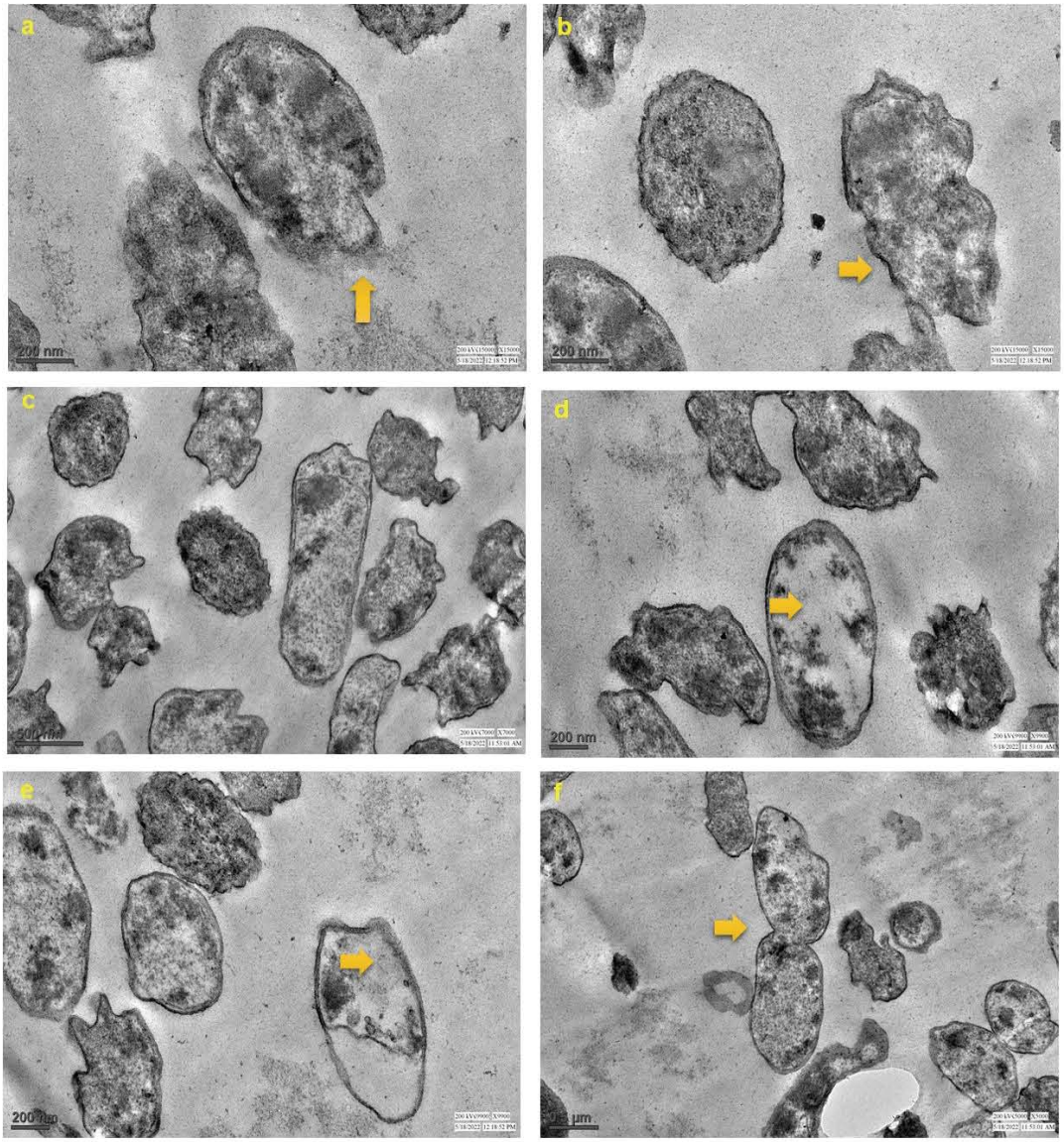


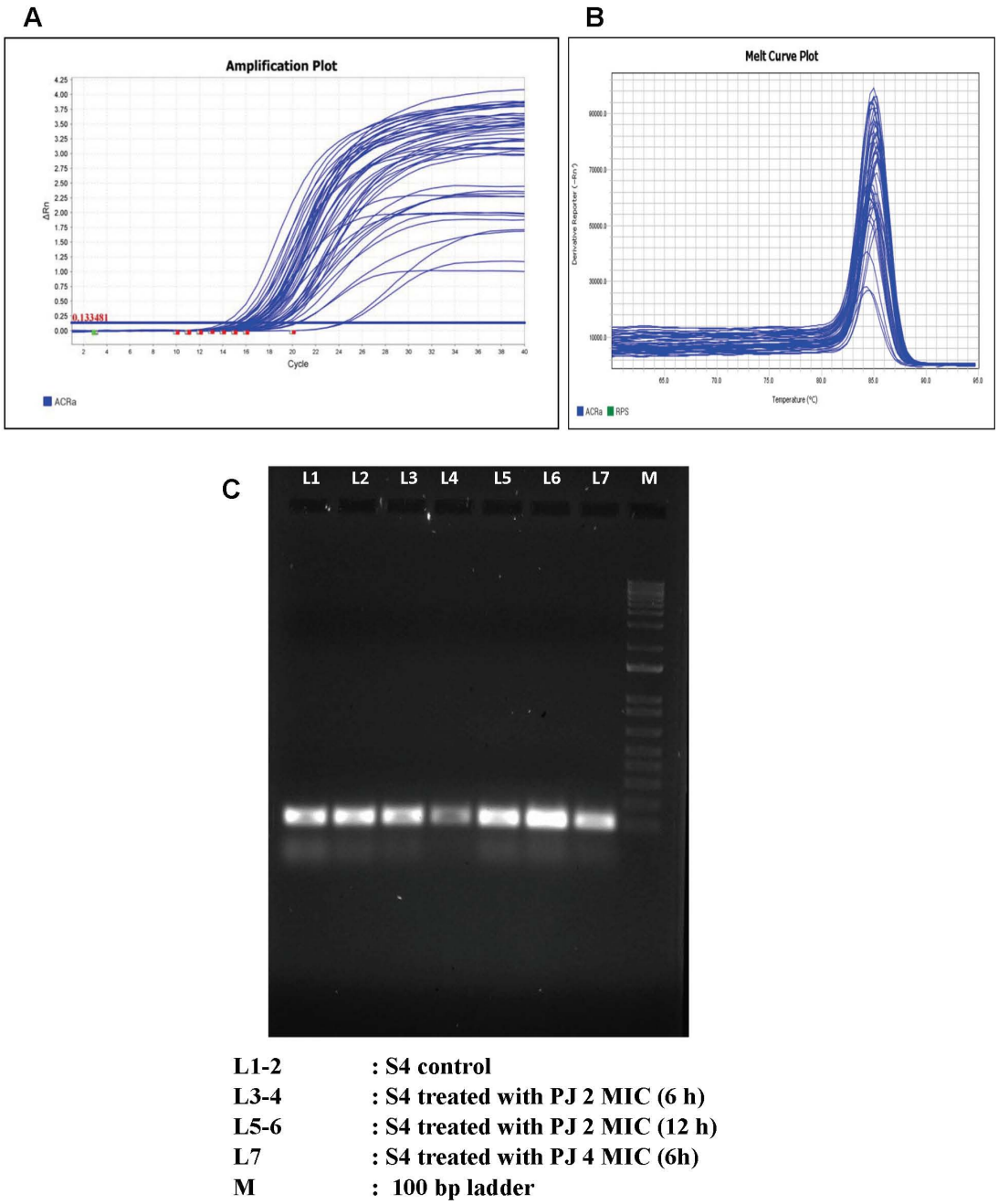
Fig. 21: Representative electron microscopy (TEM) slides from Gr-V showing abnormal bacterial morphology with disrupted cell wall and loss of integrity of cell membrane (a-f). There was leakage of cytoplasmic content and disruption of cell wall (a,b,c), appearance of misshapen cell (c), Ghost cell with no cytoplasmic content (d,e). Improper distribution of cytoplasmic content during binary fusion (f) along with clumping of cytoplasmic contents (e,f) and detachment of cell membrane (b) were seen following combined treatment with *P. juliflora* leave extract (2 MIC) and cefotaxime (2 MIC).

along with detachment of cell membrane (Fig. 19c). Appearance of misshapen cell (Fig. 19d) with cytopalsmic vacuoles were also prominent following exposure of bacterial cells to *P. juliflora* leave extract (2 MIC). Exposure to relatively higher concentration of plant extract (4 MIC, Gr-IV) produced more detrimental effect as evidenced by appearance of abnormal bacterial morphology with intensely disrupted cell wall and loss of integrity of cell membrane (Fig. 20a-d). There was clumping of cytoplasmic contents with vacuole formation (Fig. 20a & b) and loss of cell wall integrity (Fig. 20c). Detachment of cell membrane with misshapen cells (Fig. 20b & c) along with condensation and aggregation of chromatin materials were observed (Fig. 20c & d). Improper distribution of cytoplasmic contents during binary fusion along with loss of cell wall structure (Fig. 20b & d) was evident in this group. Following co-exposure to plant extract (2 MIC) and cefotaxime (2 MIC) bacterial cells appeared with disrupted cell wall and loss of integrity of cell membrane (Fig. 21a-f). There was leakage of cytopalsmic content due to disruption of cell wall (Fig. 21a-c), appearance of misshapen cell (Fig. 21c), and ghost cell with no cytopalsmic content (Fig. 21 d & e). Improper distribution of cytopalsmic content during binary fusion (Fig. 21f) along with clumping of cytoplasmic contents (Fig. 21e & f) and detachment of cell membrane (Fig. 21b) were seen following combined treatment with *P. juliflora* leave extract (2 MIC) and cefotaxime (2 MIC). However, qualitatively no additive effect was observed following co-exposure to cefotaxime along with herbal extract as compared to the effect of the individual agent.

4.11 Effect on mRNA expression of efflux pumps:

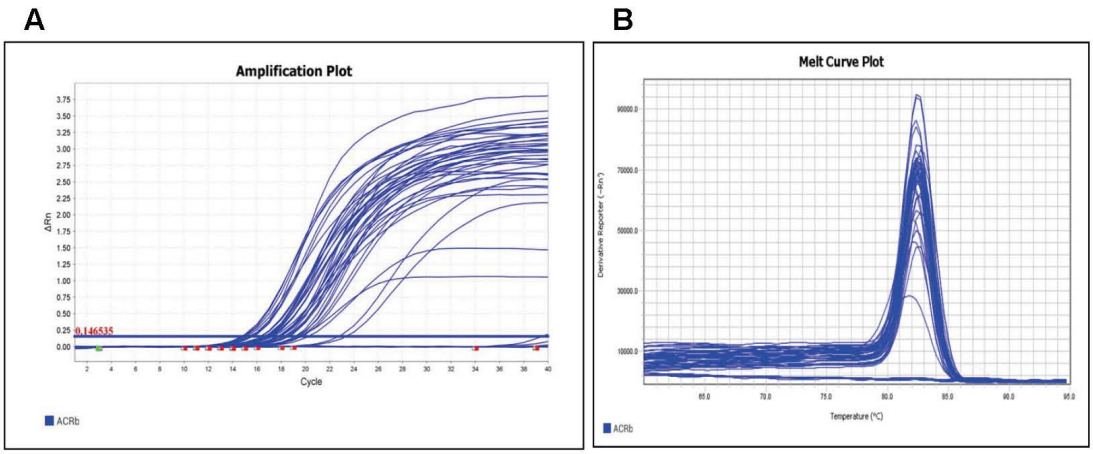
Figures 22, 23 and 24 depict the RT-qPCR analysis of acrA, acrB and RPS genes (reference gene), respectively, in S4 clinical isolate. Real time PCR amplification plot and dissociation curve of acrA are shown in Fig. 22A & B, respectively. Following RT-PCR amplification, a single product of 107 bp long amplicon was amplified in different samples from control and treated groups of S4 (Fig. 22C). Similarly, the amplification plots and dissociation curves of acrB are shown in Fig. 23A & B. Following RT-PCR amplification, the amplicon size corresponding to acrB found to be 107 bp (Fig. 23C).

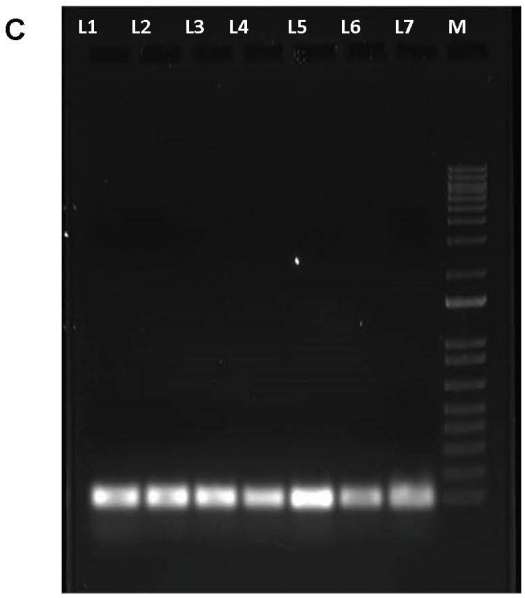
The mRNA expressions of all the efflux pump genes (acrA and acrB) in different groups were normalized to corresponding bacterial RPS gene expression from different groups. Fig. 24A & B, show the representative real time PCR



L1-2 : **S4 control**
L3-4 : **S4 treated with PJ 2 MIC (6 h)**
L5-6 : **S4 treated with PJ 2 MIC (12 h)**
L7 : **S4 treated with PJ 4 MIC (6h)**
M : **100 bp ladder**

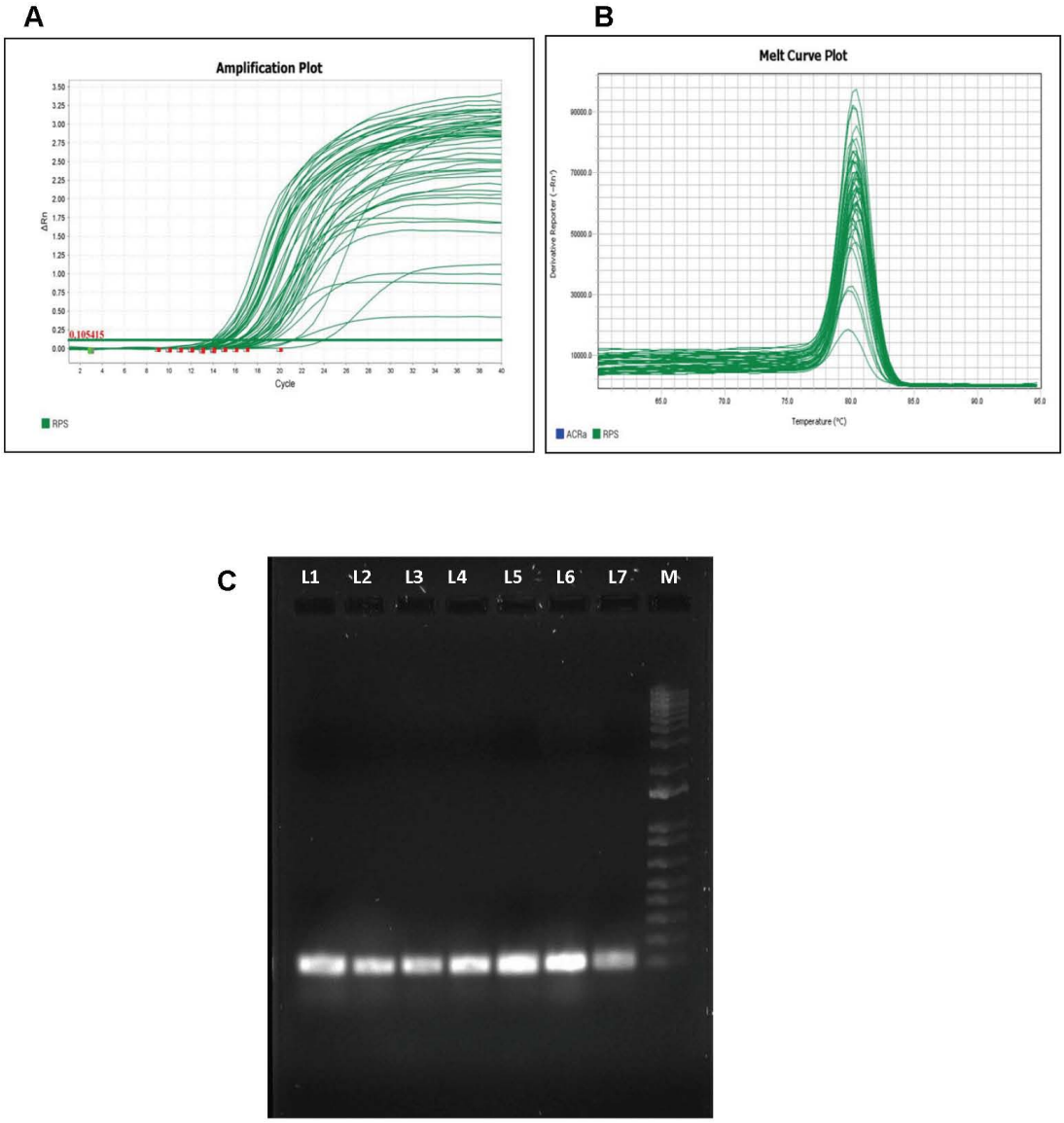
Fig. 22: RT-qPCR analysis of acrA in S4 (clinical isolate of *E. coli*). Amplification plot (A) and Melt curve plot (B) of acrA amplified from cDNA samples of different groups. Representative agarose gel image (C) showing the single amplified RT-PCR amplicon for acrA (107 bp) from different samples.





L1-2	**: S4 control**
L3-4	**: S4 treated with PJ 2 MIC (6 h)**
L5-6	**: S4 treated with PJ 2 MIC (12 h)**
L7	**: S4 treated with PJ 4 MIC (6h)**
M	**: 100 bp ladder**

Fig. 23: RT-qPCR analysis of acrB in S4 (clinical isolate of *E. coli*). Amplification plot (A) and Melt curve plot (B) of acrB amplified from cDNA samples of different groups. Representative agarose gel image (C) showing the single amplified RT-PCR amplicon for acrB (107 bp) from different samples.



L1-2	**: S4 control**
L3-4	**: S4 treated with PJ 2 MIC (6 h)**
L5-6	**: S4 treated with PJ 2 MIC (12 h)**
L7	**: S4 treated with PJ 4 MIC (6h)**
M	**: 100 bp ladder**

Fig. 24: RT-qPCR analysis of RPS (reference gene) in S4 (clinical isolate of *E. coli*). Amplification plot (A) and Melt curve plot (B) of RPS amplified from cDNA samples of different groups. Representative agarose gel image (C) showing the single amplified RT-PCR amplicon for RPS (104 bp) from different samples.

amplification plot and dissociation curve, respectively, for RPS gene. Following RT-PCR amplification, a single product of 104 bp long amplicon was amplified from different samples (Fig. 24C).

As summarized in Table 20 and illustrated in Fig.25A, exposure to PJ extract at lower concentration (2 MIC) significantly ($p<0.05$) down-regulated the mRNA expression of acrA gene in S4 clinical isolate following 6 h (0.46 ± 0.16, n=5) and 12 h (0.35 ± 0.11, n=5) post exposure as compared to untreated control (1.00 ± 0.06, n=5). Interestingly, 6 h exposure to higher concentration (4 MIC) of PJ extract although produced 39 % (0.61 ± 0.05 *vs.* 1.00 ± 0.06, n=5) decrease in mRNA expression of acrA gene, this changes did not reach to significant level as compared to untreated control.

As depicted in Fig. 25B, unlike acrA, down-expression of acrB gene was observed in S4 bacterial isolate only after 12 h post-exposure to lower concentration of PJ extract (0.56 ± 0.09, n=5) as compared to untreated control (1.03 ± 0.12, n=5). There was only 33 % (0.69 ± 0.09 *vs.* 1.03 ± 0.12, n=5) and 27 % (0.75 ± 0.15 *vs.*1.03 ± 0.12, n=5) decrease in mRNA expression of acrB gene following 6 h post exposure to PJ extract at lower (2 MIC) and higher (4 MIC) concentration, respectively (Table 20).

Table 20: Effect on mRNA expression of efflux pump related genes in clinical isolate (S4) following exposure to *P. juliflora* leaves extract (PJ) at different time interval.

Efflux Pump Gene (s)	mRNA expression in S4 isolate			
	S4 Control	S4+ PJ (2 MIC for 6 h)	S4+ PJ (2 MIC for 12 h)	S4+ PJ (4 MIC for 6 h)
acrA	1.00 ± 0.06	0.46 ± 0.16*	0.35 ± 0.11*	0.61 ± 0.05
acrB	1.03 ± 0.12	0.69 ± 0.09	0.56 ± 0.09*	0.75 ± 0.15

Data are presented as mean ± SEM, n=5. Data were analysed by one-way ANOVA followed by Tukey's *post-hoc* test. *$p<0.05$ *vs.* respective S4 control.

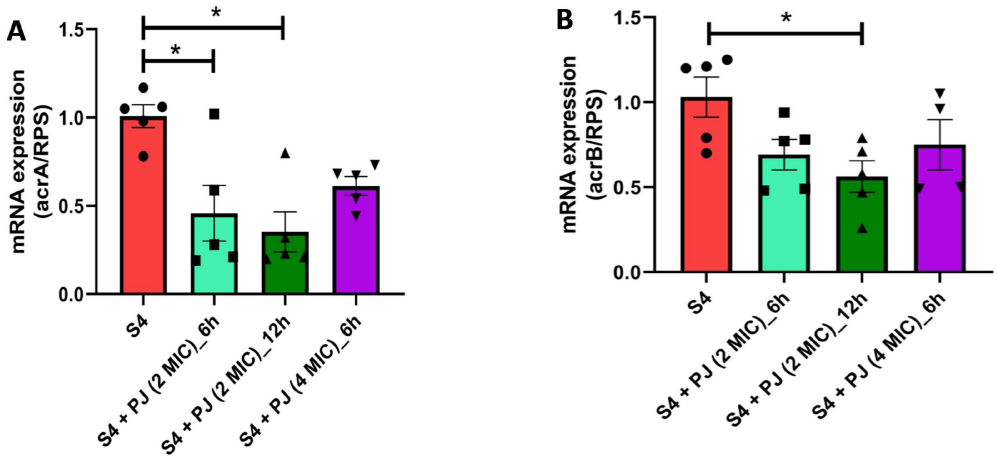
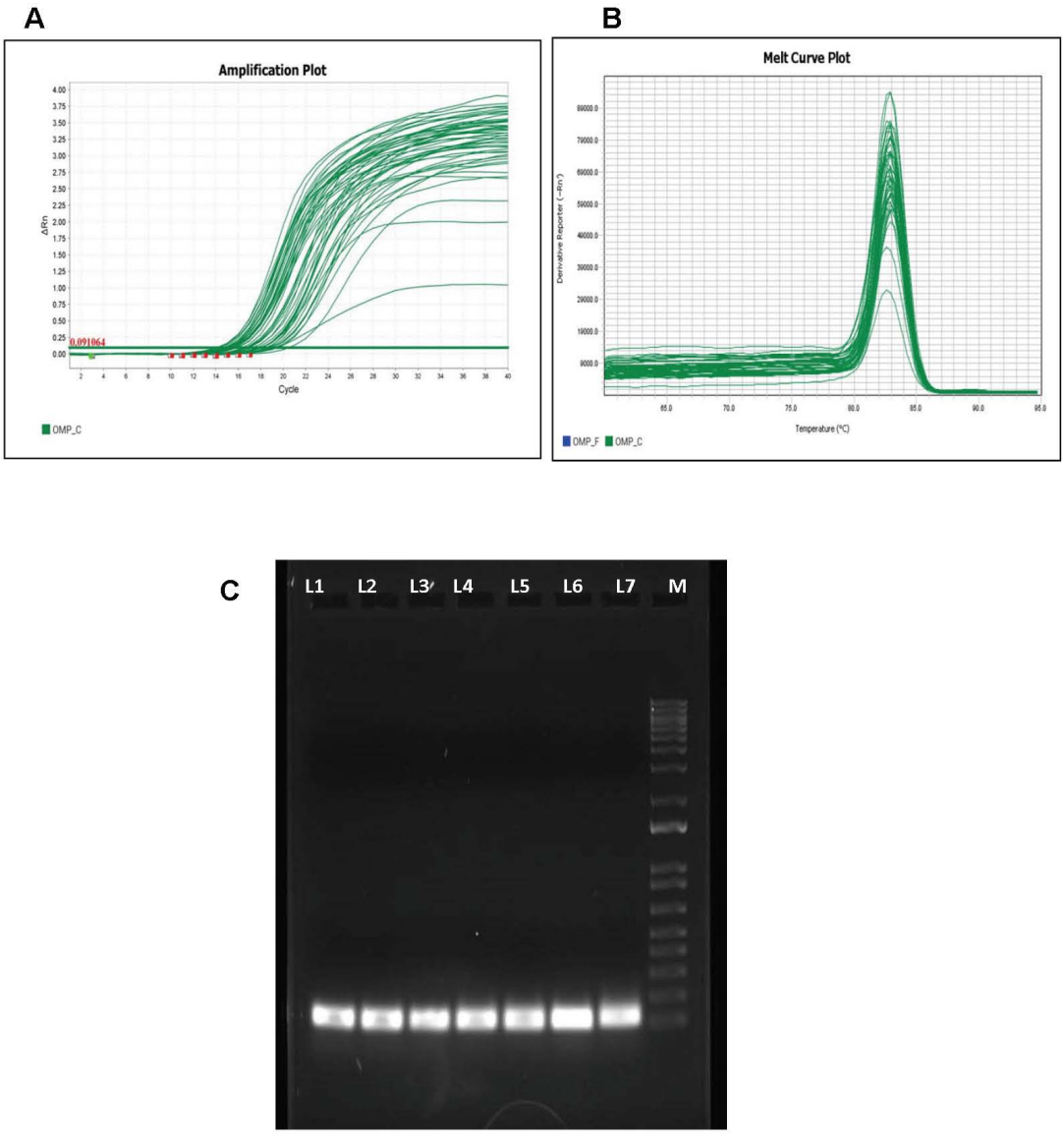


Fig. 25:Bar diagrams showing the effect of exposure to *P. juliflora* leaves (PJ) extract on mRNA expression of efflux pump related genes (A; acrA and B; acrB) in clinical isolates of *E. coli* (S4). Data are presented as mean ± SEM, n=5. Vertical bars represent SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test. *$p<0.05$ *vs.* respective S4 control.

4.12 Effect on mRNA expression of outer membrane protein (OMP):

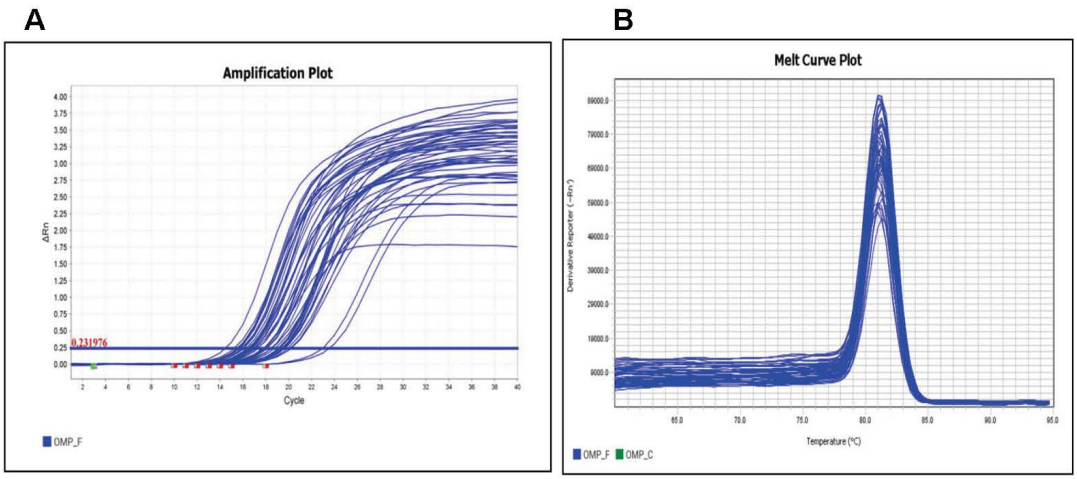
Figures 26 and 27 depict the RT-qPCR analysis of OmpC and OmpF genes, respectively, in S4 clinical isolate. Real time PCR amplification plot and dissociation curve of OmpC are shown in Fig….A & B, respectively. Following RT-PCR amplification, a single product of 125 bp long amplicon was amplified in different samples from control and treated groups of S4 (Fig. 26C). Similarly, the amplification plots and dissociation curves of OmpF are shown in Fig. 27A & B. Following RT-PCR amplification, the amplicon size corresponding to OmpC found to be 118 bp (Fig. 27C). The mRNA expressions of all the outer membrane protein genes (OmpC and OmpF) in different groups were normalized to corresponding bacterial RPS gene expression from different groups.

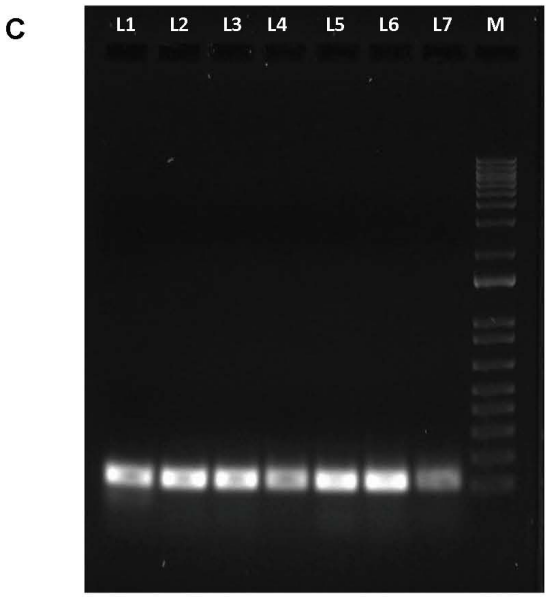
As summarized in Table 21 and illustrated in Fig. 28A &B, exposure to PJ extract either at lower concentration (2 MIC) or higher concentration (4 MIC) did not produce any significant change in the mRNA expression of OmpC as well as OmpF genes in S4 clinical isolate.



L1-2	**: S4 control**
L3-4	**: S4 treated with PJ 2 MIC (6 h)**
L5-6	**: S4 treated with PJ 2 MIC (12 h)**
L7	**: S4 treated with PJ 4 MIC (6h)**
M	**: 100 bp ladder**

Fig. 26: RT-qPCR analysis of ompC gene in S4 (clinical isolate of *E. coli*). Amplification plot (A) and Melt curve plot (B) of ompC amplified from cDNA samples of different groups. Representative agarose gel image (C) showing the single amplified RT-PCR amplicon for ompC (125 bp) from different samples.





L1-2	**: S4 control**
L3-4	**: S4 treated with PJ 2 MIC (6 h)**
L5-6	**: S4 treated with PJ 2 MIC (12 h)**
L7	**: S4 treated with PJ 4 MIC (6h)**
M	**: 100 bp ladder**

Fig. 27: RT-qPCR analysis of ompF gene in S4 (clinical isolate of *E. coli*). Amplification plot (A) and Melt curve plot (B) of ompF amplified from cDNA samples of different groups. Representative agarose gel image (C) showing the single amplified RT-PCR amplicon for ompF (118 bp) from different samples.

Table 21: Effect on mRNA expression of outer membrane protein related genes in clinical isolate (S4) following exposure to *P. juliflora* leaves extract (PJ) at different time interval.

Efflux Pump Gene (s)	mRNA expression in S4 isolate			
	S4 Control	S4+ PJ (2 MIC for 6 h)	S4+ PJ (2 MIC for 12 h)	S4+ PJ (4 MIC for 6 h)
OmpC	1.04 ± 0.14	0.63 ± 0.18	0.57 ± 0.11	0.56 ± 0.12
OmpF	1.04 ± 0.15	0.80 ± 0.19	0.69 ± 0.12	0.70 ± 0.15

Data are presented as mean ± SEM, n=5. Data were analysed by one-way ANOVA followed by Tukey's *post-hoc* test.

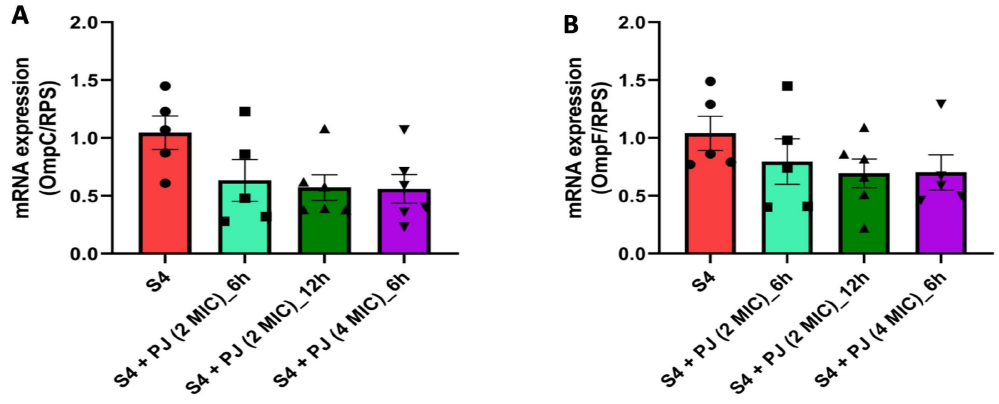


Fig. 28:Bar diagrams showing the effect of exposure to *P. juliflora* leaves (PJ) extract on mRNA expression of outer membrane protein related genes (A: ompC and B: ompF) in clinical isolates of *E. coli* (S4). Data are presented as mean ± SEM, n=5. Vertical bars represent SEM. Data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test.

4.13 Effect on bacterial biofilm production:

To evaluate the efficacy of the plant extract against bacterial biofilm production, initially the ability of biofilm production by S4 and S21 clinical isolates of *E. coli* were assessed by both congo red agar and microtitre plate assays. Though both S4 and S21 isolates were found to be positive for biofilm production in congo red agar, S4 isolates produced typical black colour colonies among these two isolates (Fig. 29). Quantitative measurement of biofilm production by both these isolates was depicted in Fig. 30 and summarized in Table 22. Perusal of the result revealed that,

S21 produced strong biofilm with significantly higher intensity of crystal violet stain (OD_{600}: 0.22 ± 0.01) as compared to the moderate biofilm production by S4 isolate (OD_{600}: 0.16 ± 0.01). Thus in the further experiment, ability of inhibition of biofilm production by PJ extract was evaluated using S21 isolate.

Table 22: The biofilm producing ability of the clinical isolates of *E. coli.* as quantified by crystal violet staining assay

SN	Bacterial isolates	OD_{600}	Biofilm Formation
1	S21	0.22 ± 0.01*	Strong
2	S4	0.16 ± 0.01	Moderate

Data are presented as mean ± SEM of three observations. Data were analyzed by unpaired student's t-test. *$p<0.05$ *vs.* S4

The quantitative measurement of anti-biofilm activity by crystal violet microtitre method revealed that PJ extract (@ MIC) caused 54.55 % inhibition of biofilm production by S21 isolate in comparison to 90.91 % inhibition by chloramphenicol at 10 µg/ml concentration (Table…..). Gentamicin (equivalent to MIC i.e. 10 µg/ml) exhibited 31.82% inhibition of biofilm production by S21 isolate.

Table 23: Effect of *P. juliflora* leaves (PJ) extract on biofilm production by S21clinical isolate.

Extract/ Drugs	OD_{600}	Biofilm inhibition (%)
PJ extract	0.10 ± 0.01	54.55
Chloramphenicol	0.02 ± 0.01	90.91
Gentamicin	0.15 ± 0.01	31.82

Data are presented as mean ± SEM of three observations.

In situ visualization of biofilm production by bacteria and anti-biofilm activity of PJ extract were assesed by scanning electron microscope (SEM). Representative SEM images from different groups are summarized in Fig. 31and 32. The untreated bacterial culture (S21) under SEM microscopy appeared as clusters of cells indicating their ability to form biofilm (Fig. 31a, b & c). The surface morphology of these bacteria appeared smooth with regular shape (Fig 31c). Exposure to chloramphenicol caused distortion of the cluster (Fig. 31d & e) along with appearance of rough and

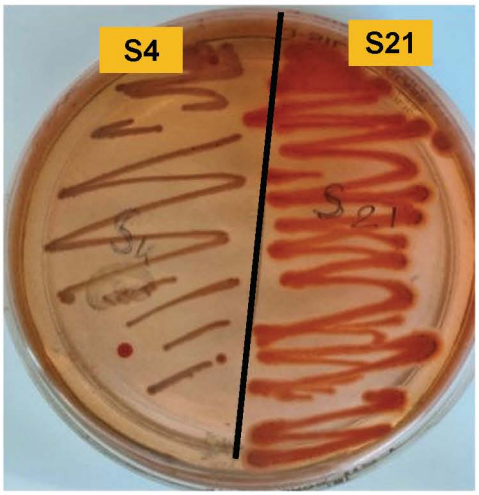


Fig. 29: Representative photograph showing the bacterial growth (S4 and S21 isolates) on Congo red agar plate. The appearance of black colour colonies indicate the biofilm forming ability of the respective isolates.

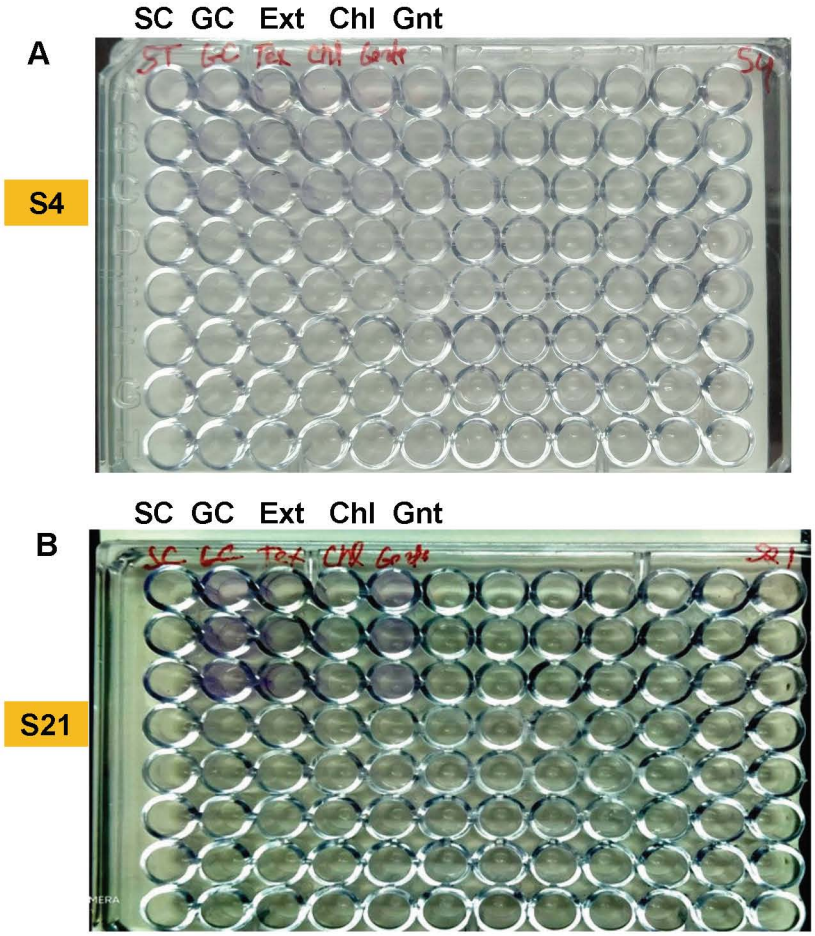


Fig. 30: Representative microtitre plate showing the quantitative measurement of biofilm by crystal violet assay. The intensity of blue colour of the well is directly proportional to amount of biofilm produced by the isolates (A: S4 and B: S21) in the absence and presence of the standard drugs or extract. SC: sterility control, Ext: PJ extract, Chl: Chloramphenicol; Gnt: gentamicin

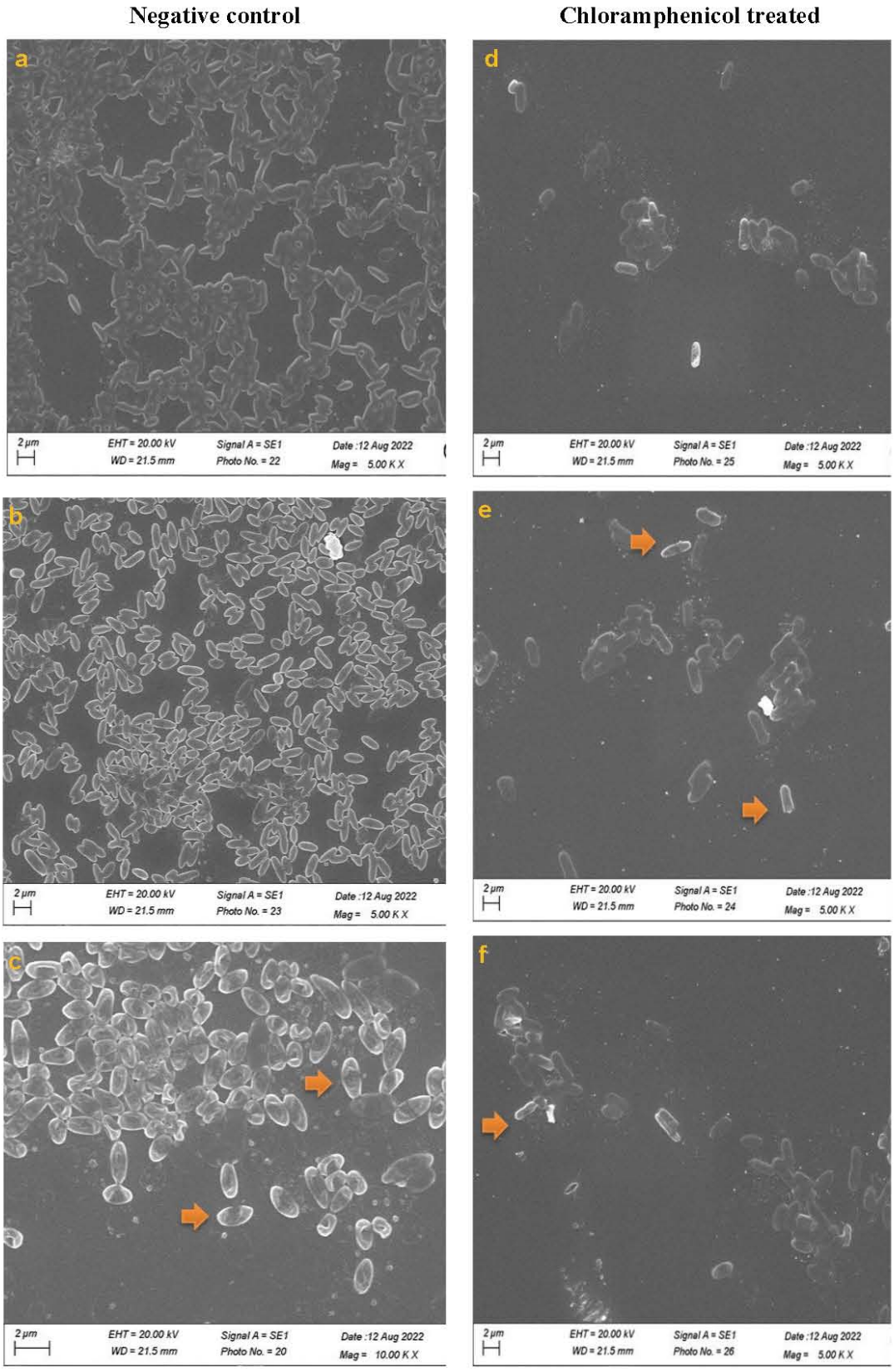


Fig. 31: Representative scanning electron microscopy (SEM) images of untreated bacterial cells (S21 isolates) showing clusters of cells indicating the biofilm production (a-c). The surface of the bacteria appeared smooth ©. Treatment with chloramphenicol reduced the cell density or clusters (d-f) with rough and wrinkled surface (e,f).

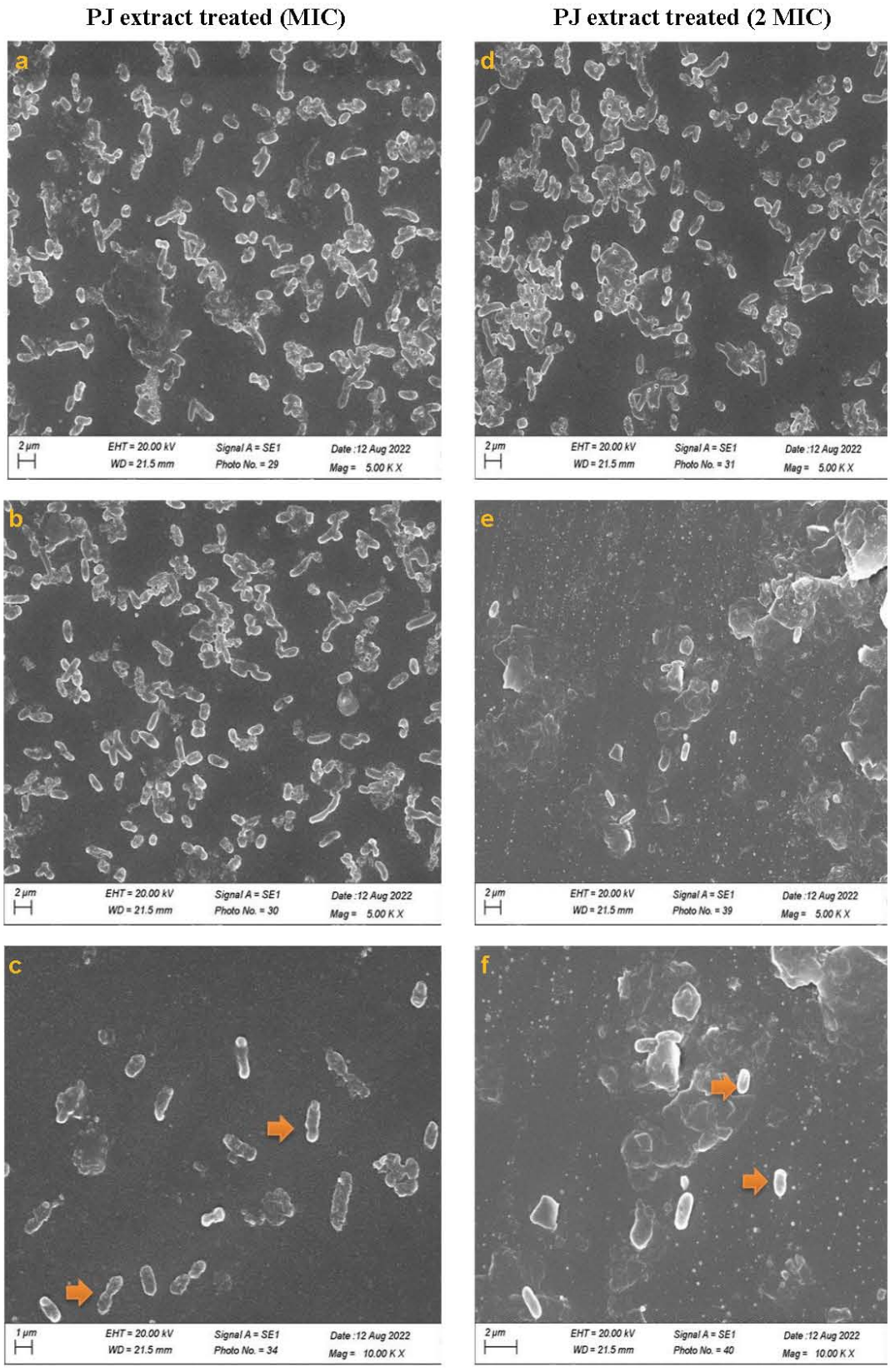


Fig. 32: Representative scanning electron microscopy (SEM) images showing the antibiofilm activity of *P. juliflora* leaves (PJ) extract . Bacterial cells (S21 isolates) treated with lower concentration (MIC) of extract showing reduced clumping of bacterial cells (a-c) with rough surface (c). Higher concentration (2MIC) also inhibited bacterial cluster formation (d,e) with invagination and protrusion on cell surface (f).

wrinkled surface of the exposed bacterial cells (Fig. 31f). Treatment with PJ extract at lower dose (MIC) reduced the cell density and clumping (Fig. 32a & b). There was change in the surface morphology of the exposed cells characterized by twisted, rough and wrinkled cell surface along with invagination and protrusion on the surface (Fig. 32c). Exposure to higher concentration of PJ extract (2 MIC) also exhibited anti-biofilm activity characterized by loosening of the cell density and clumping (Fig. 32 d & e) along with change in surface morphology (Fig. 32f).

Discussion

CHAPTER -5

DISCUSSION

Endometritis in farm animals cause great economic loss to the farmers due to its contribution in reducing reproductive efficiency, fertility and milk yield in high yielding dairy cows and buffaloes as well as in other species of animals (Fourichon et al., 2000; LeBlanc et al., 2002; Gilbert et al., 2005; Chapwanya et al., 2009). Endometritis is a polymicrobial disease involving mixed infection of microorganisms and bacteriological contamination of uterus usually seen after parturition and during metabolic changes at transition period. Inflammation of endometrium due to microbial infection causes histopathological changes in the uterine tissues resulting in abnormalities in uterine involution, ovulation and embryonic survival (Sheldon et al., 2006). Incidence of clinical endometritis in cattle ranges from 18 to 37 % (Drillich et al., 2002, 2005) whereas subclinical endometritis ranges from 12 to 94 % (Raab, 2004; Kasimanickam et al., 2004; Gilbert et al., 2005; Barlund et al., 2008). Most common pathogens involved in endometritis are *Escherichia coli, Staphylococcus aureus, Aracanobacterium pyogens* and *Fusobacterium necrophorum* (Sheldon et al., 2002; Foldi et al., 2006; Donofrio et al., 2007; Sheldon et al., 2008).

Intra-uterine antimicrobials *viz.* oxytetracycline, penicillins, aminoglycocides, quinolones and cephalosporins are commonly employed for treatment of endometritis in veterinary clinical practices. However, these therapeutic agents often leads to inconsistent results in terms of success, recovery rate, high cost of treatment, residual issues in animal-derived foods and emergence of microbial resistance (Drillich et al., 2005; Santos et al., 2010). Indiscriminate use of antibiotics results in emergence of multi-drug resistant bacterial strains; therefore, identification of alternative therapy is gaining importance in human and veterinary medicine. Phenolic compounds and flavonoids present in different medicinal and aromatic plants have been reported in scientific literature to possess great antioxidant and antimicrobial potential (Gyawali and Ibrahim, 2014; Guil-Guerrero et al., 2016; Bhuyan and Basu, 2017).

Prosopis juliflora belongs to the family Leguminosae (Fabaceae), sub-family Mimosoideae. It has 44 different species of which 40 are native to the Americas, three to Asia and one to Africa (Burkart, 1976, Pasiecznik et al., 2004). These species are

having the several properties such as soil binders, sand stabilizers, as well as its ability to grow in the poorest soils (Michel-López et al., 2013). *P. juliflora* has been used as a traditional treatment for catarrh, cold, diarrhea, dysentery, flu, hoarseness, inflammation, measles, sore throat and hepatic and ocular problems (Mazzuca et al., 2003; Singh et al., 2011). Wounds can also be treated with the decoction obtained from leaf and seed extracts (Tene et al., 2007; Singh et al., 2011). The syrup, made from ground pods of *P. juliflora*, is used traditionally for nourishment of underweight kids or those suffering from retardation in motor development and the syrup are reported to enhance lactation (Singh et al., 2011). Tea prepared from *P. juliflora* is believed to be useful in healing of digestive disorders and skin wounds (Singh et al., 2011). Several alkaloids including juliflorine, julifloricine, julifloridine, juliprosine, juliprosinene, juliflorinine, 3' oxojuliprosopine, sceojuliprosopinol, 3-oxojuliprosine and 3'- oxo-juliprosine have been isolated from various parts of *P. juliflora* and have shown to possess several promising pharmacological activities (Singh et al., 2011).

Though accumulating evidences suggested the antimicrobial activity of *P. juliflora* leaves, however, most of the studies are related to their action against food borne pathogens with little or no information against pathogens of animal origin. Further, the exact mechanism of action of *P. juliflora* leaves is yet to be explored. Thus, in the present study an attempt was made to elucidate the *in vitro* antibacterial efficacy of *P. juliflora* leaves extract against clinical isolates of *E. coli* of uterine origin as well as to unravel its possible mechanism of action.

Major findings of the present study were i) based on the morphological, biochemical and genotypic characteristics, two isolates were identified as virulent *E. coli* (S4 and S21) from 42 clinical samples collected from cows and buffaloes having uterine infection, ii) the phytochemical analysis revealed presence of large quantity of phenolic acid and flavonoids in the ethanolic extract of *P. juliflora* leaves (PJ) iii) the PJ extract showed promising *in vitro* antibacterial activity against clinical isolate of *E. coli* (S4) with minimum inhibitory concentration of 0.39 mg/ml iv) Significant antibacterial action of PJ extract was initiated at 6 h post-exposure while complete bactericidal action was achieved at 12 h post-exposure, iv) PJ extract possibly disrupt the cell membrane integrity and caused cell wall damage besides attenuating the RNA expression of efflux pump (acrA and acrB) to produce its bactericidal action, v) PJ

extract also exhibited promising anti-biofilm action against S21 isolate as evidenced by biochemical assay and *in situ* visualization of bacterial biofilm.

The postpartum environment of the uterine lumen supports the growth of variety of aerobic and anaerobic bacteria. *Escherichia coli* and *Arcanobacterium pyogenes* are the most prevalent bacteria isolated from the uterine lumen of cattle with uterine disease, followed by a range of anaerobic bacteria such as *Prevotella* species, *Fusobacterium necrophorum* and *Fusobacterium nucleatum* (Sheldon et al., 2002; Williams et al., 2005). In an earlier report from our laboratory, we have observed that *E.coli* and *Stahylococcus aureus* are the most predominant bacterial pathogens causing uterine infections in cattle and buffaloes (unpublished data). In the present study, out of 42 clinical samples 22 samples were found to be positive for *E. coli* based on the cultural, morphological and biochemical characteristics. However, for further study we selected two isolates (S4 and S21) based on the presence of virulence genes (*Pep1*, *csgA* and *csgD*).

P. juliflora possesses several compounds including alkaloid, tannin, phenolics, steroids, terpenes, flavonoid, proteins, sugars, and fatty acids and many of them are reported to exhibit antibacterial activity (Marangoni and Alli, 1988; Singh, 2012; Prabha et al., 2014). For example, juliprosinene and juliflorinine isolated from *P. juliflora* exhibit antibacterial action against *E. coli, S. aureus, Klebsiella pneumoniae* and *Shigella sonnei* (Prabha et al., 2014). In the present study too, we have observed a promising antibacterial activity of ethanolic extract of *P. juliflora* leaves (PJ) extract against clinical isolates of *E. coli* (S4 and S21) as well as reference strain (ATCC 25922) as evidenced by higher zone of inhibition at 20 mg/ml concentration in agar well diffusion test. Increasing the concentration of the extract from 20 mg/ml to 25 mg/ml, however, did not produce any significant increase in the zone of inhibition against both these isolates. Given that, ethanolic extract of *P. juliflora* leaves showed comparatively better antibacterial action (in terms of zone of inhibition) in comparison to methanolic and hydro-alcoholic extracts, and looking to the safety and stability of the extract we used ethanolic extract in the subsequent experiments to assess its mechanism of action. In consistent to our observation, Singh and co-workers (2011) also reported the antibacterial activity of ethanolic extract of the leaves, pods and fowers of *P. julifora* against different bacterial strains. The zone of inhibition of

ethanolic leaves extract (100 mg/ml) against *E. coli* and *S. aureus* were reported to be 12.81 ± 0.45 mm and 12.72 ± 0.67 mm, respectively. Recently, Saleh and Abu‑Dieyeh (2021) have also documented the antibacterial potential of *P. juliflora* leaves extract against food spoiling isolates of *E. coli* and *S. aureus* at 50 mg/ml concentration. The aqueous extract of *P. julifora* leaves was also reported to exhibit antibacterial action against *Enterococcus faecalis, Staphylococcus aureus, Prevotella intermedia, Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans* (Osuru et al., 2011). In addition, combined effect of aqueous extract of *P. juliflora* leaves with silver nano-particles encapsulated in chitosan showed higher antibacterial activity against *E. coli* possibly due to the combined antimicrobial effect of chitosan and the synthesized nanoparticles (Malini et al., 2020). Again, zinc monoxide nanoparticles derived from aqueous extract of *P. julifora* leaves (100 µg/ml) was also reported to show promising antibacterial action against *E. coli* and *B. subtilis* (Mydeen et al., 2020). Thus, further studies are warranted to evaluate these modified nanoformulations of PJ extract against clinical isolates of uterine origin. Moreover, their possible health risk and environmental safety are also to be assessed before recommending them for clinical use. Interestingly, the MIC value of PJ extract against S4 isolate was calculated to be 0.39 mg/ml whereas for S21 isolate the MIC value was found to be comparatively higher (1.56 mg/ml). Cefotaxime was also exhibited higher MIC (32 µg/ml) against S21 isolate as compared to that against S4 isolate (0.04 µg/ml). This observed difference in MIC values between these two isolates was attributed to the higher virulence of S21 isolate as evidenced by its ability to produce strong biofilm. Further, the antibacterial action of PJ extract against S4 isolate was initiated at 6 h post-exposure while the complete bactericidal action was observed at 12 h post-exposure. These observations evidently suggest that the antibacterial action of PJ extract enhanced with increase in exposure time. The alkaloid fractions of *P. juliflora* leaves extract are considered to have maximum antibacterial effect among other parts of the plant and the MIC values against most common *Gram-positive* and *Gram-negative* bacteria ranges between 25 µg/ml to 100 µg/ml (Ukande et al., 2019).

Plant-derived active principles are thought to have promising antibacterial action in comparison to conventional antimicrobial agents or pure compounds due to the notion that the multicomponent mixture of the plant extracts can act on different

target sites (Yap et al., 2014). For example, p-cymene and γ-terpinene is reported to disrupt cell membrane (Ultee et al., 2002; Oyedemi et al., 2009) while thymol can target both cell membrane as well as intracellular citrate metabolic pathway to produce antibacterial action (Trombetta et al., 2005; Di pasqua et al., 2007; Di pasqua et al., 2010). Moreover, crude extracts are often proved to be safer for animal and human health as well as for the environment as compared to the concentrated phytochemicals which might exhibit toxicity (Lima et al., 2017). In the present study too, we have observed substantially higher quantity of total phenolic acid (120.68 ± 1.36 mg GAE/g extract) and flavonoids (148.48 ± 3.27 mg QE/g extract) in the PJ extract. Moreover, phytochemical analysis by GC-MS also revealed the presence of several phytoconstituents that have promising antibacterial and anti-inflammatory potentials. *P. juliflora* leaves extract also exhibited promising *in vitro* anti-oxidant and radical scavenging activity which also confers to its antimicrobial action. This radical scavenging activity of the extract was attributed to its large quantity of total phenolic content as phenolic compounds are known to scavenge active oxygen species and free radicals such as singlet oxygen, superoxide anion, and hydroxyl radicals (Mahesh and Jayakumaran, 2010). In agreement to our findings, earlier report also documented the presence of distinct classes of secondary metabolites *viz.* tannins, phenolics, flavonoids, alkaloids, terpenes and steroids in different parts of *P. juliflora* extract (Singh, 2012). In another study the ethanolic leaves extract revealed the presence of tannins, phenolic acids, glycosides, flavonoids and alkaloids (Sathiya and Muthuchelian, 2008). Moreover, some compounds belonging to phytosterol (β-sitosterol), terpenoid diketone (prosopidione) and alkaloid bioactive molecules (secojuliprosopinal, 3'oxojuliprosopine and juliprosopine) were also identified from this plant (Lakshmi et al., 2010). Similarly, DART-MS analysis showed that *P. juliflora* leaves are a rich source of piperidine alkaloid and contain two diverse groups of alkaloids, one with an indolizidine ring in the centre of the molecule (*viz.,* juliprosopine, juliprosine and juliprosinine) and other without indolizidine ring (*viz.,* julifloridine, projuline and prosafrinine) (Singh et al., 2011). Thus, further studies are warranted to isolate the active principles of *P. juliflora* leaves extract and to assess their biological activity.

To further elucidate the mechanism of action of the test extract against S4 isolate, the cellular morphology of PJ extract-treated cells were examined. As evident

from the fluorescent microscopy and transmission electron microscopy (TEM), PJ extract mainly disrupted the cell wall and/or cell membrane of the bacterial isolate to produce its bactericidal action. Further, it also altered the cytoplasmic contents and its distribution during bacterial division. This membrane permeabilizing action of the test extract observed in the present study possibly due to the presence of bioactive compounds in the crude extract which can alkylate proteins leading to alteration in the protein conformation and cell membrane fluidity (Pelczar et al., 1988; Wink, 2008).

The disruption of cell wall and cell membrane and change in cellular permeability following exposure to PJ extract may lead to release of intracellular contents outside the cell resulting in lysis of bacteria. Thus, it may not be unreasonable to infer that PJ extract may facilitate the entry of conventional antimicrobials and increase their efficacy by altering the membrane permeability of exposed bacterial populations. Discontinuation of cell membrane leading to loss of potassium ions may result in inhibition of respiration and promote the uptake of propidium iodide (Price-Whelan et al., 2013) which was evident from the fluorescent microscopic examination in the present study. Appearance of intracytoplasmic vacuole formation following exposure to PJ extract observed in the present study was attributed to alteration in the cytoskeletal matrix. Additionally, PJ extract also caused change in the shape and size of exposed bacterial population.

Synergistic effect of various plant-derived active principles and conventional antimicrobials are of great importance in revealing new strategies to combat antimicrobial resistance. It has been reported that synergistic effect can enable to lower dosage of one or both agents and/or resensitize the bacterial strains (Rosato et al., 2007; Langeveld et al., 2014; Aleksic et al., 2014). Interestingly, the FIC value of the test extract for cefotaxime was found to be 3.0 which suggest that combination of PJ extract with cefotaxime produced indifferent or no antagonist effect. This is probably due to the similar mechanism of action (damage to cell membrane/cell wall) of these two agents. Additionally, TEM study has revealed that combination of cefotaxime and PJ extract did not produce any significant increase in the alteration in the cellular architecture although did not even reduce the antibacterial action of the individual agent. In agreement to this, Alipiah and coworkers (2015) have also

reported indifferent relation following combination of sea cucumber extract (SBE) with penicillin based on the FIC index.

Gram-negative bacteria have evolved a distinct permeability barrier to amphipathic compound due to the presence of outer membrane and expression of efflux pumps. Medicinal plants with antimicrobial properties are the potential source of novel and effective efflux pump (EP) inhibitors (Newman and Cragg, 2012). To attain low sensitivity against the bioactive compounds, bacteria reduce the membrane permeability by attenuating the number of porin channels and/or inducing the efflux pumps for outward transport of drug molecules often in a non-specific manner making the bacterial cells resistant to multiple antimicrobials (Masi et al., 2017). Identifying novel and potential EP inhibitors to revert the bacterial resistance is therefore gaining importance now-a-days. Thus to investigate the role of PJ extract in regulating the expression of efflux pump in bacteria, we have evaluated the mRNA expression of certain important efflux pumps following exposure to plant extract. Among the major five families, resistance-nodulation cell-division family (RND) of efflux pump is the well characterized in *E. coli* and is made up of three components *viz.* an inner membrane transporter (acrB), an outer membrane protein channel (tolC) and a periplasmic adaptor protein (acrA) (Du et al., 2014). This RND pumps allows transport of compounds across the membranesincluding β-lactams, tetracycline, tigecycline, chloramphenicol, trimethoprim, sulfamethoxazole and norfloxacin (Srikumar et al., 1997; Poole, 2000). In the present study, a significant decrease in the mRNA expressions of acrA and acrB were observed in S4 clinical isolate following 12 h exposure to PJ extract (@ 2 MIC). A significant decrease in acrA transcript was also observed at 6 h post-exposure of PJ extract (@ 2 MIC). Surprisingly, we did not observe any significant change in mRNA expression of either acrA or acrB when the concentration of the extract was increased to 4 MIC with 6 h exposure time. This evidently suggests that exposure time of the plant extract, rather than concentration, is the critical factor to dampen the expression of efflux pump in the exposed bacteria. Given that, acrA and acrB are having substrate specificity to fluroquinolones and β-lactams, thus suggesting a wide spectrum of efflux pump inhibiting potential of the test extract. However, further study is warranted to assess the effect of PJ extract on the protein expression and the activity of these clinically important efflux pumps. This efflux pump inhibitory action of the test extract is attributed to the presence of certain

bioactive components in the crude extract. For example, quercetin was reported to be a substrate for acrB as deletion of acrB from *E. coli* resulted in 8 fold reduction in MIC of quercetin (Al-Karablieh et al., 2009). Similarly *in silico* prediction and bioassay postulated that plumbagin, norhydroguaretic acid, shikonin and mangiferin also have potential to bind and inhibit acrB in bacteria (Ohene-Agyei et al., 2014). However, we did not find any significant alteration in mRNA expression of outer membrane protein related genes (ompC and ompF). This finding evidently suggest that outer membrane protein may not be a potential targets for PJ extract in *E. coli.*

Biofilms are considered as a virulence factor responsible for the long persistence of bacteria in invaginating organs (Costerton et al., 1999; Aminov, 2011) and they also protect the microorganism from host immune response and antimicrobial therapy (Tabibian et al., 2008). The development of biofilm begins with the initial adhesion step with the aid of exopolysaccharide (EPS), which upon maturation, forms a typical shape (Hall-Stoodley et al., 2004). On the other hand, quorum sensing (QS) is the another chief action involving in the biofilm formation, where, microbial cells can communicate to each other through signaling molecules and has been extensively studied in bacteria for controlling the biofilms (Rutherford and Bassler, 2012). Currently, natural products and plant-derived secondary metabolites are emerged as therapeutic agents against the bacterial biofilms (Adnan et al., 2018). Therefore, we evaluated the anti-biofilm activity of *P. juliflora* leaves extract against clinical isolate of *E. coli.* Though biofilm production was recorded by both the clinical isolates (S4 and S21) in the present study, quantification of biofilm production by crystal violet assay revealed the comparatively higher adherence for S21 isolate. Thus, the anti-biofilm activity of the plant extract was assessed using S21 isolate in the present study. *P. juliflora* leaves extract exhibited promising anti-biofilm action (54.55 % inhibition) against clinical isolate of *E. coli* at its MIC in a concentration dependent manner. This was supported by SEM analysis which further revealed that PJ extract decreased the multilayer growth of biofilms and free living cells by influencing the integrity of cell wall. Additionally, it was also observed that the disturbed cell wall of the bacterium led to failure in the emergence of cluster and incapable of maintaining their typical morphology in presence of the extract. This anti-biofilm action of PJ extract was attributed to the presence of flavonoids and phenolic acids as quercetin is reported to possess potent antibiofilm action against *E.*

coli, S. aureus, S. typhimurium etc. (Murakami et al., 2008). In a similar study, natural compounds like eugenol, thymol and carvacol was reported to inhibit biofilm formations by *S. typhimurium* by more than 50 % (Miladi et al., 2017). Nevertheless, our findings provide substantial evidences of anti-biofilm activity of PJ extract which have the promosing potential in designing novel therapeutic strategies against uterine infection. This pharmacological property of PJ extract may help to enhance the immunological defense of infected hosts against bacterial cell populations, especially those in biofilms, and subsequent host clearance and reduction of disease symptoms (Sathiya et al., 2018).

In conclusion, *E. coli* bacterial population are the one of the major pathogens causing uterine infections in large animals. *P. Juliflora* leaves extract exhibited promising *in vitro* antibacterial activity against the clinical isolate of *E. coli* and its bactericidal action was mediated by disruption of the cell membrane and cell wall integrity leading to formation of intra-cytoplasmic vacuole and release of cytoplasmic contents. In addition, it also produced significant down-regulation of efflux pump genes (especially acrA and acrB) of the exposed bacteria and thereby has the potential to enhance the entry of the related antimicrobial agents. Further, it also exerted promising anti-biofilm activity by altering the bacterial surface morphology characterized by rough, wrinkled and invaginated surface and thereby reducing bacterial nidus or clumping. Taken together, *P. juliflora* leaves extract have potential to use as an alternative to conventional antimicrobials against uterine infection, however, the *in vivo* efficacy of this extract against uterine disorders needs to be evaluated in future.

Summary and Conclusions

CHAPTER-6

SUMMARY AND CONCLUSIONS

Present study was aimed to isolate and identify virulent *E. coli* from cows and buffaloes with a clinical history of uterine infection. Further, an attempt was also made to unravel the mechanism of antibacterial action of *Prosopis juliflora* leaves (PJ) extract against the clinical isolates of *E. coli*. This study was performed in two phases where in phase-I, isolation and identification of clinical isolates of *E. coli* was performed while in phase-II, *in vitro* efficacy of PJ extract against the clinical isolates was assessed.

6.1 Isolation and identification of *E. coli* from clinical samples

i) Uterine discharges were collected from 23 cows and 19 buffaloes that were presented to TVCC, DUVASU, Mathura during September 2021-December 2021 with a clinical history of uterine infections. Out of these 42 samples, 23 isolates showed positive for cultural and biochemical characteristics for *E. coli.* However, based on the presence of virulence genes (*Pep1*, *csgA* and *csgD*), only two isolates (S4 and S21) were selected for further study.

6.2 Phytochemical analysis of *Prosopis juliflora* leaves (PJ) extract

i) The hot ethanolic extract of dried leaves of *P. juliflora* was prepared and the yield was calculated to be 41.42 %. The dried extract was reconstituted in ethanol and subjected to gas chromatography-mass spectrophotometry (GC-MS) analysis. The phytochemical analysis revealed the presence of 22 major phytoconstituents which are reported to have antibacterial, anti-inflammatory and anti-oxidant properties. Further biochemical analysis revealed presence of large quantity of phenolic acids (120.68 ± 1.36 mg GAE/g extract) and flavoonoids (148.48 ± 3.27 mg QE/g extract) in PJ extract. The PJ extract also exhibited appreciable *in vitro* anti-oxidant property as evidenced by its promising DPPH free radical scavenging activity and ferrous ion chelating ability.

6.3 *In vitro* antibacterial activity of *Prosopis juliflora* leaves (PJ) against clinical isolates of *E. coli*

i) The *in vitro* antibacterial efficacy of PJ extract was initially evaluated against reference strain of *E. coli* (ATCC 25922) and it was found that the ethanolic extract produced comparatively greater zone of inhibition as compared to methanolic and/or hydro-alcoholic extract. Thus, in the future experiments, ethanolic extract were used to evaluate its efficacy against clinical isolates of *E. coli* (S4 and S21). Perusal of the data revealed that ethanolic extract when dissolved in ethanol showed appreciable zone of inhibition at both the concentrations (20 mg/ml and 25 mg/ml) of the plant extract against both S4 (13.78 ± 0.55 mm and 15.11 ± 0.61 mm, n=7-9, respectively) and S21 (12.50 ± 0.50 mm and 12.75 ± 0.48 mm, n=7-9, respectively) isolates.

ii) The MIC values of PJ extracts against reference strain (ATCC 25922) as well as clinical isolates of S4 and S21 were calculated to be 0.39 mg/ml, 0.39 mg/ml and 1.56 mg/ml, respectively. The MIC values of cefotaxime against these isolates were 0.04 µg/ml, 0.04 µg/ml and 32 µg/ml, respectively. The fractional inhibitory concentration (FIC) of cefotaxime for PJ extract was found to be 3.0 thus, indicating indifferent or no antagonistic effect.

iii) To assess the effect of PJ extract on growth kinetics of S4 clinical isolates, time kill assay was performed and live bacterial counts was determined following 0, 2, 4, 6, 8, 12, 16, 18 and 24 h post-exposure of PJ extract. Significant antimicrobial action of PJ extract against S4 isolate was exhibited at 6 h post-exposure while complete bactericidal action was observed at 12 h post-exposure. Cefotaxime produced its bactericidal effect at 6 h post-exposure.

iv) In order to elucidate the mechanism of antibacterial action, the cell membrane integrity of the PJ extract-treated bacterial cells were determined by fluorescence microscopy. Following 12 h exposure to PJ extract, marked loss of bacterial cell membrane integrity was observed as most of the bacterial cells (both S4 clinical isolate and ATCC 25922) were found to be positive for propidium iodide dye indicating the dead cells with loss of membrane integrity

as compared to the untreated control group which was found to be positive for CFDA dye that emitted green fluorescence suggesting the live bacteria. Cefotaxime was used as positive reference antimicrobial agent.

v) To further confirm the mechanism of action of PJ extract against clinical isolate of *E. coli*, the cellular ultra-structure of bacterial cells was evaluated by transmission electron microscopy (TEM) following exposure to PJ extract and/or cefotaxime. The untreated bacteria showed normal bacterial structure with intact cell wall, cell membrane with uniform distribution of cytoplasmic contents. In addition, the intact cell membrane and uniform distribution of cytoplasmic contents was also observed in these cells during binary fusion. Treatment of bacterial cells with cefotaxime (positive control) produced abnormal bacterial morphology with disrupted cell wall and loss of integrity of cell membrane. The cell wall was also found to be damaged during binary fusion with non-uniform distribution of cytoplasmic contents. Similarly, exposure to *P. juliflora* leaves extracts at lower concentration (2 MIC) caused change in bacterial morphology characterized by disrupted cell wall and loss of integrity of cell membrane. There was clumping of cytoplasmic contents and loos of cell wall integrity during binary fusion with vacuolization of cytoplasmic contents. Exposure to relatively higher concentration of plant extract (4 MIC) produced more detrimental effect on bacterial cells as evidenced by intensely disrupted cell wall and loss of integrity of cell membrane. Detachment of cell membrane with misshapen cells along with condensation and aggregation of chromatin materials were observed. Following co-exposure to cefotaxime and PJ extract, no additive effect was observed, however, this combination did not produce any antagonist effect on the antibacterial action of the individual agent.

vi) Exposure to PJ extract at lower concentration (2 MIC) significantly ($p<0.05$) down-regulated the mRNA expression of acrA gene in S4 clinical isolate following 6 h (0.46 ± 0.16, n=5) and 12 h (0.35 ± 0.11, n=5) post exposure as compared to untreated control (1.00 ± 0.06, n=5). Interestingly, 6 h exposure to higher concentration (4 MIC) of PJ extract although produced 39 % (0.61 ±

0.05 *vs.* 1.00 ± 0.06, n=5) decrease in mRNA expression of acrA gene, this changes did not reach to significant level as compared to untreated control.

vii) Unlike acrA, down-expression of acrB gene was observed in S4 bacterial isolate only after 12 h post-exposure to lower concentration of PJ extract (0.56 ± 0.09, n=5) as compared to untreated control (1.03 ± 0.12, n=5). There was only 33 % (0.69 ± 0.09 *vs.* 1.03 ± 0.12, n=5) and 27 % (0.75 ± 0.15 *vs.*1.03 ± 0.12, n=5) decrease in mRNA expression of acrB gene following 6 h post exposure to PJ extract at lower (2 MIC) and higher (4 MIC) concentration, respectively.

viii) Exposure to PJ extract either at lower concentration (2 MIC) or higher concentration (4 MIC) did not produce any significant change in the mRNA expression of OmpC as well as OmpF genes in S4 clinical isolate.

ix) The congo red assay and crystal violet assay revealed the strong biofilm producing ability of S21 clinical isolate. The quantitative measurement of anti-biofilm activity by crystal violet microtitre method revealed that PJ extract (@ MIC) caused 54.55 % inhibition of biofilm production by S21 isolate in comparison to 90.91 % inhibition by chloramphenicol. Gentamicin exhibited only 31.82% inhibition in biofilm production by S21 isolate.

x) *In situ* visualization of anti-biofilm activity of PJ extract was performed by scanning electron microscope (SEM). The untreated bacterial culture (S21) under SEM microscopy appeared as clusters of cells indicating their ability to form biofilm. The surface morphology of these bacteria appeared smooth with regular shape. Exposure to chloramphenicol caused distortion of the cluster along with appearance of rough and wrinkled surface of the exposed bacterial cells. Treatment with PJ extract at lower dose (MIC) reduced the cell density and clumping. There was change in the surface morphology of the exposed cells characterized by twisted, rough and wrinkled cell surface along with invagination and protrusion on the surface. Exposure to higher concentration of PJ extract (2 MIC) also exhibited anti-biofilm activity characterized by loosening of the cell density and clumping along with change in surface morphology.

CONCLUSIONS

In conclusion, *E. coli* bacterial population are the one of the major pathogens causing uterine infections in cows and buffaloes. *P. Juliflora* leaves extract exhibited promising *in vitro* antibacterial activity against the clinical isolates of *E. coli* and its bactericidal action was mediated by disruption of the cell membrane and cell wall integrity leading to formation of intra-cytoplasmic vacuole and release of cytoplasmic contents. In addition, it also produced significant down-regulation of efflux pump genes (especially acrA and acrB) of the exposed bacteria and thereby has the potential to enhance the entry of the related antimicrobial agents. Further, it also exerted promising anti-biofilm activity by altering the bacterial surface morphology characterized by rough, wrinkled and invaginated surface and thereby reducing bacterial nidus or clumping. Taken together, *P. juliflora* leaves extract have potential to use as an alternative to conventional antimicrobials against uterine infection, however, the *in vivo* efficacy of this extract against uterine disorders needs to be evaluated in future.

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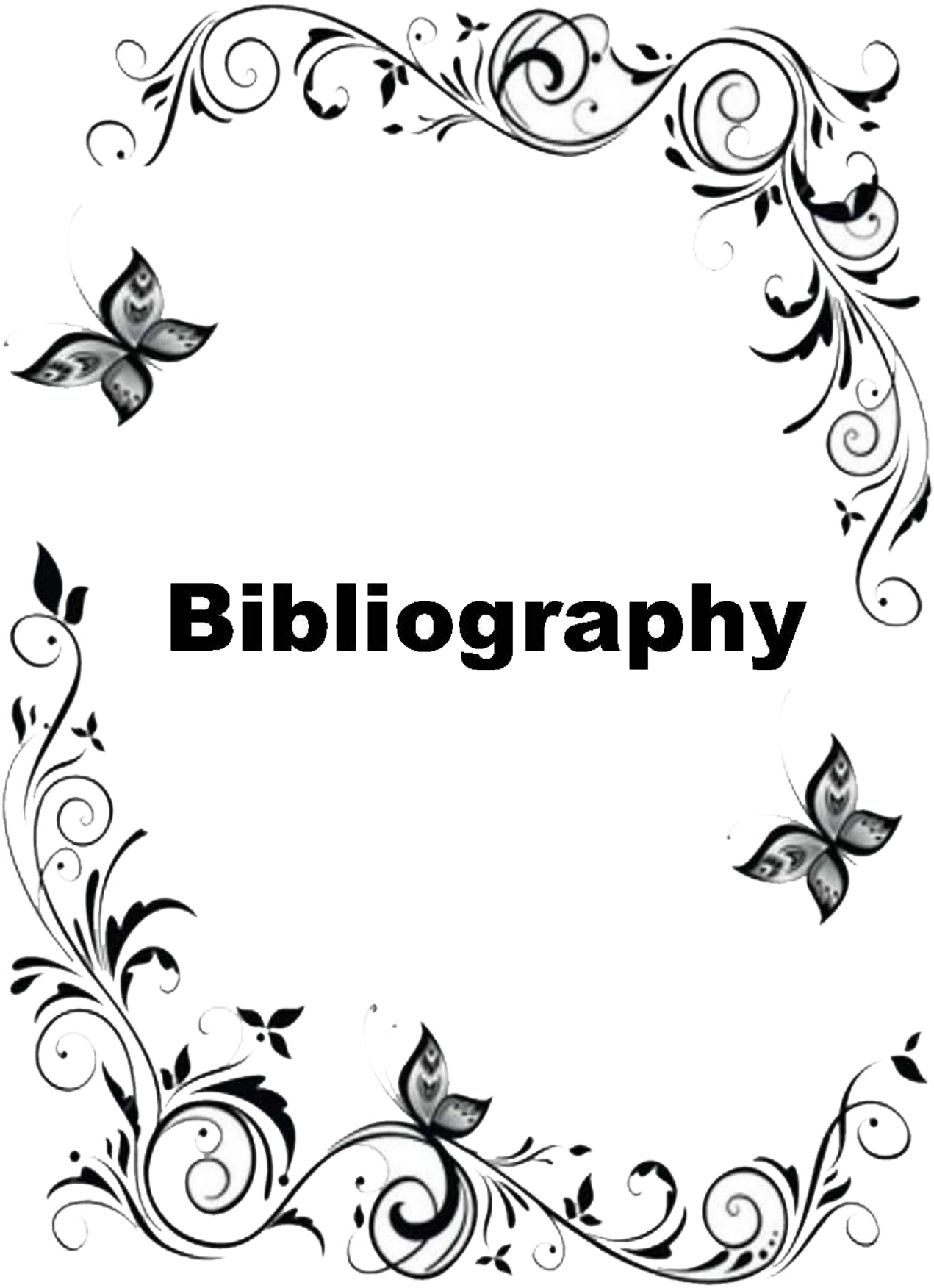

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Annexure-I

A. 10X TAE buffer (pH 8.2)

Constituents	Strength (g/l)
Tris Base	48.4
Glacial acetic acid	10.9
EDTA	2.92

B. Phosphate buffer saline (pH-7.4)

Constituents	Strength (mM)
NaCl	137 (8.0)
KCl	2.7 (0.2)
KH_2PO_4	1.4 (0.24)
Na_2HPO_4	4.3 (1.14)

The figures in parentheses indicate g/l of the constituents

C. Muller Hinton Broth

Constituents	Amount
Muller Hinton Broth	10.5 g
Distilled water	500 ml

Autoclave to sterilize the media before use

D. Muller Hinton Agar

Constituents	Amount
Muller Hinton Agar	19.0 g
Distilled water	500 ml

Autoclave to sterilize the media before use

E. Tryptic Soya Broth

Constituents	Amount
Tryptic Soya Broth Powder	15.0 g
Distilled water	500 ml

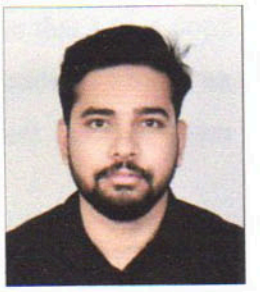
Autoclave to sterilize the media before use

F. Biofilm assay media

Constituents	Amount
Luria-Bertani broth	12.5 g
KH_2PO4	12.0 g
K_2HPO_4	28.0 g
$(NH_4)_2S0_4$	8.0 g
$MgSO_4.7H_2O$	0.25 g
D-Glucose	2.0 g
Casamino acid	5.0 g
Distilled water	1000 ml

Autoclave to sterilize the media before use

CV OF STUDENT



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Place: Mathura

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UNDERTAKING OF COPY RIGHT

I, **Dr. Rajneesh Singh,** Enrolment No.**V-2181/20** undertake that I give copy right to the DUVASU, Mathura of my thesis entitled **"Mechanistic Study on Antibacterial Efficacy of Babool Leaves Extract against *E coli* Isolated from Clinical Cases of Uterine Infections in Bovines''**

I also undertake that patent, if any, arising out of research work conducted during the programme shall be filed by me only with due permission of the competent authority of DUVASU, Mathura (UP).

Rajneesh Singh

Signature of Student