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In Vivo and In Vitro Investigation of the Antibacterial, Cytotoxic, and Immunological Effects of Aqueous Extract from *Ziziphus spina-christi* Leaves

Saif Mazeel Abed [1] ; Wafaa Ayad Al-Nuaimy [2,*] and **Sheereehan A.M. Albyati [3]**

[1]Department of Medical Laboratories, College of Health and Medical Technology, Sawa university, Al Muthana, Iraq

[2]Department of Biology, College of Science/Al-Muthana University/ Al Muthana, Iraq
Corresponding author email: wafaa.ayad@mu.edu.iq

[3]Department of Biology, College of Science/Al-Muthana University/ Al Muthana, Iraq

Abstract **Background:** The adoption of Ziziphus species medicines for the treatment of inflammatory illnesses is extensive and increasing, nevertheless, the impact of the Ziziphus genus on inflammatory illnesses has not been well studied. **Aim of study:-** study the Antibacterial, Cytotoxicity of *Ziziphus spina-christi* leaves extract of in vitro and Immunological effect *in vivo* , **Methods and material :** *Enterobacter cloacae* was obtained from patients from patient with Orthodontics **,** The ***Ziziphus spina-christi*** leaves were obtained from Iraqi market then dried for extraction and 100, 150, 200 mg/ml concentrations were prepared, the experimental design included two parts , in vitro and in vivo, *In virto* study which estimation the antibacterial activity by agar diffusion methods and Cytotoxicity using WRL-68 cell line with neutral red uptake method, in vivo study was included four groups Group I (Control): daily intraperitoneal injections of normal saline, Group II: injected Intraperitoneal with 100 µl of 1.5 x 10^8 CFU / ml of *E. cloacae*, Group III administrated orally with 200 mg/ml of extract , Group IV injected Intraperitoneal with 100 µl of 1.5x10^8 CFU/ ml of *E.cloacae* then for 14 day administrated orally with 200 mg/ml with extract then notice the clinical signs of each group evaluation the innate immunity (neutrophil activity) by Nitro-blue tetrazollum (NBT) method , Toll Like receptors-2(TLR-2) and Adaptive immunity (Arthus reaction Delayed hypersensitivity,IL-4,IL-17,IL-10). **The results**: The results showed that the extract revealed a good antibacterial activity against the studied isolates (*E. cloacae*) which that highest activity recorded in 200 mg/ml concentration , The cytotoxicity findings reported the IC50 value of *Ziziphus Rugosa* extract, which indicates the concentration at which 50% proliferation inhibition of WRL-68 cells occurs, was found to be

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280 mg/ml. At the greatest concentration of the extract (200 mg/ml), the cell viability was measured to be 80.08%. On the other hand, the lowest dosage tested (6.25 mg/ml) had no effect on the cells. Furthermore, the mice group that received *E.cloacae* presented clinical symptoms of illness with clear weight loss and diarrhea, reduction in movement activity through the cage space for 7 days while the mice groups that followed by extract administration presented clinical symptoms of illness for 3 days. On the other side the innate immunity findings that represented with the neutrophilsindex were showed the group that had a combination injection by *E.cloacae* and extract recorded high values(47.15 %) with valuable difference at ($p \leq 0.05$) comparison with other studied groups and findings of TLR-2 reported the mice that received only *E.cloacae* showed highest value (98.12±0.087) with a valuable difference at ($p \leq 0.05$). In additional the adaptive immunity that represented with the Arthus test and DHT were revealed the group that had a combination administration of extract and *E.cloacae* showed a higher value than the other group, after 48 hr but the value started decreasing after 72 hr but the highest value recorded (4.24± 0.00) after 24 hr with valuable difference at $p \leq 0.05$ with other groups. On the other side IL-17,IL-4 and IL-10 showed the groups that received extract with *E.cloacae* given a high value (424.95±0.46, 100±0.00, 72.18±0.12) respectively which higher than other groups with a significant difference at $p \leq 0.05$. **Conclusion**: the current study showed *Ziziphus spina-christi* leaves aqueous extract have positive effect to regulate the immune response by rising the IL-10 and IL-4 that played anti-inflammatory role also increase IL-17 which contributed to mobilization of neutrophil.

Keywords: aqueous, *Ziziphus spina-christi* , extract, antibacterial, cytotoxic , immunity

Introduction

Plants have the potential to serve as a solution to address the absence of new antibacteria and the increasing problem of resistance to antibiotics. Plants own a variety of efficient mechanisms of defense, such as secondary metabolites synthesis to fight against diseases and pests before they can inflict significant harm. Plants and other species have engaged in co-evolution for over 350 million years (Clarke *et al*., 2011), adapting techniques to overcome each other protection mechanisms. Plant secondary metabolites serve multiple functions, including aiding the plant in adapting to non-living environmental factors such as UV radiation, as well as facilitating interactions with neighboring plants, pathogens,herbivores, pollinators, and fruit dispersers. Consequently, these metabolites play a key part in the growth and development of plants. (Kessler and Kalske, 2018).According to scientific literature, the

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Ziziphus genus primarily utilizes six species, namely *Z.jujuba , Z. xylopyrus, Z.mauritiana, Z.nummularia, Zizyphus oxyphylla, and Z. mauritiana* (El Maaiden *et al*., 2020). The genus above has a crucial role in medicine for the treatment and management of a wide range of disorders, including antipyretic, antibacterial, analgesic, ,sedation, antioxidant, GIT protecting, anti-diabetes, cardiovascular, anti-inflammatory and antifungal properties. Compounds such as cyclopeptide alkaloids, flavonoids, polysaccharides, terpenoids, and saponins have been extracted from this species. The utilization of Ziziphus medicines for the treatment of inflammatory illnesses is extensive and increasing. Nevertheless, the impact of genus Ziziphus on inflammatory illnesses has not been well examined. Researchers have analyzed several Ziziphus species and recorded their medicinal and biological actions (Alsayari and Wahab,2021). The aim of study was to investigated the antibacterial and the cytotoxicity of the *Ziziphus spina-christi* extract in vitro and in vivo and its immunomodulation effect on the immune response.

Material and methods

Bacterial isolation

E. cloacae was obtained from patients with from the microbiology laboratory in College of Health and Medical Technology, Sawa University, Iraq which was isolated from patient with Orthodontics and identified by Vitek2 system (Olympus, Japan).

Table (1) Identification of *E. cloacae* by Vitek2 system

2	APPA	-	3	ADO	+	4	PyrA	-	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	-	18	dMAL	+	19	DMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	+	34	idTAG	-	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	+
40	ILATK	-	41	AGLU	-	42	SUCT	+	43	NAGA	+	44	AGAL	+	45	PHOS	-
45	GlyA	+	47	ODC	+	48	LDC	-	53	IHISa	-	56	CMT	-	57	BGUR	+
58	0129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Aqueous Extract preparation

The plants leaves were taken from Iraqi market then dried for extraction (100 g) of the crushed leaf particles submerged in 1 L distillate water in a conical flask and stirred while being heated for a duration of 4 hours , Whatman No.1 filter paper was used to separate the extract after it had cooled down. The filtrate was gathered and put into an ice cube tray to freeze. The cold ice cube was freeze-dried, or lyophilized, to get powdered, concentrated

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water-based extracts (Juvatkar *et al*., 2012). Then 100, 150, 200 mg/ml concentrations were prepared .

Antibacterial Activity Determination

Agar well diffusion method was used to determination antibacterial activity of plant extracts,the isolates were adjusted to 1.5×10^8 colony-forming units CFU/ml , and sub cultured on Mueller-Hinton agar had wells in diameter (6mm), these wells were filled by 0.1 ml of prepared concentrations(100 mg/ml, 150 mg/ml, 200mg/ml (Wiegand *et al*.,2008).

Cytotoxic Activity of Aqueous Extract

The cellular viability was assessed using the neutral red absorption method, as outlined by Repetto *et al*. (2008). The assay of neutral red uptake provides an accurate quantification of the number of viable cells in a culture. This procedure is dependent on the ability of live cells to selectively uptake neutral red in the lysosomes. The cells were subjected to varying concentrations of the test chemicals (125, 250, 500, and 1,000 µg/ml-1) for a duration about 48 hours. The cells were cultured in agle's minimum essential medium (EMEM) for 4 days at +37 °C and 5% CO2 humidified atmospher. at a density of 104 cells per well in a 96-well plate. The IC50 of the investigated extract was established by examining the correlation between the concentrations employed and the intensity value of neutral red.

The laboratory animals

A total of 20 male mice were obtained from Mustansiriyah University - Iraqi Center for Genetics and Cancer Research. The male albino mice (Blab-c), age was ranged between six to eight week, weight was 22±3 g and kept in bio-clean hoods at a temperature range of 20-25ºC, with alternating intervals of light and darkness lasting 14 and 10 hours, respectively.

Design of the study

The current experiment was included 20 animals (5 mice in each group) as following; Group I (Control): administered daily intraperitoneally with normal saline solution, Group II: injected Intraperitoneal with 100 µl of 1.5 x 108 CFU / ml of E. cloacae, Group III administrated orally with 200 mg/ml of extract for 14 days , Group IV injected Intraperitoneal with 100 µl of 1.5 x 108 CFU / ml of E.cloacae only once then for 14 days administrated orally with 200 mg/ml with extract, then notice the clinical signs of each group.

Note :-the study used 200 mg/ml of extract depending on the antibacterial and cytotoxic results

Blood and serum collection

After 14 days of treatment, the number of blood sample collected from the facial vein approximately 1.5 ml from each animal(20 samples),after collection the whole blood the

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serum obtained by centrifuging at 1,000–2,000 x g for 10 minutes in the serum was divided and stored at 4C .

Immunological assays

Nitro-blue tetrazollum (NBT) test for neutrophils activity:

A yellow water soluble nitro-blue tetrazolium (NBT) dye from (BDH Biochemical company, England) was used to determine the phagocytic activity of neutrophils. A modification of a method proposed by Park *et al*.(1968) was used. A solution of 0.2% of NBT was prepared. Then two equal volumes of NBT solution and phosphate buffer saline were mixed together in a test tube and the fresh blood was added in an equal volume to the NBT butter mixture and gently mixed. The resulting mixture was kept warm at 37° C for (30-40) minutes. Next a routine blood film was done, and stained with Leishman's stain. Two hundred neutrophils were counted, and the percentage of with a dark blue formazine deposite was determine Index and percentage of phagocytosis are explained by the following formulas(Park *et al*.,1968)

$$\text{Phagocytic percent} = \frac{\text{No. of phagocytic Neutrophils}}{\text{Total No. of Neutrophils}} \times 100$$

Arthus Reaction and Delayed Type Hypersensitivity

After the treatment of mice, on day five, 50 ul of *E.cloacae* was injected into the right foot pad of each mouse in the groups, while normal saline was used in the left foot pad. The arthus reaction was assessed by measuring the increase in footpad swelling after four hours, while the delayed type hypersensitivity peak occurred after twenty-four and forty-eight hours post-injection. The measurements were taken in millimeters using a digital vernia, as recommended by (Tansho *et al*, 2002).

Measurements of TLR-2 IL-17 , IL-10, and IL-4 Level

TLR-2,IL-17 , IL-10, and IL-4 levels in male of albino mice were measured by means of ELISA. Procedures were conducted in compliance with Elabscience's manufacturer's instructions which The assay employs a quantitative sandwich enzyme immunoassaytechnique. The capture antibody that specific TLR-2, IL-10 and IL-4 has been pre-coated intothe microplate. Standards or samples are added to the micro ELISA platewells and for TLR-2 combined with the specific antibody. After removing anyunbound substances, then a biotinylated detection antibody specific by the biotin-conjugated antibody specific for TLR-2, IL-10 and IL-4 was added into the wells. After washing, an avidin conjugated Horse Radish Peroxidase (HRP) was added to the wells and incubated. The following washing to remove the unbound avidin-enzyme reagent, the substrate solution is added to each well.

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Only those wells that contain Mouse TLR-2, IL-10 and IL-4 , biotinylated detection antibody, and Avidin-HRP conjugate will appear blue. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The color development is stopped and the intensity of the color is measured. The following steps were estimated according to the leaflet of the ManufactureCompany which was provided with each Kit.and utilizing the standard curves shown below in figure 1,2 and 3.

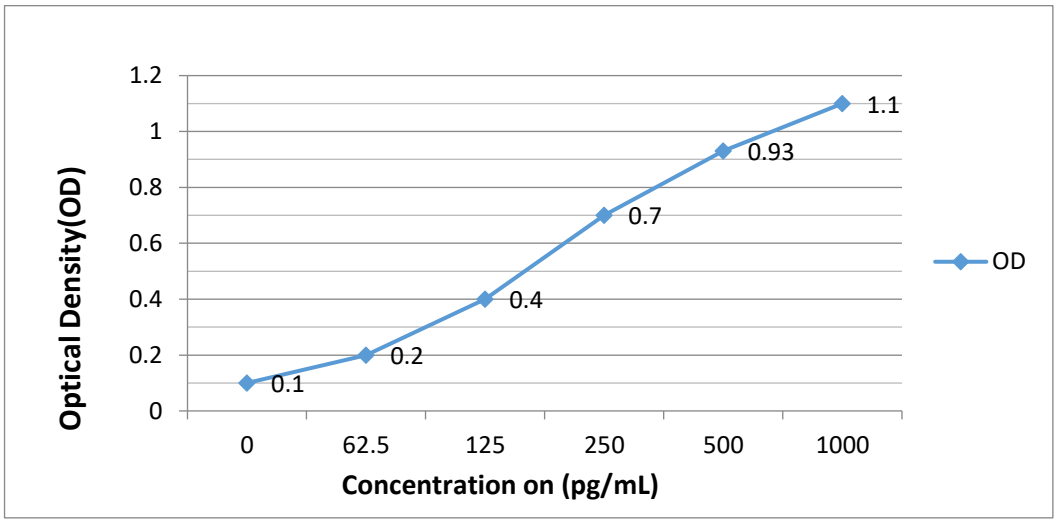

Figure (1): Standard curve of IL 17 and IL-4

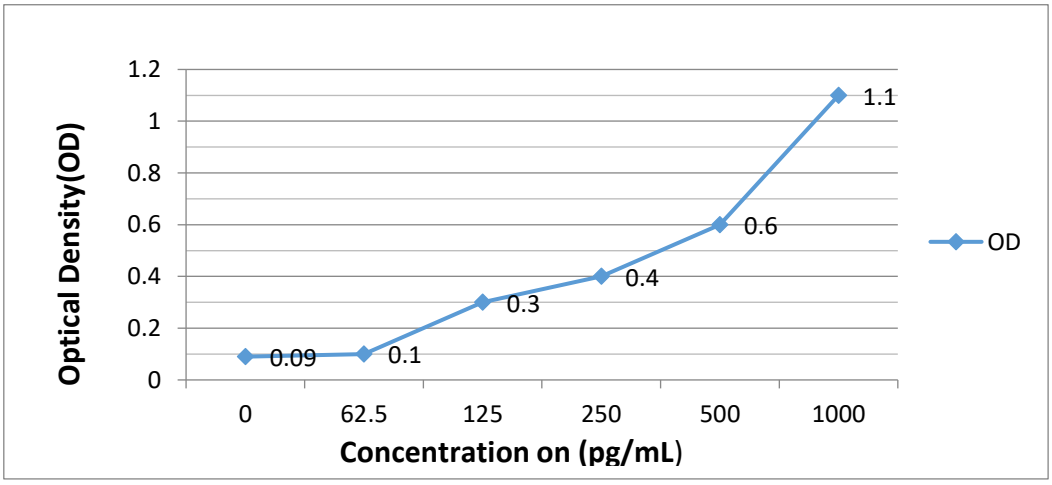

Figure (2): Standard curve of of IL 10

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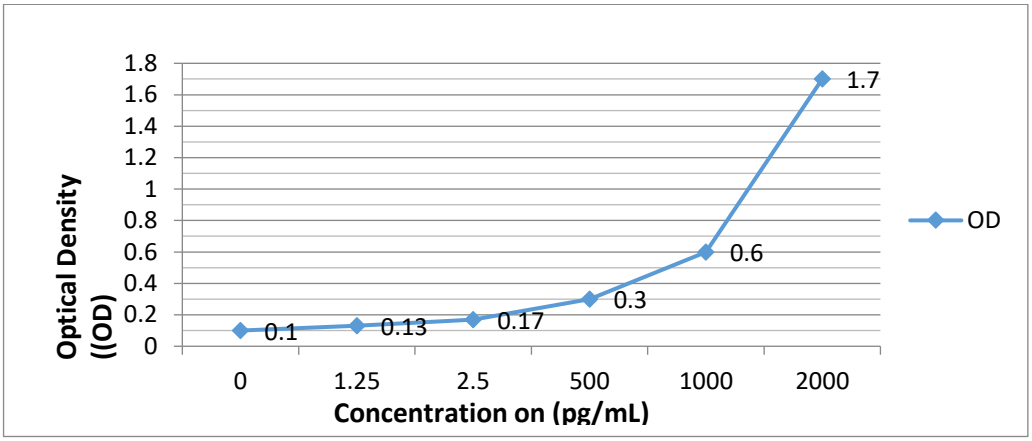


Figure (3): Standard curve of TLR-2

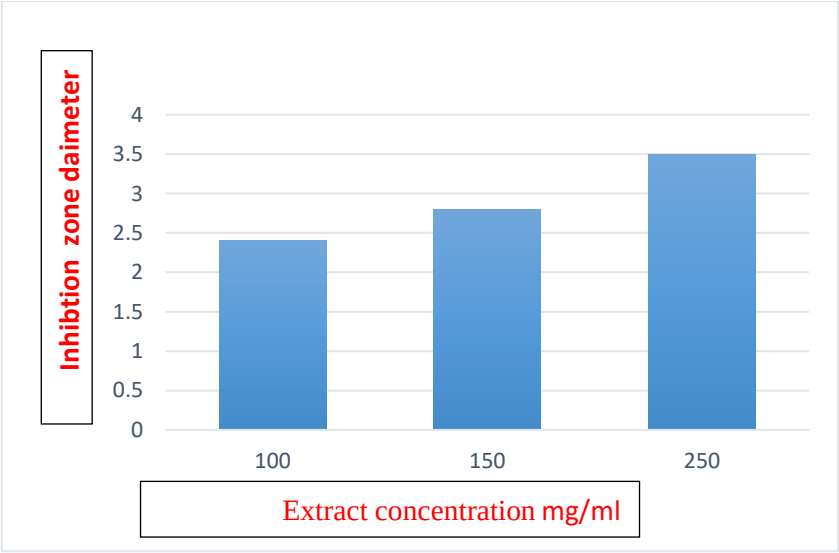
Statistical analysis

The results were expressed by difference between means were analyzed statistically according to LSD, the differences were considered significant when P≤0.05 by using the software SPSS statistic (Cary,2008).

Result and Discussion

Antibacterial activity

The results indicated that the extract exhibited significant antibacterial activity against the studied isolates. The highest activity was observed at a concentration of 200 mg/ml, which produced an inhibition zone measuring 3.5 ± 0.03. This was followed by inhibition zones of 2.8 ± 0.01 and 2.4 ± 0.04 at concentrations of 100 mg/ml and 150 mg/ml, as in figure 5 and 6.



Figure(4): Inhibition zone Diameter of different concentrations

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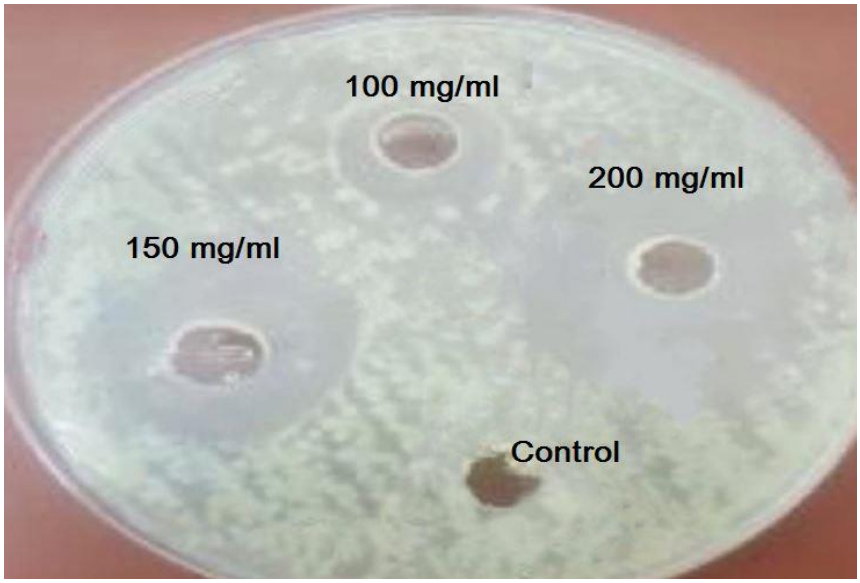

Figure (5) Inhibition zone of different concentrations extract against *E.cloacae*

The results showed an agreement with (Sofy *et al*.,2020 *)* found *Ziziphus spina-christi*, , *Origanum majorana*, *Allium sativum* and Rosmarinus officinalis were tested for their medicinal properties against highly resistant bacteria found in clinical samples. These bacteria include *K.pneumoniae*, *Escherichia coli* , P.aeruginosa (Gram negative), *S.aureus* and Methicillin-resistant Staphylococcus aureus (Gram positive). While Al-Kaabi and his colleague in 2021 reported the antibacterical activity of aqueous extracts against the gram positive and negative bacteria in 50 µg/ml, no effect was observed on any of the bacteria isolates. The highest level of inhibition, with a zone diameter of 9 mm, was observed at a concentration of 100 µg/ml against *Klebsiella oxytoca*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* exhibited delayed responses to the extract, resulting in inhibition zone of 6 mm, 4 mm, and 4 mm for the isolates respectively. These measurements were observed at a concentration of 100 µg/ml for all bacterial isolates. The highest zone of inhibition, measuring 9 mm, was observed against *Klebsiella oxytoca*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and Staphylococcus. The present findings further demonstrated the moderate antibacterial efficacy of the alkaloids component derived from *Z. spina-christi* extract. This effect is likely attributed to its capacity to form chemical bonds with the DNA of both Gram-positive and Gram-negative bacteria, thereby disrupting cell division (Bukar *et al*.,2015). Plants produce much of secondary metabolites, including phenols, quinones, flavonoids, alkaloids, tannins, and terpenoids, which have been scientifically demonstrated to have antibacterial properties (Cushnie and Lamb, 2005). Typically, plants produce these secondary metabolites as a response to being attacked by plant pathogens. The precise mechanism underlying the antimicrobial activity of plants remains poorly comprehended, despite a few studies suggesting that phytochemicals, such as flavonoids, may impede DNA synthesis (Rabaan *et al.,* 2022). Quinone, a group of plant-

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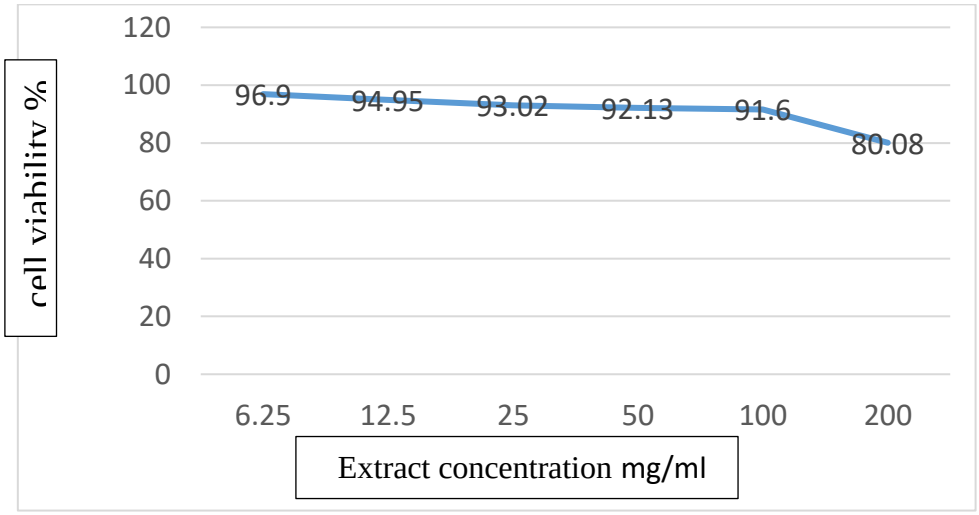
derived compounds, is recognized for its ability to generate persistent free radicals and attach to proteins, thereby permanently blocking bacterial growth (Tang *et al*.,2016).

The cytotoxicity

The cytotoxicity findings reported The minimum concentration of *Ziziphus Rugosa* extract required to inhibit WRL-68 cell proliferation by 50% (IC50) was 280 mg/ml. Simultaneously, the highest concentration of extract (200 mg/ml) resulted in 80.08 percent cell viability, while the lowest concentration (6.25 mg/ml) had no discernible impact on the cells as in figure 6.



Figure(6): WRL-68 expressed by the percentage of cell viability after 24h of exposure to extract.

It is important to note, that extracts selective cytotoxic activity derived from different species of ziziphus was mentioned, Taechakulwanijya *et al.* (2016) mentioned that jujube seeds ethanolic extract induced Jurkat cell mortality in a selective manner, an effect that was not observed in normal Vero cells. In a similar way, the *Ziziphus mauritiana* seeds exhibited inhibition of the proliferation of HeLa , HL-60, and Molt-4 cell lines, while having no discernible impact on the normal HGF cell line (Suriyavadhana *et al*., 2011) or normal rat liver cells (Plastina *et al*., 2012). In addition, *Ziziphus jujuba* pulp (chloroform-based extract) did not have any effect on normal cells., while on MCF-7 and SKBR3 cells exhibited selective cytotoxic effects (Taechakulwanijya *et al*.,2016).

Animal Activity Observation

The mice group that received *E.cloacae* presented clinical symptoms of illness with clear weight loss and diarrhea, reduction in movement activity through the cage space for 7 days while the mice groups that followed by extract administration presented clinical symptoms

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of illness for 3 days furthermore the mice group that received only normal saline and extract , no changing observed in activity and behaviors. The results agreed with (Mesaik *et al*.,2018) that used Each of the five animal groups was given 1 ml of E. coli suspension orally. As the control group, Group 1 was administered 0.2 mL of 2% tragacanth orally. In contrast, Group 2 was given (260 mg/kg Amoxicillin) and functioned as the positive control. The results obtained from Group 3, 4, and 5 which were administered *Z. Jujuba* extract at (400, 800, and 1200) mg/kg correspondingly, did not indicate any statistically significant changes in parameters including the amount of watery stool and the appearance of diarrhea when compared to the control. Nevertheless, the collected results lacked statistical significance. Out of the three concentrations of *Z. Jujuba* extract examined, only (1200mg/kg) produced a valuable reduction ($P \leq 0.01$) in the quantity of watery diarrhea produced. The observed level of significance was similar to that of the positive control in that it generated a noteworthy outcome ($P \leq 0.01$). Moreover, in comparison to the control group, *Z. Jujuba* extract at a dosage of (1200 mg/kg) was the only experimental group to exhibit a statistically valuable reduction ($P \leq 0.05$) in the overall quantity of feces these effects were attributed as Han *et al*. (2020), the polysaccharide from Ziziphus Jujuba improved gut microbiota diversity and increased levels of IL-2, IL-4, IL-10, IFN-γ, and TNF-α in serum and intestine. Impact was according to the dose of polysaccharide. JP affects intestinal barrier and peripheral immunity.

Innate immunity

Neutrophils Activity

The results in table 1 showed the phagocytic activity of neutrophils index in group IV that had a combination injection by *E.cloacae* and extract recorded high values(47.15 %) with significant differences at ($p \leq 0.05$) comparison with other studied groups, while the lowest value was recorded in the group that treated with only *E.cloacae* (13.40 %).

Table (2): showed the percentage of neutrophils (Means± S.E)

Groups	Means ± SD	Phagocytic activity%
GroupI	2.00^{a} ±0.00	2.00 %
Group II	13.40^{b} ±0.30	13.40 %
Group III	23.15^{c} ±0.20	23.15 %
Group IV	47.15^{d} ±0.20	47.15 %
LSD	**3.234**	

** the different small letters refer to the significant differences between times at ($p \leq 0.05$)*

Limited research has been conducted on the impact of Ziziphus species on phagocytic activity; however, the findings were consistent with those of (Ganachari *et al*., 2004), which examined the effect of Ziziphus jujuba leaf extract at various concentrations (5, 10, 25, 50,

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and 100µg/ml) on various in vitro phagocytic assays, including neutrophil locomotion and chemotaxis tests: Human neutrophil chemotactic, phagocytic, and intracellular killing activity was stimulated by Ziziphus jujuba leaf extract at concentrations ranging from 5 to 50µg/ml. Zou *et al*.,(2018) concluded the polysaccharides from Ziziphus , polyphenol and triterpenoid antioxidants have the potential to considerably boost immune organ indices, serum half hemolysis value (HC50), phagocytic index, and footpad thickness in mice. Additionally, these compounds have the ability to enhance cellular and humeral immunity as well as natural immunological responses.

Estimation Toll Like Receptor -2 serum concentration

The findings of TLR-2 reported as shown in figure 7 the mice that received only *E.cloacae* showed highest value (98.12±0.087) with a significant difference at p≤ 0.05

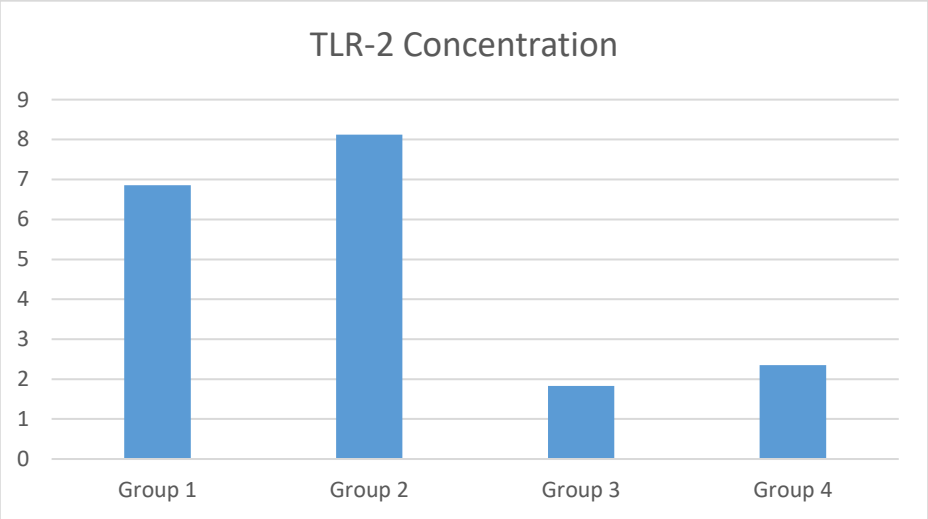

Figure (7): TLR-2 Concentration in studies groups

The findings were agreed with Xie *et al*.(2023) who reviewed the tonic chinese herbal ,tonic beverage four distinct classifications of chinese herbal medicine can be delineated according to their respective functions. These include *Ziziphus jujuba*, *Atractylodes macrocephala Koidz*., *Astragalus membranaceus*, and *Acanthopanax senticosus*, which are tonic Chinese herbal medicines for tonifying vital energy, the backbone of the entire system; *Angelica sinensis*, which is a tonic Chinese herbal medicine for tonifying blood; *Cordyceps sinensis* and *Epimedium brevicornu*, which are tonic chinese herbal medicines for tonifying body storesThe enhancement of immunity by tonic Chinese herbal medicine polysaccharides is primarily mediated through signaling pathways including MAPK, NF-κB, TLR, JAK-STAT, and others. Endosomal membrane or cell membrane Toll-like receptors (TLRs) are located. Initiating an immune response, the TLR family is capable of recognizing pathogen-associated molecules borne by microorganisms such as fungi, bacteria, viruses, and parasites (Fitzgerald

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and Kagan *et al*., 2020). Through the TLR4 signaling pathway, the immune mechanism of traditional Chinese medicine polysaccharides has been studied the most. Immune function is dependent on the activation of the TLR4 signaling pathway, which is located upstream of NF-κB and MAPK. Two mechanisms exist for activating the TLR4 signaling pathway: those that rely on MyD88 and those that do not (Ahmed-Hassan *et al*., 2018). The downstream IRAK4 and IRAK1 pathways, which are dependent on MyD88, require TRAF6 (Chen *et al*.,2017).

Adaptive immunity

Arthus Reaction and Delayed Hypersensitivity Test (DHT)

The results of the Arthus test and DHT revealed the group that had a combination administration of extract and *E.cloacae* showed a higher value than the other group, after 48 hr but the value started decreasing after 72 hr but the highest value recorded (4.24± 0.00) after 24 hr with significant differences at $p \leq 0.05$ with other groups as in table (3 and 4).

Table 3: showed results of Arthus test between groups (Means ±S.E) in studied concentrations .

Group I	Group II	Group III	Group IV
2.00± 0.00 [a]	5.00± 0.00 [c]	2.00± 0.00 [a]	4.25± 0.25 [b]
2.00± 0.00 [a]	5.00 ± 0.00 [b]	2.00 ± 0.00 [a]	5.00± 0.00 [b]
2.00± 0.00 [a]	5.00 ± 0.00 [b]	2.25± 0.25 [c]	5.00 ± 0.00 [b]
LSD	**2. 12**		

** the different small letters refer to the significant differences between times at (p≤0.05)*

Table 4 : Delayed Hypersensitivity Test after 24hr, 48hr, 72 hr (Means ± S.E, mm) studied groups.

Groups	24hr	48hr	72 hr
Group I	2.00± 0.00 [a]	4.00± 0.00 [a]	2.00± 0.00 [a]
Group II	3.00± 0.00 [a]	5.50± 0.00 [b]	5.50± 0.00 [b]
Group III	2.00± 0.00 [a]	5.00± 0.00 [b]	4.00± 0.00 [b]
Group IV	2.50± 0. 50 [a]	4.50± 0. 50 [a]	4.25± 0.25 [b]
LSD	**2.340**		

** the different small letters refer to the significant differences between times at (p≤0.05)*

The results came in an agreement with a preliminary study that revealed *the* extract *of Ziziphus mauritiana* stimulated phagocytic and intracellular killing potency of human neutrophils at different concentration. Thus from the results obtained it can be observed that alcohol and aqueous extract of Ziziphus mauritianaleaves stimulates cell-mediated immunity by increasing neutrophil function and phagocytosis (Wadekar, & Patil, 2008). Chi *et*

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al.(2015) found through detection the the efficacy of Ziziphus Jujube fruits as a treatment for chronic fatigue syndrome. JPC, or Jujube polysaccharide conjugates, were extracted from Z. Jujube fruits. The physicochemical properties of JPC in general were evaluated. Following the establishment of a four-week CFS model in rodents and the oral administration of JPC, behavior experiments were conducted. An analysis was conducted on the serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). Additionally, the proliferation of T lymphocytes, the CD4(+)/CD8(+) ratio, and the activity of natural killer (NK) cells were assessed. JPC significantly improved the behavior of CFS rodents, decreased serum MDA levels, and increased the proliferation of T lymphocytes, the ratio of CD4(+) to CD8(+), and the activities of natural killer (NK) cells. Spleenocyte proliferation was significantly enhanced by the concurrent administration of Shenzao Cha (SZC), a composite of American ginseng and Chinese jujube (1.3, 2.6, and 5.2 g/kg), and T-cell mitogen (ConA, 7.5 μg/ml). Significant lymphocyte proliferation was observed in the high-dose (5.2 g/kg) group, which was 1.4 times greater than that in the control group. The potential for SZC to augment cellular immunity is suggested by its positive impact on T-lymphocyte proliferation (Yu *et al*., 2016). Adaptive immune responses, encompassing both humoral and cellular immunity, rely predominantly on B and T lymphocytes. The investigation of the antibody response subsequent to vaccination against a foreign antigen is the initial focus (Primorac *et al.*, 2022). B-lymphocytes secrete hemolysin in response to SRBC vaccination in rodents; the concentration of this substance in the serum is utilized to assess the humoral immunity's functionality (Yang *et al*., 2009). In addition to releasing a variety of cytokines, T-lymphocytes have the ability to directly eradicate target cells; furthermore, their proliferation is essential for the initiation of a cascade of humoral and cellular immune responses (Chen *et al*., 2012). Similar to previous research (Lv *et al*., 2013), the lowest dose demonstrated the highest efficacy in inducing serum hemolysin formation. The involvement of macrophages in the processing and presentation of antigen to T and B lymphocytes implies that an increase in dosage could have potentially led to a suppression of macrophage function. Additionally, the indices of the thymus and spleen, where lymphocytes reside and endure differentiation and maturation, were significantly increased by Ziziphus. Ziziphus can enhance immune function by stimulating lymphocyte proliferation, lymphocyte cytokine secretion, and mRNA expression, according to a recent study (Li *et al*.,2021)

Estimation IL-14,IL-17 and IL-10 serum concentration

On the other side , IL-17,IL-4 and IL-10 finding in table 4 showed the groups that received extract with *E.cloacae* given a high value (424.95±0.46, 100±0.00, 72.18±0.12) respectively which higher than other groups with a significant difference at $p \leq 0.05$.

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Table (5): IL-17, IL-4 and IL-10 Concentration level (pg/ml) in studies groups

Groups	IL-17	IL-4	IL-10
Group I	216.57±0.02 [a]	45.40±0.09 [a]	68.627±0.06 [a]
Group II	162.71 ±0. 87[b]	41.341±0.07 [a]	65.388±0.21[b]
Group III	188.99±0.43[b]	64.15±0.032 [b]	69.97±0.38 [a]
Group IV	424.95±0.46[c]	100±0.00 [c]	72.18±0.12 [c]
LSD	**13.54**		

** the different small letters refer to the significant differences between times at ($p \leq 0.05$)*

The obtained results agreed with(Mishra, & Bhatia, 2010), the aqueous-ethanolic seed extract (100–400 mg kg^{-1}) of *Z. mauritiana* was investigated for immunomodulatory potential in mice revealed The extract of seed showed a substantial increase in cell-mediated and humoral immune response, as well as an up-regulation of the Th-1 mediated cytokine IFN-γ. Additionally, there was a decrease in the Th-2 mediated cytokine IL-4. The outcomes achieved with a larger dosage of the extract were similar to those obtained with levamisole. However the results agreed with (Hoseinifar *et al*.,2019) that investigated the impact of consuming Ziziphus jujube fruit extract (ZJFE) on the non-specific immunological parameters of skin mucus, as well as the mRNA levels of immune-related genes in the skin of common carp fingerlings. The study found that the fish fed a diet containing 0.5% ZJFE had the greatest levels of skin mucus total Ig, whereas the control group had the lowest levels ($P < 0.05$). No notable variations were seen across treatments in terms of skin mucous lysozyme activity ($P > 0.05$). In addition, gene expression analyses conducted on skin samples revealed a notable upregulation of il1b expression in fish that were fed a diet containing 0.5% ZJFE, as compared to the other treatment groups ($P < 0.05$). In addition, the expression of the il8 gene was significantly increased in the 0.5% and 1% treatment groups compared to the control group ($P < 0.05$). There was no significant difference in the relative expression of the il10 gene between the 0.25% JFE treatment and the control group ($P > 0.05$). However, feeding on diets containing 0.5% or 1% ZJFE dramatically decreased the expression of the il10 gene ($P < 0.05$).The text is empty. Following stimulation by antigens and cytokines, T helper cells primarily undergo differentiation into Th1, Th2, and Th17 cells. Th1 cells express T-bet transcription factors, Th2 cells express GATA-3 transcription factors, and Th17 cells express ROR-γt transcription factors (Patel & Kuchroo, (2015).).Moreover, The chemical study revealed the presence of both betulinic acid and quercetin in Z. jujuba fruit. Prior research has demonstrated that the majority of medicinal plants have the ability to mitigate dysentery and diarrheal episodes. The majority of medicinal plants contain alkaloids, saponins, flavonoids, sterols, and triterpenoids (Otshudi *et al*., 2000). Prior research indicates that betulinic acid possesses strong anti-inflammatory properties, since it effectively decreases the synthesis of TNF-α and nitric oxide in mice. In addition, betulinic acid has been discovered to enhance the concentration of IL-10 when stimulated by LPS (Park *et al*., 2014). Furthermore, quercetin

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has the ability to suppress the inflammatory response of bone marrow-derived macrophages in a laboratory setting. There is also evidence that quercetin can prevent the release of cytokines and stimulate the production of nitric oxide synthase by inhibiting the NF-κB pathway, without making any changes to activity of the Jun N-terminal kinase (Mesaik *et al*.,2018).

Conclusion:

The current study showed the *Z. spina-christi* aqueous leaves extract have antibacterial activity in vitro against gram negative bacteria that used in current study *E.cloacae* in high concentration 200 mg/ml however same concentration showed no cytotoxicity against normal cell line Finally the extract have a positive effect to regulate the immune response by rising the IL-10 and IL-4 level that played anti-inflammatory role also increase IL-17 which contributed to mobilization of neutrophil in vivo.

The Declaration of Competing Interest:

The authors have declared no conflict of interest.

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