RESEARCH ARTICLE

In vitro study on immunomodulatory compounds isolated from *Ziziphus xylopyra*

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Manuscript details:	ABSTRACT
Available online on http://www.ijlsci.in ISSN: 2320-964X (Online) ISSN: 2320-7817 (Print)	The present study aims to evaluate the efficacy of ethyl acetate extract of <i>Ziziphus xylopyra</i> bark (ZXEA) for the antibacterial activity against diarrhea causing Enterococcus fecalis. The partially purified extract 1.5 mg solubilized in 0.1 % DMSO was effective in dose dependent manner. For positive control vancomycin was used throughout. ZXEA was partially purified (ZXT) on silica
Editor: Dr. Chavhan Arvind	gel (60 – 120 mesh size) and eluted with Benzene:Ethyl acetate solvent mixture in 2:1 ratio, dried in rotary vacuum evaporator & tested for its
Cite this article as: Butle Ashwin and Patil Mandakini (2016) <i>In vitro</i> study on immunomodulatory compounds isolated from <i>Ziziphus xylopyra, Int. J. of Life</i> <i>Sciences,</i> A6:10-14.	potential towards in vitro' immunomodulatory activity. <i>Z. xylopyra</i> extract is regularly used by the tribal people for treatment of diarrhea. In this context, when tested, 'in vitro', the lymphocyte proliferation and phagocytic activity of isolated macrophages was found to get stimulated by ZXT. Thus, the mechanism of action of <i>Z. xylopyra</i> extract, 'in vivo', would be probably via the activation of macrophages in the intestinal lumen thereby inducing phagocytosis of causative microorganism, thereby preventing diarrhea. Composition of this potent fraction for the existence of specific class of compounds was subsequently detected using Gas Chromatography – Mass
Acknowledgement: Authors express sincere thanks to The Head University	Spectrometry (GC-MS). It appears to contain a major compound belonging to a class of triterpenoid group.
Department of Biochemistry; RTM Nagpur University, Nagpur for facilities and encouragement.	Keywords: <i>Ziziphus xylopyra</i> , Antibacterial activity,Lymphocyte Proliferation, Phagocytic Activity, GC-MS.
Copyright: © Author, This is an open access article under the terms of the Creative Commons Attribution-Non-Commercial - No Derives License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.	INTRODUCTION <i>Enterococcus faecalis</i> – formerly classified as part of the group D <i>Streptococcus</i> system – is a Gram-positive, commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals (Ryan and Ray, 2004). Like other species in the genus <i>Enterococcus, E. faecalis</i> can cause life- threatening infections in humans, especially in the nosocomial (hospital) environment, where the naturally high levels of antibiotic resistance found in <i>E. faecalis</i> contribute to its pathogenicity (Ryan and Ray, 2004). <i>E. faecalis</i> mainly causes diarrhea, endocarditis, bacteremia, urinary tract infections, meningitis, and other infections in humans and having gentamycin resistance (Murray, 1990; Hidron <i>et al.</i> , 2008; Huycke <i>et al.</i> , 1981).
	It is known that when antigen enters the body, the immune system gets activated and induces an immunological response by either humoral or by its

cellular arm. The immune system is remarkably versatile defense system that

has evolved to protect animals from invading pathogenic microorganisms and cancer. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders (Kuby, *et al.*, 2007).

It has been proven that plants contain essential components responsible to cure several diseases serving as natural source of medicine. The *Ziziphus xylopyra* is the medicinal plant that has large number of traditional benefit since the ancient time. *Ziziphus* commonly called, Chinese date or Bera (Pushto), belongs to family *Rhamnaceae*. The bark, leaves and fruits of several species have been used as laxatives, curing jaundice (Gul *et al.*, 2009), used to prevent malaria (Neto *et al.*, 2008), proven to be antibacterial, phytotoxic and and rich with hemagglutination activities (Ahmad *et al.*, 2011), bark decoction of *Ziziphus* is taken thrice a day for six days in diarrhea and in urinary troubles (Schomburg *et al.*, 2001).

Ziziphus species are known to possess bioactive compounds with therapeutic potential. The present work aims to evaluate the efficacy of ethyl acetate extract of Ziziphus xylopyra bark for the antibacterial activity specifically towards the diarrhea causing *Enterococcus fecalis* and '*in vitro*' immunomodulatory activity.

MATERIALS AND METHOD

Plant Material: Plant material collected from the Gadchiroli district and authenticated by the taxonomist of the region (Voucher No. 9848).

Bacterial Culture: ATCC type cell culture of *Enterococcus fecalis* was obtained from University Department of Microbiology, RTM Nagpur University, Nagpur.

Bacterial Culture Medium: Blood agar medium used as a bacterial culture medium. The medium were sterilized and prepared as per the instructions given by the manufacturer (Himedia, Mumbai).

Blood Samples: Blood samples of infected patients were collected from the Clinical Biochemistry Laboratory, University Department of Biochemistry, RTM Nagpur University, Nagpur (MS), India. *Lymphocyte's separation & culture medium*: Hi Sep LSM; Lymphocyte separation medium, XTT Cell Assay Kit; CCK015, DMSO (Himedia Mumbai), Dubecco's phosphate buffered saline (DPBS), RPMI-1640, Fetal calf serum (FCS) were purchased from Gibco laboratories. Antibiotic antimycotic solution, lipopolysaccharide (LPS), concanavalin-A (Con-A), MTT reagent, Zymosan-A, Nitroblue tetrazolium and all the cell culture medium procured from Sigma Aldrich (St. Louis, USA).

Extraction and purification of Z. xylopyra: Bioactive compounds from the bark of *Z. xylopyra* were extracted in ethyl acetate solvent (ZXEA) (Cordeiro *et al.*, 1999). Solvent was evaporated by rotary vaccume evaporator (SuperfitTM DB-3135S) and dried in hot air oven at 60 °C. ZXEA extract was column chromatographed over silica gel (60-120 mesh size) and eluted with Benzene:Ethyl acetate solvent mixture to obtain *Z. xylopyra* triterpenoids (ZXT) in 2:1 solvent mixture ratio, as mentioned by Kundu *et al.* (1989).

Antibacterial Activity assay: By using Well Assay method, antibacterial activities of ZXEA and their dilutions were determined (Ahmad *et al.*, 2011).

Isolation of Lymphocytes: Lymphocytes were isolated (1x10⁶ cells/well) according to the method of Kainthla *et al.*, 2006 in RPMI-1640 medium, containing 10% fetal calf serum (FCS) supplemented with antibiotic-antimycotic solution (complete medium). Viability was determined by trypan blue exclusion, and was consistently greater than 96%.

Lymphocyte Proliferation Assay: Effect of ZXT on proliferation was tested by XTT assay. Lymphocytes were treated with different concentration of fraction dissolved in PBS. PBS was used as a control while atropine solution was taken as a negative control. One group was treated with 10 μ g/ml LPS. After incubation for 72 h at 37 °C in 5% CO₂ humidified atmosphere (Thermo Scientific, Class-100), XTT assay was performed as per the instruction given by the manufacturer.

Phagocytic Activity Assay: Capacity of ZXT to stimulate phagocytosis was tested by NBT dye reduction assay (Rainard, 1986). Macrophages suspended in complete RPMI 1640 medium. Control was run with PBS (without isolated fraction). After incubation for 24h at 37°C in 5% CO₂ humidified

atmosphere. Zymosan – A (1 μ g/ml of PBS) was introduced along with NBT solution (1.5 mg/ml of PBS) and assay was carried out according to the method of Talmale *et al.*, 2014.

Gas Chromatography – Mass Spectrophotometry: A

Varian 4500 GC coupled with Varian MS240 ion trap mass spectrometer (Varian, Walnut Creek, USA) was employed for determination of analytes in most potent partially purified ZXT using electron ionization (EI) mode. Split less injections of 1 µl volume were carried out with a split programmable temperature injection (STI) Type 1079 kept at 270 °C. The ion trap, manifold and the transfer line were kept at 240, 40 and 250 °C, respectively. Separations were performed on Varian Chromopack Capillary column WCOT Fused Silica (30 m long, 0.25 mm ID) CP-Sil 8CB, helium (Ultra pure 99.99%) was employed as a carrier gas. Compounds were identified by direct comparison of their MS with data from the NIST library.



Fig.1: Antibacterial activity of ZXEA against E. faecalis



Statistical Analysis:

Results are expressed as mean (SD) using Microsoft Excel, 2007. The differences have been determined using Student 't' test used by MedCalc software. Significance level was set at p<0.05.

RESULTS AND DISCUSSION

Tremendous traditional medicinal usage to cure several disorders and ailments during historic time was not truly based on the knowledge of its chemical constituents (Kumari *et al.*, 2012). Later, literature reports divulged the occurrence of bioactive compounds called secondary metabolites like alkaloids, terpenoids, flavonoids, glycosides, waxes and fatty acids in medicinal plants, recognized to be responsible for their medicinal and pharmacological actions (Kalimuthu *et al.*, 2014).



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Fig. 2 (D), MS of urs-12-en-28-oic acid, 3β- hydroxy-, methyl ester, **Fig. 2 (E)**, **MS of** 2-Hydroxy-10-isopropenyl-3,3,5a,5b,12b-penta methyl octadecahydro dicyclopenta [a,i] phenanthrene-1,7a(1H)-dicarboxylic acid



Fig.3: Effect of purified ZXT to stimulate lymphocyte proliferation by XTT assay



Fig.4: Effect of purified ZXT on phagocytic index. In the figures C was compared with all different dilutions of purified ZXT and NC where, *: $p \le 0.0001$, **: $p \le 0.005$, ***: $p \le 0.05$ and #: $p \ge 0.05$.

Antibacterial Activity assay: The interest regarding the research on medicinal plants has increased over the last few decades due to onset of new infection, in particular, infections by *Enterococcus* and *Staphylococcus* species, which are agents of many intra-hospital infections and antibiotic resistance to available drugs (Ahmad *et al.*, 2011). The 1.5 mg ZXEA extract showed moderate activity against *E. faecalis*, Fig. 1(*: $p \le 0.0001$, **: $p \le 0.005$,).

Identification of Active partially purified ZXT fraction by GC-MS analysis: GC-MS analysis conducted on the effective ZXT fraction of *Z. xylopyra* bark revealed to identify four chemical compounds Fig. 2, disclosing presence of compounds belonging to triterpenoid group responsible for its bioactive properties.

Lymphocyte Proliferation Assay: Lymphocytes are the first weapon of an immune system to degrade and eliminate antigen from the host. ZXT were found to be quite effective in stimulating lymphocyte proliferation. The stimulation index shows little less than the LPS and near to the activity of Con – A which are very effective known mitogens (Fig., 3). Proliferation of lymphocytes by the ZXT suggested that it is a good stimulator for an immune system. *Phagocytic Activity Assay*: Until and unless the phagocytes are actively working, body doesn't need to switch on the acquired immunity. Lymphocytes are most important parts of the acquired immunity (Talmale *et al.*, 2014). ZXT was explored for its phagocytosis stimulating activity at different concentrations. It was observed that the effective concentration has shown its maximum activity (Fig. 4).

CONCLUSION

In recent years, researchers focused on the identification of novel biomolecules from medicinal plants. Phytochemicals have been proved to perform a significant role, amongst them triterpenoids due to the broad range of exceptional bioactive capabilities mostly receive great attention. It has been known that when antigen enters the body, the immune system gets activated and induces an immunological response by either humoral or by its cellular arm. The week or immunosuppressed patients collapse or die due to weakening of immune system of body. Thus, the triterpenoid compounds present in Z. xylopyra bark might be acting in a similar way, in vivo, to combat the diarrhea by killing the causative microorganisms by activation of macrophages. Reports on stimulation of immune system by plants are available in the literature.

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