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ASSESSMENT OF *IN VITRO* ACTIVITY OF CRUDE EXTRACTS OF *BERBERIS LYCIUM* AGAINST *SALMONELLA TYPHI*

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ABSTRACT

This Plant derived drugs have been important source of traditional health care in many parts of the world. There is increasing focus on medicinal plants as sources of new agents to combat microbial diseases. *Berberis lycium* is an important medicinal plant usually found in Azad Kashmir, Northern Khyber Pakhtunkhua, Baluchistan and hilly areas of Punjab, Pakistan. Since 1989, *Salmonella typhi* has developed resistance to conventional antibiotics of choice. The main objective of the study is to determine the *in vitro* activity of crude extracts of *Berberis lycium* against *Salmonella typhi*. Crude aqueous, methanol, chloroform and n-hexane extracts of root and stem bark were used in triplicates of each concentration to evaluate anti *Salmonella typhi* activity using Agar well diffusion method. Activities were performed against *Salmonella typhi* ATCC 6539, *Salmonella typhi* ATCC 19430, and *Salmonella typhimurium* ATCC 14028. Crude methanol extract (1000µg/ml) showed maximum zone of inhibition of 19.4±0.6mm, 19.7±0.8mm, and 18.5±0.7mm, respectively. Crude chloroform extract (1000µg/ml) showed zones of inhibition of 17.3±0.9mm, 19.4±1.2mm, and 14.7±1.3mm against three strains, respectively. Same concentration of cr. aqueous extract exhibited less activity. Zone of inhibition determined was 12±1.7mm, 13.1±0.9mm, and 16.2±1.3mm, respectively. Crude n-hexane extract exhibited no activity at all. MIC of all extracts against all strains was determined. MIC values exhibited that *S. typhi* and *S. typhimurium* strain are more susceptible to crude methanol extract than crude chloroform, aqueous or n-hexane extracts. Significant activity shown by extracts confirms the traditional use of *Berberis lycium* in typhoid fever.

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INTRODUCTION

Medicinal plants have been used over the centuries for the treatment of various human diseases for improving healthcare of the people (Oluma, 2004). Plant derived drugs have been important component of traditional health care in many parts of the world and there is increasing focus on them as sources of new agents to combat microbial diseases (Mohana, 2008; Adwan *et al*., 2009 and 2010). In developing countries, focus of recent research has been on developing natural plant products as an alternative to the present drugs (Aiyegoro, 2007). With the passage of time the development of multiple antibiotic resistant pathogens has constituted an enormous global problem for the treatment of infectious diseases. Typhoid fever caused by *Salmonella typhi* has developed resistance to conventional antibiotics of choice (Shanahan, 1998).

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Typhoid fever is a systemic infection caused by *Salmonella enterica* serotype typhi (Kidgell, 2002 and Merrell *et al*., 2004). Approximately, 21.6 million illnesses and 216,500 deaths globally have been reported due to typhoid in 2000 (Crump, 2004). Incidence of typhoid, reported in Pakistan is 451 out of 100,000 (Kothari, 2008). Chloremphenicol, ampicillin, amoxicillin and trimethoprim were drugs of choice in recent past (Bhutta, 1994). Currently fluoroquinolones are regarded as optimal for the treatment of enteric fever in adults (Chinh, 2000). These are well tolerated, relatively inexpensive, more reliably and rapidly effective than the former first line drugs of choice, viz. chloramphenicol, amoxicillin, ampicillin and trimethoprim-sulfamethoxazole. Fluoroquinolones are well tolerated in adults and produce a therapeutic response in three to five days. They present very low rates of post treatment carriage (Arnold, 1993). In children, third generation cephalosporin, cefixime is frequently prescribed (Bhutta, 1994). Now a day's extensive research is being done on multi drug resistant *S. typhi* strains. A study conducted in India showed that eleven of the *S. typhi* strains were resistant to

chloramphenicol, amoxicillin and trimethoprim. Recently, strain having a plasmid containing multi drug resistance has emerged in the *Salmonella typhi* population and has successfully been able to adapt, endure and survive the antibiotic (Shanahan, 1998). The rapid emergence of drug resistance to antibiotics emphasizes on a need to discover new agents to treat typhoid infection (Samuelson, 1999). Traditional systems of medicine have provided us with many beneficial lead compounds in the development of novel drugs for various diseases. Useful lead compounds, identified through traditional system of medicine, continue to help us in development of modern medicine system and discovery of novel therapeutic targets. Investigation of large number of less familiar plants for medicinal use is today's necessity. *B. lycium* Royle has been reported by various studies for its use in traditional system of medicine to treat infectious diseases along with fever, diabetes, rheumatism, and gingivitis. *Berberis lycium* Royle (family: Berberidaceae) is among widely used medicinal plants. It is named in as "barberry" in English (Khan, 1979) whereas, its fruit is called as "Kashmaal" (Baquar *et al*., 1989 and Usmanghani, 1997). A famous drug "Rasaunt" is prepared from this plant by boiling plant bark in water. Berberine, a major constituent of this plant is now well known to possess anti-inflammatory property. Traditional practitioners have also used this plant to treat typhoid fever and wounds (Aggarwal, 2006). Development of antibiotic resistance against the existing antibiotics of choice against *S. typhi* and growing acceptance of alternative medicines emphasizes on a need to investigate the anti *Salmonella typhi* activity of medicinal plants *Berberis lycium*. Present study has been designed to investigate the anti *Salmonella typhi* activity of n-hexane, chloroform, methanol and aqueous crude extracts of *Berberis lycium* root and stem bark and to determine the sensitivity of *Salmonella typhi* to the different concentrations of crude extract of *Berberis lycium* root and stem bark.

MATERIALS AND METHODS

Whole plants of *Berberis lycium* were collected from Gilgit, Baltistan region of Northern Pakistan, during the months of May and June 2013. The plant was identified by Department of Pharmacognosy, Riphah Institute of Pharmaceutical Sciences Islamabad. Roots and stem were cut and washed with water two to three times to remove all the unnecessary material from the plant. These clean roots and stems were dried under shade at room temperature for 60 days. When the plant was completely dry and there was no significant change in weight of plant between two different time intervals, its root and stem bark was separated and was converted into fine powder with the help of mechanical grinder.

Extraction of crude extracts

Traditionally used, effective solvents for extraction of medicinal active compounds include water, methanol, ethanol, chloroform, and palm kernel oil; however, mostly used extractive solvent is water (Musa, 2011). Crude aqueous, methanol, chloroform, and n-hexane extracts were obtained in small amounts. 5 g of the plant material was Soxhlet extracted at 90°C in 200 ml of each solvent for 2 days. Extracts were filtered and dried using rotary evaporator, operated between 40 to 50 °C. Yield was determined and dried extracts obtained were evaluated for their anti *Salmonella typhi* activity.

Anti *Salmonella typhi* activity

Anti *Salmonella typhi* activity of crude extracts

Anti *Salmonella typhi* activity of crude aqueous (Cr. Aq), crude methanolic (Cr. Me), crude chloroform (Cr. Chl), and crude n-hexane (Cr. Hex) extracts in 3 different concentrations were investigated. This experiment was conducted as a first step towards identification of the most active crude extract and towards in vivo experimentation and purification of bioactive compound. This shall also determine the concentration at which extracts shows activity.

Preparation of dilutions

Three different dilutions of 100 µg/ml, 500 µg/ml, and 1000 µg/ml were prepared in DMSO for all the crude extracts by serial dilution method. Dilutions were kept in plastic vials and from there taken for activity. Same dilutions were prepared for the standard drug ciprofloxacin.

Preparation of culture media

38 g of Mueller Hinton agar was dissolved per liter under heating and with constant stirring, to prepare the culture media. Media was sterilized by autoclaving for 15 minute at 121 °C temperature and 15 psi pressure. Sterilized culture medium was then used for, experimentation.

Sensitivity testing

Agar well diffusion method was used to investigate *Salmonella typhi* sensitivity to the extracts. 10 ml of the culture medium was poured in culture plates. Medium was allowed to solidify in laminar flow hood chamber. Culture plates were inoculated by fresh *Salmonella typhi* (ATCC 6539), *Salmonella typhi* (ATCC 19430), and *Salmonella typhimurium* (ATCC 14028), by inoculating cotton swab stick. 4 wells per plate were bored by 6mm stainless steel borer. Wells were locked from bottom by adding in them small amount of liquid agar medium. 100 µl of each dilution was filled in each well and were labeled respectively as Cr. Aq 100 µg/ml, Cr. Aq 500 µg/ml, Cr. Aq 1000 µg/ml for crude aqueous extracts, Cr. Me 100 µg/ml, Cr. Me 500 µg/ml, and Cr. Me 1000 µg/ml for crude methanol extract, Cr. Chl 100 µg/ml, Cr. Chl 500 µg/ml, and Cr. Chl 1000 µg/ml for crude chloroform extract, and Cr. Hex 100 µg/ml, Cr. Hex 500 µg/ml, and Cr. Hex 1000 µg/ml for crude n-hexane extracts. Same concentrations of the positive standard ciprofloxacin were filled in the wells too. DMSO was used as negative standard. Culture plates were incubated for 24-48 hours at 37°C. Zone of inhibition was measured using Vernier Caliper. Values were recorded and most active crude extract was identified (Agarry, 2005).

MIC determination of crude extracts

From a pure culture of *Salmonella typhi*, material from at least 3-4 colonies was picked. It was resolved totally in 4 ml Mueler Hinton agar medium in tubes. Mixed and adjusted to McFarland 0.5 standard. Turbidity of inoculums was adjusted to match the standard by comparing visually with the McFarland 0.5 standard. The inoculum suspension was used for inoculation within 15 min. 1ml of the inoculums' suspension (104 CFU) was uniformly applied on MH agar plates. Agar dilution method was used to check the MIC.

Dilutions of 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml, 160 µg/ml, and 320 µg/ml were applied in each well and zone of inhibition appearing at the minimum concentration applied was observed and recorded (Adesokan, 2007 and Oyeleke, 2008).

Statistical Analysis

Three replicates were performed for each sample. The mean of zones of inhibitions and standard deviation were calculated by using Microsoft Excel.

RESULTS

Soxhlet extraction of powdered plant material for collection of crude aqueous, methanol, chloroform and n-hexane extracts yielded dried mass of 1.2g, 1.05g, 0.94g, and 0.34g, respectively.

Anti *Salmonella typhi* activity of crude extracts

Results of anti *Salmonella typhi* activity were measured as zone of inhibition± S.D in mm. Results of anti *Salmonella typhi* activity of the extracts are listed in the Table 1. Measurement of zone of inhibition showed that crude methanol and crude chloroform extracts are the most active against *Salmonella typhi*. It was observed that value for zone of inhibition was directly proportion to the concentration of extract. 1000 µg/ml of cr. methanol extract showed zone of inhibitions 19.4±0.6mm, 19.7±0.8mm, and 18.5±0.7mm against *Salmonella typhi* ATCC 6539, *Salmonella typhi* ATCC 19430, and *Salmonella typhimurium* ATCC 14028, respectively. Cr. chloroform extract showed zone of inhibition 17.3±0.9mm, 19.4±1.2mm, and 14.7±1.3mm, respectively. Crude aqueous extract did not show activity at low concentrations while showed small zone of inhibition of

Table 1. Anti Salmonella typhi activity of crude extracts of Berberis lyceum

Extracts/ Conc.	100 µg/ml	500 µg/ml	1000 µg/ml	MIC(µg/ml)	Strain
Cr. Aqueous extract	No activity	8.6±0.5	12±1.7	80-160	ATCC 6539
	No activity	9.2±0.6	13.1±0.9	80-160	ATCC 19430
	No activity	13.6±1.1	16.2±1.3	40-80	ATCC 14028
Cr. Methanol extract	11.2±0.4	13.8±1.5	19.4±0.6	10-20	ATCC 6539
	12.5±0.8	14.2±1.2	19.7±0.8	10-20	ATCC 19430
	10.1±0.5	12.2±0.7	18.5±0.7	20-40	ATCC 14028
Cr. Chloroform extract	10.2±0.3	12.8±1.8	17.3±0.9	10-20	ATCC 6539
	11.8±1.5	13.9±1.3	19.4±1.2	20-40	ATCC 19430
	9.6±0.8	11.9±0.9	14.7±1.3	20-40	ATCC 14028
Cr. n-Hexane extract	No activity	No activity	No activity	No activity	ATCC 6539
	No activity	No activity	No activity	No activity	ATCC 19430
	No activity	No activity	7.8±0.5	No activity	ATCC 14028
Ciprofloxacin (positive control)	33.6±2.2	35.5±1.4	37.8±1.9	5-10	ATCC 6539
	31.8±1.9	32.7±2.1	35.9±1.6	5-10	ATCC 19430
	29.5±1.7	30.1±1.9	32.2±1.9	5-10	ATCC 14028
DMSO (negative control)	No activity	No activity	No activity	No activity	ATCC 6539
	No activity	No activity	No activity	No activity	ATCC 19430
	No activity	No activity	7.3±0.4	No activity	ATCC 14028

*Mean of 3 replicates ± S.E.M
*Diameter of inhibition zones are including diameter of well 6 mm.
*MIC= Minimum inhibitory concentration

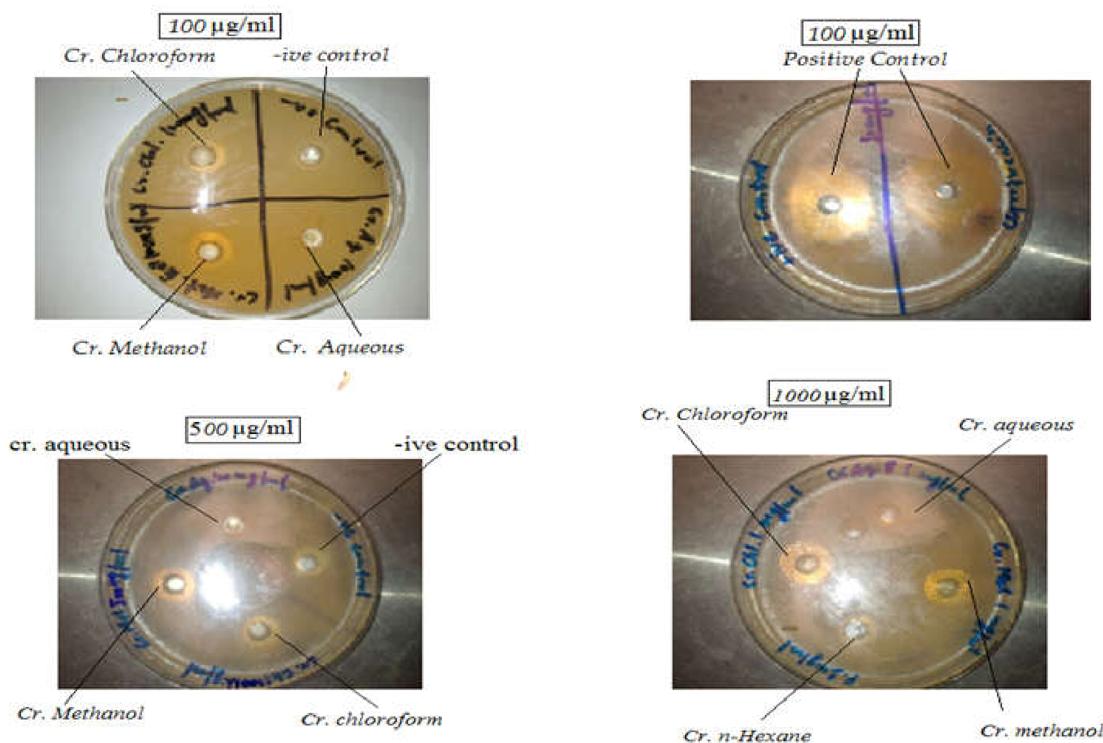

Figure 1. Anti *Salmonella typhi* Activity

12±1.7mm, 13.1±0.9mm, and 16.2±1.3mm, respectively at 1000µgml. Negative control (DMSO) was totally inactive against the *S. typhi* strains but showed a slight zone of inhibition 7.3±0.4mm at 1000µg/ml concentration against S. typhimurium. Results were compared with ciprofloxacin, used as the positive control. Methanol and Chloroform extracts were most active among all extracts but less active than positive standard (Ciprofloxacin). Crude n-hexane extract was completely inactive against *S. typhi* strains but showed little activity against *S. typhimurium* at 1000µg/ml concentration. MIC values of Cr. Aq *Berberis lycium* Root and stem bark extracts were in the range of 80-160 µg/ml, 80-160 µg/ml, and 40-80 µg/ml, against *S. typhi* ATCC 6539, *S. typhi* ATCC 14930, and *S. typhimurium* ATCC 14028, respectively. MIC values exhibited by Cr. methanol extract are 10-20 µg/ml, 10-20 µg/ml and 20-40 µg/ml, respectively. Cr. Chloroform extracts showed MIC of 10-20 µg/ml, 20-40 µg/ml and 20-40µg/ml, respectively against three strains. Lower MIC values exhibited by methanol and chloroform extracts showed Salmonella strains are more sensitive to methanol and chloroform extract.

DISCUSSION

Cr. Methanol, Cr. Chloroform and Cr. Aqueous extracts of *Berberis lycium* showed significant anti *Salmonella typhi* activity. Cr. n-hexane extract demonstrated no activity. Results reveal that crude methanol and crude chloroform extracts were the most active against *Salmonella typhi*. Results were consistent with study performed on aqueous, ethanol and pet. ether extract of *Berberis lycium* where aqueous and ethanol extracts being polar solvents exhibited higher zones of inhibition than pet. ether extract. Petroleum ether extract showed no activity at all (Hussain, 2011), Another study performed on hydroalcoholic extract of B. aristata, B. asiatica, B. chitria and B. lycium against *S. typhimurium* exhibited that B. lycium extract showed 47% activity against the gentamycin standard. Result was consistent with this study. Higher activity shown by methanol extract in our study is attributed to alkaloids. Methanol extract contains a large quantity of alkaloids due to its polar nature. Methanol extract of *Berberis lycium* is known to be rich in medicinally important quaternary alkaloid, Berberine.

Berberine possesses good anti bacterial activity against gram positive as well as gram negative bacteria. Good anti *Salmonella typhi* activity shown by Cr. Chloroform extract was interesting as chloroform dominantly contains some non polar compounds, while anti bacterial properties of *Berberis lycium* are associated mainly with alkaloids. This may be due to the presence of little amount of some tertiary alkaloids which being less polar are extracted in chloroform. n-hexane extract like pet. ether extract showed no activity. This indicated that non alkaloids have no role in showing anti *Salmonella typhi* activity. Bioactive constituents obtained from different species of genus Berberis are mostly the same. Anti *Salmonella typhi* effect of Crude methanol extract (20% in DMSO) of *Berberis baluchistanica* showed zone of inhibition 10.67±1.04mm (Kakar, 2012). This result is consistent with our findings. Antibacterial study of *Berberis aristata* conducted by disc diffusion method on gram positive and gram negative bacteria reveals no activity of aqueous and alcohol extract at 50 µg/ml against *S. typhi.* Polar solvent extracts were unexpectedly found completely inactive (Shahid, 2009). This result was not consistent with our study. This difference may be due to the fact that in our study *Berberis lycium* was studied at higher concentrations. i.e 100-1000 µg/ml and anti bacterial activity was performed using agar well method instead of disc diffusion method. Another study performed in Pakistan mentions that hot water extract of this plant is considered to be most effective against infectious diseases by herbal practitioners (Zabihullah, 2006). Our findings contradict this consideration. Methanol and chloroform extract proved better against typhoid than its hot water extract. Difference may be due to the decomposition of some polar compounds during the process of hot water extraction.

Conclusion

The results of the study confirm the present traditional use of the plant for anti typhoid effects. The plant has a great potential to serve as an alternative option to existing anti typhoid treatment. Further study and discovery of lead compound can help solving the increasing antibiotic resistance problem by providing an alternate.

Recommendations

In vivo studies and clinical trials should also be carried out to explore the potential of the plant extracts. Effort is needed to investigate the active constituents in methanol and chloroform extracts.

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