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Antibacterial effect of *Swertia chirata* against multi-drug resistant strains S. aureus and E. coli: *in vivo* and *in vitro* study

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Abstract

Misuse and overuse of antibiotics results in widespread multi-drug resistance. The major cause of increased mortality and morbidity rates is Multi-drug resistance to antibiotics and it becomes a global heath challenges now a days, therefore it becomes a necessity and need for researchers to seek for alternative and natural sources of antimicrobials. In the present study an experimental trial (*In-vitro* and *In*-vivo) was undertaken to examine the antibacterial activity of extracts of *Swertia chirata* leaf against multi drug resistant (MDR) *E.coli* and *S.aureus*. Broth dilution test for MIC and Disc diffusion method for antimicrobial susceptibility test were applied to investigate Antibacterial activity of plant methanolic extracts. Twenty five albino mice weighted between 180-200g were used for bacterial inhibitory activity (*In- Vivo).* Plant extract showed broad spectrum of antibacterial activity against *S.aureus* in comparison to *E.coli* and showed MIC values of 20µg/ml. *Swertia chirata* plant extract contains lesser inhibition zone against *E. coli* (8.7±0.80) and showed highest zone of inhibition against *S.aureus* (16.2±0.60). It can be concluded that *Swertia chirata* plant extract was significantly better ($P<0.05$) against *S.aureus* than *E. coli*. The number of bacteria in treated groups was very low, These findings have cleared demonstrated that the clearance of *bacteria* from the blood of infected mice by sub-MIC of plant extract was significant. the findings of present study provide a suitable evidences for the use of a drug composed of plant extract as a new generation of drugs to attack the antibiotic resistance of bacteria.

Keywords: Antibacterial activity, *Taxus baccata, Swertia chirata, Escherichia coli, Staphylococcus aureus*

INTRODUCTION

To combat infectious diseases, great achievements of modern science and technology is the discovery and development of antibiotics. (Ge *et al*. 2002; Nair and Chanda 2005; Neogi *et al.* 2008). However, resistance rate of pathogenic microorganisms is increasing with dangerous frequency against various antimicrobial agents. Recovery rate of resistant isolates while antibacterial therapy is growing universally (Cohen 2002; Hancock 2005). Today after 50 years of the widespread use of antibiotics many of them do not pack the same punch once they did. This is due to antimicrobial drug resistance which is defined as "Survival of the fittest" microorganism in the presence of drugs. The problem of antimicrobial drug resistance is not

new, but has increased during the last decade, creating a serious threat to the treatment of infectious diseases (Conly 2002). Frequently misuse and overuse of antimicrobials in many developing countries, had led to emergence of resistant pathogens which have been responsible for morbidity, mortality and cost of health care (Sharma *et al*. 2005).

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources; many of these isolations were based on the uses of the agents in traditional medicine. According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento *et al*. 2000). Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well-being (Iwu *et al.* 1999). Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds etc. (Gordon and David, 2001). As these plants and their products are known to possess various secondary metabolites, which showed significant inhibitory effect against the growth of pathogens, therefore, the plant and their products should be utilized to combat the disease causing pathogens. The screening of plant extracts and plant products for antimicrobial activity has shown that plants represent a potential source of new anti-infective agents (Amani *et al*. 1998; Salvat *et al*. 2001; Costa *et al*. 2008). Numerous experiments have been carried out to screen natural products for antimicrobial property (Martinez et al. 1996; Ateb and Erdourul 2003; Nair and Chanda 2006; Nair et al. 2007a; Ndhlala *et al.* 2009). Himalayas possess a rich, profuse and varied flora mainly due to the varied climatic and diverse ecological conditions. The Garhwal and Kumaon Himalayas have about 116 plant species as endemic and are considered as national heritage (Polunin *et al.* 1984).

Swertia chirata a medicinal plant indigenous to temperate Himalaya, belongs to family Gentianaceae, consist of 180 species. The trade name of *Swertia chirata* is chiretta. It is found at an altitude of 1200-3000 m (The Wealth of India, 1976; Kirtikar and Basu 1984; Pradhan and Badola 2010).It is effective against gastrointestinal infection (Mukherji 1953), used as antipyretic, hypoglycemic (Saxena and Mukherjee 1992; Bhargava *et al*. 2009; Verma 2013), antiperiodic, antifungal, (Chakravarty *et al.*1994; Rehman *et al*. 2001), hepatoprotective, anti-inflammatory (Banerjee *et al.*2000), antispasmodic (Saha and Das 2001), antibacterial (Joshi and Dhawan 2005), antioxidative (Scartezzini and Speroni 2000) and used to treat malaria and diabetes (Kumar and Staden 2016).

This study planned to investigate the antibacterial activity of *Swertia chirata* plant extract against MDR *S. aureus* and MDR *E. coli* (*In-Vitro* and *In-Vivo*).

MATERIALS AND METHODS

Plant material

Fresh Plant material were collected from Institute of Biotechnology, G. B. Pant University of Agriculture and Technology, Uttarakhand, India.Afterwashing under running tap water,plant material (leaves and fruits) were allowed to dry at room temperature for about 15 days. After complete drying, plant material were powderedby usingelectricalblenders andstored in air tight container in the refrigerator at 4ºC for further analysis.

Preparation of Extract

Dried powder (50 gm) of *Swertia chirata* leaves were placed inside two different porous cellulose thimbles and placed in an extraction chamber. Extraction was done by using 750 ml methanolfilled in the collection flask upto 72 hours. The extract was then concentrated and freed of solvent under reduced pressure at 40°C. the dried crude concentrated extracts were stored for further investigation at -4^{0}C.

Bacterial strains

Clinical isolates of bacterial strains (*S. aureus* and *E. coli*) were obtained from Microbiology Department (V.C.S.G.G.I.M.S & R, H.N.B. Base hospital, Srikot, Srinagar, Garhwal) in a lyophilized form. Nutrient agar slants at 4^{0}C were used to maintain the strains.

Antibacterial activities of plant extract *in- vitro*

Antibacterial activities of methanol extract of the plant was screened by Disk diffusion assay (Anon 1997, Bauer *et al.* 1966, NCCLS 2003) and minimum inhibitory concentration (MIC).

Disk Diffusion Assay

For disk diffusion method(Anon 1997, Bauer *et al.* 1966, NCCLS 2003), dried plant extracts was prepared by following protocol given in Gangoue*et al.* 2009. Cell suspension was made by culturing the bacterial strains (*S. aureus* and *E. coli)* on nutrient agar at 37^{0}Covernight. Cell suspension of both strains were then adjusted to 0.5 McFarland standards (108 CFU/ml). 0.1 ml of this suspension were allowed to poured and dispersed by a cotton swab on Muller Hinton agar. Plates were kept at room temperature and dried for at least 5 minutes before incubation. Now methanol extracts of the plant was added at 5, 10, 15 and 20 µg/ml concentrationon to a sterile paper disc of 5mm diameter and allowed to dry. On agar plate, each paper disc was gently tapped down to provide uniform contact. 20 µl of DMSO were poured onto a paper disk and used as a control. Now the plates were allowed for overnight incubation at 37^{0}C. The diameter of zones of inhibition were measured and same experiment was repeated thrice to maintained triplicates. Antibacterial activity was measured by calculating mean of the diameter of inhibition (mm). No inhibition zone was expressed as the absence of activity (Kohner *et al.* 1994, Mathabe *et al.* 2006).

Measurement of Minimal Inhibition Concentration (MIC)

The bacterial and fungal growth on the culture plate that is inhibited by the lesser concentration is known as the minimum inhibitory concentration (Shahidi 2004). The concentration at which no turbidity was seen is used to express the MIC value of the extract (Sambrook and Russell 2001, Ondruschka 2006). Broth dilution method was used to evaluate the MIC of the plant extract (Brantner and Grein 1994).

Antibacterial activities of plant extract *in-vivo*

In order to examine the antibacterial effect of methanolic extract of *S. chirata in vivo*, 25 Swiss albino mice (180-200g) obtained from Central university (H.N.B Garhwal Srinagar, Uttarakhand) were used, and allotted into five groups. Each group had five animals.

All mice were kept at the animal house of high altitude plant physiology research center, Srinagar, Uttarakhand, India in standard stainless steel plastic cages with wheat straw as bedding material. Animals were kept at temperature 25^{0}C ± 2^{0}C, at relative humidity 50% ± 20% having

12 hrs light and dark cycle. All the animal husbandry conditions for the use and care of laboratory animals were followed as per the guidelines. All the animals were provided with commercial mice lab diet. Standard food and water were provided to the animals ad libitum. This animal study was approved by institutional ethical committee (IEC).

Study design

Mice were allotted into five groups. Each group had five animals. After three to five day the plant extracts were given orally to the mice of the groups II and III. Standard dose were decided according to MIC and study was conducted for 14 days.
Three groups were injected subcutaneously and number of animals were used as follows:

I Group(control group): Mice of this group were injected with normal saline (0.1 ml)
II Group: Mice of this group were injected with suspension (100 µl) of *E. coli*(1x108 CFU/ml) and at the same time administered extracts of *Swertia chirata.*
III Group: Mice of this group were injected with suspension (100 µl) of *S. aureus* (1x108 CFU/ml) and at the same time administered extracts of *Swertia chirata.*

Blood Collection

After 24 hrs of the treatment 1 ml blood was collected from the treated groups daily and inoculation of it was done on nutrient agar plates. After that plates were allowed for incubation for 18-24 hrs at 37^0C.Efficacy of the treatment was evaluated by counting of emerged colonies.

Hematological analysis

The Haematological parameters like Haemoglobin, PCV, Neutrophils, Leukocytes (WBC), Total red blood cells (RBC), Monocytes, Eosinophils, Reticulocytes, Lymphocytes and platelet counts were determined at the beginning i.e 0 day, on the 5^{th} and 14^{th}day.

STATISTICAL ANALYSIS

Statistical analysis was done using MS excel. Each value is mean ± standard deviation (SD) of three replicates. ANOVA (Analysis of variance) were used for calculating P-value (Statistical significance) by applying level of significance($P<0.05$).

RESULTS AND DISCUSSIONS

Antibacterial Activity *In vitro*

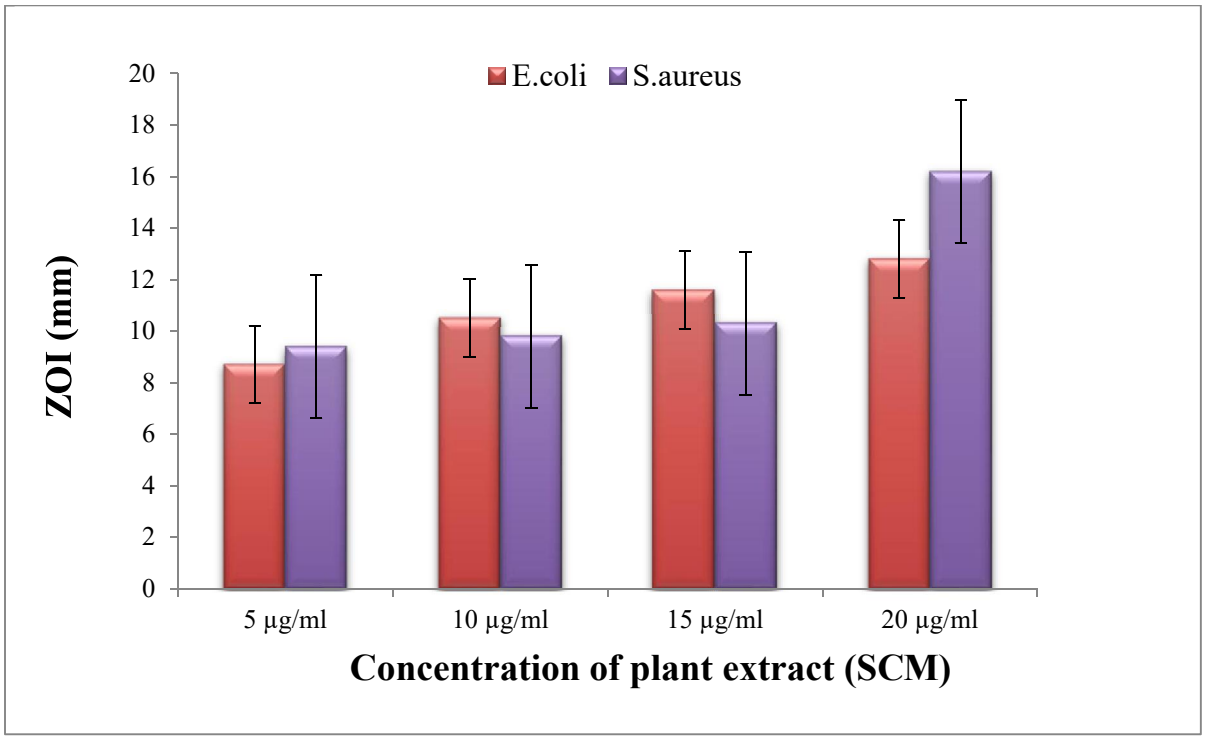
In this study, the MDR strains were sensitive to the antimicrobial activity of *Swertia chirata*. The methanol extracts of *Swertia chirata*(SCM) were active against *E. coli, S. aureus*. This result clearly indicate that the bioactive compounds responsible for the antibacterial activity were present in the plant extracts at different concentration 5, 10,15, 20 µg/ml (Table 1, figure 1). It is shown that methanolic extract of SCM have the highest effect on *S. aureus*having zone of inhibition (20.3 mm) and (16.2 mm) respectively ($p < 0.02$) and low zone of inhibition was seen against *E. coli.* (8.3±0.60 mm) and(8.7±0.80 mm). This difference of plant extract antibacterial activities between *S. aureus* and *E. coli* may be due to the difference occur in the outer membrane of both strains.

Table 1: Screening of Anti-bacterial activity from *Swertia chirata* Methanol extract tested against pathogenic bacteria

N0.	Plants extract	Concentration of plant extracts (µg/ml)	ZOI (mm)	
			E.coli	*S aureus*
1.	SCM	5 µg/ml	8.7±0.80	9.4±0.60
		10 µg/ml	10.5±0.51	9.87±0.40
		15µg/ml	11.6±0.59	10.3±0.50
		20 µg/ml	12.8±0.33	16.2±0.60

Each value is mean ±SD of three replicates.
ZOI: Zone of inhibition, **TBM:** *Taxus baccata*Methanol extract**SCM:** *Swertia chirata*Methanol extract

Figure 1: Graph showing Anti-bacterial activity of *Swertia chirata* plant methanol extract against *S. aureus* and *E. coli*



ZOI: Zone of inhibition, **TBM:** Methanolic extract of *Taxus baccata* **SCM:**Methanolic extract of *Swertia chirata*

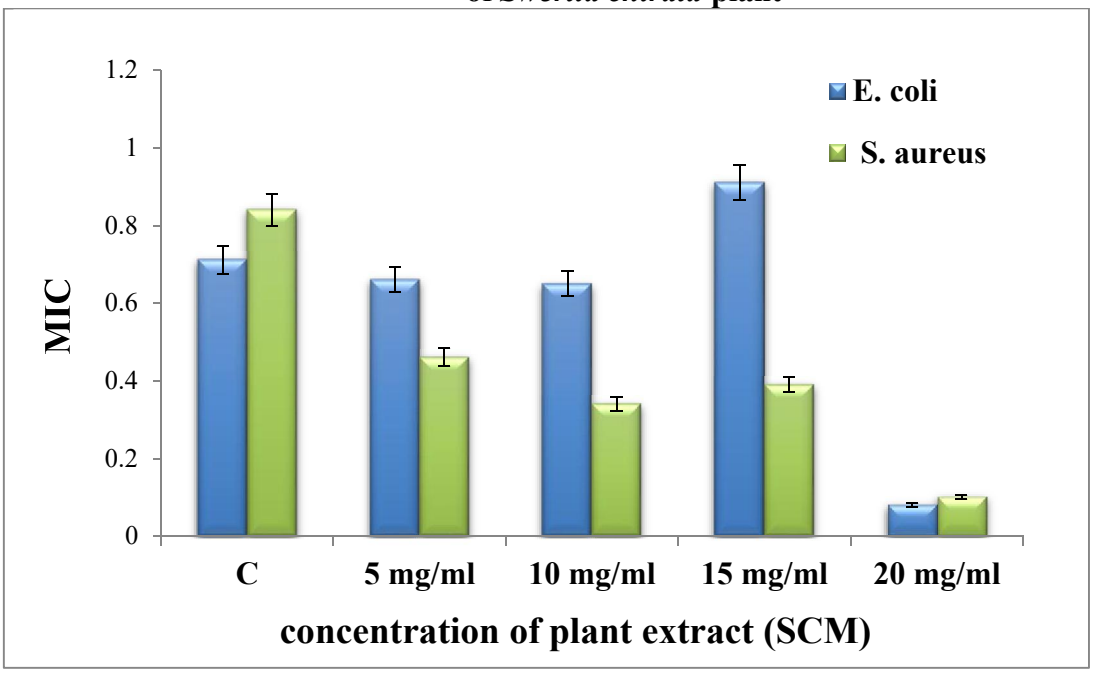
MIC (Minimum inhibitory concentration)

Broth dilution method was applied to determine the MIC of methanol extract and it is shown that the maximum activity was seen against *S. aureus* and *E. coli* at concentration of 20 µg/ml (table 2, figure 2).

Table 2: Minimum Inhibitory Concentrations of methanolic extract from the leaves of *Swertia chirata* against pathogenic bacteria

Test	Plants extract	Concentration	*E.coli*	*S aureus*
MIC	*Swertia chirata* SCM	C	0.71 ± 0.01	0.84 ±0.01
		5 µg/ml	0.66 ±0.02	0.46 ±0.01
		10 µg/ml	0.65 ±0.01	0.34 ±0.02
		15 µg/ml	0.91 ± 0.03	0.39 ± 0.03
		20 µg/ml	0.08 ± 0.02	0.10 ± 0.02

Figure 2: Graph showing Minimum inhibitory concentrations of methanolic leaves extracts of *Swertia chirata* plant



Antibacterial activity *in vivo*

According to results presented in (Table 3), it can be concluded that plant drug can be used against bacteria. Number of bacterial units measured in the mice blood samples are depicted in

table 3. Bacterial units was decreased in groups II, when mice of this group (infected with *E. coli*) were given methanolic extract of *Swertia chirata* followed by group III infected with *S. aureus* and treated with methanolic extract of *Swertia chirata*.

Table 3: Bacterial count from mice blood samples

Bacterial Count (CFU)	**Mice Groups**		
	I Group (Control)	II Group	III Group
After five day of infection	0	12	11
After giving plant extract	0	3	2

After 5 days from infection the value of total WBC raised for infected untreated while decreased for infected and treated mice after 14 days from infection and the mice become healthy. Nutrophil also raised after infection in the mice, after 14 days from infection the amount of nutrophil decreased when infected mice treated with plant extracts. These findings have cleared demonstrated that the clearance of *bacteria* from the blood of infected mice by sub-MIC of plant extract was significant.

Conclusion and recommendations

It can be clearly indicated from the present study that *S. chirata* has adequate potential to inhibit two pathogenic bacteria as it was seen from its strong inhibition against tested organisms. Now a day, many multidrug resistant strains are developing especially in hospital environment. To overcome drug resistance and to avoid side effects associated with the commonly available antibiotics, there is need of an alternative treatment method to cure such infections by use of traditional medicinal herbs like *S. chirata.* These herbs shows much promise in the development of phyto-medicine having antimicrobial properties and the drug derived from *S. chirata* may have the possibility of alternative medicinal source because of their antibacterial activity. This study also indicated that methanol extract of the plant have highest antibacterial activity against the MDR strain *S. aureus* than *E.coli.* leaf extracts of its constituents are clinically safe and economically cheap. In general, the conclusion of this study is that *S. chirata* plant methanol extract contains potential inhibition against bacterial growth *in vitro* and *in vivo*. Finally, recommendations were made for further investigation to be done for different plant parts fractionates on other bacteria for their antibacterial effects. The plant parts extraction should be done with other solvent types in order to evaluate enough quality and quantity of bioactive compounds.

The result of this study concluded that *Swertia chirata* plant extracts are safe and effective in animal models however more clinical trial are needed before licensing them as a drug and evaluating its particular therapeutic dose.

Conflict of interests

There is no conflict of interest declare by the authers.

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