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Comparison of Antimicrobial Properties of *Silybum marianum* (L) Collected from Ten Different Localities of Khyber Pakhtunkhwa Pakistan and Diversity Analysis Through RAPDs Pattern

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Abstract

The *Silybum marianum* was collected from ten different localities of Khyber Pakhtunkhwa Pakistan to investigate the effects of diverse environmental conditions on the antimicrobial profile and genetic diversity of the plant and to look into possible natural therapy. In antibacterial assay it was explored that the methanolic and *n*-hexane fractions of all the localities exhibited insignificant results, while the chloroform fraction showed profound results with inhibition percentage 58.8, 52.9 and 47.1 from Charsada, Karak and Bannu regions respectively against *E. coli* whereas the fractions from Bannu, Kohat and Lakimarwat showed 57.9%, 52.6% and 47.4% inhibition against *Vibrio cholerae*. The average percentage inhibition of the samples from Peshawar, North Waziristan, Abbottabad, Hangu and Lakimarwat was 45 percent against *Shigella* species. Moderate percentage of inhibition was noticed against *Staphylococcus aureus* by the fractions of Lakimarwat (42.1%), each 36.8 % for Waziristan and Abbottabad. No antifungal activity was showed by any fraction used. Using RAPP markers OPE7, the genetic diversity analysis results revealed two monomorphic bands of 600bp and 450bp for all the samples of *Silybum marianum* collected from different locations. All the bands observed have no differentiateable association with antibacterial and antifungal profiles. The bioactive fractions of the plants can be used against various microbial infections and can be further used for the isolation of potentials natural product for drug development.

Keywords

Silybum marianum, Antimicrobial, Antifungal, Drug Sighting, Genetic Diversity, RAPDs

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1. Introduction

Silybum marianum (L), milk thistle, is an annual or biennial herb. Its stem is 20 to 150 cm in height while its leaves ranged from 25 to 50 cm long and 12 to 25 cm wide. It belongs to the family Asteraceae having white and blue capitulum. The fruit are hard skinned achene with brown spots and are 15 to 20 mm long (Nasir, 1990). It is indigenous to North America, Asia, Southern Europe and Russian Federation. It is naturalized to South and North America, Australia, China, Central Europe (Burgess, 2003). In Pakistan, it is found wild in Khyber Pakhtunkhwa and Punjab (Shah *et al.,* 2011). The plant is known for its medicinal properties having important chemical constituents including several flavonolignans collectively

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known as silymarin. Silymarin has antioxidant properties and used in various hepatic disorders, including hepatotoxicity secondary to acute and chronic viral hepatitis and mushroom poisoning. (Sayyah *et al*., 2011)

It is hypothesized that association of the medicinal properties of *Silybum marianum* with RAPDs pattern may be helpful for the development of RAPD markers which can be used further to analyze medicinal properties in any endangered medicinal plant populations. The research was planned to observe the environmental effects on the antimicrobial profile of the plant and to see the association between the bioscreening and molecular marker used.

2. Materials and Methods

2.1. Plant Species and the Extraction

Silybum marianum were collected from ten different localities of Swat, Abbottabad, Peshawar, Charsada, Kohat, Hangu, Lakimarwat, Bannu, Karak and Waziristan of Khyber Pakhtunkhwa. Plants were identified by using taxonomical key of Asteraceae (Nasir 1990, Flora of Pakistan), followed by deposition of the voucher specimens at the Herbarium of Department of Botany, Kohat University of Sciences and Technology Kohat. The aerial parts of plants were dried in the shade at room temperature, powdered using Waringer blender and were loaded to soxhlet apparatus. The extraction was carried out using solvents such as purified methanol for 8 to 10 hours for each plant. The resulting mixture were then filtered and concentrated under vacuum at 40ºC (Buchi, Rotavapor R-210, Labortechnik, AG, Flavil, Switzerland). Methanol, *n*-hexane and chloroform extract/fractions were filter-sterilized and pipetted to biological assay discs at different concentrations to find their LD^{50}. The filter-sterilized solvents without extracts were used as negative controls and antibiotics as positive control.

2.2. Preparation of Plant Extract

The plant specimens were dehydrated and trodden to a fine residue. Around 70g powder of each plant was drenched in 550 ml of solvents (methanol, n-hexane and chloroform). The concoctions were reserved for two weeks at ordinary temperature. The jumbles were incessantly shaken two fold for a day throughout the procedure. After three weeks the drenched plant stuff was clean by usual sift paper and then by Whattmann filter paper no 41. The filtrates were then practiced by rotary evaporator to acquire semisolid extracts (Ahmad *et al.,* 2015).

2.3. Evaluation of Bioassays

2.3.1. Antibacterial Analysis

For antibacterial activities agar diffusion technique was used with little modifications as described by Khan *et al.,* (2011). In this scheme, wells were prepared in petriplates and the required concentration of stock solution were poured in these wells and after incubation of 24 hours, the halo zones were found around these wells which were measured and compared with the zones made around the standard antibiotic used.

2.3.2. Antifungal Activity

Agar tube dilution technique as reported by Khan *et al.,* (2011) with minor amendment was used for antifungal activity. These pathogens (*Fasarium*, *Aspergillus* and *Alternaria* spp.) were retained at 4°C on Sabouraud Dextrose Agar (SDA) media. 12 mg of every extract (methanol, n-hexane and chloroform) of plant specimens were mixed in 1ml of satirized distilled water to obtain 200 µg/ml. 12 mg of Terbinafine was taken in 1ml of autoclaved distilled water to arrange stock solution of antibiotic which was used as positive control.

2.3.3. Molecular Characterization

Silybum marianum was collected from ten different regions of Khyber Pakhtunkhwa. Young leaf tissues were collected and immediately grinded in CTAB buffer. Total genomic DNA was extracted from 0.1 g of leaves of each accession using standard DNA isolation method (Doyle and Doyle, 1990).

3. Results and Discussion

3.1. Antibacterial Activity of *Silybum marianum*

Approximately 80% of the developing people use medicinal plants to indulgence different disorders (Ashokkumar *et al*., 2010). It was projected from the expansion of the chemotherapeutic tactics that contagious diseases could be utterly healed but drug defiant bacteria bent an immense hitch so it is very vital to select suitable and secure antimicrobial mediators (Saga and Yamaguchi, 2009).

The present research work is a stride to genus out a harmless foundation of some antimicrobial agents. Through present analysis, the crude/methanolic, *n*-hexane and chloroform extracts/fractions of 10 chosen plant samples were tested against five bacterial species (both Gram +ve and – ve) and zone of inhibition were determined. The inhibition region for Chloromphenicol (antibiotic as control) was also firmed as standard.

The results presented in the present study revealed that crude extracts and n- hexane fractions of *Silybum marianum* at concentration of 15 mg/ml showed insignificant activities against all tested bacterial pathogens.

It was noticed that antibacterial profile of chloroform fraction of *Silybum marianum* collected from different locations against

Salmonella spp. was found highest for Peshawar and Kohat respectively (15 mm) having 37.5% inhibition while it was found lowest for Lakimarwat (10 mm). Antibacterial activity for Swat, Karak and Charsada against *Salmonella spp*. was found absent. Similarly for *Shigella spp*. it was found highest (20 mm) for Peshawar and Waziristan. For *E. coli* it was found highest (16 mm) for Bannu and Kohat while for *Staphylococcus aureus* it was found highest (16 mm) for Lakimarwat. In the same way, for *V. cholerae* it was found highest for Bannu (22 mm) having 57.9% inhibition followed by Kohat (20 mm) having 52.6% inhibition as shown in Table 4.1. Chloromphenicol was evaluated as positive control for these bacterial species showing % inhibition highest (100 mm) for *Salmonella spp*. and *Shigella spp*. as shown in Table 4.1. Plants are exercised as drugs owing to the occurrence of broad variety of biologically vigorous molecules. Therapeutic significance of the plants stand on the attentiveness of energetic molecules creating them an affluent spring of diverse medicines which play a prevailing task in the safeguarding of human fitness (Edeoga *et al*., 2005).

Table 4.1. Antibacterial Profile of Chloroform Fraction of *Silybum marianum* collected from different locations.

LOCATION	Antibacterial activity against bacterial strains (mm and %)									
	Salmonella spp.	% Inhibition	Shigella spp.	% Inhibition	E. coli	% Inhibition	Stap. aureus	% Inhibition	V. cholerae	% Inhibition
Peshawar	15	37.5	20	50	14	41.2	12	31.6	12	31.6
Abbottabad	12	30	18	45	12	35.3	14	36.8	14	36.8
Wazirstan	12	30	20	50	12	35.3	14	36.8	12	31.6
Hangu	14	35	16	40	12	35.3	12	31.6	16	42.1
Lakimarwat	10	25	16	40	14	41.2	16	42.1	18	47.4
Bannu	14	35	14	35	16	47.1	12	31.6	22	57.9
Kohat	15	37.5	12	30	16	47.1	12	31.6	20	52.6
Swat	---	---	12	30	14	41.2	14	36.8	14	36.8
Karak	---	---	15	37.5	18	52.9	14	36.8	12	31.6
Charsada	---	---	14	35	20	58.8	10	26.3	12	31.6
Chloromphenicol (Positive Control)	40	100	40	100	34	88	38	96	38	96

Table 4.2. Antibacterial Profile of *n*-Hexane Fraction of *Silybum marianum* collected from different locations.

LOCATION	Antibacterial activity against bacterial strains (mm and %)									
	Salmonella spp.	% Inhibition	Shigella spp.	% Inhibition	E. coli	% Inhibition	Stap. aureus	% Inhibition	V. cholerae	% Inhibition
Peshawar	---	---	4	10	---	---	---	---	---	---
Abbottabad	---	---	---	---	---	---	---	---	6	15.8
Waziristan	---	---	---	---	---	---	---	---	2	5.3
Hugu	---	---	3	7.5	---	---	---	---	---	---
Lakki Marwat	---	---	---	---	---	---	---	---	---	---
Bannu	---	---	---	---	---	---	3	7.9	5	14.2
Kohat	---	---	---	---	---	---	---	---	---	---
Swat	---	---	5	12.5	---	---	---	---	---	---
Karak	---	---	---	---	---	---	---	---	---	---
Charsada	---	---	---	---	---	---	---	---	---	---
Chloromphenicol Positive Control	39	98	40	100	40	100	38	96	38	96

Table 4.3. Antibacterial Profile of Methanolic Extract of *Silybum marianum* collected from different locations.

LOCATION	Antibacterial activity against bacterial strains (mm and %)									
	Salmonella spp.	% Inhibition	Shigella spp.	% Inhibition	E. coli	% Inhibition	Stap. aureus	% Inhibition	V. cholerae	% Inhibition
Peshawar	6	16.2	10	25.6	6	15.0	8	20.5	10	25.6
Abbottabad	8	21.6	6	15.4	5	12.5	7	17.9	11	28.2
Wazirstan	5	14.5	7	17.9	5	12.5	10	25.6	13	34.3
Hangu	6	16.2	8	20.5	6	15.0	10	25.6	10	25.6
Lakimarwat	3	8.1	3	7.7	4	10.0	7	17.9	10	25.6
Bannu	5	14.5	6	15.4	6	15.0	8	20.5	12	30.8
Kohat	8	21.6	6	15.4	6	15.0	7	17.9	15	38.5
Swat	7	18.9	3	7.7	5	12.5	7	17.9	10	25.6
Karak	6	16.2	2	5.1	4	10.0	6	15.4	12	30.8
Charsada	7	18.9	3	7.7	4	10.0	9	24.1	12	30.8
Chloromphenicol Positive Control	37	97	39	98	40	100	39	98	39	98

Table 4.2 showed the antibacterial profile of n-Hexane fraction of *Silybum marianum* collected from different locations. It was analyzed that antibacterial activity for most of the bacterial species was almost negligible. For *Salmonella spp.* and *E. coli,* antibacterial profile for *Silybum marianum* samples collected from different localities was not measureable while for *Shigella spp.,* it was only found for Peshawar (4 mm), Hangu (3 mm) and Swat (5 mm). Similarly, for *V. cholerae* antibacterial activity was observed for Abbottabad (6 mm) having 15.8% inhibition while for *Staphylococcus aureus* the activity (3 mm) was only noticed for Bannu collection having 7.9% inhibition.

The methanolic fraction of various samples determined insignificant below (50% inhibition) activities against all bacterial strains. The maximum percentage of inhibition was 38. 5 exhibited by Kohat samples against *Vibrio cholerae* followed by Waziristan samples with 34.3 percent of inhibition and the lowest percentage of inhibition was 25.5 against *Vibrio cholerae* showed by samples from Peshawar, Hangu, Swat and Lakimarwat (Table 4.3). The samples from Hangu and Waziristan projected percentage of inhibition was 25.6 against *Stap. aureus* and the lowest percentage of inhibition was 15.7 exhibited by Karak samples against the same pathogens. The highest percentage of inhibition was 15.0 against *E. coli* by the samples from Peshawar, Hangu, Bannu and Kohat while lowest percentage of inhibition was 10 presented by Lakimarwat, Charsada and Karak samples. The 25.6 percent inhibition was showed by samples from Peshawar followed by samples from Hangu with 20 percentage of inhibition and the lowest percentage of inhibition was given by Karak samples (5.1%) against *Shigella spp.* The maximum percentage of inhibition was 21.6 against *Salmonella* exhibited by Abbottabad and Swat samples and the samples from Lakimarwat showed minimum inhibition of 8.1 percent against *Salmonella* (Table 4.3).

From the findings it can be incidental that only the chloroform fractions of *Silybum marianum* were most effective against *Escherichia coli, V. cholerae* and *Shigella* as compared to standard drug so they could be utilized in the formulation of bioactive antimicrobial drugs. Dabur *et al*., (2007) explored seventy seven diverse plant extracts from twenty four plants beside eight bacterial species via microbroth dilution test. They establish that water extracts of *Acacia nilotica*, *Justicia zylanica*, *Lantana camara* and *Saraca asoca* reveal fine activity against all the pathogenic bacterial species and the MIC was confirmed in the series of 9.37 to 37.5 µg/ml. They also described the antimicrobial profile of *Woodfordia fruticosa* with MIC range of 75 - 1200 µg/ml. Likewise, Bajracharya *et al*., (2008) firmed the antibacterial assets of plant extracts against diverse strains of entero pathogenic bacteria like *E. coli*, *Klebsiella sp, Citrobacter* sp, *Enterobacter* sp, *Salmonella typhi, Salmonella paratyphi*. They also noted that *Woodfordia fruticosa* was established valuable against all the enteric bacteria.

3.2. Antifungal Activity of *Silybum marianum*

This dilemma annoyed the scientists of today to locate novel antibiotics from other sources including medicinal plants (Shinwari *et al*., 2013). Considering the issue, during present study, the crude/methanolic, n-hexane and chloroform extract/fractions of ten selected plant samples of *Silybum marianum* collected from different localities, were analyzed against the three fungal strains.

Results obtained revealed that extracts of *Silybum marianum* for the samples collected from different localities of Khyber Pakhtunkhwa were not found effective against any of the fungal strains. The results were not promising and showing no significant zone of inhibition against the fungal strains which confirm the results of Shah *et al.,* (2011), who reported that fungus is resistant to crude extracts of *Silybum* plant.

3.3. RAPD-PCR Results

Polymerase Chain Reaction (PCR) was carried out to investigate the genetic variation of *Silybum marianum* collected from different localities of Khyber Pakhtunkhwa and their association with antibacterial and antifungal profiles.

Using RAPD markers OPE7, the genetic diversity analysis results revealed two monomorphic bands of 600bp and 450bp for all the samples of *Silybum marianum* collected from different locations. All the bands observed have no differentiate-able association with antibacterial and antifungal profiles. Ren, *et al*., (2005) suggested low genetic level of diversity among populations of the rare Chinese endemic *Kingdonia uniflora* by using RAPD analysis. They further highlighted the fact that UPGMA analysis and the Mantel test was not able to reveal genetic differentiation between populations and have no association with geographic distribution pattern.

4. Conclusion

It is concluded from the present investigation that the chloroform fraction of the *Silybum marianum* could be use as a potential source of natural antibiotics against bacterial infections while the fungi are highly resistant to the extract of *Silybum* plant. Moreover no association was observed in

between the antimicrobial profile and genetic makeup of plant using RAPD marker OPE-7.

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