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RELATIONSHIP BETWEEN DIFFERENT AGRO-CLIMATIC CONDITIONS AND SILYMARIN PRODUCTION IN WILD MILK THISTLE (*SILYBUM MARIANUM* L. GAERT.) IN PAKISTAN

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Abstract

Milk thistle (*Silybum marianum* L. Gaert.) is an important medicinal plant that have a complex of flavonolignans mainly silymarin, which is used in the pharmaceutical industry to prepare medicines against liver diseases. Mostly, the seeds collected from wild plants are used to extract silymarin contents, although, it is also produced in leaves and roots. Silymarin contents can vary under different agro-climatic regions. This study was aimed with a novel idea to find the relationship between varied agro-climatic conditions and silymarin production that has not been established so far. To evaluate these aspects, wild plants of milk thistle were collected from five different agro-climatic regions of Pakistan during two seasons 2018-19 and 2019-20. Fluctuating agro-climatic conditions significantly changed the antioxidant activities, ion accumulation and production of silymarin contents in milk thistle. Silymarin contents were determined using high performance liquid chromatography (HPLC) from all the parts of milk thistle. Higher contents of silymarin (2.01 to 3.19%) were present in seeds and lowest concentration of silymarin contents was observed in roots (0.021 to 0.094%) and leaves (0.121 to 0.721%). Higher silymarin contents (3.19%) was found in seeds during 2019-20 at region Dina. This region was relatively different in agro-climatic conditions due to high rainfall and humidity, low temperature, soil Ec and pH. There was a positive correlation of silymarin contents with average rainfall and soil organic matter that was directly linked with silymarin production. While, temperature, humidity %, soil Ec and pH showed negative correlation with silymarin production. It was concluded that the production of silymarin consents have strong relationship with agro-climatic regions. These outcomes can be imperative for the collection and cultivation of milk thistle.

Key words: Milk thistle, Ion contents, Silymarin, Antioxidants.

Introduction

Silybum marianum (L. Gaert.) is a native of Mediterranean territory and is growing wild as well as cultivated throughout the world (Abenavoli *et al*., 2018). Purpose for the cultivation of this plant is to produce silymarin contents at commercial scale (Alemardan *et al*., 2013). It is also used in generation of bioenergy as well as oil from seeds (Dominguez *et al*., 2017). Silymarin complex typically comprises 36.3% of silybin, 15.7% silychristin, 5.9% of silydianin, and 5.1% of isosilybin that is a liver tonic (Sersen *et al*., 2006). Pakistan lies in the temperate zones which are compatible for cultivation as well as growth of milk thistle. The latitude of Pakistan is 30.3753° N, and longitude is 69.3451° E. Provinces of Punjab and Khyber Pakhtunkhwa are suitable for its better growth (Rahman *et al*., 2016).

Studies related to milk thistle revealed that the growth and crop yield strictly depend upon climatic and environmental conditions and optimum crop cultivation should, therefore, be in accordance with rainfall and temperature regime (Karkanis *et al*., 2011). The environmental factors effect on seed maturation and agronomic quality of Milk thistle (Wong *et al*., 1986). Different ecological factors for example, temperature and rainfall created variation in active constituents (Ghimire *et al*., 2006; Kumar *et al*., 2007; Ahmad *et al*., 2008 and 2009). The plants of milk thistle form a unique and strong root system as a result of which can be cultivated in light soils having periodic deficiency of water. It is adapted to grow on wide range of types of soils that is from sandy soil to substantial clay soils (Karkanis *et al*., 2011). Wild Milk thistle needs tolerance towards huge range of pH but its growth is best in soil having pH from 5.5-7.6 (Andrzejewska *et al*., 2011) with sufficient supply of nutrients produce active substances rich seeds.

Milk thistle reacts strongly to the system of thermal and moisture conditions. Deficiency and also the excess of water inhibits the accumulation of silymarin. According to Hammouda *et al*., (1993), higher accumulation of flavonolignans was noted in milk thistle when the moisture of the soil was 60–65%. Organic fertilizers or soil organic matter also play a significant role in restoration maintenance of humidity, decreasing of wind and water erosion, physical improvement of the soil structure and also cause in increased number of the beneficial organisms in the soil (Prakash *et al*., 2007). Milk thistle is a source of natural silymarin hence its cultivation has become an urgent demand now a days. The quality of final product is influenced by conditions of cultivation that influences the production of high quality milk thistle achenes (Spitzova & Stary, 1985). Activities of enzymes (CAT, POD and SOD) are a significant index to foretell the plant responses to the changing environments (Sen & Mukherji, 2009). In plants, antioxidants activities may act like a defense line for many troublesome condition (Lohrmann *et al*., 2004).

This study was designed to find out the relationship of various agro-climatic with silymarin production in milk thistle for sustainable plant cultivation that has not been established so far.

Materials and Methods

Wild Milk thistle (*Silybum marianum* L. Gaert.) plants were collected from 5 different regions of Punjab, Pakistan (Gujrat, Mandi Bauhudin, Islamabad, Rawalpindi and Dina) during emergence season 2018-19 and 2019-20. Regions lie at Latitude: 32° 34' 22.22" N and Longitude: 74° 04' 44.29" E (Fig. 1). Four plants were collected randomly from the away of road side that have no influence of automobile and industries pollutants. Meteorological data of these regions for whole experimentation period were noted with the help of Pakistan Meteorological Department (PMD), Pakistan. Soil samples were also collected from these regions for the study of soil characteristics with the help of Punjab Agriculture Soil Testing Labs, Gujrat, Pakistan.

Estimation of antioxidant activities: Chance & Maehly (1955) procedure was used to determine the estimation of antioxidant activities such as catalases (CAT), peroxidases (POD) and superoxide dismutase (SOD). For this purpose, 5g fresh leaves were crushed in 5ml of 50mM cooled Phosphate buffer having pH 7.8 and sample was poured into tubes and placed in ice bath for antioxidant enzymes extraction. After that the crushed samples were then centrifuged for 20min at 15,000RPM at 4°C. Supernatant was used for the purpose of determination of different activities of enzymes. CAT solution (3ml) was made to determine the catalase enzymes by adding phosphate buffer (50mM) having pH 7.0, 0.1ml enzyme extract and 5.9Mm H_2O_2. At 240nm, a change in the absorbance of CAT solution was determined. POD solution (3ml) was prepared by the addition of 50mM phosphate buffer having pH 5.0, 20mM guaicol, 40 Mm H_2O_2 and 0.1ml extract of enzyme. At 470nm, a change in the absorbance of the POD solution was determined. A solitary POD action was characterized utilizing an assimilation move of 0.02 units every moment. SOD activity was dictated by estimating the restraint in photo reduction of nitroblue tetrazolim by SOD enzyme. The SOD reaction was carried out by exposing the reaction mixture (containing 50 mM of phosphate buffer (pH 7.6), 0.1 mM EDTA, 50 mM sodium carbonate, 12 mM L-methionine, 50 µM NBT, 10 µM riboflavin and 100 µL of crude extract in a final volume of 3.0 mL) to while light for 15 min for incubation at room temperature then absorbance was noted at 560 nm by spectrophotometer. SOD (one unit) was the amount of enzyme causing 50% inhibition of photochemical reduction of NBT.

Ionic studies: The dried plant material was first ground into small pieces with pestle and mortar and placed in test tubes and 2 ml of conc. H_2SO_4 were added and incubated at room temperature unisng Wolf technique (1982). Then H_2O_2 (1ml) (35% Analar reagent extra pure) was poured down with the help of the sides of the digestion tubes and rotated them. After the fulfillment of the reaction, the tubes were sited in a specialized block called digestion block and heated till 350^{o}C. Process was repated by adding H_2O_2 (1 ml) till to get colorless. End volume of extract was made 50 ml with distilled water and used for the determination of NPK contents.

Total nitrogen contents were determined by Kjeldhal method (Bremner, 1965). Digested aliquot (5 ml) was poured into Kjeldhal flask and placed on the Kjeldhal ammonia distillation unit, after that 10 ml of 40% NaOH solution was added and nearly connected the flask to distillation apparatus. After that, 100 ml solution was taken in conical flask and few drops of mixed indicator was added with 5 ml of 2% boric acid solution. Distillation procedure was stoped when the distillate was about 40-50 ml. Conical flask then placed for cooling and titrated with 0.01 N standard H_2SO_4 until the pink color was appeared. Nitrogen was calculated by the following formula:

$$N\% = \frac{\text{Acid used for titration (ml) x Normality of acid x Correction factor (1.4007)}}{\text{Weight of sample (g)}}$$

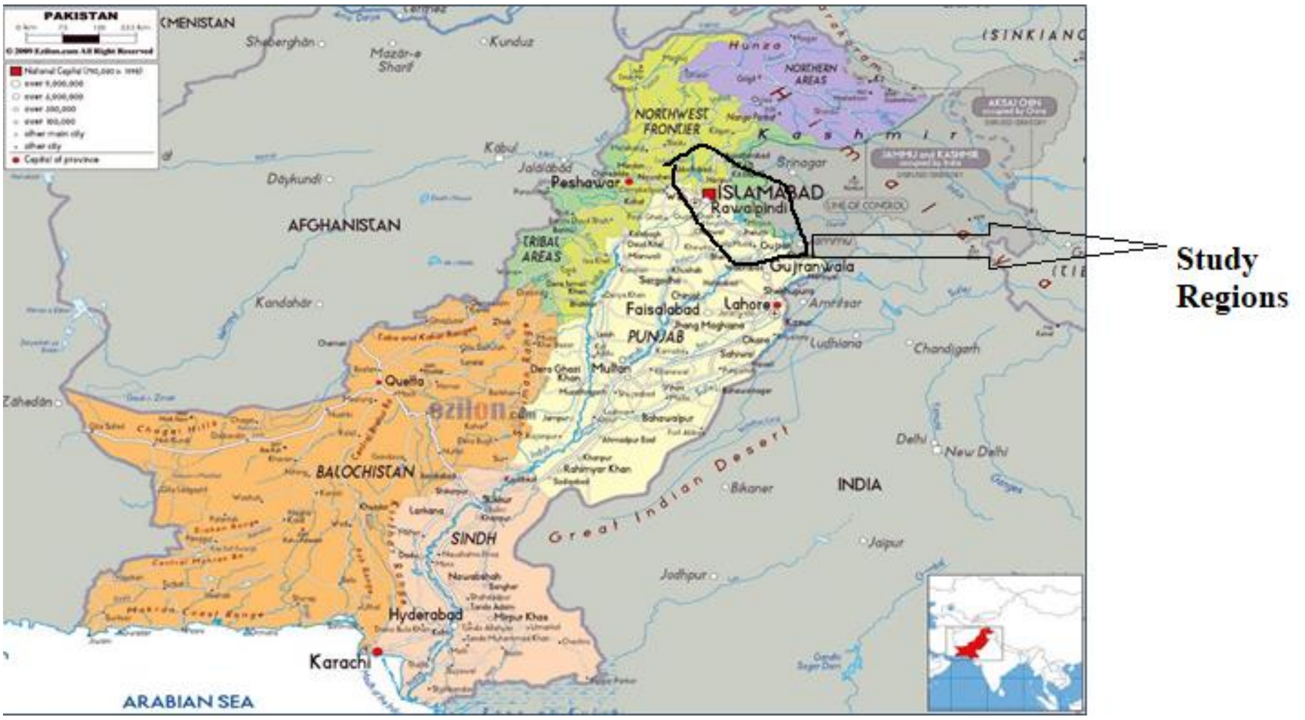

Fig. 1. Map of study area of Pakistan (source: www.worldometers.info).

Phosphorus was determined with spectrophotometer (Hitachi-220). Extracted material (5ml) was prepared and dissolved in 2 ml of Barton reagent and final volume was made upto 50 ml. The samples were placed for ½ hour and values of P were calculated using standard curve. Optical density was read at 460nm. Barton reagent was prepared as described by Jackson (1962).

Determination of silymarin: Silymarin contents from seeds, aerial parts (leaves and stem) and roots were extracted in 250mL of methanol using Soxhlet apparatus for 4 hrs. Extract was dissipated to dryness at 40^{o}C under vacuum and it was reconstituted in 25ml of HPLC (high performance liquid chromatography) grade methanol. 1ml extract (reconstituted) was diluted with methanol upto 23ml for the determination of silymarin. Silymarin was determined with the help of HPLC (HP 1100 Liquid Chromatograph, Hewlett, Germany) at PCSIR Labs, Lahore Pakistan. Separation of silymarin on chromatograph was completed using Reprosil Gold C-18 column (5.0 µm, 250×4.6 mm) at 40^{o}C by injecting 20µL of sample as describe by Arampatzis *et al.*, (2019). For solvent system purpose, formic acid (water containing 0.1%) and methanol was used. Inclination elution was conducted with expanding methanol add up to water at 1mL/min stream rate and identification was made at 288nm. At the end Silymarin standard (Sigma-Aldrich) was used when dissolving in methanol for the determination of retention time for each silymarin constitute (Fig. 2). Calibration curves were made by Silybinin standard (Sigma-Aldrich, Germany) in methanol. Silymarin contents were calculated by its peak area and the calibration curve.

Data analysis

Data were subjected to analysis of variance for three factor factorial in Minitab (Version: 19.2.0, Coventry, UK) and means were compared at $p \leq 0.05$ using Tukey's test.

Results

Agro-climatic and soil conditions: Mean data related to climate and soil properties is given in Tables 1 and 2. Data was collected for those months in which milk thistle completes its life cycle (October to April). All the regions had varied rainfall, average minimum and maximum temperature and humidity from each other mainly the region Dina that was relatively different in climatic conditions. Overall, higher rainfall (838mm) was noted in region Dina and lowest (513mm) rainfall was noted in Mandi Bauhudin. Higher average temperature (24.5^{o}C) was in region Islamabad and lowest in region Dina (16^{o}C). This region also had maximum humidity (84%). Soil was analyzed from theose regiosn from where milk thistle plants were collected. There was clay loam and sandy loam soils in most of the regions except Dina, it had loamy soil with low Ec, pH and high organic matter as compared to the soils of other regions (Table 1).

Antioxidant activities: Antioxidant activities i.e., catalases (CAT), peroxidases (POD) and superoxide dismutase (SOD) were determined under varied agro-climatic conditions. Effect of various agro-climatic regions had highly significant effect ($p<0.001$) for CAT activity (Table 3). There was also significant results for year x region interaction. Higher CAT activity was determined in the plants collected from Dina region and lowest CAT activity was present in the plants collected from Mandi Bauhudin (Fig. 3a). It was noted that impact of various agroc-climatic conditions was also highly significant ($p<0.001$) for POD and SOD activities (Table 2). It was noted that higher POD and SOD activities were present in those milk thistle plants which were collect from Dina region and lowest POD and SOD activities were in the plants taken from Islamabad region (Fig. 3b-c).

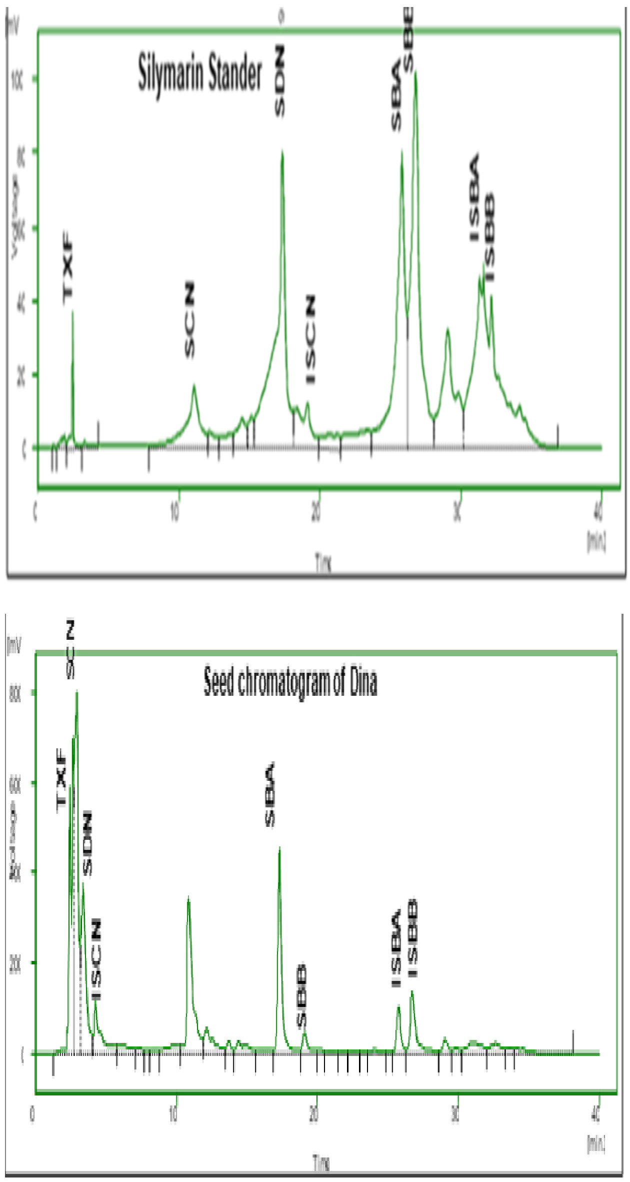

Fig. 2. HPLC Chromatograph of silymarin constituents (TXF: taxifolin; SDN: silydianin SCN: silychristin; ISCS: isosilychristin; SBA: silybin A; SBB: silybin B; ISBA: isosilybin A; ISBB: isosilybin B).

Table 1. Climatic data of various regions for 2018-19 and 2019-20.

Region	Average rainfall (mm)		Average temperature (°C)		Humidity (%)	
	2018-19	2019-20	2018-19	2019-20	2018-19	2019-20
Gujrat	677	706	21	20	70	76
Mandi Bauhudin	513	576	26	21.8	69	74
Islamabad	789	809	24.5	22.9	58	60
Rawalpindi	793	805	20.5	22.7	65	67
Dina	817	838	17.4	16	80	84

Table 2. Soil properties of various regions for 2018-19 and 2019-20.

Region	Soil texture		EC (dS/m)		pH		Organic matter (%)	
	2018-19	2019-20	2018-19	2019-20	2018-19	2019-20	2018-19	2019-20
Gujrat	Clay loam	Clay loam	0.92	0.88	7.3	7.6	0.71	0.77
Mandi Bauhudin	Clay loam	Clay loam	0.79	0.74	6.5	7.0	0.51	0.57
Islamabad	Sandy loam	Sandy loam	1.06	1.02	7.2	7.5	0.52	0.57
Rawalpindi	Sandy loam	Sandy loam	1.10	1.07	6.9	7.6	0.50	0.55
Dina	Clay loam	Clay loam	0.70	0.65	7.1	7.2	0.79	0.85

Table 3. Means squares (MS) from analysis of variance (ANOVA) for antioxidant activities of Milk thistle in response to different agro-climatic conditions.

Source	df	Catalase activity (CAT)	Peroxidase activity (POD)	Superoxide dismutase activity (SOD)
Region (R)	4	0.4321***	0.47289***	0.06421***
Year (Y)	1	19.6723*	3.6721*	0.0534*
R x Y	4	0.2913*	0.1722*	0.0618*
Error	30	0.0468	0.01031	0.0052
Total	39			

ns= Non-significant; *,**, *** = Significant at $p\leq0.05$, 0.01, or 0.001, respectively

Ion contents: Means square from ANOVA related to nitrogen, potassium and phosphorus (NPK) accumlation in different parts of the milk thistle is given in Table 4. Effect of various agro-climatic regions was highly signficant ($p\leq0.001$) for the accumlation of N contents in roots, leaves and seeds. High N accumulation was noted in roots and leaves for those plants collected from Dina region (Fig. 4a). Overall, there was higher N contents in roots and leaves as compared to seeds. There were lowest N contents in the plants collected from Rawalpindi region. Effect of varied agro-climatic conditions had highly signficant ($p\leq0.01$, 0.001) results for K accumulation in milk thistle (Table 4). There was a high accumulation of K in roots of milk thistle. Higher K contents were found in those plants collected from Dina region and lowest contents were present in the plants growing in Rawalpindi region (Fig. 4b). ANOVA for P contents revealed that different regions had highly signficant results ($p\leq0.01$). All the interactions were also significant in response to varied regions (Table 4). Seeds had lowest accumulation of P as compared to roots and leaves. Higher P contents were noted in roots and leaves for those plants collected from Dina region and lowest accumulation was in the plants taken from Rawalpindi (Fig. 4c).

Silymarin contents : ANOVA related to silymarin contents showed that the effect of agro-climatic regions was highly significant ($p\leq0.001$) in silymarin production in roots, leaves and seeds (Table 5). There were also significant results for year x region interaction. In roots and leaves, there was a low production of silymarin contents as compared to seeds. Higher silymarin contents were present in seeds of milk thistle and lowest in roots (Fig. 5). Seeds were found imperative part of the plant to produce higher silymarin contents that can be utilized in the industries for medicine purpose. Leaves can also be a source of silymarin production than roots.

Pearson correlation: Pearson correlation data showed a positive correlation of silymarin contents with average rainfall and soil organic matter (Table 6). Average rainfall and soil organic matter were directly linked with silymarin production. When these two variables were increased then the production of silymarin contents was also increased. While, average temperature, humidity %, Soil Ec and pH showed negative correlation with silymarin production. Reduction in average temperature, humidity %, Soil Ec and pH resulted high production of silymarin.

Discussion

Results have shown that silymarin production in milk thistle changed with different climatic and soil conditions. It was described that environmental factors as temperature, rainfall and humidity can effect on seed maturation and agronomic quality of Milk thistle. Various studies have reported that different conditions of agriculture can influence concentration of bioactive compounds as silymarin in milk thistle. Ghavami & Ramin (2007) studied the soil and temperature effects on Milk Thistle. It was found that germination was severely affected by these factors. Seedling emergence (50%) were achieved at level of 9dS/m soil Ec and 15°C. It also affected the concentrations of silymarin contents. Hammouda *et al*., (1993) reported that silymarin contents was influenced by the soil moisture availability. In the plants grown at 60% field capacity, highest level of silymarin (63.1% Silymarin in ethyl acetate extract) was observed. At this water level, the levels of silybin, silychristin and isosilybin were also highest silybin (Azizi *et al*., 2018).

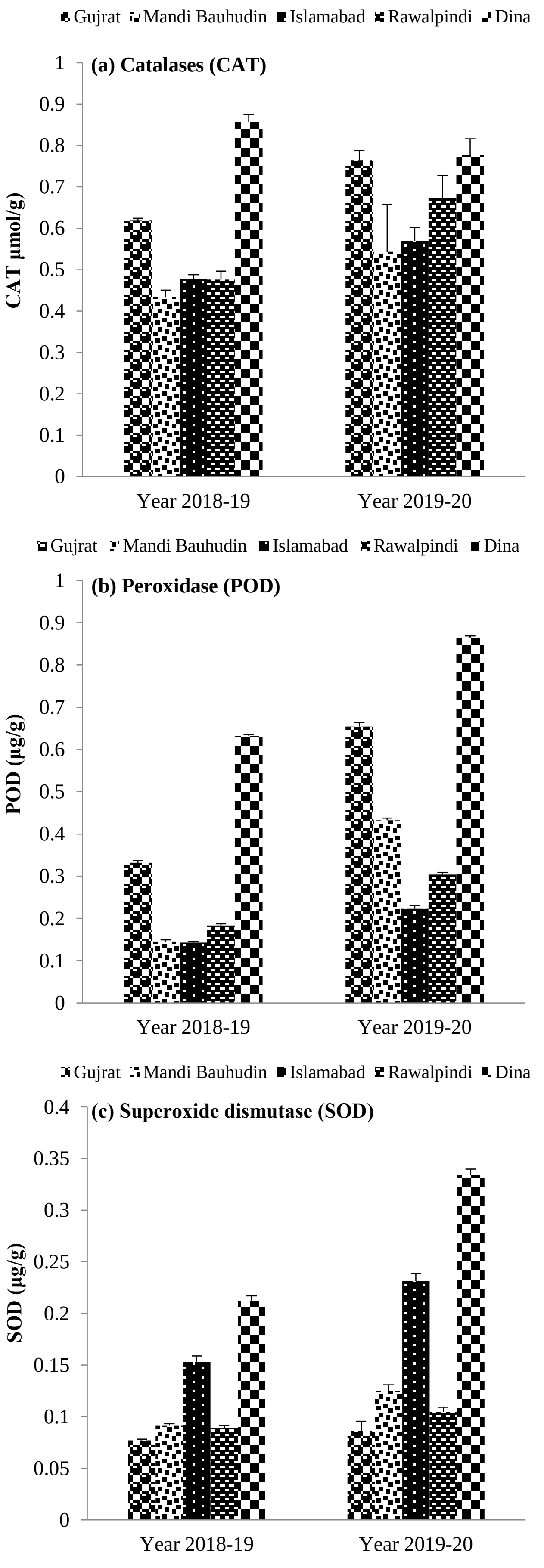

Fig. 3. Enzyme activities in Milk thistle in response to varied agro-climatic and soil conditions.

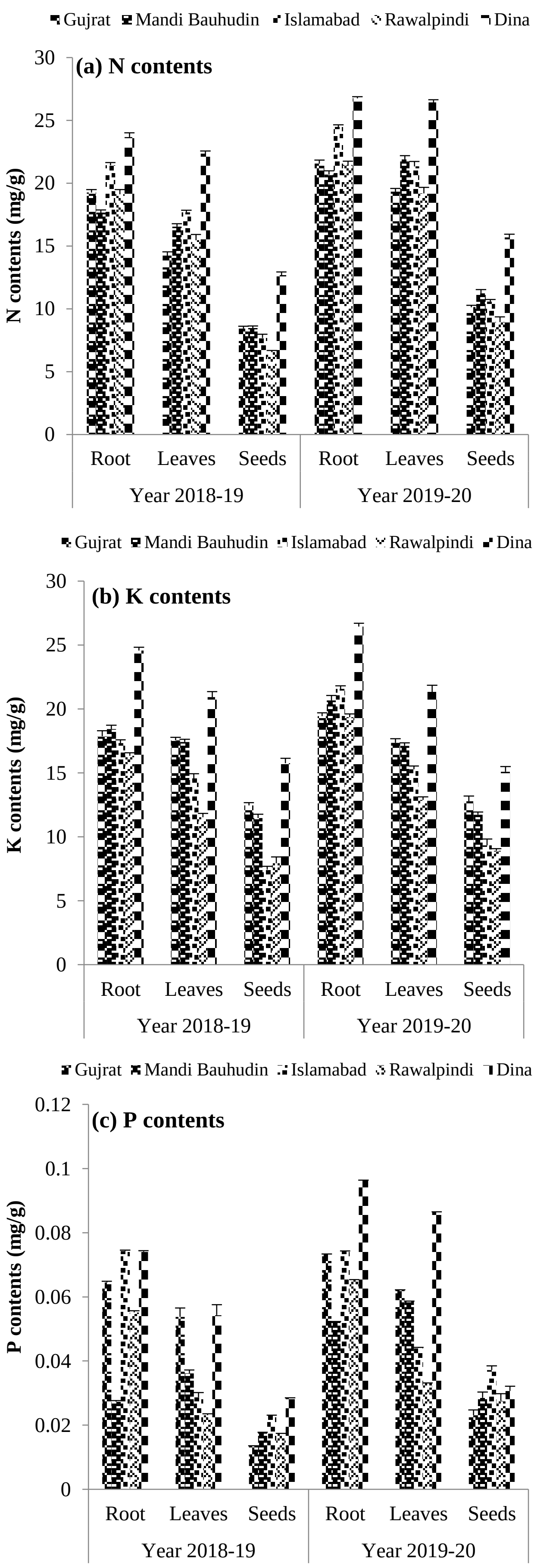

Fig. 4. Ionic contents (mg/g) in Milk thistle in response to varied agro-climatic and soil conditions.

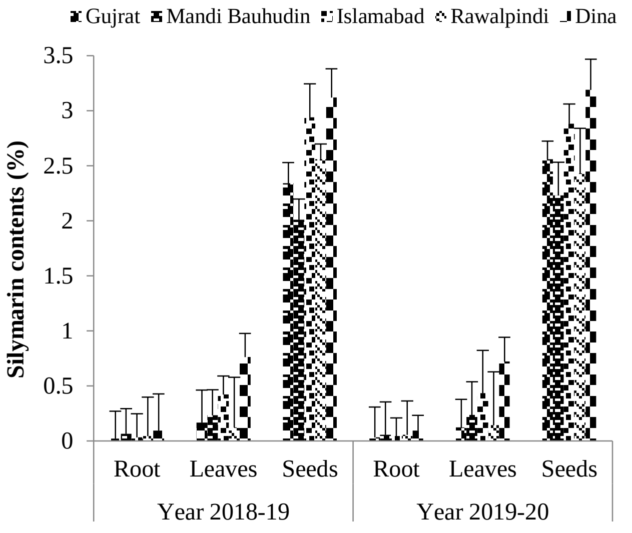

Fig. 5. Silymarin contents in Milk thistle in response to varied agro-climatic and soil conditions.

Sadowska *et al*., (2011) found the effect of weather and agrotechnical conditions on Milk thistle. Weather conditions showed a greater effect on the changes in chemical composition that agrotechnical conditions. Effects of moderate and severe soil moisture was studied on silymarin content and its composition. Variations were observed in the constituents of silymarin contents with different water regime. Low soil moisture levels reduced the silymarin contents (Afshar *et al*., 2015). It was noted that milk thistle strongly response towards changing environmental condition. Karkanis *et al*., (2011) also described the cultivation and growing requirements for Milk Thistle. Milk thistle can be grown under low water conditions and low temperature. It grows well under poor quality soil and the silymarin contents ranges 1 to 3% of achene dry matter. . Silymarin is present in all plant parts with very high concentration in leaves and seeds but seeds are the main source of its production at large scale (Saller *et al*., 2007). Andrzejewska *et al*., (2011) found the changes in yield and silymarin content of Milk thistle studied on light soil and moderate climatic conditions. They also studied its positive correlations among all these variables. Arampatzis *et al*., (2018) determined the antioxidant activities and silymarin contents in Greece and found variation for antioxidant activities and silymarin contents among different milk thistle population as well as regions.

It is now well notable that milk thistle needs a moderate range of temperature, proper rainfall and good soil conditions (low pH, Ec) and high organic matter for the production of silymarin contents (Azizi *et al*., 2018). Similarly, present study revealed that although milk thistle plant can grow in varied agro-climatic condition but silymarin production can be changed. Montemurro (2007) claimed that minimum germination temperature for milk thistle is 10°C and maximum temperature for germination is 35°C and constant temperature is 20 or 25°C. Germination was best at 20-25 or 20-30°C. According to Koshki & Karimzadeh (2003), silymarin contents are highly affected by daily average temperature from the formation of capitol to harvest in milk thistle. Milk thistle has tolerance towards huge range of pH but its growth is best in soil having pH from 5.5-7.6 (Haban *et al*., 2009).

Table 4. Means squares (MS) from the analysis of variance (ANOVA) for Ion concentrations in Milk thistle in response to different agro-climatic conditions.

Source	df	N in root	N in leaves	N in seeds	K in root	K in leaves	K in seeds	P in root	P in leaves	P in seeds
Region (R)	4	77.480***	91.247***	53.470***	94.388***	108.027***	94.804**	0.2538**	0.3674**	0.2451**
Year (Y)	1	137.124*	427.662*	165.894*	90.250**	33.640*	17.640**	0.0762**	0.0659*	0.0743*
R x Y	4	72.211*	210.780**	82.664ns	227.097*	244.357*	136.517*	0.0189*	0.0127*	0.0041*
Error	30	185.741	1595.463	783.951	1213.39	1249.844	1051.84	0.2269	0.337	0.0070
Total	39									

ns = Non-significant; *,**, *** = Significant at p≤0.05, 0.01, or 0.001, respectively

Table 5. Means squares (MS) from analysis of variance (ANOVA) for silymarin contents in Milk thistle in response to different agro-climatic conditions.

Source	df	Silymarin contents in roots	Silymarin contents in leaves	Silymarin contents in seeds
Region (R)	4	1.0943***	2.1862***	4.1563***
Year (Y)	1	0.0543*	0.7321*	0.9321**
R x Y	4	0.2771**	1.4280**	5.5802**
Error	30	0.2533	3.5124	7.5391
Total	39			

ns = Non-significant; *,**, *** = Significant at p≤0.05, 0.01, or 0.001, respectively

Table 6. Pearson correlation coefficients for silymarin with different agro-climatic regions with pooled mean data.

	Silymarin contents	Average rainfall	Average temperature	Humidity %	Soil Ec	Soil pH
Average rainfall	0.82691223					
Average temperature	-0.529650013	-0.6203025				
Humidity %	-0.478306797	-0.4789677	0.1714485			
Soil Ec	-0.691275123	-0.6061344	0.5000303	0.914656		
Soil pH	-0.844258779	-0.8929892	0.4875657	0.808913	0.872455	
Soil organic matter	0.924562928	0.6493694	-0.5055166	-0.65244	-0.85597	-0.82097

r value >0 indicates positive correlation and r value <0 indicates negative correlation

Conclusion

It was concluded that the production of silymarin have strong positive relationship with different agro-climatic regions. Higher silymarin contents are produced in seeds as compared to other plant parts. Milk thistle plants required an optimum agro-climatic conditions for cultivation and the production of flavonolignans (silymarin) resembling with Dina region.

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