



ORIGINAL ARTICLE

# Variations in genetic and chemical constituents of *Ziziphus spina-christi* L. populations grown at various altitudinal zonation up to 2227 m height



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Received 1 May 2016; revised 5 September 2016; accepted 20 September 2016

Available online 10 October 2016

## KEYWORDS

Genetic diversity;  
Chemical diversity;  
*Z. spina-christi*;  
Altitudinal gradient

**Abstract** Altitudinal gradient-defined specific environmental conditions could lead to genetics and chemical variations among individuals of the same species. By using RAPD, ISSR, GC–MS and HPLC analysis, the genetic and chemical diversity of *Ziziphus spina-christi* plants at various altitudinal gradient namely; Abha (2227.86 m), Dala Valley (1424 m), Rakhma Valley (1000 m), Raheb Valley (505 m) and Al-Marbh (147 m) were estimated. RAPD markers revealed that the highest similarity value (40.22%) was between Raheb Valley and Al-Marbh while the lowest similarity (10.08%) was between Abha and Raheb Valley. Based on ISSR markers the highest similarity value (61.54%) was also between Raheb Valley and Al-Marbh, while the lowest similarity (26.84%) was between Abha and Rakhma Valley. GC–MS results showed the presence of various phytochemical constituents in each population. The dendrogram based on chemical compounds separated the *Z. spina-christi* grown at the highest elevations (Abha) from the populations in lower elevations. HPLC analysis showed that the leaves of *Z. spina-christi* plant contain considerable amount of vitamins including B<sub>1</sub>, B<sub>12</sub>, B<sub>2</sub> and folic acid. In conclusion, there is a close relation between altitudinal gradients, genetic diversity and chemical constituents of the leaves of *Z. spina-christi* plants.

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**Abbreviations:** RAPD, random amplified polymorphic DNA; ISSR, inter-simple sequence repeats markers; GC–MS, gas chromatography–mass spectrometry; HPLC, high performance liquid chromatography

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Peer review under responsibility of National Research Center, Egypt.

<http://dx.doi.org/10.1016/j.jgeb.2016.09.001>

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## 1. Introduction

*Ziziphus spina-christi* (L.) Desf. (family, Rhamnaceae), locally known as Sidr, or Nebeq is highly respected by people throughout the Middle East. There are about 50 species distributed in the tropical Asia, Africa and America and in the temperate regions of both hemispheres [1,2]. It was considered

as one of the few native tree species of Saudi Arabia that is still growing comparatively along with many newly introduced invasive weeds species [3]. It has the ability to grow in drought conditions and to adapt to the different environmental conditions in the Kingdom of Saudi Arabia. In traditional medicine *Ziziphus spina-christi* (Sidr) is a well known source of healthy food and energy. Recently, to analyze and estimate genetic diversity in plant species, a series of molecular markers techniques have been developed, for example, the randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR). The randomly amplified polymorphic DNA (RAPD) is potentially simple, rapid, reliable, effective, no prior knowledge of DNA sequence information is required and considered as one of the most popular DNA-based approaches [4,5]. The ISSR technique is more reproducible than the RAPD technique, since ISSR markers are designed from di- or trinucleotide repeat motifs with a 5' or 3' anchoring sequence of one to three nucleotides [6], producing a high degree of polymorphism to distinguish between individuals genetically related, without prior sequence information [7]. ISSR and RAPD techniques were successfully applied to study genetic diversity in *Phoenix dactylifera* L. cultivars [8], *Astragalus oniciformis* populations [9], *Jatropha* species [10] and in many else. Because of the only published results on genetic diversity of *Ziziphus* spp. was on ber of *Z. mauritiana* (Lam.) by Singh et al. [11] and Obeed et al. [12], who used the AFLP technique to study their genetic diversity. Therefore, the aim of our study is the molecular characterization of *Z. spina-christi* using randomly amplified polymorphic DNA (RAPD) technique and inter simple sequence repeat (ISSR) and the impact of altitudinal gradient on the plant genetic diversity.

To investigate the chemicals in various plant groups gas chromatography mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) have been successfully applied. For examples, Alamri and Moustafa [13], identified chemical constituents by HPLC in ethanol extracts of fresh fruits of *Solanum incanum*, fresh leaves of *Ricinus communis* and *Allium ampeloprasum* var. *porrum*, found that they have varying amounts of phenols. Nadir et al. [14] found 78 unreported chemicals constituents from *Salvia santolinifolia* and this species was identified to be  $\alpha$ -pinene chemotype. The essential oils of 4 species namely *Hypericum linarifolium*, *Hypericum perforatum*, *Hypericum humifusum* and *Hypericum pulchrum* from Portugal analyzed by GC and GC-MS found that alpha-pinene, beta-pinene and *n*-nonane separated the four species from each other [15]. Since, there are various disciplines associated with plant taxonomy to improve the identification, classification and systematic position of plant taxa, our study also aimed to use the distribution pattern of chemical compounds for the better understanding of the phylogenetic relationships between the five populations of *Z. spina-christi* collected from various elevations in Aseer region, KSA. In addition, we will establish chromatographic fingerprints to some vitamins present in the leaves by means of HPLC collected *Z. spina-christi* plants.

## 2. Materials and methods

### 2.1. Study area

The studied plant populations covered five sites of the Aseer region, KSA at various altitudinal gradients namely; Abha

(latitude 18° 13' 40", longitude 42° 30' 11" – Alt.-2227.86 m), Dala Valley (latitude 18° 9' 35", longitude 42° 30' 41" – Alt.-1424 m), Rakhma Valley (Latitude 18° 4' 7", longitude 42° 30' 21" – Alt.-1000 m), Raheb Valley (Latitude 17° 56' 13" longitude 42° 28' 7" – Alt.-505 m) and Al marbh (Latitude 17° 45' 32", longitude 42° 19' 51" – Alt.-147 m). Contour Lines and spot elevations from the raster image (topographical map), are extracted then converted to digital vectors to calculate the elevation values using (Digital Elevation Model, DEM) (Fig. 1A and B). The topography of the study area is characterized by a series of semi arid undulating mountains, with max/min rainfall of 549.68 mm/133.47 mm with an average of 234.14  $\pm$  114.99 mm per year, whereas the period between June and October has the bulk of rainfall with some deviations from year to year [16].

### 2.2. Plant material

From each site 500 g of leaves of *Z. spina-christi* plants reaching a height of 1.5 m was randomly sampled without any prior fertilization to the plants. The sampled leaves were immediately placed in a sealed plastic bag and about 3 g from each preserved in dry ice for the DNA extraction. A voucher specimen from each population was deposited in the Biology Department, Faculty of Science, King Khalid University.

### 2.3. DNA extraction from fresh leaves *Z. spina-christi* plants

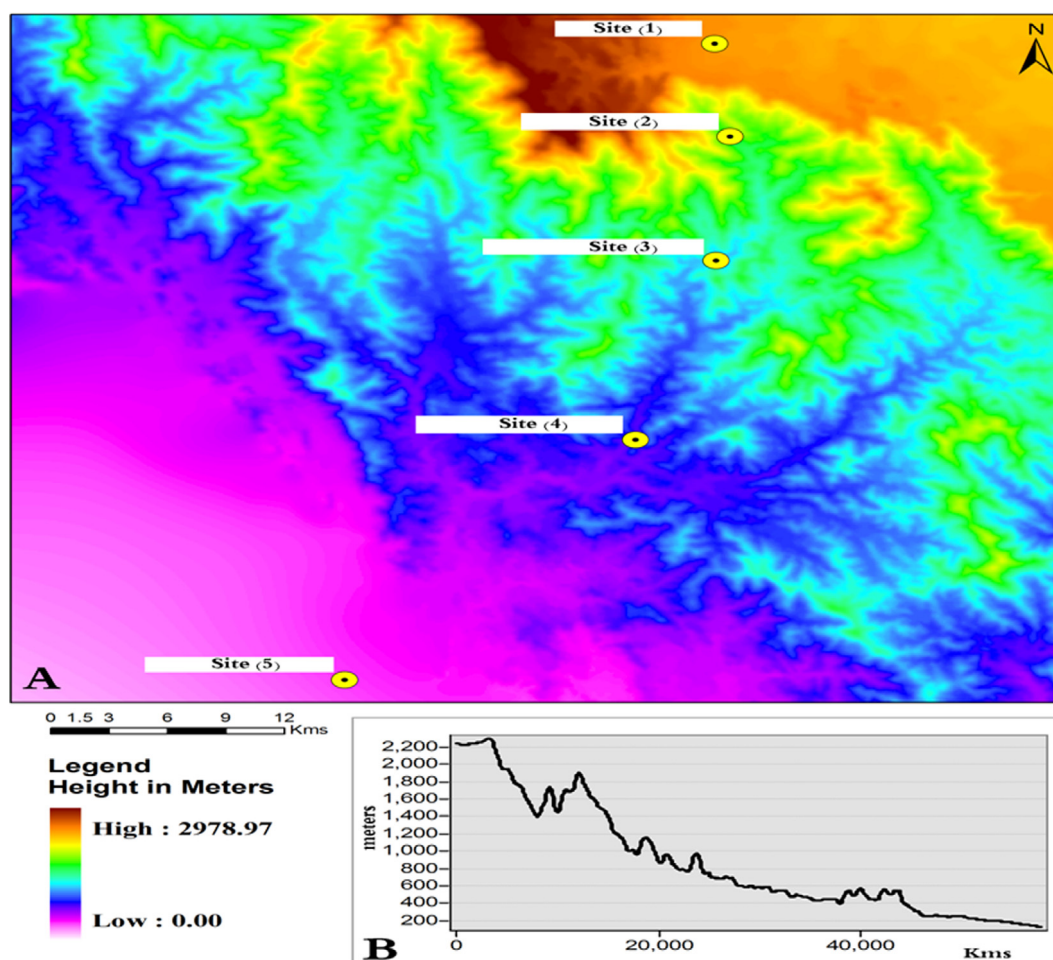
The genomic DNA was extracted using 0.80 mg from fresh leaves of *Z. spina-christi* after grinding them to a fine powder using liquid nitrogen and commercial kit, (DNeasy plant mini kit) provided by QIAGEN-USA. All the steps were indicated in the Kit.

### 2.4. The quality and quantity extracted DNA

The DNA fragments were separated using 1% agarose gels after staining with ethidium bromide in a final concentration of 0.5  $\mu$ g/ml. Agarose gels were run horizontally in 0.5 $\times$  Tris-borate-EDTA (TBE) buffer and run for 60 min at 90 V. The DNA bands were visualized by UV transilluminator at 365 nm wavelengths and its conc. was estimated using a Thermo Scientific™ BioMate 3S UV-Visible at 260 nm [17].

### 2.5. RAPD-PCR and ISSR-PCR analysis of genomic DNA

Twenty-four biomarkers from RAPD and ISSR were used as shown in (Table 1). Standard PCRs buffer were performed using GoTaq Green Master Mix (2 $\times$ ). Each PCR reaction contained 1 $\times$  GoTaq Green Master Mix, 23  $\mu$ mol from each primer (either RAPD or ISSR), 25 ng DNA template and nuclease free water to obtain a final volume of 25  $\mu$ l [18]. Cycling procedure was run using PTC 200 Peltier Thermal Cycler (MJ Research — USA) as follows: Initial denaturation at 94 °C for 5 min followed by 49 cycles of denaturation at 94 °C for 1 min, annealing temperature of 30 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 7 min. 17  $\mu$ l of PCR amplified products were separated by electrophoresis in 1.4% agarose gels for about 120 min at 100 V. A negative control reaction in each PCR experiment was set up



**Figure 1** (A and B) Digital elevation model of the study area in Aseer regions, KSA. Site (1), Abha (2227 m); Site (2) Dala Valley (1424 m); Site (3) Rakhma Valley (1000 m); Site (4) Raheb Valley (505 m); Site (5) Al marbh (147 m).

containing all components of the reaction without the DNA template. The molecular weight of RAPD-PCR and ISSR-PCR fragments were estimated by comparing them with the marker 1 kb DNA ladder 250–10,000 bp. Scored bands fewer than 250 pb were calculated by using a 100 bp DNA Ladder.

#### 2.6. Data analysis

RAPD and ISSR amplified fragments were manually scored as present (1) or absent (0) from the photographs to estimate genetic similarity between investigated *Z. spina-christi* plants. Jaccard's similarity coefficient [18], squared euclidean distance and agglomerative cluster analysis method were used to estimate the genetic distance and to build phylogenetic trees by using the Community Analysis Package Software Program (CAP). Estimation the polymorphism percentage between tested plants was done by dividing the number of polymorphic bands over the total number of bands.

#### 2.7. GC–MS of chloroform, petroleum ether, methanol, ethanol and acetone extracts of *Z. spina-christi* leaves

The GC–MS analysis was carried out using a Clarus 500 Perkin – elmer (Auto system XL) Gas Chromatograph equipped

and coupled to a mass detector Turbo mass gold – Perkin Elmer Turbomass 5.1 spectrometer with an Elite – 1 (100% Dimethyl poly siloxane) and TR-V1 column (30 m × 0.32 mm × 1.8 μm) was used. The running process was previously described [19]. The identification of mass spectrum GC–MS was conducted using the database of National Institute Standard and Technology (NIST). Unidentified spectrum was compared with the identified spectrum stored in the NIST library.

#### 2.8. Quantification of vitamins $B_1$ , $B_2$ , $B_{12}$ and folic acid by HPLC

A Shimadzu model HPLC system (Shimadzu Corporation, Kyoto, Japan) was used. Potassium dihydrogen phosphate (BDH, Anala R), formic acid (BDH, Anala R), methanol (BDH, Anala R), acetic acid (BDH, Anal R) and HCl (BDH, Anala R) have been used for sample preparation. Folic acid, vitamins  $B_1$ ,  $B_2$  and  $B_{12}$  were purchased from Sigma–Aldrich, Buchs SG, Switzerland have been used as a standard. 15.07 g of *Z. spina-christi* leaves were accurately weighted and 10 mL of 0.1 N HCl and 80 mL distilled water were added and then refluxed on a boiling water bath for 15 min. A gradient of 30–70 (in eight minutes) from methanol and potassium



**Table 1** RAPD and ISSR primers.

RAPD Primers	Sequence of primer (5'–3')
Oligo345	GCG TGA CCC G
Oligo42	TTA ACC CGG C
Oligo349	GGA GCC CCC T
Oligo211	GAA GCG CGA T
D07	TTGGCACGGG
OPE-17	CTACTGCCGT
OPL-3	CCAGCAGCTT
HB 15	GC GTC GTG GTG GC
OPE-3	CCAGATGCAC
OPK-8	GAACACTGGG
OPJ-1	CCGGCATAA
OPE-18	GGACTGCAGA
ISSR primers	Sequence of primer (5'–3')
Primer 3	TGGA TGGA TGGA TGGA
Primer 4	CA CA CA CA CA CA AG
UBC888	CAC CAC ACA CAC ACA CA
UBC823	TCT CTC TCT CTC TCT CC
Primer 2	GA GA GA GA GA GA GA GA GA GA GA
UBC824	TCT CTC TCT CTC TCT CG
UBC826	ACA CAC ACA CAC ACA CC
UBC842	GAG AGA GAG AGA GAG ACG
ISSR06	GAG AGA GAG AGA GAG AC
Primer 7	CC AG GT GT GT GT GT GT GT GT
Primer 1	GA GA GA GA GA GA GA GA GA GA
Primer 5	GA GA GA GA GA GA GA GA CG

dihydrogen phosphate (0.05 M, pH 4.2, adjusted with formic acid) was used as mobile phase. The flow rate was adjusted at 1 ml min<sup>-1</sup> and a detection wave length at 254 nm with an injection volume of 20 µL [20].

### 2.9. Leaves physical characters

To construct comparative physical study of leaves among plant populations under the prevailing growing conditions, the following physical characteristics were evaluated: LSA, leaf surface area, LPL, leaf petiole length, W/L%, leaf width (W)/leaf length (L) × 100; SS/LS%, short leaf spine (SS)/long leaf spine (LS) × 100; Moisture (%) = Fresh weigh–dry weight/fresh weight × 100; Dry matter (%) = Average dry weight (g)/Average fresh weight (g) × 100, whereas leaves were dried in an oven at 80 °C.

## 3. Results

### 3.1. RAPD analysis

The genetic similarity coefficients based on RAPD markers illustrated that the highest similarity value (40.22%) was between Raheb Valley (505 m) and Al marbh (147 m) while the least similarity value (10.08%) was between Abha (2227 m) and Raheb Valley (505 mm) (Fig. 2 and Table 2). Resulted dendrogram grouped the five genotypes into three main clusters. The first cluster comprised *Z. spina-christi* from Abha (2227 m) populations with the least similarity index value ranging from 10.08% to 15.74% with other population's

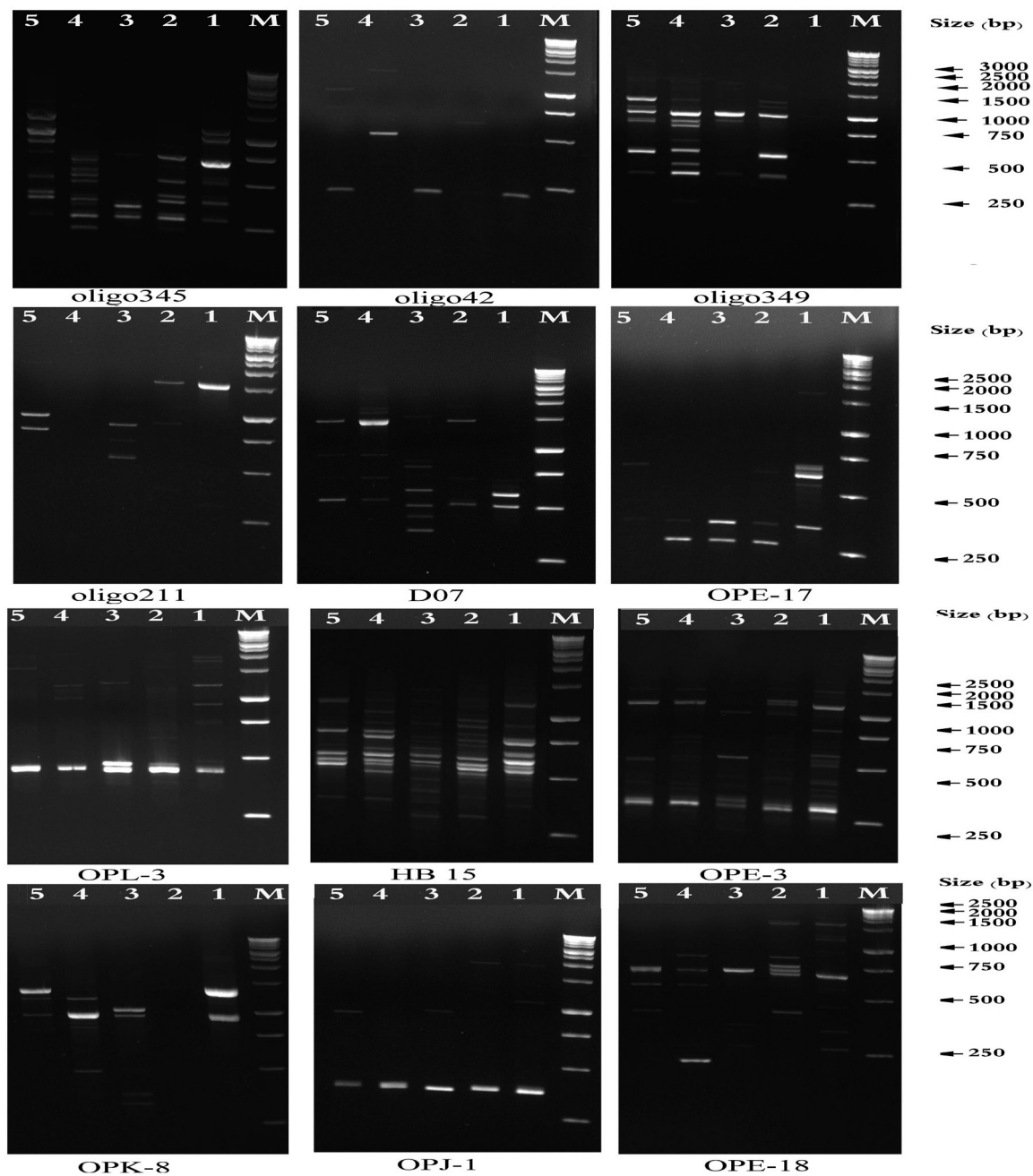
genotypes. The second cluster contained two genotypes, *Z. spina-christi* from Dala Valley (1424 m) and Rakhma Valley (1000 m) with a similarity index value of 18.81%. The third cluster comprised *Z. spina-christi* from Raheb Valley (505 m) and Al marbh (147 m) having an index similarity value of 40.22%. The twelve RAPD primers generated a total of 181 scorable bands, out of which 42.54% were found to be polymorphic, 1.10% monomorphic and 56.35% unique bands. HB 15 primer yielded the highest number of bands (26.0) while the least number of bands (7.00) was recorded from OPE-17 and OPJ-1. The percentage of polymorphism ranged from 0.00% (oligo211) to 65.0% (oligo345). The highest number of unique bands (13.0) was recorded from the primer OPE-18, followed by the primer oligo211 and HB 15, (11 bands), while the least number of unique bands recorded (3 bands) from the primer 22 (Table 3).

### 3.2. ISSR analysis

Amplification products from the twelve ISSR primers yielded a total of 257 scorable bands, out of which 60.70% were found to be polymorphic bands, 10.12% monomorphic bands and 29.18% unique bands (Fig. 4 and Table 4). Primer 3 showed the highest percentage value of polymorphism (94.4%), while the primer 1 yielded the least number of polymorphism (29.4%). The highest percentage numbers of unique bands were generated from the primer 5 (53.84%), while the least numbers were generated from primer 3 (5.55%). Resulted similarity coefficients showed that the highest similarity value (61.54%) was between Raheb Valley (505 m) and Al marbh (147 m), while the least similarity value (26.84%) was between Abha (2227 m) and Rakhma Valley (1000 m) (Table 5). The ISSR dendrogram grouped the five populations into three main clusters (Fig. 5). The first cluster clearly distinguished the out-group *Z. spina-christi* genotype grown in Abha (2227 m) from the in-group including *Z. spina-christi* genotype grown in Dala Valley (1424 m), Rakhma Valley (1000 m), Raheb Valley (505 m) and Al marbh (147 m). The second cluster contained three genotypes, *Z. spina-christi* from Dala Valley (1424 m), Raheb Valley (505 m) and Al marbh (147 m). Similarity index values between Raheb Valley (505 m) and Al marbh (147 m) were 61.54% while between Dala Valley (1424 m) and Raheb Valley (505 m) and Al marbh (147 m) between 39.79% and 44.16%. The third cluster contained *Z. spina-christi* from Rakhma Valley (1000 m) which showed slightly less relation to the genotypes from Al marbh (147 m), Raheb Valley (505 m) and Dala Valley (1424 m) with similarity index value that ranged from 30.53% to 36.21% (Table 5).

### 3.3. Chemicals in various extract of *Z. spina-christi* grown at various elevations

GC–MS chromatogram analysis of acetone, chloroform, ethanol, methanol and petroleum ether extracts of leaves of *Z. spina-christi* grown at various elevations showed the presence of various phytochemical constituents. Characterization and identification of phytochemicals found in various localities are presented in (Table 6). Twenty-one chemical compounds were identified in the acetone extracts, fourteen in the chloroform extracts, twenty-nine in the ethanol extracts, eighteen in



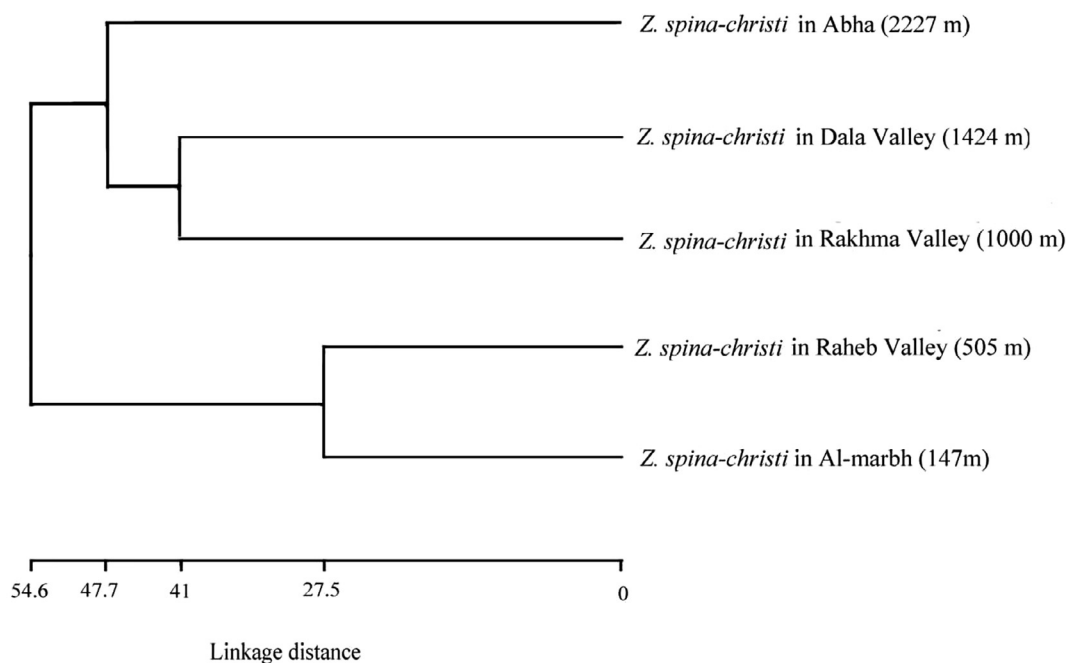
**Figure 2** RAPD profiles of five populations of *Z. spina-christi* using RAPD primers. Lane 1, Abha (2227 m) population; Lane 2, Dala Valley (1424 m) population; Lane 3, Rakhma Valley (1000 m) population; Lane 4, Raheb Valley (505 m) population; Lane 5, Al marbh (147 m) population; M, Molecular weight marker (1 kb DNA Ladder on the right side).

**Table 2** Genetic similarity among five populations of *Z. spina-christi* based on RAPD markers.

	Abha (2227 m)	Dala Valley (1424 m)	Rakhma Valley (1000 m)	Raheb Valley (505 m)	Al marbh (147 m)
Abha (2227 m)	1.00				
Dala Valley (1424 m)	0.1574	1.00			
Rakhma Valley (1000 m)	0.1058	0.1881	1.00		
Raheb Valley (505 m)	0.1008	0.2143	0.1887	1.00	
Al marbh (147 m)	0.1346	0.1827	0.1895	0.4022	1.00

**Table 3** Polymorphisms of twelve RAPD primers applied on five populations of *Z. spina-christi*.

Primer ID	Total No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Polymorphism%
Oligo345	20.0	13.0	0.00	7.00	65.00
Oligo42	9.00	1.00	0.00	8.00	11.10
Oligo349	17.0	8.00	0.00	9.00	47.06
Oligo211	11.0	0.00	0.00	11.0	0.000
D07	19.0	5.00	0.00	14.0	26.30
OPE-17	7.00	4.00	0.00	3.00	57.14
OPL-3	16.0	8.00	1.00	7.00	50.00
HB 15	26.0	14.0	1.00	11.0	53.80
OPE-3	19.0	12.0	0.00	7.00	63.16
OPK-8	11.0	3.00	0.00	8.00	27.27
OPJ-1	7.00	3.00	0.00	4.00	48.86
OPE-18	19.0	6.00	0.00	13.0	31.60
Total	181	77.0	2.00	102	40.11

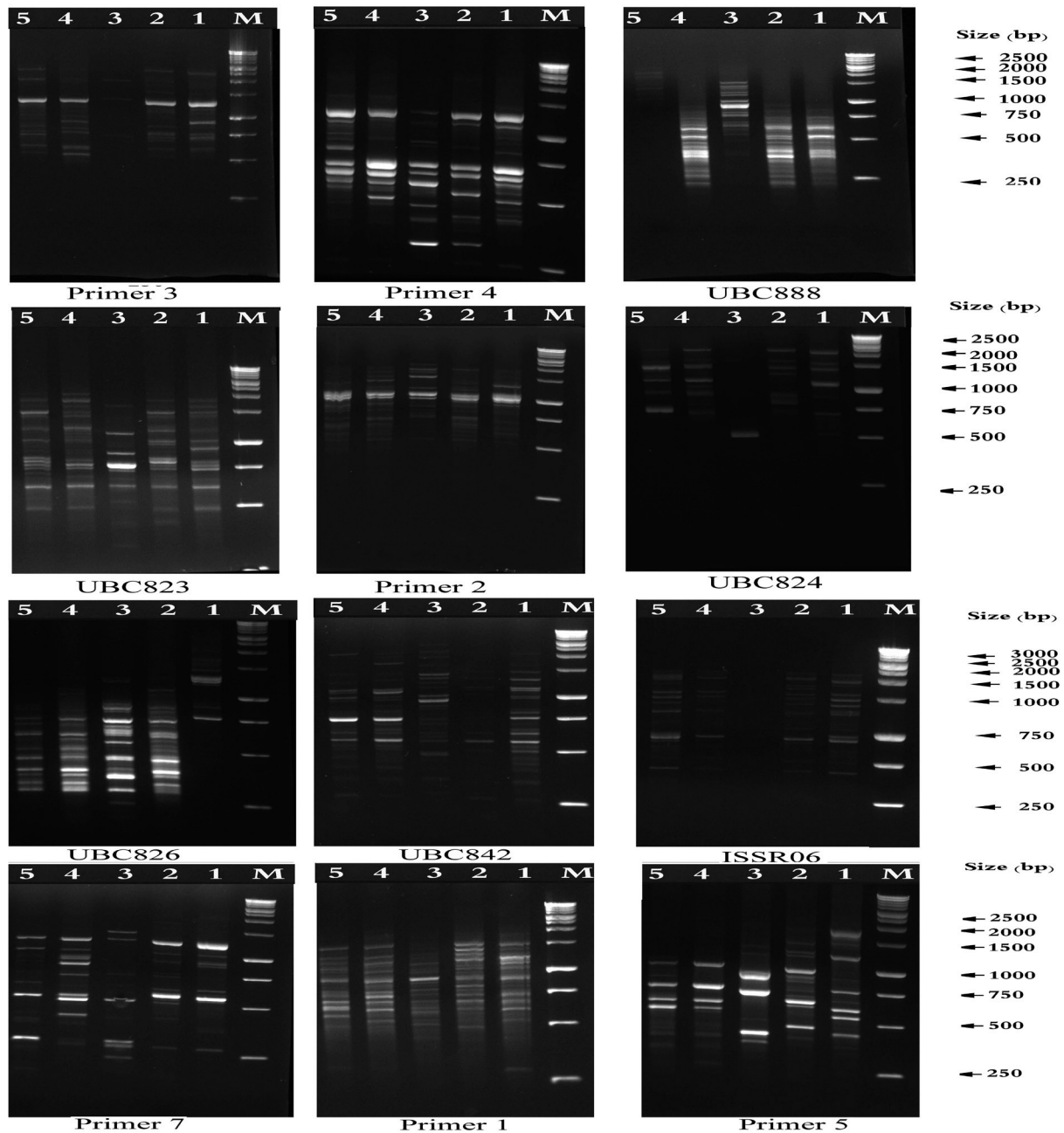
**Figure 3** Dendrogram depicting the genetic relationship among 5 populations of *Z. spina-christi* genotypes growing in various elevations in Aseer region, KSA, based on RAPD data.

the methanol extracts and twenty chemical compounds in the petroleum ether extracts of leaves of *Z. spina-christi* collected from various elevations. According to the peak of the resulted GC-MS graph the amount vary greatly in the studied localities (data not show). Constructed dendrograms based on chemical compositions found in acetone, chloroform, ethanol, methanol and petroleum ether extracts of leaves of *Z. spina-christi* growing in various habitats using Ward's methods are shown in (Fig. 6). It grouped the five populations into three main clusters. The out-group contained *Z. spina-christi* grown in the Abha (2227 m) populations with least similarity index value with other population's chemotype between 5.882% and 9.615% (Table 7). The in-group contained four chemotypes, *Z. spina-christi* from Dala Valley (1424 m), Rakhma Valley (1000 m), Raheb Valley (505 m) and Al-marbh (147 m) that

bifurcated in two other main clusters. *Z. spina-christi* chemotype from Dala Valley (1424 m) formed a separated cluster from the other three populations with similarity index ranging from 25.93% to 14.81%. The third cluster contained the chemotype of *Z. spina-christi* from Rakhma Valley (1000 m), Raheb Valley (505 m) and Al-marbh (147 m) that showed the highest similarity index (34.78) between the Rakhma Valley (1000 m) and Raheb Valley (505 m) (see Fig. 3).

#### 3.4. Analysis of water-soluble vitamins in *Z. spina-christi* leaves growing in various elevations in Aseer region, KSA

The amounts of vitamins B<sub>1</sub>, B<sub>12</sub>, B<sub>2</sub> and folic acid found in the leaves *Z. spina-christi* grown in various localities are shown in



**Figure 4** ISSR profiles of five populations of *Z. spina-christi* using twelve primers. Lane 1, Abha (2227 m) population; Lane 2, Dala Valley (1424 m) population; Lane 3, Rakhma Valley (1000 m) population; Lane 4, Raheb Valley (505 m) population; Lane 5, Al marbh (147 m) population; M, Molecular weight marker (1 kb DNA Ladder on the right side).

(Figs. 7 and 8). It is noted that vitamins B1 and Folic acid are richer in *Z. spina-christi* leaves collected from Al-marbh (147 m) population than any other populations with values of 0.363 mg/g and 1.006 mg/gm, respectively. Highest content of B12 was recorded from *Z. spina-christi* leaves collected from Abha (2227 m) population with value of 0.227 mg/g. Vitamins B2 content was found to be highest in plants growing in Raheb Valley (505 m) habitat with a value of 1.484 mg/gm, followed by those growing in Al-marbh (147 m) (1.248 mg/g) and in Rakhma Valley (1.228 mg/g).

### 3.5. Physical characters of leaves of *Z. spina-christi* in five populations

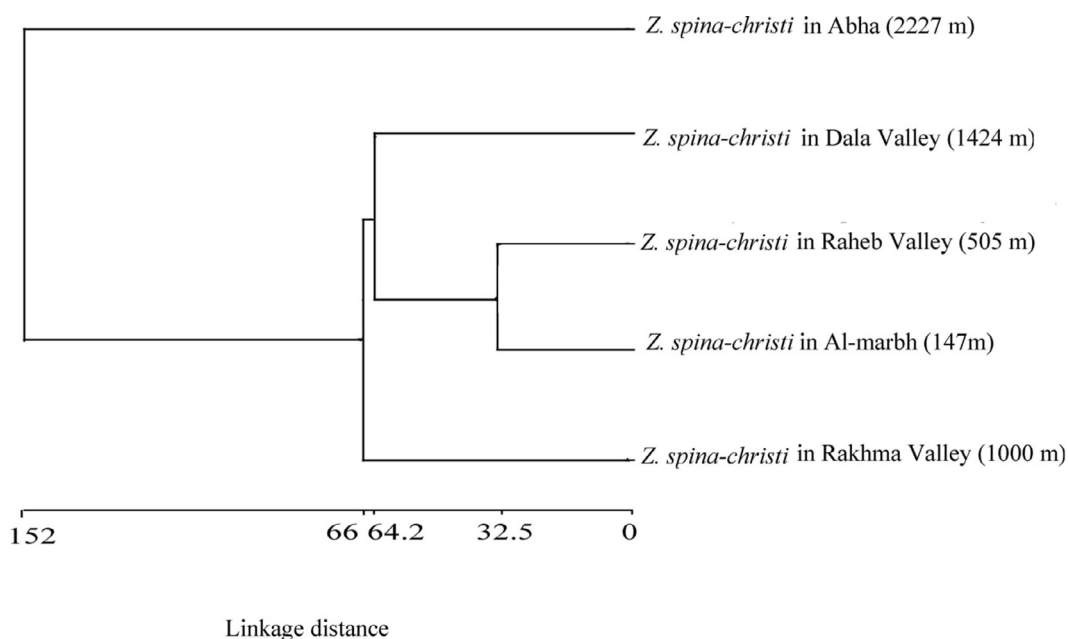
Leaves characteristics of *Z. spina-christi* grown in various habitats along an environmental gradient were evaluated (Fig. 1). There were differences among the plant phenotypes in terms of leaf surface area, leaf petiole length, percentage of leaf width/leaf length, percentage of short leaf spine/long leaf spine, percentage of moisture content and percentage of dry matter contents. Leaf surface area was in between 30.42

**Table 4** Polymorphism of twelve ISSR primers applied on five populations of *Z. spina-christi*.

Primer ID	Total No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Polymorphism%
Primer 3	18.0	17.0	0.00	1.00	94.40
Primer 4	25.0	15.0	2.00	8.00	60.00
UBC888	20.0	13.0	0.00	7.00	65.00
UBC823	21.0	12.0	4.00	5.00	57.14
Primer 2	18.0	11.0	3.00	4.00	61.10
UBC824	14.0	9.00	0.00	5.00	64.28
UBC826	23.0	13.0	1.00	9.00	56.50
UBC842	33.0	21.0	5.00	7.00	63.63
ISSR06	17.0	15.0	0.00	2.00	88.23
Primer 7	25.0	13.0	0.00	12.0	52.00
Primer 1	17.0	5.00	11.0	1.00	29.40
Primer 5	26.0	12.0	0.00	14.0	46.15
Total	257	156	26.0	75.0	61.49

**Table 5** Genetic similarity among 5 populations of *Z. spina-christi* based on ISSR markers.

	Abha (2227 m)	Dala Valley (1424 m)	Rakhma Valley (1000 m)	Raheb Valley (505 m)	Al marbh (147 m)
Abha (2227 m)	1.00				
Dala Valley (1424 m)	0.4263	1.00			
Rakhma Valley (1000 m)	0.2684	0.3053	1.00		
Raheb Valley (505 m)	0.3781	0.4416	0.3583	1.00	
Al marbh (147 m)	0.3333	0.3979	0.3621	0.6154	1.00

**Figure 5** Dendrogram depicting the genetic relationship among five populations of *Z. spina-christi* genotypes growing in various elevations in Aseer region, KSA, based on ISSR data.

$\pm 6.24 \text{ mm}^3$  and  $10.64 \pm 1.84 \text{ mm}^3$ . Leaf surface area from the Al-marbh population had the highest value followed by those collected from Abha habitat, while the lowest values were noticed in the Raheb Valley population. The highest value for leaf petiole length was noticed in the Abha population ( $12.57 \pm 1.27 \text{ mm}$ ), followed by those in Al-marbh popu-

lation ( $7.2 \pm 1.49 \text{ mm}$ ), while the lowest values were in Raheb Valley population ( $3.28 \pm 0.49 \text{ mm}$ ). Leaf width/length ratio was in between  $80.78 \pm 9.84\%$  and  $56.52 \pm 4.59\%$ , whereas those in the Rakhma Valley habitat showed the highest ration followed by that in the Al-marbh habitat while the lowest ratio in Abha habitat. The highest short leaf spine/long spine ratio



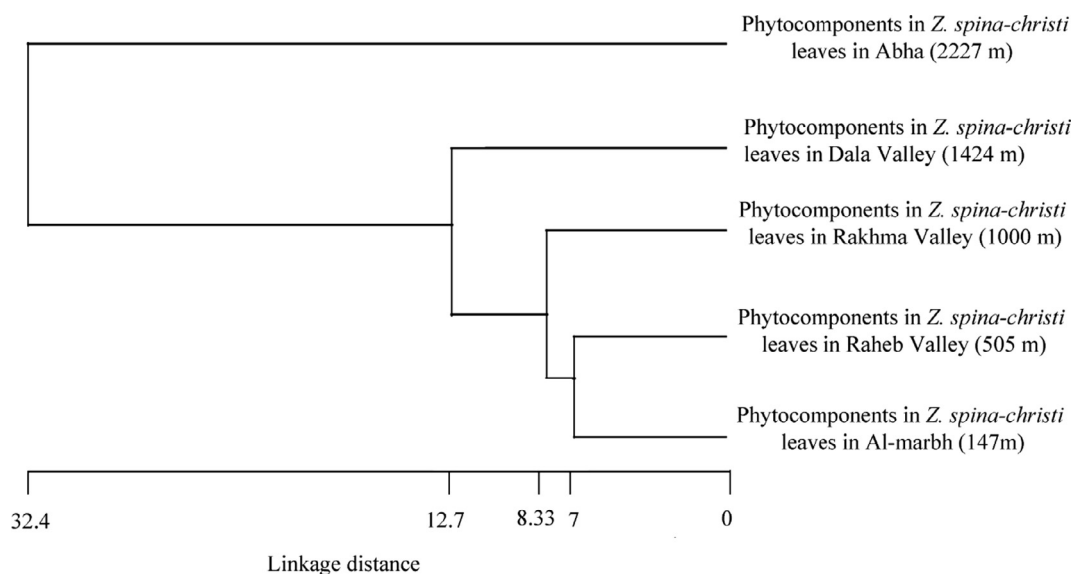
**Table 6** Chemical comparison profile detected by GC-MS analysis of solvents extract of *Z. spina-christi*.

Solvents	No.	Compounds	Abha	Dala Valley	Rakhma Valley	Raheb Valley	Al marbh
Acetone extract	1	Dimethyl sulfone	1	1	1	1	0
	2	Undecane	1	0	0	0	0
	3	Cyclopropane,octyl-	1	0	0	0	0
	4	2-Decenal, (E)-	1	0	0	0	0
	5	2,4-Decadienal, (E,E)-	1	0	0	0	0
	6	2,4-Nonadienal, (E,E)-	1	0	0	0	0
	7	Octane, 2,4,6-trimethyl-	1	0	0	0	0
	8	2,Undecenal	1	0	0	0	0
	9	4-Heptenal	1	0	0	0	0
	10	9-Octadecene, (E)-	1	0	0	0	0
	11	9,12-Octadecadienoic acid (Z,Z)-	1	0	0	0	0
	12	Cis-Vaccenic acid	1	0	0	0	0
	13	1,3,5-Triazine-2,4-diamine,N,N'-bis(1 methylethyl)-6-(methylsulfonyl)-	0	1	0	0	0
	14	Decane, 6-ethyl-2 methyl-	0	1	1	0	0
	15	Erucic acid	0	1	0	0	0
	16	1,14-Tetradecanediol	0	0	1	0	0
	17	Oxalic acid, isobutyl pentyle ester	0	0	0	1	0
Chloroform extract	1	Dimethyl sulfone	1	1	1	1	1
	2	Decane, 6-ethyl-2 methyl-	1	1	0	0	0
	3	cis-Vaccenic acid	1	0	0	0	0
	4	9-Hexadecenoic acid	0	1	1	0	0
	5	Undecane	0	0	1	1	0
	6	Oxalic acid, isobutyl pentyl ester	0	0	0	0	1
	7	Diethyl Phthalate	0	0	0	0	1
Ethanol extract	1	Decane, 6-ethyl-2 methyl-	1	0	0	0	1
	2	Phenol, 2-(1,1-dimethylethyl)-4-(1 methyl-1-phenylethyl)-	1	0	0	0	0
	3	Phthalic acid, 3,4-dimethylphenyl 3,5-dimethylphenyl ester	1	0	0	0	0
	4	Cyclooctene, 3 methyl-	1	0	0	0	0
	5	cis-Vaccenic acid	1	0	1	1	0
	6	2,4-Diphenyl-4 methyl-1-pentene	1	0	0	0	0
	7	o-Anisaldehyd, azine	1	0	0	0	0
	8	1H-Indene, 2,3dihydro-1,1,3trimethyl-3-phenyl-	1	0	0	0	0
	9	Benzene, 1,1'-(1,1,2,2-tetramethyl-1,2-ethanediyl)bis-	1	0	0	0	0
	10	Benzene, 1,1'-(3,3-dimethyl-1-butenylidene)bis-	1	0	0	0	0
	11	Benzene, 1,1'-(4,4-dimethyl-1-butene-1,4-diyl)bis-	1	0	0	0	0
	12	p menthane, 2,3-dipromo-8-phenyl-	1	0	0	0	0
	14	3-(6-phenylcyclohex-3-enyl)prop-2-enoic acid	1	0	0	0	0
	15	Terephthalic acid, 2-ethylhexyl undecyl ester	1	0	0	0	0
	16	Dimethyl sulfone	0	1	1	0	1
	17	Tridecane	0	1	0	0	0
	18	2,4-Octadienal, (E,E)-	0	1	0	0	0
	19	Cis-10-Nonadecenoic acid	0	1	0	0	0
	20	Oxalic acid, isobutyl pentyl ester	0	0	1	0	0
	21	Tetradecanoic acid	0	0	0	1	0
	22	n-Hexadecanoic acid	0	0	0	1	0
	23	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-,(all-E)-	0	0	0	1	0
	24	Squalene	0	0	0	0	1
	25	Cholesterol	0	0	0	0	1
	Methanol extract	1	n-Hexadecanoic acid	1	0	0	0
2		9,12-Octadecadienoic acid (Z,Z)-	1	0	0	0	0
3		cis-Vaccenic acid	1	1	0	0	0
4		9,17-Octadecadienal, (Z)-	1	0	0	0	0
5		Octadecanoic acid	1	0	0	0	0
6		Dimethyl sulfone	0	1	1	1	1
7		Decane, 6-ethyl-2 methyl-	0	1	0	0	0

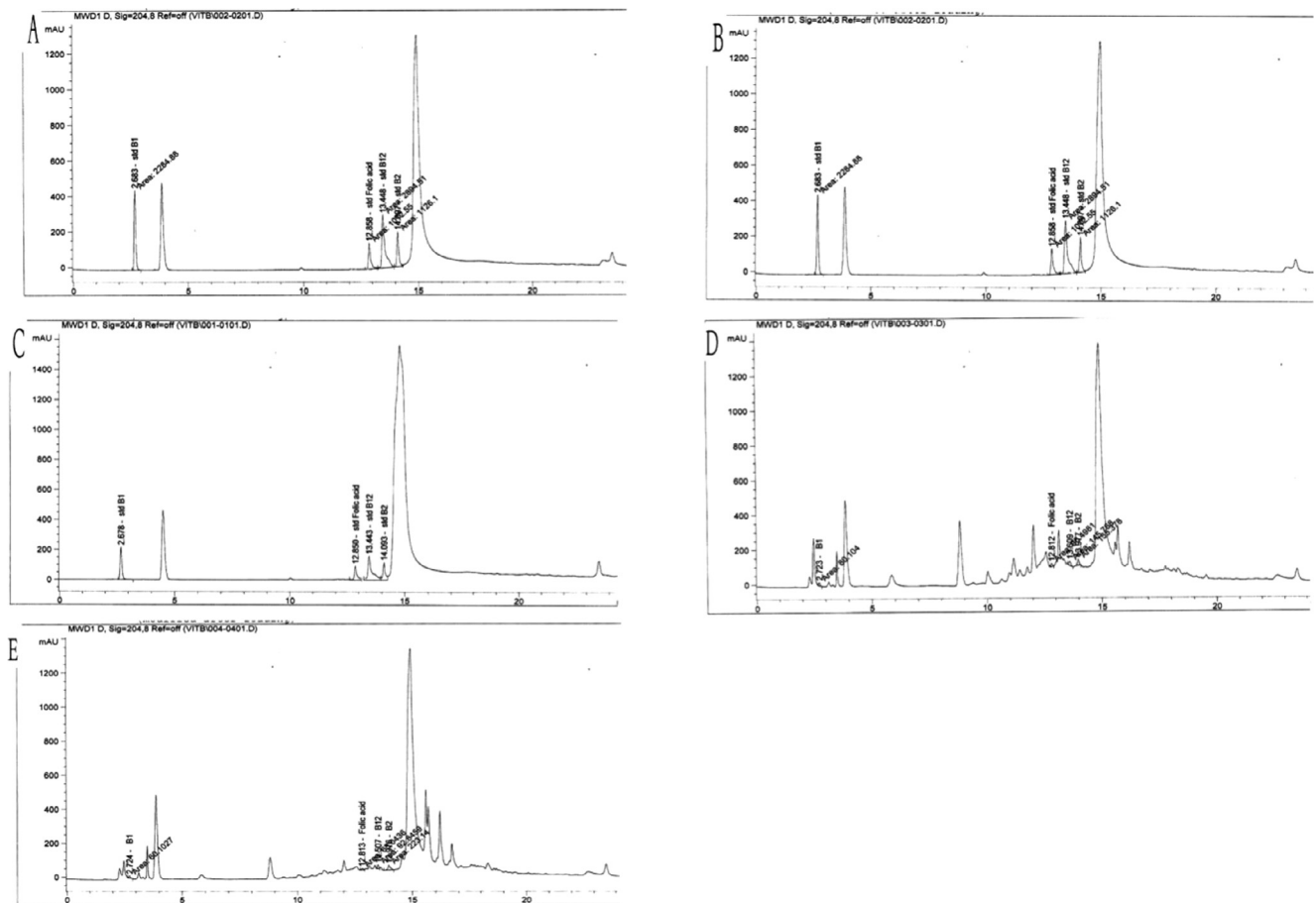
(continued on next page)

**Table 6** (continued)

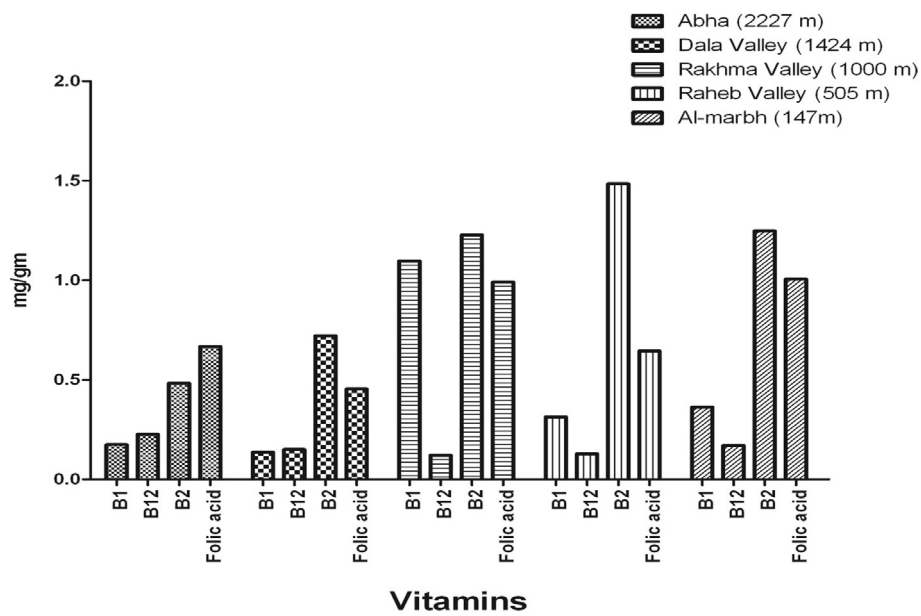
Solvents	No.	Compounds	Abha	Dala Valley	Rakhma Valley	Raheb Valley	Al marbh
	8	Dimethyl Sulfoxide	0	0	1	0	0
	9	Oxalic acid, isobutyl pentyl ester	0	0	1	1	1
	10	1-Undecanol	0	0	1	0	0
	11	Diethyl Phthalate	0	0	0	1	1
Petroleum ether extract	1	Oleyl Alcohol	1	0	0	0	0
	2	Dimethyl sulfone	1	1	1	1	1
	3	Undecane	1	0	0	0	0
	4	Cis-9-Hexadecenoic acid	1	0	0	0	0
	5	1,3,5-Triphenyl-1,5-pentanedione	1	0	0	0	0
	6	Benzene, (1,1-dimethylpropyl)-	1	0	0	0	0
	7	Decane, 6-ethyl-2 methyl-	0	1	0	0	0
	8	cis-Vaccenic acid	0	1	0	0	0
	9	Dimethyl sulfoxide	0	0	1	0	1
	10	Oxalic acid, isobutyl pentyl ester	0	0	1	1	1
	11	1-Undecanol	0	0	1	0	0
	12	Diethyl phthalate	0	0	0	1	1

**Figure 6** The dendrogram constructed from chemicals in *Z. spina-christi* leaves growing in various elevations in Aseer region, KSA.**Table 7** Jaccard's similarity coefficient among 5 populations of *Z. spina-christi* leaves growing in various elevations in Aseer region, KSA, based on phytocomponents.

	Abha (2227 m)	Dala Valley (1424 m)	Rakhma Valley (1000 m)	Raheb Valley (505 m)	Al marbh (147 m)
Abha (2227 m)	1.00				
Dala Valley (1424 m)	0.09615	1.00			
Rakhma Valley (1000 m)	0.07547	0.2593	1.00		
Raheb Valley (505 m)	0.0800	0.1481	0.3478	1.00	
Al marbh (147 m)	0.05882	0.1481	0.2917	0.3333	1.00



**Figure 7** Typical HPLC chromatogram of water soluble vitamins (B<sub>1</sub>, B<sub>12</sub>, B<sub>2</sub> and folic acid) of *Z. spina-christi* leaves growing in Abha (2227 m) (A); Dala Valley (1424 m) (B); Rakhma Valley (1000 m) (C), Raheb Valley (505 m) (D) and Al-marbh (147 m) (E).



**Figure 8** HPLC analysis of water-soluble vitamins (B<sub>1</sub>, B<sub>12</sub>, B<sub>2</sub> and folic acid) of *Z. spina-christi* leaves growing in various elevations in Aseer region, KSA.

was  $48.57 \pm 8.99$  mm in the Abha habitat followed by  $43.12 \pm 6.43\%$  in Al-marbh habitat while the lowest ratio of  $25.83 \pm 5.78\%$  was seen in the Rakhma Valley habitat. The values for leaf moisture content showed that Abha habitat having highest value ( $76.45 \pm 12.66\%$ ) followed by Al-marbh habitat ( $60.71 \pm 5.78\%$ ) and those growing in Dala Valley habitat exhibited the lowest values ( $53.48 \pm 8.51\%$ ). The dry matter content ranged in between  $46.67 \pm 13.12\%$  and  $32.54 \pm 12.66\%$ , whereas Raheb Valley populations had the highest value followed by Dala Valley and Rakhma Valley and those growing in Abha habitat showed the lowest content (see Fig. 9).

## 4. Discussion

### 4.1. Genetic diversity using RAPD and ISSR markers

The assessment of genetic diversity among the *Z. spina-christi* populations growing at various heights was evaluated using RAPD and ISSR biomarker techniques. The obtained results indicated that both of the marker systems can be effectively used in the determination of genetic relationship among *Z. spina-christi* populations. Percent of polymorphism that was obtained in this study from RAPD (40.11%) and ISSR (61.49%) indicates the presence of genetic variations among the *Z. spina-christi* populations. This variation may be due to the differences in the number of alleles per locus/or loci and their distribution within each population. The efficiency of RAPD and ISSR biomarkers determines whether the genetic diversity among plant populations was proofed [21,22]. Our results showed that each primer varied in their ability to detect the amount of genetic variation among *Z. spina-christi* populations. Herein, there were 181 bands with an average of 15.08 selected markers/primers using the twelve RAPD primers. Kernodle et al. [23] reported that many factors affected the yielded bands using oligonucleotide primers such as primer structure, template quantity and the number of annealing sites in the plant genome. It was reported that *Butea monosperma* collected from five different agro-ecological localities yielded 12 markers/primers [24]. There were eight markers/primers scored from five populations of annual caraway (*Carum carvi*) [25]. Polymorphism rate resulted in our study using 12 decamer oligonucleotide primers was almost similar to many previous studies. For example, Ahmad et al. [26] examined the genetic diversity of 20 lines of *Brassica napus* using RAPD primers and found that the level of polymorphism ranges between 21.50% and 59.41%. Sobotka et al. [27] estimated the genetic diversity of oilseed rape (*Brassica napus*) and found that polymorphic bands range between 31% and 76%. In terms of unique bands, there were clear differences among the five genotypes of *Z. spina-christi*, which may be used as DNA fingerprints for species identification and characterization under specific environmental conditions. The obtained dendrogram and genetic similarity produced from the twelve RAPD primers showed that the Abha population (2227 m) was genetically distant from the other four populations including Dala Valley (1424 m), Rakhma Valley (1000 m), Raheb Valley (505 m) and Al-marbh (147 m). The dendrogram from combined analysis of RAPD and ISSR data (data not shown) revealed that Abha population (2227 m) was genetically close to that of the Dala Valley (1424 m). This indi-

cates that *Z. spina-christi* plants showed a response to a suite of climatic conditions by using altitudinal gradients within circumscribed various localities i.e., along Aseer Mountains, Aseer region, KSA. This is in agreement with the data obtained for *Arabidopsis thaliana* in Iberia, Spain, which showed strong genetic isolation by distance among regions associated with major geographic barriers, whereas the populations in northeastern Spain belonging to a distinct genetic group are strongly differentiated from populations in the other Iberian geographic regions [28]. The relation between latitudinal clines and functional genetic variation has been noted in several traits, including flowering time [29], heat shock protein expression [30], response to vernalization [31], freezing tolerance and many else.

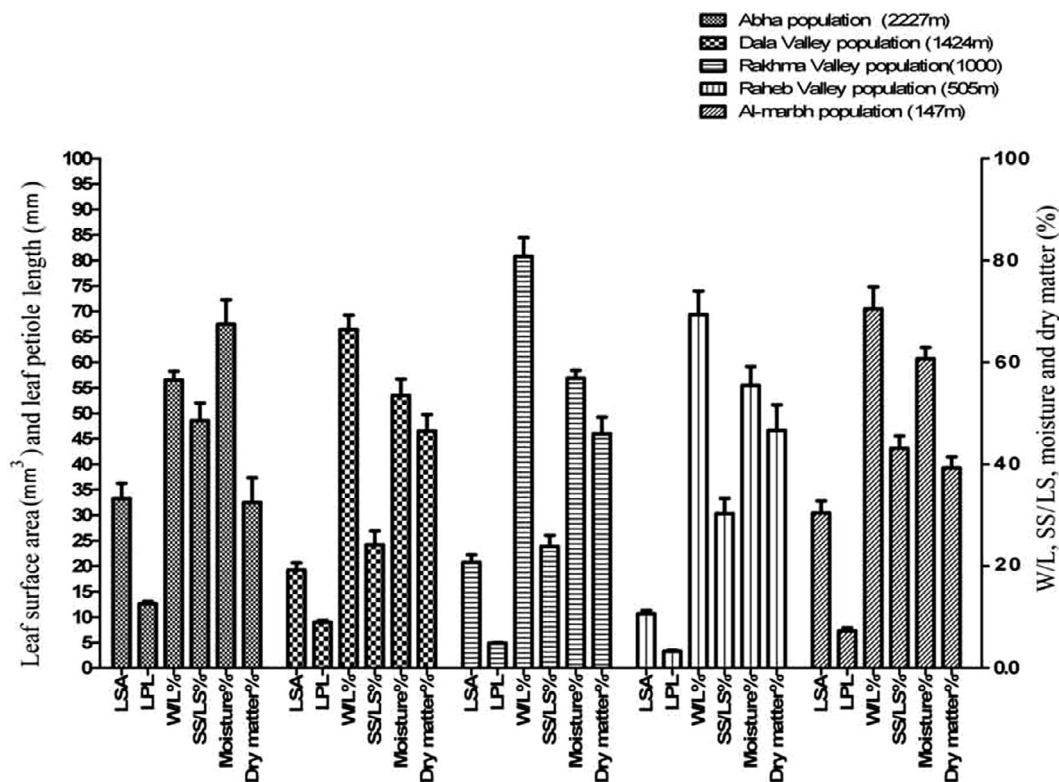
Concerning ISSR markers, the present results showed that the twelve ISSR primers have high polymorphic bands than the twelve RAPD primers applied. These results were in agreement with the results obtained by Manimekalai et al. [32] who reported that in coconut plants (*Cocos nucifera* L), the mean of polymorphic information content (PIC) value for the ISSR was higher than the polymorphic information content observed with the RAPD technique.

Our results showed that the total scrobale DNA fragments and monomorphic generated from ISSR markers were higher than that recorded from RAPD primers. These results are in agreement with the observations of El-Assal et al. [33] and Djamila et al. [34] in terms of numbers of alleles per ISSR primer. This may be due to the abundant natures of the microsatellites due to slippage in DNA replication [35].

The dendrogram that resulted from ISSR markers also revealed molecular discrimination among the five *Z. spina-christi* populations upon the effect of elevations. It showed that the lowest genetic similarity values was between Abha population (2227 m) and Rakhma Valley (1000 m) and the highest similarity between Raheb Valley (505 m) and Al-marbh (147 m) populations. This is in agreement with previous results that generated dendrograms by the ISSR matrix correlated with the genealogy of the barley cultivars [36]. Hence, both RAPD and ISSR markers could be effectively used in determination of genetic relationships among various populations of *Z. spina-christi*.

### 4.2. Chemicals in five *Z. spina-christi* populations along an elevation gradient

To characterize and verify the variations among *Z. spina-christi* plants growing in various localities based on chemical constituents present in their extract, the obtained data were analyzed using cluster analysis. The dendrogram obtained could differentiate among all the studied *Z. spina-christi* from different localities indicating the existence of chemical constituent's polymorphism. The dendrogram obtained from RAPD primers markers showed almost the same population distribution pattern along the altitudinal gradient obtained from chemical constituents of the plant. The RAPD system grouped the various population genotypes differently from ISSR markers. ISSR showed the Dala Valley (1424) meters and Rakhma Valley (1000 m) divergent from each other forming a separate cluster while those two populations were grouped together as per the RAPD primers. Therefore, both RAPD marker and chemicals constituents can be effectively



**Figure 9** Physical characteristics of leaves of *Z. spina-christi* leaves grown in Abha (2227 m) population, Dala Valley (1424 m) population, Rakhma Valley (1000 m) population, Raheb Valley (505 m) population, and Al-marbh (147 m) population. Abbreviations: LSA, leaf surface area; LPL, leaf petiole length; W/L%, leaf width (W)/leaf length (L)  $\times$  100; SS/LS%, short leaf spine (SS)/long leaf spine (LS)  $\times$  100; Moisture (%) = Fresh weight–dry weight/fresh weight  $\times$  100; Dry matter (%) = Average dry weight (g)/Average fresh weight (g)  $\times$  100.

used in determination of the genetic relationships among various populations of *Z. spina-christi* better than the ISSR marker. As mentioned in the genetic diversity part, plants growing along an elevation gradient are exposed to a range of biotic and a biotic environmental factors which in-turn affect the metabolic processes involved in the synthesis of a wide range of secondary metabolites. Similar studies found that there was a significant variation in chemical composition among different species and even among individual trees from the same species as reported in *Copaifera guianensis* Desf., *Copaifera duckei* Dwyer and *Copaifera multijuga* Hayne plants in relation to changing environmental conditions [37]. It was noted that the types of chemical compounds in *Thymus algeriensis* Boiss. et Reut., plant were changed in accordance with geographical location [38]. Many other researchers proofed that plants species exhibited significant variation in their chemical composition and this was attributed to the geographic location of the studied plants [39]. To the best of our knowledge, this is a first report indicating that there is correlation between genetic variation based upon RADPD data and chemical compounds as illustrated from the dendrogram of ISSR, RAPD markers and chemical compounds.

Because there was no available study regarding vitamin contents in the leaves of *Z. spina-christi* plants, in this research the impact of latitude on vitamin contents has been investigated. Significant differences for the content of vitamins B<sub>1</sub>, B<sub>12</sub>, B<sub>2</sub> and folic acid in five *Z. spina-christi* populations were

found. These differences are obviously due to the differences in genotypes but the growing location also may have an effect on the content of such vitamins. In general, warm and less precipitation increases the level of B<sub>2</sub> and Folic acid, whereas cooler and moist conditions favor the production of vitamins B<sub>12</sub> and moderate conditions favor the production vitamins B<sub>1</sub>. Our finding agrees well with those reported by Vlahakis and Hazebroek [40] who observed significant differences for the content of campesterol in nine canola genotypes due to differences in genotypes and to the growing location. In North America Amelung et al. [41], found that there was a parabolic relationship between amino sugars and mean annual temperature (MAT) along a climosequence. Therefore, it is a worthwhile for isolation, characterization of chemical compounds especially vitamins from leaves of *Z. spina-christi* as natural and easy available sources that will be of great pharmaceutical value.

#### 4.3. Leaf physical traits and genetic diversity

Genes influence every aspect of plant physiology, so accurate estimates of leaf surface area, leaf petiole length, percentage of leaf width/leaf length, percentage of short leaf spine/long leaf spine, percentage of moisture content and percentage of dry matter contents can assist in determining physiological status of vegetation which resulted in genetic variation. Significant differences were observed among *Z. spina-christi*



populations as a result of environmental gradient that affects both phenotypes and genotypes. However, it is obvious in our study there was no close relation between the genetic variation and studied morphological characters of the leaf among the various populations as was visible in the resulted dendrogram from RAPD and ISSR markers and chemical compounds clustering.

### Acknowledgments

The authors would like to thank: King Abdulaziz City for Science and Technology (KACST – KSA) for financial support under the grant no (A-1-34-262).

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