Correlation between the Morphological and Genetic Relationships among *Thymus syriacus* Boiss populations from NW-Syria.

***Malak Sabouh, ** Hafez Mahfoud, *Talal Ameen**

*Department of Forestry and Ecology, Faculty of Agriculture , Tishreen University , Lattakia , Syria.

** Department of biotechnology. General Commission for Scientific, Agricultural Research-Latakia-Syria.

** Corresponding author E.mail: ha.mahfoud@hotmail.com

Abstract

The genetic relationships among eighteen populations of *Thymus syriacus.* Boiss collected from two regions (Solas and Kasab) in latakia provence (Syria) were studied using morphology and molecular markers. A total of 20 morphological characters and 10 random amplified polymorphic DNA (RAPD) markers were used.

Cluster analyses of both morphological and RAPD markers demonstrated the presence of clear separation of Solas populations from Kasab ones. The highest dissimilarity coefficient of morphological and RAPD markers were 0.57 and 0.19 respectively.

The obtained dendrograms showed that the applied markers could distinguish the *Thymus syriacus* populations properly, some morphological characters, especially flower and inflorescence, seems to be of great value to estimate morphological differentiation between *thymus* populations. In addition, our study indicate that RAPD marker is suitable approach to determine the polymorphic loci and to estimate the genetic distance between the populations of thyme species.

Key words: *Thymus syriacus*, RAPD, Genetic diversity, morphological characterization, Syria.

1. Introduction:

The genus *Thymus* L. belongs to the family Lamiaceae reviles about 300 species of herbaceous perennials and small shrubs distributed mainly over Mediterranean countries, Northern part of Africa and Southern Greenland (Sunar et al., 2009). The west Mediterranean region seems to be the center of origin of the genus (Maksimovic et al., 2008; Pirbalouti et al., 2016).

This genus is presented in Syrian flora by 6 species (Mouterede, 1966), *Thymus syriacus* Boiss is a perennial aromatic and medicinal shrub grown wildly and abundantly in the north of Latakia province (Syria) as well as the near east countries (Lebanon, Jordan, Irag, Turkey and Iran). The aerial parts of *T. syriacus* Boiss are commonly used as herbal tea and have positive results for cough pertussis, bronchitis and other respiratory complaints (Al-Mariri et al., 2013). The *T. syriacus* Boiss essential oil and its components (mainly Carvacrol, dihydrocarvone, β-caryophyllene, p-cymen) have high antioxidant capacity (Zyzafoon et al., 2012), and antimicrobial activity (Al-Mariri et al., 2013; Ziani et al., 2011).

There have been very few studies on the morphology and anatomy of *Thymus* species in Syria. Mouterede(1966) gave the first detailed information on Syrian *Thymus* species, further Anatomical information on some *Thymus* species collected from Syria has been provided by (Al-Hakim, 1988; Aziz et al., 2008, Khalil et al., 2012, Zyzafoon et al., 2012). In spite of the Importance of *T. syriacus* Boiss there are no detailed studies about the status of its morphological-anatomical characteristics, genetic variation and the relationship among its populations in Syria.

In the present study we use some morphological keys and a molecular marker (RAPD: Random Amplification Polymorphic DNA) to asses morphological and genetic variation within several populations of wild *T. syriacus* Boiss from latakia province (Syria).

2. Materials and methods:

2.1. Location selection and plant Material:

Samples of *T. syriacus* Boiss were collected from wild populations at 6 locations in two regions from North-west Latakia province (Syria) during 2016 and 2017 (Table 1).

Table 1: Location, Altitude and studied populations of Syrian T. *syriacus* Boiss

Location	Altitude/M	Population*
Latakia - Solas	425	S1-1, S1-2, S1-3
Latakia - Solas	450	S2-1, S2-2, S2-3
Latakia - Solas	485	S3-1, S3-2, S3-3
Latakia - Kasab	600	K1-1, K1-2, K1-3
Latakia - Kasab	688	K2-1, K2-2, K2-3
Latakia - Kasab	752	K3-1, K3-2, K3-3

* Morphological observations were conducted on ten living plants per population.

2.2. Morphological data recording:

Morphological observations were conducted on ten living plants per population using 20 morphological features of different plant parts (leaves, flowers, steams, fruits and seeds). The list of descriptors and methods of observation and data recording were consistent with those described by (Morales, 2002; Stahl-Biskup and Saec, 2002).

2.3. DNA extraction and RAPD amplification:

For the molecular analysis, only the young leaves were collected and kept at -80°C until use. Total DNA was extracted from the frozen leaves following the method described by (Doyle and Doyle, 1987) as follows: 1 g of young leaves were grounded in liquid nitrogen into a fine powder

and extracted with CTAB buffer [50 mM Tris HCl, pH8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 2% (w/v) PVP-40 (polyvinylpyrrolidone) and 1% (v/v) b-mercaptoethanol]. The mixture was incubated at 65°C for 30 min, followed by two chloroform/isoamyl alcohol (24:1) extractions. The extracted DNA, then precipitated in 2/3 volumes of cold isopropanol at -20°C. In the final step, the pellet was dissolved in TE (Tris–EDTA) buffer and RNA was removed by using RNase enzyme. The purified total DNA was quantified and its quality verified using a spectrophotometer and according to the initial concentration of DNA, diluted solution with same concentration (15 ng/µL) was prepared by adding TE buffer and stored at 4C.

Ten RAPD primers (VBC biotech - Germany) were used for screening all the accessions and revealing the genetic diversity (Table 2).

The protocol for RAPD analysis was performed in a volume of 25-µl containing 10ng total DNA, 1xPCR buffer (Vivantis - Malaysia), 3.0mM MgCl2, 200mM deoxynucleotide triphosphates (dNTPs), 1mM primer, 1mg/mL (w/v) Bovine Serum Albumin (BSA) and 1unit Taq DNA polymerase (Vivantis - Malaysia). The amplification reactions were performed in a Biometra (Flixe-gene - Germany) thermocycler and consisted of an initial 7 min denaturation step at 94°C, followed by 40 cycles of 30 sec at 94°C, 1 min at (32 -34)°C and 2 min at 72°C. A final extension of 10 min at 72°C completed the amplification. The PCR products were separated in 1.2% agarose gels. Gels were stained with ethidium bromide, visualized with a UV trans illuminator (CAMAG Reprostar3 - Switzerland).

Table 2: List of selected RAPD primers for polymorphic DNA generations.

	Primer Name	Sequence (3` - 5`)	Temperature

1	OPH-16	3´-TCTCAGCTGG-5´	34
2	OPY-07	3´-AGAGCCGTCA-5´	34
3	OPAF-14	3´-GGTGCGCACT-5´	34
4	OPAK-06	3´-TTGGGAGATA-5´	34
5	OPAG-02	3´-CTGAGGTCCT-5´	32
6	OPAN-08	3´-AAGGCTGCTG-5´	34
7	OPBB-06	3´-TTCCCGTGAG-5´	34
8	OPTH-01	3´-GAATGCTCCG-5´	34
9	OPTH-02	3´-CTAACCGGCA-5´	32
10	OPTH-04	3´-TTGCCGTGAT-5´	34

2.4. Statistical Analysis:

To calculate RAPD polymorphism, a binary data matrix was made based on the marker data. RAPD markers were scored for the presence (1) or absence (0) of amplified bands for each of 18 samples. Similarity index was estimated using the Jaccard's coefficients. Cluster analysis was performed using the unweighted pair group method with an arithmetic mean (UPGMA), and the dendrograms were drawn using NTSYS software version 2.02 (Rohlf, 2002).

3. Results:

3.1. Morphological data:

According to our field observation and morphological characterization of 18 *T. syriacus* Boiss populations depending on 20 morphological traits (Table 3), Syrian *T. syriacus* Boiss could be described as a perennial herbs, basal parts woody, erect, much branching 30-50cm high, basal parts brown and woody, upper stems green and slender. Leaves 10-30 x 2-9mm, green to green grayish, linear to linear oblong, obtuse to subacute at apex, both surfaces covered sparsely glandular hairs, veins usually weakly prominent or not, leaf margin entire. Inflorescence 30-

80mm, 30-100 flowered. Sepals 4, green. Corolla white-pink to purplish-pink, bilabiate, upper lip 2-lobed, lower lip 3-lobed, outer surface pubescent and glands. Anthers 4, purplish-pink. Ovary 4-lobed, oblong, style white, stigma purple-pink. Fruits, more or less-oblong 0.2-0.6 x 0.1-0.2mm, brown to dark-brown. Seeds more or less-oblong 0.16-0.6 x 0.08-0.18mm, black.

Table 3: Descriptors for morphological characterization of *T. syriacus* Boiss

Code	Traits/descriptors	Score code - descriptors state
1	**leaf**	
1-1	Wide (mm)	<3 small, 3-5 medium, >5 large
1-2	Length (mm)	<17 small, 17-20 medium, >20 large
1-3	Shape of life blade	<0.13 linear, 0.13-0.2 linear-oblong, >0.2 oblong
1-4	colour	1 green, 2 greyish
1-5	Pubescence	0 absent; 1 present
1-6	Leaf margin	0 entire; 1 dentate
2	**Steam**	
2-1	Colour	1 green, 2 brown
2-2	Pubescence	0 absent; 1 present
3	**flower**	
3-1	Number per inflorescence	<58 low, 58-83 medium, high >83
3-2	Colour of sepal	1 green, 2 greenish
3-3	Colour of petal	1 violet, 2 violet purple
3-4	Stamen number	1 four stamen, 2 more or less

4 Fruit	
4-1	Wide (mm) <0.133 small, 0.133-0.166 medium, >0.166 large
4-2	Length (mm) <0.3 small, 0.3-0.5 medium, >0.5 large
4-3	Shape <0.27 more oblong, 0.27-0.34 oblong, >0.34 less oblong
4-4	Colour 1 brown, 2 dark brown
5 Seed	
5-1	Wide (mm) <0.121 small, 0.121-0.153 medium, >0.153 large
5-2	Length (mm) <0.23 small, 0.23-0.46 medium, >0.46 large
5-3	Shape <0.243 more oblong, 0.243-0.286 oblong, >0.286 less oblong
5-4	Colour 1 brown, 2 dark brown

Closter analysis of morphological data (Figure 1) indicate a high level of morphological variation exists within *T. syriacus* Boiss populations (57%).

All thymus populations were grouped in two distinct clusters. The first one included all Solas populations diverging at a morphological distance of about 45%, while the second cluster include all Kasab populations with more morphological variation ratio (49%).

Variables such as leaf shape and size, flower number per inflorescence, main color of petals, fruit shape and size explained the largest portion of the total variance observed.

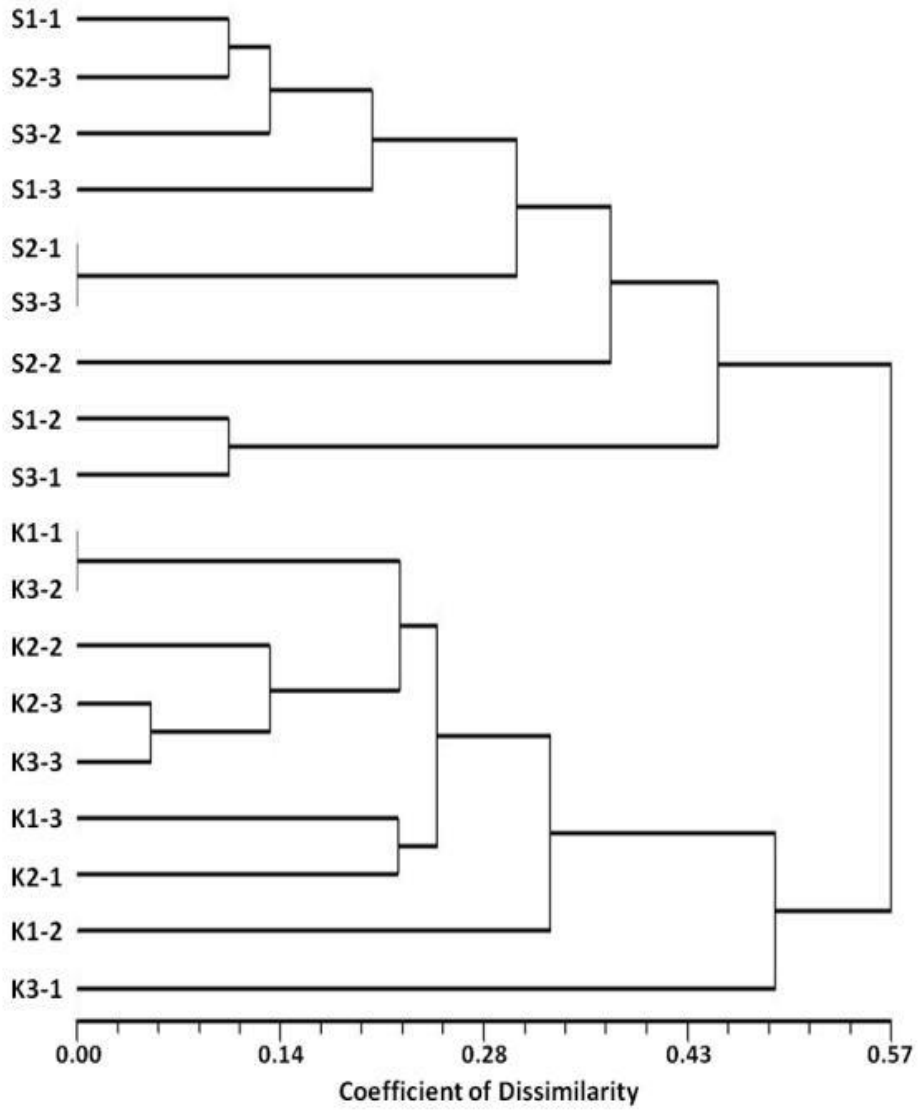

Figure 1. UPGMA dendrogram of eighteen *T. syriacus* Boiss based on morphological data.

3.2. RAPD marker data:

Total of 16 primers were screened from which 10 primers were selected for RAPD analysis. An average 5.7 bands were obtained per primer and 2.4 of these were polymorphic. OPTH-01 primer and OPBB-06 primer yielded the lowest (3) and highest (8) number of bands, respectively as shown in Table 4. Primer OPAG-02 (Figure 2) gave the highest percentage of polymorphism

(57.14%), while the lowest percentage of polymorphism (20%) was obtained by the primer OPAF-14 (Table 4).

The banding profiles for some Kasab populations of *T. syriacus* Boiss (K1-1, K1-2 and K2-1, K3-3), and two populations from Solas (S1-1, S1-2) appeared to be identical with a resemblance value of 100%. However, evident differences in banding profiles were found among Solas and Kasab populations as well as among several populations in the same localities.

Table 4: The percentage of polymorphic sequences of used primers in the RAPD analysis of *Thymus syriacus* populations

Nr.	Primer Name	Total number of bands amplified	Polymorphic bands	Percent polymorphism
1	OPH-16	5	2	40
2	OPY-07	7	3	42.85
3	OPAF-14	5	1	20
4	OPAK-06	6	3	50
5	OPAG-02	7	4	57.14
6	OPAN-08	5	2	40
7	OPBB-06	8	4	50
8	OPTH-01	3	1	33.33
9	OPTH-02	6	2	33.33
10	OPTH-04	5	2	40
Sum		57	24	42.1

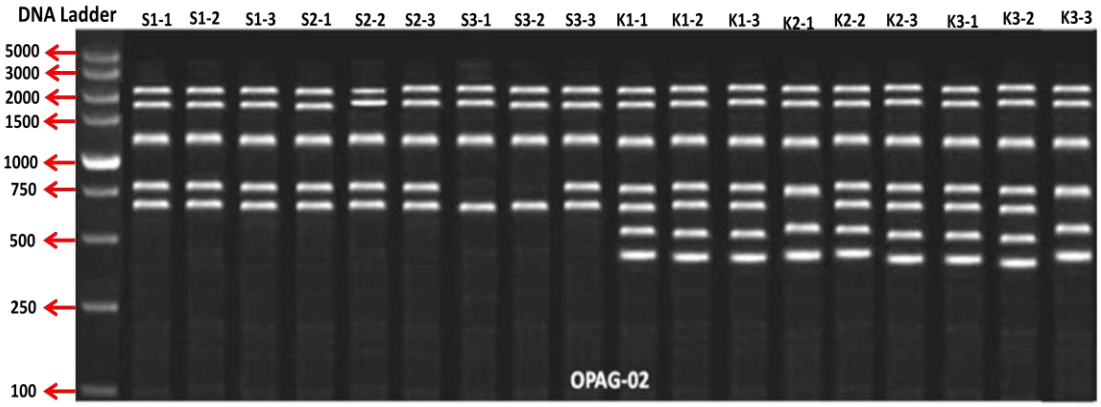

Fig.2.Example of an agarose gel showing the amplified DNA patterns obtained with a RAPD-PCR reaction with primer OPAG-02

Cluster analysis based on jaccards similarity coefficients and UPGMA method, were divided the studied genotypes into 2 well-separated clusters with a genetic variation of 19%.The first one include all *T. syriacus* Boiss populations from Solas with only a small amount of variation (6%) present differences within populations, while all Kasab populations were included in a second cluster with a genetic variation ratio of (8%) (Figure 1) .

T. syriacus Boiss Solas populations could be separated into two sub-clusters, one group representing 6 populations from three different locations, while the other group consists of 3 populations from two locations. Reassembly, *T. syriacus* Boiss Kassb populations were further divided into two smaller sub-clusters (Figure 3).

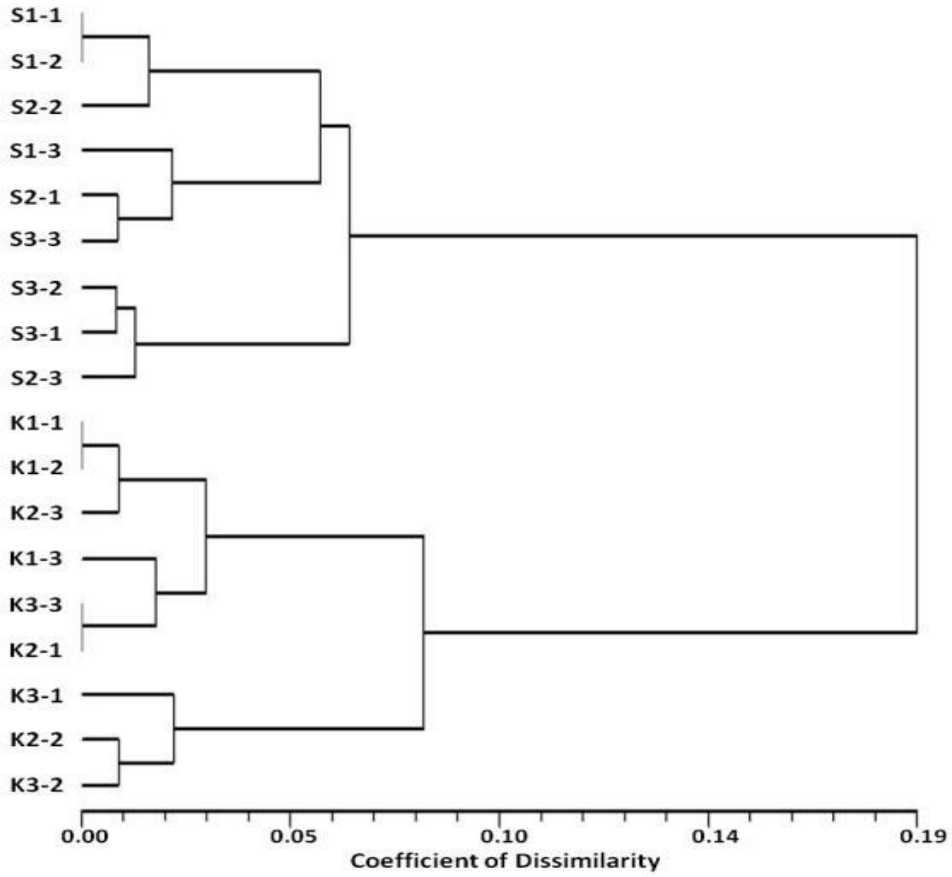

Figure 3. UPGMA dendrogram of eighteen *T. syriacus* Boiss populations based on RAPD marker data.

4. Discussion:

The genus *Thymus* is very rich in spices, with a high degree of diversity within the genus and the difficulty to differentiate them from each other, some authors have proposed morphological descriptors (Morales et al., 2005; Stahl-Biskup and Sace, 2002; Akcin, 2006) while others used molecular markers (Aziz et al., 2008; Khalil et al., 2012; El-Hadje Ali et al., 2012, Rustaiee et al., 2013). Although *T. syriacus* Boiss samples in our study from two closely related locations were separated morphologically and genetically into two different groups. Our results are in

contrast to the above mentioned studies by evaluating the use of *Thymus* morphological characters as well as molecular markers to determine the taxonomy of several *Thymus* species.

Morphological data showed the presence of two partially isolated groups, one representing all Solas populations and another one with all populations from Kasab, this is due to the fact that all *T. syriacus* populations from Solas are distinguished from Kasab populations by some morpgological characters as leaf shape and size, color of petals and flower number per inflorescence. Leaf and flower characters have been mentioned as useful taxonomic characters within the genera belongs to the family Labiatae (El-Gazzar and Watson, 1970), which is in contrast with our results.

The results presented in this study demonstrate a significant correlation between genetic and geographic distances of Syrian *T. Syriacus* Boiss populations; our results are in contrast with several previous studies referred to RAPD marker as an efficient tool to detect differentiation of geographically and genetically isolated populations (Khalil et al., 2012; Solyma and Alkowni, 2014; Panda et al., 2015). In addition, low genetic distance between the two main clusters suggests that these populations are undergoing ecological and evolutionary factors more than genetic differentiation.

5. Conclusion:

Morphological variation and genetic diversity detected within *T. Syriacus* Boiss populations in the present study is of great value in *Thymus* breeding programs to select excellent cultivars from the wild populations with high value and strong adaptation.

References:

1- Akcin, T. A. 2006. Numerical Taxonomic Studies on Some Species of the Genus *Thymus* L. (Labiatae) in Turkey. Asian Journal of Plant Sciences. vol.5, pp.782-788.

2- Al-Hakim, W. 1988 . The plants medicinals and aromatics in Syria, PHD. Fac. Of science. Montpellier, France. 280 P.

3- Al-Mariri, A., Swied, G., Oda, A. and Al-Hallab, L. 2013. Antibacterial Activity of *Thymus Syriacus* Boiss Essential Oil and Its Components against Some Syrian Gram-Negative Bacteria Isolates. Iran J Med Sci. vol. 38(2), pp. 180–186.

4- Aziz, R. A., Hamed, F. and Abdulaa, N. A. 2008. Molecular Characterization and Genetic Relatedness Between some Accessions of *Thymus serpyllum* in Syria Using RAPD Technique. Journal of Damascus university - Agricultural science. Vol 24(1), pp. 263-282.

5- Doyle, J. J. and Doyle, J. L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin, vol.19, p.11-15.

6- El-Gazzar, A. and Watson, L. 1970. A taxonomic study of Labiatae and related Genera. New. Phytol. vol. (69), pp.451-486.

7- El-Hadji Ali, I. B., Guetat, A. and Boussaid, M. 2012. Genetic diversity and structure of wild Tunisian *Thymus capitatus* (L.) Hoffm. et Link. (Lamiaceae) assessed using isozyme markers. Afrrican Journal of Ecology. Vol. 50(2), pp. 140-151.

8- Khalil, R., Khalil, R. and LI, Z. 2012. Determination of genetic variation and relationship in Thymus vulgaris populations in Syria by random RAPD markers. Plant Biosystems. Vol.146, pp. 217-225.

9- Maksimovic Z, Stojanovic D., Sostaric, I., Dajic, Z. and Ristic, M. 2008. Composition and radical-scavenging activity of Thymus glabrescens Willd. (Lamiaceae) essential oil. J Sci Food Agr vol. 88, pp. 2036-2041.

10- Morales, R. 2002. The history, botany and taxonomy of the genus *Thymus*. Pp: 1-43 in Stahl-Biskup, E & Saec, (eds), Thyme: the genus thymus: Taylor & Francis.

11- Mouterede, P. 1966. Nouvelle flore du Liban et de la Syrie. Dar el Mechreq ,Beyrouth. Liban .

12- Panda, S., Naik, D. and Kamble, A. 2015. Population structure and genetic diversity of the perennial medicinal shrub *Plumbago.* AoB Plants. vol. 7(48), pp. 1-12.

13- Pirbalouti, A. G., Bistghani, Z. E. and Malekpoor, F. 2016. An overview on genus *Thymus*. vol. 6(2), pp. 93-100.

14- Rohlf, F. J. 2002. Numerical Taxonomy and Multivariate Abalysis System. NTSYS version 2.11a.Applied Biostatistics Inc., New York, Stoy Book. N.Y., USA 231.

15- Rustaiee, A. R., Yavari, A., Nazeri, V., Shokrpour, M., Sefidkon, F. and Rasouli, M. 2013. Genetic diversity and chemical polymorphism of some *Thymus* species. Chem Biodivers. Vol. 10(6), pp. 88-98.

16- Solyman, E. and Alkowni, R. 2014. RAPD for Assessment of *Thymes* Genetic Diversity in Palestine. Palestine Technical University Research Journal. vol. 2(2), pp. 01-08.

17- Stahl-Biskup, E. and Saec, F. 2002. *Thyme*: the genus *Thymus*: Taylor & Francis Group, LLC. Boca Raton, FL 33487-2742. P332.

18- Sunar, S., Aksakal, O., Yildirim, N., Agar, G., Gulluce, M. and Sahin, F. 2009. Genetic diversity and relationships detected by FAME and RAPD analysis among *Thymus* species growing in eastern Anatolia region of Turkey. Rom. Biotechnol. vol. 14, pp. 4313-4318.

19- Zayzafoon, G., Odeh, A. and Allaf, A. W. 2012. Determination of essential oil composition by GC-MS and integral antioxidant capacity using photochemiluminescence assay of two *Thymus* leaves: *Thymus syriacus* and *Thymus cilicicus* from different Syrian locations. Herba polonica. Vol. 58(4), pp. 70-84.

20- Ziani, K., Chang, Y., McLandsborough, L. and McClements, D. J. 2011. Influence of surfactant charge on antimicrobial efficacy of surfactant-stabilized thyme oil nanoemulsions. J Agric Food Chem. Vol.59, pp. 6247–55. doi: 10.1021/jf200450m. PubMed PMID: 21520914.