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Full Length Research Paper

Genetic diversity of seed storage protein in the Ethiopian garden cress (*Lepidium sativum* L.)

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The Ethiopian garden cress (*Lepidium sativum* L.) is an important crop extensively used as food and medicine. In this study, total seed storage proteins of 112 garden cress genotypes collected from diverse growing regions in Ethiopia were investigated to assess patterns of genetic diversity and relationships. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a total of 1774 stable protein bands were identified through discontinuous electrophoresis. Of these, 1597 bands were polymorphic. A maximum of 20 protein sub-units in the range of 15 to 75 kDa were observed per genotype. The similarity coefficient among these genotypes ranged from 0.25 to 1.00 with an average genetic dissimilarity of 0.2754. On the basis of Ward Euclidian distance, the genotypes were grouped into five major clusters, the largest one comprised of 62 genotypes (Cluster III) followed by 26 genotypes in Cluster I. Cluster IV and V contained a total of 14 genotypes that were the most distantly related to other groups, and thus can be potentially used as parents for exploitation of heterotic effects in hybrid breeding programs. Our findings using SDS-PAGE profiles revealed no obvious association between geographic region of origin and germplasm clustering. However, the polymorphism and cluster analysis indicated that garden cress genotypes differed greatly in the composition of seed proteins. This shows that protein profiling could be used as a rapid and reliable method for genetic diversity studies. In order to fully explore the protein based genetic diversity in garden cress germplasm, techniques such as 2-D gel electrophoresis are recommended in future studies.

Key words: Cluster analysis, dissimilarity index, garden cress, *Lepidium sativum*, protein polymorphism, protein profiling, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), seed storage protein.

INTRODUCTION

Garden cress (*Lepidium sativum* L.) belongs to the family Brassicaceae and the genus *Lepidium* which contains about 150 species that are distributed throughout almost all temperate and subtropical regions of the world (Bermejo and Leon, 1994; Wadhwa et al., 2012; Rava, 2016). Garden cress is widely cultivated in Africa, Europe, Russia and North America. It is a fast growing, edible herb and an important medicinal plant having a wide range of desirable effects on human health since the Vedic era about 3000 years ago (Manohar et al.,

2012; Doke and Guha, 2014). Various beneficial effects have been observed with the consumption of garden cress. Different parts of the plant including roots, seeds and leaves have been used as a source of functional food and/or medicine (Rava, 2016).

The seeds and leaves of garden cress contain volatile oils and have been consumed as salad and as spice (Amare, 1976; Wadhwa et al., 2012; Rava, 2016). Its seeds are rich source of proteins, carbohydrate, fat (for example omega-3 fatty acids), dietary fiber, vitamins (tocopherol, β--carotene and ascorbic acids), minerals (K, Mg, P, Ca, Fe), and other essential nutrients and phytochemicals (Gokavi et al., 2004; Doke and Guha, 2014). This indicates that the seeds of garden cress play vital role as a promising multipurpose medicinal and nutritional plant. Nowadays, garden cress is becoming popular not only because of its superior medicinal and nutritional values but also due to its contribution in the biofortification of nutritionally inferior crops (Manohar et al., 2012; Singh et al., 2015) in order to ensure the nutritional security of the global population.

Diversity existing in germplasms of crops collected from diverse growing regions need to be properly characterized and evaluated to improve strategies for conservation and utilization towards cultivar development (Parashar et al., 2015; Sharma and Krishna, 2017). Several strategies have been adopted for germplasm characterization including the use of morphological markers (Kancherla and Bhalla, 2003), seed storage protein markers and DNA molecular markers (Rahman and Hirata, 2004). Using quantitative, biochemical and molecular markers suitable germplasm for future plant breeding programs can be identified. Studies in 49 Ethiopian garden cress landraces using morphological and yield-related traits revealed huge variability at diverse agro-ecological zones (Temesgen et al., 2013a, b). Similarly, large diversity was recorded among 85 Ethiopian garden cress genotypes using inter simple sequence repeat (ISSR) (Said and Kassahun, 2015).

Storage or structural seed proteins, encoded by families of polymorphic genes (Mandal and Mandal, 2000) have been extensively used as a genetic marker as they are largely independent of environmental fluctuations (Hameed et al., 2009). Unlike morphological markers (Siddiqui and Naz, 2009), the banding patterns of protein markers are stable (Iqbal et al., 2005; Nasar et al., 2006; Iqbal et al., 2014). Due to these benefits, protein markers have been widely applied in the analysis of genetic diversity within and between accessions, in studying plant domestication in relation to genetic resource conservation and breeding, and in establishing genome relationships (Kakaei and Kahrizi, 2011; Hameed et al., 2012; Sharma and Krishna, 2017). Seed storage protein profiling has been used in investigating diversity among selected varieties of *Brassica napus* (Nasar et al., 2006; Choudhary et al., 2015). Similarly, seed protein profiles were instrumental in the identification of intra-specific genetic divergence in rape seed (Khan et al., 2014).

The use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in studying plant proteins was found to be simple and inexpensive; hence, it has applications in the improvement of the crop of interest through breeding (Zada et al., 2013; Sharma and Krishna, 2017). However, so far, only few studies were made regarding the diversity of storage proteins in the garden cress germplasm. Gianazza et al. (2007) examined the influence of different concentrations of cadmium on the garden cress plants using seed protein storage marker. However, genetic diversity in the seed protein of garden cress was so far not made using the SDS-PAGE, at least in the Ethiopian germplasm collections. Hence, the current study was conducted to investigate using SDS-PAGE diversity in the seed storage protein of the Ethiopian garden cress accessions collected from diverse agro-ecological regions in the country.

MATERIALS AND METHODS

Plant materials and protein samples

Lists of plant materials used in the study and their origin are presented in Table 1. For extraction of proteins, mortar and pestle were used to crush and grind seeds of each genotype. Defatting of about 0.1 g of flour was carried out with chloroform, methanol and acetone in the ratio of 2:1:1 as described by Geetha and Balamurugan (2011) for mustard genotypes. Next day, about 0.05 g of flour was suspended in 1 ml extraction buffer (0.125 M Tris-Cl, 4% SDS, 20% v/v glycerol, 1% 0.2 M DTT, 25 mM EDTA, pH 6.8). Homogenized mixture was incubated at room temperature for 3 h. Protein extraction buffer was properly mixed by vortexing for 5 to 10 min intermittently. The solubilized samples were centrifuged at 14000 rpm for 10 min at 4°C, and the clear supernatant saved at 4°C until they were run gel electrophoresis following the methods of Roy and Kumar (2014) and Buckseth and Singh (2016) with some modification. The gel electrophoresis was run by mixing of 30 μl of the protein extract with 30 μl of sample loading buffer (0.1% of bromophenol blue, 2% SDS, 6% v/v glycerol, 2.5% 0.5 M Tris-HCL PH 6.8 and 1% 0.5 M DTT) followed by vortexing and heating at 65°C for 10 min to ensure complete denaturation, and then with brief vortexing and spinning at room temperature just before loading on gel (Bollag et al., 2002; Nisar et al., 2016).

The SDS-PAGE was carried out in various combinations and optimized to 12.5% acrylamide gel concentration and by loading 30 μl of samples to obtain the best resolution. The procedure

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Table 1. Names and origin of plant materials used in the study.

Accession ID	Origin		
	Regional state	**Zone**	**District/location**
229799	Amhara	East Gojam	Enbise SarMidir
229798	Amhara	East Gojam	Hulet Ej Enese
CG14	Amhara	North Gojam	Goncha Siso Enese
235892	Amhara	North Gondar	Addi Arkay
214243	Amhara	North Gondar	Debark
205163	Amhara	North Gondar	Debark
205162	Amhara	North Gondar	Debark
208030	Amhara	North Gondar	Gondar Zuria
CG12	Amhara	North Shewa	Efratana Gidim
229203	Amhara	North Shewa	Lay BetnaTach Bet
229202	Amhara	North Shewa	Lay BetnaTach Bet
229200	Amhara	North Shewa	Lay BetnaTach Bet
229201	Amhara	North Shewa	Lay BetnaTach Bet
229204	Amhara	North Shewa	Lay BetnaTach Bet
229199	Amhara	North Shewa	Siyadebrina Wayu Ens
229205	Amhara	North Shewa	Weremo Wajetuna Mid
241777	Amhara	North Wello	Guba Lafto
207542	Amhara	South Gondar	Kemekem
90004	Amhara	South Gondar	Tach Gayint
90018	Amhara	South Wello	Debresina
CG7	Amhara	South Wello	Debresina
90020	Amhara	South Wello	Dessie Zuria
212628	Amhara	South Wello	Kutaber
215714	Amhara	South Wello	Werebabu
215713	Amhara	South Wello	Werebabu
CG11	Amhara	South Wello	Werebabu
CG22	Amhara	South Wello	Werebabu
90012	Unknown		
233679	Unknown		
233370	Unknown		
240579	Unknown		
90010	Unknown		
90009	Unknown		
90014	Unknown		
90017	Unknown		
90007	Unknown		
90008	Unknown		
205141	SNNP	Gurage	Goro
242916	SNNP	Keficho Shekicho	Chena
240396	SNNP	Keficho Shekicho	Decha
240397	SNNP	Keficho Shekicho	Decha
202116	SNNP	Keficho Shekicho	Ginbo
CG17	SNNP	North Omo	Basketo
225725	SNNP	North Omo	Bonke
CG13	SNNP	North Omo	Damot Wayde
8604	SNNP	North Omo	Damot Weyde
240808	SNNP	North Omo	Damot Weyde
90016	SNNP	North Omo	Gofa Zuria
CG16	SNNP	North Omo	Goffa Zuria

Table 1. Contd.

225799	SNNP	North Omo	Kemba
CG18	SNNP	North Omo	Mareka Gena
240578	SNNP	North Omo	Melokoza
CG21	Tigray	West Tigray	Shire
233985	Tigray	West Tigray	Laelay Adiyabo
219959	Tigray	West Tigray	Medebay Zana
216885	Oromia	Arssi	Merti
216886	Oromia	Arssi	Merti
CG15	Oromia	Arssi	Tiyo
CG20	Oromia	Bale	Meliyu
237991	Oromia	Bale	Adaba
CG4	Oromia	Bale	Agarfa
CG5	Oromia	Bale	Dinsho
CG9	Oromia	Bale	Gaserana Gololcha
19001	Oromia	Bale	Ginir
212852	Oromia	Bale	Goro
19002	Oromia	Bale	Goro
212853	Oromia	Bale	Goro
90002	Oromia	Bale	Sinanana Dinsho
CG6	Oromia	Bale	Sinanana Dinsho
230524	Oromia	East Hararghe	Girawa
CG10	Oromia	East Shewa	Akaki
90006	Oromia	East Hararghe	Deder
90005	Oromia	East Hararghe	Deder
230831	Oromia	East Hararghe	Girawa
208693	Oromia	East Hararghe	Gursum
216816	Oromia	East Hararghe	Gursum
230830	Oromia	East Hararghe	Jarso
208669	Oromia	East Hararghe	Kersa
234828	Oromia	East Wellega	Diga Leka
CG8	Oromia	Jimma	Limu Seka
18843	Oromia	North Shewa	Debre Libanos
208666	Oromia	West Hararghe	Mieso
18841	Oromia	West Shewa	Bako
90021	Oromia	West Shewa	Cheliya
19000	Oromia	West Hararghe	Chiro
CG2	Oromia	West Hararghe	Chiro/Wachu
CG3	Oromia	West Hararghe	Gemechis
208667	Oromia	West Hararghe	Habro
CG19	Oromia	West Hararghe	Mesela
CG1	Oromia	West Shewa	Wolmera
90022	Oromia	West Wellega	Dale Lalo
208769	Oromia	West Wellega	Sayo
215808	Oromia	West Wellega	Sayo
215807	Oromia	West Wellega	Sayo
230829	Somalia	Jigjiga	
230523	Somalia	Jigjiga	
216815	Somalia	Jigjiga	
231210	Somalia	Jigjiga	
233982	Tigray	Central Tigray	Adwa
237512	Tigray	Central Tigray	Adwa

Table 1. Contd.

219961	Tigray	Central Tigray	Adwa
207910	Tigray	Central Tigray	Adwa
219958	Tigray	Central Tigray	Laelay Maychew
233984	Tigray	Central Tigray	Werielehe
233983	Tigray	Central Tigray	Werielehe
238273	Tigray	Central Tigray	Adwa
219962	Tigray	Central Tigray	Naeder Adet
233981	Tigray	East Tigray	Ganta Afeshum
242609	Tigray	East Tigray	Ganta Afeshum
234355	Tigray	East Tigray	Ganta Afeshum

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developed by Laemmli (1970) was followed for gel preparation and running. 30 µl of samples digested was loaded into the wells of 4% acrylamide stacking gel (1.5 mm thick) for protein separation. Electrophoresis was carried out at a constant 250 V for the medium slab vertical gel apparatus until bromophenol blue marker crossed bottom of the gel. Pre-stained protein marker, ranging from 10 to 250 kDa (precision plus protein dual color standard supplied by BIO-RAD) was run for reference to molecular weight of respective protein bands in KDa. After complete run, the gels were fixed and stained with 0.5% coomassie brilliant blue (CBB) R-250 in acetic acid: methanol: water (10:40:50 volume ratio) for 3 h and destained in the same acetic acid-methanol-water solution except CBB for overnight (Bollag et al., 2002; Sadia et al., 2009) with constant and gentle shaking.

Data analysis

Gel evaluation for data scoring was done on a light box and rechecked by using photograph that was taken by high resolution camera supported by white light illuminator. The experiment was repeated twice to check the reproducibility of the protein bands. A band presence was coded (1), while the absence of bands scored as (0). Only reproducible bands occurring in high frequency were scored by identifying each protein band carried out according to standard proteins. The intensity of bands was not taken into consideration but only the presence of the bands was taken as indicative. Presence and absence of the bands were entered in a binary data matrix. Based on results of electrophoretic band spectra, similarity index was calculated for all possible pairs of protein type's electrophore-grams. Similarities among genotypes were estimated using Jaccard coefficient of similarity (Jaccard, 1908). Depending on the electrophoretic band spectra, similarity index (S) was designed for all pairs of protein band pattern by the subsequent formula: $S = a/(a + b)$ where S = similarity index, a = Number of bands common to a and b protein types, b = Number of bands in protein type 'b'. The similarity matrix was generated and converted to a dissimilarity matrix. The different bands in the range of 15-75 kDa were used for calculation of similarity indices. Polymorphism % was calculated using the formula:

Polymorphism (%) = [Number of polymorphic bands/Total number of bands] ×100.

The generated data matrix was then used for descriptive statistics and for constructing dendrogram by the ward method using Darwin version 6 Software.

RESULTS AND DISCUSSION

Genetic diversity in seed storage protein

A typical electrophoretic banding patterns and their distribution is presented in Figure 1. A maximum of 20 protein sub-units were observed per genotype within the range of protein molecular weight of 15 to 75 kDa. A total of 1774 polypeptide bands with an average of 15.8 bands per genotype were obtained (Table 2). Out of the total of 1774 bands, 1597 were polymorphic while 177 were monomorphic (data not shown). The banding patterns revealed large variations among genotypes in the low molecular weight protein profiles. This revealed considerable variations in five regions (A to E) (Figure 1). Region A contains relatively high molecular weight proteins ranging from 50 to 75 kDa while in Region B, three protein sub-units ranging in size from 37 to 50 kDa are observed. Five protein bands were found for Region C which ranged from 25 to 37 kDa while Region D comprised of four protein sub units ranging from 20 to 25 kDa. The last part, Region E, contains small size proteins with molecular weight ranging from 15 to 20 kDa. All these five regions showed both light and dark stained bands and were polymorphic except for the last two bands of Region E which were monomorphic (Table 1). This indicates that the proportion of polymorphic bands over the total bands detected were 90%. Ten genotypes (namely, 241777, 229203, 235892, 229205, 214243, 208030, 229199, 237991, 216816 and CG2) showed the highest number of protein bands (each 20 bands) followed by 12 genotypes (207542, 229202, 229799, 212628, 229204, CG14, 238273, 90021, 208667, 219961, 242609, and CG6) each with 19 bands. Three genotypes (90002, 229799 and 90022) had minimum number of protein bands ranging from 7 to 9. Protein sub units located on band number 19 and 20 were the most frequent (Table 2). Similar banding patterns were reported for Brassica species (Rabbani et al., 2001; Nasar et al., 2006; Turi et al., 2010).

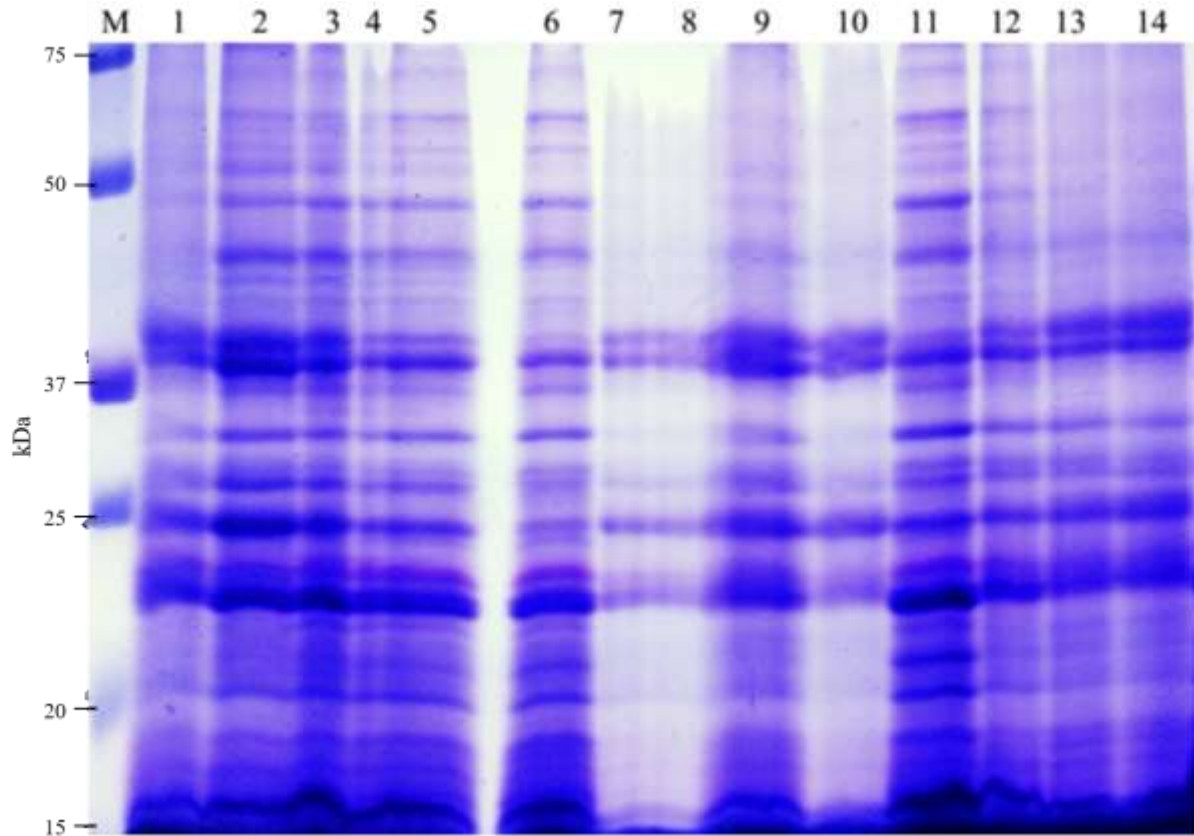

Figure 1. Atypical electrophoretic banding pattern of genotypes generated through SDS-PAGE M represents molecular size marker, while numbers from 1-14 represent selected land cress genotypes.

SDS-PAGE has of paramount importance in separating and characterizing the proteins and estimating the extent of genetic diversity in the present set of garden cress germplasm. The banding pattern in the total seed protein showed close relationships among these studied genotypes. Similarity coefficient among these genotypes ranged from 25 to 100%. This is in agreement with earlier study on *Brassica carinata* where a similarity coefficient of 50 to 100% was reported (Zada et al., 2013).

Proteins have been used as markers for the assessment of genetic diversity in many crops (Iqbal et al., 2005; Nisar et al., 2016; Singh et al., 2017). The seed protein fragments exhibited appreciable polymorphism among the Ethiopian genotypes of garden cress, being used for the study of variability (Table 2). Consequently, electrophoretic analysis of the seed proteins had direct relationship to the genetic background of the proteins, and hence it is a potential marker for the study of genetic diversity and varietal identification. Similar studies have been carried out using protein marker for the study of genetic diversity and/or varietal identification in many crops, as mentioned above (Iqbal et al., 2005; Netra and Prasad, 2007; Nisar et al., 2016; Singh et al., 2017). In general, DNA markers are more robust to detect variability among different genotypes. The only diversity study on garden cress using DNA markers was the one using less efficient inter simple sequence repeat (ISSR) (Said and Kassahun, 2015).

Cluster analysis of seed storage proteins

The cluster analysis showed that the genotypes were divided into three main groups (SG1-SG3) consisting of five major clusters and several sub-clusters (Figure 2). According to the magnitude of this genetic distance, genotypes from Cluster IV and V (SG3) were most divergent from the other two groups (SG1 and SG2). Such divergent genotypes should be used for designing effective breeding programs for evolving genetically vigor and variable breeding lines. Similarly, Cluster I and II represent relatively diverse group as compared to Cluster III. Thus, crossing between genotypes from Cluster I, II, III and genotypes from Cluster IV and V gene pools could create more genetic variability than crosses within genotypes of each main group (Figure 2). They are used

Table 2. The distribution and presence of bands in SDS-PAGE for 112 garden cress genotypes

Region	Code of protein band	Number of genotypes	
		Present	Absent
A	1	56	56
	2	41	71
	3	48	64
	4	49	63
B	5	94	18
	6	93	19
	7	97	15
C	8	88	24
	9	107	5
	10	104	8
	11	99	13
	12	108	4
D	13	101	11
	14	65	47
	15	101	11
	16	90	22
E	17	106	6
	18	103	9
	19	112	0
	20	112	0
Total		1774	
Mean per genotypes		15.84	

to develop desirable recombinant breeding lines and cultivars for future breeding programs.

Cluster III was the largest among all five clusters and consisted of 62 most similar genotypes (55% of the total), revealing low genetic diversity at genomic level (Table 3). However, the results might indicate the limitation on the number of markers used in the current study. In this connection, Opond-Konadu et al. (2005) reported the absence of large genetic difference among cowpea genotypes which hindered the use of protein electrophoresis to investigate diversity. Hence, genotypes in cluster III need to be further investigated in combination with 2D electrophoresis to minimize the lower variability detection efficiency of SDS-PAGE (Javaid et al., 2004; Jan et al., 2016). In this case, the integration of the usual electrophoresis separation with isoelectric focusing point electrophoresis to maximize the resolving power of seed storage protein markers due to amphoteric nature of amino acids is useful.

The cluster analysis also revealed that genotypes from different zones were observed to be closely related and genotypes from the same zone had different genetic background. This suggests that different selection pressures have been applied to yield and other biochemical properties in different genotypes. The high diversity among the genotypes from same region also shows the high exchange of germplasm among garden cress farmers although the exact mechanism of seed exchange among farmers from different regions has not yet been reported. According to Sihag et al. (2004) and Faisal et al. (2009) the cluster pattern for soybean genotypes showed that genetic diversity and geographic distribution were independent of each other and there was no definite relationship existed between them.

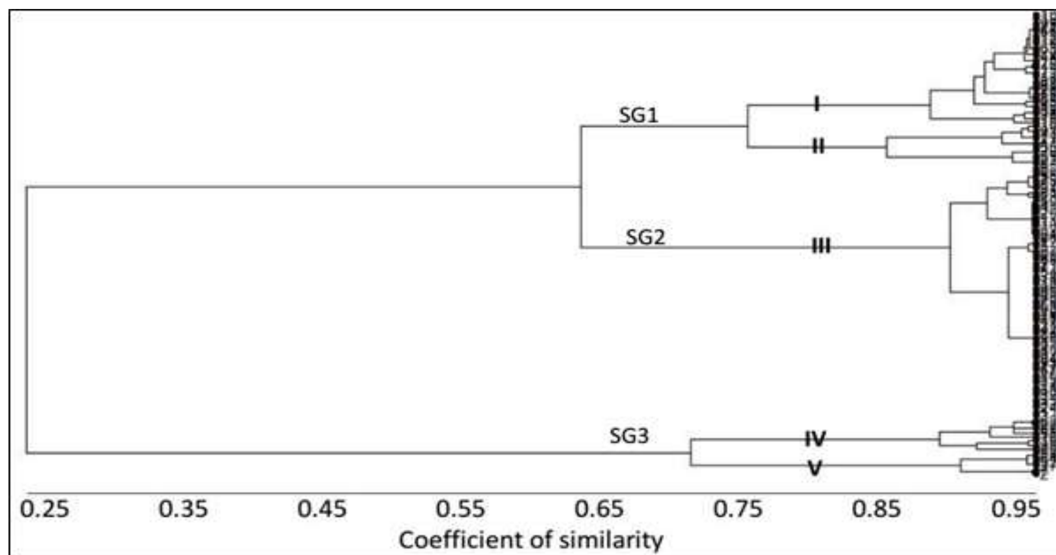

Figure 2. Dendrogram showing the relationships among genotypes based on SDS-PAGE and Ward method. Names of accessions under the five groups are shown in Table 3.

Table 3. Grouping of tested genotypes using data derived from SDS-PAGE analysis.

Cluster[1]	Accession ID	Origin of genotype[2]
I (26)	241777, 225725, 219962, 212852, 240396, 229203, 237991, 229205, 90022, 212628, 90010, 90009, 229201, 240397, 231 210’ 229204, 208667, 215713, 90004, 18843, CG10, CG12, G16, CG18, CG19, CG20	1, 2, 3, 4, 5, 6
II(10)	233984, 237512, 229798, CG2, CG17, 219960, 233982, 90016, 240578, 242609	1, 2, 3, 5
III (62)	207542, 208693, 8604, 205141, 202116, 238273, 90006, 230831, 219959, 235892, 234355, 233983, 229202, 233370, 216885, 219958, 214243, 216816, 230829, 229200, 242916, 240579, 90002, 205163, 215714, 233985, 215807, 216886, 19001, 19000, 219961, 208030, 90021, 230523, 234828, 208666, 240808, 229199, 90018, 90014, 19002, 207910, 216815, 90005, 212853, 230830, 208669, 90007, 230524, 205162, 233981, CG1, CG4, CG5, CG6, CG7, CG8, CG11, CG13, CG15, CG21, CG22	1, 2, 3, 4, 5, 6
IV(8)	90012, 233679, 225799, 229799, 90017, 18841, 90008, CG14	1, 2, 3, 6
V(6)	208769, 215808, 233986, 90020, CG3,CG9	1, 2, 5

[1]Values in parenthesis indicate number of genotypes.
[2]Origin of genotype. 1, Amhara; 2, Oromia; 3, Southern Nation, Nationalities and of People Regional State (SNNP); 4, Somali; 5, Tigray; 6, unknown source.

In general, the seed storage protein profiling generates wide array of polymorphism, hence could serve as a valuable tool in determining the extent of genetic diversity. Thus, SDS-PAGE marker data provided more sub-groupings and revealed considerable amount of genetic diversity.

Conclusions

Based on similarity indices, the dendrogram divided the genotypes into three groups and five clusters, indicating the genetic relationships among genotypes. The grouping of genotypes into clusters did not associate with their geographic distribution. Seed storage protein profile could be economically useful marker to assess genetic diversity in garden cress germplasm. Predominately polymorphic proteins were noted in SDS-PAGE analysis used for selection of desirable genotypes in the garden cress improvement programs. However, the study revealed that nearly half of the genotypes were grouped into similar clusters requiring further analysis with a combination of 2D electrophoresis. The hybridization among the genotypes from distantly related groups is

suggested in order to enhance future breeding programs towards the development of desirable varieties.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Identification and evaluation of secondary metabolites by gas chromatography-mass spectrometry (GC-MS) in native strains of *Trichoderma* species

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***Trichoderma* species is a heterotrophic saprobion organism, which competes with other microorganisms, preventing them from developing. This fungus has mechanisms of action, among which are: production of antibiotics and metabolites, and hyperparasitism, which also promotes the induction of systemic resistance in plants. In this study, five native *Trichoderma* strains which were collected from agricultural land in Tamaulipas, Mexico were evaluated. The objective of this research was to identify the secondary metabolites produced by native strains of *Trichoderma* spp. under *in vitro* conditions and evaluation of the effect of these compounds on the growth of the pathogen *Fusarium oxysporum* and the germination of tomato seeds (*Solanum esculentum*). The production of *Trichoderma*'s metabolites was performed by culturing in 150 ml of potato broth supplemented with sucrose (5%) and yeast extract (5%). Inoculation was performed by the addition of a 4 mm disk of active mycelium and allowed to grow at 25C ± 2°C and 12 h light/dark on a rotary shaker at 150 rpm for three weeks. Subsequently, the extraction of metabolites was performed using liquid: liquid phase which consisted of the filtrate and methylene chloride in a ratio of 1:3, and then the organic phase was recovered. The organic fraction was evaporated at 40°C with the aid of a rotary evaporator (Buchi®); the sample was recovered in 1 ml of methylene chloride. Analysis by GC-MS indicates that *Trichoderma* isolates produced 41 secondary metabolites of volatile and semi-volatile molecules and was observed, such that the number of compounds varies from the species analyzed and the collection site. The evaluation of the antagonistic activity of the extracts of *Trichoderma* spp. on *F. oxysporum* showed no significant differences between treatments (P=0.05); however, it was observed that pigmentation decreased in the mycelium of the pathogen. Regarding the evaluation of the effect of the extracts on the seeds of tomato, it was observed that the compounds of *Trichoderma asperellum* (TV1) induced the germination and the development of the seedling.**

Key words: Volatile compounds, *Trichoderma,* gas chromatography-mass spectrometry secondary metabolites, *Solanum esculentum.*

INTRODUCTION

Trichoderma species is a fungus found in the rhizosphere and organic matter of soils, with carbon as its main source of energy *(*Galarza et al., 2015). *Trichoderma harzianum, Trichoderma viride, Trichoderma virens* and *Trichoderma koningii* are the species more utilized in agriculture, pharmaceutical industry and biotechnology, because *Trichoderma* spp. produces secondary metabolites being the main peptides, peptaiboles, polyketides, pironas and volatile and non-volatile molecules (Vinale et al., 2009; Muller et al., 2011) that have functional processes such as biofungicides, plant growth promoters, enzyme generation, biofuels production, proteins, pigments, and antibiotics (Gajera et al., 2013). The effects of these molecules on plants are diverse, including: induction of resistance, elimination of toxins and deactivation of enzymes produced by phytopathogens during the infection process; in addition to the solubilization of nutrients, which in their elemental form are not bioavailable to plants, the processes are conferred to the antagonist of additional characteristics when used in the field (Dudareva et al., 2013). It has been reported to stimulate plant growth, due to the production of enzymes linked to tolerance to water stress (Mukherjee et al., 2013).

The success of *Trichoderma* spp. in the rhizosphere is due to its reproductive capacity, as well as its efficiency in the use of soil nutrients; the effectiveness of the genus has been reported against a variety of plant pathogenic fungi (Szabó et al., 2012). Some species of *Trichoderma* have the potential to synthesize and release enzymes (polysaccharases, cellulases, xylanases and chitinases) that have been implemented in industrial bioprocesses (Nikolajeva et al., 2012). It has also been reported that *Trichoderma* spp. are capable of producing auxin and gibberellin type growth regulators reported as promoters of the growth of some agricultural crops (Garnica-Vergara et al., 2016).

The antagonic effects of the genus *Trichoderma* have been reported in several phytopathogens of economic importance (Woo et al., 2014) including *Phytophthora infestans* (Kerroum et al., 2015), *Fusarium fujikuroi* (Ng et al., 2015), *Pestalotia theae*, *Fusarium solani, Colletotrichum gloeosporioides* MTCC 3439, *Colletotrichum lindemuthianum* MTCC 8474, *Colletotrichum capsici* MTCC 3414, *Curvularia senegalensis* MTCC 8463 and *Alternaria alternata* MTCC 8459 (Naglot et al., 2015).

Trichoderma has been reported to colonize the root epidermis and cortical outer layers, releasing volatile compounds such as ethylene, alcohols, aldehydes, ketones, and non-volatile compounds as peptides and enzymes capable of inhibiting the growth of the fungi (Yang et al., 2011).

Currently, commercial strains of *Trichoderma* spp. are used in Mexico; however, its effectiveness is diminished by abiotic factors such as the type of climate and soil, which are different from the collection site where they were isolated. Furthermore, there are limited studies in the country which focused on the identification of native compounds of *Trichoderma* spp. and the evaluation of the compounds in the impact on the inhibition of *Fusarium oxysporum* and the effect of germination and growth of plants.The objective of this study was to identify the secondary metabolites produced by native strains of *Trichoderma* spp. under *in vitro* conditions and the evaluation of *Trichoderma* extracts on *F. oxysporum* growth and in the germination of seeds of tomato.

MATERIALS AND METHODS

Cultivation of fungal strains

The native strains of *Trichoderma* were obtained from the Microbiology Laboratory of the Faculty of Engineering and Sciences, belonging to the Autonomous University of Tamaulipas. The organisms were isolated from lots located in the municipalities of the state of Tamaulipas: Padilla, Victoria and Jaumave. The municipality of Padilla is at latitude of 23° 59'26.1" N, 98°56'39.0" W, in which the semi-arid climate (BS1hw) predominates, tempered with summer rains and scarce along the year temperatures range from 1 to 43°C. The average rainfall is of the order of 700 mm^3, the predominant soils are vertisols with aptitude for agriculture.

The municipality of Victoria is located at 23°66'37" N, 99°10'81" W. The climate of the area is dry of steppe, very warm (BS1hw) with temperatures ranging from 2 to 40°C. Rainfall occurs in summer with an average precipitation of 926 mm, the soil type is loamy. The municipality of Jaumave is located at 23°24'46.1 "N, 99°23'03.4" W, which has a temperate semi-arid climate (BS1hw), with an annual average temperature of 18 and 22°C; with rainfall in summer from 5 to 10.2% per year. The type of soil is lithosol (LPq) and presents a shallow soil, all the native strains of *Trichoderma* were isolated from the soil where the *Aloe barbadensis* Miller is cultivated (CONABIO, 2016).

The cultures were identified by molecular level and maintained on potato dextrose agar (PDA) (Difco®) at 27 ± 2°C. *Trichoderma* Victoria (TV1) corresponds to *Trichoderma asperellum*, *Trichoderma* Padilla (TP1) identified as *T. harzianum* and the isolated *Trichoderma* Jaumave corresponds to *T. asperellum.*

Production and extraction of secondary metabolites by *Trichoderma* strains

The isolates were grown on solid PDA medium for 7 days prior to the assay. To evaluate the production of metabolites, each isolate was inoculated into 150 ml of potato broth supplemented with sucrose (5%) and yeast extract (5%). The inoculation was performed by the addition of a 4 mm disk of active mycelium. The culture was grown at 25 ± 2°C, 12 h light/dark on a rotary shaker

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(Labco®) at 150 rpm for three weeks. Each isolate was inoculated in triplicate.

The extraction of metabolites was performed using liquid: liquid phase which consisted of 10 ml of the filtrate and methylene chloride (J.T. Baker®) in a ratio of 1: 3. The sample was stirred until a homogeneous mixture was obtained, then the organic phase was recovered. The organic fraction was evaporated at 40°C with the aid of a rotary evaporator (Buchi®), and then the sample was recovered in 1 ml of methylene chloride. Each concentrated sample was stored at -80°C for further analysis. The procedure was performed in triplicate in each of the samples.

Analysis of secondary metabolites of native strains of *Trichoderma*

To determine the presence/absence of metabolites, each sample was subjected to thin layer chromatography (TLC) with a mobile phase of hexane: acetone (50:50) (Analytyka®) and as a stationary phase with a silica gel matrix in aluminium plate (Whatman®). 200 µl of non-inoculated culture medium (by several successive applications of 20 µl at the same point, allowing drying between each application) was added to each plate, which was used as a reference, and at another three points, 200 µl of the culture of each isolate was added by successive application. The plate was then air dried for 20 min. The development was performed by exposure of the plate to iodine vapors for 15 min. The procedure was repeated in triplicate for each sample.

The qualitative analysis of the *Trichoderma* extracts was performed from 3 weeks old samples by sas chromatography-mass spectrometry (GC-MS) on an Agilent® 6890 chromatograph, with an Agilent® 5973N mass detector using a 5% phenyl-methylpolysiloxane column with helium mobile phase. Analysis of each sample was performed in triplicate.

Evaluation of *Trichoderma* extracts on *F. oxysporum* growth

The pathogen, *F. oxysporum* was isolated from the wilt infected roots of *Aloe barbadensis* Miller plants, which were collected from the Microbiology Laboratory of the Faculty of Engineering and Sciences, Autonomous University of Tamaulipas. The culture was identified by molecular level and maintained on potato dextrose agar (PDA) (Difco®) at 27 ± 2°C. In this experiment, the *Trichoderma* extracts (TV1, TP1, TJ1) were evaluated.

The antagonist effect was evaluated in Petri dishes with sterile PDA medium, 100 µL of the extract of *Trichoderma* spp. was later dispersed throughout the surface of the Petri dish, then a 4 mm disc of *F. oxysporum* was placed in the center of the Petri dish and incubated at 28 ± 2°C for 7 days. This procedure was performed in triplicate for each *Trichoderma* extract. A control was added to the evaluation, which consisted of applying 100 µL of sterile distilled water to a Petri dish with PDA medium and then placing a 4 mm disc of *F. oxysporum* in the middle of the surface. The variables to be evaluated were growth and coloration of the mycelium.

The mycelium growth inhibition (%) of *F. oxysporum* was measured at 7 days of incubation. The antagonistic effect of the test fungi was estimated by measuring their radial growth in comparison with the control plates by the following formula: $I = [(C-T) / C] \times 100$, where I is the % inhibition in mycelia growth; C is the growth of pathogen in control plates; T is the growth of pathogen in dual culture plates.

Evaluation of *Trichoderma* extracts in the germination of seeds of tomato (*Solanum esculentum)*

Seeds of tomato were purchased from commercial market and were surface disinfected in 80% ethanol for 60 s followed by a 20% bleach solution for 2 min with constant agitation. In Petri dishes with sterile PDA medium (Difco®), 200 µL of the *Trichoderma* extract was added, then dispersed over the entire surface of the Petri dish, then 5 tomato seeds were previously disinfected, incubated at 28 ± 2°C for 7 days.

Five replicates were used per volatile exposure condition and the experiments were repeated three times. A control was added to the evaluation, which consisted in applying 200 µL of sterile distilled water to a Petri dish with PDA medium. The variables to be evaluated were percentage of germination, length of root and stem. The data were analyzed using an analysis of variance (ANOVA), when it was necessary, the comparison of means was performed with the Tukey method (P=0.05). The analysis was analyzed with the statistical program Statistical Analysis System (SAS, version 9).

RESULTS

Production and extraction of secondary metabolites by *Trichoderma* strains

Differences in the content and diversity of metabolites were observed between strains selected according to thin layer chromatography (TLC) analysis. Comparisons of the samples at the 3rd week showed the presence of more metabolites as compared to the first and second week extracts evaluated. The analysis of metabolites by GC-MS showed that all the native strains of *Trichoderma* produced secondary metabolites; it was also found that the similarity of the metabolic profiles presented was greater among the isolates of the same species than when the isolate of *T. harzianum* was compared with any of *T. asperellum*. The analyzed metabolites are volatile and semi-volatile, such as aromatics, fatty acids, alcohols and hydrocarbons in general, including the alkanes (C4-C9) shown in the three isolates (Table 1).

Analysis of the spectra showed that the number of compounds depended on the species analyzed. The species of *T. asperellum* (TJ1 and TV1) produced the highest amount of compounds; however, *T. harzianum* (TP1) produced molecules with higher percentage of abundance. The compounds detected, 10 corresponded to hydrocarbon compounds, 5 were phenolic compounds, 6 corresponded to organic acids, and 5 were unidentified compounds. In this study, six compounds shared between two of the isolates of *T. asperellum* and eight compounds of exclusive distribution for a single isolated (TP1) was found

The isolated *T. asperellum* (TV1) produced 35 compounds with relative abundances between 0.38 and 7.38% and retention times between 6.19 and 52.27 min, of which 17 are hydrocarbon, 4 phenolic, 6 organic acids and 8 molecules were unidentified. In the case of *T. asperellum* (TJ1), 23 compounds was detected, with relative abundances varying between 0.78 and 19.97% with retention times between 6.19 and 48.14 min of which two are hydrocarbon, one phenolic, five organic acids, four alcohols and one was unidentified. The analysis of the *T. harzianum* (TP1) isolate showed the presence of

Table 1. Identification of the metabolites of *Trichoderma* spp and its abundance by CG-MS.

S/N	Isolate	Chemical compound	Abundance	RT*
1	TP1	Xylene or 1,3-dimethylbenzene	6.73	6.19
2	TP1	Phenol, 2,4 bis, (1,1-dimethyl ethyl)	3.35	6.78
3	TP1	Tricosane	2.75	36.2
4	TP1	Tetracosane	3.12	39.63
5	TP1	Pentacosane	5.87	41.28
6	TP1	Hexacosane	9.07	42.87
7	TP1	Heptacosane	12.44	44.52
8	TP1	Octacosane	14.34	45.94
9	TP1	Triacontane	13.55	47.11
10	TP1	Unidentified	12.26	48.17
11	TP1	Unidentified	8.38	49.33
12	TP1	Unidentified	5.1	50.67
13	TP1	Unidentified	2.98	52.25
1	TJ1	1,3-dimethyl benzene	19.97	6.2
2	TJ1	Xylene	9.93	6.78
3	TJ1	2, butoxy ethanol	6.06	7.16
4	TJ1	Meta octane 3 ethyl	3.33	8.19
5	TJ1	Cyclooctane 1,4-dimethyl trans	2.4	12.13
6	TJ1	Unidentified	2.34	12.26
7	TJ1	Meta octanol 3,7 dimethyl	3.07	18.59
8	TJ1	Unidentified	4.58	18.82
9	TJ1	Phenol, 2,4 bis, (1,1-dimethyl ethyl)	3.16	19.05
10	TJ1	Unidentified	7.26	23.88
11	TJ1	R Hexadecanol, 3,7 11,15 tetramethyl	2.07	24.13
12	TJ1	M Acetic acid, 3, 7, 11, 15, tetramethyl hexadecyl ester	1.66	24.36
13	TJ1	Hexadecane, 2,6,11,15-tetramethyl	3.03	24.53
14	TJ1	R Hexadecanol, 3,7 11,15 tetramethyl	2.46	24.79
15	TJ1	R 1,2, benzenedicarboxylic acid, butyl 8-methylnonyl ester	5.52	33.59
16	TJ1	M 9, 12 octadecanoic acid	0.78	36.77
17	TJ1	Octadecanoic acid	1.08	37.29
18	TJ1	M tetracosane	1.91	41.26
19	TJ1	M pentacosane	2.65	42.84
20	TJ1	R Hexacosane	3.33	44.47
21	TJ1	R Heptacosane	4.37	45.91
22	TJ1	M Octacosane	4.31	47.08
23	TJ1	M Nonacosane	3.97	48.14
1	TV1	1,3-dimethylbenzene	4.56	6.19
2	TV1	M cyclooctane, 1,4, dimethyl trans	2.2	6.78
3	TV1	M Nonadecane	1.83	12.14
4	TV1	M 1-Nonadecane	1.69	12.26
5	TV1	R phenol 2,4, bis (1,1-dimethyl ethyl)	2.95	18.6
6	TV1	R Hexadecanol, 3,7 11,15 tetramethyl	3.7	18.84
7	TV1	Unidentified	2.76	19.06
8	TV1	R Hexadecanol, 3,7 11,15 tetramethyl	1.05	19.13
9	TV1	Unidentified	5.53	23.89
10	TV1	M Nonadecane	1.69	24.15
11	TV1	Unidentified	1.37	24.37
12	TV1	R Tetradecanoic acid	2.55	24.55
13	TV1	Unidentified	2.43	24.8
14	TV1	M 3-heptadecane	0.9	25.04
15	TV1	Unidentified	1.51	29.02

Table 1 Contd.

16	TV1	R 1,2, benzenedicarboxylic acid, butyl 8-methylnonyl ester	0.77	29.34
17	TV1	Unidentified	0.9	29.52
18	TV1	M Octadecanoic acid	2.17	29.64
19	TV1	R Octadecanoic acid	1.6	29.83
20	TV1	M tetracosane	1.28	30.34
21	TV1	R Hexacosane	3.04	33.6
22	TV1	M Octacosane	2.24	33.65
23	TV1	M Pentadecane	0.67	36.78
24	TV1	Unidentified	0.38	36.89
25	TV1	M hexadecane, 2,6, 11,15 tetramethyl	2.66	37.34
26	TV1	R 1 Hexadecanol 3,7,11,15-tetramethyl	1.5	39.63
27	TV1	M hexadecane, 2,6, 11,15 tetramethyl	2.94	41.29
28	TV1	M 1-Nonadecane	4.31	42.88
29	TV1	M 3-heptadecane	6.49	44.52
30	TV1	Hexadecanoic acid	7.38	45.95
31	TV1	R Oleic acid	6.98	47.11
32	TV1	R Tricosane	6.81	48.17
33	TV1	R Octacosane	5.13	49.34
34	TV1	Unidentified	3.5	50.68
35	TV1	R Heptacosane	2.32	52.27

*Retention time.

13 compounds, with relative abundances ranging from 2.75 to 12.44% with retention times between 6.19 and 52.25 min of which eight are hydrocarbon, one phenolic and four unidentified.

In this study, a correspondence of compounds between the two species of *Trichoderma* was found when sharing a compound between both species, such as phenol, 2,4 bis, 1,1-dimethyl ethyl. The correspondence of compounds produced by each isolate of *T. asperellum* was 11.56%, since six compounds were shared, among 1,3-dimethyl benzene, M octadecainoic acid, M tetracosan, R octadecainoic acid, R heptacosan and R hexacosane. All volatile compounds, which act in an antibiotic way against plant pathogenic fungi and can promote the growth of the same, as well as grant them systemic resistance. In this study, volatile metabolites of n-hydrocarbons type *T. harzianum* and *T. asperellum* were identified (Table 1). According to these results, C4 to C9 alkanes were identified, with a predominance of C5-C8 (Table 2).

The native strains of *Trichoderma* produced secondary metabolites, the compounds analyzed are volatile and semi-volatile in nature, these molecules are related to the interaction between the fungi and the environment, it was observed that the number of compounds varies between species, intraspecific variation as a possible result of the different environmental conditions to which they were submitted at their respective collecting sites, *T. asperellum* species (TJ1 and TV1) produced the highest amount of compounds, however, *T. harzianum* (TP1) produced molecules with greater percentage of abundance.

Evaluation of *Trichoderma* spp. extracts on *F. oxysporum* growth The evaluated extracts of *T. asperellum* (TV1, TJ1) and *T. harzianum* (TP1) did not inhibit the growth of the phytopathogen *F. oxysporum*, because growth percentages were observed for 81 to 94.37% (Table 3); however, in all treatments with extracts of *Trichoderma* spp. the color of the phytopathogen mycelium turned white, this is in comparison with the control, which is red-pink color (Figure 1).

Evaluation of *Trichoderma* extracts in the germination of seeds of tomato *S. esculentum*

The evaluated extracts of *T. asperellum* (TV1, TJ1) and *T. harzianum* (TP1) promoted the germination of the tomato seeds; however, the control treatment did not germinate. Seedlings that were germinated (root and stem) were measured; it was observed that the treatment *T. asperellum* (TV1) showed the highest growth in the variable stem with 5.5 cm and root 3.10 cm, in contrast with the treatment *T. asperellum* (TJ1) that belongs to the same species that registered a stem growth of 0.51 and 0.46 cm of root. The treatment TP1 belonging to *T. harzianum* promoted a growth of 0.9 cm of stem and root of 0.45 cm (Table 3). In this study, it was observed that tomato seedlings exposed to the metabolites of *Trichoderma* showed a development in the main root as

Table 2. Secondary metabolites produced *in vitro* by *Trichoderma* spp.

Chemical compound	*T. harzianum* (TP1)	*T. asperellum* (TJ1)	*T. asperellum* (TV1)
1,3-dimethyl benzene	-	*	*
2, butoxy ethanol	-	*	-
Hexadecanoic acid	-	-	*
Cyclooctane 1,4-dimethyl trans	-	*	-
Phenol, 2,4 bis, (1,1-dimethyl ethyl)	*	*	-
Heptacosane	*	-	-
Hexacosane	*	-	-
Hexadecane, 2,6,11,15-tetramethyl	-	*	-
M 1-Nonadecane	-	-	*
M 3-heptadecane	-	-	*
M 9,12 octadecanoic acid	-	*	-
Acetic acid, 3,7,11,15, tetramethyl hexadecyl ester	-	*	-
M octadecanoic acid	-	*	*
M cyclooctane, 1,4, dimethyl trans	-	*	-
M hexadecane, 2,6,11,15-tetramethyl	-	*	-
M Nonacosane	-	*	-
M Nonadecane	-	-	*
M Octacosane	-	*	*
M pentacosane	-	*	-
M Pentadecane	-	*	-
M tetracosane	-	*	*
M octane 3 ethyl	-	*	-
M octanol 3,7 dimethyl	-	*	-
Octacosane	*	-	-
Pentacosane	*	-	-
R 1 Hexadecanol 3,7,11,15-tetramethyl	-	-	*
R 1,2, benzenedicarboxylic acid, butyl 8-methylnonyl ester	-	*	*
R octadecanoic acid	-	*	*
R Oleic acid	-	-	*
R Tetradecanoic acid	-	-	*
R phenol 2,4, bis (1,1-dimethyl ethyl)	-	-	*
R Heptacosane	-	*	*
R Hexacosane	-	*	*
R Hexadecanol, 3,7,11,15 tetramethyl	-	*	*
R Octacosane	-	-	*
R Tricosan	-	-	*
Tetracosane	*	-	-
Triacontane	*	-	-
Tricosane	*	-	-
Xylene	-	*	-
Xylene or 1,3-dimethylbenzene	*	-	-

*Presence.

shown in Figure 1.

DISCUSSION

In this study, 3 *Trichoderma* spp. were screened and demonstrated that all of these strains produced secondary metabolites. However, no effect was observed on the decrease in growth of the phytopathogenic mycelium. On the other hand, the evaluation of the effect of metabolites in tomato seeds showed that it increases the percentage of germination, the growth of root and stem. The study

Table 3. *In vitro* evaluation of the effect of the metabolites of *Trichoderma* spp. on *F. oxysporum* and *Solanum esculentum.*

Treatment	Tomato (*S. esculentum*)		*F. oxysporum*
	Stem	Root	
TV (*T. asperellum*)	**5.500^{A}***	**3.100^{A}**	7.551^{A}
TJ (*T. asperellum*)	0.511^{B}	0.466^{B}	6.487^{A}
TP (*T. harzianum*)	0.900^{B}	0.455^{B}	7.262^{A}
Control	0.000^{B}	0.000^{B}	7.469^{A}

Effect of secondary metabolites isolated from *Trichoderma* spp. on *F. oxysporum* and tomato seeds at 7 days after treatment. *Treatments with the same letter are statistically equal according to the Tukey test (P = 0.05).

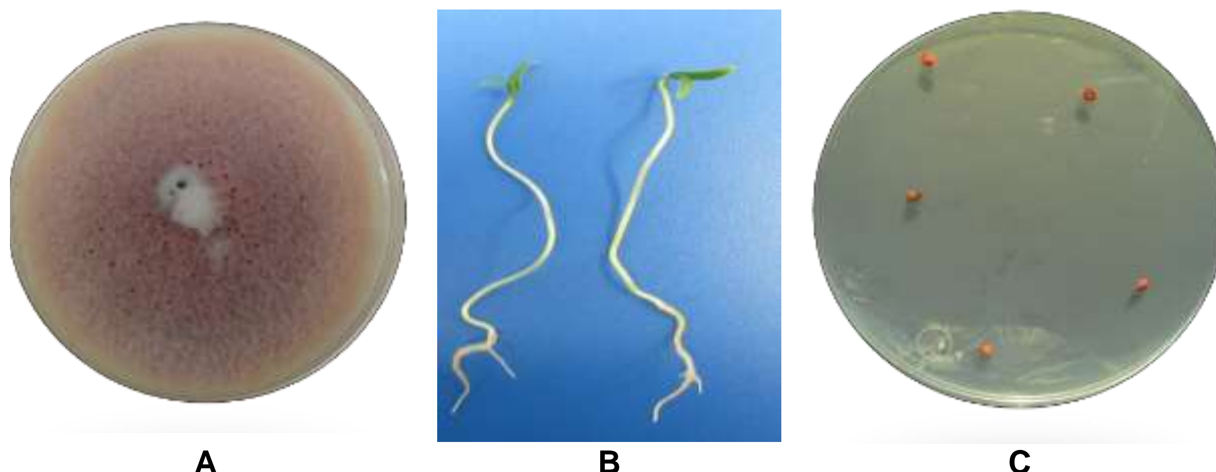

Figure 1. *In vitro* evaluation of the effect of the metabolites of *Trichoderma* spp. on *F. oxysporum* and *Solanum esculentum.* Effect of secondary metabolites isolated from *Trichoderma* spp. on *F. oxysporum* and tomato seeds at 7 days after treatment. **(A)** Mycelium of *F. oxysporum* exposed to the extract of *T. asperellum* (TP1). **(B)**Tomato seeds exposed to the extract of *T. asperellum* (TV1). **(C)** Control treatment where seeds that did not germinate are observed.

showed that a 3-week of fermentation *Trichoderma* spp. has a greater amount of metabolites, compared to the production of metabolites of the extracts of weeks 1 and 2.

The isolated *T. asperellum* (TV1) produced 35 compounds with relative abundances between 0.38 and 7.38%, of which 17 are hydrocarbons, 4 phenolics, 6 organic acids and 8 molecules unidentified. Twenty three *T. asperellum* (TJ1) compounds were detected, with relative abundances varying between 0.78 and 19.97%, of which two are hydrocarbons, one phenolic, five organic acids, four alcohols and one was unidentified. The analysis of the *T. harzianum* (TP1) isolate showed the presence of 13 compounds, with relative abundances ranging from 2.75 to 12.44%, of which eight are hydrocarbons, one phenolic and four unidentified. These data are consistent with the studies reported (Lee et al., 2016) where there are differences in metabolite production even among organisms of the same species.

The spectrum analysis showed that the number of compounds depended on the species analyzed. The species of *T. asperellum* (TJ1 and TV1) produced the highest amount of compounds; however, *T. harzianum* (TP1) produced molecules with higher percentage of abundance. Of all the compounds detected, 10 corresponded to hydrocarbon compounds, five were phenolic compounds, six corresponded to organic acids, five were unidentified compounds, and some of them to date have reported plants, nevertheless, none has been attributed to *Trichoderma* spp. This study has found six compounds shared between two of the isolates of *T. asperellum* and eight compounds of exclusive distribution for a single isolate (TP1). These results are consistent with the studies (Mukherjee et al., 2012) which reported that *Trichoderma* possesses fungistatic mechanisms that prevent the development of phytopathogenic fungi, as well as the capacity to synthesize volatile substances involved in the complex responsible for this phenomenon, these components are: carbon dioxide, ethanol, acetaldehyde, acetone, propanol, isobutanol and isopentanol, which in different concentrations intervene in the regulation of the fungistatic mechanism.

In this study, a correspondence of compounds between the two species of *Trichoderma* was found when sharing

a compound between both species, such as phenol, 2,4 bis, 1,1-dimethyl ethyl. The correspondence of compounds produced by each isolate of *T. asperellum* was 11.56%, since six compounds are shared, among which are 1,3-dimethyl benzene, M octadecainoic acid, M tetracosan, R octadecainoic acid, R heptacosan and R hexacosane. All volatile compounds act in an antibiotic way against plant pathogenic fungi and can promote the growth of the same, as well as grant them systemic resistance. In the present work, *T. asperellum* isolates (TJ1 and TV1) were produced, even when the same species were involved. This is due to the fact that these organisms were collected at different sites, where interaction with environmental factors, the type of soil, precipitation, among others would be affecting the mechanisms of action of *Trichoderma*. It has been elucidated that the diversity of metabolites produced by *Trichoderma* have evolved for communication or defense, against phytopathogens, in addition to these, stress conditions influence the expression of the volatile metabolites (Polizzi et al., 2011).

In the present work, *T. asperellum* isolates (TJ1 and TV1) produced different type of volatile compounds; this is due to the fact that these organisms were collected at different sites, where interaction with environmental factors, the type of soil, precipitation, among others would be affecting the mechanisms of action of *Trichoderma*, so it has been elucidated that the diversity of metabolites produced by *Trichoderma* have evolved for communication or defense, against phytopathogens, in addition to these, stress conditions influence the expression of the volatile metabolites.

In this work, volatile metabolites of n-hydrocarbons type *T. harzianum* and *T. asperellum* were identified (Table 1). According to these results, C4 to C9 alkanes were identified, with a predominance of C5-C8 (Table 2). It has been reported that C8 (octanone) compounds induce conidiation and also have an antimicrobial and antiviral action (Polizzi et al., 2011). In addition, *Trichoderma* species such as *T. harzianum*, *Trichoderma atroviride* and *T. asperellum* are used as plant protection agents and as growth promoters (Stoppacher et al., 2010). Studies focusing on bioremediation with *Trichoderma* strains in soils contaminated by hydrocarbons showed that *T. harzianum*, *Trichoderma pseudokoningii* and *T. viride* possess the ability to degrade pyrene and to use it as a carbon source (Ravelet et al., 2000). Others studies report that *T. harzianum* contributes in pollutant degradation in 65 and 33.7% of monoaromatic compounds in concentrations of 50 and 100 mg L^{-1}, respectively (Saraswathy and Hallberg, 2002). The implementation of chromatographic techniques, studies have been carried out on the chemical profile of the secondary metabolites produced by *Trichoderma* and its effect on the biochemical, molecular and physiological processes of plants with potential in the field (Stoppacher et al., 2010).

The native strains of *Trichoderma* produced secondary metabolites, the compounds analyzed are volatile and semi-volatile in nature, these molecules are related to the interaction between the fungi and the environment; it was observed that the number of compounds varies between species, intraspecific variation as a possible result of the different environmental conditions to which they were submitted at their respective collecting sites. *T. asperellum* species (TJ1 and TV1) produced the highest amount of compounds; however, *T. harzianum* (TP1) produced molecules with greater percentage of abundance. Studies carried out by Martínez-Medina et al. (2014) reported that the effectiveness of the native strains of the antagonist differs due to the associated abiotic factors, highlighting mainly the site of collection, type of soil, average temperature and humidity.

Specific criteria, such as mass spectral fusion factors and retention indices, the application of the method resulted in the identification of 41 volatile and semi-volatile compounds. However, studies report the identification of more than 141 compounds when analyzing 11 species of *Trichoderma* by chromatographic methods (Lee et al., 2016). Other works reported more than 278 compounds when using different columns and mobile phases in the chromatograph; in this sense, the mobile phase is related to the detection of the compounds analyzed, due to the polarity level that is present (Siddiquee et al., 2012). The number of compounds identified may be influenced by the number of species analyzed, the number and variety of culture conditions and conditions of analysis (preparation method, separation and analysis conditions).

Among the metabolites observed, the presence of the 1,3-dimethyl benzene compound in the TJ1 and TV1 isolates, with 19.97 and 4.56% of abundance, respectively was found. For TP1 isolates, the triacontane alkane stands at 13.55%, which has been reported as a plant growth regulator (Cai et al., 2013) (Table 1). Studies reported have demonstrated that volatile secondary metabolites play a key role in *Trichoderma* mycoparasitism and its interaction with plants (Piechulla and Degenhardt, 2014).

Regarding the evaluation of the effect of the secondary metabolites produced by the isolates of *Trichoderma* spp., it was observed that the phytopathogen mycelium coloration turned white in comparison with the control, which is red-pink colored. This effect is possibly due to the production of secondary metabolites of *Trichoderma* spp. which have an antibiosis effect; so the mycelia of the phytopathogen decreased, because the color of the phytopathogen is correlated with the virulence of it (Hermosa et al., 2012).

Studies that focused on the interaction of *Trichoderma* with the roots of plants have reported the appearance of auxin-like metabolites released by *Trichoderma* and perceived by the roots, altering multiple hormonal mechanisms that control the growth and development of plants in "normal" or stress conditions (Bae et al., 2009; Garnica-Vergara et al., 2016). Therefore, when the root

system is colonized, the association is enhanced, providing protection in this area against pathogenic microorganisms, and also develops a root system that improves the absorption of nutrients and water in the plant which have demonstrated that volatile metabolites play an important role in mycoparasitism (Contreras-Cornejo et al., 2015).

Conclusion

The analysis by GC-MS indicates that *Trichoderma* isolates produced 41 secondary metabolites of volatile and semi-volatile molecules and it was observed that the number of compounds varied from the species analyzed and the collection site. The metabolites analyzed are volatile and semi-volatile, such as aromatic compounds, fatty acids, alcohols and general hydrocarbons, including the alkanes (C4-C9) shown in all the three isolates.

The evaluation of the antagonistic activity of the extracts of *Trichoderma* spp. on *F. oxysporum* showed no significant differences between treatments (P=0.05); however, it was observed that pigmentation decreased in the mycelium of the pathogen. Moreover, in the evaluation of the effect of the extracts on the seeds of tomato, it was observed that the compounds of *T. asperellum* (TV1) induced the germination and the development of the seedling. The present study shows the wide range of compounds produced by native *Trichoderma* spp. However, it is necessary to search for strains to evaluate them as potential agents of biological control due to their possible specific adaptations, since they constitute a reservoir of genetic material and potential synthesis of particular antagonistic compounds. This underscores the importance of exploring the chemical diversity of compounds secreted by *Trichoderma* strains for the purpose of implementing them in potential agricultural applications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Heterozygote polymorphisms of *ARG16GLY* and *GLN27GLU ADRB2* gene is risk protective for obesity in Javanese population of Indonesia

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The relationship between obesity and insulin resistance is influenced by various factors, including genetics. *ADRB2* gene polymorphisms of *Arg16Gly* and *Gln27Glu* showed inconsistent results toward obesity and insulin resistance in some populations. This study aimed to investigate the frequency of the genetic polymorphisms of *Arg16Gly* and *Gln27Glu* of the *ADRB2* gene and to analyze risk factors for obesity and insulin resistance in Javanese sample population. This study was conducted on 100 control and 100 obese subjects. Subjects were measured for body weight and height to determine body mass index. Waist hip ratio was calculated by dividing the size of waist circumference by hip circumference. Fasting blood glucose was measured with GOD-PAP methods, and plasma insulin levels was measured using ELISA. *Arg16Gly* and *Gln27Glu ADRB2* gene polymorphisms were detected using the PCR-RFLP method. Results of polymorphisms of *Arg16Gly* showed that AG genotype decreased the risk of obesity with OR 0.264 (CI 95% : 0.119 – 0.585), p = 0,001 compared to the AA genotype. CG genotype of *Gln27Glu* increased the risk of obesity with RR 2.082 (CI 95% : 1.786 – 2.427). CC and CG genotypes toward GG genotype in the obese group had significant differences for plasma insulin, but were not significantly different for BMI, waist hip ratio, fasting blood glucose, and HOMA-IR. The combination of *Arg16Gly* and *Gln27Glu* AG+CG decreased the risk of obesity compared to AA+GG; however, it was not associated with insulin resistance.

Key words: Obesity, insulin resistance, *Arg16Gly polymorphisms, Gln27Glu polymorphisms.*

INTRODUCTION

Obesity is caused by an imbalance between food intake and energy use. This imbalance is due to the complex interactions between dietary habits, lack of physical exercise and genetic background (Marti et al., 2004). Data from the Indonesia Basic Health Research (*Riskesdas*) in 2013 showed that the prevalence of

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obesity in the adult age group is 15.4% (Kemenkes, 2003). Insulin resistance is a decrease in insulin activity in skeletal, liver, and adipose tissue (Saltiel and Kahn, 2001). Insulin and catecholamines are the major hormonal regulators of fat cell metabolism in humans. The function of hormones in fat cells changes in obese people. Insulin stimulates glucose uptake, lipid synthesis of glucose (lipogenesis) and inhibits the hydrolysis of triglycerides into glycerol and free fatty acids (Large and Arner, 1998). Catecholamines stimulate lipolysis through adrenoreceptors-β1, β2, and β3, but inhibit the effects of lipolysis mediated by α2-adrenoreceptors (LaFontan and Berlan, 1995).

The β2-adrenergic receptor gene (*ADRB2*) is expressed in many tissues including the liver, adipose tissue, smooth muscle in the bronchus and intestine. The *ADRB2* gene regulates energy use by stimulating lipolysis and thermogenesis through activation of catecholamine induction from adenylate cyclase through the action of G protein (Barbe et al., 1996; Naka et al., 2013). Common *ADRB2* polymorphisms are changes of glutamine into glutamic acid at codon 27 (*Gln27Glu*) and arginine to glycine at codon 16 (*Arg16Gly*). Some studies indicated that *Arg16Gly* and *Gln27Glu* have been shown to be associated with both obesity and body mass index (BMI) (Gjesing et al., 2007), but other studies found no association between *Arg16Gly* and *Gln27Glu* with obesity or BMI (Pereira et al., 2003; Bengtsson et al., 2001).

The *Gln27Glu* polymorphism has a significant association with increased waist circumference and free fatty acids (Park et al., 2008). One of meta-analysis for *Arg16Gly* and *Gln27Glu* showed that the 27 Glu allele was a significant risk factor for obesity in Asia Pacific and American populations (Jalba et al., 2008).

Based on this background, the aim of this study was to determine the difference of genotype frequency encoding *ADRB2* gene on codon 16 *Arg16Gly* and codon 27 *Gln27Glu* between obese and control groups, and to study *Arg16Gly* and *Gln27Glu* *ADRB2* gene polymorphism as a risk factor for obesity and insulin resistance.

METHODOLOGY

Subjects

The subjects included 200 healthy volunteers: 100 obese (50 males and 50 females) and 100 control (50 males and 50 females) with age 18 - 35 years' old, Javanese people. Subjects were categorized as control group with BMI 18.5 – 22.9 kg/m^2 and obesity group with BMI >25 kg/m^2. These subjects were recruited in the Universitas Gadjah Mada and University of Muhamadiyah Purwokerto. The study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada with reference number: KE/FK /0644/EC/2017. Subjects were excluded if they were not Javanese people, under 18 years and over 35 years, had diseases associated with metabolic syndrome, weight loss program, smoking, and had fasting blood glucose ≥ 125 mg/dL.

Anthropometric measurements

Height and weight of subjects were first measured and their BMI scores calculated. Waist hip ratio was calculated by dividing the size of waist circumference by hip circumference (Chan et al., 2003).

Measurements of blood glucose, plasma insulin levels and HOMA-IR

Five milliliters fasting blood was collected from study subjects and inserted in the EDTA test tube. Blood was centrifuged to get plasma and buffy coat. Plasma is used to measure the blood glucose and insulin levels. Buffy coat was taken for DNA isolation and genotyping. Measurements of blood glucose used GOD-PAP enzymatic colorimetric tests. Measurements of plasma insulin concentrations were based on ELISA KIT Insulin DRG® protocol. The HOMA-IR was calculated by multiplying plasma Insulin (µIU/mL) x fasting blood glucose (mmol/L) and dividing by 22.5 (Yamada et al., 2012).

Genotyping

Genomic DNA was prepared from buffy coat using PROMEGA kit. For single nucleotide polymorphisms (SNPs) in the *Arg16Gly* and *Gln27Glu ADRB2* gene, the primers were as follows: (forward primer 5' CAGCGCCTTCTTGCTGGCACCCCAT-3' and reverse primer 5'-CTGCCAGGCCCATGACCAGATCAG-3'. Polymerase chain restriction (PCR) process began with an initial denaturation of 94°C for 2 min, followed by 35 cycles with temperature of 94°C for 30 s, annealing 65°C for 45 s, elongation at 72°C for 1 min, and final elongation at 72°C for 7 min. The resulting PCR product was 242 bp (Aynacioglu et al., 1999). The PCR product was then cut by restriction enzyme and incubated for 16 h at 37°C. The Eco130I restriction enzyme was used for detection of the *Arg16Gly* polymorphism and Fnu4HI restriction enzyme for detection of the *Gln27Glu* polymorphism. Digestion products were for Arg16 = 242 bp, Gly16 = 214 bp, Gln27 = 181 bp, and Glu27 = 236 bp.

Statistical analysis

Characteristics of subjects were first tested for normality with the Kolmogorov-Smirnov test. The comparison of BMI, waist hip ratio, fasting blood glucose, insulin level, and HOMA IR mean between obese and control groups used independent sample t-tests when the data were normally distributed. Differences in allele and genotypes frequencies in each obese and control group were analyzed with Chi-square tests. Risk factors for each genotype to BMI and HOMA-IR between obese and control groups were tested with odds ratios. The comparison of BMI, waist hip ratio, fasting blood glucose, plasma insulin levels and HOMA IR mean between groups of genotypes was performed with independent sample t-test.

RESULTS

This study was conducted with 200 people who had met the inclusion criteria and signed an informed consent form. Characteristics of obese and control group were presented in Table 1. The subjects were divided into two groups: control group (non obese) with body mass index

Table 1. Characteristics of obese and control groups.

Variable		Obese (n = 100)	Control (n = 100)
Sex	Men	50 (50%)	50 (50%)
	Women	50 (50%)	50 (50%)
Ages		21.99 ± 4.006	21.26 ± 3.778
Height (m^2)		162.85 ± 7.882	160.68 ± 7.304*
Body Weight (kg)		83.26 ± 14.944	53.69 ± 8.204*
Body Mass Index (kg/m^2)		31.24 ± 4.076	20.73 ± 2.307*
Waist hip ratio		0.86 ± 0.128	0.77 ± 0.166*
Fasting blood Glucose (mg/dL)		93.40 ± 14.905	89.30 ± 12.039*
Plasma Insulin (μIU/mL)		3.54 ± 3.073	1.90 ± 1.642*
HOMA-IR		2.66 ± 2.086	1.43 ± 1.413*

n, Number of subjects. Data were presented in mean ± SD. *Independent sample t test, p<0.05 was significantly different.

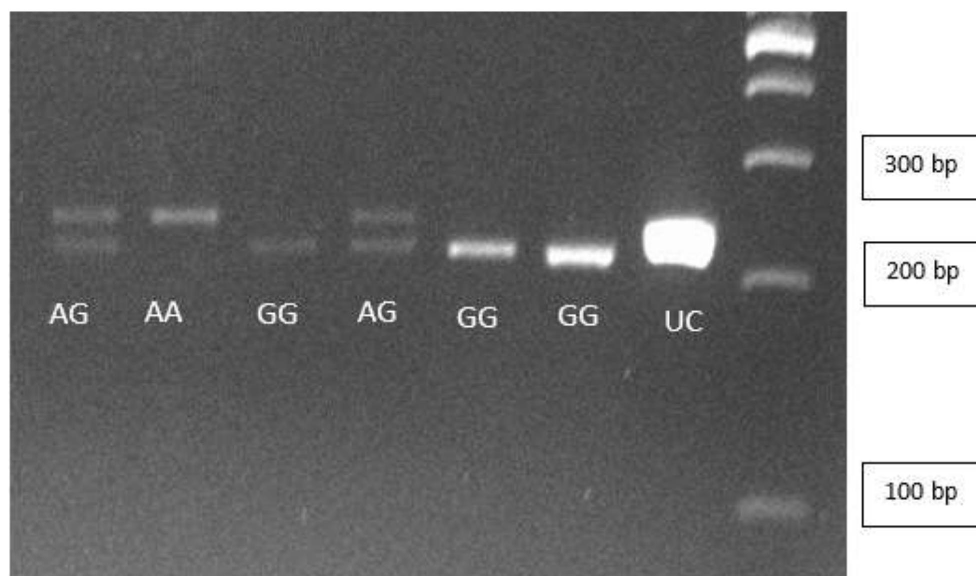

Figure 1. The AA genotype was showed with fragment length (242bp), AG genotype (242 bp and 214 bp), and GG genotype (214bp), UC (uncut)..

of 18.5 - 22.9 kg/m^2 and case group (obese) with body mass index ≥ 25 kg/m^2. Characteristics of subjects based on BMI, waist hip ratio, fasting blood glucose, plasma insulin, and HOMA-IR were significantly different and had higher mean values in the obese group.

The genotyping of *ADRB2 Arg16Gly* gene was performed by PCR-RFLP method. The resulting PCR product was 242 bp; thereafter, it was cut with the Eco130I enzyme. Genotypes found in *ADRB2 Arg16Gly* gene polymorphism in this study were AA, AG, and GG. The result of analysis of *ADRB2 Arg16Gly* genotype polymorphism can be seen in Figure 1, whereas the genotypes found in *ADRB2 Gln27Glu* gene polymorphism in this study were CC, CG, and GG. GG genotype in this research were wild type because they appeared more than the CC genotype with 236 bp fragment length, CG genotype with 236 bp and 181 bp fragments, and CC with 181 bp fragment length. The results of genotype analysis of *ADRB2 Gln27Glu* polymorphism can be seen in Figure 2.

Based on the data in Table 2, the genotype frequency distribution of obese was AA (Arg16Arg): 27%; AG (*Arg16Gly*): 62%; and GG (Gly16Gly): 11%. The genotype distribution of the control group was AA (10%), AG (87%) and GG (3%). Genotype AG can reduce the risk of obesity by 0.264 (CI: 95% = 0.119 - 0.585) with a value of p = 0.001 compared to genotype AA. The genotype distribution of the obese group was GG (Glu27Glu): 85%;

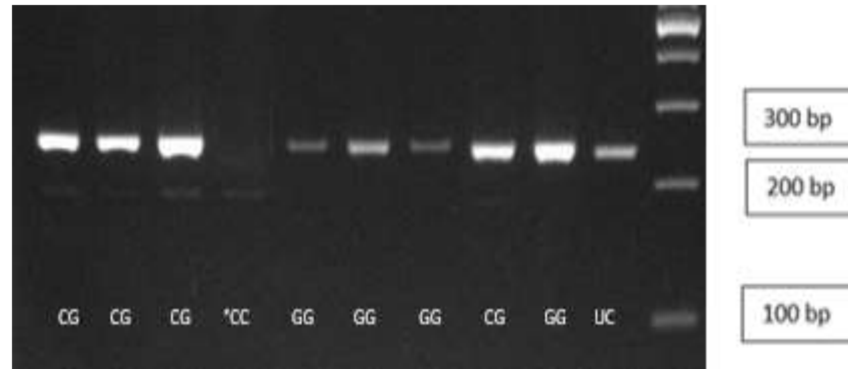

Figure 2. The CC genotype was showed by a fragment length of 181 bp, CG genotype (236 bp and 181 bp), GG genotype (236 bp)..

Table 2. Codon 16 and 27 polymorphisms of *ADRB2* gene in obese and control subjects.

Codon 16 genotype	**AA**	**AG**	**GG**
Obese	27 (27%)	62 (62%)	11 (11%)
Control	10 (10%)	87 (87%)	3 (3%)
OR (CL 95%)	Reference	0.264 (0.119 – 0.585)*	1.358 (0.313 – 5.897)

Codon 27 genotype	**GG**	**CG**	**CC**
Obese	85 (85%)	11 (11%)	4 (4%)
Control	92 (92%)	0	8 (8%)
OR (CL 95%)	Reference	2.082 (1.786 – 2.427)**	0.541 (0.157 – 1.862)

OR, odds ratio; CL, confidence limit. **Chi square test*: p<0.05; was significantly different; ***Continuity correction*: p<0, 05; was significantly different.

CG (*Gln27Glu*): 11%; CC (Gln27Gln): 4%, whereas the genotype distribution of control group was GG (92%) and CC (8%). The genotype frequency of CG has an obesity risk of 2.082 (CI: 95% = 1.786 - 2.427) compared to GG genotype.

The distribution of genotypes observed was compared with expected results by Hardy-Weinberg calculations. Based on $\chi 2$ test, it was found that the result of observation and expected result shows that the result was not significant. Hardy-Weinberg equilibrium test results on the genotype of *ADRB2* gene polymorphism *Arg16Gly* and *Gln27Glu* in Javanese population showed no significant differences between the genotype distributions of the research result (Table 2) with the population of HWE (Table 3).

The role of *ADRB2 Arg16Gly* gene polymorphism on BMI, waist hip ratio, fasting blood glucose, plasma insulin, and HOMA-IR in control and obesity group were tested using independent sample t-test. The genotype AA with genotype GG and AG did not have significant differences in body mass index, waist hip ratio, fasting blood glucose, plasma insulin and HOMA-IR in the control group and obese group. The CC and CG genotypes toward GG genotype in the obese group had significant differences in plasma insulin (p : 0.011), however, it was not significantly different for BMI, hip ratio, fasting blood glucose levels and HOMA-IR. The CC + CG genotype has a tendency towards insulin resistance with a higher mean HOMA-IR value than the GG genotype although not different.

Based on Table 5, the results of the analysis combination of AG+CG toward AA+GG showed OR value of 0.280 (CI 95%: 0.119 – 0.659) with p = 0.002. GG+GG haplotype toward AA+GG haplotype showed the OR value 1.929 (CI 95%: 0.345 – 10.767) with p value = 0.371 was not significantly different between obese and control groups.

DISCUSSION

The combination analysis of *ADRB2* gene genotypes *Arg16Gly* and *Gln27Glu* aimed to know the difference in frequency between the obese and control groups and their relation to obesity risk. The combination of genotypes in *Arg16Gly* and *Gln27Glu* (AG +CG) *ADRB2*

Table 3. Observed and expected genotype distribution of *ADRB2* in obese and control group.

Genotype	Observed (n)	Expected (n)	*p* (x^2)
Obese group			
AA (Arg16Arg)	27	33	
AG (*Arg16Gly*)	62	49	7.31
GG (Gly16Gly)	11	18	
Total	100	100	
CC (Gln27Gln)	4	1	
CG (*Gln27Glu*)	11	17	11.23
GG (Glu27Glu)	85	82	
Total	100	100	
Control group			
AA (Arg16Arg)	10	29	
AG (*Arg16Gly*)	87	50	55.24
GG (Gly16Gly)	3	21	
Total	100	100	
CC (Gln27Gln)	8	1	
CG (*Gln27Glu*)	0	15	50.44
GG (Glu27Glu)	92	84	
Total	100	100	

Chi square test; $p < 0.05$: was significantly different.

gene polymorphisms toward AA + GG was significantly different between obese and control groups with OR 0.280 (CI: 95% : 0.119 - 0.659), *p* = 0.002. The combination of AG + GG had a protective risk on obesity compared to the AA + GG haplotype. This study was shows Haplotype AG + CG had a protective risk to obesity. Haplotypes that provide protective risks are associated with catecholamine reduction by inducing cAMP production in recombinant cells and decreasing insulin sensitivity in human fat cells (Drysdale et al., 2000; Eriksson et al., 2004). The mismatch between single nucleotide polymorphism (SNP) analysis and haplotype analysis of the *ADRB2* gene against obesity provides a debatable outcome (Rosmond, 2003).

Protective haplotypes and other haplotypes had no effect on cAMP production on mononuclear cells in peripheral blood (Lipworth et al., 2002). Human fat cells have four ADRB subtypes that regulate lipolysis (Large and Arner, 1998). The mechanism is related to the function of other adrenoceptors in adipocytes. Another thing is the existence of gene and environment interactions such as fitness and diet (Jiao et al., 2005). *ADRB2* haplotype may have a direct influence on glucose metabolism. For example, *ADRB2* triggers blood flow (Barbe et al., 1996). Differences in blood flow between haplotypes *ADRB2* will affect the delivery of glucose and insulin in the peripheral tissues, thus affecting glucose tolerance. The β-Adrenergic stimulus also produces insulin resistance in skeletal and fatty muscles (Prior et al., 2011). This causes a different stimulus of the haplotype *ADRB2* to explain the independent relationship of the haplotype *ADRB2* with insulin resistance. The *ADRB2* genotype may affect receptor function, receptor density, or efficiency. Reduced expression of the receptor may result in inefficient lipolysis of adipose tissue and the accumulation of excess fat over time (McGraw et al., 1998). The combination of *Arg16Gly* and *Gln27Glu* did not appear to affect being overweight and obese, or subjects with BMI > 30 kg/m^2 when compared with subjects with BMI <25 kg/m^2 (Gjesing et al., 2007). The *ADRB2* haplotype study conducted by Lima et al. (2007) in both white and black male and female subjects was not associated with BMI.

The obese group had a higher mean than the control group (Table 1). Body mass index was associated with high fasting glucose levels in men and elevated concentrations of insulin in women (Patel and Abate, 2013). Fasting blood glucose levels and plasma insulin levels were elevated in obese subjects (Nguyen and Nguyen, 2008). When BMI increases, HOMA-IR will also increase (Martinez et al., 2017). The data in this study support the previous studies where if the obesity group had a higher mean of BMI, then the value of HOMA IR will also be high when compared with the control group. The combination of *ADRB2* genotype to insulin resistance calculated by HOMA-IR in this study did not differ

Table 4. BMI, waist hip ratio, fasting glucose, fasting insulin, and HOMA-IR between genotype groups in control and obese with *Gln27Glu* (CG), Gln27Gln (CC), and Glu27Glu (GG).

Variable	Obese		Control	
	GG (n = 85)	CC+CG (n = 15)	GG (n = 92)	CC (n = 8)
BMI (kg/m^2)	30.96±3.77	32.86±5.36	20.77±2.32	20.19±2.14
Waist hip ratio	0.86±0.13	0.85±0.08	0.77±0.17	0.77±0.06
Fasting Blood glucose (mg/dL)	92.43±14.30	98.93±17.45	89.45±11.98	87.50±13.32
Plasma Insulin (μIU/mL)	3.21±2.13	5.38±5.92*	1.88±1.57	2.13±2.42
HOMA-IR	2.51±1.90	3.49±2.84	1.40±1.31	1.74±2.41

n, Number of subjects. Data were presented in mean ± SD. **Independent t-test:* p < 0.05: was significantly different.

Table 5. Haplotype of *Arg16Gly* and *Gln27Glu* polymorphisms in *ADRB2* gene in obese and control subjects.

Genotype	n	Obese	Control	OR (CL 95%)
AA+GG	30	21	9	1.00
AG+CG	134	53	81	0.280 (0.119 – 0.659)*
AG+CC	9	3	6	0.214 (0.044 – 1.052)
GG+GG	11	9	2	1.929 (0.345 – 10.767

**Chi square test*: p<0.05; was significantly different.

high insulin resistance. In several previous studies, *ADRB* gene polymorphisms were associated with serum insulin levels and insulin resistance but were not associated with obesity in Swedish women (Mottagui et al., 2008). The HOMA IR in the CC+CG genotypes of the obese group had a mean value (3.49±2.84 μIU/mL) greater than GG genotype (2.51±1.90 μIU/mL). Association between the combination of polymorphism and metabolic characteristics requires a larger number of samples (Tsunekawa et al., 2011). *ADRB2* gene polymorphism is often found in obese individuals (Large et al., 1997), although the link between polymorphism with body weight, glucose tolerance, insulin sensitivity has shown mixed results (Oberklofer et al., 2000; Prior et al., 2011; Echwald et al., 1998).

In this study, CC and CG genotypes had a higher mean BMI when compared to the GG genotype (Table 4). Research conducted by Kawamura et al. (2001) showed no association between the ADRB polymorphism of *Gln27Glu* gene against obesity. The results of research conducted by Oberklofer et al. (2000) also showed the polymorphism of *Gln27Glu* gene *ADRB2* not related to obesity in Austrian women. Similar results in a study conducted by Gjesing et al. (2007) suggests that the OR allele Glu27 is 0.92 and is not associated with obesity. Frequency of the CG (*Gln27Glu*) genotype in this study increased obesity risk of 2.082 compared to GG genotype. These results indicated that the subjects with *Gln27Glu* variation were at risk of becoming obese by 2.082 times compared to the GG (wild type) genotype. The genotype frequency of CG was most found in case group of Austria (47.5%) and in Spain with (47.3%). The genotype frequency of CG was found higher in the control group in Sweden (53.4%) and Denmark (46.5%) (Zhang et al., 2014). The GG genotypes in Sweden and Denmark were found higher in the case group with significant comparisons in Sweden (24.4%) vs (3.4%), and Denmark (30.2%) vs (16.5%), whereas in Austria this study showed the genotype frequency of GG most commonly found in the control group. Research Kim et al. (2002) did not find GG genotypes in both case and control groups. There was no association between the polymorphism of *Gln27Glu* of *ADRB* gene and obesity (Kawamura et al., 2001). The obese phenotype in humans appears to be determined not only by a number of different genes but also because of links to other diseases, genetic variations, and environmental factors (Gjesing et al., 2007). The Glu27 allele has a tendency to increase BMI (Large et al., 1997), waist-to-hip ratio (Hellstrom et al., 1999), type 2 diabetes mellitus (Ishiyama-Shigemoto et al., 1999), and can suppress lipid oxidation (Macho-Azcarate et al., 2003). Echwald et al. (1998) found that *ADRB2* Glu27 polymorphisms were not associated with obesity in the Ducati Caucasian male population. The Glu27 allele group had low lipolysis as measured by plasma glycerol. Echwald (1998) and Oberkofler et al., (2000) found no association between *ADRB2* and obesity.

The AG (*Arg16Gly*) genotype decreased the risk of obesity by 0.264 compared to the AA (Arg16Arg) genotype. *Arg16Gly ADRB2* gene polymorphism in some populations in the world showed different results. The

genotype frequency of AG in some countries were more found in the control group such as in Austria (47.2%), Korea (51.2%) (Zhang et al., 2014) and in this study (87%). While in Sweden, Japan, Brazil and Taiwan found many in the case group. The genotypes of GG in Sweden were 53.4%, Japan (25%), Austria (36.1%), Brazil (31.2%), and Taiwan (20.9%) were found in control group compared to case groups (Zhang et al., 2014). Polymorphism at codon 16 was not significantly associated with variations in obesity variables. *Arg16Gly* polymorphism associated with changes in *ADRB2* protein function (Large et al., 1997). Polymorphism at codon 16 affects the bond between catecholamines and *ADRB2* genes thus preventing lipolysis (Daghestani et al., 2012). Research conducted by Kim et al. (2002) showed that the Gly16 allele associated with a low BMI when compared with the Arg16 allele.

The distribution and affinity of adrenergic receptors in human adipocytes is believed to play an important role in lipid metabolism and may contribute to differences in the distribution of fats between the sexes. Lipolysis is controlled by a number of receptors on the cell surface, including ADRB1, *ADRB2*, ADRB3, and ADRA2 receptors. *ADRB2* activation stimulates lipolysis (triglycerides are hydrolyzed into glycerol and free fatty acids), whereas the activation of α2-receptors inhibits lipolysis (Ellsworth et al., 2002).

Conclusion

Based on the results of this study, The AG genotype of *ADRB2* gene polymorphism decreased the risk of obesity. The genotype CG gene polymorphism *ADRB2 Gln27Glu* increased the risk of obesity. The combination of AC+CG was protective risk for obesity but it was not associated with insulin resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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The technological influence of the different methods of extraction on the initial concentration of thymol in the essential oil of thyme (*Thymus satureioides*)

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The present study deals with the influence of the technological factor in relation to the technique used for the extraction of essential oil of thyme, its chemical composition, and in particular, the concentration of thymol as the principal chemo-typical active substance of this variety of thyme from the Moroccan High Atlas. The working protocol includes the extraction of the essential oil by three different methods (First method: Academic hydro-distillation; Second method: Training with water vapor using artisanal alembic; and Third method: Traditional hydro-distillation using artisanal alembic) and the comparative study of the individual thymol areas in the three spectrograms corresponding to the three essential oil samples and calculation of the differences. It emerges that the first method produces an essential oil very rich in thymol, followed by second method which produces an essential oil moderately concentrated in thymol, while the latter method produces an essential oil with very low concentration of thymol. It has been deduced that an important influence of the technological factor in relation to the type of the technique used for the extraction of the essential oil of thyme was exerted on the initial chemical composition, in particular, on the concentration of thymol.

Key words: Concentration, essential oil, *Thymus satureioides*, thymol.

INTRODUCTION

Among the critical points highlighted during the elaboration of the general strategic policy (GSP) of the national system of management (NSM) of the S: Safety/E: Efficiency/Q: Quality/E: Ecology ("SEQE") relating to medicinal plants (MP) and health products based on medicinal plants (HP.b.MP) abbreviated to “The P.S.G of N.S.M-S.E.Q.E relating to M.P/H.P.b.M.P.”, especially in its axis dedicated to production itself noted the question "the performance of different methods and techniques of extraction of essential oil (EO)". Of course, this question becomes even more critical when it highlights, in the light of the day, the performance of the technology (technique, method and equipment) used in the extraction of EO and its influence on quality. Of the latter, knowing that this is a very sensitive point that falls within the framework of the professional secrecy of the producers in the field and who exercise their trade in artisanal, academic or industrial (modern) ways.

It was in this context that the present study was conducted to test some techniques (methods) of

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extractions of EO: one academic (academic hydro-distillation) and two sub-techniques falling under a traditional method (training with water vapor using artisanal alembic and traditional hydro-distillation using artisanal alembic).

The main raw material used is a medicinal plant "thyme = *Thymus satureioides*", chosen to represent its category at the national level, both in terms of its endemism, nationwide (Morocco) production and its phyto-pharmacological interest.

The species "thyme" has more than 200 varieties with an endemism of about 46.6% in Morocco and it is represented by 21 species, 12 of which are endemic (Fennane et al., 2007; Benabid, 2000). The essential oil of thyme has various pharmacological properties, including antibacterial properties (Possas et al., 2017), antifungal (Anzlovar et al., 2017), and antioxidants (Bektas et al., 2016).

MATERIALS AND METHODS

Plant

After completing all the administrative requirements and procedures and following the Good Harvesting Practice Guidelines, sufficient quantity of the raw material "flowering aerial parts of thyme" at the level of the zone (Bin Elouidane in the province of Azilal/Beni Mellal, Morocco) was gathered during a two-day period, June 10 and 11, 2016, with the help of local collectors.

Identification the specimen

A preliminary identification of the plant was made on the spot, even before harvesting in accordance with the specific botanical monograph of *T. satureioides* resulting from the manual of determination of the vascular plants of Morocco, Practical flora of Morocco (Fennane et al., 2007).

After the harvest, a representative sample of the plant was deposited at the Scientific Institute of Rabat, Department of Herbal Botany and Ecology, for official botanical identification.

Extraction of essential oil of *T. satureioides*

After about 5 days of drying the raw material, the extraction of the essential oil of the saturated thyme was carried out by three methods, during three successive days, at the rate of one extraction per method and per day.

Three techniques (methods) of extraction of the essential oil include: one academic and two sub-techniques of a traditional method:

The academic method (method 1)

Academic, called this way because it is practiced in the context of academic research within the university (Laboratory of the Faculty of Medicine and Pharmacy of Rabat). Essential oil extraction method of thyme (aerial flowered parts) by academic hydro-distillation, ecological variant.

This was done using hydro-distillation apparatus, "Clevenger" type (Figure 1) and its principle consists of immersing the plant raw material, which in this case consists of aerial parts of thyme, in a bath of water boiled. Once released outside the cells, under internal pressure and chemical action of water, the essential oil of thyme is transported by stream of water vapor, cooled and condensed, further, through a condensation system and special refrigeration and subsequently discharged into a receiving container. During settling, the difference in the density between the water and the aromatic compounds causes the formation of an aqueous phase which composed essentially of hydrolate and an organic phase which composed essentially of essential oil. The latter and in the present case (ecological procedure) is separated, physically, from the first aqueous phase, using a simple separating funnel or other equivalent physical separation device, apart from any other chemical substance or solvent of separation. This gives the present procedure the title ecological.

The traditional methods

Extraction of the essential oil using artisanal alembic which has two sub-techniques.

The traditional sub-technique of training with water vapor (method 2)

Essential oil extraction method of thyme (aerial flowered parts) by training with water vapor using artisanal alembic (Figure 2), ecological variant.

This technique is different from that of the hydro-distillation, because the contact "steam/plant" is done "*ex situ*" in the case of the present technique (by training with the steam) and which is explained by the fact that the plant is not put in direct contact with water (*in situ*) as the case of hydro-distillation, but it is placed, at a level apart, in a perforated tank or special grid and the contact can only be done with water vapor (and not directly with water) and can only take place after evaporation of water in the boiler located at the lower level of the still. In fact, water is boiled in the lower tank also called "boiler" to generate water vapor that is passed through the plant to recover the oil and placed at the level of the perforated tank also called central tank-grid. Steam, by the effect of its temperature and its osmotic tension exerted on the plant, will destroy the cellulosic structure of the plant cells which will release their essential oils. Under the effect of heat, the oil evaporate and as two gases are always miscible, the water and oil vapors will mix and the water vapor carries with it the essential oils of the plant. Once vaporized, the volatile compounds of the essential oil are transported by steam flow, cooled and condensed, further, through a special condensing and refrigeration system (through the cooling water tank) and subsequently discharged into a receiving container. The decantation and separation steps are the same as in method 1.

The traditional sub-technique of hydro-distillation (method 3)

Method of extraction of essential oil of thyme (aerial flowered parts) by traditional hydrodistillation using artisanal alembic (Figure 2), ecological variant.

In essence, this technique is identical to that of academic hydro-distillation (method 1) with the only difference compared to the latter; it is done in a traditional way using traditional alembic (Figure 2). It is therefore and above all a difference of form between the two techniques. Indeed, the raw material is brought into direct contact with water (*in situ*) in the lower tank also called "boiler". The perforated central tank, also called a tank-grid or a “couscoussière”, will remain vacant throughout the extraction process unless it has played the role of intermediation and passage of the volatile mixture between the lower tank (or boiler) and the upper tank cooling. The

Figure 1. Special laboratory hydro-distillation apparatus (Clavenger).
Source: Elkacimi (2016).

Figure 2. The artisanal alembic - type "Alquitara" used in this study.
Source: Elkacimi (2016).

mixture of water vapor and the essential oil obtained by heating is generated. This time directly in the boiler and by passing through the central tank, it is cooled at the level of the upper tank to recover the level of the harvesting container. The decantation and separation steps are the same as in the two previous methods.

Raw materials

The raw vegetable material is the flowering aerial parts of thyme (50 g for method 1, 1000 g for method 2, and 1250 g for method 3) in the semi-fresh state (only 5 days of drying in the open air and at room temperature). The parallel raw material, source of water vapor, is the tap water (1000 ml for method 1 and 8000 ml for methods 2 and 3).

The conditioning

The quantity of essential oil of *T. satureoides* (EO/TS), thus recovered, was put in vial(s), as it was, using a sterile syringe. No specific method of preservation (such as the sub vacuum or under nitrogen) was considered in this case study.

Storage

The three essential oil samples were kept separately, at a temperature of 4°C in the dark to preserve their original composition.

Initial chemical characterization of the essential oil extract HE/TS obtained

A first analysis (at t = 0) of the chemical composition of the essential oil (EO/TS) was carried out at the National Center for Scientific and Technical Research "CNRST"/Division of Technical Support Units for Research Scientific "UATRS", Rabat, Morocco, under the following conditions:

(1) GPC/MS Analysis conditions: UATRS Standard Conditions (Essential Oil)
(2) Apparatus: Gas chromatograph (TRACE GC ULTRA) coupled with a mass spectrometer (Polaris Q MS with ion trap).
(3) Type of analysis performed: Qualitative and quantitative.
(4) Type of ionization: Electronic impact (70 eV)
(5) Solvent type: n-Hexane or ethyl acetate
(6) Column type: VB-5 (Methylpolysiloxane 5% phenyl), 30 m × 0.25 mm × 0.25 μm.
(7) Injection conditions : See table 1
(8) Separation conditions : See table 2

Data processing

The data obtained after the analysis, were processed on computer, in the form of digital data thus allowing the development of a "spectrogram" which is a graphical presentation of the spectral map (peaks) of the various components of the analyzed product.

Special software, integrated with the GC-MS system, manages the digital data from the detector, by making an automatic comparative study with reference scientific data contained in computerized libraries belonging to the laboratory.

Comparism of the individual thymol areas at the three spectrograms corresponding to the three essential oil samples and calculating the percentage difference

All the parameters of the analysis (GC/MS) of all the samples at t = 0 months were fixed and identical, in particular, those linked to the technique of analysis, to the used equipment, to the manipulation of the analyst, to the analytical environment (temperature, humidity, pressure, etc.), and the automatic interpretation of the software.

A very simple calculation approach was used by direct comparison of the individual areas corresponding to the thymol fractions, in the three different samples of the thyme essential oil. The calculation was done as follows:

$A_{T\,1}$: Thymol area in spectrogram 1 corresponding to sample 1 of the essential oil extracted by method 1.
$A_{T\,2}$: Thymol area in spectrogram 2 corresponding to sample 2 of the essential oil extracted by method 2.
$A_{T\,3}$: Thymol area in spectrogram 3 corresponding to sample 3 of the essential oil extracted by method 3.

The largest of the three areas (A_T X) corresponds to 100% of the substance. The percentages (Y) and (Z) of the two remaining areas (A_T Y) and (A_T Z) relative to (A_T X) were calculated, using the equation (rule of three):

$Y = A_T Y / A_T X \times 100\%$
$Z = A_T Z / A_T X \times 100\%$

Subsequently, the difference "E_T A" (in percentage) between A_T X and A_T Y was calculated by:

$E_T A = 100\% - Y$

The difference "E_T B" (in percentage) between A_T X and A_T Z:

$E_T B = 100\% - Z$

RESULTS

Harvesting stage

At the harvest level, about 20 kg of the raw material was collected which composed of the aerial parts of the fresh plant of the saturated thyme, gathered from the mountainous area of Bin Elouidane (Morocco), in accordance with the good practices of harvests recommended by World Health Organization (WHO). An image of the plant sample is as shown in Figure 3.

Identification stage

The working sample was officially identified by the Scientific Institute of Rabat with the reference: "*T. saturioides* Cosson in Bull.Soc.Bot. France 58: 436. 1911 AS AA MA MA Mam -2-3 and registered at the herbarium of the same institute under the index number: RAB98065.

Extraction step

Approximately 1.5 ml of EO/TS essential oil was extracted from almost 50 g of the raw material in the laboratory of the faculty and in accordance with the aforementioned academic procedure. 19 ml of the essential oil was extracted by method 2 from 1000 g of

Figure 3. Photo taken on the premises of the raw vegetable material.
Source: Elkacimi (2016).

thyme, while using method 3 (artisanal), only 13 ml of essential oil was extracted from 1250 g of the plant.

Initial analysis step: Initial chemical characterization (at t = 0) of the essential oil EO/TS

The results of initial qualitative analysis (at t = 0) of the various samples of the essential oil, performed using gas chromatography coupled with mass spectrometry (GC/MS), are summarized in the following spectrograms:

(1) Spectrogram 1 (Figure 4): Corresponds to the chemical characterization of sample 1 of the EO/TS (extracted by method 1);
(2) Spectrogram 2 (Figure 5): Corresponds to the chemical characterization of sample 2 of the EO/TS (extracted by method 2);
(3) Spectrogram 3 (Figure 6): Corresponds to the chemical characterization of sample 3 of the EO/TS (extracted by method 3).

Calculation of individual percentages and deviations

The calculated difference results between thymol areas ($A_{T\ 2}$ and $A_{T\ 3}$ versus $A_{T\ 1}$) are listed in Table 3. These results are in accordance with the following basic principles of calculation:

$A_{T\ 1}$: Thymol area in spectrogram 1 = 302397036
$A_{T\ 2}$: Thymol area in spectrogram 2 = 157845574.
$A_{T\ 3}$: Thymol area in the spectrogram 3 = 70322589

Given the importance of its area relative to the other two, $A_{T\ 1}$ is considered to be 100% of the substance. The percentages (Y) and (Z) of the two remaining areas ($A_{T\ 2}$) and ($A_{T\ 3}$) are calculated on the basis of ($A_{T\ 1}$), through the equation (rule of three):

For Y

$Y = A_{T\ 2} / A_{T\ 1} \times 100\%$
$Y = 157845574 / 302397036 \times 100\% = 52.19\%$
$Y = 52.19\%$

For Z

$Z = A_{T\ 3} / A_{T\ 1} \times 100\%$
$Z = 70322589 / 302397036 \times 100\% = 23.25\ \%$
$Z = 23.25\%$

The differences are calculated subsequently as follows:

(1) The difference "$E_{T\ A}$" (in percentage) between $A_{T\ 1}$ and $A_{T\ 2}$:

$E_{T\ A} = 100\% - Y$
$E_{T\ A} = 100\% - 52.19\% = 47.81\%$
$E_{T\ A} = 47.81\%$

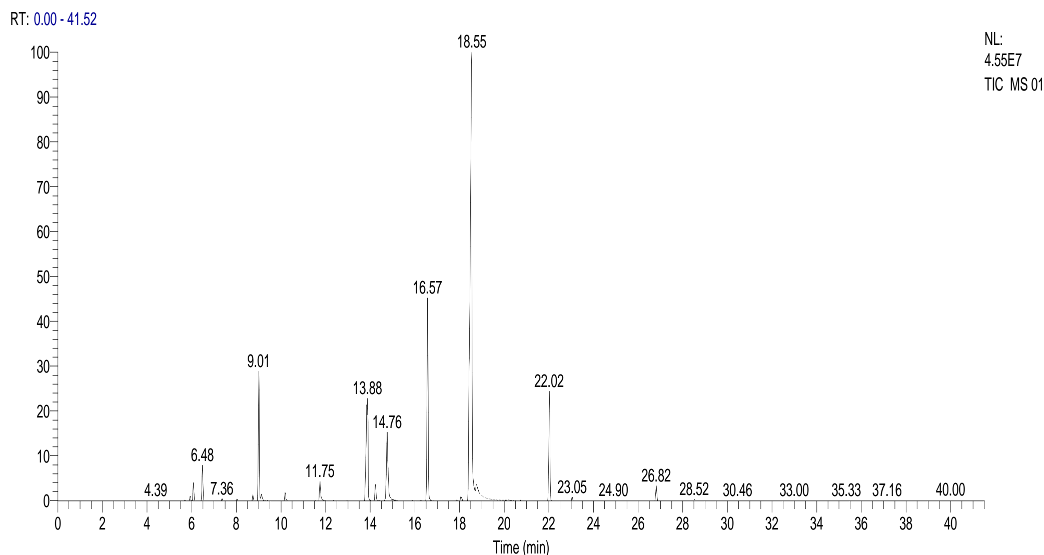

Figure 4. Initial spectrogram (graphic presentation of the spectral map (peaks)) of the different components of the *Thymus satureioides* essential oil extracted by method 1, at t=0. Retention times: RT = 18.55 correspond to "Thymol".
Source: Elkacimi (2016).

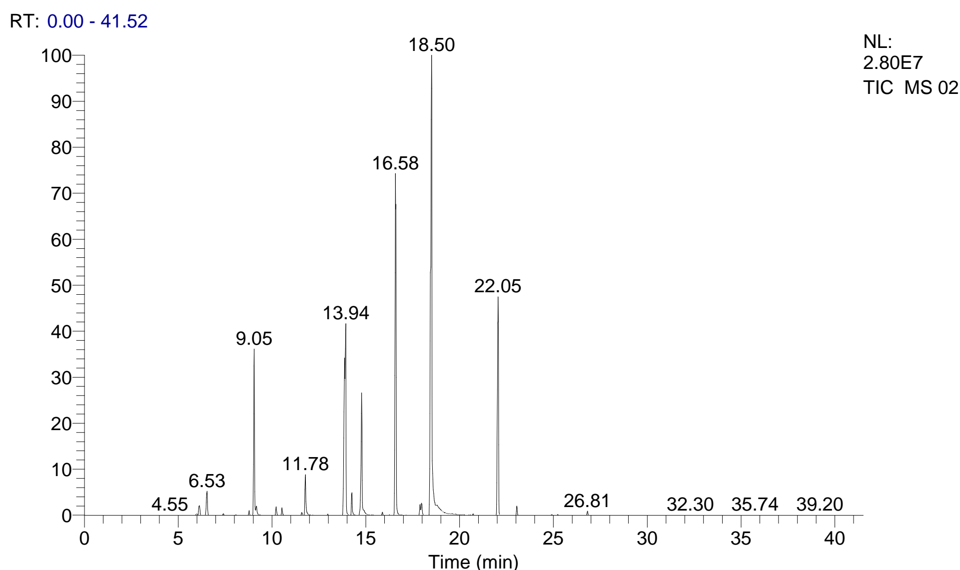

Figure 5. Initial spectrogram (graphic presentation of the spectral map (peaks)) of the different components of the *Thymus satureioides* essential oil extracted by method 2, at t=0. Retention times: RT = 18.50 correspond to "Thymol".
Source: Elkacimi (2016).

(2) The difference "$E_{T B}$" (in percentage) between $A_{T 1}$ and $A_{T 3}$:

$E_{T B}$ = 100% - Z
$E_{T B}$ = 100% - 23.25% = 76.75%
$E_{T B}$ = 76.75%

DISCUSSION

Making a comparative study of the quantitative results in relation to the differences in the thymol concentrations obtained for the three different samples of the essential oil obtained using different methods (1, 2 and 3) of

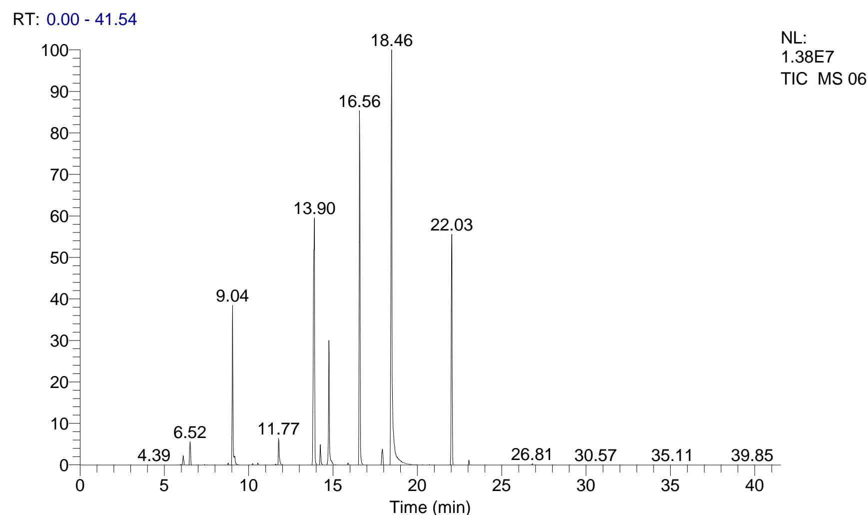

Figure 6. Initial spectrogram (graphic presentation of the spectral map (peaks)) of the different components of the *Thymus satureioides* essential oil extracted by method 3, at t=0. Retention times: RT = 18.46 correspond to "Thymol".
Source: Elkacimi (2016).

Table 1. Representation of injection conditions.

Injection volume	1 µl
Injection temperature	220°C
Interface temperature	300°C
Injection mode	SPLIT
Vector gas	Helium
Flow rate	1.4 ml/min

Table 2. Representation of separation conditions.

Ramp (°C/min)	Final temperature (°C)	Time (min)
-	40	2.00
4	180	0.00
20	300	2.00

Table 3. Representation of the differences (%) of the thymol areas, successively $A_{T\,2}$ and $A_{T\,3}$ compared to $A_{T\,1}$.

Deviation $E_{T\,A}$	Deviation $E_{T\,B}$
Between $A_{T\,1}$ and $A_{T\,2}$	Between $A_{T\,1}$ and $A_{T\,3}$
47.81%	76.75%

extraction of thyme, a significant decrease of about 47.81% was noticed in the thymol concentration in sample 2 as compared to that of the same substance in sample 1 and even greater decrease was noticed in the thymol concentration approaching 76.75% in sample 3 as compared to the same reference in sample 1.

This means that essential oil extraction methods: method 2 (artisanal extraction method by steam distillation) and method 3 (artisanal extraction method by hydro-distillation), produce essential oils of thyme, less concentrated in thymol (approximately 47% less for method 2 and about 75% less for method 3), as compared to method 1 (method of academic extraction by hydro-distillation). It is deduced that an important influence of the technological factor in relation to the type of technique and the method used for the extraction of the essential oil of thyme, was exerted on the initial chemical composition of the oil and in particular, on the concentration of thymol.

Conclusion

In view of the foregoing, it can be concluded that the technological factor in relation to the type of technique and method used for the extraction of thyme essential oil has a direct and important influence on the qualitative and quantitative aspect of the production of thyme essential oil, and therefore, its characteristics and consequently its properties, both physico-chemical and phyto-pharmacological.

The technological factor consists of a set of sub-factors and/or interactive cofactors that are related, as the case may be, to the different elements of the extraction method (or procedure), in particular and mainly the following elements:

(1) The equipment used for extraction: Its nature (main component materials), for example copper, aluminium, stainless steel, galvanized iron, glass, etc. The technology of its manufacture (technological quality of its different parts as well as their arrangement and their sequence etc.) and technique of its use;
(2) The technique itself, the extraction procedure: Is the choice of such a technique favorable or compatible with the medicinal plant;
(3) The environment: Is the environment of the procedure favorable or not to the extraction of the plant, for example, the climatic conditions such as: temperature, pressure, humidity, etc;

The ability and competence of the personnel responsible for carrying out the extraction procedure

Faced with such a problem, both scientific, technical and practical, it is hoped the present study should open the way (at least at the national level) to other similar or even more advanced and/or more specialized studies, to better elucidate this enigmatic set that constitutes the technological factor and its influence on the qualitative and quantitative process of the extraction.

The results of this type of study will serve the cause of the national production of essential oils, in general, since they will form a basis of main guiding elements for better selection of suitable extraction equipment, to better target the exact procedure and optimal exercise environment and better performance and for a rational and intelligent production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Anti-inflammatory and antioxidant potentiality of *Solanum xanthocarpum*

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Inflammation is the body's immune response to combat suspected harmful effect by a stimulus. Antioxidants prevent chain of reaction that would result in the production of free radicals. Prolonged usage of steroids as anti-inflammatory agents is unsafe, demanding search for anti-inflammatory compounds with sustainable future. In this study, seeds and leaves of *Solanum xanthocarpum* were explored *in vitro* for their anti-inflammatory and antioxidant activity. The ethanolic extract of seeds and leaves of *S. xanthocarpum* was prepared by maceration. Anti-inflammatory activity of ethanolic extract of seeds (SE) and leaves (LE) was evaluated in terms of percent inhibition of albumin denaturation, membrane stabilization and protease inhibition. The antioxidant activity was estimated by 2,2-diphenyl-1-picrylhydrantiiazyl (DPPH) free radical scavenging assay by exploiting 1,1-diphenyl-2-picrylhydrazine. Successive solvent extraction with maceration of seeds was carried out by using solvents ethanol, ethyl acetate, acetone and water. The ethanolic extracts of seeds and leaves of *S. xanthocarpum* demonstrates anti-inflammatory and antioxidant activity. The acetones extract exhibited potent anti-inflammatory activity than ethyl acetate and aqueous extract. Results presented here suggest that the *S. xanthocarpum* exhibits anti-inflammatory and antioxidant potentiality.

Key words: *Solanum xanthocarpum*, anti-inflammatory, phytochemical and antioxidant activity.

INTRODUCTION

Inflammation is a complex response to local injury or other trauma; it is characterized by redness, heat, swelling, and pain. Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical and/or microbial agents. It is the body's response to inactivate or destroy the invading organisms, to remove the irritants and set the stage for tissue repair (Leelaprakash and Dass, 2011; Chandra et al., 2012).

The response can sometimes be alarming including allergies; autoimmune diseases, microbial infections, transplants, and burns may initiate a chronic inflammatory response. Various therapeutic approaches are available for reducing long-term inflammatory responses and thus the complications associated with them. The major approach used presently includes use of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs). The duration of their use is limited by gastrointestinal side effects that include unease and abdominal pain and in a

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few serious cases bleeding or perforation of the stomach or upper GI tract (Karthik et al., 2013; Bacchi et al., 2012). The NSAIDs has been reported to cause transient imbalance in electrolyte and water levels as well as liver and renal toxicity (Bacchi et al., 2012; Feldman et al., 1997).

In many inflammatory disorders there is excessive activation of phagocytes, free radical, hydroxyl radical as well as non-free radical species, which can harm surrounding tissue either by powerful direct oxidizing action or indirectly with hydrogen peroxide (H_2O_2) and hydroxyl radicals; those that initiates lipid peroxidation and membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors. The reactive oxygen species are also known to activate matrix metalloprotease (MMP), causing increased destruction of tissues for example collagenase damage seen in various arthritic reactions. Hence, agents that can scavenge these reactive oxygen species would be beneficial in treatment (Sakat et al., 2010). Plants have been used as traditional medicinal source. Plants have bioactive compounds with medicinal value and with low side effects.

The *Solanum xanthocarpum* is a very prickly diffused bright-green, perennial herb of the family *Solanaceae*. It is found abundantly throughout India in plains of dry regions, by roadsides, wastelands and rubbish heaps (Gangwar et al., 2013). The *S. xanthocarpum,* a plant from dashmula (More et al., 2013) of Ayurvedic system has been found to have anti-asthematic, hypoglycemic, hepatoprotective, anti-inflammatory, antipyretic and nephron-protective activities (Hussain et al., 2012; Solapure et al., 2016). This work was aimed to estimate whether or not seeds from raw fruits and leaf of *S. xanthocarpum in vitro* exhibits anti-inflammatory and antioxidant activity.

MATERIALS AND METHODS

Collection of plant

Plant material was collected from Varud, Aurangabad (MS, India) from its natural habitat during May 2017. Authentication of plant was conducted by Herbarium center at Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. The leaves of *S. xanthocarpum* were washed by distilled water and dried under shade. Fine powder was made in mixer grinder (Kenstar). The seeds were collected from the fruit and washed with distilled water to remove fruit impurities. The seeds were then dried at 40°C for overnight. Fine powder of dried seeds was prepared in mortar and pestle.

Ethanolic extraction

Three grams powder of seed and leaf was extracted with 30 ml ethanol. Maceration was carried out for 24 h. The extracts were filtered through muslin cloth and afterwards by Whatman paper 1. Extract was dried in oven at 40°C for 1 h. Dried extract was dissolved in 1% DMSO. The stock of extract 1 mg/ml was prepared in 1% DMSO.

Solvent extraction and phytochemical analysis

Three grams of dried seeds were extracted with 30 ml of various solvents successively in the order ethyl acetate, acetone and distilled water. The maceration was carried out for 24 h for each solvent at room temperature. The extracts for each solvent were collected separately, filtered and dried in oven at 40°C for 1 h and re-suspended in 1% DMSO. Phytochemical analysis was carried out for detection of carbohydrate, phenolics. Tannins, falvonoids, terpenoids, glycosides, alkaloids and saponins were determined as described by More and Kharat (2013).

Inhibition of albumin denaturation

The method given by Govindappa et al. (2011) with slight modification was used. The reaction mixture contained 500 µl of 1% aqueous solution of bovine serum albumin fraction prepared in phosphate buffer saline of pH 6.4 and extract at concentration 100 µg/ml. The reaction mixture was incubated at 37°C for 20 min and then heated to 50°C for 20 min, reaction mixtures were cooled to room temperature and turbidity was measured spectrophoto-metrically at 660 nm. Diclofenac was used as the comparative standard (Geetha et al., 2013; Osman et al., 2016). The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:

% inhibition= [{Abs control- Abs sample}/Abs control] ×100

Where, Abs control is the absorbance of the bovine serum albumin fraction, Abs sample is the absorbance of bovine serum albumin fraction with extract/standard (Govindappa et al., 2011).

Membrane stabilization

Fresh whole human blood (10 ml) from volunteers as per University guidelines was collected and transferred to centrifuge tubes, mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) and was centrifuged at 3,000 rpm. The packed cells were washed with isotonic saline for three times and a 10% suspension was made in isotonic saline (Pant et al., 2012). Heat induced hemolysis studied with modified method of Govindappa (2011) and Kamlesh Pant (2012) was carried out. The reaction mixture consisted of 100 µg/ml of extract and 500 µl of 10% RBCs suspension; instead of test sample only saline was added to the control test tube. Diclofenac was taken as a standard drug (Osman et al., 2016). All the tubes containing reaction mixture were incubated in water bath at 50°C for 30 min. At the end of the incubation the tubes were cooled. 500 µl of isotonic saline was added to reaction mixture. The reaction mixture was centrifuged at 3000 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the extracts. Percent membrane stabilization activity was calculated by the formula mentioned as follow:

% Protection = 100-(Optical density of drug treated sample/Optical density of control) × 100 (Chowdhury et al., 2013)

Protease inhibitory action

Modified method of Govindappa (2011) was carried out. The reaction mixture contained 0.06 mg trypsin, 500 µl of 20 mM Tris-

Table 1. Various phytochemicals present in ethanolic and acetone extracts of seed taken from raw fruits of *S. xanthocarpum.*

Test	LE	SE	AC	Aq
Carbohydrate	-	-	-	-
Phenolic compound	+	+	+	+
Tannins	+	-	-	-
Flavanoids	-	+	-	+
Terpenoids	-	-	-	-
Glycosides	-	-	-	-
Alkaloids	+	-	+	+
Saponin	-	-	-	+
Quinols	-	+	-	+

HCl buffer (pH 7.4) and 100 µg/ml extract. The action mixture was incubated at 37°C for 5 min and then 1 ml of 0.8% (W/V) casein was added. The mixture was inhibited for an additional 20 min, 2 ml of 70% acetic acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as a blank (Govindappa et al., 2011). The experiment was performed in triplicate. The percentage of inhibition of protease inhibitory activity was calculated as follow:

Percentage inhibition = (Abs control –Abs sample) × 100/ Abs control (Leelaprakash and Dass, 2011)

Free radical scavenging activity

Modifications in method given by Kumar et al., 2012 were used. The free radical scavenging activity of the extract was measured *in vitro* by using 1,1diphenyl2 picryl hydrazine (DPPH). The reaction mixture contained 1 ml of DPPH (0.004% in methanol), 100 µg/ml of extract in DMSO (Kumar and Pandey, 2014). The content was mixed and allowed to stand at room temperature for 30 min in dark. The reduction of DPPH free radical was measured by recording the absorbance at 517 nm (Kumar et al., 2012; Patil, 2013) ascorbic acid served the comparison standard (Kumar et al., 2012; Maharana et al., 2010). The percentage scavenging activities (% Inhibition) of the extract was calculated using the following formula.

$$(\%)\ I = [(AC - AS) / AC] \times 100$$

Where, I is inhibition, AC and AS are the absorbance values of the control and the sample respectively. For each sample, three replicates were made, standard deviation were calculated, and indicated as standard error on the graph.

RESULTS AND DISCUSSION

The leaves and fruit of *S. xanthocarpum* showed evidence of anti-inflammatory activity, which was reported by careenage induced mice (Poongothai et al., 2011). The leaves were raised to form callus and its methanolic extract was used to detect antioxidant activity by thiobarbituric acid reactive substances (Poongothai et al., 2011).The whole plant was evaluated for anti-inflammatory activity. The sequential extraction with hexane, benzene, chloroform, ethyl acetate, acetone, ethyl alcohol and water of *S. xanthocarpum* roots exhibited antioxidant activity (Kumar et al., 2012). In this study for the first time, *S. xanthocarpum* raw seeds were evaluated for their anti-inflammatory as well as antioxidant potentiality.

Phytochemical analysis

The *S. xanthocarpum* ethanolic extract of leaf (LE), seed (SE), acetone (AC) and aqueous (Aq) seed extracts were subjected for phytochemical analyses. Results shown in Table 1 indicates LE contained phenolics, tannins and alkaloids, SE contained phenolics, quinols and flavonoids, the AC contained phenolics, alkaloids whereas aqueous seed extracts Aq contained phenolics, flavonoids, alkaloids and quinols.

Invariably in all of the extracts, existence of phenolic compounds was confirmed. With an exception of seed ethanolic extract, all other extracts contained alkaloids. Curiously, carbohydrates were not found to exist in the detectable threshold concentration.

Inhibition of albumin denaturation

The denaturation of proteins as one of the causes as inflammation has been well documented. A number of anti-inflammatory drugs have been known to inhibit protein denaturation (Perumal et al., 2008). To investigate the ability of anti-inflammatory activity of *S. xanthocarpum,* albumin denaturation inhibition assay was performed. The inhibition of albumin denaturation by *S. xanthocarpum* at concentration 100 µg/ml for leaf ethanolic extract was effective (25%) than seed ethanolic extract (8.33%) (Figure 1). The standard drug used diclofenac, which served a positive control could efficiently inhibit bovine serum albumin denaturation than that of ethanolic seed extract (13.88%). Successive solvent extraction of seeds with ethyl acetate, acetone and water of seeds was also found to be inhibiting albumin

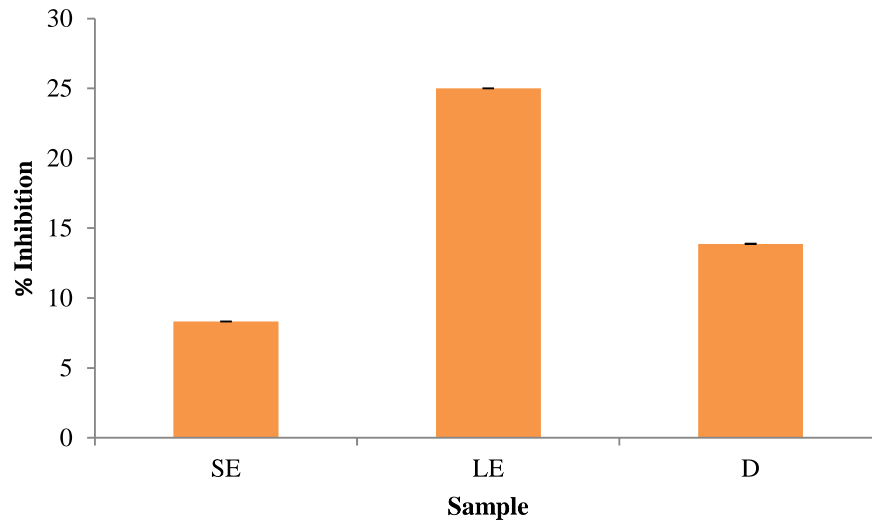

Figure 1. Inhibition of albumin denaturation ethanolic extract. SE, Seed extract; LE, Leaf extract; D, diclofenac. The black bars on histogram denotes standard error.

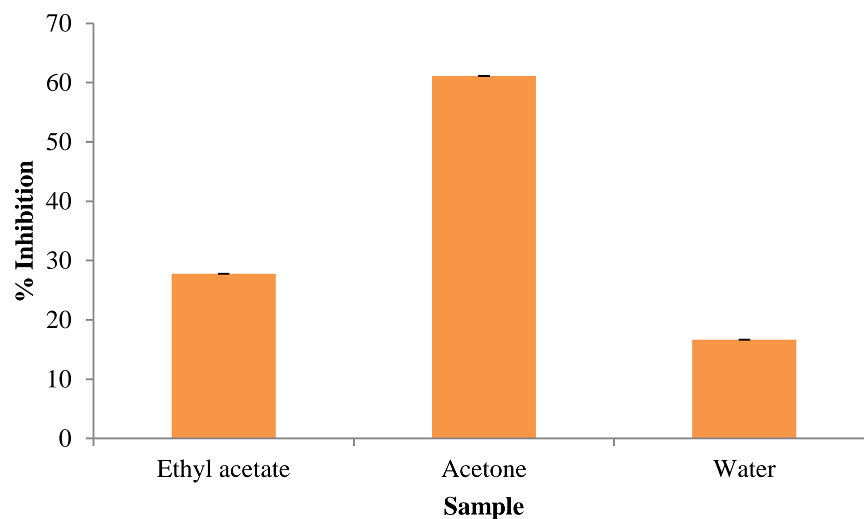

Figure 2. Inhibition of albumin denaturation solvent extraction. The black bars on histogram denotes standard error.

denaturation. It is evident from Figure 2, acetone extract has maximum inhibition (61.11%) compared to ethyl acetate (27.77%) and aqueous extract (16.66%).

Membrane stabilization

Inflammation is a complex response to local injury or other trauma; it is characterized by redness, heat, swelling, and pain. Lysosomal enzymes released during inflammation produces a variety of disorders which leads to the tissue injury by damaging the macromolecules and lipid peroxidation of membranes. Stabilization of lysosomal membrane is important in limiting the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release or by stabilizing the lysosomal membrane. Human red blood cells (HRBC) or erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of HRBC by heat induced membrane lysis can be taken as an *in vitro* measure of anti-inflammatory activity of the drugs or plant extracts (Chippada et al., 2011. Erythrocyte membrane stabilization was studied by incubating erythrocyte with SE and LE. Results shown in Figure 3 denote that LE

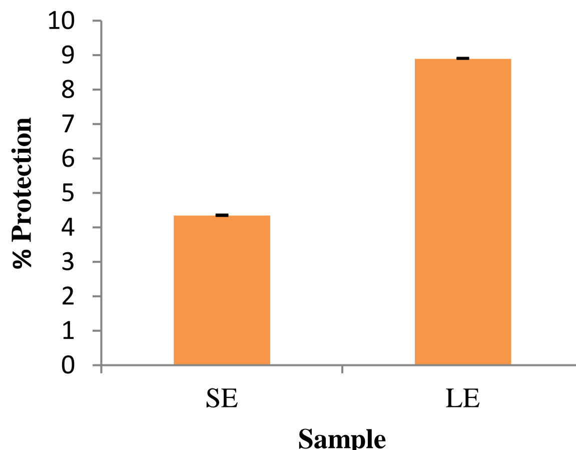

Figure 3. Membrane stabilization ethanolic extract . SE, Seed extract; LE, Leaf extract. The black bars on histogram denotes standard error.

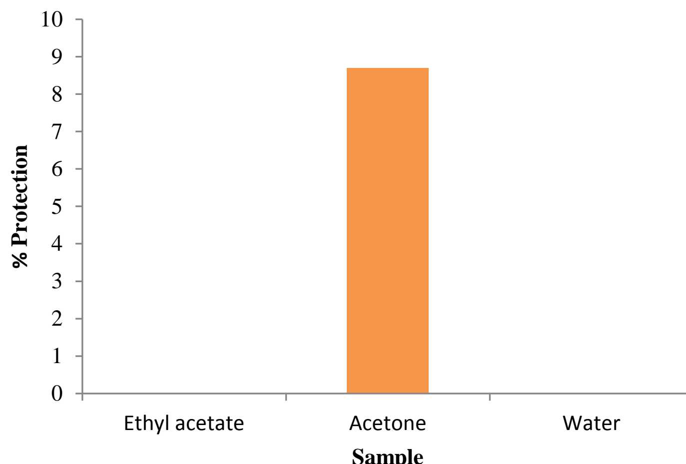

Figure 4. Membrane stabilization Solvent extraction. The black bars on histogram denotes standard error.

was able to stabilize erythrocyte membrane better to that of SE. In a parallel experiment, erythrocyte membrane stabilization studies were carried with acetone, ethyl acetate and aqueous extracts prepared from seeds. Results shown in Figure 4 demonstrate that membrane stabilization was found with acetone extracts only whereas both ethyl acetate and aqueous extracts failed to stabilize erythrocyte membrane.

Protease inhibition

Proteases play a crucial role in inflammation process. The serine proteases have been known to be involved in tissue damage thus triggering inflammatory response. The protease inhibitors thus were anticipated to reduce inflammatory response which would be caused by mediators released by leukocytes in response to proteases. To investigate serine protease inhibitor activity, we sought to test anti-trypsin activity in *S. xanthocarpum* extracts. Results depicted in Figure 5 indicate that anti-trypsin activity was noticed more for SE (3.14%) than for LE (1.26%). The experiment with successive solvent extraction of seeds with acetone, ethyl acetate and water illustrated 4% protease inhibitory activity for acetone and aqueous extract while poor 1% activity was seen for ethyl acetate extract, shown in Figure 6.

Free radical scavenging activity

It is known that during inflammatory disorders there is an excessive activation of phagocytes, production of reactive

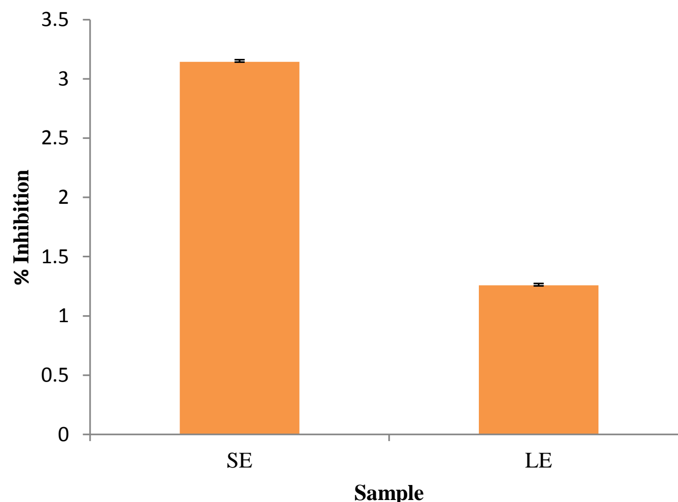

Figure 5. Protease inhibition ethanolic extract. SE, Seed extract; LE, Leaf extract. The black bars on histogram denote standard error.

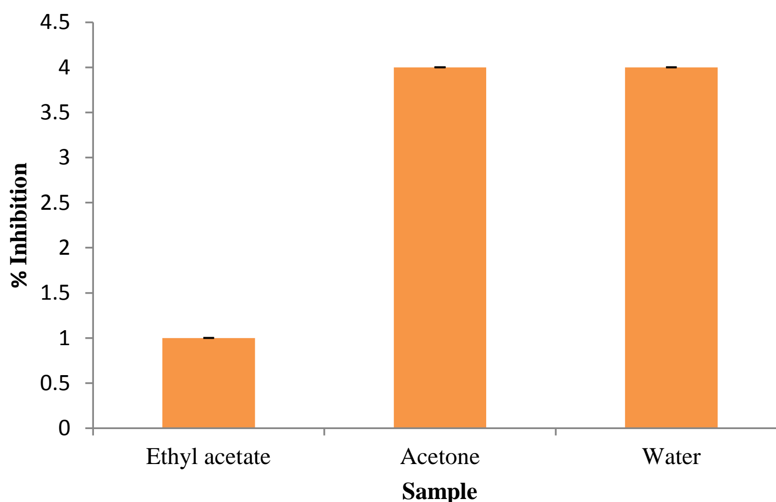

Figure 6. Protease inhibition solvent extraction. The black bars on histogram denote standard error.

oxygen species, nascent oxygen, hydroxyl and hydrogen peroxide, which could harm surrounding tissue. This in turn would initiate lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors. The reactive oxygen species are also known to activate MMP. The collagenase and MMP is known to cause increased tissue destruction demonstrated in various arthritic reactions. Hence, the agents that can scavenge these reactive oxygen species would be of greater interest in treatment (Sakat et al., 2010). The DPPH assay for free radical scavenging activity by the first time as per references cited using 1,1-diphenyl-2-picrylhydrazine was performed. The scavenging of free radicals results shown in Figure 7 indicates highest activity for SE (44.68%) in comparison to LE (36.17%) and data were parallelized with a standard compound ascorbic acid (23.4%). Curiously, both LE and SE exhibited superior activity than that of the positive control ascorbic acid.

Conclusions

Solanum species have been known to exhibit various bioactivity in leaf, root and ripened fruits. Our preliminary

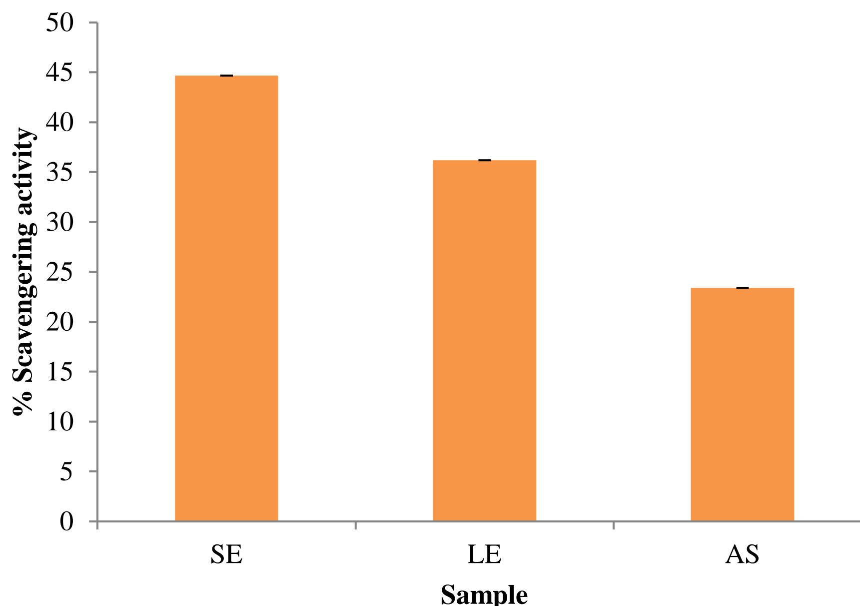

Figure 7. Free radical scavenging activity ethanolic extract. SE, Seed extract; LE, Leaf extract; AS, Ascorbic acid. The black bars on histogram denote standard error.

results suggest that raw fruits and in particular seeds of *S. xanthocarpum* exhibit strong anti-inflammatory and anti-oxidation potentiality. Our experimental data suggest that either phenolic compounds or alkaloids are likely to be the active pharmaceutical ingredient (API). Experiments are underway to dissect more on biochemical characterization of an API from the SE and acetone seed extracts.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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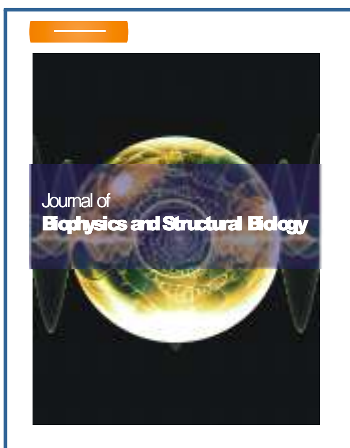

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