Genetic and chemical analysis of Carthamus species growing in Egypt and Libya

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Introduction:

Carthamus L. is a genus belonging to the tribe Cynareae (thistle), subfamily Tubuliflorae and family Compositae. The genus includes about 25 species, distributed from Spain and North Africa across the Middle East to northern India (Ashri & Knowles, 1960). *Carthamus tinctorius* L. "known as Safflower" is one of the most famous species and is the only species that is widely cultivated. Safflower (known in Egypt as Kurtum and in Japan as Beni bana) was prized in ancient Egypt as a source of red-yellow and orange dyes for cotton and silk, as well as ceremonial ointment and to anoint mummies prior to binding. Safflower seeds and packets and garlands of florets have been found with 4000-year-old mummies. Safflower has been used in the Middle East, India and Africa for purgative and alexipharmic (antidote) effects, as well as in a medicated oil, to promote sweating and cure fevers (Weiss, 1971). Safflower is currently cultivated in Egypt, mainly for its seeds which are used as source of edible oil.

The dried florets of *C. tinctorius* have been used extensively for over 2,500 years in Traditional Chinese Medicine (TCM) to treat stroke, coronary heart disease and angina pectoris (Zheng, Dong, & She, 1998). In the Compendium of Materia Medica, it is described as being able "to invigorate the circulation of blood", which suggests its potential in circulatory system (Zhu, 1998). As well, Safflower was also found to demonstrate a number of other biological activity e.g. antioxidant (Jun et al., 2011), anti-inflammatory (Choi, Kim, & Lee, 2010) and hepatoprotective activities (Zhang et al., 2011).

Major active constituents isolated from Safflower are the C-glycoside pigments; hydroxysafflor yellow A, safflor yellow A, tinctormine, safflor yellow B, carthamin and safflomin A (Kazuma et al., 2000; Meselhy et al., 1993; Onodera, Obara, Osone, Maruyama, & Sato, 1981; Takahashi et al., 1984; X. Zhou, Tang, Xu, Zhou, & Wang, 2014). All of these compounds have been classified into the quinochalcone family of flavonoid which have a unique structure with a

C-glycosylated cyclohexanonedienol moiety **(Fig. 1)**. In addition several kaempferol, quercetin, luteolin and apigenin mono-, di- and triglycosides have been reported from the florets and the leaves of *C. tinctorius* (Hattori et al., 1992; Lee, Chang, Kim, Park, & Choi, 2002; Lim, 2007) as well as alkaloids (Jiang, Lu, Yang, Zhang, & Zhang, 2008) and cinnamic acid derivatives (Y.-Z. Zhou et al., 2008).

Regarding *C. lanatus* L., few reported the isolation of flavonoids, phenolic acids and sesquiterpene glycosides from the aerial parts (Mikhova, Duddeck, Taskova, Mitova, & Alipieva, 2004; San Feliciano, Medarde, Del Rey, Miguel Del Corral, & Barrero, 1990; Taskova, Mitova, Mikhova, & Duddeck, 2003). Nothing was reported concerning the chemical composition of *C. divaricatus* (Beg. et Vacc.) Pamp. or *C. leucocaulos* Sibthorp Smith.

C. tinctorius has several varieties which are cultivated widely in the Middle East, India, Africa, China and Japan. However, genetic and chemical diversity of *Carthamus* species remains unclear, which affects therapeutic effects.

The aim of this work is to present a comparative phylogenetic study and metabolite profiling of several *Carthamus* species from Egypt and Libya.

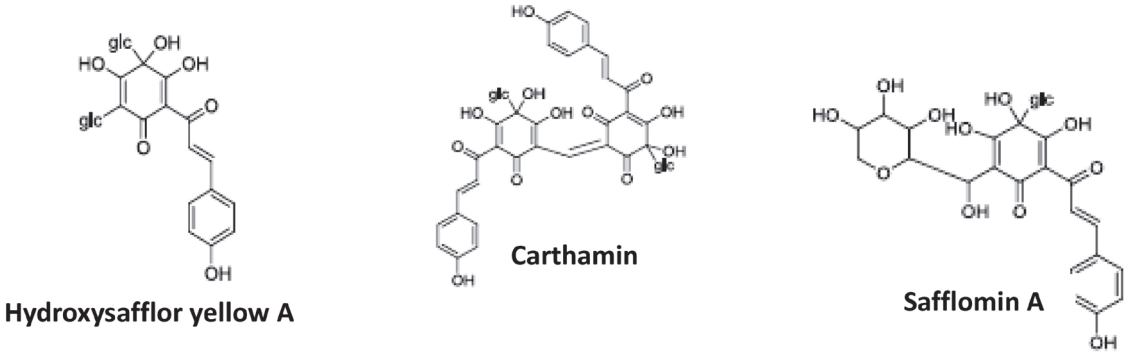

Fig. 1: Structures of common compounds isolated from *C. tinctorius* L.

Part I: Genetic Characterization based on ITS sequences

Materials and Methods:

Plant Material

Three *Carthamus* species were collected from Libya, and two varieties were collected from Egypt **(Table 1)**. Vouchers are stored at the Museum of Materia Medica, Institute of Natural Medicine, University of Toyama, Japan.

Genomic DNA Extraction

About 20-100 mg of the samples were cut to small pieces, frozen for 30 min at -80 ˚C and ground using Tissuelyser for 2 min at 30 Hz. Total DNA was then extracted by DNeasy Plant Mini Kit (Qiagen, Germany). Gel electrophoresis was performed to determine the quality and quantity of the DNA.

Table 1: Sample list of *Carthamus* species used in genetic study

	Code	Species	Source	Part Used	Date of Collection
1	DA-1	*Carthamus tinctorius* var. *typicus*	Shandaweil, Sohag district, Upper Egypt	Leaves	May, 2013
2	DA-3	*C. tinctorius* var. *inermis*	Shandaweil, Sohag district, Upper Egypt	Leaves	May, 2013
3	DA-5	*C. lanatus* L.	Barqa district "near borders of Benghazi", Libya	Flower head	May, 2013
4	DA-6	*C. leucocaulos* Sibthorp Smith	Barqa district "near borders of Benghazi", Libya	Flower head	May, 2013
5	DA-7	*C. divaricatus* (Beg. Et Vacc.) Pamp.	Barqa district "near borders of Benghazi", Libya	Leaves	May, 2013
6	DA-9	*C. tinctorius* L.	Europe	Leaves	July, 2014
7	DA-10	*C. tinctorius* L.	Japan	Seeds	Sep, 2014

Polymerase Chain Reaction (PCR) Amplification

Using the tDNA as a template, ITS region **(Fig. 2)** of all the samples was amplified by polymerase chain reaction (PCR) using Takara PCR Thermal Cycler. The reaction mixture contained: 0.5 µl KOD-Plus-DNA polymerase, 2.5 µl dNTPs, 1 µl MgSO4, 13.5 µl dH_2O, 2.5 µl 10 × Buffer, 2 µl of each primer (ITS-1F, 18S-25S-3' R) and 1 µl template DNA. The thermal cycling conditions for amplification were as follows: initial denaturation at 96 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s, extension at 68 °C for 1 min and final extension at 68 °C for 7 min. The PCR product was detected by 1.0% agarose gel electrophoresis followed by purification using SV gel and PCR clean up system (Promega, U.S.A.) and the DNA concentration was determined spectrophotometrically.

Sequencing and Molecular analysis

Sequencing reaction of purified PCR products was carried out using ABI PRISM Bigdye Terminator v3.1 Cycle sequencing kits (Applied Biosystems, U.S.A.) with the primers (ITS-1F, In18S-25S-3'R, In18S-25S-5'F, 18S-25S-3'R) **(Table 2, Fig. 2)**. The thermal cycling condition was as follows: Initial denaturation at 96 °C for 1 min followed by 26 cycles of denaturation at 96 °C for 10 s, annealing and extension at 50 °C for 5 s and final extension at 60 °C for 4 min.

The products were purified using SAM solution and X Terminator, centrifuged and the sequence was determined directly by an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems, U.S.A.). The obtained sequences were edited and assembled and a consensus sequence of each sample was reached. The DNA sequences were compared and aligned using the

program MULTALIGN (http://multalin.toulouse.inra.fr/multalin/multalin.html).

The borders of ITS1, 5.8S and ITS2 regions were determined by comparison with the known sequences of mung bean and rice (Schiebel & Hemleben, 1989; Takaiwa, Oono, & Sugiura, 1985).

Further, the DNA sequences of the tested samples were subjected to BLAST (http:// www. ncbi.nlm.nih.gov/blast/blast.cgi) for better identification of sequence at species level and the resulting GenBank data were compared and aligned with the DNA sequences of the tested samples.

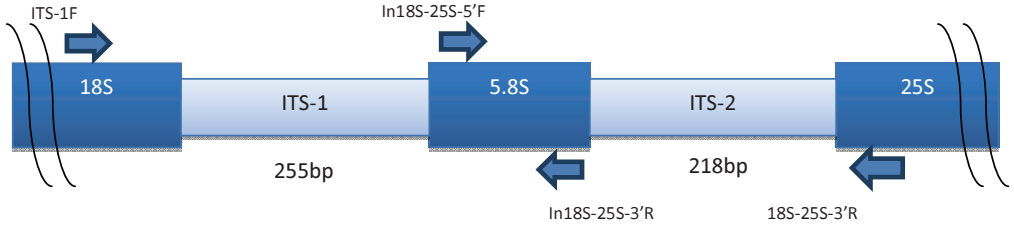

Fig. 2: Approach for Sequence Determination of ITS region

Table 2: Primers used for PCR and Sequencing Reaction

Primer	Sequence (5'→3')	Length(bp)
Forward primer		
ITS-1F	TCC ACT GAA CCT TAT CAT TTA G	22
In18S-25S-5'F	TCT CGC ATC GAT GAA GAA CG	20
Reverse primer		
In-18S-25S-3'R	GAC TCG ATG GTT CAC GGG ATT CT	23
18S-25S-3'R	CCA TGC TTA AAC TCA GCG GGT	21

Results and Discussion:

Seven plant samples from Egypt, Libya, Europe and Japan **(Figs. 3-6)** were studied. The nucleotide sequence of internal transcribed spacer (ITS) region of all samples were clearly determined and compared.

The sequences differed in length by one base pair, in which the ITS1 region was 255 bp, the 5.8S rRNA gene region was 163 bp, and the ITS2 region was 218 bp for *C. tinctorius* samples (DA-1, DA-3 and DA-10) and 219 bp for the other samples (DA-5, DA-6 and DA-9) **(Table 3)**. It is worthy to mention that both samples from Egypt, DA-1 and DA-3, *C. tinctorius* var. *typicus* and var. *inermis* had the same sequence, meanwhile, DA-5 *C. lanatus* and DA-6 *C. leucocaulos* showed the same sequence.

The ITS sequences of *C. tinctorius* deposited in International Nucleotide Sequence Database (INSD: DDBJ/EMBI/NCBI) included 6 main types, which differed from each other by three nucleotides at positions 464, 531 and 624 **(Table 3)**. Type 1 (JQ230977 (1)) shows **C-C-C**, type 2 (HQ112166 (2)) shows **C-C-Y**, type 3 (HQ112178 (3)) shows **C-C-T**, type 4 (HQ112171 (4)) shows **Y-C-C**, type 5 (GU969649 (5)) shows **T-C-C** and finally Type 6 (GU969651 (6)) shows **T-T-C**.

On comparing the GenBank data available for *C. lanatus, C. leucocaulos* and *C. divaricatus*, they showed a somewhat similar sequences but which are different from that of *C. tinctorius*, where *C. divaricatus* (GU969623) differed in 23 nucleotides positions, *C. lanatus* (GU969619 *C. lanatus* subsp. *baeticus* and HM921409 *C. lanatus* CAULA-7) in 20 positions, while *C. leucocaulos* (GU969634 *C. leucocaulos* and GU969635 *C. leucocaulos*) differed in 24 nucleotide positions.

Regarding the studied samples, they showed ITS sequences that could be separated into two main groups, the first group comprises the *C. tinctorius* samples from Egypt and Japan where they all showed similar sequence, to that found in GenBank differing at nucleotides positions 464 and 624, where *C. tinctorius* var. *typicus* (DA-1) and *C. tinctorius* var. *inermis* (DA-3) exhibited additive peaks of thymine and cytosine (Y). In the case of *C. tinctorius* sample from Japan (DA-10), additive peaks of thymine and cytosine (Y) were as well observed at nucleotide positions 464 and 531, while position 624 showed cytosine.

The second group contained sequences of *C. lanatus* (DA-5), *C. leucocaulos* (DA-6) and *Carthamus* sample from Europe (DA-9). Samples DA-5 and DA-6 showed similar sequence to *C. divaricatus* (GU969623), *C. turkistanikus* (GU969653) and *C. lanatus* ssp. *baeticus* (GU969619) from GenBank. Their patterns differed at nucleotide positions 117, 131, 201, 204, 211, 229, 255, 387, 439, 490 and 491.

In the case of *Carthamus* sample from Europe, it showed close similarity with *C. lanatus* ssp. *baeticus* (GU969619), differing only in 3 nucleotides at positions 133, 597 and 617, where DA-9 showed peaks of **(Y-W-Y)** instead of **(T-T-C)**. These findings clearly show that this sample is most probably *C. lanatus* L. sample not *C. tinctorius* L. as previously identified.

Concerning sample DA-7, previously identified as *C. divaricatus* (Beg. Et Vacc.) Pamp., it clearly possessed quite a different sequence from all *Carthamus* samples, as well as the reported data in GenBank. The sample also showed different morphological characters than that of *Carthamus* species, where the size of the flower head was significantly smaller and showing differences in shapes of bracts. The ITS sequence of the sample showed >99% homology to *Eryngium campestre* (family Apiaceae) HE602451 from GenBank as well as similar morphological characters.

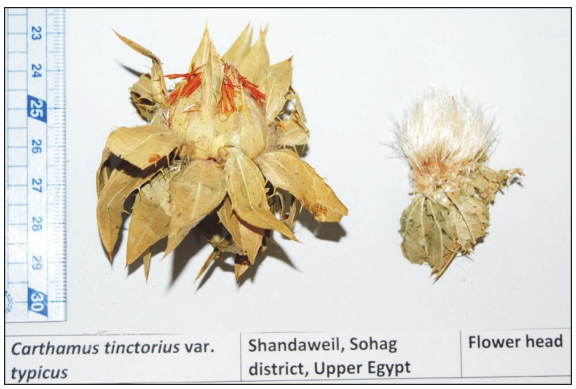

Fig. 3: *C. tinctorius* var. typicus flower head

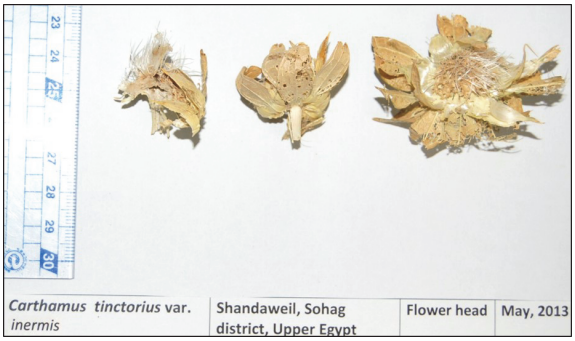

Fig. 4: *C. tinctorius* var. *inermis* flower head

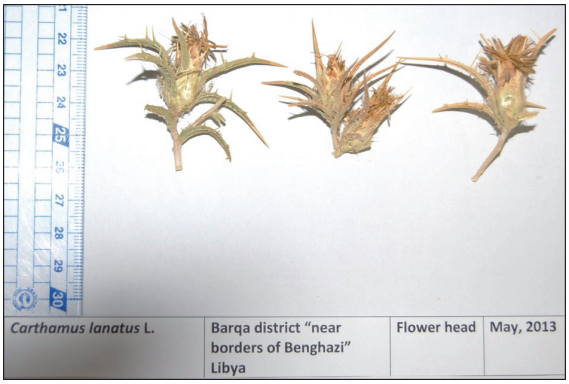

Fig. 5: *C. lanatus* flower head

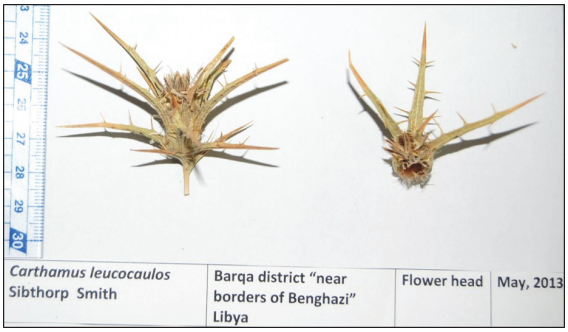

Fig. 6: *C. leucocaulos* flower head

Table 3: Comparison of ITS sequence among various samples of *Carthamus* species

Samples	ITS-1													5.8S	ITS-2																		
	5	9	1	3	3	3	9	0	0	1	1	2	5	8	2	3	3	5	6	7	7	8	9	9	3	5	5	5	6	7	9	9	1	2	2
	0	8	7	1	3	4	8	1	4	1	9	9	5	7	2	8	9	4	4	2	6	9	0	1	1	2	5	7	5	5	0	7	7	0	4
C. tinctorius [a] JQ230977(1)	T	T	C	C	T	C	T	T	C	C	G	C	T	C	C	G	G	A	C	A	G	T	C	G	C	A	G	A	A	T	T	T	C		C
C. tinctorius [b] HQ112178(3)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	-	T
C. tinctorius [c] HQ112166(2)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	-	Y
C. tinctorius [d] HQ112171(4)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	Y	*	*	*	*	*	*	*	*	*	*	*	*	*	*	-	*
C. tinctorius [e] GU969649(5)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	-	*
C. tinctorius [f] GU969651 (6)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	T	*	*	*	*	*	*	*	*	-	*
***C. divaricatus* (GU969623)**	C	C	T	T	*	T	G	*	T	*	T	*	C	T	*	*	T	C	*	G	C	C	T	C	*	C	*	C	T	C	C	*	*	T	*
***C. turkestanicus* (GU969653)**	C	C	*	Y	*	T	G	C	T	*	T	*	*	*	*	*	*	C	*	G	C	C	T	C	*	C	*	C	T	C	C	*	*	T	*
***C. lanatus ssp. baeticus* (GU969619)**	C	C	*	T	*	T	G	*	*	*	T	*	C	*	*	*	*	C	*	G	C	C	T	C	*	C	*	C	T	C	C	*	*	T	*
***C. lanatus* CAULA-7 (HM921409)**	C	C	*	T	*	T	G	*	*	*	T	*	C	*	*	*	*	C	*	G	C	C	T	C	*	C	*	C	T	*	C	A	*	T	*
[g]***C. leucocaulos* (GU969634)**	C	C	*	*	*	T	G	*	T	*	T	*	C	*	T	C	*	C	*	G	C	C	T	C	*	C	A	C	T	C	C	T	C	T	*
DA-1, DA-3	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	Y	*	*	*	*	*	*	*	*	*	*	*	*	*	*	-	Y
DA-10	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	Y	*	*	*	*	*	Y	*	*	*	*	*	*	*	*	-	*
DA-5 , DA-6	C	C	Y	Y	*	T	G	Y	Y	Y	T	M	Y	Y	*	*	K	C	*	G	C	C	Y	S	*	C	*	C	T	C	C	*	*	T	*
DA-9	C	C	*	T	Y	T	G	*	*	*	T	*	C	*	*	*	*	C	*	G	C	C	T	C	*	C	*	C	T	C	C	W	Y	T	*

DA-1: *C. tinctorius* var. *typicus*, **DA-3:** *C. tinctorius* var. *inermis*, **DA-5:** *C. lanatus*, **DA-6:** *C. leucocaulos*, **DA-9:** *C. tinctorius* (Europe), **DA-10:** *C. tinctorius* (Japan)

K = G&T; M = A&C; R = A&G; S = C&G; W = A&T; Y = C&T

[a]: JQ230977 (1): JQ230977, *C. tinctorius*, SBB-1162; GU724280, *C. tinctorius*; HM921410, *C. tinctorius,* CAUTI-19; GU969650, *C. tinctorius,* DAV:27591; GU969648, *C. tinctorius*; GU969647, *C. tinctorius*; EF483946, *C. tinctorius* cultivar, BJ1063; EF483943, *C. tinctorius* cultivar, W66730; HQ407426, *C. palaestinus*; GU969643, *C. palaestinus*; GU969642, *C. palaestinus* voucher DAV:24847; HQ112177, *C. glaucus,* HW-024; HQ112172, *C. glaucus,* HW-019; HQ112154, *C. glaucus,* HW-001; HQ112176 *C. glaucus,* HW-023; GU969644, *C. persicus*; GU969639, *C. oxyacanthus* voucher GH:244792; GU969641, *C. oxyacanthus*; GU969640, *C. oxyacanthus* (Malik & Babbar , Berner & McMahon and (Bowles et al) .

[b]: HQ112178(3): HQ112178, *C. glaucus,* HW-000; HQ112168, *C. glaucus,* HW-015 (Huh et al).

[c]: HQ112166 (2): HQ112166, *C. glaucus,* HW-013; EF483948, *C. tinctorius* cultivar BJ1067; EF483947, *C. tinctorius* cultivar BJ673 (Huh et al and Chapman et al).

[d]: HQ112171(4): HQ112171, *C. glaucus* HW-018; HQ112164, *C. glaucus,* HW-011; HQ112155, *C. glaucus* HW-002; EF483950, *C. tinctorius* cultivar ToziSpiny; EF483949, *C. tinctorius* cultivar BJ2701; EF483945, *C. tinctorius* cultivar ENANA ; EF483944, *C. tinctorius* cultivar LESAF (Huh et al and Chapman et al).

[e]: GU969649 (5): GU969649, *C. tinctorius*; FJ539127, *C. tinctorius*(Bowles et al and Daniel & Knoess).

[f]: GU969651 (6): GU969651, *C. tinctorius.*

[g]: GU969634: GU969634, *C. leucocaulos*; GU969635, *C. leucocaulos.*

Part II: Chemical Profiling

The aim of the work reported here is to define and compare the phytochemical composition of the different parts of the Carthamus flower heads, as well as the leaves for the samples under investigation using HPLC-DAD and HPLC-DAD-MS.

An HPLC analysis was conducted first to determine the optimal conditions for analysis using *C. tinctorius* sample provided from Kanazawa University and the samples under study.

Plant Material:

The dried flower heads of the samples under investigation were separated into florets, pappus, inner bracts and outer bracts, in addition to the leaves **(Table 4, Fig. 7)**, they were ground separately using an electric crusher or in a mortar using liquid nitrogen for samples of small amounts.

Different quantities of plant material were used for extraction; 20 mg, 50 mg and 100 mg to determine the suitable amount. The powders were then vortexed and homogenized separately with 5 ml MeOH using an ultrasonic bath for 30 min. Extracts were centrifuged at 4000 rpm for 10 min and filtered through 22 µm membrane filter.

HPLC Apparatus:

Shimadzu HPLC (**pump**: LC-10AD vp, **Autosampler**: SIL-10AD vp, **Detector**: SPD-M20A multiwavelength detector)

Column: Zorbax SB-C18, 250 × 4.6 mm, 5 µm, Agilent.

Solvent: A: Acetonitrile, B: 0.1% Aqueous formic acid

Flow rate: 1 ml/min

Column temp: 25 ˚C

Injection volume: 20 µl

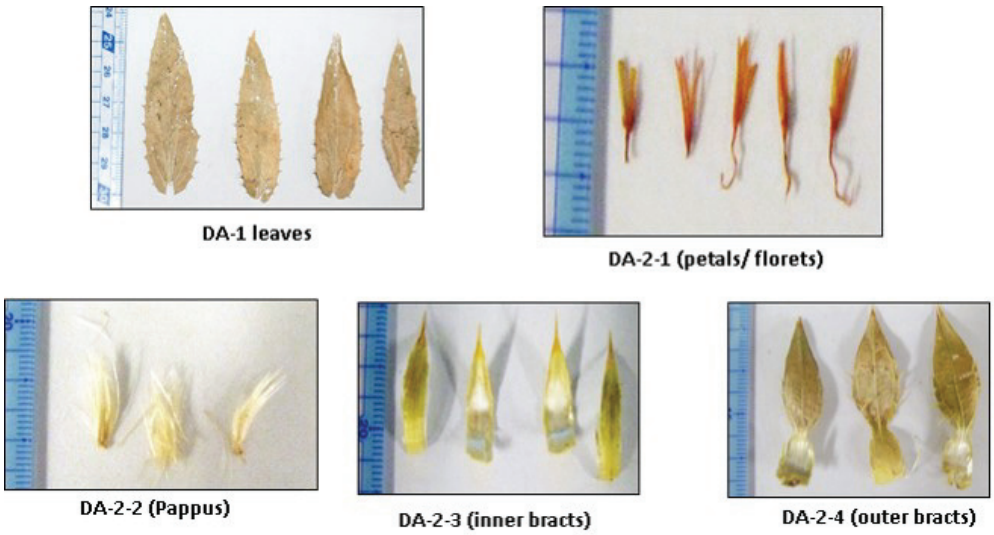

Fig. 7: Different parts of *C. tinctorius* var. *typicus* used in Chemical study

Table 4: Sample list for Chemical profiling:

Code	Species	Site	Part Used
DA-1	*C. tinctorius* var. *typicus*	Shandaweil, Sohag district, Upper Egypt	Leaves
DA-2-1	*C. tinctorius* var. *typicus*	〃	Florets
DA-2-2	*C. tinctorius* var. *typicus*	〃	Pappus
DA-2-3	*C. tinctorius* var. *typicus*	〃	Inner bract
DA-2-4	*C. tinctorius* var. *typicus*	〃	Outer bract
DA-3	*C. tinctorius* var. *inermis*	〃	Leaves
DA-4-1	*C. tinctorius* var. *inermis*	〃	Pappus
DA-4-2	*C. tinctorius* var. *inermis*	〃	Inner bract
DA-4-3	*C. tinctorius* var. *inermis*	〃	Outer bract
DA-5-1	*C. lanatus* L.	Barqa district "near borders of Benghazi", Libya	Florets
DA-5-2	*C. lanatus* L.	〃	Pappus
DA-5-3	*C. lanatus* L.	〃	Inner bract
DA-5-4	*C. lanatus* L.	〃	Outer bract
DA-6-1	*C. leucocaulos* Sibthorp Smith	〃	Florets
DA-6-2	*C. leucocaulos* Sibthorp Smith	〃	Pappus
DA-6-3	*C. leucocaulos* Sibthorp Smith	〃	Inner bract
DA-6-4	*C. leucocaulos* Sibthorp Smith	〃	Outer bract
DA-12	*C. tinctorius* L.	Kanazawa University	Leaves
DA-13-1	*C. tinctorius* L.	〃	Florets
DA-13-2	*C. tinctorius* L.	〃	Pappus
DA-13-3	*C. tinctorius* L.	〃	Inner bract
DA-13-4	*C. tinctorius* L.	〃	Outer bract

Results:

- Extraction:

Results of HPLC analysis of the tested samples showed that 50 mg were suitable for samples of the leaves and florets, while 100 mg of powdered plant material was optimal for pappus, inner and outer bract samples.

- Chromatographic Conditions:

Mixtures of Acetonitrile in combination with 0.1% aqueous formic acid were tried as mobile phases and good separation was achieved with the following conditions:

Mobile phase: A: Acetonitrile, B: 0.1% Aq. Formic acid

Program: 0-30 min, 10-30% A; 30-35 min, 30-35% A; 35-50 min, 35-70% A; 50-55 min, 70-10% A finally 55-60 min, 10%A.

Injection volume: 20 µl, Flow rate: 1 ml/min, Column temp.: 25˚C

- Two chromatograms were examined from the DAD spectral data: the 407 nm chromatogram for detecting quinochalcones and the 350 nm chromatogram for detecting flavonols.

Discussion:

Comparing the chromatograms of the different tested organs (**Figs. 8-14**), it was clear that quinochalcones are mainly present in the samples of the florets, while all other samples (pappus, inner bracts, outer bracts and leaves) showed peaks having characteristic UV pattern of flavonols which is in accordance with previous references (Lee et al., 2002; Yue, Tang, Li, & Duan, 2013).

It is worthy to mention that the two *C. tinctorius* varieties (*typicus* and *inermis*) from Egypt exhibited similar pattern in all tested samples, which was different from those samples of *C. lanatus* and *C. leucocaulos* from Libya (**Fig. 9-14**). These results are in accordance with the results obtained from the genetic study where these samples showed identical patterns.

As well, the extracts of the inner and outer bracts showed similar patterns to each and to that of the leaves (**Fig. 8**).

Up to date, there is no report dealt with chemical constituents of the different parts of *Carthamus* florets viz; pappus, inner bracts and outer bracts, so it is of interest to compare and identify chemical components using HPLC-DAD-MS. Preliminary data was obtained from HPLC-DAD-MS analysis, and annotation of peaks and quantification of major constituents are undergoing.

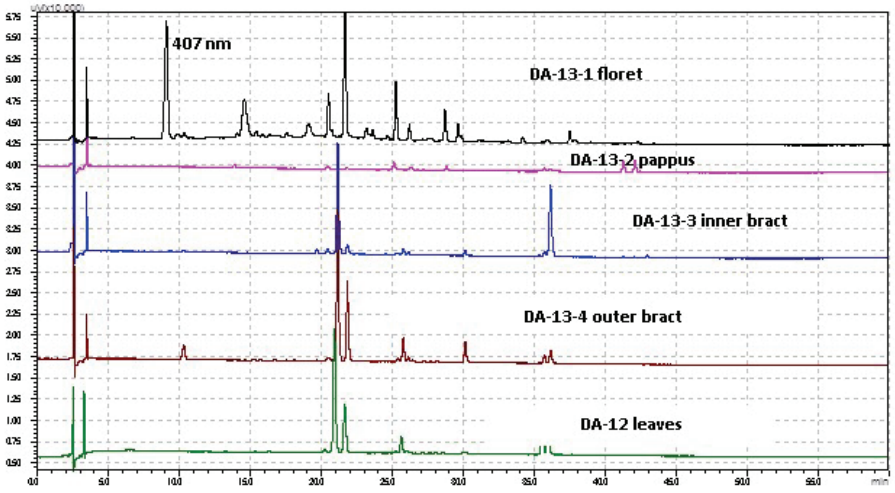

Fig 8: Chromatograms of different parts of *C. tinctorius* (Kanazawa Univ.) at 407 nm

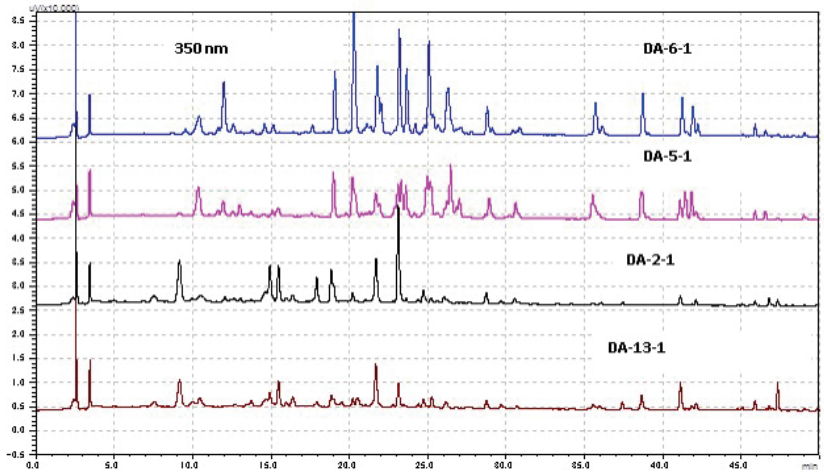

Fig. 9: Chromatograms of *Carthamus* floret samples at 350 nm

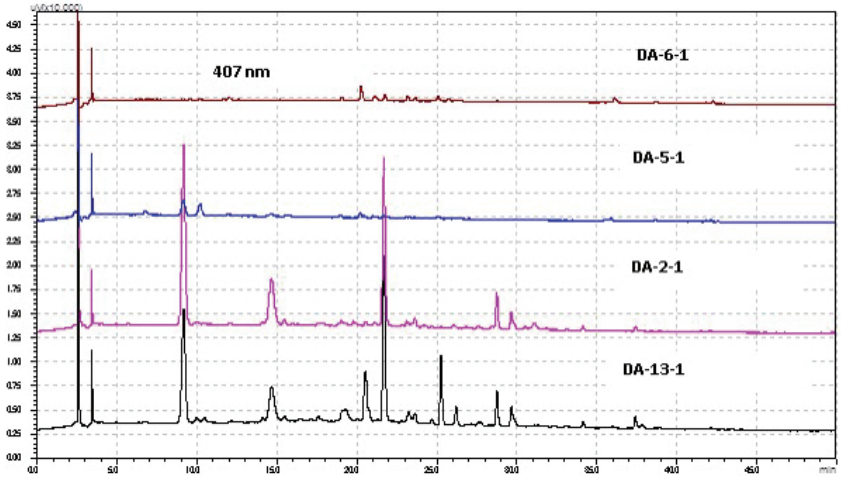

Fig. 10: Chromatograms of *Carthamus* floret samples at 407 nm

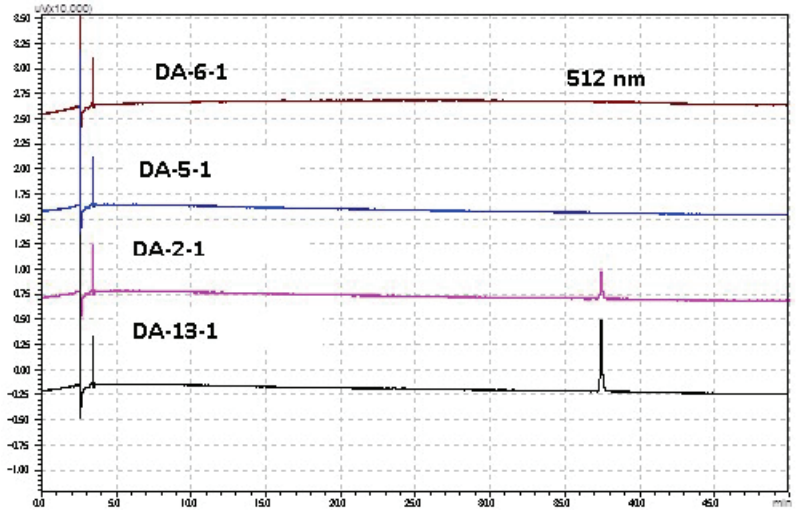

Fig. 11: Chromatograms of *Carthamus* floret samples at 512 nm

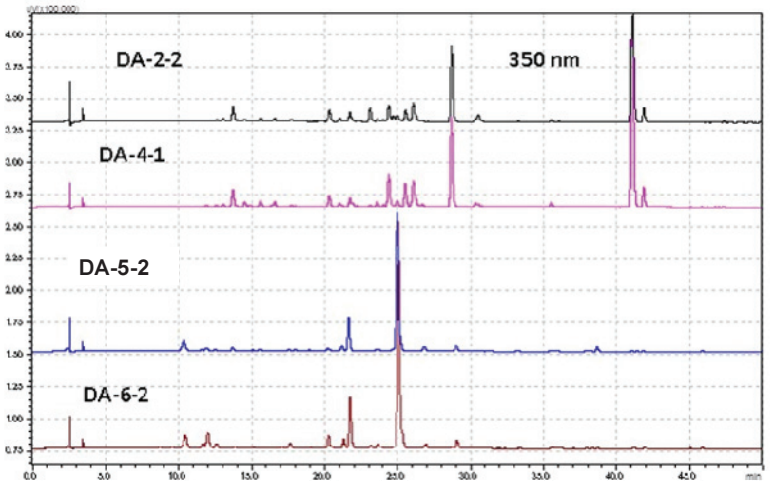

Fig. 12: Chromatograms of *Carthamus* pappus samples at 350 nm

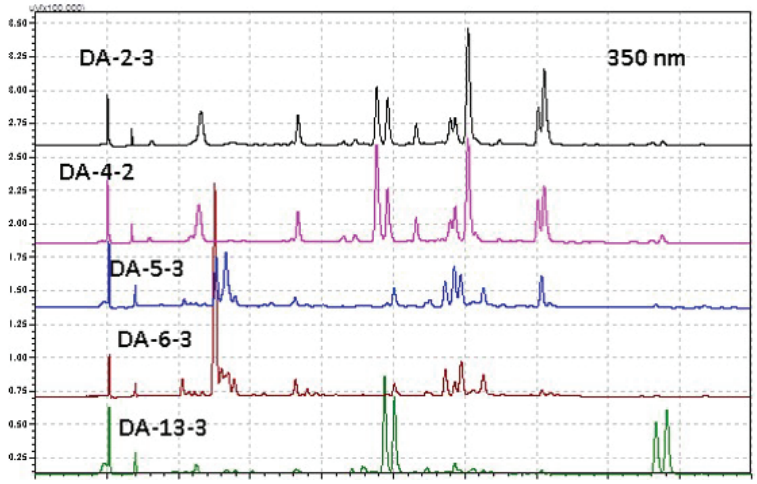

Fig 13: Chromatograms of *Carthamus* inner bracts samples at 350 nm

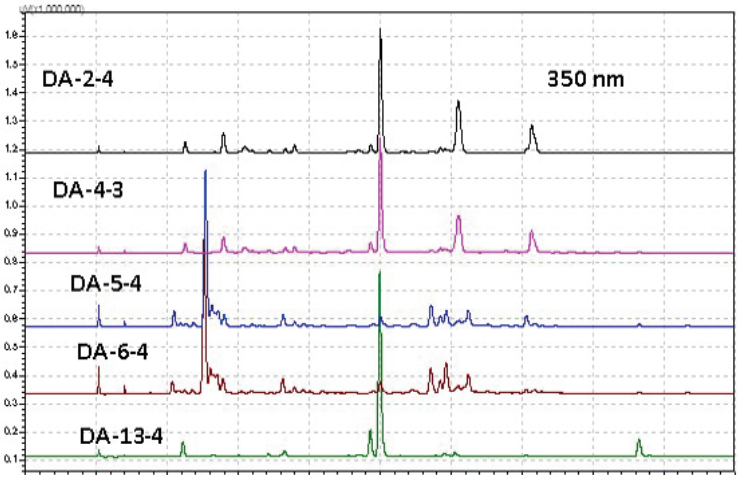

Fig 14: Chromatograms of *Carthamus* outer bracts samples at 350 nm

Experiences gained and Significance of the young researcher fellowship:

1- Learn the protocol of gene sequence analysis in authentication and quality evaluation of herbal drugs/medicinal plants, using *Carthamus* species collected from Egypt and Libya as a case study.
2- Clarify the differences/similarities among *Carthamus* species in rDNA ITS sequence.
3- Operate an LC-MS instrument and obtain chemical profiling of the *Carthamus* species to explore the relation between genotypes and chemo-types.
4- Strengthen the collaboration between ICCO in Egypt and Japan.

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