

## Five isolated phytochemicals from Iraqi *Medicago sativa* with tissue cultured comparative study by reverse-phase High Performance Liquid Chromatography (RP-HPLC)

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### Abstract

*Medicago sativa* L. (alfalfa) is one of the most cultivated plants which has high nutritional value as fodder since antiquity and to treat many health disorders related to secondary metabolites with antioxidant and anti-inflammatory effects. For the first time, a comparative study was applied for bioactive constituents of tissue cultured and their ordinary counterparts of Iraqi *Medicago sativa*. Two types of agriculture techniques were employed, plant tissue cultured (three different tissue culture types) and ordinary agriculture (two different aerial parts and growth stages). The reverse - phase high-performance liquid chromatography applied for qualitative and quantitative of different phytochemicals in this plant giving an idea about the differences in the availabilities of these phytochemicals like steroids (Beta-sitosterol, Stigmasterol), phenolic acids (Caffeic acid, Chlorogenic acid, Gallic acid), and flavonoids (Myricetine, Naringinine) by the differentiated techniques.

**Keywords:** *Medicago sativa* L, tissue culture, reverse – phase high-performance liquid chromatography (RP-HPLC)

### Introduction

The perennial plant *Medicago sativa* L., also known as alfalfa, belongs to the Fabaceae family. It has been cultivated since antiquity and is utilized in both cookery and phytotherapy. Over time, it has been regarded solely as a fodder plant, but it is now gaining popularity because of the minerals and bioactive chemicals it contains, which can have a favorable impact on health. Alfalfa can be regarded as a natural multivitamin because of its nutritional qualities and vitamin and mineral content. The plant's above-ground sections are high in beta carotene, vitamins C and E, as well as minerals like potassium, iron, calcium, magnesium, and silicon.

Furthermore, alfalfa has a cleansing impact and helps to prevent intestinal malignancies due to its high chlorophyll content. The root of this plant contains triterpene saponins that decrease cholesterol without damaging the good fraction (HDL), protect against arteriosclerosis, and induce atherosclerosis regression. Furthermore, the European Food Safety Authority (EFSA) has approved this species as a safe dietary supplement (Rafińska et al., 2017, Gualt et al., 1995). Scientists are devoting increasing attention to Lucerne or Alfalfa (*Medicago sativa* L.) in the food sector recently since the demand for green food has increased. Lucerne is a drought-tolerant, perennial grass (Lenné & Wood, 2004). Aherbaceous legume forage ( Lucerne protein, which is derived from lucerne leaves, is a suitable source for generating healthy and functional foods (Mielmann, 2013). However, alfalfa was mentioned in Babylonian texts in 700 BC, [5]; Additionally, the Mesopotamian plain (Iraq) was a traditional meeting ground for ancient races from Asia, Africa, and Europe (Zubair et al., 2017). Implying it was

cultivated by then. From its origins, Alfalfa spread across much of Europe, North Africa, the Middle East, and Central and Northern Asia. It was introduced into North and South America in the 16th century, and Australia in the 1800s (Barnes, 1977). The growth of cells, tissues, and organs in a specified solid or liquid medium is known as plant tissue culture. Plant tissue culture technologies are used to mass-produce plants on a large scale. In suspension cultures and bioreactors, axillary buds and somatic embryos are only employed in small amounts. Micropropagation includes steps such as prepropagation, explant initiation, explant proliferation culture, shooting and rooting, and hardening (Savangikar, 2004). RP-HPLC is a chromatographic process that can separate a mixture of substances and is used in phytochemical and analytical chemistry to identify, quantify, and purify the different components of the mixture (Cannell, 1998). This methodology is currently gaining traction as the recommended method for fingerprinting investigations for herbal plant quality control, according to various analytical techniques (Fan et al., 2006).

### 1. Material and methods

#### 2.1. In vivo and in vitro growth protocol, plant materials and extraction:

##### 1.1.1 In Vivo-plant material:

Aerial parts (stem, leaves and flowers) of Alfalfa (*Medicago sativa*) which grow as cultivated plant in Iraq, were collected during the months of (June-November) for leaves and flowers from district AL\_Hilla city farms located at Babylon-Iraq, then authenticated by the

herbarium of the department of Horticulture and landscape\College of Agriculture\Al-Qasim Green University. These parts were left to dry in shade, then grinded using an electrical blender, weighted and then extracted.

### 1.1.2 In Vitro-Tissue culture materials:

The type of tissue culture done in this study was seed culture and callus culture, at different culture media and different circumstance, the work done at Collage of agriculture\University of Al Kufa, then the explant of seed culture propagates at different type of light:

- White light, mimic the ordinary light
- Red light, to make the explant propagate at tensive circumstances, to know the explant ability to make differences in concentration and availability of secondary metabolites in

comparison to white light.

- Callus culture, was done at different type of culture media and different circumstances in which the explant propagates at dark chambers, then explants of all types mentioned above collected individually, washed, dried and grinded to fine particles.

### 1.1.3 Study design

Comparison of different type, stages and parts of Iraqi *Medicago sativa* for ordinary plants and propagated seeds tissue culture explants, taking two different stages of growth for plant aerial parts (leaves and stems), while the flowers were collected later on, as illustrated in table (1).

**Table 1.** List of Iraqi *Medicago sativa* samples.

| Sample                               | Types            | Abbreviation |
|--------------------------------------|------------------|--------------|
| Mature aerial plants                 | Leaves and stems | A            |
| Un mature aerial plants              | Leaves and stems | B            |
| Flowers of mature plants             | Flowers          | C            |
| Explant propagated under white light | Leaves and stems | D            |
| Callus tissue culture                | Callus mass      | E            |
| Explant propagated under red light   | Leaves and stems | F            |

### 1.1.4 Extraction

Approximately 10gm of dried grounded aerial part of mature(45 day and more), 11gm of un-mature (25 day and less) and 12gm flowers alone (mature plant top flower) and approximately 2.9gm of explant tissue culture for normally propagated (under white light), 1gm of explant propagated under tensive light (red light) and 0.7gm of dried grounded callus type of this technique, were all subjected to extraction using Soxhlet apparatus with 85% ethanol (150ml) (Abdul-Jalil et al., 2020), then crude extracts was filtered, concentrated under reduced pressure using rotary evaporator and suspended in distilled water then partitioned successively with petroleum ether for defatting ( 100ml of petroleum ether) fraction 1 (F1).

After that the aqueous layer fraction concentrated under reduced pressure by the rotary evaporator to form (F2) fraction 2 ( Abdul-Jalil, 2013, Hamad, 2016). The above step was done to make differentiated comparisons between different stages of the plant (mature and un-mature), different parts of the plant (aerial parts, flowers) and different applied types of tissue culture techniques for the evaluation of tissue culture on the concentrations of secondary metabolites (phytochemicals) selected to be

identified here which were Beta-sitosterol and Stigmasterol as steroids, Caffeic acid, Chlorogenic acid and Gallic acid as phenolic acids, lastly Myricetin and Naringenine as flavonoids.

### 2.2. Identification of different bioactive constituents

Identification of different bioactive constituents was performed by:

- Thin layer chromatography (TLC).
- Reverse phase high performance liquid chromatography (RP-HPLC).

#### 2.2.1. Qualitative identification by thin layer chromatography

An analytical TLC was performed for the six Iraqi *Medicago sativa* samples identification in which Silica gel TLC (GF 254) plate was used as a stationary phase, diffent developing solvent system were applied and the best were Chloroform: ethyl acetate (80:20) and N-butanol: acetic acid: water (40:10:50), the six Iraqi

Medicago sativa samples were applied on TLC plate. After development, detecting the chromatogram was done by UV spectrum 254nm and 365nm (Raj, 2020).

### 2.2.2. Qualitative and quantitative identification by reverse phase high performance liquid chromatography

Qualitative and quantitative determination of expected bioactive constituents in their six samples (A-F) obtained from different stages, parts and propagation of Iraqi Medicago sativa was carried out using reverse-phase high performance liquid chromatography (RP-HPLC). The identifications were made by comparison between retention times and UV/V is spectrum matching obtained at identical chromatographic conditions of analyzed samples and authentic standards (Smith, 2021).

The following equation was used to calculate the percentage of the compound in the plant: -

$$\text{Percentage of compound in the plant} = \frac{(\text{AUC of plant sample} \times \text{Conc. St} \times \text{DF})}{\text{Weight of the dried pla}}$$

Where:

AUC = Area under the curve.

DF = Dilution

factor.

Conc. St. = Concentration of the standard used in HPLC

### 2.2.3. HPLC conditions of petroleum ether fractions (F1)

- Mobile phase: linear gradient: methanol (solvent A): acetonitrile (solvent B) (0-20min A:30% B:70%,20-30min A:0% B:100%,30-45min A:30% B:70%)
- Column: Knauer/Germany LC C18 (250 mm x 4.6 mm, 5 µm particle size).
- Sample: petroleum ether fractions of six Iraqi *Medicago sativa* samples (A-F)
- Standards: β-sitosterol and stigmasterol

**Table 2.** Fraction extract percent of different parts, stages and type of ordinary and tissue culture propagated Iraqi Medicago sativa seeds.

| Fractions                 | A%     | B%     | C%      | D%      | E%     | F%    |
|---------------------------|--------|--------|---------|---------|--------|-------|
| Petroleum ether fractions | 3.28%  | 1.645% | 0.583%  | 3.793%  | 9.57%  | 4.6%  |
| Aqueous layer fractions   | 15.89% | 23.35% | 30.516% | 18.89%  | 25.85% | 18.3% |
| Total %                   | 19.175 | 25%    | 31.1%   | 22.683% | 35.4%  | 22.9% |

The appliance of percent extract yielded comparison gave an idea about the preferred propagation technique, plant part and germination stages required to reach the best

- Flow rate: 0.7 ml / min
- Injection volume: 50 µL
- Injection concentration: 1 mg /ml
- Detection: UV Detector at λ 210 nm (Lee et al., 2018).

### 2.2.4. HPLC conditions of aqueous layer fractions (F2)

- Mobile phase: gradient: solvent A (1% acetic acid in HPLC grad water) and solvent B (acetonitrile) (0-5min A:90% B:10%,5-20min A:60% B:40%,20-40min A:40% B:60%,40-50min A:10% B:90%,50-55min A:90% B:10%,)
- Column: Knauer/Germany LC C18 (250 mm x 4.6 mm, 5 µm particle size).
- Sample: aqueous layer fractions of six Iraqi Medicago sativa samples (A-F)
- Standards: Caffeic acid, chlorogenic acid, myricetin, gallic acid and Naringenine
- Flow rate: 0.7 ml / min
- Injection volume: 50 µL
- Injection concentration: 1 mg /ml
- Detection: UV Detector at λ 272, 280 and 310 nm ( Seal, 2016).

## 3. Results and discussions:

Different parts, stages and types of ordinary and tissue culture propagated Iraqi Medicago sativa extractions with different solvents were applied for the purpose to get the best extract with the highest concentration of the active constituents depend on the specific nature of these compounds. Table 2 shows the fractions percentage of yield of extracts obtained from different parts of the plant, ccomparison between these percentages gave a good idea about extractions yield in which the flower of mature plant(C) gave the highest percent of extraction than the other areal parts of the plant, while tissue culture explant propagated at the Callus technique (E) gave the highest percent of extraction than the other tissue culture techniques.

desired concentration of phytochemicals and open many gates for researchers to develop the desired one (Wakte et al., 2011).

### 3.1. Qualitative identification of active

### constituents by thin layer chromatography (TLC):

Thin layer chromatography (TLC) is a primary beneficial analytical technique applied for the identification and qualification of different phytochemicals present in the targeted extract samples of different plant parts, germination stages and propagation technique(8). TLC of different fractions obtained from Iraqi *Medicago sativa* plant by different developing systems are explained in figure (1) and the following:



**Figure 1.** Iraqi *Medicago sativa*, ordinary plant and tissue cultured at different circumstances.

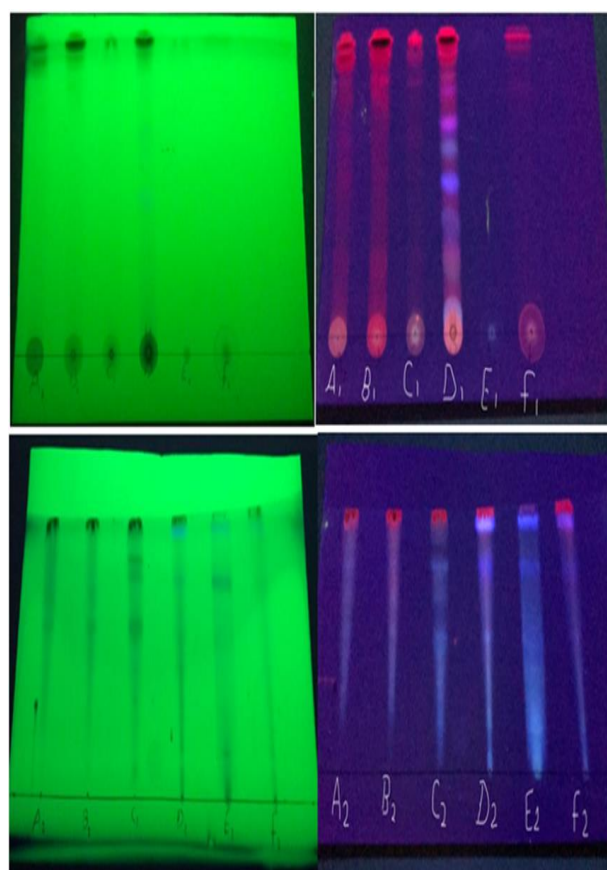
- Petroleum ether fractions 1:

The best yielding developing solvent system was Chloroform: ethyl acetate (80:20), in which the D1 fraction (plant tissue culture propagated at white light)

gave more spots than others, while the E1 fraction callus tissue culture gave the least as illustrated in figure (2).

- Aqueous layer fractions 2:

The best yielding developing solvent system was the N-butanol: acetic acid: water (40:10:50) in which the E2 fraction (callus tissue culture) gave more spots than others, while the A2 fraction (ordinary mature plant) gave the least asb illustrated in figure (2).



**Figure 2.** TLC chromatogram of six Iraqi *Medicago sativa* at petroleum ether and Aqueous layer fraction samples, at UV spectrum 254nm and 365nm.

### 3.2. Fingerprinting of some phytochemicals by RP- HPLC analysis

Identification of some bioactive constituents by RP-HPLC in ordinary plant (different stages and parts) and propagated tissue culture explants (two for seeds and one for callus) of Iraqi *Medicago sativa* as bellow.

### 3.3. RP-HPLC analysis for petroleum ether fraction

According to the RP-HPLC analysis of the six Iraqi

Medicago sativa samples, Beta-sitosterol and stigmasterol were presented in sample A, while stigmasterol was present in all except sample E and F as apparent in Figure 3, showing the specific steroid compound profiles from different Iraqi Medicago sativa samples.

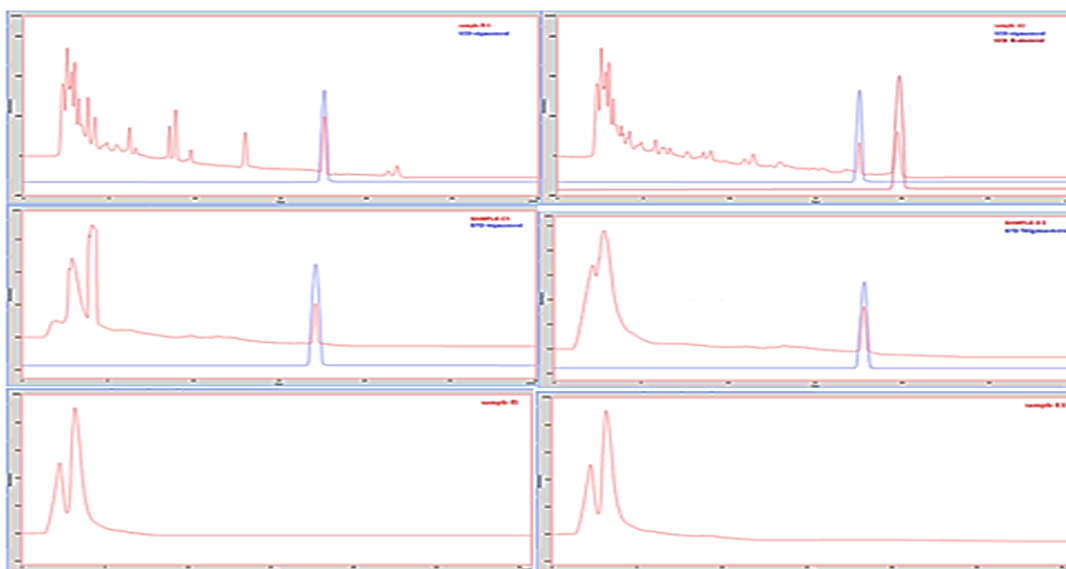
Concerning quantitative determination of Beta-sitosterol and stigmasterol in the desired fractions serial dilutions of

the standards were prepared. A plot of area vs concentration of Beta-sitosterol and stigmasterol standards shows a linear fit which was subsequently used for the quantification of Beta sitosterol and stigmasterol in the analyzed fractions as illustrated in table (3).

**Table.3.** The conc. and peak area of Stigma sterol and Beta-sitosterol. At different parts, stages and type of propagated Iraqi Medicago sativa ordinary plant and tissue culture seeds in petroleum ether fractions.

| Phytochemical          | HPLC results |                        |                       |                           |
|------------------------|--------------|------------------------|-----------------------|---------------------------|
| <b>Stigmasterol</b>    | Sample       | Stigmasterol peak area | STIGMASTEROL<br>μg/ml | μg/gm dry powder of plant |
|                        | A1           | 2032.957               | 30.1445421            | 1.581985569               |
|                        | B1           | 3124.523               | 46.8455019            | 0.308328576               |
|                        | C1           | 312.172                | 3.8165316             | 0.06167064                |
|                        | D1           | 3370.281               | 50.6055993            | 1.481113219               |
|                        | E1           | Nd                     | Nd                    | Nd                        |
|                        | F1           | Nd                     | Nd                    | Nd                        |
| <b>Beta-sitosterol</b> | Sample       | B-sitosterol peak area | Beta-SITerol μg/ml    | μg/gm dry powder of plant |
|                        | A1           | 3327.305               | 194.64734             | 194.6473425               |
|                        | B1           | Nd                     | Nd                    | Nd                        |
|                        | C1           | Nd                     | Nd                    | Nd                        |
|                        | D1           | Nd                     | Nd                    | Nd                        |
|                        | E1           | Nd                     | Nd                    | Nd                        |
|                        | F1           | Nd                     | Nd                    | Nd                        |

\*Nd=Not detected

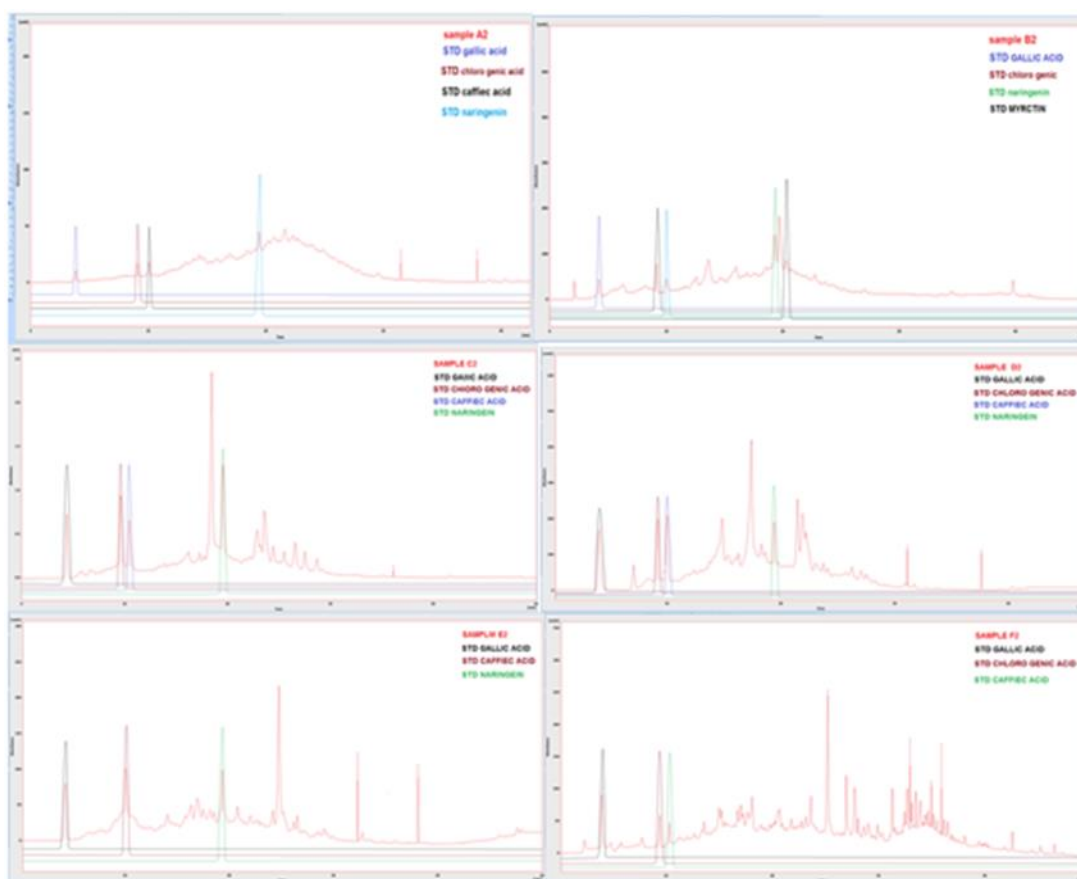


**Figure 3.** RP-HPLC for beta-sitosterol and stigmasterol at six petroleum ether fractions at different parts, stages and types of propagated Iraqi *Medicago sativa*.

### 3.4. RP-HPLC analysis for aqueous layer fractions

According to the RP-HPLC analysis of the six Iraqi *Medicago sativa* samples, Caffeic acid, chlorogenic acid, myricetin, gallic acid and Naringenin was the targeted analytes, in which caffeic acid and gallic acid present in all fractions while myricetin were present only in fraction B2, chlorogenic acid disappear from fraction E2 and Naringenin disappear from fraction F2, as apparent in Figure 4, showing the specific phenolic acids and flavonoids compound profiles from different Iraqi

*Medicago sativa* samples. Concerning quantitative determination of Caffeic acid, chlorogenic acid, myricetin, gallic acid and Naringenin in the desired fractions serial dilutions of the standards were prepared. A plot of area vs concentration of Caffeic acid, chlorogenic acid, myricetin, gallic acid and Naringenin standards shows a linear fit which was subsequently used for the quantification of Caffeic acid, chlorogenic acid, myricetin, gallic acid and Naringenin in the analyzed fractions.



**Figure 4.** RP-HPLC spectroscopy for caffeic acid, chlorogenic acid, myricetin, gallic acid and Naringenin at six aqueous layer fractions at different parts, stages and types of propagated Iraqi *Medicago sativa* ordinary plant and tissue culture.

### 3.5. Comparative correlation between different fractions constituents.

Comparison between different parts, stages and type of ordinary and tissue culture propagated Iraqi *Medicago sativa* relating on phytochemical concentrations yield by RP- HPLC (micro gram/gram of plant dry powder) for

identified constituent steroids, phenolic acids and flavonoids.

#### 3.5.1. Comparison between two different stages.

Comparison between mature (fraction A) and un mature

(fraction B) extracts of ordinary Iraqi *Medicago sativa* revealing the following:

- Steroids (Beta-sitosterol, stigmasterol) was affected by the maturation of the plant in which the mature plants have higher concentration than un mature. Caffeic acid concentration wasn't affected by maturation, so the cut time have no effect on the concentration of caffeic acid.
- Chlorogenic acid was affected by the maturation, in which the un mature plant has higher concentration than the mature, so the maturation will affect the amount present in the plant.
- Myricetin present at the early stages of plant maturation and disappear at the maturation, so the maturation decreases its concentration (Oh & Rajashekar, 2009).
- Gallic acid concentration wasn't affected by the maturation of plant.
- Naringenine with higher concentration at the early stages of maturation of the plant, at which the concentration decreases in mature plant.
- The cut time have approved effect on the concentration of phytochemicals present in the ordinary propagated Iraqi *Medicago sativa* (Bora & Sharma, 2011).

### 3.5.2. Comparison between two different parts.

Comparison between top flowers (fraction C) extracts of mature plant and the aerial parts extracts (fraction A) of the same mature ordinary Iraqi *Medicago sativa* revealing the following.

- Steroids (beta-sitosterol, stigmasterol) will disappear or decreased at the tope flower of the mature plant.
- Caffeic acid, chlorogenic acid, gallic acid and Naringenine present with higher concentration at the top flower than the aerial parts of the mature plant.
- Myricetin disappear from the top flower and the aerial parts extracts.
- The top flower of the Iraqi *Medicago sativa* have appreciable conc. of phytochemical in comparison with the other aerial parts of the plant (Haq, 2004).

### 3.5.3. Comparison between two different propagations.

Comparison between aerial part extracts of mature plant (fraction A) and the aerial parts extracts of the same mature seed tissue culture (fraction D) propagated at white light of Iraqi *Medicago sativa* is revealing the following:

- Steroids, beta- sitosterol wasn't found in fraction D,

stigma- sterol has the same conc. of A, so there is no benefit of doing tissue culture if the desire compounds were steroids.

- Caffeic acid, chlorogenic acid, gallic acid and Naringenine concentrations increased more than one double in fractions D than fraction A, so there is more benefit of doing tissue culture technique to get oriented increased concentration of them.
- Myricetin still absent at fraction A and D.
- The application of advanced techniques of propagation like tissue culture may give appreciable results in orientation to increase the phytochemicals concentrations desired like phenolic compounds and may open many gates to benefit from the secondary metabolites present in the plant (Adams et al., 2007).

### 3.5.4. Comparison between two different propagations types of tissue culture.

Comparison between aerial part extracts of mature seed tissue culture (fraction D) propagated at white light and the same one but propagated at red light (fraction F) of Iraqi *Medicago sativa* revealing the following.

-There was no benefit of doing this technique (propagation at red light) to get more yield concentrations of phytochemical, in which there was a noticeable decrease or absence in concentration yield of phytochemicals.

### 3.5.5. Comparison between two different type of tissue culture.

Comparison between aerial part extracts of mature seed tissue culture (fraction D) propagated at white light and the same one but propagated at different technique called callus tissue culture (fraction E) of Iraqi *Medicago sativa* revealing the following.

- There was no benefit of doing this technique (callus tissue culture) to get oriented increase concentrations of phytochemicals, in which some of them absent and the other still have the same concentration except Naringenine have double concentration in fraction E than fraction D.
- At this article, application of different techniques other than propagation at white light of tissue culture wasn't gave a good result unless some changes in callus tissue culture may have an appreciation for other researchers at this plant or others (Gray & Flatt, 1997).

## Conclusions

The investigated Iraqi *Medicago sativa* which propagated by different techniques, such as soil propagation and tissue culture propagation, plant parts and time to harvest

yielding different phytochemicals and suggest reasons for their varying chemical compositions. This is the first study in Iraq with results providing a comprehensive evaluation for the quality and quantity of some phytochemicals in ordinary *Medicago sativa* and tissue cultured ones, such as steroids, phenolic acids and flavonoids which play an important role in medicinal uses of these species over the past centuries, spreading all over the world and providing for Iraqi as do for worldwide researchers to have enormous and inexpensive sources of many phytochemicals required to produce nutraceuticals, antioxidants, and other therapeutic drugs. The TLC and RP-HPLC help in qualification and quantification of different phytochemicals at different plant parts and types assisting in clearing the fog between tissue cultured and ordinary plants chemical constituents, although they have similar genetic information and homoplastic morphologies with increasing or decreasing some phytochemical existence and concentration.

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