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Identification of alkaloids in methanol extract from leaves of *Semenovia suffruticosa* when humic substances were added to its root soil in the primary growth

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Abstract

Alkaloids are a group of natural compounds originally found in plants. *Suffruticosa* is a species of the genus *Semenovia*, the *Apiaceous* family, a native herb in Iran. In this family, alkaloids are found rarely. Humic substances application to the root soil at the beginning of plant growth improves quality, productivity and physical properties of the soil. Accordingly, humic substances were added separately to the root soil of the samples and other samples were selected as the blank plant sample in the distance of 50 cm. Methanol extracts were prepared from dried and powdered leaf samples. The crude alkaloid solution was extracted from methanol extract with 2 % sulfuric acid, dense ammonia and chloroform. The crude alkaloid solution was passed gradiently with mixture solvents of methanol and chloroform through a chromatography column. The output samples of the chromatography column were analyzed by FT-IR, UV-Vis, and TLC and GC-MS techniques. Pyrimido [1,2-a] quinoxalinone and 1, 3,8-triisopropyl-6-methyl-2,7-naphthyridine alkaloids were identified in both samples and the extracted humic acid (0.513% and 0.979% respectively) and the Sapropel solution (0.449% and 0.678% respectively)were added to their plants soil. Also another alkaloid named 9-benzyloxypyrido [1,2-a]pyrimidin-4-one(9.538%) was identified in the sample and Sapropel was sprayed onto its root soil. No alkaloid was found in the blank plant sample. The results showed that humic substances had considerable effects on the effective substances of the plant *Semenovia. suffruticosa*.

Keywords: Crude alkaloid solution; Sapropel-solvent water; GC-MS technique; Pyrimido [1, 2-a] quinoxalinone; 1, 3, 8-triisopropyl-6-methyl-2,7-naphthyridine

Introduction

The genus *Semenovia* has 11 species in Iran [1]. *Semenovia suffruticosa* is a perennial plant species of *Apiaceae* (*Umbeliferae*) family that grows only in altitudes of 2300 - 2500m Taftan Mountain (Sistan & Baluchestan province, Iran). The plant *Semenovia suffruticosa* has comb-like leaves or bifurcate divisions and long petioles with hard pods. The stems are almost cylindrical trunk and 45 – 70 cm long, with low bifurcate branches, and sometimes with shallow grooves on the surface. Terminal Umbels are within 7 – 10 cm radiuses with almost equal parts and glabrous, and 40 - 50 cm long in fruit-containing state *Umbellules* have 12 - 15 flowers and peduncle is shorter than the ripe fruit [2]. *Apiaceae* family plants contain compounds such as coumarin, furanocoumarin, cromenocoumarin, terpene, sesquiterpene, triterpenoid saponins and acetylene compounds. Alkaloids are rarely found in this family.

Humic substances (HS) are heterogeneous organic compounds that constitute the major organic matrix in surface water, soil and other environmental compartments. HS are critical components of water and soil ecosystems, which are essential to soil genesis and the global cycling of carbon and nutrients. HS are formed by a process called humification. The humification process is chaotic with innumerable reactions occurring under different conditions. The interactions among microbes, clays and minerals are dependent upon HS [3,4]. Humification is a decay process involving the transformation of biomolecules, originating from dead organisms, and microbial activities where HS are formed and nonhumic substances are decomposed [5]. HS increase the quality of compounds, and enhance plant tolerance against both biotic and abiotic stresses [6].

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HS could influence, both directly and indirectly, the physiological processes of plant growth [7]. Regarding their direct effects, nutrients, permeability increases in cell membrane [8]. protein biosynthesis [9]. plant root growth increases [10,11]. respiration, enzyme activities and hormone-like activity [12].They also increase the cation exchange capacity, holding more water in soil, forming complexes with heavy metal ions [13,14]. on the other hand, they increase the buffering capacity of the soil pH, and generally, improve the structure and fertility of soils [15,16]. HS comprise relatively high molecular mass compounds with mixed aliphatic and aromatic characteristics [17,18]. The molecular weight of HS varies from 100 –300 KDa [19]. Based on solubility in acids and alkalis, they can be divided into three main fractions: humic acid (HA), fulvic acid and humin [20]. In most of the proposed structures, it is assumed that oxygen is present in different forms such as: carboxylic, phenolic, alcoholic hydroxyl; carboxylic esther and ethers; nitrogen is present in heterocylic structures and nitriles [17,21]. HA is a group of organic compounds that is formed with heterogeneous accumulation of microbiological and plant matters, and animal origin with high molecular weight or small humic units that have different nature and chemical origin [22,23]. Nutrient uptake is an important part of plant growth. Plant nutrient availability depends on physicochemical and biological factors. If nutrients exist but plants cannot absorb them, the nutrients do not benefit the plant growth. No plant is able to absorb nitrogen directly from the air. HA does not directly and indirectly supply the plants with nutrients, but it does make soil nutrients easier for plants to access [24,25,26]. Rhizobium (small bacteria that live near plants roots) plays an important role in absorption and air nitrogen fixation. If there were no such bacteria and other bacteria living outside the roots of plants and in soil (Azotobacter), agriculture would not have been possible, because they are the only organisms that can take nitrogen from air and stabilize it in soil [27,28]. HA is their nutrition and growth promoter. HA constitutes the stable fraction of carbon, thus regulating the carbon cycle and releasing nutrients, including nitrogen, phosphorus and sulphur in soil [29]. In 2015, Sardashti et al. sprayed humic substances onto root soil around the base of plant, and measured metal ions in plant organs before growth and after HS spray during growth, the results indicated a reduction in the amount of metal ions and better growth of plant [30]. In another research, Sardashti et al. added humic substances (HS) to the base of plants root soil in the primary growth and extracted essential oil with better quality from their aerial organs [31].

HA is naturally present in all agricultural soils and it makes up about 80 percent of soil organic matter. The ideal amount of organic matter in agricultural soils is between four to six percent. This amount in Europe is two to four percent and in some parts of Eastern Europe, including Ukraine it reaches to six percent. But in dry areas, soil organic matter and consequently HA is very low. Except for the northern coastal strip, the amount of soil organic material in most parts of Iran is under one percent and in many places it is even under 0.1 percent [32]. Most nitrogen-containing compounds in plants are alkaloids. Alkaloids are alkaline compounds which contain one or more nitrogen atoms (usually in a heterocyclic ring). In general, producing secondary metabolites of medicinal plants are affected by three main factors including heredity (genetically), different growth stages and environmental conditions whose effects are more quantitative. Alkaloids, percentage in alkaloid-containing plants is more in wet areas than in dry areas. This also depends on the soil type, because soil nitrogen is low in dry areas. Provided that the nitrogen sources are more available for plant, the alkaloids, percentage would increase in it. Growth stage of plant is important in alkaloid production [33].

Extraction methods of alkaloid are different and depend on the amount of alkaloid, its extraction and the situation of crude material. If a large amount of alkaloids is needed, their separation and final extraction are sometimes performed by using fractional precipitation method of their salts such as oxalates, tartarates and/or picrates. If that mixture of alkaloids is complex and small amounts of the alkaloids are required, chromatography methods are used for separation. In many studies, chromatography techniques are used because of their improved performance in terms of convenience, sensitivity, accuracy and rapidity.

Materials and methods

Plant material

In March 2012, two samples of the plant *Semenovia suffruticosa* were selected in the distance of 50 cm. The sapropel solution (a 20±0.01 g package in 400±0.1 ml water) was sprinkled on one of the samples along with 3 L water on the base of its root soil and another sampling was selected as a blank plant. Then extracted humic acid (HA), from Nahakhoran forest soil of Gorgan in north of Iran, was added to the base of the other sampling. This action was repeated for several sampling. In June 2013, the plants were collected from Taftan Mountain located 30 km away from Khash city of Sistan and Baluchestan province, Iran. For preparing a laboratory sample, the leaves were freeze-dried in the shade at the ambient temperature and stored in double-layer paper bags at the room temperature (20 days) and then grounded it into powder by a grinder, protected from the light, direct until further analysis.

Other materials

CH_3OH, CCl_4, H_2SO_4, NH_3, Na_2SO_4, KI, HCl, HF, bismuth sub nitrate, acetic acid, n-hexane, petroleum ether, acetone and TLC were all purchased from Merck Company. Sapropel-solvent water (pH=6.35) and pelosilt blank-humic acid 30 % were purchased from HUMIN TECH Company, Germany and silica gel (sized 60, 230-400 mesh), from Sisco Research Laboratories PVC LTD Company, India.

Instrument

The instruments applied are as follows: Rotary evaporator apparatus Model IKA, made in Germany; Utraviolet-visible spectrometer (UV/Vis) apparatus model A160, made in Shimadzu company (Japan); FT-IR spectrum of the preparation sample by (disk); Fourier transform-infrared apparatus model 460 plus, made in JASCO Company (Japan); gas chromatography/mass spectrometry (GC-MS) apparatus model HP Agilent Technology, made in USA and agitator (ELM 1400 rpm, Germany).

Humic acid extraction

Humic acid (HA) was extracted from the soil sample collected from Nahakhoran forest of Gorgan north of Iran, according to the International Humic Substances Society (IHSS) protocol and then it was purified [18,34]. The efficiency of extracted humic acid was 1 % (w/w) dry weight. Since, Sapropel has not more than 30% humic acid implying that our extracted humic acid has stronger effect.

Preparation of methanol extract

After achieving the optimum conditions (weight of powdered plant leaves , amount of methanol and extraction time), the extraction was done using 300±0.1 ml methanol solvent, 40±0.01 g powdered leaf sample for 12 h by Soxlet apparatus under controlled temperature (ca. 45 C). This function was performed separately for each of the three plant samples.

Alkaloid extraction

2 % Sulfuric acid (150±0.1 ml) was added to methanol extract and filtered by a filter paper and Buchner funnel. Drops of ammonia were added to the filtrate solution until pH of 8-9 was obtained. Extraction was performed with chloroform (3 ×50±0.1 ml). The chloroform phases were collected and concentrated under reduced pressure using a rotary evaporator apparatus. A little sodium sulfate (Na_2SO_4) was added to the concentrated chloroform solution and filtered and stored at 4 C. This process was performed for other methanol extracts. The crude solutions of alkaloid were analyzed by FT-IR, UV-Vis, TLC and GC-MS.

Chromatography column (CC) (5 cm diameter and 1 m length) was filled with a uniform slurry of silica gel (sized 60, 400-230 mesh) and petroleum ether. To prevent silica gel from eluting to the mobile phase or solvent mixture, it was plugged to the bottom end of column with some cotton. The column was subjected to gentle tapping and the petroleum ether was passed through the column for 2 days for compact packing of the stationary phase. Mixture of the crude solutions of alkaloid and silica gel were placed separately in rotary evaporator apparatus to obtain a uniform powder. Then it was loaded on the column with caution. First, the column was eluted with 250±0.1 ml n-hexane solvent (the plant sample, to the root of which was added HA) or 250±0.1 ml petroleum ether (the plant sample, to the root of which was added Sapropel), and then solvent mixture of methanol and chloroform was passed gradiently from non-polar position to the polar one on the column. Exodus solutions of the column were collected and analyzed by FT-IR, UV-Vis, TLC and GC-MS [35].

Preparation of Dragendroff' Reagent

This reagent was prepared as two different solutions, known as A and B. Solution A contained 2±0.01 g of bismuth sub nitrate, 25±0.1 ml of acetic acid and 100±0.1 ml of water. Solution B consisted of 40±0.01 g of potassium iodide in 100±0.1 ml of water. 10±0.1 ml of each solution was added to 20±0.1 ml of acetic acid and 100±0.1 ml of water to complete the reaction. The reagent was sprayed to TLC plates prior to heating in autoclave to develop a dark orange color to confirm the presence of alkaloids.

Gas chromatography/mass spectrometry (GC-MS) analysis

The following analysis systems were used: system GC: Hewlett-Packard 6890 Network, system GC: Hewlett- Packard 6890 Network, detector: (MS) 5973 Network mass selective detector, Ion source: Electron Impact (EI) 70 eV, Analyzer: Quadrupole. Capillary column: HP-1, length 30 m, internal diameter (id) 0.250 mm and 25 μm film thickness. Temperature program: first, column temperature was held at 50 C for 1 min and then it was programmed from 50 to 250 C at a rate of 10 C min^{-1} and finally held isothermally for 20 min. The flow rate of gas carrier was (He, 99.99 %) 1$ml.min^{-1}$.

Identification of compounds

Most alkaloids have an absorption in the range between 220 to 280 nm in UV-Vis spectrum. Alkaloids have nitrogen and carbonyl groups in their molecular structure which are component of the main functional groups. Also, peaks were observed at 3000 cm^{-1} for the nitrogen group and ca, and 1750 cm^{-1} for the carbonyl group in their FT-IR spectrum.

The linear retention indices for all compounds were calculated by retention times and using a solution containing the homologous series that were injected at the same chromatographic conditions according to Van Den Dool and Kratz method [36].

Moreover, a comparison of the identified compounds in four samples (the three samples before and after column chromatography (CC) in which HS was added to their root soil) shows that their changes are influenced by humic substances (Table. 1 and Figure. 1).

The lowest percentages of these identified compounds are hydrocarbon sesquiterpenes in four samples with no identified hydrocarbon sesquiterpenes (Tables 1 and 2). Also, the highest

percentage of the identified compounds is coumarin in four samples.

Compounds were identified by comparing the retention indices of the peaks on the HP-5 MS column relative to the homologous series and the WILEY library, and by comparing the fragmentation patterns of the mass spectrum with those reported in the literature 7 [37].

Results and discussion

When humic substances (HS) was added to the root soil of plants in the initial growth phase and the extraction was carried out along with the analysis of samples, some compositions were totally removed and displaced by new compositions as compared to those of the blank sample (Table1). Identifying the percentage of the classification compounds indicates that their variations have been affected by addition of HS compared to all the four samples (the blank sample and the three samples, before and after their column chromatography (CC) in which HS was added to their root soil (Tables 1, 2 and Figure 1).

The lowest percentages of these identified compounds are hydrocarbon sesquiterpenes in four samples whit no identified hydrocarbon sesquiterpenes (Tables 1 and 2). Also, the higest percentage of the identified compounds is coumarin in four samples.

When the Sapropel solution was sprayed on the root, the percentage of hydrocarbon monoterpenes did not vary in the sample before CC, compared with its percentage in the blank sample, but these compounds, percentage reached zero after CC. This is due to n-hexane and petroleum ether, which were passed before the solvent mixture of methanol and chloroform on chromatography column. These two solvents eluted the hydrocarbon monoterpenes, oils, and paints in the samples and removed them from the chromatography column. The percentage of oxygenated monoterpenes increased in two alkaloid solution samples (the alkaloid solution of plant with HA in root after CC and the alkaloid solution of plant with Sapropel in root before CC decreased to zero in alkaloid solution of plant with Sapropel in root after CC, compared to the blank sample. The percentage of oxygenated sesquiterpenes in the alkaloid solution of plant with Sapropel in root after CC and the alkaloid solution of plant with Sapropel at root before CC and the alkaloid solution plant with HA in root after CC were decreased to 0.713%, 0.563% and 1.174%, respectively, compared to the alkaloid solution of the blank sample with a percentage of 0.669.Oxygenated terpenoids, coumarin and all of terpenoids were increased significantly when HA was added to the soil compared with other samples (before and after CC).The total percentages of the identical alkaloids were 1.491% and 10.919% in the plants to the soil of which HA and Sapropel solution were added.

In general, a comparison of the compounds identified in the alkaloid solutions of the treated plants showed that seven compounds in all four samples (with different percentages), 13 compounds in the samples to the soil of which HS (HA and Sapropel) was added after CC and 12 compounds in the samples to the root soil of which Sapropel was added before and after CC are similar.

In UV-Vis spectrum for the crude alkaloid solution to the root soil of which Sapropel was added, a peak at 225nm is observed before CC indicating the possible presence of alkaloid in its root (Figure 2). In its FT-IR spectrum, peaks were observed at 3421cm^{-1} and 1729 cm^{-1}, for the nitrogen and carbonyl groups, respectively (Figure 3). Dark orange colors appeared in TLC plates confirming the presence of alkaloids in this solution. According to table 3, this solution was analyzed by GC-MS and a nitrogen compound (alkaloid) was identified as Croton amide, 3-methyl-N-purin-6-yl- which contained a purine ring of 0.254%. This alkaloid is biologically important. Also, 0.773% terpenoid and 20.074% coumarin were identified in its crude alkaloid solution (Table 2).

For the crude alkaloid solution of plant with Sapropel in its root soil, after CC, peaks were observed at 3430 cm^{-1} and 1730 cm^{-1} in FT-IR spectrum (Figure 4), principal absorption peak at 247nm in UV-Vis spectrum (Fig. 2) and dark orange colors appeared in TLC plates all confirming the presence of alkaloids. Three alkaloids as 1, 3, 8-triisopropyl-6-methyl-2, 7-naphthyridine, Pyrimido [1,2-a] quinoxalinone and 9-benzyloxypyrido [1,2-a] pyrimidin-4-one were identified with percentages of 0.678 %, 0.449 % and 9.538%, respectively, by GC-MS analysis of this sample (Table 3 and Figure 5). Analysis revealed that for the crude alkaloid solution of plant with HA in its root soil, after CC, peaks were observed at 3421cm^{-1} and 1730cm^{-1} in FT-IR spectrum (Figure. 6), principal absorption peak at 245nm in UV-Vis spectra (Figure 2) and the dark orange colors appeared in TLC plates confirming the presence of alkaloids. According to table 3, two nitrogen-containing compounds contain two alkaloids, 0.979% of 1, 3, 8-triisopropyl-6-methyl-2,7-naphthyridine and 0.513 % of Pyrimido[1,2-a]quinoxalinone were identified by GC-MS analysis (Figure 5). These two alkaloids were exactly similar to two alkaloids identified in the plant with Sapropel in its root soil after CC, while the percentage of these compounds were higher in the crude alkaloid solution with HA added to its plant root. Also, there were 2.824% terpenoids and 80.4.14% coumarin in its crude alkaloid solution (Table 2).

In FT-IR spectrum of the alkaloid solution of the blank plant at 1750 cm^{-1} and 3000 cm^{-1} and in UV-Vis spectrum in the range between 220 to 280 nm, there was no significant peak confirming the presence of alkaloid (Figures 2 and 7). Also, the dark orange color was not observed in TLC plate with respect to its alkaloid solution.

The alkaloid solution of the blank plant was analyzed by GC-MS analysis and identified 0.877% terpenoids (0.669% whit regard to sesquiterpenes) and 24.111% coumarin, and there was no nitrogen-containing compound in its compounds (Table 2).

When humic substances (HS) were sprinkled to the plant root soil, these plants developed more growth compared with the blank plants. So the plant to the root soil of which humic acid (HA) was added underwent more growth compared to the other two plants.

Table 1. .GC-MS analysis for classification of compounds in the crude alkaloid solutions from treated plants

No	Category of compounds	Alkaloid solution of blank plant sample before CC	Alkaloid solution of plant with Sapropel at root before CC	Alkaloid solution of plant with Sapropel at root after CC	Alkaloid solution of plant with extracted humic acid at root after CC
1	Hydrocarbonated monoterpenes (%)	0.189	0.102	—	—
2	Oxygenated monoterpenes (%)	0.089	0.108	—	1.650
3	Hydrocarbonated sesquiterpenes (%)	—	—	—	—
4	Oxygenated sesquiterpenes (%)	0.669	0.563	0.713	1.174
5	Other hydrocarbonated compounds (%)	1.252	1.050	3.778	—
6	Other oxygenated compounds (%)	32.989	28.582	71.379	88.914
7	Other compounds (%)	1.914	1.607	11.537	1.174

Table. 2. Classification of important compounds in the crude alkaloid solutions from treated plants.

N0	Category of important compounds	Alkaloid solution of blank plant sample before CC	Alkaloid solution of plant with Sapropel at root before CC	Alkaloid solution of plant with Sapropel at root after CC	Alkaloid solution of plant with extracted humic acid at root after CC
1	Total of terpenoids (%)	0.877	0.773	0.713	2.824
2	Sesquiterpenes (%)	0.669	0.563	0.713	1.202
3	Coumarin (%)	24.111	20.074	58.891	80.414
4	Total of alkaloid (%)	—	0.254	10.665	1.491

Table. 3. Percentage of alkaloids in the crude alkaloid solutions from treated plants.

N0	Alkaloids	Alkaloid solution of blank plant sample before CC	Alkaloid solution of plant with Sapropel at root before CC	Alkaloid solution of plant with Sapropel at root after CC	Alkaloid solution of plant with extracted humic acid at root after CC
1	9-benzyloxypyrido[1,2-a]pyrimidin-4-one (%)	—	—	9.538	—
2	1,3,8-triisopropyl-6-methyl-2,7-naphthyridine (%)	—	—	0.678	0.979
3	Pyrimido[1,2-a]quinoxalinone (%)	—	—	0.449	0.513
4	Crotonamide, 3-methyl-N-purin-6-yl- (%)	—	0.254	—	—

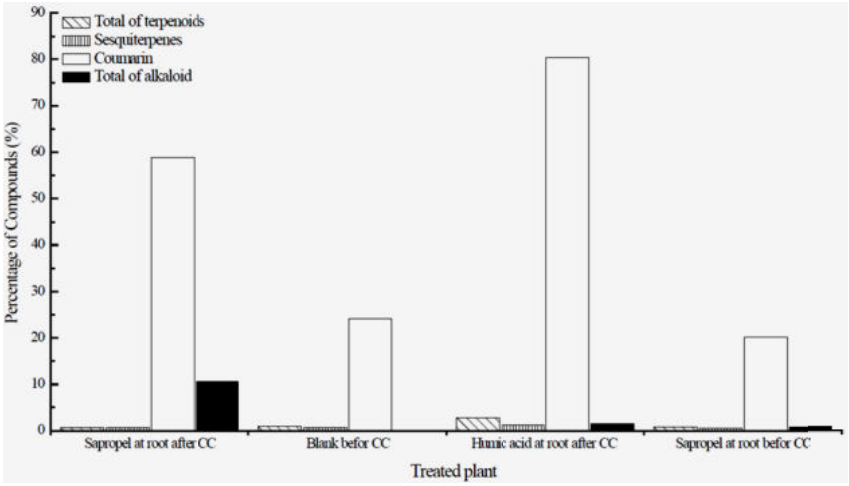


Figure 1. The percentage of important compounds in the crude alkaloid solutions from treated plants.

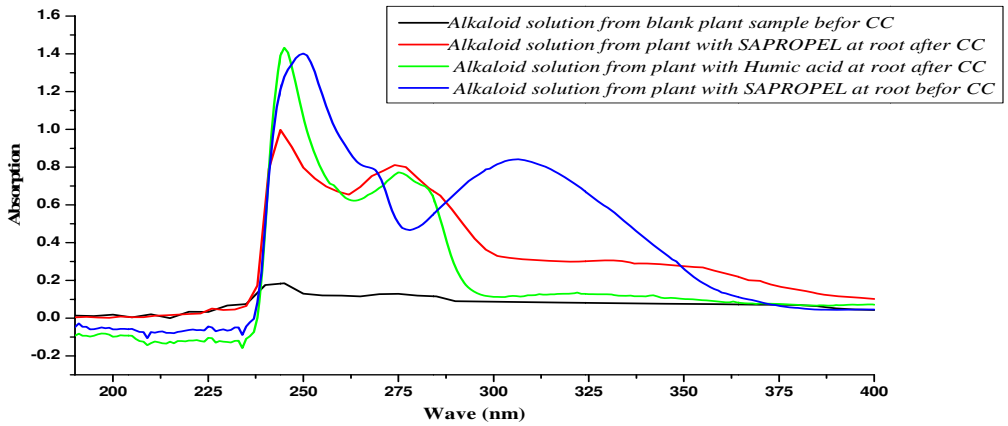
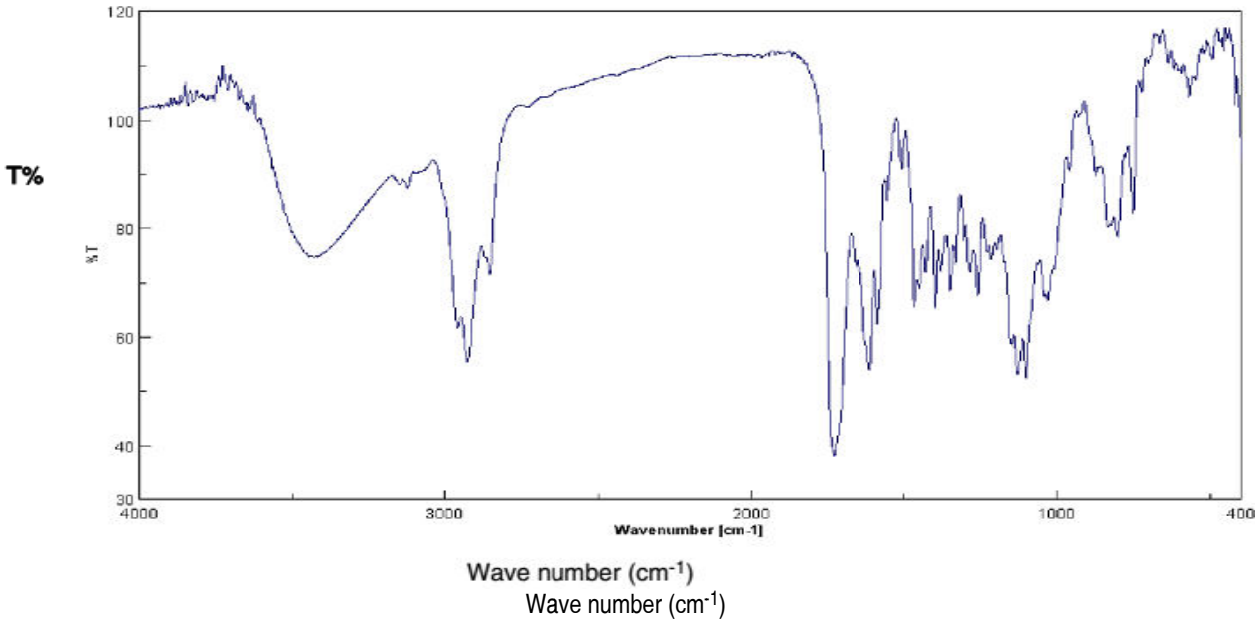


Figure 2. UV-Vis spectrum relative to the crude alkaloid solutions from treated plants.



Wave number (cm^{-1})

Figure 3. FT-IR spectrum relative to the crude alkaloid solutions which sprayed Sapropel solution on the root of plant before CC.

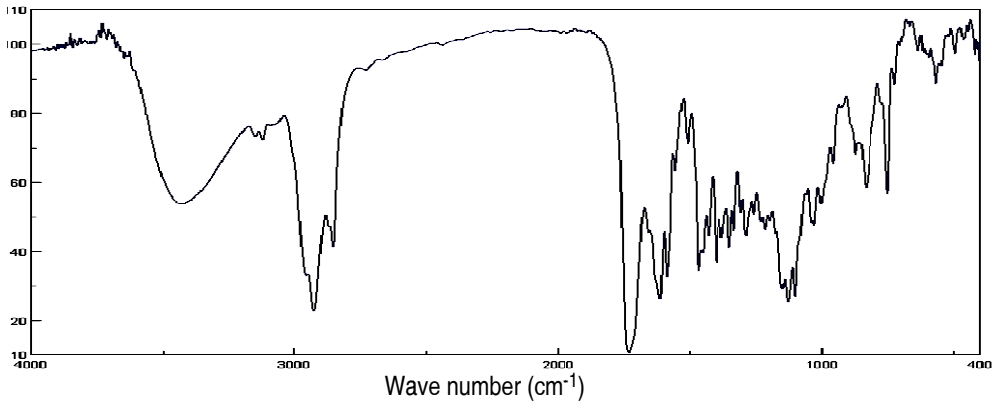


Figure 4. FT-IR spectrum relative to the crude alkaloid solutions which sprayed Sapropel solution on the root of plant after CC.

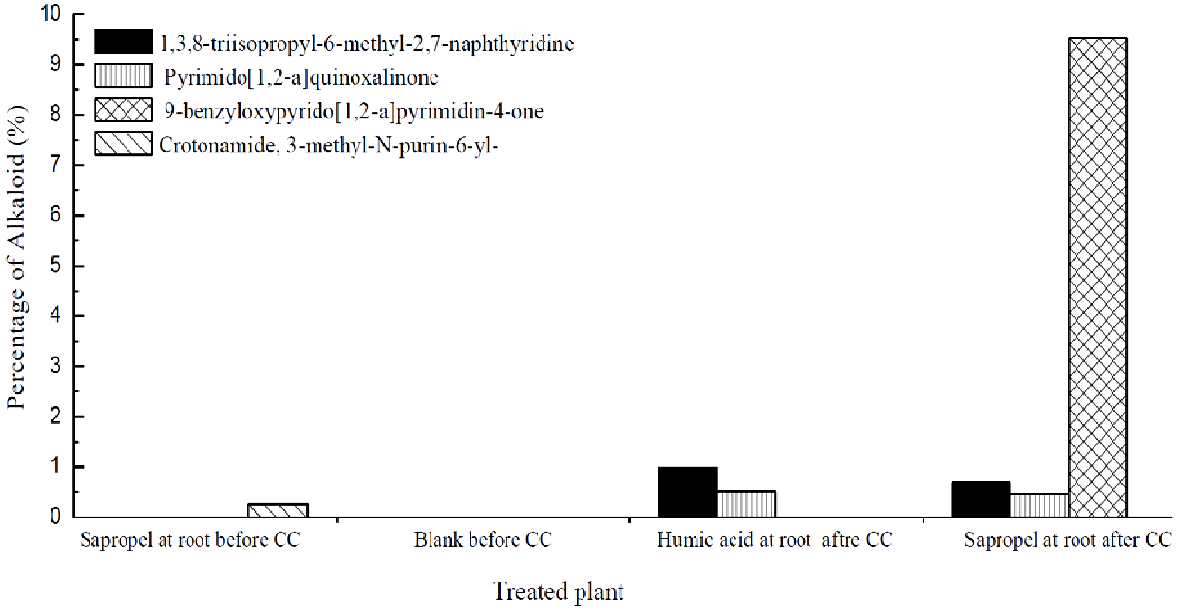


Figure 5. Percentage of identified alkaloids in treated plants

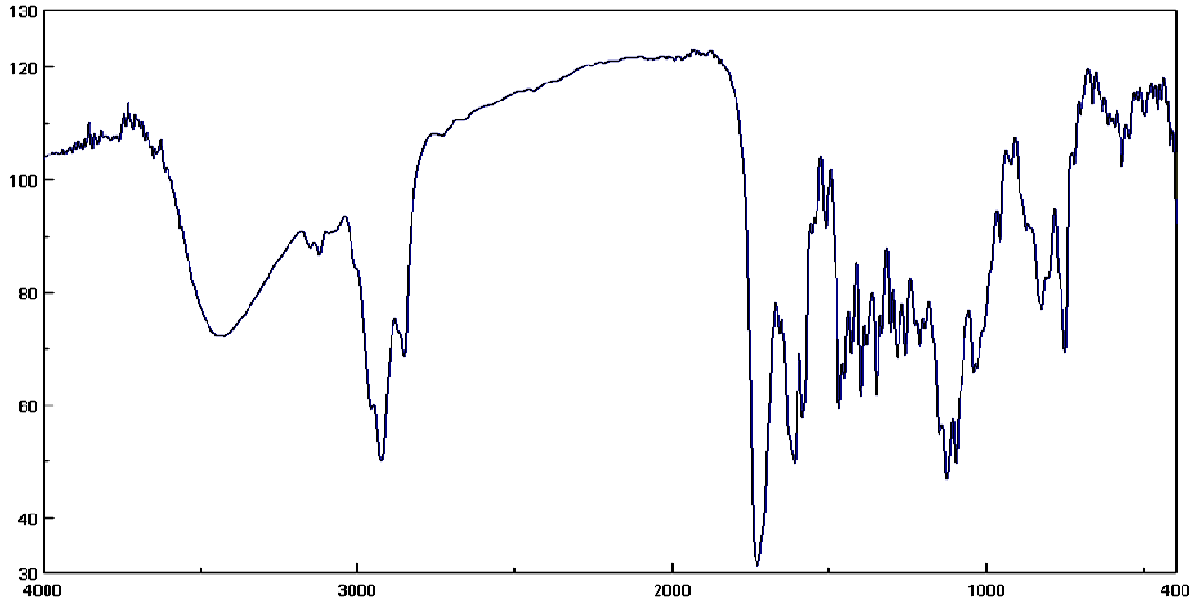


Figure 6. FT-IR spectrum relative to the crude alkaloid solutions on the root of which was added HA after CC.

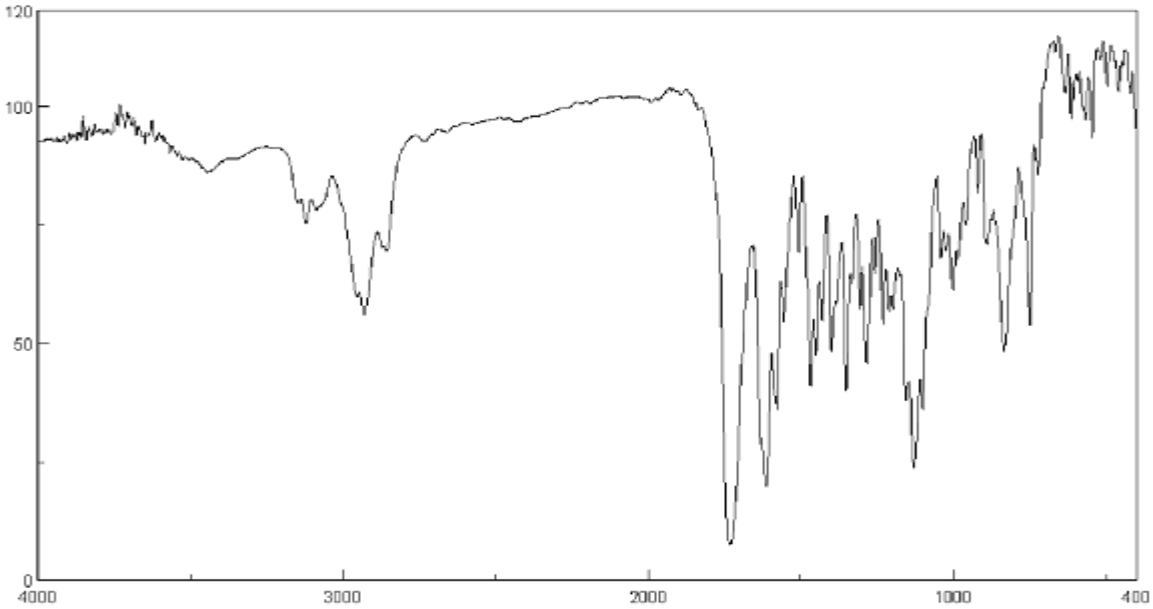


Figure 7. FT-IR spectrum relative to the crude alkaloid solution from plant leaves of the blank sample

Conclusion

Addition of HS (extracted humic acid and Sapropel solution) to the base of the plant *S. Suffruticosa* established a significant increase in growth, quantity and quality of compounds in the plant. HS provide suitable conditions whereby nutrients, minerals, beneficial nutrients (such as nitrogen) and the new compounds, absorption by plant roots is not possible in their absence since the plant is naturally unable to absorb these substances, and HS also make the plant roots unable to absorb other materials.

According to the results, addition of HS to the root in studied plants increases available nitrogen in the soil, and the absorption of nitrogen by plants produced alkaloids in the plants, compared to the blank plant. The amounts of produced alkaloids in the plants to the soil of which was added HA increased compared to the plant to the root soil of which was added Sapropel. Also, the superior complex–making properties of HA than Sapropel resulted in better elimination and assimilation of heavy metals such as Cd and Pb ions (these ions inhibit the plant growth) increased the plant growth and improve the quality and quantity of its compositions.

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