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Research Paper

Primary metabolites and flavonoid constituents of *Isatis microcarpa* J. Gay ex Boiss.

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ABSTRACT

Phytochemical studies on *Isatis microcarpa* J. Gay ex Boiss (Family-*Cruciferae*) revealed that the plant water content was high at March, while the total ash, acid soluble and insoluble ash, beside the crude fibers of *Isatis microcarpa* were high at May. Meanwhile the total carbohydrates, nitrogen contents and lipids contents reach its high values at April. Preliminary phytochemical screening indicated that the plant contains coumarins, tannins, sterols and/or terpens, flavonoids, glycosides and/or carbohydrates and resins. The plant contained five free sugars, eight combined sugars, ten free amino acids and fifteen protein amino acids. While GLC of Saponifiable matter revealed the presence of seven saturated fatty acids and two unsaturated fatty acids. Chromatographic investigation of flavonoids constituents of *Isatis microcarpa* revealed the presence of eight flavonoids (apigenin-7-O-rutinoside, apigenin-7-O-glucoside, luteolin, luteolin-7-O-glucoside, kaempferol, kaempferol-7-O-glucoside, kaempferol-3-O-glucoside and naringenin). These compounds were identified through R_f values, acid hydrolysis, enzymatic hydrolysis, UV spectral data, ^{1}H-NMR analysis and mass spectral data. The microbiological activities (six bacterial and fungal strains) of the plant extracts on microorganisms revealed that the ethyl alcohol (96% and 70%) and water extracts showed the best effect on all the tested bacteria and fungi.

Keywords: *Isatis microcarpa*; Primary metabolites; Flavonoids.

INTRODUCTION

The Egyptian deserts are very rich with medicinal plants belonging to many families. *Cruciferae* was represented in Egypt by 53 genera and 107 species (Boulos, 1999). *Lepidium crassifolium* accumulated both carbohydrates and amino acids, while its leaves were contained a very high amount of proline, associated with a high level of soluble carbohydrates was found (Murakeozy, et al., 2003). The oil in *Camelina sativa* has a combined linolenic acid content that is greater than 50% and a relatively low

saturated fatty acid content (10%), while the plant sterols were separated and identified as: cholesterol, brassicasterol, compesterol, stigmasterol and sitosterol using GC-MS (Shukla, et al., 2002). Flavonoids are important dietary compounds, having a capacity to inhibit DNA damage and lipid peroxidation, and quench free radicals. They also have anticarcinogenic and antiproliferative properties (Romanova and Vachalkova, 1999). Naringenin is recognized as phyto-estrogen which could affect sex hormone, cause mediate biological response by several different mechanisms, including binding to estrogen receptors. Also naringenin could be used as antioxidant, anticarcinogenic and blood lowering lipid activities (Brev, et al., 2004). Leaves of *Isatis tinctoria* contain a blue dye called woad and its seeds contain fatty oil. Plant used for ulcers (Kumer, 2005). Heinemann et al., (2004) found that lipophilic extracts of *Isatis tinctoria* exhibit significant activity against several clinically relevant targets of inflammation which make skin diseases.

MATERIALS AND METHODS

Isatis microcarpa J. Gay ex Boiss (annual plant) was identified by Prof. Dr. Sayed Farag Khalifa (Prof. of Plant Taxonomy and Flora, Botany Department, Faculty of Science, Ain Shams University) and Prof. Dr. Adel Kamel Youssef (Prof. of Plant Ecology and Phytochemistry, Desert Research Center). The plant was collected from Wadi Om Sheihan, Abo-Ogeila, North Sinai identified by [Egypt map (Sinai map)] during the period of investigation (from March to May 2005). They were air dried, then ground to fine powder and kept to be used for different analysis.

***Determination of plant water content*:** by the method of Rowell (1994).

Determination of certain pharmacopial constants

Determination of percentages of inorganic (Ash) and organic matter (Brower and Zar, 1984).

Determination of acid-soluble and acid-insoluble ash (Askar and Treptow, 1993).

Determination of water-soluble and water- insoluble Ash (Askar and Treptow, 1993).

Determination of crude fibres (British pharmacopoeia, 1980).

***Preliminary phytochemical screening*:** by the method of Balbaa, et al., (1981).

This includes testing for volatile oil by steam distillation method, steroids, coumarins, flavonoids, phenolics compounds, alkaloids using Dragend-orrf's, Mayer's and Wagner's reagents, glycosids and/or carbohydrates, resins and saponins.

Metabolic products

Carbohydrates content: by the method of Chaplin and Kennedy (1994).

Determination of total carbohydrates, soluble and insoluble carbohydrates.

Identification of free sugars: HPLC of the free sugars according to Nagel (1992).

Identification of combined sugars: HPLC according to Nagel (1992).

Nitrogen content

Determination of total nitrogen: using Kjeldahl method (James, 1995).

Investigation of free and protein-amino acids**:** according to Pellet and Young (1980), using Amino Acid Analyzer.

Lipids content

Determination of total lipids

***Physical properties of lipids*:** The lipid fraction was studied physically with regard to its odor, color and physical nature. Its solubility in petroleum ether, diethyl ether, benzene, chloroform, acetone, carbon tetrachloride and warm alcohol was tested.

Fundamental chemical properties of lipids: Acid value (A.V), saponification value (S.V.) ester value (E.V.) and iodine value (I.V) were determined according to British Pharmacopoeia (1993).

***Determination of unsaponifiable matter and fatty acids*:** their components were determined using GLC according to Farag *at al*. (1986) and Eaton (1989).

Investigation of flavonoids

Preparation of flavonoids extract: The dried plant powder of *Isatis microcarpa* was extracted with 70 % aqueous ethanol several times. The alcoholic extracts were evaporated till very small volume under reduced pressure, treated with excess of ethanol, filtered to remove inorganic salts and nonphenolic compounds then concentrated. The aqueous layer was shaken with chloroform in a separating funnel, where the chlorophyll was transferred to the chloroform layer and the phenolic compounds remained in the aqueous layer. The phenolic compounds were separated and evaporated under reduced pressure to a very small volume as described by Liu et al. (1989). Two dimensional paper chromatography was performed on whatmann No.1 paper chromatography using the solvent systems; butanol: acetic acid: water (4:1:5 v/v/ v) (S1) followed by the solvent system; acetic acid: water (15: 85 v/v) (S2). The paper chromatograms were examined under visible and ultraviolet light, then exposed for 2-3 minutes to ammonia vapors and immediately re-examined to observe the appeared changes in color of fluorescence. The ethanolic extract of *Isatis microcarpa* was mixed with polyamide, and then evaporated under reduced pressure till dryness. The mixture was applied on the top of a polyamide column. Elution was started with distilled water followed by water containing gradual increasing of ethanol 5 %, 10 %, 15 % until finally 96 % ethanol. The separated bands were observed under visible and ultraviolet light. The eluted fractions received were evaporated and concentrated, where one dimensional paper chromatography of the different fractions were performed using the solvent systems S1 and S2. Similar fractions were pooled together, and evaporated under reduced pressure. Preparative paper chromatography of the fractions of flavonoid nature was applied on whatmann No. 3 MM paper chromatography using BAW as eluent system for 48 hours. The separated flavonoid compounds were purified using Sephadex LH-20 column. The pure flavonoid compounds were identified by chemical method and confirmed by physical method.

Physical tests

Ultraviolet spectrophotometric analysis (UV:) (Mabry et al., 1970).

Using Schimadzu UV 240 spectrophotometer.

Reagents for UV spectroscopic analysis of flavonoids

Aluminum chloride, boric acid, hydrochloric acid, sodium methoxide solution and sodium acetate.

^{1}H- and ^{13}C-Nuclear Magnetic Resonance Analysis (NMR) (Mabry, et al., 1970).

The NMR measurement was carried out on A JEOL EX-270 NMR spectrometer apparatus (270 MHz for ^{1}H- NMR and 67.5 MHz for ^{13}C-NMR).

Mass Spectrometric Analysis (MS) (Mabry, et al., 1970).

The spectra were conducted using mass spectrometer Varian Mat 711, Finnigan SSQ 7000 and MM 7070 E.

Chemical Reactions

Controlled (Mild) Acid Hydrolysis (Harborne, et al., 1975). Using 0.1N aqueous HCl at 100ºC for 15 minutes.

Complete (Normal) Acid Hydrolysis (Harborne, et al., 1975).

Using 2N HCl at 100ºC for 2 hours or in 2N HCl in aqueous MeOH (1:1) in vaccuo.

***Enzymatic hydrolysis*:** Using corresponding enzyme in an acetate buffer (P^{H}=5) and the mixture was worked up for 24 hours at 37-40°C.

***Estimation of Flavonoid Content*:** By the method of Karawya and Aboutable, 1982.

***Microbiological Activities*:** The effect of different successive-selective extracts of *Isatis microcarpa* plant using the following solvents; Ether, petroleum ether, chloroform, acetone, ethyl acetate, 96% ethyl alcohol, 70% ethyl alcohol and water on some pollutant micro-organisms were achieved.

Microorganisms*:* The following six bacterial and fungal strains were tested:

Bacterial strains of *Bacillus subtilis, Staphylococcus aureus, Micrococcus leteus, Escherichia coli, Salmonella sp.* and *Pseudomonas sp.*

Fungal strains of *Aspergillus niger, A. flavus, Fusarium solani, Fusarium oxysporium, Microsporum fulvum* and *Alternaria tennius.*

The microorganisms were obtained from Plant Pathology and Microbiology Department, the National Research Center, Cairo, Egypt. It was checked for purity and identity and regenerated to obtain active microorganisms. The cultures were stored in refrigerator at 5ºC and reactivated on the media suitable for each microorganism.

Preparation of the Spore Suspension*:* According to Padwal, et al., (1976).

Agar Diffusion Method: As described by Booth (1972).

RESULTS

The studied area during the period of investigation (2005) for El-Arish characterized by moderate temperature during the growth period, which were favorable for plant growth. Mean while the total annual amount of rainfall was 21.8 mm during the period of investigation. The soil supporting *Isatis microcarpa* indicated that the soil was alkaline in reaction with pH 8.20 at surface layer (0-20 cm) and 8.42 at bottom layer and sandy in texture at Wadi Om Sheihan, North Sinai.

***Plant water content*:** The percentages of water content of *Isatis microcarpa* decreased from 39.30% at March to 31.60 % at April and finally decreased to 28.50 % at May, respectively.

Determination of certain pharmacopoeial contents of plant material

***Inorganic and organic matter*:** The percentage of total ash content of *Isatis microcarpa* decreased from 14.72% at May to 11.43% at April and finally decreased to 9.23% at March, respectively. While the percentage of organic matter decreased from 90.77% at March to 88.57% at April and finally decreased to 85.28% at May, respectively.

***Acid-soluble and acid-insoluble ash*:** The percentage of acid-soluble ash of *Isatis microcarpa* decreased from 9.53% at May to 7.63% at April and finally decreased to 5.62% at March, respectively. While the percentage of acid-insoluble ash of *Isatis microcarpa* decreased from 6.80% at April to 5.19% at May and finally decreased to 3.61% at March, respectively.

***Water-Soluble and Water-Insoluble Ash*:** The percentage of water-soluble ash of *Isatis microcarpa* decreased from 7.12% at May to 5.87% at April and finally decreased to 4.11% at March, respectively. While the percentage of water-insoluble ash of *Isatis microcarpa* decreased from 7.60% at May to 5.56 % at April and finally decreased to 5.12% at March, respectively.

***Crude Fibers*:** The percentage of crude fibres content of *Isatis microcarp* decreased from 30.66% at May to 25.10% at April and finally decreased to 20.89% at March, respectively.

Preliminary phytochemical screening: The preliminary phytochemical screening of *Isatis microcarpa* plant showed that it contains coumarins, tannins, sterols and/or terpens, flavonoids, glycosides and/or carbohydrates and resins. Neither saponins nor volatile oil were detected

Metabolic product

Carbohydrates content

***Determination of total, soluble and insoluble carbohydrates*:** The percentage of total carbohydrates of *Isatis microcarpa* decreased from 3.58% at April to 2.04% at March and finally decreased to 1.94% at May, respectively. While the percentage of soluble carbohydrates decreased from 1.21% at April to 0.87% at March and finally decreased to 0.69% at May, respectively. The percentages of insoluble carbohydrates decreased from 2.37% at April to 1.25% at May and finally decreased to 1.17% at March, respectively.

Investigation of free sugars: The separation of the free sugars contents of *Isatis microcarpa* when achieved using High Pressure Liquid Chromatography (HPLC), the following sugars were obtained; arabinose, ribose, fructose, glucose and sucrose as free sugars. Where the concentration of glucose (37.5%) was the highest one of the separated free sugars in *Isatis microcarpa*, (Table 1).

***Investigation of combined sugars*:** The separation of the hydrolyzed combined sugars of *Isatis microcarpa* when achieved using HPLC the following sugars were obtained: xylose, arabinose, ribose, fructose, glucose, sucrose, cellobiose and raffinose as combined sugars. Where the concentration of glucose (22.4%) was the highest percentage of the separated sugars at the plant (Table-1).

Nitrogen content

***Total nitrogen and protein contents*:** The percentage of total nitrogen of *Isatis microcarpa* decreased from 3.87% at April to 2.65% at March and finally decreased to 2.36% at May, respectively. The percentages of total protein decreased from 24.19% at April to 16.56% at March and finally decreased to 14.75% at May, respectively.

***Investigation of free amino acids*:** The separation of free amino acids of *Isatis microcarpa* when achieved using amino acid analyzer, where ten free amino acids (Theronine, Glutamic acid, Glycine, Isoleucine, Argenine, Phenylalanine, Lysine, Leucine, Methionene and Proline) were detected. The concentration of glutamic acid (25.5%) and proline (18.06%) were the highest percentages of the separated free amino acids of *Isatis microcarpa* (Table-1).

***Investigation of protein amino acids*:** The investigation of hydrolyzed protein-amino acids of Isatis *microcarpa* when achieved using amino acid analyzer, where fifteen protein-amino acids (Aspartic acid, Theronine, Serine, Glutamic acid, Glycine, Alanine, Valine, Isoleucine, Argenine, Leucine, Tyrosine, Cystiene, Methionene, Proline and Histidine) of different types were detected. The concentration of proline (20.5%) and glutamic acid (18.4%) were the highest percentages of the separated protein amino acids in the plant (Table-1).

Lipids content

***Total lipids content*:** The percentages of total lipids of *Isatis microcarpa* decreased from 3.15% at April to 2.61% at March and finally decreased to 1.82% at May, respectively.

Physical Properties of lipids: The obtained lipid was faint yellow in color, semi-solid and disagreeable taste. It was soluble in benzene, petroleum ether, diethyl ether, chloroform, acetone, warm methyl and ethyl alcohol.

Fundamental Chemical Properties of the extracted lipids: The fundamental chemical properties of the extracted lipids of *Isatis microcarpa* values were 21.95, 38.87, 238.91 and 260.86 for acid value, iodine value, ester value and saponification value, respectively.

Investigation of unsaponifiable matter fraction (hydrocarbons and sterols): The unsaponifiable matter content of *Isatis microcarpa* was determined using Gas Liquid Chromatography technique (GLC), where the obtained results indicated that the

plant contained ten hydrocarbons Dodecane, Tetradecane, Hexadecane, Heptadecane, Octadecane, Eicosane, Docosane, Tricosanem, Octacosane, Squalene two sterols, Cholestrol and Stigmasterol with different range of concentration. The highest percentage was Tricosane (22.1%) (Table-1).

***Investigation of saponifiable matter (fatty acids)*:** The fatty acid contents of the lipids of *Isatis microcarpa* were determined using GLC technique, where the obtained results revealed the presence of the saturated fatty acids, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid and arachidic acid beside the unsaturated fatty acids, linoleic acid and linolenic acid with different range of concentrations. Arachidic acid (34.6 %,) was the highest percentage of fatty acids, while capric acid was the lowest percentage (0.1 %) in the plant (Table-1).

Investigation of flavonoids

Identification of compound (1): The purified compound (1) appeared on paper chromatography as purple spot changed to yellow-green, with ammonia. R_f values 0.53 and 0.43 in systems; S1 and S2 respectively. Complete acid hydrolysis gave an aglycone apigenin and two sugar residues identified as glucose and rhamnose (comparative paper chromatography using authentic markers). Enzymatic hydrolysis using β-glucosidase gave negative results (no intermediates) which confirmed that the glucose moiety is directly linked to the flavonoid nucleus and that rhamnose is terminally located in compound (1), where UV spectral data of compound (1) were recorded at Table (2), where UV spectral data with methanol gave band I at 335, 333 and band II at 265 proving that it was a flavone, while on addition of NaOAc indicating the occupation of 7- position. Addition of H_3BO_3 indicated the absence of catecholic hydroxyl groups. Addition of $AlCl_3$ indicating the presence of free OH at C-5(Mabry, et al., 1970).

^{1}H-NMR spectral data: Aglycone moiety: δ (ppm) 7.95 (d, J=7.5Hz for H-2′, H-6′), 6.95 (d,J=7.5 Hz for H-3′, H-5′), 6.8 (d, J=2.5 Hz for H-8), 6.75 (S, H-3) and 6.45 (d,J=2.5 Hz for H-6). Sugar moieties: δ (ppm) 5.05 (d, J=7.5 Hz for H-1′′ glucose), 4.55 (d, J=2.5 Hz for H-1′′′ rhamnose at 7- position) 3.1-3.9 (m) for the rest of the two sugar protons and 0.95 (d, J=6 Hz for rhamnosyl CH_3). By comparing the obtained data with that of the literature compound (1) was identified as apigenin -7-O-rutinoside.

***Identification of compound (2)*:** The purified compound (2) appeared on paper chromatography as purple spot changed to yellow-green, with ammonia. R_f values 0.61 and 0.23 in systems; S1 and S2 respectively. Complete acid hydrolysis gave an aglycone apigenin and one sugar residue identified as glucose. UV spectral data of compound (2) (Table 2), where UV spectral data with methanol indicated that it was a flavone, while on addition of NaOAc indicating the occupation of 7- position. Addition of H_3BO_3 indicated the absence of catecholic hydroxyl groups. Addition of $AlCl_3$ indicating the presence of free OH at C-5 (Mabry, et al., 1970).

^{1}H-NMR spectral data: Aglycone moiety : δ(ppm) 7.95. (d, j =7.5 Hz H-2̀ and H-6̀) . 6.95 (d, j= 7.5 Hz H-3̀, H-5̀) 6.8 (d, j =2.5 Hz H-8), 6.75 (S, H-3) and 6.4 (d, j =2.5 Hz , H-6). Sugar moiety: δ (ppm) 5.1 (d, J = 7.5 Hz H" -glucose), 3.2-3.8 (m, glucose protons). From the previous data compound (2) was identified as apigenin-7-O-glucoside.

Identification of compound (3): The purified compound (3) appeared on paper chromatography as brown spot changed to yellow with ammonia. R_f values 0.78 and 0.09 in systems; S1 and S2 respectively. UV spectral data of compound (3) (Table 2), where UV spectral data with methanol indicated that it was a flavone, while on addition of NaOAc indicating free OH at 7- position. Addition of H_3BO_3 indicated the presence

of catecholic hydroxyl groups. Addition of $AlCl_3$ indicating the presence of free OH at C-5 (Mabry, et al*.,* 1970).
^{1}H-NMR spectral data δ (ppm): 6.19 (d, J = *2.5* Hz, H-6), 6.46 (d,J=2.5 Hz, H-8), 6.85 (S, H-3), 6.89 (d, J=8Hz, H-5'), 7.4(d, J=2.5 Hz and J=8Hz, H-2' and H-6').
El-mass spectrum showed molecular ion peak (M^+) at m/z 286 as the base peak. From the previous data compound (3) was identified as luteolin.
Identification of compound (4): The purified compound (4) appeared on paper chromatography as purple spot changed to yellow with ammonia. R_f values 0.45 and 0.17 in systems; S1 and S2 respectively. Complete acid hydrolysis gave an aglycone luteolin and one sugar residue identified as glucose. UV spectral data of compound (4) (Table 2), where UV spectral data with methanol indicated that it was a flavone, while on addition of NaOAc indicating the occupation of 7- position. Addition of H_3BO_3 indicated the presence of catecholic hydroxyl groups. Addition of $AlCl_3$ indicating the presence of free OH at C-5 (Mabry et al*.,* 1970). ^{1}H-NMR spectral data showed signals for luteolin type and signals for glucose protons. From the previous data compound (4) was identified as luteolin-7-O-glucoside.
Identification of compound (5): The purified compound (5) appeared on paper chromatography as yellow spot changed to florescence yellow with ammonia. R_f values 0.86 and 0.14 in systems; S1 and S2 respectively. Complete acid hydrolysis gave an aglycone kaempferol and one sugar residue identified as glucose. UV spectral data of compound (5) (Table-2), where UV spectral data with methanol indicated that it was a flavonol, while on addition of NaOAc indicating free OH at 7- position. Addition of H_3BO_3 indicated the absence of catecholic hydroxyl groups. Addition of $AlCl_3$ indicating the presence of free OH at C-5 (Mabry, et al*.,* 1970).
^{1}H-NMR spectral data δ (ppm): 8.0 (2H, d, J= 8 Hz, H2', H6'), 6.9 (2H, d, J= 8 Hz, H3', H5'), 6.4 (1H, d, J= 2.5 Hz, H8), 6.2 (1H, d, J= 2.5 Hz, H6). EI- mass spectrum of compound (5) revealed a molecular ion peak (M^+) at m/e 286 and other important ions. m/e 285, 258, 229, 121 and 93. From the previous data compound (5) was identified as kaempferol.
Identification of compound (6): The purified compound (6) appeared on paper chromatography as brown spot changed to yellow with ammonia. R_f values 0.82 and 0.53 in systems; S1 and S2 respectively. Complete acid hydrolysis gave an aglycone kaempferol and one sugar residue identified as glucose. UV spectral data of compound (6) (Table-2), where UV spectral data with methanol indicated that it was a flavonol, while on addition of NaOAc indicating the occupation of 7- position. Addition of H_3BO_3 indicated the absence of catecholic hydroxyl groups. Addition of $AlCl_3$ indicating the presence of free OH at C-5 (Mabry, et al*.,* 1970).
^{1}H-NMR spectral data δ (ppm): 8.0 (2H, d, J = 8.5 Hz, H 2' and H 6') , δ 6.9 (2H, d, J = 8.5 Hz, H3 ' and H5'), δ 6.3 (I H , d, J = 2.5 Hz, H8), δ 6.1 (l H , d , J = 2.5 H z , H 6), δ 5.3 (l H , d , J = 8 H z , H 1"glucose, anomeric proton) and δ 3-4 (m, remaining sugar protons). From the previous data compound (6) was identified as kaempferol -7-O-glucoside.
Identification of compound (7): The purified compound (7) appeared on paper chromatography as brown spot changed to yellow with ammonia. R_f values 0.84 and 0.53 in systems; S1 and S2 respectively. Complete acid hydrolysis gave an aglycone kaempferol and one sugar residue identified as glucose. UV spectral data of compound (7) (Table-2), where UV spectral data with methanol indicated that it was a flavonol with 3-OH substitution , while on addition of NaOAc indicating free OH at 7- position. Addition of H_3BO_3 indicated the absence of catecholic hydroxyl groups. Addition of $AlCl_3$ indicating the presence of free OH at C-5 (Mabry, et al., 1970). ^{1}H-NMR spectral

data δ (ppm): 7.9 (2H, d, J = 9 Hz, H 2̀ and H 6̀) , δ 6.7 (2H, d, J = 9 Hz, H3 ' and H5'), δ 5.6 (I H , d, J = 1.5 Hz, H8), δ 5.5 (l H , d , J = 1.5 H z , H 6) , δ 5.1 (d , J = 7 H z , H 1"glucose) and δ3.1-3.8 (m, remaining sugar protons). From the previous data compound (7) was identified as kaempferol -3-O-glucoside.

Identification of compound (8): The purified compound (8) appeared on paper chromatography as deep purple spot changed to greenish purple with ammonia. R_f values 0.88 and 0.33 in systems; S1 and S2 respectively. UV spectral data of compound (8) (Table-2), where UV spectral data with methanol exhibited a major band at λ_{max} 290 and shoulder at λ_{max} 325 (characteristic range of flavanone), while on addition of NaOAc indicating free OH at 7- position. Addition of H_3BO_3 indicated the absence of catecholic hydroxyl groups. Addition of $AlCl_3$ indicating the presence of free OH at C-5 (Mabry, et al*.,* 1970). ^{1}H-NMR spectral data δ (ppm): 7.3 (2H, dd, J= 8.5, 2.3 Hz, H2', H6'), 6.8 (2H, dd, J= 8.5, 2.3 Hz, H3', H5'), 6.0 (1H, d, J= 2.5 Hz, H8), 5.8 (1H, d, J= 2.5 Hz, H6), 5.2 (1H, dd, J= 5, 11 Hz, H2), 2.5 (cis and trans, 2H, 2d, J= 17 Hz, H3) (Mabry, et al*.,* 1970). From the previous data compound (8) was identified as naringenin.

***Estimation of total flavonoids*:** The percentage of total flavonoids of *Isatis microcarpa* reached its maximum value of 2.64 at May in flowers and its minimum value of 0.87 at March in stem. Data also indicates that the percentage of total flavonoids of *Isatis microcarpa* stem decreased from 1.95% at May to 1.00% at April and finally decreased to 0.87% at March, respectively. While for *Isatis microcarpa* leaves the percentage of total flavonoids were 2.21%, 1.12% and 1.05% at May, April and March, respectively. Mean while for *Isatis microcarpa* flowers the percentage of total flavonoids were 2.64%, 1.30% and 1.17% at May, April and March, respectively.

***Microbiological activities*:** The effect of different successive extracts of following organic solvents (chloroform, ethyl acetate, acetone, ethyl alcohol 70% and 96 % and water) of *Isatis microcarpa* on the inhibition of bacteria and fungi with different dilutions are illustrated at Table- 3 and 4.

***Anti-bacterial activity*:** Table-3 showed that extracts of ethyl alcohol (96% and 70%) and water extracts of *Isatis microcarpa* showed the best effect on all tested bacteria with different dilutions (250, 500, 1000 and 2000 ppm). Meanwhile it was found that ether and acetone extracts of *Isatis microcarpa* had a significant effect on all the tested bacteria with different dilutions (250, 500, 1000 and 2000 ppm) except acetone extract (250 ppm) which produced no effect on *Bacillus subtilis*. On the other hand chloroform and petroleum ether extracts showed the lowest effect on most of the tested bacteria at different dilutions. At the same time petroleum ether extract (0 and 250 ppm) produced no effect on all tested bacteria, at the same time concentration of (500 ppm) caused no effect on *Bacillus subtilis* and *Bacillus spharicus*. Chloroform extract (250 and 500 ppm) caused no effect on *Bacillus subtilis*.

***Anti- fungal activity*:** Table-4 showed that extracts of ethyl alcohol (96% and 70%) and water followed by ethyl acetate produced the best effect on all the tested fungi in different dilutions (250, 500, 1000 and 2000 ppm) of *Isatis microcarpa*. Meanwhile chloroform extract (250 and 500 ppm) caused no effect on *Fusarium oxysporium* at the same time concentration of (250 ppm) has no effect on *Aspergillus niger*. Petroleum ether extract (250 ppm and 500) has no effect on *Fusarium oxysporium* at the same time concentration of (250 ppm) caused no effect on *Aspergillus flavus* and *microsporum fulvum*. Acetone extract (250 ppm) caused no effect on *Fusarium solani*. On the other hand, petroleum ether and chloroform extracts in different dilutions (250, 500, 1000 and 2000 ppm) produced the lowest effect on *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporium*, *Fusarium solani*, *Alternaria tennius* and *microsporum fulvum*.

DISCUSION

It is obvious that there was a steady and successive increase in ash content, acid soluble, acid insoluble, water soluble and water insoluble ash from March, April to May, beside a steady increase in crude fiber contents from March, April to May.
The rise in ash content during at May at the studied habitat may be due to the increase of total ion accumulation as a result of increasing soil moisture stress, which agreed with Stocker's assumption (1960).

Ali et al., 1992, declared that the decrease of total nitrogen attributed to the decrease in water content, which was found to be linked with an accumulation of some amino acids (e.g. proline), that may play an important role in increasing cell osmoregulation.

The increase in the soil moisture stress may remarkably decrease the assimilation and accumulation of nitrogenous compounds of *Isatis microcarpa*, a behavior similar to the response of carbohydrates to soil drought conditions. These results are in agreement with those obtained by El-Monayeri et al. (1981). The increase in soil moisture stress decreased the photosynthesis, which was associated with an increase in respiration rate and led to the reduction of the total carbohydrates concentration in plant. These results are in agreement with those obtained by El-Monayeri et al., (1981).

The highest percentage of total lipid content at April may be due to the increase in carbohydrates concentration which is converted to lipid by oxidation reaction which agreed with Meayer and Andrson (1952). It was observed that the highest amount of total nitrogen content was recorded at April, which may be due to the increase in metabolic rate of *Isatis microcarpa* as a result of high water resources at April than that at May, which accounts to Stocker's assumption (1960).

Taha (2009) isolated the isoleucin, phenylalanine, tyrosine, proline, valine, argenine and histidine as free and protein amino acids, while leucine, methionine, alanine, theronine, glutamic acid, glycine, serine, aspartic acid and lysine as protein amino acids from the aerial part of *Sisymbrium irio* L. These compounds were recorded in *Isatis microcarpa*. Mean while he estimated the free and combined sugars using paper chromatography. GLC investigation of fatty acids revealed the presence of capric, lauric, palmitic, linoleic, linolenic and arachidic acids from the aerial part of *Sisymbrium irio.* These compounds were recorded in *Isatis microcarpa*. The chromatographic investigation revealed that *Isatis microcarpa* contained eight flavonoid compounds. Flavonoids play apart in the physiology of tendril contraction because of their ability to inhibit enzymes (Harborne, 1973). Some flavonoids act as growth regulators, phytoalescenins, animal toxins and sweetning agents while other have estrogenic action, reduce capillary fragility and employed as genetic marker (Watt and Breyer-Brandaijk,1962). The air dried powder of aerial parts of *Sisymbrium irio* L. was contained luteolin-7-glucoside, apigenin and six compounds of apigenin derivatives (Taha, et al., 2009). Apigenin has markedly augmented the cytotoxicity of tumor necrosis factor-alpha (TNF) which induced cytotoxicity in murine fibblast L-929 cells and is useful in chemoprevention, plays a role in the prevention of carcinogensis, inhibits the proliferation, and to a lesser degree, the migration of endothelial cells, and capillary formation in vitro, independently of its inhibition of hyaluronidase activity (Romanova and Vachalkova, 1999).

Isatis microcarpa extracts have antimicrobial activity especially at concentration (96% and 70%) ethyl alcohol and water followed by ethyl acetate and ether extracts. This may be due to the different soluble biochemical compounds in each extract. Flavonoids are ubiquitous in photosynthesising cells and are commonly found

in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. Flavonoids possessing antifungal, antiviral and antibacterial activity.The robinetin, myricetin, apigenin, rutin and galangin compounds represent novel leads, and future studies may allow the development of a pharmacologically acceptable antimicrobial agent or class of agents (Cushnie and Lamb, 2009). Hartleb and Seifert (1994) reported that *I. tinctoria*, which has been used as a dye plant for centuries, also exhibits antifungal and insecticidal activities. *Isatis indigotica* root extract showed antimicrobial activity against *Bacillus subtilis* and Saccharomyces (Lin, et al., 2005).

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Table-1: Relative percentages of free, combined sugars, free and protein amino acids, hydrocarbons, sterols and fatty acids of *Isatis microcarpa*.

Sugars	Free sugar %	Combined sugars %	Amino acids	Free amino acids %	Protein amino acids %	Item	Hydro-carbons and sterols (%)	Fatty acids	Fatty acids (%)
Xylose	-	4.1	Aspartic acid	-	1.3	Dodecane	0.5	Cprylic	1.6
Arabinose	24.4	18.8	Theronine	6.2	1.9	Tetradecane	7.5	Capric	0.1
Ribose	22.7	14.5	Serine	-	2.2	Hexadecane	0.1	Lauric	0.4
Fructose	8.6	10.4	Glutamic acid	25.5	18.4	Heptadecane	9.0	Myristic	2.1
Glucose	37.5	22.4	Glycine	5.6	1.6	Octadecane	12.6	Palmitic	27.2
Sucrose	6.1	8.9	Alanine	-	2.7	Eicosane	17.8	Stearic	5.3
Cellobiose	-	8.2	Valine	-	13.6	Docosane	7.6	Arachidic	34.6
Raffinose	-	12.3	Isoleucine	6.8	0.9	Tricosane	22.1	Linolenic	19.4
			Argenine	14.8	1.6	Octacosane	3.3	Linoleic	8.4
			Phenylalanine	7.6	-	Squalene	5.3		
			Lysine	3.6	-	Cholestrol	5.8		
			Leucine	8.4	15.7	Stigmasterol	4.9		
			Tyrosine	-	2.03				
			Cystiene	-	1.4				
			Methionene	2.7	1.05				
			Proline	18.06	20.5				
			Histidine	-	14.8				

Table-2: UV spectral data, λmax, nm of flavonoid compounds of *Isatis microcarpa*.

Compounds	MeOH	NaOMe	NaOAc	NaOAc +H_3BO_3	$AlCl_3$	$AlCl_3$+HCl
Apigenin- 7-O- rutinoside	265 , 330 (sh), 335	--	265, 310(sh), 334, 390	268, 320, 335	270, 295 , 335, 382	270, 295 , 335, 382
Apigenin-7-O-glucoside	226, 266, 333	240, 272, 305 (sh), 390	252 (sh), 263, 350, 385(sh)	266, 338	274, 300(sh), 345, 385	275, 294, (sh), 340, 380
Luteolin	253, 300 (sh), 349	266, 330(sh), 400	268, 326(sh), 384	262, 301 (sh), 370, 426 (sh)	272, 300 (sh), 330, 426	272, 300 (sh), 355, 385
Luteolin-7-O-glucoside	251,263,330, 345	271,326, 401	266,300(sh), 400	264,300(sh), 398	256,310,377	257,307(sh), 362
Kaempferol	253 (sh), 268, 342 (sh), 367	280,318, 420	275, 302(sh), 385	267, 296(sh), 320 (sh), 370	266, 305 (sh), 350, 422	266, 305 (sh), 350 , 422
kaempferol-7-O-glucoside	275, 330 (sh), 360	275, 330 (sh), 390	275, 330 (sh), 360	275, 330 (sh), 360	275, 300 (sh), 360	275, 300 (sh), 360
kaempferol-3-O-glucoside	265, 325, 350	275, 325, 400	223, 265, 304 (sh), 370	224, 265, 377	265, 290, 420	265, 300, 420
Naringenin	290, 325 (sh)	247, 267 (sh), 327	290 (sh), 327	292, 327 (sh), 330 (sh)	310, 375	310 , 375

Table-3: The effect of the selected plant extracts in different concentrations on the growth of some bacteria (inhibition zone in mm).

Plant extract	Conc. ppm	Inhibition zone (mm)					
		Bacillus subtilis **+ ve**	***Bacillus sphaericus*** **+ ve**	***Staphylococcus aureus*** **+ ve**	***Psedomonas sp.*** **-ve**	***Escherichia coli*** **- ve**	***Salmonella spp.*** **-ve**
Pet. Ether	0	0	0	0	0	0	0
	250	0	0	0	0	0	0
	500	0	0	3.9	4.1	6.7	4.2
	1000	6.1	7.6	7.2	7.8	8.3	7.7
	2000	8.3	10.6	8.2	9.4	9.9	9.0
Ether	0	0	0	0.3	0	1.1	0
	250	9.8	10.9	11.2	13.5	12.2	15.4
	500	15.3	13.7	14.1	16.4	14.2	16.6
	1000	17.4	18.0	16.2	19.4	16.5	17.1
	2000	18.6	18.7	18.5	21.2	19.7	20.5
Chloroform	0	0	0.2	0.1	0	0	0.1
	250	0	0.6	3.4	3.2	4.2	7.6
	500	0	5.3	8.0	7.9	10.2	11.1
	1000	11.3	10.6	9.3	10.4	11.9	12.5
	2000	12.5	11.2	12.7	11.3	15.6	14.1
Ethyl acetate	0	1.1	0.1	0	0	0.2	0
	250	4.6	5.9	5.4	7.1	9.3	12.2
	500	5.1	11.3	10.0	9.9	11.2	12.9
	1000	9.6	11.9	12.3	11.4	15.6	13.3
	2000	10.4	12.5	13.7	14.3	15.9	14.8
Acetone	0	0	0	0	0	1.0	0
	250	0	8.2	6.7	8.8	10.4	13.5
	500	9.2	10.4	10.8	11.3	13.0	14.2
	1000	10.9	11.1	13.4	12.4	14.5	16.4
	2000	13.5	13.8	14.5	15.3	17.1	17.2
Ethyl alcohol 96%	0	0	0.4	0.3	0	0	1.1
	250	17.4	20.2	21.6	23.2	21.9	22.6
	500	20.2	21.5	26.4	27.9	24.6	24.4
	1000	25.4	26.1	35.9	34.5	31.4	28.4
	2000	27.4	29.4	44.1	42.1	44.1	34.8
Ethyl alcohol 70%	0	0.2	0	0.1	0	0	0.1
	250	19.7	21.6	25.6	23.8	27.2	22.4
	500	23.1	23.6	30.5	32.5	33.2	24.7
	1000	30.0	28.1	41.5	42.1	39.2	32.7
	2000	35.4	35.7	50.3	49.4	47.4	38.4
Water	0	0	0	0	0	0	0
	250	0	10.9	12.2	14.9	12.9	9.6
	500	10.9	14.4	15.5	21.1	18.7	16.3
	1000	16.9	15.8	18.6	23.6	23.4	20.7
	2000	19.7	19.3	20.2	29.1	31.5	24.2

Table-4: The effect of the selected plant extracts in different concentrations on the growth of some fungi (inhibition zone in mm).

Plant extract	Conc. ppm	Inhibition zone (mm)					
		Aspergillus niger	***Aspergillus flavus***	***Fusarium oxysporium***	***Fusarium solani***	***Alternaria tennius***	***Microsporum fulvum***
Pet. Ether	0	0	0	0	0	0	0
	250	3.3	0	0	3.1	6.1	0
	500	5.8	5.5	0	3.8	7.5	3.7
	1000	7.3	7.9	5.7	7.4	8.8	8.7
	2000	8.6	9.1	8.5	8.5	9.7	11.3
Ether	0	0	0.3	0	0.1	0	0.1
	250	7.1	16.2	15.4	13.9	13.7	14.8
	500	12.9	18.5	17.4	16.2	15.3	16.9
	1000	15.3	20.6	20.2	17.0	17.9	19.4
	2000	18.6	22.5	22.4	21.3	20.5	23.5
Chloroform	0	0.4	0	0	0.5	0	0
	250	4.5	0	0	3.5	6.6	7.4
	500	8.7	3.9	0	6.9	8.3	8.7
	1000	10.4	9.9	7.6	8.6	9.1	10.3
	2000	11.1	11.4	11.5	9.7	11.1	12.0
Ethyl acetate	0	0	0	0.3	0.1	0	0.1
	250	14.5	14.7	14.0	14.8	15.2	16.7
	500	16.6	16.5	19.1	17.7	17.1	19.7
	1000	21.4	20.3	24.2	20.4	21.7	24.3
	2000	25.2	27.4	27.5	22.6	28.4	26.9
Acetone	0	0	0	0.1	0	0	0.2
	250	5.8	11.3	8.7	0	8.4	11.3
	500	11.6	13.8	12.5	8.6	11.7	13.0
	1000	12.5	16.1	15.0	13.3	12.1	14.5
	2000	14.4	17.3	17.2	14.3	14.8	16.6
Ethyl alcohol 96%	0	0	0.5	0	0.2	0.1	0
	250	20.1	17.4	18.3	18.3	20.2	21.7
	500	23.4	22.2	24.1	23.2	26.2	26.1
	1000	29.7	28.3	28.8	26.8	31.6	31.8
	2000	36.1	36.0	39.6	35.1	38.6	38.9
Ethyl alcohol 70%	0	0.1	0	0.4	0	0.2	0.1
	250	22.9	22.3	23.5	21.5	23.9	25.4
	500	28.4	25.1	30.2	27.7	28.9	30.1
	1000	35.7	30.9	34.1	35.8	34.7	37.2
	2000	39.5	38.3	43.5	40.9	41.7	43.6
Water	0	0	0	0	0	0	0
	250	13.2	15.3	15.5	12.5	14.7	14.4
	500	17.4	20.2	21.2	17.3	19.7	18.6
	1000	21.3	23.5	25.8	19.8	24.9	23.2
	2000	25.6	29.9	30.1	25.4	31.8	29.9

