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Research Article

Phytochemical and Biological Investigation of *Ipomoea carnea* Jacq. Grown in Egypt

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ABSTRACT
Total phenolic and flavonoid contents of the leaves and flowers of *Ipomoea carnea* Jacq. were carried out using Folin-Ciocalteu's and aluminum chloride assays, respectively. Resulted in 6.59 and 9.37 mg gallic acid equivalent (GAE)/g. dry wt. and 1.336% and 0.885% rutin equivalent (RE)/g for leaves and flowers, respectively. The concentration of rutin and *β*-sitosterol in leaves and flowers extracts of *I. carnea* were also estimated by HPLC analysis resulting in 9.174 and 2.733 mg/g dry wt. (rutin) and 0.463, 17.085 mg/g dry wt. (*β*-sitosterol) for leaves and flowers, respectively. Chromatographic separation of the leaves and flowers ethanol extracts led to the isolation of a new biflavonoid compound: 3', 3"", 5, 5"', 7, 7"'-*O*-*β*-D-glucosyl-4', 4""-biflavonoyl ether [4'-*O*-4"" Bis-isoquercetin] (Ipomoeflavoside) from leaves and other four known compounds namely; caffeoyl ethyl ester, caffeic acid, rutin, lycopene isolated for the first time from leaves and *β*-sitosterol from flowers. The leaves and flowers ethanol extracts showed antioxidant, antihyperglycemic and hepatoprotective activities. They were also evaluated for their anticancer and antimicrobial activities. The ethanol leaves extract showed the highest cytotoxic activity against the breast cancer cell line, with (IC_{50}: 7.4 µg/ml) while it showed weak cytotoxic effect on liver and colon cancer cell lines (IC_{50}:23 and 35 µg/ml) respectively, The ethanol flowers extract showed weak or no anticancer cytotoxic activity against the tested cancer cell lines. The leaves and flowers ethanol extracts showed significant antimicrobial activity against *Streptococcus pneumonia, Bacillus subtillis*, *Escherichia coli* and *Aspergillus fumigatus* while showing no activity against *Candida albicans* and *Pseudomonas aeurginosa*.

Keywords: Convulvulaceae, flavonoids, phenolics, cytotoxic activity, antioxidant, antihyperglycemic, hepatoprotective.

INTRODUCTION

Convolvulaceae, commonly known as the bindweed or morning glory family, is a family of about 60 genera and more than 1,650 species of mostly herbaceous vines, but also trees, shrubs and herbs, more than one-third of the species are included in two major genera, *Ipomoea* and *Convolvulus*. Although the family is best known in temperate regions for its weedy representatives, many tropical species are as valuable ornamentals, medicinals, and food crop[1]. *Ipomoea* is the largest genus in the family Convolvulaceae, with over 500 species, among which; is *Ipomoea carnea* Jacq. species, (known as morning glory). This plant propagates vegetative by stems which are capable of rooting within a few days, and farmers use it as ornamental and hedge plant along the banks of irrigation and drainage canals edges in the Nile Delta. This plant propagates vegetatively by stems which are capable of rooting within a few days. The farmers use it as ornamental and hedge plant along the banks of irrigation and drainage canals[2]. *Ipomoea carnea* contain variety of bioactive components such as phenolic acid, alkaloids, flavonoids, coumarins and sterols[1-4] Also having an immense biological and pharmacological activities as an anti-inflammatory, antioxidant, antidiabetic, antimicrobial, wound healing, immunomodulatory, antifungal and hepatoprotective activities[5-9]. The biological importance and the few phytochemical studies reported on *Ipomoea carnea* encouraged the author to undertake this study to validate scientifically its reported uses in folk medicine and to trace new biological activities.

MATERIALS

Plant material

Leaves and flowers of *Ipomoea carnea* were collected from canal edges at Al-Sharquia, Egypt, during the flowering stage in August and September 2013 and kindly identified by Dr. Abd El Halim Abd El Mogaly, Professor of Plant Taxonomy, Department of Flora, Agricultural Museum, Giza, Egypt. A dry voucher specimen has been deposited in the Phytochemistry laboratory, National Organization for Drug Control and Research (NODCAR), Egypt.

Chemicals and biochemical kits

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DPPH (1, 1-diphenyl-2-picrylhydrazyl, ascorbic acid, acidified isopropanol**,** MTT {3-[4, 5- dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide} solution, Folin-Ciocalteu's reagent, gallic acid, rutin, *β*-sitosterol, aluminum chloride, sodium carbonate, were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All chemicals and solvents were of analytical grade. The following biochemical kits were purchased from their respective sources: Glutathione kit (Wak-Chemie Medical, Germany) and Transaminase kits (BioMerieux Co.) were used for the assessment of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) enzymes. Alloxan: Sigma Co., Vitamin E (dlα-tocopheryl acetate): Pharco Pharmaceutical Co. were used in the present investigation.

Materials for chromatography

Sephadex LH-20 for column chromatography (25-100 µm, Sigma-Aldrich, Sweden); Cellulose Microcrystalline for column chromatography (LOBA Chemie PVT. Ltd., India); Polyamide for column chromatography (50-160 µm, Fluka Chemie GmbH, Switzerland). Solvent systems S_1: *n*-Butanol–Acetic acid–Water (BAW) (4:1:5 v/v/v, top layer), S_2: Acetic acid–Water 15:85 v/v, S_3: *n*-Butanol–Isopropyl alcohol–Water (4:1:5 v/v/v, top layer)

Materials for in-vitro cytotoxic activity

The ethanol extracts (0-50 µg/ml) of *I. carnea* Jacq. leaves and flowers were tested for cytotoxic activity against HEPG2 (liver carcinoma), MCF7 (breast cancer) and HCT116 (colon cancer) cell lines were obtained frozen in liquid nitrogen (-80°C) from the American Type Culture Collection. The tumor cell lines were maintained in the National Cancer Institute, Cairo University, Cairo, Egypt, by serial subculturing. Doxorubicin®: Sigma-Aldrich Co., USA.

Materials for microbiological studies

Plant extracts were tested against two standard Gram positive bacteria: *Streptococcus pneumonia* (RCMB 010010) *Bacillis subtilis* (RCMB 010067); two standard Gram negative bacteria: *Escherichia coli* (RCMB 010052); *Pseudomonas aeruginosa* (RCMB 010049); the fungal strain: *Aspergillus niger*. and yeast: *Candida albicans* (RCMB 05036). The culture media used for bacteria was nutrient agar medium, malt extract agar medium used for cultivation of *Candida albicans* and Czapeks Dox agar medium was used for *Aspergillus niger*. Standards with the concentration of 1 mg/ml were used as positive controls Ampicillin (Sedico Co, Egypt) for Gram positive, Gentamicin (Sedico Co, Egypt) for Gram negative bacteria, Amphotericin B (Sedico Co, Egypt) for fungi.

Experimental animals

Male Wistar albino rats (100–120 g) were obtained from the Animal House, National Research Center (NRC), Egypt. All animals were kept in a controlled environment of air and temperature with access to water and diet ad libitum. Anesthetic procedures and animal handling were in compliance with the ethical guidelines of Medical Ethics Committee of the National Research Centre in Egypt and performed to ensure that animals did not suffer at any stage of the experiment (Approval No. #10021).

METHODS

General experimental procedures

The structure of the compounds was identified by spectroscopic methods including: UV/VIS (Schimadzu 160 A; UV spectrophotometer; National Organization for Drug Control and Research, Giza, Egypt) for measuring UV spectral data of the isolated compounds, in the range of 200–500 nm in methanol and with different diagnostic shift reagents. NMR (Nuclear Magnetic Resonance Spectrophotometer, JEOL JNM-ECA, 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR), NMR(JEOL Delta2 spectrometer ,500 MHz for ^{1}H-NMR), NMR(Bruker High Performance Digital FT-NMR Spectrometer Advance III,400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR), Mass spectrometer: Schimadzu QP-2010 plus, Micro Analytical Center, Cairo University was used for determination of molecular weight of compounds, HPLC apparatus Agilant 1200 with column ODS HYPERSIL C18 and ZOBRAX Eclipse plus C_{18} for quantitative assay of major isolated compounds rutin and *β*-sitosterol respectively.

Conditions of HPLC analysis

Conditions for HPLC analysis of rutin: Column type: ODS HYPERSIL C18 Column, dimensions length: 250mm x ID 4.6 mm, particle size (5 µm), mobile phase: Isocratic elution with (methanol: water: phosphoric acid) (100: 100: 1), flow rate: 1.5 ml/min, sample size: 20 µl, temperature: 20°C, detector UV: at 270 nm.

Conditions for HPLC analysis of *β*-sitosterol: Column type ZOBRAX Eclipse plus C18 Column dimensions :(100 x 4.6) mm 3.5µ, mobile phase: Isocratic elution with (Acetonitrile: Ethanol: water) (45: 40: 15), flow rate1.5 ml/min, injection volume: 50 µl, temperature: 40°C Detector

UV at 210 nm.

Phytochemical investigation

Determination of total phenolic content

Total polyphenol content (TP) of the leaves and flowers was determined according to the procedure adopted by Kumazawa *et al*., 2002[10] using Folin-Ciocalteu's reagent. TP content was expressed as mg of gallic acid equivalent (GAE)/g of the dry plant material. Also, the percentage of

TP in leaves and flowers ethanol extracts of *I. carnea* was compared with the percentage of total polyphenol in green tea and grape seed extracts available in Egyptian market (obtained from Phytochemistry Laboratory, NODCAR). Ten grams of the powdered plant leaves and flowers under investigation was separately exhaustively extracted with 80% ethanol, the filtrate was evaporated to give crude polyphenol (CPP) extract of leaves (CPP=3g) and crude polyphenol extract of flowers (CPP=4.21g). A stock solution of leaves and flowers ethanol extracts in addition to green tea and grape seed extracts were prepared in water with a final concentration of 1 mg/ml. Results were expressed as mg gallic acid equivalents (GAE)/g extract. Each assay was carried out in triplicate.

Determination of total flavonoid content.

The total flavonoid content was determined of the leaves and flowers by the method described by Chang *et al*., 2002[11] with some modifications. A 500 μL of each plant sample was separately placed in a 25mL flask and 500 μL of $AlCl_3$ 0.5% were added and the volume was completed with water. It was incubated for 40 minutes at room temperature and protected from light. Absorption was measured, at 415 nm. The results were expressed in mg/g equivalents of rutin. Each assay was carried out in triplicate.

Quantitative HPLC assay of β-sitosterol

Quantification of *β*-sitosterol was carried out using method validated at PentaPharma Pharmaceutical Industries and was registered in National Organization for Drug Control and Research (NODCAR) A.R.E. for assay of *β*-sitosterol from sesame extract in Pentaburn® topical preparation[12]. The chloroform extracts were prepared by hot maceration of the dried leaves and flowers material (50 g of each) in chloroform then dried under reduced pressure. The yield of leaves was (1.003 g) and flowers (4.987 g) of ethanol extracts. A solution of ethanol extract (1 mg/ml) was prepared in 40% of tetrahydrofuran and a solvent mixture (Acetonitrile: ethanol: water) (45:40:15) and subjected to HPLC analysis. A stock solution of the *β*-sitosterol (USP reference standard) (1 mg/ml) was prepared in 40% tetrahydrofuran in solvent mixture. Aliquots of this stock solution were diluted with solvent mixture, and a calibration curve was constructed over the range of 0.04-0.2 mg/ml. *β*-sitosterol content of leaves and flowers chloroform extracts expressed as mg/ml of the solution used.

Extraction and isolation of active constituents from I. carnea leaves and flowers

The air dried powdered *I. carnea* leaves (2 kg) and flowers (1kg) were extracted by cold maceration with 70% ethanol till exhaustion. The concentrated extracts of leaves and flowers (600 and 400 g, respectively) were separately suspended in water and successively fractionated with petroleum ether, chloroform, ethyl acetate and butanol. The concentrated fractions yielded 19.27, 40.12, 50.87and 77.603 g for leaves and 4.6986, 99.74, 9.7083 and 117.135 g for flowers, respectively. By TLC examination, ethyl acetate fraction from leaves extract and chloroform fraction from flowers extract contained number of spots more than other fractions suggesting the presence of valuable phytochemical constituents. They were fractionated on chromatographic columns as illustrated in flow charts (Fig. 1 and 2). Five compounds were isolated from leaves ethyl acetate fraction and one compounds was isolated from flowers chloroform fraction.

Biological study

Determination of Median Lethal Dose (LD_{50})

The leaves and flowers ethanol extracts of *Ipomoea carnea* Jacq. were separately subjected to determination of the LD_{50} according to the method described by Karber procedure (1931)[13]. Male albino mice of (20-30g) were divided into groups each of six animals. Preliminary experiments were done to determine the minimal dose that kill all animals (LD_{100}) and the maximal dose that fails to kill any animal. Several doses at equal logarithmic intervals were chosen in between these two doses, each dose was injected in a group of 6 animals by subcutaneous injection. The mice were then observed for twenty four hours and symptoms of toxicity and mortality rates in each group were recorded and the LD_{50} was calculated.

In-vitro antioxidant activity

The in-vitro antioxidant activity was assessed using a modified quantitative 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay[14]. The DPPH was prepared at a concentration of 0.004 % in ethanol (HPLC grade). The lyophilized 70 % ethanol extract of the leaves and flowers of *I. carnea* Jacq. were separately dissolved in 50% methanol at a concentration of 2, 3, 4, 6, 8 mg/ml with 200 µl of each test solution added to 6 ml DPPH solution. Blank samples were run using 99.9 % ethanol. After 30 min. incubation period at room temperature, the absorbance was read against the blank at 517 nm. Gallic acid was used as a positive control at a concentration of 0.005, 0.01, 0.02, 0.04, 0.06 mg/ml.

In-vivo Antioxidant activity (Determination of blood glutathione)

The antioxidant activities of the ethanol extracts of *I. carnea* leaves and flowers and pure compound 4 (rutin) isolated from ethyl acetate fraction of leaves ethanol extract were evaluated by the determination of glutathione in blood of alloxan-induced diabetic rats, using vitamin E as a positive control[15]. Male albino rats of the Sprague Dawely Strain (130-140 g) were divided into six groups (6 animals each). One group was kept as negative control,

diabetes mellitus was induced by injecting a single dose of 100 mg alloxan / kg of b.wt. intra-peritoneally in each animal[16] in the other groups. Hyperglycemia was assessed after 72 hours by measuring blood glucose[17]. Animals are divided into 5 groups; first group: healthy untreated rats as negative control, second group: diabetic rats and kept untreated, third group: rats received reference drug (Vitamin E, 7.5 mg/kg b.wt.), fourth and fifth groups: rats received the ethanol leaves and flowers extracts of *I. carnea* Jacq. orally in a dose of (100 mg/kg b.wt. each) and sixth group: rats received compound 4 (20 mg/kg). Blood samples were taken after a week for the determination of glutathione.

Antihyperglycemic activity

Rats were weighed and injected intraperitoneally with alloxan (150 mg/kg) dissolved in distilled water. After 48 h blood samples were withdrawn from the retro-orbital venous plexus under light ether anesthesia and the serum was separated by centrifugation for the determination of glucose level. Only rats with serum glucose levels more than 250 mg/dl were selected and considered as hyperglycemic animals[18]. The hyperglycemic rats were then divided into six groups (10 rats each); first group: normal healthy control group, second group: diabetic rats that served as positive control, third group: diabetic rats that received 100 mg/kg b.wt. of leaves ethanol extract, fourth group: diabetic rats that received 100 mg/kg b.wt. of flowers ethanol extract, fifth group: diabetic rats that received 20 mg/kg b.wt. of rutin, sixth group: diabetic rats that received 100 mg/kg b. wt. of Metformin®. orally for 10 consecutive days.The extracts and Metformin were started 48 h after alloxan injection at which time hyperglycemia was confirmed. Twenty-four hours after the last dose of either drug treatment, a blood sample was withdrawn from the retro-orbital venous plexus from 18 h food-deprived rats and was centrifuged at 3000 rpm for 10 min. The serum was obtained for determination of the serum glucose level, as quinineamine using a test reagent kit (Biodiagnostic, Egypt)[17]. The absorbance was measured at 510 nm and the results were expressed as mg/dl.

Hepatoprotective activity

Liver damage in rats was induced by intraperitonial injection of 5 ml/kg of 25% carbon tetrachloride (CCL_4) in liquid parafen[19]. Animals were randomly divided into four groups each of ten rats; first group: the control group received a daily oral dose of 1 ml saline for one week before and after liver damage; second and third group: the liver damaged rats pretreated with daily oral dose of (100 mg/kg b.wt.) leaves and flowers ethanol extracts of *I. carnea*, respectively for one week and the administration of extracts was continued after liver damage for another one month; fourth group: the liver damaged rats pretreated with daily oral dose of (25 mg/kg b. wt.) silymarin as a reference standard drug and the administration of the drug was continued after liver damage for another one month. After that, the whole blood was obtained by an overnight fast from the retro orbital venous plexus through the eye canthus of anaesthetized rat. Blood samples were collected at zero time, one month and seventy two hours after carbon tetrachloride injection and after one month intervals. Serum was isolated by centrifugation. Serum alanine aminotransferase (ALT, GPT), aspartate aminotransferase (AST, GOT)[20] and alkaline phosphatase (ALP)[21]. The data obtained were analysed using the Student T test[22].

Antimicrobial activity

The antimicrobial activity was performed against *Streptococcus pneumonia* (RCMB 010010), *Bacillus subtillis* (RCMB 010067), *Pseudomonas aeurginosa* (RCMB 010049), *Escherichia coli* (RCMB 010052), *Aspergillus fumigatus* (RCMB 02568) and *Candida albicans* (RCMB 05036). They were maintained in the regional center for mycology and biotechnology, Al Azhar University. Bacterial strains were cultured overnight at 37 °C in Mueller Hinton agar, and yeasts were cultured overnight at 30 °C in Sabouraud dextrose agar. The agar disc diffusion method was employed for determination of the antimicrobial activities of the leaves and flowers ethanol extracts of *Ipomoea carnea* Jacq.[23]. A suspension of the tested microorganism (0.1 mL of 10^8 cells per mL) was spread on solid media plates. Aliquots of 100 µg of the leaves and flowers ethanol extracts dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) were applied on sterile paper discs (6 mm diameter). Ampicillin, Gentamycin and Amphotericin B were used as positive control standard drugs for gram positive, gram negative bacteria and antifungus, respectively, and DMSO without the extracts was used as a negative control. The discs were deposited on the surface of inoculated agar plates. These plates were held at 4°C for 2 h, followed by incubation at 37 °C for 24 h for bacteria, or at 30 °C for 48 h for fungi. The diameters of the inhibitory zones were measured in millimeters. All tests were performed in triplicate. Results are recorded as mean zone of inhibition in mm ± Standard deviation beyond well diameter (6 mm) produced on a range of environmental and clinically pathogenic microorganisms using (1 mg/ml) concentration of tested samples[24].

Minimum Inhibitory Concentration (MIC) Determination

The Minimum Inhibitory Concentration (MIC) of the samples was estimated for each of the tested organism in triplicates by microdilution broth susceptibility assay described by National Commitee for Clinical Laboratory Standards (NCCLS), 1993[25]. Tests for bacterial strains

were performed in Mueller Hinton Broth supplemented with Tween 80 detergent at a final concentration of 0.5% (v/v), while those for fungal strains were performed in Sabouraud dextrose broth with Tween 80. The bacterial strains were cultured overnight at 37 °C in Mueller Hinton Broth, and the yeasts were cultured overnight at 30 °C in Sabouraud dextrose broth. Test strains were suspended in Mueller Hinton Broth at a final density of 5×10^5 cfu/mL, which was confirmed by viable counts. Geometric dilutions ranging from 0.035 to 72.0 mg/mL of the leaves and flowers ethanol extracts were prepared in a 96-well micro liter plate. The plates were incubated under normal atmospheric conditions at 37 °C for 24 h for bacteria, or at 30 °C for 48 h for yeasts. Bacterial growth was indicated by the presence of a white pellet at the well bottom.

Cytotoxic activity

Cytotoxicity determinations were carried out in National Cancer Institute, Cairo University, Cairo, Egypt. Cytotoxic activity was tested using Sulforhodamine-B assay[26]. IC_{50} was determined and compared with that of doxorubicin as reference standard.

Statistical analysis

All data were expressed as mean ± SE and the statistical significance was evaluated using the ANOVA test followed by Duncan's multiple range tests. A probability value of less than0.05 was considered statistically significant ($P < 0.05$ was considered statistically significant)

RESULTS AND DISCUSSION

Total phenols and flavonoids contents

Total phenols and flavonoids contents of *I. carnea* leaves and flowers were investigated. The flowers extract showed higher amount of total polyphenols (9.375 ± 0.0065 mg (GAE)/g dry wt.) than leaves extract (6.5976 ± 0.00754 mg (GAE)/g dry wt.). The leaves and flowers ethanol extracts of *I. carnea* contain significant contents of flavonoid compounds. It was found that leaves ethanol extract contain higher concentration of total flavonoids (1.3356%) than flowers ethanol extract (0.8855%). Previously, Khatiwora *et al.,* 2010 and Adsul *et al.,* 2012[27,6] found that flowers ethanol extract contains highest amount of polyphenols (73 mg catechol equivalent/ g dry wt.) compared with leaves ethanol extract (45 mg catechol equivalent/ g dry wt.) and stem ethanol extract (30 mg catechol equivalent/ g dry material) which was in agreement with the current results obtained that showed the flowers ethanol extract contain maximum amount of total polyphenols compared to leaves ethanol extract. Our results of total flavonoids were in contrary to previous literature[27, 6] reporting the flavonoid content of the flowers was quite high compared to the leaves and the stem.

Assay of β-sitosterol

The present investigation revealed the leaves and flowers chloroform extracts of *I. carnea* contain significant amount of *β*-sitosterol. The results were (0.463, 17.085 mg *β*-sitosterol/g dry wt., respectively). It was the first time for the quantitative HPLC assay of *β*-sitosterol in leaves and flowers chloroform extracts of *I. carnea* species grown in Egypt.

Characterization and identification of isolated compounds

The isolated pure compounds were identified on the basis of acid hydrolysis, comparative PC, UV, ESI-MS, ^{1}H-, ^{13}C-NMR and in some cases 2D-NMR spectroscopic analyses and comparing with previous reported data Fig. 3.

Compound 1

Off-white fine powder (13.3 mg). It gave a blue fluorescence in UV light which appeared as intensified yellow fluorescence by ammonia vapour. it showed a UV absorption spectrum in methanol (λ_{max}: 244, 295, 326), confirming presence of phenyl propene system.^{1}H-NMR(600 MHz, CD_3OD, δ ppm): 7.51 (1H, d, *J*= 16.26 Hz, H-7), 7.01 (1H, d, *J*= 2.1 Hz, H-2), 6.91 (1H, dd, J^1= 2.04 Hz,J^2= 8.3 Hz, H-6), 6.75 (1H, d, *J*=8.22 Hz, H-5), 6.22 (1H, d, *J*=16.26 Hz, H-8), 4.18 (2H, q, *J*= 7.56, 6.84 Hz, H-CH_2), 1.28 (3H, t, *J*= 6.9 Hz, H-CH_3) , ^{13} C-NMR (150 MHz, CD_3OD, δ ppm): 168.0 (C-9), 148.21 (C-4), 145.48 (C-3), 145.39 (C-7), 126.38 (C-1), 121.55 (C-2), 115.14 (C-8), 113.91 (C-6), 113.73 (C-5), 60.9 (C-CH_2), 13.3 (C-CH_3). ^{1}H-NMR spectra of compound 1 showed an ABX spin coupling system of three resonances at δ 7.01 (d, *J*= 2.1 Hz), 6.91 (dd, *J*= 2.04 and 8.3 Hz), 6.75 (d, *J*=8.22 Hz) each integrated for one proton attributed to H-2, H-6 and H-5 respectively of 3,4-dihydroxy benzene ring together with two doublets signals with large *J* values at δ 7.51 and 6.22 (*J*= 16.25 HZ) for trans olefinic double bond of α, *β*-unsaturated system indicating caffeoyl structure. The signals at δ 4.15 (2H, q, *J*= 7.56, 6.84 Hz, H-CH_2) and 1.28 (3H, t, *J*= 6.9 Hz, H-CH_3) suggesting an acyl group in the structure. The ^{13}C-NMR spectra of compound 1 showed the presence of nine characteristic carbon signals of caffeoyl moiety at δ ppm: 168.0 (C-9), 148.21 (C-4), 145.48 (C-3), 145.39 (C-7), 126.38 (C-1), 121.55 (C-2), 115.14 (C-8), 113.91 (C-6), 113.73 (C-5)[28]. The additional two carbon resonance signals at δ 60.9 and 13.3 indicating an ethyl ester moiety. Final assignment of the signals in the^1 H-NMR and ^{13}C-NMR spectra was confirmed based on ^{1} H-^{1} H shift correlation spectroscopy (COSY) and Heteronuclear Multiple-Bond Correlation spectrum (HMBC) that showed different ^{2}J and ^{3}J set of correlation between hydrogens and carbons. The most important correlations are between H-CH_2 of ethyl moiety (4.18) with C-CH_2 (60.9) and H-CH_3 of ethyl moiety (1.28) with C-

CH_3 (13.3). ^3J – coupling between H-CH_2 (4.18) and C-9 (168.0) that confirmed a caffeoyl ethyl ester structure. From above data and by comparison with previous published data, compound 1 is identified as **caffeoyl ethyl ester** which was isolated and identified previously in *I. batatas* leaves[4]. But, it was isolated for the first time from the *I. carnea* species grown in Egypt.

Compound 2

Off-white fine powder (14.36 mg). It gave a blue fluorescence under short UV-light turned to blue colour with ferric chloride spraying reagent which indicate phenolic nature. Compound 2 presented a UV absorption spectrum in methanol (λ max: 242, 296, 325) that was characteristic of phenyl propene system. ^{1}H-NMR(600 MHz, CD_3OD, δ ppm): 7.5 (1H, d, *J*= 15.84 Hz, H-7), 7.01 (1H, d, *J*= 2.04 Hz, H-2), 6.91 (1H, dd, *J*= 8.25, 2.1 Hz, H-6), 6.75 (1H, d, *J*= 8.25 Hz, H-5), 6.2 (1H, d, *J*= 15.84 Hz, H-8). ^{13}C-NMR (150 MHz, CD_3OD, δ ppm): 171.36 (C-9), 149.68 (C-4), 147.19 (C-7), 147.04 (C-3), 128.03 (C-1), 123.04 (C-6), 118.57 (C-2), 116.69 (C-5), 115.27 (C-8). ^{1}H-NMR spectra of compound 2 showed an ABX spin coupling system of three resonances at δ 7.01 (d, *J*= 2.04 Hz), 6.91 (dd, *J*= 2.04 and 8.3 Hz), 6.75 (d, *J*=8.25 Hz) each integrated for one proton attributed to H-2, H-6 and H-5 respectively of 3,4-dihydroxy benzene ring together with two doublets signals with large *J* values at δ 7.5 and 6.2 (*J*= 15.84 Hz) for trans olefinic double bond of α, *β*-unsaturated system indicating caffeoyl structure. The ^{13}C-NMR spectra of compound 2 showed the presence of nine characteristic carbon signals of caffeoyl moiety at δ ppm: 171.36 (C-9), 149.68 (C-4), 147.19 (C-7), 147.04 (C-3), 128.03 (C-1), 123.04 (C-6), 118.57 (C-2), 116.69 (C-5) and 115.27 (C-8). HPLC chromatogram of compound 2 gave the same retention time as caffeic acid standard at the same conditions together with the previous identification by co-TLC. From the previous data and comparing those data with the published data[28], compound 2 was identified as **caffeic acid** which was isolated from the genus *I. batatas*[4,29,30] and *I. muricata*[30] and it is isolated for the first time from the leaves of *I. carnea* species grown in Egypt.

Compound 3 (A new natural product)

Canary yellow amorphous powder (14.5 mg). It gave a dark purple spot under long UV-light which appeared intensified yellow spot by ammonia vapour and turned to blue colour with ferric chloride spraying reagent indicating phenolic nature also it gave positive Molish's test, indicating it's glycosidic nature[31]. M.P:213°C, R_f:0.756 (S_3) UV: λ_{max}(nm): MeOH: 256, 353, NaOMe:270, 353, $AlCl_3$:269, 365, $AlCl_3$/HCl:269, 364, NaOAc:273, 404, NaOAc/H_3BO_3:260, 360,^{1}H-NMR(600 MHz, CD_3OD, δ ppm): δ ppm: 7.82 (1H, d, *J*= 2.04, H-2'), 7.68 (1H, d, *J*= 2.04, H-2''''), 7.56 (1H, dd, *J*= 1.8, H-6', H-6''''), 6.84 (1H, dd, J^1= 5.52, J^2= 1.8, H-5', H-5''''), 6.38 (1H, d, *J*= 2.76, H-8, H-8'''), 6.18 (1H, d, *J*= 2.04, H-6, H-6'''), 5.24 (1H, d, *J*= 7.56, H-1'' glucose), 5.15 (1H, d, *J*= 8.22, H-1'''' glucose), 3.83-3.32 (m) remaining sugars. ^{13}C-NMR(150 MHz, MeOH) δ ppm: 178.2 (C-4, 4'''), 164.71 (C-7, 7'''), 161.68 (C-5, 5'''), 157.43 (C-2, 2'''), 157.1 (C-9, 9'''), 148.61 (C-4', 4''''), 144.57 (C-3'), 144.47 (C-3''''), 134.42 (C-3), 134.26 (C-3'''), 121.84 (C-6'), 121.57 (C-6''''), 116.43 (C-2'), 116.19 (C-2''''), 114.74 (C-5'), 114.64 (C-5''''), 104.35 (C-10), 104.29 (C-10'''), 104.03 (C-1'' glucose), 102.92 (C-1'''' glucose), 98.53 (C-6, 6'''), 93.53 (C-8, 8'''), 77.05 (C-5''), 76.77 (C-5''''), 75.84 (C-3'', 3''''), 74.38 (C-2''), 73.75 (C-2''''), 69.86 (C-4''), 68.68 (C-4''''), 61.19 (C-6''), 60.59 (C-6'''').

UV spectral data in methanol of compound 3 showed two major absorption bands; band I at 353 nm and band II at 256 nm, which are characteristic for flavonol nucleus. The addition of sodium methoxide resulted in bathochromic shift in band I and band II which suggested a substituted hydroxyl group at C-4' and this shift resulted due to the presence of free hydroxyl group at C-3'. The presence of a bathochromic shift with increase in intensity of colour in band II on addition of sodium acetate compared with the same band in methanol suggested the presence of a free hydroxyl group at C-7. On addition of boric acid to sodium acetate showed no shift suggested the absence of any *ortho*-dihydroxyl groups. The bathochromic shift with increase in intensity of colour in band I on addition of alumminium chloride which was not affected by addition of hydrochloric acid indicated the presence of free hydroxyl group at C-5 and confirmed the absence of any *ortho*-dihydroxyl groups[32,33]. Compound 3 was also subjected to complete acid hydrolysis by treating 4-5 mg of compound 3 with 4 N HCl in aqueous methanol (50%) for 2 hours at 100 °C. The mixture was then extracted with ethyl acetate and the extract was subjected to CoPC investigation along with authentic aglycones yielding glucose moiety in the aqueous layer and unknown aglycone in the organic layer. The ^{1}H-NMR spectrum of compound 3 showed the presence of duplication in proton signals suggesting the presence of symmetrical biflavonoid structure. The aromatic region exhibited an ABX system for the biquercetin moiety at δ 7.82 (1H, d, *J*= 2.04, H-2'), 7.68 (1H, d, *J*= 2.04, H-2''''), 7.56 (1H, dd, *J*= 5.52, H-6', H-6''''), 6.84 (1H, dd, J^1= 8.3, J^2= 1.8, H-5', H-5'''' due to a 3', 3'''' and 4', 4'''' disubstitution of ring B1 and B2, and a typical *meta*-coupled pattern for H-8, 8''' and H-6, 6''' protons [6.38 (1H, d, *J*= 2.76, H-8, H-8'''), 6.18 (1H, d, *J*= 2.04, H-6, H-6''')]. Also, ^{1}H-NMR spectral data of compound 3 showed two symmetrical signals for two *β*-anomeric protons at δ 5.24 (1H, d, *J*= 7.56, H-1'' glucose), 5.15 (1H, d, *J*= 8.22, H-1'''' glucose) with the rest of the

symmetric remaining sugar protons that appeared as multiplets at δ (3.83 - 3.32) ppm gave evidence of a bis isoquercetin structure[30] with one glucose sugar moiety in each side of the symmetric biflavonoid structure. The ^{13}C-NMR spectra of compound 3, revealed a typical 15 ^{13}C-resonance signals for 3-*O*- substituted quercetin compound[34]. The duplication of signals confirmed the presence of bis-isoquercetin compound. ^{13}C-NMR spectra showed a ketanoic carbon at 178.2(C-4, 4''') and the acidic carbons at 164.71 (C-7, 7'''), 161.68 (C-5, 5'''), 148.61 (C-4', 4''''), 144.57 (C-3'), 144.47 (C-3''''). From ^{13}C-NMR spectrum, the two *β*-anomeric carbon resonance of sugars at 104.29 and 102.92 ppm for C-1'' and C-1'''' respectively, indicating the disaccharides *β*-glucoside nature of compound 3. The duplication of carbon resonance signals from 77.0589 to 60.5903 indicates the remaining carbons of two sugar moieties. The attachments was confirmed to be (4'-*O*-4'''') by comparing those data with structurally related compound[35,36] previously reported. Final confirmation was carried out by negative ESI-MS spectrum revealed molecular ion peak at m/z 909.32 [M-H]$^-$ ($C_{42}H_{38}O_{23}$), characteristic peaks were m/z 463.86 [M-isoquercetin]$^-$ ($C_{21}H_{19}O_{12}$) and m/z 300.22 [quercetin-2H]$^-$ ($C_{15}H_9O_7$), also this result is another confirmation for the symmetrical bi-isoquercetin structure of compound 3. Based on the UV spectra, ^{1}H-NMR, ^{13}C-NMR and negative ESI-MS data and by comparison with previously reported data of structurally related compound, the structure of compound 3 was identified as 3', 3'''', 5, 5''', 7, 7'''-*O*-*β*-D-Glucosyl-4', 4''''-biflavonoyl ether [4'-*O*-4'''' Bis-isoquercetin] (Ipomoeflavoside) which was identified for the best of our knowledge as a new compound in nature.

Compound 4

Yellow amorphous powder (158 mg). It gave a dark blue spot under long UV-light which appeared intensified yellow spot by ammonia vapour and turned to green colour with ferric chloride spraying reagent indicating phenolic nature also it gave positive Molish's test, indicating it's glycosidic nature[31]. M.P:190°C, Rf: 0.53 (S_3), UV: λ_{max}(nm): MeOH: 256, 358, NaOMe:270, 408, $AlCl_3$:269, 400, $AlCl_3$/HCl: 267, 362 NaOAc:270, 392, NaOAc/H_3BO_3:260, 375,^{1}H-NMR(500 MHz, DMSO, δ ppm): δ ppm: 7.57 (1H, d, *J*=2.1 Hz, H-2'), 7.54 (1H, dd, *J*= 9, 2.1 Hz, H-6'), 6.85 (1H, d, *J*= 9 Hz, H-5'), 6.39 (1H, d, *J*= 2.1 Hz, H-8), 6.2 (1H, d, *J*=2.1 Hz, H-6), 5.35 (1H, d, *J*= 7.5 Hz, H-1'' glucose), 4.4 (1H, brs, d, *J*= 2.0 Hz, H-1''' rhamnose), 3.16-3.65 (m, remaining sugar protons), 1.01 (3H, d, *J*=6.3 Hz, CH_3-6''' of rhamnose). UV spectral data showed two major absorption bands in methanol band I at 358 nm and band II at 256 nm, which indicate the presence of a flavonol nucleus[32,33]. Sodium methoxide made a bathochromic shift in band I with an increase in intensity, indicated the presence of a free hydroxyl group at position C-3' and C-4'. The addition of sodium acetate make a bathochromic shift in band II compared with the same band in methanol and the presence of a shoulder at 327 nm in sodium methoxide suggested the presence of a hydroxyl group at position C-7. The bathochromic shift in band I on addition of alumminium chloride indicated the presence of free hydroxyl group at position C-5, while the hypthochromic shift which occurred on the addition of hydro-chloric acid in band I indicated the presence of a free ortho-dihydroxyl groups in the B-ring[37]. Compound 4 was subjected to complete acid hydrolysis by treating 4-5 mg of compound 4 with 4 N HCl in aqueous methanol (50%) for 2 hours at 100 °C. the mixture was then extracted with ethyl acetate and the extract was subjected to CoPC investigation along with authentic aglycones yielding glucose and rhamnose moieties in the aqueous layer and quercetin in the organic layer. The ^{1}H-NMR spectrum of compound 4, the aromatic region exhibited an ABX coupling system at δ 7.57 (1H, d, *J*=2.1 Hz, H-2'), 7.54 (1H, dd, *J*= 9, 2.1 Hz, H-6'), 6.85 (1H, d, *J*= 9 Hz, H-5') due to a 3', 4' dihydroxy B ring, and a typical *meta*-coupled pattern for H-6 and H-8 protons [6.2 (1H, d, *J*=2.1 Hz, H-6) and 6.39 (1H, d, *J*= 2.1 Hz, H-8)] of 5, 7-dihydroxy A ring. In aliphatic region, the presence of rhamnosyl glucoside moiety was indicated from the presence of *β*-anomeric proton signal of glucoside moiety at δ 5.35 (*J*= 7.5 Hz) with a characteristic anomeric proton of terminal α-L-rhamnosyl at δ 4.4 together with signal of CH_3-6'' at δ 1.01 (6.3 Hz). From the ^{1}H-NMR data, by comparison with previously reported data[38] and by direct comparison with authentic sample on TLC and HPLC against reference standard**,** compound 4 was identified as Quercetin-3-*O*-α-L-1 C4-rhamnopyranosyl (1'''→6'') *β*-D-4C1-glucopyranoside (Rutin). Previously, Guan *et al.*, 2006[39] reported the presence of rutin in the tubers of *I. batatas.* Also, Furtado *et al.*, 2016[40] reported the presence of rutin in aqueous leaves extract of *I. asarifolia.* Thus, the rutin was isolated for the first time from the leaves of *I. carnea* species grown in Egypt.

Compound 5

It was isolated as dark red amorphous powder sparingly soluble in methanol. It gave a greenish yellow fluorescence spot under UV-light turned to red colour with methanolic $AgNO_3$ spray reagent[41]. In order to identify the lycopene, a few crystal of extracted lycopene was dissolved in concentrated sulfuric acid, imparting an indigo blue colour to the solution. In another test, by adding a solution of antimony trichloride in chloroform to a solution of lycopene in chloroform, an intense unstable blue colour appeared. These tests primarily proved the presence of

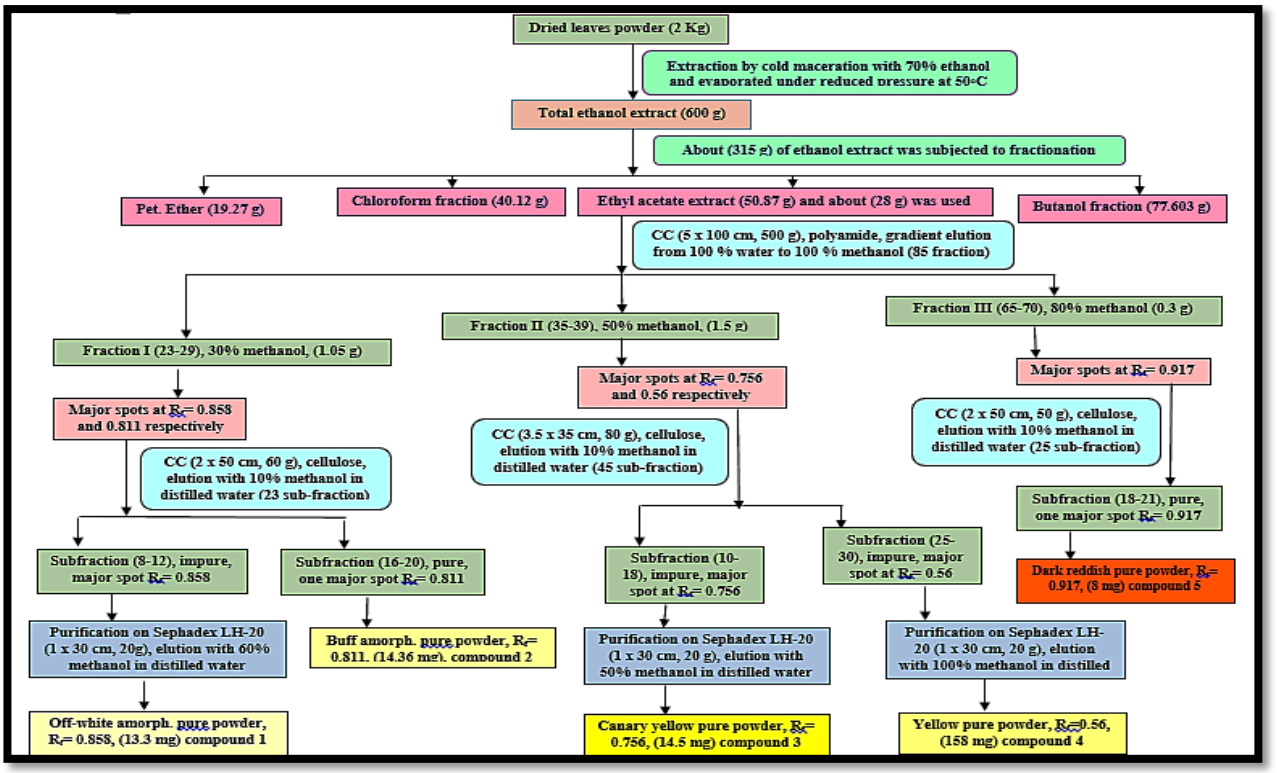


Figure 1: Scheme for extraction, chromatographic fractionation and purification of compounds isolated from the ethyl acetate fraction from leaves ethanol extract of I. carnea.

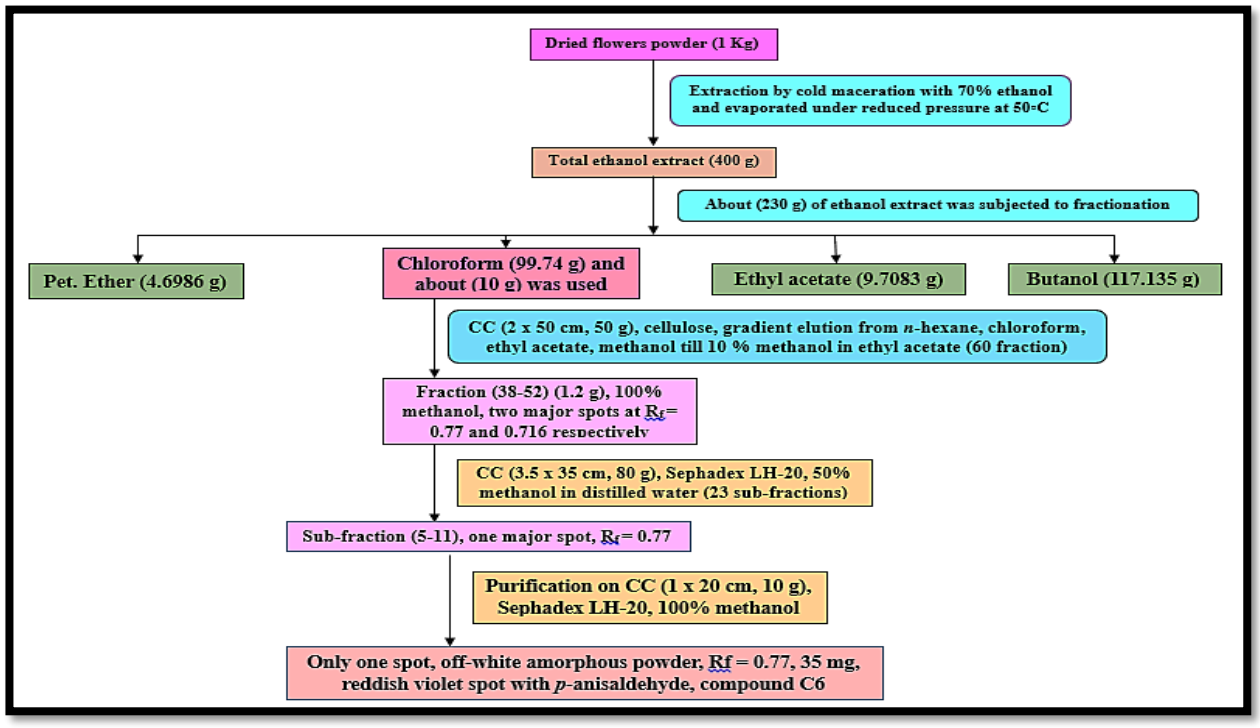
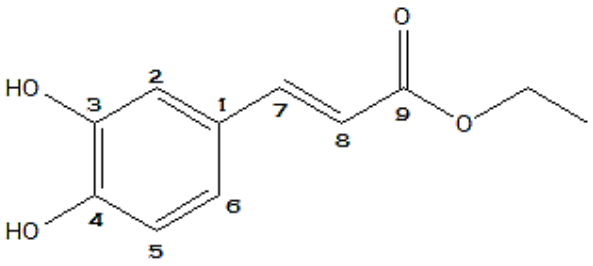
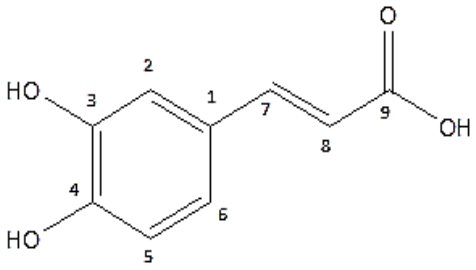


Figure 2: Scheme for extraction, chromatographic fractionation and purification of compounds isolated from chloroform fraction from flowers ethanol extract of *I. carnea*.

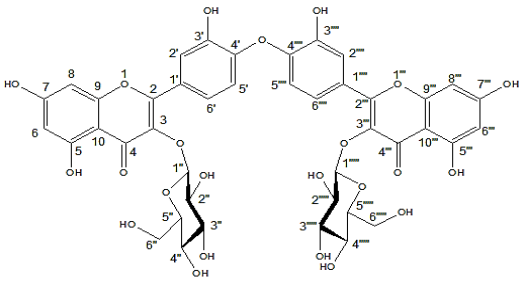
lycopene in the extract[42]. UV Spectrum of compound 5 showed the λmax at 446, 472 and 503nm, which is the maximum wavelengths of pure lycopene reported in the literatures[42]. Positive ESI-MS showed spectral data at *m/z* 534 [M^+], 465 [M-69], 442 [M-92], 135, 105[43]. The data was identical to those published for lycopene and the identification was confirmed by direct comparison with authentic sample on TLC chromatogram against authentic



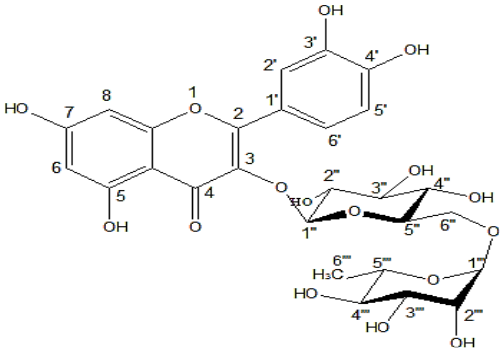
Compound 1: Caffeoyl ethyl ester



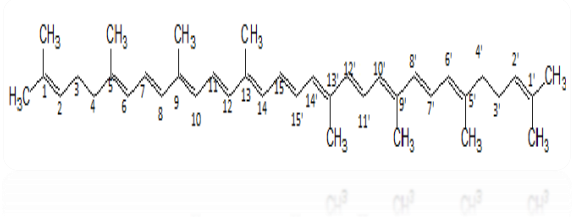
Compound 2: Caffeic acid



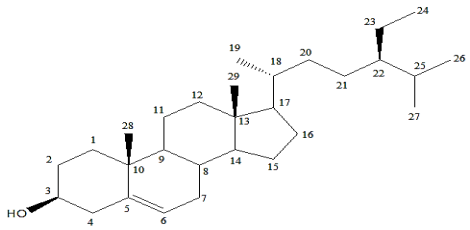
Compound 3: 3', 3"", 5, 5"', 7, 7"'-*O*-*β*-D-Glucosyl-4', 4""-biflavonoyl ether [4'-*O*-4"" Bis-isoquercetin] (Ipomoeflavoside)



Quercetin-3-*O*-α-L-1 C4-rhamnopyranosyl (1"'→6") *β*-D-4C1-glucopyranoside (Rutin



Compound 5: Lycopene



Compound 6:*β*-Sitosterol

Figure 3: Chemical compounds isolated from fractionation of the ethanol leaves and flowers extracts.

Table 1: Antioxidant activity assayed by DPPH method of the 70 % ethanol extracts of the leaves and the flowers (expressed as % Inhibition ± SD).

Ethanol extract	% Inhibition* ± SD					IC_{50} (mg/ml)* ± SD
	2 mg/ml	3 mg/ml	4 mg/ml	6 mg/ml	8 mg/ml	
Leaves	47.798 ± 1.207	61.264 ± 0.397	65.702 ± 0.793	79.137 ± 0.618	85.63 ± 0.385	1.608 ± 0.026
Flowers	19.768 ± 0.481	29.475 ± 0.243	37.897 ± 0.687	68.932 ± 0.575	80.006 ± 0.655	4.861 ± 0.038

*Average of the three determinations.

Table 2: Anti-oxidant effect of leaves and flowers ethanol extract of *Ipomoea carnea* Jacq and Vitamin E drug on GSH diabetic female albino rats (n=6).

Group	Blood Glutathione (mg/dL)	% of change	Relative Potency
Normal	36.4 ± 1.2	-	-
Diab. Control	21.8 ± 0.4*	40.109	60.387
Diab. Treated with leaves ext. (100 mg/kg)	35.8 ± 0.6	1.648	99.168
Diab. Treated with flowers ext.(100 mg/kg)	35.1 ± 0.7	3.571	97.22
Diabetic + Vitamin E(7.5 mg/kg)	36.1 ± 1.1	0.841	100
Compound B (Rutin) (20 mg/kg)	33.2 ± 0.5	8.79	91.96

*Statistically significant from control group at P<0.01.
Potency calculated relative to vitamin E.

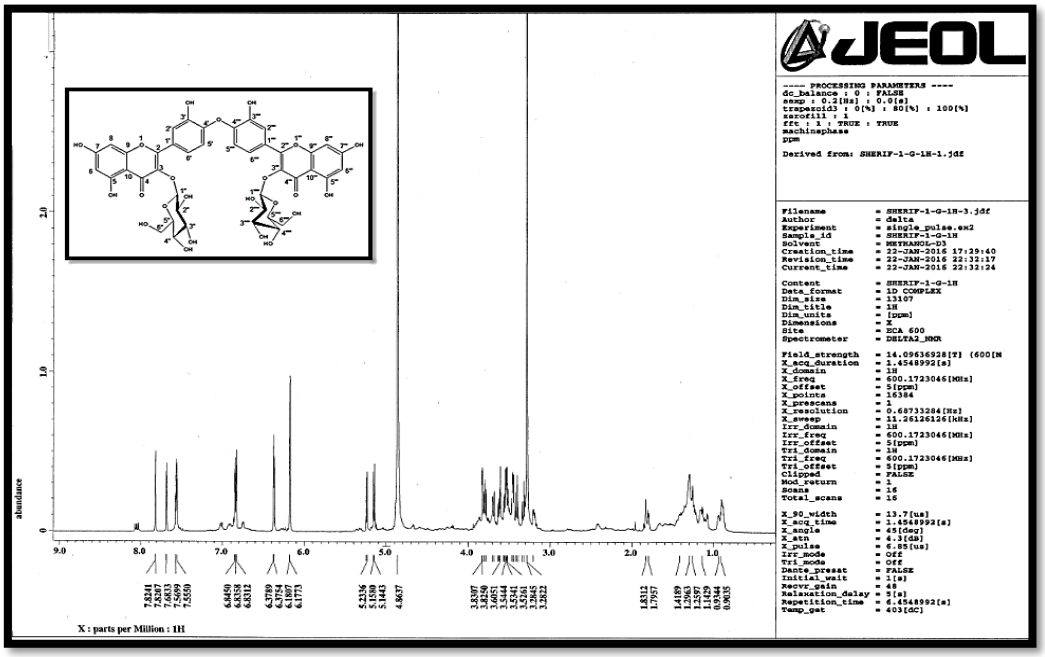


Figure 4: ^{1}H-NMR spectrum of compound 3.

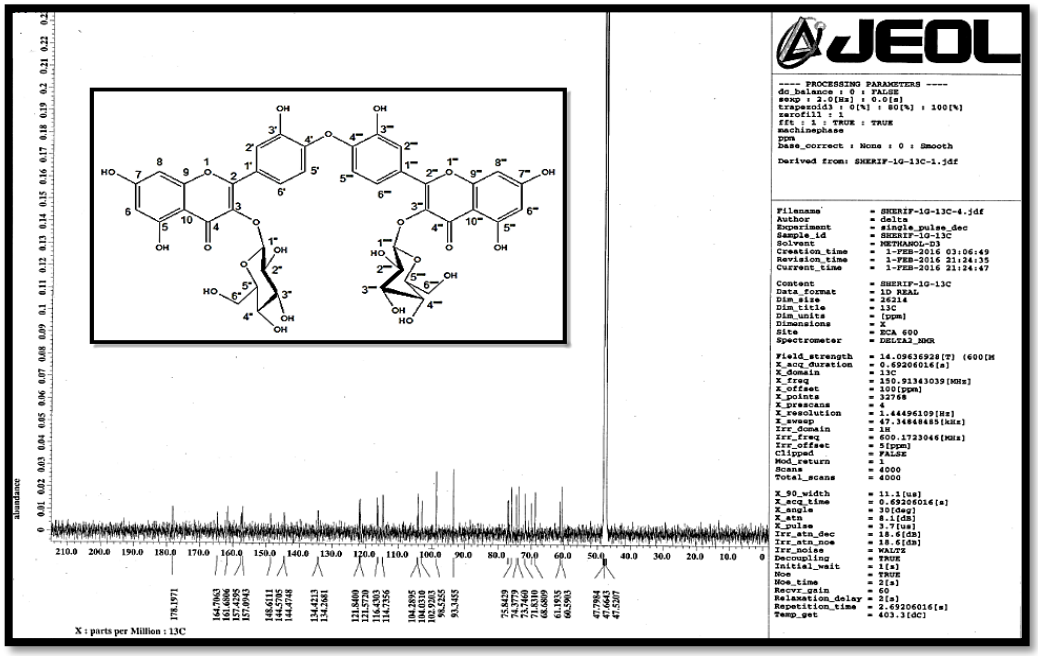


Figure 5: ^{13}C-NMR spectrum of compound 3.

reference standard. So, from the previous data, compound 5 was identified as lycopene. Previously, Dagawal, 2015[44] reported that lycopene was identified in leaves of *I. alba* L. So, lycopene was first isolated from the family Convovulaceae.

Compound 6

Off-white amorphous powder (35 mg), it gave a blue fluorescence in UV light which appeared as reddish violet spots by spraying with *p*-anisaldehyde which steroidal compound [45]. M.p. :140°C, R_f:0.77 (S_3), ^{1}H-NMR(400 MHz, DMSO, δ ppm) : 5.33 (1H, m, Olefenic proton H-6), 3.47 (1H, m, H-3), 1.51 (3H, t, *J*= 14.34, H-29), 1.24 (1H, s, H-19), 0.95 (1H, d, *J*=10.32, H-21), 0.9 (1H, d, *J*= 6.48, H-26), 0.84 (1H, d, *J*= 6.84, H-27), 0.66 (1H, s, H-18), ^{13}C-NMR(400 MHz, DMSO, δ ppm): 140 (C-5), 121.69 (C-6), 70.58 (C-3), 56.64 (C-17), 50.07 (C-9), 61.57 (C-14), 39.78 (C-20), 39.57 (C-12), 31.89 (C-25), 40.41 (C-13), 42.33 (C-4), 25.9 (C-28), 24.33 (C-11), 19.09 (C-19), EI-

Table 3: Chronic antihyperglycemic activity of leaves and flowers ethanol extracts of *Ipomoea carnea* Jacq plant compared to metformin in female albino rats

Group	Zero time	2 Weeks		4 Weeks	
	Mean ± S.E.	Mean ± S.E.	% of change	Mean ± S.E.	% of change
Prediabetic healthy group	82.7 ± 2.1	81.9 ± 2.5	-	82.4 ± 1.9	-
Diab. non treated	249.8 ± 8.2	251.3 ± 6.8	-	266.4 ± 5.7	-
Diab. Treated with ethanol leaves extract (100mg/kg)	258.1 ± 8.1	191.6 ± 7.2*	25.765 %	128.5 ± 3.8*	50.21%
Diab. Treated with ethanol flower extract (100 mg/kg)	261.4 ± 7.2	203.2 ± 5.8*	22.264 %	151.4 ± 4.9*	42.081 %
Compound C4 (rutin) (20 mg/kg)	251.3 ± 6.8	218.6 ± 5.9*	13.01 %	178.4 ± 6.3*	29.01%
Diab. Treated with Metformin (100 mg/kg)	267.2 ± 8.4	171.8 ± 7.6*	35.703 %	88.3 ± 3.6*	66.953 %

*Statistically significant different from control group at P<0.01.

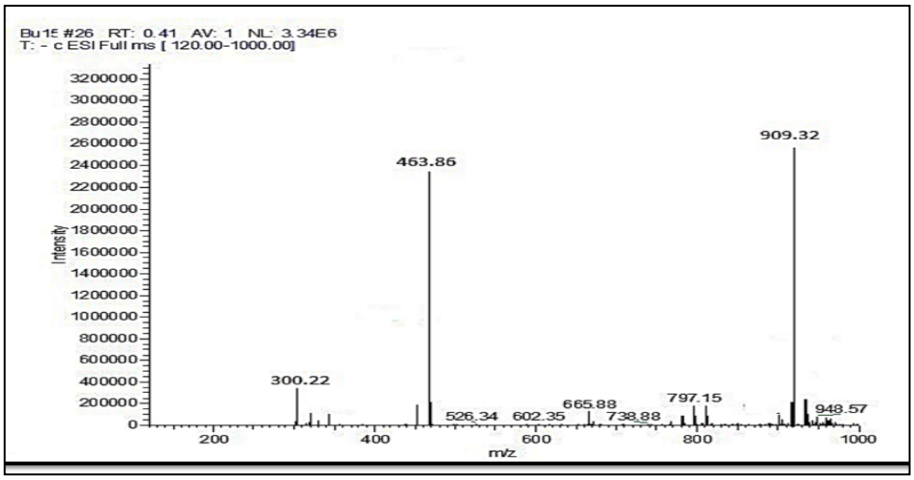


Figure 6: Negative ESI-MS spectrum of compound 3.

Table 4: Effect of leaves and flowers ethanol extracts of *I. carnea* Jacq and silymarin drug on serum enzymes level (ALT, AST and ALP) in liver damaged rats (n=6).

Group	AST (u/l)				ALT (u/l)				ALP (KAU)			
	zero	7d	72h	7d	zero	7d	72h	7d	zero	7d	72h	7d
Control	38.8± 1.1	38.3± 1.3	139.7± 4.9*	145.6± 6.2*	37.1± 1.2	36.8±1.5	146.1± 7.8*	151.2± 6.8	7.4± 0.1	7.3± 0.1	51.3± 3.4*	58.2± 3.8*•
Leaves Eth. Ext.	38.1± 1.6	37.9± 1.4	65.5± 2.1*	52.8± 2.2*•	38.1± 1.9	37.9±1.1	69.8± 2.5*	56.6± 2.1*•	7.5± 0.1	7.4± 0.1	26.2± 0.9*	15.3± 0.8*•
Flowers Eth. Ext.	38.5± 1.3	38.1± 1.4	77.2± 2.6*	63.7± 2.5*•	36.6± 1.5	36.1±1.3	82.3± 2.7*	79.8± 2.5*•	7.6± 0.1	7.5± 0.1	34.3± 2.1*	23.6± 0.8*•
Silymarin (25mg/kg)	37.6± 1.5	37.2± 1.2	53.2± 2.2*	37.2± 1.4	39.4± 1.7	39.1±1.8	63.7± 1.8*	38.9± 1.4*	7.8± 0.1	7.6± 0.1	18.9± 0.6*	7.6± 0.1*

*Statistically significant from zero group at P <0.01

•Statistically significant from 72h after CCl_4 at P <0.01

KAU: King-Armestrong Unit for Alkaline Phosphatase

MS: 414 ($C_{29}H_{50}O$), 396, 381, 367, 329, 314, 303, 281, 273, 255, 213, 199, 185, 173, 159, 145, 133, 119, 105, 91, 81, 57, 55.

The ^{1}H-NMR of compound 6 revealed the presence of an olefinic proton at δ 5.33 (m, *br*, H-6) at H-6, and a multiplet proton shift at δ 3.47 correspond to H-3, in addition to six methyl groups, two singlet proton shifts at δ 0.65 and 1.24 assigned for Me-18 and Me-19 respectively, three doublet proton shifts at δ 0.9, 0.84, 0.96 assigned for Me-26, Me-27 and Me-21 respectively and one triplet proton shift at δ 1.51 assigned for Me-29.The ^{13}C-NMR has shown recognizable signals at 140.92 ppm and 121.69 ppm which are assigned (C-5) and (C-6) double bonds respectively. The value at 19.41 ppm corresponds to angular carbon. The EI-MS spectrum revealed weak molecular ion peak at m/z 414 ($C_{29}H_{50}O$), characteristic peaks were m/z 396 (M-H_2O) and at m/z 367 that corresponds to (M-47) or loss of HO^{+}= CH-CH_3. A prominent peak appeared at m/z 329 which is characteristic for sterols with C_5 double bond. Other ion peaks were m/z 273, 255 due to the formation of

Table 5: Antimicrobial activity and minimum inhibitory concentration (MIC) of leaves and flowers ethanol extracts of *Ipomoea carnea* Jacq.

Microorganism	Leaves extract		Flowers extract		Standards					
	DD M ± SD	MIC (µg/ml)	DD M ± SD	MIC	Ampicillin DD M ± SD	Ampicillin MIC	Gentamycin DD M ± SD	Gentamycin MIC	Amphotericin B DD M ± SD	Amphotericin B MIC
Gram (+) bacteria										
Streptococcus pneumonia	16.7 ± 0.58	31.25	20.4 ± 0.58	3.9	23.8 ± 0.58	0.98	-	-	-	-
Bacillus subtillis	18.4 ± 0.63	15.63	22.8 ± 1.2	0.98	32.4 ± 1.5	0.49	-	-	-	-
Gram (-) bacteria										
Pseudomonas aeurginosa	-	-	-	-	-	-	21.8 ± 0.63	1.95	-	-
Escherichia coli	13.3 ± 1.5	125	19.3 ± 0.72	3.9	-	-	19.9 ± 0.72	3.9	-	-
Fungi										
Aspergillus fumigatus	18.6 ± 1.2	7.81	18.3 ± 0.63	15.63	-	-	-	-	23.7 ± 1.2	0.98
Candida albicans	-	-	-	-	-	-	-	-	25.4 ± 0.72	0.98

DD, agar disc diffusion method. Diameter of the inhibition zone (mm) including the disc diameter of 6 mm; MIC, minimum inhibitory concentration; values are given as µg/mL; NT, not tested; M ± S.D., mean of three

Table 6: Potential cytotoxicity of leaves and flowers ethanol extracts of *I. carnea* Jacq against liver tumer cell lines.

Cell line	Extract	Conc.µ/ml	SF	IC_{50}	IC_{50} of Doxorubicin µg/ml
Liver tumor cell line HEPG2	Ethanol leaves extract	0.0	1.000	23	4.73
		5.0	0.611		
		12.500	0.534		
		25.000	0.496		
		50.000	0.580		
	Ethanol flower extract	0.0	1.0	_	
		5.0	0.840		
		12.500	0.748		
		25.000	0.550		
		50.000	0.603		

Sf = Surviving fraction.
IC_{50} = Dose of the extract which reduces survival to 50%.

carbocation by *β* bond cleavage of side chain leading to the loss of molecular ion, the most characteristic peak was at m/z 55 (base peak). ^{1}H-NMR, ^{13C}-NMR, EI-MS spectral data, by comparison with published data[46] and by comparison of authentic sample against reference standard on TLC, compound 6 could be identified as *β*-Sitosterol. It was isolated from the petroleum ether leaves extract of *I. carnea*[3]. However, it is the first report of isolation of *β*-Sitosterol from chloroform flowers extract of *I. carnea* species. The larvicidal activity of *I. carnea* stem against Culex quinquefasciatus reported in the literature (Khatiwora et al., 2014)[27] may be due to the presence of *β*-Sitosterol. Also, Sen *et al*., 2012[47] reported that *β*-Sitosterol has higher antimicrobial activity against Escherichia coli followed by Staphylococcus aureus, Pseudomonas aeurginosa, and Klebsiella pneumonia

Acute toxcicity (LD_{50})

The LD_{50} of the leaves and flowers ethanol extracts were 4.2 and 3.1 g/kg body weight respectively, which indicates that they are slightly toxic according to Ahmed, 2015[48] who indicated that LD_{50} ranged between 500-5000 mg/kg body weight are considered slightly toxic. This toxicity may be due to the presence of polyhydoxylated alkaloids in leaves, flowers and seeds of *I. carnea* Jacq[3-49].

Determination of antioxidant activity

It was found that the ethanol extracts of both the leaves and the flowers showed significant antioxidant activity expressed as IC_{50} (1.608 mg/ml and 4.861 mg/ml) respectively. The ethanol extract of the leaves parts showed higher antioxidant activity with a lower IC_{50} value (1.608 mg/ml) this result was in agreement with Hasan *et al.,* 2015[3] who reported that the leaves ethanol extract has more antioxidant activity than flowers and seeds extracts. Ambeiga and Jeyaraj, 2015[50] noticed that the flowers extract of *I. carnea* has potent scavenging activity with lower IC_{50} (99.4 µg/ml) nearly equal to the standard ascorbic acid. Also, Adsul *et al.,* 2012[6] reported that the

Table 7: Potential cytotoxicity of leaves and flowers ethanol extracts of *I. carnea* Jacq against breast tumer cell lines.

Cell line	Extract	Conc.µ/ml	SF	IC_{50}	IC_{50} of Doxorubicin µg/ml
Breast tumor cell line MCF7	Ethanol leaves extract	0.0	1.000	7.4	4.13
		5.0	0.536		
		12.500	0.400		
		25.000	0.373		
		50.000	0.427		
	Ethanol flower extract	0.0	1.0	44.2	
		5.0	0.636		
		12.500	0.600		
		25.000	0.587		
		50.000	0.482		

Sf = Surviving fraction.
IC_{50} = Dose of the extract which reduces survival to 50%.

Table 8: Potential cytotoxicity of leaves and flowers ethanol extracts of *I. carnea* Jacq against colon tumer cell lines.

Cell line	Extract	Conc.µ/ml	SF	IC_{50}	IC_{50} of Doxorubicin µg/ml
Colon tumor cell line HCT116	Ethanol leaves extract	0.0	1.000	35	3.73
		5.0	0.611		
		12.500	0.534		
		25.000	0.496		
		50.000	0.580		
	Ethanol flower extract	0.0	1.0	_	
		5.0	0.840		
		12.500	0.748		
		25.000	0.550		
		50.000	0.603		

Sf = Surviving fraction
IC_{50} = Dose of the extract which reduces survival to 50%.

acetone extract of *I. carnea* leaves has more pronounced scavenging activity with lower IC_{50} (195 µg/ml) than ethanol extract (215 µg/ml), ethyl acetate extract (270 µg/ml) and leaves itself (398 µg/ml) compared with ascorbic acid (IC_{50} 25 µg/ml). The antioxidant property may be related to the polyphenols and flavonoids present in the extract[50].

The antioxidant activity

Being a chronic disease, diabetes mellitus reduces serum glutathione level, so, the diabetic animals show a high percentage of change from control non diabetic group = 40.109%. Diabetic animals treated with vitamin E (7.5 mg / kg b. wt.) restored the level of blood glutathione and showed lowest percentage of change from control non diabetic group = 0.841%. Leaves and flowers ethanol extracts showed significant increase in blood glutathione level in diabetic rats. Oral administration of leaves ethanol extract of *I. carnea* (100 mg / kg b. wt.) showed larger increase in blood glutathione level in diabetic rats and thus showed lower percentage of change from the control non diabetic group = 1.648% than flowers ethanol extracts = 3.571%, generally leaves extracts are slightly more potent than flowers extract. Compound 4 (rutin) (20 mg/kg b. wt.) showed significant increase in blood glutathione level in diabetic rats and thus showed lower percentage of change from the control non diabetic group = 8.79. These results are in accordance with Khan *et al.*, 2015[9] since they had proved that *I. carnea* leaves extract possess significant antioxidant activity but it was the first time to establish the antioxidant activity for flowers ethanol extract and the compound 4 (rutin) of *I. carnea* in Egypt. The antioxidant activity of *I. carnea* leaves ethanol extract may be attributed to the presence of high concentration of compound 4 (rutin) in leaves ethanol extract more than flowers ethanol extract[51].

Antihyperglycemic

It was found that the leaves and flowers ethanol extracts (100 mg/kg b. wt) found to have significant antihyperglycemic activity on alloxanized diabetic rats. The ethanol leaves extract of *I. carnea* reduced the blood glucose level in alloxan induced diabetic rats after two and four weeks by 25.765% and 50.21% repectively; while the same dose of the ethanol flowers extract reduced it by 22.264% and 42.081% respectively. Also, it was found that the compound 4 (rutin) (20 mg/kg b. wt) isolated from ethyl acetate fraction of leaves ethanol extract reduce blood glucose level in alloxan induce diabetic rats after two and four weeks by 13.01% and 29.01% respectively. These results are in accordance with previous literatures[7,9]; since they had proved that *I. carnea* leaves extract possess significant antihyperglcemic activity but it was the first time to investigate the antihyperglycemic activity for flowers ethanol extract of *I. carnea* in Egypt. The

antihyperglycemic potential of *I. carnea* leaves extract may be due to the high concentration rutin[52]. The ethanol leaves extract induced higher antihyperglycemic activity of the blood sugar level than ethanol flowers extract in the alloxan induced diabetic rats and rutin was found to be responsible for the antihyperglycemic activity.

Hepatoprotective activity

The results showed that the leaves and flowers ethanol extracts of *I. carnea* significantly had protective activity against liver injuries as well as oxidative stress, resulted in decreased serum biochemical parameters such as AST, ALT and ALP in intoxicated rats. Pretreatment with leaves ethanol extract significantly reduced the raised serum biochemical parameters more than flowers ethanol extract nearly to the normal levels and to the silymarin standard drug level. These results are in accordance with Gupta *et al.,* 2012 and Gupta *et al.,* 2013;[53,8] since they had proved that *I. carnea* leaves extract possess significant hepatoprotective activity but it was the first time to investigate the hepatoprotective activity for the flowers ethanol extract of *I. carnea* in Egypt. The leaves ethanol extract of *I. carnea* has proved more significant hepatoprotective activity than flowers ethanol extract which rationalizes the importance of this herb and this could be attributed to the high content of rutin in the plant which found to have powerful hepatoprotective activity[54].

Antimicrobial activity

The leaves and flowers ethanol extracts showed significant antimicrobial activity against *Streptococcus pneumonia, Bacillus subtillis*, *Escherichia coli* and *Aspergillus fumigatus* while showing no activity against *Candida albicans* and *Pseudomonas aeurginosa*. Diameter of the inhibition zone (mm) of The leaves and flowers ethanol extracts against G +ve bacteria ranged from 16.7 to 22.8 mm, the flowers ethanol extract showed stronger antibacterial activity against *Bacillus subtillis and Streptococcus pneumonia* (22.8 and 20.4 mm. respectively) than the leaves ethanol extract (18.4 and 16.7 mm. respectively) compared to Ampicillin. The flowers ethanol extract showed stronger effect against *Escherichia coli* than leaves ethanol extract (19.3 and 13.3 mm. respectively) compared to Gentamycin. The leaves ethanol extract showed slightly more antifungal activity against *Aspergillus fumigatus* than the flowers ethanol extract (18.6 and 18.3 mm. respectively) compared to Amphotericin B. The minimum inhibitory concentration (MIC) of the ethanol extracts from the leaves and flowers of *I. carnea* Jacq is shown in Table 5. Gram (+ ve) bacteria: The MIC of leaves and flowers extracts ranged from (0.98 – 31.25 µg/ml); the lowest concentration was (0.98 µg/ml) for flowers ethanol extract against *Bacillus subtillis* followed by *Streptococcus pneumonia* with concentration (3.9 µg/ml compared to Ampicillin. Gram (- ve) bacteria: the flowers extract showed lower concentration against *Escherichia coli* than leaves extract (3.9 and 125 µg/ml respectively) compared to Gentamycin. Fungi: the leaves extract showed lower concentration against *Aspergillus fumigatus* than flowers extract (7.81 and 15.63 µg/ml respectively) compared to Amphotericin B. From the previous literature, it was found that the ethanol leaves extract exhibited activity against *Pseudomonas aeurginosa* which was in the agreement of the current results[6]. Hasan *et al.,* 2015[3] reported that methanol extract of the leaves showed higher antibacterial activity than the methanol extracts of the flowers and seeds against *Staphylococcus aureus, Escherichia coli, Candida albicans and Penicillium chrysogenum* which was in agreement of the current results except for *C. albicans* which found to have slightly more potent antifungal activity in leaves than in flowers. Also, Alam and Chowdhury, 2015[55] reported the antimicrobial activity of *n*-hexane, dichloromethane and methanol extracts of *I. carnea* leaves against *Pseudomonas aeurginosa, Staphylococcus aureus, Escherichia coli, Salmonella paratyphi and Klebsiella pneumonia* which found to have significant antimicrobial activity in to amoxicillin as reference drug standard. The higher antimicrobial activity of flowers ethanol extract of *I. carnea* may be attributed to the anthocyanin composition which was identified by HPLC/MS/MS of flowers[56]. Also, Sen *et al.,* 2012[47] reported that *β*-Sitosterol has higher antimicrobial activity against *Escherichia coli* followed by *Staphylococcus aureus, Pseudomonas aeurginosa*, and *Klebsiella pneumonia* and this antimicrobial activity almost equivalent to the standard drug gentamycin and this result was found to be in accordance with the result of this study. From the assay of *β*-sitosterol which was found to contain (17.085 mg/g dry wt.) in flowers chloroform extract compared with (0.463 mg/g dry wt.) in leaves chloroform extract and was isolated from flowers chloroform extract of *I. carnea*, It was suggested that the powerful antibacterial activity of *I.carnea* flowers extract resulted from the high content of *β*-sitosterol. Flowers ethanol extract showed higher antibacterial activity than leaves ethanol extract while leaves ethanol extract showed slightly higher antifungal activity than flowers ethanol extract.

Cytotoxic activity

The ethanol leaves extract showed the highest cytotoxic activity against the breast cancer cell line, with (IC_{50}: 7.4 µg/ml) while it showed weak cytotoxic effect on liver and colon cancer cell lines (IC_{50}:23 and 35 µg/ml) respectively, comparing to the doxorubicin as standard. The ethanol flowers extract showed weak cytotoxic activity against breast cancer cell lines (IC_{50} 44.2 µg/ml) while showing no anticancer activity against colon and liver cancer cell lines. Hasan *et al.,* 2015[3] reported that the leaves methanol extract of *I. carnea* Jacq showed the highest cytotoxic activity compared to methanol extracts of flowers and seeds against breast and liver cancer cell lines and this result was found to be in agreement with the result of our study but it was the first time to investigate the cytotoxic activity of leaves and flowers ethanol extracts against colon cancer cell line (HCT116) for this species in Egypt. It was found that caffeic acid which was isolated and identified in ethyl acetate fraction of ethanol leaves extract inhibit growth and modify estrogen receptor and insulin-like growth factor 1 receptor levels in human breast cancer

and this found to be the purpose for the strongest activity of leaves against breast cancer cell lines[57]. Also, Gloria *et al.,* 2014[58] reported that carotenoids as lycopene which was isolated from leaves ethanol extract of *I. carnea* showed significant decrease in the number of viable breast cancer cells on treatment with lycopene (Table 6-8).

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