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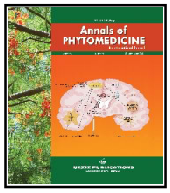

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Isolation, characterization and evaluation of antioxidant and anticancer activities from isolated components of *Ixora chinensis* Lam. flowers

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Abstract

This research sought to isolate potent phytoconstituents from *Ixora chinensis* Lam. flowers and evaluate their usefulness to treat cancer, as well as reducing oxidative radicals. Several solvents were used in the extraction of the flowers, including petroleum ether, ethyl acetate, and ethanol. In this study, compounds were separated from extracts using column chromatography, and their properties were determined using ^{13}C NMR, 1H, IR, and mass spectrometry. Compounds were tested for antioxidant activity *in vitro* and biochemical activity. Breast cancer (MCF-7) and colon cancer (CACO-2) cell lines were used to test the extract's anticancer potential. The extract was found to have anticancer activity. On the other hand, *in vivo* anticancer action was tested on animals by measuring the levels of ferritin and CEA in their bloodstream. Oleanolic acid (1), 5,7-dihyroxy-,3'4'-dimethoxy flavone from petroleum ether extract, (2), 3-acetoxy-olean-12-ene-30-oic acid (3) from ethyl acetate extract, and masticadienoic acid (4) were isolated from ethanolic extract of *I. chinensis* flowers, and these compounds were discovered for the first time in the flowers of this plant. Results exhibited that the separated compounds (1-4) shown considerable anticancer activity against both the MCF-7 and the CACO-2 cell lines. Following clinical research, it is possible that I. chinensis flower extract may be employed as an alternative medication therapy in the treatment of breast and colon cancer.

1. Introduction

On a global scale, cancer is the second greatest cause of death. Due of its variability at the tissue level, it is difficult to determine its particular diagnosis, which in turn makes determining the efficacy of treatment a huge obstacle as well (Hassanpour and Dehghani, 2017). It is the third most common cancer after lung and breast cancer. Surpassing lung cancer in terms of cancer-related mortality, on average, 5% of people with colon cancer also have another primary malignancy. However, breast cancer is the most frequent cancer among women worldwide, accounting for 30% of all cancer diagnoses (Weissman *et al.,* 2019).

Food and free radical exposure are major environmental factors in the development of cancer. There has been a dramatic reduction in morbidity and mortality in ER positive breast cancer due to the development of selective estrogen replacement modulators (SERMs) and aromatase inhibitors. However, these medicines might cause major side effects such as thromboembolism, uterine cancer, cataracts, and perimenopausal symptoms (Chaudhary *et al.,* 2015). New medicines are required due to the existing state of cancer research and dismal prognosis necessitates. Cancer cells have abnormal cell cycle progression and apoptosis resistance that causing excessive cell proliferation. Cancer cells lose their ability to negatively regulate the cell cycle and develop the ability to evade apoptosis (Labi and Erlacher, 2015). Many compounds found in medicinal plants have been shown to have anticancer activity by regulating signalling cascades related to cell cycle regulation and /or apoptosis in cancer cells in reported works (Wanner *et al*., 2021). Inhibiting the course of cancer cell cycle is thought to be a promising therapeutic method for treating tumours and metastases.

In the family Rubiaceae, *I. chinensis* is a tropical evergreen shrub endemic to southern China and Malaysia that grows as an evergreen shrub. Sanskrit words for “Iswara” and “Shiva deity” are used to describe the genus “Ixora”, however, “chinesis” is a Latin word that means “Origin of China,” which refers to the seed’s origin. Medicinal purposes in the area are served by harvesting this plant. A popular ornamental plant, it is prized for its long-lasting blossoms, which attract a lot of attention. When used after childbirth or for bronchial issues, a decoction of the root is beneficial. Traditionally, an infusion of fresh flowers has been used to treat incipient tuberculosis as well as bleeding.

For centuries, it has been used to cure cancer, amenorrhea, and high blood pressure among other ailments. There is a fatty acid known as ixoric acid, which is found in the seed oil of the plant (Minquan, 1990). Takeda *et al.* (1975), extracted two new iridoid glucosides from the twigs and leaves of *I. chinensis.* These compounds are ixoroside (1) and ixoside (7,8-dehydroforsythide) (2), which were

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combined with geniposidic acid (3) to form ixoroside (1). Despite this, little is known about the flower of *I. chinensis*. Because of this, a methodology was developed for the study in order to isolate components from *I. chinensis* flower extract, and then evaluate their efficacy as a cancer treatment. This is a kind of first study to investigate the traditional value of *I. chinensis* flowers in a scientifically rigorous setting.

2. Materials and Methods

2.1 Plant material

The flowers of *I. chinensis* were collected from a Nursery at Tirupati, Andhra Pradesh, India. Dr. K. Madhava Chetty, Plant Taxonomist, Department of Botany, Tirupati authenticated the collected plant material. Voucher specimen with the No. 1591 was kept at Malla Reddy College of Pharmacy, Maisammaguda, Dhullapally, Secunderabad, Telengana, India.

2.2 Extraction and purification of plant material

I. chinensis were used for extraction; the fine powder (4 kg) was subjected Soxhlet apparatus and extracted with ethyl acetate, petroleum ether, and ethanol in 15 batches. The extractives were subjected to evaporation to remove solvent and concentrated through flash evaporator. It was then weighted and yield was determined; it was found to be 14 g.

2.3 Preliminary phytochemical investigation

The extracts of different solvents were subjected to investigation of secondary metabolites present in them. To identify secondary metabolites in *I. chinensis*, the extract was evaluated according to the methods of Harborne (1998).

2.4 Column chromatography

TLC plates (20 x 20 cm; Merck, silica gel 60-F254) and aluminium sheets were utilized for compounds isolation in a column chromatography. In this experiment, we only employed analytical grade solvents (Sigma Aldrich 32213). One-hour NMR spectra were recorded on Varian NMR instruments at 300 MHz, using $CDCl_3$ as the solvent; ^{13}C NMR spectra were recorded on Bruker instruments at 300 MHz; and the mass spectrum was recorded on an EI-MS, data on E:ISO/21184-1.QGD and IR spectra was recorded on Thermo Nicolet Nexus 670.

2.4.1 Isolation and characterization of petroleum ether extract

We prepared an *I. chinensis* flower petroleum ether extract (31 g) by dissolving it in a tiny amount of the same solvent, then applying it to silica gel and then charging it into a column. The open end of the column was plugged with cotton to keep it from drying out overnight. As soon as the column was fully saturated, the elution process began with a 90:10 and 85:15 graded mixture of petroleum ether and acetone. Fraction A was isolated from these elutions. TLC established the identity of the sole component eluted from petroleum ether: acetone (90:10) as MeOH: $CHCl_3$ (MeOH: $CHCl_3$; 7:3). (82 mg). Compound ICFPE-1 (compound 1) was the product's designation.

2.4.2 Isolation and characterization of ethyl acetate extract

Excessive amounts of 33 g of the concentrated ethyl-acetate extract were mixed with 15 ml of chloroform, which was then used to chromatographically separate the silica gel 60-120 mesh LR from the solution. Chloroform was used in a graded mixture of 90:10, 85:15, 80:20, 70:30, 60:40, and 50:50 to extract 24 sub-fractions from the column. It was determined by TLC that the silica gel column eluted with EtOAC: CHCl3 (80:20) (3.4 g) contained a single component from sub-fractions 02 to 16 (3.4 g). Compound ICFEA-2 (78 mg) (compound 2) was produced by evaporation of solvents and named as such. Using silica gel and ethyl acetate: chloroform (60:40), a single chemical was eluted from sub-fractions 18 to 24 (2.6 g) and this was confirmed by TLC as the source of the new compound. A solid compound was formed due to reaction and elute was gathered and condensed into a crude residue (73 mg). It was designed as Compound ICFEA-3 (compound 3).

2.4.3 Isolation and characterization of ethanol extract

N-Butanol is eluted from the column first, followed by methanol. Column was run with an n-butanol: methanol mixture of 95:5, 90:10, 85:15, 80:20, and finally 100 % methanol, which resulted in 12 sub-fractions of n-butanol:methanol mixture. TLC verified the identity of a single component eluted from the n-butanol: methanol (80:20) mixture (chloroform: methanol; 9:1). Re-crystallization of those elutes in acetone followed collection and concentration to crude residue. Compound ICFEt-4 (compound 4) was the designed to the product.

2.5 Pharmacological screening of *I. chienesis* flower extracts

2.5.1 *In vitro* antioxidant activity

The antioxidant activity of the *I. chinensis* flower was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl), superoxide radical, and nitric oxide radical scavenging methods (Olaoye *et al.* 2021).

2.5.1.1 DPPH radical scavenging activity

The DPPH technique was employed to determine oxidative stress reducing property of *I. chinensis* extracts. After mixing separate aliquots of extracts ranging from 50 to 250 µg/ml in methanol; 2 ml of DPPH (0.1 mM) solution was added to the extracts, and the mixture was allowed kept overnight. In a dark place, the solutions were allowed to stand for 30 to 45 min before being measured at 517 nm (UV-1800 UV-spectrophotometer, Shimadzu Corp. Japan) against a blank solution containing an equal amount of DPPH and methanol (Wali *et al.*, 2019). In order to calculate the radical scavenging efficiency per cent (RSA per cent) of DPPH, the following equation was used:

$$\% \text{ Inhibition} = \left(\frac{A0 - A1}{A0}\right) \times 100$$

Equation (1) where,

A_o = absorbance of the control and

A_1 = absorbance of the test extracts.

2.5.1.2 Scavenging activity for superoxide radical

Robak and Gryglewski (1988) described the method for determination of superoxide anion scavenging activity was followed in this study. A PMS-NADH system was used to create superoxide radicals, which were then measured using the reduction of NBT. Briefly, sodium phosphate buffer (100 mm, pH 7.4, 3 ml) was used in this experiment to create superoxide radicals, which contained NBT solution (1 ml, 150 µm), NADH (1 ml, 468 µm), and varied concentrations of the

CRE (25-250 µg / ml) in water. PMS solution (60 µm) was added in the mixture to start the reaction and then incubated for 5 min at 25°C to measure its absorbance on spectroscopy. L-ascorbic acid was used as a positive control. The amount of NBT reduction that can be assessed as reduction absorbance of reaction mixture that was directly proportion to the reduction of superoxide radical scavenging activity by *I. chinensis*. The percentage of superoxide radical scavenging activity was determined using following formula:

$$\text{Superoxide radical scavenging activity (\%)} = \left(\frac{A0 - A1}{A0}\right) \times 100$$

where A_0 is the absorbance of the control and

A_1 is the absorbance of *I. chienesis* or the standard sample.

2.5.1.3 Nitric oxide radical scavenging method

The extracts were tested for their ability to neutralise free radicals, according to Sreejayan and Rao (1997). Griess reagent was used to measure nitric oxide radicals; it was a mixture of sulphailamide (1 %), N-1-napthylethylenediamine dihydrochloride (0.1 %) and orthophosphoric acid (2 %). At physiological pH, sodium nitroprusside creates nitric oxide radicals which interfere with oxygen to produce nitrite ions. Sodium nitroprusside (10 m Mol /l, 3 ml) was added to the extract in the concentration of 50-250 µg/ml. It was then incubated for 150 min at 25°C and then centrifuged. The absorbance (UV-1800 UV-spectrophotometer, Shimadzu Corp. Japan) was measured at 546 nm by adding Griess reagent (500 µl) (Equation 1). Nitric oxide radical scavenging activity was indicated by low optical density values. The experimental DPPH, ABTS, and NO scavenging activity assay methods were used to generate IC_{50} values.

2.5.2 Effect of *I. chinensis* on biochemical parameters

In order to estimate the amount of antioxidant enzymes present in liver tissues, biochemical assays were performed. The livers of the breast cancer and colon cancer generated mice were removed in order to determine the level of lipid peroxidation in the blood. In order to make dissection easier, the liver tissues were thoroughly cleansed in the presence of ice-cold, pH-7.2 PBS solution before being sliced into small pieces by means of a heavy-duty blade. The tissues were then homogenised in cold PBS using a glass homogenizer tube, and spun using a Remi cool centrifuge to remove any leftover debris (8000 rpm for 30 min). Supernatants were then employed to determine various biochemical properties.

2.5.2.1 Lipid peroxidation

Fresh sample was prepared using ethyl acetate extract (0.25 ml). It was necessary to dilute acetic acid (20 %, 1.5 ml) with pH 3.5 to make the solution volume to 4 ml, and then diluted the solution volume to 4 ml using deionized water. A light pink color was achieved by heating the mixture at 95! for 60 min in a water bath. A UV - visible spectrophotometer at 532 nm was then used to measure the absorbance of the combination and then allowed to cool. There was a 156 nm MDA level in the wet tissue, it was expressed in terms of nmol /g wet tissue (Ohkawa *et al.* 1979).

The following formula was used to determine MDA concentration:

Absorbance/156 × [total volume (4 ml)/sample volume (0.25 ml)] × dilution factor (10) × 1000

2.5.2.2 Reduced glutathione (GSH)

GSH concentration in the tissues was détermined according to the method of Ellman *et al.* (1959). For the purpose of preparing the standard graph, reduced glutathione was used as a reference standard. To 2 ml of 0.1 M potassium phosphate buffer with a pH of 8.4, 0.1 ml of standard or sample solution was added, followed by adding 0.5 ml of DTNB, and diluted to 3 ml with distilled water to obtain the desired volume. A UV-visible spectrophotometer was used to detect the absorbance at 412 nm, and the GSH content was calculated using a standard graph after 10 min of incubation at room température.

GSH = DA412 /min (sample) X dil / DA412/min (1 nmole) X vol DA412 /min (sample) = slope generated by sample (after subtracting the values generated by the blank reaction).

DA412/min (1 nmole) = slope calculated from standard curve for 1 nmole of GSH

dil = dilution factor of original sample, vol = volume of sample in the reaction in ml.

2.5.2.3 Glutathione peroxidase (GPx)

The rate of glutathione oxidation at 420 nm was used to determine the total amount of glutathione peroxidase in the sample. Measurements were made in real-time using spectrophotometric techniques. A 0.1 ml solution of 10 mM sodium azide, 0.2 ml of homogenate, 0.02 ml of EDTA, and 0.1 ml of 2.5 Mm H_2O_2 were added to the reaction mixture and the tubes were centrifuged at 2000 rpm for 15 min. An immediate colour change occurred after the addition of three millilitres of disodium hydrogen phosphate (DHP) and one millilitre of dithiothyronine (DTNB) to the supernatant. Glutathione peroxidase activity was estimated as follows:

mole of oxidised glutathione /min /mg protein

GPx = Test O.D × 20 × Total volume/0.11 × Sample volume × mg protein per ml

2.5.2.4 Catalase (CAT)

Catalase (CAT) is a key enzyme in the breakdown of H_2O_2 to water and oxygen in all living creatures. CAT activity in liver epidermis was found to be 22 % lower in malignant conditions like cancer (Mason *et al*., 1960). Because of the increase in intracellular H_2O_2 that resulted from the decrease in CAT activity, there was a more favourable intracellular environment for DNA damage and the development of cancer (Asaduzzaman *et al.,* 2010). Tissue breakdown of hydrogen peroxide (H_2O_2) was utilised to measure catalase activity (Aebi *et al*., 1974). To create the reaction mixture, 50 l of sample and 1 ml of H_2O_2 were added to a 1.95 ml phosphate buffer with a pH of 7. To determine activity in units of K/min, the following formula was used to compute absorbance changes at 240 nm over the course of one min at a time, with 15 sec intervals between readings.

$$\text{Catalase activity (K/min)} = (1/\Delta t) \times \ln (S_1/S_2) = (2.3/\Delta t) \times \log (S_1/S_2)$$

where, $\Delta t = t_2 - t_1$ (time interval)

S_1 and $S_2 = H_2O_2$ concentrations at times t_1 and t_2.

2.5.3 Acute toxicity study

The fixed dosage method of CPCSEA, OECD guideline no. 423, was used to conduct an acute toxicity study to determine the lethal dose

(LD_{50}) of *I. chinensis* flowers ethyl acetate extract. Mice (Swiss albino mice) were employed in the study weighing 20-25 g of both sexes. A total of four groups of three mice each were formed from the group of three mice. 5 mg/kg (Group I) (oral); Group II: 50 mg/kg (oral); Group-III received 300 mg/kg (orally), while Group-IV received 2000 mg/kg (oral).

2.5.4 Anticancer activity

2.5.4.1 Cell lines

The National Institute of Nutrition provided the cancer cell lines MCF-7 breast cancer and CACO-2 colon cancer for testing *in vivo* anticancer effectiveness. FBS medium and 10 % glycerol-calf serum were added to the harvested cells, as well as the cells themselves.

2.5.4.2 Experimental animals

Mice (Swiss albino mice) were employed in the study weighing 20-25 g of both sexes for screening anticancer activity of extract against breast cancer and colon cancer. The National Institute of Nutrition (NIN) in Hyderabad provided the animals. A steady room temperature was maintained for all of the animals. Observations were made during the experiments in accordance with the Institutional Protocols for Animal Care. The Institutional Animal Ethical Committee accepted the experimental procedure with the registration number MRCP/CPCSEA/IAEC/2015-16/PHD/1 (IAEC).

2.5.4.3 Experimental protocol

The anticancer activity of *I. chinensis* flowers was determined using ethyl acetate extract. The animals were separated into 5 groups with each group consisting of 6 animals. One per cent tween 80 (1 ml/100 g) was given to the animals in the control group for 30 days. Group 2 animals received cancer cell lines on the first day of the trial. One day after implanting a cancer cell line, Group 3 mice received 5-fluorouracil intraperitoneally every three days for the next 30 days. After receiving a cancer cell line transplant, animals in groups 4 and 5 were given 200 mg/kg and 400 mg/kg p.o. dosages of *I. chinensis* extract on the third day of treatment, respectively, until the end of the experiment on the 30th day.

2.5.4.4 Tumor transplantation

Female mice were implanted with 0.2 ml of MCF-7 cell lines (2×10^6 cells /mouse) subcutaneously under the mammary fat pads to induce breast cancer. In order to induce colon cancer in mice, 0.2 ml of CACO-2 cell lines (2106 cells /mouse) were injected intraperitoneally into the mice.

2.5.4.5 Serum parameters

A little ether anaesthetic was used to sacrifice the animals on 31st day of the experiment. Carotid artery bleeding was used to collect blood. A Remi cood centrifuge was used for centrifugation of the blood for 15 min at 4000 rpm. Ferritin and carcino embryonic antigens were measured in serum (CEA).

2.5.4.6 Histopathological estimation

On day 31 of the experiment, all animals were anaesthetized with light ether and their breast and colon tissues were removed, sectioned with a microtome and stained with hematoxyline and eosin after being fixed in 10 % formalin solution. To examine the slides for histological changes, they were examined using a light microscope at a magnification of 40 times.

2.6 Statistical analysis

In order to perform the statistical analysis, we used Graph pad prism 8.02 (Graph pad Software, San Diego, California). It is all provided as the mean ± SEM. Following an unpaired t-test for statistical significance, Dunnet's posthoc approach was used to assess the data for statistical significance. There was a statistically significant difference between the experimental group and the control group at $*p<0.05$, $**p<0.01$, and $***p<0.001$.

3. Results

3.1 Phytochemical investigation of *I. chinensis* flowers extracts

Phytochemical tests on *I. chinensis* flower extracts are shown in Table 1. Extracts from the flowers contained alkaloids, glycosides, steroids, tannins, flavonoids, carbohydrates, and triterpenoids. However, all the three solvent extracts, ethanolic flower extract had the highest concentration of phytochemicals. However, all solvent extracts lacked amino acids and proteins.

Table 1: Preliminary qualitative chemical investigation of different extracts of *I. chinensis* flowers

S No.	Chemical test	Pet. ether extract	Ethyl acetate extract	Ethanol extract
1	Alkaloids	–	–	+
2	Glycosides	–	+	+
3	Steroids	+	–	+
4	Amino acids	–	–	–
5	Proteins	–	–	–
6	Tannins	–	–	+
7	Flavonoids	–	+	+
8	Carbohydrates	–	+	+
9	Triterpenoids	+	+	+

'+' =Positive '-'= Negative

3.2 Identification and characterization of isolated compounds

ICFPE-1 : IR (KBr, cm^{-1}) **:** 3402.52 (O-H stretching), 2931.15 and 2853.32 (CH stretching in CH_3 and CH_2 groups), 1727.34 (C=O, stretching), 1407.40 (CH_2, bending), 1256.66 (alkaline, CH_3 bending), 1074.56 (C-O stretching of secondary alcohol), 815.85 (Exocyclic CH_2); ^{1}HNMR**:** 10.35 (s, 1H, -COOH group H-28), 5.35 (d, 1H, vinylic proton, H-12), 3.35 (s, 1H, OH group, H-3), 2.80 to 1.50 (m, -CH_2 and -CH group), 1.24 (m, 3H, -CH_3 group, H-25), 1.13 to 1.03 (m, 9H, 3×CH_3 group, H-23, 24 and 27), 0.98 to 0.72 (m, 2×CH_3 group, H-29 and 30). ^{13}C NMR ($CDCl_3$, 125 MHz): Table 2; $C_{30}H_{48}O_3$; EIMS m/z: 456 [M^+], 479 [M+Na]. From the spectral data compound ICFPE-1 was ascertained as **"Oleanolic acid" (3β-hydroxyolean-12-en-28-oic acid).** Figure 1 shows the structure of isolated compound-1**.**

ICFEA-2 : IR (KBr, cm^{-1})**:** 3339.72 (-OH group, stretching), 2923.57, 2852.90 (C-H, stretching) 1737.24 (C=C, stretching), 1611.24, 1534.90 (C=O, stretching), 1448.45, 1418.36 (CH_2, stretching), 1037.39 (C-O-C, stretching), 951.01 to 756.61 (C=C, deformation); ^{1}HNMR ($CDCl_3$, 500 MHz): δ 12.83 (1H, br, s, OH group, H-5), δ7.37 (1H, d, H-6´) , δ 6.90 (1H, d, H-5´), δ 6.82 (1H, d, H-2´), δ 6.71 (1H, s, H-3), δ 6.14 (1H, s, H-8), δ 5.32 (1H, br, s, OH group, H-7), δ 3.80 to 3.81 (6H, s, –OCH_3 group); ^{13}C NMR ($CDCl_3$, 125 MHz): Table 2; ($CDCl_3$, 125 MHz): Table 1; $C_{17}H_{14}O_6$; EIMS *m/z*: 314 [M^+], 337[M + Na]. From the spectral data compound ICFEA-2 was ascertained as **"Quercetin"**. Figure 1 shows the structure of isolated compound-2**.**

ICFEA-3: IR (KBr, cm^{-1})**:** 3415.62 (O-H stretching), 2925.37 and 2853.35 (CH stretching in CH_3 and CH_2 groups), 1686.32 (C=C stretching), 1487.39 (C-H deformation in CH_3), 1379.12 (C-H deformation in gem dimethyl) 1166. 82 (C-O stretching of secondary alcohol), 746.16 (Exocyclic -CH_2); ^{1}H NMR: 10.82 s, 1H, -COOH group H-28), 6.32 (m, 1H, vinylic proton H-1), 6.01 (m, 1H, vinylic proton, H-2), 5.65 (m, 1H, vinylic proton , H-9), 2.42 and 1.21 (m, CH_2 and CH group protons), 1.23 (m, 2×CH_3 group, H-23 and 24), 1.25 (m, 9H, 3×CH_3 group, H-25, 26 and 27), 0.99 to 0.98 (m, 2×CH_3 group, H-29 and 30); ^{1}HNMR ($CDCl_3$, 500 MHz): δ 10.82 (s, 1H, COOH group H-28), 6.32 (m, 1H, vinylic proton, H-1), 6.01 (m, 1H, vinylic proton, H-2), δ 5.65 (m, 1H, vinylic proton , H-9), δ 2.42 to 1.21 (m, protons of triterpenoid skeleton), δ 1.23 (m, 2×CH_3 group, H-23 & 24), δ 1.25 (m, 9H, 3×CH_3 group, H-25, 26 & 27), δ 0.99 - 0.89 (m, 2×CH_3 group, H-29 & 30). ^{13}C NMR ($CDCl_3$, 125 MHz): Table 2; $C_{30}H_{42}O_4$; EIMS m/z: 466 [M^+], 489 [M+Na]. From the spectral data compound ICFEA-3 was ascertained as **"3,12-dioxo-olene 1,9-diene 28-oic acid"**. Figure 1 shows the structure of isolated compound-3**.**

ICFEt-4: IR (KBr, cm^{-1})**:** 3477.09 (O-H stretching), 2992.21 and 2850.60 (CH stretching in CH_3 and CH_2 groups), 1637.36 (C=C stretching), 1379.06 (C-H deformation in CH_3), 1379.66 (alkane, CH_3 bending), 1134. 66 (C-O stretching of secondary alcohol), 871.87 (exocyclic -CH_2); ^{1}HNMR: 10.39 (s, 1H, -COOH group H-28), 6.48 (m, 1H, olefinic proton H-25), 5.41 (d, 1H, vinylic proton, H-7) 3.54 (m, 1H, H-3), 2.42 and 1.22 (m, CH_2 and CH group protons), 1.96 (m, CH_3 group, H-27), 1.52 to 0.99 (m, 12H, 4×CH_3 group, H-18, 19, 20, 22), 0.99 to 0.88 (m, 2×CH_3 group, H-29 and 30); ^{13}CNMR ($CDCl_3$, 125 MHz): **Table 2;** ($CDCl_3$, 125 MHz): Table 1; $C_{30}H_{48}O_3$; EIMS *m/z*: 456 [M^+], 479[M + Na]. From the spectral data compound **ICFEt-4** was ascertained as **"Masticadienoic acid"**. Figure 1 shows the structure of isolated compound-4.

Table 2: ^{13}C-NMR Spectral data of isolated compounds (1- 4) *I. chinensis* flowers

Carbon No.	ICFPE-1	ICFEA-2	ICFEA-3	ICFEt-4
1	29.2	-	128.3	29.0
2	26.7	163.6	143.6	37.0
3	71.7	104.4	203.2	82.1
4	37.1	182.1	56.1	39.9
5	42.5	153.9	52.9	51.1
6	18.9	131.3	22.5	28.8
7	29.7	157.5	33.3	121.3
8	29.8	95.0	40.9	148.8
9	38.6	151.1	162.1	55.5
10	21.7	106.5	35.8	37.1
11	46.5	-	119.8	26.2
12	121.7	-	200.7	34.4
13	141.9	-	43.7	48.1
14	39.1	-	40.3	150.1
15	25.6	-	26.9	30.2
16	24.2	-	31.6	19.2
17	50.1	-	33.6	17.8
18	31.7	-	35.1	47.1
19	34.3	-	38.5	-
20	26.29	-	25.1	-
21	36.1	-	35.5	33.2
22	22.9	-	30.6	-
23	-	-	-	16.7
24	-	-	-	39.3
25	-	-	-	143.2
26	-	-	-	128.5
1'	-	122.3	-	-
2'	-	108.8	-	-
3'	-	149.7	-	-
4'	-	149.0	-	-
5'	-	111.7	-	-
6'	-	121.1	-	-
COOH	179.2	-	180.3	170.7
CH_3	19.7	-	17.1	21.1
CH_3	19.6	-	17.2	17.7
CH_3	20.2	-	21.3	33.5
CH_3	18.4	-	20.7	16.7
CH_3	18.2	-	15.7	17.8
CH_3	27.3	-	27.3	19.3
CH_3	27.2	-	37.2	19.2

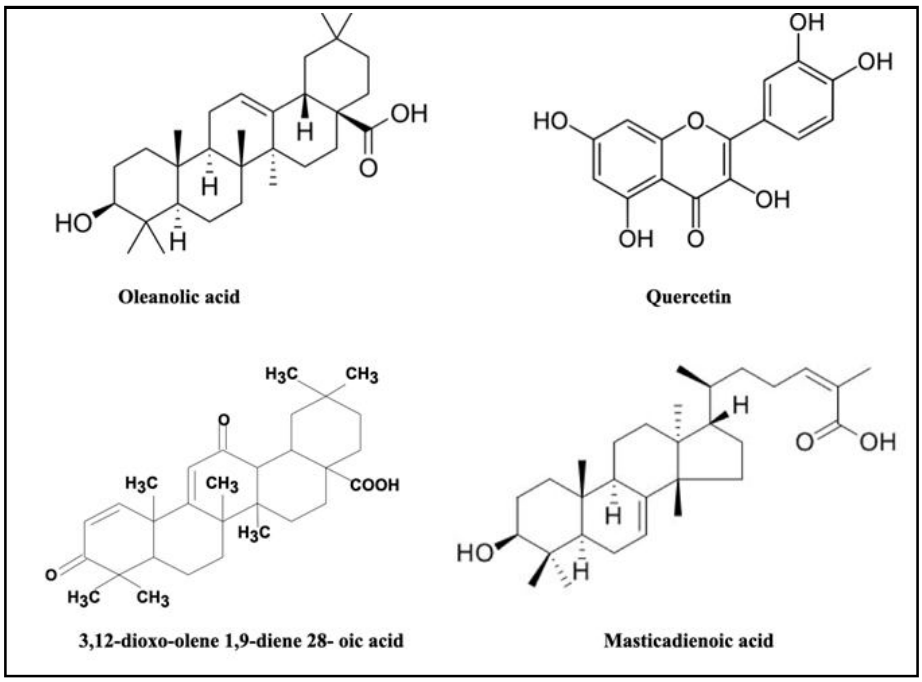

Figure 1: Structure of compounds isolated from plant extracts.

3.3 *In vitro* antioxidant activity

Antioxidant activity of *I. chinensis* floral isolates is shown in Table 3. In this study, the compounds (2-3) were found to have the greatest effect on oxidative radical inhibition among all the isolated compounds. Compound 3 (77.90 ± 3.32) had the highest DPPH radical scavenging activity, while compound 2 (75.96 ± 2.32) had the highest nitric oxide radical scavenging activity. However, of all the groups examined, ascorbic acid, the standard drug was the most significant (Figure 2).

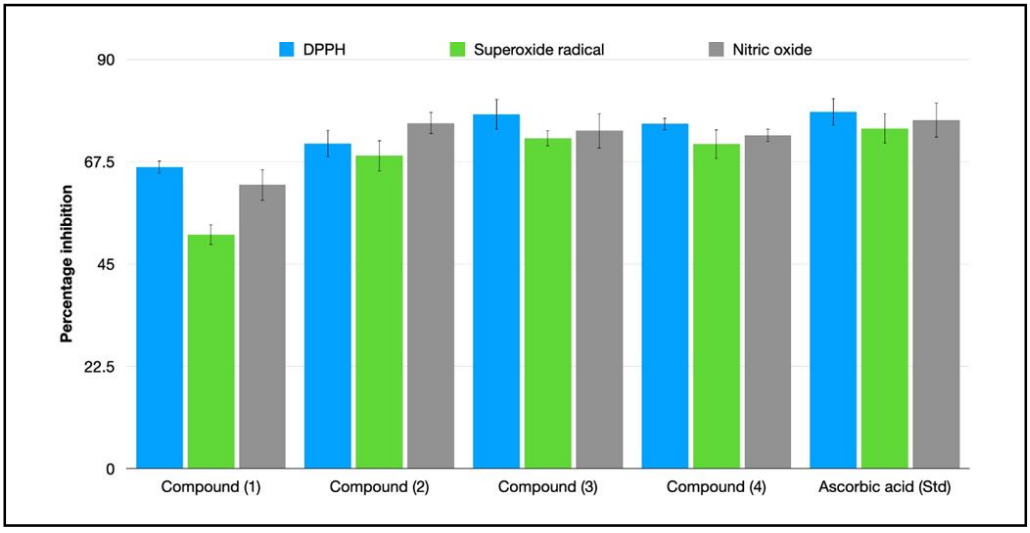

Figure 2: Percentage inhibition of isolated compounds from *I. chinensis* flowers.

3.4 Effect of *I. chinensis* on biochemical parameters

Isolated compounds from *I. chinensis* flowers treated with MCF-7 and CACO-2 cell lines reveal biochemical enzymatic levels in Tables 4 and 5. The biochemical enzymatic level of cancer cell lines was greatly restored by all of the compounds (1-4). MDA, catalase, GSH, and GPx biochemical enzyme levels were significantly recovered by compounds 1 (75.4 ± 1.64 nmol/g), compound 3 (0.41 ± 0.15 µm/min / mg), compound 2 (13.12 ± 0.02 µm/min/mg) and compound 3 (7.51 ± 0.27 µm/min/mg), respectively, in comparions to the control group (Table 4) on MCF-7 cell line (Figure 3). For MDA, catalase, GSH, and GPx on CACO-2 cell line; the highest biochemical enzymatic levels were reported for compound 1 (82.4 ± 2.3 nmol/g), compound 3 (0.43 ± 0.027 µm/min/mg), compound 3 (12.3 ± 0.073 nmol/min/mg) and the compound 3 (7.18 ± 0.141 µm/min/mg) (Figure 4). However, in both MCF-7 and CACO-2, the standard drug 5-FU was most significant in restoring all biochemical parameters.

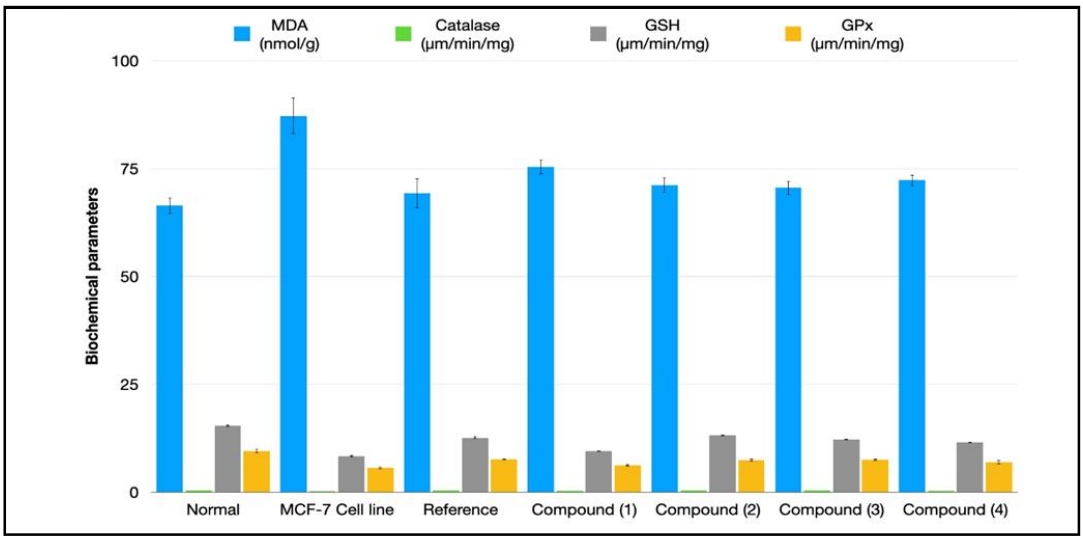

Figure 3: Effect of *I. chinensis* isolated compound on biochemical enzymes in MCF-7 cancer cell lines.

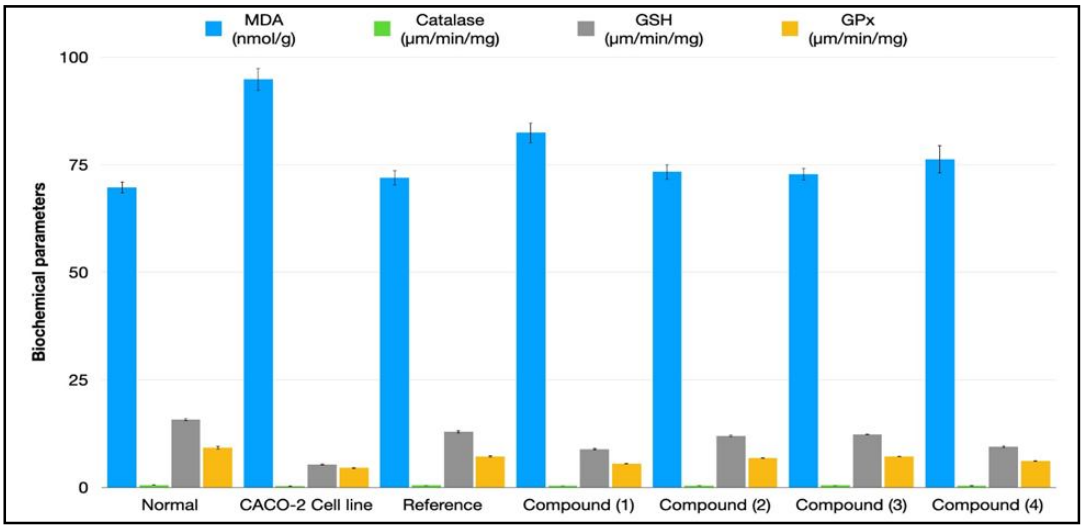

Figure 4: Effect of *I. chinensis* isolated compound on biochemical enzymes in CACO-2 cancer cell lines.

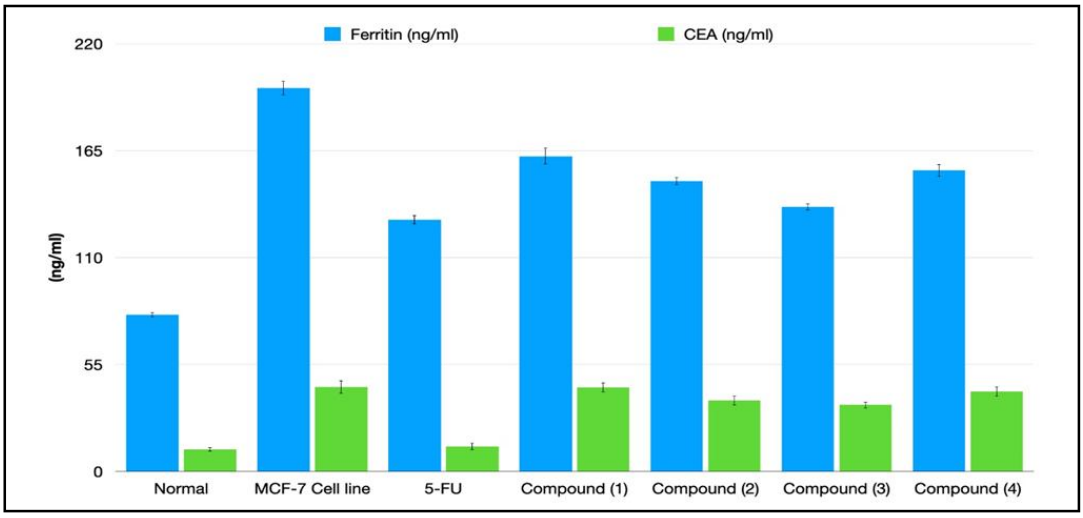

Figure 5: Effect of *I. chinensis* flower extracts on ferritin and CEA in breast cancer.

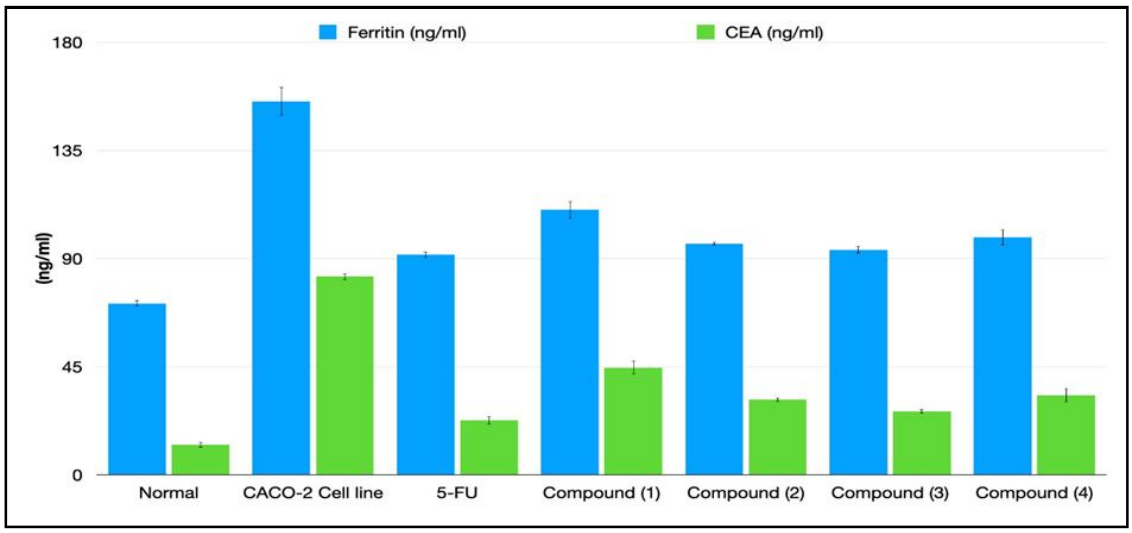

Figure 6: Effect of *I. chinensis* flower extracts on ferritin and CEA in colon cancer.

3.5 Acute toxicity study

Even at the highest dose of 2000 mg/kg body wt, no mortality was reported. However, autonomic responses increased in amplitude with increasing dose.

3.6 Anticancer activity

Anticancer activity on ferritin and CEA levels in breast (Figure 5) and colon cancer (Figure 6), respectively, by compounds (1-4) on animals. Animal ferritin and CEA levels were significantly improved by all of the study drugs. Nevertheless, the 5-FU standard group was most important in recovering ferritin (129.5 ± 2.3 ng/ml) and CEA (12.9 ± 1.75 mg/ml) levels.

4. Discussion

In the present study, the flower extracts of *I. chinensis* were evaluated for antioxidant and anticancer activities. The phytochemical study of the flowers extracts showed that ethanol solvent extract had more number of secondary metabolites as compared to petroleum ether and ethyl acetate. The ethanol solvent has good solubility profile to be used for extraction of plant secondary metabolites (Ragunathan and Jayaprakash, 2016). Our study, was in agreement with the findings of Akanji *et al*. (2018) reported highest number of phytoconstituents from *I. chinensis* in ethanolic extract. The isolation of flowers extracts using different solvents revealed four isolates in our study *i.e.,* ICFPE-1, ICFEA-2, ICFEA-3, and ICFEt-4. ICFPE-1 gave positive Liebermann-Burchardt and anisaldehyde test, pale green powder with formula $C_{30}H_{48}O_3$ on the basis of positive-ion peak EIMS (m/z); 456 [M^+]. IR spectra absorption peak was 2931.15 and 2853.32 cm^{-1} (CH stretching in CH_3 and CH_2 groups) (Elvira *et al.,* 2014). The ^{1}HNMR spectrum of compound 1 exhibited the tertiary methyl groups in between the δ 01.13 to 0.72 on an oleanane skeleton. One protron of doublet of doublet was found at δ 3.35 and vinyl proton of triplet at δ 5.35 were given H-12, position, signifying an olea12-ene skeleton (Gohari *et al.,* 2009). The carboxyl (-COOH group) signal emerged at δ 179.2 in the ^{13}C NMR spectrum. According to the results, the spectral data was indistinguishable from that of the acid.

ICFEA-2 showed positive response for "Shinnoda test, yellow color crystals. The combined spectra data analysis using ^{1}H, ^{13}C-NMR, compound 2 shows that it is a pentacyclic triterpene (Mahato and Kundu, 1994; Ogunkoya, 1981; Agarwal, 1989). ICFEA-3 gave positive Liebermann-Burchardt test, obtained as green sticky mass. The appearance of signals at δ128.7 and 145.3 indicated the presence of a double bond (C-1, 2) for another double bond (C-9,11) signals at δ162.6 and 119.8 in olean-12-ene triterpenoid (Moghaddam *et al.,* 2007; Seebacher *et al.,* 2003; Hamza and Lajis, 1998). ICFEt- 4 gave positive Liebermann-Burchardt test, obtained as brown sticky mass. The ^{1}HNMR spectrum further exhibited a broad singlet peak at ô 3.14 at 3rd position for proton geminal of a secondary hydroxyl group. The ^{13}C NMR spectrum of compound 4, the peaks were associated to the absence of peak at δ 143.2 and 128.2 for C-24th and C-25th position, respectively, shows the hydrogenation occurs absolutely.

Results of antioxidant activity revealed that all the isolated compounds significantly reduced free radicals levels. Interestingly, compounds 2 and 3 were the most significant in their action. For the most part, natural antioxidants like tocopherols and flavonoids are derived from plants (Deshpande and Kadam, 2013). Flavonoids are able to interact with extracellular and soluble proteins and with the bacterial cell wall because of this property (Marjorie, 1996). Additionally, flavonoids have strong anticancer activity (Salah *et al.,* 1995).

Biochemical enzymatic levels in MCF-7 and CACO-2 cell lines were restored by all identified substances. The cytotoxic effect of stimulated cell lines was significantly reduced by ethyl acetate solvent compounds 2-3. No significant differences were seen between MCF-7 and CACO-2 cell lines when the investigated substances were compared to untreated cells. As a result of these findings, we found that isolated chemicals are safe because of their natural herbal sources. MCF-7 and CACO-2 cells, on the other hand, displayed increased ROS levels, which may be linked to the higher cell viability loss. Starting with pathological modifications ranging from tiny mucosal lesions to tumour growth, the process of colon cancer is multifaceted. When it comes to the development and progression of cancer, however, oxidative stress plays an important role. When malignant

growth begins and progresses, oxidative stress is a life-threatening trait that affects the tumour microenvironment, where ROS functions as a secondary messenger to alter numerous signalling pathways. Colon and breast tissue homeostasis are harmed by a reduction in antioxidant machinery or enzymes of the detoxication system, as well as an increase in ROS. Ferritin and CEA levels in both breast and colon cancer were restored by the use of all the substances in this investigation. Patients with metastases show a considerable increase in ferritin levels. According on the results of CEA and ferritin serial measurements during radiotherapy and chemotherapy, the assay may be useful for evaluating the outcomes of treatment (Gropp *et al.,* 1978).

The antioxidant and anticancer properties of *I. chinensis* flower extracts were investigated in this work. Ethanol solvent extract had more secondary metabolites than petroleum ether and ethyl acetate, according to a phytochemical examination of the flower extracts. To extract secondary metabolites from plants, ethanol has a favourable solubility profile (Ragunathan and Jayaprakash, 2016). Ethanolic extract was found to contain the maximum amount of phyto-constituents from *I. chinensis,* according to our investigation. ICFEA-2, ICFEA-3, and ICFEt-4 were the four isolates discovered in our investigation by the isolation of floral extracts using different solvents. ICFPE-1 passed the Liebermann-Burchardt and anisaldehyde tests, yielding a light green powder whose formula was determined by EIMS (m/z) 456 as $C_{30}H_{48}O_3$. For the CH_3 and CH_2 groups, the IR spectra absorption peak was 2931.15 and 2853.32 cm^{-1} (Elvira *et al*., 2014). Compound-1 ^{1}HNMR spectra revealed tertiary methyl groups on an oleanane skeleton between δ 01.13 to 0.72. A vinyl proton of a triplet was detected at δ 5.35 and one of a doublet's protrons was found at δ 3.35, indicating an olea12-ene skeleton (Gohari *et al.,* 2009).

The carboxyl (-COOH) signal showed in the ^{13}C NMR spectrum at δ 179.2. These findings are in line with those reported for oleanic acid. The "Shinnoda test, yellow colour crystals" revealed a good reaction from ICFEA-2. Based on the positive-ion peak EIMS (m/z) 314 [M^+], its chemical formula is $C_{17}H_{14}O_6$. At 3470 cm^{-1} and 1645 cm^{-1}, respectively, IR data shows absorption bands for hydroxyl and carbonyl groups. Using ^{1}H NMR data, it was discovered that compound 2 contains two hydroxyl (-OH) groups, one in the 5th position (δ 12.83) and the other in the 7th position (δ 5.32). Three protons were found to be singlet in each of the two methoxy groups a δ 3.80 and δ 3.81, respectively, for the 3' and 4' positions of the flavone skeleton. At δ 6.14 for the 8th position, a proton was revealed to be a doublet. In the ^{13}C NMR spectra of 3, there were 18 carbon signals, each with a ppm value (Table 1).

^{13}C NMR spectrum investigations validated the structure much more. For a total of 18 carbons, the compound's ^{13}C NMR spectra showed 16 signals. At a wavelength of 182.1 GHz, C-4 received a signal. The two –OCH_3 groups at C-3' and C-4' were shown to be responsible for the δ 56.6 and 56.4 nm signals, respectively. Additional signals resonating at δ108.8 GHz and δ 121.2 GHz, corresponding to C-2' and C-6', were also discovered to exist (Agarwal *et al.* 1989). Compound 2 's ^{1}H and ^{13}C NMR spectra revealed that it is a pentacyclic triterpene (Mahato and Kundu, 1994; Ogunkoya, 1981).

The Liebermann-Burchardt test was positive for ICFEA-3, which was a green sticky mass. A positive-ion peak EIMS (m/z) 466 [M+] was used to identify its chemical formula as $C_{30}H_{42}O_4$. There were absorption bands for OH of hydroxyl groups and carboxylic acid OH in the IR spectral data (Seebacher *et al.* 2003). The C-O stretching of secondary alcohol (Hamza and Lajis, 1998) at 1166 cm^{-1} and the C-O stretching of exocyclic CH_2 group (Hamza and Lajis, 1998) at 2853 cm^{-1} were present. There were four vinylic multiplets found in Compound-3 ^{1}HNMR data: one at δ 10.82 in the COOH group, three at δ 6.32 (H-1), δ 6.01 (H-2), and δ 5.65 (H-9). It was discovered as multiplets that the aliphatic proton of the triterpenoidal skeleton may be found in the range of δ 2.42 and δ 1.21 as multiplets, δ 1.23 two CH_3 groups, δ 1.25 of three CH_3 groups, and δ 0.98 to 0.89 of two methyl groups as multiplets. Seven quaternary carbons for seven methylsignals identified between δ 27.3 and δ 18.7 deduced from the DEPT experiments are found in the ^{13}C NMR spectrum, which exhibits 30 signals. The carboxylic acid is responsible for the downfield signal's resonant frequency of δ 180.3 (C-30). There was a double bond (C-1, 2) at positions δ 128.7 and δ 145.3 in olean-12-ene triterpenoid that was responsible for the development of signals at δ 162.6 and δ 119.8 (C-9, 11) (Moghaddam *et al.,* 2007).

The Liebermann-Burchardt test was positive for ICFEt-4, which was a brown, sticky substance. Compound ICFEt-4 had a [M+] peak at 456 with the formula $C_{30}H_{48}O_3$ in its mass spectra. The proton geminal of a secondary hydroxyl group showed a large singlet peak at δ 3.14 in the 3rd position in the ^{1}HNMR spectrum. There was no peak at δ 143.2 and δ 128.2 in compound 4 ^{13}CNMR spectrum for the C-24th and C-25th positions, respectively, indicating that hydrogenation occurred completely. All of the separated compounds were found to have a considerable impact on reducing the amounts of free radicals. The most important compound in their function was compound (2-3). Tocopherols and flavonoids, two types of phenolic chemicals found in plants, are the most common natural sources of natural antioxidants (Deshpande and Kadam, 2013). Flavonoids ability to interact with extracellular and soluble proteins, as well as the bacterial cell wall, accounts for most of their potency (Marjorie, 1996). Additionally, flavonoids have strong anticancer activity (Salah *et al.,* 1995).

Both MCF-7 and CACO-2 cell lines were restored to their normal biochemical enzymatic levels by the isolated chemicals. Induced cell lines cytotoxicity was reduced the most by ethyl acetate solvent components 2-3. MCF-7 and CACO-2 cell lines were found to have no significant variations in cell percentage between drugs and untreated cells. We can deduce from these findings that the low toxicity of the separated chemicals is attributable to their natural herbal sources. MCF-7 and CACO-2 cells treated with TNF-alpha displayed late apoptosis/necrosis and elevated ROS levels, which may be linked to the greater loss of cell viability. Starting with pathological modifications ranging from tiny mucosal lesions to tumour growth, the process of colon cancer is multifaceted.

When it comes to the development and progression of cancer, however, oxidative stress plays an important role. When malignant growth begins and progresses, oxidative stress is a life-threatening trait that affects the tumour microenvironment, where ROS functions as a secondary messenger to alter numerous signalling pathways. Colonic and breast tissue homeostasis is negatively impacted by the loss of antioxidant machinery or detoxication system enzymes, as well as an increase in ROS. Against both breast and colon cancer in mice, all of the drugs tested were effective in restoring ferritin levels and CEA concentrations. With metastases, ferritin levels rise dramatically. In the course of radiotherapy and chemotherapy, the

CEA and ferritin assays were used to monitor treatment outcomes (Gropp *et al.,* 1978).

5. Conclusion

The findings of the study indicated that breast and colon cancer may be prevented with the use of *I. chinensis* flower extract. Isolated chemicals with antioxidative activity may be useful in suppressing tumour growth. *I. chinensis* displayed the best antioxidant and anti-proliferative effects making this species a prospective source of anticancer drug development and thus worthy of future exploration. An essential future study would be to investigate the mechanism of action of isolated drugs against tumours *in vivo* utilizing experimental models.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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