Indian Journal of Natural Products and Resources
Vol. 12(2), June 2021, pp. 181-194

Novel anticancer drug delivery system based on zeolite encapsulating *Hamelia patens* leaf and flower extracts

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Received 20 July 2018; Revised 06 April 2021

Nanotechnology had placed an impact in improving the antiproliferative activity of crude leaf (CL) and flower (CF) extracts of *Hamelia patens* on the liver (HEPG2) and breast (MCF7) carcinoma cell lines, after their encapsulation onto zeolite (ZSM-5) nanopores, which acts as a drug delivery system (DDS). The cytotoxicity of the new formulas showed remarkable improvement in the activities on HEPG2. CL had low activity on HEPG2, at the tested concentrations, but the ZSM-5 loaded one (CLZ) showed enhanced action with IC_{50}=42 µg/mL. Also, the cytotoxicity of CF improved after Zeolite incorporation, where the IC_{50} has been changed from 48 µg/mL for CF to 25 µg/mL for CFZ. Moreover, the polar fraction of leaves extracted by methanol (Me.L) showed improvement on the cytotoxicity on MCF7 for zeolite loaded formula (Me.Z) (IC_{50} had changed from 65 to 44.4 µg/mL). Quantitative estimations for polyphenolic contents as well as the phenolic profiles for CF and CL extracts were inspected using HPLC-DAD to explore the bioactive phytochemical compounds. Acute toxicity test showed that LD_{50} was 1500 and 2333 mg/kg b.wt. for CL and CF, respectively. The cytogenetic study was also done to detect the chromosome aberrations in somatic and germ cells after CL and CF administration to mice. DPPH antioxidant capacity assay revealed that CL has high redox-active effect than CF. This study is a contribution to the development of a creative new DDS that help in cancer treatment.

Keywords: Anthocyanins, Antiproliferative, Chromosome aberrations, Flavonoids, *Hamelia patens*, Zeolite.

IPC code; Int. cl. (2015.01)- A61K 36/00, A61K 36/74, A61K 127/00, A61K 133/00, A61P 35/00

Introduction

Cancer is one of the most common destructive disease affecting millions of people per year. Cancer still represents the second cause of mortality in developed countries, after cardiovascular diseases, despite significant progress in prevention, diagnosis and development during the last 25 years. Discovering new drugs that are more active, more selective and less tumour multi-drug resistance is a challenge for the 21st century[1].

Herbal medicines have been widely used around the world since ancient times for curing various diseases. In modern times, natural products have been used for the prevention and treatment of cancer. Scientists are still attempting to discover the bioavailability of anti-cancerous compounds in unexplored plant species.

Hamelia patens Jacq., family Rubiaceae, is an ornamental, large perennial evergreen shrub, indigenous to America and grown almost worldwide in warm areas[2]. Ethnobotanically, it was used for the treatment of a wide range of disorders. In Mexican traditional medicine, leaf decoction was used as antimicrobial, analgesic and anti-inflammatory[3,4].

H. patens extract proved to possess antibacterial activity[5], antioxidant activity[6,7], anthelmintic, antimicrobial activity[8] and Leishmanicidal activity[9]. Concerning the anticancer activity, Mena-Rejon *et al*[10], performed an MTT assay on methanol extracts of leaves, stem and root bark on four cancerous cell lines; nasopharynx (KB), laryngeal (Hep-2), cervix adenocarcinoma (HeLa) and cervix squamous carcinoma cells (SiHa). Only root bark extract of *H. patens* exhibited cytotoxic activity on HeLa cells where the IC_{50} was 13 µg/mL.

It had become obvious that major problems are being faced with the use of herbal extracts in the treatment of human diseases. Despite numerous natural products have shown great activities in preclinical *in vitro* cytotoxic tests, many of them are of little use in the chemoprevention for human

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treatment. The reasons for that are the presences of many problems which face the use of natural products for clinical uses. Poor solubility, low stability in body fluids and the large molecular sizes of some active compounds lead to inefficient systemic delivery and poor bioavailability of the active agents[11]. Moreover, It will not be suitable to deliver the effective petroleum ether, chloroform, acetone, or methanol extracts as such, they need to be incorporated into the plain polymer to be more specifically targeted to the site of action, more soluble and stable in biological fluids and hence more bioavailable[12].

One of the solutions to overcome the drawbacks of the use of herbal medicines is the incorporation of active herbal extract into novel formulation systems. The need for a drug delivery system (DDS), a carrier for herbal remedies, either in nano-form or micro-form, is a must. This will help in dose reduction instead of using bulk drugs and hence decreasing patient compliance and side effects. DDS helps to deliver the extract in smaller particle size and higher surface area leading to faster dissolution in blood[13].

Materials in micro and nanometer sizes are considered excellent carriers to incorporate active substances, like natural products and the final preparations have great advantages[14]. Hence, incorporation of natural products onto a microporous plain carrier, like zeolites, could improve the efficacy, bioavailability of them as the new extract DDS may solve many problems, like poor stability, solubility and toxicity, associated with nutraceuticals. This binding could also lead to sustained release of the chemopreventive agents and targeting the effective material to the desired action[15].

There are about 40 known natural zeolite minerals and more than 150 synthetic types. Zeolite is a microporous crystalline alumino-silicate that contains alkaline metal ions and water molecules. It is composed of a three-dimensional framework of SiO_4 and AlO_4 tetrahedra. This structure results in an extended, uniform network of channels and pores on a nano- and sub-nanometer scale of definitely regular dimensions[16].

Zeolites have become a challenging matter in different aspects of scientific research and are now widely applied in biological, agricultural, environmental and industrial technology[17]. The reason for that is the presence of AlO_4 tetrahedra which makes zeolite framework is negatively charged[18]. These negative charges are balanced by an extra-framework of alkaline ions that keeps the overall structure neutral. Besides, those water molecules are loosely bound and can be easily removed upon heating resulting in large accessible pore volume and high surface area. Zeolite possesses a well organized and regular system of channels and pores. Hence, they constitute ideal matrices for hosting micro and nano-sized particles[19].

Zeolites and other related mesoporous silicates have comprised enormous applications in the encapsulation of different ions and molecules into them to obtain a consequently delayed release[20]. Also, Zeolite has a great potential for medical use owing to its stability in biological environments. Numerous toxicological studies proved that natural zeolites are nontoxic and safe materials hence they can be used in human and veterinary medicine[21]. It has been examined as a carrier for delayed release of anthelmintic and antitumor drugs[22]. They could be used as antibacterial agents or as adjuvant treatment of cancer[23,24]. A pharmaceutical preparation composed of zinc; erythromycin and zeolite have been used in the treatment of acne[25].

This research study aims to improve the cytotoxic effect of *H. patens* crude flower and leaf extracts as well as the polar methanol fraction of leaves (Me.L) and the non-polar chloroform fraction of leaves (Chl.L) on HEPG2 and MCF7 cancerous cell lines before and after the incorporation to microporous-based drug delivery system (DDS) made from ZSM-5. Phytochemical investigation for the polyphenolic contents for CL and CF were done. Acute toxicity, cytogenetic assay as well as antioxidant activity were also determined.

Materials and Methods

Plant material

Fresh aerial parts (leaves and flowers) of *Hamelia patens* Jacq. family Rubiaceae were collected from the garden of National Research Centre, Dokki, Giza, Egypt and were kindly identified by the agricultural engineer, Mrs Trease Labib, Consultant of plant taxonomy, Ministry of Agriculture and the ex-director of Al-Orman Botanical Garden, Giza, Egypt and further confirmed by Dr Mohamed El-Gebaly, Senior Botanist, National Research Centre, Egypt. The plant was dried, ground and a voucher specimen (no. 10/2015) was kept at Pharmacognosy Department, National Research Centre, Cairo, Egypt.

Preparation of plant extracts

Crude extracts

About 100 g of each of air-dried powdered leaves and flowers of *H. patens* were exhaustively extracted

by reflux in ultrasound instrument for 30 minutes, amplitude %: 60, cycles: 0.8 with 70% methanol. Each extract dried at 40 °C under reduced pressure to give crude leaf (CL) and crude flower (CF) extracts.

Successive extracts

About 100 g of dried powdered *H. patens* leaves were successively extracted with chloroform then with methanol in ultrasound instrument in 30 minutes, amplitude %: 60, cycles: 0.8. Each successive extract was filtered; the filtrates were dried at 40 °C under pressure on a rotary evaporator, weighed and kept in vials. Those extracts represented the non-polar and the polar successive fractions of leaves, respectively.

Quantitative determination of total phenolic content

The total phenolic content was determined according to the Folin-Ciocalteu procedure[26]. The sample (100 µL) was transferred into a test tube and the volume adjusted to 3.5 mL with distilled water and oxidized with the addition of 250 µL of Folin-Ciocalteau reagent. After 5 minutes, the mixture was neutralized with 1.25 mL of 20% aqueous sodium carbonate (Na_2CO_3) solution. After 40 minutes, the absorbance was measured at 725 nm against the solvent blank. The total phenolic content was determined using a calibration curve prepared with gallic acid and expressed as milligrams of gallic acid equivalent (mg GAE) per gram of sample. Additional dilution was done if the absorbance value measured was over the linear range of the standard curve.

Quantitative determination of total flavonoid content

Exactly 250 µL of 5% $NaNO_2$ was mixed with 500 µL of extract. After 6 minutes, 2.5 mL of a 10% $AlCl_3$ solution was added. After 7 minutes, 1.25 mL of 1 M NaOH was added, and the mixture was centrifuged at 5000 rpm for 10 minutes. The absorbance of the supernatant was measured at 510 nm against the solvent blank. The total flavonoid content was expressed as mg of catechin equivalent (CE) per g of sample[27].

Quantitative determination of total anthocyanin content in fresh flower sample

Total anthocyanin content was determined by the pH differential method for the determination of total monomeric anthocyanins in *H. patens* flower[28]. Anthocyanin was extracted from fresh tissues of *H. patens* flower by maceration in MeOH: HCl (99:1, v/v) solution for 24 h. The extract was clarified by cooling centrifugation at 10,000 rpm for 10 min at 4 °C. The obtained supernatant was used for the assay of anthocyanin content. Quantification of anthocyanin was done spectrophotometrically at 527 nm. Absorbance readings were converted to the total amount of anthocyanin as a cyanidin-3-glucoside equivalent using a molar extinction coefficient (ε) of 2.96×104[ref. 29]. Results were expressed as mg of cyanidin-3-*O*-glucoside (Cy3G) equivalent per gram of fresh weight.

HPLC analysis of phenolic compounds

HPLC analysis was carried out according to Kim *et al.*[30], with slight modifications using HPLC (Agilent Technologies, 1100 series LC, USA) equipped with an autosampler and a diode-array detector (DAD). The analytical column was Agilent Eclipse XDB C18 (150 x 4.6 mm; 5 µm) with a C18 guard column. The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 mL/min for a total run time of 70 minutes and the gradient program was as follows: 100% B to 85% B in 30 minutes, 85% B to 50% B in 20 minutes, 50% B to 0% B in 5 minutes and 0% B to 100% B in 5 minutes. There were 10 minutes of post-run for reconditioning. The injection volume was 10 µL and peaks were monitored simultaneously at 280, 320, and 360 nm for the benzoic acid, cinnamic acid derivatives, and flavonoids, respectively. All samples were filtered through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectrum and compared with those of the standards.

HPLC analysis of anthocyanin compounds in fresh flower sample

The analysis was carried out on HPLC (Agilent Technologies, 1100 series LC, USA) equipped with an autosampler and a diode-array detector (DAD). The analytical column was an Agilent Lichrospher 5RP select column (250 x 4 mm id, 5 µm) with a flow rate of 1 mL/min at room temperature. The mobile phase used was a binary gradient eluent (solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile). Acetonitrile (MeCN) used was of HPLC grade (Sigma/Aldrich) and was degassed in an ultrasonic bath before use. The water was distilled using a Milli-Q system (Millipore). Fifty milligrams of fresh flower extract were dissolved overnight with 5 mL of 0.1% trifluoroacetic acid in methanol at 4 °C in a blender.

The sample was centrifuged at 3000 rpm for 10 min. The supernatant was collected and filtered through a Millipore membrane (0.45 μm). The filtrate was twice diluted with purified distilled water. One hundred microliters (100 μL) of the filtrate were injected by an Agilent 1100 series autosampler and chromatograms were monitored at 521 nm. The elution program was 5-15% B (0-5 min), 15-25% B (5-15 min), 25-100% B (15-30 min) and 100% B (30-40 min)[31]. A reference library of compounds was performed previously with 2 commercially available anthocyanidin standards (cyanidin and delphinidin). Compounds were identified by comparing their retention times with those of the used standards.

Encapsulation of extracts in ZSM-5

The encapsulation method was prepared by 'diffusion in liquid phase'[32] in the void spaces of the host Zeolite (ZSM-5). 500 mg of ZSM-5 was reacted with a solution of 500 mg of each extract (CF, CL, Chl.L and Me.L), with a concentration of 10 mL of 50% MeOH/ H_2O as a solvent. Mixing was carried out by stirring at (495 rpm) at room temperature for 24 hours. During this time, the original white colour of ZSM-5 was changed to the brownish colour of the extract, indicating that it was effectively entrapped inside the host. Samples then dried in the oven at 60 °C for 24 hours to get rid of the solvent to yield the new formulae; CFZ, CLZ, Chl.Z and Me.Z).

Characterization

Microstructures of the synthesized material were characterized using FTIR, XRD, SEM and EDX elemental analysis. All extracts, before and after the encapsulation process were characterized by the mineral analysis. The prepared zeolites were investigated by X-ray diffraction method, using BRUKUR D 8 ADVANE with secondary monochromatic beam Cu K radiation at keV = 40 and mA =40. Microstructures of the prepared materials were scanned using SEM Model Quanta 250 FEG (Field Emission Gun) attached with EDX Unit (Energy Dispersive X-ray Analyses), with accelerating voltage 30 K.V., magnification 14x up to 1000000 and resolution for Gun.1n). FEI Company, Netherlands.

The specific surface area, pore and particle size distribution of the prepared zeolites were determined from nitrogen adsorption-desorption isotherms were measured with a high-speed gas sorption analyzer (NOVA 2000 series, chromatic, UK) at 77 K. Before measurements, the samples were out-gassed at 150 ºC in vacuum for 6 h. The Barrett–Emmett–Teller (BET) method was utilized to calculate the specific surface areas. The total pore volume was derived from the adsorption branches of the isotherms using the Barrett–Joyner–Halanda (BJH) method. The total pore volume was estimated from the amount adsorbed at maximum relative pressure.

Room temperature Fourier transform infrared, FTIR spectra of the samples in KBr pellets were measured using MB154S Bomem spectrometer, Quebec, Canada equipment, in the range 4000–500 cm^{-1} by averaging 20 scans at a maximum resolution of 4 cm^{-1}.

***In vitro* cytotoxicity assay**

Investigation of the antiproliferative activity for all samples (CL, CLZ, CF, CFZ, Chl.L., Chl.Z, Me.L and Me.Z) was done using SulphoRhodamine-B (SRB) Cell survival assay[33] at National Cancer Institute (NCI), Cairo, Egypt. Two cell lines were tested; human breast carcinoma (MCF7) and human liver carcinoma (HEPG2) cell lines. As breast and liver cancers have become one of the most common types of cancers in Egypt, they were chosen for the study. Doxorubicin (Pharmacia, Belgium) cytotoxic drug was used as a references drug.

***In vitro* antioxidant activity**

Free radical scavenging capacities of CL and CF were determined on the stable 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) using Trolox as a standard (Vit. E analogue)[34]. The final concentration was 200 μM for DPPH and the final reaction volume was 3 mL. The absorbance was measured at 517 nm against a blank of pure methanol after 60 minutes of incubation in a dark condition. Then the absorbance was taken at 517 nm using the spectrophotometer. The standard curve was prepared using Trolox. Results were expressed as mg Trolox equivalents (TE)/g sample.

Pharmacological *in vivo* studies

Experimental animals

Albino mice weighing 20-25 g of Swiss strain obtained from the animal house colony of National Research Centre, Cairo, Egypt. They were kept under the same hygienic conditions and well-balanced diet and water. All experiments had followed ethical animal care and approved by The Medical Research Ethics Committee, National Research Centre (Approval no. 15/100).

Normal diet

It consisted of vitamin mixture (1%), mineral mixture (4%), corn oil (10%), sucrose (20%), cellulose (0.2%), casein 95% (10.5%) and starch (54.3%).

Cytogenetic study

About 1/10 mg of LD_{50} of each extract was I.P. administered to healthy mice for 7 days in the two following tests.

Chromosome aberrations in somatic cells

Chromosome preparations from bone - marrow (somatic cells) carried out[35]. 100 well spread metaphases were analyzed per mouse. Metaphases with gaps, chromatid break and fragments were examined and recorded under 100X magnification with a light microscope (Olympus, Saitama, Japan).

Chromosome abnormalities in germ cells

Chromosome preparations from spermatocytes (germ cells) were made[36]. 100 well spread diakinase-metaphase I cells was analyzed per animal for chromosomal aberrations. Metaphases with X-Y univalents and autosomal univalents were recorded. For statistical analysis, the difference between treated groups and controls were tested with the t-test.

Determination of acute toxicity

CL and CF were tested on uninfected healthy mice to determine the acute toxicity via calculation of the median lethal dose (LD_{50})[37]. Five groups each of 6 mice, weighing 20-25 g each, were used. Different doses expressed by g/kg b.wt of each extract were administered. The toxic symptoms and mortality rate in each group were recorded after 24 hours. LD_{50} of each extract was calculated according to the formula:

$$LD_{50} = Dm - \Sigma (Z \times d)/ n$$

where, Dm: is the minimum dose that kills all animals in the group; Z: is the mean of dead animals in two successive groups; d: is the constant factor between two successive groups; n: is the number of animals of each group, and Σ: is the sum of (Z x d).

Results

Quantitative phytochemical investigation

Total phenolic, total flavonoids, and total anthocyanin contents

Quantitative estimations of the amount of total phenolic (TP) and total flavonoid (TF) contents in CL and CF extracts, besides total anthocyanin (TA) contents in CF fresh sample, are shown in Table 1.

The CL extract contains TP contents more than that of CF extract, while the case is reversed concerning TF contents. TA contents were determined for the fresh flowers owing to their red colour and it gave 0.391 mg/g fresh wt. calculated as cyanidine-3-*O*-glucoside equivalent.

Identification of compounds by HPLC-DAD

Some phenolic acids and flavonoids, in both CL and CF extracts, as well as 2 anthocyanidins, in fresh flower sample, were identified by comparing their retention times (Rt) with those of pure standards. The results were expressed as area % of each identified compound from the total area. Results are presented in Tables 2 and 3. It was revealed that there are 16 compounds were identified out of 25 standards analyzed in CL extract. Chlorogenic acid was present

Table 1 — Total amounts of phenolic, flavonoid and anthocyanin contents *H. patens* crude extracts

Sample	TP (mg GAE/g)	TF (mg CE/g)	TA (mg Cy3GE/g)
CL ext.	123.518	69.022	Not detected
CF ext.	113.455	77.667	0.391

Table 2 — Phenolic and flavonoids compounds identified in CL and CF extracts and their concentrations as detected by HPLC-DAD against standards

Standard compound	Rt (min.)	Conc. (µg/g)	
		CL	CF
Pyrogallol	4.7	9.908	ND
Gallic acid	5.7	ND	0.053
Protocatechuic acid	9.9	1.922	0.141
p-hydroxybenzoic acid	15.1	ND	1.097
Gentisic acid	16.8	ND	ND
Catechin	18.6	25.603	0.248
Chlorogenic acid	20.6	78.244	2.169
Caffeic acid	21.4	2.707	0.127
Syringic acid	23	1.772	ND
Vanillic acid	24.8	0.607	0.132
Scopolatine	30.9	ND	ND
Ferulic acid	32.4	0.414	0.056
Sinapic acid	33.8	ND	ND
Rutin	36.2	14.480	1.030
p-coumaric	37.2	2.413	3.916
Naringin	37.8	3.719	0.128
Hisperidin	38.5	5.115	0.902
Apigenin-7-glucoside	38.8	ND	ND
Myrcetin	39.5	ND	0.714
Rosmarinic acid	40	15.827	5.163
Cinnamic acid	42.8	0.076	ND
Quercetin	43.5	0.656	0.281
Apigenin	46	ND	ND
Kaempferol	46.5	1.302	ND
Chyrsin	52	ND	ND

in the highest amount (78.244 µg/g), followed by catechin (25.6 µg/g), rosmarinic acid (15.8 µg/g), rutin (14.48 µg/g), and pyrogallol (9.9 µg/g). Also, hesperidin, naringin, caffeic, *p*-coumaric acid, protocatechuic acid, syringic acid and kaempferol are present in a lower amount. Traces of quercetin, vanillic, ferulic and cinnamic acids are also detected. For CF extract; 15 compounds were identified out of 25 standard compounds analyzed. Rosmarinic acid was present in the highest amount (5.16 µg/g), followed by *p*-coumaric acid (3.9 µg/g), chlorogenic acid (2.17 µg/g), *p*-hydroxybenzoic acid (1.097), and rutin (1.03 µg/g). Traces of hesperidin, myricetin, quercetin, catechin, protocatechuic acid, vanillic, naringin, caffeic, ferulic, pyrogallol are also detected.

Table 3 — Anthocyanin compounds identified in CF extracts and their concentrations analyzed by HPLC-DAD against standards

Standard compound	Rt (min.)	Conc. (ug/g)
Delphindin	16.486	30.557
Cyanidin	17.166	258.419

HPLC analysis of CF extract against the 2 available anthocyanidin standards revealed that it contains cyanidin in a higher amount than delphinidin.

Extract-DDS encapsulation

ZSM-5 was used for the preparation of a new DDS to host CF, CL, Chl.L and Me.L extracts of *H. patens*. It acts by trapping the extract molecules into its internal framework pores and channels in the liquid phase. The new formulations of crude flower extract-zeolite (CFZ), crude leaf extract-zeolite (CLZ), non-polar "chloroform" fraction of leaves-zeolite (Chl.Z) and polar "methanol" fraction of leaves-zeolite (Me.Z) were confirmed by some analytical data; XRD, SEM coupled to EDX and IR.

XRD analysis

X-Ray diffraction patterns of CF, CL, Chl.L and Me.L extracts before and after ZSM-5 incorporation, as well as ZSM-5 alone, were represented in Fig. 1a-e. It was clear that similar XRD zeolite profiles were

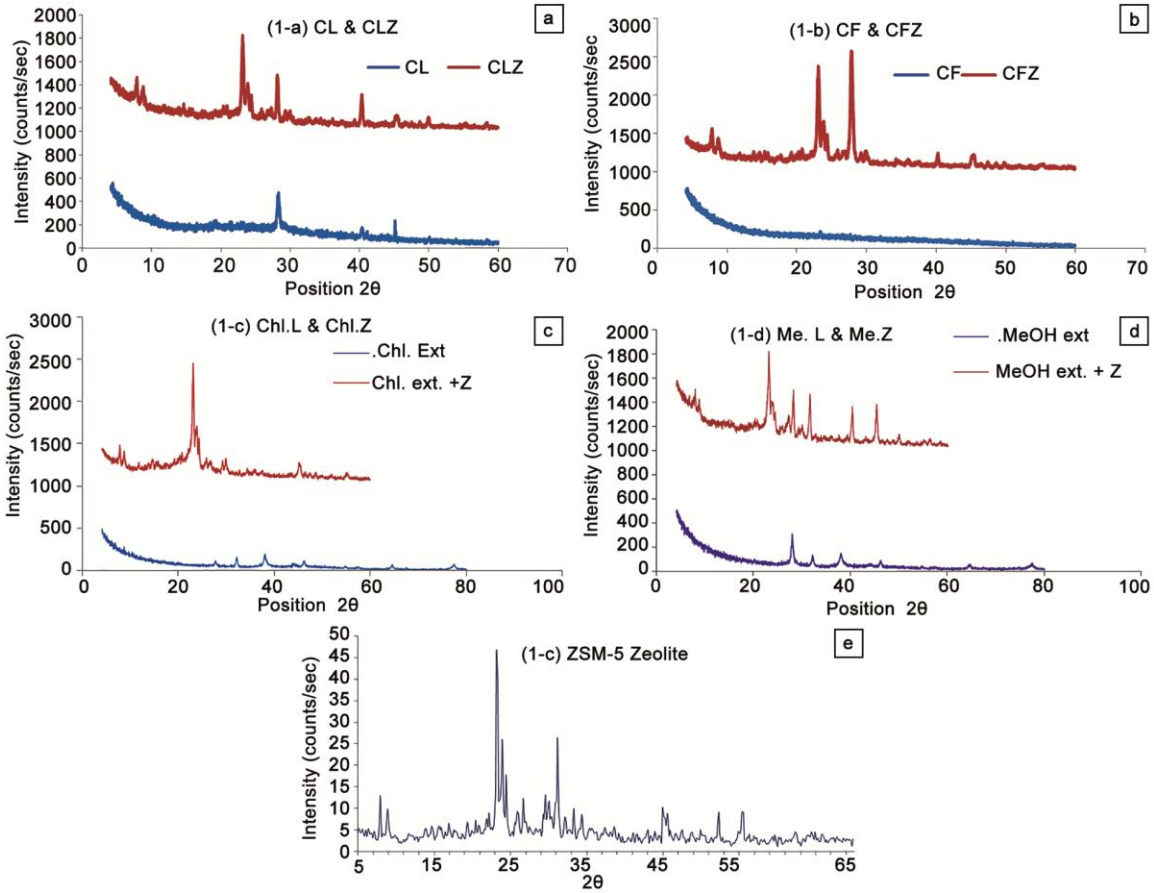

Fig. 1 — a) XRD pattern for CL and its zeolite-incorporated versions CLZ, b) XRD pattern for CF and its zeolite-incorporated versions CFZ, c) XRD pattern for Chl.L and its zeolite-incorporated versions Chl.Z, d) XRD pattern for Me.L and its zeolite-incorporated versions Me.Z, e) XRD pattern for zeolite ZSM-5.

detected in samples after the extract loading with no changes in the zeolite peaks positions. This indicates the successful drug backing into zeolite nanopores with complete stability of zeolite structure.

Scanning electron microscope (SEM) and EDX

SEM and EDX micrographs for CFZ, CLZ, Chl.Z and Me.Z were shown in Fig. 2a-e and Table 4. Fig. 2a is showing the microstructures of ZSM-5 zeolite wherein, the characteristic cross-twinned crystals appeared with nearly 5-7µm in size. EDX microanalysis indicated a pure ZSM-5 with only silicon, oxygen and sodium constituents (Table 4). The previous chemical composition is altered after extract loading and the presence of Carbon (Fig. 2b-e), which indicated and proved the invasion of both extracts into zeolite channels and pores.

In Table 4 zeolite constituents, in terms of weight and atomic percentages (wt% and At%), ascertained the previous SEM and EDX results were nearly equal amounts of carbon atoms (indicating the presence of

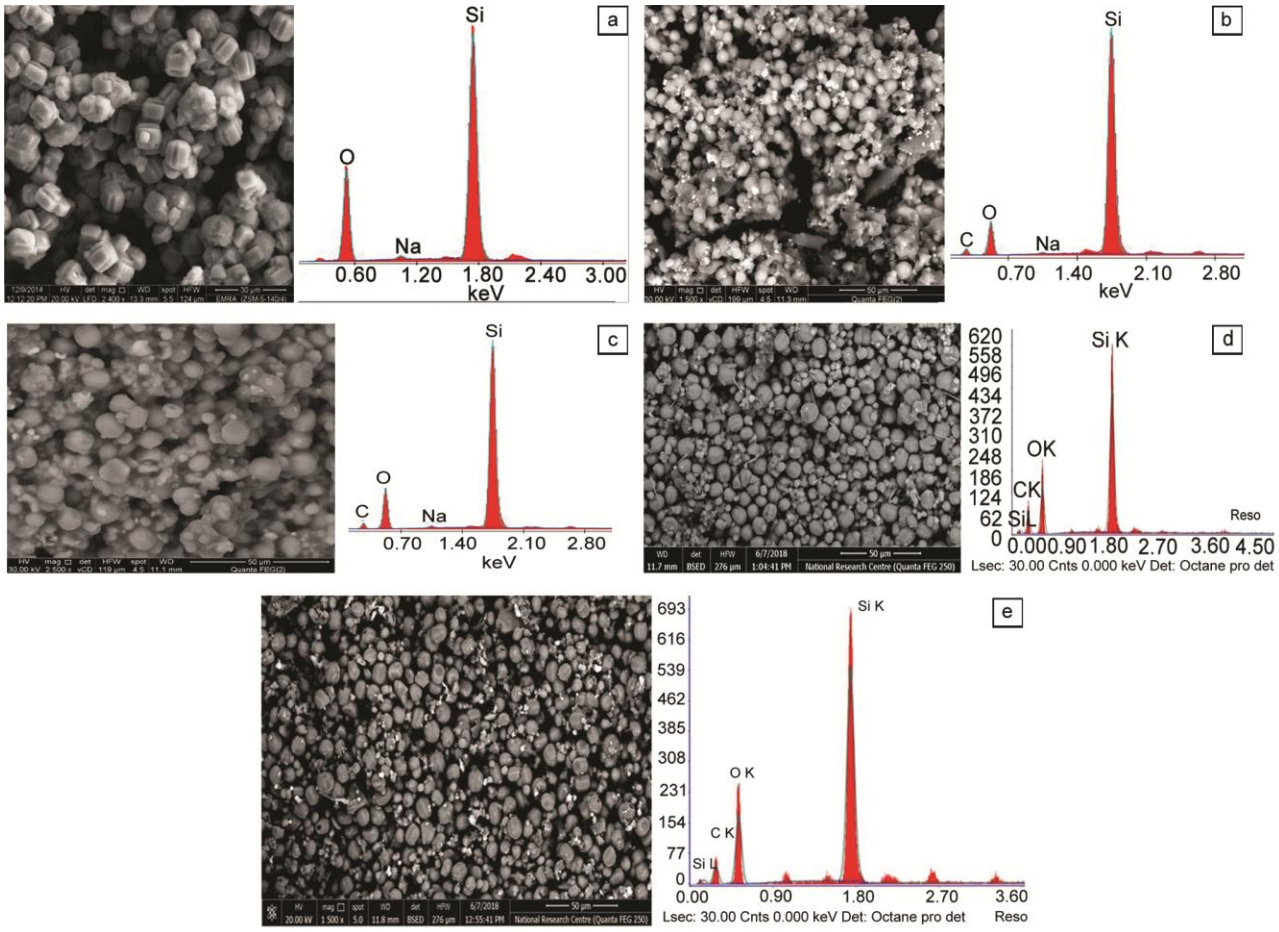

Fig. 2 — a) SEM and EDX of ZSM-5, b) SEM and EDX of CLZ, c) SEM and EDX of CFZ, d) SEM and EDX of Chl.Z, e) SEM and EDX of Me.Z.

Table 4 — Chemical microanalysis for zeolite ZSM-5 before and after loading with CFZ, CLZ, Chl.Z and Me.Z extracts

Element	ZSM-5		CFZ		CLZ		Chl.Z		Me.Z	
	Wt %	At %	Wt %	At %	Wt %	At %	Wt %	At %	Wt %	At %
O	38.25	52.01	43.80	43.80	32.03	37.34	40.33	39.59	42.66	43.43
Na	0.97	0.91	0.97	0.76	0.67	0.55	-	-	-	-
Si	60.78	47.08	42.65	27.81	47.70	31.68	23.51	13.14	27.29	15.82
C	-	-	18.12	27.63	19.60	30.44	36.16	47.27	27.29	15.82
Total	100	100	100	100	100	100	100	100	100	100

Wt: weight, At: Atom

organic material) were contained in the micro-analysis of zeolite crystallites when immersed into CF, CL, Chl.L and Me.L extracts. This indicated the successful process of extract-loading into zeolite inorganic framework channels with appreciable amounts of nearly 20% weight and more than 27-30% atoms of the drug- zeolite package.

IR data

FTIR measurements for ZSM-5, CL, CF, Chl.L and Me.L extracts before and after ZSM-5 incorporation were represented in Fig. 3a-e.

Fig. 3a shows the FT-IT spectral bands of the prepared ZSM-5 zeolite. In IR spectra, in the range of 400 to 4000 cm^{-1}, the complete set of ZSM-5 bands were presented; the characteristic T-O bending vibration of the internal SiO_4 and AlO_4 tetrahedra appeared at 454 cm^{-1}, the external symmetrical and asymmetrical stretching bands related to the T-O-T (where T is Si or Al) were typically positioned at 793 and 1086 cm^{-1}, respectively. On the other hand, the five-membered pentasil ring structure was assigned as the double ring vibration at 547 cm^{-1} in the same figure[38].

The band at 1230 cm^{-1} is attributed to one of the case sensitive bands of zeolite structures and related to the asymmetric stretch vibration of the external linkage between tetrahedra[39]. The band at 3500 cm^{-1} is due to hydroxyl groups (Si-OH) that are usually found in zeolite structures, as they are known as a group of hydrated minerals. So, it is clear from the figures that, zeolite structure was preserved and has a stable silicate structure that capable of hosting the plant extract efficiently.

***In vitro* cytotoxicity SRB assay**

Potential cytotoxic activities of *H. patens* CF, CL, Chl.L and Me.L extracts were tested using SRB assay before and after ZSM-5 encapsulation against MCF7 and HEPG2 cell lines. Results of IC_{50} of each sample

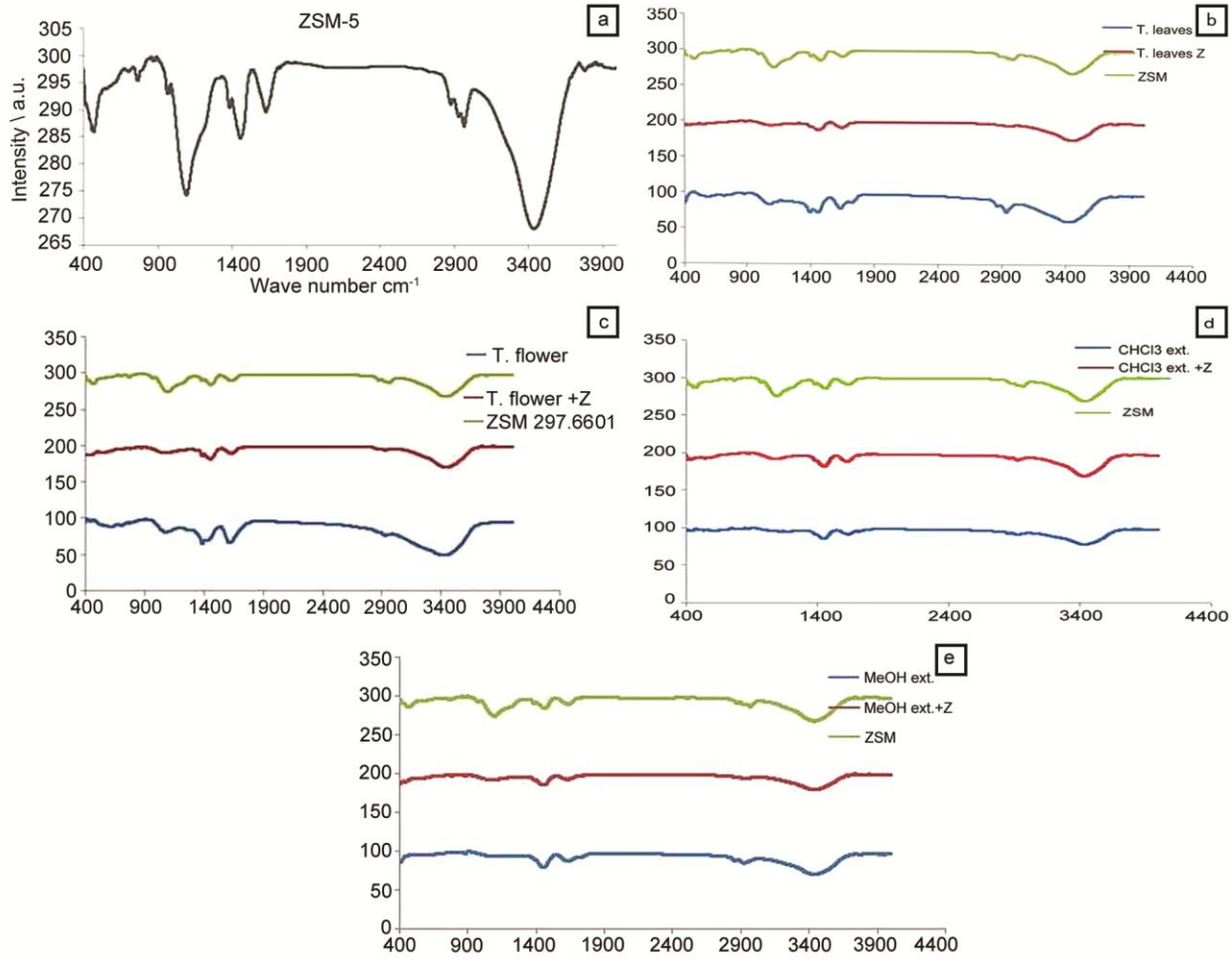

Fig. 3 — a) IR data for the ZSM-5, b) IR data for *H patens* CL before and after ZSM, c) IR data for *H patens* CF. before and after ZSM, d) IR data for *H patens* Chl.L before and after ZSM, e) IR data for *H patens* Me.L before and after ZSM.

are shown in Table 5. Results revealed that CL and CF extracts exhibited a decline in the MCF7 cancerous cells viability in a dose-dependent manner and hence potent cytotoxic effect. Their potency was enhanced after ZSM-5 incorporation on HEPG2. The polar fraction of leaves (Me.L) had moderate cytotoxic action against MCF7 but this activity was improved for the encapsulated formula (Me.Z)

On the other hand, the non-polar fraction of leaves (Chl.L) showed extremely powerful cytotoxic action against MCF7 and HEPG2 but the new formula (Chl.Z) didn't enhance the activity.

Table 5 — IC_{50} of *H. patens* CL and CF before and after Zeolite encapsulation

Sample extract	IC_{50} µg/mL against tested Cell lines			
	MCF-7		HEPG-2	
	Before ZSM-5	After ZSM-5	Before ZSM-5	After ZSM-5
CL	26	66.7	>100	42
CF	24	45	48	25.3
Chl.L.	18	40.6	29	44.4
Me.L	65	44.4	44	65.5
Doxorubicin	3.8	---	5.87	---

ZSM-5 alone has weak or no activity on cancer cell lines as it acts as a plain, neutral incorporating agent that only help in decreasing the particle size of the effective materials[40]. The results support that *H. patens* leaves and flowers have great potential to be efficient agents in treating breast and liver cancer.

***In vitro* antioxidant activity**

The potential antioxidant activities for CL and CF extracts were assessed based on the free radical scavenging capacities of the stable DPPH spectrophotometrically using Trolox as a standard antioxidant. Results revealed that the antioxidant activities were 204.147 and 19.004 mg TE/g for CL and CF, respectively.

***In vivo* cytogenetic assays**

Chromosomal aberrations in mice bone marrow cells (somatic cells) and spermatocyte cells (germ cells) were examined after i.p. treatment of mice with CL and CF for a different period time *in vivo*. Types and percentage of metaphases with chromosomal aberrations induced in somatic and germ cells after treatment with each extract are illustrated in Tables 6 to 9. Results showed the number and percentage of

Table 6 — Chromosomal aberrations induced in mice bone marrow cells after treatment with CL

Treatment dose (mg/kg b.wt)	Time of treatment	Abnormal metaphases		No. of different types of aberrations		
		No.	Mean (%) ± SE	Chromatid gap	Chromatid break	Fragment
I. Negative control	--	16	3.20±0.49	9	3	4
II. Single treatment						
250	24 h	21	4.20±0.48	7	6	8
500	24 h	24	4.80±0.56	8	5	11
750	24 h	23	4.60± 0.52	11	5	7
III. Repeated treatment						
150	7 days	21	4.20±0.60	9	8	4

The total number of examined metaphases is 500 (5 animals/ group)

Table 7 — Chromosomal aberrations induced in mice spermatocyte cells after treatment CL

Treatment dose (mg/kg b.wt)	Time of treatment	Abnormal metaphases		No. of different types of aberrations	
		No.	Mean (%)±SE	XY-uni	Auto. Uni.
I. Negative control	--	17	3.40±0.48	11	6
II. Single treatment					
250	24 h	22	4.40±0.7	15	7
500	24 h	24	4.80±0.58	16	8
750	24 h	25	5±0.48	19	6
III. Repeated treatment					
150	7 days	23	4.60±0.68	15	8

Total number of examined metaphases is 500 (5 animals/ group)

XY-uni: XY- univalent; Auto. Uni.: Autosomal univalent

Table 8 — Chromosomal aberrations induced in mice bone marrow cells after treatment with CF

Treatment dose (mg/kg b.wt)	Time of treatment	Abnormal metaphases		No. of different types of aberrations		
		No.	Mean(%) ± SE	Chromatid gap	Chromatid break	Fragment
I. Negative control	--	16	3.20±0.48	7	5	4
II. Single treatment						
250	24 h	21	4.20±0.37	9	8	4
500	24 h	21	4.20±0.58	8	7	6
750	24 h	25	5.00±0.70	9	10	6
III. Repeated treatment						
250	7 days	24	4.80±0.58	7	7	10

The total number of examined metaphases is 500 (5 animals/group)

Table 9 — Chromosomal aberrations induced in mice spermatocyte cells after treatment with CF

Treatment dose (mg/kg b.wt)	Time of treatment	Abnormal metaphases		No. of different types of aberrations	
		No.	Mean (%) ± SE	XY- uni	Auto. Uni.
I. Negative control	--	12	2.40±0.50	10	2
II. Single treatment					
250	24 h	15	3.00±0.44	11	4
500	24 h	17	3.40±0.67	13	4
750	24 h	21	4. 20±0.48	12	9
III. Repeated treatment					
250	7 days	22	4.40±0.60	16	6

The total number of examined metaphases is 500 (5 animals/ group); XY-uni: XY- univalent; Auto. Uni.: Autosomal univalent

Table 10 — Median lethal dose (LD_{50}) of CL

Dose (mg/kg b.wt.)	Number of dead mice (of 6 mice)	Z	d	(Z)x(d)
250	0	0	250	0
500	0	0	250	0
1000	1	0.5	500	250
1500	2	1.5	500	750
2000	6	4	500	2000

Z: is the mean of dead animals in two successive groups, d: is the constant factor between two successive groups.

Table 11 — Median lethal dose (LD_{50}) CF

Dose (mg/kg b.wt.)	Number of dead mice (of 6 mice)	Z	d	(Z)x(d)
500	0	0	500	0
1000	1	0.5	500	250
1500	1	1	500	500
2000	2	1.5	500	750
2500	2	2	500	1000
3000	4	3	500	1500

Z: is the mean of dead animals in two successive groups; d: is the constant factor between two successive groups.

the chromosomal aberrations induced in the -ve control group and treated animal groups with CL and CF, at the doses 250, 500, and 750 mg/kg b.wt. for 24 hours and 150 and 250 mg/kg b.wt (1/10 LD_{50}), for CL and CF, respectively, for one week. The percentage of aberrant cells in all treated groups with the extracts were statistically not significant in comparison to the -ve control groups in somatic and germ cells (P <0.01).

Determination of acute toxicity

The CL and CF were tested for the determination of the acute toxicity via calculation of the median lethal dose (LD_{50}). Results are shown in Tables 10 and 11. LD_{50} of CL and CF of *H. patens* found to be 1500 and 2333 mg/kg b.wt., respectively.

Discussion

The antiproliferative activity of crude leaf (CL) and flower (CF) extracts as well as the polar (Me.L) and non-polar fraction (Chl.L) of leaves of *H. patens* Jacq. on the liver (HEPG2) and breast (MCF7) carcinoma cell lines, using SRB assay were investigated. An attempt was made to develop the bioactivity of these extracts by their encapsulation onto zeolite (ZSM-5) pores, which acts as a drug delivery system (DDS) to bring the molecules in a smaller size. Reinvestigating the cytotoxic activity of the new formulas on the same cancerous cells showed remarkable improvement in the activities on HEPG2. CL had low activity on HEPG2, at the tested concentrations, but the ZSM-5 loaded one (CLZ) showed enhanced action with

IC_{50}=42 µg/mL. Also, the cytotoxicity of CF improved after Zeolite incorporation, where the IC_{50} has been changed from 48 µg/mL for CF to 25 µg/mL for CFZ. Also, The polar fraction of leaves (Me.L) revealed improved activity against MCF7 after zeolite incorporation (Me.Z). Meanwhile, the plain non-polar fraction of leaves (Chl.L) showed potent cytotoxic action against MCF7 and HEPG2.

Acute toxicity determination experiment showed that CL and CF are safe. This indicates the low toxicity of the plant *in vivo* hence its use in herbal medicine is most probably useful. Traditionally, its leaf decoctions and infusions were taken safely by oral routes[41]. The acute toxicity of ethanol extract of *H. patens* leaves has been measured and its LD_{50} found to be 2964 mg/kg b.wt i.p. and more than 5000 mg/kg b.wt for oral doses[42]. This is the first determination of acute toxicity for *H. patens* flowers.

Investigating CL and CF by *in vivo* chromosomal aberrations assays either in somatic (bone marrow) and germ (sperm) cells in mice indicate the safety of the plant leaves and flower on normal cells. To the best of our knowledge, this is the first study to prove the safety of the plant in both somatic and germ cells. The chromosomal aberrations *in vivo* assays measure the ability of a substance to be carcinogenic and hence predict if the tested substance is clastogenic[43]. Chromosomal aberrations are the mutations that may occur in chromosomes due to the exposure of the cell to a carcinogenic material. These carcinogens, which are responsible for the induction of neoplasm in humans or animals, increase the incidence of tumours. Chromosomal aberrations are visible changes in the morphology and structure of chromosomes[43]. Different structural changes occur in chromosomes due to abnormal divisions. That results in the reunification of segments in combinations that differ from those in the original chromosomes. These changes comprise deletion; due to loss, duplication; due to gain, or translocation; due to exchange of chromosomal segments. Also, chromosomal inversion may occur through the turning of the chromosomal segment by 180 °C and reinserted at the same position.

Measuring the antioxidant capacity of CL and CF extracts by evaluating their ability to convert the violet colour of DPPH to the yellow coloured α,α-diphenyl-β-picryl hydrazine which causes a decrease in the absorbance wavelength[44]. The extent of discolouration demonstrates the activity of the sample as a free radical scavenging agent. Results revealed that CL has a good antioxidant effect and much higher activity than CF. That effect is most probably due to the presence of a high amount of flavonoids and phenolics in the plant. As it is well known, plants rich in polyphenols are considered highly redox-active antioxidants as these compounds struggle against dangerous oxidative damage of the cell components and hence can protect cells from various types of cancers[44].

Khandelwal *et al.*[8] have investigated the quantitative estimation of phenol contents of different organs of *H. patens*; leaves, stems and barks and it was found that phenolic contents were 104.6±1.12, 50.7±1.41, and 47.2±0.89 respectively. To the best of our knowledge, this is the first time investigating flavonoid contents in CL. Also, there was no previous study that investigated the quantitative estimation of phenolics, flavonoids, or anthocyanin contents in CF.

There are previous studies that have investigated the chemical composition *H. patens* leaves extracts especially phenolic acids and flavonoids. Narirutin and rosmarinic acid were isolated from the methanol extract of the aerial parts of the plant[45]. Also, two flavonoids were isolated from the ethyl acetate extract: kaempferol-3-O-rutinoside, and (-) epicatechine[9]. Caffeoylquinic acid (chlorogenic acid) was detected in high amount along with quercetin 3-O-rutinoside (rutin) and Kaempferol-3-O-rutinoside as major compounds that were detected by HPLC/ESI-MS analysis of *H. patens* 70% ethanol leaves extract, obtained by 3 different methods of extractions; maceration, percolation and soxhlet[46] Where hydroxycinnamic acid and catechin were present only in the extract obtained by maceration.

The promising cytotoxic activity of *H. patens* leaves and flowers, against liver and breast cancer cells, may be attributed to the presence of a high amount of polyphenolic compounds. Polyphenolic compounds have been reported to possess many biological activities. One of which being potent anticancer agents.

Polyphenols are a bulky group of compounds present abundantly in plants and can stop or treat a wide variety of human ailment such as cancer[47]. Numerous studies have proven that the anticancer effect of phenolics is due to their interference with each of the stages of carcinogenesis; either in initiation, promotion or progression of the disease[48].

The most abundant compound detected in CL is chlorogenic acid (CQAs; caffeoylquinic acid ester). It

is not surprised as the plant is belonging to the coffee family, Rubiaceae. It has many significant biological activities and present in many plant species. Gouthamchandra *et al.*[49] have inspected chlorogenic acid complex (CGA7), a decaffeinated coffee bean extract, for their anticancer activities against different cell lines. The MTT assay results indicated CGA7 has higher activity against mouse tumour cells (EAC and B16F1), followed by human colon cancer cells (HCT-116 and HCT-15), then moderate activities against MCF7, Hela and HEPG2 human cancer cells. Another study has been carried out to investigate the molecular mechanism of chlorogenic acid antitumor activity. It was revealed that up-regulation in the expression of GSK-3β and APC genes and a down regulation in the expression of β-catenin gene occurred in the mice group of 20 mg/kg dosage (P <0.05). Based on the fact that, inducing genes GSK-3β and APC and inhibiting gene β-catenin will promote the apoptosis of tumor cells[50].

The major phenolic compound detected in CF is rosmarinic acid. Several studies recorded the efficiency of rosmarinic acid in the inhibition of different tumour cells like liver, breast, stomach and colon[51]. It showed a decrease in the colony formation of many kinds of colon cancer cells where it decreases CaCo-2 cells at dose 30 µg/mL after 24 hours[52], SW480 colon cancer cells at dose 31.25 µg/mL after 28 hours[53]. It also found to enhance the cytotoxic effects of 5-fluorouracil (5-FU), the chemotherapeutic drug, on the growth of 5-FU resistant cells[54].

Catechin and its related, more complex flavanol compounds, are known to acquire anticarcinogenic effect[55]. Polyphenols belonging to green tea catechins were found to induce apoptosis which aid in the treatment of numerous types of cancer as human colon and stomach cancer cells. From a structure-activity relationship viewpoint, the presence of a pyrogallol-type structure in a molecule is a minimum requirement for apoptosis induction of catechin compounds[56].

Boundless research studies have proven that flavonoids have diverse therapeutic effects. The most prominent action is their anticancer properties. Dietary flavonoids, such as quercetin, genistein, apigenin, found to be able to inhibit *in vitro* and *in vivo* proliferation of cancerous cells[48].

Rutin, the abundant flavonol present in various plants, possess many pharmacological actions. It demonstrated anticancer effects against different cell lines. It acts on reduction in tumour size of HL-60 Leukemic cells[57]. It acts by arresting the cell cycle and apoptosis of colorectal cancer[58]. It also has a cytotoxic effect against HTC hepatic carcinoma cells[59].

Having orange-red flowers, *H. patens* (syn. Firebush) can recruit insects, butterflies and hummingbirds for pollination. These flowers are found to be rich in anthocyanin. The detected anthocyanidins; cyanidin and delphinidin, are recorded to demonstrate inhibitory effects on breast cancer cells proliferation. Generally, anthocyanidins found to inhibit tumours proliferation by blocking the activation of the mitogen-activated protein kinase (MAPK) pathway in TPA-induced cultured mouse JB6 cells[60].

Conclusion

In the present research, crude extracts of *H. patens* were incorporated in a micro-porous delivery carrier; zeolite ZSM-5. Each extract was loaded into the nanopores and channels characteristic of zeolite species. The loading was confirmed by XRD analysis, EDX, SEM and IR spectra. All data proved that the encapsulation was successful and ZSM-5 structure wasn't altered. The capacity of zeolite in carrying the extract was about 20% of the net weight of the zeolite phase. the antiproliferative activity was enhanced after loading the plant extracts on ZSM-5 on HEPG2 cancerous cell lines. The investigated *in vivo* studies proved that the plant is safe for therapeutic administration and not toxic either to the somatic or genetic material. Inspections of the phytochemical contents for these extracts were done to explore the bioactive compounds and some compounds were detected for the first time. it was revealed that they are rich in active antioxidant and anticancer polyphenolic compounds. To the best of our knowledge, this is the first study using Zeolite as DDS for plant extract incorporation and testing them as an anticancer agent. This study is a contribution to the development of a creative new DDS with active cytotoxic agents from natural sources that help in cancer treatment.

Conflict of interest

All authors declare that there are no conflicts of interest.

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