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RESEARCH ARTICLE

5-HYDROXYMETHYL FURFURAL FROM THE FRUIT PART OF *GREWIA ASIATICA* POSSESSES ANTI-COLON CANCER ACTIVITY

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Abstract: The present research work aims to investigate the *in vitro* cytotoxic potential of *Grewia asiatica* (phalsa) fruit against human cancer cell lines and further isolation of active ingredient. The methanolic extract was prepared and further fractionated with hexane, chloroform and butanol in the order of increasing polarity. The extract and fractions were evaluated against eight human cancer cell lines (A-549, HCT-116, HT-29, SW-620, PC-3, MCF-7, MDAMB-231, MIAPACA) from five different tissues (lung, colon, prostate, breast, pancreatic) respectively at the conc. of 100 µg/ml *via* SRB assay. The results revealed that chloroform fraction of *G. asiatica* exhibited *in vitro* cytotoxicity against colon cancer cell line (SW-620) with growth inhibition of 70% whereas hexane fraction also inhibited the growth of same cell line by 76%. A compound namely 5-hydroxy methyl furfural (5-HMF) was isolated from chloroform fraction through column chromatography and characterized *via* NMR (^{1}H and ^{13}C) and mass spectroscopy (HRMS). It was tested against SW-620 cancer cell line and the IC_{50} value was calculated that showed growth inhibitory potential of the component against colon cancer cells.

Keywords: *Grewia asiatica*, 5-HMF, SRB assay, *In vitro* cytotoxicity, Cancer cell lines

INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells that is the common cause of death in humans, worldwide (Wand *et al*., 2014; Goyal, 2012; Ghali *et al*., 2014). Despite of developments in the tools of disease diagnosis and treatment, it is one of the leading causes of death and only modest progress has been made in reducing the morbidity / mortality of this disease (He *et al*., 2014; Hail, 2005). *Grewia asiatica*, locally known as phalsa, is well-known for its nutritional and therapeutic attributes. The fruits are claimed to be beneficial for heart, blood / liver disorders, anorexia, indigestion, thirst, toxemia, stomatitis, hiccough, asthma, spermatorrhoea, fever, diarrhea and are used for treating throat, tuberculosis, sexual debility troubles (Sharma and Sisodia, 2009; Pallavi *et al*., 2011; Mishra *et al*., 2012). The root bark is used for the treatment of rheumatism and urinary tract problems while the stem bark is used in sugar refining (Sisodia and Singh, 2009; Muhammad *et al*., 2006; Haq *et al*., 2012). Some phalsa species have free radical scavenging activities which may be responsible for therapeutic action against tissue damage (Kshirsagar and Upadhyay, 2009). *Grewia*'s extracts are also supposed to be helpful in curing hepatitis and used as herbal antidiabetic (Tripathi *et al*., 2011). *In vitro* cytotoxic activity was determined by methylthiazolyltetrazolium (MTT) assay using epidermal kidney (HEK-293), breast (MCF-7), cervical (HELA), lung (NCI-H522) and laryngeal (Hep-2) cancer cell lines. The fruit extract was found to be active on lung (IC_{50} = 59.03 µg/mL) and breast (IC_{50} = 58.65 µg/mL) cancer cell lines, while the leaf extract was active against breast (IC_{50} =50.37 µg/mL) and Hep-2 (IC_{50} = 61.23 µg/mL) cancer cell lines (Marya *et al*., 2011). Therefore, the fruits of *G. asiatica* are reported for their antitumor and cytotoxic activity (Kakoti *et al*., 2011). The search for novel drugs is still a priority target for cancer therapy due to the fact that chemotherapeutic drug resistance is becoming more and more frequent and very little work has been reported on *Grewia asiatica*

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(phalsa) fruit regarding medicinal application and biological activity of its phytochemicals. In view of the above, *in vitro* cytotoxic potential of methanolic extract / fractions of *Grewia asiatica* fruit was tested against human cancer cells for further isolation of bioactive compound.

MATERIALS AND METHODS

Chemicals

RPMI-1640 medium, dimethyl sulfoxide (DMSO), EDTA, fetal bovine serum (FBS), sulphorhodamine blue (SRB) dye, phosphate buffer saline (PBS), trypsin, gentamycin, penicillin and 5-flurouracil were purchased from Sigma Chemical Co., USA. All other chemicals were of high purity and obtained locally with the brand Sigma-Aldrich Chemicals Pvt. Ltd. and S.D. Fine Chemicals Pvt. Ltd. from Ramesh Traders, Panjthirthi-Jammu, J&K.

Fruit material and preparation of extract

Grewia asiatica, fruit part, was authenticated at site by Professor Vijay Bahadhur Singh and enough quantity of fresh fruits were collected from Rainfed Research Sub-station for Sub-tropical Fruits, Raya, SKUAST-Jammu. The freshly collected fruits were chopped, shade-dried and ground into powdered form. The methanolic extract of the fruit was prepared by percolating the dried ground plant material (400 g) with 95% methanol and then concentrating it to dryness under reduced pressure to yield a crude extract of 101 g (Kandil *et al.*, 1994). Stock solutions of 20 mg/ml were prepared by dissolving 95% methanolic extract in DMSO. Stock solutions were prepared at least one day in advance and were not filtered. The microbial contamination was controlled by addition of 1% gentamycin in complete growth medium *i.e.*, used for dilution of stock solutions to make working test solutions of 200 µg/ml.

Fractionation, isolation and characterization

The crude (methanolic) extract was fractionated with hexane, chloroform and butanol solvents. For fractionation, water was added to dissolve the extract and the same volume of hexane was used for solvent-solvent partitioning, and the upper phase (hexane) was removed (3.85 g). The residue was further partitioned using chloroform (3.38 g) and *n*-butanol (37.18 g). Chloroform fraction was purified by column chromatography (silica gel, 100-200 mesh), eluted with a gradient of hexane-ethyl acetate (250 mL collected volumes of each fraction) and concentrated, giving sixty fractions based on TLC profile. Fractions (Fr. 35-39) showed same pattern in TLC were pooled and concentrated. Obtained compound was subjected to TLC using 50% ethylacetate: hexane as mobile phase and detected a single spot after spraying with anisaldehyde reagent and heating the plate at 100 ^{0}C for 5 min. Spot on TLC obtained was dark blackish green in colour and the nature of compound was oil type (yellow colour).

All NMR spectral data were recorded on a Bruker 400 MHz spectrometer. Chemical shifts (δ) were referenced internally to the residual solvent peak ($CDCl_3$: ^{1}H-7.26, ^{13}C-77.0 ppm) and the reference point was TMS (δ_H and δ_C: 0.00 ppm). HR-ESIMS spectra were recorded on an Agilent 1100 LC-Q-TOF mass spectrometer and HRMS-6540-UHD machines. HPLC purifications were performed on an Agilent 1260 Infinity II HPLC system with UV detector. HPLC solvents like MeOH and ACN were procured from Merck chemicals and water for extractions and HPLC analysis was obtained from high-purity Milli-Q Advantage A10 water system (Millipore, Molsheim, France). Column chromatography was done using silica gel (100-200 mesh). Thin layer chromatography (TLC) analysis were performed using Merck Kieselgel (Aufoilen) 60 F_{254} plates.

Cell lines / cultures and positive controls

The human cancer cells-A-549 (lung), HCT-116, HT-29 & SW-620 (colon), MCF-7 & MDAMB-231 (breast), MIAPACA (pancreatic), PC-3 (prostate) were obtained from National Centre for Cell Science, Pune, India and National Cancer Institute, Frederick, USA. These human cancer cells were further grown and maintained in RPMI-1640 medium.Doxorubicin, 5-Fluorouracil, Mitomycin-C and Paclitaxel were used as positive controls.

In vitro assay for cytotoxic activity

Extract, fractions and the compound were subjected to *in vitro* anticancer activity against various human cancer cell lines (Monks *et al.*, 1991). In brief, the cells were grown in tissue culture flasks in growth medium at 37 ^{o}C in an atmosphere of 5% CO_2 and 90% relative humidity in a CO_2 incubator (Hera Cell, Heraeus; Asheville, NCI, USA). The cells at sub-confluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in growth medium. Cells with more than 97% viability (trypan blue exclusion) were used for determination of cytotoxicity. An aliquot of 100 µl of cells (10^5 cells/ml) was transferred to a well of 96-well tissue culture plate. The cells were allowed to grow for 24 h. Extracts (100 µl/well) were then added to the wells and cells were further allowed to grow for another 48 h.

The anti-proliferative SRB assay which estimates cell number indirectly by staining total cellular protein with the dye SRB was performed to assess growth inhibition. The SRB staining method is simpler, faster and provides better linearity with cell number. It is less sensitive to environmental fluctuations and does not require a time sensitive measurement of initial reaction velocity (Skehan *et al.*, 1990). In brief, the cell growth was stopped by gently layering 50 µl of 50% (ice cold) trichloroacetic acid on the top of growth medium in all the wells. The plates were incubated at 4 ^{o}C for 1 h to fix the cells attached to the bottom of the wells.

Liquid of all the wells was then gently pipetted out and discarded. The plates were washed five-times with distilled water and air-dried. SRB 100 µl (0.4% in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 30 min. The unbound SRB was quickly removed by washing the cells five-times with 1% acetic acid. Plates were air-dried, tris buffer (100 µl, 0.01 M, pH 10.5) was added to all the wells to solubilize the dye and then plates were gently stirred for 5 min on a mechanical stirrer. The optical density (OD) was recorded on ELSIA reader at 540 nm. Suitable blanks (growth medium and DMSO) and positive controls (prepared in DMSO and distilled water) were also included. Each test was done in triplicate and the values reported were mean values of three experiments.

Calculations

The cell growth was determined by subtracting average absorbance value of respective blank from the average absorbance value of experimental set. Percent growth in presence of test material was calculated as under:

OD Change in Presence of Control = Mean OD of Control – Mean OD of Blank

OD Change in Presence of Test Sample = Mean OD of Test sample – Mean OD of Blank

% Growth in Presence of Control = 100/OD change in presence of control

% Growth in Presence of Test Sample = (% growth in presence of control) × OD change in presence of test sample

% Inhibition by Test Sample = 100 – % growth in presence of test sample

Statistical Analysis

The experiments were done in triplicates and each data represents the average of at least three independent experiments. The data was expressed as mean±S.D. IC_{50} value was calculated by Graph PAD Prism software version 5.0.

RESULTS AND DISCUSSION

The phalsa fruit extract did not exhibit any significant cytotoxicity (growth inhibition of 70% or more) against any of the human cancer cell lines mentioned above and the overall growth inhibition was seen in the range of 0-54%. For colon (HCT-116 & HT-29) and lung (A-549) cancer cell line, the extract was completely considered as inactive as growth inhibition was 0% whereas the results produced in fractions were quite interesting as the n-hexane fraction and chloroform fractions were found to be active specifically on colon cancer cell line *i.e.*, SW-620 with 76% and 70% growth inhibition respectively. The butanol fraction was not found to be active on any of the human cancer cell lines as the growth inhibition was in the range of 0-35% (Table 1). Chloroform fraction was further taken up and a compound namely 5-hydroxy methyl furfural (5-HMF) was isolated *via* column chromatography and characterized by NMR (^{1}H and ^{13}C) and mass spectroscopy (HRMS). It was tested against SW-620 cell line at different conc. of 50, 30, 10 and 1 µM. The compound exhibited 40% (50 µM) and 0% growth inhibition (30, 10 and 1 µM) with IC_{50} value calculated as > 50 ±0.77 µM for SW-620 (Table 2).

Yellowish oil; ^{1}H NMR ($CDCl_3$, 400MHz); 9.50 (1H, s, H-7), 7.19 (1H, d, *J*=3.6 Hz, H-3), 6.47 (1H, d, *J*=3.2 Hz, H-4), 4.65 (2H, d, *J*=3.2 Hz, H-6); ^{13}C NMR ($CDCl_3$, 100MHz); 178.1 (C-7), 161.4 (C-5), 151.8 (C-2), 124.2 (C-3), 110.0 (C-4), 56.8 (C-6), ESIMS *m/z*: 127.1000 $[M+H]^+$ (calcd for $C_6H_6O_3$, 126.10). The structural feature of 5-HMF contributes to its anticancer potential. The ^{1}H NMR spectrum exhibited an aldehyde group at δ_H 9.50 (1H, s, H-7), an aromatic group at δ_H 7.19 (1H, d, *J*=3.6 Hz, H-3) & 6.47 (1H, d, *J*=3.2 Hz, H-4) and CH_2 group at δ_H 4.65 (2H, d, *J*=3.2 Hz, H-6). The ^{13}C NMR spectrum exhibited total six carbon signals including CHO group, two aromatic carbon, two quartnery carbon and CH_2 group. The compound obtained was profiled by HPLC (High-performance liquid chromatography) with an intense peak at R_t= 14.56 and confirming its presence in chloroform fraction of *Grewia asiatica* at same R_t (Fig. 1, 2, 3, 4 & 5)

Grewia asiatica provides a unique source of various phytochemicals, is known to be a versatile genus of medicinal plants. 5-HMF isolation from chloroform fraction of fruit part of *G. asiatica* possess anticancer potential specifically on colon tissue. A growing interest is arising around phytochemicals role in cancer prevention and treatment. Recent research suggests that the investigation of "new" phytochemicals and related molecular targets can be exploited to identify novel anti-cancer drugs, following sequential steps. This approach includes preliminary selection of phytochemical candidates for cancer prevention or therapy, based on the pre-clinical results related to cell-transformation and antitumorigenic activity assays. Further *in vivo* studies are required for exploring the mechanism of action of 5-HMF, responsible for cytotoxicity and thereby helps in the managment of colon carcinoma.

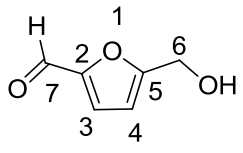


Table 1. Growth inhibitory effect of *Grewia asiatica* (extract & fractions) against different human cancer cell lines

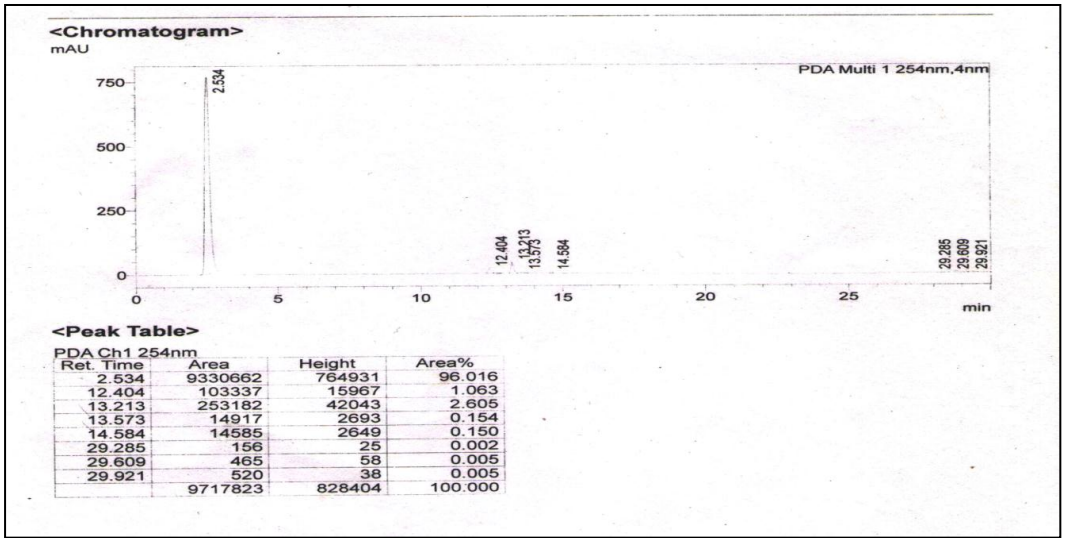
Generic name of the fruit	**Conc. (µg\ml)**	**Human cancer cell lines from five different tissues**							
Grewia asiatica		Breast	Breast	Colon	Colon	Colon	Lung	Pancreatic	Prostate
		MCF-7	MDAMB-231	HCT-116	HT-29	SW-620	A-549	MIAPACA	PC-3
		Growth Inhibition (%)							
Extract (Methanolic)	100	31	34	0	0	54	0	19	0
Fractions (n-Hexane)	100	23	44	0	0	**76**	0	17	0
Chloroform	100	19	29	0	0	**70**	0	0	0
Butanol	100	29	35	0	0	32	0	1	0
Positive controls (standard drugs)	Conc. (µM)								
Doxorubicin	1	65	65	-	-	-	-	-	-
5-Fluorouracil	20	-	-	52	52	65	-	-	-
Mitomycin-C	1	-	-	-	-	-	-	-	66
Paclitaxel	1	-	-	-	-	-	78	-	-
Paclitaxel	50	-	-	-	-	-	-	87	-

Growth inhibition of 70% or more in case of fractions has been indicated in bold numbers
Mark (-) indicates that particular human cancer cell line was not treated with that particular positive control

Table 2. Growth inhibitory effect of 5-HMF from *Grewia asiatica* against colon cancer cell line

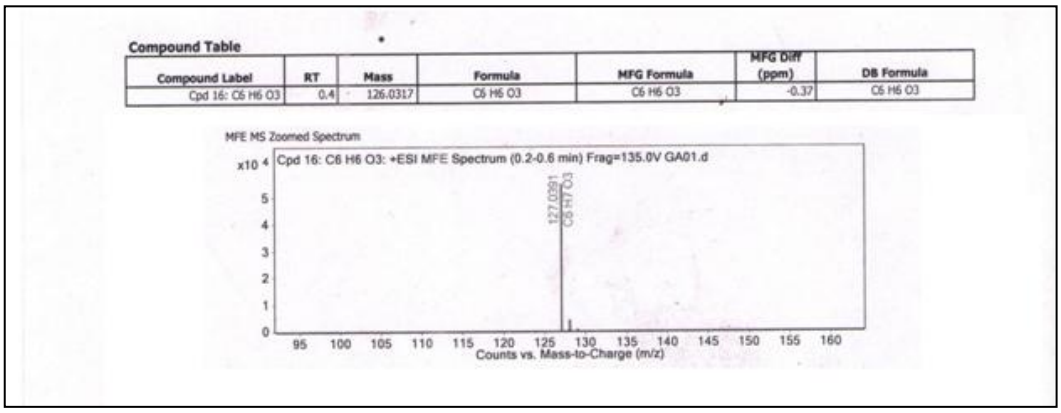
Compound	**Conc.**	**Colon cancer cell line**
		SW-620
	Growth Inhibition (%)	
5-HMF	50	40
	30	0
	10	0
	1	0
IC_{50} (µM)		> 50±0.77
Positive control (standard drug)		
5- Fluorouracil	20	65

Fig. 1. TLC profile of 5-hydroxy methyl furfural isolated (35 & 36) and crude extract of *Grewia asiatica* with solvent system of 50% EtoAc/hex.



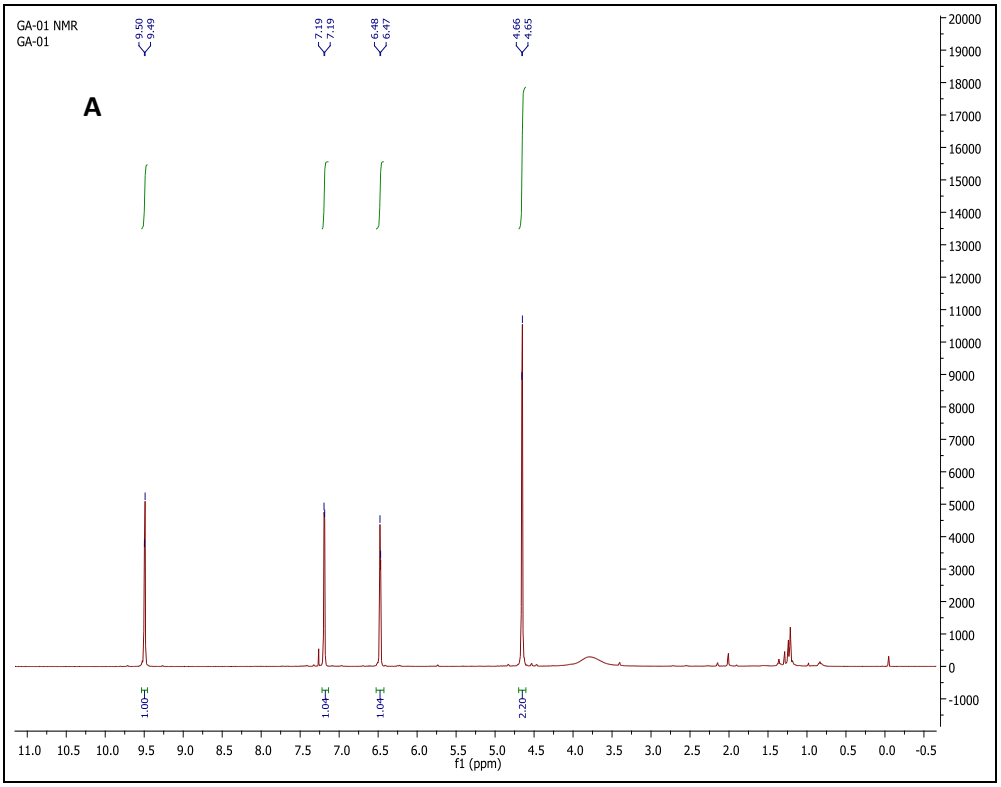
Ret. Time	Area	Height	Area%
2.534	9330662	764931	96.016
12.404	103337	15967	1.063
13.213	253182	42043	2.605
13.573	14917	2693	0.154
14.584	14585	2649	0.150
29.285	156	25	0.002
29.609	465	58	0.005
29.921	520	38	0.005
	9717823	828404	100.000

Fig 2. HPLC chromatogram of 5 - hydroxy methyl furfural isolated from *Grewia asiatica*



Compound Label	RT	Mass	Formula	MFG Formula	MFG Diff (ppm)	DB Formula
Cpd 16: C6 H6 O3	0.4	126.0317	C6 H6 O3	C6 H6 O3	-0.37	C6 H6 O3

Fig. 3. HRMS chromatogram of 5 - hydroxy methyl furfural isolated from *Grewia asiatica*



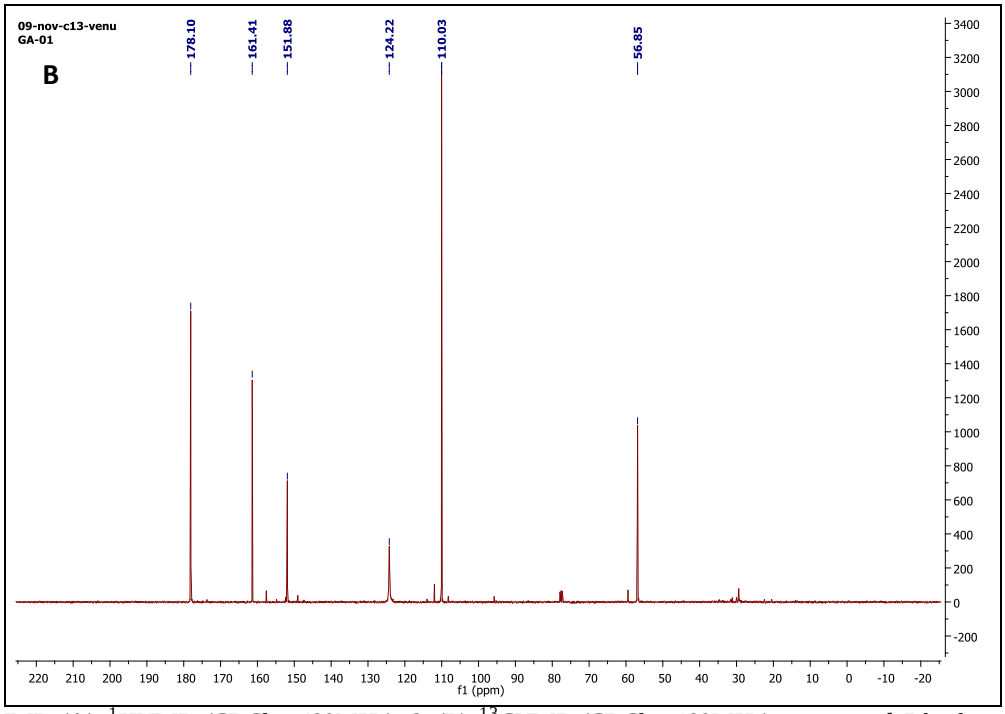


Fig 4. NMR (A) ^{1}HNMR ($CDCl_3$; 400MHz) & (B) ^{13}CNMR ($CDCl_3$; 100MHz) spectra of 5-hydroxymethyl furfural isolated from *Grewia asiatica*

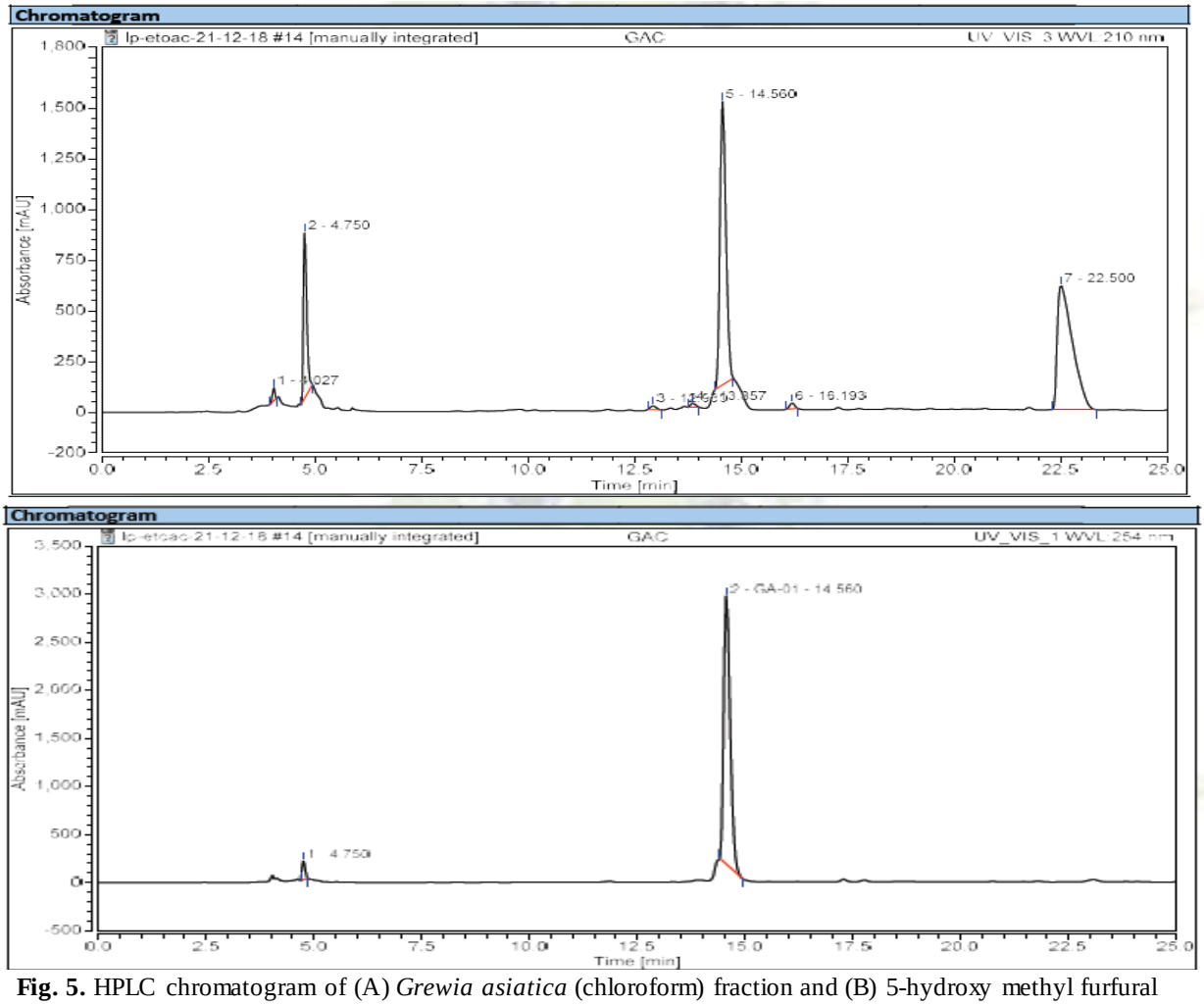


Fig. 5. HPLC chromatogram of (A) *Grewia asiatica* (chloroform) fraction and (B) 5-hydroxy methyl furfural isolated from *Grewia asiatica*

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