Bull. Pharm. Sci., Assiut University, Vol. 48, Issue 1, 2025, pp. 481-496

Bulletin of Pharmaceutical Sciences
Assiut University
Website: http://bpsa.journals.ekb.eg/

BULL. PHARM. SCI. Assiut Univ.

DEATH OF THP-1 LEUKAEMIA CELLS BY *CASSIA FISTULA* FLOWER EXTRACT

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The bioactive components of the plant Cassia fistula L. possess immense biological activities showing anti-inflammatory, anti-microbial, anti-tumor, anti-diabetic, and hepato-protective roles; therefore, this plant-derived formulations are used in many traditional medicinal systems like Ayurveda and Chinese traditional medicine. In this study, we have explored the death-inducing role of the crude ethanol extract of the flowers of the plant on acute monocytic leukemia derived cell line THP-1 cells. The cytotoxicity of the crude ethanol extract of Cassia flowers was observed on a normal human embryonic kidney cell line (HEK 293). The anti-cancer efficacy was studied based on the relative fold change in gene expressions of CD95 (Fas), Caspase 3 (CAS 3), Caspase 9 (CAS 9) as well as of cytokines such as Interleukins - IL-6, IL-8, IL-10, IL-1β; Transforming Growth Factor - TGF β1, TGF β3, Tumor Necrosis Factor (TNF α), Tumor Necrosis Factor Receptor 1 (TNFRSF1A) amongst the different experimental sets. We have also studied the detailed phytochemical profiling of the extract using liquid chromatography followed by positive ion electrospray ionization mass spectrometry. The study results indicated a mild up-regulation of the Cas 3 gene, along with a down-regulation of the IL-8 gene. Mixed changes were observed among the other cytokines. The phytochemical profiling of the extract revealed 16 compounds such as aloin A, Neoisoliquiritin, Rutin, Apigenin-6,8-di-C- glycoside, Myricetin hexoside, Patuletin, which might contribute towards the anti-cancer property of the plant

Keywords: *Cassia fistula, Flower extract, anti-cancer activity, THP-1 cell line, LC-MS analysis, aloin, Neoisoliquiritin, Apigenin, Myricetin*

INTRODUCTION

Plants are potential sources of biomedicine and although many bioactive chemicals known as Natural Products are already documented, there is a plethora of such chemicals which are hitherto unknown to us. *Cassia fistula* L. is an important medicinal plant having its role both in Traditional Chinese Medicine and also in *Ayurveda* [1]. It is a deciduous tree of medium size with bright yellow colored flowers and elongated fruit. As the inflorescence of hundreds of bright-yellow flowers cascades down the stem, it is classically called "Yellow Shower"[1]. Medicinal properties of various functionalities are associated with the different parts of the plant which include antimicrobial, anti-oxidant, anti-diabetic, anti-inflammatory, anti-cancer, and hepato-protective activities[2]. Natural Products wield beneficial action in human and animal diseases as well as in the field of agronomics[3].

The anti-cancer activities of Rhein – a Natural Product present in the ethyl acetate extract of *C. fistula* flowers revealed in human colon adenocarcinoma cell line COLO 320DM[4]. This particular anthraquinone showed minimal cytotoxicity on the Vero cell line. Another study performed the green synthesis of the extract of *C. fistula* leaf and fruit pulp and it showed anti-tumor activity against the MCF-7 breast cancer cell line[5]. The maximum cancer cell growth was 18% and 28% with *C. fistula* leaf and fruit extract respectively[5]. In a previous study, we explored the anti-cancer activity of ethanolic extract of fruit and pulp of *the C. fistula* plant against the hepatic carcinoma cell line (HepG2) and a control

Received : 2/9/2024 & Accepted : 16/12/2024

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normal cell line HEK 293 [6]. It was observed that following the addition of *C. fistula* fruit and leaves extract, the cells started to lose their usual morphological characteristics, they became round-like apoptotic bodies and had detached from the base indicating their loss of viability. The methylene blue assay stated the formation of apoptotic bodies in most of the cells while the control cells were mostly normal [6].

The phytochemical profiling of the plant showed the presence of anthraquinones, tannins, and flavonoids. The detailed phytochemical profiling of the crude ethanolic extract of the flower *Cassia fistula* was determined by scanning the extract in the range of 200 – 800 nm by UV-Vis Spectrophotometer followed by LC –ESI-MS/MS analysis. Several literatures have pointed out the presence of novel chemicals such as epiafzelechin-3-O-glucoside along with other biologically important flavonoids [7].

In the present study, we have explored the anti-cancer efficacy of crude ethanol extract of the *Cassia fistula* flower on the THP-1 leukemia cell line. This cell line was prepared from isolated and cultured cells from an acute monocytic leukemia patient. The anti-cancer efficacy was determined in terms of targeted gene expression changes of Tumor Necrosis Factor Receptor 1 (TNFRSF1A), CD95 (Fas), Caspase 3 (CAS 3), Caspase 9 (CAS 9) genes, along with cytokine gene expression changes of Interleukins - IL-6, IL-8, IL-10, IL-1β, Transforming Growth Factor - TGF β1, TGF β3, Tumor Necrosis Factor (TNF α).

MATERIAL AND METHODS

Collection of *Cassia fistula* flower

The flower sample was collected from the horticulture garden of the Heritage Institute of Technology (22°31′6.72″N, 88°24′58.85″E) on 15th March 2023. The fresh weight of the flower sample was recorded **(Fig. 1)**.

Flower sample processing and extraction

The flower was kept in a hot-air oven at 40ºC for 2 – 3 days. The dry weight of the flower was noted. After drying the flower, it was ground into powder form in a mixture grinder and stored in a sterile air-tight glass container. Extraction was carried out with 70% ethanol (molecular biology grade) in a 1:10 ratio of the powder in dark conditions for 72 hours. Thereafter, the extract was filtered through Whatman filter paper No. 1 and the filtrate was passed through a 0.22-micron syringe filter. The sterile filtrate was stored in an amber-colored glass bottle with an air-tight screw cap [6] **(Fig. 2)**.

Fig. 1: Full bloom flower of *C. fistula*.

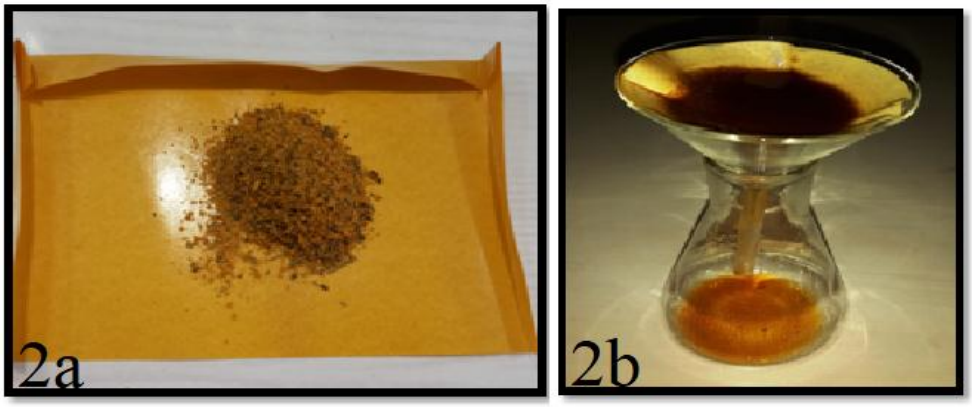


Fig. 2 : Powder form of dried flower of *C. fistula*; 2 b Ethanolic extract of dried *C. fistula* flower

Procurement of Cell Lines and Medium for Culturing and Maintenance

The human monocyte leukemia cell line, THP-1 of passage number 22 was procured from the National Centre for Cell Science (NCCS), Pune, India. For the transportation and culturing of the cell line, sterile Roswell Park Memorial Institute 1640 liquid medium (RPMI 1640 medium; Gibco; Catalog No. 11-875-093) with L-glutamine, and without sodium bicarbonate, suitable for cell culture with 10% Fetal Bovine Serum (FBS, Gibco; Catalogue No. 11573397) was used. Another cell line, the human embryonic kidney cell line (HEK 293 Passage Number- 27) was also procured from NCCS, Pune to observe any detrimental action of the extract on the normal cells. The medium used for the culture and transportation of this cell line was sterile Dulbecco's Modified Eagle Medium with L-glutamine/GlutaMAX supplementation (DMEM) media (Gibco; Catalogue No. 10-566-016).

The cell lines were analyzed using GeneMapper® ID-X v1.5 software (Applied Biosystems) and appropriate positive and negative controls were kept during the assay. The cell lines were tested for *Mycoplasma* contamination by Hoechst staining and/or by PCR method at NCCS, Pune during previous passages before sending the cell lines.

Culturing conditions of the Cell lines

THP-1 is a non-adherent cell line, cultured in a ventilated sterile T 25 cm2 cell culture flask (Himedia Pvt. Ltd., India) with RPMI-1640 medium supplemented with 10% FBS and Penicillin and Streptomycin solution (100 µL for 100Ml; Gibco, Lot No. 2321103). The cell line was kept for growth within a 5% carbon-dioxide environment (Carbon-dioxide incubator, ESCO, Singapore) and 85% - 95% humid conditions. HEK 293 cell line is both an adherent and non-adherent cell line depending on the medium used to culture the cell line. It can be cultured in both ways. The cells were in suspended condition with the absence or low concentration of serum in the media. We used a DMEM medium with Penicillin and Streptomycin solution (Gibco, Lot No. 2321103; 100 µL for 100mL). The cell line was kept for growth within a 5% carbon-dioxide environment (Carbon-dioxide incubator, ESCO, Singapore) and 85% - 95% humid condition in a ventilated sterile T25 cm2 cell culture flask (Himedia Pvt. Ltd., India)[7, 8, 9].

MTT assay to determine the cytotoxicity of the *C. fistula* flower extract

THP-1 and HEK 293 cell lines were seeded in a 96-well micro-titre plate. Each well contains nearly 1x104 cells. The extract was inoculated on both the cell lines in the dosage – 0, 20 µL, 40 µL, 60 µL, and 80 µL per mL concentration. A controlled study was also done with only media and media plus extract of the same concentration without the cell lines to find out the baseline optical density (O.D.) levels. All sets were replicated in triplicates to standardize the findings. The 96-well micro-titre plate was kept in a sterile 5% CO_2 environment with 85 – 95% humidity for about 18 hours. 10µL of MTT reagent was added to each well and the plate was agitated manually. The plate was kept in incubation at the above-mentioned conditions for the next 4 hours. After that, each well was visualized under 400x magnification of an inverted microscope to

observe the formazan crystal formations. Following the crystal formation, 100 µL solubilizing agent was added to each well, and again the plate was shaken well for proper mixing. The plate was kept for overnight incubation at a sterile 5% CO_2environment with 85 – 95% humidity for another 18 hours and absorbance was taken at 570nm. The percentage of cell death was calculated with the formula mentioned below:

% cell death = 100- [A_t / A_c ×100]

Where A_c was the absorbance of the control and A_t was the absorbance of the treated cells. The IC50 value was calculated as half of the maximum inhibitory effect [6].

Extract Inoculation to cell lines

Both THP-1 and HEK 293 cell lines were seeded in a 12-well culture plate, where the cell concentration in each well was approximately $1x10^5$ – $1x10^6$. Based on the MTT assay, IC50 dosage value, i.e., 20 µL of the *C. fistula* flower extract was inoculated upon the THP-1 and HEK 293 cell lines in 12 well plates respectively. The following sets were prepared – a) Control set – cell lines with media in the absence of extract; b) Alcohol control set – cell lines inoculated with 20 µL of 70% ethanol; c) treated cells – cell lines treated with 20 µL of the *C. fistula* flower extract. The plate was shaken gently in a clockwise and anti-clockwise manner and was kept in incubation for 18 hours in a sterile 5% CO_2 environment with 85 – 95% humidity. The next day the cells were observed under an inverted microscope (400 x magnification) and the pictures were recorded. Cell cytopathic effect (CPE) was studied [6].

Gene expression assay by RT-PCR of the targeted cytokines

The cells were harvested using RNA isoplus for 5 minutes to extract the RNA following the steps of the manufacturer. The total RNA was dissolved in 60µL of nuclease-free water at 56ºC in a water bath for 10 minutes. The yield was measured using an A260/280 ratio in a UV-Vis spectrophotometer. The total RNA was then converted to cDNA using a cDNA synthesis kit (Bio-Rad, USA). The gene expression of selected cytokines namely Interleukins - IL-6, IL-8, IL-10, IL-1β, Transforming Growth Factor - TGF β1, TGF β3, Tumor Necrosis Factor (TNF α), Tumor Necrosis Factor Receptor 1 (TNFRSF1A), CD95 (Fas), Caspase 3 (CAS 3), Caspase 9 (CAS 9) were calculated as relative fold change in gene expression against house-keeping gene β-actin using RT-PCR (CFX-96 model, Bio-Rad, USA). The relative fold change in gene expression was calculated using the formula $2^{-(\Delta\Delta Ct)}$ [6, 10]. Here $\Delta\Delta Ct = \Delta Ct1 - \Delta Ct2$, i.e., ΔCt = Ct (target gene) – Ct (reference gene); Therefore, $\Delta\Delta Ct = \Delta Ct$ (target sample) - ΔCt (Reference Sample). Here Ct stands for cycle threshold. The gene expression of the reference sample is usually normalized to 1 as because $\Delta\Delta CT$ is equal to 0 and therefore 2^0 is equal to 1[10].

The primer sequences of the cytokines studied are given below [9]

IL-6 F: AGACAGCCACTCACCTCTTCAG; R: TTCTGCCAGTGCCTCTTTGCTG

IL-8F: GAGAGTGATTGAGAGTGGACCAC; R: CACAACCCTCTGCACCCAGTTT

IL-10 F: TCTCCGAGATGCCTTCAGCAGA; R: TCAGACAAGGCTTGGCAACCCA

IL-1β F: CCACAGACCTTCCAGGAGAATG; R: GTGCAGTTCAGTGATCGTACAGG

TGF β1 F: TACCTGAACCCGTGTTGCTCTC; R: GTTGCTGAGGTATCGCCAGGAA

TGF β3 F: CTAAGCGGAATGAGCAGAGGATC; R: TCTCAACAGCCACTCACGCACA

TNF α F: CTCTTCTGCCTGCTGCACTTTG; R: ATGGGCTACAGGCTTGTCACTC

TNFRSF1A F: CCGCTTCAGAAAACCACCTCAG; R: ATGCCGGTACTGGTTCTTCCTG

CD95(FAS) F: GGACCCAGAATACCAAGTGCAG; R: GTTGCTGGTGAGTGTGCATTCC

Cas 3 F: GGAAGCGAATCAATGGACTCTGG; R: GCATCGACATCTGTACCAGACC

Cas 9 F: GTTTGAGGACCTTCGACCAGCT; R: CAACGTACCAGGAGCCACTCTT

β-actin F: CACCATTGGCAATGAGCGGTTC; R: AGGTCTTTGCGGATGTCCACGT

Phytochemical Analysis using UV-Vis Spectrophotometer

The extract was analyzed using UV-Vis Spectrophotometer using a quartz cuvette with a slit width of 2nm, at room temperature. The extract was diluted in a 1:1 ratio with 70% ethanol and scanned in the range of 200 – 800 nm. The peaks were analyzed based on the previous scientific reports [11].

LC –ESI-MS/MS of ethanolic extract of *C. fistula* flower

The ethanolic extract of *C. fistula* flower was analyzed using LC –ESI-MS/MS (Waters Acquity TM, USA). 100µL of the sample was infused through reverse-phase ultra-performance liquid chromatography and electrospray ionization mass spectrometry. The MS system operates in the positive ion mode. Based on the m/z value of the analyte, the compounds **(Table 1)** were compared in the standard database and probable presence was reported [12].

Statistical Analysis

The changes in relative fold change in cytokine expressions were analyzed by two-way ANOVA using the statistical software Graph-Pad Prism version 9.3.1. The result showed significant changes between the row factors and column factors individually **(Table 2)**.

Table 1: Comparative relative fold change in gene expression analysis of of Interleukins – IL-6, IL-8, IL-10, IL-1β, Transforming Growth Factor - TGF- β1, TGF- β3, Tumor Necrosis Factor Receptor-1 (TNFRSF1A), Caspases – Cas 3 and 9 against control gene β-actin.

Sets (IL 6)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.06 ± 0.05	1.05 ± 0.05
Cassia	3.52 ± 0.18	2.62 ± 0.13
Ethanol Control	3.89 ± 0.19	0.63 ± 0.03
Sets (IL 8)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.04 ± 0.052	1.00 ± 0.050
Cassia	0.12 ± 0.006	0.97 ± 0.050
Ethanol Control	0.22 ± 0.011	0.27 ± 0.010
Sets (IL 10)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.00 ± 0.050	1.185 ± 0.059
Cassia	0.28 ± 0.014	0.59 ± 0.030
Ethanol Control	0.32 ± 0.016	1.68 ± 0.084
Sets (IL 1β)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.06 ± 0.05	1.185 ± 0.059
Cassia	1.60 ± 0.08	7.96 ± 0.39
Ethanol Control	25.03 ± 1.25	0.41 ± 0.021
Sets (TGF β1)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.04 ± 0.052	1.185 ± 0.060
Cassia	0.068 ± 0.003	2.65 ± 0.133
Ethanol Control	0.61 ± 0.031	0.61 ± 0.031
Sets (TGF β3)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.00 ± 0.050	1.00 ± 0.050
Cassia	1.86 ± 0.09	0.05 ± 0.00
Ethanol Control	2.34 ± 0.012	0.02 ± 0.00
Sets (TNF α)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.00 ± 0.050	1.185 ± 0.06
Cassia	0.10 ± 0.001	3.34 ± 0.17
Ethanol Control	0.67 ± 0.03	0.18 ± 0.01

Table 1: Continued.

Sets (TNFRS)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.00 ± 0.050	1.12 ± 0.056
Cassia	0.17 ± 0.008	0.95 ± 0.048
Ethanol Control	0.71 ± 0.035	0.72 ± 0.036
Sets (CD95)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.00 ± 0.050	1.01 ± 0.051
Cassia	0.036 ± 0.02	2.69 ± 0.135
Ethanol Control	1.68 ± 0.084	7.70 ± 0.385
Sets (Cas 3)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.06 ± 0.053	4.25±0.050
Cassia	1.78 ± 0.089	2.36±0.028
Ethanol Control	4.43 ± 0.222	2.93±0.034
Sets (Cas 9)	**THP-1 (Mean ± SD)**	**HEK 293 (Mean ± SD)**
Control	1.06 ± 0.053	1.185 ± 0.059
Cassia	0.21 ± 0.011	7.91±0.396
Ethanol Control	1.73 ± 0.087	4.20±0.210

Table 2 : LC-MS analysis table of ethanolic flower extract of *C. fistula.*

Compound ID	**Adducts**	**Formula**	**Theoretical Isotope Distribution**	**Description**	**m/z**	**Charge**	**Retention time (min)**
1	M+H	C24H50NO7P	100 - 27.2 - 4.99 - 0.687	MFCD00036904	496.3500878	1	11.04986667
2	M+H	C21H22O9	100 - 23.3 - 4.44 - 0.614	aloin A	419.1373883	1	7.182116667
3	M+H	C21H22O9	100 - 23.3 - 4.44 - 0.614	Neoisoliquiritin	419.1373883	1	7.182116667
4	M+H	C21H22O9	100 - 23.3 - 4.44 - 0.614	3-Hydroxy-4-[(2E)-3-(4-hydroxyphenyl)-2-propenoyl]phenyl hexopyranoside	419.1373883	1	7.182116667
5	M+H	C21H22O9	100 - 23.3 - 4.44 - 0.614	3-Hydroxy-4-[(2E)-3-(4-hydroxyphenyl)-2-propenoyl]phenyl D-glucopyranoside	419.1373883	1	7.182116667
6	M+K	C24H26O4-2	100 - 26.4 - 4.17 - 0.488	(2E,4Z,6E,8E,10E,12E,14E,16E,18E)-4,8,13,17-Tetramethyl-2,4,6,8,10,12,14,16,18-icosanonaenedioate	417.1394867	1	7.182116667
7	M+K	C20H26O7	100 - 22.2 - 3.79 - 0.476	(1alpha,2beta,4bbeta,10beta)-2-Hydroxy-1-methyl-8-methylenegibbane-1,4a,10-tricarboxylic acid	417.1394867	1	7.182116667

Table 2: Continued.

8	M+K	C20H26O7	100 - 22.2 - 3.79 - 0.476	gibberellin A17	417.1394867	1	7.182116667
9	M+H	C30H26O12	100	Procyanidin B2	552.3313837	1	7.491883333
10	M+H	C27H30O16	100 - 0.397	Rutin	304.3344351	1	8.713816667
11	M+H	C33H40O14	100 - 0.588	200-O-Rhamno-sylicariside II	496.3500878	1	11.04986667
12	M+H	C27H30O15	100	Apigenin-6,8-di-C- glycoside	473.2352966	1	11.42293333
13	M+H	C15H12O4	100 - 0.117	Furoaloesone	170.1393674	1	9.4485
14	M+H	C21H20O13	100	Myricetin hexoside	318.3325795	1	8.08265
15	M+H	C16H12O8	100	Patuletin	332.35	1	19.865
16	M+H	C13H16O10	100	1-Galloyl-β-D-glucose	332.41	1	4.6

The important phytochemicals isolated from the flower extract were aloin A, Neoisoliquiritin, Rutin, Apigenin-6,8-di-C- glycoside, Myricetin hexoside, Patuletin, etc. which might contribute towards the varied medicinal properties of the plant.

RESULTS AND DISCUSSION

Results

MTT assay

By assessing the mitochondrial activity of a viable cells, the colorimetric MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay determines the amount of live cells present. The tetrazolium ring of MTT is broken down by dehydrogenases found in the mitochondria of living cells. Viable cells convert the MTT chemical into a purple formazan precipitate, which builds up in the culture media and cells. The wavelength at which the formazan absorbs the greatest, typically about 570 nm, is used to quantify the optical density (OD) of the MTT-formazan solution. The intracellular reduction of MTT and the formazan concentration are presumed to be represented by the OD value. MTT assay was done to analyze the cyto-toxicity of ethanolic extract of *C. fistula* flower on THP-1 and HEK 293 cell lines. 20µL/ mL of extract was considered to be concentration cyto-toxic to THP-1 in comparison to HEK 293 cell line **(Fig. 3)**.

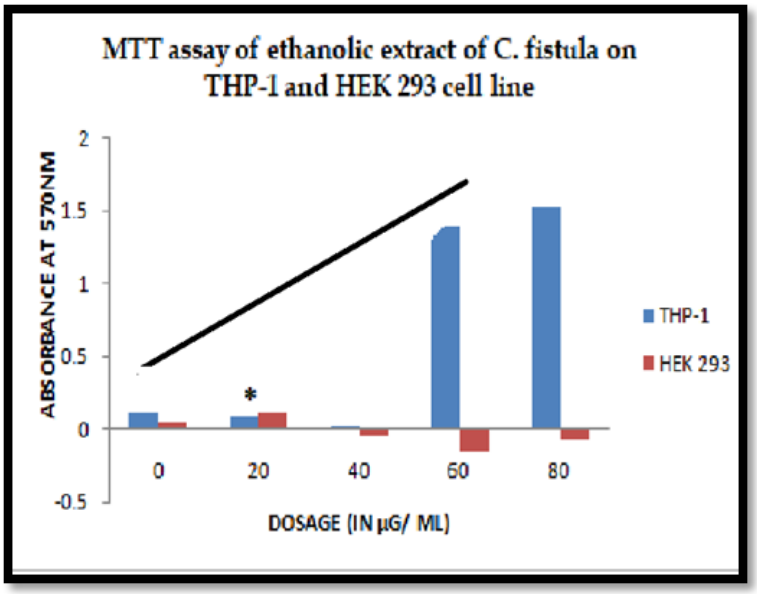
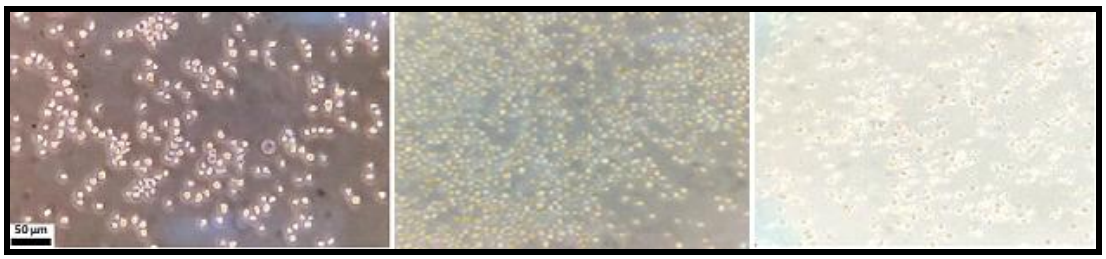


Fig. 3: MTT assay to analyze the cyto-toxicity of ethanolic extract of *C. fistula* flower on THP-1 and HEK 293 cell lines. 20µL/ mL of extract was considered to be concentration cyto-toxic to THP-1 in comparison to HEK 293 cell line.

Cell Cytopathic Effect

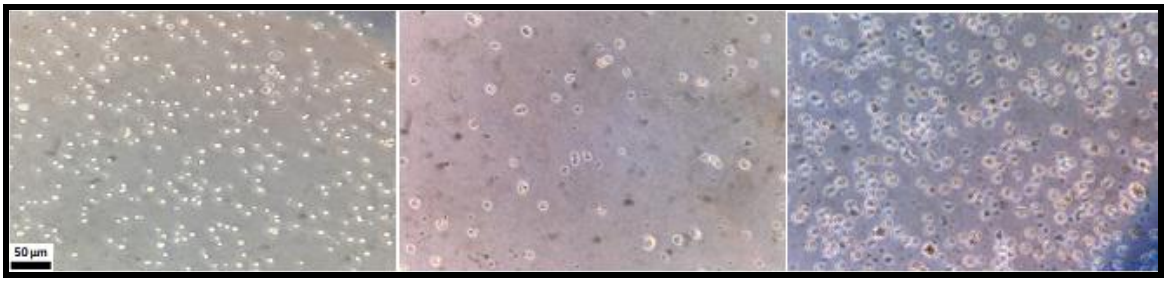
In **Fig. 4a** - Control THP-1 cells were round to oval in shape with mild pleo-morphism, big nuclei with fine reticulum; cytoplasm was abundant. No degenerative change was noted; **Fig. 4b** cells showed degenerative changes along with membrane defect, mild karyorrhexis and irregular outline; **Fig. 4c** - The cell-like round bodies were smaller in size, with prominent outline, mostly without nuclei like necrotic cells and apoptotic bodies. On the basis of the cytopathic effects, it can be said that in the test sample monocytic leukemia cell death occurred because of the inoculation of *Cassia fistula* flower extraction against the control cell line (without any inoculation). The Ethanol (70%) vehicle control also did not show as destructive properties as *Cassia fistula* flower extract. **Fig. 4d** - Control HEK 293 cells in suspension culture was round to oval in shape with big nuclei and proper cell membrane; cytoplasm was abundant. No degenerative changes were observed; **Fig. 4e** - cells showed degenerative changes along with membrane defect, irregular outline, and smaller in size than usual; **Fig. 4f** – The HEK 293 cells in suspension proliferated in huge numbers with the inoculation of *C. fistula* flower extract and the cell morphology was much healthier with proper nuclei and margin **(Fig. 4a – 4c and 4d – 4f)**.



4 a 4b 4c

Fig. 4a: - THP-1 cells 24 hours control; 4b - THP-1 cell line with 70% ethanol treatment (vehicle control); 4c - THP-1 cell line with 70% ethanol extract of flowers of *C. fistula* treatment (Same scale bar for all pictures).

4a - Control THP-1 cells were round to oval in shape with mild pleo-morphism, big nuclei with fine reticulum; cytoplasm was abundant. No degenerative change was noted; 4b - cells showed degenerative changes along with membrane defect, mild karyorrhexis and irregular outline; 4c - The cell-like round bodies were smaller in size, with prominent outline, mostly without nuclei like necrotic cells and apoptotic bodies. On the basis of the cytopathic effects, it can be said that in the test sample monocytic leukemia cell death occurred because of the inoculation of *Cassia fistula* flower extraction against the control cell line (without any inoculation). The Ethanol (70%) vehicle control also did not show as destructive properties as *Cassia fistula* flower extract.



4d 4e 4f

Fig. 4d: – HEK 293 cells in suspension culture, 24 hours control; 4e - HEK 293 cells with 70% ethanol treatment (vehicle control); 4f - HEK 293 cells with 70% ethanol extract of flowers of *C. fistula* treatment (Same scale bar for all pictures).

4d - Control HEK 293 cells in suspension culture were round to oval in shape with big nuclei and proper cell membrane; cytoplasm was abundant. No degenerative changes were observed; 4e - cells showed degenerative changes along with membrane defect, irregular outline, and smaller in size than usual; 4f – The HEK 293 cells in suspension proliferated in huge numbers with the inoculation of *C. fistula* flower extract and the cell morphology was much healthier with proper nuclei and margin.

Gene expression assay by RT-PCR

The cytokine gene expression of IL-6 had increased in both THP-1 (3.5 fold) and HEK-293 cells (2.6 fold) with inoculation of *C. fistula* flower extract, however, with inoculation of vehicle control IL-6 expression got further increased in THP-1 cells (3.89 fold). In-case of IL-8, the gene expression got down-regulated in THP-1 cells (0.12 fold) with the inoculation *C. fistula* flower extract but in HEK 293 cells (3.00 fold) the gene expression was comparative to control set. IL-10 gene expression was down-regulated in both the cell lines with respect to control set (0.29 fold in THP-1 cells and 0.59 fold in HEK 293 cells), however, ethanol control had mildly increased its expression in HEK 293 cells (1.69 fold). The gene expression of IL-1β had mildly increased (1.60 fold) in the *C. fistula* set but the expression was around 7.96 fold in the HEK 293 cells. Gene expression of TGF-β1 was down-regulated in the THP-1 cells in the *C. fistula* flower extract set, but it got enhanced (2.66 fold) in the HEK 293 cell line by the flower extract. In the vehicle control set, the gene expression was lower than the control set. The expression of TGF-β3 got up-regulated in the flower extract set (1.86 fold) in the THP-1 cells; however the up-regulation could be attributed to vehicle control. The gene expression of TNFRSF1A got markedly down-regulated (0.17 fold) among the THP-1 cells in comparison to control. Similarly, the gene expression of CD95 was also down-regulated in the flower extract set (0.04 fold) in comparison to the control set. The gene expression of Cas 3 had mildly enhanced (1.79 fold) in the *C. fistula* flower extract set and it got highly up-regulated in the vehicle control set (4.43 fold) in the THP-1 cell line. However, *C. fistula* flower extract set down-regulated the expression of Cas 9 gene expression in the THP-1 cell line (0.22 fold), but it got up-regulated in the vehicle control set among both the cell lines (1.73 fold in THP-1 cell line; 4.2 fold in HEK 293 cells) **(Table 1)**.

Table 3: Statistical analysis.

Table Analyzed	Data 1				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	51.22	<0.0001	****	Yes	
Row Factor	38.05	<0.0001	****	Yes	
Column Factor	10.54	<0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	439551	30	14652	F (30, 84) = 774.9	P<0.0001
Row Factor	326573	6	54429	F (6, 84) = 2879	P<0.0001
Column Factor	90456	5	18091	F (5, 84) = 956.8	P<0.0001
Residual	1588	84	18.91		
Data summary					
Number of columns (Column Factor)	6				
Number of rows (Row Factor)	7				
Number of values	126				

The statistical analysis of the data by Two-way ANOVA was found to be significant with P- value <0.0001.

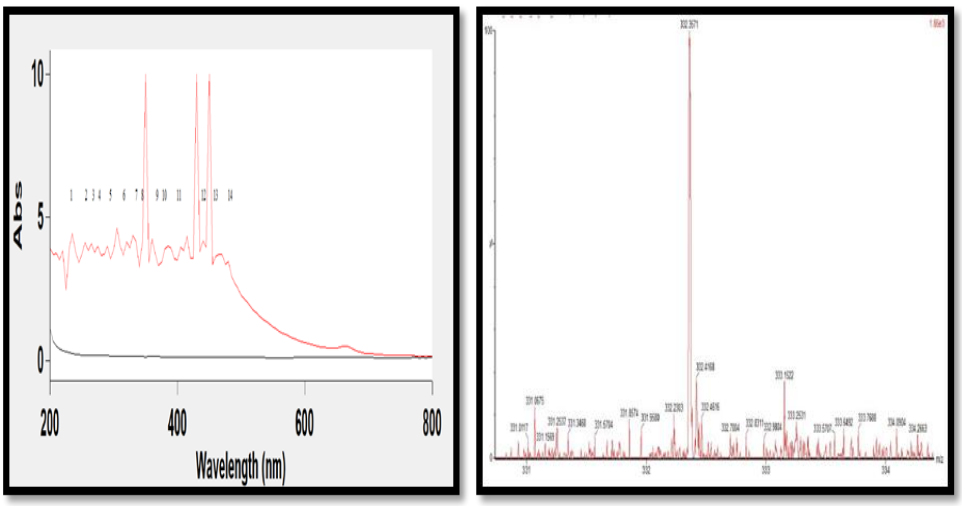
Discussion

Acute Myeloid Leukemia (AML) is a heterogeneous hematological cancer that is exceedingly aggressive. The particular cancer is associated with molecular and cytogenetic mutations among the hematopoietic stem and/or progenitor cells (HSPCs) which lead to the development of leukemic stem cells (LSCs) [13]. 29.5% of the patients who suffer from AML undergo an extreme clinical treatment course not having more than a 5-year survival rate as per the surveillance report of the year, 2021 [14]. The associated risk factors are gender, age of the patient, any history of prior exposure to chemo and radiation therapy, along any other genetic inclination[15, 16]. During the diseased condition, an array of cytokines Interleukin IL-3, IL-1β, IL-6, Tumor necrosis factor-α (TNF-α), and Interferon (IFN) together with hematopoietic growth factors (HGFs) such as M-CSF, G-CSF and Granulocyte-macrophage colony-stimulating factor (GM-CSF) suddenly bounds from the normal state to crisis level for neoplastic hematopoiesis[17,18]. In general, a relatively higher age group is affected by AML, therefore, it is also known as the most common acute leukemia in adults. The survival prognosis of elderly patients is awfully poor [19].

The MTT assay was done to analyze the cyto-toxicity of ethanolic extract of *C. fistula* flower on THP-1 and HEK 293 cell lines. 20µL/ mL of extract was considered to be concentration cyto-toxic to THP-1 in comparison to HEK 293 cell line **(Fig. 3)**. Previous anti-cancer efficacy of the leaf and fruit extract of the same plant showed 50µL to be the IC_{50} value upon hepatic carcinoma (HepG2) cell line [6]. When the cell cytopathic study was done, it was observed that the Control THP-1 cells were round to oval in shape with mild pleo-morphism, big nuclei with fine reticulum; cytoplasm was abundant. No degenerative change was noted. In the THP-1 cell line with 70% ethanol treatment (vehicle control) set, cells showed degenerative changes along with membrane defect, mild karyorrhexis and irregular outline. In the THP-1 cell line with 70% ethanol extract of flowers of *C. fistula* treatment set, the cell-like round bodies were smaller in size, with prominent outline, mostly without nuclei like necrotic cells and apoptotic bodies. On the basis of the cytopathic effects, it can be said that in the test sample monocytic leukemia cell death occurred because of the inoculation of *Cassia fistula* flower extraction against the control cell line (without any inoculation). Therefore, ethanol (70%) vehicle control also did not show as destructive properties as *Cassia fistula* flower extract. In control HEK 293 cells in suspension culture was round to oval in shape with big nuclei and proper cell membrane; cytoplasm was abundant. No degenerative changes were observed; Whereas in the HEK 293 cells with 70% ethanol treatment (vehicle control) cells showed degenerative changes along with membrane defect, irregular outline, and smaller in size than usual; At last in HEK 293 cells with 70% ethanol extract of flowers of *C. fistula* treatment set, the HEK 293 cells in suspension proliferated in huge numbers with the inoculation of *C. fistula* flower extract and the cell morphology was much healthier with proper nuclei and margin. When similar study was conducted upon liver cancer cell line (HepG2), with the *Cassia* plant leaves and fruit extract, it was observed that after being treated with the ethanolic plant fruit and leaf extract, the HepG2 cells began to lose their integrity, developed blebs, and round out, all of which were signs of apoptosis and cell death. After a day of drug inoculation, the cells also separated from the surface. Both apoptotic and non-apoptotic bodies were observed in the vehicle control set (alcohol). In the vehicle control set, membrane blebs were observed and the cell size was decreased. The majority of the cells were dead, showing necrosis or apoptosis, while some cells demonstrated their vitality [6].

Studies have also shown that there is an induction of pro-inflammatory microenvironment due to the abnormal regulation of cytokines and this is considered an important hallmark of Acute Myeloid Leukemia (AML)[20]. This is marked by the hyper-production of pro-inflammatory cytokines such as Interleukin-6 and Tumor Necrosis Factor-α along with abnormal IL-1β signaling which as a whole leads to the formation of dysregulation of cytokine network among the AML patients. Certain anti-inflammatory cytokines namely transforming growth factor β (TGF-β) and Interleukin- 10 are the two important signaling molecules that cause peripheral tolerance and re-establish homeostasis [21]. Interleukin-6 is a pro-

inflammatory cytokine that remains elevated in the plasma and partially supports the growth and proliferation of AML cells [22]. In our experimental results, the cytokine gene expression of IL-6 has increased in both THP-1 (3.5 fold) and HEK-293 cells (2.6 fold) with inoculation of *C. fistula* flower extract, however, with inoculation of vehicle control IL-6 expression got further increased in THP-1 cells (3.89 fold). Thus, the up-regulation of IL-6 gene expression is triggered by vehicle control (70% ethanol). Among the AML patients, the chemokine, and IL-8 gene expressions are high in the peripheral blood and bone marrow. Previous scientific studies reported that anomalous hyper-expression of the IL-8 gene is responsible for myeloid malignancies[23]. IL-8 binds with CXCR1/CXCR2 receptors which in turn activates the oncogenic signaling pathway. The outcome of anomalous hyper-expression of the IL-8 gene is a worse prognosis of AML and MDS patients along with enhanced bone marrow fibrosis in Myelofibrosis [24]. In the case of IL-8, the gene expression got down-regulated in THP-1 cells (0.12 fold) with the inoculation *C. fistula* flower extract and in HEK 293 cells (0.97 fold) the gene expression is comparative to the control set. However, in the ethanol control set, the gene expression of IL-8 got down-regulated concerning control among both the cell lines. IL-10 is an anti-inflammatory cytokine that remains elevated in the peripheral blood (PB) [25]. The function of the particular cytokine is that it inhibits the proliferation of AML cells and down-regulates the production of IL-6, IL-1α, IL-1β, GM-CSF, and TNF-α expression. Here in our experimental data, IL-10 gene expression was down-regulated in both the cell lines concerning the control set (0.29 fold in THP-1 cells and 0.59 fold in HEK 293 cells), however, ethanol control has mildly increased its expression in HEK 293 cells (1.69 fold). This data does not support the anti-cancer activity of flower extract of *C. fistula*. Similarly, TGF-β is another anti-inflammatory cytokine that remains down-regulated in the PB and BM levels. The property of this cytokine is to restrain the proliferation and survival of AML cells[21]. Gene expression of TGF-β1 is down-regulated in the THP-1 cells in the *C. fistula* flower extract set, but it got enhanced (2.65 fold) in the HEK 293 cell line by the flower extract. In the vehicle control set, the gene expression was lower than the control set. However, the expression of TGF-β3 got up-regulated in the flower extract set (1.86 fold) in the THP-1 cells.



6 a 6 b

Fig. 5: UV-Vis spectral scan (200 nm – 800 nm) and LC-MS (of ethanolic extract of *C. fistula*.

According to the previous research report, the gene expression of IL - 1β either shows no change or remains enhanced in the PB, and it remains unchanged in the BM. This is a pro-inflammatory cytokine that helps in the growth and proliferation of AML cells and their survival [26]. It also increases the expression of IL-6, GM-CSF, and TNF expression [26]. The gene expression of IL-1β has mildly increased (1.60 fold) in the *C. fistula* set but the expression is around 7.96 fold in the HEK 293 cells. The expression of this cytokine is marginally influenced by *C. fistula* flower extract concerning the control set. The gene TNFRSF1A denotes tumor necrosis factor receptor 1 (TNFR1). The gene expression of TNFRSF1A got markedly down-regulated (0.17 fold) among the THP-1 cells in comparison to the control. CD95 (FAS/APO-1) is the protein antigen that acts as a new prognostic marker for blast cells of acute lymphoblastic leukemia patients [27, 28]. The gene expression of CD95 was also down-regulated in the flower extract set (0.036 fold) in comparison to the control set among the THP-1 cells. Caspase-3 belongs to the member of cysteine protease family that plays an important role in apoptosis. The gene expression of Cas 3 had mildly enhanced (1.78 fold) in the *C. fistula* flower extract set and it got highly up-regulated in the vehicle control set (4.43 fold) in the THP-1 cell line. However, *the C. fistula* flower extract set down-regulated the expression of Cas 9 gene expression in the THP-1 cell line (0.21 fold), but it got up-regulated in the vehicle control set among both the cell lines (1.73 fold in THP-1 cell line; 4.2 fold in HEK 293 cells).

The important phytochemicals isolated from the flower extract were aloin A, Neoisoliquiritin, Rutin, Apigenin-6,8-di-C-glycoside, Myricetin hexoside, Patuletin, which might contribute towards the varied medicinal properties of the plant. Aloin is considered to be an active constituent among the medicinal plants of Genus Aloe which is reported to have several medicinal properties such as anti-cancer, anti-inflammatory, anti-oxidative, etc[29]. Compound Neoisoliquiritin belongs to a class of licorice (Glycyrrhiza) which is a flavonoid possessing tumor-suppressive properties, acts as an anti-depressant, and shows anti-inflammatory action. It shows chemo-preventive effectiveness against prostate cancer and it is included within the commercial combination of eight herbs named PC-SPES [30]. Apigenin is another flavonoid that is found within several vegetables and fruits demonstrated anti-tumor activity against several types of cancer specifically prostate cancer by blocking the IKKα and IKKβ activity, stimulating apoptosis, autophagy, resulting in cell cycle arrest, blocking the migration of cells and metastasis and thus overall enhance the immune response [31]. The phytochemical rutin belongs to the class of flavonoids and can be extracted from several natural sources like fruits (grapes, apples, lemons), vegetables (potatoes, carrots), and beverages like tea and wine [32]. The compound is reported to have several medicinal properties antidiabetic, anti-inflammatory, antioxidant, antimicrobial, vasoprotective, and anti-cancer activities. It inhibits the growth of tumors by arresting the cell cycle process, thereby inducing apoptosis in several cancer cell models [32]. However, due to a major drawback, this particular compound is not suitable for pharmacological application as it is not absorbed in its native form. It hydrolyzes into quercetin which gets absorbed as conjugated metabolite in the blood circulation. Rutin also demonstrates low solubility which weakens its bio-availability and inhibits the formation of a delivery system [32]. Myricetin is a hexahydroxyflavone and is present in natural sources like berries, fruits, vegetables, medicinal herbs, beverages, etc [33]. The reported medicinal properties are anti-inflammatory, anti-diabetic, antioxidant, and hepato-protective, along with other disease management activities. Myricetin demonstrates anti-cancer activity by the modulation of several cell-signaling molecules, thereby inhibiting cell proliferation, angiogenesis, and metastasis, and stimulating the apoptotic pathway[34]. Past scientific reports have shown that myricetin reveals pro-apoptotic and anti-proliferative effects upon K562 human leukemia cells in a concentration-dependent manner. Thus myricetin has anti-leukaemic activity following the inhibitory mechanism of biosynthesis of purine nucleotides and suppresses the catalytic potential of hIMPDH1/2 [34]. Patuletin or 3, 5, 7, 3′, 4′-pentahydroxy-6-methoxy-flavone is a natural

flavonoid present in the genus *Eriocaulon* [35]. The compound is reported to have anti-cancer efficacy against breast cancer (studied on human breast cancer cells, particularly in SK-BR-3 cells), by inhibiting intracellular FASN (Fatty acid synthase) activity which stimulates breast cancer cell apoptosis[36].

Thus, the extract of the flower, *C. fistula* may have anti-cancer activity against the human monocytic cell line, isolated and cultured from an acute monocytic leukemia patient based on the overall relative fold change in the gene expression of the targeted cytokines where the detrimental effect of one cytokine is getting ameliorated by the other cytokines in the *in-vitro* model.

Limitations of The Study

Further study in animal models is necessary to correlate the above-discussed findings within an *in-vivo* model before clinical application.

Conclusions

Flower extract of *Cassia fistula* possess anti-cancer activity which might be due to the presence of an array of phytochemicals such as aloin, Neoisoliquiritin, Apigenin, Myricetin, Patuletin, 3-Hydroxy-4-[(2E)-3-(4-hydroxyphenyl)-2-propenoyl]phenyl hexopyranoside, 3-Hydroxy-4-[(2E)-3-(4-hydroxyphenyl)-2-propenoyl]phenyl D-glucopyranoside, 1alpha,2beta,4bbeta,10beta)-2-Hydroxy-1-methyl-8-methylenegibbane-1,4a,10-tricarboxylic acid, gibberellin A17, Procyanidin B2, Rutin, Apigenin-6,8-di-C-glycoside, Myricetin hexoside, Furoaloesone,

Abbreviations

AML: Acute Myeloid Leukemia
THP-1: Tohoku Hospital Pediatrics-1 Cells
IL: Interleukins
TGF: Transforming Growth Factor
TNF: Tumor Necrosis Factor
TNFRSF1A:Tumor Necrosis Factor Receptor 1
CAS 3: Caspase 3
CAS 9: Caspase 9
LC-MS: Liquid Chromatography- Mass spectrometry
CD95: Cluster of Differentiation 95
COLO320 DM cells: Colorectal Adenocarcinoma Cancer
VERO cell line: Normal, Adult African Green Monkey Kidney Cell Line
MCF-7: Michigan Cancer Foundation-7 Breast cancer cell line
HEK 293: Human Embryonic Kidney cell line
NCCS: National Centre For Cell Science
RPMI 1640: Roswell Park Memorial Institute 1640 liquid medium
FBS: Fetal Bovine Serum
DMEM: Dulbecco's Modified Eagle Medium
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay
CPE: Cell cytopatheic effect
cDNA: complementary Deoxy Ribonucleic Acid
RT-PCR: Reverse Transcriptase Polymerase Chain Reaction
LSCs: Leukemic Stem Cells
HGFs: hematopoietic growth factor
GM-CSF: Granulocyte-macrophage colony-stimulating factor
PB: Peripheral Blood
BM: Bone Marrow
CXCR1/CXCR2: C-X-C Motif Chemokine Receptor ½
PC-SPES: a patented herbal mixture that was sold as a dietary supplement and used as a complementary and alternative medicine (CAM) treatment for prostate cancer.
IKKα: IκB kinase α/β
SK-BR-3 cells: a human breast cancer cell line isolated by the Memorial Sloan–Kettering Cancer Center

Acknowledgements

The authors would like to acknowledge the Heritage Institute of Technology for providing the infrastructure to carry out the research work.

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Bull. Pharm. Sci., Assiut University, Vol. 48, Issue 1, 2025, pp. 481-496.

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موت خلايا اللوكيميا THP-1 بواسطة مستخلص زهرة كاسيا فيستيولا

ديباسميتا تشاترجي – كريشنيندو بايرا – ساتادال داس*

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تمتلك المكونات البيولوجية الفعالة لنبات كاسيا فيستيولا لأنشطة بيولوجية هائلة حيث تظهر خصائص مضادة للالتهابات، مضادة للبكتيريا، مضادة للأورام، مضادة للسكري، وحامية للكبد؛ ولذلك، يتم استخدام المستحضرات المشتقة من هذا النبات في العديد من أنظمة الطب التقليدي مثل الأيورفيدا والطب الصيني التقليدي. في هذه الدراسة، قمنا باستكشاف دور المستخلص الخام للإيثانول من زهور النبات في تحفيز موت الخلايا في خط الخلايا المشتقة من اللوكيميا الأحادية الحادةTHP-1 . تم ملاحظة السمية الخلوية للمستخلص الخام من زهور كاسيا على خط خلايا الكلى البشرية الجنينية الطبيعية (HEK 293). تم دراسة فعالية مكافحة السرطان بناءً على التغير النسبي في تعبيرات الجينات لـ CD95 (Fas)، كاسبيز ٣ (CAS 3)، كاسبيز ٩ (CAS 9)، بالإضافة إلى السيتوكينات مثل الإنترلوكينات IL-6 -، IL-8، IL-10، IL-1β؛ عامل النمو المحول TGF β1 -، TGF β3، عامل نخر الورم (TNF α)، مستقبل عامل نخر الورم ١ (TNFRSF1A) بين المجموعات التجريبية المختلفة. كما قمنا بدراسة كيمياء العقاقير التفصيلي للمستخلص باستخدام كروماتوغرافيا السائل تليها تقنية التحليل الطيفي الكتلي باستخدام التأين بالرذاذ الكهربائي للأيونات الموجبة. أظهرت نتائج الدراسة تنظيمًا خفيفًا للجين Cas 3، بالإضافة إلى تنظيم سلبي لجين IL-8. تم ملاحظة تغييرات مختلطة بين السيتوكينات الأخرى. كشفت دراسة الكيمياء العقاقيرية للمستخلص عن ١٦ مركبًا مثل الوين A، نيوإيزوليكويريتين، روتين، أبيجينين-٦،٨-دي-سي-جليكوسيد، هكسوزيد ميريستين، باتوليتين، و التي قد تساهم في خاصية مكافحة السرطان للنبات.