RESEARCH ARTICLE

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Mechanistic Exploration of the anticancer activity of *Justicia adhatoda* Plant Leaf Ethanolic Extract against Colon Cancer Cells: An *in silico* and *in vitro* Approach

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Abstract

Objective: Being the 3rd most prevalent cancer, colorectal cancer (CRC) has a high rate of mortality, a poor prognosis, and a clinical outcome. Treatment with commercial synthetic anti-cancer drugs also imparts a number of co-morbidities, further complicating the condition. Hence, there is a need to identify a potential anticancer agent to decrease the mortality rate and provide better clinical outcome in CRC patients. Pharmacologically active agents from natural sources are the currently recognized alternatives to synthetic drugs due to their minimal toxicity. In this study, a widely recognized plant, Justicia adhatoda (JA), traditionally used for treating respiratory ailments and rich in anti-inflammatory potential, was used to investigate its antiproliferative property against CRC. **Materials & methods:** Preliminary phytochemical screening, GCMS, Antioxidant assay, anti-inflammatory assay, cell culture -MTT assay, fluorescence staining assay, and protein and gene expression analysis. **Results:** The ethanolic extract of JA showed a strong cytotoxic effect on the cancer cells. The results of fluorescence staining show that the JA extract impart reactive oxygen species (ROS) -mediated apoptosis in proliferating cells by increasing ROS generation. This was further confirmed by evaluating the protein level of the antioxidant reservoirs and oxidative/inflammatory markers, which upon treatment were found to decrease and increase, respectively. This imbalance is attributed to the activation of the intrinsic apoptosis pathway, by altering the levels of pro- and anti- apoptotic genes in the cells and ultimately leading to cell death. The *in silico* analysis of pharmacokinetics, toxicity and molecular docking suggests that JA phytoconstituents show drug-like qualities and a promising activity against cancer. **Conclusion:** Overall, the findings suggest that JA ethanolic extract could be an effective anticancer agent against HT-29 colon adenocarcinoma cells.

Keywords: Colon adenocarcinoma- Natural source- GCMS- Apoptosis- ROS generation- Anti-inflammatory

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Introduction

Cancerous lesions or mass in the latter part of intestinal lining, collectively referred as CRC, is the 3rd most prevalent cancer around the world [1]. This cancer holds second in cancer related deaths globally and is expected to increase upto 60% and 71.5% for rectal and colon cancer respectively, by 2035 [2]. Based on gender differences, CRC is found to be 3rd and 2nd most likely cancer among men and women respectively [3]. However, these numbers may tend to vary depending on the socioeconomic development of a country as this change among the society is directly or indirectly linked with factors influencing CRC promotion. Some noted risk factors of CRC compiling with socioeconomic status of a country and lifestyle are overweight and obesity, altered dietary patterns which includes increased consumption of red - processed meat, alcoholic beverages, animal fats, poor dietary fiber and sedentary lifestyle [4]. Apart from these, other risk factors include age, sexuality, hereditary incidence, and medical conditions. Around 65% of CRC cases are sporadic whereas others are due to genetic predisposition [5]. Recognition of suspected CRC are linked to symptoms such as bloody stools, abdominal gain with or without pain, abnormal bowel patterns, asymptomatic shedding pounds, and iron-

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deficiency anemia, unexplained appetite loss and deep vein thrombosis, is also noted [4].

CRC development is a slow and tedious process which involves several years for precipitating clinical symptoms. Its onset is divided into three parts, namely initiation, promotion and progression. This prolonged process starts with damage in the epithelial lining of the intestinal mucosa that results in genetic predisposition of mucosal cells and enhancing the formation of preneoplastic or neoplastic lesions at the vicinity. Then these cells tend to multiply drastically resulting in lumps of cell masses with an abnormal growth resulting in a polyp. Polyps tend to advance in different forms namely hyperplastic polyp, traditional serrated adenoma, sessile serrated adenoma and mixed polyp. Of which the hyperplastic polyp (~ 80 – 90%) is the most common one in the gut region. Indisputably, CRC carcinogenesis initiates with mild inflammation, later advancing into adenomatous polyps in the epithelium, and ultimately evolving into adenocarcinoma except for certain medical condition namely, Lynch syndrome, where the progression is fast. This shift from polyp to adenoma and adenocarcinoma involves a great number of genetic alterations and mutations. Genes with high frequency of mutation in CRC are adenomatous polyposis coli (APC), kirsten rat sarcoma viral oncogene homolog (KRAS), B-Raf proto-oncogene serine/threonine kinase (BRAF) tumor protein *p53* (*TP53*) and PIK3CA [6,7]. Aberrant signaling pathways are central to CRC pathogenesis, promoting tumorigenesis, sustaining proliferation, and facilitating metastatic spread such as Wnt/β-catenin, RAS/RAF/MEK/ERK, phosphoinositide 3-kinase (PI3K)/AKT, and transforming growth factor-beta (TGF-β) signaling [6]. All these pathways and gene mutations are not only involved in the progression of disease but also in the alterations of host immune system and tumor microenvironment thereby challenging the intervention techniques.

Justicia adhatoda L. (JA) widely seen in the south and south east Asia originating from family of Acanthaceae. This familiar plant, rich in phytochemicals is remarked for its distinct medicinal properties. Some noted pharmacological properties are anti-microbial activity, antioxidant activity, anti-inflammatory activity, antipyretic activity, insecticidal properties, hepatoprotective activity, anti-diabetic activity, anti-tubercular activity, anticancer and radioprotective activity, anti-ulcer activity and activity against respiratory ailments [8]. Commonly, JA is well recognized for its benefits against respiratory ailments including cough, inflamed bronchus, asthma, and increased phlegm [9]. In some parts, the leaves are extracted and used to treat reproductive problems in females. In addition, there were also used for ailments like impotence, sexual disorders, bleeding piles, leprosy, jaundice, diarrhea, dysentery, malaria, gonorrhea, and vomiting [10]. Focusing on the anticancer properties of this plant, studies are very limited. Few studies focus on anti-hyperproliferative activity of a specific compound extracted from the plant, namely, vasicine after its acetylation manually against A549 cells [11] and 2-acetyl benzylamine against leukemia cells including CEM, NB-4, MOLM-14, Jurkat, IM-9, K562 and HL-60 [12]. Another study against PA1 cells showed a good anticancer activity of ethanolic leaf extracts [13]. Similarly, methanolic leaf extract of the plant tested against human breast cancer cells proves the potential of anticancer activity of JA [14]. However, all these studies lack focus on mechanistic pathway by which JA acts in reducing the cancer burden to the cells.

Hence in this article, we would like to elaborate mechanistically with evidence that J.adhatoda has immense potential in fighting cancer cells, specifically on colorectal cancer.

Materials and Methods

Plant collection and extraction

JA leaf powder was commercially purchased from herbal nursery and was subjected to extraction with 70% ethanol and acetone. For extraction, 25g of leaf powder was weighed and mixed with 100 ml of solvents in separate bottles and placed in orbital shaker for 48 hrs at 100 rpm (revolutions per minute), which was later passed through Whatmann No.1 filter paper, concentrated using rotary evaporator and preserved at -20^{o} for further investigations.

Yield = (Weight of extract after solvent evaporation / Weight of the plant material used)*100

Phytochemical analysis

The qualitative screening of phytochemicals namely, alkaloids [15], flavonoids [15], glycosides [16], cardiac glycosides [17], terpenoids/steroids [18], saponin, tannins/phenols [19], proteins and carbohydrates [20] for leaf ethanolic and acetone extract was carried out using standard protocols reported in earlier studies.

In vitro Antioxidant assay:
DPPH radical scavenging activity

Scavenging of DPPH radical was assessed by following [21]. DPPH (1, 1-Diphenyl –2-picrylhydrazyl) solution was prepared with methanol; 50µl of ascending concentrations of plant extract and standard (Ascorbic acid) were taken and the final volume was made 3ml with methanol and 150µl of DPPH solution was added. Control tube consists of only DPPH solution and methanol. Absorbance was measured using UV-visible spectrometer Shimadzu, UV-1601, Japan at 517nm after 15mins.

% scavenging = (control absorbance – sample absorbance/control absorbance) X 100

In vitro anti-inflammatory assay
Protein denaturation inhibition assay

This assay was done as instructed in [22]. A 5ml reaction solution was prepared with 0.2ml of 1% bovine serum albumin (BSA), 4.78ml of phosphate buffered saline (PBS, pH 6.4), and different concentration of extract. The solution was placed in a water bath (37°C) for 15 min, and then at 70°C for 5 min. The resulting turbidity was read at 660 nm using a Shimadzu, UV-1601, Japan. PBS was used as the control.

% inhibition = (control absorbance – sample absorbance/control absorbance) X 100

Gas chromatography and mass spectrophotometry analysis

GC–MS analysis of leaf extracts was performed using a Perkin-Elmer Clarus 680 system (Perkin-Elmer, USA) with an Elite-5MS capillary column (30 m × 250 µm × 0.25 µm). Helium (99.99%) served as the carrier gas at a constant flow rate of 1 mL/min. Detection was carried out using electron ionization at 70 eV, with a scan time of 0.2 s and a mass range of 40–600 m/z. A 1 µL sample was injected in split mode (10:1), with the injector temperature set at 250 °C. The oven temperature was initially held at 50 °C for 3 min, increased at 10 °C/min to 280 °C, and then held at 300 °C for 10 min. Phytochemicals were identified by comparing retention times, peak areas, and mass spectra with those in the NIST database [23].

Cell culture analysis

Cell procurement

HT-29 and IEC-6 cells was obtained from Cell Repository at National Centre for Cell Science, Pune, India. For cell viability test, HT – 29 cells, were seeded as 1x10^5 cells/well in 96-well plate. After incubation, different concentration of plant ethanolic extract were dissolved in 2% DMEM medium and kept for 24 hrs and 48 hrs at 37 degree Celsius in CO2 incubator. 50 µl of MTT reagent (10mg/ml) was added and stored for 4 hrs; then 100µl of DMSO was added and kept in shaker for 20 mins and read at 570nm in plate reader. Doxirubicin was used as positive control. IEC- 6, normal intestinal cells were used for toxicity testing of plant extract. The inhibitory concentration 50 (IC_{50}) dose was fixed.

Secondly, $2x10^5$ HT-29 cells seeded in two 6-well plate allow adherence to reach 70–80% confluency. IC_{50} concentrations of JA ethanolic extracts were added and incubated for 24 and 48hr. Later 10 µM 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) working solution was introduced to first plate, and acridine orange/ethidium bromide (AO/EB) solution mixture (10 µg/mL) was introduced to second plate. After 30 min the cells were analysed using the green fluorescent protein (GFP) channel on a EVOS inverted fluorescent microscope and imaged under 20X magnification.

Protein expression studies

JA extract treated and untreated cells were subjected to protein expression analysis using commercially procured ELISA kits. The cells were stored in radioimmunoprecipitation assay (RIPA) buffer and centrifuged prior to use. The cell supernatant is used to check the levels of nuclear factor erythroid derived – 2 (Nrf-2), glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), matrix metalloprotease – 2, 9 (MMP-2, MMP-9), vimentin (Vim), and interleukin – 6, 1β (IL-6, IL-1β) beta following the kit manual.

Gene expression studies

The cells incubated with IC_{50} dose for 24/48 hrs, trypsinized and centrifuged to get a pellet. The pellet was then subjected to PBS wash and then RNA was isolated using Trizol/chloroform/ isopropanol. RNA was reverse transcribed using a Qiagen kit following the kit manual. Then, 2 µl of 2 µg concentration cDNA mixed with 10 µl PCR master mix, forward and reverse primers for *GAPDH, Bax, BAD, Bcl-xl*, and *Bcl-2* genes and made up to 20 µl with RNase free water. The reactions were carried out in CFX96 Touch™ Real-Time PCR Detection System (Biorad, Hercules, California, USA). The fold change in the expression of the target gene with reference to house-keeping gene were calculated using 2-ΔΔCT.

Insilico analysis

The phytocompounds identified via GCMS analysis were subjected to virtual screening to analyze its drug like properties. Initially, the phytocompounds were searched in pubchem database to retrieve its SMILES format and 3D conformation. Using the SMILES format pharmacological properties of the identified phytocompounds were assessed using Swiss-ADME server (http:// www.swissadme.ch/) and the toxicity profiling were analyzed using ProTox-II (http://tox.charite.de/protox II).

Next, to perform molecular docking analysis the obtained, protein of interest was downloaded from RCSB Protein data bank (https://www2.rcsb.org/). All the proteins were first prepared using Discovery studio before docking and saved in pdb format. PyRx virtual screening Autodock vina was used to perform molecular docking by creating protein receptor grid for the desired proteins and target ligands. Then the docked protein ligand complex is visualized using Discovery studio.

Statistics

Statistical analysis was carried out using one-way ANOVA with a follow up test of multiple comparison using Tukey's test, with $P < 0.05$ indicating statistical significance in Graph pad prism version 8.0. All the investigations were done in triplicates and were represented as mean ± SD.

Results

Invitro plant analysis

Extraction of JA leaves yielded 12.2% and 2.5% of ethanolic and acetone extracts respectively. The qualitative presence of phytochemicals namely alkaloids, glycosides, cardiac glycosides, flavonoids, phenols, steroids, tannins, proteins and carbohydrates proves the possession of medicinal properties (supplementary).

In continuation to this, the acetone and ethanolic extracts of JA leaves, showed a good antioxidant and anti-inflammatory activity (supplementary). DPPH scavenging activity showed good antioxidant property by the extracts with a highest inhibition of 79.74% and 71.43% for ethanolic and acetone extracts respectively (Figure 1a). In respect to protein denaturation inhibition assay, both the extracts showed a good but similar anti-inflammatory action of 76.21% and 76.80% for ethanolic and acetone extracts respectively (Figure 1b). The IC_{50} values for DPPH activity was 323.25 and 335.76 (µg/ml) and for protein denaturation inhibition assay 329.94 and 412.05 (µg/ml) for ethanolic and acetone extracts respectively (Figure 1c). There was gradual increase in the percentage inhibition

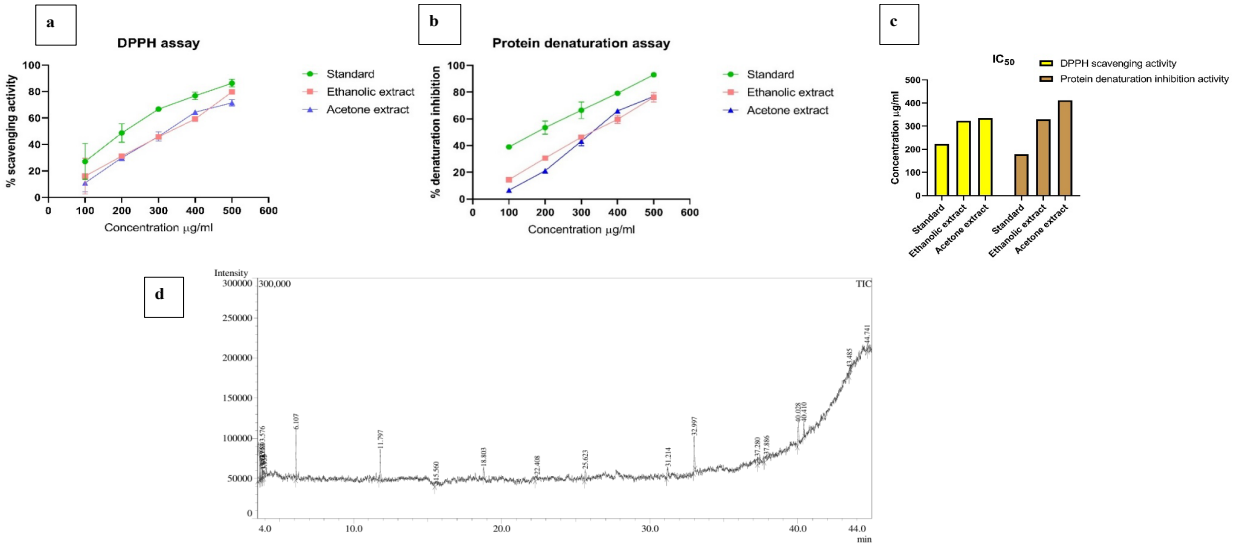

Figure 1. *In vitro* Pharmacological Activity of JA Extract. (a) Anti-oxidant assay. (b) Anti-inflammatory assay. (c) IC50 value of DPPH and Protein denaturation assay. (d) GCMS chromatogram. The error bars represent mean±SD of 3 individual values.

of the plant extracts upon increase in concentration in comparison with standard.

Chromatographic analysis of JA ethanolic extract

Here, JA displayed a total of 20 peaks each denoting unique phytoconstituents present in it. From the obtained results individual compounds were distinguished by relating the obtained peak retention time, peak area (%), height (%) and mass spectral fragmentation patterns to known compounds described by the National Institute of Standards and Technology (NIST) library. The phytoconstituents were found to be silane methyl-; 4-amino-1,2,5-oxadiazole-3-carboxamide; propane, 1,1,3-triethoxy-; 3,4-dimethyl-2-(6-oxo-7,11-diazatricyclo[7.3.1.0(2,7)]trideca-2; dihydroxymaleic acid; 1-methyl-2-phenoxyethylamine; heptadecane; pentadecane; cis-aconitic anhydride; pentadecane; 2-(isopropyldisulfanyl)butane; heptadecane, 2,6,10,15-tetramethyl-; quinazoline-4-carboxylic acid, 1,2-dihydro-2-oxo-; phytol; d-mannitol, 1-decylsulfonyl-; 3-(2-benzyl-benzoimidazol-1-yl)-propane-1,2-diol; benzyldiethyl-(2,6-xylylcarbamoylmethyl)-ammonium benzoate; bis(2-ethylhexyl) phthalate; cis-4-methylcyclohexanol, dimethyl penta-fluorophenylsilyl; adenine, n,n,o-trimethyl. The results show four major plant compounds detected in relation to obtained retention time and peak area. They are heptadecane (8.51%), pentadecane (7.94%), phytol (8.66%) and quinazoline-4 carboxylic acid (2.89%) (Table 1; Figure 1 d).

Cellular alteration in HT-29 cells upon treatment with J.A ethanolic extract

To examine anticancer property of JA at cellular level, we performed cell culture analysis. Initially, cytotoxicity testing for JA plant extracts were performed by testing in normal IEC-6 cells. The results showed difference in % of cellular viability at highest concentration of 500µg/ml as 86.47% and 46.48% upon 24 hrs and 82.37% and 33.32% upon 48 hrs for ethanolic and acetone extracts respectively (Figure 2a). Based on these results, ethanolic extract showed a better viability and hence used for the rest of investigations. Thus, to investigate the anticancer activity of JA, HT-29 cells co-cultured with JA ethanolic extract at different concentrations (Figure 2b). Findings of MTT assay revealed a dose dependent decrease in HT-29 cell viability upon treatment with plant extract with an IC_{50} value of 171.71 and 111.74 µg/ml for 24 and 48 hrs respectively (Figure 2c). In accordance to this, the occurrence of cell death was investigated using staining procedures, DCFH-DA and AO/EtBr (Figure 2d). Figure 2e displays live/dead staining in HT-29 cells untreated and treated with JA extract at IC_{50} dose revealed that the control cells showed a uniform green luminescence with a condensed nucleus whereas the IC_{50} treated cells after 24 hrs showed the presence of green and yellow staining with a granulated nucleus indicating the apoptosis. There was also presence of orange staining at sides of cells indicating late apoptosis. Whereas, upon 48 hrs treated cells showed a majority of yellow, deep orange red stain and a scarcely present green stain. This indicates that all cells are undergoing apoptosis and are in early and late stages. As an add-on, the staining images of DCFH-DA showed the increased generation of ROS in treated cells and less in untreated cells. This proves that the mode of apoptosis could be ROS mediated HT-29 cell death owing to anticancer property of JA ethanolic extract.

Protein expression and gene expression studies

To check the alterations in transcriptional and translational level, we performed RT-PCR and sandwich ELISA respectively using cell samples. The antioxidant enzymes Nrf-2 ($p=0.0023$), SOD ($p<0.0001$), CAT ($p=0.0043$), GSH ($p=0.0007$), and GPx ($p=0.0080$) were found to be down regulated in JA incubated cells than in control cells (Figure 3 a). The levels of oxidative stress marker, MDA and inflammatory marker IL-1β were

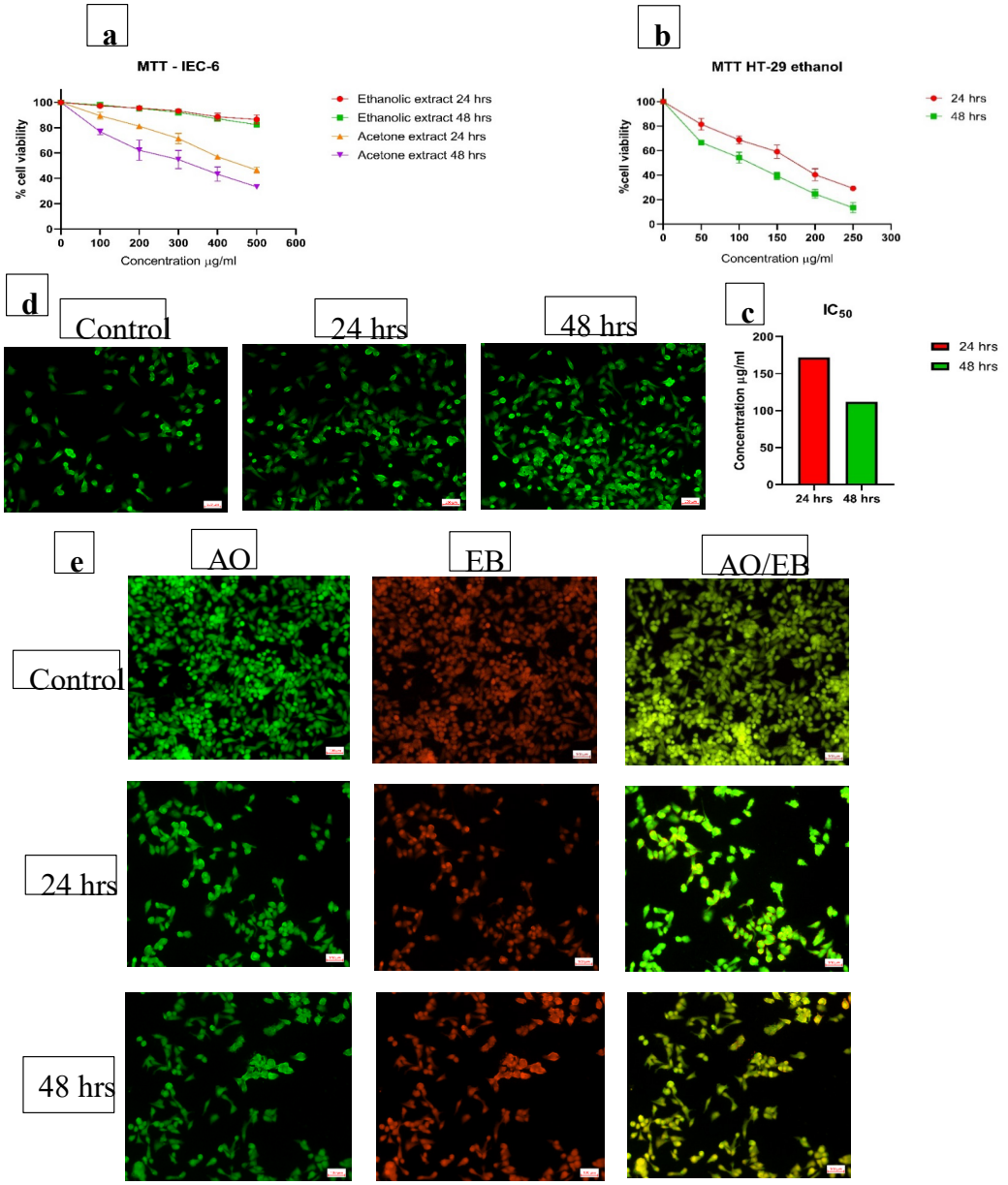

Figure 2. The Anti-Colon Cancer Activity of JA Ethanolic Extract in 24 - 48 hrs. (a) Represents the cytotoxicity of JA extracts against normal IEC-6 cells. (b) (c) Shows the % cell viability and IC50 values of HT-29 cells treated with ethanolic JA extract. (d) Depiction of ROS generation upon JA extract treatment. (e) Displays the live/dead staining of HT-29 cells incubated with JA ethanolic extract. Scale bar 100µM. The error bars represent mean±SD of 3 individual values.

found to be upregulated in treated cells than in untreated cancer cells with $p<0.0001$ and $p=0.0006$ respectively (Figure3 b). This proves that due to excessive generation of oxidative stress markers creates an imbalance in the redox system in the cancer cells owing to its death. Additionally, the gene expression studies on the pro- and anti- apoptotic genes showed that the levels of Bax and Bad were upregulated in the JA treated cells upto 1.33 and 1.61 fold ($p=0.0005$) for the former and 1.31 and 1.43 ($p<0.0001$) fold for the latter when compared to untreated cancer cells. Simultaneously, the levels of *Bcl-2*, and *Bcl-xl* were found to be downregulated in the JA treated cells to 0.75 and 0.58 fold ($p<0.0001$) for the former and 0.89 and 0.64 ($p<0.0001$) for the latter (Figure 3 c).

in silico analysis

Initial assessment of drug likeliness, pharmacological property and toxicological property of the four phytocompounds pentadecane, heptadecane, phytol and quinazoline -4 – carboxylic acid. The scoring obtained from ADME server for physiochemical characteristics namely hydrophilicity, soluble, pharmacokinetics, medicinal chemistry, and drug-likeness; these phytocompounds showed that all the above mentioned phytocompounds shown to conserve an optimal pharmacological property. All the compound showed molecular weight of > than 300 Da which is an important property for a compound to be regarded as a lead compound. Regarding the lipophilicity, except quinazoline, other three showed good lipophilicity hence parallelly the water solubility showed poor to moderate solubility for phytol, pentadecane

Table 1. List of Phytochemical Constituents Present in Ethanolic Leaf Extract of Justica Adhatoda Using GC-MS Analysis

Peak	R.Time	Area	Area%	Phytocompound
1	3.576	315723	19.87	SILANE, METHYL-
2	3.68	88040	5.54	4-AMINO-1,2,5-OXADIAZOLE-3-CARBOXAMIDE
3	3.753	94871	5.97	Propane, 1,1,3-triethoxy-
4	3.825	64128	4.04	3,4-DIMETHYL-2-(6-OXO-7,11-DIAZATRICYCLO[7.3.1.0(2,7)]TRIDECA-2
5	3.876	42936	2.7	Dihydroxymaleic acid
6	3.955	44029	2.77	1-Methyl-2-phenoxyethylamine
7	6.107	135273	8.51	HEPTADECANE
8	11.797	126200	7.94	PENTADECANE
9	15.56	39415	2.48	cis-Aconitic anhydride
10	18.803	40675	2.56	Pentadecane
11	22.408	41248	2.6	2-(ISOPROPYLDISULFANYL)BUTANE #
12	25.623	49542	3.12	HEPTADECANE, 2,6,10,15-TETRAMETHYL-
13	31.214	45913	2.89	Quinazoline-4-carboxylic acid, 1,2-dihydro-2-oxo-
14	32.997	137594	8.66	Phytol
15	37.28	38802	2.44	d-Mannitol, 1-decylsulfonyl-
16	37.886	51680	3.25	3-(2-Benzyl-benzoimidazol-1-yl)-propane-1,2-diol
17	40.028	95151	5.99	Benzyldiethyl-(2,6-xylylcarbamoylmethyl)-ammonium benzoate
18	40.41	50983	3.21	Bis(2-ethylhexyl) phthalate
19	43.485	38756	2.44	Cis-4-methylcyclohexanol, dimethylpentafluorophenylsilyl
20	44.741	48059	3.02	ADENINE, N,N,O-TRIMETHYL-

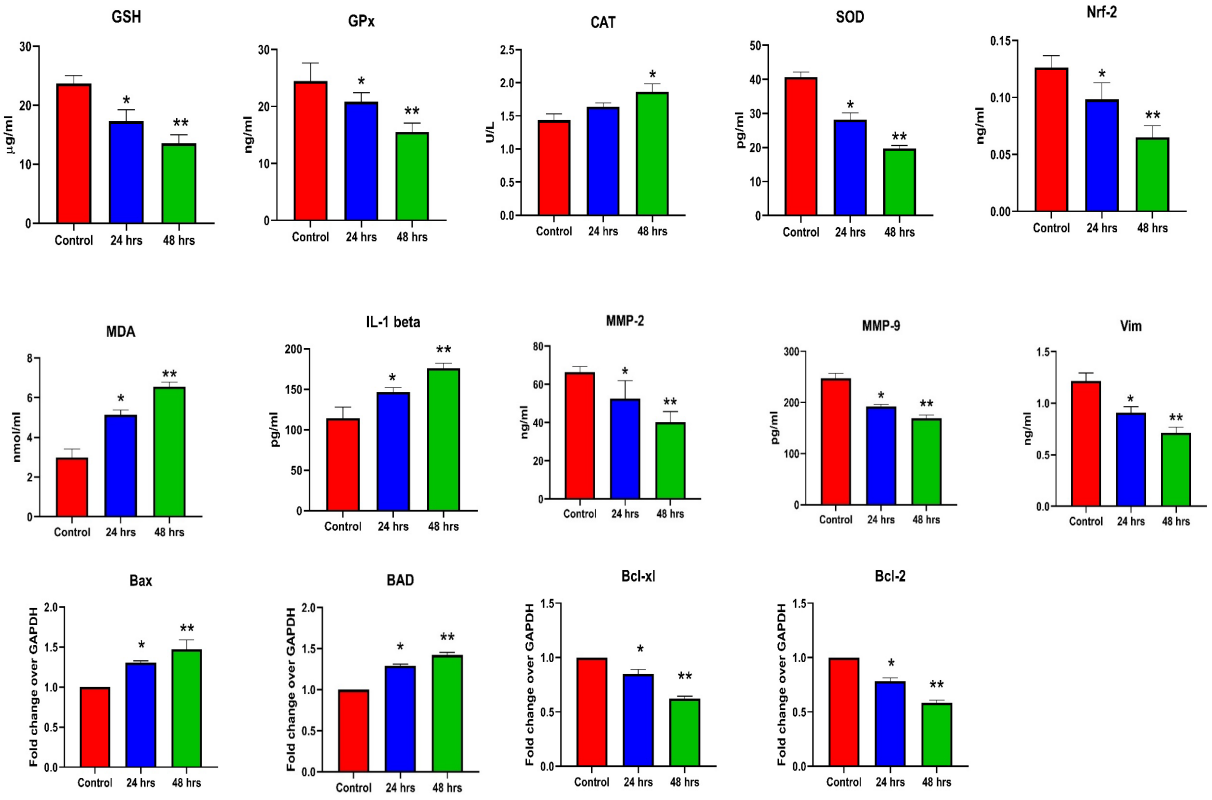

Figure 3. (a) Levels of antioxidant Enzyme Levels in HT-29 Treated and Untreated Cells. (b) Levels of ROS, inflammatory, and tumor markers in colon cancer cells. (c) RT-PCR analysis - mRNA expression of Pro- and anti-apoptotic genes. The error bars represent mean±SD of 3 individual values. Significance denoted by - * compared to control, ** compared to control and 24 hrs treatment.

Table 2. Rules for Druglikeliness and Bioavailability Score of Phytocompounds of JA Extract.

Compound	Drug likeliness					Bioavailability score
	Lipinski	Ghose	Veber	Egan	Muegge	
Phytol	Yes; 1 violation: MLOGP>4.15	No; 1 violation: WLOGP>5.6	No; 1 violation: Rotors>10	No; 1 violation: WLOGP>5.88	No; 2 violations: XLOGP3>5, Heteroatoms<2	0.55
Heptadecane	Yes; 1 violation: MLOGP>4.15	No; 1 violation: WLOGP>5.6	No; 1 violation: Rotors>10	No; 1 violation: WLOGP>5.88	No; 2 violations: XLOGP3>5, Heteroatoms<2	0.55
Pentadecane	Yes; 1 violation: MLOGP>4.15	No; 1 violation: WLOGP>5.6	No; 1 violation: Rotors>10	No; 1 violation: WLOGP>5.88	No; 2 violations: XLOGP3>5, Heteroatoms<2	0.55
Quinazoline – 4 – carboxylic acid	Yes; 0 violation	No; 1 violation: #atoms<20	Yes	Yes	No; 1 violation: MW<200	0.85

and heptadecane leaving quinazoline with a good solubility. All these factors contribute to the ADME of the compounds (supplementary). Pharmacokinetics parameters showed that overall, all the phytocompounds showed good absorption, distribution, excretion properties (supplementary). The drug likeliness property is the main factor to depict a compound to possesses a drug like properties. Lipinski rule of 5 is considered as the foremost rule to scrutinize compounds which has led-like properties. In this investigation, all the compounds passed the Lipinski law with 1 or 0 violations (Table 2). Moreover, quinazoline also passed Veber and Egan algorithms of drug likeliness. Also, the scoring of bioavailability for phytol, heptadecane and pentadecane were 0.55, while quinazoline showed a score of 0.85. This proves that at physiological pH the phytocompounds can stay 55% and 85% in its active form. From these results, we can virtually predict that the major compounds present in this extract

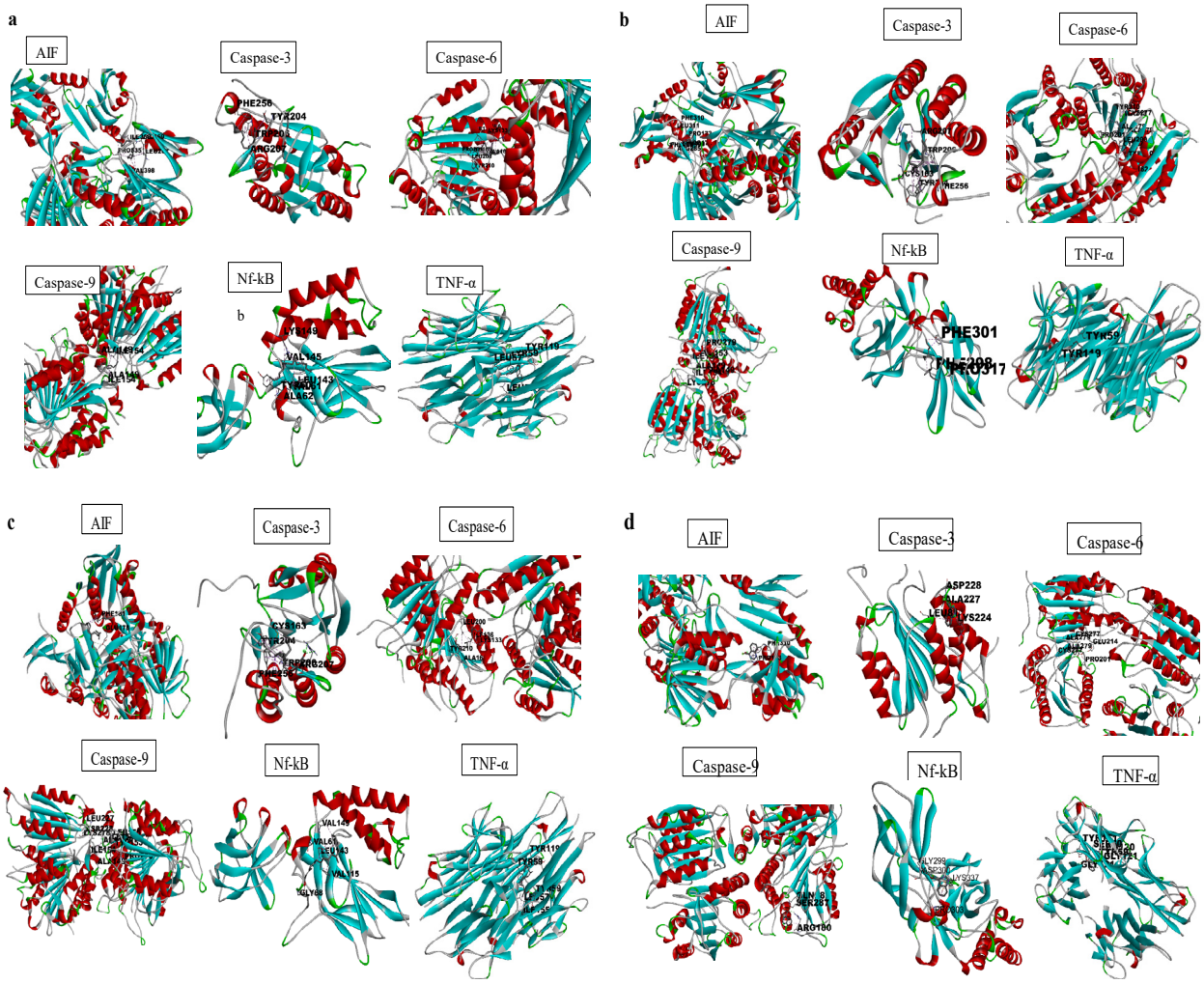

Figure 4. 3D Image of Phytoligands Bound with Target Proteins. (a) Pentadecane (b) Heptadecane (c) Phytol (d) Quinazoline.

Table 3. Molecular Docking Results of Phytocompounds

Compound	Protein	Binding affinity (kcal/mol)	Types of interaction	Amino acid residues
Pentadecane	AIF	-3.8	Alkyl	ILE, PRO, VAL, LEU, VAL
	Caspase-3	-4.2	Pi-alkyl Alkyl Pi-sigma	PHE, ARG, TYR, TRP
	Caspase-6	-4.7	Pi-alkyl Alkyl	ILE, PRO, LEU, TRY, ALA
	Caspase-9	-4.7	Alkyl	ALA, ILE
	Nf-kB	-4	Pi-alkyl Alkyl	LYS, VAL, LEU, ALA, TYR
	TNF-α	-4.9	Pi-alkyl	LEU, TRY
Heptadecane	AIF	-5.8	Pi-alkyl Alkyl	LEU, PHE, ARG, PRO
	Caspase-3	-4.3	Pi-alkyl Alkyl	PHE, TRP, TYR, ARG, CYS
	Caspase-6	-5.4	Pi-alkyl Alkyl Pi-sigma	ALA, CYC, TYR, ALA, PRO, LEU
	Caspase-9	-5.5	Pi-alkyl Alkyl	LYS, ALA, ILE, PRO, TRY
	Nf-kB	-3.8	Pi-alkyl Alkyl Pi-sigma	PRO, PHE
	TNF-α	-4.7	Pi-alkyl	TYR
Phytol	AIF	-4.8	Pi-alkyl Pi-sigma	PHE
	Caspase-3	-5	Conventional hydrogen bond Pi-alkyl Alkyl Pi-sigma	ARG, PHE, TRP, TYR, CYS
	Caspase-6	-5.6	Pi-alkyl Alkyl	LEU, ILE, TYR, ALA, LYS
	Caspase-9	-6.5	Conventional hydrogen bond Carbon hydrogen bond Alkyl Pi-alkyl	PRO, TRY, LEU, ALA, ILE, ASP, LYS
	Nf-kB	-5.4	Alkyl Unfavorable Acceptor - acceptor	LEU, VAL, GLY
	TNF-α	-6	Pi-alkyl Alkyl Pi-sigma	ILE, TYR, LEU
Quinazoline – 4 – carboxylic acid	AIF	-6.7	Pi-Pi T-shaped Pi-alkyl	PRO, PHE
	Caspase-3	-5.7	Conventional hydrogen bond Pi-alkyl Pi-sigma	LEU, LYS, ASP, ALA
	Caspase-6	-6.4	Conventional hydrogen bond Pi-alkyl Unfavorable acceptor-acceptor	PRO, CYC, ALA, GLU
	Caspase-9	-7.4	Conventional hydrogen bond Pi-alkyl	ARG, GLN, SER
	Nf-kB	-5.3	Conventional hydrogen bond Pi-cation Pi-alkyl	ASP, GLY, PRO
	TNF-α	-6.6	Conventional hydrogen bond Pi-Pi stacked Amide-Pi stacked	TYR, GLY, SER, LEU

according to GCMS data possess desired properties to impart pharmacological action inside a living system.

The outcome of protox database showed the scoring of possible adverse effects by the phytocompounds in terms of LD50, organ toxicity and toxicological end points (supplementary). There was no predicted toxicity for phytol, pentadecane and heptadecane. Whereas in case of quinazoline, the results suggest that there might be a possibility for hepatoxicity, neurotoxicity and nephrotoxicity. On the whole, these predictions propose that above mentioned phytochemicals possess qualities to be a potent drug with minimal toxicity. Molecular docking of ligands to target proteins (*Caspase – 6, Caspase – 9, Caspase – 3*, apoptosis inducing factor, nuclear factor – kappa B (Nf-kB), tumor necrosis factor (TNF-α)) showed that phytol and quinazoline were more likely to bind actively with the proteins based on the obtained affinity scores (Table 3, Figure 4).

Discussion

Cancer is a field of medicine which needs a separate attention as it is diverse and has undifferentiated characteristics. Moreover, this field lacks therapeutic opportunities to completely revive the patients with a minimal or no side effects. Colon cancer is regarded as deadly as the symptoms are only distinct in its late stage making it difficult to cure [24]. There are a number of synthetic anticancer drugs and repurposed drugs approved by FDA to treat CRC patients, but due to multi-diversity between each individual there is still lacking in the prognostic outcome among patients. Additionally, the associated adverse effects and co-morbidities of these chemotherapy drugs pose a great challenge in treating cancer [25]. This creates a need to identify a potential anticancer drug from a natural source so as to reduce the comorbidities.

In this article, we have enlisted the evidences for JA, to be a potent anti-proliferative agent against colon cancer using HT-29 cells. Initially, based on the literature extraction was done with two solvents namely ethanol and acetone. They were then assessed for detecting preliminary phytochemicals, antioxidant and anti-inflammatory potential. The GCMS analysis reveals the presence of a compound when correlated with retention time and corresponding peak area. From the obtained GCMS data, there was presence of a number of phytoconstituents and organic compound along with which four phytocompound were detected, namely heptadecane, pentadecane, phytol and quinazoline – 4-carboxylic acid. These phytocompounds are noted for its number of pharmacological activities [26-29]. The results of antioxidant and anti-inflammatory activity showed a good free radicle scavenging potential and protein denaturation ability of the plant extracts which may be contributed by the phytochemicals and phytoconstituents present in the leaf extracts.

Next, cytotoxicity of the two extracts was checked against normal IEC-6 cells. The results showed a good cellular viability for ethanolic extract without affecting normal cells whereas the acetone extract showed a decrease in the cell viability. Therefore, acetone being toxic to normal cells they were not further used in study. Now the antiproliferative property of JA ethanolic extract against CRC was assessed against the HT-29 cells. The treatment for 24 hrs and 48 hrs showed an increase in % inhibition of oncogenic cell viability in a dose dependent manner. Under physiological conditions, cancer cells possess an aberrant redox potential. ROS acts as a double ended sword as they can play a dual role as pro-tumorigenic or cytotoxic [30]. In general, increase in the metabolic rate simultaneously increases the generation of ROS. Cancer cells being in hyperproliferative state, tend to generate a humpty amount of ROS which promotes ROS induced proliferation meanwhile they also maintain the antioxidant levels to avoid ROS induced senescence, apoptosis and ferroptosis [31]. Hence, this experimentation checked the generation of ROS in untreated and treated HT-29 cells. The DCFH-DA assay showed that when compared to untreated cells there was an increase in the ROS generation in the JA treated cells which may be the contributing factor for cell death upon extract treatment. We also checked the levels of oxidative stress marker, MDA and inflammatory marker IL-1β. Supporting the staining images, there was a lowering of antioxidant enzymes SOD, GSH, GPx, and Nrf-2, while an increase in the levels of CAT in treated cells than in control cells. Parallelly, there was an increase in the levels of MDA, and IL-1β in the JA treated cells than in the control cells. A study employing cobolt (III) Schiff bases in amelioration of lung cancer showed that cancer cells exposed with cobolt-III showed a mark increase in the ROS generation complementing the shift in apoptotic and anti-apoptotic genes thus resulting in cell death [32]. They also checked the levels of antioxidant enzymes at mRNA level, as antioxidants enroll in maintaining redox balance of cancer cells thus preventing ROS-mediated cell death. Their study indicated downregulation of SOD, CAT, and GPX contributing to death of A549 cells. Another study by [33]; on sertaconazole, a repurposed drug, nano-particles (Nps) against lung cancer showed that upon treatment the cancer cells showed a rise in ROS levels thereby imparting cell death, whereas those cells co-incubated with N-acetyl cysteine (NAC), a potent antioxidant, showed a reduction in release of ROS by the cancer cells. Thus, the author suggests that sertaconazole Nps bear the potential to increase the ROS by disrupting the redox balance and diminishing the antioxidant reserves in A549 cells leading to death, which was not noted when cotreated with NAC, as NAC restores the antioxidant ambience in tumor environment. These evidences support the claim that JA ethanolic extract collapses the redox balance in the colon cancer cell which promotes the shift from ROS-induced proliferation to ROS-induced cell death.

Cellular mortality takes place either as necrosis or programmed cell death. Programmed cell death covers both apoptosis, a caspase dependent pathway and autophagy/necroptosis, a caspase independent pathway [34]. During apoptosis, there is a shift in the levels of tumor promoting and de-promoting genes paving its path towards death [35]. In our study, we checked the levels of pro- and anti- apoptotic gene in the 24 hrs and 48 hrs

treated cells and compared it with the untreated cells. The results showed an upregulation in pro-apoptotic genes Bax and BAD, and a downregulation of *Bcl-2* and *Bcl-xl* in the extract treated cells. This increase in Bax and BAD can trigger the release of cytochrome c from the mitochondrial membrane which in turn activates the release of caspases resulting in apoptosis [32]. Also, we checked the levels of MMP-2 and MMP-9 in the treated and untreated cells. Matrix metallo-proteinases are group of enzymes that play a major role in the cell proliferation, survival, metastasis and angiogenesis [36]. In epithelial mesenchymal transition, as tumor progresses the cells exhibit downregulation of E-cadherin promoting loosening of tight, gap and adherent junctions to undergo mesenchymal transition [37]. This results in increase of vimentin, N-cadherin, and MMPs which promotes tumor metastasis and invasion. In this study, the protein expression of VIM, MMP-2, and MMP-9 were elevated in the untreated cells and upon treatment there was a marked decrease in their levels with $p=0.0002$, $p=0.008$ and $p<0.0001$ respectively (Figure 3 b). From the above investigations, it is evident that treatment of HT-29 cancer cells with pharmacologically active JA extracts induced apoptosis of cancer cells with a minimal effect on the normal cells via ROS generation thereby altering the levels of pro- (Bax, BAD) and anti- (*Bcl-2*, *Bcl-xl*) apoptotic genes.

Computer aided drug designing is a growing field to design, predict, and model various natural and synthetic compounds to develop it into an active pharmacological agent. The current investigation also employed virtual prediction of pharmacokinetics, toxicity and molecular docking for phytochemicals present in the plant extract. Knowing the physicochemical constituents of the lead compound makes it easy to determine the drug's fate and mode of action [38]. This study employed Swiss ADME, freely available bioinformatic tool, which has a number of algorithms in-built in it to determine the physical and chemical properties, hydrophobicity, hydrophilicity, absorption, distribution, metabolism and excretion, drug-likeliness. The ADME prediction showed that phytol, quinazoline, heptadecane and pentadecane surpasses all the laws of Lipinski rule making it a drug like agent. Also, the bioavailability score was good as it is an important characteristic for a drug as it shows the amount of drug which will be available to act pharmacologically in its active form [39]. Similarly, any compound or agent with no or minimal toxicity makes it a potent pharmacological agent. In this investigation, the toxicity testing for these phytochemicals showed no toxicity proving its favorable nature in being developed into a drug. Moreover, the docking results of these four phytochemicals with caspase proteins, apoptosis inducing factor, and inflammatory proteins suggest that quinazoline> phytol>heptadecane>pentadecane have more affinity. Overall finding evidence that JA ethanolic extract has the potential to inhibit cancer cell growth and initiates intrinsic apoptosis pathway in HT-29 cells, thereby can act as a potential therapeutic agent in treating colon cancer.

There are certain limitations to the study, firstly, protein level validation using Western blot method was not performed; secondly, absence of animal experimentation and thirdly, we have narrowed down to only apoptosis pathway. Future directions focusing invivo animal experimentation helps in validating our findings under a physiological context also exploring other pathways (Wnt/β-catenin, PI3K/mTOR) may provide a deeper understanding on multiple pathways involved.

Our investigations evaluated the ability of JA leaf ethanolic extract to act as an anti-proliferative agent. The invitro plant analysis showed that the leaf extract has a good potential to scavenge free radicle and inhibit protein denaturation owing to its antioxidant and anti-inflammatory potential. This property of the plant extract could be contributed by the rich pool of phytochemicals present in it. In vitro cell line studies show that the extract induced cytomorphological changes in the HT-29 cells thereby imparting cell death. The resulting cytotoxicity was mediated via increase ROS generation and imbalance in the redox potential by lowering the endogenously present antioxidant enzyme stores. This imbalance further initiates the intrinsic apoptotic pathway by elevating the levels of pro- apoptotic genes and downregulating the anti-apoptotic gene levels. All of these results obviously specify the anti-colon cancer strength of JA ethanolic extracts. Being a natural source of anticancer therapeutic agent JA extracts could act as a capable anticancer agent with a minimal or no adverse effects.

Author Contribution Statement

Bhuvaneswari Ponnusamy – Preparation of methodology, experimentation, manuscript writing. Selvaraj Jayaraman – Idea sharing, critical thinking, correction. Vishnupriya Veeraraghavan – Manuscript review and correction. Ponnulakshmi Rajagopal-Review and Editing. Chella Perumal Palanisamy- Review writing and formal analysis.

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Conflict of interest

None.

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