



Metabolomic profiling of cancer cells to *Aloe vera* extract by ^1H NMR spectroscopy

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Abstract

Background: The modern life style despite its comforts has made cancer a leading public health problem. As chemotherapeutics has many side effects including resistance, recent attention has focused on plants which may provide a good opportunity for complementary cancer treatment. *Aloe vera* has been described as a wonder plant due to its many advantages. It has been reported to have anticancer properties. Metabolomics is the measurement and analysis of metabolites in a biological sample by the use of throughput technology like ^1H Nuclear Magnetic Resonance and Mass Spectrometry. The results are analyzed by multivariate analysis methods. The metabolome reveals the final interaction between the genome of an organism and its environment. We studied the inhibitory effects and metabolomic profile of *Aloe Vera* extract on Raji cells (cancerous lymphoma cells) using ^1H NMR spectroscopy.

Methods: The gel was removed from *Aloe vera* leaves by a sharp knife and chopped by a blender, warmed to 50°C with ascorbic acid as antioxidant and finally cooled to almost 20°C . After filtering, the gel was lyophilized and dissolved in RPMI medium ($2\text{mg}/\text{ml}$) and added to Raji cells and 50% inhibitory concentration determined by MTT. Large scale culture of Raji cells were carried out using 50% inhibitory concentration dose of *Aloe vera* gel. The Raji cells were collected, metabolites extracted using water or chloroform/methanol, lyophilized and sent for ^1H Nuclear magnetic resonance analysis by NOESY technique.

Results: 50% inhibitory concentration of *Aloe vera* was seen to be $40\ \mu\text{g}/\text{ml}$. The differentiating metabolites were identified and the metabolic cycles were detected.

Conclusions: Changes were observed mainly in the amino acid metabolites and the main metabolic cycles involved were protein biosynthesis, mitochondria transport chain, catecholamine biosynthesis and pentose phosphate pathway.

Keywords: Metabolomics, *Aloe Vera*, Raji cells, ^1H NMR spectroscopy

Introduction

Aloe Vera is a plant that belongs to the *Aasphodelacea* (Liliaceae) family has been used as an herbal medicine for centuries. It is native to Africa and many Mediterranean countries and is used for several healing purposes, like treating various skin disorders such as abscesses, burns and eczema. Its juice may help people with ulcerative colitis, an inflammatory bowel disease. *Aloe Vera* has been used as a remedy for coughs, wounds, diabetes, gastritis, headaches, arthritis, ulcers, cancer and also immune-system deficiencies [1]. There are over 100 active biological

components found in *Aloe Vera*, including: anthraquinones, vitamins, minerals, enzymes, amino acids, polysaccharides and plant sterols [2].

Cancer after cardiovascular disease is the second leading cause of death in the world. Approximately 10 million people are diagnosed with it every year and 6 million die of it [3]. Treatment options for cancer include chemotherapy, surgery, radiation and immunotherapy. The use of any of these treatments depends upon the location, grade, tumor stage as well as the general state of a person's health. Chemotherapy and

radiation in addition to cancer cells, destroy normal cells [4] and recently resistance to chemotherapeutic drugs has been reported in some cases and this has renewed an interest in alternative and herbal methods to complement conventional treatment. In fact, medicinal plants are more available, cheaper and possess less toxicity compared to modern (allopathic) drugs. Biological active components of plants can be important sources for new drugs which may lead to new and better treatments for cancer [3].

In this study, Raji cells were used as a model system to study the anti-cancer properties of *Aloe vera*. Raji cells are a series of cell lines derived from B lymphocyte of a patient with Burkitt's lymphoma (BL) which is an aggressive form of B cell non Hodgkin's lymphoma [5] with reports of chemotherapy resistance.

Metabolomics comprehensively analyzes hundreds of metabolites in a biological sample simultaneously using high throughput technology. Mass spectrometry (MS) and ¹H Nuclear Magnetic Resonance spectroscopy (¹HNMR) are used in the measurement of metabolites in the sample with chemometrics analysis. Although metabolomics comprises the smallest domain (approximately 5,000 metabolites) as compared to genomics (30,000 genes) and proteomics (100,000 or more proteins), it is more diverse and involves many different biological molecules, making it chemically and physically more complex than the other two. The importance of metabolomics is in disease diagnosis and drug discovery as the changes in the metabolome are a direct outcome from perturbations in cellular activity [6].

The purpose of this research is to study the effect of *Aloe vera* gels on Raji cells and to obtain the difference in their metabolic pattern by ¹HNMR spectroscopy so as to identify metabolites and modified metabolic cycles. Earlier work has been done on the effect of ginger on the metabolic pattern of Raji cells and the pathways detected were mainly glucose cycles [6,7].

Materials and methods

Preparation of *Aloe vera* extract

Aloe vera leaves were prepared fresh from herbarium and identified by Tehran University. They were first washed with a solution of sodium hypochloride and rinsed with distilled water at room temperature for 4h. A sharp knife was used for separation of the leaf crusts and the gel collected by a spatula-like knife gel from the lower crust. The gels were chopped by a blender and warmed to 50°C using ascorbic acid as antioxidant and then cooled to 20°C. The *Aloe Vera* extract was filtered through gauze and the filtrate lyophilized [8].

Cell culture

The Raji cell lines (B-cell lymphoma) were purchased from Pasteur Institute of Iran. The cells were obtained from NCBI code C127 with ATCC number CCL-86, but are DNA typed and karyotyped periodically by the Pasteur Institute of Iran. The

culture medium contained 10% fetal calf serum and RPMI-1640, penicillin and streptomycin and incubated at 37°C with 5% CO₂ at 95% humidity in a 25 cm² flask [9].

50% inhibitory concentration (IC₅₀)

1 mL of culture medium containing 1.6X10⁴ Raji cells were added to each well and treated with different concentrations of 0.2, 2, 20, 40, 100 mg/ml *Aloe Vera* extract with duplicates using controls without extract. The cells were treated for 24, 48, 72 hours.

Viability test

Trypan blue was used to stain dead cells and their viability assessed by counting under an invert microscope using a hemocytometer at 24, 48 and 72 h [10].

MTT

[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay: was performed at 24, 48 and 72 h in 96-well plates with the same concentrations of *Aloe Vera* as viability test. At the end of the time periods, the culture medium supernatant was discarded and the cells were incubated with 50 µg/mL MTT stock solution in PBS for 3 to 4 hours at 37°C. Then, 100 µL formazan was added in dimethyl sulfoxide to the wells and shaken for 30 min and absorbance read at 570 nm using ELISA Reader (Anthos 2020) [11].

Culture of Raji cells for ¹HNMR

After determining IC₅₀ concentration, Raji cells were cultured with IC₅₀ titer of *Aloe Vera* extract in 75 cm² flask for 48 h and then collected by centrifugation at 6,000Xg for 5min and stored in freezing solution containing 90% Fetal Bovine Serum and 10% DMSO at -70°C.

Cell extraction

The method of extraction using methanol-chloroform-water was done as described previously. The temperature of the extraction procedure was maintained at 4°C by working in a crushed ice bath. Cells were washed in 1X PBS and centrifuged at 6,000g for 5 min and resuspended in 500 µL of ice-cold 2:1 (v/v) methanol-chloroform solution. It was then transferred into a 1.5mL Eppendorf tube, 250 µL of ice-cold H₂O 1:1 (v/v) chloroform/H₂O was added and vortexed. The cells were sonicated on ice for 10min and centrifuged for 5min at 18000Xg.

The lower lipophilic and the upper hydrophilic extracts were separated and collected in different Eppendorf tubes and lyophilized and stored at -20°C [7].

Preparation for ¹HNMR

Lyophilized hydrophilic cell extracts (n=10) were resuspended in 200 µL of buffer (150mM potassium phosphate at pH 7.4, 1mM NaN₃, and 0.01% trimethylsilyl propionate (TSP) (Sigma, CA, USA) in 100% D₂O and the lipophilic cell extracts (n=10) were resuspended in 200 µL deuterated chloroform [12].

¹HNMR spectroscopy

The cell suspensions were placed in 5mm probes for analysis and one dimensional spectroscopy was performed on a ¹HNMR spectrometer (Bruker AV-500) with filed gradient operating at 500.13 MHz for observation of proton at 298K. One dimensional ¹HNMR spectra were acquired with 6009.6 Hz spectral width, a 10- μ s pulse 0.1 s mixing time, 3000 transients and 3.0 s relaxation delay, with standard 1D NOESY (nuclear Overhauser spectroscopy) pulse sequence to suppress the residual water peak [13]. The ¹HNMR spectrum comprising of Fourier Transformed information about metabolites in the control and treated groups(both hydrophilic and lipophilic extracts of each) were imported into MATLAB (v.7.8.0.347) software and first analyzed by ProMetab software (version 1.1) Chemical shifts between 0 and 10 ppm were normalized and spectra binned in 0.004units and the water peak (4.7) removed. The Excel files were then assessed by PLS-Toolbox version 3.0 and Partial Least Square Discriminant Analysis (PLS-DA) was applied [14].

Identification of metabolites

The differentiating metabolites related to these resonances were identified by chemical shift determination using Human Metabolome Database Data Bank (HMDB) (<http://www.hmdb.ca/metabolites>). The metabolic cycles were determined using Metabo-Analyst software (<http://www.metaboanalyst.ca/>).

Results

IC₅₀ of *Aloe Vera* extract on Raji cells is shown to be 40 μ g/ml at 48 h (Figure 1). Superimposed ¹HNMR spectra of hydrophilic layer and lipophilic layer between experimental and control groups can be seen in (Figures 2A and 2B). The analysis of ¹HNMR spectra are depicted as score plot (Figures 3A and 3B) and loading plot (Figures 4A and 4B) of hydrophilic layer and lipophilic layer between experimental and control groups. Altered metabolites of hydrophilic phase and lipophilic phase are identified in Tables 1 and 2 respectively. Metabolite enrichment analysis is shown in Figure 5 and 6 depicting the main pathways affected by the extract.

Discussion

Aloe vera has many beneficial properties. Anti- cancer properties of *Aloe vera* have been reported from 2010 when its anti-proliferation effects on some types of cancer cells *in vitro* such as lung, squamous, glioma, and neuro-ectodermal were described [15,16]. It also has anti tumorigenic properties and increases expression of p53 gene and Bcl2 as demonstrated on human hepatocellular carcinoma cells [16,17]. Ehrlich ascites carcinoma in Swiss albino mice were apparently healed by *Aloe vera* extract by reversion of their hematological profiles towards normal levels and a decrease of peroxidation enzymes of lipid peroxidation and increased the levels of reduced glutathione and other antioxidant enzymes, like superoxide dismutase, catalase and glutathione peroxidase [16]. The anti-

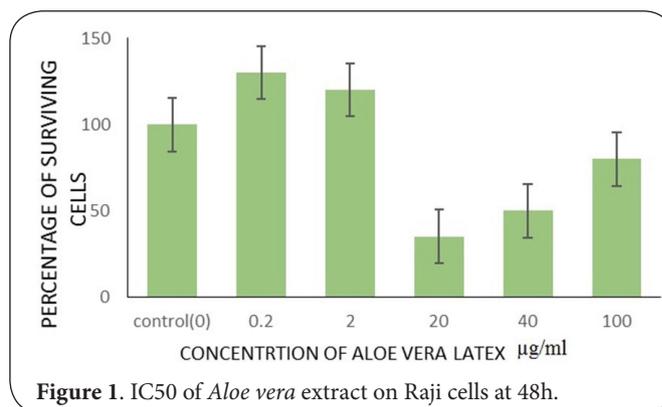


Figure 1. IC₅₀ of *Aloe vera* extract on Raji cells at 48h.

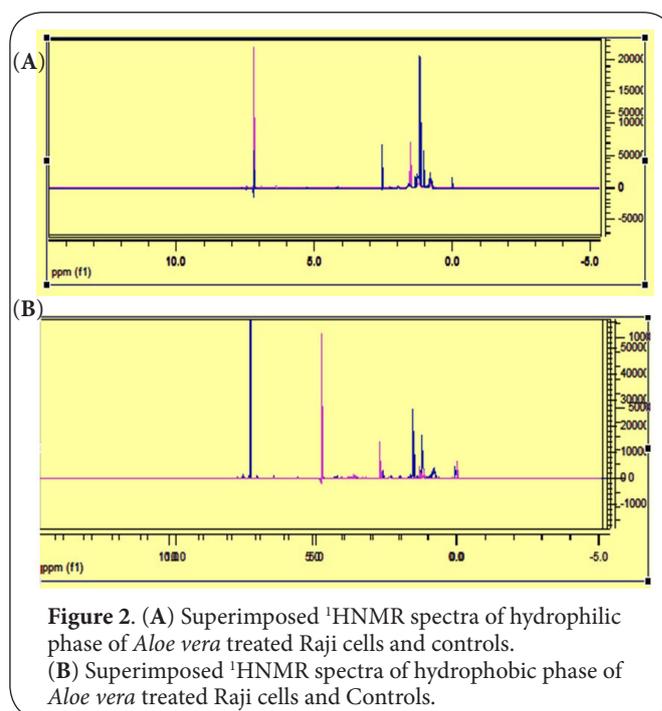
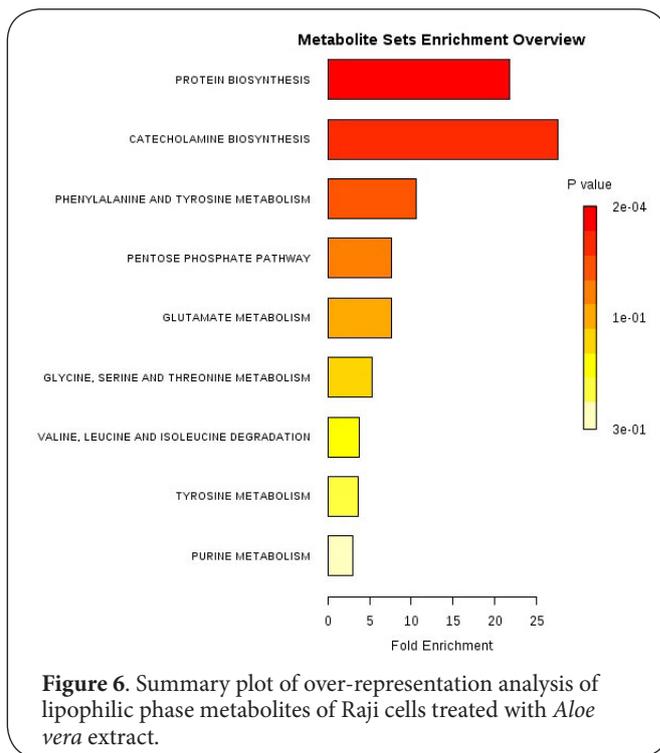
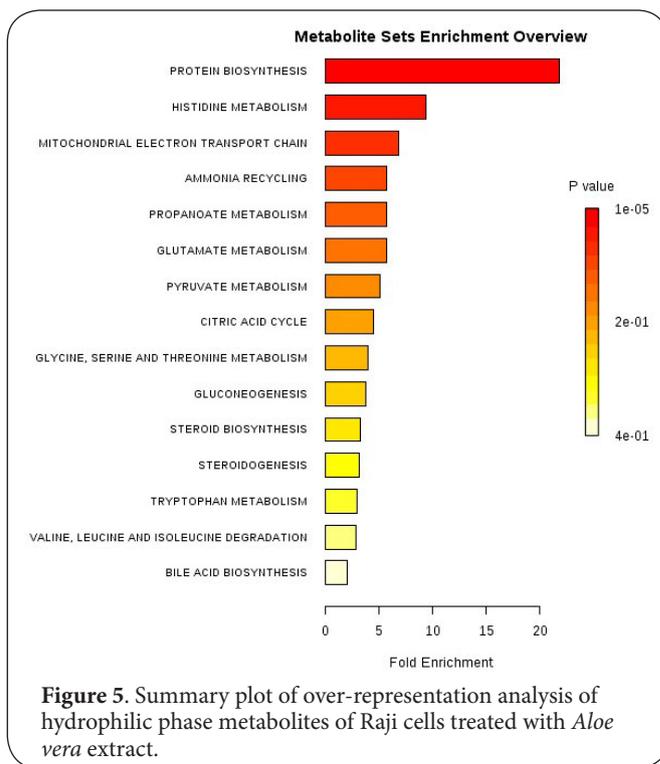
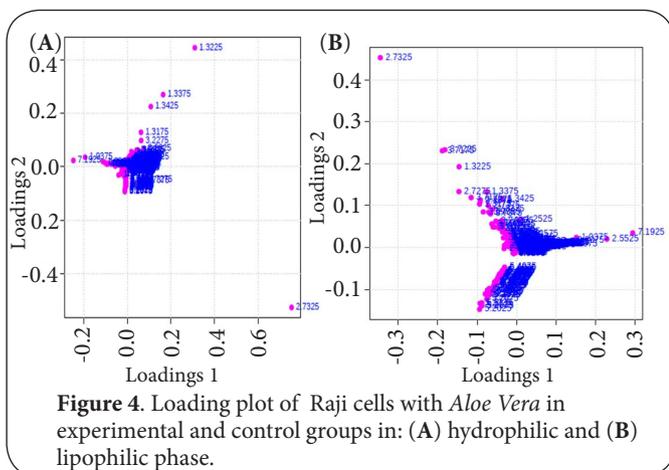
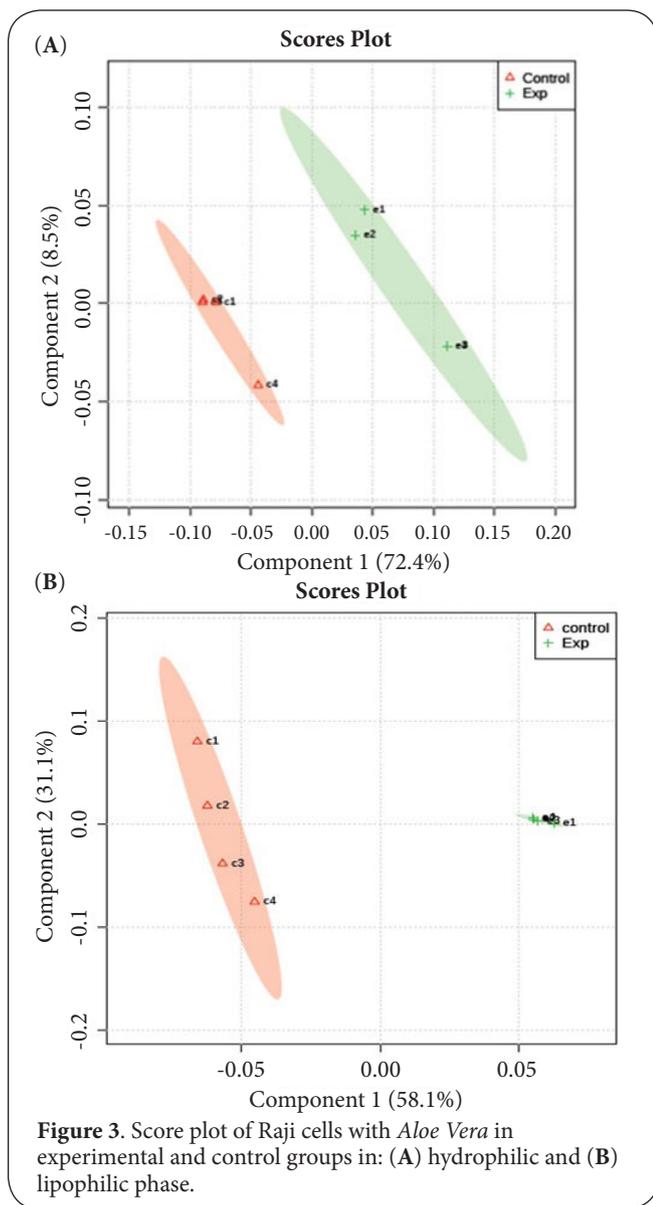


Figure 2. (A) Superimposed ¹HNMR spectra of hydrophilic phase of *Aloe vera* treated Raji cells and controls. (B) Superimposed ¹HNMR spectra of hydrophobic phase of *Aloe vera* treated Raji cells and Controls.

cancer activity of *Aloe vera* on Raji cells was exhibited by IC₅₀ of 40 μ g/ml *in vitro* confirming the above results.

Metabolomic studies done in this survey also revealed the mechanism of activity of *Aloe vera* extract on Raji cells. Levels of some amino acids had altered between the two groups (normal and treated) and the metabolic cycles in which they participated were identified by the Metaboanalyst website. These amino acids were involved primarily in protein biosynthesis, a cycle which was affected in both the hydrophilic and hydrophobic extracts. A number of glycoproteins present in *Aloe vera* gel have been described as antitumor and anti-ulceric and seen to increase proliferation of normal human dermal cells [18]. A substance named *Aloin*, an anthroquinone and the main ingredient of *Aloe vera* has been shown to possess anticancer potential activities, as it blocks signal transducers and is an activator of transcription 3 activation by inhibiting



tumor angiogenesis and growth demonstrating its potential as a drug candidate for cancer therapy [17]. Investigations carried out in mice fed with fresh leaf pulp extract have shown *Aloe vera* to be an inducer of phase-II enzyme system which

Table 1. Altered metabolites of treated group with drug in hydrophilic phase.

No.	Chemical shift	Metabolite name	HMDB No.
1	1.3175, 1.3225	L-Lactic acid	HMDB00190
2	3.5825	L-Threonine	HMDB00167
3	0.7575,0.7675,0.7725	Cholesterol	HMDB00067
4	7.1875	L-Tryptophan	HMDB00929
5	1.0375, 1.3425, 1.0325, 1.0425	L-Valine	HMDB00883
6	1.3375	L-Alloisoleucine	HMDB00557
7	7.0925	L-Histamine	HMDB00177
8	2.732 5	Succinic acid	HMDB00254

Table 2. Altered metabolites of treated group with drug in lipophilic phase.

No.	Chemical shift	Name of metabolite	HMDB No.
1	0.2225,0.2025	D-Ribose 5-phosphate	HMDB01548
2	0.8075	2-Hydroxy-3-methylbutyric acid	HMDB00407
3	3.7125	L-Leucine	HMDB00687
4	7.1975	L-Tyrosine	HMDB0605
5	1.3225	L-Threonine	HMDB00167
6	2.7275	Succinic acid semialdehyde	HMDB01259

help in detoxification and are associated with carcinogen metabolism. The microsomal and cytosolic proteins were increased in the *Aloe vera* treated mice indicating the possibility of its involvement in the initiation of protein synthesis [19].

Aloin treatment is shown to inhibit the secretion of vascular endothelial growth factor (VEGF), thus inhibiting proliferation and migration of endothelial cells. VEGF is one of the most important proangiogenic cytokines known and well distinguished as a provoker of tumor neovascularization. VEGF production is shown to be regulated by histamine which is produced from histidine via the enzyme L-histidine decarboxylase [20,21]. Histidine metabolism is one of the cycles detected by our analysis.

L-tyrosine is involved in the catecholamine cycle, another metabolic cycle seen to differ with *Aloe vera's* action on Raji cells. There have been reports since 1991 that catecholamines and their metabolites are present in single lymphocytes and extracts of T-and B-cell clones as detected by capillary electrophoresis and electrochemical detection. Pharmacological inhibition of tyrosine hydroxylase diminishes perceived catecholamine levels, suggesting catecholamine synthesis by lymphocytes [22]. L-tyrosine is the substrate for tyrosine hydroxylase and is detected in this study.

Succinic acid participates in the mitochondria electron transport chain system. The protective effect of *Aloe vera* gel on the permeability transition pore in the inner membrane of rat liver mitochondria *in vitro* has been described. These findings indicate that *Aloe vera* gel modulates the effect of calcium by moderating the mitochondrial pore opening which

is needed in situations that requires tissue wastage such as in cancer treatment [23].

Degradation of leucine, isoleucine and valine identified in this study has been reported earlier as an important step in cancer metabolism. An investigation carried out on breast cancer cells using microarray has shown this to be the second most important metabolic cycle involved [14,24]. These amino acids are also present in the *Aloe vera* gel and are likely to be utilized by Raji cells [25].

Gutamate is an antioxidant and is also a source of amino groups for nonessential amino acids like glycine, aspartate, alanine and serine which are required for macromolecular synthesis [26]. The tricarboxylic acid cycle (TCA), the main source of α -ketoglutarate in glutamine consuming cells is glutamate which is also an intermediate for TCA cycle and dioxygenases substrate which can cause protein and DNA modifications. These dioxygenases enzymes include histone demethylases, 5-methylcytosine hydroxylases and prolyl hydroxylases whose requirement for α -ketoglutarate maybe just a fraction of total α -ketoglutarate consumption which makes this metabolite an essential component of cell signaling and epigenetic networks [27]. *Aloe vera* affects glutamate metabolism by disturbing these crucial networks.

Phenylalanine and tyrosine metabolism was one of the cycles involved and there are reports of its importance in oesophageal cancer in a serum metabolic study ([28]). Infact, limitation of pheylalanine and tyrosine amino acids is seen to inhibit prostrate cancer [29].

The pentose phosphate pathway(PPP) is also seen in this study. Energy metabolism is readjusted in many human cancers that bestows many advantages to cancer cells, which include ATP generation, promotion of biosynthesis, detoxification and support of rapid proliferation. Glucose catabolism is carried out by the PPP. It directs the flux of glucose to its oxidative branch and produces a reduced form of nicotina-mide adenine dinucleotide phosphate (NADPH), an essential reductant in anabolic processes. It is now obvious that PPP has a very important role in regulation of growth of cancer cells by supplying cells with ribose-5-phosphate and also the NADPH required, reductive biosynthesis, detoxification of intracellular reactive oxygen species and ribose biogenesis. It can be concluded that any disturbance in the PPP directly contributes to proliferation of cancer cells, their endurance and weakness. Recent work has proved that regulation oncogenically/or metabolically of PPP is by numerous factors, including proteins such as oncoproteins, tumor suppressors and intracellular metabolites. Cancer cell growth and its survival is due to disturbance of PPP flux. PPP is one of the significant cycles identified by *Aloe vera* [30].

Earlier work implemented on the metabolic effect of ginger on Raji cells with an IC50 of 1mg/ml. It is interesting that the only common metabolic pathway identified by *Aloe vera* and ginger is protein biosynthesis which is directly involved in cell proliferation. Ginger seemed to act primarily on the

different glucose cycles, whereas *Aloe vera* acts mainly on amino acid cycles and the mitochondria. It seems that the two medicinal herbs act on different metabolic pathways to inhibit Raji cell growth [7].

Conclusion

Aloe vera exhibited inhibitory effect on Raji cells with an IC50 of 40 µg/ml. It affects primarily the protein biosynthetic pathway, different amino acid pathways, catecholamine synthesis, mitochondrial transport chain and pentose phosphate pathway exhibiting its unique mechanism of action.

List of abbreviations

¹HNMR: 1H Nuclear Magnetic Resonance
 MTT: MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 MS: Mass spectrometry
 NOESY: Nuclear Overhauser Spectroscopy
 MATLAB: Matrix laboratory
 PLS-DA: Partial linear square-discriminate analysis
 TCA: Tricarboxylic acid cycle
 PPP: Pentose phosphate pathway

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	SMN	SS	MM	MA	NAR	MA2	FV	HH	SM	ZZ
Research concept and design	--	--	--	--	--	--	--	✓	--	✓
Collection and/or assembly of data	✓	--	✓	--	✓	--	--	--	--	--
Data analysis and interpretation	--	--	--	✓	--	✓	--	--	--	--
Writing the article	--	--	--	--	--	--	--	--	--	✓
Critical revision of the article	--	✓	--	--	--	--	--	--	--	--
Final approval of article	--	--	--	--	--	--	--	--	✓	--
Statistical analysis	--	--	--	--	✓	--	✓	--	--	--

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