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1
2 # Detection of the altered metabolites of Raji cells in the presence of Epstein-Barr virus (EBV) using 1HNMR Spectroscopy

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12

13 **Abstract**

14 EBV is one of the most common viruses in humans and is directly implicated in carcinogenesis. The present study
15 aimed to detect the altered metabolites of Raji cells in the presence of Epstein-Barr virus (EBV) using 1H NMR by
16 NOESY technique. The cells and EBV were maintained in RPMI 1640 and 1 ml of cells were transfected into falcon
17 tubes containing cells and were incubated at 37°C for 2 hours. Metabolites extracted using water or
18 chloroform/methanol, lyophilized and sent for 1H NMR analysis by NOESY technique. The NMR spectrum
19 comprising of Fourier Transformed information about metabolites in the control and treated were imported into
20 MATLAB (v.7.8.0.347) software and the metabolic cycles were determined using Metabo-Analyst software. The
21 data have demonstrated that infected cells leads to proliferation and subsequent immortalization of cell lines through
22 cellular replication machinery recruitment and changes the metabolic profile and promotes vital metabolites such as
23 the carbohydrates engage in pentose phosphate and glycolactic, biosynthesis of nucleotide and amino acid
24 pathways. The results also indicate that essential amino acids are required for protecting viral structure and the
25 function of viral genes. Therefore, EBV infection of cells leads to the sustained elevation of cell growth and cell
26 immortalization.

27 **Keywords**:Raji cells, Epstein-Barr virus, 1H NMR Spectroscopy, Metabolites

28 ## Introduction

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29 Epstein-Barr virus (EBV) is one of eight known human herpes virus types in the herpes family
30 that was first isolated by Epstein in 1964 from cultured cells of Burkett's lymphoma frequently
31 found in children of equatorial Africa[1,2]. It is a double-stranded DNA virus of about 170 kb,
32 and encodes about 80 genes. EBV is one of the most common viruses in humans and is directly
33 implicated in carcinogenesis [3] which is associated with the nasopharynx[4], salivary gland [5],
34 breast [6], bladder [7], kidney [8], uterine cervix [9], colon [10] and lung [11] cancers cell lines.
35 EBV can cause infectious mononucleosis, also called mono, and other illnesses. EBV has
36 coevolved and become ubiquitous in all human populations through its different hosts, its ability
37 to establish lifelong latency, intermittent reactivation after primary infection and limited clinical
38 symptoms in the majority of infected individual[12].

39 The Raji cell line of lymphoblast-like cells was established from a Burkitt's lymphoma of the left
40 maxilla of an 11-year-old Negro male which have become invaluable tools for hematological
41 research as they provide an unlimited amount of cellular material. Cells of the Raji line do not
42 contain virus particles as demonstrated by electron microcopy and although the cells are resistant
43 to vesicular stomatitis virus, this resistance is not transferred to other normally susceptible test
44 cultures and an interferon-like inhibitor has not been found[13].

45 Metabolomics is broadly defined as the large-scale study of systematic identification and
46 quantification of the small molecule metabolic products (the metabolome) of a biological system
47 (cell, tissue, organ, biological fluid, or organism), which are the end products of cellular
48 processes. Metabolome refers to the complete set of small-molecule metabolites (such as
49 carbohydrate, amino acids, nucleotides, phospholipids, steroids, fatty acids, metabolic
50 intermediates, hormones and other signaling molecules, and secondary metabolites)[14]. The
51 metabolome forms a large network of metabolic reactions, where outputs from one enzymatic
52 chemical reaction are inputs to other chemical reactions. Mass spectrometry and NMR
53 spectroscopy are the techniques most often used for metabolomics profiling[15,16].

54 Various studies have shown that EBV have been an important cause of cancer in human and is
55 associated with a broad-spectrum of human cancers originating from epithelial cells,
56 lymphocytes and mesenchymal cells. Recent advances in cancer research have demonstrated that
57 Epstein Barr virus can alter the metabolites of infected cells and may result in the development
58 of cancer in humans[4-11]. In recent years, LC-MS and 1H NMR have been established as the
59 gold standard technique for metabolites analysis because of the technique's inherent analytical
60 specificity and sensitivity[16,17]. The purpose of these efforts is for identification and
61 quantification of metabolites that are uniquely correlated with an individual disease in order to
62 accurately diagnose and treat the morbidity. Previous reports revealed that some metabolites
63 (tripenoides) in raji cells may have inhibitory effects on the induction of Epstein-Barr virus early
64 antigen (EBV-EA) by 12-O-tetradecanoylphorbol-13-acetate (TPA) which metabolites were
65 determined on the basis of spectroscopic methods[18,19].

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66 The purpose of precision medicine is to design disease prevention and treatment methods taking
67 into account individual variability in environment, lifestyle, genetics, and molecular phenotype
68 which metabolic phenotyping has the potential to generate high-volumes of complex spectral
69 data[20]. The present study aimed to detect the altered metabolites of Raji cells in the presence
70 of Epstein-Barr virus (EBV) by using 1H NMR Spectroscopy.

71 Materials and methods

72 *Cell line*

73 The Raji (B-cell lymphocyte) and EBV-producing marmoset B-cell (B95-8) cell lines were
74 obtained from national Cell Bank of Iran (Pasteur Institute, Tehran, Iran)[21,22].

75 *EBV preparation*

76 B95-8 cells were cultivated in RPMI 1640 with 15% fetal bovine serum at 37°C and in 5% CO2
77 humidified atmosphere. After 48 hours, the cells were centrifuged at 1300 rpm for 4 min to
78 separate EBV-containing culture supernatant from cells[22].

79 *Cell culture*

80 The Raji cells were maintained in RPMI 1640 supplemented with L-glutamine, 10% FBS and
81 1% antibiotics (penicillin/streptomycin). The cells (1×10^6 cells/ml) were plated in T-25 flasks
82 containing 5 ml of CGM and grown in a humidified incubator under an atmosphere of 95% air
83 and 5% CO2 at 37°C to sub confluence (90 - 95%). The Raji cells were transferred to 6 falcon
84 tubes and were centrifuged at 1300 rpm for 5 min[23].

85 *Virus transfection*

86 Viruses were obtained from B95-8 cells. 1 ml of cells had transfected into falcon tubes
87 containing Raji cells and incubated at 37°C for 2 hours. After incubation, 4 ml of CGM
88 supplemented with 5% fetal bovine serum was added to each falcon tubes and the contents of the
89 falcon tubes were transferred to a new flask. After one week, Raji cells were infected by
90 EBV[23].

91 *PCR analysis*

92 Raji cells were seeded in dishes at 500,000 cells/10 mL/ 75 cm2. One day after seeding, the
93 medium was changed, and the cells were incubated with the test compounds for 12 h. At the end
94 of the incubation, DNA was extracted using QIAGEN kit (QIA amp® DNA Mini & Book Mini
95 Handbook) and was refrigerated at -20°C. PCR for EBNA I gene was carried out using the
96 specific primers which forward and reverse primers were prepared from LIGO company. The
97 results of the PCR productshave been confirmed by an agarose gel electrophoresis.

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98 *Cell extraction*

99 The method of extraction using methanol-chloroform-water was done as described previously.
100 The temperature of the extraction procedure was maintained at 4°C by working in a crushed ice
101 bath. Cells were washed in 1X PBS and centrifuged at 6,000g for 5 min and resuspended in 500
102 µL of ice-cold 2:1 (v/v) methanol-chloroform solution. It was then transferred into a 1.5mL
103 Eppendorf tube, 250 µL of ice-cold H2O 1:1 (v/v) chloroform/H20 was added and vortexed. The
104 cells were sonicated on ice for 10min and centrifuged for 5min at 18000X*g*. The lower lipophilic
105 and the upper hydrophilic extracts were separated and collected in different Eppendorf tubes and
106 lyophilized and stored at −20°C [24].

107 *1H NMR preparation*

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

112 *1H NMR spectroscopy*

113 The cell suspensions were placed in 5mm probes for analysis and one dimensional spectroscopy
114 was performed on a 1H NMR spectrometer (Bruker AV-500) with filed gradient operating at
115 500.13 MHZ for observation of proton at 298K. One dimensional 1H NMR spectra were
116 acquired with 6009.6 Hz spectral width, a 10-µs pulse 0.1 s mixing time, 3000 transients and 3.0
117 s relaxation delay, with standard 1D NOESY (nuclear Overhauser spectroscopy) pulse sequence
118 to suppress the residual water peak . The 1H NMR spectrum comprising of Fourier Transformed
119 information about metabolites in the control and treated groups(both hydrophilic and lipophilic
120 extracts of each) were imported into MATLAB (v.7.8.0.347) software and first analyzed by
121 ProMetab software (version 1.1) Chemical shifts between 0 and 10 ppm were normalized and
122 spectra binned in 0.004units and the water peak (4.7) removed. The Excel files were then
123 assessed by PLS-Toolbox version 3.0 and Partial Least Square Discriminant Analysis (PLS-DA)
124 was applied.

125 *Identification of metabolites*

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

130 **Results**

131 Figure 1 shows Superimposed 1H NMR spectra of hydrophilic and lipophilic layers between
132 experimental and control groups.

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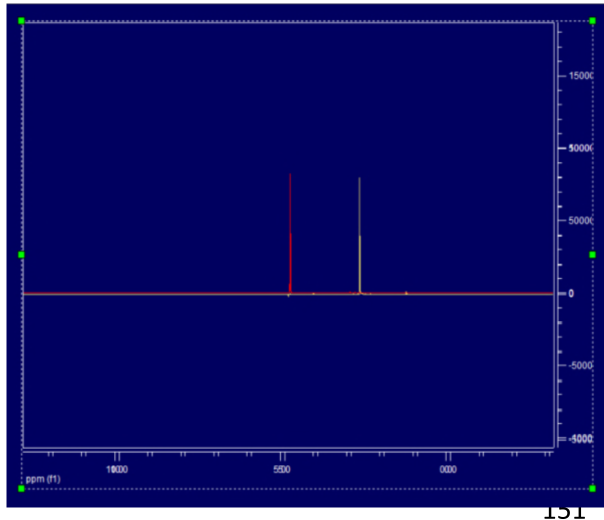

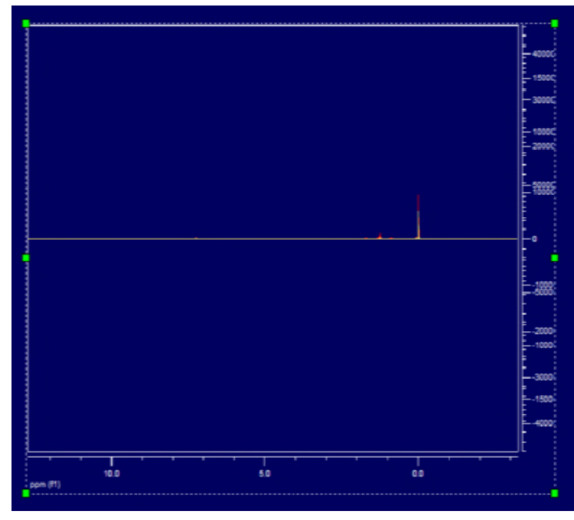

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153 Figure A Figure B

154 **Fig.1.** Superimposed 1HNMR spectra ofhydrophilicphase (A) and lipophilic phase (B) of EBV treated Raji cells and
155 controls.

156
157 Figure 2 Indicates the analysis of 1H NMR spectra of hydrophilic and lipophilic layers is
158 depicted as score plot using Partial Least Squares – Discriminant Analysis (PLS-DA) method.
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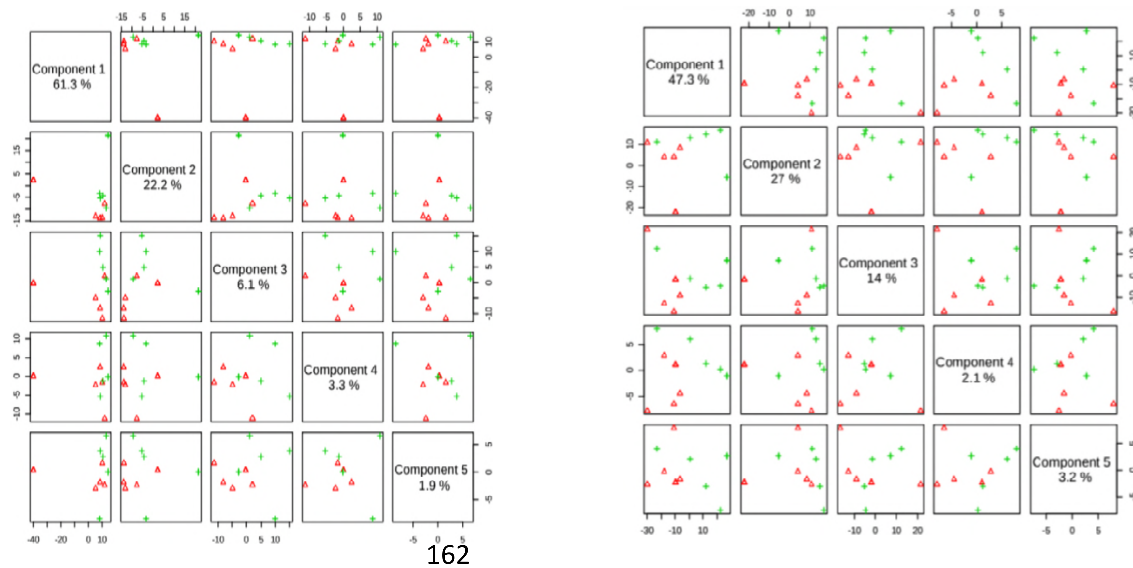

162

163 Figure AFigure B
164
165 **Fig.2.** Score plot of Raji cells with EBV inexperimental and control groups inhydrophilic phase (A) and lipophilic
166 phase (B) using PLS-DA method.

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Figure 3 shows loading plot of hydrophilic and lipophilic layers between experimental and control groups using PLS-DA method.

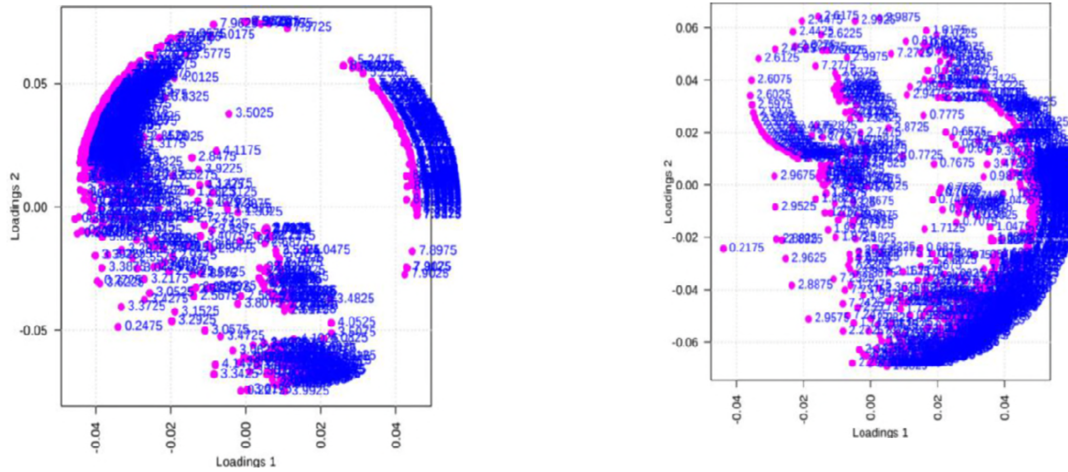

Figure A Figure B

Fig.3. Loading plot of Raji cells with EBV inexperimental and control groups inhydrophilic phase (A) and lipophilic phase (B) using PLA-DA method.

Figure 4 shows the affected metabolites of the raji cells in hydrophilic and lipophilic phase treated by EBV using enrichment analysis.

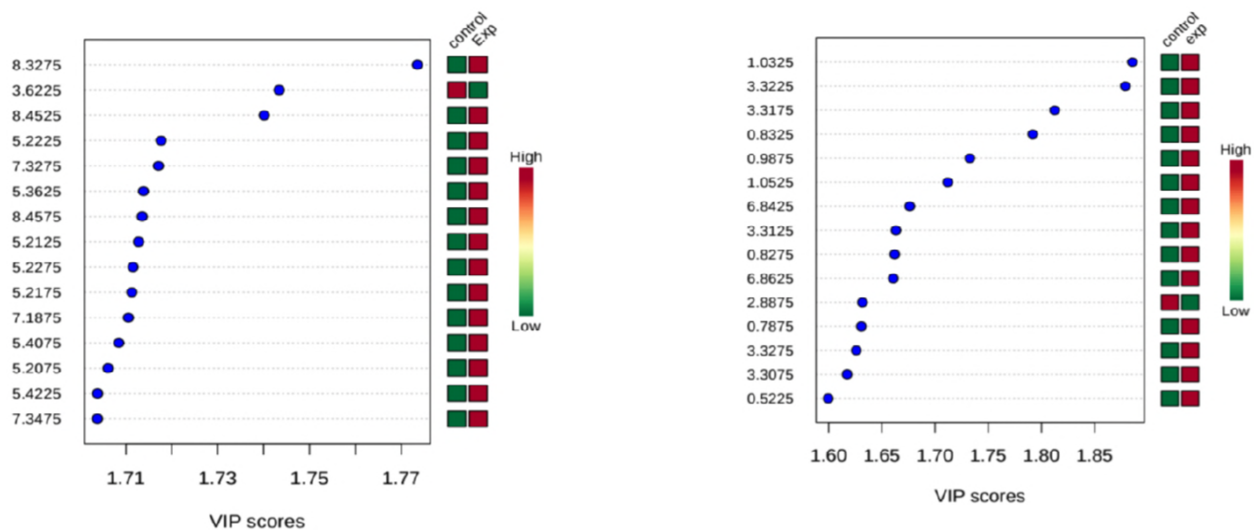

Figure AFigure B

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190 **Fig.4.** Summary plot of over-representation analysis ofhydrophilic phase (A) and lipophilic phase (B) metabolites of
191 Raji cells treated with EBV.
192
193 Table 1 showed the results of metabolites of Raji cells in hydrophilic phase treated by EBV
194 which obtained from human metabolites data base (HMDB).

195

196 **Table.1.** Altered metabolites of treated group with EBV in hydrophilic phase on the base of HMDB.

Metabolite	HMDB	Chemical Shift	levels	Abbreviation
L-Phenylalanine	HMDB00159	7.3475	↑	L-Phe
Glucose 1-phosphate	HMDB01586	5.4225	↑	Glu 1-P
L-Fucose	HMDB00174	5.2075	↑	L-Fu
D-Ribose 5-phosphate	HMDB01548	5.4075	↑	D-Rib
L-Tyrosine	HMDB00158	7.1875	↑	L-Tyr
L-Fucose	HMDB00174	5.2175	↑	L-Fuc
Glucose 6-phosphate	HMDB01401	5.2275	↑	Glu 6-p
1-Methyladenosine	HMDB03331	5.2125	↑	1.Methyladenosine
PC(16:0/16:0)	HMDB00564	8.4575	↑	PC
L-Phenylalanine	HMDB00159	5.3625	↑	L- Phe
Glucose 6-phosphate	HMDB01401	7.3275	↑	Glu 6-p
D-Ribose 5-phosphate	HMDB01548	5.2225	↑	D-Rib 5-p
1-Methyladenosine	HMDB03331	8.4525	↑	1.Methyladenosine
Fructose 6-phosphate	HMDB00124	3.6225	↓	Fru 6-p
Nicotinamideribotide	HMDB00229	8.3275	↑	Nicotinamideribotide

197

198 Demonstration of the alteration of metabolites of Raji cells in lipophilic phase treated by EBV
199 which obtained from human metabolites data base (HMDB) showed in Table 2.

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206
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210 **Table.2.** Altered metabolites of treated group with EBV in lipophilic phase on the base of HMDB.

Metabolite	HMDB	Chemical Shift	levels	Abbreviation
Cholesterol	HMDB00067	0.5225	↑	Chol
L-proline	HMDB00162	3.3075	↑	L-Pro
Beta-Leucine	HMDB03640	3.3275	↑	ß- Ala
Cholesterol	HMDB00067	0.7875	↑	Chol
L-Arginine	HMDB0000168	2.8875	↓	L-Arginine
5-Hydroxy-L-tryptophan	HMDB00472	6.8625	↑	5-Hydroxy-L-Try
Tryptophan	HMDB00929	6.8625	↑	Try
Cholesterol sulfate	HMDB0000653	0.8275	↑	Chol
L-Proline	HMDB00162	3.3125	↑	L-Pro
S-Adenosylhomocysteine	HMDB00939	6.8425	↑	SAH-Cys
2-Ketobutyric acid	HMDB00005	1.0525	↑	2- Ketobutyric acid
L-Valine	HMDB00883	0.9875	↑	L-Val
Cholesterol	HMDB00067	0.8325	↑	Chol
L-Proline	HMDB00162	3.3175	↑	L-Pro
L-Proline	HMDB00162	3.3225	↑	L-Pro
L-Valine	HMDB00883	1.0325	↑	L-Val

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212
213 Figure 5 shows the affected metabolic pathways of the raji cells in hydrophilic phase treated by
214 EBV using the pathway analyzing tool (MetaboAnalyst 3.0).

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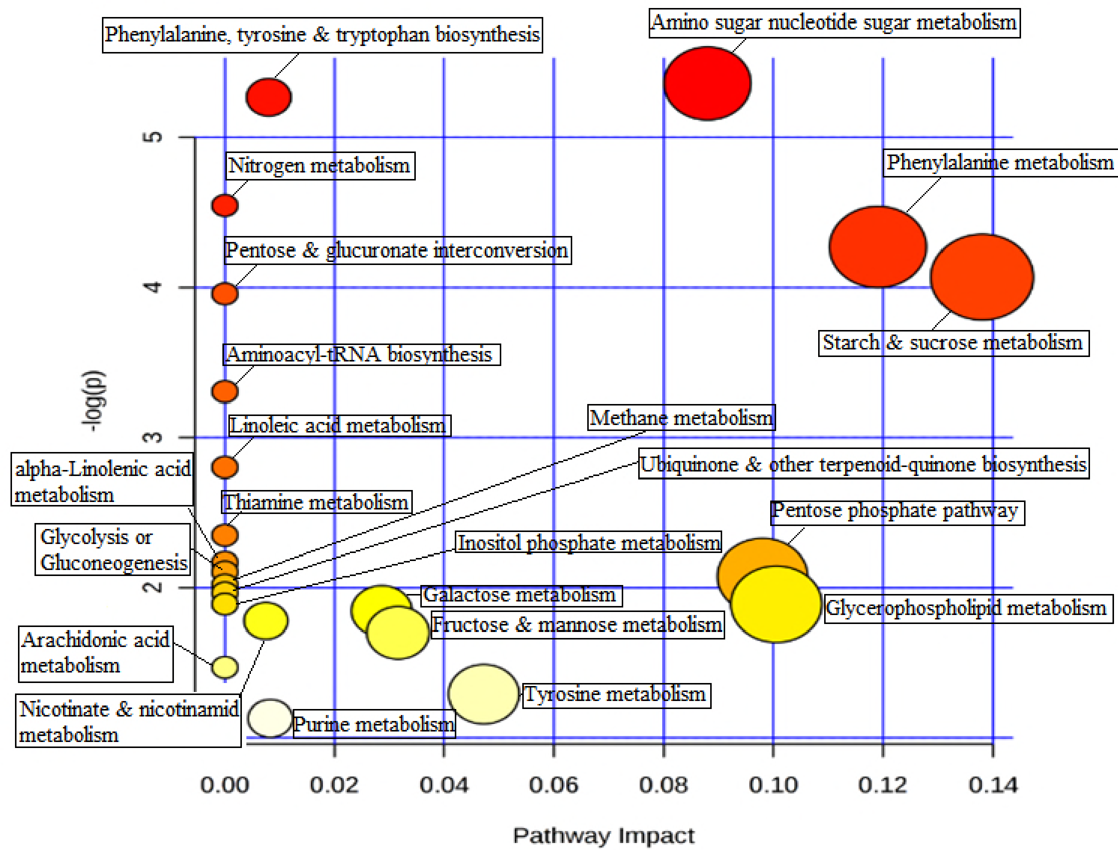

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216 **Fig.5**.Important metabolicpathways in the hydrophilic of Raji cells treated with EBV using MetaboAnalyst 3.0.
217
218

219 Table 3 indicates the alteration of metabolic pathways of Raji cells in hydrophilic phase treated
220 by EBV using the pathway analyzing tool (MetaboAnalyst 3.0).
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234 **Table.3.** Altered metabolic pathways of treated group with EBV in hydrophilic phase using MetaboAnalyst 3.0.

235

Pathway	Total	Expected	Hits	Raw p	-log(p)	Metabolites
Amino sugar & nucleotide sugar metabolism	88	0.37	3	4.70E-03	5.36E+00	Glu1p,Fru6p,Fuc
Phenylalanine,tyrosine& tryptophanbiosynthesis	27	0.11	2	5.16E-03	5.27e+00	Tyr, Phe
Nitrogen metabolism	39	0.16	2	1.06E-02	4.55E+00	Tyr, Phe
Phenylalanine metabolism	45	0.19	2	1.40E-02	4.27E+00	Tyr, Phe
Starch and sucrose metabolism	50	0.21	2	1.71E-02	4.07E+00	Glu1p,Glu 6p
Pentose and glucuronateinterconversion	53	0.22	2	1.91E-02	3.96E+00	Fru6p, Glu1p
Aminoacyl-tRNA biosynthesis	75	0.31	2	3.67E02	3.31E+00	Tyr, Phe

236

237 Figure 6 indicates the affected metabolic pathways of the raji cells in lipophilic phase treated by
238 EBV using the pathway analyzing tool (MetaboAnalyst 3.0).

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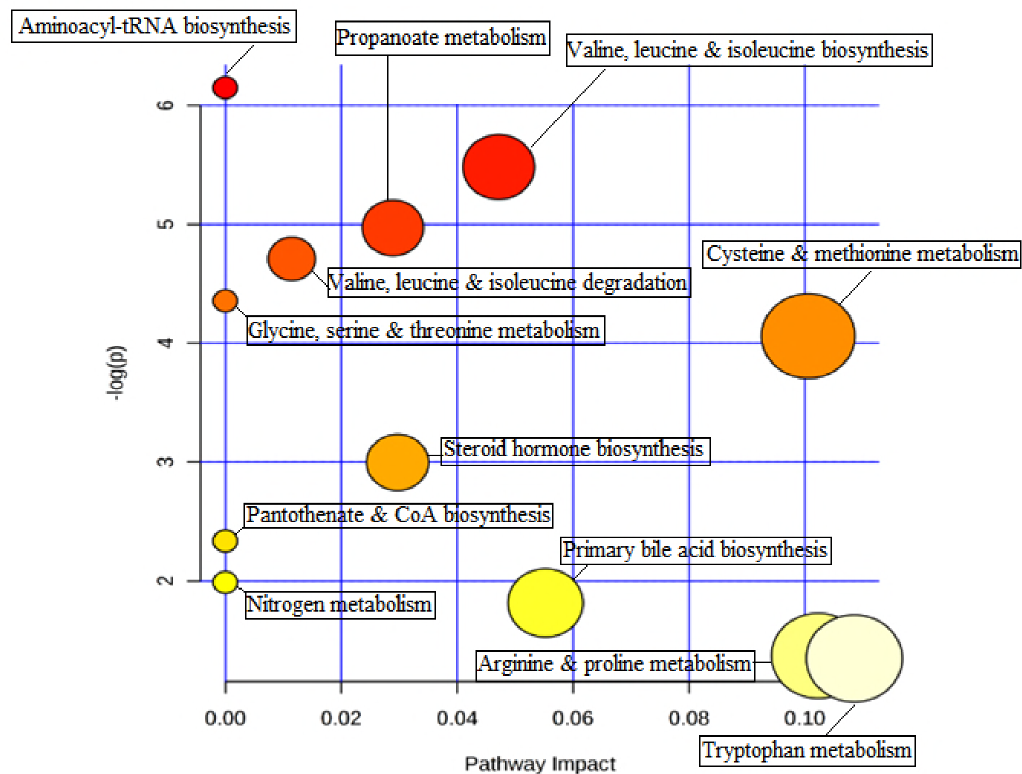

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241 **Fig.6.**Important metabolicpathways in the lipophilic phaseofRaji cells treated with EBV using MetaboAnalyst 3.0.

242

243 Table 4 demonstrates the alteration of metabolic pathways of Raji cells in lipophilic phase
244 treated by EBV using the pathway analyzing tool (MetaboAnalyst 3.0).

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Table.4.........................

Pathway	Total	Expected	Hits	Raw p	-log(p)	Metabolites
Aminoacyl-tRNAbiosynthes	75	0.28	3	2.31E-03	6.15E+00	Pro,Tyr, tRNA(Val)
Valine, leucine& isoleucine biosynthesis	27	0.1	2	4.16E-03	5.48E+00	Val, Ketobutyric acid
Propanoate metabolism	35	0.13	2	6.94E-03	4.97E+00	Val, Ketobutyric acid
Valine,leucine and isoleucine degradation	40	0.15	2	9.01E-03	4.71E+00	Val, Leu
Glycine,serine& threonine metabolism	48	0.18	2	1.28E-02	4.36E+00	Trp, Ketobutyric acid
Steroid hormone biosynthesis	99	0.37	2	4.99E-02	3.00E+00	Chol,L-Arg

Discussion

A wide range of metabolites and chemical structure were measured upon extraction and samples preparation, which berecognizedsignificantly, altered metabolic pathways according to metaboAnalyst website.

According to the obtained results for hydrophilic phase, glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate have been the most frequency among metabolic pathways including amino sugar and nucleotide sugar reaction, starch and sucrose metabolism and pentose-glucuronateinterconversion. In lipophilic phase, proline, valine, tyrosine, triptophane, and ketobutyric acid also have the most alteration among metabolic pathway including amino acyl tRNA, valine, leucine and isoleucine biosynthesis, glycine, serine, threonine and propaonate metabolism and valine, leucine and isoleucine degradation aromatic amino acids (tyrosine and phenylalanine) also may be changed among tyrosine, phenylalanine, tryptophane and nitrogen metabolic pathways. Although,the alteration in metabolic pathways have been resulted in difference between Raji cells and EBV-infected cells.

The results indicated that amino sugar and nucleotide sugar metabolism are the most important metabolic pathways in hydrophilic phase. The metabolites conversions, especially glucose 1-

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273 phosphate,fructose 6-phosphate and fucose were observed in these pathways, which they are the
274 most valuable metabolites in glycolysis pathway. The result from this study also support that
275 changes in metabolite concentration, particularly rising glucose and L-fucose levels were found
276 in EBV-infected raji cells in comparison with control cells. It also hasbeenshown that fructose 6-
277 phosphate and glucose 1-phosphateare involved in pentose-glucuronateinterconversion pathway.
278 In line with this investigation, some virological studies showed that EBV lead to change in
279 metabolic assay from early infection to long-term outgrowth that may be stimulate glucose
280 import and surface glucose transporter-1 (Glut-1) levels, result in induction of glycolysis,
281 oxidative phosphorylation and suppression of basal autophagy[25]. There are some reports
282 showing that serum glycoprotein L-fucose levels have two-fold rise in head and neck neoplasma
283 compared to control group[26]. Advanced in cancer have been shown that fucose may be is
284 useful in breast cancer treatments and α-L-fucose has pivotal role in construction of malignant
285 and metastaric phenotype of various human breast cancer cell lines. Additionally, some breast
286 cancer cell lines biomarkers are fucose-rich[27].

287 Metabolomics approach demonstrate alteration in glycolysis metabolites is associated with EBV
288 in nasopharyngeal carcinoma (NPC) which overexpression of EBV-encoded latent protein 1
289 (LMP1) may lead to glycolysis induction. Some glycolysis genes (i.e. hexokinase 2) have central
290 roles in LMP1-mediated glucose metabolism reprogramming in NPC cells. Additionally, positive
291 correlation was existed between HK2 and LMP1 in NPC biopsies, and the HK2 induction was
292 associated with poor survival of NPC patient after radiation therapies[28]. Therefore, there is a
293 potential correlation between glycolysis pathway and EBV infected cells. Furthermore, the
294 pentos phosphate pathway is required for ribonocleotide synthesis and NADPH production
295 which branches from glycolysis [29].At first this research suggests phenylalanine and tyrosine
296 are involved in nitrogen metabolism pathway. According to virologycal studies, two
297 phenylalanine (F600, F605) are located in R transactivator (Rta) c-terminal which play pivotal
298 role in DNA binding to target cells. These two phenylalanine are essential for Rta expression
299 which Rta activates the EBV lytic cycle. If two other aromatic amino acids (Tryptophan and
300 Tyrosine) are substituted with two phenylalanine, maintenance of mRNA activity of the BMLF1
301 gene have been seen. However, substitution of Tryptophan and Tyrosine with non-aromatic
302 amino acid including Alanine and Valine, lead to capacity elimination of Rta activity. Valine and
303 glycine substitution instead of phenylalanine in Rta protein act as inhibitor and may prevent its
304 DNA-binding function. The EBV BZLF1 protein (ZEBRA, Zta) aromatic amino acids including
305 (phenylalanine, Tyrosine and Tryptophan) are crucial components of activation
306 domain,thereforeRta play important role in EBV lytic cycle by the subsitution of cellular
307 signaling pathway and synergy with EBV ZEBRA protein [30].

308 Moreover, the results in lipophilic phase revealed that proline, valine, tyrosine, tryptophane
309 amino acids and ketobutyric acid are altered in metabolic pathway of tRNA, valine, leucine,
310 isoleucine, serine, glycine, threonine and propanoate biosynthesis, and degradation of valine,
311 leucine and isoleucine. Amino acyletRNA biosynthesis could be among the first pathways in

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312 lipophilic phase which several amino acids such as proline, tyrosine and valine can be altered in
313 thesepathways. N-terminal domain of LMP2A contains eight phosphorylated tyrosine
314 residue.Two of which constitute an immunoreceptor tyrosine activation motif (ITAM)[31].
315 ITAM consist of a pared tyrosine and leucine residues and play a pivotal role in signal
316 transduction of B-cell receptors (BCR) and T-cell receptors, lymphocyte proliferation and
317 activity of kinase families. Although, LMP2A ITAM motif participate in BCR signal
318 transduction as an inhibitor[32]. Several lines of studies suggest that LMP2A prevents BCR
319 signal transduction trough engaging Nedd-4 ubiquitin protein ligases to promote the degradation
320 of Lyne and LMP2A by an ubiquitin- dependent mechanism[33].

321 The results showed in lipophilic phase also indicating that valine and acid butyric are involved
322 and altered in metabolic pathway of valine, leucine and isoleucine biosynthesis. Several
323 investigation consider that the C-myc transcription factor is acting as a proto-oncogene which
324 activation of MYC lead to promotion of cell cycle transition and is recognized as a leucine zipper
325 protein which is activation by mitogenic factors under normal circumstances[24]. On this base,
326 previous studies on EBV genome have shown that multi-nucleonal proteins have a highly
327 charged N-terminus which may provide nuclear signals and contains heptad repeats of leucine,
328 isoleucine or valine that can act as dimerization domain; the third exon includes leucine and
329 isoleucine heptad repeats which make possible intraction of coiled-coild and facilities in
330 hemodimerization of BZLF1[35].

331 In present study tryptophane level has dramatically increased in glycine, serine and threonine
332 metabolic pathways. Valine and ketobutyric acid also have seen to be engaged in propaonate
333 metabolism'srecent data, indicating that Tryptophane is essential for virus penetration in cells
334 and crosses lipid bilayers without pore formation[36].

335 In steroid hormone metabolism, the most alteration was observed in amount of cholesterol
336 metabolites. Some investigation has revealed that cholesterol enriched- domains in plasma
337 membranes may are required for infection of human B-cells with EBV and is necessary for
338 membrane fusion, receptor localization in membrane micro-domain and early viral signaling
339 events. Lipid rafts are also involved in MHC class II protein function[37].

340 Additionally, the finding results demonstrate that L-Argenine level has significantly reduced in
341 cells exposed to EBV compared to control cells. There is various reports indicating L-Argenine
342 supplementation may be lead to inhibition of spontaneous EBV reactivation in another Burkitts
343 lymphoma cell line EB1 and B lymphoblastic cell line OB. L-Argenine also can induces
344 inducible NO synthase and generates NO, which inhibits EBV reactivation in EBV-possitive
345 cells[38].

346 The conclusionof this research showed that infection of Rajicells with EBV leads to proliferation
347 and subsequent immortalization of cell lines through cellular replication machinery recruitment
348 andchanges the metabolic profile and promotes vital metabolites such as the carbohydrates (i.e.

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349 glucose-1-phosphate and fructose 1-phosphate) engage in pentose phosphate and glycolactic,
350 biosynthesis of nucleotide and amino acid pathways. Theresults also indicate that essential amino
351 acids are required for protecting viral structure and the function of viral genes. Furthermore, rates
352 of proteins synthesis and function of glycolysis pathway give rise to increase in EBV-infected
353 Raji cells compare with control cells. Therefore, EBV infection of Raji cells leads to the
354 sustained elevation of cell growth and cell immortalization.

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360 **Conflict of interest statement**

361 The authors state that there are no conflicts of interest regarding the publication of this article.

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