Iranian J Biotech. 2020 July;18(3): e2336 DOI: 10.30498/IJB.2020.141102.2336

Research Article

An Integrative *In Silico* Mathematical Modelling Study of The Anti-Cancer Effect of Clove Extract *(Syzygium aromaticum)* Combined with *In Vitro* Metabolomics Study Using ^{1}HNMR Spectroscopy

Mehri Azadi[1], Fatemeh Alemi[2], Sedigheh Sadeghi[1], Maryam Mohammadi[1], Nowrooz Ali Rahimi[1], Sako Mirzaie[3], Farideh Vahabi[1], Shirin Parvaz[1], Mohammad Arjmand[1] and Zahra Zamani[1*]

[1] Biochemistry Department, Pasteur Institute of Iran, Tehran, Iran.
[2] Chemical Engineering Department, Islamic Azad University, Tehran, Iran.
[3] Biology Department, Islamic Azad University, Pasdaran Avenue, Sanandaj, Iran.

**Corresponding author: Zahra Zamani, Biochemistry Department, Pasteur Institute of Iran, Tehran, Iran. TelFax:+982164112101; E-mail: zamani@pasteur.ac.ir P.O. Box: 1316943551*

Background: Clove oil is known for its medicinal properties. The mechanism of anti-cancer properties of *Syzygium aromaticum* were investigated by mathematical modelling on the genome scale with metabolomics using ^{1}H Nuclear Magnetic Resonance spectroscopy on Raji cells.
Objectives: An integrative analysis correlated the metabolites identified by ^{1}HNMR and genes with the detected pathways.
Materials and Methods: Raji cells treated with clove oil were collected and sent for ^{1}HNMR spectroscopy and the spectra analyzed by MATLAB and Human Metabolome Database for metabolite identification. Pathway and topology analysis was implemented using the genes and metabolites in the integrative analysis of Metaboanalyst software.
Results: 50% inhibitory concentration of clove oil was 50 µg/ml and the model anticipated 74 genes with differentiating metabolites being some amino acids, cholesterol and fucose.
Conclusions: The integrative study predicted that the anti cancer mechanism of clove oil involves novel enzymes, as likely drug targets, 24-dehydrocholesterol reductase and 7-dehydrocholesterol reductase in cholesterol biosynthesis, dehydrofolate reductase in one carbon metabolism and serine palmitoyl-transferase long chain in sphingolipid biosynthesis.
Keywords: Genes and pathways, *In silico* mathematical model, Methanol clove extract, Raji cells.

1. Background

Any drug whether natural or synthetic, preventing, delaying or supressing a disease such as cancer is described as chemo-preventive. It is well known that a wide range of spices have anti-cancer properties and this is substantiated by laboratory evidence (1).

Clove extract has been used for treatment of respiratory diseases by Indian traditional medicine, Ayurveda, since ancient times. Cloves have also been used in Chinese medicine for treating indigestion, diarrhea, ring worms and fungal infections. Even, modern medicine employs cloves to heal digestive disorders, nausea and its oil is used to relieve toothaches (1). Cloves possess anti-septic, antibiotic, antiviral and anaesthetic properties; and inhibit the growth of spore germination of the bacillus *Subtilis enterides* and also pathogens in the intestine like *Salmonella* and *Escherichia coli* (1). Moreover, although there have been reports of anti-cancer activity of clove oil on different cancer cell lines *in-vitro*, a large number of questions have arisen about its mechanism (2).

In cancer research, mathematical modelling approaches have become significantly more numerous. The intricacy seen in cancer is well matched to quantitative analysis as it can result in new approaches and can provide challenges and opportunities for new progress. Mathematical models can be a balance between clinical and experimental investigations, but they also defy present standard prototypes and reconsider the comprehension of different mechanisms steering tumorigenesis and hence chart out new ways for further research in cancer biology (3).

In this study, the modified Recon-1 model was used on the presumption that altered concentrations of a metabolite represent the different expression of the gene(s) responsible for enzymatic reaction(s) for synthesis of that metabolite. Its model was based on phenotypic variations of acute leukaemia (4). To corroborate the *in*

Copyright *© 2019 The Author(s); Published by National Institute of Genetic Engineering and Biotechnology. This is an open access article, distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (http://creativecommons.org/licenses /by-nc/4.0/) which permits others to copy and redistribute material just in noncommercial usages, provided the original work is properly cited.*

silico model with *in vitro* mechanisms, the model was combined with a metabonomics study of the effect of clove extract on Raji cells, a lymphoblast line of B cell cancer seen in Hodgkin's disease (5).

Metabonomics is a simultaneous study of metabolites using high- throughput technology like Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) in biological samples like cells and their organelles, tissues and their extracts, biofluids or entire organisms. It is a non-invasive method which is used to identify biomarkers in bio-fluids such as plasma, urine and saliva (6).Cancer cell lines are widely used to study drugs which could have therapeutic potential, to identify proteins with altered expression levels, gene mutations, and inhibition or activation of metabolic pathways (7). Several metabolomics studies have shown variances in metabolic profiles among different cancer cell lines, during apoptosis and cell cycle phases and have helped in better understanding their anti-cancer mechanisms (8). The mechanism for anti cancer effect of clove oil is unclear.

2. Objectives

In the present study, a novel *in silico* prediction based on a mathematical model constructed on silencing of the metabolic genes was carried out. This was integrated with the metabolites obtained in the analysis of the inhibitory potential of clove treated Raji cells and controls *in vitro* using ^{1}HNMR spectroscopy. This investigation may help in predicting the anti cancer targets of clove oil for Raji cells.

3. Materials and Methods

3.1. In silico Mathematical Modelling

Detection of the genes responsible for alteration of metabolites was carried out by mathematical modelling. For this, we used an *in silico* approach which is based on analysis of the genome-scale metabolic model of cancer cell lines by modified Recon-1 model. Our method was based on the premise that the altered concentration of a metabolite represents the differential expression of the gene(s) encoding the enzymatic reaction(s) responsible for synthesis of that metabolite. We employed the human genome-scale metabolic model of Ruppin (4) known as modified Recon-1 which includes 1,496 ORFs, 3,742 reactions and 2,766 metabolites. The metabolic network is shown as an $m|n$ stoichiometric matrix S, where the number of metabolites is m, the number of reactions is n, and the stoichiometric coefficient of metabolite i in reaction j is represented by Sij. The definition of the biomass reaction was as the objective function which is biomass by the Constraint based method (CBM) and Flux Balance Analysis (FBA). FBA studies for a flux distribution v that maximizes the objective function (Equation 1) subject to steady-state, thermodynamic and growth medium constraints:

$$Max_{v}\ biomass \quad (1)$$

Subject to

$$S.v=0 \quad (2)$$

$$v_{min} \leq v \leq v_{max} \quad (3)$$

Equation 2 imposes the constraints of the steady state on the system, presuming that the metabolite concentrations remain constant in time. Thermodynamic constraints deciding the reaction directionalities are arranged via the flux limits v_{min} and v_{max} in Equation 3. The uptake and excretion of a pre-defined set of metabolites from and to the surrounding is assisted via the definition of exchange reactions in the stoichiometric matrix (4).

The genes whose silencing resulted in loss of proliferation (from 50% to 100% in the IC50 range) were identified from their numbers by NCBI genes website and the enzymes and metabolic pathways which they affect were detected by KEGG pathway analysis.

The model was obtained from modified Recon 2 for leukaemia and the supplementary files were courtesy the authors (Tomer Shlomi 2011) showing the genome model MATLAB file, excel files for the enzyme molecular weight data for reactions, enzyme turnover number data and human biomass composition (Supplementary data # 1) obtained from their paper. Validation of the model was carried out by the same authors (Tomer Shlomi 2011) where different criteria of the model was validated with variations in different conditions and compared with data obtained by proteomics.

3.2. Preparation of Samples

50g air-dried powdered clove buds obtained from Mumbai, India was subjected to hydro-distillation for 3h using a Clevenger-type apparatus. The essential oil was carefully collected in a separated sealed container to avoid evaporation. The isolated essential oils were re-extracted with organic solvent dichloromethane and dried over anhydrous sodium-sulfate and preserved in a sealed vial at 4°C until further analysis (8). Then 1 gram of the sticky extract was dissolved in 20 mL RPMI medium to give 50mg/mL methanolic clove extract (MCE).

3.3. Identification of Active Ingredient of Clove Extract by Thin Layer Chromatography (TLC)

Silica gel slurry was prepared on a slide and toluene and ethyl-acetate (93:7) were used as mobile phase. Standards

such as clove oil and standard eugenol were used. The spotting was done in such a way that approximately 1 mm-sized spot of the solution could be seen. The plate was kept slightly tilted in the solvent system so as to run the solvent system to 3/4th of the plate. The spots became visible by incubation in an iodine chamber and photographs were recorded and Rf calculated (8).

3.4. Cell Culture

B cell lymphoma named Raji cells were purchased from the Cell Bank of Pasteur Institute of Iran. The cells were cultured in 25 mL flasks in RPMI-1640 medium containing 10% foetal calf serum, penicillin, and streptomycin (complete medium). Incubation was at 37 ˚C with 5% CO2, 95% humidity and the medium changed every 48h (8).

3.5. Treatment of Raji Cells with Clove

1 mL of culture medium and 1.6×10^4 Raji cells were added to each well, in three 12 well plates, and treated with different concentrations of MCE, 5000 $\mu g.ml^{-1}$, 500 $\mu g.ml^{-1}$, 50 $\mu g.ml^{-1}$ and 5 $\mu g.ml^{-1}$ was added for 24, 48, and 72 hours. The test was carried out in duplicate and negative controls without MCE (9).

3.6. Viability Test

Viability was tested using Trypan blue method at 24, 48, and 72 hours (9).

3.7. MTT Assay

180 µl of culture medium were applied to 96 well plates to which were added 10 µl of MCE and 10 µl containing 1×10^4 cells; and then they were left to incubate overnight. The next day, 20µl/$well^{-1}$ of MTT (5mg⁄ml^{-1}) was added and the cells kept at 37°C for 3 to 4 hours. The crystals were dissolved in 100µl of dimethyl sulfoxide for 30 min and the absorbance read at 570nm using Microplate Reader, (BIOTEK U.S.A)(10).

3.8. Cell Extraction for ^{1}HNMR Spectroscopy

Methanol-chloroform-water extraction was done at 4 ˚C on a crushed ice bath. The cell pellets were re-suspended in 500 µL of ice-cold 2:1 (v/v) methanol:chloroform solution and then transferred to a 1.5mL Eppendorf tube, vortexed and placed in a mixer for 10 min at 4 ˚C. Next, 250 µL of ice-cold H2O 1:1 (v/v) chloroform/ H20 was added and once again vortexed. The tubes were sonicated on ice for 10 min and centrifuged at 18000×g for 10 mins. The top hydrophilic and the bottom lipophilic extract were separated into different Eppendorf tubes. The samples were lyophilized to remove water as its presence could interfere in the spectra and then stored at −20 ˚C until analysis (11).

3.9. Preparation of ^{1}HNMR Extracts

Lyophilized hydrophilic cell extracts were re-suspended in 200µL of 150mM potassium phosphate buffer of pH 7.4, 1 mM NaN3, and 0.01% Trimethyl-silyl-propionate (TSP) in 100% D2O (deuterium oxide: the required quantity of buffer for each sample was originally prepared in H2O, lyophilized, and reconstituted in 100% D2O), and the lipophilic cell extracts were re-suspended in 200µL deuterated chloroform. The hydrophilic extract will be denoted as (hyd-ext) and lipophilic extract as (lip-ext). Both the extracts were analyzed via ^{1}HNMR analysis (12).

3.10. ^{1}HNMR Spectroscopy

The cell suspensions were placed in 5mm probes (Bruker) for analysis. A Bruker spectrometer operating at 400 MHZ recorded the results by 1D NOESY method. The temperature of the sample was maintained at 298 K. For each sample 3000 transients and 3.0s relaxation delay, with standard 1D NOESY (Nuclear Overhauser Spectroscopy) pulse sequence to suppress the residual water peak was considered (11).

The spectra were then analyzed by MestReC Nova software. Automatic phase correction was used as baseline correction and chemical shifts were referenced to external 0.1% TSP in D2O. All spectra were binned into 1000 parts and their normal intensity and chemical shift were added to Excel files (8).

3.11. Data Processing

Chemometrics analysis: MATLAB 6.5 was employed and PLS was implemented with the PLS-Toolbox version 3.0 with Orthogonal Signal Correction and Partial Linear Square (OSC-PLS) analysis.

3.12. Identification of Metabolites

Metabolites corresponding to these resonances were then recognized by assigning the chemical shifts of the spectra based on comparison with chemical shifts of metabolites in Human Metabolome Database (HMDB) (*http://www.hmdb.ca/metabolites*) and in other available data bases (13). Analysis of metabolites was carried out using Metabo-Analyst software (*http://www.metaboanalyst.ca/*) (14).

3.13. Combination of Modelling and Pathway Analysis

Integrative analysis option on the Metaboanalyst website was used for combination of modelling and pathway analysis. The list of gene accession numbers obtained from our mathematical model and the list of metabolites from ^{1}HNMR spectroscopy was entered

into the integrative analysis option which were then converted into csv excel files and enrichment analysis and topology analysis was carried out. Enrichment analysis evaluated whether the observed genes and metabolites in a particular pathway were significantly enriched (appear more than expected by random chance) within the dataset. The topology analysis however calculated whether a given gene or metabolite plays an important role in a biological response based on its position within a pathway.
The result is depicted as a graph showing the *p* values of the significance of the metabolite pathways and the genes participating in them.

3.14. Statistical Analysis

Statistical analysis was performed by SPSS version 19 and results showed as mean ± SEM. Student's t-test; the significance in differences stood at $p< 0.05$.

4. Results

4.1. In Silico Mathematical Modelling

The modified Recon 1 model predicted 74 genes to participate in the IC50 inhibition of Raji cells and our metabolomics study identified 11 metabolites which when integrated with the genes, obtained 29 pathways with different p values and the result of the metabolomics analysis will be presented first followed by the integrated study.

4.2. TLC Analysis

Thin layer chromatography of MCE and standard clove oil had the same Rf value **Figure 1** and MCE was seen to be reasonably pure. (**Fig. 1a**)

4.3. MTT Assay

The effect of MCE on percentage viability of Raji cells after 48h. IC50 of MCE on Raji cells is 50 µg.ml^{-1}.(**Fig.1b**)

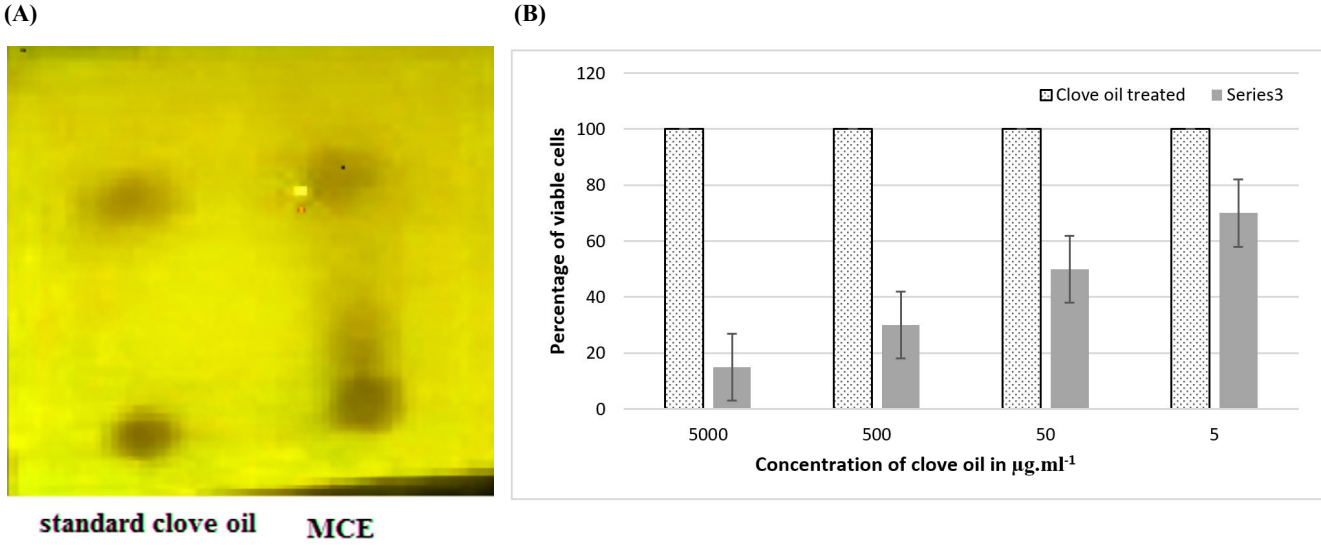

Figure 1. (A) TLC of methanolic clove extract (left lane) with standard clove oil (Right lane). **(B)** Effect of clove oil extract on Raji cells. MTT assay showing effect of MCE on Raji cells after 48 h. IC50 obtained was 50µg.ml^{-1}

4.4. ^{1}HNMR Spectra Analysis

The superimposed spectra of the hydrophilic phases of control and Raji cells exposed to MCE are seen in **Figure 2a** and b and that of the lip-ext of control and Raji cells exposed to MCE in **Figure 2C and 2D** with differentiating metabolites marked on the spectra.

4.5. Data Processing

OSC-PLS was carried out and the score plot of both the hydrophilic and lipophilic phases show good separation of samples in **(Fig. 3A, 3B)**. The biplot depicting the loading plot and score plot together of both the phases are shown in **Figure 3C and 3D** where the overlapping variables are common in the controls and the treated samples and the outliers are the differentiating ones.

4.6. Identification of Metabolites

The chemical shifts of the differentiating metabolites were identified using the reference databank of HMDB as depicted in **Table 1**.

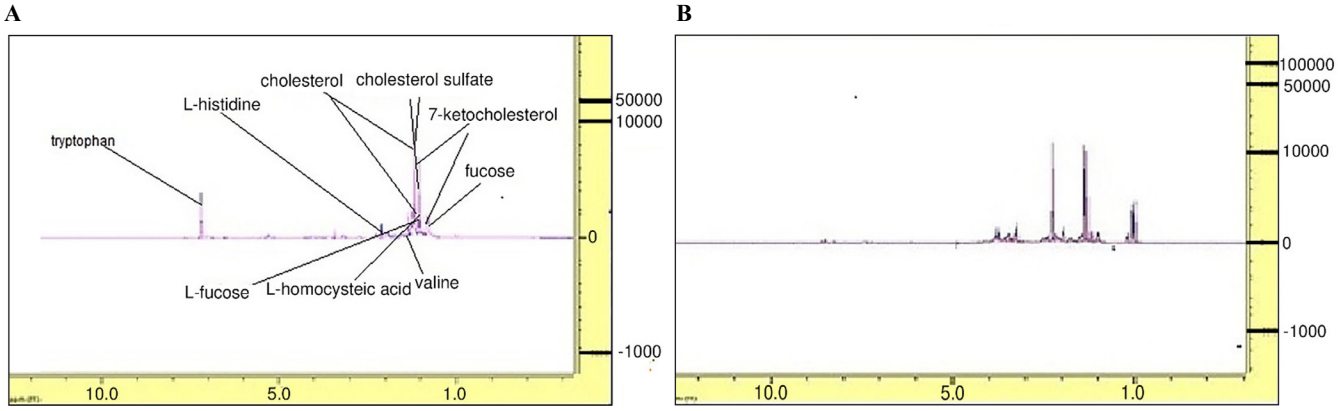

Figure 2. (A) Superimposed spectra of hyd-ext of control and experimental Raji cells exposed to MCE showing differentiating metabolites. **(B)** Superimposed spectra of lip-ext of control and experimental Raji cells exposed to MCE exhibiting differentiating metabolites.

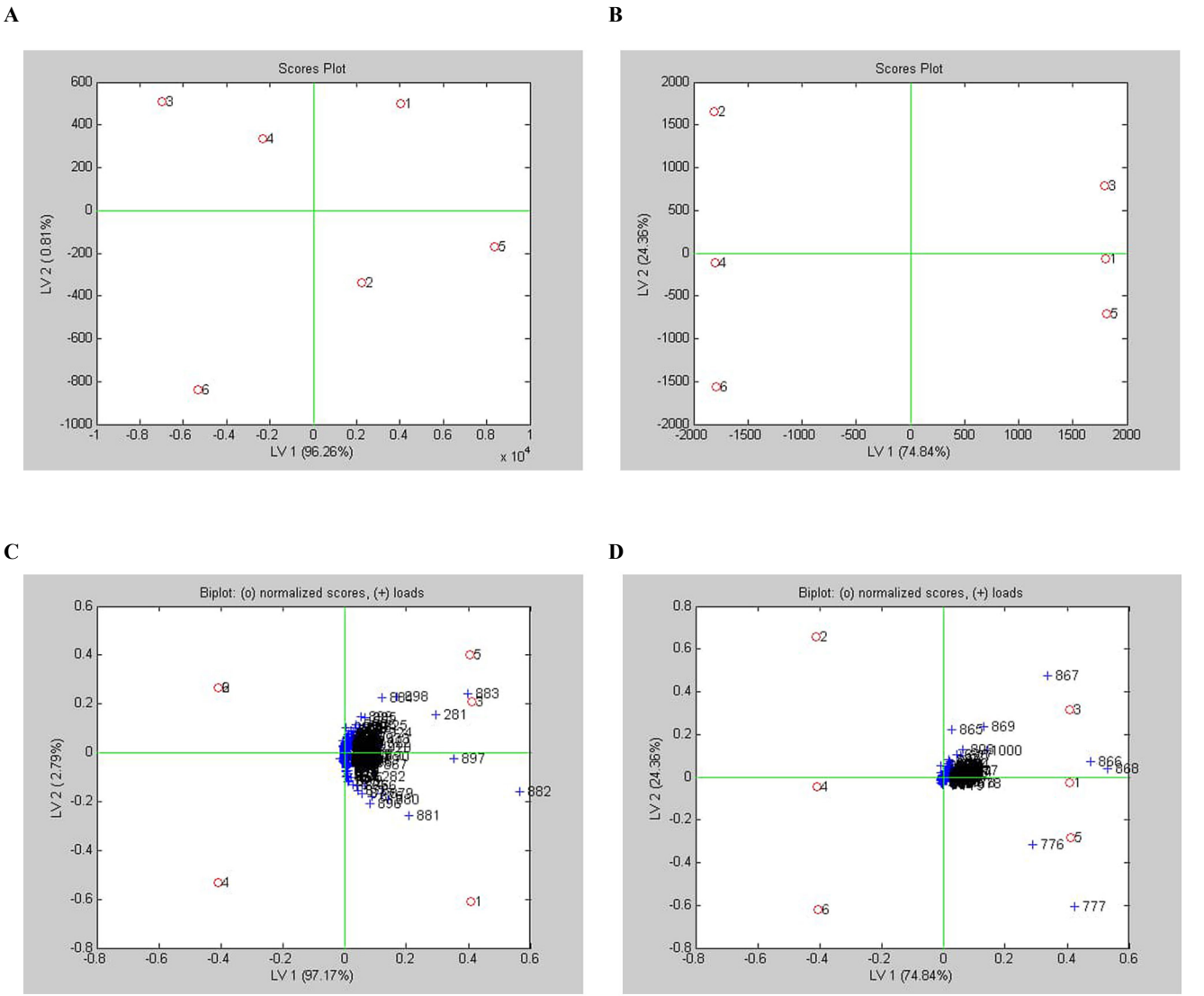

Figure 3. A) Score plot of OSC-PLS of hyd-ext. **B)** Score for lip-ext of the control and MCE group. Odd numbers indicate control group and even numbers are related to the treated extract.**C)** Biplot plot of OSC-PLS of hdy-ext **D)** Biplot of OSC-PLS of lip-ext.

Table 1. Metabolites identified by their chemical shifts

Number	Chemical shifts	Level	Location	List of metabolites
1	7/2003 7/19	↓	lip-ext&hyd-ext	L-Tryptophan
2	2/1103 2/2501 2/2398	↓	lip-ext	L-Homocysteine 7-Ketocholesterol Cholesterol sulfate
3	1/3403 1/35 1/33 1/3203	↓	lip-ext&hyd-ext	7-Ketocholesterol Cholesterol sulfate Cholesterol
4	1/1992 1/1901	↓	lip-ext	7-Ketocholesterol Cholesterol sulfate
5	1/2101	↓	lip-ext	L-Isoleucine Fucose
6	1/0302	↓	lip-ext	L-valine
7	3/2501	↓	both phases	L-histidine
8	2/3099	↓	both phases	L-Homocysteic acid
9	2/2498 2/24	↓	lip-ext&hyd-ext	Cholesterol
10	1/93	↓	lip-ext&hyd-ext	Cholesterol sulfate 7-Ketocholesterol
11	1/3497 1/3399 1/3199	↓	lip-ext&hyd-ext	Cholesterol sulfate Cholesterol
12	1/2001	↓	lip-ext&hyd-ext	Cholesterol sulfate
13	1/18	↓	lip-ext&hyd-ext	7-Ketocholesterol
14	1/1698	↓	lip-ext&hyd-ext	L-Fucose

4.7. Combination of Modelling and Pathway Analysis

For identification of the affected pathways, all the detected metabolites along with the Entrez number of the genes were copied into the MetaboAnalyst software using integrated pathway analysis with pathway enrichment analysis and pathway impact values from pathway topology analysis, as seen in **Figure 4**. The identified genes in the pathways are shown in **Table 2**. The main pathways are shown in Supplementary data #1, 2.

5. Discussion

The modified Recon-1 model analyzes a very precise network of 2766 metabolites and 1496 ORFs along with 3,742 pathways. This model provides information on known biochemical changes in the target species to produce a biochemical and genomic knowledge base (4). This model is used for checking the Warburg effect which is a characteristic of cancer cells. The modified model was used for the first time in an integrative study with metabolomics on the effect of MCE on lymphoblastic B cells of Burkitt's lymphoma throwing light on its mechanism. The model identified the relevant genes in the obtained metabolic pathways whose levels changed as a result of the MCE and hence the altered

Table 2. Metabolic pathways and the genes affected

Number	Pathway name	Gene name	P values
1	Steroid biosynthesis	DHCR-34, DHCR7, SOAT-1, EBP, CYPS1A1, SC5DL, DHCR-24. TM7SF2, NSDML, EBF, SOAT1, SSDL,	00
2	Terpenoid backbone biosynthesis	PMVK, DHCR24, DHCR7, SOAT, SCSDL, CYP51, LSS, SC5OL, TM7F2, FDPS	00
3	Pyrimidine metabolism	DHODH	0.01
4	One-carbon pool by folate	DHFR, TYMS,	0.01
5	Sphingolipid metabolism	SPTLC1	0.03
6	Valine, leucine, isoleucine biosynthesis	BCAT1	0.44
7	Folate biosynthesis	DHFR	0.44
8	Purine metabolism	ATIC, SATCAR, ARM2B, PAIC, PRPS	0.06
9	Alanine, aspartate and glutamate metabolism	CAD, PPAT	0.27
10	Primary bile acid biosynthesis	HSD17B4	0.32
11	Glycine, serine and threonine metabolism	DLD, PHDGH	0.35
12	Glycerophospholipid metabolism	PISD, AGPAT6, PISD	0.36
13	Glycerolipid metabolism	PISD, AGPAT6, GPDIL	0.38

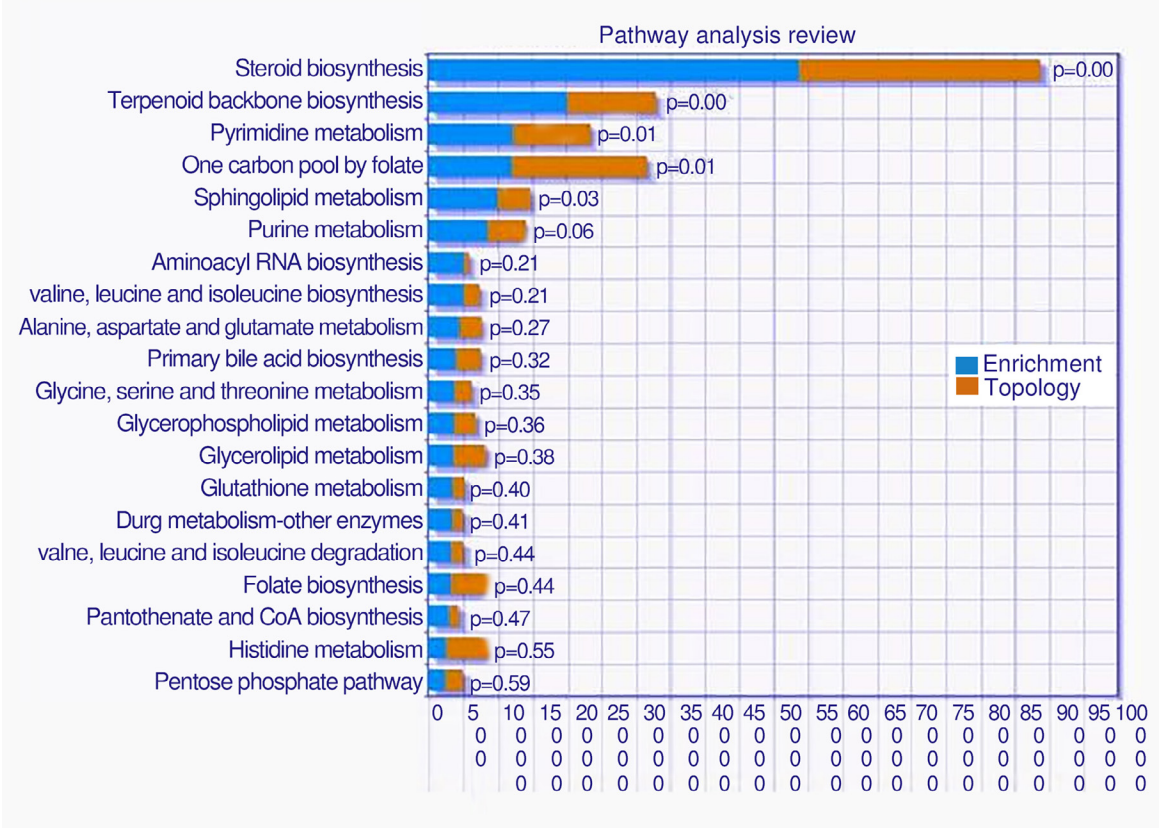

Figure 4. The pathway analysis overview showing the integrated analysis of all matched pathways with reported genes (according to p values) from pathway enrichment analysis and pathway impact values from pathway topology analysis. Pathway analysis utilized the hypogeometric test in the over-representation analysis and relativeness between centrality in the pathway topology analysis.

metabolic state was mapped onto the genetic level and then associated with the metabolites obtained from the metabolomics studies, using integrated analysis from two platforms consisting of pathway enrichment analysis and pathway topology analysis by the *http://www.metaboanalyst.ca/* website with novel results.

Different investigations have demonstrated the effect of clove extract on various anatomical cell lines *in vitro* showing anti-proliferative and apoptosis properties (15). In lung cancer-induced mice, the effect of clove oil infusion has been reported with a significant reduction in the number of proliferating cells and an increase in the number of apoptotic cells, along with a boost in pro-apoptotic proteins p53 and Bax which causes down regulation of anti-apoptotic protein Bcl-2, as detected by western blot (16).

The IC50 of clove extract on Raji cells was 50 $\mu g.ml^{-1}$ revealing its cytotoxic effect with 80% cells killed at less than 5mg/ml. It is noteworthy that the dosage obtained in this study is far less than the ones reported earlier which was 300µ/ml from a stock of 100mg/ml (1).

Our analysis predicted 72 genes, whose knockout would partially or completely inhibit cancer cell growth for which there was 100% match between the identified metabolites and the genes recognized. The integrated study showed different pathways, the most significant ones are discussed in detail below.

The first two important pathways depicted in the integrative study are steroid biosynthesis and terpenoid backbone synthesis, which are related. The metabolites participating in these pathways are cholesterol, cholesterol sulfate and 7-ketocholesterol. Investigations in the last few years have linked low plasma cholesterol levels to early cancers diagnosis (17). In a recent metabolomics study on the plasma of Non-Hodgkin's lymphoma using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry, cholesterol was the main metabolite detected and steroid biosynthesis was identified as the most important cycle(18). Another new report has indicated that targeting cholesterol with lipid lowering drugs like lovastatin enhances chemo immune-sensitivity in lymphoid malignancies(19). In the present integrated study, the metabo-analyst figure shows the presence of two enzymes which participate in many reactions. The two enzymes are 24-dehydrocholesterolreductase (1.3.1.72) (DHCR24) which is seen in 7 reactions and DHCR-7 is also seen in 5 reactions, (Supplementary data #1) both are potential drug targets in cancers. DHCR24 is an oxido-reductase enzyme acting on the CH-CH group of donors and takes part in the formation of cholesterol and has been associated with endometrial cancer, advanced clinical stage of lymphatic metastasis and reduced overall survival (20). There are reports that its gene expression is increased in melanoma metastases and is related to resistance to apoptosis induced by oxidative stress (21). The other enzyme is DHCR-7, also seen to participate in three reactions and in 2011 a new inhibitor of DHCR7 was tested on various human hepatocytes in vitro with differing results (22). These are the enzymes affected by clove extract, and as the modified Recon-1 model shows an increase or decrease in the genes, it can be concluded from our integrative studies that clove extract targets these important enzymes.

The next 3 cycles predicted by our integrative analysis are closely related, the pyrimidine metabolism, one carbon folate cycle and purine metabolism (Supplementary data #2). All three cycles are involved with the amino acids present in our metabolic table with differing *p* values (23). One-carbon metabolism encompasses a complex metabolic network that is based on the chemical reactions of folate compounds with dehydrofolate reductase DHFR as the enzyme detected by our model. DHFR has been recognized as vital in cancer as its interference results in inhibition of cancer cells (24). The enzyme dihydorotate dehydrogenase (DHOH) is a mitochondrial enzyme involved in different stages of purine and pyrimidine metabolism. It has recently been identified as an anticancer drug target (25). Hence, MCE affects these enzymes which are known drug targets for cancer.

The next cycle identified is the sphingolipid cycle with one gene detected by our modelling study of (supplement 2) serine palmitoyl-transferase long chain base (SPTLC), a key enzyme in sphingolipid biosynthesis pathway (26). Sphingolipids are a class of lipids synthesized in the ER from non-sphingolipid precursors such as serine. Besides its participation in the one carbon folate metabolism, serine is required for a number of biosynthetic and signalling pathways including the synthesis of other amino acids such as glycine and cysteine and the production of phospholipids such as sphingolipids and phosphatidylserine. This cycle has been reported as a new target for anti-cancer therapy because modification of biosynthesis of sphingolipid and its homeostasis cause an increase of ceramide levels and programmed cell death. A water-soluble sphingosine analogue FTY720 acting on the nutrient transporter proteins via ceramide generation in cancer cells is being tested (27), but, more research will have to be carried out to observe its therapeutic value in Burkitts lymphoma. SPTLC2 is the next important enzyme on which MCE acts.

The next two cycles identified in the integrative study are again closely related; t-RNA biosynthesis and valine, leucine, isoleucine biosynthesis due to the presence of the amino acids, L-valine, L-isoleucine, L-histidine and L- tryptophan which were detected in the pathway. These amino acids singly have been linked to regulation of anti-tumour immune response, especially tryptophan. Branched chain amino acids (BCAAs), leucine, isoleucine, and valine make up 40% of the essential amino acids and are important in protein synthesis as they are major nitrogen donors for alanine and glutamine synthesis. BCAAs signal the regulation of mammalian target of rapamycin (m-TOR) pathway, which controls protein translation, cell growth, proliferation, and autophagy and is recognized as a critical regulator of cellular function (28).

Cytoplasmic branched chain aminotransferases (BCATc) participating in amino acid biosynthesis may play a role in immunosuppression in the cellular micro-environment continuing to tumour escape mechanisms. BCATc may play an immunosuppressive role in the tumour microenvironment possibly contributing to different tumour escape mechanisms (29).

The model detected the amino acids and the enzymes which are involved in t-RNA biosynthesis called aminoacyl-tRNAsynthetases (ARSs) found in the cytoplasm and the mitochondria that are in charge of cellular protein synthesis and help in ligation of amino acids to their cognate tRNAs. In mammals, there are additional domains that cause interaction with three auxillary factors named ARS-interacting multifunctional protein 1 (AIMP1), AIMP2 and AIMP3 which is a complex known as the multi-synthase complex (MSC) whose systemic analysis of the expression of ARSs and AIMPs shows that these proteins are associated with cancer. MCE is seen to affect these 2 cycles of prime importance (30).

Our mathematical model predicted many more cycles but these were of importance due to the p-values and the number of genes involved. The Ruppin model has been constructed on acute leuakemia, but as there was no genomic data available on Hodgkin's disease, this was used as the closest model and the most significant cycles were discussed. It can be said that MCE affects many different genes and cycles and exhibits an anti-cancer effect on Raji cells. However, as metabolomics identifies only a few metabolites, probably all the genes will be identified by the analysis, but a few metabolites would correlate with them. The important enzymes affected by MCE were DHCR-7 and DHCR-24 in cholesterol biosynthesis, DHFR in one carbon metabolism and STLC1 in sphingolipid biosynthesis. These enzymes could make good cases for further studies as likely drug targets in B cell lymphoma.

Finally, it can also be safely concluded that not only can integrative mathematical modelling and metabolomics be used to detect genes involved in cancers and to study their mechanisms but also to identify new drug targets.

6. Conclusions

The integrative study predicted that the anti cancer mechanism of clove oil involves novel enzymes, as likely drug targets, 24-dehydrocholesterol reductase and 7-dehydrocholesterol reductase in cholesterol biosynthesis, dehydrofolate reductase in one carbon metabolism and serine palmitoyl-transferase long chain in sphingolipid biosynthesis.

Conflict of interests

The authors declare that they have no conflict of interests with each other.

Acknowledgements

The authors wish to thank Pasteur Institute of Iran for funding the research.

References

1. Dwivedi V SR, Hussain S, Ganguly C, Bharadwaj M. Comparative Anticancer Potential of Clove (Syzygium aromaticum) - an Indian Spice - Against Cancer Cell Lines of Various Anatomical Origin. *Asian Pac J Cancer P*. 2011;**12**:1989-93.
2. Nandini Gautam AKM, 2 and Sunil Mittal1. Essential Oils and Their Constituents as Anticancer Agents: A Mechanistic View. *Biomed Res Int.* 2014; doi: 10.1155/2014/154106
3. Nielsen ANaJ. Genome scale metabolic modeling of cancer *Metabol Eng.* 2017;**43(B)**:103-12, doi.org/10.1016/j.ymben.2016.10.022.
4. Tomer Shlomi TB, Eyal Gottlieb, Roded Sharan. Eytan Ruppin Genome-Scale Metabolic Modeling Elucidates the Role of Proliferative Adaptation in Causing the Warburg Effect *PLoS Comput. Biol.* 2011**;7**(3):e1002018, doi.org/10.1371/journal.pcbi.1002018.
5. Karpova MB SJ, Ernberg I, Henter JI, Nordenskjöld M, Fadeel B. Raji revisited: cytogenetics of the original Burkitt's lymphoma cell line. *Leukemia.* 2005; **19**(1):159-61.
6. Larive CK1 BGJ, Dinges MM. DOI: NMR spectroscopy for metabolomics and metabolic profiling. *Anal Chem.* 2015**;87**(1):133-46. doi: 10.1021/acs.
7. Vogel MSAPaHJ. The Future of NMR Metabolomics in Cancer Therapy: Towards Personalizing Treatment and Developing Targeted Drugs?. *Metabolites.* 2013;**3**(2): 373–96. Pubmed Central PMCID: 3901278.8, doi: 10.3390/metabo3020373.
8. N. Parvizzadeh SS, S. Irani, A. Iravani, Z. Kalayee, N. A. Rahimi, M. Azadi, and Z. Zamani. A Metabonomic Study of the Effect of Methanol Extract of Ginger on Raji Cells Using 1HNMR. *Spectroscopy. Biotech Res Int*. 2014;**2014**:(8), doi.org/10.1155/2014/572534.
9. Strober W. Trypan blue exclusion test of cell viability. *Curr Protoc Immuno*l. 2001;Appendix **3:**(Appendix 3B.), doi.org/10.1002/0471142735.ima03bs21

10. Thomas M. Buttke JAMaTCO. Use of an aqueous soluble tetrazolium/formazan.assay to measure viability and proliferation of lymphokine-dependent cell lines. *J. Immunol Methods* 1993;**157**(1-2):233-40, doi.org/10.1016/0022-1759(93)90092-L
11. Gottschalk M IG, Collins DM, Eustace A, O'Connor R, Brougham DF. Metabolomic studies of human lung carcinoma cell lines using in vitro (1)H NMR of whole cells and cellular extracts. *NMR Biomed*. 2008;**21**(8):809-19, doi.org/10.1002/nbm.1258
12. DuEm HGFMYZCY. Conformation and dynamics of polyoxyethylene lauryl ether (Brij- 35) chains in aqueous micellar solution studied by 2D NOESY and 1H NMR relaxation Science in China Series B. *Chemistry.* 2002;**45**(2):143–50.
13. Wishart DS TD, Knox C, Eisner R, Guo AC, Young N, Cheng D, Jewell K, Arndt D, Sawhney S, Fung C, Nikolai Lry *et al.* HMDB: the Human Metabolome Database. *Nucleic Acids Res*. 2007;**Jan**(35):D521-6, doi.org/10.1093/nar/gkl923.
14. Xia JN WD. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. *Curr Protoc Bioinformatics.* 2016;**7**(55):1410.1-14.1:0.91, doi: 10.1002/cpbi.11.
15. Haizhou Liu JCS, Jianteng Wei, Shousong Cao, Jan H. Beumer, Sandra Strychor, Linyou Cheng, Ming Liu, Cuicui Wang, Ning Wu, Xiangzhong Zhao, Yuyan Zhang, Joshua Liao, Edward Chu,and Xiukun Lin. Clove Extract Inhibits Tumor Growth and Promotes Cell Cycle Arrest and Apoptosis. *Oncol Res.* 2014;**21**(5):247–59, doi: 10.3727/096504014x13946388748910.
16. Banerjee S PC, Das S. Clove (Syzygium aromaticum L.), a potential chemopreventive agent for lung cancer. *Carcinogenesis.* 2006;**27**(8):1645-54, doi.org/10.1093/carcin/bgi372
17. Naik PP GM, and Raste AS. Lipid profile in leukemia and Hodgkin's disease. *Indian J Clin Biochem.* 2006;**21**(2):100–2, doi: 10.1007/BF02912921.
18. Zhou J WY, Yu S, Yan C. Serum metabolite profiling of B-cell non-Hodgkin's lymphoma using UPLC-QTOFMS and GC-TOFMS. *Metabolomics.* 2014;**10**(j):677-87.
19. Benakanakere I JT, Sleightholm R, Villeda V, Arya M, Bobba R, Freter C, Huang C. Targeting cholesterol synthesis increases chemoimmuno-sensitivity in chronic lymphocytic leukemia cells. *Exp Hematol Oncol*. 2014;**26**(3):24.
20. Dai M ZX, Liu F, Xu Q, Ge Q, Jiang J, Xiao-Mei Yang, Jun Li, Ya-Hui Wang, Qing-Kai Wu, Zhi-Hong Ai, Yin-Cheng Teng & Zhi-Gang Zhang, . Cholesterol Synthetase DHCR24 Induced by Insulin Aggravates Cancer Invasion and Progesterone Resistance in Endometrial. *Sci Rep*. **Jan 23**(7)41404. doi: 10.1038/srep41404.
21. Di Stasi D VV, Campi V, Ranzani T, Daniotti M, Chiodini E, Fiorentini S, Greeve I, Prinetti A, Rivoltini L, Pierotti MA, Rodolfo M. DHCR24 gene expression is upregulated in melanoma metastases and associated to resistance to oxidative stress-induced apoptosis. *Int J Cancer.* 2005;**115**(2):224-30, doi.org/10.1002/ijc.20885
22. Acimovic J KT, Seliskar M ,Bjorkhem I ,Monostory K ,Szabo P ,Pascussi JM ,Belic A ,Urleb U , Kocjan D ,Rozman D. Inhibition of human sterol Δ7-reductase and other postlanosterol enzymes by LK-980, a novel inhibitor of cholesterol synthesis. *Drug Metab Dispos* .2010;**39**(1):39-46, doi: 10.1124/dmd.110.035840.
23. Tedeschi PM ME, Gounder M, Lin H, Dvorzhinski D, Dolfi SC, Chan LL, Qiu J, DiPaola RS, Hirshfield KM, Boros LG, Bertino JR, Oltvai ZN, Vazquez A. Contribution of serine, folate and glycine metabolism to the ATP, NADPH and purine requirements of cancer cells. *Cell Death Dis.* 2013;**24**(4:e877).
24. Neradil J PG, Veselska R. New mechanisms for an old drug; DHFR- and non-DHFR-mediated effects of methotrexate in cancer cells. *Klin Onko*l. 2012;**25**(Suppl 2):2S87-92.
25. Lolli MLS, Stefano; Pippione, Agnese C.; Giorgis, Marta; Boschi, Donatella; Dosio, Franco. Use of human Dihydroorotate Dehydrogenase (hDHODH) Inhibitors in Autoimmune Diseases and New Perspectives in Cancer Therapy. *Recent Pat Anti-Canc*. 2018;**13**(1):86-105(20), doi.org/10.2174/1574892812666171108124218
26. Taouji S HA, Delom F, Palcy S, Mahon FX, Pasquet JM, Bossé R, Ségui B, Chevet E. Phosphorylation of serine palmitoyltransferase long chain-1 (SPTLC1) on tyrosine 164 inhibits its activity and promotes cell survival. *J Biol Chem.* 2013;**288**(24):17190-201. Epub 2013 Apr 29.
27. Ponnusamy S M-NM, Senkal CE, Saddoughi SA, Sentelle D, Selvam SP, Salas A, Ogretmen B. Sphingolipids and cancer: ceramide and sphingosine-1-phosphate in the regulation of cell death and drug resistance. *Future Oncol*. 2010;**6**(10), doi.org/10.2217/fon.10.116.
28. Yoon M-S. The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance and Metabolism. *Nutrients.* 2016**;8**(7):405, doi: 10.3390/nu8070405.
29. Ananieva E. Targeting amino acid metabolism in cancer growth and anti-tumor immune response. *World J Biol Chem*. 2015;**6**(4):281-9, doi: 10.4331/wjbc.v6.i4.281.
30. Kim S YS, Hwang D. Aminoacyl-tRNA synthetases and tumorigenesis: more than housekeeping. *Rev Cancer.* 2011;**11**(10):708-18, doi: 10.1038/nrc3124.