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Cinnamaldehyde and eugenol change the expression folds of AKT1 and DKC1 genes and decrease the telomere length of human adipose-derived stem cells (hASCs): An experimental and in silico study

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ABSTRACT

Objective(s): To investigate the effect of cinnamaldehyde and eugenol on the telomere-dependent senescence of stem cells. In addition, to search the probable targets of mentioned phytochemicals between human telomere interacting proteins (TIPs) using *in silico* studies.

Materials and Methods: Human adipose derived stem cells (hASCs) were studied under treatments with 2.5 µM/ml cinnamaldehyde, 0.1 µg/ml eugenol, 0.01% DMSO or any additive. The expression of TERT, AKT1 and DKC1 genes and the telomere length were assessed over 48-hr treatment. In addition, docking study was conducted to show probable ways through which phytochemicals interact with TIPs.

Results: Treated and untreated hASCs had undetectable TERT expression, but they had different AKT1 and DKC1 expression levels (CI=0.95; $P<0.05$). The telomere lengths were reduced in phytochemicals treated with hASCs when compared with the untreated cells ($P<0.05$). Docking results showed that the TIPs might be the proper targets for cinnamaldehyde and eugenol. Data mining showed there are many targets for cinnamaldehyde and eugenol in the intracellular environment.

Conclusion: The general effect of cinnamaldehyde and eugenol is their induction of stem cell senescence. Therefore, they could be applicable as chemo-preventive or antineoplastic agents.

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Introduction

Telomerase prevents stem cell aging via telomere lengthening (1). Some herbal ingredients have been proposed as anti-aging, and some others, aging-inducing agents. Anti-aging effect of medicinal plants is as a result of the antioxidant or free radical scavenging properties of their ingredients (2-4). Cinnamon (*Cinnamomum zeylanicum*) and clove (*Syzygium aromaticum*) are rich in two active ingredients, which are, cinnamaldehyde and eugenol, respectively (5, 6). These two phytochemicals have different biological activities including, cytotoxic and apoptotic effects on the cancer cell lines (6-8). Anti-mutagenicity, anti-oxidant, anti-inflammatory and anti-depressant activities have also been reported for cinnamaldehyde and eugenol (5, 9-11). The effect of phytochemicals on telomere length and telomerase interacting proteins of proliferating stem cells have not been reported yet. Gene folds and cell characteristic changes could manifest the ultimate effect of any interaction between chemical components and intracellular proteins. Recently, we have reported that cinnamaldehyde and eugenol affect the human adipose derived stem cells (hASCs) viability, doubling time and differentiation (12) using cheminformatics and experimental approaches. In the present work, the effect of cinnamaldehyde and eugenol on hASCs telomere length was determined. Also, three important telomere/telomerase interacting proteins (TIPs) including TERT, protein kinase B isoform 1 (AKT1) and DKC1 gene expressions under cinnamaldehyde and eugenol treatment were assessed. TERT and DKC1 gene's products are necessary for whole enzyme telomerase activity (13); protein kinase B phosphorylates and activates the telomerase enzyme (14).

A probable mechanism of action of herbal ingredients may be the direct binding of phytochemicals to the cell signaling molecules; this has been shown for some phytochemicals, since the last decade (15, 16). Further, experimental methods are becoming more complicated, sensitive and accurate,

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but they are not empirical to study the ligand-protein docking. Computational biology tools help in exploring and assessing the probable ligand-protein interactions in a virtual environment. Therefore, in the present investigation, using computational biology and docking tools, the probable cinnamaldehyde and eugenol interactions with TIPs were explored. This approach can help in finding suspected reasons for the present study experimental results.

Materials and Methods

Cell culture, gene folds and telomere length

Cell culture materials were from Gibco-Invitrogen. Cinnamaldehyde (HPLC, >99.9%), eugenol (HPLC grade; >98%), DMSO and antibiotics were from Sigma-Aldrich. The plasmid DNA of *psiCHECK-2*, used in telomere length determination, was a gift from Dr Farideh Talebi. The qPCR master mix was from Solis BioDyne. Human ASCs were prepared, after filling a consent form by a 34-year-old woman who was admitted to a hospital for caesarean section. The hASCs preparation and stem cell confirmation methods were as described method in our recent publication (12).

Experimental design

Four groups of hASCs studied were treated for 48 hr: 2.5 µM/ml cinnamaldehyde treated, 0.1 µg/ml eugenol treated, 0.01% DMSO treated and untreated hASCs. Cinnamaldehyde and eugenol were primarily dissolved in 0.01% DMSO in phosphate buffer, which is the solvent. We have selected this concentrations according to the cheminformatics and experimental information about the best results of doubling time of hASCs denoted in recently published work. According to mentioned study, 2.5 µM/ml cinnamaldehyde and 0.1 µg/ml eugenol exert better cell viability and doubling time on hASCs (12). Treated and untreated hASCs were assessed for the telomere length and the expression of TERT, AKT1 and DKC1 genes. RNA extraction (GeneAll), complementary DNA (cDNA) synthesis (Viogene) and total DNA extraction (QIAGEN) were performed according to the kit instructions. Semi-quantitative PCR was done for TERT, AKT1, DKC1 and Actin-Beta gene expression, and quantitative PCR (qPCR) was done for telomere length determination. Primers and PCR programs are shown in Tables 1 and 2. For absolute telomere length by diploid genome measurement using qPCR, previously described method was used (17).

Protein tertiary structure prediction and docking study

Docking study needs 3DS structures of the proteins and ligands. The telomerase interacting proteins (TIPs) were selected according to the telomerase database (http://telomerase.asu.edu/) (18). Therefore, 3DSs were downloaded from PDB or modeled using Swissmodel server (http://swissmodel.expasy.org/) (19, 20). Table 3 shows the 3DS of modeled TIPs using Swissmodel server. For docking analysis, the mol2 format of cinnamaldehyde and eugenol were prepared from zinc database (http://zinc.docking.org) (21). Molegro Virtual Docker Ver.4.2 was used for docking analysis; this software has about 87% docking accuracy (22).

Statistical analysis

The gene folds, absolute quantities of telomere length and docking scores were analyzed by SPSS software Ver.20. The mean±SD was compared using analysis of variances (ANOVA) and Tukey-HSD with a confidence interval of 95%. Also, cluster analysis was performed on the docking scores to select the best scores presentation as graphic views of docking. For clustering, the algorithm of nearest neighbor method and squared Euclidean distance that was standardized with Z score in SPSS software were used.

Data mining using protein-protein association search tools

String database at the URL http://string-db.org/ predicts and plots the protein-protein direct or indirect association networks. String-db was used for TIPs association network plotting. The string confidence of association net tools was adjusted on the lowest confidence level (0.15) to cover more associations.

Results

Gene expression and telomere length

Untreated hASCs were compared with 0.01% DMSO, 2.5 µM/ml cinnamaldehyde or 0.1 µg/ml eugenol treated ones. The TERT gene was not detectable in any untreated or treated hASCs group. K562 cells, telomerase positive cell lines, were used to confirm whether the designed primers are competent for amplification of detectable TERT in qPCR reaction. The results of primer functionality were acceptable for TERT gene expression determination. 0.01% DMSO and

Table 1. Primer pairs used for expression analysis of human TERT, AKT1, DKC1 and housekeeping Actin-Beta genes

Gene name	Forward primer	Backward primer	Amplicon size
TERT	5'-GCTGTACTTTGTCAAGGTGGAT-3'	5'-GCTGGAGGTCTGTCAAGGTA-3'	197 bp
	PCR program: 50 cycles at 95 °C for 15 min, 95 °C for 20 sec, 60 °C for 20 sec and 70 °C for 20 sec		
AKT1	5'-GCTCAAGAAGGAAGTCATCG-3'	5'-GTACTTCAGGGCTGTGAGGA-3'	106 bp
	PCR program: 45 cycles at 95 °C for 15 min, 95 °C for 20 sec, 55.7 °C for 20 sec and 72 °C for 20 sec		
DKC1	5'-GCCAAGATTATGCTTCCAGG-3'	5'-CTTCTGACTTGCCTTTGGAC-3'	231 bp
	PCR program: 45 cycles at 95 °C for 15 min, 95 °C for 20 sec, 52 °C for 20 sec and 72 °C for 20 sec		
Actin-Beta	5'-AAACTGGAACGGTGAAGGTG-3'	5'- TATAGAGAAGTGGGGTGGCT-3'	174 bp
	PCR program: 45 cycles at 95 °C for 15 min, 95 °C for 20 se, 55.7 °C for 20 sec and 72 °C for 20 sec		

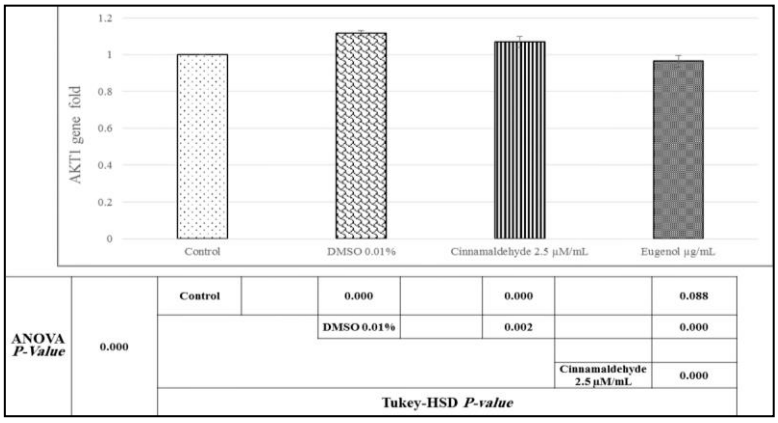


Figure 1. Tukey-HSD and ANOVA analysis for AKT1 folds comparison, using qPCR. The Figure shows the 0.01% DMSO or 2.5 µM/ml cinnamaldehyde increase the AKT1 expression folds in hASCs. Nevertheless, the 0.1 µg/ml eugenol did not affect AKT1 expression significantly

2.5 µM/ml cinnamaldehyde treated hASCs had higher AKT1 folds (P<0.05) when compared with the untreated cells. But the AKT1 folds did not change in the 0.1 µg/ml eugenol treated cells as compared to the untreated hASCs (P>0.05) (Figure 1). The 0.01% DMSO and 2.5 µM/ml cinnamaldehyde significantly increased the DKC1 gene folds in hASCs (P<0.05). But 0.1 µg/ml eugenol decreased the DKC1 folds (P<0.05) compared with the untreated cells (Figure 2). Untreated hASCs has the longest telomere length as compared to 0.01% DMSO, 2.5 µM/ml cinnamaldehyde or 0.1 µg/ml eugenol treated hASCs (Figure 3). There was no statistical difference among 0.01% DMSO and untreated or phytochemicals treated cells (P>0.05). The shortest telomere length was observed in eugenol treated cells. Generally, the phytochemicals treated hASCs had shortened telomeres as compared to the untreated cells (P<0.05).

Protein tertiary structure modeling and docking results

Figure 4 shows the gene name, number of docking sites and the best minimum docking scores of cinnamaldehyde and eugenol with 22 TIPs. The 3DS prediction was done for human GAR1P, NHP2, NOP10, PINX1, STAU2, TERT and Dyskerin that had no 3DS on the protein data bank. Table 3 shows prediction information from the Swissmodel software.

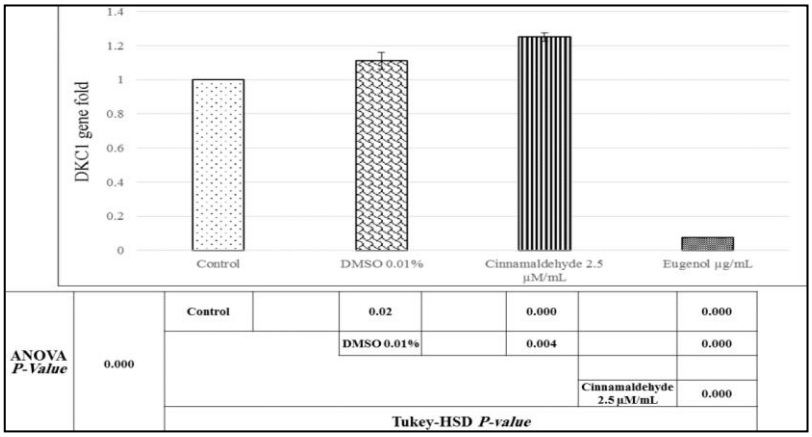


Figure 2. Tukey-HSD and ANOVA analysis for DKC1 folds comparison. The Figure shows the 0.01% DMSO or 2.5 µM/ml cinnamaldehyde increase the DKC1 expression folds in hASCs. But the 0.1 µg/ml eugenol decreased DKC1 expression significantly

Table 2. Oligomers used for absolute quantification of hASCs telomere length

	Oligomer name	Oligomer sequence 5'-… to… -3'	Length or amplicon size
Standard sequences for human species	*Telomere*	$(TTAGGG)_{14}$	84 bp
	36B4	CAGCAAGTGGGAAGGTGTAATCCGTCTCCACAGACAAGGCCAGGACTC GTTTGTACCCGTTGATGATAGAATGGG	75 bp
PCR primers	*Telomere forward*	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	>76 bp
	Telomere revers	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	
	36B4 forward	CAGCAAGTGGGAAGGTGTAATCC	75 bp
	36B4 revers	CCCATTCTATCATCAACGGGTACAA	

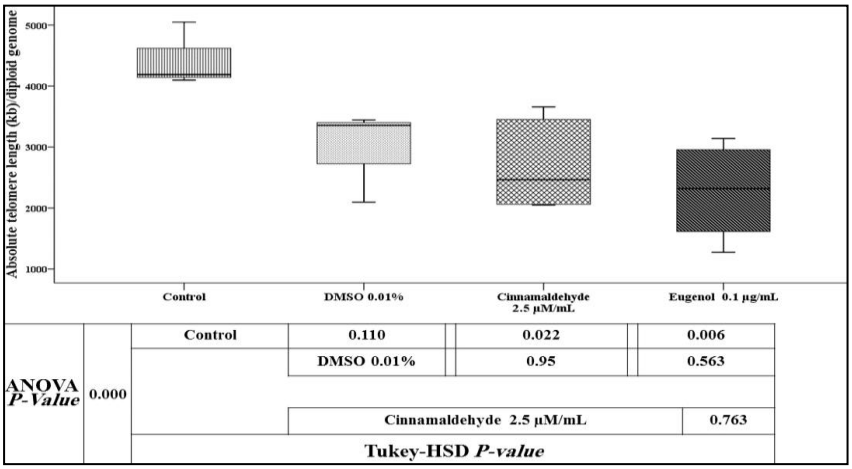
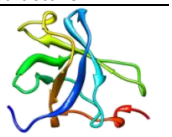
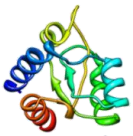
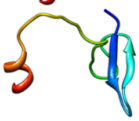
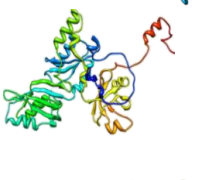
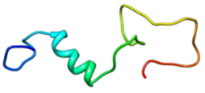
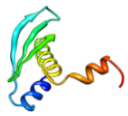
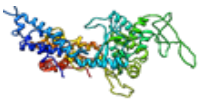


Figure 3. Telomere length comparison between examined groups. The control hASCs, 2.5 µM/ml cinnamaldehyde and 0.1 µg/ml eugenol significantly decreased the telomere length (*P-value*< 0.05) but not in comparison with 0.01% DMSO group (*P-value*> 0.05)

Table 3. The predicted tertiary structures of some TIPs or unites

Number	Gene name	Interaction with	Function	Predicted protein structure
1	GAR1P (GAR1)	Telomerase	TR and RNP stability	
2	NHP2	Telomerase	TR and RNP stability	
3	NOP10	Telomerase	TR and RNP stability	
4	Dyskerin (DKC1)	Telomerase	TR and RNP stability	 
5	PINX1 (orthologue of SOX7)	Telomerase	Inhibition of Telomerase activity	
6	STAU2	Telomerase	Not known	
7	TERT (Telomerase gene)	Other telomeric proteins	Telomere lengthening	

TR: telomerase enzyme; RNP: ribonucleoprotein; TERT: telomerase gene

Category	N of Binding Sites	Minimum docking Score	Protein
1	100	-80.18	La
	610	-80.42	14-3-3
	81	-79.87	DKC1
	19	-77.07	Gar1
	53	-77.70	AKT1
	55	-76.28	SMG5
2	240	-75.21	KIP
	32	-74.17	Pinx1
	18	-72.94	NHP2
	40	-73.33	p23
	160	-71.70	STAU2
3	2542	-89.74	HSP90AA1
	22	-88.94	Nucelolin
	156	-87.70	SMG6
4	138	-82.83	HNRNPA1
	195	-84.96	Ku70-Ku80
5	1664	-101.81	RPL22
6	180	-95.6	TERT
7	26	-57.70	RNPC1
	88	-58.96	SMN1
	72	-54.35	NOP10
8	22	-62.49	RNPC2

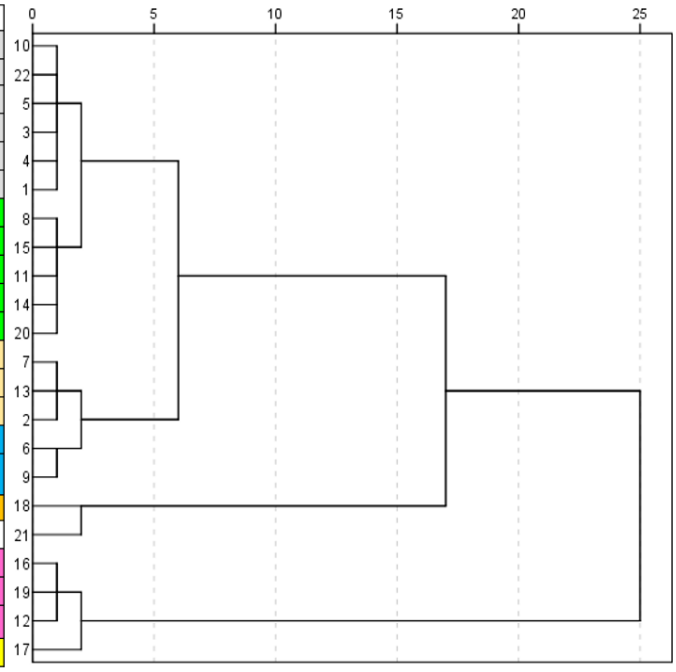


Figure 4. Dendrogram of cluster analysis for 22 TIPs plus telomerase (TERT); Left table represents number of binding sites on each protein for both cinnamaldehyde and eugenol and minimum docking scores. Clustering for docking scores performed by the nearest neighbor method and the measure of Squared Euclidian distance. Some important docking results are shown in Figure 6-14

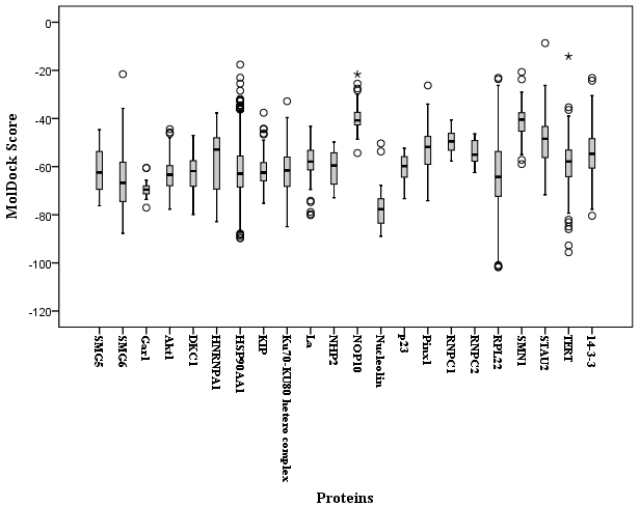


Figure 5. Comparison of docking scores for different ligand-proteins interactions. The cinnamaldehyde and eugenol docked with TIPs in Molegro Virtual Docker (MVD) software. Analysis of variance (ANOVA) shows there is a significant difference between all scores (CI= 0.95; *P*= 0.000)

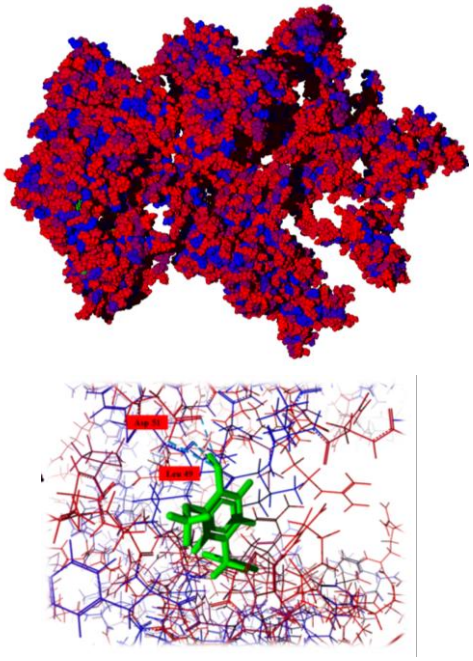


Figure 6. Docking result of RPL22 (PDB ID: 3J3A) protein with eugenol; Above: Binding site of the eugenol (green) on the protein; Below: Hydrogen bounds of ligand-protein interaction (striated lines) in binding site pocket. MolDock Score for this interaction= -101.80. It is the best score of dockings and belong to the category 5 of clustering

Docking scores of both cinnamaldehyde and eugenol were analyzed using cluster analysis (Figure 4) and one-way ANOVA (Figure 5). Eugenol had more hydrogen bonds than cinnamaldehyde. Cinnamaldehyde tend to inhabit the hydrophobic parts of the proteins, although in some cases, it formed hydrogen bonds with hydrophilic domains of TIPs. According to the cluster analysis, the ligand-protein docking scores were divided into 8 distinct clusters. The dendrogram of cluster analysis represents calculated distances between the best phytochemicals-TIPs docking scores (Figure 4).

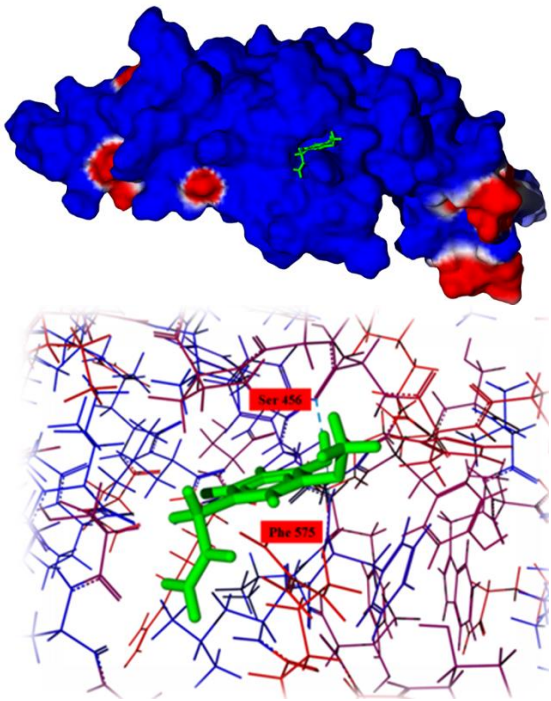


Figure 7. Docking result of TERT (modeled structure) protein with eugenol; Above: Binding site of the eugenol (green) on the protein; Below: Hydrogen bounds of ligand-protein interaction (striated lines) in binding site pocket. MolDock Score for this interaction= -95.6. This score belongs to category 6 of clustering

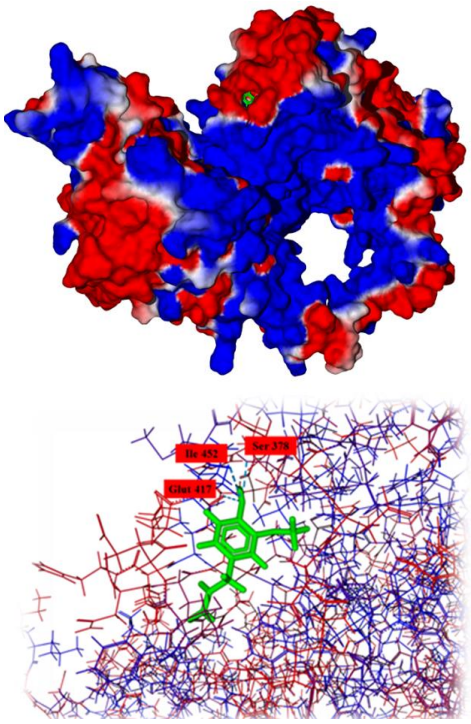


Figure 9. Docking result of KU70-KU80 hetero-complex (PDB ID: 1JEQ) protein with eugenol; Above: Binding site of eugenol (green) on the protein; Below: Hydrogen bounds of ligand-protein interaction (striated lines) in binding site pocket. MolDock Score for this interaction= -84.95. This score belongs to category 4 of clustering

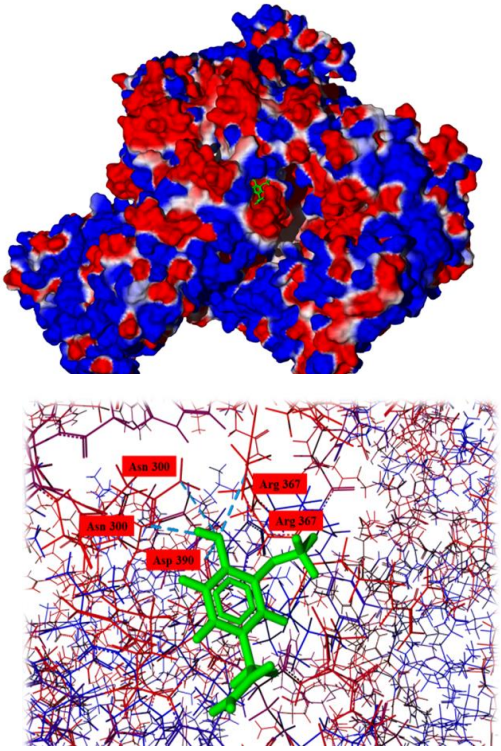


Figure 8. Docking result of HSP90AA1 (PDB ID: 3Q6N) protein with eugenol; Above: Binding site of eugenol (green) on the protein; Below: Hydrogen bounds of ligand-protein interaction (striated lines) in binding site pocket. MolDock Score for this interaction= -89.73. This score belongs to category 3 of clustering

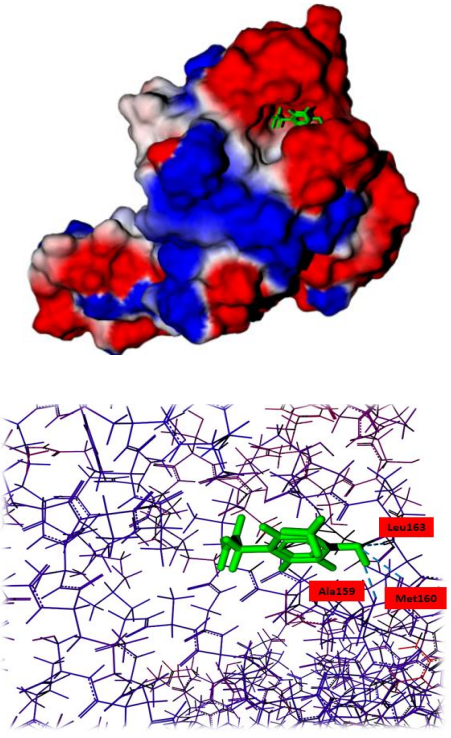


Figure 10. Docking result of 14-3-3 (PDB ID: 2BR9) protein with eugenol; Above: Binding site of eugenol (green) on the protein; Below: Hydrogen bounds of ligand-protein interaction (striated lines) in binding site pocket. MolDock Score for this interaction= -80.42. This score belongs to category 1 of clustering

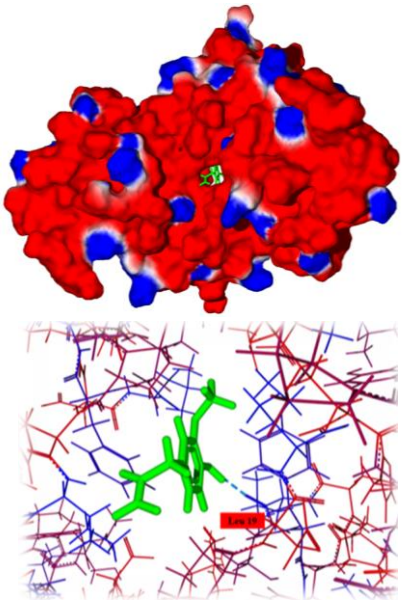


Figure 11. Docking result of KIP (PDB ID: 1Y1A) protein with eugenol; Above: Binding site of eugenol (green) on the protein; Below: Hydrogen bounds of ligand-protein interaction (striated lines) in binding site pocket. MolDock Score for this interaction= -75.21. This score belongs to category 2 of clustering

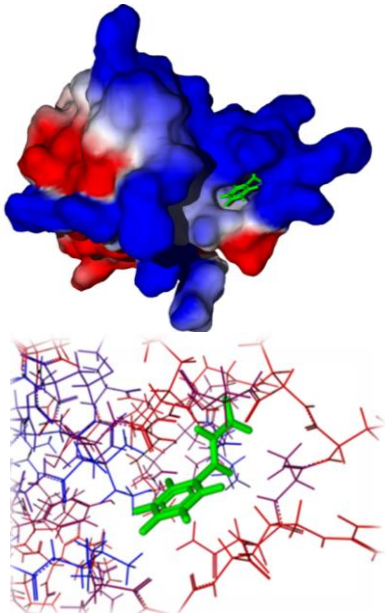


Figure 12. Docking result of RNPC2 (PDB ID: 2CQ4) protein with cinnamaldehyde; Above: Binding site of cinnamaldehyde (green) on the protein; Below: binding pocket has not hydrogen bounds. MolDock Score for this interaction= -62.42. This score belongs to category 8 of clustering

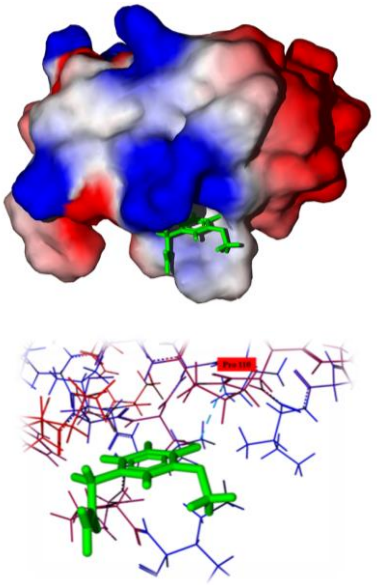


Figure 13. Docking result of SMN1 (PDB ID: 1G5V) protein with eugenol; Above: Binding site of eugenol (green) on the protein; Below: Hydrogen bound of ligand-protein interaction (striated line) in binding site pocket. MolDock Score for this interaction= -58.96. This score belongs to category 7 of clustering

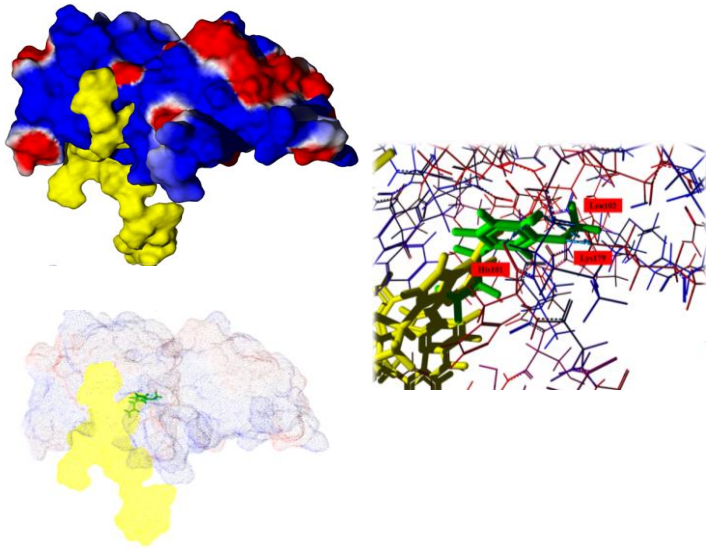


Figure 14. Docking result of HNRNPA1 (PDB ID: 1PGZ) protein with eugenol; Left: Binding site of eugenol (green) on the protein; Right: Hydrogen bounds of ligand-protein interaction (striated lines) in binding site pocket. MolDock Score for this interaction= -82.83. The molecule eugenol locates in a deep binding pocket of protein

There was a significant difference between docking scores of TIPs-phytochemicals (CI= 0.95; *P-value*= 0.000). As an example of docking results and according to the cluster analysis, only the best interactions from each category are presented in this article (Figure 6-13). An interesting result was obtained for eugenol and telomeric DNA binding domain of HNRNPA1 protein (PDB ID: 1PGZ). The eugenol was exactly docked in the binding site of the telomeric DNA purines of HNRNPA1 (Figure 14).

Association network information

Figure 15 shows an association network between TIPs and telomerase; this is plotted using string data base (see materials and methods). The thicker lines represent the stronger association. A low score (0.15) was selected for drawing the association net;

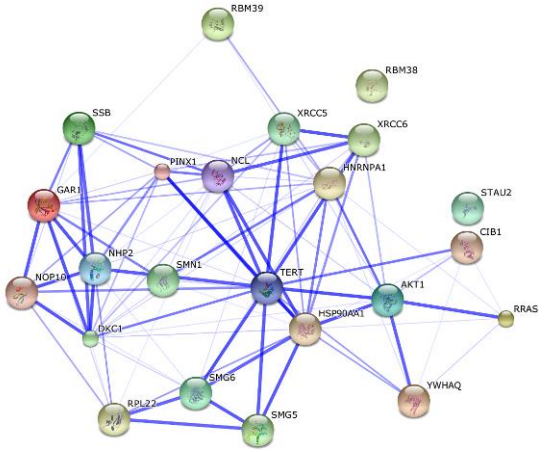


Figure 15. Association network of 23 TIPs; the thicker lines represent stronger confidence of association. Some abbreviations in the string-db plotted net were not mentioned in the text; they include: RBM38 equals RNPC1; RBM39 equals RNPC2; XRCC6 equals Ku70; XRCC5 equals Ku80; NCL equals nucleolin; La equals SSB; CIB1 equals KIP; YWHAQ equals 14-3-3; TMED10 equals p23

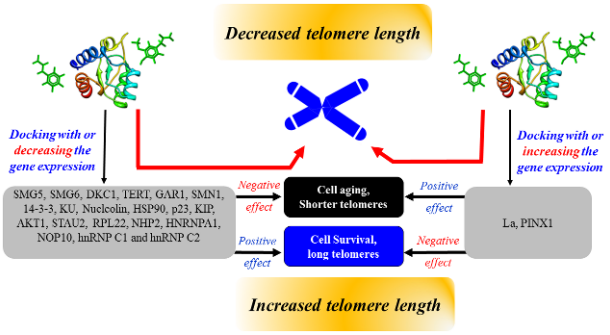


Figure 16. The probable effects of the cinnamaldehyde and eugenol on the senescence and expected events after the cell treatment. The cinnamaldehyde and eugenol may bind to TIPs or act on their gene expression and impel the signal transduction pathways. This effect strength or postponed the cellular aging. The left gray box shows the gene names of proteins, which have positive effect on the telomere or telomerase. The left gray box shows two proteins with negative effects on the telomere or telomerase. In our work, we have seen that phytochemicals induce the senescence in hASCs. Then, the red lines show the probable occurred events in the current work

apart from the STAU2 protein, all the 30 proteins had at least a weak association. According to the string-db biological function analyzer (23), the most important biological pathways, the TIPs, play roles include: intrinsic apoptosis pathway, intracellular transport, cellular localization, membrane organization, cell organelle organization, protein localization, etc. Also, molecular functions affected by TIPs include: the telomeric DNA binding, poly(A) RNA binding, telomerase activity, RNA binding, RNA-directed DNA polymerase activity and telomeric RNA binding. Besides, protein kinase C inhibitory activity, ion channel binding, enzyme binding and nitric oxide synthase regulatory activity.

Discussion

Recently, we have reported that cinnamaldehyde and eugenol affect the hASCs doubling time and differentiation (12). In the current study, cinnamaldehyde and eugenol did not increase the TERT expression to a detectable level in hASCs. However, human mesenchymal stem cells had been previously reported to be telomerase negative (24). We have used K562 cell lines, as a positive cell line model for TERT gene expression during real-time PCR method validation. As far as we know, there is not known chemical material with a definite positive effect on the telomerase expression in ADSCs.

Eugenol decreased the AKT1 expression but cinnamaldehyde increased or decreased it as compared to the untreated or DMSO treated cells, respectively. When 0.01% DMSO is also present in the cinnamaldehyde and eugenol groups, as the solvent of phytochemicals (see materials and methods), the ultimate effect of both phytochemicals on the AKT1 gene expression will decrease. Eugenol docks TIPs better than cinnamaldehyde. This evidence confirms more potent effect of eugenol on the decrease of the AKT1 expression. Cinnamaldehyde or DMSO-treated hASCs increased the DKC1 gene expression folds but the eugenol decreased it severely as compared to the untreated cells. These findings suggest that the final targets of cinnamaldehyde and eugenol are different in the intracellular environment. The effect of cinnamaldehyde and eugenol on some TIPs or cell aging was reported previously. For example, cinnamaldehyde up-regulates the STAU2 expression in MUTZ-3 cell line and human peripheral blood monocyte-derived dendritic cells (25). According to the comparative toxicogenomics database (http://ctdbase.org/), eugenol decreases the DKC1 expression and cinnamaldehyde increases the AKT1 phosphorylation. Overall, these bioinformatics findings are similar to the findings of the present study. King and colleagues reported that cinnamaldehyde induces the DNA damage in HCT116, a human colon cancer cell line (9). The result of the present study on telomere length showed that damage such as telomere shortening may be obvious (Figure 3). The hallmark of some publications is the insignificance of the effect of solvents on the gene expression of treated cells. King and coworkers did not report the effect of DMSO on the HCT116 cell line. In the current study, it was shown that a low concentration of DMSO increases both AKT1 and DKC1 gene folds when compared with the untreated cells. The findings of this study infer the importance of the solving agent (DMSO) effects on the gene expression and cell aging.

Cinnamaldehyde and eugenol reduced the telomere lengths as compared to the untreated cells notably, but insignificantly when compared with DMSO treated hASCs. Then, a low concentration of DMSO had a negative effect on the hASCs telomeres,

although not significantly (Figure 3, *P-value*= 0.110). Cinnamaldehyde and eugenol treated hASCs telomeres were not meaningfully different from DMSO treated cells (Figure 3, *P*> 0.05), although the telomere lengths were shortened. It is assumed that the broad ranges of telomere lengths in the studied groups (see standard deviations), are responsible for the results of telomere length comparisons insignificance. Absolute quantification of the telomere length using quantitative polymerase chain reaction (PCR) showed high coefficient of variations percent (CV %). Aviv and colleagues described this fact previously when comparing Southern blot and qPCR techniques (26). However, the qPCR is more rapid, lesser time consuming, with lower costs, without radioactive hazards and acceptable regression with Southern blot (17). The decreased telomere length, in the presence of phytochemicals, in the present work could be logical when considering the docking results. The docking results showed that cinnamaldehyde and eugenol attached well within the binding pockets on the TIPs.

Chemical chaperones known as small molecules bind proteins and stabilize their folding. Some herbal ingredients change the gene expression folds of molecular chaperons such as heat shock proteins; therefore, medicinal plants ingredients are proposed as chaperon-based medications (27). Based on the docking results, it is assumed that cinnamaldehyde and eugenol interact with chaperons, such HSP90AA1, or chaperon inducers; then they change the behaviors of TIPs. This may affect the stem cell aging via a telomere or telomerase dependent pathway. Grover and coworkers, using a virtual study, reported that Withaferin A, a phytochemical, targets the Hsp90/Cdc37-chaperone or co-chaperone complex. They proposed that the Withaferin A is a HSP90/Cdc37 inhibitor and a potent anticancer agent (28). In addition, the findings of the present work proposed that cinnamaldehyde and eugenol might exert similar effects on the telomerase and TIPs. The molecular effects of cinnamaldehyde and eugenol on the hASCs were telomere shortening and the gene expression fold changes. Logically, such changes did not occur without involving regulatory events of the cell aging. Researchers have shown that the active ingredients of some herbs inhibit the telomerase enzyme dose-dependently (29, 30) but others increase its gene expression (31, 32). The role of herbal ingredients on the telomere or telomerase dependent anti-aging or aging induction is not well known. The study on direct interaction of ligands-proteins or biomolecules needs certain tools and professional operators. Nevertheless, computational biology tools and analysis, such as docking, helps in predicting the probable direct bindings. Intracellular environment has dynamic properties (33); therefore, the chemicals interact with biomolecules in a dynamic way. This infers that the best docking scores are of interest, especially if the dynamic binding site conformations change whenever it is necessary.

Elizabeth H. Blackburn is a noble prizewinner for her works on the telomere and telomerase enzyme. She believes the telomerase and the telomere interacting enzymes exert four distinct biochemical effects on the telomeric DNA *in vivo*: "1. in fusion with another telomere or DNA end; 2. in replication of telomeric sequence by telomerase; 3. in degradation of telomeric DNA; 4. in recombination at and near a telomere. Such controls are integral to functional telomere capping"(34). In the current study, it has been shown that using computational tools, the cinnamaldehyde and eugenol may target TIPs efficiently. After cinnamaldehyde and eugenol docking with TIPs or regulating proteins, three effects are probable: telomere shortening, lengthening or no length change. The experiments of this study confirmed the first event. Figure 16 proposes a subjective map for the *in silico* and experimental results of this study. According to the map, the TIPs were divided into positive or negative factors for cell aging. The blocking effect of phytochemicals on the positive TIPs results to the senescence.

The HNRNPA1 activates the telomerase (35), but as shown in Figure 14, it also interacts with telomeric repeats (36). The eugenol docking with HNRNPA1 is the most significant result of docking, which occurred exactly in the telomeric DNA binding pocket. Such result may suggest a competitive inhibition between telomeric repeats of the DNA and the eugenol for binding to the HNRNPA1. Same result was derived for cinnamaldehyde and HNRNPA1 docking and exactly in the binding pocket (the result is not shown). Thompson and coworkers showed that eugenol binds covalently to the hepatic proteins. They believed that eugenol metabolism products exert toxic effect on the rat hepatocytes dose-dependently (37). Also, eugenol inhibits the monoamine oxidase A (MAOA), a metabolic enzyme, through the covalent binding (11). All mentioned studies show negative effect of eugenol on the cellular proteins and are in accordance with the experimental and virtual findings of the present study.

Weibel and Hansen have shown that the cinnamaldehyde binds to the nucleophilic groups of proteins (38). Chao and colleagues reported that cinnamaldehyde inhibits some cellular signaling pathways (10). Python and coworkers evaluated the effect of cinnamaldehyde on 80 gene expression profiles in dendritic cells and MUTZ-3 cell line. Using DNA microarray and qPCR, they showed that some genes were down-regulated but others were up-regulated (25). All the mentioned studies confirm the present study results of cinnamaldehyde effect on the gene expression changes. In this work, the

cinnamaldehyde increased the DKC1 and AKT1 gene folds when compared with untreated cells. However, in comparison with the only DMSO treated hASCs, cinnamaldehyde decreased AKT1 and increased DKC1 folds. These findings are in accordance with Python and coworkers results on the discrepant behavior of cinnamaldehyde in the gene expression folds.

Association net (Figure 15) helps in interpreting the experimental results. A single ligand-protein docking, as cinnamaldehyde or eugenol to any TIP, is not enough for interpreting experimental results. In fact, other protein-protein interrelations are important for their impacts on the special pathway members. The ligand-protein interaction may exert direct or indirect effect on protein-protein interactions in a pathway. TIPs are good examples of such interactions because of the different roles they play in the cell signaling (33, 39). The network shows that the protein-protein interactions may be weak or strong; therefore, the docking affects the quantities of signaling changes. Mount and Pandey reported on the importance of the systems biology application for new drug finding. They emphasized the inter-relations between the different biomolecules in an event, such as cancer development (40). In fact, such interrelationships between members of a biological network make sequential steps logical. Curcumin, the active ingredient of turmeric, makes sequentially, activation or inactivation in the signaling pathways (16). According to the *in silico* analysis of the present study, the cumulative effects of cinnamaldehyde or eugenol were predicted. Therefore, any prediction or estimation on phytochemical targets is not true without considering pre or post events in a signaling pathway. Curcumin down-regulates the cell division, apoptosis and metastatic genes in NF-kappa B signaling in a sequential order (16). Cinnamaldehyde is proposed as a chemopreventive agent and has toxic effect on cancer cell lines (8, 41). Eugenol is an apoptosis inducer for colon cancer cell line (7). Altogether, cinnamaldehyde and eugenol proposed as anticancer agents which affect the human cells through the telomere shortening; they change the gene folds and have many targets, of which some were checked virtually and 3, experimentally in the present study.

Conclusion

Combining the *in silico* and experiments offer a novel view of the phytochemicals effects on the stem cell aging. This study is a novel one as it focused on the phytochemicals effects on hASCs. However, these results need to be followed by animal or clinical trials. The results of this study revealed that cinnamaldehyde and eugenol might be useful in cancer therapy or chemoprevention because of the induction of stem cell aging characteristics. *In silico* predictions, confirmed the results of *ex-vivo* interventional results. The aims of this study were to explore the stem cell anti-aging properties of the phytochemicals but surprisingly, the adverse results were obtained. The cinnamaldehyde and eugenol could be considered as the potential chemotherapeutic agents for cancer prevention via telomere shortening or telomerase blockade in proliferating cells such as cancer stem cells. However, the field is so large for complementary works on the Shelterins, regulatory and epigenetics agents.

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Conflict of interest

The authors declare that there is no conflict or competing interest for the publication of the present data.

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