EFFECTS OF MULBERRY HEARTWOOD EXTRACT ON THE GENE EXPRESSION OF NF-kB AND TWO PROINFLAMMATORY CYTOKINES IN A CELL CULTURE MODEL OF OXIDATIVE STRESS

Milena Pasheva, Milka Nashar, Desislava P. Ivanova, Oskan Tasinov, Diana G. Ivanova
Department of Biochemistry,Molecular Medicine and Nutrigenomics
Medical University Prof. Dr. Paraskev Stoyanov,
9002 Varna, 55 M. Drinov Str., Bulgaria;
Corresponding author: pashaeva19@gmail.com

ABSTRACT

Oxidative stress in adipose tissue is closely associated with low-grade inflammation which can lead to chronic metabolic disorders, particularly obesity-related insulin resistance and type 2 diabetes. In the present study a model of oxidative stress in 3T3-L1 preadipocyte cell culture was used to investigate the effect of *Morus nigra* tree heartwood ethanol extract on gene expression of nuclear factor kB (NF-kB) and the two pro-inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). It was estimated that mulberry tree heartwood extract inhibited the expression of NF-kB, TNF-α and IL-6 genes in a concentration depended manner. To our knowledge, these results reveal for the first time the anti-inflammatory potential of the mulberry heartwood extract. This effect could be contributed to the earlier established antioxidative capacity of the extracted compounds.

Key words: *Morus nigra; 3T3-L1 preadipocyte cell; oxidative stress; inflammation.*

INTRODUCTION

Systemic oxidative stress, defined as a persistent imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses, correlates with metabolic disorders, including insulin resistance, obesity and type 2 diabetes [13]. Increased cytoplasmic ROS contribute to the activation and nuclear translocation of nuclear factor kappa B (NF-kB), which can induce the transcription of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) [7]. Adipose tissue produces large amounts TNF-α and IL-6 and it is a target to study the effects of various biologically active compounds with potential antioxidant activities. TNF-α was the first cytokine to be implicated in the pathogenesis of obesity and insulin resistance [29]. IL-6 was considered to be a stress-induced cytokine with varying effects in different tissues and it is known to be an acute phase protein in the inflammatory response [35].

Morus nigra L. (mulberry tree) is traditionally applied by the folk medicine for various complaints. According to some authors extracts from mulberry leaves exhibit diuretic, hypoglycemic, and hypotensive activities, whereas the fruits and root bark are known to possess antiinflammatory, antitussive, and antipyretic properties [5, 17, 26, 37]. Along with the knowledge about the medicinal properties of the plant, mulberry is known to have also other traditional application – its heartwood is used as a material for barrels manufactured for storage of alcoholic beverages and respectively for their aging. Although the ethanol extracts of mulberry heartwood have specific phytochemical composition [8], to our knowledge their biological effects still remain unrevealed. Moreover, our recent study estimated a high antioxidant activity of the ethanol infusion from heartwood of the plant, strongly correlating with its high polyphenol content [25].

The aim of this study was to determine the ability of ethanol infusion from *Morus nigra* heartwood to affect the expression of inflammatory cytokines on transcription levels.

MATERIALS AND METHODS

Heartwood processing and extract preparation

Heartwood samples were subjected to fumigation following the popular technology for aging of beverages: the wooden chips were boiled for 10 minutes and then saturated with cold water for 24 hours. Finally, the material was dried for 15 minutes at 150-190°C. Ethanol infusion from *Morus nigra* heartwood was prepared following the traditional recipe for coloring high alcoholic beverages: 2g dried material from heartwood was placed in 1L 40% ethanol for 40 days.

Experimental procedure

Mouse 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin mixture to final concentration of 100U/ml each at 37°C in a humidified chamber containing 5% CO_2. After reaching 80% confluence the cells were collected and seeded in 6 well flasks at density $2x10^5$ cells/well for differents treatments. The *Morus nigra* extract (M) or 40 % EtOOH (Et) were dissolved as follows: 6,25µl, 12,5µl or 25µl to 2 ml in phenol red- free DMEM to a final content in the nutrient medium of 0,3%, 0,6% and 1,25% respectively. For induction of oxidative stress tert-BuOOH (tB) was applied as described earlier [34]. Depending on the treatment, the following groups were defined: Group C (nontreated cells in nutritional medium), Group tB (cells in nutritional medium treated with tert-BuOOH), groups M 0.3/0.6/1.25 and M 0.3+tB/0.6+tB/1.25+tB (cells treated with final concentrations of *M. nigra* extract, respectively 0.3, 0.6 and 1.25 %, without or in presence of oxidant), groups Et 0.3/0.6/1.25 and Et 0.3+tB/0.6+tB/1.25+tB (cells treated with ethanol at final concentrations of 0.3, 0.6 and 1.25 % respectively, without or in the presence of the oxidant). After 24 h- incubation with the respective compounds the cells were harvested for total RNA.

Quantitative real-time PCR analysis

Total RNA was extracted from differentiated 3T3-L1 cells using TRI Reagent according to the manufacturer's protocol (Ambion). In order to remove contamination of genomic DNA, DNA-ase I treatment was performed using the recommended protocol of the manufacturer (Sigma). Complementary DNA was synthesized using Revertaid™ First Strand Synthesis Kit with oligo $(dT)_{18}$ primers and RevertAid™ reverse transcriptase (Fermentas). The synthesis reaction was performed on GeneAmp PCR System 9700 (Applied Biosystems). Two-step real-time PCR analysis was performed (ABI PRISM 7500, Applied Biosystems) to estimmate gene expression level in cultured cells. Maxima SYBR Green qPCR Kit (Fermentas) was used for sample analysis. The cDNA was amplified using forward and reverse primers of target genes (Table 1) commercially synthesized (Invitrogen Alpha DNA, Canada). Beta-actin was used as endogenous control. All samples were analyzed in triplicates. Gene expression levels were calculated by $2^{-\Delta\Delta Ct}$ method [20] and expressed as relative units (RU) mRNA compared to the untreated controls where the level of gene expression of interest was considered to be equal to 1.

Table 1. Sequences of primers used for RT-PCR analysis

Genes	**Nucleotide sequence**
β-Actin	F: 5'-ACG GCC AGG TCA TCA CTA TTG-3' R: 5'-CAA GAA GGA AGG CTG GAA AAG- 3'
NFKB1	F: 5'-ATGGCAGACGATGATCCCTAC- 3' R: 5'- TGTTGACAGTGGTATTTCTGGTG- 3'
TNFα	F: 5'- CCCTCACACTCAGATCATCTTCT -3' R: 5'- GCTACGACGTGGGCTACAG -3'
IL-6	F: 5'- GAGTTGTGCAATGGCAATTCTG -3' R: 5'- GCAAGTGCATCATCGTTGTTCAT -3'

Statistical Analysis

Data are presented as mean ± standard error of mean (*SEM*). Differences between means of groups were analyzed using Student's *t*-test or one-way ANOVA with Dunnett's multiple comparison test (GraphPad Prism 5.0). Values of $P<0.05$ were considered to be statistically significant

RESULTS AND DISCUSSION

Effect of the oxidant on gene expression

A model of oxidative stress induced in preadipocite cell culture was used with the aim to explore the effects of three concentrations of mulberry heartwood ethanol extract on genes related to inflammatory response. The same concentrations of ethanol or ethanol + oxidant were applied in order to distinguish the effects of the plant-derived active compounds from the effects of ethanol solely. The oxidative stress was induced with tertiary-Butyl hydroperoxide which is commonly used in developing cellular oxidative stress. The mechanisms of its metabolism involves membrane lipid peroxidation and depletion of cellular storage of glutathione [21, 28, 16]. The addition of the oxidant to the nutrition medium (group tB) in our study resulted in a significant increase in mRNA levels of the three tested genes, as compared with the non-treated cells (**$p<0.01$ vs. group C). Results are summarized in figures 1, 2 and 3.

Expression of NF-kB

None of the applied concentrations of the extract changed the NF-kB expression. Ethanol applied alone stimulated the expression of the gene in concentration dependent manner (*$p<0.05$). Upon induction of oxidative stress (groups M+tB) only the highest concentration of the extract significantly inhibited the expression of NF-kB as compared with the two lower concentrations (groups M0.3+tB and M0.6+tB). Furthermore, this inhibitory effect was confirmed in comparison with the cells treated with oxidant alone (group tB) and the same concentration of ethanol with oxidant (Et1.25+tB). It could be suggested that the concentration-dependent manner of the effects is due to the active compounds extracted in the solution from the plant. Data are summarized in figure 1:

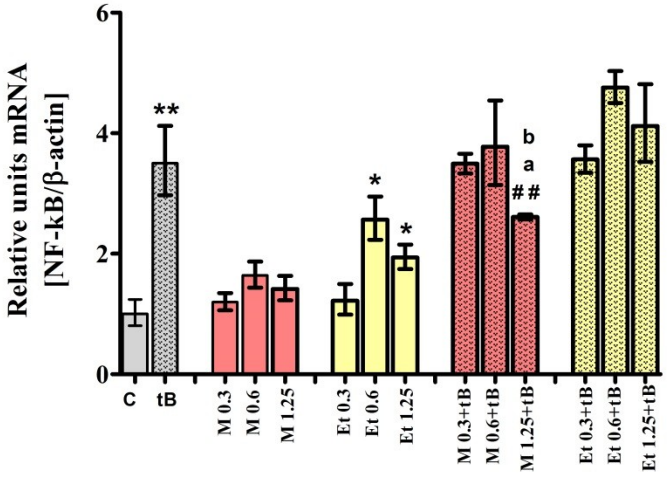

Figure 1. Expression of NF-kB in preadipocytes, treated with different concentrations of *M. nigra* extract or ethanol in normal medium or under induced oxidative stress. Data are presented as mean±standard eror of mean (SEM); *$p<0.05$, **$p<0.01$ versus group C; $^{\#\#}p<0.01$ versus groups M 0.3+tB and M 0.6+tB; $^{a}p<0.05$ versus group tB; $^{b}p<0.05$ versus group Et 1.25+tB.

NF-kB has been described as a redox-sensitive transcriptional factor since it may be activated by oxidative stress and inhibited by various antioxidants [31]. In a previous study we estimated that the mulberry heartwood extract possesses high antioxidant activity *in vitro*, correlating with high total polyphenol content [25]. The activation of NF-kB system is associated with chronic pathological conditions including CVD, type 2 diabetes, obesity and neurodegenerative disorders [2, 33, 32] and these conditions are often accompanied by oxidative stress [18, 22]. The results described above revealed the mulberry heartwood extract as a potent NF-kB inhibitor under oxidative stress and thus as a source of active compounds for prophylaxis and treatment of oxidative based-chronic diseases.

Expression of TNFα

The changes of TNFα transcriptional levels are presented in figure 2. Significant increase in mRNA levels of the gene was measured in the cells treated with highest concentration of the extract (M1.25), compared with the two lower concentrations and with the untreated cells (group C). This effect could be attributed rather to the ethanol contented in the extract since ethanol applied alone in all concentrations stimulated TNFα expression (groups Et 0.3, Et 0.6 and Et 1.25). Moreover, the ethanol and the oxidant applied together had a cumulative stimulatory effect on the mRNA levels of the gene ($^{sss}p<0.001$, vs. group Et1.25). This effect of ethanol is not surprising. Several studies reported that the basic mechanism of alcohol-induced cellular damage involves upregulation of proinflammatory cytokines via excessive generation of reactive oxygen species [1, 10, 11].

The highest concentration in the extract, applied in the oxidatively stimulated cells significantly inhibited the expression of TNFα ($^{ccc}p<0.001$ vs. Et1.25+tB). This inhibitory effect of the extract observed under induced oxidative stress is not unexpected, regarding its high polyphenol content. Many authors reported similar effects of the polyphenols or polyphenol-rich extracts on the proinflammatory cytokines (including TNFα) in various experimental models of oxidative stress and inflammation [6, 23, 30, 40, 41].

Adipose tissue is the predominant source of the elevated TNF-α in obesity and it is known as a mediator of obesity-related insulin resistance and type 2 diabetes [12, 36, 38]. Based on the results commented above it could be suggested that the mulberry extract has a potential to ameliorate adipose tissue metabolic disorders.

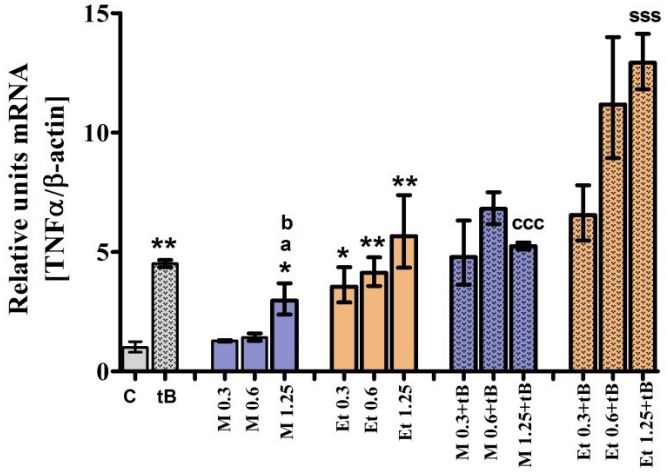

Figure 2. Expression of TNFα in preadipocytes, treated with different concentrations of *M. nigra* extract or ethanol, in normal medium or under induced oxidative stress. Data are presented as mean±standard eror of mean (SEM); *$p<0.05$, **$p<0.01$ versus group C; $^{a}p<0.05$ versus group M 0.3; $^{b}p<0.05$ versus group M 0.6; $^{ccc}p<0.001$ versus group Et 1.25+tB; $^{sss}p<0.001$ versus group Et 1.25.

Expression of IL-6

Unexpectedly, the levels of IL-6 mRNA were significantly increased in cells treated with the highest concentration of the extract (M1.25), compared with the two lower concentrations and the controls (fig. 3). This stimulatory effect of the extract could be attributed mainly to the active compounds extracted from the plant than to the ethanol. The results received for the ethanol treated cells (groups Et0.3, Et0.6 and Et1.25) support this suggestion. No changes in mRNA levels were estimated in these groups.

Many of the studies focused on the mulberry extracts from leaves, fruits and root bark demonstrated the potential of the investigated preparations to inhibit proinflammatory cytokins, including IL-6 [9, 19, 24]. However, bearing in mind the specific phytochemical composition of the heartwood as compared to that of other parts of the plant, it could be suggested that along with the polyphenols extracted in the solution, other specific active compounds probably contributed to the effects on cytokines gene expression, including the IL-6 upregulation.

According to the scientific literature, some plant preparations have a potential to activate the healthy immune system by increasing inflammatory and anti-inflammatory cytokines production *in vivo* and *in vitro,* suggesting the immunomodulatory properties of the investigated plants [3, 4, 27, 39, 14, 15].

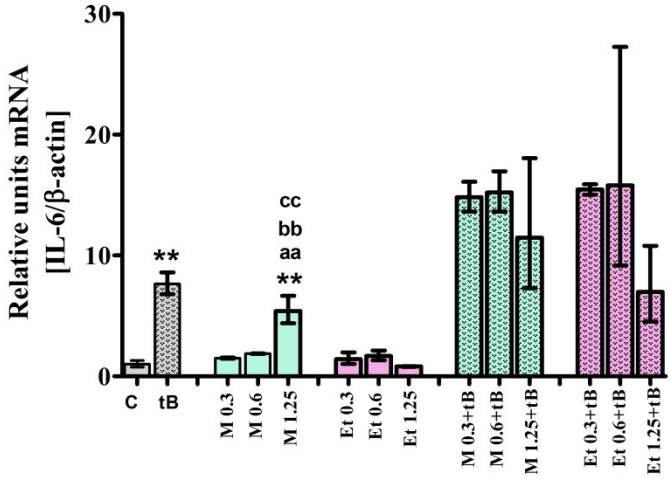

Figure 3. Expression of IL-6 in preadipocytes, treated with different concentrations of *M. nigra* extract or ethanol, in normal medium or under induced oxidative stress. Data are presented as mean±standard eror of mean (SEM); **p<0.01 versus group C; ^{aa}p<0.01 versus group M 0.3; ^{bb}p<0.01 versus group M 0.6; ^{cc}p<0.01 versus group Et 1.25

CONCLUSION

To our knowledge this is the first investigation of the anti-inflammatory properties of the mulberry heartwood extract. Along with the inhibitory effects on the NF-kB and TNFα gene expression, the mulberry extract stimulated IL-6 mRNA levels. This dual effect reveals the immunomodulatory properties of the investigated extract. Based on the results presented above, we concluded that the studied preparation could have beneficial effects in prophylaxis and therapeutics in oxidative stress-based pathological conditions.

REFERENCES

1. Ambade A. and Mandrekar P., 2012. Oxidative Stress and Inflammation: Essential Partners in Alcoholic Liver Disease. Int J Hepatol (2012), Article ID 853175, 9 pages

2. Baker RG, Hayden MS, Ghosh S., 2011. NF-κB, inflammation, and metabolic disease. Cell Metab 13(1):11-22

3. Barak V, Birkenfeld S, Halperin T, Kalickman I., 2002. The effect of herbal remedies on the production of human inflammatory and anti-inflammatory cytokines. Isr Med Assoc J 4(11 Suppl):919-22.

4. Barak V, Halperin T, Kalickman I., 2001. The effect of Sambucol, a black elderberry-based, natural product, on the production of human cytokines: I. Inflammatory cytokines. Eur Cytokine Netw 12(2):290-6.

5. Chan KC, M.Y. Yang, M.C.Lin , Y.J. Lee, W.C. Chang, C.J. Wang, 2013. Mulberry leaf extract inhibits the development of atherosclerosis in cholesterol- fed rabbits and in cultured aortic vascular smooth muscle cells. *J Agric Food Chem*., 61(11):2780–2788.

6. Choi EM, Koo SJ., 2003. Effects of soybean ethanol extract on the cell survival and oxidative stress in osteoblastic cells. Phytother Res. 17(6):627-32.

7. Csiszar, A., M. Wang, E. G. Lakatta and Z. Ungvari, 2008. Inflammation and endothelial dysfunction during aging: role of NF-KB. *J Appl Physiol* , 105, 1333–1341

8. De Rosso M.D., D. Cancian, A. Panighe A.D. Vedova, R. Flamini, 2009. Chemical compounds released from five different woods used to make barrels for aging wines and spirits: volatile compounds and polyphenols. *Wood Sci Tech*., 43, 375-385

9. Eo HJ, Park JH, Park GH, Lee MH, Lee JR, Koo JS, Jeong JB., 2014. Anti-inflammatory and anti-cancer activity of mulberry (Morus alba L.) root bark. BMC Complement Altern Med. 14:200.

10. Gutierrez-Ruiz MC, Gomez Quiroz LE, Hernandez E, Bucio L, Souza V, Llorente L, Kershenobich D., 2001. Cytokine response and oxidative stress produced by ethanol, acetaldehyde and endotoxin treatment in HepG2 cells. Isr Med Assoc J. (2):131-6

11. Hoek JB, Pastorino JG., 2002. Ethanol, oxidative stress, and cytokine-induced liver cell injury. Alcohol 27(1):63-8.

12. Hotamisligil G.S., Shargill N.S., Spiegelman B.M., 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science, 259, 87–91

13. Houstis, N., Rosen, E.D., and Lander, E.S. 2006. Reactive oxy-gen species have a causal role in multiple forms of insulin resistance. Nature 440, 944–948

14. Hsieh TC, Kunicki J, Darzynkiewicz Z, Wu JM., 2002. Effects of extracts oi Coriolus versicolor (I'm-Yunity) on cell-cycle progression and expression of interleukins-1 beta. -6. and -8 in promyelocytic HL-60 leukemic cells and milogcnically stimulated and nonstimulated human lymphocytes. J Altcrn Complement Med:8:591 -602.

15. Johnson VJ. He Q. Osuchowski MF, Sharma RP., 2003. Physiological responses of a natural antioxidant flavonoid mixture, silymarin. in BALB/c mice: III. Silymarin inhibits T-lymphocyte function at low doses but stimulates inflammatory processes at high doses. Planta Med:69:44-49.

16. Kučera O., Endlicher R., Roušar T., Lotková H., Garnol T., Drahota Z, and Červinková Z., 2014. The effect of tert-butyl hydroperoxide-induced oxidative stress on lean and steatotic rat hepatocytes *in vitro*. Oxid Med Cell Longev. Article ID 752506, 12 pages

17. Kumar A, P.K.Chauhan , V.S.Bhardwaj , R. Kumar , A.Tyagi, 2011. In vitro antioxidant and phytochemical investigations of ethanolic extracts of Viola serpens and Morus nigra. *J Chem Pharm Res.*,3(4),166-171.

18. Lay S.L., Simard G., MartinezM.C., Andriantsitohaina R., 2014. Oxidative stress and metabolic pathologies: from an adipocentric point of view. Oxid Med Cell Longev Volume 2014 (2014), Article ID 908539, 18 pages

19. Lim HH, Lee SO, Kim SY, Yang SJ, Lim Y., 2013. Anti-inflammatory and antiobesity effects of mulberry leaf and fruit extract on high fat diet-induced obesity. Exp Biol Med (Maywood) 238 (10):1160-9.

20. Livak, K. J.,T.D. Schmittgen , 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔC(t) method. Methods, 25 (4), 402-408.

21. Martín C, Martínez R, Navarro R, Ruiz-Sanz JI, Lacort M, Ruiz-Larrea M.B., 2001. tert-Butyl hydroperoxide-induced lipid signaling in hepatocytes: involvement of glutathione and free radicals. Biochem Pharmacol. Sep 15;62(6):705-12.

22. Ogihara T, Asano T, Katagiri H, Sakoda H, Anai M, Shojima N, Ono H, Fujishiro M, Kushiyama A, Fukushima Y, Kikuchi M, Noguchi N, Aburatani H, Gotoh Y, Komuro I, Fujita T., 2004. Oxidative stress induces insulin resistance by activating the nuclear factor-kappa B pathway and disrupting normal subcellular distribution of phosphatidylinositol 3-kinase. Diabetologia 47(5):794-805.

23. Park CM, Cha YS, Youn HJ, Cho CW, Song YS., 2010. Amelioration of oxidative stress by dandelion extract through CYP2E1 suppression against acute liver injury induced by carbon tetrachloride in Sprague-Dawley rats. Phytother Res. 24(9):1347-53.

24. Park E, Lee S.M., Lee J., Kim J.H., 2013. Anti-inflammatory activity of mulberry leaf extract through inhibition of NF-κB. J Funct Foods 5 (1), 178–186

25. Pasheva, M., M. Nashar, D. Pavlov, S. Slavova, D. Ivanov, D. Ivanova, 2013. Antioxidant capacity of different woods traditionally used for coloring hard alco-holic beverages in Bulgaria.-*J. Sci. Technol*., 3, (1), 123-127.

26. Pawlowska A.M., W. Oleszek , A. Braca, 2008. Quali-quantitative analyses of flavonoids of *Morus nigra* L. and *Morus alba* L. (Moraceae) fruits. *J Agric Food Chem*., 56, 3377–3380

27. Rininger JA. Kickner S. Chigurupati P. et al., 2000. Immunopharmacological activity of Echinacea preparations following simulated digestion on murine macrophages and human peripheral blood mononuclear cells. J Leukoc Biol 68:503-510.

28. Roy A., Sil P.C. 2012. Tertiary butyl hydroperoxide induced oxidative damage in mice erythrocytes: Protection by taurine. Pathophysiology 19 (2), 137–148

29. Ruan, H. and H. F. Lodish, 2003. Insulin resistance in adipose tissue: Direct and indirect effects of tumor necrosis factor- α. Cytokine Growth Factor Rev.,14, 447–455

30. Sabu M.C., Kuttan R., 2002. Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. J Ethnopharmacol 81:155-160

31. Salminen A, Kauppinen A, Kaarniranta K., 2012. Phytochemicals suppress nuclear factor-κB signaling: impact on health span and the aging process. Curr Opin Clin Nutr Metab Care. 15(1):23-8

32. Sell H, Eckel J., 2010. Adipose tissue inflammation: novel insight into the role of macrophages and lymphocytes. Curr Opin Clin Nutr Metab Care. 13(4):366-70.

33. Surh YJ, Kundu JK, Na HK, Lee JS., 2005. Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. J Nutr 135(12 Suppl):2993S-3001S.

34. Tasinov O., Kiselova-Kaneva Y., Ivanova D., 2013. *Sambucus ebulus* L. fruit aqueous infusion modulates GCL and GPx4 gene expression. Bulg J Agricult Sci 19 (2), 143-146

35. Trayhurn, P., I. S. Wood, 2004. Adipokines: inflammation and pleiotropic role of white adipose tissue. *Br. J. Nutr*. , 92, No 3, 347-355

36. Uysal K.T., Becherer J.D., Arner P., Hotamisligil G.S., 2002. Altered tumor necrosis factor-alpha (TNF-alpha) processing in adipocytes and increased expression of transmembrane TNF-alpha in obesity. Diabetes, 51, 1876–1883

37. Venkatesh K.R., S. Chauhan, 2008.Mulberry: Life enhancer. *J Med Plants Res*., 2(10), 271-278

38. Ventre J., Doebber T., Wu M., MacNaul K., Stevens K., Pasparakis M., Kollias G., Moller D.E., 1997. Targeted disruption of the tumor necrosis factor-alpha gene: metabolic consequences in obese and nonobese mice. Diabetes, 4, 1526–1531

39. Waihenya RK. Mtambo MM. Nkwengulila G, Minga UM., 2002. Efficacy of crude extract oX Aloe sectiiuliflora against Salinoiwlla i^aUiuarniu in experimentally infected free-range chickens in Tanzania. J Ethnopharmacol:79:317-323.

40. Yen GC, Chen YC, Chang WT, Hsu CL., 2011. Effects of polyphenolic compounds on tumor necrosis factor-α (TNF-α)-induced changes of adipokines and oxidative stress in 3T3-L1 adipocytes. J Agric Food Chem 59(2):546-51.

41. Zhang Y, Huang C., 2012. Targeting adipocyte apoptosis: a novel strategy for obesity therapy. Biochem Biophys Res Commun 417(1):1-4.