Int J Biol Med Res. 2014; 5(4): 4648-4653

BioMedSciDirect Publications

Contents lists available at BioMedSciDirect Publications

International Journal of Biological & Medical Research

Journal homepage: www.biomedscidirect.com

Original Article

Protective Effect of Cocoa Extract on Albino Mice Skin Tumor After Exposure to 7,12 Dimethylbenz (□) Antrasena (DMBA) and 12-O-Tetradecanoylphorbol-13-Acetate (TPA)Malondyaldehyde (MDA) analysis and Histopathology

S Wahab[a]*, MN Massi[b], A Gemini[c], K Djawad[d]

[a]*Hasanuddin University, Department of Dermatovenereology, Faculty of Medicine, Jl. Perintis Kemerdekaan Km.10 Tamalanrea, Makassar, South Sulawesi, Indonesia*
[b]*Hasanuddin University, Department of Microbiology, Faculty of Medicine, Jl. Perintis Kemerdekaan Km 10 Tamalanrea, Makassar, South Sulawesi, Indonesia*
[c]*Hasanuddin University, Department of Pharmacognosis Phytochemistry, Faculty of Pharmacy, Jl. Perintis Kemerdekaan Km 10 Tamalanrea, Makassar, South Sulawesi, Indonesia*
[d]*Hasanuddin University, Department of Dermatovenereology, Faculty of Medicine, Jl. Perintis Kemerdekaan Km 10 Tamalanrea, makassar, South Sulawesi, Indonesia*

ARTICLE INFO

Keywords:
Cocoa
DMBA
histopathology
MDA
TPA

ABSTRACT

Aims.Oxidative stress result in DNA destruction in the induction cells. If this damage is irreversible, DNA will undergo mutation and may lead to cancer. One of cancer caused chemical and often use in experimental study is DMBA/TPA. Thus the chemical is utilized in this study to examine protective effect of cocoa extract on mice. Method. 30 albino mice, age 6-9 weeks, weight 20-30 gr were used as sampel study. The animal were divided into 6 groups with different treatment; group I, is negative control which receive no treatment; group II is control group receiving DMBA/TPA; group III receiving aceton, and group IV,V, and VI each receiving cocoa extract of 200,400 and 800 ppm, respectively. Group II-VI expose to DMBA three times a week with every other day fashion in week 1, followed by application of 4µg TPA on week 2-12. All the animal were sacrificed 24 h after last exposure and samples were taken and proccess for MDA and histopathology examination. Result. MDA level in 400 and 800 ppm cocoa extract groups were higher than control, while it is lower in 200 ppm group than in control. Anova result showed no signicant differences among control and cocoa groups ($p > 0.05$). Histopathology examination result on DMBA/TPA control, aceton and 200 ppm cocoa groups consist of severe dysplasia and squamous cell carcinoma, whereas in 400 and 800 ppm cocoa groups some samples exhibit mild dysplasia. Kruskall Wallis statistical analysis indicates significant differences among control and cocoa groups ($p \leq 0.05$) Conclusion.Topically applied 400 and 800 ppm cocoa exhibit protective effect on cancer formation with the best protection effect shown in concentration of 800 ppm.

1. Introduction

Cancer occurences, including skin cancer, is a multiphase process. In vitro and epidemiology study indicate the carcinogenesis process occur in several phases. Apoptotic and cell proliferation occur in a different rates but still in balance condition throughout initiation and promotion phase. In progression phase the changing in balance condition enable cancer formation.

One of chemicals known to induce carcinogenesis phase is 7,12-dimethylbenz[a]anthracene (DMBA) as inisiator and12-O-tetradecanoylphorbol-13-acetat (TPA) as promotor. Inisiator agent is genotoxic which contribute to the increase in skin cancer risk in human and experimental animal, thus it is often use to observe carcinogenesis processespescially as mutagen prototype and modulator of signal transduction.

Exposure to chemical carcinogenic agent induce the formation of reactive oxygen species (ROS) which will provoke DNA, RNA and protein damage through chemical reaction such as oxidation, nitration, and halogenation. These events will increase mutation, altered protein and essential enzyme function. Several studies have indicate that ROS formation occur at initiation, promotion and progression phase through genotoxicity. Cell membrane is sensitive to ROS and become the target of destruction caused by ROS induction. Unsaturated fatty acids generally consist of methylene groups between two double bonds,thus make it more sensitive to oxidation. ROS react with fatty acids on cell membrane forming lipid peroxyde, furthermore forming specific and carcinogenic malondialdehyde (MDA). This product is

* Corresponding Author : **S Wahab**
Hasanuddin University,
Department of Dermatovenereology, faculty of Medicine,
Jl. Perintis Kemerdekaan Km 10 Tamalanrea, Makassar,
South Sulawesi, Indonesia.
E-mail: siswantowahab@ymail.com

endogenous genotoxic agent made through enzymatic process and oxygen radicals induced by lipid peroxide, thus it can be used to evaluate DNA oksidative damage after DMBA exposure to the skin.Double-stranded DNAexposure to MDA modified this DNA and increase mutation rate if it repalicates in mammalian cells. Destruction of DNA from the exposure indicate mutagenic nature of MDA in relation to oksidative stress and lipid peroxide. The result of this study indicates specific DNA destruction caused by oksidative stress is the formation of 8-OHdG. DMBA exposure to breast epithelial stem cell has proven the increasing oksidative damage on nuclear DNA (8-OH-dG).

DMBA application onto skin as initiator indicates by specific mutation on oncogen. Furthermore, latent phase of tumor formation decrease significantly with application of TPA, thus both agents will formed benign tumor (papilloma). However, the feasibilities of papilloma formation in mice strain depends on carcinogen types, application doses and evaluation periode. Study using 1 mg DMBA in 50 µl aceton with 3 days interval followed by application of TPA 10 µg twice a week for 8-32 weeks result in skin tumor 34,9% from sample. Our previous study indicates DMBA exposure of 50µg three times a week in the first week followed by application of 4 µg TPA three times a week in the second untill week 10 on mice induce formation of papilloma and squamous cell carcinoma, thus enabling the use of the dose for the following study to induce tumor formation and protective effect trials on variuos natural ingredients which possess antioxidant and antiproliferative effect.

Skin tumor formation after DMBA/TPA exposure is caused by DNA mutation, and alteration in gene regulates apoptotic balance, which play role in cell death if DNA damage is irreversible. Apoptotic occur in two main pathways, that is extrinsic or sitoplasmic pathway and intrinsic or mithocondrial pathway which enable the releasing of cytochrom C from mithocondria and apoptotic activation signal when the pathway is stimulates. Both pathways will induce caspase which later caused apoptotic. One of the most important regulator gene in the intrinsic pathway is BCL-2. It is first identified on translocation of chromosome 18 to 14 in folliculer non-Hodgkin lymphoma, and act as key regulator in apoptotic. Kedua jalur tersebut akan menginduksi caspase yang akhirnya menginduksi apoptosis. Apoptotic is controlled by gene that induce apoptotic (e.g. BAX) and antiapptotic gene (BCL-2). Overexpression of BCL-2 in the intrinsic pathway inhibit the extrinsic one. Various studies have proven that the increasing in BCL-2 expression which inhibit apoptotic play roles in the tumor formation induced by DMBA/TPA. Administration of phenolic garlic extract indicates protective effect on tumor through induction of proapoptotic gene p53 and BAX, and inhibition of antiapoptotic gene expression BCL-2.

Flavonoid is the greatest group of polyphenol which possess specific antioxidant nature beneficial for human healthiness. Various natural ingredients included in the group are green tea, fruits and vegetables has biologic effect on proliferation and angiogenesis and decreasing cardiovascular and cancer risk. Other natural ingredient with strong antioksidant activities is cocoa. Cocoa extract has some active antioksidant ingredients includingflavonoids, catechins, epicatechins dan proanthocyanidins. Exvivo and invivo studies indicates that epicathecin is the main polyphenol in cocoa and cocoa extract, and exhibit inhibitory effect on lipid peroxide plasma because of its ability to bind on low density lipoprotein. Stress oxidative induction with H2O2 induce BCL-2 downregulation result in apoptotic. Cocoa procyanidin as antioxidant has`proven inhibit apoptotic through inhibition of downregulation BCL-2 expression on tissue induced with H202. On the contrary, cocoa rich diet has proven inhibit tumor formation at the early stage of colon carcinoma on experimental animal. This inhibition effect attained through antioxidant effect, and increasing of endogenous enzymatic and nonenzymatic defence. Cocoa also exhibit antiproliferative effect through decreasing extracellular regulated kinases, kinase protein B dan cyclin D1 together with proapoptotic effect as indicated with decreasing Bcl-xL levels dan increasing level of Bax dan caspase-3 activities.

Despite the already established effect of cocoa as antioksidant on various diseases, protective effect of various topical application concentration on carcinogenesis process as antioksidant has not so far been established, especially protective effect of cocoa extract on skin tumor formation by evaluating MDA. Thus, further study are needed on theeffect of topical cocoa seed extract on mice receiving various concentration of cocoa extract and DMBA/TPA application.

MATERIAL AND METHODS

Study design. This study is a true experiment post design with control group.It was conducted at Animal laboratory of Medical faculty of Hasanuddin University (Makassar, Indonesia). Histopathology preparation was made at the Pathology anatomy and biotechnology laboratory of Hasanuddin University's Hospital.

Study subject. The subject of this study were 6-9 weeks old albino mice from one mother purchased from Balitbang veterinary centre in Maros, South Sulawesi. The mice were housed in standard condition for 1 week: room temperature (28 ± 2°C), humidity 50 ± 10% and subjected to a 12 h light/ 12 h dark cycle.

Materials and Equipments.Cocoa extract with concentration of 200, 400 and 800 PPM in lotion preparation, 50 µg 7,12-Dimethylbenz () Antrasena (DMBA), 4 µg 12-O-Tetradecanoylphorbol-13-Asetat (TPA) and DP12 type of olympus microscope were used.

Study sample. The sample of this study consist of 30 mice which were divided into several perpetration groups. Inclusion criteria were healthy female albino mice spesies swiss albino mice aged 6-9 weeks old, weighing 20-30 g. Mice which became sick or dead throughout the study were excluded.

Methods. Sixty mice were divided into 6 groups, group I were expose to DMBA/TPA (10 mice), group IIwere treated topically with lotio base (control) together with exposure of DMBA/TPA (15

mice), group III-V each consisted of 10 mice were treated topically with 200, 400 and 800 PPM cocoa extract, respectively, 20 minutes before exposed to DMBA/TPA. DMBA/TPA were exposed in an every other day fashion for 12 weeks. The mice were terminated 24 hours after the last exposure and examined for MDA level and histopathologic feature.

Intervention and outcome measurements. Documentation were obtained from all the mice included in this study for initial data and throughout the study.The mice were acclimatized 1 week before use.They were randomly put in groups according to each treatment. DMBA/TPA and cocoa extract were applied onto shaved interscapular area. The perpetration were done for 12 weeks, and at the end of week 10 all the mice were sacrificed. Exsicional biopsies with 2 cm diameter were takenfor MDA and histopathological examination. For histopathology, the sample were taken from mice's ear, fixate in formaline buffer 10%, and cut in blocks with 4-5 µM thickness. It were deparaffinated with xylene and stained with hematoxylin eosin (HE) for standard evaluation using olympus CV microscope.MDA were calculated using Wright methods.The last concentration were calculated using molar coefficient of 1,56X105/M/cm.Lipid peroxide inhibition determined by measure the decreasing number of MDA in the incubation compare to acetone control.

RESULTS

Sample characteristics. This study was performed on 30 albino mice eligible for inclusion criteria weighing 20-30 g (.097 ± 2.680) randomly divided into groupsof each treatment. Natural ingredients used in this experiments were already tested for the flavonoid content, with concentration of 100, 200, 400 and 800 ppm of cocoa extract contain 7,42; 14,48; 29,68; and 59,36 µg of total flavonoid content respectively.

MDA level in every treatment groups expose to DMBA/TPA and cocoa extract.

Data from tabel I shows no significant differences among the treatment groups ($p \geq 0.05$). Nevertheless, there are increasing level of MDA in treatment group with topically applied 400 and 800 PPM cocoa compare to DMBA/TPA control and aceton. All treatment groups had a higher MDA level than the negative control.Graphic I shows the feature of MDA level on study sample.

Histopathologic feature of skin tissues of every treatment groups expose to DMBA/TPA and cocoa extract.

Data from table II shown frequency of distribution from sample after DMBA/TPA exposure for 12 weeks. In the control group without protection (DMBA/TPA control and DMBA/TPA + acetone control) and group of 200 ppm cocoa extract, severe dysplasia and squamous cell carcinoma occur, while in 400 and 800 ppm of cocoa extract, mild dysplasia occur in 20% and 40% of samples, respectively. Statistical analysis result indicates significant differences among treatment groups $p<0.05$. The result of histopathologic feature as shown in figure 1 – 5.

Tabel I. Level of tissue MDA after exposure with DMBA/TPA for 12 weeks according to cocoa concentration in aceton solution groups as protector

Treatment Group	Mean ±SD	P
DMBA/TPA control	3.677±0.598	0.386*
Aceton control	2.313±3.252	
Cocoa 200	2.484±2.068	
Cocoa 400	4.379±3.194	
Cocoa 800	4.395±4.028	
Negative control	1.165±1.338	

*Anova statistical analysis

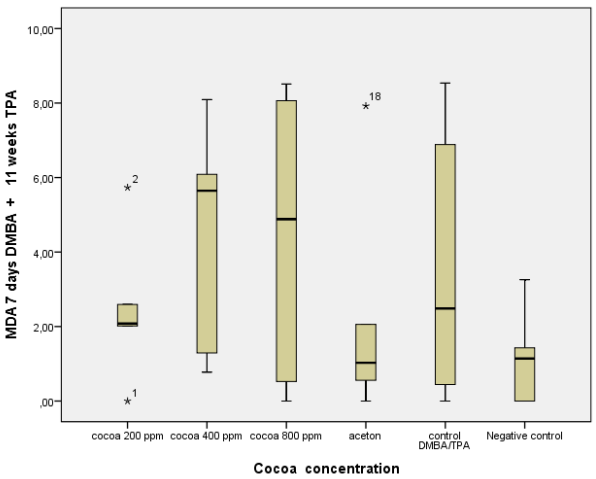
Tabel II. Frequency of histopathologic feature distribution according to treatment

Treatment groups	Histopathology examination									
	Normal		Mild Dysplasia		Severe Dysplasia		Squamous cell Ca.		Total	
	N	%	N	%	N	%	N	%	N	%
DMBA/TPA control	0	0	0	0	3	60	2	40	5	100
DMBA/TPA + Acetone	0	0	0	0	4	40	1	20	5	100
Acetone + Cocoa 200	0	0	0	0	4	40	1	20	5	100
Aceetone + Cocoa 400	0	0	1	20	2	40	2	40	5	100
Acetone + Cocoa 800	0	0	2	40	2	40	1	20	5	100
Negative control	5	100	0	0	0	0	0	0	5	100

groups after exposure with DMBA and TPA for 12 weeks

*Kruskall Wallis statistical analysis

Mice group expose to DMBA for 7 days and TPA for 11 weeks Graphic I. Level of MDA tissue expose to DMBA/TPA for 12 weeks based on cocoa concentration in acetone solution as protector



Graphic II. The effect of cocoa extract on mice expose to DMBA/TPA for 12 weeks based on histopathologic feature

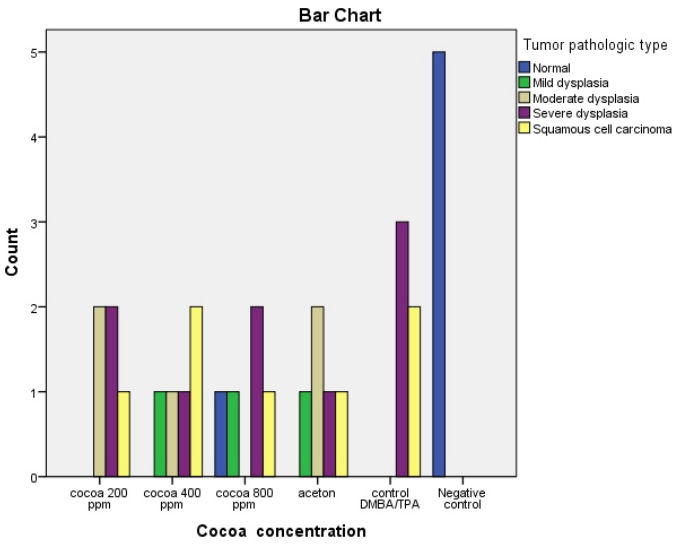


Figure 1. Normal skin tissue, Cell dysplasia was not found, cell polarity is still good

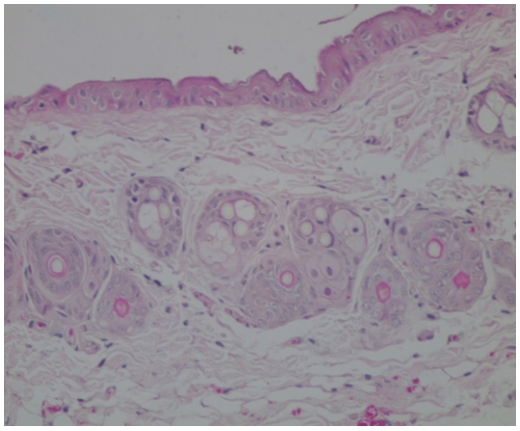


Figure 2. mild dysplasia at less than 1/3 of the epidermis

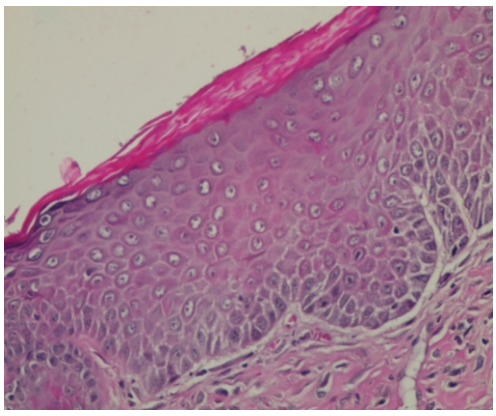


Figure 3. moderate dysplasia at 1/3 - 2/3 of the epidermis

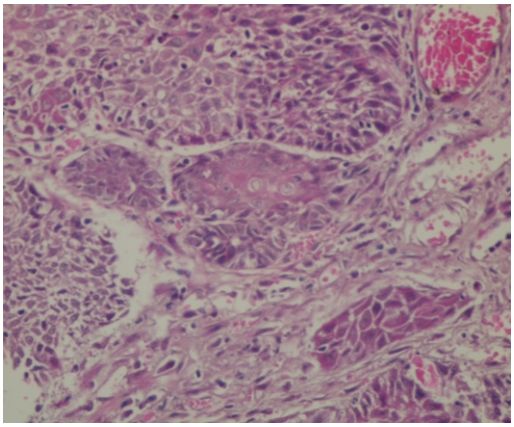


Figure 4. severe dysplasia at more than 2/3 the epidermis of the epidermis

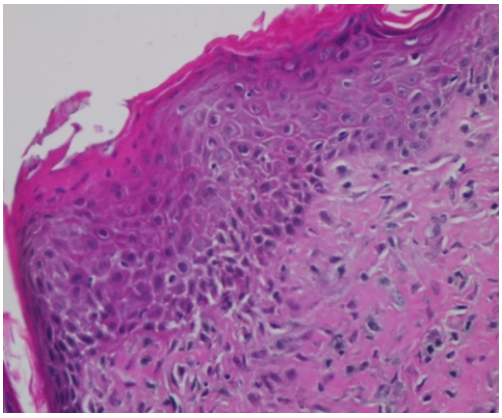
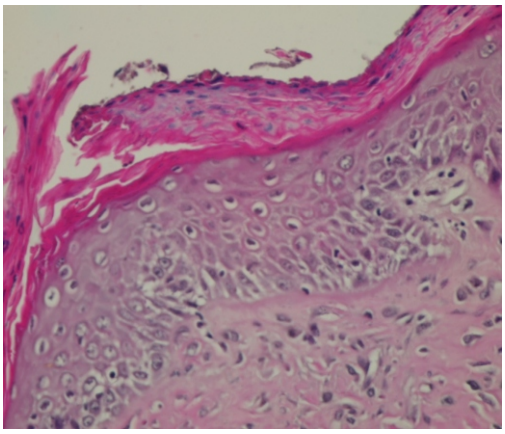


Figure 5. squamous cell carcinoma, showed expansion of atypical keratinocytes that pass through the basal membrane and into the dermis to form clumps of cells were detached from the epidermis



Discussion

Oxidant role in skin cancer development is a complex staging process, comprising of three phases: initiation, promotion and progression, which is mediated by various alteration in moleculer, celluler and biochemistry. ROS is involved in these three phases. ROS is generally found in cell membrane as malondialdehyde, product of enzymatic process and lipid peroxide oxidation. Various studies indicates that MDA induce mutation that result in cancer. This study utilize MDA level measurements to evaluate the extent of oxidative stress induced by DMBA exposure to mice. ELISA result indicates that DMBA induce oxidative stress in DMBA/TPA control group with higher level than negative control. Whereas in Anova statistical analysis, MDA level in cocoa group is not statistically significant with negative control. Despite this result, there is tendencies of higher level in cocoa group compare to DMBA/TPA control which indicates the effect of cocoa extract on stress oxidative induction. This result is different to other studies which indicates that cocoa may inhibit stress oxidative formation by heat exposure on leucocyte winstar. The differencies may be caused by cell target on this study is the cell which is already undergo transformation resulting from DMBA/TPA induction. On invivo and exvivo studies, exposure of cocoa polyphenol extract with concentration 10-100μM on RLE cell may substantiate hydrogen peroxide induction on oxidative stress accumulation. Other study on cancer cells indicates increasing oxidative stress on camcer cell induced by mitogen-activated protein kinases (MAPKs) inhibitor which enables prematur senescence. Thus, protective effect of cocoa extract may occur through apoptotic and or premature senescence pathways.

DMBA induction in initiation phase, followed by TPA application as promotor agent result in histopathologic alteration as severe dysplasia and squamous cell carcinoma compare to unexposed mice skin. Several experiment indicate that diet rich in cocoa possess antiproliferative effect on winstar rat colon cancer induced by azoxymerthalne. Cocoa procyanidin and B2 procyanidin in concentration of 5μg/mL and 40μM, respectively, inhibit induction of neoplastic cell (JB6P) by TPA as high as 47% and 93% through suppression on kinase activity. Cocoa also inhibit COX-2, AP1 and NF-KB expression related to cancer. Antimutagenic effect of cocoa extract has already proven in invitro studies using benzo[a]pyrene as mutagene, which indicate cocoa inhibition on cancer initiation through metabolic activation inhibition by cytochrom p450 (CYP), typically CYP1. In the histopathologic graphic, it is shown that 200 ppm cocoa group has similarity in relation to cancer occurences with control group which exhibit severe dysplasia and squamous cell carcinoma, thus it is ineffective as cancer protector in the promotion/pregression phase in carcinogenesis study. Topically applied 400 and 800 ppm cocoa group exhibit inhibition effect on skin tumor. In these concentration, some sample still exhibit mild dysplasia. It is as shown in the result of Kruskall Wallis statistical trials which is significantly different with control group ($p<0,05$) with concentration of 800 ppm as the best protective concentration. In association with MDA level in this concentration, there maybe a tendency that the increasing MDA level has a role in cocoa protection effect, evenmore in comparison to 200 ppm cocoa which has lower MDA level. Increasing level of MDA probably induce apoptotic and or senescence pathway. Further study in association with both pathways will be required.

CONCLUSIONS

Topically applied 400 and 800 ppm cocoa exhibit protective effect on cancer formation with the best protection effect shown in concentration of 800 ppm.

ACKNOWLEDGEMENT

We thank the laboratory of Pharmacognosis and Phytochemistry of Pharmacy Faculty and Microbiology Laboratory of Medical Faculty of Hasanuddin University for the support.

REFERENCES

1. Kumar N, Sharma P, A.K B, A.K M. Expression in Cytologic Scrapes from Oral Squamous Cell Carcinoma before and after 24 Gray Radioteraphy-a Study on 43 Patients. Oral med and pathol. 2007;10:15-7.
2. Kimoto T, Arai S, Kohguchi M, al e. Apoptosis and suppresion of tumor growth by artepillin C extracted from Brazilian propolis J of Cancer detec and prevent. 1998;22:506-15.
3. Yusuf N, T.H N, Meleth S, C.A E. Resveratrol enchances cell-mediated immune response to DMBA through TLR4 and prevents DMBA induced cutaneous carcinogenesis. J Mol Carcinog. 2009;48:713-23.
4. Meeran SM, T MP, K K. Skin Tumor Promotion 7,12-dimethylbenz (a) anthracene in Mouse Skin, which is associated the inhibition of inflammatory. 2009;30:500-9.
5. Grigorov B. Reactive Oxygen Species and Thei Relation to Carcinogenesis. Trakia J of Science. 2012;10(3):83-92.
6. Niedernhofer LJ, Daniels JS, Rouzer CA, al e. Malondialdehyde, a Product of Lipid Peroxidation, is Mutagenic in Human Cells. JBC 278.33:31426-33.
7. Muqbil I, Azmi AS, Banu N. Prior exposure to restraint stress enhances 7,12-dimethylbenz (a) anthracene (DMBA) induced DNA damaged in rats. FEBS Letters. 2006;580(2):3995-99.
8. Mackie RM, Quinn AG. Non-Melanoma Skin Cancer and Other Epidermal Skin Tumors. Burns, Breathnach, Blackwell Science. 2004.
9. Salvadori D, Ribeiro D. Mediumterm tongue carcinogenesis assays: a comparative study between 4-nitroquinoline 1-oxyde (4NQO)-induced rat and dimethylbenz (a) anthracene (DMBA)-induced hamster carcinogenesis. J Exp Animal Sci. 2006;43(3):219-27.
10. Mun'im A, Andrajati, Susilowati H. Uji hambatan tumorgenesis sari buah merah (Pandanus conoideus lam) terhadap tikus putih betina yang diinduksi 7,12 dimetilbenz (a) antrasen (DMBA). MIK III. 2006;3:153-61.
11. Arora A, Siddiqui IA, Shukla Y. Modulation of p53 in 7,12-dimethylbenz (a) anthracene-induced skin tumors by diallyl sulfide in Swiss albino mice. Mol cancer Ther. 2004;3(11):1459-66.
12. Cho ES, Lee KW, Lee HJ. Cocoa procyanidins protect PC12 cells from hydrogen-peroxide-induced apoptosis by inhibiting activation of p38 MAPK and JNK. J of Mutation Research. 2008;640:123-30.
13. Vinson JA. Chocolate is a Powerfull ex vivo and in vivo antioxidant, an antiatherosclerotis agent in an animal model, and a significant contributor to antioxidants in the European and american diets. J Agric Food Chem. 2006;54:8071-6.
14. Romiro IR, Ramos S, Oliva EL. Cocoa-rich diet prevents azoxymethane-induced colonic preneoplastic lessions in rats restraining oxidative stress and cell proliferation and inducing apoptosis J of Mol Nutr. 2011;55(12):1895-9.

15. Suzuki JS, Nishimura N, Zhang B. Metallothionein Deficiency Enhances skin Carcinogenesis Induced by 7,12-dimethylbenz (a) anthracene and 12-0-tetradecanoylphorbol-13-acetate in Metallothionein-Null mice. J of Carcinogen. 2003;24(6):1123-32.

16. Lee JJ, Lee JH, Ko YG. active oxygen prevention of premature senescence requires JNK regulation of Bcl-2 and reactive oxygen species. J of Science. 2010;29:561-75.