ORIGINAL ARTICLE

Poguntano effect on cell viability in cholesteatoma keratinocyte of chronic suppurative otitis media

Harry A. Asroel[1], Aznan Lelo[2], Delfitri Munir[1], Jenny Bashiruddin[3], Restu Syamsul Hadi[4]

[1]Ear, Nose and Throat - Head and Neck Surgery Department, School of Medicine, Universitas Sumatera Utara, Medan, [2]Pharmacology Department, School of Medicine, Universitas Sumatera Utara, Medan, [3] Ear, Nose and Throat - Head and Neck Surgery Department, School of Medicine, Universitas Indonesia (UI), Jakarta, [4]Stem Cell Research Centre, Universitas Yarsi, Jakarta; Indonesia

ABSTRACT

Aim Chronic Suppurative Otitis Media (CSOM) with or without cholesteatoma is a global major problem and it is becoming a burden especially in developing countries. Studies have found that ethanol extracts of Puguntano leaves obtained by the percolation and socletation methods have phytochemical contents that provide anti-inflammatory effect. The aim of this study was to investigate the effects of ethanol extract and *Puguntano* on the viability of cholesteatoma keratinocytes.

Methods This *in vitro* experimental study included 8 groups of the cholesteatoma keratinocyte culture: not given puguntano leaf ethanol extract, three positive control groups given different concentrations of dexamethasone, and four groups that were given different concentrations of ethanol extract of leaf Puguntano.

Results A statistically significant decrease in procalcitonin level ($p<0.001$) and an amount of bacterial colonies ($p<0.001$) in four groups were found.

Conclusion This study showed that Puguntano leaf extract has the same effect as dexamethasone in terms of suppressing cell viability and has lower side effects compared to dexamethasone as other herbal medicines. Thus, the ethanol extract of Puguntano leaves can be used as an alternative for the prevention of cholesteatoma.

Key words: inflammation, otitis, treatment

Corresponding author:
Asroel, Harry Agustaf
ENT-Head and Neck Surgery Department,
School of Medicine,
Universitas Sumatera Utara
20155 Medan, Indonesia
Phone: +62 812 6517 710
Fax: +62 61 8216264
E-mail: harry.aa@usu.ac.id
ORCID ID: https://orcid.org/0000-0003-3096-2166

Original submission:
04 February 2020;
Revised submission:
29 April 2020;
Accepted:
19 June 2020
doi: 10.17392/1155-20

Med Glas (Zenica) 2020; 17(2): 451-456

INTRODUCTION

Chronic suppurative otitis media (CSOM) with or without cholesteatoma is a global major problem and becomes a burden especially in developing countries (1)Cipro HC. The World Health Organization (WHO) reported that there are 65-330 million people with CSOM globally and 94% of them are in developing countries (1). About 60% (39 – 200 million people) died because of CSOM (1,2)Cipro HC. Commonly, CSOM prevalence in developing countries, such as India, is higher with total 5.2% (2). In Indonesia, CSOM prevalence is reported as many as 3.6% (2). There are two types of CSOM: tubotympanic (without cholesteatoma) and atticoantral (with cholesteatoma) (3).

Atticoantral type of CSOM can cause a serious complication such as intratemporal complication (facial nerve paralysis) and intracranial complication (meningitis, cerebral abscess) which increases morbidity and mortality rates significantly (2,4). The CSOM resulted from acute otitis media, which is characterized with continuous otorrhea from the middle ear through perforated tympanic membrane (1). This process is the main cause of hearing disorder (1)Cipro HC.

Cholesteatoma characterized by hyperproliferation is associated with chronic inflammation and bone destruction (2). Ossicle resorption occurs in local area surrounding cholesteatoma perimatrix or granulation tissue (5). Cholesteatoma perimatrix contains lymphocytes, monocytes, fibroblasts and endothelial cells, which are sources of proinflammation cytokines, such as TNF-α and IL-6 (5). TNF-α and IL-6 expression in cholesteatoma tissue is higher compared to granulation tissue (5). TNF-α plays a direct role in bone destruction through osteoclast differentiation and through opened bone matrix, indirectly (4). This process occurs simultaneously with IL-1 expression (4).

Puguntano (*Curanga fel-terrae* [*Lour*] *Merr*.) is a herb from *Scrophulariaceae* family that grows in Asian regions, such as Indonesia, Malaysia, the Philippines, China and India (6). In Indonesia, in the Tiga Lingga village community of Dairi District in North Sumatra province Puguntano leaf is empirically used as a medicinal herb (7). This plant is believed to be efficacious as a painkiller, it increases host immunity, and is even used in an anti-aging process (7). This plant has begun to be widely cultivated by the local community as a medicinal plant (7). In some areas, this plant is known as "*kukurang, tamah raheut"* and "*empeduh tanah*" (6). Juwita et al. (8) and Harfina et al. (9) reported an anti-inflammatory potential of Puguntano leaf in the form of ethanol extract.

Studies have found that ethanol extracts of Puguntano leaves obtained by the percolation and socletation methods have phytochemical contents, such as alkaloids, flavonoids, glycosides, saponins, tannins and steroids/terpenoids (6). One of the classes of chemical compounds responsible for anti-inflammatory effects is steroids/terpenoids (8).

A study in Jakarta which conducted research on the effect of dexamethasone on proinflammatory cytokines, namely TNF-α and IL-1α, got an average cell viability of 92.95% when planted, and 96.60% after 48h cultivation (9)tissue culture methods can be applied also to cholesteatoma of chronic suppurative otitis media patients that can be used to study the nature of cholesteatomas to the cellular level. Purpose: The purpose of this study was to get tissue culture methods for cholesteatomas, so that the steps, techniques and specific tools for culturing cholesteatoma can be determined, also the number of cells that were planted, good environment in order to obtain cell viability and good culture results. Methods: This study consists of three phases: 1; with 0.05 significance level, from the matrix it was seen that cell viability was significantly different between the control group and 40 mg and 100 mg dose groups (9).

The aim of this study was to investigate the effects of ethanol extract and *Puguntano* on the viability of cholesteatoma keratinocytes.

MATERIALS AND METHODS

Materials and study design

This study was conducted at the Universitas Sumatera Utara (USU), Medan, from January 2019 to September 2019.

This *in vitro* experimental study included 8 groups of the cholesteatoma keratinocyte culture: one group which was not given puguntano leaf ethanol extract, three positive control groups given different concentrations of dexamethasone, and four groups that were given different concentrations of ethanol extract of leaf Puguntano.

Cholesteatoma tissue was taken during the surgery of the patients with atticoantral type of CSOM in the Central Surgery Installation at the Tangerang General Hospital. Cholesteatoma keratinocyte cultivation was carried out at the Integrated Laboratory Research Institute of YARSI Universitas, Jakarta. Cell viability tests are carried out in the same laboratory.

The approval for the investigation was obtained from the Ethics Committee of the Faculty of Medicine, Universitas Sumatera Utara.

Methods

Samples. Patients with atticoantral type of CSOM, who had agreed to be included into the study and had undergone surgery in Tangerang General Hospital, were included in the study. Patients were not allowed to take steroids at least 1 week before the surgery. Cholesteatoma tissues were immediately stored in a 50 mL conical bottle containing sterile phosphate buffer saline plus antibiotics-antimycotics in a cold state. Then the samples were cultured using a keratinocyte growing medium, Epilife plus Human Keratinocyte Growth Supplement (HKGS) (10–12)

Ethanol extract preparation. The preparation of ethanol extract of leaf Puguntano was done at Faculty of Pharmacy of USU. Puguntano extracts were made by the maceration method (13). Puguntano leaves that had been washed and dried, were then ground into powder. Puguntano extracts were made by the maceration method. Ten parts of Simplisia powder were incorporated into a closed container and left for 5 days protected from lights while stirred occasionally. The mixture was then squeezed until the pulp was left. The pulp was washed so that 100 parts of a solution were obtained. The solution was transferred into a clean container and then left for two days to be filtered.

Keratinocytes cells were cultured in Epilife plus HKGS medium and then stained using Hoecsht protocol (14,15)the capacity to simultaneously measure DNA content in cells being tracked over time remains challenged by dye-associated toxicities. The ability to measure DNA content in single cells by means of LCFM would allow cellular stage and ploidy to be coupled with a variety of imaging directed analyses. Here we describe a widely applicable nontoxic approach for measuring DNA content in live cells by fluorescence microscopy. This method relies on introducing a live-cell membrane-permeant DNA fluorophore, such as Hoechst 33342, into the culture medium of cells at the end of any live-cell imaging experiment and measuring each cell's integrated nuclear fluorescence to quantify DNA content. Importantly, our method overcomes the toxicity and induction of DNA damage typically caused by live-cell dyes through strategic timing of adding the dye to the cultures; allowing unperturbed cells to be imaged for any interval of time before quantifying their DNA content. We assess the performance of our method empirically and discuss adaptations that can be implemented using this technique. Results: Presented in conjunction with cells expressing a histone 2B-GFP fusion protein (H2B-GFP. Puguntano extract was diluted within 5 mL ethanol. We then examined the cells viability under fluorescent microscope.

Cholesteatoma keratinocyte culture that was suitable for the analysis, was divided into 8 groups using microplate based on the treatment obtained (11,12): Group 1- control group that was not given puguntano ethanol extract, Group 2 - positive control group by giving dexamethasone solution at a dose of 1 µM, Group 3 - positive control group by giving dexamethasone at a dose of 10 µM, Group 4: positive control group with dexamethasone at a dose of 100 µM, Group 5 - cholesteatoma keratinocytes given ethanol extract of puguntano at a dose of 1%, Group 6 - cholesteatoma keratinocytes were given ethanol extract of puguntano at a dose of 2%, Group 7 - cholesteatoma keratinocytes given ethanol extract of puguntano at a dose of 4%, and Group 8 - cholesteatoma keratinocytes treated with ethanol extract of puguntano at a dose of 8%.

Cell viability test. Cell viability is the number of living cells and it is usually expressed as a percentage of the number in the control group. A cell viability assay is performed based on the ratio of live and dead cells. This assay is based on an analysis of cell viability in cell culture for evaluating in vitro drug effects in cell-mediated cytotoxicity assays for monitoring cell proliferation (16). In this study, we assayed cell viability with EVOS fluorescence microscopy.

Statistical analysis

The data obtained were processed and analysed with univariate analysis (normal or abnormal dis-

tribution) using the Kolmogorov Smirnov test, by variate analysis (comparing before and after the treatment) using t-test for normal data and Wilcoxon test for non-data normal, as well as multivariate analysis (comparing many groups) using the ANOVA test for normal distribution and the Kruskal-Wallis test for abnormal distribution. Bonferroni test was performed to see whether there are differences in each of the two experimental groups. A $p < 0.05$ was used as statistically significant.

RESULTS

Cell viability in the control group showed the highest mean far above the group given dexamethasone and puguntano with a mean of 45.6. The mean of viability between groups given dexamethasone and puguntano did not show significant differences in mean viability ($p > 0.05$). Compared to the control group, we found that the more dexamethasone was given, the lower number of cells' viability was noticed (Figure 1).

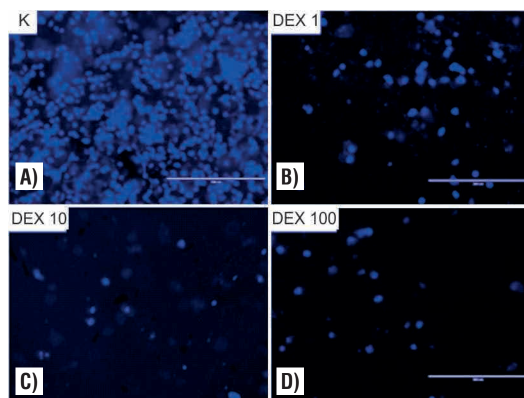

Figure 1. Dexamethasone effect on cells viability of cholesteatoma keratinocytes with Hoecst staining. A) Control group (K) (it was not given Puguntano ethanol extract) where cells' viability was 100%; B) there was a decreasing number of cells' viability in group DEX1 (positive control group adding dexamethasone at a dose of 1 μM) compared to group DEX10; C) there was a decreasing number of cells' viability in group DEX10 (positive control group adding dexamethasone at a dose of 10 μM) compared to group DEX1; C) there is a decreasing number of cells' viability in group DEX100 (positive control group adding dexamethasone at a dose of 100 μM) compared to group DEX10

The same effect was also seen in samples that were given Puguntano ethanol extract (Figure 2). There was an increasing number of cell's viability on samples given 2% of Puguntano extract. However, when the samples were given 4% and 8% of Puguntano extract, the number of cell's viability was decreasing (Figure 3).

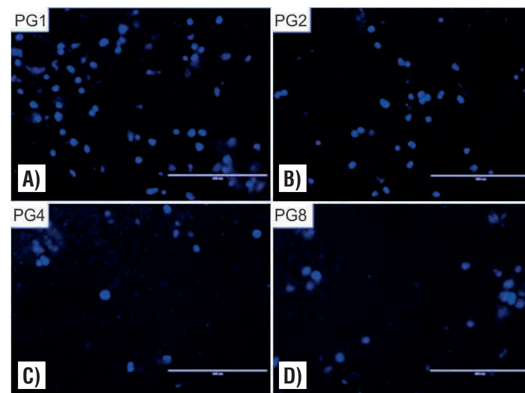

Figure 2. Puguntano effect on cells viability of cholesteatoma keratinocytes with Hoecst staining. A) there was a decreasing number of cells' viability in PG1 group (when added ethanol extract of Puguntano at a dose of 1%) compared to group K (it was not given Puguntano ethanol extract – Figure 1); B) there was a decreasing number of cells' viability in PG2 group B (when added ethanol extract of puguntano at a dose of 2%) compared to PG1 group ; C) there was a decreasing number of cells' viability in PG4 group (when added ethanol extract of Puguntano at a dose of 4%) compared to PG2 group; D) there was a decreasing number of cells' viability in PG8 group compared to PG2 group

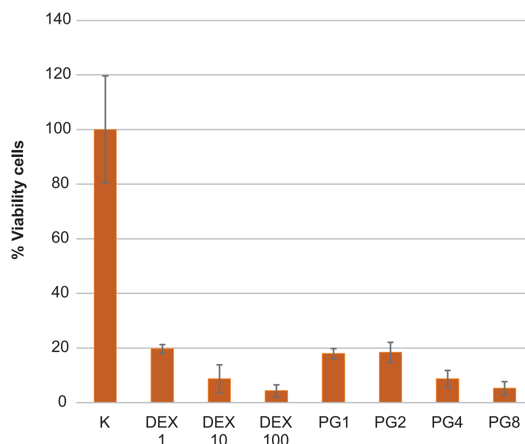

Figure 3. Number of cell's viability in the control group and samples, which were given dexamethasone and Puguntano extract in various doses. K, total percentage of cells' viability; Dex1, cell's viability after given 1 μM dexamethasone; Dex10, cell's viability after given 10 μM dexamethasone; Dex100, cell's viability after given 1000 μM dexamethasone; PG1, cells' viability after given Puguntano extract 1%; PG2, cells' viability after given Puguntano extract 2%; PG4, cells' viability after given Puguntano extract 4%; PG8, cells' viability after given Puguntano extract 8%

DISCUSSION

Studies that assess cell viability, especially in cholesteatoma are still very rare. This research may be a novel study that can be used as a pilot study to see the effect of Puguntano ethanol extract on the viability of cholesteatoma culture cells. We found that the culture group with the highest cell viability rate was the control group (group 1 that was

not given Puguntano ethanol extract) with a mean of 45.6 %. This value is significantly greater than either the culture group that received dexamethasone or those who received ethanol extract from Puguntano leaves. Kara et al at Turkish Kocaeli University (2019) in their 3D cholesteatoma cell culture model study using sodium diclofenac 1% and 2%, 5-fluorouracil 1% and negative controls received lower cell viability and higher apoptosis in the sodium diclofenac group compared to cholesteatoma cell cultures group without additional drugs (18)and there is no medical treatment for this disease. Exploring new medical treatment options May help to create treatment alternatives instead of surgery. Materials and Methods: Required cholesteatoma tissues for cell culture were excised from 4 different participants who underwent surgery in our clinic and agreed to give tissue for the study. Cholesteatoma-derived keratinocytes and fibroblasts were cocultured in temperature-sensitive culture dishes to make a three-dimensional (3D. In our study, on the other hand, cells' viability, when samples were given dexamethasone, was not significantly different when compared to the culture groups that received Puguntano ethanol extract.

Puguntano ethanol extract contain chemical compounds of glycoside, flavonoid, saponin, tannin, and steroid/triterpenoid (19). Yassine et al. (20) in their research have demonstrated flavonoid role in anti-inflammation process. The early step where histamine and serotonin release is followed with oedema caused by bradykinin and prostaglandin release (this phase is known to be sensitive with anti-inflammatory agent, both steroid and non-steroid), flavonoid is shown to inhibit important enzyme that has a role in biosynthesis of tissue activator, especially prostaglandin and nitric oxide (20). Flavonoid shows several biological effects, such as anti-inflammation, anti-hepatotoxic, anti-allergy, anti-virus, and anti-cancer (21). Flavonoid is also effective in inhibiting arachidonic acid metabolism that mediates prostaglandin biosynthesis through gene expression of cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) inhibition. Active substance of flavonoid is also considered as an effective scavenger in reactive oxygen species due to its phenolic hydroxyl group, and thus, is considered to be a strong anti-oxidant (13,20). Furthermore, effect of the drug on COX-2 decrease in cell viability and the presence of necrotic processes shown by Fourier transform infrared analysis, suggesting a direct correlation of prostanoids in cellular homeostasis and survival (22). Currently, there is evidence that prostaglandins produced by COX-2 intervene in tumour cell proliferation as NSAIDs and selective COX-2 inhibitors inhibit proliferation of different cancer cell types expressing COX-2(23). NS-398, a COX-2 specific inhibitor, was described to reduce cell proliferation of MC-26 cell line, a highly invasive mouse CRC cell model expressing constitutively COX-2 (24),(25).

Appropriate host immune response could offer protection against infectious threats; however, excessive inflammatory immune response can lead to the uncontrolled growth and proliferation of cholesteatoma (26).

In conclusion, this study showed that Puguntano leaf extract has the same effect as dexamethasone in terms of suppressing cell viability compared to dexamethasone as other herbal medicines. Thus, the ethanol extract of Puguntano leaves can be used as an alternative for the prevention of cholesteatoma.

ACKNOWLEDGEMENT

We thank Ashadi Budi, MD, Otologic Surgeon (Tangerang General Hospital) for dedication and support to the cholesteatoma matrix for this research.

FUNDING

No specific funding was received for this study.

TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

REFFERENCES

1. World Health Organization. Chronic suppurative otitis media - Burden of Illness and Management Options. Geneve: World Health Organization, 2004 https://apps.who.int/iris/handle/10665/42941 (14 January 2020)
2. Yarisman L, Asroel HA, Aboet A, Zaluchu F. Hubungan ekspresi RANKL dengan derajat destruksi tulang akibat kolesteatoma pada otitis media supuratif kronik (The relationship of RANKL expression with the degree of bone destruction due to cholesteatoma in chronic suppurative otitis media) [In indonesian] Oto Rhino Laryngol Indones 2017; 47:1–9.

3. Park M, Lee B, Lee J. Expression of tumor necrosis factor-like weak inducer of apoptosis in human middle ear cholesteatoma. J Otorhinolaryngol Relat Spec 2013;75:221–7.
4. Vitale RF, Ribeiro FDAQ. The role of tumor necrosis factor-alpha (TNF-alpha) in bone resorption present in middle ear cholesteatoma. Rev Bras Otorrinolaringol (English ed) 2007; 73:123–7.
5. Kuczkowski J, Sakowicz-Burkiewicz M, Iycka-Świeszewska E, Mikaszewski B, Pawełczyk T. Expression of tumor necrosis factor-α, interleukin-1α, interleukin-6 and interleukin-10 in chronic otitis media with bone osteolysis. ORL J Otorhinolaryngol Relat Spec 2011; 73:93–9.
6. Hijriani A, Chandra A, Hardiansyah N, Riki TA. Which Potential as Antiasma) Profil Fitokimia Ekstrak Etanol Daun Puguntano [Curangafel-terrae (Merr.) Lour.] (Phytochemical Profile of Ethanol Extract of Puguntano Leaves [Curangafel-terrae (Merr.) Lour.] [In indonesian] Semin Nas Sains Teknol 2013;422–6.
7. Depkes RI. Inventaris Tanaman Obat Indonesia Inventaris Tanaman Obat Indonesia. 1st ed. (Inventory of Indonesian Medicinal Plants Inventaris Tanaman Obat Indonesia. 1st ed.) Jakarta: Departemen Kesehatan Indonesia, 2000.
8. Barnes PJ. How corticosteroids control inflammation:Quintiles Prize Lecture 2005. Br J Pharmacol 2006;148:245–54.
9. Restuti RD. Metode biakan jaringan kolesteatoma pasien otitis media supuratif kronik tipe bahaya (Cholesteatoma tissue culture method for chronic suppurative otitis media patients with hazard type) [In indonesian] Oto Rhino Laryngol Indones 2013; 43:13–8.
10. Raynov AM, Choung Y-H, Park HY, Choi SJ, Park K. Establishment and characterization of an *in vitro* model for cholesteatoma. Clin Exp Otorhinolaryngol 2008; 1:86–91.
11. Iwamoto Y, Nishikawa K, Imai R, Furuya M, Uenaka M, Ohta Y, Morihana T, Itoi-Ochi S, Penninger JM, Katayama I, Inohara H, Ishi M. Intercellular communication between keratinocytes and fibroblasts induces local osteoclast differentiation: a mechanism underlying cholesteatoma-induced bone destruction. Mol Cell Biol 2016; 36:1610–20.
12. Raffa S, Leone L, Scrofani C, Monini S, Torrisi MR, Barbara M. Cholesteatoma-associated fibroblasts modulate epithelial growth and differentiation through KGF/FGF7 secretion. Histochem Cell Biol 2012; 138:251–69.
13. Rambe AYM, Munir D, Sembiring RJ, Ilyas S. Effect of poguntano leaves extract (Picria fel-terrae merr.) to procalcitonin level in acute bacterial rhinosinusitis model of wistar mice. Med Glas (Zenica) 2020;17:110–6.
14. Gomes CJ, Harman MW, Centuori SM, Wolgemuth CW, Martinez JD. Measuring DNA content in live cells by fluorescence microscopy. Cell Div 2018; 13:1–10.
15. Chan LLY, McCulley KJ, Kessel SL. Assessment of cell viability with single-, dual-, and multi-staining methods using image cytometry. Methods Mol Biol 2017; 1601:27–41.
16. Mukherjee PK. Bioassay-Guided Isolation and Evaluation of Herbal Drugs. In: Quality Control and Evaluation of Herbal Drugs. Neederlands: Elsevier Mosby, 2019: 515–37.
17. Kara A, Duman BO, Yazır Y, SinanYilmaz M, Halbutogulları ZSU, Demir D, Kara RO, Bayraktar H, Guven M. Evaluation of the effect of diclofenac sodium and 5-fluourasil in a 3D cholesteatoma cell culture model. Otol Neurotol 2019; 40:1018-25.
18. Sibangriang. HE. Pengaruh pemberian ekstrak daun Poguntano (Picria fel-terrae (Lour.) Merr.) terhadap Kadar Superoxide Dismutase (SOD) pada penderita diabetes mellitus tipe 2 (Effect of administration of Poguntano (Picria fel-terrae (Lour.) Merr.) Leaf extract to levels of Superoxide Dismutase (SOD) in patients with type 2 diabetes mellitus) [In indonesian] Medan: Universitas Sumatera Utara, 2017.
19. Yassine EZ, Dalila B, Latifa EM, Smahan B, Lebtar S, Sanae A.. Phytochemical screening, anti-inflammatory activity and acute toxicity of hydro-ethanolic, flavonoid, tannin and mucilage extracts of *Lavandula stoechas* L. from Morocco. Int J Pharmacogn Phytochem Res 2016; 8:31–7.
20. Satria D, Silalahi J, Haro G, Ilyas S, Hsb PAZ. Antioxidant and antiproliferative activities of an ethylacetate fraction of picria fel-terrae Lour. herbs. Asian Pacific J Cancer Prev 2017; 18:399–403.
21. Sakane KK, Monteiro CJ, Silva W, Silva AR, Santos PM, Lima KF, Moraes KCM. Cellular andmolecular studies of the effects of a selective COX-2 inhibitor celecoxib in the cardiac cell line H9c2 and their correlation with death mechanisms. Brazilian J Med Biol Res 2014; 47:50–9.
22. Cao Y, Prescott SM. Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. J Cell Physiol 2002; 190:279-86.
23. Yao M, Lam EC, Kelly CR, Zhou W, Wolfe MM. Cyclooxygenase-2 selective inhibition with NS-398 suppresses proliferation and invasiveness and delays liver metastasis in colorectal cancer. Br J Cancer 2004; 90:712–9.
24. Sobolewski C, Cerella C, Dicato M, Ghibelli L, Diederich M. The role of cyclooxygenase-2 in cell proliferation and cell death in human malignancies. Int J Cell Biol 2010; 2010:215158.
25. Kuo CL, Shiao AS, Yung M, Sakagami M, Sudhoff H, Wang CH, C-H, Lien C-F. Updates and knowledge gaps in cholesteatoma research. Biomed Res Int 2015.