International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 9 | Special Issue 1- NCBTIL | 2019 | 112-117
National Conference on Biochemistry –Transcending and Integrating Life Sciences (NCBTIL) -2019
Held @ Kristu Jayanti College, K Narayanapura, Kothanur PO, Bengaluru-560077, January 17th & 18th
| Conference Proceedings | Research Article | Biological Sciences |Open Access |MCI Approved|
|UGC Approved Journal|

Kristu Jayanti College
AUTONOMOUS Bengaluru

In Vitro Evaluation of Anti-bacterial And Biological Activity of Phytochemicals from Spices

Bindu I[1] and Elcey C. D[2*]

[1]Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu, India.
[2]Department of Life science, Kristu Jayanti College (Autonomous), Kothanur, Bangalore-560077. Karnataka, India

Received: 10 Dec 2018 / Accepted: 30 Dec 2018 / Published online: 10 Jan 2019
Corresponding Author Email: elcey@kristujayanti.com

Abstract
The treatment of ulcer and related diseases are still challenging. Macrophages have a major role in the initiation of inflammatory response by the release of Nitric oxide (NO), TNF α etc. The adhesion of *H. pylori* results in inflammatory response which is possibly inhibited by the bio actives. *In vitro* antimicrobial activity against *Helicobacter pylori and* anti-inflammatory activity was evaluated using phyto extracts of selected medicinal plants, *Cinnamomum zeylanicum* and *Punica granatum L*. The methanol extracts of the plants have potential role in inhibiting the growth of *H. pylori* and act as anti-inflammatory agents, cinnamaldehyde, eugenol and linalool from *C. zeylanicum* and ellagic acid from *P. granatum*. Antibacterial activity of the biomarkers was evaluated by MIC against *H. pylori* at different concentration ranging from 4mg to 1.95 ng. The results indicate that the methanol extracts of *C. zeylanicum and P. granatum* and it could be used as an agent for *H. pylori* inhibition.

Keywords
Helicobacter pylori, Antibacterial, Cinnamomum zeylanicum, Punica granatum,

*****

INTRODUCTION
Phytochemicals have been used as bioactive compounds against various chronic and infectious diseases from the ancient time. Herbal drugs are considered safer as compared to synthetic drugs due to rare or zilch side effects.

Role of *Helicobacter pylori* as a causal organism for gastritis and the duodenal and peptic ulcers was well studied [1, 2]. Elimination of the organisms from the site of infection become tedious as it acquires antibiotic resistance during the course of treatment [3]. The infection caused by *H. pylory,* a gram- negative spirally shaped bacterium, is a lifelong disease until it

is specifically treated. The acute infection of this may lead to the peptic ulcer, gastric carcinoma and neurodegenerative disorders [4-7]. Gastritis is a commonly occurring inflammatory condition during the infection of *H. pylori*. The organism also tops the list of bacteria induced oncogenesis. Strategies of treatment for the complete elimination of *H pylori* are still a challenge [8]. It is essential to develop integrated therapies which can associate with food supplements with biological activities. Daily intake of food and its ingredients are highly significant as the organisms are directly exposed to the same. Phyto-molecules from various Medicinal plants were traced for in the treatments of *H. pylori* infection as it was reported evolving as drug resistant organisms [9]. Consumption of natural food has an advantage of reduced incidents of gastritis [10]. Phyto constituents present in food supplements are considered as an important substance for many biological activities. Natural products are of high preference due to the less toxicity [11]. Hence, utilization of natural compounds for many clinical applications has been a continuous effort.

Inflammation is a natural mechanism which helps in the refurbishing of normal structure and function of cell [12]. Prolonged inflammatory response may lead to various chronic diseases [13- 15]. Macrophages play an important role in the initiation of inflammatory response by the release of Nitric oxide (NO), TNF α etc. Nitric oxide has the biological properties of endothelium-derived relaxing factor generated by NO synthase. The inflammatory process initiates with pro-inflammatory cytokines influenced by TNF α [16].

The plants, *Cinnamomum zeylanicum and Punica granatum L* were commonly used as condiments to elevate the flavours of food or fruits. Extracts of these plants were evaluated against *H. pylori, in vitro cytotoxicity and* NO inhibitory activity

MATERIALS AND METHODS

The barks of *C. zeylanicum* were procured from the different sources cleaned and segregated. Fresh fruits of pomegranate were picked from orchards and brought to the laboratory. These fruits were washed, peeled and rinds were cut into smaller pieces and then washed thoroughly with distilled water. It was then dried under sunlight; the pericarp was then kept in hot air (80°c) oven for 2-3 days. Dried pericarp is then ground to get fine powder. All the other plant samples also were dried, powdered and stored in a sterile container until use. The solvents and chemicals used were of analytical grade. The standard of ellagic acid, cinnamaldehyde, linalool, and eugenol were procured from Sigma-Aldrich.

Preparation of Extracts

Plant materials were subjected to extraction processes. To each 100g of powdered materials, 600 ml of purified water was added, and pH was adjusted to 9 using 10% of potassium hydroxide and allowed to stand for an hour and adjusted the pH to 7 using 5% hydrochloric acid during which precipitate was formed. To this 4 L of methanol was added with manual stirring and allowed to stand for 30 minutes. The methanol soluble fraction was filtered through ordinary filter paper and concentrated using water bath at 90°c under fume hood. The obtained extracts were used for further studies.

HPLC and GC chromatographic conditions for the estimation of active compounds.

Cinamaldehyde, eugenol and linalool from *Cinomum zeylanicum*

Phyto-compounds from *C. zeylanicum* were extracted by Chromatographic separation, achieved by Gas chromatography using ZB-5 (30m x 0.25mm ID, 0.25µ film thickness) and nitrogen as carrier gas with 1.0ml flow rate. Injector and detector temperature were set at 220 °c and 250°c respectively using FID as detector (Fig.1).

Ellagic acid from *Punica granatum*

Chromatographic separation of ellagic acid was achieved by the gradient elution method using Reverse-phase chromatography with Hypersil ODS column (5µ, 250 x 4.6). The mobile phase consisted of acetonitrile and different concentrations of phosphoric acid in water ranging from 0.1 to 50%. Different Concentrations of phosphoric acid were used for gradient elution, with a flow rate of 1mL/min *i.e.*, 5, 10, 30 and 50 % with an interval of 0.01, 10, 15, 18, 20 and 22 min. and the peaks were measured at 254 nm. The phyto-compounds were separated using HPLC equipped with a degasser (DGU-20A5), binary pump (LC-20AD), autosampler (SIL-20ACHT) and diode array detector (SPD-M20A) of Shimadzu. The data were processed using LC solutions software version 1.2.

The content of ellagic acid was calculated from the respective peak areas according to individual standard

curves and the content of the compound was 25.0 %(w/w) Fig.2.

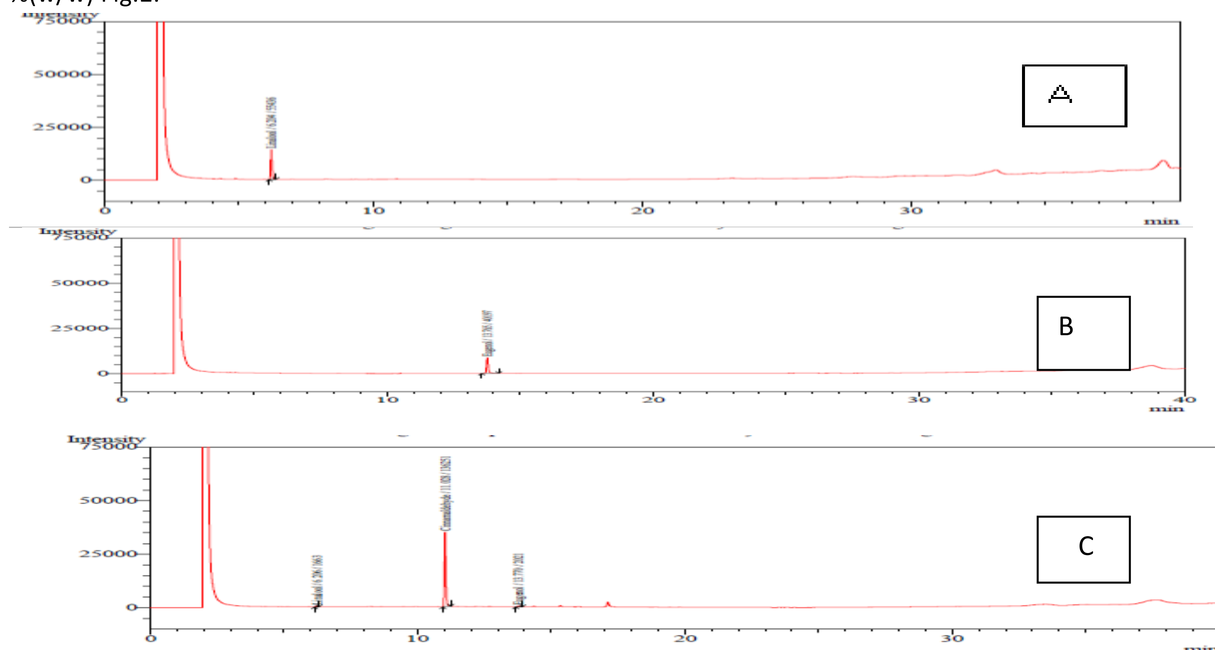

Fig: 1 Chromatograms of Standards of Linalool (A), Eugenol (B) and Extract of *Cinnamomum zeylanicum* barks (C)

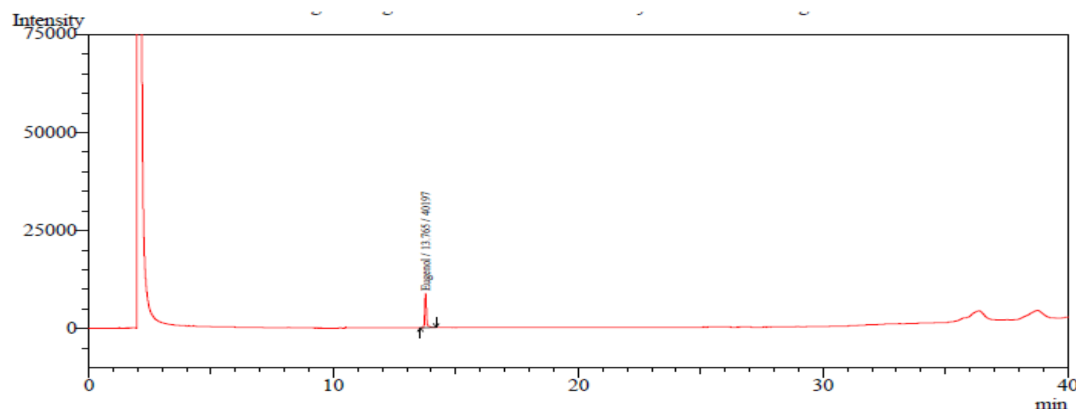

Fig: 2 Chromatograms of *Punica granatum* extract

The *in vitro* cytotoxicity tests were carried out for extracts on Mouse macrophages (RAW) and Mouse fibroblasts (L929) to find its inhibitory concentration and further to evaluate the anti-inflammatory activity.

Preparation of Extract Solution

The purified plant extracts of 10µl each were diluted, and volume was made up with MEM supplemented with 2% inactivated Foetal Bovine Serum (FBS) to obtain a stock solution of 1% v/v (1 mg/ml) concentration and sterilized by 0.22µ syringe filtration. Serial two-fold dilutions were prepared from this for carrying out cytotoxic studies.

Cytotoxicity Studies

The monolayer cell culture was trypsinized and the cell count was adjusted to10^5 cells/ml using MEM/DMEM containing 10% FBS. Each well of the 96 well microtitre plate was loaded with 0.1 ml of the diluted cell suspension. After 24 h incubation, when a partial monolayer was formed, the supernatant was skimmed off, the monolayer washed once with medium and 100 µl of different test concentrations of the extracts were added onto the partial monolayer in microtitre plates. The plates were then incubated at 37° C for72 h in 5% CO_2 atmosphere. Microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the extracts in the wells were discarded and

50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. (Fig. 1)

Anti- Inflammatory Activity

RAW cells were seeded in to 6 well culture dishes at a cell population of 1.5 to 2 x10^5 cells/ml in DMEM with 10% FBS. After 24 h, the cells were treated with known non-toxic concentration of the extracts along with 1µg/ml of lipopolysaccharide (LPS) and incubated at 37°C with 5% CO_2 for 4 h. After incubation, the cell supernatant was collected, centrifuged, separated and stored at -20°c till use.

Nitric Oxide Inhibition

RAW cells were treated with LPS and the processed plant extracts as described above and incubated for 24 h and conditioned media collected were used for nitrite determination which is a biomarker for NO. Equal volume (50µL each) of 0.1% N-1-Napthylethylenediamine dihydrochloride prepared in water, 1% sulfanilamide prepared in 5% Phosphoric acid and cell culture media were mixed in flat bottom 96-well plate incubated for 10–15 min. Coloured end product was measured at 530 nm. Percentage of nitric oxide inhibition was calculated over LPS control (Fig.7).

Antibacterial activity by MIC against *H. pylori*

The purified plant extracts were evaluated for antibacterial activity by MIC against *H. pylori at* different concentration ranging from 4mg to 0.00195mg. The MIC values of test substances were compared with the activity of a standard antibiotic.

Preparation and standardization of stock cultures

Broth culture *of H. pylori* was prepared to evaluate the activity of the plant extract. The culture was inoculated in Brain Heart Infusion Broth (BHIB) containing 5% horse blood and incubated at 37°C under microaerophilic conditions for 3 days. Cell density of the culture was adjusted to 0.17 absorbance at 600 nm (corresponding to approximately 10^8cfu/ml and 0.5 McFarland Standard) using spectrophotometer. Further, it was diluted to a concentration of 10^7 cfu/ml.

Preparation of Resazurin and standard antibiotic solution

The stock solution of Resazurin was prepared by dissolving 2.7mg in 4 ml of sterile saline. The working solution was prepared by dissolving 1ml of stock solution in 5ml of sterile saline. The standard antibiotic, Ciprofloxacin, was prepared by dissolving it in sterile distilled water to 1% concentration.

Preparation of plant extracts

Purified extracts of the plants were prepared for the test by dissolving 40 mg in 1ml of sterile Brain Heart Infusion Broth containing 5% horse serum. Samples were mixed using cyclomixer for 5minutes.

Cytotoxicity

The microtitre plates were loaded with 100μl respective sterile Brain Heart Infusion broth containing 5% horse serum except first three wells ($A_1B_1C_1$) to which 200μl the plant extract was added. The extracts were further diluted serially by removing 100 μ l each from the wells from $A_1B_1C_1$ and constituted in corresponding wells till $A_{12}B_{12}C_{12}$. *H. pylori* broth culture was inoculated to all the wells to obtain 10^6 cfu/ml cell density (10μl bacterial suspension of approximately 10^7 cfu/ml). Control was maintaining parallel to the test ie., 10μl bacterial cell suspension in 100μl broth medium from the well, G1 to G12 and broth control ie., broth medium 100μl, from H1 to H12. A positive control that consists of the 0.1% ciprofloxacin was also loaded in the plate. The plates were incubated at 37°c under microaerophilic condition for 3days. After incubation, 10μl of Resazurin solution was added to all wells. The plates were wrapped with aluminium film and incubated at 37°c for 1hour.The colour change was then assessed visually. Any colour change from purple to pink or colourless was recorded as positive ie., growth of the organism. The lowest concentration at which there is no colour change occurred was taken as the MIC value.

RESULT AND DISCUSSION

The oxidative stress is due to the production of excess reactive oxygen species (ROS) which can initiate signalling pathways of the inflammatory system. The immune system has Macrophages which are the white blood cells that can decrease the immune reactions through the release of cytokines. Macrophages are very important in anti-inflammatory processes by the release the molecules like NO, TNF-α, which in excess amount may lead to several inflammatory diseases.

The present study revealed that the cell lines treated with different concentrations of the plant extracts has

significant toxicity. Higher concentrations of the extract had graded impact on the mortality of the cells. Inhibition of Nitric oxide was significant with graded concentrations of the plant extract (Fig.1) Concentrations ie., 30.44 µg/ml of *P. granatum*, 41.52 µg /ml of *C. zeylanicum* and 57.78 µg/ ml of the sample with an equal ratio of 1:1plant extract showed the maximum inhibition (Fig.2). There is a distinct inhibition of Nitric oxide with the mixed sample in 1:1 proportion against their highest respective individual concentrations.

The MIC value of *C. zeylanicum*, *P. granatum* and the combination were found to be 0.5, 4.3 and 2.0 mg respectively against *H. pylori* (table-1). The MIC of antibiotic, Ciprofloxacin was less than 0.488 µg against the test organism. The secondary metabolites in plants are considered as the molecules involve in their defence mechanisms. It is evident that the phytochemicals present in the plant extracts can resist the growth and activity of *H. pylori*. The above tested plant and their product are consumed as part of regular food ingredients as spices or condiments. Hence the daily use of these ingredients in food might help the body to prevent oxidation and inhibit microbial growth.

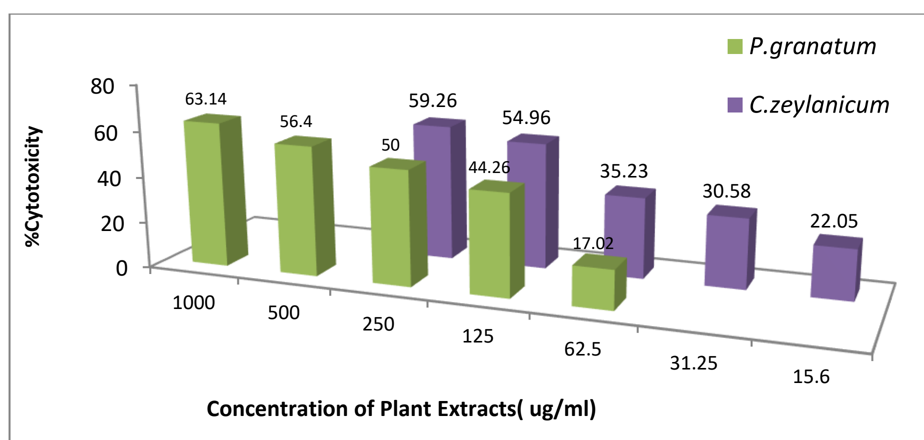

Fig .3 Cytotoxicity of the plant extracts

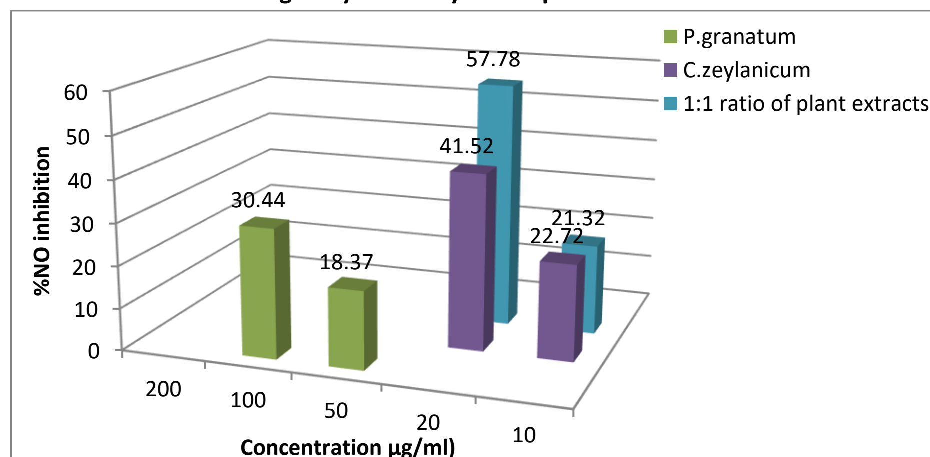

Fig .4 Effect of Plant extract on *Nitric O*xide inhibition

Table1. MIC of plant extract against human *H. pylori*.

Plant extracts	Concentration (%)	MIC (mg) against H. *pylori* n=3)
Cinnamomum zeylanicum (Cinnamaldehyde, Eugenol & *linalool)*	04	0.5
Punica granatum (Ellagic acid)	04	4.3
1:1 ratio of Cinnamomum zeylanicum and Punica granatum	04	2.0
Ciprofloxacin standard	01	0.000488

CONCLUSION

Gastritis and ulcer are due to the host-microbial interactions result in the development of mucosal inflammation. The increased side effect and resistance to antibiotic against *H. pylori* may be one of the reasons for the consideration of natural sources as an alternative way. The use of plant extract can control the infection caused by *H. pylori* which may be due to the presence of secondary metabolite in the plant extract. The *in vitro* studies have been conducted with the plant extracts to find out the anti-*H. pylori* activity. These extracts are able to reduce the growth of this pathogen, which is considered as the main causal agent responsible for human gastritis. The present investigation has demonstrated that the commonly used spices have several phyto constituents including phenolic compounds, flavonoids and alkaloids which are the dynamic factor for the selection of these plants and can be used as the anti-inflammatory and anti-bacterial drug. Safavi *et al* also emphasised the use of traditional folk medicines for the treatment of gastric infections and antibacterial activity of plant extracts against H. pylori [8]. The active substances which are identified in the plant extract screened in this experiment have considerable properties against the inhibition of Nitric Oxide and *H. pylori*. The present study concludes that the consumption of commonly used plant such as, *Cinnamomum zeylanicum* and *Punica granatum* can prevent gastrointestinal inflammation, and its daily consumption may have remarkable beneficial effects on human health.

REFERANCES

1. Megraud, F. and Lehours P., *H. pylori* detection and antimicrobial susceptibility testing. Clin. Microbiol. Rev., 20: 280-322 (2007).
2. Marshall, B.J. and Warren J.R., Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet, 1: 1311-1315 (1984).
3. Megraud, F., H. *pylori* antibiotic resistance: prevalence, importance and advances in testing. Gut, 53:1374-1384 (2004).
4. Marshall B J. and Armstrong, J. Marshall BJ, Armstrong JA, McGechie DB, Glancy RJ. Attempt to prove Koch's postulates for pylori campylobacter.*Med. J. Aust.*, 142, 436–439 (1995).
5. Parsonnet J., Friedman G.D., Vandersteen D.P., Chang Y., Vogelman J.H., Orentreich N., Sibley R.K., *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.*, 325, 1127–1131(1991).
6. Wotherspoon, A. C., Ortiz-Hidalgo, C., Falzon, M.R., Isaacson, P. G., *Helicobacter pylori*- associated gastritis and primary B-cell lymphoma. *Lancet*, 338, 1175–1176(1991).
7. Diaconu S., Predescu A., Moldoveanu A, Pop C.S., and Braticevici C. F., *Helicobacter pylori* infection: old and newJ Med Life. Apr-Jun; 10(2): 112–117 (2017).
8. Weyermann M, Rothenbacher D., Brenner H., Acquisition of Helicobacterpylori infection in early childhood.: independent contributions of infected mothers, fathers, and siblings. Am J Gastroenterol.104:182-189 (2009).
9. Safavi M., Sabourian R., Foroumadi A..Treatment of Helicobacter pylori infection: Current and future insights. World J Clin Cases. Jan 16;4 (1):5-19 (2016).
Parsonnet J. *Helicobacter pylori*: the size of the problem. Gut. 43: S6–S9 (1998).
10. Gullett N.P., Ruhul Amin A.R., Bayraktar S., Pezzuto J.M., Shin D.M., Khuri F.R., Aggarwal B.B., Surh Y.J., Kucuk O. Cancer Prevention With Natural Compounds. Semin Oncol. ; 37:258–281 (2010).
11. Naik, G.H., Priyadarsini K.I, Satav,Banavalikar J.G., Sohani D.P.,. Biyani M.K., Mohan H., Comparative antioxidant activity of individual herbal components used in ayurvedic medicine. Phytochemistry, 63: 97-104 (2003).
12. Galli S.J., Grimbaldeston M., Tsai M. Immuno modulatory mast cells: negative, as well as positive, regulators of immunity.Nat Rev Immunol 8: 478-486 (2008).
13. Hofseth L.J. and Ying L..Identifying and defusing weapons of mass inflammation in carcinogenesis. Biochim BiophysActa .1765: 74-84 (2006).
14. Leung D.Y. Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic diseases. J Allergy Clin Immunol 96:302-319 (1995).
15. McMahon B. J., Bruce M. G., Koch A., Goodman K. J., Tsukanov V., Mulvad G., Borresen M. L., Sacco F., Barrett D., Westby S. and Parkinson A. J. The diagnosis and treatment of Helicobacter pylori infection in Arctic regions with a high prevalence of infection: Expert Commentary. Epidemiol Infect. 144(2): 225–233 (2016).
16. Möller B., Villiger P.M. Inhibition of IL-1, IL-6, and TNF-αin immune-mediated inflammatory diseases. Springer Semin Immunopathol. 27: 391-408 (2006).