Video Article

# Determination of Tolerable Fatty Acids and Cholera Toxin Concentrations Using Human Intestinal Epithelial Cells and BALB/c Mouse Macrophages

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## Abstract

The positive role of fatty acids in the prevention and alleviation of non-human and human diseases have been and continue to be extensively documented. These roles include influences on infectious and non-infectious diseases including prevention of inflammation as well as mucosal immunity to infectious diseases. Cholera is an acute intestinal illness caused by the bacterium *Vibrio cholerae*. It occurs in developing nations and if left untreated, can result in death. While vaccines for cholera exist, they are not always effective and other preventative methods are needed. We set out to determine tolerable concentrations of three fatty acids (oleic, linoleic and linolenic acids) and cholera toxin using mouse BALB/C macrophages and human intestinal epithelial cells, respectively. We solubilized the above fatty acids and used cell proliferation assays to determine the concentration ranges and specific concentrations of the fatty acids that are not detrimental to human intestinal epithelial cell viability. We solubilized cholera toxin and used it in an assay to determine the concentration ranges and specific concentrations of cholera toxin that do not statistically decrease cell viability in BALB/C macrophages.

We found the optimum fatty acid concentrations to be between 1-5 ng/µl, and that for cholera toxin to be < 30 ng per treatment. This data may aid future studies that aim to find a protective mucosal role for fatty acids in prevention or alleviation of cholera infections.

## Video Link

The video component of this article can be found at https://www.jove.com/video/50491/

## Introduction

The health benefits of fatty acids, such as oleic, linoleic and linolenic acids have been and continue to be documented. For example, oleic acid helps facilitate penetration of lipophilic drugs in the body[1,2], reduces coronary heart disease by 24% when substituted for saturated fatty acids[3], and is used to treat metabolic diseases such as Adrenoleukodystrophy[4] which is an X-linked genetic disorder of fatty acid metabolism. While a necessary precursor for arachidonic acid in mammals, linoleic acid (unlike oleic acid) is not synthesized by the body and must be obtained through outside sources such as by flax seed consumption.[5] Studies show several beneficial health effects of linoleic acid such as: anti-aging properties for the skin;[6] anti-inflammatory properties;[7] reduced proliferation of colorectal and prostate carcinoma cells;[8] and the ability to fight obesity and promotion of cardiovascular health.[9] Linolenic acid plays a role in reducing periodontal inflammation,[10] and modulating thromboxane and prostacyclin biosynthesis.[11]

Arpita[12] studied the influence of bile fatty acids and cholesterol on *V. cholerae*'s expression of virulence factors and motility. Yamasaki[13] indicated that methanol extract from red chili peppers, and other naturally extracted compounds, can potentially decrease cholera toxin production. It is conceivable to consider the use of food products that are rich in the above fatty acids (such as flax seeds) in the prevention and alleviation of infectious disease such as cholera through providing mucosal immunity. We conducted investigations to solubilize fatty acids and to determine, using cell proliferation assays, the maximum concentration of fatty acids that human intestinal epithelial cells can tolerate without detrimental effects on cell viability. We hypothesized that oleic, linoleic and linolenic acids provide a beneficial effect on cell viability at lower concentrations, but that at higher concentrations they will be toxic to the cells. We also solubilized the cholera toxin and determined the maximum concentration of cholera toxin that BALB/C mouse macrophages can tolerate without a significant decrease in cell viability. We hypothesize a toxic effect of cholera toxin on cell viability even at very low level. The method of solubilizing a cholera toxin and using it to determine the maximum amount of the toxin that the cells can tolerate without a significant decrease in survivability provides an advantage for future studies. For example, a combination of the above methodologies can be used to determine whether fatty acids provide cells with mucosal immunity against cholera infections. To the best of our knowledge, this rational and methodology has not been explored.

We discuss how our preliminary data can be used in later investigations to determine if oleic, linoleic and linolenic acids provide cells with mucosal immunity against cholera infection.

## Protocol

### 1. Tissue Culture

1. Use *Mus musculus* macrophages (BALB/c mice) for cholera toxin determinations. Initially culture all of *M. musculus* cells following vendor's instructions.
2. Propagate BALB/c mice cells in Dulbecco's Modified Eagle's Medium with L-glutamine completed with 10% fetal bovine serum, and 1% antibiotic/antimycotic or RPMI 1640 base media completed with 10% fetal bovine serum, 5% L-glutamine, and 1% antibiotic/antimycotic reagent.
3. Grow cells in 75 $cm^2$ corning flasks with vented caps at 37 °C, 95% air and 5% $CO_2$.
4. Bring up human intestinal epithelial cells within 24 hr of delivery as per manufacturer's instructions for the determination of fatty acid determinations.
5. Propagate the human intestinal epithelia cells in 75 $cm^2$ corning flasks with vented caps in HybriCare media completed with 10% FBS and 30 ng/ ml human EGF at 37 °C. Do not use Antibiotic/antimycotic reagents as per manufacturer's instructions.
6. Split all cells (1:3) at approximately 70% confluency.
7. Freeze and bring up all cells throughout the study using standard freezing protocols.
8. **Note:** For larger scale concentration determination of fatty acids, use the faster growing, easier to maintain, mouse macrophages initially. For fine scale fatty acid concentration determination, use human epithelial cells. Use mouse macrophages for all cholera toxin treatments (see discussion).

### 2. Fatty Acid and Cholera Toxin Treatments

1. Transfer oleic, linoleic and linolenic acids from company-provided glass ampules to sterilized glass vials.
2. Transfer each fatty acid to a separate sterilized Eppendorf tube and dissolve it first in 100% ethanol at a dilution of 1:6, and then in RPMI 1640 incomplete media for a final concentration of 10 µg/µl.
3. Vortex each solution and transfer it to glass vials for storage in the freezer (-20 °C).
4. Plate cells at 2,500 cells/well in 96 well tissue culture plates.
5. Add the appropriate complete media to bring the total well volume to 200 µl for treatment of cells with fatty acids in preparation for MTT assays.
6. After a 24 hr proliferation period, remove the media and replace it with fresh media.
7. Add the appropriate concentrations of each fatty acid to be tested to the wells (see results). Add complete media to bring the total well volumes to 200 µl.
8. Incubate the treated plates for 24 hr before beginning the MTT assay.
9. Dissolve the cholera toxin in PBS at a concentration of 1 mg/ml and aliquot the solution in cryovials and store the cryovials at 2-8 °C.
10. For cell treatments, dilute the toxin 1:100 in either sterilized PBS (pH 7.4) or incomplete DMEM for a final working concentration of 1 ng/µl.
11. To treat cells with cholera toxin in preparation for MTT assays, plate cells as described above.
12. After a 24 hr proliferation period, add the appropriate concentrations of cholera toxin (for our concentrations, see results) to be tested to the wells following the procedures used in fatty acid applications (above).
13. Incubate the treated plates for 24 hr before beginning the MTT assay.
14. As a positive (positive c throughout the paper) and negative control (negative c), for both fatty acid and cholera toxin treatments, incubate cells with complete medium and 70% ethanol, respectively.
15. For all samples and treatments use a minimum of three replications (n=3), with more replications for positive controls (n=6 in this study).

### 3. MTT Assay

1. Make a MTT stock solution to a concentration of 2.5 mg/ml.
2. Remove the solution in each well of the 96 well plate to be tested and replace it with 200 µl of fresh complete media solution.
3. Add 10 µl of the MTT solution to each well.
4. Incubate the plate at 37 °C for 3-4 hr.
5. Discard the media solution and add 100 µl of 0.04 M HCl in isopropanol to each well.
6. Incubate the plate at room temperature for five minutes.
7. Transfer the solution from each well to a new centrifuge tube.
8. Centrifuge at 20,000 x g, at room temperature, for 1 min, or until a pellet was formed.
9. Transfer between 20-40 µl of each sample to a microplate reader.
10. Read absorbance at 570 nm using a spectrophotometer.

## Representative Results

**Determination of the Optimum Concentration of Fatty Acids**

The optimum concentration for fatty acids is defined as the maximum concentration at which cell growth is comparable to or exceeds that of control cells, with relatively low variability in results. To determine the optimum concentration of oleic, linoleic and linolenic acids cells were initially treated with varying concentrations of each fatty acid in large increments and later with smaller increments. **Figure 1** shows the survival

of cells depicted as a function of the positive control for treatments using increasing concentrations of the fatty acids. It is evident that the cells do not tolerate concentrations of above 100 ng/µl for any of the fatty acids. A one way ANOVA showed that for each of the treatments, one or more of the means are different from the others ($p$<0.001). A post-hoc test (Tukey's) showed that for oleic acid all treatments except the 1 ng/µl were different from the positive control. Interestingly, due to high variability, this treatment was marginally not significantly different from the negative control either. For both linoleic and linolenic acids, one way ANOVA showed that at least one mean is different than the others ($p$<0.001). A Tukey's post-hoc test revealed that the mean of the 10 ng/µl treatment for either fatty acid is not significantly different from the positive control, but are from the negative control ($p$<0.05).

This experiment was repeated for concentrations that were lower than 100 ng/µl, in 10 ng/µl increments (up to 70 ng/µl, using human epithelial cells). Cell survival is provided for each concentration in **Figure 2**. For oleic acid (**Figure 2a**), one way ANOVA showed that one or more of the means were different (p<0.001). However, it is only the treatments at higher concentrations (40 and 50 ng/µl) that rendered means statistically different from that of the positive control. Interestingly, the differences observed were due to an increase in cell viability (with more variability, **Figure 2a**). One way ANOVA yielded similar results for linoleic and linolenic acids. Specifically, none of the treatment means were different from the mean of the positive control. Once again, variability seems to increase for some of the higher concentrations (**Figures 2b** and **2c**).

**Determination of the Optimum Concentration of Cholera Toxin**

The optimum concentration for cholera toxin is defined as the concentration at which cell viability is not affected or just begins to decrease as compared to untreated cells. To assess the minimum concentration of cholera toxin that minimally decreases cell viability, cells were initially treated with varying concentrations of cholera toxin in large increments and later with smaller increments. **Figure 3a** shows a decreasing viability trend for cell survival as a result of cholera toxin treatments for toxin levels at relatively large increments (between 100-1,000 ng, $R^2$; = 0.5436). As expected, cell viability varied greatly for each treatment (**Figure 3a**). The large observed variability in viability resulted in no significance difference between the mean of our positive control with any of the treatments (one-way ANOVA). Based on these results, we determined that toxin levels <100 ng should be tested further. **Figure 3b** shows cell viability for the smaller toxin level treatments. Cell viability is highly variable for almost all toxin concentrations, albeit slight increases were observed in viability as a result of the treatments. These increases (**Figure 3b**) are not statistically significant for any of the treatments compared to the mean of the positive control (one way ANOVA, Tukey's).

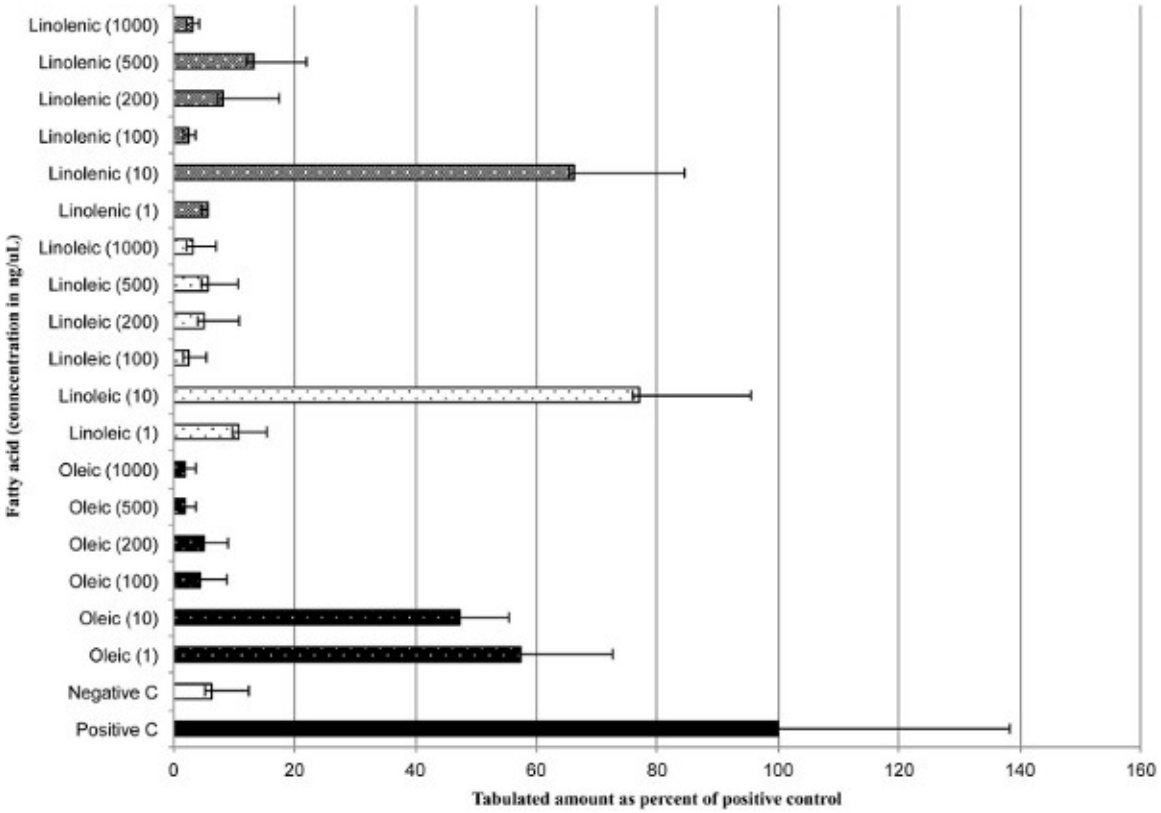


**Figure 1. Determination of usable concentrations of fatty acids: Effect of varying concentrations of fatty acids in relatively large increments (100-1,000 ng/µl) for oleic acid, linoleic acid and linolenic acids.** Click here to view larger figure.

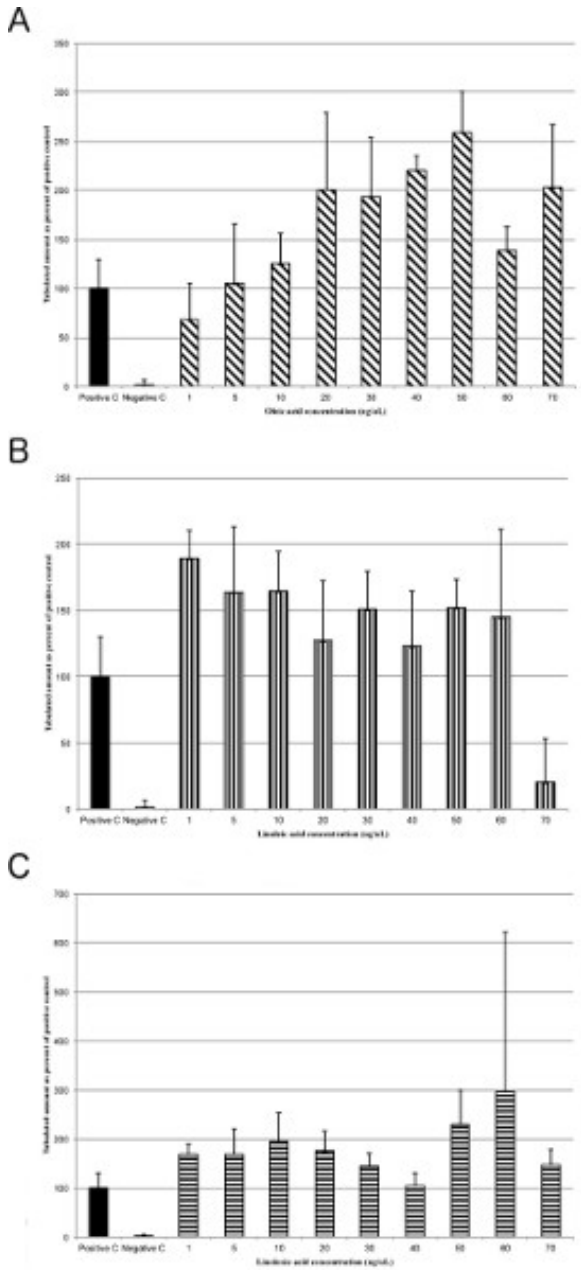


**Figure 2. Fine scale fatty acid concentration determination: Effects of varying concentrations of fatty acids in smaller increments (10 ng/µl) on human intestinal epithelial cell survival.** Intestinal epithelial cells were treated with varying concentrations of oleic **(a)**, linoleic acid **(b)** and linolenic acid **(c)**. The positive control is cells treated with complete media only, and the negative control with 70% ethanol. For each trial, n=3 for all treatments except positive control (n=6). Click here to view larger figure.

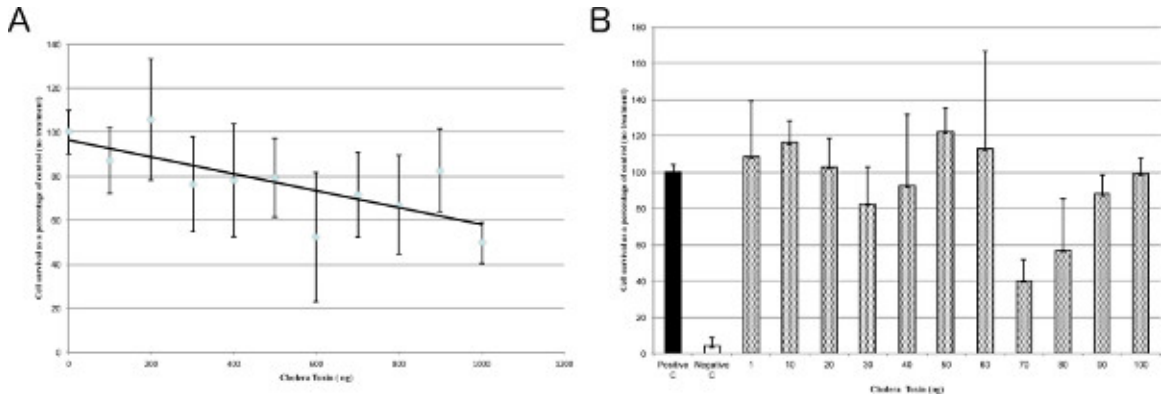


**Figure 3. Determination of usable cholera toxin concentrations based on assessment of application of varying concentrations of cholera toxin on mouse macrophage survival: Mouse macrophages were treated with varying concentrations of cholera toxin in larger increments (a) then smaller ones (b).** The positive control is cells treated with complete media only, and the negative control with 70% ethanol. For each trial, n=3 for all treatments except positive control (n=6). Click here to view larger figure.

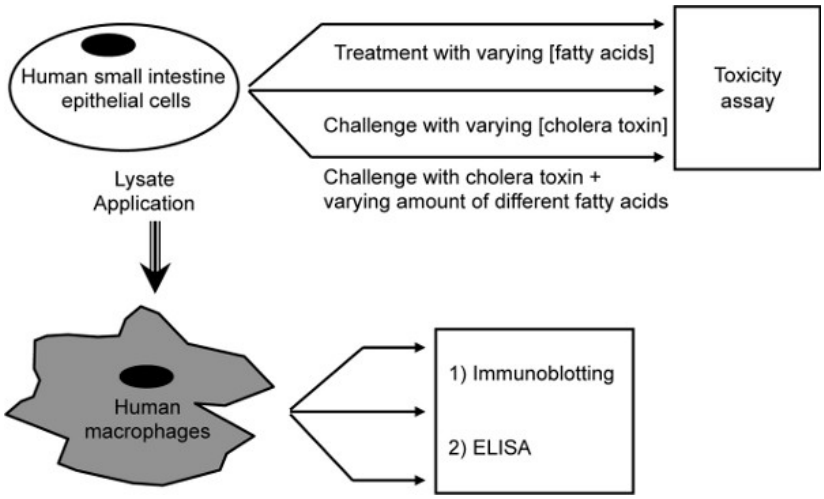


**Figure 4. Experimental design-determination of appropriate fatty acid and toxin concentrations for use in future studies: Cell lysates can be used to treat macrophages and cytokine dynamics can be qualitatively and quantitatively assayed by immunoblotting and ELISA using antibodies against specific cytokines as markers for infection.**

## Discussion

### Suggestion of Concentration of Fatty Acids and Cholera Toxin

While the exact mechanism of how fatty acids enhance mucosal immunity is unknown, several studies have attempted to investigate their beneficial effects. Our study aims to provide methodology to determine the maximum concentration of fatty acids that cells can tolerate as well as the maximum concentration of cholera toxin that cells can tolerate without a significant influence on cell survival.

To determine the concentration of three fatty acids (oleic, linoleic and linolenic) as well as that of cholera toxin for treatment of cells without very significant adverse effect on cell viability we solubilized the fatty acids and the cholera toxin and treated tissue cultured cells to differing concentrations followed by an MTT assay to assess viability. Based on our methodology and results we provide ranges of fatty acids and cholera toxin useful for future investigations that aim to use these fatty acids or cholera toxin (see below). We recommend concentrations of 1, 5-10, and 5-10 (ng/µl) for oleic acid, linoleic acid, and linolenic acids, respectively, for using human intestinal epithelial cells. We also recommend the use of <30 ng (per 200 µl, see methods) of cholera toxin using BALB/C mouse macrophages. We recognize that, at least for cholera toxin, no significant difference was observed between cell viability for the positive control compared to the treatments. However, our recommendation is based on the relatively high variability in cell viability that was observed as a result of higher toxin level exposures.

### Future Studies

The data provided in this study for fatty acids and cholera toxin levels can be used in future studies. We provide an example of one such investigation here.

Cholera is a disease associated with developing countries that do not have proper sewage and water treatment plants and safe drinking water. In 2009, a death toll of over 3,000 was reported for a five month period due to cholera related symptoms in Zimbabwe alone.[14] As of 2011, cholera remains an epidemic in different regions of the world including an outbreak in Haiti (CDC, 2010). A molecular genetics study in 2010 showed evidence that the bacterium in this outbreak was of Nepalese origin.[15] The disease is caused by ingestion of fecal-contaminated food or drinking

water and colonization of the intestine by *V. cholerae*, followed by the release of an enterotoxin. While cholera vaccinations do exist, they are not always effective in preventing infection during outbreaks. Effective and inexpensive interventions are needed for populations where cholera outbreaks occur. One approach is to study whether food sources that are rich in derivatives such as oleic, linoleic and linolenic acids provide or enhance mucosal immunity against cholera infection. Mucosal immunity functions through the use of protein receptors found on the surface of cells, such as Toll-like receptors[16] (TLRs) and nucleotide-binding domains (NLRs). Through changes in the membrane composition and the release of ligands that interact with TLRs and NLRs, the addition of fatty acids to the intestinal epithelium may enhance the receptor function in recognizing hazardous microbes and recruiting the appropriate immune responses. To determine fatty acids effects on cholera infection, cytokine dynamics, such as the cell's release of TNF- α, IL-6, IL-10 and IL-12, must be studied more so in human intestinal epithelial cells than in mouse macrophages (**Figure 4**). Such knowledge from future studies will help us better understand the mechanism by which fatty acids might work to prevent infection, in order to ultimately provide effective interventions to inhibit cholera outbreaks. It is possible that treatment of cells with fatty acids prior to cholera toxin infection will help induce and/or enhance the mucosal immune response in the intestinal epithelium, ultimately increasing cell viability during microbial cholera infection.

## Disclosures

The authors of this study have no financial interest from the results of this study. Funding for this study was provided to FT by Kean University; however, FT is currently an employee at Kingsborough Community College.

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## References

1. Franceur, M.L., Golden, G.M., & Potts, R.O. Oleic Acid: Its effects on stratum corneum in relation to (trans) dermal drug delivery. *Pharm Res.* **7** (6), 621-7 (1990).
2. Tandon, P., *et al.* X-ray diffraction and spectroscopic studies of oleic acid-sodium acetate. *Chem. Phys. Lipids.* **109** (1), 37-45 (1990).
3. Kris-Etherton, P.M. The debate about n-6 polyunsaturated fatty acid recommendations for cardiovascular health. *Journal of the American Diabetic Association.* **110** (2), 201-204 (2010).
4. Rizzo, W.B., Phillips, M.W., *et al.* Adrenoleukodystrophy: dietary oleic acid lowers hexacosanoate levels. *Annals of Neurology.* **21** (3), 232-9 (1987).
5. Bozan, B. & Temelli, F. Chemical composition and oxidative stability of flax, safflower and poppy seed and seed oil. *Bioresource Tech.* **99**, 6354-6359 (2005).
6. Krein, S., Meldurm, H., Hawkins S., & Foy V. Clinical benefits of conjugated linoleic acid to 3-dimensional wrinkle morphology. *J. American Academy of Dermatology.* **60** (3), AB30 (2009).
7. Yu, Y., Correll P.H., & Heuvel. J.P. Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: Evidence for a PPARy dependent mechanism. *Biochimica et Biophysica Acta.* **1581** (3), 89-99 (2002).
8. Palombo, J., Ganguly, A., Bistrian, B., & Menard, M. The anti-proliferative effects of biologically active isomers of conjugated linoleic acid on human colorectal and prostatic cancer cells. *Cancer Letters.* **177**, 163-172 (2002).
9. Granda, M. & Sinclair, A.J. Fatty acids and obesity. *Current Pharm. Design.* **15** (36), 4117-25 (2009).
10. Rosenstein, E., Kushner, L., Kramer, N., & Kazandjian G. Pilot study of dietary fatty acid supplementation in the treatment of adult periodontitis. *Prostaglandins, Leukotrienes and Essential F.A.* **68** (3), 213-218 (2003).
11. Ferretti, A. & Flanagan, V. Antithromboxane activity of dietary alpha-linolenic acid: a pilot study. *Prostaglandins Leukot. Essent. Fatty Acids.* **54** (6), 451-455 (1995).
12. Arpita, C., Pradeep, K.D., & Chowdhury, R. Effect of Fatty Acids and Cholesterol Present in Bile on Expression of Virulence Factors and Motility of *Vibrio cholera. Infection and Immunity.* **75** (4), 1946-1953 (2007).
13. Yamasaki, S., Asakura, M., Neogi, S.B., Hinenoya, A., Iwaoka, E., & Aoki, S. Inhibition of virulence potential of *Vibrio cholerae* by natural compounds. *Indian J. Med. Res.* **133** (2), 232-239 (2011).
14. World Health Organization. Efforts must be intensified to control Zimbabwe cholera outbreak [WHO PRESS RELEASE]. http://www.who.int/csr/don/2009_01_30/en/ Accessed on: 10 January 2010, (2010).
15. Hendriksen, R.S., Price, L.B., *et al.* Population Genetics of *Vibrio cholerae* from Nepal in 2010: Evidence on the origin of the Haitian outbreak. *mBio.* **2** (4), 1-6 (2011).
16. Schaeffler, A., Gross, P., *et al.* Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappa B pathway in adipocytes links nutritional signaling with innate immunity. *Immunology.* **126** (2), 233-245 (2009).

 