Journal of Sustainability Science and Management
Volume 12 *Number* 2, December 2017: 96-102
ISSN: 1823-8556

WOUND HEALING PROPERTIES OF BIOTRANSFORMED ASIATICOSIDE BY *Aspergillus niger*

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Abstract: Biotransformation is extensively used to create useful metabolites from various natural products and as an alternative to chemical synthesis for the preparation of pharmacologically-active compounds. This study investigated the effects of asiaticoside and its biotransformed product on wound healing activities. Asiaticoside, the bioactive constituent of *Centella asiatica* has been reported to possess wound healing properties. Microbial transformation of asiaticoside using *Aspergillus niger* was carried out to produce an asiaticoside biotransformed product and the wound healing activities of asiaticoside and its biotransformed product were investigated. Their effects on transforming growth factor-beta 1 (TGFβ1) and tissue inhibitor of metalloproteinase 1 (TIMP1) gene expression were examined to understand the mode of action and on the cell proliferation and wound healing using human keratinocytes. Results of *in vitro* study showed that asiaticoside concentrations between 7.5 and 120 µg/mL gave higher value of cell proliferation than the negative control. On the other hand, the biotransformed product concentrations between 0.058 and 3.75 µg/mL exhibited high cell viability but the viability was lowest at 15.0 µg/mL, suggesting cytotoxic effects on the cells. In wound healing assays, there were significant differences on wound closure in comparison to the negative control ($P<0.05$). Both asiaticoside and the biotransformed product increased the expression of TGFβ1 and TIMP1 respectively, with the latter showing more enhanced expressions of both genes. The biotransformed product also showed faster migration and healing rate under microscopic observation.

Keywords: *Centella asiatica,* wound healing, asiaticoside, biotransformation.

Introduction

Biotransformation using microorganisms has been extensively used since the early days of mankind for the production of dairy products, alcoholic beverages and bread. Microbial transformation is used to create new and useful metabolites from terpenes, steroids and herbal extracts. This can be used as an alternative route to chemical synthesis for the preparation of pharmacologically-active compounds (Omar *et al*., 2012; Chen & Chen, 2013). Today, there is a great interest to exploit whole-cell catalysis using microorganisms as natural reagents in organic synthesis especially for the production of drug metabolites, carbohydrates and amino acids.

Biotransformation has been demonstrated to be very useful to obtain considerable volumes of metabolites for pharmacological and toxicological studies. For example, asiaticoside (AS) has previously been transformed by some enzymes extracted from different types of microorganisms (Monti *et al*., 2005). AS is a triterpene glycoside isolated from *Centella asiatica* leaves and has been reported to exhibit anti-microbial effects and wound healing properties (Grimaldi *et al*., 1990).

The present study was aimed to establish better understanding on wound healing activities of AS and AS-biotransformed product (ABP). Their mode of actions was determined by examining the correlation between the phenotypic results and the expression of wound healing-related genes such as TGFb1 and TIMP1 by using semi-quantitative PCR technique.

Materials and Methods

All reagents were used as received from the suppliers without further purification. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen USA), penicillin-streptomycin from GIBCO (USA), phosphate buffered saline (PBS) – magnesium and calcium-free, from Fisher Scientific (New Hampshire, USA), MTT reagent from Sigma-Aldrich (USA) and TrypLE express (1X) from Thermo Fisher Scientific (USA). The HaCaT cell-line, the transformed aneuploid immortal keratinocyte cell line from adult human skin was obtained from ATCC (USA).

Biotransformation of Asiaticoside

AS was isolated from *C. asiatica* leaves and then converted using *Aspergillus niger* into ABP according to the previously reported method (Alfarra & Omar, 2014).

MTT Cell Viability Assay

The cytotoxicity of AS and ABP was evaluated using the MTT assay according to the established procedures with slight modifications (Mossmann 1983; Roy *et al.*, 2010; Lopez-Garcia *et al*., 2014). Briefly, a confluent HaCaT cells (95-100%) were trypsinized. Cells were then seeded (60% of the 95% confluent) into 96-well plates and incubated at 37 °C for 24 h. After incubation, the old media was removed and the cells were exposed to different concentrations of AS and ABP. The HaCaT cell proliferation and viability was observed after 24 h of exposure to the compounds using MTT assay.

Cell Migration and Wound Healing Assay

Scratch assay was done with trypsinization of the 95%-100% confluent HaCaT cells according to Yang *et al*. (2011). The cells were seeded in 12-wells plate at 60% of the 95%-100% confluent. One mL of cells was added into each well and the plate was shaken gently to for well-distribution of cells. The plates were incubated at 37 °C and 5% CO_2 for 24 h. The outer bottom surface of the plate was scratched with a needle to mark the area of the scratch. After 24 h, the 70-80% confluent monolayer was checked under the inverted microscope.

Gene Expression Analysis

HaCaT Cells Preparation and Exposure

HaCaT cells were distributed into five plates of 10 cm^2 area and incubated at 37 ^oC, 5% CO_2 for 24 h. After that, three plates were treated with the concentrations of each product that gave the best results in the scratch assay. Another plate was treated with DMSO-DMEM while the remaining plate was incubated with fresh DMEM for 24 h.

RNA Extraction and cDNA Synthesis

RNA isolation was carried out using TRIzol isolation protocol (Chomczynski & Mackey, 1995). RNA was reverse-transcribed according to the Omniscript Reverse-Transcription kit protocol (Qiagen GmbH, Hilden). The reaction tube was incubated for 60 min at 37 ^oC and then removed. Another 20 mL diethyl pyrocarbonate (DEPC)-treated H_2O (DEPC-H_2O) was added to the tube and the reaction tubes were stored at -20 ^oC.

Sample Preparation for PCR Reactions

Qiagen RT-PCR was used to amplify the target genes to examine them by gel electrophoresis. The SYBR Green method was used for PCR reactions and the reagents included 2X Quantitect SYBR Green (Qiagen, Germantown, MD), Roche Light Cycler Capillaries (Thermo Fisher Scientific, Singapore), Blocks and Primers Main Stock (Forward and Reverse, 100 mM each) were purchased from Integrated DNA Technologies (IDT, Iowa, USA). The master mixture was mixed thoroughly and 18 µL was dispensed into 0.1 mL tubes for each reaction. Then, 2 µL of sample was added per reaction. The tubes were capped securely and arranged in rotor and then the PCR was started. The PCR conditions were optimized first according to the properties provided in the primers' specification sheets and some runs were performed to obtain the optimum melting, annealing and elongation

time for each gene, for a clear band to appear on the agarose gel.

Gel Electrophoresis

PCR products were separated by 2% agarose gel electrophoresis for 30 min at 160V. Ethidium bromide 0.005% was used to visualize the PCR products in a UV chamber. Photos were taken and gene expressions were quantified using ImageJ software (US National Institute of Health, Bethesda, MD).

Statistical Analysis

Experiments were done in triplicate and all the data were expressed as mean ± standard deviation (SD). Differences between control and experimental groups were analyzed by two-way analysis of variance and the student's t-test using GraphPad Prism software package version 5.03 (2009) (GraphPad, San Diego, CA).

Results and Discussion

MTT Assay

The HaCaT cells were used in this study to investigate the effects of AS and ABP on cell proliferation and migration and wound closure. As shown in Figure 1, AS concentrations between 7.5-120 µg/mL gave higher cell proliferation than the control (100% cell viability). The 240 µg/mL concentrations gave the lowest viability, though no indication of cytotoxic effects. The IC_{50} determined by Instat software package (GraphPad Software Inc., San Diego, CA) suggested the value of ~446.2 µg/mL for AS which was close to the results reported by de Oliveira *et al*. (2013), taking into consideration that IC_{50} may be affected by the cell types. The results showed that AS had indeed low toxicity on human HaCaT cell lines at the highest used concentration of 240 µg/mL. On the other hand, the ABP concentrations between 0.058-3.75 µg/mL resulted in reasonably high viability, whereas the viability was lowest at 15.0 µg/mL. The tested concentrations between 15-240 µg/mL had severe toxicity towards HaCaT cells and the cells were almost totally killed (Figure 1). The IC_{50} of the ABP was ~ 11.23 µg/mL. According to Andriani *et al.* (2011), cytotoxic activity criteria are obtained if the IC_{50} value is less than 30 µg/mL. Thus, it can be concluded that AS was not cytotoxic to HaCaT cells at any concentration, but ABP showed extreme cytotoxicity on the cells suggesting that the changes were due to the biotransformation process.

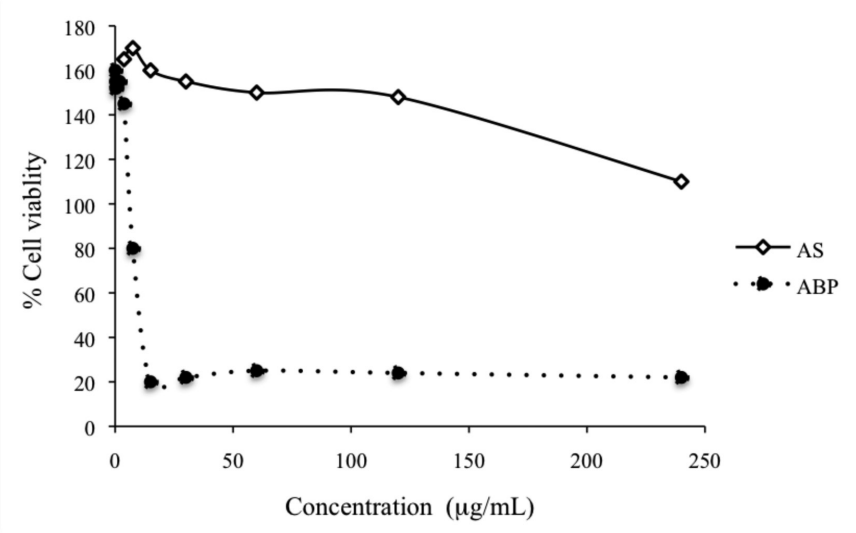

Figure 1: Effects of Asiaticoside and its Biotransformed Product (ABP) on HaCaT cell viability

Scratch Assay

Figure 2 shows the effect of the different concentrations of AS and ABP on wound closure in comparison to the negative controls. After 48 h, the negative control promoted only about 55% of wound closure while AS and ABP accomplished wound healing closure to 96% and 85% respectively. Statistical analysis on the phenotype observation showed that there were significant differences in comparison to the negative control (P<0.05), but no significant variations between AS and ABP on wound closure. The main difference was that ABP could provide the same results at very low concentrations (about 13 times lesser) in comparison to the AS. The high cytotoxicity of ABP was probably the main reason for lower wound closure than AS. It also suggests that ABP may contain other compounds that could inhibit or slightly reverse the wound healing activity of the other compounds. However, ABP with its cytotoxic and growth promoting compounds can be used for external wound dressing.

Effects of AS and ABP on TGFβ1 and TIMP1 Expression in Human Keratinocytes

Figure 3 shows the gel electrophoresis results of the semi-q-PCR products. The results, normalized using conversion factors of the ACTB gene, suggested that both AS and ABP significantly increased the TGFβ1 gene expression in keratinocytes. The statistical analysis showed that AS and ABP had significant variations with the negative control with P values of 0.01 and 0.007 respectively (Figure 4) and significant variation between the AS and ABP with P value of 0.009.

The AS and ABP also considerably enhanced the TIMP-1 gene expression with statistically significant variations (P<0.05) when compared to the negative control (Figure 4) and significant difference (P<0.05) between AS and ABP. The genotypic result of TIMP-1 therefore supported the phenotypic results on the effects of the tested AS and its biotransformed product.

The *in vitro* assay results of AS supported previous reports that asiaticoside elevates collagen synthesis via activating TGFβ1 (Wu *et al*., 2012). However, ABP increased the expression of TGFβ1 and TIMP1 genes more than AS. The ABP also showed faster migration and healing rate under microscopic observation. In wound healing development, it is proposed that TGFβ first binds to the TGFb type II receptor (TβRII) and then the heterodimer is used by TGF b type I receptor (TβRI), leading to the phosphorylation of TβRI. The molecular pathway of wound healing may involve SMAD

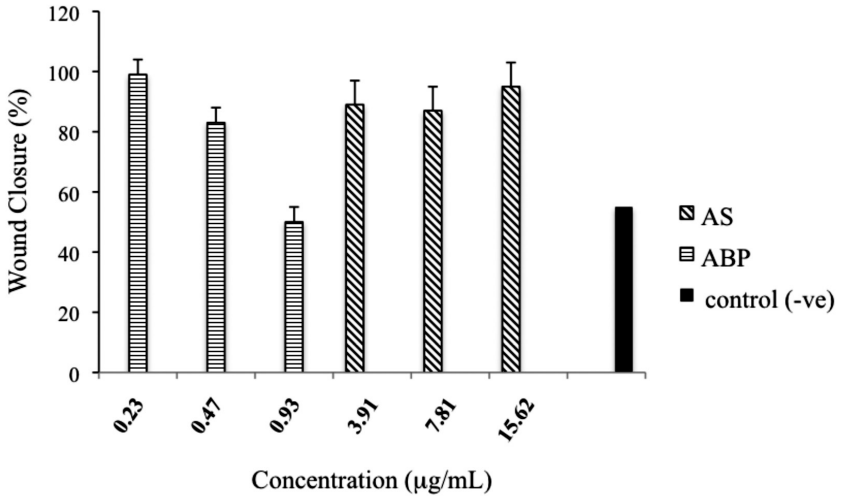

Figure 2: Effects of the different concentrations of AS and ABP on wound closure of HaCaT cells

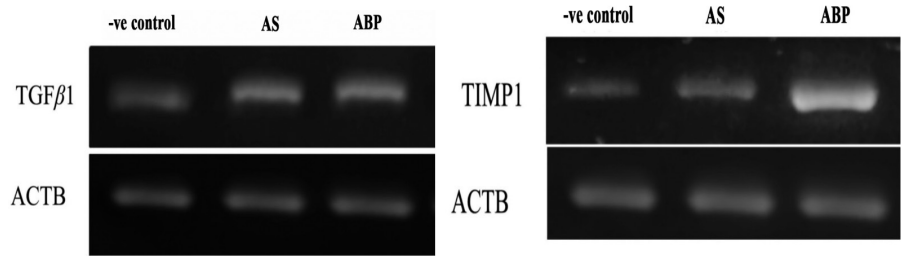

Figure 3: Effects of AS and ABP on the TGFβ1 and TIMP1 expressions in HaCaT cells

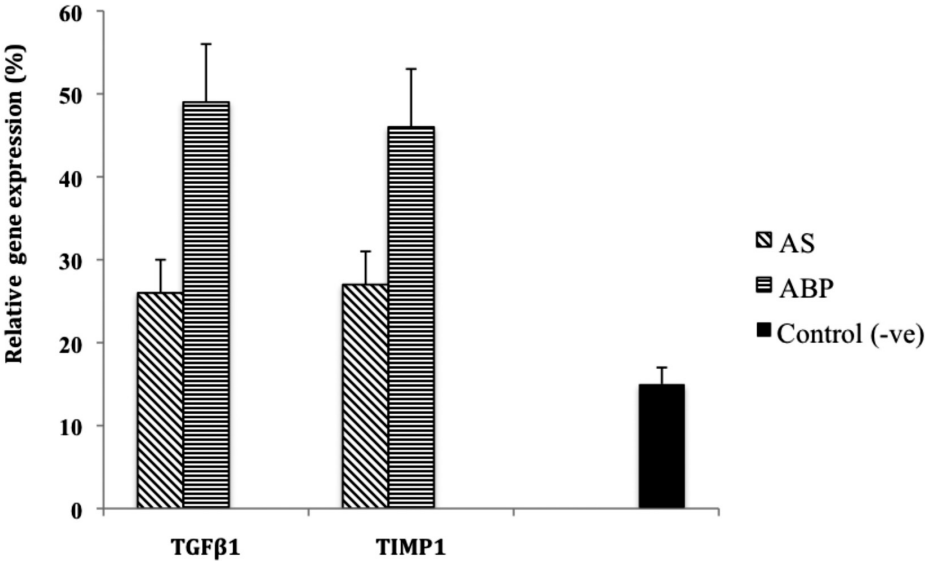

Figure 4: Effects of AS and ABP on relative TGFβ1 and TIMP1 expressions in HaCaT cells

genes family that work as co-regulators. SMADs are intracellular proteins that transduce extracellular signals by transforming GFβ ligands to the nucleus where they activate gene transcription (Leask & Abraham, 2004). The TGFβ1 stimulation helps in the proliferation and secretion of extracellular matrix (ECM), mostly collagen types I and III. In addition, it helps in the differentiation into myofibroblasts, which contributes towards accelerating cells migration and closure of the wounds (Tomasek *et al*., 2002). The TGFβ is an important signal in the phase of tissue development and helps in increasing the collagen, proteoglycans and fibronectin gene transcription, with the net result ofenhanced total manufacturing of matrix proteins. The TGFβ also works in the reduction of protease secretion and in the induction of TIMP, the protease inhibitor (Diegelmann & Evans, 2004; O'Connor & Gomez, 2013).

Conclusion

In conclusion, the *in vitro* study showed that the ABP could accelerate wound healing effectively at lower concentration as compared to it parent compound (AS). The healing process most possibly through the activation of fibroblast, and not only by increasing the cell proliferation. In addition, the up-regulation of TGFβ1 and TIMP1 and their linked pathways may correlate with the accelerated effects of AS and ABP on wound healing. Microbial transformation has produced a biotransformed product that changes the bioactivity of the original metabolite of natural product.

Acknowledgement

This research was supported by International Islamic University Malaysia (IIUM) under EDW B14-109-0994.

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