Decrease in Barbaloin and Product of Butyric acid Fermented by Endophytic Bacteria in Aloe Arborescens leaves

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ABSTRACT
Quantitative analysis of phenolic compounds in Aloe arborescens fermented extract revealed that barbaloin concentration was found to be 14 ug/mL (14 ppm) by HPLC analysis. Seven phenolic compounds could be identified: aloesin, 8-C-glycosyl-7-O-methyl-S-aloesol, alonin, isobarbaloin, barbaloin, 2’-O-feruloylaloesin, and aloe-emodin based on previous HPLC analysis. Short chain fatty acids such as acetic, propionic, butyric, lactic acid, were identified in the fermented extract of A. arborescens by GC/MS analysis. Reduction of barbaloin concentration and production of butyric acid clearly indicate a potential efficacious role of endophytic bacteria in A. arborescens leaves. Dietary intake of the extract fermented by endophytic bacteria in A. arborescens highlights immune-stimulating activity to human health and strongly influences an importance of host-microbial crosstalk to ensure maintenance of homeostasis.

Key words: Aloe arborescens; Endophytic bacterial fermentation; Barbaloin; Short chain fatty acids

INTRODUCTION
Aloe arborescens is native to southern Africa and is a vernacular name of shrubby aloe. In Japan where it was first introduced in the 17th century, it became naturalized. The taxonomy of A. arborescens is complicated by occurrence of interspecific hybrids both in the wild and in cultivation. Chromosome number of A. arborescens is 2n = 14 and it is a morphologically very variable species[1,2]. On A. arborescens var. natalensis (kidachi aloe) in Japan, Reynolds[3] concluded that it is regarded A. arborescens as being a variable species, than to attempt to uphold varietal names (A. arborescens var. natalensis) on overseas growth forms. Furthermore, Reynolds suggested that this is presently the best approach to allow for natural variation found within and between populations of A. arborescens.

A 2-year carcinogenicity study of A. arborescens, a health-food additive in Japan, was conducted by Yokohira et al[4] for assessment of toxicity and carcinogenic potential in the diet at doses of 4 or 0.8% in groups of male and female Wistar Hannover rats. Both sexes receiving 4% A. arborescens showed diarrhea, with loss of body weight gain. The survival rate in the 4% female group was

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significantly increased compared with control females after 2 year. Adenomas or adenocarcinomas in the cecum, colon, and rectum were observed in 4% male but not in the 0.8% and control male groups. Similarly, in females, adenomas in the colon were also observed in the 4% but not 0.8% and control groups. A. arborescens used as a food additive, exerted equivocal carcinogenic potential at 4% high-dose level on colon in the 2-y carcinogenicity study in rats. A. arborescens is not carcinogenic at nontoxic-dose levels and that carcinogenic potential in at 4% high-dose level on colon is probably due to irritation of the intestinal tract by diarrhea. Furthermore, the authors demonstrated that the no observed adverse effect level (NOAEL) for A. arborescens was 0.16% in diet, which is equivalent to 87.7 and 109.7 mg/kg/day in males and females, respectively. Taxonomical study of barbaloin (aloin) was performed by Boudreau et al. Groups of 10 male F344/N rat were administered aloe at 0, 6.95, 13.9, 27.8, 55.7, 111, 223 and 446 mg/kg drinking water for 13 weeks. Aloe indicated dose-related increased incidences and severities of mucosal and goblet cell hyperplasia that extended from the cecum to the rectum, with increased incidences and severities detected at aloe doses ≥ 55.7 mg/kg drinking water. International Aloe Science Council (IASC) certified Aloe vera products from gel for oral consumption are analytically tested for aloe content to ensure compliance with a maximum limit of 10 parts per million. FDA issued a final rule stating that the stimulant laxative ingredients of aloe whole leaf in OTC drug products are not generally recognized as safe or are misbranded. The German Federal Institute for Risk Assessment (BfR: Bundesinstitut für Risikobewertung) has ruled whole-leaf aloe preparations: A. arborescens preparations, containing anthranoids not suitable for use in foods, including food supplements.

In previous HPLC analysis, phenolic compounds in Aloe species indicated that the content of phenolic compounds increases clearly in the order: base, center and point, suggesting the relation to the part of the leaf decreases with age in aloe. The content per fresh weight of inner epidermal layer was 1.25-3 times higher than those of gel. Li et al. reported relationship between leaf structure and aloe content, and aloe content in leaves of A. arborescens is three times higher than that of A. vera. The stability and degradation products of aloe A, one of barbaloin isomer, under various pH, temperature and light conditions usually encountered in processing was explored by Ding et al. The stability of aloe A was significantly affected by temperature and pH. Aloe-emodin, elegonica-dimers A and B (aloe-emodin-aloin A and B dimers) were characterized as major degradation products of aloe A at pH 5.0 or below, and elegonica-dimers were mainly formed at 4°C as well. 10-Hydroxyaloin A and B were found under any condition except at pH 2.0 and 3.0, and they were mainly formed under high temperature, neutral-basic and any light conditions. Shindo et al. investigated the contents of barbaloin, barbaloin, aloin-dimers A, B, C, D and aloe-emodin in aloe drinks. When aloe drinks were stored for 4 weeks at 5°C after opening the bottle, the decrease of barbaloin and isobarbaloin were observed by using of LC-MS analyses, suggesting that barbaloin and isobarbaloin in aloe drinks is converted to the dimer and then to the trimer during storage.

Chiodelli et al. evaluated the effects of different extracts of Aloe vera and A.arborescens in fermented milk, taking into account both the prebiotic effect of aloe polysaccharides. The results showed a beneficial effect of 5% aloe inner gel on growth of lactic acid bacteria, Lactobacillus spp. Bourriau et al. assessed the role of D- and L-lactate as a precursor for butyrate biosynthesis in human colonic microbiota. The study suggests that the butyrogenic capability of colonic prebiotics could be related to lactic acid availability. The human intestinal microbiota can utilize both D- and L-lactate as precursors for butyrate synthesis. Inter-individual variation was found, suggesting the presence of the lactate-utilizing bacteria in the human intestinal microflora. Munoz-Tamayo et al. provided a mathematical model to analyze the production of butyrate by lactate-utilizing bacteria from the human colon. The findings of the mathematical model adequately matched those from the bacterial batch culture experiments.

In previous paper we described that aloe vera gel with endophytic bacteria, Bacillus cereus, B. licheniformis and Lactobacillus paralimentarium, produced butyric acid, and suggested that daily consumption of Aloe vera gel juice may be beneficial to putative prophylaxis for health and QOL as an immune modulation. Furthermore, Al-Madboly et al. investigated that the probiotic activity of Aloe vera in in vitro fermentation with L. fermentum isolates provided acetic, propionic and lactic acid. Fakhry et al. found that sequence comparisons between human gut intestinal tract and plant root isolates using multiple genes, implying that human and plant isolates are closely related strains. The authors suggested that the isolated Bacillus species do not transit, but rather colonize this specific habitat, such as sporulate in anaerobic conditions, and proposed them as probiotic strains for human use. Furthermore, Torres et al. analyzed a subset of probiotic microbes in genus Bacillus to evaluate the possibility that they may move from plant to human hosts after consumption of food plants. Sequence comparisons between human and plant root isolates using multiple genes indicate that human and plant isolates are closely related strains. Consumption of microbes in plants may be one way the human gut microbiome becomes established.

Potential efficacious role of barbaloin, aloe-emodin and short chain fatty acids fermented of Aloe vera and A. arborescens needs further scrutiny and evidence-based documentation.

Present investigation aims to evaluate the concentration of barbaloin by HPLC/UV, and production of short chain fatty acids by GC/MSD analysis for the extract fermented by endophytic bacteria in Aloe arborescens leaves.

**MATERIALS AND METHODS**

**Aloe arborescens sample preparation**

Aloe arborescens leaves collected from the herbal garden of Fukuyama university, A. arborescens leaves, 300g, were washed with 0.02% hypochlorite solution and rinsed with water. After trimming off butts and tips, the whole leaves were cut into pieces containing 20% W/W sucrose in closed bottle at room temperature for one month. The pH of exuded filtrate, 220ml, was 3.38-3.42 at 19.4-12.0°C. Automatic Water Still (Sci Finetech, Seoul, South Korea). Standard materials: Acetonitrile of HPLC grade was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water was double distilled using Automatic Water Still (Sci Finetech, Seoul, South Korea). Standard barbaloin and aloe-emodin were purchased from Wako.

**HPLC/UV detection of phenolic compounds in Aloe arborescens leaves**

**Determination of phenolic compounds in Aloe arborescens by HPLC/UV detection**

**Materials:** Acetonitrile of HPLC grade was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water was double distilled using Automatic Water Still (Sci Finetech, Seoul, South Korea). Standard barbaloin and aloe-emodin were purchased from Wako.
Instrumentation: The separation was performed on an Ultimate 3000 rapid separation instrument (Dionex, Sunnyvale, CA, USA) equipped with a VWD-3400RS UV detector and a WPS-3000TPL RS auto-sampler, Chromleon® software (Ver.7.2) was employed for data processing. The column was X-Bridge™ C18 (250 mm × 4.6 mm, 5 µm particle size, Waters Corp., Milford, USA) and the detector was set at λ = 293 nm.

Stock solutions: Barbaloin, 1.9mg, were weighed, transferred into a 10mL volumetric flask, dissolved in 3 mL methanol and completed to volume with methanol (190 µg/mL). Mixed standards of phenolic compounds, 10mg, were weighed, transferred into a 10mL volumetric flask, dissolved in 5 mL methanol and completed to volume with methanol (1 mg/mL).

Methodology

HPLC conditions: Mobile phase; acetonitrile/water (50:50, v/v), Chromatographic mode: gradient elution (12 to 50% acetonitrile in 50 min), Flow rate; 1 ml/min, Injection volume 10 µL, column; Xbridge™ C18 (4.6 × 150mm, 5 µm), column temperature; 35°C, Detector; UV at 293 nm.

RESULTS

Quantitative results of phenolic compounds in A. arborescens fermentation extract revealed that the amount of barbaloin was found to be 14 µg/mL (1.4ppm). Based on previous analysis, seven phenolic compounds could be identified: aloesin; 8-C-glucoyl-7-O-methyl-(S)-aloesol; aloesin; isobaloradin, barbaloin; 2’-O-feruloylaloesin and aloe-emodin. The extract does not contain any detectable amounts of aloeresin A, isoaloresin D, or aloeresin E.[30]

GC-MS analysis

Sample preparation: The ether extract of the fermentation broth, 0.1g, was dissolved in 2ml hexane, sonicated for 10 minutes, methylated by sodium methoxide, then clear hexane layer filtered through PTFE membrane. Sample was diluted 1:50 with hexane before injection under SIM conditions.

Instrument used: GC/MSD 5977A, Agilent, USA. The column used: Agilent, DB 225 ms 60m × 250µm × 0.25 µm. Oven program: 35°C for 3.7 min, then 7°C/min to 150°C. Inlet: Split mode. Split ratio 25:1. Liner Agilent 5190-2294: 990µL. Inlet temperature: 100°C for 1 min, then 100°C/min to 225°C. Auxiliary temperature: 230°C.

MS information:
Acquisition mode: SCAN; Scan parameters: Low Mass 45, High Mass: 550.00
Environmental condition: 24°C, humidity: 51%.
SIM Parameters: illustrated in the table 1.

Results

The fermentation extract of Aloe arborescens leaves exhibited the production of acetic, propionic, butyric, and lactic acid as short chain fatty acids (SCFAs) by GC/MSD analysis under SIM mode. The concentration of SCFAs was acetic acid (0.024%), lactic acid (19.12%), propionic acid (0.01%), and butyric acid (0.15%).

DISCUSSION

The interaction of endophytic fungi with host plants results in a compromise between mutualism and antagonism to create a harmonious symbiotic system. In the previous papers we described the benefits of endophytic bacteria producing butyric acid in Aloe vera leaves[17,18]. Asai et al[19] reported new short-branched fatty acid dimers in the cultures of endophytic fungi, Mycosphaerella sp., isolated from Aloe arborescens leaves. As biological active constituents of leaves of A. arborescens, Hirata and Sugai[20] found several bioactive substances, such as magnesium lactate, succinic acid, aloesin, aloe-emodin, and barbaloin. Magnesium lactate inhibits histidine decarboxylase, which prevents the formation of histidine. This may partially explain the antiinflammatory and antiinflammatory effect of A. arborescens.

In the present study, we found that acetic, propionic, butyric, and lactic acid were produced from the fermentation extract of A. arborescens leaves with endophytic bacteria by GC/MSD analysis. Fermented extract of A. arborescens leaves exhibited a possible promotion of the growth of host plant to achieve a balanced living environment by Josi et al[21]. It is important to note that endophytic bacteria in A. arborescens produced short chain fatty acids (SCFAs), such as acetic, propionic, butyric, and lactic acid in high concentration in in vitro fermentation extract. Butyric acid is preferred energy source for colonocytes and plays an important role in gastrointestinal tract homeostasis. Production ratio of endophytic bacterial metabolic SCFAs, butyric acid, in A. vera and A. arborescens, which direct the butyrogenic capability of colonic prebiotics related to lactate availability[22], could be suggested as one of the metabolic difference between A. vera and A. arborescens, because magnesium lactate was isolated from A. arborescens leaf[23]. Some ingredients, such as aloesin and lactate, in the extract of A. arborescens antagonize or synergize with each other to produce a harmonizing efficacy between wound-healing and anti-inflammatory activity through an immunoreaction. On the study of A. arborescens leaf pulp, we isolated a partially acetylated β-D-mannan, named aloe-mannan having molecular weight: 15,000 by equilibrium ultracentrifugation as a pure state. The water-soluble partially acetylated β-D-mannan (aloemannan) exhibited the inhibiting activity for the implanted salcoma-180 in mouse[24]. A homogeneous glycoprotein (Mw: 40,000) containing 50.7% of protein showed Bradykinin-degrading activity[25], DNA synthesis stimulation, and lectin properties[26]. Clinical trial of A. arborescens extract[27] demonstrated that the active compounds, such as glycoprotein, clearly enhance activity of both phagocytosis and NBT reduction, and polysaccharide act as an immune-potentiator and promote phagocytosis in adult bronchial asthmatics. The actual phagocytosis step is really a cleaning up operation after the actual phagocytosis process. Randomized study of chemotherapy versus biochemotherapy with chemotherapy plus A. arborescens in 240-patients with metastatic cancer was demonstrated by Lissoni et al[28]. The study suggests that A. arborescens may be successfully associated with chemotherapy to increase its efficacy in terms of both tumor regression rate and survival time.

Endophytic microbial-derived butyric acid in A. arborescens leaf could exert anti-cancerous effects by several mechanisms, highlighting the importance for health maintenance[29]. Regulation of the epigenome in particular inhibition of histone deacetylases,
such as butyric acid, impacts pathogenic mechanisms involved in chronic disease. Kim et al. demonstrated that probiotics mixture and sodium butyrate increased Th1 and Treg cell differentiation in mesenteric lymph nodes and spleen tissues in mouse model. These results suggest that the probiotic mixture and sodium butyrate can prevent and alleviate allergic symptoms.

Monthly variations and different parts in barbaloin content of A. arborescens were studied by Beppu et al. The barbaloin content in the supernatant of homogenized leaves showed 294.8 ± 24.31 ppm in average annual value and 288.5 ppm in apex part on the same stalk. Barbaloin content in methanol supernatant of homogenized leaves of A. vera and A. arborescens showed high in A. arborescens (600 ppm in fresh tissue) and low in A. vera (400 ppm in fresh tissue). In order to remove of barbaloin, various treatments are utilized: the active charcoal adsorption process and cellulase process for viscosity reduction in industrial processing. On the microbiology of A. vera and A. arborescens, one should be cognizant of two sources: endogenous and exogenous microbiota. Endophytes, endogenous microorganisms, include bacteria and fungi living within plant tissues without causing any overt negative effects. Barbaloin content in the fermented extract by endophytic bacteria in A. arborescens was reduced to 14ppm in present experiment. It is important to note that endophytic bacteria in A. arborescens provided 14ppm barbaloin in in vitro fermentation extract, which shows near concentration to IASC carcinogenic activity standard (less than 10ppm of barbaloin). IASC certified that aloe vera products for oral consumption are analytically tested for barbaloin content to ensure compliance with a maximum limit of 10 ppm, and aloe vera products made to IASC standards for barbaloin content are safe. The concentration of barbaloin 14ppm in original fermented extract in A. arborescens could be diluted to less than 10 ppm when it is used as aloe soft-drinks and beverage.

Dietary intake of extract fermented by endophytic microbiota in A. arborescens leaves provides for beneficial influences to human health maintenance. Further study is needed to identify the endophytic microbiota in the fermented extract of A. arborescens.

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