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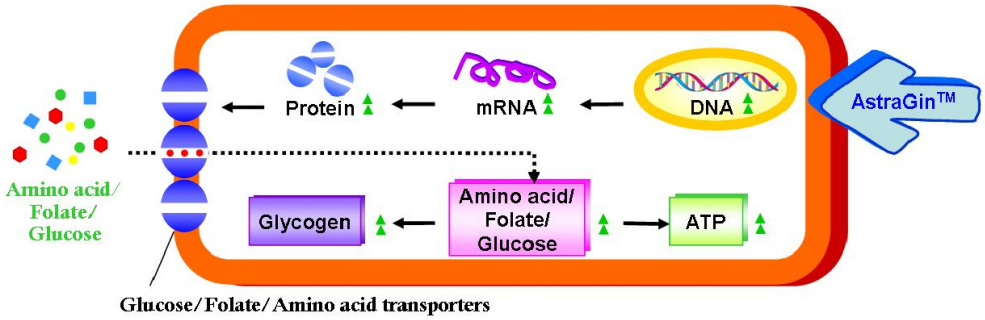
AstraGin™

Clinically Demonstrated to Significantly Increase the Absorption of Amino Acids, Peptides, Vitamins, and Other Nutrients in Intestinal Cells to enhance the Nutritional Value of Targeted functional Foods and Supplements



AstraGin®'s Nutrients Absorption Model

Contents

What Is AstraGin®?

AstraGin® is a proprietary all natural plant derived compound extracted from highly fractionated *Panax notoginseng* and *Astragalus membranaceus* using a proprietary pharmaceutical extraction and processing technology. AstraGin® has demonstrated in twelve *In vitro* studies to significantly enhance the absorption of many important nutrients. Specially, AstraGin® increased the absorption of three amino acids, Arginine, Citrulline, and Tryptophan; one vitamin, Folate; an amino sugar, Glucosamine; and Glucose in Caco-2 intestinal cell, the gold standard used by pharmaceutical companies to measure the absorption of drugs. AstraGin® also increased the ATP production in HepG2 liver cell.

AstraGin® has also demonstrated in multiple *In vivo* studies in a normal and IBD (Inflammatory Bowel Disease) rat model to increase the absorption of cationic amino acids, Arginine, Lysine, and Histidine in normal and colitis rats. Human uses began in late 2010 and results so far have been very positive. A twenty eight day sub-acute toxicity study in rats was completed Q4 2011. AstraGin® is a self-affirmed GRAS and NDI nutraceutical ingredient.

2. How AstraGin® Works

AstraGin® up-regulates both the mRNA and protein expression levels of the nutrient transporters that regulate the amount of amino acids, glucose, vitamins, and other nutrients entering into the intestinal cells. Once inside the cells, these nutrients are transported by portal vein to liver and the rest of human body. Those nutrients that do not enter into the intestinal cells will be excreted from the human body. (Sections 6)

3. Specific Functions of AstraGin®

- Increases the steady-state absorption rate of arginine, citrulline, agmatine, β-alanine, creatine, tryptophan and peptides by 66.7%, 45%, 36%, 26%, 33%, 53% and 40% (section 6.4, 6.5, 6.6, 6.7, 6.8, 6.9)
- Increases vitamins absorption such as folate by 50% (section 6.10)
- Increase glucosamine absorption by 23% (section 6.11)
- Increases omega-7 fatty acid (Palmitoleic acid) by 39% (section 6.12)
- Increase curcumin absorption by 92% (section 6.13)
- Increase ATP production in liver by 18% (section 6.14)

4. Applications

- Weight Management - protein powders, meal replacements, dietary supplements
- Sport Nutrition – body building, fitness and energy formulas, protein supplements, energy bars, ready-to- drink supplements
- Functional Beverages - energy drinks, smoothies, powder drink mixes
- Nutraceuticals - all categories of dietary supplements in capsule, liquid, powder, softgel, and tablet forms
- Medical Food - Nutrition Supplements
- Baked goods - breads, cereals, pastas, snacks
- Pet Foods - enhances pets' nutritional status through enhanced absorption of nutrients in the foods they eat
- Animal Nutrition and Natural Feed supplement products

5. Research Summary

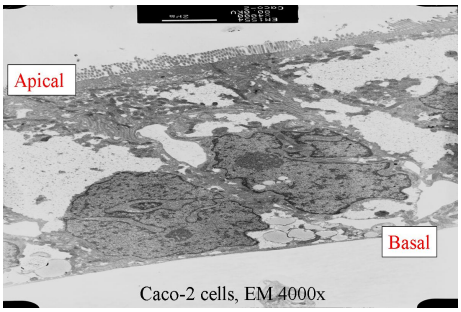
In a total of seventeen (17) *In vitro* studies (section 6), AstraGin® has demonstrated to upregulate the mRNA expression levels of folate and glucose, the transporter protein of cationic amino acids cAT1, folate and SGLT1, the uptake of arginine, citrulline, agmatine, β-alanine, creatine, tryptophan, peptides, folate, glucosamine, omega-7 fatty acid, curcumin and ATP by 66.7%, 45%, 36%, 26%, 33%, 53%, 40%, 50%, 23%, 39%, 92% and 18% when compared to the control groups. The *In vitro* study on AstraGin™ is similar to the *In vitro* study published in *J. Agric. Food Chem*., Vol. 55, No. 5, 2007 by T.C. Chang, etc. that was sponsored and funded by NuLiv Science.

6. *In Vitro* Studies

6.1 Cell Culture

Caco-2 cells

The established human intestinal epithelial Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) containing glucose, glutamine, fetal bovine serum, sodium bicarbonate, penicillin, streptomycin, and non-essential amino acids. The medium was changed every 2 days and the cells were inspected daily. For transport experiments, the cells were seeded into polycarbonate filter cell culture chamber inserts (Transwell, 24-mm diameter, 3 μm; Costar, Corning Inc., Corning, NY) .The cells were left to differentiate for 15-17 days after confluency; the medium was regularly changed three times a week. The integrity of the Caco-2 cell monolayers and the full development of the tight junctions were monitored before every experiment by determining the transepithelial electrical resistance (TEER) of filter-grown cell monolayers by use of a commercial apparatus (Millicell ERS; Millipore, Bedford, MA). Only cell monolayers with TEER values of 400-600 Ωcm^2 were used for experiments.



Caco-2 cells, EM 4000x

6.2 Quantitative Analysis of Nutrient Transporter Transcripts

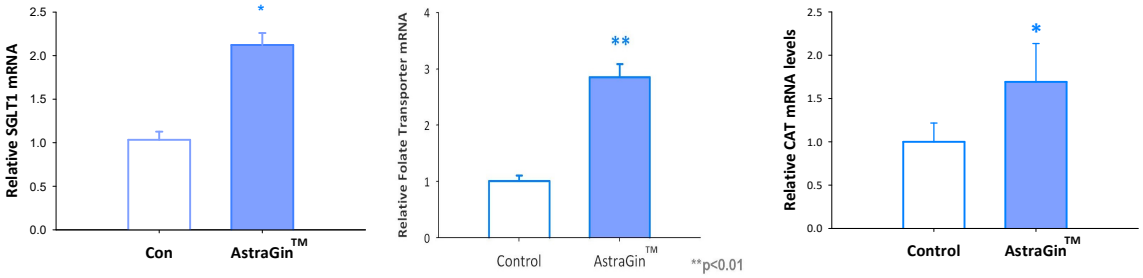
Materials and Methods

Relative levels of glucose transporters expressed in human Caco-2 cells were determined by real-time quantitative PCR (Q-PCR). Total RNAs were isolated from the cultured human cells by use of TRIzol reagent (Invitrogen, Irvine, CA). RNA was reverse-transcribed at 37°c for 60 min in transcription mixture containing dNTP, oligo-dT, RNasin, 1x PCR buffer, and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Q-PCR was performed on Applied Biosystems 7500 system with predeveloped Taqman gene expression assays (Applied Biosystems, Foster City, CA). The reaction mixtures contained of serially diluted cDNA, Taqman universal PCR master mix (Applied Biosystems), and primer mix of either SGLT1, FR, CAT or GAPDH (Applied Biosystems). Two independent triplicate experiments were performed for the selected genes, and the obtained threshold cycle (Ct) values were

averaged. According to the comparative Ct method described in the ABI manual, gene expression was normalized to the expression of the housekeeping gene GAPDH, yielding the ΔCt value.

Results

Glucose transporter mRNA **Folate transporter mRNA** **Cationic amino acids transporter**

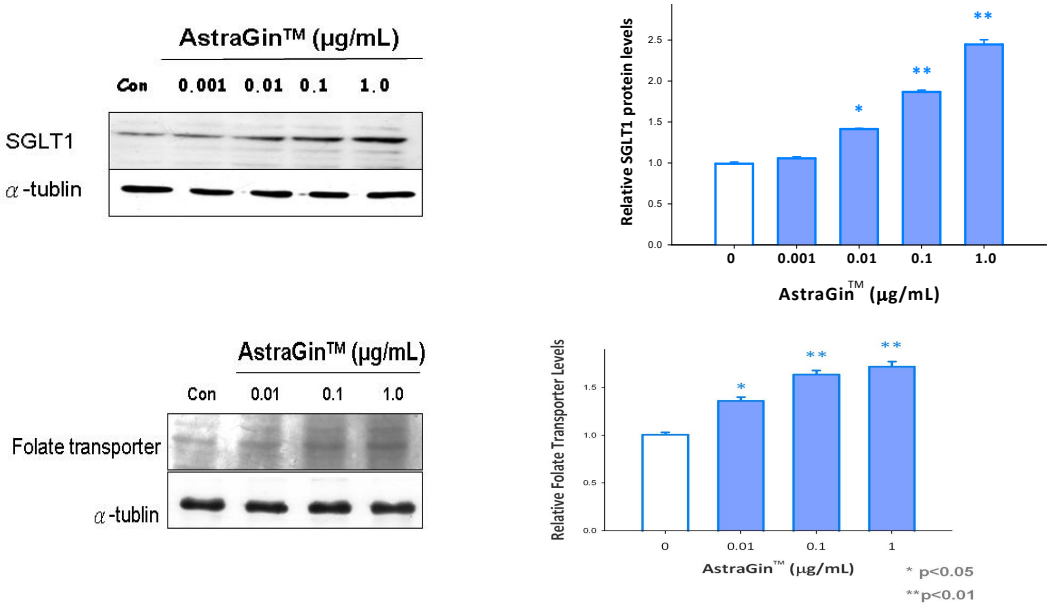


6.3 Western Blot Analysis

Materials and Methods

Human Caco-2 cells were plated on culture dishes (6 cm diameter) at a density of 1×10^5 cells/dish and designated as day 0. The cells were left to undergo differentiation for 8 days prior to treatment with AstraGin™ for another 24 h. The cells were then washed and lysed of lysis. Protein concentrations of the samples were measured by the bicinchoninic acid (BCA) protein assay kit according to the manufacturer's protocol (Pierce, Rockford, IL). Equal amount of protein samples of cell lysate were separated by SDS- PAGEl electrophoresis and transblotted onto PVDF membrane. Immunoblotting was performed with anti-human antibodies for SGLT1, FR and α-tubulin (Abcam, Cambridge, MA). Signals were visualized with an enhanced chemoluminescence kit (ECL, Amersham, U.K.) followed by exposure to X-ray films.

Results



6.4 Arginine and Tryptophan Uptake Assay

Materials and Methods

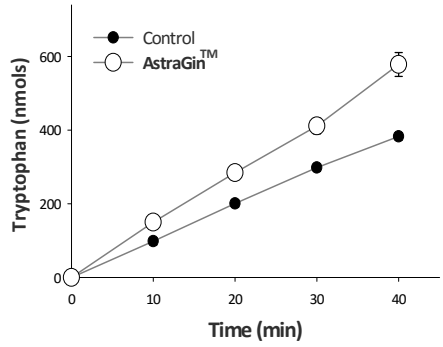
In measuring the transport of arginine or tryptophan across the Caco-2 cell monolayer, both sides of the transwells were washed with incubation buffer. Then, the cell monolayer was preincubated in the incubation buffer at 37ºC for 1 h and replaced with fresh incubation medium right before transport experiment. The transport experiment was initiated by replacing the incubation solution on the apical side with solution containing 10 mM of L-arginine or L-tryptophan in which 0.125 µCi/mL of L-[^{3}H]-arginine or L-[^{3}H]-tryptophan was included. At designated time intervals, 10 µL-solution samples were removed from the basolateral side and radioactivity of each sample was counted using a microplate liquid scintillation counter (TopCount, Packard NXT). The uptake of [^{3}H]-mannitol was used to correct for nonspecific transport of molecules across the monolayer membrane. Results are expressed as the steady-state rate of arginine or tryptophan transport across (nanomoles per minute) across the Caco-2 monolayers in mean ± SD (*n* = 3-5). Differences between means of groups were assessed by the *t*-test.

Radiolabelling method was used to determine the amount of arginine and tryptophan absorbed/transported from outside into the Caco-2 cell membrane. Therefore, more radioactivity across the Caco-2 cell membrane from apical side to the basal side indicates that more arginine or tryptophan was absorbed and transported.

Relative absorption rate is calculated based on total amount of arginine or tryptophan absorbed and transported in the first 10 minutes, the most efficient time period.

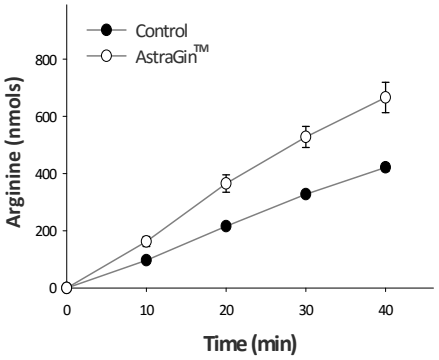
Results

Tryptophan absorption



Control: 11.34±0.943 nmol/min
AstraGin™: 16.79±1.516 nmol/min*

Arginine absorption



Control: 8.66±0.3 nmol/min
AstraGin™: 14.15±0.29 nmol/min*

Relative Absorption Rate of Arginine and Tryptophan for the Control and 3 AstraGin® Groups

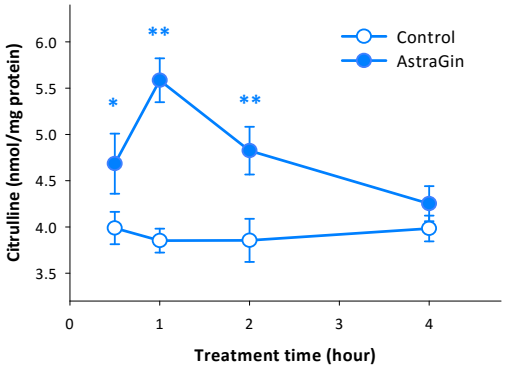
Group	Dosage (µg/mL)	Accumulated tryptophan In basolateral side (nmols at 10 min)	Accumulated arginine In basolateral side (nmols at 10 min)
Control		98.47 ± 10.20	97.29 ± 10.33
AstraGin®	1.0	152.02 ± 18.86*	145.24 ± 24.24*
	0.10	150.17 ± 9.51**	162.25 ± 17.6**
	0.01	148.63 ± 19.83*	136.89 ± 18.95*

* p<0.05; **p<0.001

6.5 Citrulline Uptake Assay

In the citrulline uptake test, the Caco-2 cells were first deprived of amino acids by incubation for 15 min at 37℃ in Krebs buffer adjusted to pH 7.4 with Tris base. The buffer was then aspirated and the cells were rinsed three times with this buffer. Citrulline uptake was initiated by adding Krebs buffer containing 200 mM citrulline (10 mM L-[^{14}C]-citrulline and 190 mM unlabeled L-citrulline) in the compartment. After incubation at controlled temperature for the appropriate times (see Results), uptake was stopped by removing the Krebs buffer and immediately washing the cells three times with ice-cold buffer. Cells were harvested in 0.5N NaOH and removed by scraping after 1 h at room temperature. Intracellular uptake of ^{3}H-citrulline was determined by transferring 20 µL of the cell lysate to the filter-bottomed UniFilter plates (Perkim-Elmer) and counting as described previously in Example 1. The amount of citrulline accumulated in the cells was calculated and normalized to protein concentration, and uptake was expressed as nanomoles of citrulline per milligram of cell protein (nmol /mg). Protein concentration was determined by a standard Bicinchoninic acid (BCA) protein assay.

Results



Treat time (hr)		Relative citrulline absorption (%)
Control	0	100.0
AstraGin®	0.5	117.44*
	1.0	144.97**
	2.0	125.14**
	4.0	106.76

*p<0.05, ** p<0.01, when compared to control

6.6 Agmatine Uptake Assay

Materials & Methods

Cell culture

The Caco-2 cell line was obtained from ATCC (Philadelphia, PA, USA). The Caco-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco Life Technology), nonessential amino acids, L-glutamine and penicillin/streptomycin. The Caco-2 cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2. The cells used in the experiments were between passages 10 and 20. Caco-2 cells were subcultured weekly by trypsin and were seeded at a ratio of 1:3 upon reaching 80% confluence. The culture medium was changed every 2–3 days. For the transport experiments, the cells were reached confluence in 3 days after seeding, and the cells were differentiated for at least an additional 14 days prior to the transport experiments.

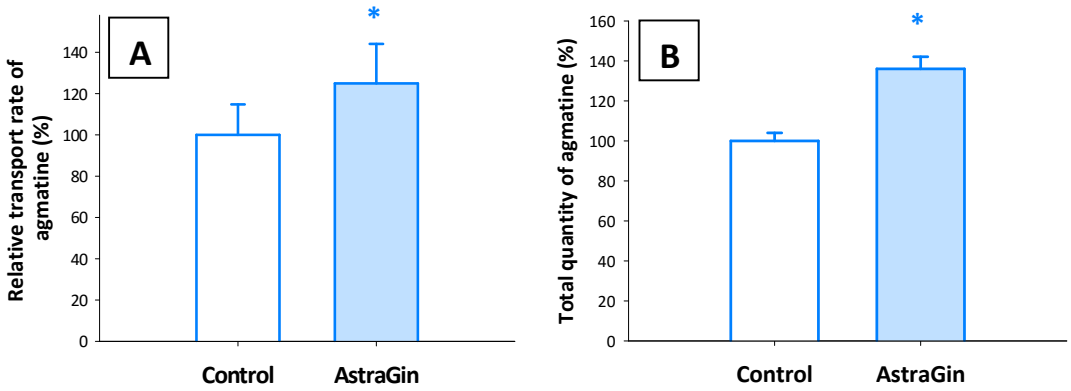
Agmatine Transport Experiment

After removal of the growth medium, cells were washed and then preincubated at 37°C for 20 min with 3 ml Krebs-Ringer-HEPES (KRH) buffer containing (in mM) NaCl 125, KCl 4.8,

KH_2PO_2 1.2, Mg_2SO_4 1.2, D(+)glucose 5.6, HEPEs 25.0 (pH 7.4), and 0.1% (w/v) bovine serum albumin (BSA). Cells were then incubated at 37°C for 15 min in KRH buffer containing 120 nM [^{14}C]agmatine. At the end of each experiment, cells were washed three times with 3 ml BSA-free, ice-cold KRH buffer and then lysed in 1.5 ml 0.1% Triton X-100. The protein and the tritium contents were determined from the cell lysates. Protein concentrations were determined by the method of Bradford [27] using BSA as standard, and tritium content was determined by liquid scintillation counting.

Results

The effect of AstraGin® on agmatine uptake in Caco-2 cells after 24h treatment.



Relative transport rate (A); total quantity of agmatine absorbed by AstraGin® in Caco-2 cells after 24h treatment.

Analysis of the effect of AstraGin® on agmatine transport rate and total quantity absorption after 24h treatment.

Groups	Relative agmatine transport rate in 90min (%)	Total quantity of agmatine absorption (AUC) in 180min (%)
Control	100.00±14.78	100.00±4.03
AstraGin®	124.95±19.11*	136.03±6.08*

* $p<0.05$, when compared to control group

This study has demonstrated that, after 24 hour treatment of Agmatine in Caco-2 cells, AstraGin® was able to increase the transport rate of agmatine by 25% in 90 minutes and the total amount of agmatine (AUC) absorbed by 36% in 3 hours when compared to the control group.

6.7 β-Alanine Uptake Assay

Materials & Methods

Materials

Alanine, β-[2,3-^{14}C] (specific activity 110 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc (ARC), and β-Alanine was obtained from Sigma-Aldrich. All other chemicals were from Sigma-Aldrich and were of the highest quality available. Cell culture media and supplements were obtained from Gibco Life Technologies.

Cell Culture

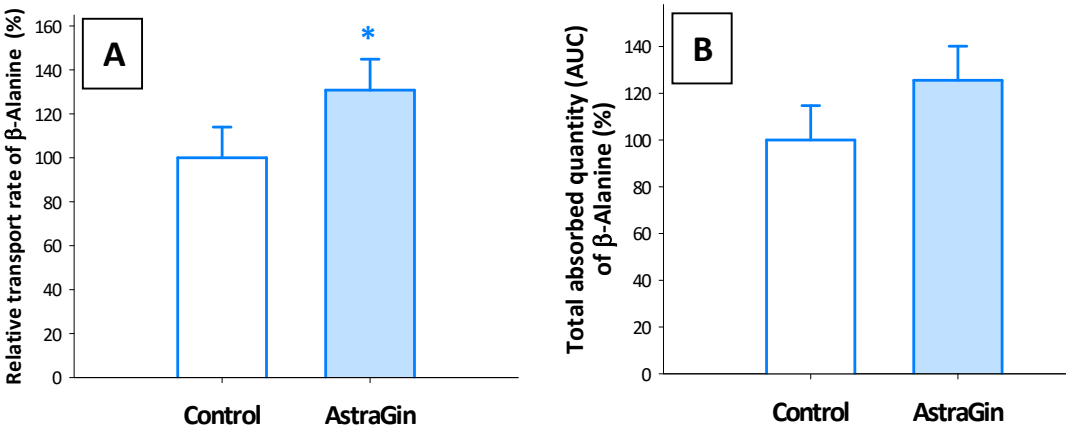
The Caco-2 cell line was obtained from ATCC (Philadelphia, PA, USA). The Caco-2 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco Life Technology), nonessential amino acids, L-glutamine and penicillin/streptomycin. The Caco-2 cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells used in the experiments were between passages 10 and 20. Caco-2 cells were subcultured weekly by trypsin and were seeded at a ratio of 1:3 upon reaching 80% confluence. The culture medium was changed every 2–3 days. For the transport experiments, the cells were seeded at a density of $9x10^5$ cells/cm^2 in 6-well filter support inserts with polyethylene membranes (0.4 μm pore size, 24 mm diameter, 4.67 cm^2 growth surface area; Costar, Corning Inc., Corning, NY). The monolayers reached confluence in 3 days after seeding, and the cells were differentiated for at least an additional 14 days prior to the transepithelial transport experiments. The integrity of the Caco-2 cell monolayers and the tight junctions were monitored before every experiment by determining the transepithelial electrical resistance (TEER) measurements using an epithelial Volt-Ohm Meter (Millicell ERS-2, Millipore, Bedford, MA). Only the Caco-2 monolayers with TEER values higher than 700Ω cm^2 were used for the experiments.

Transepithelial Transport Studies

After TEER measurement, the differentiated Caco-2 monolayers were gently rinsed twice with modified Krebs buffer (buffer composition, in mM: NaC1, 140; KCl, 5.4; $CaCl_2$, 2.8; $MgS0_4$, 1.2; NaH_2P0_4, 0.3; KH_2P0_4, 0.3; HEPES, 10; glucose, *5* ; pH 7.4) and equilibrated for 15 min at 37°C. The transport experiment was initiated by replacing the incubation solution on the apical side with solution containing 0.1 mM of β-alanine in which ^{14}C-β-alanine was included. The basolateral medium were sampled at the designated time intervals and analyzed via liquid scintillation spectrometer (Perkin Elmer). During and at the end of the experiments, TEER was measured and data were recorded only from experiments in which TEER was higher than 250Ω cm^2. Results are expressed as the initial rate of β-alanine transport across (picomoles per minute) across the Caco-2 monolayers in mean ± SD (*n* = 3-5). Differences between means of groups were assessed by the *t*-test.

Results

The effect of AstraGin® on β-alanine absorption in Caco-2 cells after 24h treatment.



Relative transport rate (A); total β-alanine absorption (AUC) by AstraGin® in Caco-2 cells after 24h treatment.

Analysis of the effect of AstraGin® on β-alanine transport rate and total β-alanine absorption (AUC) after 24h treatment.

Groups	Relative β-alanine transport rate in 30min (%)	Total β-alanine absorption (AUC) in 90min (%)
Control	100.00±14.00	100.00±14.72
AstraGin®	130.82±14.05*	125.56±14.60

* p<0.05, when compared to control group

As in Figure 1 & Figure 2 (in supplement), after 24 hour treatment in Caco-2 cells, AstraGin® increased the initial transport rate by 31% in 30min and total β-alanine absorption by 26% in 90min.

The effect of AstraGin® on β-alanine absorption in Caco-2 cells.

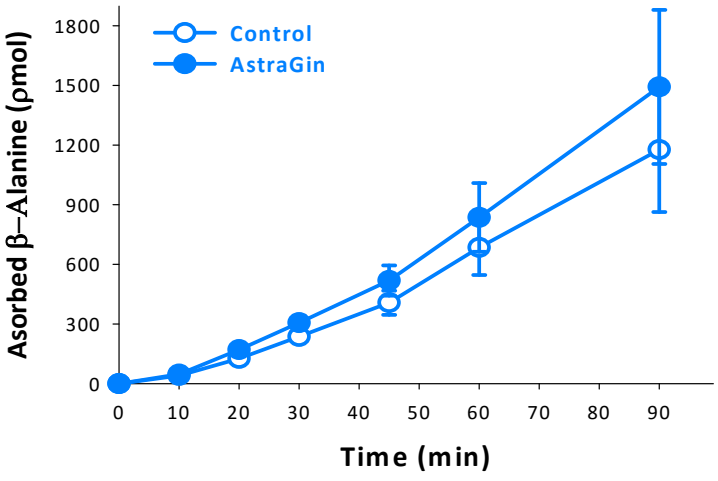


Figure2. Effect of AstraGin® on β-alanine absorption in Caco-2 cells between 0-90 min after 24h treatment.

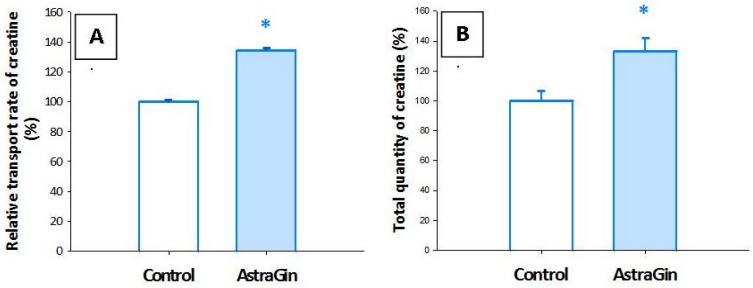
6.8 Creatine Uptake Assay

Materials and methods

After TEER measurement, the differentiated Caco-2 monolayers were gently rinsed twice with Krebs–Ringer's solution and equilibrated for 30 min at 37℃. The transport experiment was initiated by replacing the incubation solution on the apical side with solution containing 0.5 mM of creatine in which ^{14}C- creatine was included. The basolateral medium were sampled at the designated time intervals and analyzed via liquid scintillation spectrometer (Perkin Elmer). During and at the end of the experiments, TEER was measured and data were recorded only from experiments in which TEER was higher than 250Ω□ cm^2. Results are expressed as the initial rate of creatine transport across (picomoles per minute) across the Caco-2 monolayers in mean ± SD (*n* = 3-5). Differences between means of groups were assessed by the *t*-test.

Results

The effect of AstraGin® on creatine absorption in Caco-2 cells after 24h treatment.



Analysis of the effect of AstraGin® on creatine transport rate and total quantity absorption after 24h treatment.

Groups	Relative creatine transport rate in 90min (%)	Total quantity of creatine absorption in 180min (%)
Control	100.00±1.38	100.00±6.78
AstraGin®	134.39±1.56*	133.08±8.78*

* p<0.05, when compared to control group

After 24 hour treatment in Caco-2 cells, AstraGin® displayed positive effect in enhancing the initial transport rate and total creatine absorption in the observed time.

6.9 Peptides Uptake Assay

Materials and methods

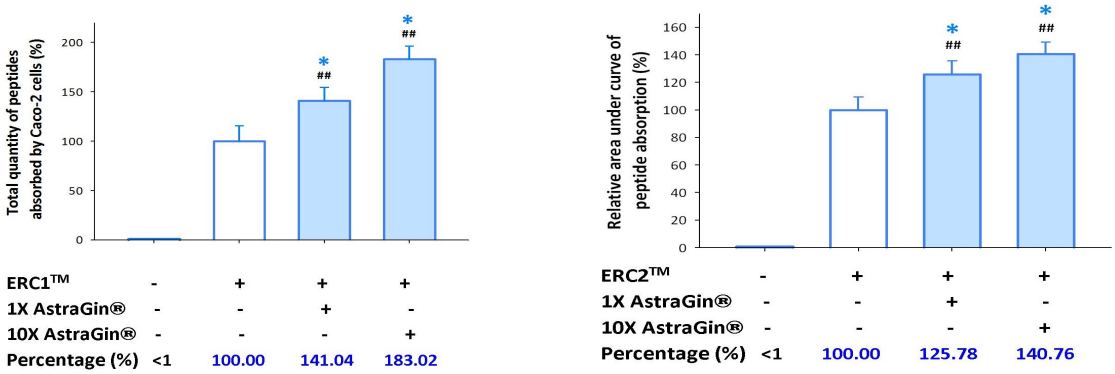
In peptide uptake test, Caco-2 cells were seeded into 6-well plate at a density of 4.5 x 10^5

cells/well. After TEER measurement, the differentiated Caco-2 cells were then treated in the absence (solvent control) or presence of AstraGin® at various doses for 24 h. The cells were gently rinsed twice with Hank's balanced salt solution (HBSS) and equilibrated for 30 min at 37℃. Then the medium was replaced with fresh HBSS containing the peptides derived from bovine hemoglobin hydrolyzed by either ERC1™ or ERC2™, a ERC proprietary blend of protease enzyme. The transwells were incubated at 37℃ for 120 min and the apical and basolateral medium were sampled at the designated time intervals and analyzed by fluoraldehyde (OPA) peptide assay. During and at the end of the experiments, TEER was measured and data were recorded only from experiments in which TEER was higher than 250Ω· cm^2

Peptide concentration was measured by the method of fluoraldehyde reaction using bovine serum albumin (BSA) as a standard. BSA (2mg/ml) was digested by incubation with 1% (w/w) trypsin for 72 h at 37℃ in a orbital incubator. The undigested protein was removed by the addition of TCA, followed by centrifugation at 10,000g for 20 min, the peptide content of the supernatant was measured. Peptide concentrations of the samples were measured by the fluoraldehyde (OPA) assay kit according to the manufacturer's protocol (Pierce, Rockford, IL). Briefly, prepare samples, blanks and standards in an opaque plate. Add optimal volume of OPA reagent solution to each well and mix well. Measure the fluorescence at excitation 330-390nm and emission at 436-475nm. The peptide concentrations were determined by Polarstar Galaxy (BMG LABTECH, DE).

Results

Effect of AstraGin® on the total quantity of peptides derived from bovine hemoglobin hydrolyzed by ERC2™ in 45 minutes



Analysis of the total quantity of peptides absorbed by Caco-2 cells in 45 minutes.

Relative area under curve of peptide absorption (%)		ERC1™		ERC2™	
		–		–	
AstraGin®	–	<1	100.00±15.82##	<1	100.00±9.45##
	1X		141.04±13.48*,##		125.78±10.00*,##
	10X		183.02±13.57*,##		140.76±8.71*,##

*p<0.05, when compared to ERC1™ only or ERC2™ only group.
p<0.01, when compared to Blank group (No ERC1™, no AstraGin® added; No ERC2™, no AstraGin® added)

Our results indicated that AstraGin® increased the initial absorption rate of peptides derived from bovine hemoglobin hydrolyzed by ERC1™ by 30.39% and 65.87% with 1X and 10X AstraGin® respectively when compared to the ERC1™ only group. AstraGin® also increased the initial absorption rate of peptides derived from bovine hemoglobin hydrolyzed by ERC2™ by 24.87% and 37.45% with 1X and 10X AstraGin® respectively when compared to the ERC2™ only group (detail data not shown).

When bovine hemoglobin was not hydrolyzed by ERC1™, the total quantity of peptides absorbed in Caco-2 cells was minimum with or without AstraGin®. After bovine hemoglobin was hydrolyzed by ERC1™, the total quantity of peptides absorbed in Caco-2 cells was 41% and 83% with 1X and 10X AstraGin® respectively when compared to the ERC1™ only group. AstraGin® also increased the total quantity of peptides absorbed in Caco-2 cells was 25.78% and 40.76% with 1X and 10X AstraGin® respectively when compared to the ERC2™ only group.

6.10 Vitamin Uptake Assay

Materials and Methods

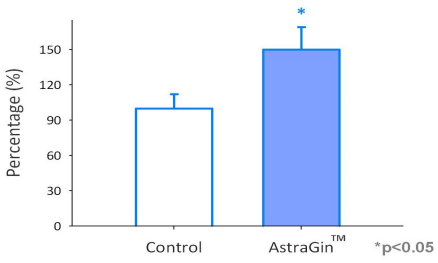
In the folate uptake test, the Caco-2 cells were cultured in a folate uptake buffer (Hank's balanced salt solution, supplemented with 0.14 g/L $CaCl_2$, 0.1 g/L $MgCl_2$, and 0.1 g/L $MgSO_4$, pH6.0) for 1 hour. The buffer was then aspirated, and uptake was initiated by adding 0.2 ml of fresh folate uptake buffer containing 2 µCi / mL radioactive folate (3,5,7,9-^{3}H-folic acid, 25 mCi / mmol, ARC) and cold, unlabeled folate giving a final folate concentration of 5 µM. Folate uptake was terminated by removing the uptake buffer at designated time intervals. The cells were then washed three times with ice-cold PBS and lysed by adding 0.2 mL of 0.2N NaOH, followed by incubating at 65°C for 20 min. Intracellular uptake of ^{3}H-folate was determined by transferring 20 µL of the cell lysate to the filter-bottomed UniFilter plates (Perkim-Elmer) and counting as described previously in Example 1. The amount of folate accumulated in the cells was calculated and normalized to protein concentration, and uptake was expressed as picomoles of folate per milligram of cell protein (pmol /mg). Protein concentration was determined by a standard Bicinchoninic acid (BCA) protein assay.

Results

Relative Absorption Rate of Folate for the Control and AstraGin™ Group

Group	Dosage (µg/mL)	Accumulated folate in Caco-2 cell monolayers (pmol/mg of protein at 1 min)
Control		53.140 ± 3.544
AstraGin®	0.10	79.899 ± 1.883

*** p<0.05**



6.11 Glucosamine Uptake Assay

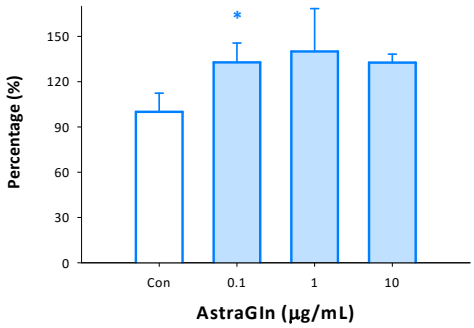
Materials and methods

In glucosamine uptake test, Caco-2 cells were seeded into 24-well plate at a density of 3 x 10^4 cells/well and cultured for 24 h. The cells were then treated in the absence (solvent control) or presence of the ingredients at various doses for another 24 h. The treated cells were then washed twice with PBS and incubated in glucose and serum free medium (GSFM). After 2 h, the cells were replaced with fresh GSFM containing 0.2 µCi of [^{14}C]-Glucosamine (American Radiolabelled Chemicals Inc, ARC, St. Louis, MO, USA). At the designated time interval, the cells were washed twice with GSFM containing cold glucosamine and then lysed in 200 µL of 2% SDS. Cell lysates were centrifuged at 15000 *g* for 15 min. Intracellular uptake of glucosamine was determined by transferring 10 µL of the cell lysate to filter-bottomed UniFilter plates (Perkim-Elmer) and counting. The samples were measured for their protein concentration using the BCA protein assay kit as described above. The amount of glucosamine accumulated in the cells was calculated and normalized to protein concentration and uptake rate was expressed as counts per minute per microgram of cell protein (mmol / mg of protein).

Results

Group	Dosage	Accumulated glucosamine In Caco-2 cell monolayers (mmol / mg of protein at 10 min)
Control		1.165 ± 0.145
	0.1 µg/mL	1.437 ± 0.184*
AstraGin®	1.0 µg/mL	1.660 ± 0.471
	10 µg/mL	1.181 ± 0.066

*** p<0.05**



6.12 Omega-7 fatty acid (Palmitoleic acid) Uptake Assay

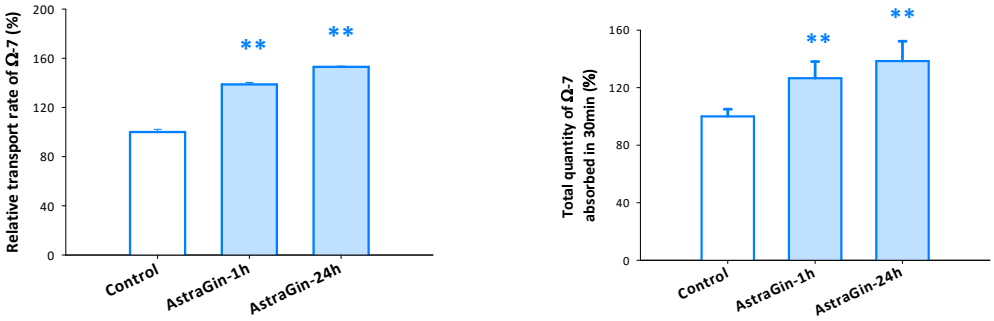
Materials and methods

Radiolabeled palmitoleic acid was dried under N_2. The dried lipids were dissolved in 0.5% (v:v) of ethanol relative to final volume, dispersed in 10 mmol/L sodium taurocholate in PBS, pH 7.4, to obtain the desired concentration, and were further incubated for 1 h at 37°C with shaking at 90 rpm to obtain a homogenous solution.

After TEER measurement, the differentiated Caco-2 monolayers were gently rinsed twice with phosphate-buffered saline (PBS, pH 7.4) supplemented with 25 mM glucose, 10 µM $CaC1_2$ and 1 mM $MgC1_2$ (PBS-GCM) and equilibrated for 30 min at 37℃. The transport experiment was

initiated by replacing the incubation solution on the apical side with solution containing 0.1 mM of palmitoleic acid in which ^{3}H- palmitoleic acid was included. The transwells were incubated at 37°C for 30 min and the basolateral medium were sampled at the designated time intervals and analyzed via liquid scintillation spectrometer (Perkin Elmer). During and at the end of the experiments, TEER was measured and data were recorded only from experiments in which TEER was higher than 250Ω cm^{2}. Results are expressed as the initial rate of palmitoleic acid transport across (picomoles per minute) across the Caco-2 monolayers in mean ± SD (*n* = 3-5). Differences between means of groups were assessed by the *t*-test.

Results



Analysis of the effect of AstraGin® on omega-7 fatty acid transport rate and total quantity absorption after 1h and 24h treatment.

Groups	Relative omega-7 fatty acid transport rate in 15min	Total quantity of omega-7 fatty acid absorption in 30min
Control	100.00±2.190	100.00±5.026
AstraGin®-1h	138.72±1.642**	126.567±11.553*
AstraGin®-24h	152.994±0.650**	138.498±13.758**

In the immediate effect, AstraGin® increased the initial transport rate and total quantity of omega-7 fatty acid absorption by 39% and 27% respectively when compared to control group. In another 24h treatment, AstraGin® increased the initial transport rate and total quantity of omega-7 fatty acid absorption by 53% and 38% respectively when compared to control group. In summary, the results of the study indicate AstraGin® has good ability to promote omega-7 fatty acid absorption in differentiated intestinal cells.

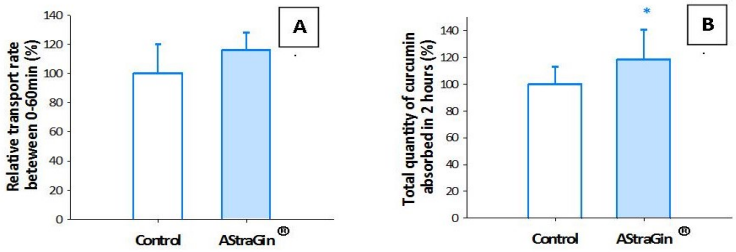
6.13 Curcumin Uptake Assay

Materials and methods

After TEER measurement, the differentiated Caco-2 monolayers were gently rinsed twice with Hank's balanced salt solution (HBSS) and equilibrated for 30 min at 37°C. Then the medium was replaced with fresh HBSS containing the curcumin solution. The transwells were incubated at 37°C for 180 min and the basolateral medium were sampled at the designated time intervals and analyzed via fluorescence (Ex: 450 nm; Em: 540 nm; Enspire 230, Perkin Elmer) . During and at the end of the experiments, TEER was measured and data were recorded only from experiments in which TEER was higher than 250Ω· cm^{2}

Results

A. Effect of AstraGin® on Curcumin absorption in Caco-2 cells
Relative transport rate (A); total quantity of curcumin absorbed by AstraGin® in Caco-2 cells.



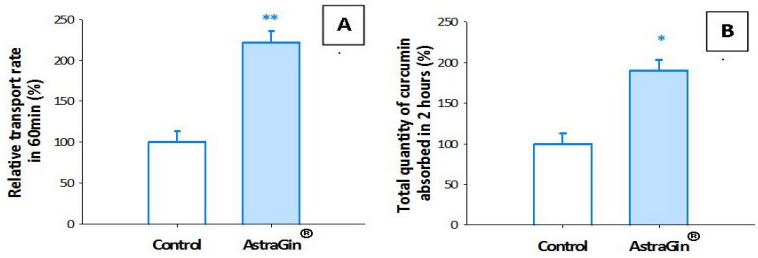
Analysis of the effect of AstraGin® on curcumin transport rate and total quantity absorption.

Groups	Relative curcumin transport rate in 60min	Total quantity of curcumn absorption in 2 hour
Control	100.00±20.12	100.00±12.68
AstraGin®	116.02±12.30	118.06±22.30*

* p<0.05, when compared to control group.

After 24 hour treatment in Caco-2 cells, we can observe that simple curcumin had poor absorption in the study, and AstraGin® displayed moderately effect on enhancing the transport rate and total curcumin absorbed in the observed time.

B. Effect of AstraGin® on Curcumin-Bioperine absorption in Caco-2 cells
Relative transport rate (A); total quantity of curcumin-bioperine absorbed by AstraGin® in Caco-2 cells



Analysis of the effect of AstraGin® on curcumin-bioperine transport rate and total quantity absorption.

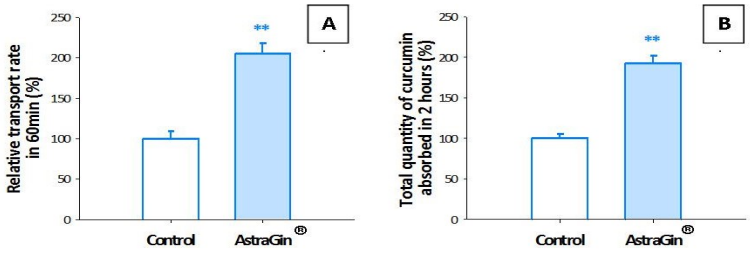
Groups	Relative curcumin transport rate in 60min	Total quantity of curcumn absorption in 2 hour
Control	100.00±13.67	100.00±12.89
AstraGin®	221.73±13.67**	189.77±13.94*

* p<0.05, when compared to control group
** p<0.01, when compared to control group

The curcumin-piperine formulation is the best documented and has been shown to almost double the human bioavailability of curcumin. After 24 hour treatment in Caco-2 cells, we can observe that curcumin-bioperine had excellent absorption in the study, and AstraGin®

displayed strong effect on enhancing the transport rate and total curcumin absorbed in the observed time.

C. Effect of AstraGin® on Curcumin-Lecithin absorption in Caco-2 cells
Relative transport rate (A); total quantity of curcumin-lecithin absorbed by AstraGin® in Caco-2 cells



Analysis of the effect of AstraGin® on curcumin-lecithin transport rate and total quantity absorption.

Groups	Relative curcumin transport rate in 60min	Total quantity of curcumn absorption in 2 hour
Control	100.00±9.18	100.00±5.87
AstraGin®	205.32±12.79**	192.43±9.96**

** p<0.01, when compared to control group

We choose another curcumin-lecithin formula to see if AstraGin® efficiently improves the curcumin absorption. After 24 hour treatment in Caco-2 cells, we can also observe that curcumin-lecithin had excellent absorption in the study, and AstraGin® displayed strong effect on enhancing the transport rate and total curcumin absorbed in the observed time.

6.14 ATP Fluorometric Assay

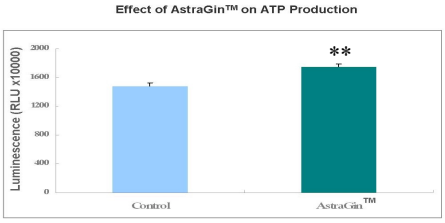
Materials and Methods

HepG2 cells were lysed in 100 µl of ATP Assay Buffer. The cell lysates were centrifuged at 15000 cpm for 2 minutes at 4°C to pellet insoluble materials. Collect supernatant. Add 2-50 µl of collected supernatant to 96-well plate. Bring final volume to 50 µl/well with ATP Assay Buffer. Mix well. Incubate at room temperature for 30 minutes, protecting from light. Measure Ex/Em = 535/587 nm for fluorometric assay in a micro-plate reader.

Results

Group	Dosage (µg/mL)	ATP Luminescence (RLU)
Control		14815572.6667 ± 438284.5035
AstraGin®	0.10	17454155.7778 ± 450089.0108**

** p<0.001



7. In Vivo Studies (in Normal and IBD Rats)

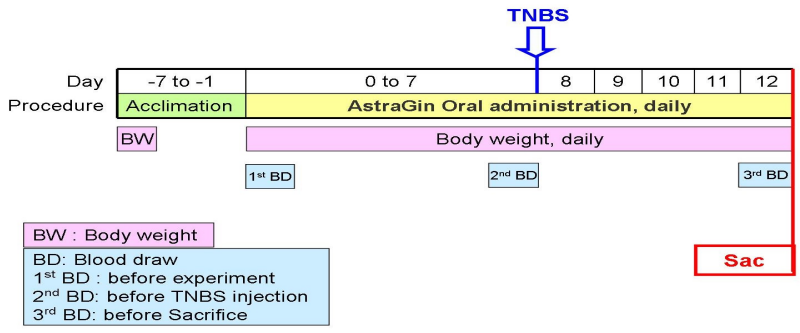
7.1 Animals Care and Feeding

Adult male Sprague-Dawley rats (6-8 weeks) were purchased from LASC Inc (AAALAC accreditation, Taipei, Taiwan). The rats were subsequently fed and maintained under specific pathogen-free conditions at the Animal Center of National Defense Medical Center (Taipei, Taiwan), which is accredited by AAALAC. The rats were individually housed in plastic cages with grated stainless steel floors. The temperature in the colony room was maintained at 24 ± 1℃ with 60% atmospheric humidity and a 12 h light-dark cycle with *ad libitum* access to food and water. The care of the animals were carried out in accordance with institutional and international standards (Principles of Laboratory Animal Care, National Institutes of Health), and the protocol has been approved by the Institutional Animal Care and Use Committee of National Defense Medical Center, Taiwan, R.O.C.

Composition of the diet, expressed as g/100g feed (w/w dry matter)

Non-amino acids component	Amino acids components	
Glucose: 63.9	Alanine: 0.61	Lysine: 1.81
Cellulose: 5.0	Arginine: 0.71	Methionine: 0.59
Soybean oil: 5.0	Aspartic acid: 1.39	Phenylalanine: 0.99
$CaCO_3$: 1.24	Glutamic acid: 4.64	Proline: 1.79
$NaH_2PO_4 \cdot 2H_20$: 0.34	Cysteine: 0.53	Serine: 1.18
$MgCO_3$: 0.14	Glycine: 0.36	Threonine: 0.88
KCl: 0.11	Histidine: 0.57	Tryptophan: 0.25
KH_2PO_4: 1.05	Isoleucine: 1.01	Tyrosine: 1.09
Vitamin/mineral mi: 2.2	Leucine: 1.87	Valine: 1.24
Total: 79.0	Total: 21.0	

7.2 Experimental Design



7.3 Induction of Colitis and Oral Intake

Group	Averaged food intake (%)		
	Before TNBS Induction	After TNBS Induction	
Normal Placebo	100.0±3.9	100.0±3.8	-
TNBS Placebo	94.1±2.8	28.3±9.5**	100.0±33.6
TNBS+AstraGin®	108.8±3.6	54.8±7.7*	193.6±27.2#

Following anesthesia, colitis was induced in rats in TNBS placebo and TNBS+AstraGin® groups by intracolonic instillation of TNBS dissolved in 50% ethanol (Sigma, St. Louis, MO). The solution was injected into the colon, 8 cm proximal to the anus using a catheter. The instillation procedure required only a few seconds and the rats were maintained in a vertical position until they recovered from the anesthesia.

Dosage of AstraGin®

AstraGin®	For Human (mg of AstraGin™ /60 Kg BW)	For Human (mg of AstraGin™ /Kg BW)	For Rat (mg of AstraGin™ /Kg BW)
1.0X	50	0.83	5.14

7.4 Histological Assay

7.4.1 Physical Observation

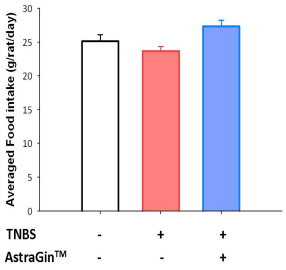
Materials and methods

Daily clinical follow-up included survival, mobility, food intake, and stool consistency. Body weight was recorded daily.

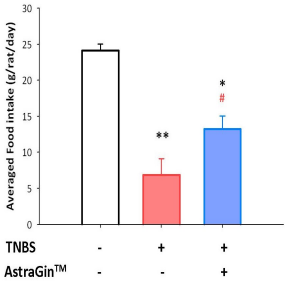
Results

*p<0.05, **p<0.01, when compared to Normal placebo
# p<0.05, when compared to TNBS placebo

Food intake before TNBS induction

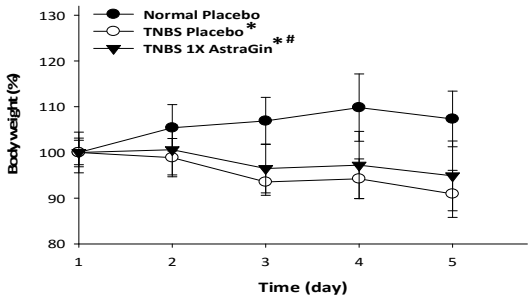


Food intake after TNBS induction



Food intake was significantly lower in TNBS placebo group right after TNBS induction but significantly recovered in TNBS+AstraGin® group.

Body weight after TNBS induction



*p<0.05, when compared to Normal placebo
# p<0.05, when compared to TNBS placebo

Body weight was significantly lower in TNBS placebo group after TNBS induction but recovered to an extent in TNBS+AstraGin™ group.

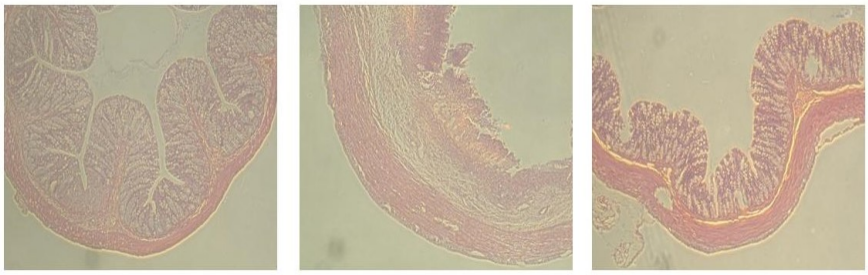
7.4.2 Hematoxylin-eosin Stain

Materials and methods

Colons were removed immediately from animals after they were euthanized by cervical dislocation. The specimens were fixed in 10% buffered formalin and embedded in paraffin. Two sections of 4µm in thickness were cut and stained with hematoxylin–eosin (H&E) for histological evaluation.

Results

- AstraGin™ reduces inflammation in colon bowel wall of colitis rats



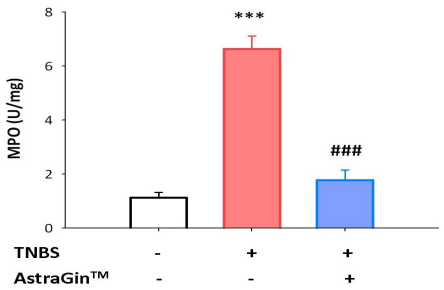
Normal | TNBS induced colitis | TNBS + AstraGin™

7.4.3 MPO Activity Assay

Materials and methods

Colon samples obtained longitudinally from a site of macroscopically detectable inflammation were homogenized in 0.5% (w/v) hexadecyltrimethylammonium bromide in potassium phosphate buffer, pH 6.0. For the myeloperoxidase (MPO) assay, 50µL of each sample were added to 200µL of o-dianisidine solution in phosphate buffer, pH 6.0 immediately prior to reading the change in absorbance at 460nm over 5min using a microplate reader

Results



	Relative MPO activity (%)	
Normal Placebo	100.0	-
TNBS Placebo	592.2***	100.0
TNBS+AstraGin®	158.7	26.8###

***p<0.001, when compared to Normal Placebo
###p<0.001, when compared to TNBS Placebo

MPO activity was significantly lower in TNBS placebo group after TNBs induction but recovered in TNBS+AstraGin® group.

7.5 Intestinal Transporter Analysis

7.5.1 Western Blot Analysis of Intestinal Transporter Expression

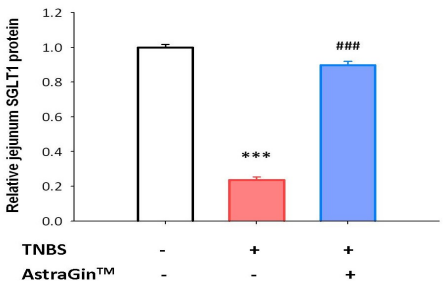
Materials and methods

Rat ileum and jejunum segments were cleansed and immersed in Krebs solution at room temperature. The mucosa and submucosa were removed under magnifying glass. Circular muscle strips (2 mm x10 mm) were cut along the circumferential axis and mounted in 5-ml muscle baths filled with 5 ml Krebs solution at 37°C

Rat tissue fragments were excised and immediately homogenized in solubilizing buffer at 4 ◦C. Insoluble material was removed by centrifugation for 20 min, at 9000×g, at 4◦C. The protein concentration of the supernatants was determined by BCA kit. Equal amount of protein samples of cell lysate were separated by SDS- PAGEl electrophoresis and transblotted onto PVDF membrane. Immunoblotting was performed with anti-human antibodies for SGLT1, CAT1 and β-actin. Signals were visualized with an enhanced chemoluminescence kit (ECL, Amersham, U.K.) followed by exposure to X-ray films.

7.5.1.1 Jejunum SGLT1 Protein Expression

Results



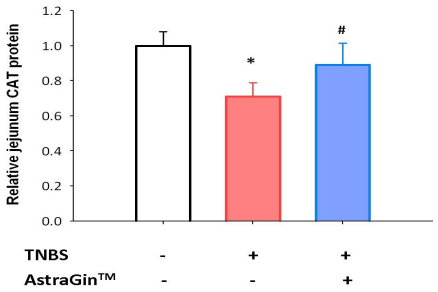
	Relative fold of SGLT1 protein	
Normal Placebo	1.00±0.02	-
TNBS Placebo	0.24±0.02***	1.00±0.02
TNBS+AstraGin®	0.90±0.02	3.75±0.02###

***p<0.001, when compared to Normal + Placebo
###p<0.001, when compared to TNBS + Placebo

TNBS suppressed jejunum SGLT-1 protein expression. AstraGin® enhanced jejunum SGLT1 protein expression in colitis rats.

7.5.1.2 Jejunum CAT1 Protein Expression

Results



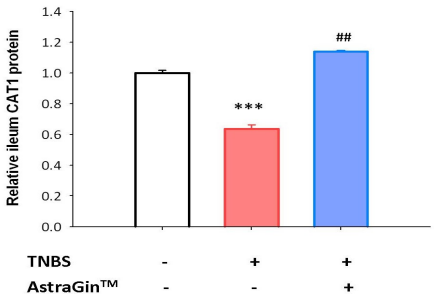
	Relative fold of CAT protein	
Normal Placebo	1.00±0.08	-
TNBS Placebo	0.71±0.08*	1.00±0.08
TNBS +AstraGin®	0.89±0.13	1.25±0.13#

*p<0.05, when compared to Normal + Placebo
#p<0.05, when compared to TNBS + Placebo

TNBS suppressed jejunum CAT1 gene expression. AstraGin® enhanced jejunum CAT1 protein expression in colitis rats.

7.5.1.3 Ileum CAT1 protein Expression

Results



	Relative fold of CAT protein	
Normal Placebo	1.00±0.02	-
TNBS Placebo	0.49±0.07***	1.00±0.07
TNBS+AstraGin®	1.23±0.04**	2.51±0.04##

**, p<0.01, *** p<0.001, when compared to Normal placebo group.
##p<0.01, when compared to TNBS placebo group.

TNBS suppressed ileum CAT1 protein expression. AstraGin® enhanced ileum CAT1 protein expression in colitis rats.

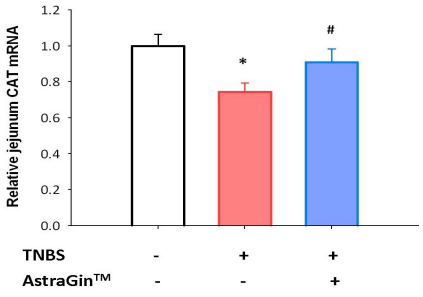
7.5.2 Quantitative Analysis of Intestinal Transporter Transcripts

Materials and methods

The method used was the same as the one described above（section 6.2）.

7.5.2.1 Jejunum CAT1 transcripts Expression

Results



	Relative fold of CAT mRNA

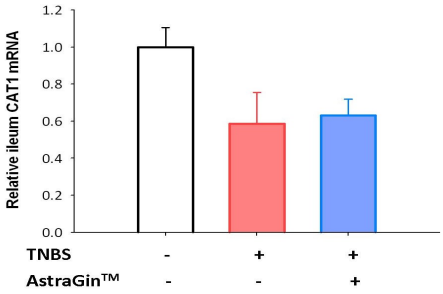
Normal Placebo	1.00±0.07	-
TNBS Placebo	0.74±0.05*	1.00±0.05
TNBS+AstraGin®	0.91±0.07	1.22±0.07#

*p<0.05, when compared to Normal + Placebo
#p<0.05, when compared to TNBS + Placebo

TNBS suppressed jejunum CAT1 mRNA gene expression. AstraGin® enhanced jejunum CAT1 mRNA gene expression in colitis rats.

7.5.2.2 Ileum CAT1 transcripts Expression

Results



	Relative fold of CAT mRNA	
Normal Placebo	1.00±0.10	-
TNBS Placebo	0.59±0.17	1.00±0.17
TNBS+AstraGin®	0.63±0.09	1.08±0.09

TNBS suppressed ileum mRNA CAT1 gene expression. AstraGin® enhanced ileum CAT1 mRNA gene expression in colitis rats.

7.6 Plasma Sample Collection and Process

Blood was collected from tail vein into heparinised tubes. Plasma specimens (0.1 ml) were mixed with (5µl) β-mercaptoethanol (β-ME) and allowed to stand for 5 minutes at room temperature followed by precipitation with ice-cold methanol (395µl) while vortexing. Tubes were allowed to stand for 15 minutes in an ice bucket before centrifuging (5000 rpm / 15 minutes) and the supernatant was collected. The protein free supernatants were processed immediately for assaying total amino acids and HPLC analysis or stored at -70° C until further analysis.

7.7 Amino Acids Quantification by HPLC

Materials and methods

A HPLC system（Waters Inc, USA） was used and the system included two HPLC pumps 600, high pressure mixer, autosampler 717+, dual λ absorbance detector 2487, and a refractive bindex detector 2414. Separation was achieved by using a 4µm AccQ.Taq C_{18} column（150x3.9 mm I.D.）with a guard column.

Microliters of filtered hydrolysated samples or standard were transferred to amber glass vial, borate buffer were added, and the solution was briefly vortexed. The derivatizaton reaction was then initiated by adding AccQ Fluor reagent, and the mixture was immediately vortexed for several seconds. After 1 min incubation at room temperature, the mixture was transferred to an autosampler vial. The vial was placed in a heating block for 10 min at 55℃

Separations were carried out on a AccQ.Taq C_{18} reverse-phase column, with a flow-rate of 1.0 ml/min. Mobile phase A consisted of AccQ.Tag elutent AccQ.Tag A concentrate-Milli-Q water

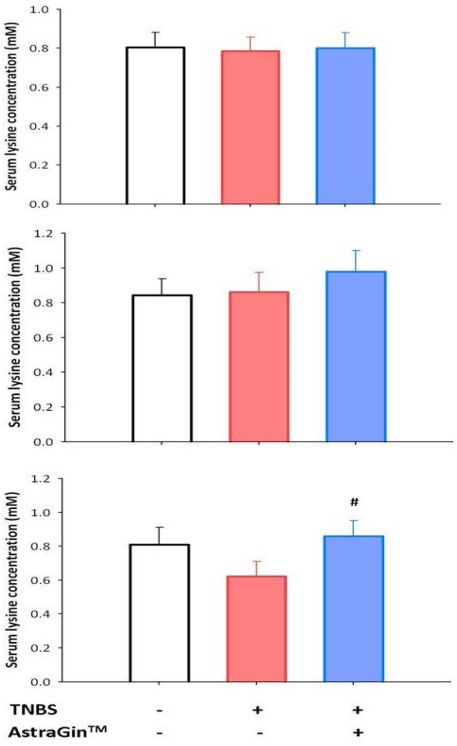
（1:10, v/v）, pH 5.02）. Mobile phase B was acetontrile （acetontrile- Milli-Q water（6：4, v/v））.

Gradient profile

Time（min）	A (%)	B (%)
0	100	0
0.5	98	2
15	93	7
19	88	12
26	68	32
35	60	40
50	60	40
51	0	100
52	0	100

7.7.1 Lysine Uptake Assay

Results



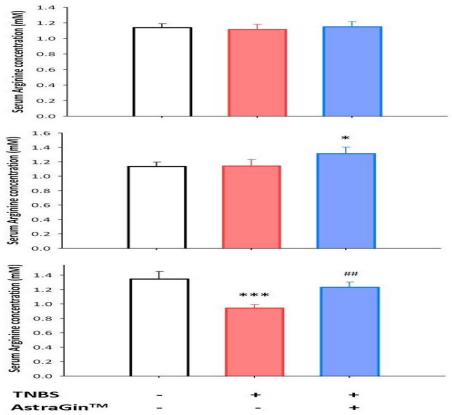
Before Experiment	
	Relative serum lysine (%)
Normal Placebo	100.0±9.57
TNBS Placebo	97.62±8.84
TNBS+AstraGin™	99.39±9.82

1 Week of oral intake before TNBS induction	
	Relative serum lysine (%)
Normal Placebo	100.0±11.35
TNBS Placebo	102.25±12.35
TNBS+AstraGin™	116.12±14.52

After TNBS induction		
	Relative serum lysine (%)	Relative serum lysine (%)
Normal Placebo	100.0±12.53	-
TNBS Placebo	76.90±11.00	100.0±14.31
TNBS+AstraGin™	106.18±11.58	138.09±15.06#

7.7.2 Arginine Uptake Assay

Results



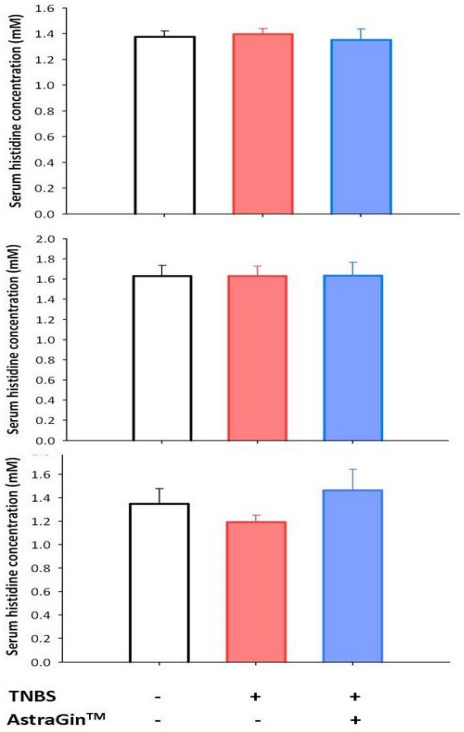
Before Experiment	
	Relative serum arginine (%)
Normal Placebo	100.0±4.57
TNBS Placebo	98.08±5.72
TNBS+AstraGin™	100.99±2.78

1 Week of oral intake before TNBS induction	
	Relative serum arginine (%)
Normal Placebo	100.0±5.34
TNBS Placebo	100.49±8.19
TNBS+AstraGin™	116.12±8.01*

After TNBS induction		
	Relative serum arginine (%)	Relative serum arginine (%)
Normal Placebo	100.0±7.79	-
TNBS Placebo	70.02±3.60	100.0±5.14
TNBS+AstraGin™	91.51±5.52	130.69±7.88

7.7.3 Histidine Uptake Assay

Results



1 Week of oral intake before TNBS induction	
	Relative serum histidine (%)
Normal Placebo	100.0±6.47
TNBS Placebo	99.92±6.18
TNBS+AstraGin™	100.17±8.18

Before Experiment	
	Relative serum histidine (%)
Normal Placebo	100.0±3.34
TNBS Placebo	101.55±3.05
TNBS+AstraGin™	98.29±2.63

After TNBS induction		
	Relative serum histidine (%)	Relative serum histidine (%)
Normal Placebo	100.0±9.67	-
TNBS Placebo	88.64±4.03	100.0±4.55
TNBS+AstraGin™	108.53±13.44	122.44±15.16

8. Toxicity Studies

8.1 Toxicity Studies Published in Peer-Reviewed Scientific Articles

Ingredient	Safety Study	Outcome	Ref.
Astragalus membranaceus	Acute toxicity (mice)	LD50: 79.98 g/Kg	1, 7, 8, 9
	Embryotoxicity (rats, rabbits)	Positive (dosage >1.0 mg/Kg)	2
	Chronic toxicity (rats)	Negative (45, 90, 180 g / Kg)	6, 8, 10
	Micronucleus (mice)	Negative (2.75, 5.5, 11 g / Kg)	7
Panax ginseng	Acute toxicity (mice, rats)	LD50 >21.5 g/ Kg (mice, rats)	3
	Ames test	Negative	11
	Chronic toxicity (rats)	Negative (dosage: 0.625, 1.25, 2.5 g/Kg, 6 months)	4, 11
	Micronucleus (mice)	Negative (2.5, 5, 10 g / Kg)	11
	Mutagenicity (mice)	Negative (dosage: 2.15, 4.3, 8.6 g/Kg)	3
	Embryotoxicity (embryonic stem cell from mouse)	ID50D3:114±8.21 µg/ml (Rb1) ID50D3: 80±5.75 µg/ml (Rg1)	5

8.2 Twenty-eight Days Repeated Oral Toxicity Study in Rats

NuLiv Science commissioned AIBMR Life Sciences in 2010 to carry out a full study and evaluation on the safety of AstraGin®. A report issued by AIBMR indicated that published scientific articles in peer-review journals on orally administered *Astragalus membranaceus* extract showed a NOAEL of 22 g / Kg and 90 g / kg in rats and 1100 mg / Kg in a 30-day repeated oral toxicity study on a *Panax notoginseng* extract. AIBMR concluded that the totality of evidence does not give significant concern for toxicity with oral consumption but recommended one twenty-eight days repeated oral toxicity study in rats for a self-affirmation of GRAS.

The twenty-eight days repeated oral toxicity study in rats was completed in December, 2011 at a AIBMR affiliated GLP lab following the OCED 407 protocol that is approved by FDA. The study was carried out at doses from 0 up to 1000 mg per Kg of body weight, and the NOAEL, in rats, was determined to be 1000 mg/kg bw/day which was the highest level tested. To extrapolate the equivalent safe human dietary addition level, a 100-fold safety factor is used to account for

intra– and inter–species difference. The acceptable daily intake (ADI) for AstraGin™ was thus determined as 10 mg/kg bw/day, or 700 mg per day for the average 70 kg human.

No mortality was observed in any animal in the control or test groups during the study period. Also, there were no clinical signs or abnormality in behavior, motor activity, or general state in control or test group animals in the daily general and weekly detail clinical observations.

8.3 AstraGin™ received GRAS status in March, 2012

The Expert Panel at ABIMR has independently and collectively critically evaluated the safety assessment of AstraGin® , and unanimously conclude that the intended use of AstraGin® as a food ingredient, produced in accordance with Good Manufacturing Practice, and meeting the specifications presented in the document that is the basis for the GRAS determination, is generally recognized as safe. The Expert Panel further concludes that the intended use of GRAS based upon scientific procedures and corroborated by an extensive history of safe use (exposure). The Expert Panel believes that other experts qualified by training and experience to evaluate the safety of food ingredients would concur with this GRAS conclusion.

The expert panel consisted of a former FDA and EPA toxicologist Dr. Judith Hauswirth, PhD, as well as Dr. john R. Endres, Dr. Amy Clewell, Dr. Tennille Marx, and Dr. Tim Murbach at AIBMR.

9. Dosage and Indications

Dosage

Percent increase in amino acid absorption at 5 mg, 10 mg, and 25 mg are estimations based on available data as of the date of this article. 50 mg per day is the dosage used in NuLiv Science *in vitro* studies that demonstrated the results given in the “Indication” section.

NuLiv considers these estimations to be reasonably correct. But only actual assays on these different dosages can finally determine their respective actual percent increase. This table is given for the benefit of those users who are interested in knowing the relative levels of efficacy at this time and realize that they are still best estimations based on available data.

Dosage	5 mg	10 mg	25 mg	50 mg
% Increase in Amino Acid Absorption	37.2%	41.8%	52.3%	62%

Indications

Based on the result of NuLiv Science *in vitro* study, at 50 mg per day, AstraGin® will increase the absorption of amino acids, glucose, and vitamins at the cellular level by 67%, 57%, and 50%.

10. Discussion

The small intestine is the absorption organ that controls the degree and rate of transport of amino acids coming from dietary protein via the portal vein to the liver and the systemic circulation. However, not all amino acids that pass the gut enter the circulation as 30-50% of them will be used by the intestine itself for local metabolism (e.g., oxidation, protein synthesis)

before releasing the rest to the circulation. [12, 13, 14]

Protein homeostasis is a continuous process in which proteins are synthesized from amino acids (protein synthesis) and degraded to amino acids (proteolysis). The amino acids pool is considered to be in dynamic equilibrium with tissue proteins. The metabolic need for amino acids is essentially to maintain stores of endogenous tissue proteins within an appropriate range, allowing protein homeostasis to be maintained. Physical illness can reduce the amount of amino acids absorption and release to the circulation due to its effect on enhancing whole body protein breakdown.

AstraGin® has shown in NuLiv Science's *In vitro* studies to increase the absorption of arginine, citrulline, tryptophan, folate, glusosamine, and glucose in CaCo-2 cells.

In a normal and TNBS-induced IBD rat model, AstraGin® has shown to enhance the gene expression of SGLT1 protein in Jejunum, CAT1 mRNA and protein in both Jejunum and Ileum. CAT1 is the main absorption transporter of cationic amino acids in intestinal tract, which includes Arginine, Lysine, and Histidine. Arginine, Lysine and Histidine uptake assays showed higher rates of absorption in normal and particularly TNBS-induced colitis rats when comparing to the control group.

The increase of absorption rates from the *In Vitro* and *In Vivo* studies are comparable when factors of local metabolism of the intestinal tract and homeostasis are taken into consideration. [12, 13, 14]

In summary, AstraGin® has clearly demonstrated both *In Vitro* and *In Vivo* to enhance the gene expression of many important nutrient mRNA and transporter protein and their absorption. Moreover, AstraGin® has shown *In Vivo* to repair impaired intestinal wall to restore its absorption function. As such, AstraGin™ is a scientifically validated natural nutritional substance that can be taken by everyone to optimize their absorption function to lead to better nutritional status, the foundation of good health. AstraGin® is especially suitable for people who are under above normal physical and mental stress, children growing up, seniors whose absorption functions are impaired to various degrees, and health compromised individuals. Considering the incidence of mal-absorption due to modern diet, overly processed foods, and sedentary lifestyle is rising in the world, especially in the western and developed countries, incorporating AstraGin® on a regular basis will better assure of adequate nutritional status for good health.

11. Conclusion

Sustainability of human life requires the constant availability of necessary nutrients to the trillion cells in human body. Nutrients in the foods must first be broken down by the digestive process to the forms that are utilizable by these cells and then "absorbed" by the intestinal cells and lymphatic ducts before becoming "available" to the entire body.

Improving digestion using digestive enzymes and creating a balanced eco-system in the digestive tract through the use of beneficial bacteria have been widely used. But the most critical phase in the long and complicated nutrient uptake processes occurs at the final absorption, which truly decide how much of these nutrients will be available for utilization by the human body. Even with a nutritious balanced diet, there are no guarantees that these nutrients will be absorbed at an optimal rate.

Absorption determines the amount of nutrients available for maintaining normal bodily functions as well as repairing and rebuilding of cells, tissues, and organs in human body. Absorption plays a very critical role in maintaining life and promoting health in humans and animals.

AstraGin® is an ideal ingredient to improve the nutritional value of a wide range of foods and

dietary products.

12. COA (Certificate of Analysis)

A NuLiv Proprietary All Natural Absorption Enhancing Ingredient

(A NuLiv Proprietary Pharmaceutical Extraction and Formulation from Astragalus Membranaceus and Panax Notoginseng)

Certificate of Analysis

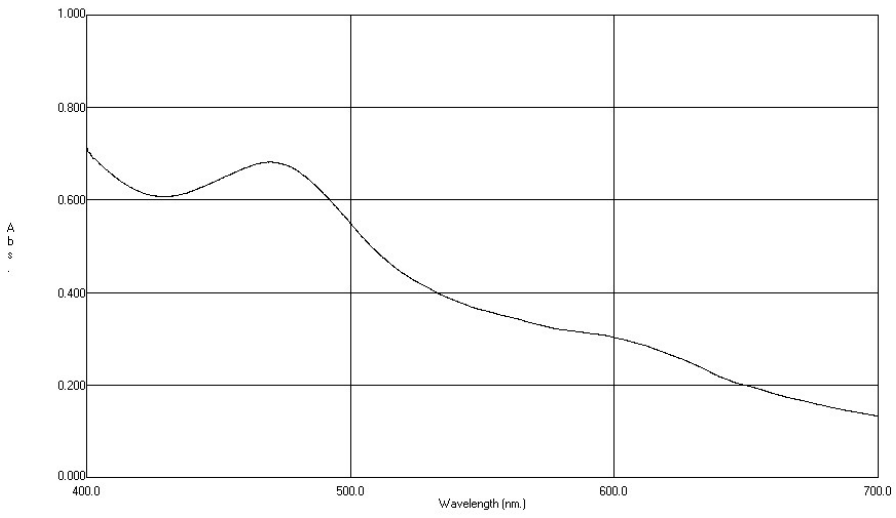
Product Name	AstraGin™	Manufacturing Date	2015/12/09
Batch No.	C20151209	Sampling Date	2015/12/11
Batch Quantity	1500 kg	Expiration Date	2018/12/08

Item	Specification	Result	Method
Marker Compounds			
Total saponin	≧1.5%	4.10%	NLS-Complex-002（UV）
Identifications			
TLC profile	Identical with reference standard	Conform	NLS-Complex-002 (TLC)
Organoleptic Data			
Appearance	Powder	Conform	NLS-QCS-1008
Color	Beige to light brown yellow	Conform	NLS-QCS-1008
Odor	Characteristic	Conform	NLS-QCS-1008
Taste	Characteristic	Conform	NLS-QCS-1008
Process Data			
Plant Name & Part Used	Panax notoginseng (root) Astragalus membranaceous (root)	Conform	
Method of Processing	Extraction/Ethanol & Water	Conform	
Carrier	None	Conform	
Drying Method	Vacuum Dried	Conform	
Excipient	Non-GMO Maltodextrin	Conform	
Physical Characteristics			
Particle Size (80 mesh)	≧80% pass through	100.0%	GB/T 5507-2008
Loss on Drying	≦8.0%	5.3%	GB/T 14769-1993
Ash Content	≦5.0%	0.9%	AOAC 942.05, 18th
Bulk Density	0.30~0.60g/ml	0.59g/ml	NLS-QCS-1013
Solubility in Water	Soluble (1g in 30ml)	Soluble	NLS-QCS-1009
Pesticide Residues			
179 kinds	No More Than Detection Limits	Conform	NLS-QCS-1010(GC/MS/MS, LC/MS/MS)

Heavy Metals			
Total Heavy Metals	≦10 ppm	Conform	USP <231>, Meth. II
Arsenic	≦0.5 ppm	0.19ppm	ChP 2010, App. IXB (ICP-MS)
Lead	≦0.5 ppm	<0.1ppm	ChP 2010, App. IXB (ICP-MS)
Mercury	≦0.025 ppm	<0.01ppm	ChP 2010, App. IXB (ICP-MS)
Cadmium	≦0.1 ppm	<0.01ppm	ChP 2010, App. IXB (ICP-MS)
Other Residues			
Organic solvent	≤0.5% (ethanol)	Conform	NLS-QCS-1007(GC-MS)
Gluten	≤5 ppm	<5ppm	NLS-QCS-1020(ELISA)
Microbiological Tests			
Total Plate Count	≤1,000cfu/g	Conform	AOAC 990.12, 18th
Total Yeast & Mold	≤100cfu/g	Conform	FDA (BAM) Chapter 18, 8th Ed.
Enterobacteriaceae	≤100cfu/g	Conform	AOAC 2003.01, 18th
E. Coli	Negative	Conform	AOAC 997.11, 18th
Salmonella	Negative	Conform	FDA (BAM) Chapter 5, 8th Ed.
Staphylococcus aureus	Negative	Conform	FDA (BAM) Chapter 12, 8th Ed.

Tested by: Amy Ji Date: 2016.01.29

Approved by: Sunnie Sun Date: 2016.01.29



UV-Vis spectrum for measuring total saponins in AstraGin®

13. Nutrition Facts

Nutrient	UOM	Per 100 g
Calories (Kcal)	**Kcal**	377
Calories from Fat	**g**	3
Total Fat (by GC)	**g**	0.35
Monounsaturated Fat	g	0.03

Polyunsaturated Fat	g	0.12
Saturated Fat	g	0.19
Trans Fat	g	0.00
Cholesterol	mg	<2.0
Sodium	mg	35
Carbohydrates	g	91.2
Total Dietary Fiber (TDF)	g	0.3
Total Sugar	g	9.8
Fructose	g	0.8
Glucose	g	0.5
Lactose	g	<0.5
Maltose	g	2.1
Sucrose	g	6.4
Protein (Combustion)	g	2.2
Vitamin A (Retinol + Carotene)	IU	<20
Vitamin C	mg	<0.5
Calcium	mg	11
Iron	mg	0.60
Moisture	g	5.8
Ash	g	.43

Analysis performed by OMIC USA Inc. Portland, Oregon, USA 97210

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