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Assessing the Efficacy of Natural Pet Products in Protecting Gastric Cells and Reducing Cytotoxicity under Hyperacidity Conditions: An In Vitro Study

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Abstract:

Gastritis in pets necessitates effective acid suppression for successful treatment. However, the synergistic potential of antacid salts within natural feed products remains underexplored. In this *in vitro* study, we aimed to compare six supplements comprising natural ingredients for their ability to safeguard gastric cells and mitigate cytotoxicity under hyperacidity conditions. While Product 1 showed ineffectiveness in cell protection, Products 2, 3, 4, and 5 exhibited varying degrees of reversal of hyperacidity-induced cytotoxicity. Notably, Product 6 demonstrated superior efficacy in shielding gastric cells from acidic pH-induced cytotoxicity, displaying a dose-dependent response. These findings highlight the potential of natural supplements, particularly Product 6, as promising candidates for mitigating gastritis-related conditions in pets. Further research, including *in vivo* studies, is warranted to validate these observations and explore their clinical applicability.

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INTRODUCTION

The management of gastritis heavily relies on acid suppression to prevent damage from exposure to the acidic environment and gastric ulcers [1-3]. Gastritis is a gastric disease characterized by acute or chronic vomiting, secondary to inflammation of the gastric mucosa [4-7].

In instances of gastric inflammation, chemical injury, ischemia, infection, or antigens can trigger the release of inflammatory mediators and vasoactive compounds, leading to the disruption of gastric epithelial cells, increased gastric acid secretion, and impaired gastric barrier function [5,8,9]. This inflammatory cascade further stimulates acid secretion, induces mucosal damage, increases cell membrane permeability, and alters microvascular blood flow [8,10].

Normal gastric secretions, comprised of acid, mucus, bicarbonate, and antibacterial substances, constitute the first line of defense against acidity, detergents, bacteria, and changes in temperature [8,9,11].

Additionally, the gastric epithelium acts as a barrier to acid diffusion and undergoes repair following cell injury [8,12].

Various therapeutic options are currently employed in veterinary medicine to reduce gastric acidity and/or promote mucosal protective mechanisms, aiming to prevent mucosal damage [13]. These include Histamine-2 (H2) receptor antagonists, proton pump inhibitors [1,2], misoprostol, sucralfate, and insoluble salts [13].

A recent *in vitro* study investigated the synergistic effects of antacid salts and certain natural feed products in suppressing acid activity. The study utilized a solution designed to simulate the acidic pH levels (2 and 4.5) of the stomach [3,14].

In particular, the product composed by the combination of carbonate calcium carbonate with magnesium hydroxide showed higher suppressant activity than the other products and confirmed gel formation for protectant activity [14]. However, this *in vitro* study can only provide information about the acid-suppressing activity through gel formation of these products. It does not assess their potential anti-inflammatory, antioxidant, and cytoprotective activities [14].

Gastric juices constitute a vital element in the digestive system, and their acidity is a natural aspect of the digestive process's physiology [15]. Nevertheless, instances of hyperacidity are not uncommon and may arise from various factors such as infections, or medications. At the cellular level, gastric juices produced under such conditions can harm the stomach lining, triggering inflammation and leading to clinical symptoms like burning sensations, gastroesophageal reflux, and dyspepsia, negatively affecting daily life. Gastroprotective products play a crucial role in neutralizing the excessively acidic environment, reinstating the gastric milieu to physiological conditions, and, consequently, enhancing functionality. They serve to shield the gastric mucosa from cytotoxic damage induced by hyperacidity.

The aim of our *in vitro* study is the comparison of six supplements based on a combination of natural ingredients. We evaluated the efficacy of the different products on the protection of gastric cells and cytotoxicity reduction in hyperacidity conditions.

MATERIAL & METHODS

Cellular Lines and Culture Conditions

The experiments were conducted on a cell line of immortalized human gastric epithelial cells (AGS cells). The cells were cultured at 37 °C, 5% CO_2 in MEM/F12 medium, supplemented with 1% L-glutamine and 10% Fetal bovine serum (FBS). Due to physiological similarities among mammalian species, biomedical research for human pathophysiology often relies on animal models, both *in vitro* and *in vivo*. For the same reason, the largely available human cell lines can be used for the generation of data inferring animal (mammalian) data. Referring to the gastric human cell line used for the experiment, the lack of availability of animal gastric cell lines allows the human model a valuable and reliable alternative to the use of the animal ones.

Products and Preparation of Treatments

Six natural supplements for pets were used in this project (Table **1**, list of ingredients). Products were purchased through the usual commercial channels of retail sales. They all have a similar composition (protein source, buffering salts, plant extract, alginate, or guar). In particular, Product 5 and Product 1 are the only ones not to have an animal-origin protein source. Product 1 does not contain plant extracts; therefore, Product 5 seems to be the most similar to Product 6 (although the composition is still different). Unlike Product 4, Product

Table 1: Ingredients of the Six Products Tested in the Study

PRODUCT 1		
Ingredients	mg/1g	%
Guar gum	18.8	1.88
Kaolinitic clays	18.8	1.88
Magnesium oxide	18.6	1.86
Calcium carbonate	15.7	1.57
Pectine	15.6	1.56
Chondroitin sulfate	12.3	1.23
Potassium bicarbonate	9.0	0.9
L-alanine	9.2	0.92
Vitamin B1	8.8	0.88
Vitamin B2	4.4	0.44
Vitamina B6	2.2	0.22
Total weight (1gr paste)		
PRODUCT 2		
Ingredients	mg/1g	%
Calcium carbonate	80	8.0
Sodium bicarbonate	40	4.0
Chondroitin sulfate	40	4.0
Althea officinalis root	40	4.0
Sodium alginate	5	0.5
Total weight (1g of paste)		
PRODUCT 3		
Ingredients	mg/1g	%
Sodium alginate	250	25.0
Sodium bicarbonate	125	12.5
Calcium carbonate	35	3.5
Total weight of a tablet (1g)		
PRODUCT 4		
Ingredients	mg/1.5g	%
Matricaria recutita e.s. (camomile)	83.25	5.55
Licorice (Glycyrrhiza glabra L.) extract	83.25	5.55
Ginger (Zingiber officinale)	63.27	4.218
Glycerol tributyrate	4.442	0.2961
Total weight of a tablet (1.5g)		
PRODUCT 5		
Ingredients	mg/1g	%
Calcium carbonate	150	15

(Table 1). Continue

Sodium bicarbonate	100	10
Altea (Althaea officinalis) root	100	10
Carob powder	100	10
Carvi (Carum carvi) fruit	75	7.5
Sodium alginate	35	3.5
Ananas (Ananas comosus) extract titrated 0,3% bromelin	30	3.0
Licorice (Glycyrrhiza glabra L.) extract titrated 10% Acid glycyrrhizic	15	1.5
Ginger (Zingiber officinale Rosc.) extract titrated 1% Gingeroli	10	1.0
Total weight of a tablet (1g)		
PRODUCT 6		

Ingredients		mg/2g	%
Microcrystalline cellulose		770	38.5
Yeasts, inactivated		268	13.4
Palatability enhancer (Optimizor Uranus)	Sodium pyrophosphate	114	5.7
	Yeasts, inactivated	60	3
	Lupin protein meal	24.2	1.21
	Sunflower oil	1.8	0.09
Guar meal		150	7.5
L-threonine		126	6.3
Calcium carbonate		100	5
Magnesium hydroxide		76	3.8
Methyl sulphonyl methane		62	3.1
Thea sinensis L. = Camellia thea Link. = Camellia sinensis (L.) O. Kuntze: Tea extract		60	3
Mono and diglycerides of fatty acids (Glyceryl dibehenate)		40	2
Psyllium – Plantago Ovata L. – powder cuticle		38	1.9
Colloidal silica		30	1.5
Magnesium stearate		30	1.5
Trigonella foenum-graecum L.: Fenugreek extract		26	1.3
Products from the processing of plant (Aloe vera)		12	0.6
Glycyrrhiza glabra L.: Licorice extract (wb)		12	0.6
Total weight of a tablet (2g)			

5 does not contain inactivated yeast and has fewer ingredients in its formulation.

Products were crushed (when tablets) using a mortar to obtain homogeneous powders or used as they are (when paste). The desired quantity was then weighed based on the daily dose/10 Kg of animal weight (Product 1 - 3ml, Product 2- 2g, Product 3- 2g, Product 4- 3g, Product 5- 2g, Product 6- 2g) dissolved in Phosphate-buffered saline (PBS) to reach a total of 6 liters (lt), and filtered through 0.22 µm filters to obtain a sterile product suitable for *in vitro* use.

The liquid product was used by extracting the desired volume, subsequently diluted in PBS, and filtered through a 0.22 µm filter to obtain a sterile product suitable for *in vitro* use.

Concentrations to be tested were selected for each product based on usage indications and by predicting them relative to the average gastric volume.

Cellular Viability Assay

AGS cells were plated in 96-well multiwell plates at a density of 10^4 cells/well in a growth medium

supplemented with 10% FBS. The next day, the medium was removed, and 100 µL of complete medium with 1% FBS was added for treatments with the 6 products under examination. Specifically, cells were treated with 10 scalar concentrations obtained through 1:2 serial dilutions to identify non-toxic doses for conducting the bioactivity test. Sodium dodecyl sulfate (SDS) at a concentration of 1mg/ml was used as a positive control. Cells were incubated for 24 hours at 37°C and 5% CO_2. The next day, the reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well at a final concentration of 0.5 mg/ml, and the plate was incubated for an additional 2 hours at 37°C and 5% CO_2. After incubation, reduced MTT crystals were solubilized by removing the medium and adding 100 µL of DMSO to each well. The absorbance at 595 nm of each sample was finally measured using an Infinite M NANO+ plate reader (Tecan). Cellular viability is expressed as a percentage of the absorbance at 595 nm of untreated (NT) cells.

Acidic pH Gastroprotection Test

AGS cells were plated in 96-well multiwell plates at a density of 10^4 cells/well in growth medium supplemented with 10% FBS. The next day, the medium was removed and replaced with a complete medium supplemented with 1% FBS. The cells were then pre-treated with the products at the first three concentrations found to be non-toxic in the previous cellular viability test for 1 hour. Subsequently, an acidic condition was simulated by treating AGS cells with 1M HCl until achieving an acidic pH (pH=2). This incubation was maintained for 1 hour, after which an MTT test was conducted as described above. The test included an experimental group of untreated cells (NT, negative control) and an experimental group of cells under conditions of hyperacidity but without products. The gastroprotection of the product was then assessed by comparing the viability of cells in wells treated only with HCl (the "pathological" condition of gastric hyperacidity) to the viability of cells treated with the products [16,17]. The data were statistically analyzed by conducting a one-way ANOVA, followed by Dunnett's post-test (vs pH2)

RESULTS & DISCUSSION

Cellular Viability, Cytotoxicity Test

Cellular viability following treatment with the product was analyzed to identify concentrations without toxic effects on AGS cells for use in subsequent experiments. AGS cells were treated with the 6 products at 10 scalar concentrations obtained through 1:2 dilutions for 24 hours. Cellular viability was inferred from the cell replication rate, measured by cell counting with a hemocytometer. SDS 1 mg/ml was used as a positive toxicity control. The results of the test are shown in Figure **1**. Specifically, none of the products exhibited cytotoxic effects at the tested concentrations. In another study by Sánchez and colleagues [16], the tested oleanolic acid derivatives [18] showed different cytotoxicity and some of them were even less toxic than the parent compound. In our study, the cellular viability remained around 100% compared to the untreated control. As expected, the positive toxicity control (SDS 1 mg/ml) showed a significant reduction in cellular viability, approximately 40%, confirming the validity of the test.

Acidic pH Gastroprotection Test

In vitro studies evaluating the gastroprotective efficacy of natural products have become a crucial precursor to *in vivo* investigations. For instance, research conducted by Sánchez and colleagues (2006 [16]) demonstrated the gastroprotective and ulcer-healing effects of triterpene oleanolic acid and its semisynthetic derivatives using human epithelial gastric cells (AGS) and human lung fibroblasts (MRC-5). Other studies reported that several terpenes have been confirmed as gastroprotective compounds [16]. Similarly, Astudillo and colleagues (2002 [19]) reported the gastroprotective properties of oleanolic acid and its derivatives in rodent models. In our study, based on the previous cytotoxicity test, cells were treated with the three highest concentrations of the products, according to Table **2**.

As expected, and as shown in Figure **2**, the hyperacidity condition resulted in a 20% reduction in cellular viability. To generate a hyperacidity model, AGS cells were treated with 1 M HCl. Simultaneously, treatment with 3 concentrations of the 5 products was administered.

From Figure **2**, it can be observed that:

For the Product 1, no cytoprotective effect was observed for any of the three concentrations used. Cellular viability remained around 80%, similar to that of cells under hyperacidity conditions.

The Product 2 showed a dose-response trend in cytoprotection, although not statistically significant.

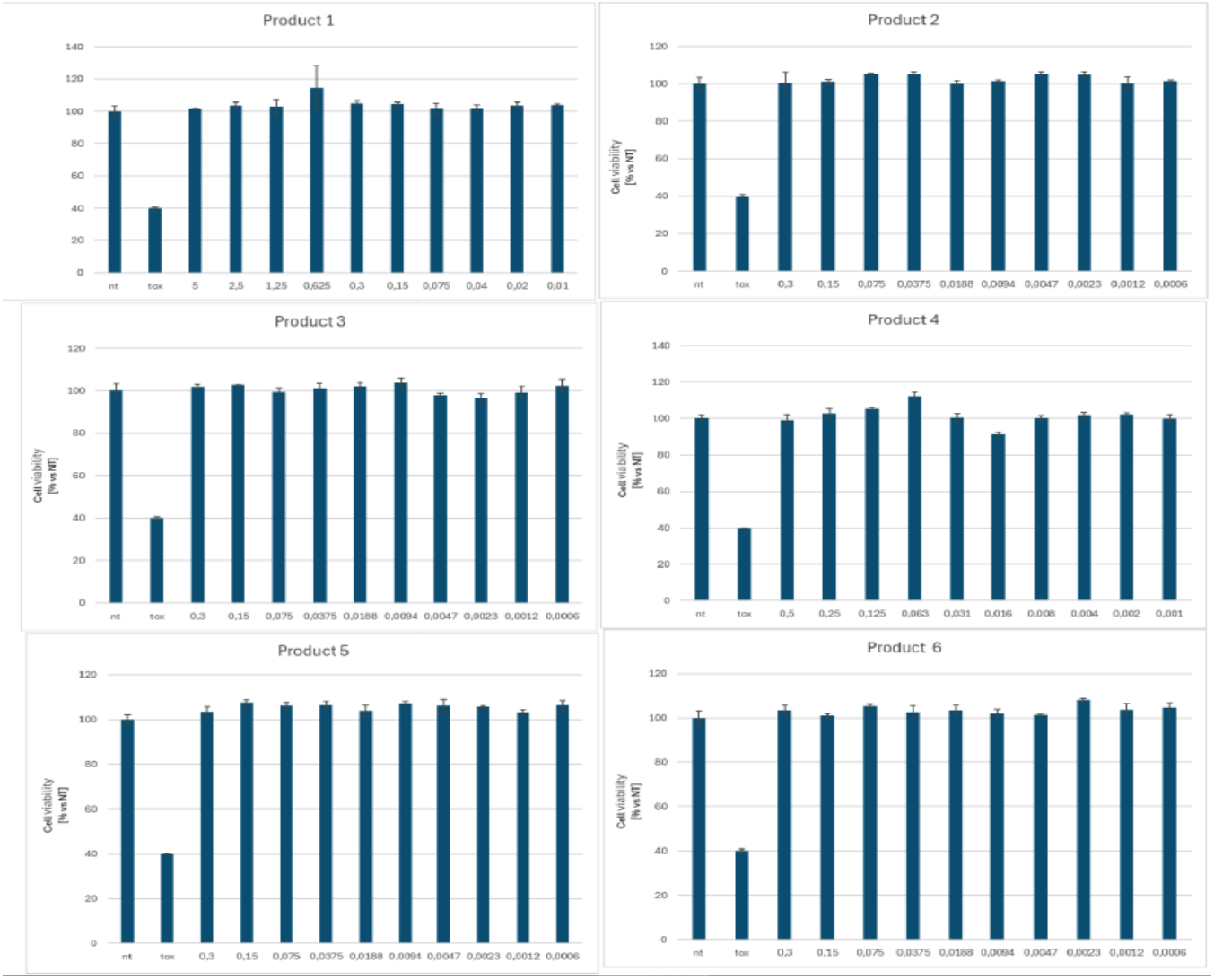

Figure 1: Cytotoxicity Test. The result is expressed as cellular viability (%) compared to the untreated control. X-axis shows the concentrations of the product used. NT is the control group (not-treated).

Table 2: List of the Three Highest Concentrations of the Tested Products

Product	Concentration A	Concentration B	Concentration C
Product 1	0,05 %	0,025%	0,0125%
Product 2	0,3 mg/ml	0,15 mg/ml	0,075 mg/ml
Product 3	0,3 mg/ml	0,15 mg/ml	0,075 mg/ml
Product 4	0,5 mg/ml	0,25 mg/ml	0,125 mg/ml
Product 5	0,3 mg/ml	0,15 mg/ml	0,075 mg/ml
Product 6	0,3 mg/ml	0,15 mg/ml	0,075 mg/ml

The Product 3 exerted a protective effect at concentrations of 0.3 and 0.15 mg/ml, while no protective effect was observed at a concentration of 0.075 mg/ml.

The Product 4 exerted a protective effect at a concentration of 0.25 mg/ml.

The Product 5 exhibited a protective effect at a concentration of 0.3 mg/ml, with a subsequent dose-response trend, although not significant.

The Product 6 exerted a protective effect at a concentration of 0.3 mg/ml, with a subsequent dose-response trend.

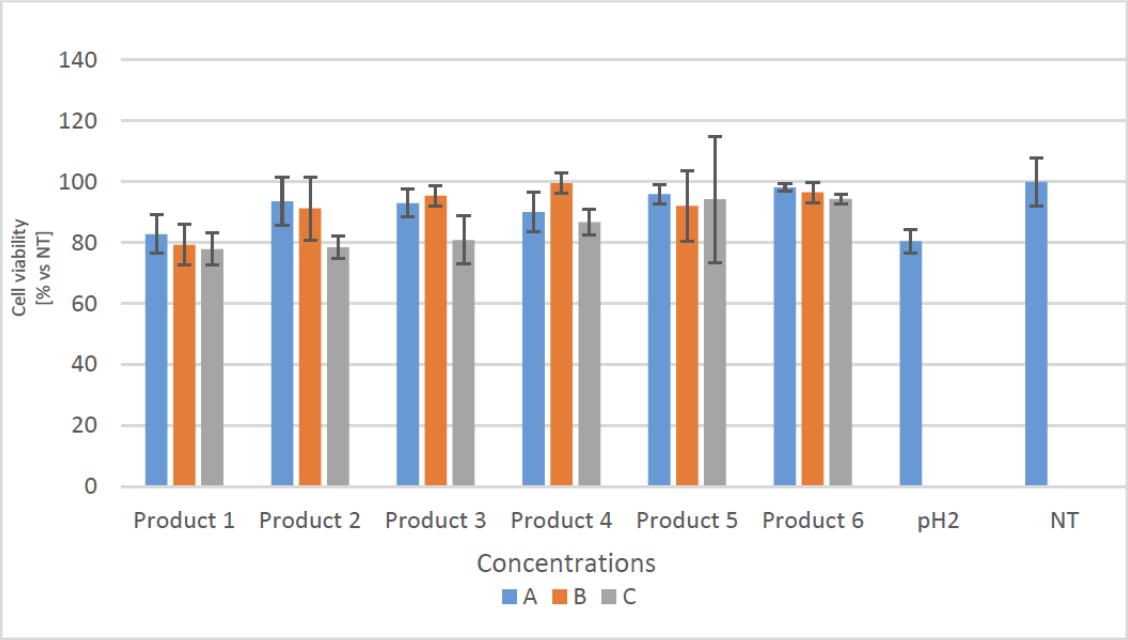

Figure 2: Acidic pH Gastroprotection Test. The result is expressed as cellular viability (%) compared to the not-treated control (NT). For each experimental group, statistical analysis was conducted compared to the positive hyperacidity control (pH2).

The establishment of robust *in vitro* models for assessing the gastroprotective effects of compounds not only reduces reliance on laboratory animals but also holds significant promise for predicting outcomes in pharmacological studies. The validation of such *in vitro* models could thus serve as invaluable tools in advancing research on gastroprotective compounds and refining drug development processes.

Based on the results of this work, out of the six products tested, one product (Product 1) appears to be ineffective in protecting gastric cells from cytotoxicity induced by hyperacidity at the tested concentrations. On the contrary, the products Product 3, Product 4, and Product 5 are effective, as they can completely or partially reverse the cytotoxicity induced by hyperacidity. The product Product 2 shows an efficacy trend that was not statistically significant. Product 6 proves to be more effective in protecting gastric cells from cytotoxicity induced by acidic pH compared to the other products, showing a dose-dependent trend. The better efficacy of Product 6 could derive from the ant inflammatory properties of its plant extracts combination and the presence of calcium carbonate ad magnesium hydroxide which increase stomach pH value [13,20].

CONCLUSIONS

The results of this *in vitro* trial, indicate that different natural products for pets have variable effects on protecting gastric cells from cytotoxicity induced by hyperacidity, with Product 6 demonstrating the highest efficacy among those tested being a promising product to be tested *in vivo* trials in pets.

SUPPLEMENTARY TABLE

The supplementary table can be downloaded from the journal website along with the article.

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