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# IMMUNIES, a unique polyherbal extract, exhibits antiproliferative activity and improves tumor-bearing canine patients: a pilot study

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**Abstract** Dog owners seek treatment when their pets develop cancer. IMMUNIES is traditional herbal medicine-based figment made of 10 natural herbs, designed to maintain host immune function. The major component of IMMUNIES is *Dendropanax morbiferus*. This clinical pilot study monitored the toxicity and efficacy of IMMUNIES. Four senile dogs with spontaneously occurring mammary and liver cancers were enrolled in this study and treated orally daily for 3 months, and their blood/urine biochemical profiles were examined each month. IMMUNIES was well tolerated during the treatment period. Blood urea nitrogen, creatinine, alanine aminotransferase, alkaline phosphatase, and C-reactive protein levels decreased in all four dogs, whereas red blood cells and hematocrit were within the normal range. IMMUNIES also changed the expression of several molecular targets in the anticancer pathway, such as pro-NAG-1, p53, and cyclin D1. Although the tumors did not completely respond to IMMUNIES, the biochemical profiles and clinical examination showed a stabilized cancer status for 3 months. Thus, IMMUNIES was found to be safe and well-tolerated in the dosage range tested and exhibited cancer antiproliferative activity in canine cancer. Future studies should address other potential benefits of IMMUNIES, including correlative assessments of immune function, quality of life, and owner satisfaction.

**Keywords** Canine cancer · Cyclin D1 · IMMUNIES · p53 · pro-NAG-1

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## Introduction

Plant-derived compounds have been used for centuries in the treatment of many diseases with few adverse effects [1]. Plant extracts can directly inhibit the proliferation of cancer cells, indicating their potential use in drug development or adjuvant therapy for cancer patients [2]. The broad range of activities of medicinal plants has been attributed to their ability to affect multiple targets, indicating that the mechanism of action is still largely unknown.

IMMUNIES is a polyherbal preparation made of 10 different herbs, many of which have antineoplastic activity and enhance the immune system (Table 1). Among them, *Dendropanax morbiferus* (DM) is a major component and has been used in folk medicine as a healthy functional food for the treatment of inflammatory and metabolic diseases [3]. However, the mechanisms underlying the varying activities of DM and other components of IMMUNIES are largely unclear. Several studies have indicated that DM extract has cytotoxic activity against several human cancers, such as breast, lung, and hepatocellular cancers [4,5]. Recent data suggest that the oral consumption of DM extract has no adverse effects in humans [6], and DM extract encapsulated into ZnO nanoparticles exhibits better anticancer activity in lung cancer cells [7]. In addition to DM, other plant extracts in IMMUNIES also exhibit many biological activities, including anticancer activity. Thus, the combination of 10 different herb extracts in IMMUNIES may have better anticancer activity than 1 herb alone. IMMUNIES also contains three species of lactic acid bacteria, which may help metabolize herbal ingredients in the gut. The addition of lactic acid bacteria to herbal extracts may increase the beneficial activity of the extracts [8]. However, the synergistic effect of lactic acid bacteria with other herbal ingredients has not been determined.

Cancer is the leading cause of death in dogs. Like humans, dogs are at risk for developing different types of cancer, including mammary and liver cancers. The incidence of canine cancer has been gradually increasing over the past two decades due to the prolonged lifespan of dogs [9]. Various types of cancer, such as

solid tumors, hematological tumors, adenocarcinoma, and soft-tissue sarcoma, can be seen in dogs, and cancer therapy in dogs generally follows the same principles as in humans. Comparative oncology has long held an important role in our understanding of the mechanisms underlying disease pathogenesis, with a recent increase in focus on the use of companion animals in translational studies to assess the efficacy of emerging therapies [10]. Conventional therapy includes surgery, chemotherapy, radiation therapy, and immunotherapy and often causes collateral damage to the body, with mild-to-severe adverse effects [11]. In contrast, herbal medicine may provide comprehensive treatment, considering the overall condition of the patient. Like human cancer patients, dogs with cancer are being increasingly treated with complementary and alternative therapies, including herbal medicines and nutritional supplements [12]. Herbal medicine works with the body rather than against it, its components maintain the body balance, and its adverse effects are typically mild. Herbal medicine may be used as a stand-alone treatment or an adjuvant treatment for cancer patients. It also provides the veterinary practitioner with the ability to not only treat active cancer but also potentially prevent recurrence and metastasis [13]. Indeed, several herbal formulas have been suggested as complementary medical options for dogs undergoing conventional therapy in order to alleviate the adverse effects of these treatments or to increase the effects of conventional therapy [14]. Furthermore, herbal medicine can also be used as an alternative option for feline cancer patients that cannot undergo conventional therapy [15].

The cancer antiproliferative activities of many single plant extracts, such as *Alpinia oxyphylla* [16], *Marrubium vulgare* [17], and green tea [18] have been previously demonstrated. It could be hypothesized that a combination of plant extracts may enhance anticancer effects; therefore, the current survey was conceived as a clinical pilot study on dogs with spontaneously occurring cancers in order to investigate the efficacy and tolerability of IMMUNIES and the documented evidence of the antitumor activity of this polyherbal preparation.

## Materials and Methods

### IMMUNIES

IMMUNIES is a powdered blend derived from 10 different herbs and 3 species of lactic acid bacteria; *Lactobacillus acidophilus*, *Streptococcus thermophilus*, and *Bifidobacterium longum*. IMMUNIES was purchased from HerbCiti LLC (Cupertino, CA, USA). The herbal ingredients in IMMUNIES are listed in Table 1. IMMUNIES was provided in the form of capsules, which were dissolved in dimethyl sulfoxide (DMSO) for *in vitro* experiments.

### Cell culture

The D-17 canine osteosarcoma, NIH/3T3 murine fibroblast, U2OS human osteosarcoma, and HepG2 human hepatocellular carcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). DSN canine osteosarcoma cell line was previously described [19]. HaCaT human keratinocyte line was purchased from Cell Line Services (Eppelheim, Germany). CPEK canine keratinocyte cell line is kindly provided by Dr. Hwang (Seoul National University, Seoul, South Korea). D-17, DSN, NIH/3T3, HaCaT, HepG2, and CPEK cells were cultured in Dulbecco's Modified Eagle's Medium (Welgene, Gyeongsan, South Korea) supplemented with 10% fetal bovine serum (FBS; Life Technologies Corporation, Grand Island, NY, USA) and 1% penicillin-streptomycin (Life Technologies). U2OS cells were cultured in McCoy's 5A Medium (Welgene) supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% sodium pyruvate. All cells were maintained in a humidified atmosphere of 5% $CO_2$ at 37 ºC. In all *in vitro* experiments, 0.1% DMSO was used as the control.

**Table 1** Composition and doses of herbal extracts and lactic acid bacteria in IMMUNIES used *in vitro* and *in vivo*

| Compounds | Percentage (%) | Dose (mg) in 650 mg IMMUNIES capsule |
|---|---|---|
| *Dendropanax morbiferus* | 47.2 | 306.80 |
| Asian lizard's tail (*Saururus chinensis* Baill.) | 9.4 | 61.10 |
| Asiatic plantain (*Plantago asiatica*) | 7.3 | 47.45 |
| Purslane (*Portulaca oleracea*) | 8.6 | 55.90 |
| Honeysuckle flower (*Lonicerae flos*) | 4.3 | 27.95 |
| Green tea leaf (*Camellia sinensis*) | 9.1 | 59.15 |
| Black bean (*Phaseolus vulgaris*) | 4.9 | 31.85 |
| Dandelion (*Taraxacum officinale*) | 3.4 | 22.10 |
| Dong quai (*Angelica sinensis*) | 2.9 | 18.85 |
| Licorice (*Glycyrrhiza uralensis*) | 2.9 | 18.85 |
| Lactic acid bacteria | CFU in 650 mg IMMUNIES capsule | |
| *Lactobacillus acidophilus* | 100,000,000 CFU | |
| *Streptococcus thermophilus* | 133,333,333 CFU | |
| *Bifidobacterium longum* | 100,000,000 CFU | |

### Cell viability assay

The CellTiter 96 $AQ_{ueous}$ One solution (Promega, Madison, WI, USA) was used to assess relative cell viability according to the manufacturer's protocol. D-17, DSN, HepG2, U2OS, NIH/3T3, HaCaT, and CPEK cells were plated in complete culture media in 96-well culture plates and grown overnight. Next, the cells were treated with various concentrations of IMMUNIES in complete media and incubated for 0, 24, and 48 h. After removing the media, a mixture of 100 μL of complete media and 20 μL of the reagent were added to each well and incubated for 1 h at 37 ºC in a 5% $CO_2$ incubator. Cell viability (absorbance) was measured at 492 nm using a microplate spectrophotometer.

### Western blot

U2OS and HepG2 cells were cultured on a 60 mm dish and treated with various concentrations of IMMUNIES. After 24 h treatment, the cells were harvested with radioimmunoprecipitation assay Lysis Buffer (BIOMAX, Seoul, South Korea) supplemented with 0.5% Universal Protease Inhibitor Cocktail (BIOMAX) and 1 mM sodium fluoride and sodium orthovanadate. The protein concentration of the lysates was quantified using the bicinchoninic acid Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Cell lysates were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) transfer membranes (GVS Filter Technology, Sanford, ME, USA). The PVDF membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature, followed by incubation in TBST containing primary antibodies overnight at 4 ºC. The membranes were washed thrice with TBST and incubated with anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP; 1:5,000) for 1 h at room temperature, followed by washing thrice with TBST again. Protein expression was detected using the ECL Western Blotting Substrate (Thermo Fisher Scientific). Western blot images were taken using Alliance Q9 mini (UVITEC, Cambridge, England, UK). Cyclin D1 (2978S; Cell Signaling Technology, Danvers, MA, USA), p53 (sc-126; Santa Cruz Biotechnology, Dallas, TX, USA), GAPDH HRP-conjugated antibody (sc-47724; Santa Cruz Biotechnology), and β-actin (sc-47778; Santa Cruz Biotechnology) antibodies were purchased, and pro-NAG-1 antibody was as previously reported [18,20].

### Patient data, treatment administration, and blood tests

Four senile female dogs (three pure-bred [cases 1, 3, and 4] and one mixed-breed dog [case 2]) diagnosed with cancer at the Dr. Oh Hwanggum Animal Medical Center (Daegu, South Korea) with spontaneously occurring solid tumors were enrolled in this study. Patient information is summarized in Table 2. IMMUNIES was administered as 650 mg capsules per 5 kg of body weight twice daily for 3 months, followed by observation of changes in patient weight, body temperature, blood pressure, clinical symptoms, blood tests, and urinalysis.

### Statistical analysis

The results are presented as the mean ± standard deviation (SD). For two-group comparisons, Shapiro-Wilk test was performed to check the normal distribution of data. Student's *t*-test was performed for statistical analysis. Differences were considered statistically significant at $p < 0.05$.

## Results

### IMMUNIES inhibited cell proliferation of canine and human cancer cells

To investigate the potential role of IMMUNIES in cancer cell growth inhibition, the antiproliferative effect of IMMUNIES in D-17, DSN, HepG2, and U2OS cells was investigated. Cell viability was determined using the CellTiter 96 $AQ_{ueous}$ One solution assay. IMMUNIES inhibited cell growth in a dose- and time-dependent manner in D-17, DSN, and HepG2 cells (Fig. 1A-C). The cell morphology significantly changed, and the cell numbers dramatically decreased in D-17 and DSN cells after treatment with 25 and 50 μg/mL of IMMUNIES for 48 h. In HepG2 cells, 50 μg/mL of IMMUNIES significantly decreased the cell viability. But cell proliferation of U2OS cells were not decreased by 50 μg/mL of IMMUNIES (Fig. 1D). These results demonstrate that IMMUNIES inhibits cancer cell growth probably by inducing cell cycle arrest or apoptosis.

**Table 2** Patient information of dogs supplemented with IMMUNIES for 3 months

| Case # | Breed | Body weight (kg) | Age (yrs) | Sex | Diagnoses | Life expectancy (month) | Dose (650 mg IMMUNIES/capsule) |
|---|---|---|---|---|---|---|---|
| 1 | Spitz | 13.3 | 8 | FS | Mammary gland tumor, Pyometra (treated w/ hysterectomy), Mastitis, Sepsis, Azotemia, Anemia | 3~6 | 1 capsule BID |
| 2 | Mix | 6 | 13 | FS | Mammary gland tumor, Pyometra (treated w/ hysterectomy), Mastitis, Sepsis, Azotemia | 6~12 | 1 capsule BID |
| 3 | Maltese | 2.3 | 10 | FS | Liver cancer, Hepatitis, Heart disease, Seizure, Azotemia, Anemia | 1~2 | 1/2 capsule BID |
| 4 | Yorkshire terrier | 4.2 | 14 | FS | Liver cancer, Hepatitis, Heart disease, Azotemia, Diabetes mellitus, Cystitis, Pyelonephritis | 1~2 | 1/2 capsule BID |

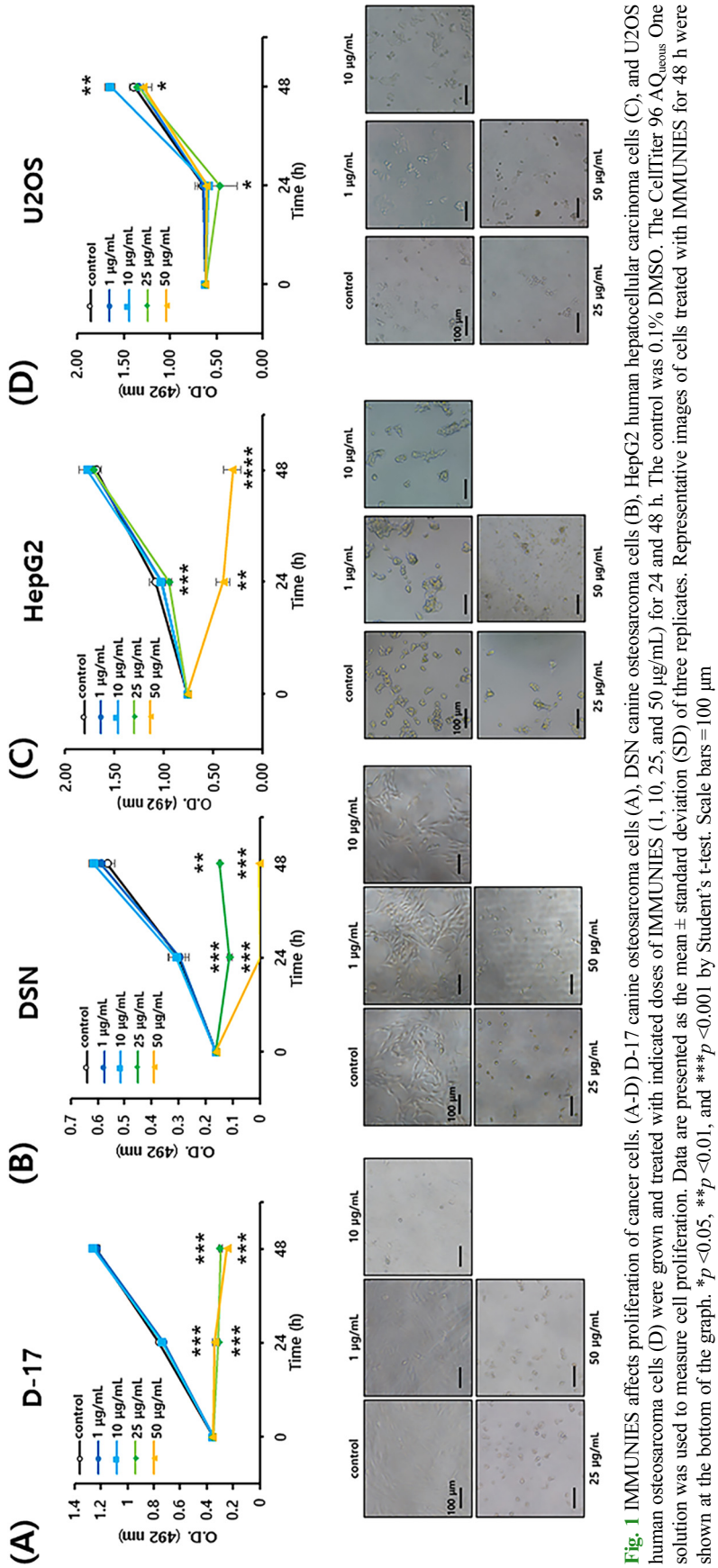


**Fig. 1** IMMUNIES affects proliferation of cancer cells. (A-D) D-17 canine osteosarcoma cells (A), DSN canine osteosarcoma cells (B), HepG2 human hepatocellular carcinoma cells (C), and U2OS human osteosarcoma cells (D) were grown and treated with indicated doses of IMMUNIES (1, 10, 25, and 50 µg/mL) for 24 and 48 h. The control was 0.1% DMSO. The CellTiter 96 $AQ_{ueous}$ One solution was used to measure cell proliferation. Data are presented as the mean ± standard deviation (SD) of three replicates. Representative images of cells treated with IMMUNIES for 48 h were shown at the bottom of the graph. *$p$ <0.05, **$p$ <0.01, and ***$p$ <0.001 by Student's t-test. Scale bars = 100 µm

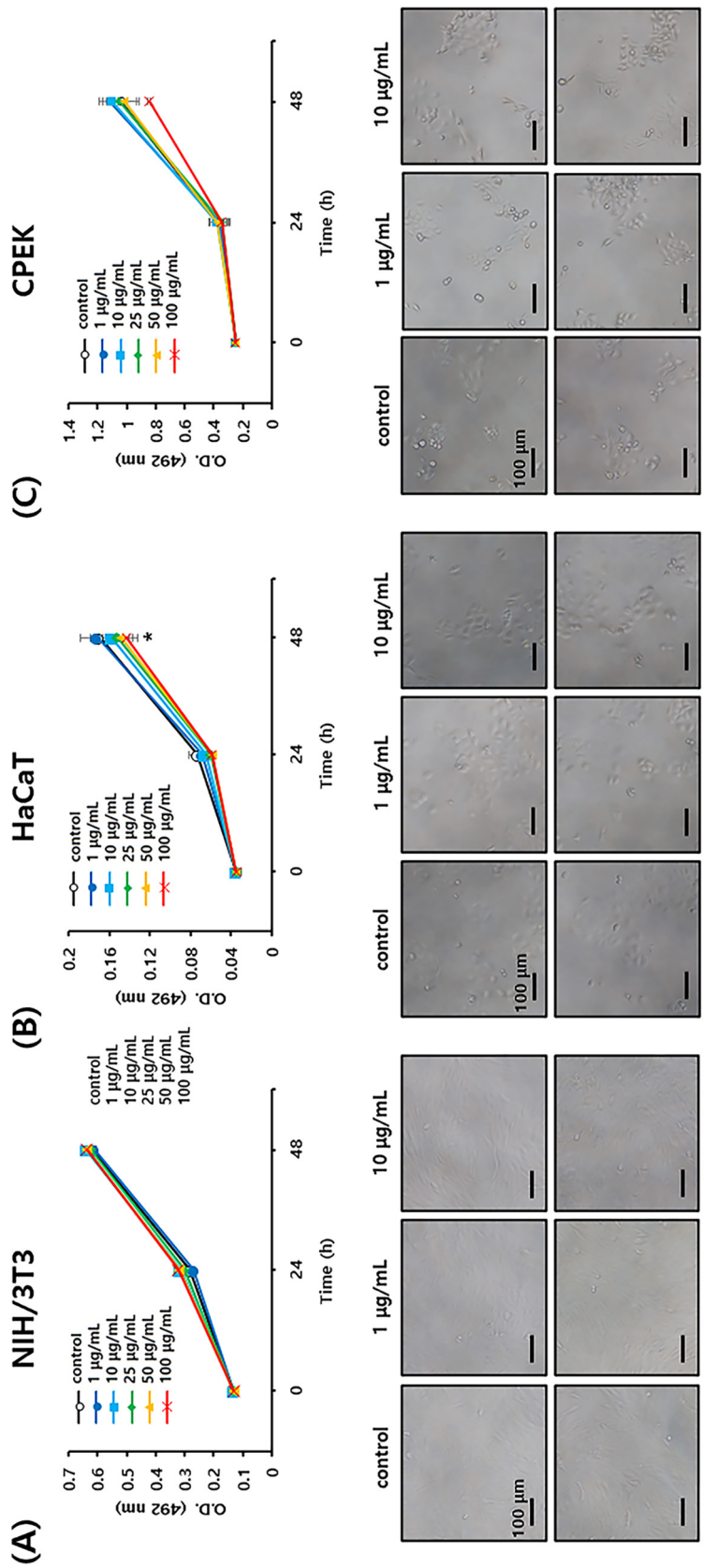


**Fig. 2** IMMUNIES affects proliferation of fibroblast and keratinocytes. (A-C) NIH/3T3 cells (A), HaCaT cells (B), and CPEK cells (C) were treated with indicated doses of IMMUNIES (1, 10, 25, 50, and 100 µg/mL) for 24 and 48 h. The control was 0.1% DMSO. CellTiter 96 $AQ_{ueous}$ One solution cell viability assay were performed. The error bars represent the mean ± standard deviation (SD) of three replicates. Representative images of cells treated with IMMUNIES for 48 h were shown at the bottom of the graph. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ by Student's t-test. Scale bars = 100 µm

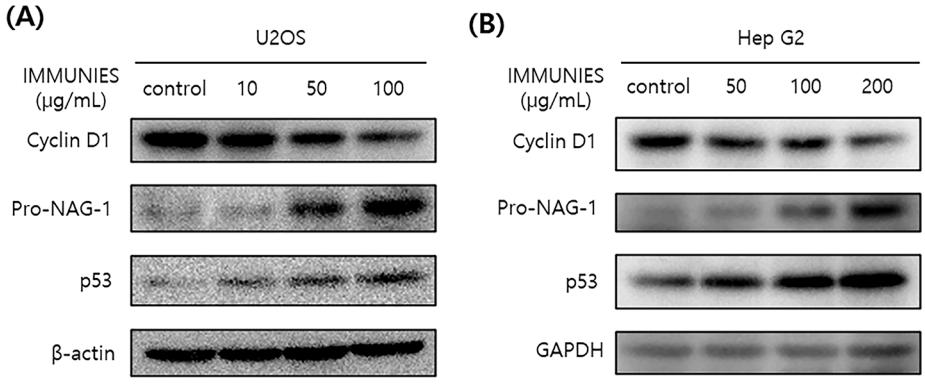


**Fig. 3** IMMUNIES alters protein expression related to cell proliferation. (A, B) U2OS osteosarcoma cells (A) (10, 50, and 100 μg/mL) and HepG2 human hepatocellular carcinoma cells (B) (50, 100, and 200 μg/mL) were treated with various doses of IMMUNIES, respectively. The control was 0.1% DMSO. After treatment, cell lysates were subjected to Western blot analysis using cyclin D1, pro-NAG-1, p53, β-actin, and GAPDH antibodies

The safety of IMMUNIES for noncancerous cells was subsequently evaluated. Treatment of NIH/3T3 murine fibroblasts (Fig. 2A), HaCaT human keratinocytes (Fig. 2B), and CPEK canine keratinocytes (Fig. 2C) with IMMUNIES revealed limited cytotoxicity up to 100 μg/mL concentration for 48 h in NIH/3T3 and HaCaT cells, whereas no statistical difference was found in CPEK cells. This result indicates that only cancer cells are effectively affected and noncancerous cells are relatively safe after IMMUNIES treatment.

**IMMUNIES increases pro-NAG-1 and decreases cyclin D1 expression in cancer cells**

To elucidate the molecular mechanism by which IMMUNIES affects anticancer activity, pro-NAG-1 and cyclin D1 expression was determined. In this study, U2OS osteosarcoma and HepG2 human hepatocellular carcinoma cells were examined. IMMUNIES treatment increased pro-NAG-1 and p53 expression and decreased cyclin D1 expression in both cancer cell lines (Fig. 3). This result suggests that IMMUNIES may inhibit cancer cell proliferation by elevating pro-NAG-1 and p53 expression and decreasing cyclin D1 expression.

**Clinical study and results**

This pilot study evaluated the safety and efficacy of IMMUNIES for dogs with cancer. The mean age of the four dogs was 11.25 years, and the mean weight was 6.45 kg. The expected lifespan of cases 1, 3, and 4 was 1-6 months, whereas case 2 was expected to live for 6-12 months, as assessed by clinical data. Blood and urine biochemical profiles were evaluated. Cases 1 and 3 seemed to have anemia before IMMUNIES treatment; however, their red blood cells and hematocrit (HCT) were restored after 1-month treatment and stayed within the normal range (Fig. 4A, B). Thus, IMMUNIES may contribute to improving anemia by enhancing hematopoietic expansion and function, increasing erythropoietin production, and improving dehydration. With regard to inflammation, white blood cells in cases 1 and 2 gradually decreased to within the normal range during treatment, whereas C-reactive protein (CRP) levels in cases 1, 2, and 3 significantly decreased to nearly the normal range. These results indicate that IMMUNIES may increase anti-inflammation activity as well as enhance immune function (Fig. 4C, D). We also examined blood urea nitrogen (BUN) levels to measure the blood nitrogen concentration. All four dogs exhibited high BUN levels, representing azotemia (Fig. 4E). After IMMUNIES treatment, the BUN level of case 4 reached the normal range and cases 1, 2 and 3 also showed improvement. Thus, IMMUNIES may contribute to improving BUN excretion, causing nitrogen detoxification. In all four dogs, creatinine levels decreased after IMMUNIES treatment, which may be linked to improved kidney function (Fig. 4F). Lastly, liver function was examined. Before IMMUNIES treatment, case 3 had elevated alanine aminotransaminase (ALT) and alkaline phosphatase (ALP) levels (800 and 3500, respectively). After 3 months of IMMUNIES treatment, the ALT level significantly decreased (Fig. 4G), indicating that IMMUNIES may contribute to protecting liver cell function, while the ALP level decreased in all four dogs, suggesting that IMMUNIES may delay fatty liver progress (Fig. 4H). In addition, the four dogs seemed to have common clinical conditions, such as anorexia, depression, and insomnia. However, IMMUNIES treatment gradually improved body conditions, including appetite, activity, and deep sleep in all four dogs for 3 months.

## Discussion

Canine mammary tumors are the most common neoplasms in female dogs [21]. However, primary canine hepatocellular carcinomas are rare in dogs and often are a result of metastatic cancer [22]. Herb therapy for canine cancer patients is being researched due to its low toxicity. Many single phytochemicals derived from plant extracts have anticancer activity; however, plant extracts in combination sometimes exhibit better activity

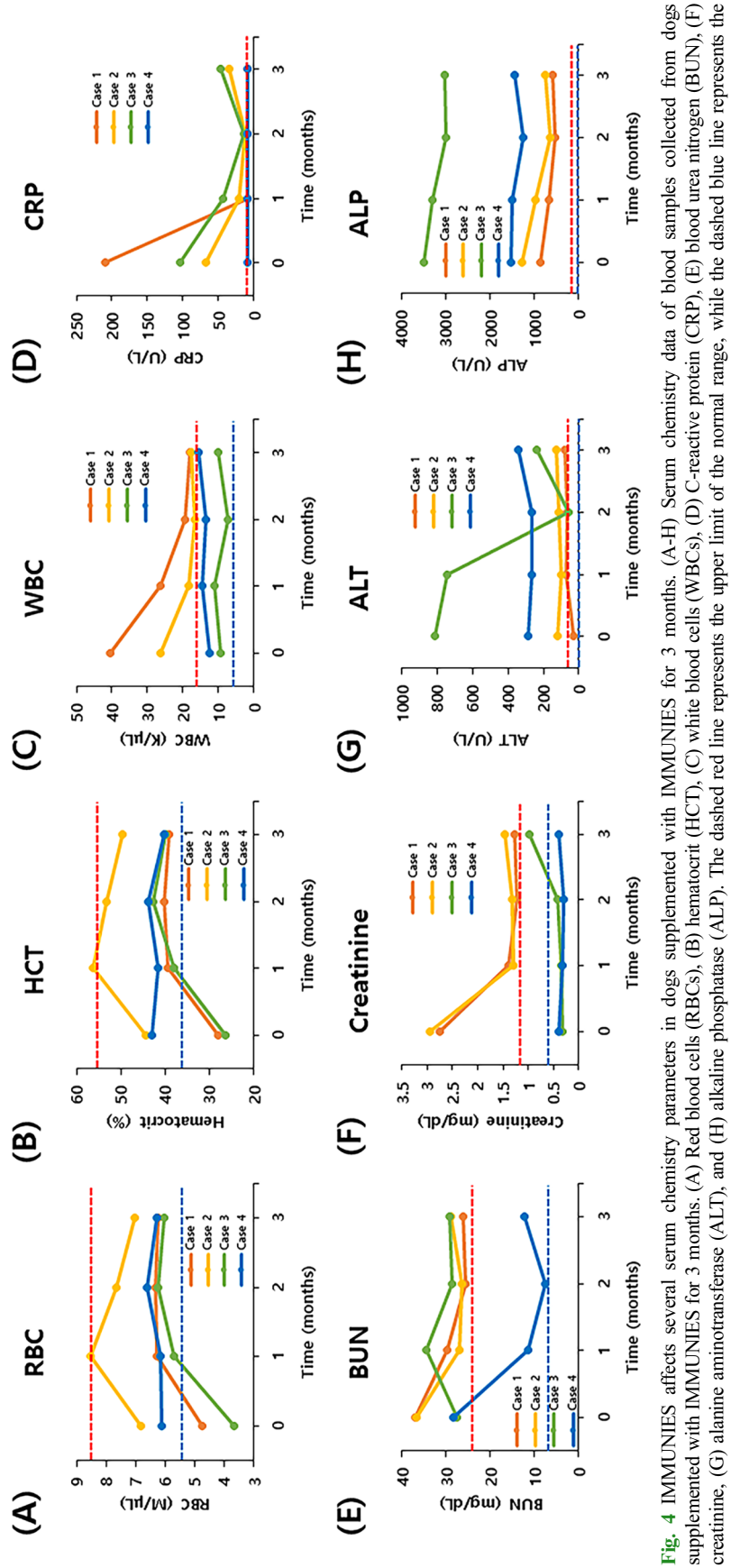


**Fig. 4** IMMUNIES affects several serum chemistry parameters in dogs supplemented with IMMUNIES for 3 months. (A-H) Serum chemistry data of blood samples collected from dogs supplemented with IMMUNIES for 3 months. (A) Red blood cells (RBCs), (B) hematocrit (HCT), (C) white blood cells (WBCs), (D) C-reactive protein (CRP), (E) blood urea nitrogen (BUN), (F) creatinine, (G) alanine aminotransferase (ALT), and (H) alkaline phosphatase (ALP). The dashed red line represents the upper limit of the normal range, while the dashed blue line represents the lower limit of the normal range

compared to a single compound because of the resulting synergistic effect [23]. IMMUNIES was synthesized to synergistically enhance the biological activity of single compounds.

According to the findings obtained in the *in vitro* assay, IMMUNIES seemed to have cancer anti-proliferative activity in canine osteosarcoma cells (D-17 and DSN) and human hepatocellular carcinoma cells (HepG2), whereas NIH/3T3 murine fibroblasts, HaCaT human keratinocytes, and CPEK canine keratinocytes did not respond to IMMUNIES in the cell viability assay. This suggests that IMMUNIES exhibits cytotoxicity in a cancer cell-specific manner. In humans, pro-NAG-1 is highly induced by several phytochemicals and its expression is linked to anti-tumorigenesis [24,25], whereas a large number of anticancer chemicals downregulate cyclin D1 in various cancer cells [16]. The p53 protein linked to NAG-1 expression is a well-known tumor suppressor [26]. Cyclin D1 downregulation and p53/pro-NAG-1 induction were observed in U2OS and HepG2 cancer cells. Cyclin D1 overexpression is often observed in many cancer cells, and plant extracts decrease cyclin D1 expression, leading to cell growth arrest [16,27], whereas pro-NAG-1 expression is linked to anticancer, anti-inflammatory, and anti-obesity activities, leading to induction of apoptosis [24,25]. It's worth noting that IMMUNIES comprises lactic acid bacteria, indicating a potential for heightened anti-cancer activity, consistent with previous findings [28]. Thus, IMMUNIES may inhibit cell growth by the similar mechanism by which other plant extracts affect anticancer activity. In the dogs' clinical profile, CRP, a marker of acute systemic inflammation, mildly decreased in all dogs. CRP is produced by the liver in response to inflammation and is often used as an inflammatory marker [29]. Case 1 dog showed significantly decreased CRP levels after 1 month of treatment, suggesting that IMMUNIES may decrease inflammation in the body and lead to better outcomes. Monitoring kidney function in patients with solid tumors is vital to the safe administration and follow-up of therapeutic agents [30]. Although measuring serum creatinine levels to assess kidney function may be insufficient because there is often a discrepancy between serum creatinine levels and the actual creatinine clearance rate [31], the serum creatinine level may indicate the safety of IMMUNIES. Indeed, all four dogs showed decreased serum creatinine levels (Fig. 4). Taken together with BUN data, IMMUNIES improved kidney function after treatment. Serum ALT, AST, ALP, total bilirubin, albumin, and total protein levels are significantly higher in cancer patients [32]. In addition, IMMUNIES seemed to marginally decrease ALT and ALP levels in all four dogs. On the basis of the clinical profile, most biochemical results indicated that all four dogs were moving toward better body functions and clinical conditions. Furthermore, urine tests performed on 4 dogs with terminal cancer showed a trend toward improvement in several values. Albumin and UPC (urine protein-creatinine ratio) levels excreted in the urine after administration decreased in all cases, suggesting that proteinuria was improved. In the case of blood cells excreted in the urine, the number of leukocytes excreted in the urine decreased in cases 1, 2, and 3, and the occult blood decreased in all cases. In addition, considering that urobilinogen was also decreased in all cases, the administration of IMMUNIES helped to improve urine test values and overall condition.

This study had a few limitations. One was the small sample size. Four dogs were administered IMMUNIES, but this number is not sufficient for a full evaluation of its efficacy. In addition, the IMMUNIES dose used was 80 mg/kg/day based on the dose recommended for humans. Appropriate doses should be optimized in further clinical trials in dogs. Long-term administration of IMMUNIES and long-term observation in a large clinical cohort would elucidate the detailed clinical efficacy and safety of IMMUNIES. In future studies, the evaluation of the effect of this product on the interleukin panel should be involved in the complex tumorigenic process, as some of them are involved in creating the favorable microenvironment for tumor growth.

The current survey aimed to assess the efficacy of IMMUNIES in dogs with cancer. According to the findings herein obtained IMMUNIES inhibited cell proliferation in the in vitro experiment, probably in a cancer cell-specific manner, and modulated cell proliferation-related proteins, such as p53/pro-NAG-1 and cyclin D1. A preclinical trial study on four dogs suggested that the patients tolerate IMMUNIES treatment and IMMUNIES may increase disease stability. Furthermore, though the clinical response observed in this study was only a blood/urine biochemical profile, it could be suggested that IMMUNIES can be safely administered without severe adverse events. The promising results obtained in this survey suggest that IMMUNIES is worthy of further investigation in large clinical trials in order to better clarify the efficacy of this polyherbal preparation in canine cancer patients and to better understand its mechanism of action.

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**Author contributions** WO and SJB contributed to the study conception and design. Material preparation and data collection were performed by WO, IK, and JM. Data analysis was performed by WO and SJB. The first draft of the manuscript was written by SJB and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Declarations**

**Ethics approval and consent to participate** All owners received an explanation of the study in oral and written form and provided written informed consent for participation before the study commenced. The experimental protocol was established according to the ethical guidelines of the Helsinki Declaration and the study was approved by the Institutional Animal Care and Use Committee of the Seoul National University (SNU; approval no. SNU-210727-2). The study was also carried out in compliance with the ARRIVE guidelines.

**Competing interests** The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. PETXELL Bio Co Ltd had no connection to the IMMUNIES formulation and was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

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