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In Vitro Evaluation of the Anti-Inflammatory Activity of a Supplement (Evexia Fast®, Candioli Srl) on Human Immune Cells

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Abstract

Osteoarthritis (OA) is a chronic, progressive inflammatory disease affecting both humans and animals, representing a major cause of pain and reduced mobility. In dogs, OA affects more than 20% of individuals older than one year, highlighting the need for effective long-term anti-inflammatory strategies. The product evaluated a multi-ingredient dietary supplement formulated to modulate inflammation through complementary mechanisms. Its composition includes *Boswellia serrata* Roxb, *Cannabis sativa* L. oil and crude fibrous flour, Pineapple (*Ananas comosus* (L.) Merr.) containing Bromelain, white willow (*Salix alba* L.), and Mitalgol® (*Zingiber officinale*, *Acmella oleracea*). The anti-inflammatory potential of this formulation was evaluated *in vitro* using THP-1 human immune cells differentiated into macrophages and stimulated with lipopolysaccharide (LPS). Cytotoxicity was assessed by MTT assay to identify non-toxic concentrations. Anti-inflammatory activity was determined by measuring the release of interleukin-6 (IL-6) via ELISA following treatment at three concentrations (3.13, 1.56, and 0.78 mg/mL). The product showed no cytotoxic effects and significantly reduced IL-6 production in inflamed immune cells at all tested concentrations ($p < 0.05$), with IL-6 levels restored to baseline values at the higher doses. These results support the biological plausibility of the supplement as a multimodal anti-inflammatory option for dogs affected by osteoarthritis.

Keywords: Inflammation, Interleukin, Natural products, Pet

Introduction

Osteoarthritis (OA) is a chronic, progressive, and inflammatory disease that affects both humans and animals, representing one of the leading causes of pain, reduced mobility, and impaired quality of life (Barbeau-Grégoire *et al*., 2022). In dogs, OA prevalence exceeds 20% in individuals older than one year, highlighting the clinical relevance of effective long-term anti-inflammatory strategies. Disease management is primarily aimed at controlling inflammation, alleviating chronic pain, and preserving joint function. Conventional pharmacological therapies, particularly nonsteroidal anti-inflammatory drugs (NSAIDs), remain the cornerstone of OA treatment; however, their long-term use is often limited by incomplete efficacy, lack of disease-modifying effects, and the risk of adverse events (Serni *et al*., 1999). Consequently, increasing attention has been directed toward nutraceuticals and dietary supplements with anti-inflammatory potential and improved safety profiles. Evexia Fast® (Candioli Srl) is a multi-ingredient dietary supplement formulated to target inflammation through complementary and synergistic mechanisms. Its ingredients were selected for their well-documented biological activities. One key component is *Boswellia serrata* Roxb., whose resin is rich in boswellic acids, pentacyclic triterpenes known to inhibit pro-inflammatory enzymes and mediators (Reichling *et al*., 2004). In particular, β-boswellic acid modulates inflammatory pathways by inhibiting cathepsin G and microsomal prostaglandin E2 synthase-1 (mPGES-1), which helps reduce leukocyte infiltration and inflammatory signalling in affected joints. In Evexia Fast®, *Boswellia serrata* is delivered in a Phytosome® system (Indena), enhancing its bioavailability and maximizing its anti-inflammatory effects. Another important ingredient is *Cannabis sativa* L., included both as fast-absorbing oil and crude fibrous flour. This CBD-rich fraction from industrial hemp engages the endocannabinoid system, which plays a key role in modulating inflammation and pain, especially in osteoarthritis (Gamble *et al*., 2018). Activation of peripheral CB2 receptors is associated with reduced inflammatory responses, while CBD itself modulates cytokine production, oxidative stress, and immune cell activity, providing analgesic and anti-inflammatory support. Pineapple (*Ananas comosus* (L.) Merr.) naturally contains bromelain, a mixture of proteolytic enzymes present mainly in the stem, as well as in the fruit and roots. These enzymes break down proteins, are readily absorbed in the intestine without interfering with digestion, and exhibit antibacterial and anti-inflammatory activity (Pavan *et al*., 2012). White willow (*Salix alba* L.) is rich in salicin and related phenolic glycosides, offering antioxidant, anti-inflammatory, and analgesic properties. *In vitro* studies suggest it may act as a chondroprotective agent, supporting joint health (Shakibaei *et al*., 2012). Finally, Mitalgol® (Candioli Srl),

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included in Evexia Fast®, combines the complementary properties of *Zingiber officinale* (ginger) and *Acmella oleracea* (both supplied by Indena) to support musculoskeletal and gastrointestinal health in dogs. Ginger helps reduce nausea and supports gastrointestinal function while offering also anti-inflammatory and antioxidant activity (Ballester *et al*., 2022). *Acmella oleracea* delivers spilanthol, a natural compound with analgesic and anti-inflammatory effects that may aid tendon reorganization and mobility (Moro *et al*., 2021). The anti-inflammatory potential of Evexia Fast® was investigated in the present study using an *in vitro* model of human immune cells (THP-1) exposed to a pro-inflammatory stimulus. The study specifically aimed to evaluate the ability of the product, tested at three non-toxic concentrations, to reduce the production of the pro-inflammatory cytokine interleukin-6 (IL-6), a key mediator involved in OA-associated inflammation and pain. The observed modulation of IL-6 production can help supporting the biological plausibility of Evexia Fast® as an effective anti-inflammatory supplement.

Materials and Methods

Cell Line and Culture Conditions

The experiment was performed using the human immune cell line THP-1. The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; ATCC® TIB-202™).

Cells were maintained in RPMI medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin, under humidified conditions at 37°C and 5% CO2.

Product Preparation

The product Evexia Fast® tablets were grounded using a mortar, solubilized in PBS, and sonicated to ensure maximum solubilization. The solution was subsequently filtered through a 0.22 μm filter to ensure sterility.

Table 1. Ingredients (in %) of Evexia Fast®, Candioli srl.

Ingredients	%
Cannabis sativa oil (fast-absorbing)	34.2000
Microcrystalline Cellulose Type 2	33.3000
Boswellia Serrata Roxb. in a Phytosome® system	9.6000
Dibasic Calcium Phosphate Dihydrate	5.0000
Pineapple stem, *Ananas comosus* L. Merr. (hydrolyzed)	4.0000
Colloidal Silica (E551b)	2.5000
Cannabis sativa crude fibrous flour	2.0000
White willow Bark, *Salix alba* L. (dried powder extract)	2.0000
Mitalgol® (Zingiber officinale and Acmella oleracea extract)	2.0000
Optimizor Uranus	2.0000
Magnesium Stearate	1.0000
Glyceryl behenate	1.0000
Brewer's Yeast	0.9000
Vitamin C	0.5000
Total	**100.0000**

Cytotoxicity Test (MTT Assay)

THP-1 cells were seeded in 96-well plates at a density of 103 cells/well in complete medium supplemented with PMA, a stimulus that induces cellular differentiation from suspension monocytes to adherent macrophages. After differentiation, the medium was removed and replaced with 100 μl of RPMI supplemented with 1% FBS. Treatment was performed using 1:2 serial dilutions starting from a concentration of 25.07 mg/ml. The range of concentrations used in the assay considered both the solubility limit of the product and the theoretical bioavailable concentrations following oral administration. Untreated cells (CTR) were included as a negative control. Cells were incubated with the product for 24 h at 37°C. At the end of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added at a final concentration of 0.5 mg/ml and the plate was incubated for 2 h. The reduced MTT crystals were solubilized by removing the medium and adding 100 μl of isopropanol. Absorbance was measured at 595 nm using an Infinite M NANO+ plate reader (Tecan). Cell viability was expressed as a percentage of absorbance at 595 nm relative to untreated cells (100%).

Anti-inflammatory Activity

THP-1 cells were seeded in 48-well plates at a density of 5×104 cells/well in complete medium supplemented with PMA to induce differentiation from suspension monocytes to adherent macrophages. The following day, the medium was removed and replaced with 500 μl of RPMI supplemented with 1% FBS. Cells were incubated with LPS to simulate an inflammatory condition and treated with the product Evexia Fast®. The doses of Product used for treatment **(Table 2)** were selected based on theoretical bioavailability following oral administration of the maximum daily dose of the product (considering an animal body weight of 15 kg), together with the results of the cytotoxicity assay.

Table 2. Concentrations of Evexia Fast® (Candioli srl) used in the anti-inflammatory activity test.

Product	Concentration 1 (mg/ml)	Concentration 2 (mg/ml)	Concentration 3 (mg/ml)
Evexia Fast®	3.13	1.56	0.78

Untreated cells were included as a negative control (CTR), while cells treated with LPS alone were included as a positive control. Treatments were incubated for 24 h, and each condition was analyzed in triplicate. At the end of incubation, the supernatant was collected and used to quantify the pro-inflammatory cytokine IL-6. Cytokine quantification was performed using a specific ELISA kit, following the manufacturer's instructions.

Statistical Analysis

All experimental data are presented as mean ± standard deviation (SD) of at least three independent replicates. Statistical analysis

was performed using one-way analysis of variance (one-way ANOVA) to compare multiple groups. When appropriate, post hoc multiple comparison tests were applied. Differences were considered statistically significant when p-values were less than 0.05. Statistical significance is indicated in the figures as follows: *** $p < 0.001$; **** $p < 0.0001$.

Results and Discussion

Cytotoxicity Test (MTT Assay)

The cytotoxicity test was performed to select non-cytotoxic *in vitro* concentrations to be used in the anti-inflammatory assay. THP-1 cells were treated with ten serial concentrations of the product for 24 h, and cell viability was analyzed using the MTT assay. The results are shown in **(Figure 1)**. The results indicated that the product did not show cytotoxic effects on immune cells starting from the dose of 12.5 mg/ml. The preliminary cytotoxicity assessment confirmed that Evexia Fast® did not exert toxic effects on THP-1 macrophages at concentrations relevant for the anti-inflammatory assay, thereby supporting that the observed reduction in IL-6 release reflects a genuine immunomodulatory effect rather than reduced cell viability.

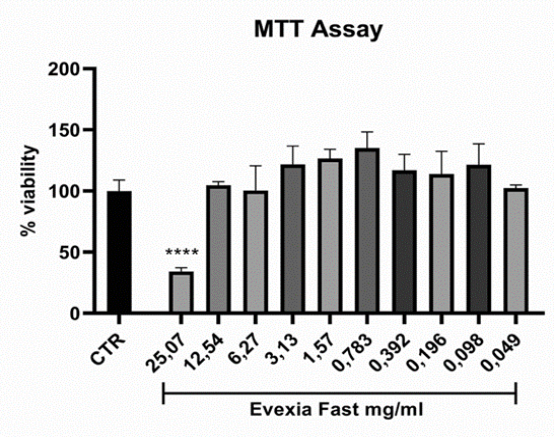

Figure 1. Cytotoxicity test.

THP-1 cells were treated for 24 h with ten serial concentrations of Evexia Fast® (Candioli srl). At the end of treatment, cell viability was assessed by MTT assay. Results are expressed as percentage of cell viability relative to untreated control (CTR). **** p-value < 0.0001 (one-way ANOVA).

Anti-inflammatory Activity

To perform the assay, an inflammatory immune cell model was generated by treating cells with LPS to simulate an inflammatory stress condition. The doses of Evexia Fast® used in the assay are reported in **(Table 2)**. As shown in **(Figure 2)**, the Product exerted a statistically significant effect in reducing IL-6 levels released by inflamed immune cells. Anti-inflammatory efficacy was observed at all tested doses. Evexia Fast® demonstrated strong anti-inflammatory activity by significantly reducing IL-6 release in LPS-stimulated immune cells, restoring cytokine levels to baseline at doses of 3.13 and 1.56 mg/ml. The results demonstrated that Evexia Fast® exerted a marked anti-inflammatory effect at all three non-toxic concentrations tested (3.13, 1.56, and 0.78 mg/mL), significantly reducing IL-6 production in inflamed human immune cells. These findings indicate that the product can effectively modulate key inflammatory pathways involved in chronic inflammatory responses.

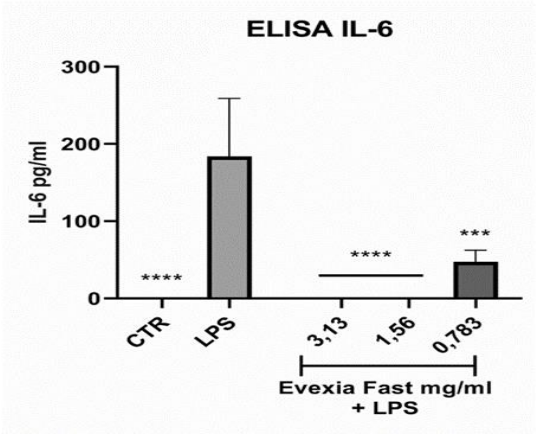

Figure 2. Anti-inflammatory activity.

THP-1 cells inflamed with LPS and treated with Evexia Fast® (Candioli srl) for 24 h at three different concentrations. CTR: non-inflamed control; LPS: inflamed control. **** p-value < 0.0001, *** p-value = 0.0006 (one-way ANOVA).

Although these in vitro findings on THP-1 macrophages provide valuable preliminary mechanistic insights into the anti-inflammatory action of Evexia Fast®, a primary limitation of this study is the reliance on a simplified single-cell model. In vitro assays cannot fully simulate complex physiological processes such as gastrointestinal metabolism, systemic bioavailability, and joint-specific microenvironments. Furthermore, osteoarthritis involves intricate crosstalk between multiple articular cell types, including chondrocytes and synoviocytes. Therefore, while these results establish the biological plausibility of the product, prospective in vivo clinical trials in canine patients are necessary to confirm its therapeutic efficacy and long-term joint benefits.

Conclusion

The relevance of Evexia Fast® lies in its multimodal anti-inflammatory profile, combining botanicals and antioxidants with complementary mechanisms of action. Although these *in vitro* results on THP-1 immune cells provide valuable preliminary mechanistic insights, a key limitation of this study lies in the use of a simplified cellular system. Future in vivo clinical studies are necessary to account for complex physiological processes in osteoarthritic target species. Overall, the observed reduction in IL-6 supports the biological plausibility of Evexia Fast® as a functional anti-inflammatory option in canine OA. The observed reduction in IL-6 supports the biological plausibility of Evexia Fast® as a functional alternative or adjunct to conventional anti-inflammatory therapies in canine OA, warranting further investigation *in vivo* to confirm its clinical efficacy and long-term benefits.

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Conflict of interest: None

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Ethics statement: This study was performed entirely *in vitro* using the commercial THP-1 human immune cell line. Since no live animals or human subjects were directly involved, institutional ethical approval reference numbers are not required.

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