Toxicity Study of *Ananas Comosus* and *Centella Asiatica* as Active Ingredients in Anti-inflammatory Lotion

N S A Rahman\(^1\), N Saadon\(^1\), F M Tap\(^1\), N A Yusof\(^2\)

\(^1\)Faculty of Chemical Engineering, Universiti Teknologi MARA Cawangan Terengganu, Kampus Bukit Besi, 23200 Dungun Terengganu

\(^2\)Academy of Language Studies, Universiti Teknologi MARA Cawangan Terengganu, Kampus Bukit Besi, 23200 Dungun Terengganu

*Corresponding author: nursyukriah@uitm.edu.my*

**Abstract.** Inflammation, a common disease faced by people, could lead to chronic inflammatory diseases, and worst, cancer. As a result, many inflammatory patients use nonsteroidal anti-inflammatory drugs (NSAIDs) as to relieve their inflammation, which are available in the market. However, these medicines do not cure the disease but relieve it temporarily, and the patients are exposed to their dangerous long-term effects. Since Malaysia is rich with fruits and herbs that are rich in therapeutic properties, more natural remedies from the local fruits and herbs should be discovered. Based on the previous research done, the combination of *Ananas comosus* and *Centella asiatica* could have the potential in relieving inflammation. Therefore, this preliminary research was aimed in determining the toxicity in combining the two main active ingredients using *in vitro* MTT assay against HaCat Cell lines. The findings proved that at the highest concentration (10000 μg/ml), no cytotoxic effect has been recorded. The findings from this research would encourage more natural remedies to be offered to the inflammation patients, who eventually could avoid themselves from depending solely on the harmful medicines.

1. **Introduction**

Inflammation is said to be experienced by everyone [1]. It is a response from our immune system to protect our body from infection, injury or disease. Our body's white blood cells release chemicals into the blood or affected tissues in order to protect our body from foreign substances. As a result of the release, the blood flows to the injured or infected area increases, and redness and warmth may occur [2]. Inflammation has two types, which are acute and chronic inflammation and the later can have long-term and whole-body effects, which could lead to diseases like heart attack, stroke and cancer [3]. Many acute inflammatory patients use nonsteroidal anti-inflammatory drugs (NSAIDs) as to relieve their inflammation, like rashes. NSAIDs are also found to inhibit the protein target such as COX-1 [4].

The NSAIDs are chemical-based drugs often used as the inhibitors for both acute and chronic inflammation [5]. They are chemically synthesized and designed to block the production of inflammatory mediators. There are many NSAIDs available in the market, such as aspirin and ibuprofen, but these medicines do not cure the disease but relieve it temporarily, and the patients are exposed to their dangerous long-term effects, such as stomach ulcers, kidney damage and increase the risk of heart attack or stroke [6]. Based on a previous study, NSAIDs were used to relieve the pain rather than to cure it [7]. Besides that, these drugs are also associated with several complications. The long-term used of NSAIDs often leads to a negative impact on the liver and renal system. The most common side effect is
gastric bleeding [8]. The long-term use of steroid, either alone or combined with NSAIDs, will disturb the immune system of our body rigorously [9]. Thus, the negative complications of the usage give a clear signal for more discoveries of natural remedies, specifically in the treatment of inflammation.

Abundant of bioactive compounds can be found in food such as fruits and herbs. Researchers have high interest in phytochemicals because of their roles in the prevention of degenerative diseases such as cancer [10]. Furthermore, these compounds also have many active substances that can control the activities of a wide range of enzymes and cell receptors [11]. Bioactive compound, such as polyphenol, is also a natural antioxidant found in food [12]. It is capable of scavenging radicals such as hydroxyl (OH') and peroxyl (ROO') [13]. Protein in plant, such as bromelain in *Ananas comosus*, has attracted high attention in therapeutic application because of its non-toxic properties. The use of bromelain as a hydrolysing agent in releasing the antioxidant and antimicrobial peptides had also been reported [14].

Treatment with a single anti-inflammatory drug often causes multiple adverse effects such as hepatotoxicity, gastrointestinal haemorrhage, meningitis and asthma [15]. Therefore, a novel strategy combining two or more potential compounds that exhibit inhibitory effects against inflammation activity has been proposed to overcome the problem. Moreover, this approach has previously been shown to enhance the thermal stability of the combination [16]. Inflammation is categorised as a complex disease, thus it needs a multi target to reduce the inflammation. The designing of multi-targeted drug combination is crucial in the development of a sustainable treatment on inflammation. Drug combination is a method of combining two or more active pharmaceutical ingredients into a single dosage form and this is a promising approach in interfering a complex disorder [17]. For example, a drug combination was used for treating complex diseases such as cancer [18] and HIV [19]. Combinations of natural products, such as alkaloids, have been discovered as new potential compounds that can treat sleeping sickness (trypananomiasis) [20]. Previous study claimed that the combination compound also provides synergistic effect on inhibition of inflammation [21].

Since Malaysia is rich with fruits and herbs which are rich in therapeutic properties, more natural remedies should be discovered from the local fruits and herbs. Based on the previous research done, the combination of *Ananas comosus* and *Centella asiatica* could have the potential in relieving inflammation since studies have been conducted on them separately and each was found to be an anti-inflammatory agent. The leaf extract of *Ananas comosus* shows significant lesser inflammation effect as compared to that in the carrageenan control group. At 48 hours post injection of carrageenan, a significant decrease in paw volume was observed with 500 mg/kg b.w. (0.78 ± 0.13 ml) of *Ananas comosus* leaf extract as compared to that in the carrageenan control group (1.3 ± 0.18 ml) [15]. Meanwhile, *Centella asiatica* exhibited promising effect on inflammatory responses by macrophages in an atopic dermatitis (AD) mouse model. The histopathological analysis showed that *Centella asiatica* inhibited hyperkeratosis, proliferation of mast cells and infiltration of inflammatory cells [22].

Extracts of plant components can be achieved using different processes and using different medium such as alcohol, glycerine, oil and vinegar [23] and some of them use very low or no heat to produce extracts. To avoid damages of a plant’s components, glycerine is used as it requires no heating and it dissolves the soluble components of the plant. As a result, the active ingredients which are soluble to glycerine will be recovered in the extract. In a skincare product, an active ingredient is the chemical in the skincare that fulfils the function or purpose of the product [24] and in this research, *Ananas comosus* and *Centella asiatica* are the active ingredients since they are anti-inflammatory agents.

Hence, based on the needs to discover more natural remedies, this research was conducted to determine the toxicity in combining the two main active ingredients, which are *Ananas comosus* and *Centella asiatica*, before they are used in an anti-inflammatory lotion formulation. Ensuring the safety of the product is part and partial of the manufacturer’s responsibility on the consumers [25].
2. Research method

2.1. Materials
The emulsifier derived from pure castor and glycerin were purchased from Gobiotics, while sweet almond oil, virgin olive oil, virgin grape seed oil, rice bran oil, jojoba oil, and coconut oil were purchased from an organic oil shop. MgCl₂ and glycerine were purchased from a chemical supplier. The rose hydrosol, distilled water, ananas glycerite, *Centella asiatica* glycerite were prepared by the formulator. Preservative ECO or Geogard ECT which consists of Benzyl Alcohol, Salicylic Acid, Glycerin and Sorbic Acid was obtained from Aromantic Limited. Phosphate buffer saline, (3-(4,5-dimethyl-thiazol-2y) 2,5-diphenyl-tetrazolium bromide (MTT), and acetic acid were purchased from Merck Sdn. Bhd., Malaysia. Modified eagle’s medium (MEM contains amino acids, vitamins, inorganic salts, dextrose and phenol red), fetal bovine serum, platelet derived growth factor, phosphate buffer saline (PBS), trypsin-EDTA (0.25% trypsin, EDTA 4Na), and dimethyl sulfoxide (DMSO) were purchased from Gibco®, USA.

2.2. Preparation of Extract
Fresh *Ananas comosus* was peeled and *Centella asiatica* leaves were cleaned using distilled water. The plant materials were allowed to dry and later, chopped in a thin and small size. The *Ananas comosus*, *Centella asiatica*, glycerine and preservative ECO were weighted. The 100 g of *Ananas comosus* and *Centella asiatica* infusions were prepared separately. All ingredients were mixed in an airtight and sanitized glass container. The container was placed away from the direct light and agitated for 10 days and on the 11th day of infusion, the mixtures were strained using a coffee filter. The strained liquid or the extract was then transferred into a final container and stored at 2°C.

2.3 Cell cultures
Human Skin epidermal keratinocytes (HaCaT) were purchased from Elabscience and were cultured according to the protocol provided by the depositor. HaCaT cell lines were grown in DMEM supplemented with 10% FBS and 10% FCS with additional 1% PS at 37°C under humidified atmosphere of 5% CO₂.

2.4 MTT assay
The cytotoxicity study on the combined extract of *Ananas comosus* and *Centella asiatica* was examined by using 3-[4, 5-dimethyl-thiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide (MTT) assay [26].

2.5 Toxicity test
The cells were seeded at a density of 2x10⁵ cells/well in a 96-well plate, excluding the first row, and incubated in humidified atmosphere for 24 hours prior to the treatment. The test samples were prepared by dissolving the combined extract in DMEM to give the final concentrations of the crude extracts at 10000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39.06, and 0 μg/ml using serial dilution calculation. The medium was replaced after 24 hours with 200 μL of complete DMEM and a serial dilution of the combined plant extract. The cells with and without the serial dilution of combined extract were incubated again in humidified atmosphere for 24 hours. After the incubation, the cells were washed using PBSA, which was removed later. 20 μL of freshly prepared MTT solution (5 mg/ml dissolved in PBS) was added into each well and the cells were incubated again at 37°C for 5 hours. The MTT solution was then removed and replaced with 200 μL DMSO for 15 minutes to allow the dissolution of the purple MTT formazan crystal. The absorbance was measured at 570 nm and samples were run in 6 replicates. Untreated cells were used as the control.

2.6 Statistical analysis
All data were expressed in mean±SEM. Statistical analysis was performed using Graphpad prism with one way ANOVA and Tukey test. Significant differences were considered as p<0.05.
3. Result and discussion

The effect of different concentrations of the combined extract on cell viability was measured by using MTT assay. MTT assay is a useful in vitro model for testing cytotoxicity because it can show the ability of the compound to stimulate the proliferation of the human dermal keratinocytes (HaCaT). HaCaT is one of the cells that will be affected by toxic substances also known to be involved in various immune responses and inflammatory of the skin. Therefore, HaCaT has been selected as it is the most appropriate target cells for evaluating sensitivity of the skin to toxicants. Basically, toxicants will affect basic functions of the cells and the toxicity can be determined by assessing cellular damage. Thus, the effect of the combined extract on the HaCaT proliferation was determined and the results were tabulated in figure 1.

![Figure 1](image)

**Figure 1.** The Effects of the combined extract of *Ananas comosus* and *Centella asiatica* on HaCaT cell proliferation during treatment for 24 hours.

In overall, all of the extracts give percentage of cell viability higher than 80% with the highest percentage of cell viability was shown at the concentration of 39.0625 µg/ml while the lowest cell viability was recorded at 78.125 µg/ml. However, no significant difference (p>0.05) has been observed among all the concentrations and control (concentration = 0 µg/ml) which indicates no cytotoxic effect was observed even at the highest concentration.

In vitro MTT assay was performed in order to determine the toxicity effect of active ingredients towards the basic cellular functions and generally based on the enzymatic conversion of MTT in the mitochondria. Thus, the cytotoxicity effect of the plant extracts at high concentration can be related to the alterations of the basic functions of mitochondria which might cause the apoptosis and necrosis occurred, thus resulted in the death of cells [27,28]. Surprisingly, at the highest concentration (10000 µg/ml), no cytotoxic effect has been recorded which showed that the combination extract does not alter the basic functions of mitochondria thus possesses a wide margin safety level upon exposure of high dose.

This current finding is in accordance with the findings from previous studies, where they found that the extract of *Ananas comosus* and *Centella asiatica* exhibited low cytotoxicity effect against human ovarian SKOV-3 and human dermal keratinocyte respectively[29,30]. Combined crude extracts only contain natural ingredients in various amounts which may explain its low cytotoxicity effect against HaCaT. Furthermore, the use of herbal in the combination is believed to produce synergistic therapeutic activity but with a lesser side effect. This recent finding suggests that the combined extract of *Ananas*
comosus and Centella asiatica is safe on human dermal keratinocytes where all tested concentrations showed above 80% on cell viability. The high percentage of cell viability showed that the combined extract could enhance the proliferation of HaCaT cell lines thus initiate the release of repair and inflammatory mediators in response to Interleukin-1β and TNFα.

With the said results, the ethnopharmacological approach in selecting this combination for study is useful for current knowledge contribution and this arises the needs for future exploitation on its potential anti-inflammatory activity.

4. Conclusion
As a conclusion, the finding from this preliminary study gives a secure assurance that Ananas comosus and Centella asiatica are a suitable combination as active ingredients in an anti-inflammatory lotion formulation. The finding provides alternatives in providing a remedy for inflammation and could encourage more natural remedies to be offered to the inflammation patients, who eventually could avoid themselves from depending solely on the harmful medicines. Further research should be conducted on the formulated lotion, which has the combination of Ananas comosus and Centella asiatica as its active ingredients, to determine its effectiveness in reducing inflammation.

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