RESEARCH ARTICLE

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# *Phallus indusiatus* Extracts Promoted MCF-7 Apoptosis Under TNFα-induced Tumor Microenvironment by Attenuating NF-kappaB and Akt Activation

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## Abstract

**Objective:** To evaluate the anti-inflammatory and anticancer effects of *Phallus indusiatus* extracts, particularly in modulating inflammatory microenvironments and sensitizing lapatinib-induced cytotoxicity in breast cancer cells. **Methods:** *Phallus indusiatus* (bamboo mushroom) extracts were prepared using water extraction from fresh and dried specimens. The anti-inflammatory effects were assessed using RAW246.7 cells by measuring NO production, while cytotoxicity and proliferation effects were evaluated in MCF-7 and MCF-10A breast cell lines. To mimic the inflammatory microenvironment of breast cancer, MCF-7 cells were treated with TNFα to induce lapatinib resistance. The effects of the extracts on cell viability, apoptosis, and NF-kappaB signaling were evaluated under inflammatory microenvironment-mimicking conditions. Co-treatment with lapatinib and the extracts was analyzed for synergistic cytotoxic effects and pathway modulation. **Results:** *P. indusiatus* extracts demonstrated anti-inflammatory effects by reducing NO production in RAW246.7 cells. The extracts showed no cytotoxicity on MCF-7 and MCF-10A cells, with an observed proliferation increase in MCF-10A but not MCF-7. Under inflammatory microenvironment-mimicking conditions induced by TNFα, the extracts slightly reduced MCF-7 cell viability in a dose-dependent manner. Additionally, the extracts sensitized lapatinib-induced cytotoxicity by increasing apoptotic cell populations. Mechanistically, co-treatment with the extracts attenuated AKT and NF-kappaB activation in a dose-dependent manner. **Conclusion:** These findings highlight the potential of *P. indusiatus* extracts as supplementary food for breast cancer patients, particularly in inflammatory microenvironment conditions.

**Keywords:** *Phallus indusiatus*- breast cancer- apoptosis- Inflammation- microenvironment

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## Introduction

Breast cancer (BC) is still high on the world ranking as has been reported by World Health Organization WHO). In Thailand, the incidences of breast cancer were 3-times higher than in western countries [1]. The major problem probably caused by different factors such as ethnicity, environmental exposures, or genetic background. Epidemiological and clinical outcome data revealed increasing mortality rate in Asia higher than Western countries caused by different genetic background. Long-term treatment of chemotherapy has led to a drug resistant occurrence [2]. Non-responders have developed some mutations or acquired a drug resistance leading cancer progression with poor prognosis [3]. Therefore, alternative medicines probably would be a promising choice for reducing this effect.

Secreting cytokines/chemokines directly/indirectly modulated cell-cell communication that related to tumorigenesis and drug response as tumor microenvironment. Elevated TNFα levels in plasma/serum have been associated in chemoresistance of breast cancer patients [4, 5]. TNFα is one of pro-inflammatory cytokines that is responsible in both apoptotic and survival pathway

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of breast cancer patients [6]. TNFα binds to both TNFR1 and TNFR2 leading to cellular signaling activation [7]. TNFα was reported to be a chemosensitizer of breast cancer by apoptosis induction. Meanwhile, some incidences demonstrated TNFα treatment enhanced various MDR-related protein upregulation [8, 9]. In the presence of TNF-alpha, PI3K/Akt activation promoted chemoresistance in MCF-7 [10]. IL-8-mediated therapeutic resistances primarily occur through the activation of the PI3K/Akt and NF-kappaB signaling pathways [11]. Elevated TNFα levels enhance IL-6 and IL-8 by NF-kappaB activation [12]. TNFαtreatment increased breast cancer stem-like cell population by upregulating TAZ through the non-canonical NF-kappaB pathway [13]. Activation of NF-kappaB induced ERα downregulation in BC leading to reducing therapeutic efficacy [14]. NF-kappaB activation likely plays a role in drug response and chemoresistance. TNF-α-induced activation of the NF-kappaB signaling pathway has been implicated in the development of resistance to apoptosis in tumors. Therefore, targeting pro-survival factors or NF-kappaB signaling present an appealing strategy to desensitize chemoresistance in BC patients .

Edible mushrooms currently become to be interested in breast cancer research. Water extraction have drawn our attention to demonstrate their culinary values [15]. Water extraction of basidiomycete fugus (B. edulis, C. alexandri, K. mutabilis, L. tigris) exhibited anti-proliferative effect against MCF-7 cell line [16-19]. Aggressive phenotype of breast cancer cells was contributed by the constitutive activation of NF-kappaB [20, 21]. Water-extracted G. lucidum attenuated aggressive phenotype of estrogen-dependent growth MCF-7 by targeting ER and TNFα-induced NF-kappaB signaling [22]. Targeting AKT activation have widely been studying in breast cancer [23, 24]. Once, AKT activation proceeded various cancer progressions traits such as cell cycle progression, invasion/metastasis, and angiogenesis . Aqueous extraction of P. lintues modulated aggressive phenotype of BC through AKT inhibition [25]. Although, some mushrooms crude extracts have been identified but seemingly exhibited potential therapeutic functions against BC.

Bamboo mushroom (Dictyophora indusiata or *Phallus indusiatus*) is a fungus that belong to the family Phallaceae. Antioxidant, neuroprotective, anti-inflammatory, and anti-cancer effects of *P. indusiatus* have extensively reviewed elsewhere. Crude extraction of *P. indusiatus* directly exhibited cytotoxicity against various cancer cells in board range concentration (10-1000µg/mL) [26]. Activation of multi-caspase proteins (Caspase-3, -8, and -9) was proposed as a possible mechanism in MCF-7 by the extracts [27]. Aqueous extracts of *P. indusiatus* decreased NO-production and pro-inflammatory cytokines (IL-1, IL-6, TNFα) leading to reducing inflammatory phenotype. MMP-2 inhibition by *P. indusiatus* extracts increased collagen stimulation through MMP-2 inhibition [28]. However, inhibitory mechanism of the aqueous extract is still unclear. The aim of the study is to investigate the anti-inflammatory and anticancer properties of *Phallus indusiatus* (bamboo mushroom) extracts, with a focus on their effects within inflammatory microenvironments and their potential to enhance lapatinib sensitivity in breast cancer cells. Herein, aqueous extracts of fruiting body and mycelium *P. indusiatus* were investigated their therapeutic effects. Antioxidant activity was first assessed by DPPH and FRAP assay. Cytotoxicity was done in both cancerous and non-cancerous cells. Combination treatment was performed to monitor the effect of each *P. indusiatus* extract. Anti-inflammation was confirmed by determining released NO in RAW 264.7 cells. To elucidate a possible inhibitory mechanism upon combination treatment, Akt and NF-kappaB activation were assessed by western blotting along with apoptosis analysis. Taken together, the aqueous extracts of *P. indusiatus* not only serve as a nutritional source but also offer potential health benefits for breast cancer patients.

## Materials and Methods

*Mushroom extraction*

*P. indusiatus* cultivation was conducted following AnonBiotec Inc.'s instruction. Briefly, the mushroom was cultured on solid medium which composed of sawdust and grain-like. Each separated part was then grinded. At the equivalent amount (~50 mg) of dried mushroom, fresh mushroom, dried mycelium, and fresh mycelium were then extracted with distilled water at 37°C for 24h. After the incubation time, all samples were centrifuged to collect the supernatant. All clear supernatants were dehydrated at 80°C until solvent residues were eliminated. Extracted fractions were then weighted and reconstituted with dimethyl sulfoxide (DMSO).

*Antioxidant analysis*

2,2-diphenyl-1-picrylhydrazyl or DPPH ethanol-based assay was performed to determine scavenging activity of the extracts. Briefly, DPPH working solution (0.02mg/mL) was freshly prepared with light protection before the experiment setting up. 10µL of each extract (10mg/mL) was mixed with 190 µL of the DPPH working solution and incubated at room temperature in the dark for 30 min. At the indicated time, the mixture was measured at OD 517 nm. The DPPH working solution and ethanol were used as negative and positive control, respectively.

$Fe^{3+}$ reduction capacity of each extract was assessed by FRAP assay following the previous study with minor modifications. Briefly, 10 µL of the extracts (10mg/mL) were mixed with 25 µL of phosphate buffer pH 6.0. and 25 µL of 1% potassium ferricyanide. The mixture was incubated at 50 oC for 30 min. 10 µL of 10% TCA was then added to stop the reaction before eliminating all precipitates by centrifugation. 25 µL of distilled water and 10 µL of 0.1% $FeCl_3$ was added. The reaction was measured the absorbance at 700 nm. $Fe^{3+}$ reduction capacity was calculated in term of reducing power from FeSO4 calibration curve.

*Cell culture*

All cell lines were purchased from American Type Culture Collection. MCF-7 (HTB-22TM); breast cancer cell lines and RAW 264.7 (TIB-71TM) were cultured with DMEM medium supplemented with 10% fetal bovine

serum and 1% penicillin and streptomycin solution. MCF-10A (CRL-10317); Human breast epithelial cell lines were cultured in DMEM/F12 supplemented with 0.25% MEGS (Gibco, MA), 5% horse serum and 1% penicillin and streptomycin solution. Both cell lines were grown at 37°C, 5% $CO_2$ and humidity. The cells were replenished with fresh medium every 3 days.

*NO production determination*

RAW 264.7 cells ($1x10^5$ cells) were plated on a 96-well plate and incubated for 16-18h. After cell attachment, the cells were replenished with fresh complete medium containing mushroom extracts at the desired concentrations. The cells were treated with the extracts for an hour before LPS stimulation for another 24h. Diclofenac (75µg/mL as a final concentration) was used as a positive control. At the indicated time, the treated medium was harvested and quantified released NO by Griess's reagent. The treated cells were further assessed cytotoxicity by MTT.

*Cytotoxicity assessment*

MCF-7 ($7x10^3$ cells/well) and MCF-10A (7x103 cells/well) were seeded into a 96-well plate. For cytotoxicity screening, various concentrations (0.03-1 mg/mL) of the extracts or Lapatinib or TNFαcontaining medium were replenished and incubated for another 72h. For co-treatment experiment, fresh culture medium containing 10 ng/mL TNFαand 10 µM Lapatinib was used as co-treatment medium. Various concentrations of the extracts were prepared with the co-treatment medium. After 72h treatment, the treated cells were assessed survival rate by MTT assay. Briefly, the treated cells were replenished with fresh medium containing 0.5mg/mL MTT and incubated for another 3h. Formed formazan crystal was then dissolved by DMSO. Cytotoxicity was expressed as %Survival rate compared to untreated condition.

*Apoptosis assay*

MCF-7 ($4x10^4$ cells/well) were plated into a 24-well plate before the experiment started. After cell attachment, the cells were replenished with the co-treatment medium in the presence of each extract. After 72h, the treated cells were harvested and stained with MUSE™ Annexin V& Dead cell kit following the user's guide instruction. MUSE® Cell Analyzer was used for determining cell populations.

*Western blotting analysis*

After cell attachment, MCF-7 were treated with various concentration of the extracts in fresh medium containing 10 ng/mL TNFα and 10µM Lapatinib. After 72h, cell lysate was harvested and quantified using BCA assay. 10 µg of cell lysate were separated and blotted on NC membrane. The blotted membrane was blocked, washed and stained primary antibody regarding to anti-phospho NFkappaB (S536) (CST) (1:1000), anti-phospho Akt (CST)(1:1000), and anti-Actin(CST)(1:2000) for 16-18h. The stained membrane was probed with HRP-conjugated Goat-anti mouse IgG (1:5000). Specific protein bands were developed using ECL substrate and visualized using Biorad Gel Doc XR+ gel documentation system.

*Statistical analysis*

All in vitro experiments were done in triplicate. $IC_{50}$ curves were fitted following nonlinear regression (curve fit) in log inhibitor vs response-variable slope (four parameters) equation. All bar graphs were also plotted using GraphPad 6.0 software (GraphPad Software, CA). Significant data were performed using two-way ANOVA following Dunnett's multi-comparison test compared to control group at $p<0.05$.

## Results

*Antioxidant properties and anti-inflammatory activity assessment of aqueous P. indusiatus extraction*

Edible mushrooms are safe for consumption that have been enjoyed worldwide through various cooking methods. Water, a readily available and convenient solvent, is commonly used in households. In this study, we utilized distilled water to explore the potential therapeutic benefits of *P. indusiatus*. To exploit the potential of the *P. indusiatus*, *P. indusiatus* was divided into 2 parts—(i) mycelium and (ii) fruiting body. Fresh and dried forms of both samples were also used. Our primary focus was to investigate its antioxidant properties as a health-promoting food. The extracts of *P. indusiatus* showed a modest percentage of scavenging activity in reducing artificial colored free radicals or DPPH (Figure 1A). Among the various extracts, dried mycelium exhibited the highest scavenging activity. Consistent with the DPPH results, the extracts also displayed a similar trend in their reducing capacity on $Fe^{2+}$ ions (Figure 1B). To further explore the cellular antioxidant properties of the extracts, their anti-inflammatory activity was evaluated. LPS-induced inflammation in murine macrophages (RAW264.7 cells) has been extensively studied, with the release of nitric oxide (NO) serving as a determinant. Interestingly, all extracts exhibited a significant reduction in the release of NO in a dose-dependent manner when exposed to LPS stimulation without any cytotoxicity (data not shown) (Figure 1C). Notably, the dried mycelium extract displayed the highest anti-inflammatory activity, which aligns with its antioxidant activity.

*Growth-modulatory effect of aqueous P. indusiatus extracts cancerous and non-cancerous*

For this study, the cellular models MCF-7 and MCF-10A were employed. The cytotoxicity of the extracts was assessed using different concentrations. It was observed that the *P. indusiatus* extracts led to a dose-dependent decrease in the survival rate of MCF-7 cells, with the survival rate dropping below 50% (Figure 2A-D) compared to the control at the highest concentration. Surprisingly, in the case of MCF-10A cells, an increase in the survival rate was observed following the treatment by increasing the concentration (Figure 2A-D). Noted, both fresh and dries mycelium extracts dramatically increased 3-fold cell survival rate in MCF-10A compared to MCF-7 at the highest concentration (Figure 2C-D).

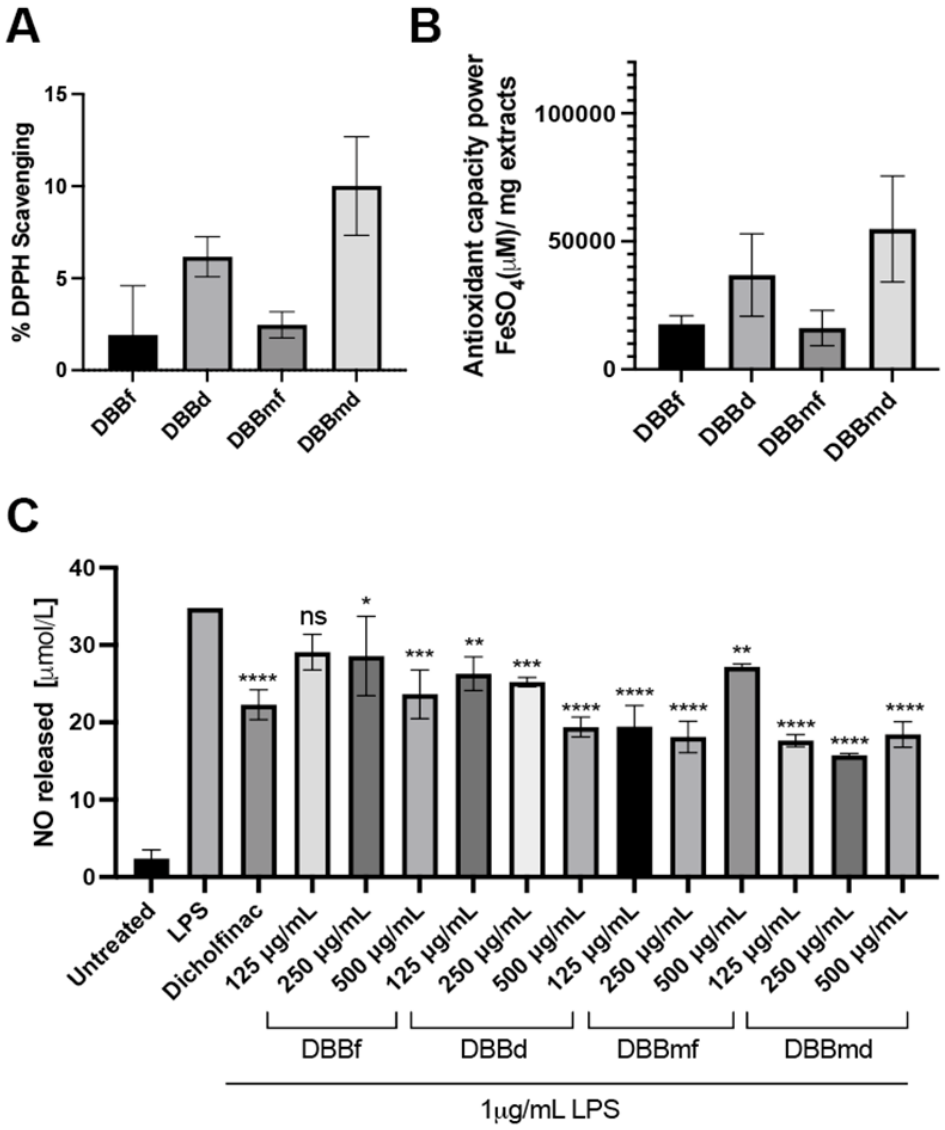


Figure 1. Antioxidant Activity and Anti-Inflammatory Effect on Murine Macrophage Cells (RAW264.7) of the extracts, Radical scavenging activity (A) and $Fe^{2+}$ Reducing capacity (B) of *P. indusiatus* was demonstrated by DPPH and FRAP, respectively. Released nitric oxide (NO) by RAW264.7 was quantified in the presence of the extracts under LPS stimulation. All independent experiments were done in triplicate. (*) indicated significant data ($p<0.05$)

This observation suggests that these extracts may have a promising therapeutic benefit in modulating cancerous cell growth while simultaneously promoting non-cancerous cell proliferation.

*Combination treatment of TNFα and Lapatinib mimicking breast cancer microenvironment*

Extensive research has been conducted on the complex cellular environment within tumors, known as the tumor microenvironment, which plays a significant role in enabling cancer cells to evade therapeutic interventions. we sought to investigate the effect of lapatinib under TNFα -mimicking microenvironment. We first determined $IC_{50}$ of Lapatinib and Lapatinib combined with TNFα. The combination treatment seemingly demonstrated greater $IC_{50}$ (~11μM) than single treatment does (~17μM) (Figure 3A). To gain further insight into the effects of the treatments, we conducted independent experiments to monitor cell growth. Plasma level of both Lapatinib and TNFαwas used in this study. In our study, we conducted independent experiments to monitor cell growth using plasma levels of Lapatinib and TNF-alpha. TNFαtreatment led to decreased cell survival rate compared to Lapatinib treatment, while the combination treatment exhibited insignificant variations in cell survival rates (Figure 3B).

*Aqueous P. indusiatus extracts sensitizes Lapatinib-induced MCF-7 cytotoxicity in the presence of TNFα*

As mentioned previously, TNFα influenced the overall survival rate of the extracts in a manner that was not dependent on the dosage. This suggests that TNFα might directly impact MCF-7 cells by inducing the extrinsic apoptosis pathway through its functional role . Lapatinib treatment did not demonstrate any cytotoxicity effect, even

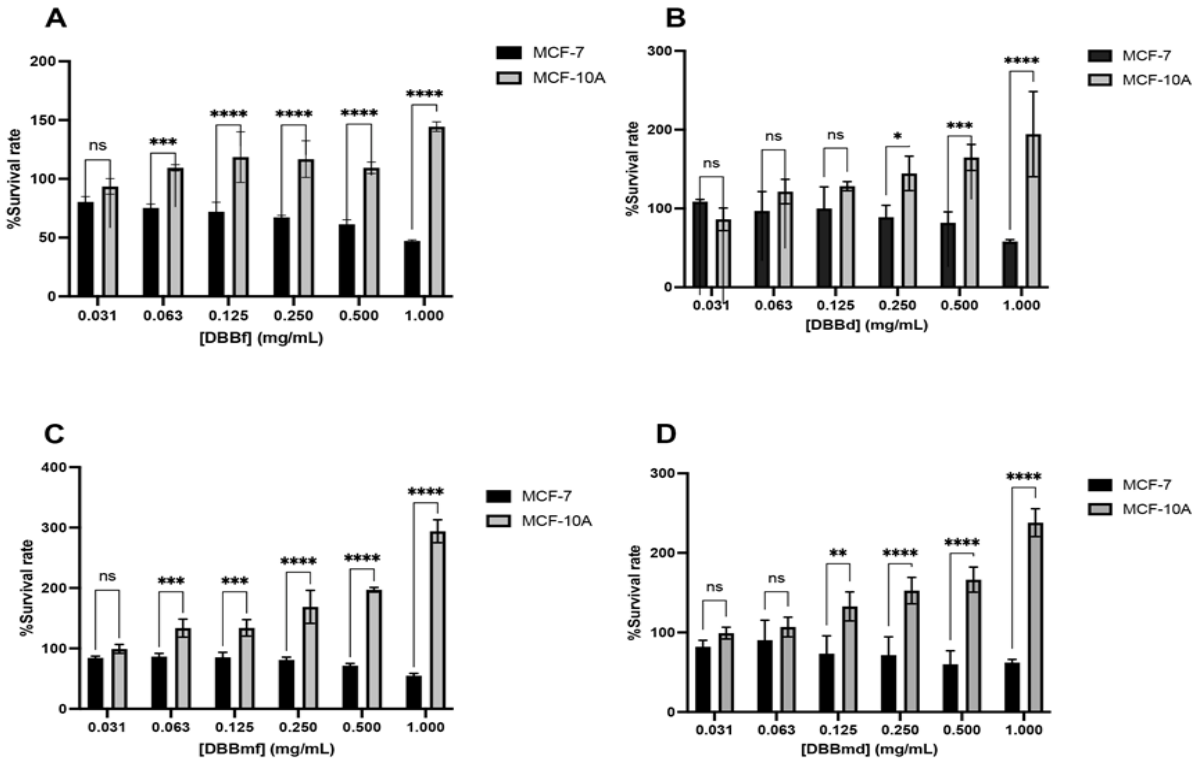


Figure 2. Cytotoxicity of the *P. indusiatus* Extracts against MCF-7 and MCF-10A, Various Concentrations of each Extracts were assessed Their Cytotoxicity by MTT. (*) significant data ($p<0.05$)

in the presence of the extracts. Interestingly, in the presence of TNFα, Lapatinib-induced cytotoxicity significantly increased in a dose-dependent manner when combined with the extracts (Figure 3C-F). Fresh mushroom and dried mycelium extract seemingly exhibited a significant enhancement of Lapatinib-induced cell death (Figure 3C and 3F). These findings demonstrated synergistic effect of the aqueous *P. indusiatus* extracts on chemotherapy by enhancing the effectiveness through sensitization.

*Aqueous P. indusiatus extracts increased Lapatinib-induced MCF-7 apoptosis in the presence of TNFα*

To evaluate the population of apoptotic cells in response to the treatment, concentrations of 500 and 250 µg/mL were utilized for each extract. Under the presence of TNFα, lapatinib-induced apoptosis was observed when compared to the untreated condition. Interestingly, in the presence of each extract, there was a dose-dependent increase in the population of apoptotic cells under the desired conditions (Figure 4). These results were consistent with the cytotoxicity findings, highlighting that both the fresh mushroom and dried mycelium extracts sensitized lapatinib-induced apoptotic cell population in the presence of TNFα.

*Aqueous P. indusiatus extracts sensitizied Lapatinb-induced cell death through suppressing NF-kappa and Akt activation*

NF-kappaB plays a central role as an inflammatory responding hub upon board range stimuli. Phosphorylation of NF-kappaB leads nuclear translocation of p65 to bind to specific promoter for responding-gene upregulation. As expected, both TNFα treatment and the combination treatment resulted in an increased activation of NF-kappaB through phosphorylation at the S536 site (Figure 5). However, Lapatinib exhibited a slight decrease in NF-kappaB activation. In the presence of the extracts (fresh mushroom, dried mushroom, and dried mycelium) downregulated NF-kappaB activation in a dose-dependent manner. Fresh mushroom completely abolished NF-kappaB activation at 250 µg/mL. Akt activation has been reported to be associated with the development of drug resistance in cancer. Collectively, the presence of the extracts resulted in the attenuation of Akt activation, thereby sensitizing Lapatinib-induced cell death in the presence of TNFα. Remarkedly, both fresh and dried mycelium dramatically downregulated Akt activation compared to the others under inflammatory environment. These findings indicated that the extracts have the potential to overcome drug resistance by targeting the Akt and NF-kappaB pathway.

## Discussion

Mushrooms have survived in harsh conditions which are enriched with tremendous microbes. Fortunately, mushrooms have to defend themselves by producing some bioactive compounds against the microbes. For decades, *P. indusiatus* have been known as a medicinal mushroom due to their activity such as an antioxidant [29], neuroprotective activity [30], immunomodulator [31, 32], immunostimulator [33, 34], including anticancer [35-37].

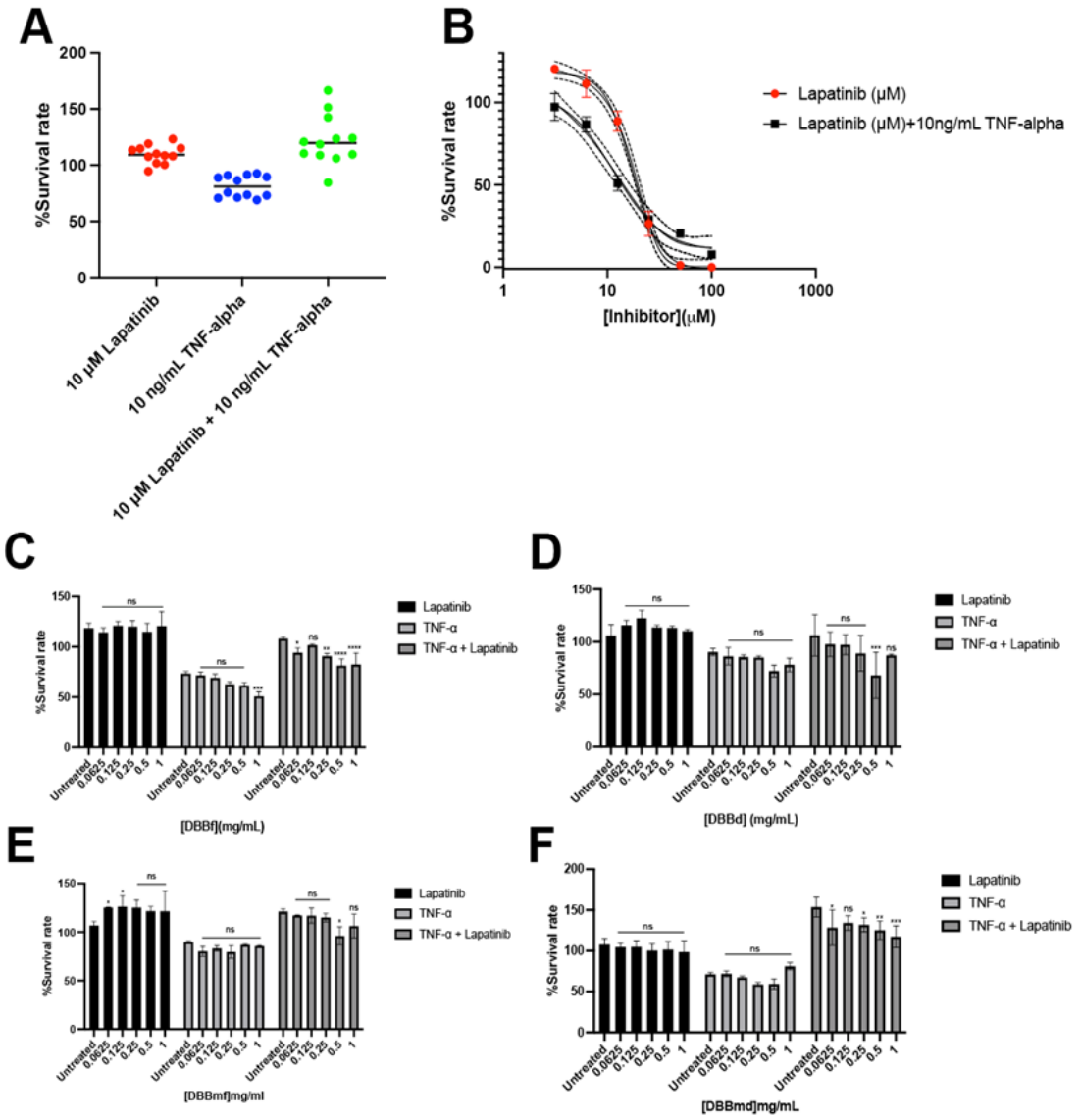


Figure 3. Cytotoxicity of TNF-alpha-induced Tumor Microenvironment of MCF-7, $IC_{50}$ of the Conditions were Demonstrated on MCF-7 by MTT (A) and comparison of co-treatment of Lapatinib and TNFα on MCF-7(B) . Dose-dependent effect of the extracts was assessed on TNF-α induced MCF-7 condition (C-F).

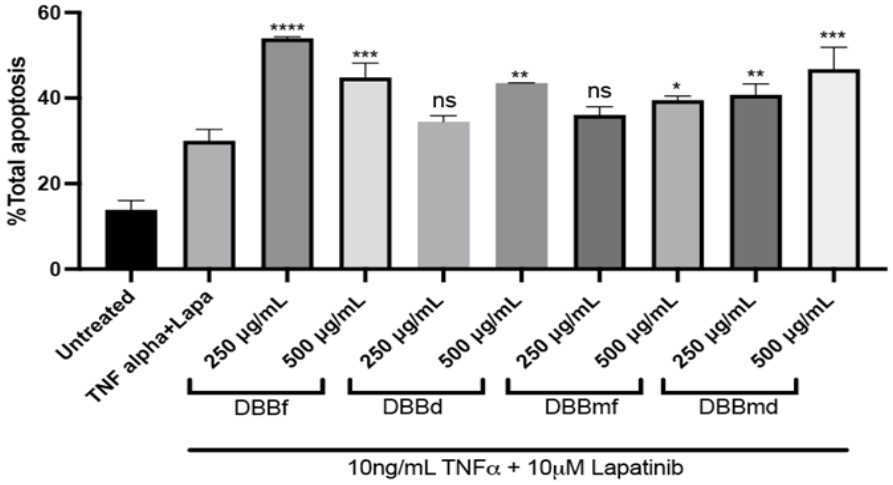


Figure 4. Apoptosis Induction of *P. indusiatus* Extracts on TNF-alpha-induced Tumor Microenvironment of MCF-7, Annexin V-FITC and 7-AAD staining was used to analyze apoptotic cells in the presence of the extracts by MUSE® Cell Analyzer.

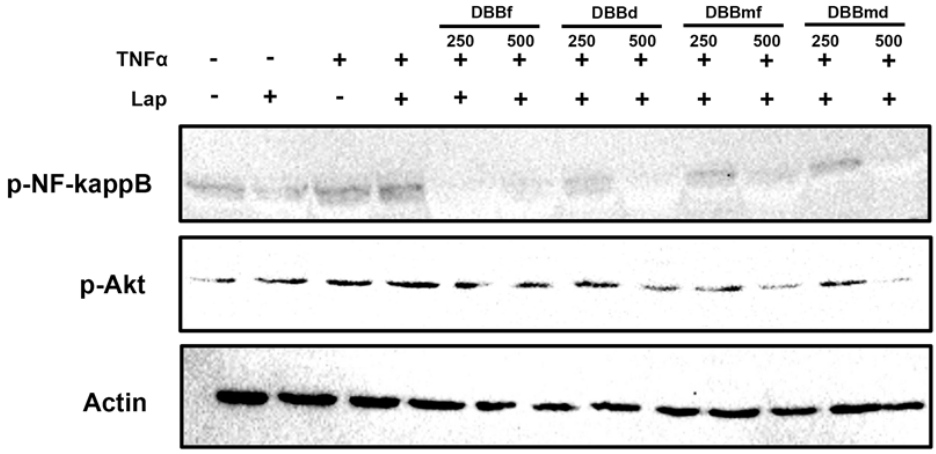


Figure 5. Effect of *P. indusiatus* Extracts on TNF-alpha-induced Tumor Microenvironment of MCF-7, the Cell were treated for 72h under the Desired Conditions (10μM Lapatinib (Lap), 10ng/mL TNFα ). Representative protein bands were showed protein expression level.

However, morphological development of *P. indusiatus* would accumulate various type of bioactive compounds. Bioactive accumulation in each part of the mushroom would be different. Dried mycelium and dried fruiting bodies exhibited greater antioxidant activity that fresh form of *P. indusiatus*. As anticipated, *P. indusiatus* contains very high-water content (90.9%) [38]. Dehydrated raw materials would be promising strategy to enrich bioactive compounds with high nutrition contents. Moreover, dried *Phallus indusiatus* also decreased released NO under LPS stimulation. It probably explains in term of antioxidant activity by scavenging LPS-induced ROS. Although, aqueous extraction mostly liberated some functional polysaccharides that act as an immunostimulant but some displayed anti-inflammatory effects [34, 39]. These findings demonstrated a simple method to extract dried *P. indusiatus* that still retain its nutraceutical properties.

Breast cancer patients have been suffering during chemotherapy. Non-responding patient developed chemoresistance after 2-3 cycles by any means such as MDR/survival factor upregulation resulting in cancer progression [40]. Tumor microenvironment in breast cancer have currently been announced to increase chemoresistant occurrence regarding to clinical observation [41, 42]. Regulatory molecules such cytokines/chemokines have been reported to contribute chemo/radio-resistance as a microenvironment factor [5]. Elevated TNFα levels was clinically detected compared to serum of healthy woman. Although, the effect of TNFα on breast cancer is still controversial in term of antitumorigenic and protumorigenic effect [43]. Our studies demonstrated that TNFα-treated MCF-7 cells slightly affected the survival rate compared to Lapatinib-treated MCF-7 cells. However, under the combination treatment condition, the survival rate seemingly increased. TNFα would exhibit modulatory effect on breast cancer in both protumorigenic and antitumorigenic effect. Although, cytotoxicity and $IC_{50}$ did not show any significant evidence. Insightly, cellular signaling is different. In the presence of TNFα, Akt and NF-kappaB activation was increased to induce cell apoptosis via canonical pathway [44]. Conversely, Akt and NF-kappaB activation was increased under lapatinib treatment. HER-2 is a key modulator to activate NF-kappaB in breast cancer. Lapatinib inactivates NF-kappaB through via blocking the PI3K/Akt cascade [45]. Interestingly, the combination treatment led to the activation of both Akt and NF-kappaB, which are associated with the increase in the survival rate (Figure 3B). TNFα would recapitulate breast cancer microenvironment. NF-kappaB and Akt seemed to be a critical determinant of drug resistant occurrence [46, 47]. Interestingly, overall survival rate was reduced by *P. indusiatus* treatment under the condition. Akt and NF-kappaB activation was attenuated in a dose-dependent manner. Regarding to our preliminary result, aqueous extraction of *P. indusiatus* exhibited inhibitory effect against recombinant TK-EGFR activity (data not shown). The extracts probably inhibited kinase activity of HER family through PI3K-Akt inactivation leading to NF-kappaB inactivation. However, anti-tumorigenic activity of *P. indusiatus* is still limited. a medicinal mushroom; Phellinus linteus elicited anti-cancer activity by targeting Akt activation resulting in reduce breast cancer progression [25].Our study first demonstrated the hidden a nutraceutical value of *P. indusiatus*. Noted, aqueous extraction of *P. indusiatus* increased cell viability of non-cancerous cell; MCF-10A. Our studies could never convince the mitogenic effect of the extract on MCF-10A. However, some edible mushroom exhibited strong mitogenic activity on mouse T-cell by mushroom lectins [48]. 18-kDa lectin of Ganoderma capense potentially increased mouse splenocyte but exhibited antiproliferative effect against cancerous cells (L1210, M1, and HepG2 cells) [49]. Mitogenic effect of *P. indusiatus* would be further investigated to better understand its mechanisms. These findings demonstrate that aqueous *P. indusiatus* extracts contain a mixture of modulatory effects on breast cancer cells, which could potentially improve the efficacy of chemotherapy for breast cancer patients.

## Author Contribution Statement

SS, KC and LT; Conceptualization and experimental design, AA; resource and mushroom cultivation SS; Extraction, SS, PS, MHHT and PrS; Cell-based experiment, LT; Data analysis and draft, LT and KC; Discussion and editing

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*Availability of data*

The data that support the findings of this study are available, upon reasonable request.

*Conflict of interest*

None of conflict of interest.

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