GSU/CMU Biotech symposium 2012

ANTI-INFLAMMATORY ACTIVITIES OF PHYSALIN A FROM *PHYSALIS ANGULATA* THROUGH THE INHIBITION OF MMP-9, NF-KB, AND MAPK ACTIVATION *IN VITRO* AND *IN VIVO*

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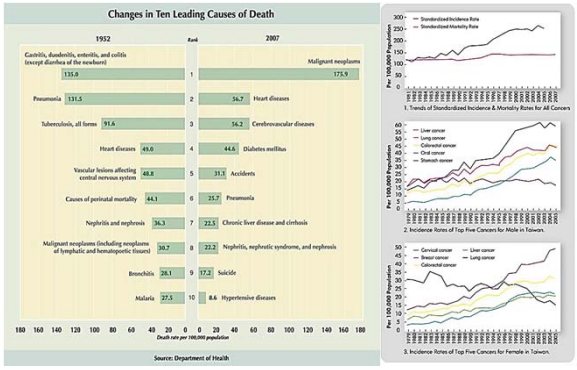
2012.11.15

First part

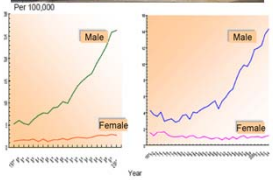
Anti-cancer research in Our Lab

Background of our lab. in anti-cancer research



Risk factors of Oral Cancer

- Betel nut (the Institu
- Tobacco (J Oral Pa
- Alcohol (Global c
- Diet and nutrition,
- Ultraviolet light,
- HPV infection



Michael Ericksen
Dean, institute of public Health

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Anticancer research in TCM and folk herbs

1. 山芙蓉 *Hibiscus taiwanensis*
2. 山豆根 Sophora subprostrata
3. 龍葵 Solanum nigrum
4. 香茅 *Cymbopogon citratus*
5. 艾草 *Artemisia indica*
6. 一枝黃花 *Solidago decurrens*
7. 鉤藤 *Viburnum odoratissimum*
8. 苦蘵 *Physalis angulata*
9. 金剛纂 *Euphorbia antiquorum*
10.……







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Physalis angulata (Solanaceae)
Usage: Herb tea
涼茶（清涼退火）
Extinguish the fire inside the body

1992 Chiang et al., Inhibitory effects of physalin B and physalin F on various human leukemia cells in vitro.

2001 Lee et al., Effects and mechanisms of PA on cell death in human lung squamous cell carcinoma.

2003 Makino et al., Cytotoxic activity of physalins possessing modified skeletal structures against HeLa cells.

2005 Vieira AT et al., Mechanisms of the anti-inflammatory effects of the natural secosteroids physalins in a model of intestinal ischaemia and reperfusion injury.

2007 Castro DP et al., Immune depression in Rhodnius prolixus by seco-steroids, physalins.

1992s | 2001s | 2002s | 2003s | 2004s | 2005s | 2006-2011 | 2012s

2001 Ismail et al., A novel cytotoxic flavonoid glycoside from *Physalis angulata*.

2002 Kuo et al., The antiproliferative activity of PA is through p53-dependent and p21-dependent apoptotic pathway in human hepatoma cell lines.

2004 Wu et al., Antihepatoma activity of PA and P. peruviana extracts and their effects on apoptosis in human Hep G2 cells.

2006 Magalhs HI, et al., In-vitro and in-vivo antitumour activity of physalins B and D from Physalis angulata.

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Available online at www.sciencedirect.com
SCIENCE DIRECT
Food and Chemical Toxicology 44 (2006) 974–983
www.elsevier.com/locate/foodchemtox

Physalis angulata induced G2/M phase arrest in human breast cancer cells

Wen-Tsong Hsieh [a], Kuan-Yuh Huang [a], Hui-Yi Lin [b], Jing-Gung Chung [c,*]

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Food and Chemical Toxicology
journal homepage: www.elsevier.com/locate/foodchemtox

Oxidative stress involvement in *Physalis angulata*-induced apoptosis in human oral cancer cells

H.-Z. Lee [a,*], W.-Z. Liu [a], W.-T. Hsieh [b], F.-Y. Tang [c], J.-G. Chung [d], Henry W.-C. Leung [e]

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[b] Department of Pharmacology, China Medical University, Taichung, Taiwan
[c] Department of Nutrition, China Medical University, Taichung, Taiwan
[d] Department of Microbiology, China Medical University, Taichung, Taiwan
[e] Department of Radiation Oncology, Chi Mei Hospital, Liouying, Tainan, Taiwan

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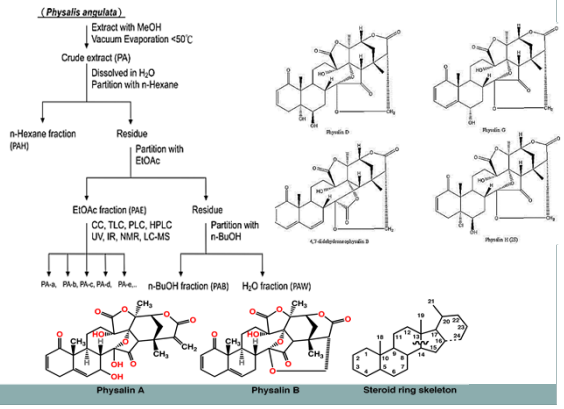
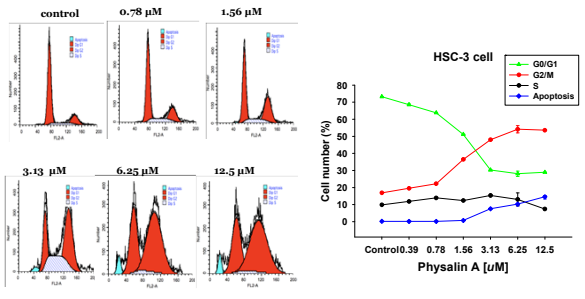
Isolation physalins from *physalis angulata*



Methods

1. Proliferation
   - Morphology
   - MTT assay
   - Cell cycle analysis (Flow cytometry) Western blot
2. Apoptosis
   - Flow cytometry
     - Ca^{++} production
     - Mitochondrial membrane potential ($\Delta\Psi$)
   - DAPI stain
   - DNA ladder assay
   - Western blot
3. Metastasis
   - Migration assay
     - Wound healing assay
     - Transwell migration assay
   - Matrigel invasion assay
   - Confoal image
   - Western blot

Physalin A on cell cycle analysis by flow cytometer



Physalin A induced G2/M arrest and apoptosis in HSC-3 cells in dose & time dependent.

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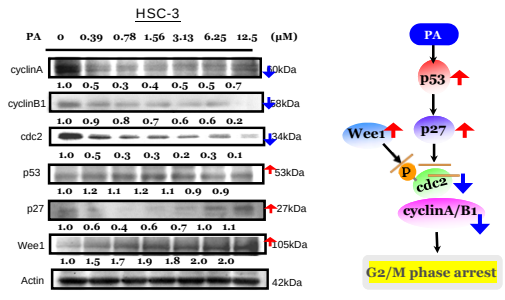
Physalin A on G2/M phase of cell cycle relative proteins



Physalin A induced G2/M arrest with decreasing cyclin A, cyclin B1 and cdc2 activity, but increasing wee1, p27 and p53 levels.

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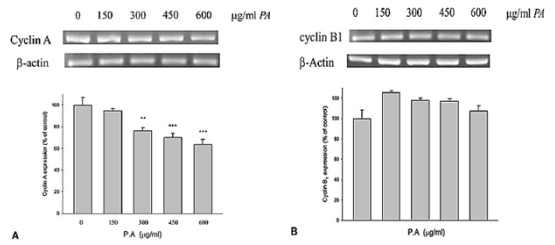
Effects of PA on mRNA expression of cyclin A and cyclin B1 in HSC-3 cells.



Physalin A induced G2/M arrest with decreasing mRNA expression in cyclin A levels, dose dependently .

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Methods

1. Proliferation
   - Morphology
   - MTT assay
   - Cell cycle analysis (Flow cytometry) Western blot
2. Apoptosis
   - Flow cytometry
     - Ca^{++} production
     - Mitochondrial membrane potential ($\Delta\Psi$
   - DAPI stain
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3. Metastasis
   - Migration assay
     - Wound healing assay
     - Transwell migration assay
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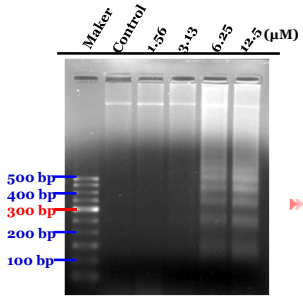
   - Confoal image
   - Western blot

Result-DNA fragmentation

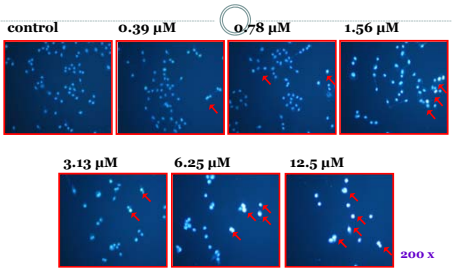
Physalin A on DNA damage with DNA ladder assay



To analyze the DNA fragmentation, HSC-3 cells were treated with Physalin A for 24 hr. Fragmented DNA was extrcted and analyzed on 2 % agarose gel electrophoresis with containing EtBr.

Physalin A could induced DNA damage with DNA ladder assay (apoptosis) in HSC-3 cells.

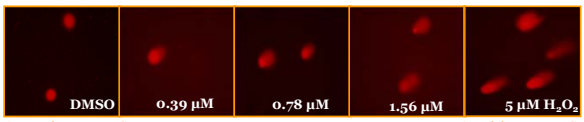
Effects of PA on DNA condensation with DAPI stain



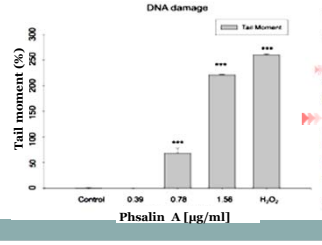
Cells were harvested after 24 h of PA treatment. After fixing, the cells were stained with DAPI. Stained nuclei were then observed under a fluorescent mircroscope. PA induced DNA condensation in HSC-3 cells.

Result-DNA damage

Physalin A on DNA damage by Comet assay



(Negative control) (positive control)

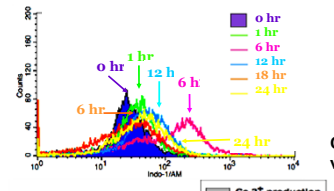


HSC-3 cells were treated with Physalin A (0.39, 0.78, 1.56 μg/ml) for 24 hr and applied to Single cell electrophoresis

Physalin A induced DNA damage dose dependent by the Comet assay in HSC-3 cells

Result-Ca 2+ production

Effects of PA-42 on Ca 2+ production by flow cytometry



Cells were harvested various hour of PA

PA-42 could increase Ca^{++} production at 6-12 h in HSC-3 cells mostly.

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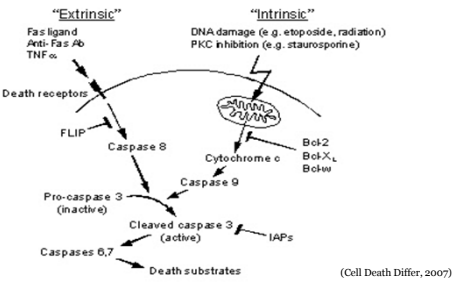


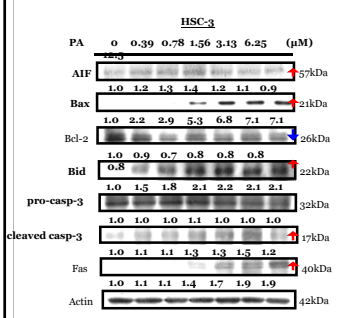
Measurement of Ca^{2+}

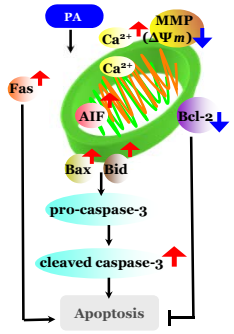
Ca^{2+} was determined by Indo 1/AM → flow cytometry.

Measurement of mitochondrial membrane potential ($\Delta\Psi m$)

Mitochondrial energization was determined by the retention of the dye $DiOC_6$3'-dihexyloxacarbocyanine iodide → flow cytometry.

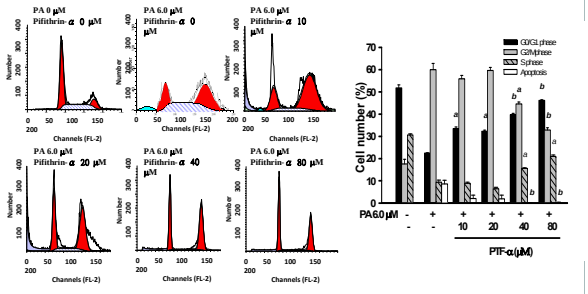
Effects of Physalin A on apoptotic proteins



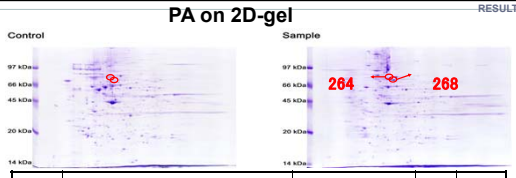


Physalin A triggered apoptosis with an increase in Fas, Bax, Bid, and cleaved caspase-3 but a decrease in Bcl-2.

AIF: apoptosis-inducing factor
MMP : mitochondrial membrane



PA on 2D-gel



	Protein Name	Accession Number	pI	MW (Da)
264	Heat shock 70kDa protein 8 [Homo sapiens]	gi|16741727	5.37	70854
268	HSP70-2 [Homo sapiens]	gi|4529892	5.48	69982

PA(μM)	0	1.6	3.1	6.3	12.5	
HSP90	1.00	1.04	1.80	1.97	2.58	90 kDa
HSP70-2	1.00	0.95	1.13	1.11	1.11	40 kDa
β-actin						42 kDa

Methods

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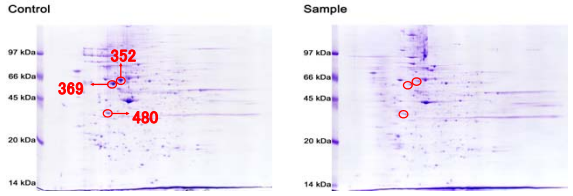
3. Metastasis
   - Migration assay
     - Wound healing assay
     - Transwell migration assay
   - Matrigel invasion assay
   - Confoal image
   - Western blot

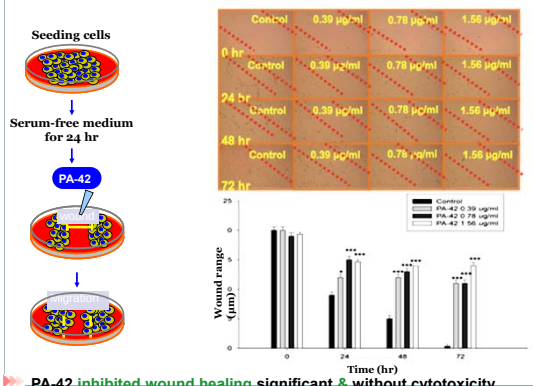
PA in 2D-gel analysis



	Protein Name	Accession Number	pI	MW (Da)
352	alpha-tubulin [Homo sapiens]	gi|340021	4.94	50120
369	Tubulin, beta [Homo sapiens]	gi|14043231	4.78	49639
480	Nucleophosmin (nucleolar phosphoprotein B23, numatrin) [Homo sapiens]	gi|16876872	4.64	32555

Result-wound healing migration

Effects of PA-42 on by wound healing assay



PA-42 inhibited wound healing significant & without cytotoxicity.

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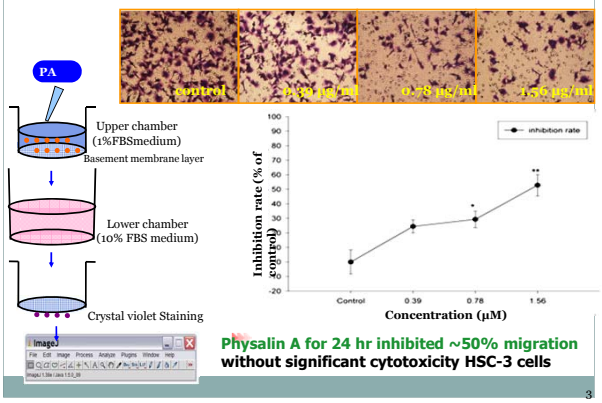
Result-transwell migration assay

Physalin A on cell migration by Transwell Migration assay

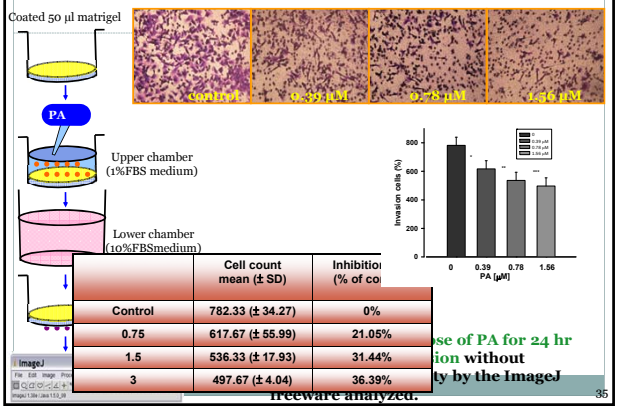


Physalin A for 24 hr inhibited ~50% migration without significant cytotoxicity HSC-3 cells

3

Result-matrigel invasion assay

Effects of PA 0.75~1.56 μM on matrigel invasion assay



	Cell count mean (± SD)	Inhibition (% of co
Control	782.33 (± 34.27)	0%
0.75	617.67 (± 55.99)	21.05%
1.5	536.33 (± 17.93)	31.44%
3	497.67 (± 4.04)	36.39%

ase of PA for 24 hr
ion without
ty by the ImageJ freeware analyzed.

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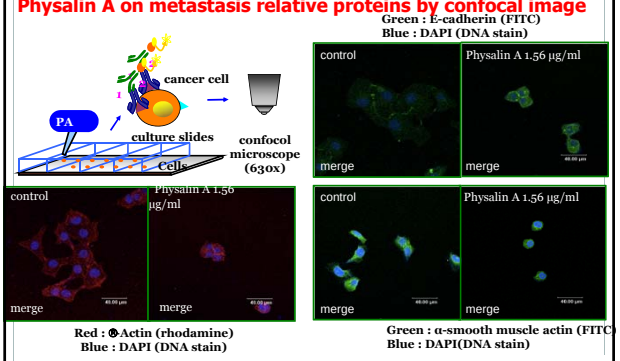
Result-cytoskeleton proteins

Physalin A on metastasis relative proteins by confocal image

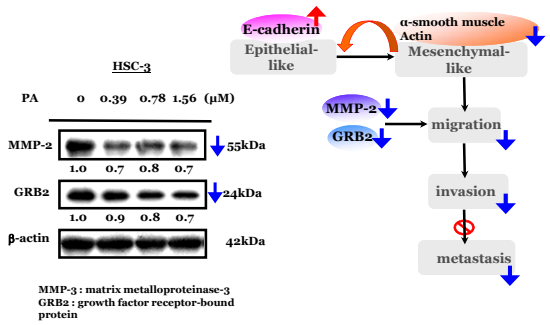


Physalin reversed cytoskeleton from mesenchymal-like to epithelial-like with increased E-cadherin (epithelial marker) and decreased α-smooth muscle actin (mesenchymal marker) in HSC-3 cells.

3

Result-metastasis proteins

Physalin A on metastasis relative proteins



MMP-3 : matrix metalloproteinase-3
GRB2 : growth factor receptor-bound protein

Physalin A inhibited metastasis with a decrease in MMP-3 and GRB2.

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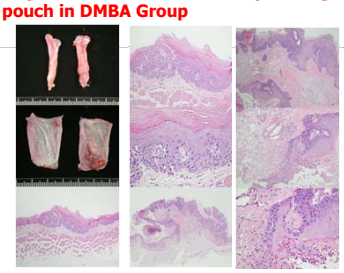
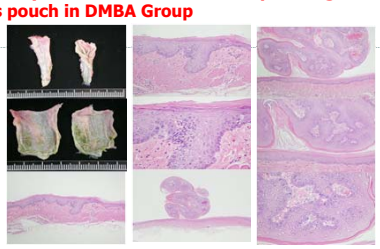
The effect of Physalin A on Gross and histopathological findings of hamster's pouch in DMBA Group



Gross and histopathological findings of hamster's pouch in (7,12-dimethylbenz [α]anthracene)DMBA Group. Gross of pouches presented tumor masses on the mucosa (A. closed, B. opened). Microscopically, moderate (3) hyperplasia with moderate (3) dysplasia, thickness of the epidermal layer (C. 100x, D. 200x, E. 400x). Severe (4) papilloma showed cauliflower-like growth, elongated and protruded with necrosis from epidermis to form papillary folding (F. 40x), and moderate (3) multicentric squamous cell carcinoma (SCC) invaded into the submucosa (G. 100x) and increased mitoses (H. 200x, I. 400x) in the epidermal epithelium of the pouch, H&E stain.

30

The effect of Physalin A on Gross and histopathological findings of hamster's pouch in DMBA Group



Gross and histopathological findings of hamster's pouch in DMBA + PA 50 mg/kg Group. Gross of pouches presented tumor masses on the mucosa (A. closed, B. opened, animal code. 202). Microscopically, slight (2) hyperplasia with moderate (3) dysplasia, thickness of the epidermal layer (C. 100x, D. 200x, E. 400x). Moderate (3) papilloma showed cauliflower-like growth, elongated and protruded with necrosis from epidermis to form papillary folding (F. 40x), and no (0) multicentric squamous cell carcinoma (SCC) invaded into the submucosa (G. 100x) but increased mitoses (H. 200x, I. 400x) in the epidermal epithelium of the pouch (animal code. 207), H&E stain.

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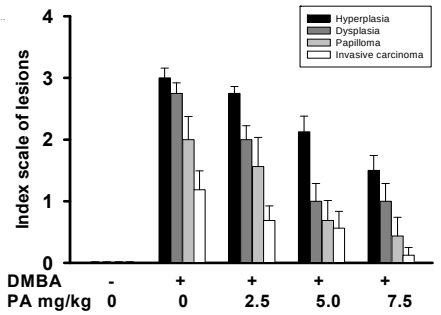
Physalin A in Hamster pouch tumor expression



Degrees of lesions
(1= minimal; 2=slight; 3=moderate; 4=severe)

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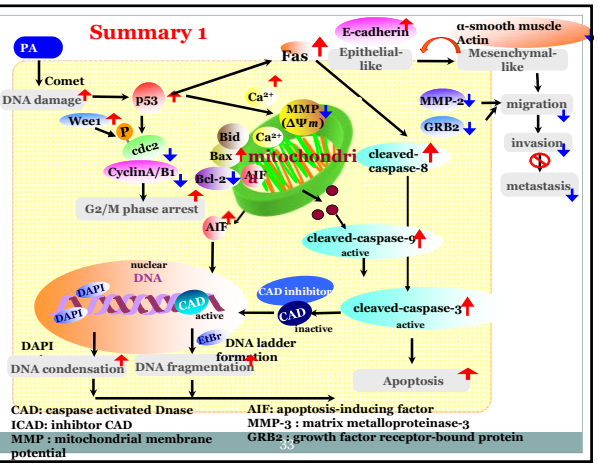
Summary 1



CAD: caspase activated Dnase
ICAD: inhibtor CAD
MMP : mitochondrial membrane potential

AIF: apoptosis-inducing factor
MMP-3 : matrix metalloproteinase-3
GRB2 : growth factor receptor-bound protein

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Second part

Anti-inflammtory research of PA

by Ya-Sing Hsiau
Undergraduate student study
from 2011/3 to 2012/6

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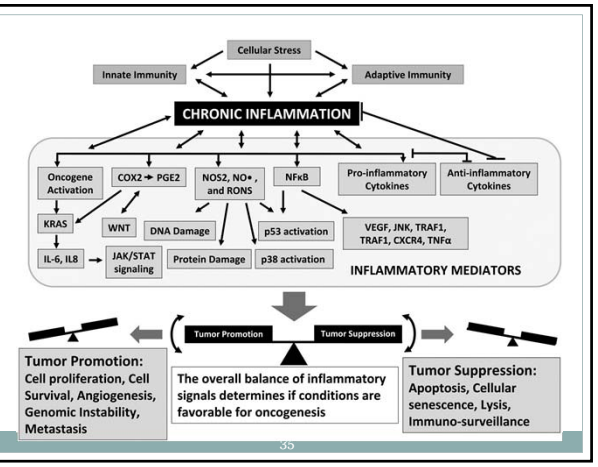

Tumor Promotion: Cell proliferation, Cell Survival, Angiogenesis, Genomic Instability, Metastasis

The overall balance of inflammatory signals determines if conditions are favorable for oncogenesis

Tumor Suppression: Apoptosis, Cellular senescence, Lysis, Immuno-surveillance

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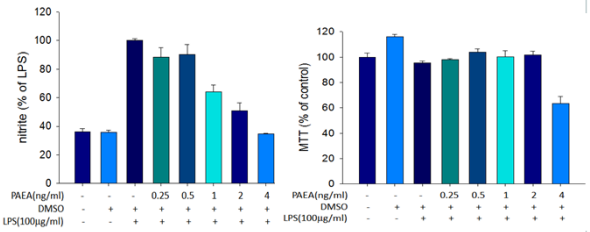
Cell Viability and the Effect of Physalin A on LPS-induced NO Production in Macrophages



PA significantly decreased the NO production and shown low cell cytotoxicity in RAW 264.8 cells, but the its mechanism still does not understand.

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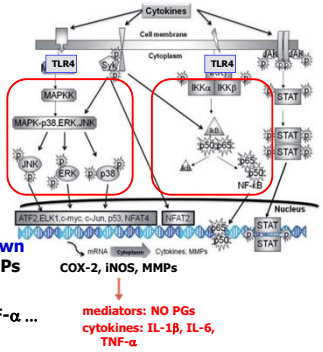
Inflammation Pathway

Inflammation Pathway

- TLR4 pathway (Upstream)
  CD14, MD2, MyD88
- MAPK pathway
  JNK, ERK, p38…
- IkK, IkB pathway
  p-IkK, p-IkB…
- NF-κB pathway
  p50, p65
- Transcription proteins (Down stream): COX-2, iNOS, MMPs
- mediators: NO, PGs…
- cytokines: IL-1β, IL-6, TNF-α …



COX-2, iNOS, MMPs

mediators: NO PGs
cytokines: IL-1β, IL-6, TNF-α

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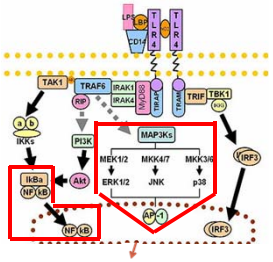
Aim

- In this study, we want to evaluation the anti-inflammatory effects of physalin A.
- *In vitro:* using lipopolysaccharide (LPS)-stimulated mouse macrophage RAW264.7 cells
- *In vivo:* using λ-carrageenan (Carr)-induced hind mouse paw edema model.



COX-2, iNOS, MMPs

mediators: NO PGs
cytokines: IL-1β, IL-6, TNF-α

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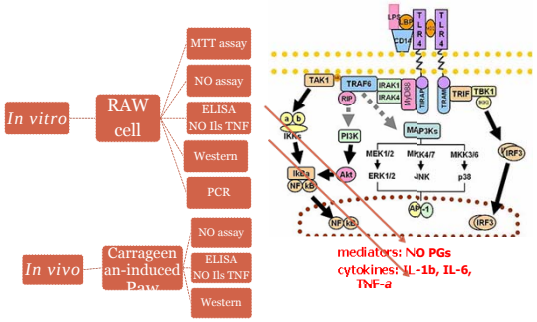
METHODS



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RESULTS

Inhibition of LPS-induced iNOS and COX-2 Protein by Physalin A

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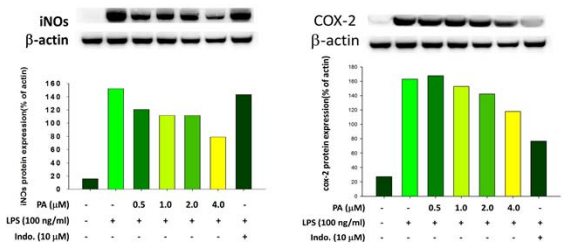
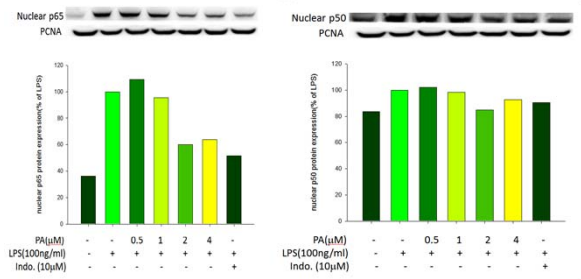


Physalin A treatment could down-regulation of iNOS and COX-2 proteins expression in RAW 264.7 cells.

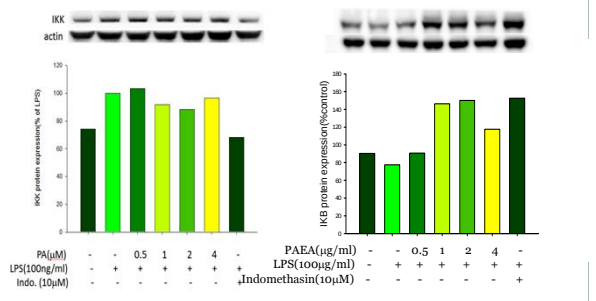
Inhibition of LPS-induced NF-κB Proteins by Physalin A

42

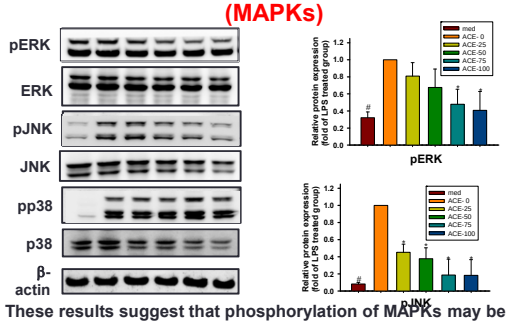


Down-regulation of p65 proteins, respectively, after the treatment with physalin A at 2 μM compared with the LPS-alone.

Physalin A Inhibits the levels of IKK , IKB Inflammatory Proteins

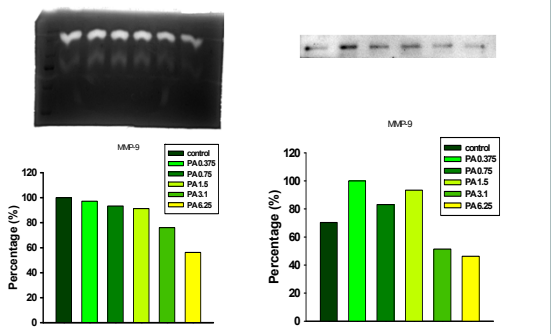


Effects of Physalin A on the LPS-stimulated Activation of Mitogen-activated Protein Kinases (MAPKs)



These results suggest that phosphorylation of MAPKs may be involved in the inhibitory effect of physalin A on LPS-stimulated NF-κB binding in RAW 264.7 cells.

Physalin A-Reduced MMP-2 & MMP-9 expression in zymography and western blotting



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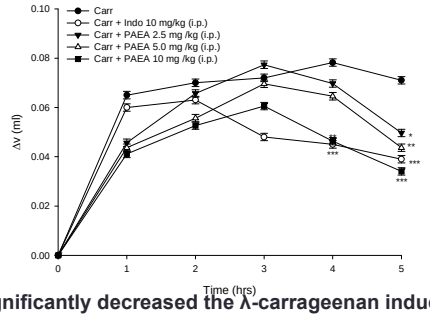
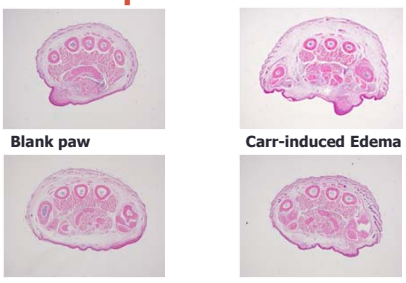
Effects of Physalin A on Carr-induced Mouse Paw Edema



PA significantly decreased the λ-carrageenan induced paw edema at the 4th h and the 5th h.

Histopathological findings of foot paw edema in mice



Blank paw | Carr-induced Edema

Physalin A in Carr-induced Edema | Indo. in Carr-induced Edema

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Summary 2

1. In *in vitro* tests, RAW264.7 macrophages were treated with physalin A together with LPS, a significant concentration-dependent inhibition of NO production was detected.
2. Western blotting revealed that physalin A blocked the protein expression of iNOS, COX-2, Ikk, NF-κB, and MMP-9 in LPS-stimulated RAW264.7 macrophages, significantly.
3. Physalin A also inhibited LPS-induced ERK, and JNK phosphorylation.
4. In *in vivo* tests, physalin A decreased the paw edema at the 4th and the 5th h after λ-carrageenan administration, that physalin A significantly attenuated the iNOS level and edema in the mice hind paw at the 3th-5th h after λ-carrageenan injection.

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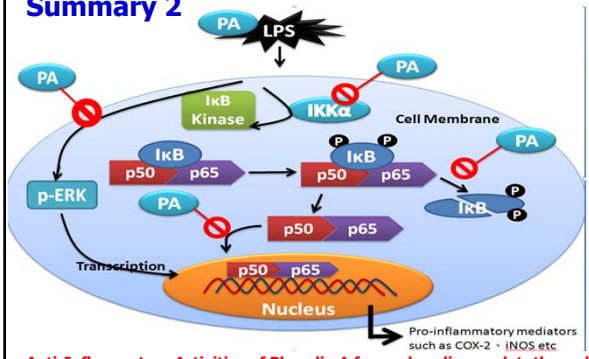
Summary 2



Anti-Inflammatory Activities of Physalin A from *physalis angulata* through the Inhibition of MMP, NF-κB, and MAPK Activation *In Vitro* and *In Vivo*

Acknowledgments

Thanks for your attention!

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