In Vitro Antioxidant, Anti-inflammatory, Cytotoxic Activities against Prostate Cancer of Extracts from *Hibiscus sabdariffa* Leaves

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Background: *Hibiscus sabdariffa (HS) leaves are a vegetable, which is used as a healthy sour soup for protection against chronic diseases in Thai traditional medicine.*
Objectives: *To investigate antioxidant, anti-inflammatory and cytotoxic activities of Hibiscus sabdariffa leave extracts from different extraction methods.*
Material and Method: *Fresh and dry Hibiscus sabdariffa leaves were extracted by various methods such as maceration with 95% and 50% ethanol, squeeze, and boiling with water or decoction. All extracts were tested for antioxidant activity by using DPPH radical scavenging assay, anti-inflammatory activity by determination on inhibitory effect of nitric oxide production on RAW264.7 cell. Cytotoxic activity also tested against human prostate cancer cell line (PC-3) by using sulforhodamine B (SRB) assay. Total phenolic content determined by the Folin-Ciocalteu colorimetric method.*
Results: *The results found that the 95% ethanolic extract of Hibiscus sabdariffa dried leaves (HSDE95) showed the highest antioxidant activity with an EC_{50} of 34.51±2.62 µg/ml and had the highest phenolic content (57.00±3.73 mg GAE/g). HSDE95 also showed potent cytotoxicity against prostate cancer cell line with an IC_{50} of 8.58±0.68 µg/ml whereas HSDE95 and all of extracts of Hibiscus sabdariffa leaves had no anti-inflammatory activity.*
Conclusion: *The obtained results revealed that HSDE95 extract showed potent cytotoxic activity against prostate cancer cells but low antioxidant and anti-inflammatory activities. This extract should be further isolated as active compounds against prostate cancer.*

Keywords: *Hibiscus sabdariffa leaves, Antioxidant, Anti-inflammatory, Cytotoxicity*

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Antioxidants are chemicals that block the activity of other chemicals known as free radicals. The free radicals have the potential to cause damage to cells and may also lead to cancer. Therefore, reduction of superoxides by free radical scavengers could be beneficial for prevention of cancer[1]. Moreover, the outcomes from chronic inflammation are cause of destruction of tissue. Chronic inflammation also made connective tissue thick and scarred or fibrosis, cells or tissue death or a necrosis pathway, so chronic inflammation can eventually cause several diseases, including some cancers[2].

Hibiscus sabdariffa L. or Roselle belongs to the Malvaceae family. The calyces of the plant are used in the manufacture of beverages and jam. However, *Hibiscus sabdariffa* L. is also used in many other applications[3]. Among the nurturing diligences, the leaves were used like vegetables in the preparation of healthy sour soup for protection against chronic diseases in Thai traditional medicine. Previous studies have demonstrated that an extract of *Hibiscus sabdariffa* leaves possess hypolipidaemic[4], antioxidant[5], and an aqueous extract of *Hibiscus sabdariffa* leaves was an apoptosis inducer in LNCaP cells[6]. There have been limited reports on the isolation

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of the *Hibiscus sabdariffa* leave extracts and its biological activity. In this study, the *Hibiscus sabdariffa* leave extracts from different extraction methods were investigated in vitro antioxidant, anti-inflammatory and cytotoxic activities.

Material and Method

Chemicals and reagents

Antioxidant assays

2, 2-diphenyl-1-picrylhydrazyl (Fluka, Germany), Butylated hydroxytoluene (Fluka, Germany), Folin-Ciocalteu's reagent (Fluka, Germany), Gallic acid (Sigma-Aldrich, USA), Sodium Carbonate (Merck, Germany).

Assay for NO inhibitory effect

Dimethyl sulfoxide (RCI Labscan, Thailand), Fetal bovine serum (Biochem, Germany), Hydrochloric acid (Univar, Australia), N-(1-Naphthyl) ethylene-diamine dihydrochloride (Sigma, USA), Penicillin-Streptomycin (Gibco, USA), Phosphate buffered saline (Amresco, USA), Phosphoric acid solution (Sigma, USA), RPMI medium 1640 (Gibco, USA), Sodium bicarbonate (Gibco, USA), Sodium hydroxide (Univar, Australia), Sulfanylamide (Sigma, USA), Trypan blue stain 0.4% (Gibco, USA), Trypsin-EDTA (BHD, England).

Sulforhodamine B (SRB) colorimetic assay

Dimethyl sulfoxide (RCI Labscan, Thailand), Fetal bovine serum (Biochem, Germany), Hydrochloric acid (Univar, Australia), Penicillin-Streptomycin (Gibco, USA), Phosphate buffered saline (Amresco, USA), RPMI medium 1640 (Gibco, USA), Sodium bicarbonate (Gibco, USA), Sodium hydroxide (Univar, Australia), Sulforhodamine B sodium salt (Sigma-Aldrich, USA), Trichloroacetic acid (Merck, Germany), Tris (hydroxymethyl) aminoethane (Sigma-Aldrich, USA), Trypan blue stain 0.4% (Gibco, USA), Trypsin-EDTA (BHD, England).

Plant materials and extraction

Fresh leaves of *Hibiscus sabdariffa* L. were obtained from Amphur Pichai, Uttaradit province, Thailand in October 2012. The plant material was authenticated and a voucher specimen (No. SKP 1090819) has been deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The extraction procedures used were similar to those practiced by Thai traditional doctors.

Ethanolic extraction

Fresh and dried leaves were macerated with 95% ethanol and 50% ethanol at room temperature for 3 days and filtrated through a Whatman No. 1 filter paper. The residue was further macerated with the same volumes of solvent for two times. The combined solvent extracts were then evaporated to dryness by using a rotary evaporator. The extracts were then dried to constant weight in a vacuum dry.

Water extraction

The fresh leaves were squeezed to yield juice. Fresh and dried leaves were boiled in distilled water. The duration of decoction was 15 min, three times. The extracts were filtrated by a Whatman No.1 filter paper and dried by lyophilizer. The residues from maceration of fresh and dried leaves were further boiled in distilled water. Then filtrated by a Whatman No. 1 filter paper and dried by lyophilizer.

Assay for antioxidant activity

DPPH radical scavenging activity assay

Antioxidant activity was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), according to the modified method of Yamasaki et al 1994[7]. Samples for testing were dissolved in absolute ethanol or distilled water to obtain the final concentration of 100 µg/ml. That extract will be determined as IC_{50} values by each extract was further diluted to obtain at least 4 solutions at lower concentrations (two-fold dilutions), final concentrated will be 100, 50, 10, 1 µg/ml, respectively. Each concentration was tested in triplicate. Then a segment of the extract solution (100 µl) was mixed with an equal volume of $6x10^{-5}$ M DPPH (in absolute ethanol) and allowed to place in the dark at room temperature for 30 minutes. The absorbance was then measured at 520 nm. Butylated hydroxytoluene (BHT) was used as a positive standard. The scavenging activity of the samples is the ability to reduce the color intensity of DPPH. Inhibition (%) was calculated using the following equation:

% inhibition = [(Abs. control-Abs. sample)/ Abs. control] x 100

Where Abs. control is the absorbance of the control reaction and Abs. sample was the absorbance of the tested compound. EC_{50} values were calculated from linear regression analysis of the graph of percentage inhibition against extract concentration. All tests were carried out in triplicate on any one day, and repeated with freshly prepared samples a further two times (n = 3).

In vitro for anti-inflammatory activity

Assay for NO inhibitory effect and cytotoxicity test in RAW264.7 cells

Inhibitory effects on NO production by murine macrophage (RAW264.7) cells is evaluated using a modified method from Tewtrakul and Itharat 2008[8]. This cell line was cultured in RPMI 1640 medium supplemented with 10% heated fetal bovine serum (FBS) and 50 IU/ml penicillin and 50 µg/ml streptomycin. Briefly, RAW264.7 cells were seeded in 96-well plate with $1x10^5$ cells/well and incubated for 24 hours in a CO_2 incubator at 37°C, in a 5% CO_2 atmosphere with 95% humidity. After that, the medium was removed and replaced with RPMI medium containing 10 ng/ml of LPS (100 µl/well) into the control and sample blank wells. Sample solutions were prepared at various concentrations 1-100 µg/ml that added to 100 µl in the wells of sample and blank of sample. The wells of control and blank of control were added with RPMI medium. The cells were incubated for 24 hours in a CO_2 incubator at 37°C, in a 5% CO_2 atmosphere with 95% humidity. Finally, 100 µl of supernatant was removed into another 96-well plate and added with Griess reagent 100 µl/well. NO production determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent and detected the color at a wavelength of 570 nm. Percentage of inhibition was calculated by using the following equation and IC_{50} values was determined graphically.

$$\text{Inhibition (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

$OD_{control}$ = Mean of control media (+LPS) - Mean of control media (-LPS)

OD_{sample} = Mean of sample (+LPS) - Mean of sample (-LPS)

Cytotoxicity was determined by using the MTT colorimetric method. After supernatant removed, the cells were tested for their cytotoxicity using MTT assay. MTT solution was added to the wells (10 µl/well) and incubated for 2 hours in a CO_2 incubator at 37°C, in a 5% CO_2 atmosphere with 95% humidity. The medium was removed, and isopropanol containing 0.04 M HCl was added to dissolve the formazan production (100 µl/well). The formazan solution was measured at 570 nm. The test sample was considered cytotoxic when the optical density of the sample-treated group was less than 70% of that in the control group.

$$\text{Cytotoxicity (\%)} = [(OD_{control} - OD_{sample})/OD_{control}] \times 100$$

$OD_{control}$ = Mean of control media (-LPS)

OD_{sample} = Mean of sample (-LPS)

In vitro assay for cytotoxic activity

Cell lines and treatment

The human prostate cancer cell line (PC-3, ATCC CRL-1435) was used in this study. It was cultured in RPMI-1640 medium (GIBCO™) supplement with 10% heated fetal bovine serum, 50 IU/ml penicillin and 50 µg/ml streptomycin and was maintained at 37°C in an incubator with 5% CO_2 and 95% humidity.

Sulforhodamine B (SRB) colorimetic assay[9]

The SRB assay is used for cell density determination, based on the measurement of cellular protein content. The SRB assay remains one of the most widely used methods for in vitro cytotoxicity screening. This assay relies on the ability of SRB to bind with protein components of cells that have been fixed to tissue culture plate by trichloroacetic acid (TCA). The amount of dye extracted from stained cells is directly proportional to cell mass.

According to their growth profiles, the optimal plating densities of PC-3 was determined to be $1x10^3$ cells/well. These cell suspensions 100 µl/well was seed in 96-well microplate and incubate at 37°C in an incubator with 5% CO_2 and 95% humidity to allow for cell attachment. After 24 hours, cells were treated with the extracts. The ethanolic extracts was dissolved in DMSO and the water extract was dissolved in sterile distilled water. The extracts was diluted in medium. That extract was determined as IC_{50} values by preparing as four serial dilutions, final concentrations were 100, 50, 10, 1 µg/ml, respectively. Add 100 µl of each concentration to the plate. The extracts were tested initially against all cancer cell line. The plates were incubated for 72 hours. After that, the media were removed and washed by PBS. The 200 µl of fresh medium was added. The plates were incubated at 37°C in an incubator with 5% CO_2 and 95% humidity for a recovery period of 6 days and cell growth was analyzed using SRB assay to determine the cytotoxicity of the extracts. The cells were fixed by 100 µl of ice-cold 40% TCA and incubated at 4°C for 1 hour. Next, the plates were washed five times with water, the excess water drained off and the plates left to dry in air. The 50 µl of SRB stain was added to each wells and allowed to be in contact with the cell for 30 minutes. They were washed with 1% acetic acid until only dye adhering to the cells is left. After that, the plates were dried and added to 100 µl of Tris base pH 10.5 to each well to solvabilize the dye. The plates were shaken gently for 20 minutes. Read the absorbance at 492 nm. Cell survival were

measured as the percentage of inhibition. Percentage of inhibition was calculated by using the following equation and IC_{50} values were determined graphically. All determinations were carried out in triplicate.

The percentage of inhibition = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$

IC_{50} value is the sample concentration required to inhibit 50% of the cell proliferation and was then calculated from the Prism program to obtain by plotting the percentage of survival versus the concentrations, interpolated by cubic spine. According to National Cancer Institute extracts guidelines with IC_{50} values <20 µg/ml will be considered active[10].

Determination of total phenolic content

Total phenolic content of all extracts was determined using colorimetric measurement[11] by the Folin-Ciocalteu's reagent[12], according to the modified method of Miliauskus 2004[13]. About 10 mg of crude extracts were weighed in centrifuge tubes and diluted in absolute ethanol or distilled water to a concentration of 1 mg/ml. Bit of the extract solution (20 µl) were mixed thoroughly with 20 µl of the Folin-Ciocalteu's reagent and 80 µl of sodium carbonate in 96-well microplates. The samples in the plates were mixed and then allowed to be placed at room temperature for 30 minutes. The absorbance was measure at 765 nm, using microplate reader spectro-photometers. All test were executed in triplicate on any one day, and repeated with freshly prepared samples a further two times (n = 3). Results are expressed as mg GAE/g, which is mg of gallic acid per gram of extract sample, obtained from a calibration curve of gallic acid, standard solution, shown in Fig. 1.

Statistical analysis

All determinations were carried out on three separate occasions, each time in triplicate. The results are reported as mean ± standard error of mean (SEM). Calculations of EC_{50} and IC_{50} values were done using the GraphPad Prism 4.03. The statistical significance was calculated.

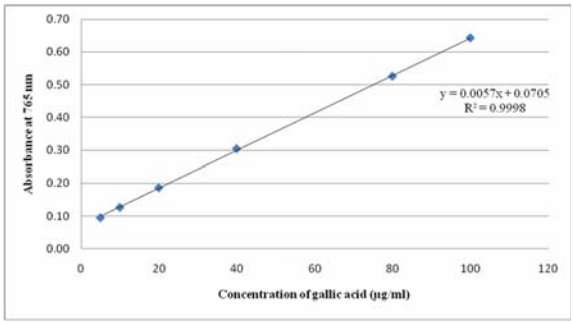

Fig. 1 Linear correlation between a series of gallic acid concentrations (µg/ml) as a standard and absorbance at 765 nm (n = 3).

Results

The percent yield and the total phenolic content of the plant extracts were shown in Table 1 the obtained results indicated that the maceration method gave the highest yield and dried leaves gave higher yield than fresh leaves.

All extracts showed significant amounts of phenolic compound in the range of 14.21±0.54 mgGAE/g to 57.00±3.73 mgGAE/g. The HSDE95 and HSDW contained the highest phenolic content of 57.00±3.73 mgGAE/g and 54.17±0.79 mgGAE/g, respectively.

The results of antioxidant activity showed that the ethanolic extract of *Hibiscus sabdariffa* leaves (HSDE95) demonstrated the highest EC_{50} (EC_{50} = 34.51±2.62 µg/ml), but its EC_{50} was less than BHT which used as a positive control for antioxidant activity (EC_{50} = 15.49±1.27 µg/ml).

The anti-inflammatory activity by NO inhibitory effect and cytotoxicity test in RAW264.7 cells, the results were shown in Table 1 that every plant extracts had no anti-inflammatory activity. In screening test of NO inhibitory effect and cytotoxicity test in RAW264.7 cells, the percentage of inhibition at concentration 100 µg/ml of every plant extract was lower than 50. The cytotoxic effect of all extracts was also determined using the MTT assay. None of the extracts showed any cytotoxicity or more than 70% viable cells were detected.

The cytotoxicity evaluation of all plant extracts as IC_{50} were shown in Table 1. This data showed that none of the water extracts showed any cytotoxic activity (IC_{50} >100 µg/ml). The 95% ethanolic extracts of *Hibiscus sabdariffa* dried leaves showed potent cytotoxicity against prostate cancer cell line with an IC_{50} value of 8.58±0.68 µg/ml.

Discussion

According to these results, the antioxidant activity and total phenolic content were related. The 95% ethanolic extracts of *Hibiscus sabdariffa* dried leaves (HSDE95) showed highest contained phenolic content of 57.00±3.73 mgGAE/g and also demonstrated the highest antioxidant activity with EC_{50} of 34.51±2.62 µg/ml. However, the antioxidant effects of *Hibiscus*

Table 1. Pecentage of yield, antioxidant activity, anti-inflammatory, cytotoxic activities and total phenolic content of *Hibiscus sabdariffa* leaves extracts

Method	Part used	Extract	Code	%Yield	Antioxidant activity* EC_{50}(µg/ml)	Total phenolic content* (mgGAE/g)	Anti-inflammatory** % Inhibition at Conc. 100 µg/ml (% Survival)	Cytotoxic activity* IC_{50} (µg/ml)
Squeez	Fresh leaves	Water	HS	3.85	>100	27.44±1.54	-1.90±0.95 (87.46±7.24)	>100
Boiling with water	Fresh leaves	Water	HSW	6.19	>100	41.75±1.25	0.45±2.71 (94.48±5.53)	>100
	Dry leaves	Water	HSDW	38.27	>100	54.17±0.79	4.15±4.04 (93.59±6.41)	>100
Decoction of residue after maceration (water)	Fresh leaves	Water	RHS50	3.06	>100	25.86±0.73	7.12±5.08 (99.01±0.01)	>100
		Water	RHS95	14.20	>100	14.21±0.54	3.47±1.63 (97.57±2.43)	>100
	Dry leaves	Water	RHSD50	6.52	>100	21.23±0.37	2.73±2.86 (89.40±10.60)	>100
		Water	RHSD95	22.23	>100	26.42±0.93	2.34±4.63 (88.44±11.56)	>100
Maceration (95% and 50% ethanol)	Fresh leaves	EtOH	HSE50	6.29	>100	33.17±3.13	28.55±6.28 (94.89±5.11)	52.12±4.25
		EtOH	HSE95	6.01	64.81±1.26	51.65±3.65	28.74±2.48 (100.00±0.00)	36.87±1.76
	Dry leaves	EtOH	HSDE50	31.70	>100	44.31±1.34	25.31±5.71 (91.56±8.44)	>100
		EtOH	HSDE95	31.88	34.51±2.62	57.00±3.73	35.17±7.06 (91.31±8.70)	8.58±0.68
BHT					15.49±1.27			

* Mean of three measurements ± SEM (n = 3); BHT was used as a positive control for antioxidant activity
** Mean of two measurements ± SEM (n = 2), screening test for anti-inflammatory

sabdariffa leave extracts were not stronger than positive standard. In addition, all extracts of *Hibiscus sabdariffa* leaves had no anti-inflammatory activity in Nitric oxide inhibitory pathway, whereas the 95% ethanolic extracts of *Hibiscus sabdariffa* dried leaves (HSDE95) showed high potent cytotoxicity to against prostate cancer cell line with an IC_{50} value of 8.58±0.68 µg/ml. From these results, it can be classified as "active" according to the NCI guideline[10]. This result was agreed with the previous report, the ethanolic extract of *Hibiscus sabdariffa* dried leaves showed cytotoxicity against prostate cancer independent on hormone LNCaP[6]. Thus, it was concluded that this extract had good cytotoxic activity against both types of prostate cancer, which were independent and hormone dependent. This extract had also the highest antioxidant and high total phenolic content, which was useful for cancer treatment. This result is the first report and it can support using roselle leaves to be food for prostate cancer patients. No water extracts had any cytotoxic and/or antioxidant activities, and had less total phenolic content. Maceration in ethanol showed the highest cytotoxic and antioxidant activities and maceration in 95% ethanol showed higher both activities than in 50% ethanol. The cytotoxic and antioxidant compound should be less polarity compounds. Thus, extraction technique of cytotoxic and antioxidant compound should be maceration in nonpolar solvent. The HSE95 was less cytotoxic activity than HSDE95 for 4.5 times, and HSE95 also less yield than HSDE95 for 5 times which this result related with cytotoxic activity. Thus, the water in roselle leaves makes them decrease in all biological activities.

Conclusion

In conclusion, the optimized method for roselle leaf extraction gives the highest benefit for health; it should be macerated in 95% ethanol. This extract affects the prostate cancer independent or hormone-dependent. In addition, this extract had benefits for the prevention of cancer cells because it had a high content of phenolic compound and antioxidant activity. Its dried leaves also obtained a high yield and high benefit for cancer prevention and cancer patients. This extract should be further investigated for isolated active compounds against prostate cancer.

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Potential conflicts of interest

None.

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การศึกษาฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านการอักเสบและฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งต่อมลูกหมากของสารสกัดจากใบกระเจี๊ยบแดง

ภัสสร วรวัฒนนุทัย, อรุณพร อิฐรัตน์, ศรีโสภา เรืองหนู

ภูมิหลัง: *ใบกระเจี๊ยบแดงเป็นผักที่นิยมใช้ในการประกอบอาหาร เช่น ใช้เป็นส่วนประกอบให้รสเปรี้ยวในแกงส้มซึ่งช่วยในการป้องกันโรคเรื้อรังได้ตามทฤษฎีทางการแพทย์แผนไทย*

วัตถุประสงค์: *เพื่อศึกษาฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านการอักเสบ และฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งของสารสกัดใบกระเจี๊ยบแดงด้วยวิธีการสกัดที่แตกต่างกัน*

วัสดุและวิธีการ: *ใบกระเจี๊ยบแดงสดและแห้ง นำมาสกัดโดยวิธีการสกัดที่แตกต่างกัน เช่น การหมักด้วย 95% และ 50% เอทานอล, การคั้นเอาน้ำสดและต้มกับน้ำ จากนั้นนำสารสกัดทั้งหมดไปทำการทดสอบฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH assay ศึกษาฤทธิ์ต้านการอักเสบโดยการยับยั้งการผลิตไนตริกออกไซด์ในเซลล์ RAW264.7 การศึกษาฤทธิ์ความเป็นพิษต่อเซลล์มะเร็ง โดยทำการทดสอบกับเซลล์มะเร็งต่อมลูกหมากของมนุษย์ (PC-3) ด้วยวิธี Sulforhodamine B (SRB) assay และการศึกษาหาปริมาณสารกลุ่มฟีนอลด้วยวิธี Folin-Ciocalteu's reagent*

ผลการศึกษา: *สารสกัดจากใบกระเจี๊ยบแดงแห้งหมักด้วย 95% เอทานอล (HSDE95) แสดงให้เห็นว่ามีฤทธิ์ต้านอนุมูลอิสระสูงที่สุดโดยมีค่า EC_{50} เท่ากับ 34.51±2.62 μg/ml และมีปริมาณสารกลุ่มฟีนอลสูงสุด (57.00±3.73 mgGAE/g) สารสกัดจากใบกระเจี๊ยบแดงแห้งหมักด้วย 95% เอทานอล ยังแสดงให้เห็นอีกว่ามีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งต่อมลูกหมากโดยมีค่า IC_{50} เท่ากับ 8.58±0.68 μg/ml ในขณะที่สารสกัดจากใบกระเจี๊ยบแดงทุกชนิดไม่มีฤทธิ์ต้านการอักเสบ*

สรุป: *ผลการศึกษาแสดงให้เห็นว่าสารสกัดจากใบกระเจี๊ยบแดงแห้งหมักด้วย 95% เอทานอลมีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งต่อมลูกหมากที่ดีและมีฤทธิ์ต้านอนุมูลอิสระที่ไม่ดีนัก สารสกัดนี้ควรนำไปทำการทดสอบต่อไปในด้านของการแยกสาร เพื่อให้ได้สารบริสุทธิ์และนำไปทดสอบกับเซลล์มะเร็งต่อมลูกหมากต่อไป*