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Anti-Inflammatory and Anticancer Activities of *Hedyotis capitellata* Growing in Vietnam

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Abstract: *Hedyotis capitellata* has widely used in traditional medicine for treatment of toxication, inflammation, diuretic, stomachache. *H. capitellata* has been showed to have antioxidant, anti-inflammatory and antibacterial effects. In this study we presented the *acute and chronic* anti-inflammatory and anticancer activities on HeLa, MCF7 and N87 cells line of *H. capitellata*. Our data showed that *H. capitellata* aqueous total extract has acute and chronic anti-inflammation activity in dose-dependent manner. Ethyl acetate fraction and Water fraction of *H. capitellata* showed strong cytotoxicity on MCF7 cells line with $IC_{50}$of 64.50 µg/ml and 34.38 µg/ml, respectively; but did not have effect on cervical cancer cell line (HeLa) and stomach cancer cell line (N87).

Key words: *Hedyotis capitellata* · Anti-Inflammation · Cytotoxicity

INTRODUCTION

Hedyotis capitellata Wall Ex G. Don are growing mostly in midland and mountainous provinces in Vietnam. This plant is easy to cultivate, extract, process and use. *H. capitellata*is well known in traditional medicine for treatment of toxication, pain, inflammation, phlegm, diuretic, hemostasis, stomachache, mouth sore, tongue sore, throat sore, skin ulcer and pimple. *H. capitellata* has been showed to have antioxidant, anti-inflammatory and antibacterial effects [1, 2]. Qian has demonstrated the anti-inflammatory activity of *H. capitellata* related to NO inhibition [3]. The results also showed that *H. capitellata* could inhibit the NO synthaseSeveral studies have shown that *H. capitellata* contains alkaloid, saponin, tanninand anthraquinones [4, 5]. Although *H. capitellata* is used for clinical treatment, in-depth studies on biological activity of *H. capitellata* leaves are limited. This study reported the anti-inflammatory and anticancer activities of *H. capitellata*.

MATERIALS AND METHODS

Plants: The samples of *H. capitellata* were collected in August 2015 in Thai Nguyen province. The leaves were dried and stored in sealed plastic bags. A voucher specimen (SMP-04) wasidentified as*Hedyotis capitellata* Wall. Ex G. Don (family *Rubiaceae*).

Method of Extraction and Isolation: The leaves of *H. capitellta* were collected, rinsed and dried at 50°C, then 2.2kg dry leaf power was extracted with 8L water at room temperature for 48 hours for 5 times. The extracts were filtered, combined and evaporated under low pressure to afford the aqueous extract (205g) (HC-total extraction). The aqueous extract was suspended in water and successively partitioned with n-hexane, chloroform and ethyl acetate. Combined solvent was then evaporated under low pressure to obtain the n- hexane fraction (HC-Hex, 18g), chloroform fraction (HC-CCl_3, 25g), ethyl acetate fraction (HC-EtOAc, 62 g) and water fraction (HC-Water, 22g).

Regents:

- Aspirin package 500mg (Aspegic) (Sanofi-Synthelabo Vietnam).
- Methylprednisolone tablet 4mg (Medrol) (Pfizer, Italy).
- Paracetamol effervescent tablet 500mg (Efferalgan) (Mayers Squibb, France).
- Carrageenan (Carrageenan sodium salt and naturally occurring polysaccharide) (BDH Chemicals Ltd. Poole England). Batch number: 6149710, content = 99%.
- D-Mannitol PA Bio Basic, Canada. Batch number: 130802S335, content: 98-102%.

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- Ascorbic acid PA Prolabo. Batch number: J320, content: 98-102%.
- Deoxyribose PA Sigma, USA. Batch number: BCBL0604V, content: 98-102%.
- TBA (2-thiobarbituric acid) PA Sigma, USA. Batch number: BCBK7728V, content: 99.5%.
- TCA (Trichloroacetic acid) PA Scharlau, Spain. Batch number: AC31320250, content = 99%.
- Taxol PA Tocris Bioscience.
- Cell culture medium: cell lines were cultivated in DMEM or RPMI medium supplemented 10% fetal bovine serum and 1% penicilin/streptomycin.
- Cell Titer 96 Non-Radioactive Cell Proliferation Assay Kit, MTS solution, PMS solution (Promega)

Instruments:

- WNB14 Water heater thermostat (Memmert, Germany).
- ELMA S 180/HUltrasonic cleaning tank (Germany).
- AB-265S Analytical balance (Mettler Toledo, Swiss) (d=0, 00001g).
- XS-204Analytical balance (Mettler Toledo, Swiss) (d=0, 0001g).
- Leica Microscope, KrussStereo microscope connected to Canon S40 Digital camera (Germany).
- Kika rotary vacuum machine (Germany).
- SevenCompactS220 pH meter(Mettler Toledo, Swiss).
- XC-55Semi-automatic biochemistryanalyzer (Italy).
- Stomacher® 80 homogenizer machine (UK).
- ELISAspectrograph machine(Bio-Rad, US).
- Shellab1430D-2Evacuum drying cabinet (Germany).
- UNB 400Memmert vacuum cabinet(Germany).
- Suction blast cabinet, Cell counter, Incubator5%CO2.

Animals:

- Swiss mice were purchased from National Institute of Hygiene And Epidemiology (Vietnam). Mice of either sex weighting 20g were maintained in standard conditions in School of Medicine and Pharmacy, Vietnam National University, Ha Noi 5 days during the study.
- Cancer cells lines:

Cervical cancer cell line (HeLa): ATCC-CCL-2
Breast cancer cell line (MCF7):ATCC-HTB-22
Stomach cancer cell line (NCI-N87):ATCC-CRL-5822

Methods

Determination of Chronic Anti-Inflammation Activity: According to Carrageenan-induced mouse paw edema test by Winter *et al*. (6), mice were divided into 4 groups with 10 mice of each group.

- Group 1 (Control) was given orally 0.2ml/10g distilled water.
- Group 2 was treated orally with 200 mg/kg body weight of aspirin.
- Group 3 was treated orally with 7.2 g/kg body weight of HC total extraction.
- Group 4 was treated orally with 21.6 g/kg body weight of HC total extraction.

Mice were treated orally for 4 days. After 1 hour of the fourth day, paw edema was induced using 0.5 ml of 1% carrageenan sodium salt. Carrageenan was injected into the right hind foot of each mouse under the plantar aponeurosis.

Measurement of foot volume was performed by Plethysmometer at different times: before (V0) and after 2 h (V2), 4 h (V4), 6 h (V6) the injection of Carrageenan.

+ The percentage of increasing in paw volume was calculated using the formula:

$$\Delta V\% = \frac{V_t - V_o}{V_o} x100$$

where,
Vt: the paw volume before Carrageenan injection.
Vo: the paw volume after Carrageenan injection.
+Level of inhibitory activity was calculated using the following formula:

$$1\% = \frac{\Delta \overline{V}_c\% - \Delta \overline{V}_t\%}{\Delta \overline{V}_c\%} x100$$

where,
$\Delta \overline{V}_c\%$: the average percentage of increasing in paw volume of control mice.
$\Delta \overline{V}_t\%$: the average percentage of increasing in paw volume of treated mice.
I%: Level of inhibitory activity

Determination of Acute Anti-Inflammation Activity: Acute anti-inflammation was determinedas described previously by Belman *et al.* (7). Mice of either sex weighting 20g were divided into 4 groups, 10 mice of each group.

- Group 1 (Control) was given orally 0.2ml/10g distilled water.
- Group 2 was treated orally with 10 mg/kg body weight of methylprednisolone.
- Group 3 was treated orally with 7.2 g/kg body weight of HC-total extraction.
- Group 4 was treated orally with 21.6 g/kg body weight of HC-total extraction.

Samples were diluted in distilled water. The volume was 0.2 ml/10 g body weight. The asbestos fibers weighting 6 mg/fiber (dry at 120°C in 1 hour) were soaked in 1% carrageenan. Acute inflammation was caused by implanting asbestos under the skin of the nape of each mouse. Mice were given orally distilled water or treatment during 7 days. In the eighth day, mice were killed to remove granulomas. The remaining granulomas were dried at 56°C in 18 hours and weighted.

The percentage of inhibition of granuloma was calculated using the following formula:

$$I(\%) = \frac{T_C - T_t}{T_C} x100$$

where:

T_c: The average weight of controlled mice (mg)

T_t: The average weight of treated mice (mg)

I: The percentage of inhibition of granulomas(%)

Determination of Cytotoxicity on Cancer Cell for HC-EtOAc and HC-Water Fraction: *In vitro* cytotoxicity MTS assay using reagent 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoliumwas conducted in HeLa, MCF7 and N87 cells line.

Principles: MTS, in the presence of phenazinemethosulphate (PMS), produces a formazan product that has an absorbance maximum at 490 nm. The quantity of formazan (presumably directly proportional to the number of viable cells) can be measured by the absorbance at 490 nm. Using this reagent is highly convenient because it is added to the cell culture medium without any washing step or other preparation (8).

Experimental Procedures:

- Cells Preparation

Activation of cells from liquid nitrogen: cells stored in liquid nitrogen (-196°C) were defrosted by 37°C water heater thermostat. Cells were dispersed in cell culture (7 ml medium) in 15 ml falcon tube and centrifuged 1000 rpm for 5 minutes. Cells were dispersed in culture medium supplemented with 20% fetal bovine serum and incubated at 37°C with 5% CO_2. Cultivated cells were used after surfacing 75-90% cell culture. Trysin was used to separate cells from plate. Number of cells was measured by Thoma cell counter.

- Plating cells in 96 well plates: Activated cells were plating in 96 well plates (180 µl medium) with 5000-7000 cells of each plate. The plates were incubated for 24 hours in 37°C with 5% CO_2
- Incubating with reagent: After 24 hours, each plate was added 20 µl sample. Drug was distributed in plates. The plates were incubated for 48 hours in 37°C with 5% CO_2. Samples and positive controls were weighted separately and diluted in DMSO. The concentrations of solutions were shown in Table 1.
- Incubating MTS and measuring optical density:

Each plate containing 200 µl medium solutions was added 30 µl MTS/PMS solution at the ratio of 20:1. The plates were incubated for 3 hours at 37°C with 5% CO_2. Optical density was measured by the absorbance at 490 nm by ELISA spectrograph machine. The OD values andconcentration response curve were processed by GraphPad Prism 5.0statistical software.

Cell proliferative index A% was calculated using the following formula:

$$A(\%) = \frac{T}{VH} x100$$

where,

A : Cell proliferative index(%).

VH : The average value of optical density in plate (solvent)

T : The average value of optical density in plate (active element)

IC50 index was calculated as the solution of the regression equation of (proliferative index A%) on x (dose or log dose of reagent).

The American National Cancer Institute guidelines (NCI) set IC_{50} of crude extract (9).

- IC_{50}<100µg/ml is considered to have inhibitory activity on cancer cell.
- $IC_{50}\leq$ 20µg/ml is considered to have strong inhibitory activity.

Pure extract with $IC_{50}\leq$ 5 µg/ml is considered to have inhibitory activity.

Table 1: Sample and Taxol concentration

Concentration (µg/ml)	C1	C2	C3	C4	C5	C6	C7
HC-EtOAc fraction concentration(µg/ml)	5000	2500	1250	625	312, 5	156, 25	78, 125
HC-Water fraction fractionconcentration(µg/ml)	1000	500	250	125	62, 5	31, 25	15, 625
Taxol(positive control)	300	30	3	0, 3	0, 03		

RESULTS

Chronic Anti-Inflammatory Activity: The results of chronic anti-inflammatory activity were shown in Table 2.

From Table 2, we observed that:

Aspirin 200 mg/kg have chronic anti-inflammatory effect at all times of study, mostly after 2 h ($p < 0.001$) and 4 h ($p < 0.05$) after the injection of Carrageenan.

Low dose HC-total extraction (7.2 g/kg body weight) reduced paw edema after 2 h ($p < 0.05$), but not statistically significant after 4 h ($p > 0.05$). After 6 h, low dose HC-total extraction did not have this effect.

High dose HC-total extraction (21.6 g/kg body weight) reduced paw edema at all times of study, mostly after 2 h, 4 h ($p <0.01$) and 6 h ($p < 0.05$) after the injection of carrageenan. This effect after 4 h and 6 h was strong when compared to aspirin 200 mg/kg, but not statistically significant ($p>0.05$).

Acute Anti-Inflammatory Activity: The results of acute anti-inflammatory activity were shown in Table 3.

From Table 3, we observed that:

A significant decrease in granuloma weight was observed in group 2 when compared to control group ($p < 0.05$).

Low dose HC-total extraction (7.2 g/kg body weight) showed significant decrease in granuloma weight when compared to control group and caused effect on acute anti-inflammation ($p <0.05$). This effect was equivalent to the one of methylprednisolone 10 mg/kg ($p > 0.05$).

High dose HC-total extraction (21.6 g/kg body weight) also showed significant decrease in granuloma weight when compared to control group and caused effect on acute anti-inflammation ($p < 0.001$). This effect was strong when compared to methylprednisolone 10 mg/kg ($p < 0.05$).

The result of microscopic pathology was showed in Table 4 and Fig. 1.

Table 2: H. capitellata aqueous extract effect on chronic anti-inflammation

	2h(V_1)		4h(V_2)		6h(V_3)	
Group	Edema (%)	Decreased edema compared to control (%)	Edema (%)	Decreased edema compared to control (%)	Edema (%)	Decreased edema compared to control (%)
Group 1 (control)	60, 14± 15, 91	42, 01± 6, 68	60, 14± 15, 91			
Group 2 (aspirin: 200mg/kg)	35, 00± 9, 45		37, 00± 3, 87		35, 00± 9, 45	
	p1-2 = 0, 0004	42, 15	p1-2 = 0, 041	12, 82	p1-2 = 0, 0004	42, 15
Group 3(HC-total extraction: 7.2 g/kg)	43, 74± 12, 03		39, 38± 9, 10		43, 74± 12, 03	
	p1-3= 0, 018	27, 27	p1-3 = 0, 471	6, 26	p1-3 = 0, 018	27, 27
Group4 (HC-total extraction: 21.6 g/kg)	41, 51± 10, 63		32, 05± 7, 90		41, 51± 10, 63	
	p1-4= 0, 009		p_{1-4}= 0, 008		p_{1-4}= 0, 009	
	p2-4= 0, 163	30, 97	p_{2-4}= 0, 121	23, 72	p_{2-4}= 0, 163	30, 97

Table 3: H. capitellata aqueous extract effect on granuloma weight

Group	Granuloma weight(mg)	p compare to group 1	P compare to group 2	Percentage of inhibition(%)
Group 1 (control)	51, 45± 10, 66			
Group 2 (methylprednisolone: 10 mg/kg)	41, 18± 11, 63	0, 043		19, 96
Group 3(HC-total extraction : 7.2 g/kg)	36, 90± 9, 17	0, 003	0, 364	28, 29
Group4 (HC-total extraction: 21.6 g/kg)	30, 11± 7, 82	0, 0001	0, 026	41, 48

Table 4: The result of microscopic pathology

Group	The result of microscopic pathology
Group 1 (control)	The central zone of abscess contains polymorphonuclear leukocytes, necrosis and degenerated tissues. Abscess wall contains inflammated fibrous tissues. The outer zone contains adipose tissues and inflammated fibrous tissues.
Group 2 (methylprednisolone: 10 mg/kg)	The central zone of abscess contains polymorphonuclear leukocytes and necrosis. Abscess wall contains fibrous tissues and polymorphousnuclear leukocytes. The outer zone contains adipose tissues.
Group 3(HC-total extraction: 7.2 g/kg)	The central zone of abscess contains polymorphonuclear leukocytes and scattered necrosis. Abscess wall contains fibrous tissues and polymorphous nuclear leukocytes. The outer zone contains adipose tissue of less inflammatory cell infiltration.
Group4 (HC-total extraction: 21.6 g/kg)	The central zone of abscess contains polymorphous nuclear leukocytes and necrosis. Abscess wall contains fibrous tissue including less polymorphous nuclear leukocytes. The outer area contains none of inflammatory aditope tissues and fibrous tissues.

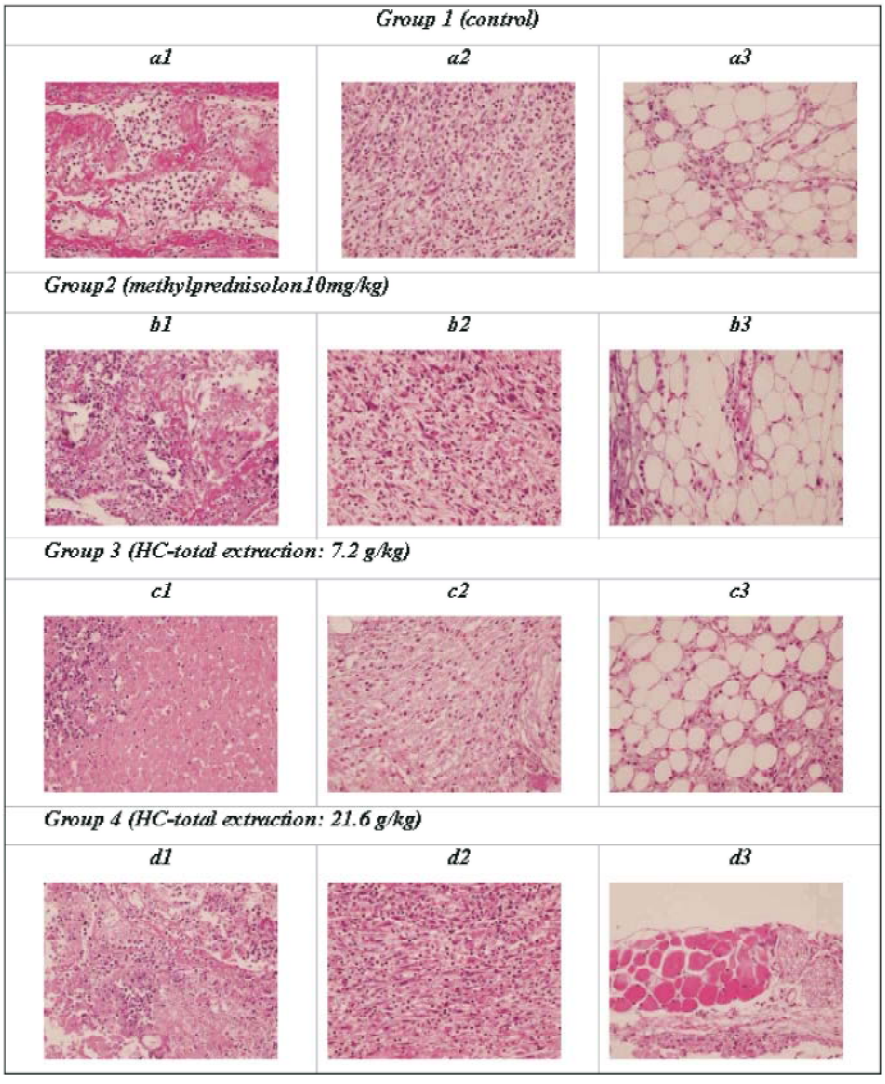

Fig. 1: Image of microscopic pathology in acute inflammation

a_1)Necrotic abscess, polymorphonuclear leukocyte; a_2)Granular abscess; a_3)Adipose tissue inflammation; b_1)Necrotic abscess; b_2)Abscess wall; b_3)Inflammatory infiltration in the outer area; c_1)Necrotic abscess; c_2)Abscess wall contains macrophages; c_3) Adipose tissue inflammation; d_1)Abscess; d_2) Abscess wall contains fibruos tissue; d_3) The outer area contains none of inflammatory cells

Cancer Cell Line Cytotoxic Activity: Concentration response curve of sample towards three cancer cell lines (Fig. 2).

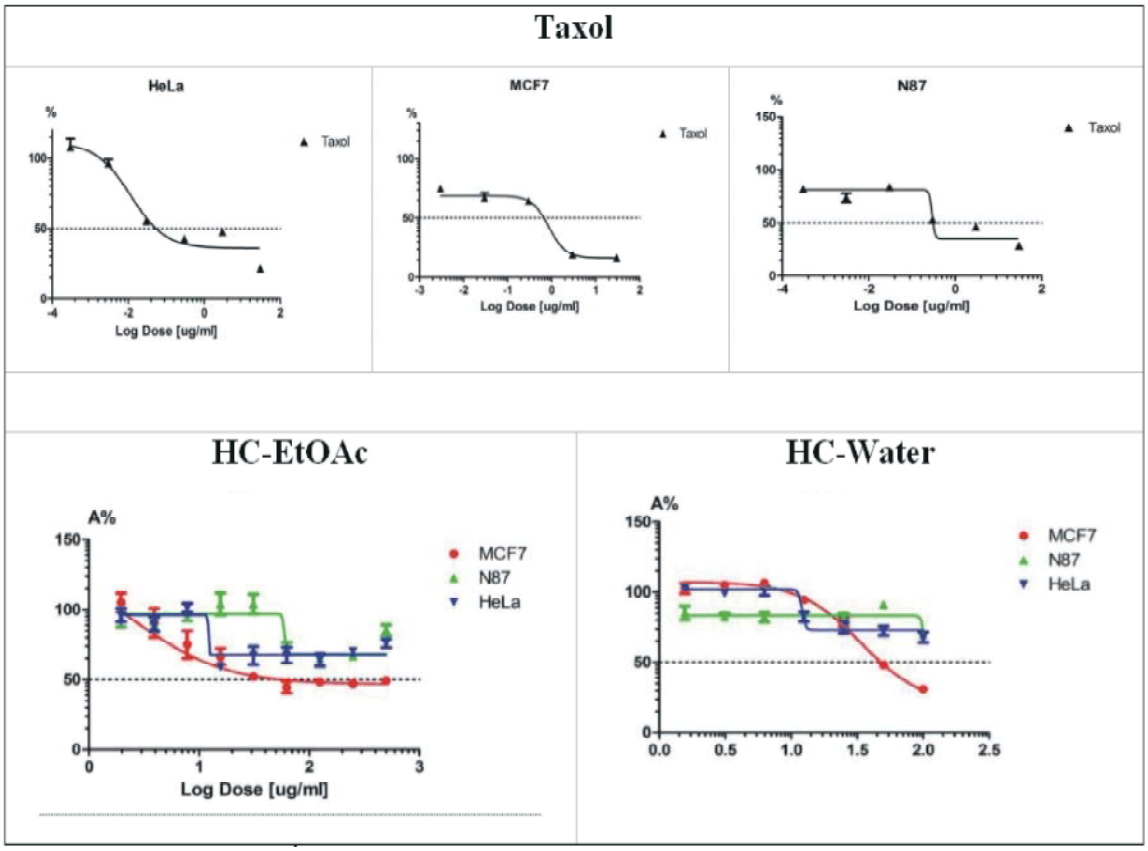

Fig. 2: Concentration response curve of three cancer cell lines

IC_{50} values (the concentration required for a 50% inhibition of viability) were determined inTable 5.

Table 5.: IC50 value of samples and taxol positive controls

	HeLa (µg/ml)	MCF7 (µg/ml)	N87 (µg/ml)
HC-EtOAc fraction fraction	>500 µg/ml	64, 50±3, 70	>500 µg/ml
HC-Water fraction	>500 µg/ml	34, 38±1, 80	>500 µg/ml
Taxol	0, 011±0, 004	0, 59±0, 04	0, 29±0, 02

The results showed that IC_{50} values of two samples towards MCF7 (Breast cancer cell line) were determined, but not to HeLa (cervical cancer cell line) and N87 (Stomatch cancer cell line) due to concentration > 500 µg/ml. HC-EtOAc fraction with IC50 of 64.50 µg/ml and HC-Water fraction water fraction with IC50 of 34.38 µg/ml inhibit cancer cell. According to NCI guidelines, HC-EtOAc fraction inhibited 50% amount of cells at concentration <100µg/ml. Thus, we can use this fraction to isolate active compounds for inhibiting cancer cells.

DISCUSSION

Anti-Inflammatory Activity: According to Carrageenan-induced mouse paw edema test, *H. capitellata* aqueous extract at dose of 7.2 g/kg and 21/6 g/kg has chronic anti-inflammatory effect on mice. Aqueous extract reduced paw edema after 2 h ($p < 0.05$), then decreased after 4 h ($p > 0.05$) and ineffective after 6 h. *H. capitellata* aqueous extract reduced paw edema at all times of study, mostly after 2 h, 4 h ($p < 0.01$) and 6 h ($p < 0.05$). This effect after 4 h and 6 h was strong when compared to aspirin 200 mg/kg, but not statistically significant ($p>0.05$).

According to chronic anti-inflammatory assay, both *H. capitellata* aqueous extract 7.2 g/kg and 21/6 g/kg have chronic anti-inflammatory effect. The effect of aqueous extract 7.2 g/kg was equivalent to the one of methylprednisolone 10 mg/kg ($p > 0.05$). The effect of aqueous extract 21.6 g/kg was strong as compared tomethylprednisolone 10mg/kg ($p<0, 05$).

Anticancer Effect: In this study, we chose 2 samples ethyl acetate fraction and water fraction of *H. capitellata* towards 3 cancer cells lines, including HeLa, MCF7 and N87. Both samples ethyl acetate fraction and water fraction had effect on MCF7 with IC_{50} of 64.5 µg/ml and 34.38 µg/ml, respectively. According to NCI guidelines (10), HC-EtOAc fraction was choosing for further studies.

CONCLUSIONS

H. capitellata aqueous extract has acute and chronic anti-inflammation activity in dose-dependent manner. Ethyl acetate fraction and water fraction of *H. capitellata* showed strong cytotoxicity on MCF7 cells line with IC_{50} of 64.50µg/ml and 34.38 µg/ml, respectively; but did not have effect on cervical cancer cell line (HeLa) and stomach cancer cell line (N87).

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