HIỆU QUẢ BẢO VỆ GAN CỦA THÀNH PHẦN ĐƯỢC PHÂN LẬP TỪ *POLYGONUM TOMENTOSUM* CHỐNG LẠI CHẤT ĐỘC CARBON TETRACHLORIDE GÂY TỔN THƯƠNG GAN

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TÓM TẮT: *Phân đoạn ethyl acetat của cây Nghể Polygonum tomentosum Willd. được tách phân đoạn bằng phương pháp sắc ký cột chân không, kết quả thu được 11 phân đoạn và được ký hiệu từ PTE1 → PTE11. Đánh giá tác dụng chống oxy hóa của 11 phân đoạn trên bản sắc ký lớp mỏng cho thấy các phân đoạn của cây Nghể đều có hoạt tính chống oxy hóa mạnh. Kết quả sàng lọc tác dụng bảo vệ gan ex vivo của các phân đoạn cho thấy PTE7, PTE8 và PTE9 có hiệu quả bảo vệ gan cao nhất so với 8 phân đoạn còn lại. Phân đoạn PTE8 được tiến hành sắc ký cột thu được hợp chất tinh khiết A1 được xác định là quercitrin. Đây là chất đầu tiên được công bố là thành phần của P. tomentosum. Quercitrin có hiệu quả bảo vệ gan trên cả hai mô hình ex vivo và in vivo của tế bào gan chuột bị tổn thương bởi carbon tetracloride (CCl_4). Những nghiên cứu trên góp phần chứng minh hoạt tính sinh học bảo vệ gan của P. tomentosum.*

Từ khóa: *Polygonum tomentosum, hoạt tính bảo vệ gan, DPPH, CCl_4, quercitrin.*

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HEPATOPROTECTIVE EFFECT OF ISOLATED CONSTITUENT FROM *POLYGONUM TOMENTOSUM* AGAINST CARBON TETRACHLORIDE INDUCED TOXICITY

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ABSTRACT: *The ethyl acetate fraction (Et-F) of Polygonum tomentosum Willd was separated by silica gel vacuum chromatography to give 11 subfractions (PTE1-PTE11). The antioxidative activity of subfractions was determined by qualitative assay by TLC assays. The result of qualitative assay showed that all 11 subfractions had antioxidative activity. The results of ex vivo hepatoprotection of 11 subfractions showed that PTE7, PTE8 and PTE9 had higher ex vivo hepatoprotective activities than those of the other subfractions. PTE8 was further studied by silica gel chromatography to obtain A1 compound, which was identified as quercitrin. (To the best of our knowledge) This is the first report on the occurrence of quercitrin in P. tomentosum. Quercitrin had signifying both ex vivo and in vivo hepatoprotective effects on injury liver mice induced by carbon tetracloride (CCl4). The results in the present study indicated that P. tomentosum is a potential source of natural hepatoprotection*

Keywords: *Polygonum tomentosum, antioxidative activity, DPPH, CCl4, hepatoprotective effect, quercitrin.*

INTRODUCTION

Liver, an important organ actively related to metabolism, secretion and storage has a great capacity to detoxicate toxic substances. Therefore, most of hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damage [2]. Lipid peroxidative process has been shown to augment collagen synthesis and fibrosis [3]. In the background of the above, it is realized that antioxidative activity which inhibits genaration of free radicals plays a crucial role in providing protection against such damage. The antioxidative effect is mainly due to phenolic compound. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from ageing-related diseases has intrigued scientists for a long time [10].

Herbs belonging to *Polygonum* species have been long used internally as antihaemorrhoidal, astringent and antirheumatic agents [8], to reduce liver discomfort and to soothe inflammation [1], etc. Phenolic compounds,

such as flavonoids, phenolic acids, stilbenes, lignans and tannins have multiple biological effects including antioxidative activity [5], [10]. In the previous paper, we have reported antioxidative and hepatoprotective activities of fractions of *Polygonum tomentosum* Willd, the result showed Et-F fraction had strongest effects [9]. In the present study, we further studied hepatoprotection of subfractions and isolated compound from Et-F fraction of *P. tomentosum.*

MATERIALS AND METHODS

Chemicals

Type I collagenase was purchased from Gibco, dimethyl sulfoxide (DMSO), trypan blue, were obtained from Merck. Bovine serum albumin (BSA), Eagle's minimum essential medium, Fetal bovine serum, dexamethasone, insulin, penicillin, streptomycine, silymarin, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma and kit of alanine aminotransferase (ALT) from Diagnosticum Zrt, L(+)-ascorbic acid from Scharlau. All other chemicals were of analytical grade.

Animals

Male Swiss albino mice weighing 20-25g (6-8 weeks old), were provided by Nha Trang Pasteur Institute (Nha trang, Vietnam).

Plant materials

Aerial parts of *P. tomentosum* were collected freshly from Long An province, Vietnam. Sample was identified by comparison its botanical characteristics with those discribed in literatures. Voucher was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy Ho Chi Minh City. The sample was dried in shade and ground into coarse powder.

Five kilogram of powdered material was macerated with 90 % aqueous alcohol for 24 h and filtered. The extract was concentrated under reduced pressure to get aqueous extract. This extract was suspended in the water and partitioned different solvents in the increasing order of their polarity with cloroform, ethyl acetat and n-buthanol successively to obtained chloroform, ethyl acetat (Et-F) and n-buthanol fractions, respectively. Et-F fraction showes strong antioxidant and hepatoprotective activities and subjected to silica gel column vacuum chromatography and elution with EtOAc in $CHCl_3$ and MeOH to give 11 subfractions (PTE1- PTE11). These fractions were used to test for their antioxidative and *ex vivo* hepatoprotective activities.

Antioxidant TLC assays (Qualitative assay)

Samples were applied on a TLC plate (the amount of sample approximately10μg in every spot) and sprayed with 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution. Yellow spots against a purple background indicated the antioxidant activity.

Hepatocyte isolation and ex vivo hepatoprotection

Liver cells were isolated by using a modified procedure of that of Kiso *et al* [7]. The mouse was cleaned thoroughly using rectified alcohol, then anaesthetized with ether. Dissection of the mouse was carried out using sterilized instruments. A midline incision was made on

the abdomen, superior vena cava was tied off and inferior vena cava was cut below the renal vein. The portal vein was canulated with needle connected to an infusion set. Perfusion of the liver was started immediately with PBS solution. When the liver was thoroughly perfused (liver has turned white), the flow of PBS was stopped and the needle was removed. The liver was transferred to a beaker containing 0.075% collagenase in PBS and shaked 100 rpm for 5' at 37°C then gently dispersed with two forcep. The cell suspension was shaked again 100 rpm for 10' at 37°C then cooled for 15' at 8-16°C, and filtered gently through cotton gauze into centrifuge tube. The preparation was centrifuged at 1000 rpm for 10'. The supernatant was removed and the pellet of cells was suspended in the Ca^{2+} free Hank's buffer. The cells were washed 3 to 5 times and counted in the presence of trypan blue dye. Viability of the cells in each of the experiment was found to be greater than 90%. The isolated hepatocytes were incubated (2 hr) in Eagle's MEM supplement with fetal bovine serum (10%), gentamycin (50 µg/L), dexamethasone (10^{-6}M) and insulin (10^{-8}M), DMSO (1%) at density of 0.75×10^{6} cells/ml in sterile disposable culture bottles and incubated in a humified incubator at 37 ^{o}C under 5% CO_2.

After incubation, the hepatocytes were exposed to medium containing 1.5% CCl_4 with or without sample to be tested for determining the hepatoprotective activity. After the exposure to CCl_4 for 45' the culture medium was collected. ALT concentration in the medium was measured as an indicator of hepatocyte injury.

In vivo hepatoprotection

Liver injury was induced by CCl_4 in mice by using a modified procedure of that of Hostettmann [4]. Hepatoprotective activity of isolated compound from *P. tomentosum* was carried out against CCl_4. Male mice were divided into six groups of six animals each. Group 1 served as vehicle control was administered with olive oil and 1% DMSO. Group 2 received 25% CCl_4 solution in olive oil and 1% DMSO. Group 3 and 4, male mice received 25% CCl_4 and treated with isolated compound from *P. tomentosum* in 1% DMSO (1.6 and 8.0 mg/kg), concomitantly. Group 5 and 6, mice received 25% CCl_4 solution and treated with silymarin in DMSO 1% (1.6 and 8.0 mg/kg). Blood was collected from the tail in all animals 24 h after last treatment and serum separated for testing ALT enzyme.

Statistical analysis

Results were expressed as mean ± S.D. The statistical significance of the difference was analysed through one way analysis of variance (ANOVA). The difference between the test group and control was determined by least significant difference method at $p<0.05$ confidence levels

RESULTS AND DISCUSSION

Antioxidant TLC assays

Antioxidant activity of subfraction was discovered by running a TLC plate with these samples. The plates were dried, sprayed with a

0.2% DPPH· solution in methanol. Antioxidant compounds of all 11 subfractions appeared as yellow spots against a purple background. The result showed that all of 11 subfractions possessed antioxidative activity.

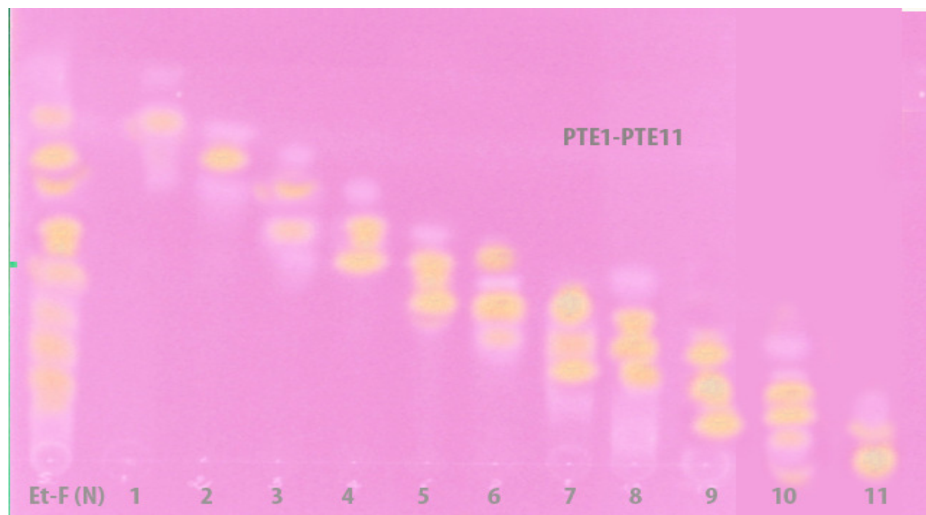

Figure 1.Antioxidants of 11 subfractions (PTE1-PTE11) are identified by antioxidant TLC assay

Ex vivo hepatoprotection

The treatment of the cells with different concentrations of fractions were examined, as result, most of fractions had a strongest hepatoprotective effect in concentration of 0.5 mg/ml (reported in the other paper). Therefore, subfractions of Et-F were also tested at concentration of 0.5 mg/ml. The ALT activity in the culture medium was measured as an indicator of hepatocyte injury after 45 minutes of hepatocyte incubation and the medium ALT activity increased 344% when treating with 1.5% CCl_4. Hepatoprotection of different subfractions of *P. tomentosum* in the presence of 1.5% CCl_4 gave the results shown in Table 1. PTE1-PTE6 showed weak protection effect against CCl_4 damage on hepatocytes with ALT activity were decreased 21% to 35% compared to toxic group (control group treated with CCl_4). PTE7, PTE8 and PT9 provided the strongest hepatocyte protection when compare to the other subfractions, ALT activity were decreased 50%, 43% and 57%, respectively.

The protection effect of 11 subfractions was found comparable to that of Et-F. Et-F reduced ALT activity was 68% compared to toxic group. Et-F possessed a stronger protective effect than that of 11 subfractions and Et-F continuously provided the strongest hepatocyte protection against the cytotoxicity of CCl_4.

Table 1. Effects of 11 subfractions and Et-F against *ex vivo* CCl_4 induced hepatocyte injury

Group	Sample conc. (mg/ml)	ALT (U/L)
Control	-	**68 ± 5** ^{a}
Control + CCl_4	-	234 ± 13 ^{i}
PTE1 + CCl_4	0.5	186 ± 8 ^{h}

PTE2 + CCl_4	0.5	178 ± 5 [gh]
PTE3 + CCl_4	0.5	171 ± 6 [g]
PTE4 + CCl_4	0.5	158 ± 6 [f]
PTE5 + CCl_4	0.5	151 ± 4 [ef]
PTE6 + CCl_4	0.5	155 ± 4 [f]
PTE7 + CCl_4	0.5	117 ± 10 [c]
PTE8 + CCl_4	0.5	133 ± 12 [d]
PTE9 + CCl_4	0.5	**101 ± 9 [b]**
PTE10 + CCl_4	0.5	137 ± 5 [d]
PTE11 + CCl_4	0.5	140 ± 9 [de]
A1 compound	0.5	86 ± 10 [ab]
Et-F + CCl_4	0.5	**75 ± 9 [a]**

(Values were mean ± SD of 3 replicates, values within the column of same concentration with the different superscript letters were significantly different at $p<0.01$).

In above assays, PTE7, PTE8 and PTE9 of *P. tomentosum* had higher *ex vivo* hepatoprotective activities than those of the other subfractions. PTE7 and PTE9 were further studied for isolating pure compounds which were reported in the other paper. In the present study, PTE8 was further studied by silica gel chromatography. The result of column chromatography was that A1 compound isolated from PTE8 is a yellow powder. A1 coumpound had signifying hepatoprotective effect the same as that of Et-F ($p>0.05$).

Identification of A1

The IR spectrum showed important absorptions attributable to OH (3420,42 cm^{-1}), C-H (2933,86 cm^{-1}), C=O (1656,78 cm^{-1}), C=C (1604,95 and 1506,50 cm^{-1}) and C-O-C (1060,90 cm^{-1}).

MS: Negative ES-MS gave a $[M-H]^-$ ion at m/z = 447 corresponding to the molecular mass of 448. ^{13}C-NMR of A1 showed signals that the compound contained 21C that were 10 quaternary C, 10 methine C and a methyl C.

^{13}C-NMR spectrum (125 MHz, MeOH-d_3): δ 179,6 (*s,* C-*4*); 165,8 (*s,* C-7); 163,2 (*s* , C-5); 159,5 (*s*, C-2); 158,5 (*s*, C-2); 158,5 (*s*, C-9); 149,7 (*s*, C-4');146,4 *s*, C-3');136,2 (*s*, C-3); 122,8 (*d*, C-6');116,9 (*d,* C-2');123,0 (*s,* C-1');116,3 (*d*, C-5'); 105,9 (*s*, C-10); 103,5 (*d,* C-1"); 99,8 (*d,* C-6); 94,7 (*d,* C-8); 71,9 (*d,* C-2"); 73,3 (*d,* C-4"); 72,1 (*d;* C-3"); 72,0 (*d*, C-5"); 17,6 (*t,* C-6"). ^{1}H-NMR spectrum (500 MHz, MeOH-d_3): 7,36 (1H, d, $J_{2'-6'}$ = 2, H-2'); 7,33 (1H, dd, $J_{6'-5'}$ = 8,0, H-6'); 6,93 (d, 1H, H-5'); 6,39 (1H, d, J_{8-6} = 2,5, H-8); 6,22 (1H, d, H-6); 5,38 (1H, d, $J_{1''-2''}$ = 1,5, H-1"); 4,24 (1H, dd, $J_{2''-3''}$ = 3,2, H-2"); 3,77 (1H, dd, $J_{3''-4''}$ = 9,2, H-3"); 3,44 (1H, dq, $J_{5''-6''}$ = 6,0, H-5"); 3,34 (1H,dd, $J_{4''-5''}$ = 9,5, H-4"); 0,97 (3H,d, H-6")

On the basis of the spectroscopic data, including MS, ^{1}H-NMR and ^{13}C-NMR in above spectrums, A1 was idetified as a flavonoid compound named quercetin-3-*O*-*α*-L-rhamnopyranoside or quercitrin (figure 2). Quercitrin is known as a strong antioxidant in several plants. This is the first report on the occurrence of quercitrin in *P. Tomentosum.*

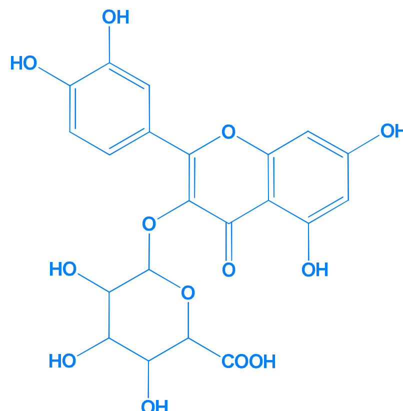

Figure 2. Structure of quercitrin isolated from PTE8

In vivo hepatoprotection

CCl_4 is one of the most powerful hepatotoxin in experimental hepatopathy. At 24 h after administration of CCl_4 induced acute liver damage, ALT activity, marker of hepatic injury in plasma was compared with control mice (Figure 3.)

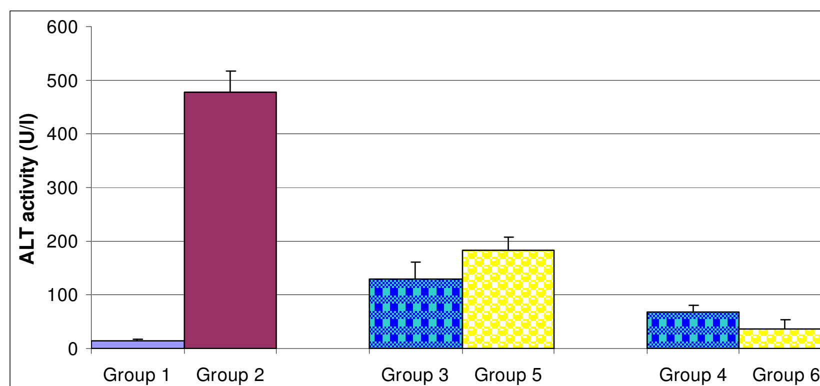

Figure 3. Effect of quercitrin and silymarin on ALT activity in plasma of CCl_4 treated mice

Blank control - Normal mice administered olive oil (group 1): 14 ± 4^{a} U/l.

Toxic control – Mice were treated with CCl_4 (group 2): 478 ± 38^{d} U/l

Quercitrin groups – Mice were digested CCl_4 and treated with quercitrin at doses of 1.6 and 8 mg/kg (groups 3 and 4, respectively): 130 ± 30^{c} and 68 ± 13^{b} U/L, respectively.

Silymarin groups – Mice were digested CCl_4 and treated with silymarin at dose of 1.6 and 8 mg/kg (groups 5 and 6, respectively): 182 ± 26^{c} and 36 ± 18^{a} U/l, respectively. Data were mean ± SD of values from 6 mice, values with the different superscript letters were significantly different at $p<0.01$.

As shown in Figure 3, the elevation of ALT activity was depressed by quercitrin treatment. The protection effect of quercitrin were found comparable to that of silymarin, a mixture of 3

flavonoids isolated from milk thistle (*Silybum marianum*) and commercelly used as hepatoprotective agent against hepatotoxicity of various chemicals including CCl_4 [6]. Quercitrin in group 3 and group 4 had protection effect against CCl_4 damage. The serum ALT concentrations were decreased 73% at dose 1.6 mg/kg (group 3) and decreased significantly 85% at dose 8.0 mg/kg (group 4) when compared with that of the toxic control group while silymarin significantly decreased 92 % at dose 8.0 mg/kg (group 6) and 62% at dose 1.6 mg/kg (group 5). Hepatoptective activity of group 3 and group 5 was equivalent ($p>0.05$). It is therefore suggested that quercitrin scavenge CCl_4-derived radicals resulting in depressing the toxicity

CONCLUSION

The data presented here indicated that fractions and isolated compound from *P. tomentosum* had hepatoprotective effect. Et-F possessed a stronger protective effect than that of its subfractions against CCl_4 induced toxicity. PTE8 was further studied for isolating pure compound that is quercitrin. This is the first report on the occurrence of quercitrin in *P. tomentosum*. Quercitrin had signifying both *ex vivo* and *in vivo* hepatoprotective effects. The result obtained in the present study indicate that *P. tomentosum* is a potential source of natural hepatoprotection.