



Bioactive Components and Hepatoprotective Activity of *Acanthopleura vaillantii* (Chiton) against Carbon Tetrachloride-Induced Hepatotoxicity in Mice

Eman Khalil¹*, Hamdy Swelim¹, and Mohd Azmuddin Abdullah²

1-Department of Zoology, Faculty of Science, Ain Shams University, Cairo, Egypt

2-Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

E.Mail : eman.a.khalil@gmail.com

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ABSTRACT

Marine animals provide a wide range of bioactive compounds that may offer new and innovative therapeutic tools for use in various diseases. In this study, the bioactive compounds in the extracts of *Acanthopleura vaillantii* (chiton) were identified, their antioxidant property assayed and the hepatoprotective activity evaluated in a carbon tetrachloride-induced hepatotoxicity model in mice. The gas chromatography-mass spectrometry (GC-MS) analysis identified mostly the polyphenolic compounds. The antioxidant assay showed that the chiton extract had radical scavenging activity. Intraperitoneal injection of the chiton extract in mice increased the hepatic enzymes and albumin content at the dose level of 10 mg chiton /kg of body weight. While administration of 20 mg chiton extract in mice was with the normalized serum albumin (Alb), alanine aminotransferases (ALT) and aspartate (AST). The histological examination of the liver sections from the 10 and 20 mg chitons treated groups showed substantial attenuation of the degenerative cellular changes, recovery from necrosis and reversal of liver fibrosis induced by the carbon tetrachloride intoxication.

INTRODUCTION

The oceans comprise more than 70% of the earth's surface and the diversity of the marine environment contributes to the presence of potent bioactive molecules. The marine shellfish extracts have exhibited biological properties including anti-tumor, hepato-protective, anti-inflammatory and antioxidant effects (Pan *et al.*, 2007).

Chitons (a class of polyplacophora) are crawling marine mollusks, found in all seas worldwide with more than 940 extant and 430 fossil species, protected by eight dorsal shell plates made of aragonite (Connors *et al.*, 2012). Chitons can be a good source of bioactive compounds attributable to the existence of chitin and chitosan components in the shell. The shell extract of *Chiton lamyi* investigated on the angiogenesis in the chick embryo chorioallantoic membrane (CAM) model has been found to decrease the number and length of vessels around the treated area (Javan-Jaafari-Bojnourdi and Baharara, 2015).

The present study aims to identify the active components in the foot extract of chiton and to evaluate its hepatoprotective potential against carbon tetrachloride (CCl₄)-induced oxidative stress and hepatotoxicity in mice.

MATERIALS AND METHOD

Sample Collection and Preparation:

The chitons (*Acanthopleura vaillantii*) were collected from the Suez Gulf (Suez harbor, El Adabia), Red sea in April – May 2016. According to Yeh *et al.* (2012), the specimens were transported alive in a container containing saltwater. The animals were dissected, and all visceral organs were removed. The specimens were rinsed thoroughly of any internal organs or body fluids, whereas the foot was taken and stored at -20°C until processing. The tissues were cut into small pieces, homogenized in a blender and extracted with pure ethyl acetate (1 ml = 1 gm tissue). The homogenate was centrifuged at 8000 rpm for 15 min, and then the resulting supernatant was filtered and concentrated by rotary evaporator working under a vacuum at 50 °C. The yield of extract was stored at -20 °C until the time of use.

GC-MS Analysis:

Biologically active components of Chiton extract were separated by GC-MS using a gas chromatograph (Agilent Technologies 7890A, USA), interfaced with a mass-selective detector (MSD, Agilent 7000 Triple Quad, USA) and equipped with Agilent HP-5ms (5%-phenyl methyl poly siloxane) capillary column (30 m × 0.25 mm i.d. and 0.25 µm film thickness). The carrier gas was helium with a linear velocity of 1.0 mL/min. The split ratio was 1:50. The injector and detector temperatures were 200° C and 250° C, respectively. The mass spectra operating parameters were as follows: ionization potential 70 eV, interface temperature 250° C, and acquisition mass range 50–600. The volume of the sample injected was 1 µl. The identification of components was based on a comparison of their mass spectra and retention time with those of the authentic compounds and by computer matching with NIST and WILEY libraries, as well as, by

comparison of the fragmentation pattern of the mass spectral data with those reported in the literature (Wang *et al.*, 2009).

Antioxidant Assay:

The free radical scavenging activity of the chiton extract samples and standards were determined by 2,2-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA) method as described before with some modifications (Blois *et al.*, 1958). A volume of 2 ml diluted extract of different concentrations (10, 15, 20, 30 & 40 mg/ml) was prepared in ethanol, and 0.5 ml aliquot was then added to the tube containing 1.5 ml of DPPH solution (0.02 mM). The mixtures were incubated for 30 min. in the dark at room temperature. The absorbance of all tubes was measured using spectrophotometer against absolute ethanol (as blank) at 517 nm. The Butylhydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (40 mg/ml) were used as a reference standard.

Experimental Animals:

Sixty adult male Swiss Albino mice weighing 25-30 g were obtained from the Theodor Bilharz Research Institute (TBRI), Giza, Egypt. The animals were grouped into four groups for the pilot study and six groups for treatment. Each group contains six mice housed in polyacrylic cages in the well-ventilated animal house of the Department of Zoology, Faculty of Science, Ain Shams University. The animals were maintained at 12 h light/dark cycle, given a commercial pellet diet (protein, fibers, minerals, and vitamins) and tap water *ad libitum* for one week before the start of the experiment as an acclimatization period. All animal experiments were performed under protocols approved by the local institutional animal ethics committee of Ain Shams University.

Pilot Study:

Overnight-fasted twenty-four mice received chiton extract by

intraperitoneal (i.p) injection with two doses - the low dose (10 mg/kg of body weight) and the high dose (20 mg/kg of body weight) for 7 consecutive days. The implemented doses were chosen based on a preliminary assessment of acute toxicity. The acute toxicity for the four random doses of the extract (5, 10, 12 and 20 mg/kg of body weight) was carried out on four groups. The animals showed neither mortality nor toxicity symptoms after 24 h of treatment.

Treated Groups:

The treated groups (thirty-six mice) were divided into six groups:- the 1st group (control) was injected with saline and olive oil; the 2nd group was injected twice a week for 6 weeks with CCl₄ (1 ml/kg of body weight, dissolved in olive oil to reach a final concentration of 20%) (Fan *et al.*, 2013); the 3rd group was injected by a low dose of chiton extract (10 mg/kg of body weight dissolved in saline) for 7 consecutive days; the 4th group was injected with chiton extract (10 mg/kg of body weight dissolved in saline) for 7 consecutive days, and then intoxicated 2 h later with CCl₄ for 6 weeks; the 5th group was injected with a high dose of chiton extract (20 mg/kg of body weight dissolved in saline) for 7 consecutive days, and the 6th group was injected with chiton extract (20 mg/kg of body weight dissolved in saline) and then intoxicated 2 h later with CCl₄ for 6 weeks.

Blood Collection and Biochemical Assays:

At the end of the experimental period, all the animals were anaesthetized using diethyl ether and sacrificed after 48 h from the last dose to collect blood samples. Blood samples were collected in non-heparinized tubes and were allowed to clot at room temperature for 1 h. Serum samples were obtained by centrifugation at 3000 rpm for 20 min. The level of serum ALT, AST, and Alb concentrations were measured by

Automated Biosystems analyzer A25 using commercially available specific kits (Biosystems S.A., Spain) and the method followed was as reported before (Gella *et al.*, 1985; Doumas *et al.*, 1997).

Histological Examination:

The livers of dissected mice were firstly washed in 0.85 saline solutions to remove the blood. Small pieces (5 mm × 5 mm) were fixed in 4% paraformaldehyde for 24 h, dehydrated with ascending series of ethanol, cleared in terpineol for at least 48 h and infiltrated with paraplast at 60 °C. Sections of 5–6 μm thick were cut using an electronic digital microtome, and then affixed on clean glass slides using dilute egg albumin solution and dried in an incubator for at least 24 h at 38 °C. The sections were stained with Mayer's haematoxylin and counterstained with 1 % Eosin (Humason, 1979). Then, the sections were dehydrated, cleared, and mounted using DPX (Sigma-Aldrich, USA). Microscopic examination was done using a Philips photographic microscope and photography was achieved with the digital camera (Optika B5, Italy).

Statistical Analysis:

All values were expressed as means ± standard deviation and the results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Post-hoc Bonferroni's test for multiple comparisons. All the statistics were processed by using SPSS 17 for Windows (SPSS, Inc., Chicago, IL, USA). Statistically significant differences between groups were defined at $p \leq 0.05$.

RESULTS

GC-MS Analyses and Antioxidant Activity:

Twenty-three components were identified in the foot of the chiton extract and most of them are polyphenolic compounds (Table, 1) where the flavonoids represented the major components of them. Table (2)

illustrates the DPPH· radical scavenging activity of chiton and the standards where the extract concentrations at 40 g/L achieved 61.3% activity, as compared to 84-85% attained by the standards.

Effects of Chiton Extract on The Liver Functions:

The treatment with chiton extract at 10 and 20 mg/kg significantly ameliorated the undesirable impact of CCl₄ toxicity on serum ALT activity.

The intraperitoneally injection of chiton extract at 20 mg/kg b.w to the normal mice insignificantly changed the serum ALT, AST, and Alb. The injection with chiton extract 10 mg/kg b.w resulted in a significant increase in the serum ALT and AST, with a decrease in the Alb level as compared to the control groups. However, these were significantly lower than the CCl₄ group as shown in table (3).

Table 1. GC-MS analyses of the Chiton extract.

Compound	Retention time (min)	Concentration (%)	Molecular Formula	Activity	References
L-Serine	3.8	6.15	C ₃ H ₇ NO ₃	Play role in mental & physical health	Mori et al., 2002
p-Allylphenol	4.7	4.6	C ₉ H ₁₀ O	Antioxidant	Murakami et al., 2015
Sinapic acid	7.17	0.36	C ₁₁ H ₁₂ O ₅	Antioxidant	Yun et al., 2008
6-Hydroxyflavone	7.4	0.6	C ₁₅ H ₁₀ O ₃	Antioxidant, inhibitor of cytochrome P-450	Cermak and Wolfrum, 2006
Hexestrol	7.65	0.52	C ₁₈ H ₂₂ O ₂	Antioxidant	Inamori et al., 1988
Scopoletin	13.57	0.32	C ₁₆ H ₈ O ₄	Antioxidant, hepatoprotective	Thuong et al., 2010
Isochroman	13.888	0.71	C ₉ H ₁₀ O	Antioxidant	Lorenz et al., 2005
3-Hydroxy-7,8-dihydro-β-ionol	14.2	1.36	C ₁₃ H ₂₀ O ₂	Anticancer	Duncan et al., 2004
4-Flavanol	14.28	5.34	C ₁₅ H ₁₄ O ₂	Antioxidant, radical scavenging	Mishra et al., 2013
Resveratrol	15.02	0.5	C ₁₄ H ₁₂ O ₃	Antioxidant	Reinisalo et al., 2015
Patchoulol	15.1	0.53	C ₁₅ H ₂₆ O	Anti-inflammatory	Nakatsu et al., 2000
Luteolin 6,8-C-diglucoside	15.51	1.53	C ₂₇ H ₃₀ O ₁₆	Antioxidant	Mishra et al., 2013
n-Hexadecanoic acid	15.65	3.23	C ₁₆ H ₃₂ O ₂	Antioxidant	Yogeswari et al., 2012
α-Tocopherol	15.83	0.45	C ₂₉ H ₅₀ O ₂	Antioxidant	Rizvi et al., 2014
Phytol	16.47	0.27	C ₂₀ H ₄₀ O	Anti-inflammatory	Yu et al., 2005
cis-6-Octadecenoic acid	16.8	3.02	C ₁₈ H ₃₄ O ₂	Anti-inflammatory	Alaluf et al., 2002
3',4',7-Trimethylquercetin	17.05	0.94	C ₁₈ H ₁₆ O ₇	Antioxidant	Materska, 2008
Cannabinol	19.9	8.47	C ₂₁ H ₂₆ O ₂	Antioxidant, Anti-inflammatory	Massi et al., 2008
Flavidin	21.25	26.4	C ₁₅ H ₁₂ O ₃	Antioxidant	Jayaprakasha et al., 2004
Zeaxanthin	21.41	16.48	C ₁₀ H ₅₆ O ₂	Antioxidant, hepatoprotective	Stahl and Sies, 2005
6,7-dimethoxy-Coumarin	21.6	6.57	C ₁₁ H ₁₀ O ₄	Antioxidant, Anti-inflammatory	Fang et al., 2003
3,5,7-Trimethoxyflavone	22.55	2.8	C ₁₈ H ₁₆ O ₅	Antioxidant	Pandey et al., 2012
cis-9, cis-12-Octadecadienoic acid	23.5	0.77	C ₁₈ H ₃₂ O ₂	Antioxidant, hepatoprotective	Rajeswari et al., 2013

Table 2. Effects of Chiton extracts on the DPPH free radical scavenging activities. Butylated hydroxyanisole, BHA; Butylhydroxytoluene, BHT; 1,1-diphenyl-2-picrylhydrazyl hydrate, DPPH·. * Experiments were carried out in duplicate

Dose	DPPH radical scavenging activity (%)
Chiton extract	
10 g/L	19.4
15 g/L	24.9
20 g/L	41.3
30 g/L	50.6
40 g/L	61.3
Standards	
BHA (40 g/L)	85.6
BHT (40 g/L)	84.3

Table 3. Effects of Chiton extract and CCl₄ on serum ALT, AST and Albumin levels in mice. ALT, alanine aminotransferase; AST, aspartate aminotransferase; Alb, albumin; CCl₄, carbon tetrachloride. Data are expressed as Mean ± SD (*n*=6 mice per group), Values sharing the superscripts (a) and (b) are significantly different. (a) Significantly different from the control group; *p*≤0.05. (b) Significantly different from the CCl₄ model group; *p*≤0.05.

Groups	ALT (U/L)	AST(U/L)	Alb (g/dL)
Control			
Mean ± SD	49± 5.48 ^b	108.83 ± 5.74 ^b	3.18 ± 0.12 ^b
CCl₄			
Mean ± SD	149.67±22.88 ^a	276.67±32.22 ^a	2.8 ± 0.14 ^a
Change (%)	205.44	154.21	-11.95
Chiton 10 mg			
Mean ± SD	70.17±15.17 ^{a,b}	138.5±8.87 ^{a,b}	3.03±0.11 ^{a,b}
Change (%)	43.2	27.26	-4.72
Chiton 10 mg + CCl₄			
Mean ± SD	102.5±10.21 ^{a,b}	174.83±8.42 ^{a,b}	2.91±0.1 ^a
Change (%)	109.2	60.64	-8.81
Chiton 20 mg			
Mean ± SD	61.76±7.6 ^b	123.3±12.04 ^b	3.2±0.19 ^b
Change (%)	26.04	13.29	0.63
Chiton 20 mg + CCl₄			
Mean ± SD	80.8±7.2 ^{a,b}	149.83±9.28 ^{a,b}	3.1±0.15 ^b
Change (%)	64.89	37.67	-2.52
F ratio	54.06	99.93	7.270

Histological Results:

The histologic examination of the liver sections from the control group stained with hematoxylin and eosin showed a normal hepatic architecture covered by fibrous Glisson’s capsule (Fig. 1 a). The hepatocytes and sinusoids, as well as phagocytic Kupffer cells, were within the normal range (Fig. 1 b). In contrast, the liver sections of CCl₄ mice showed obvious dilated blood sinusoids, a large number of pyknotic nuclei, focal necrosis, focal accumulation of inflammatory cells. Branches of the portal vein were filled with blood, and hepatocytes in the right side suffered from microvesicular fatty degeneration with faintly stained karyolytic nuclei (Fig. 1 c), with irregular dilated sinusoids (Fig. 1 d).

The liver sections from the mice injected only with chiton extract (10 mg/kg) showed liver cell strands around the central vein, with active

Kupffer cells and the appearance of few numbers of pyknotic nuclei in which most were surrounded by perinuclear space, with increased cytoplasmic granularity, and cell swelling (Fig. 1 e,f). The liver sections from the mice injected with chiton 10 mg/kg, followed by CCl₄ showed hydropic degeneration and the portal areas showed massive fibroplasia associated with the accumulation of leucocytes (Fig. 1 g, h). The liver sections from the mice injected only with chiton extract 20 mg/kg, showed normal hepatic architecture with the normal portal area (Fig. 1 i, j). The liver sections from the mice injected with chiton 20 mg/kg, followed by CCl₄ also showed the liver strands arranged regularly around the central vein, while the Kupffer cells showed some activity, and most of the nuclei exhibited wide perinuclear space (Fig. 1 k, l).

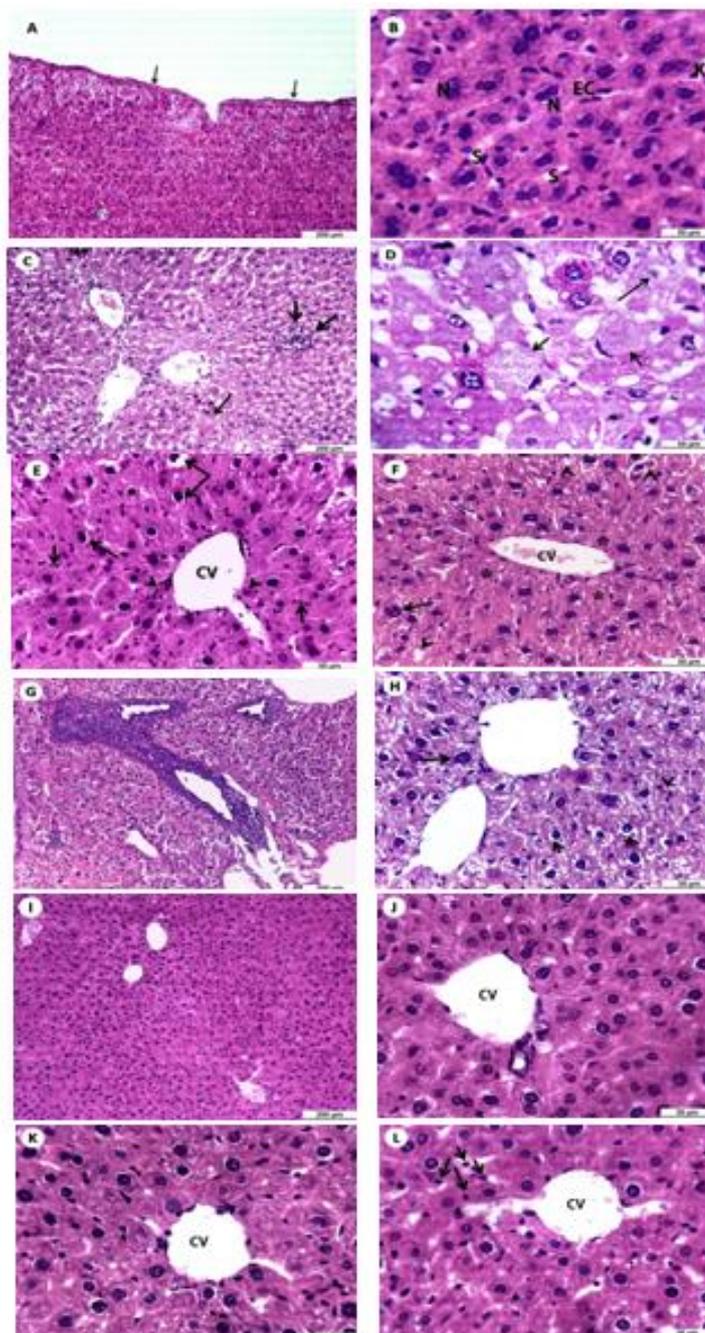


Fig. 1. Photomicrographs of liver sections stained with hematoxylin and eosin: **(A)** The control mouse showing Glisson's capsule (arrow) (100x); **(B)** The control mouse showing the hepatocytes with two nuclei. Kupffer cells bulging into the sinusoidal lumen (400x). Liver sections of CCl₄-intoxicated mice showed **(C)** dilated blood sinusoids, pyknotic nuclei, focal necrosis (arrow), focal accumulation of inflammatory cells (arrows) (100x), **(D)** microvesicular fatty degeneration with faintly stained karyolytic nuclei (long arrow), others were devoid of their nuclei (short arrows cell) (400x). Liver sections of the chiton 10 mg/kg group showing **(E)** cell strands set up around the central vein (CV), active Kupffer cells (short arrows), and appearance of few numbers of pyknotic nuclei in which most of them were surrounded with perinuclear space (long arrows). Notice, bile pigmentation in some hepatocytes (arrow head) and hydropic degeneration in the upper right corner (400x) and **(F)** increased cytoplasmic granularity, eosinophilia (asterisks) and hydropic degeneration at the periphery (arrow head). Also, slight existence of active Kupffer cells (short arrow) (400x). Liver sections of the 10 mg/kg Chiton + CCl₄ group showing **(G)** hydropic degeneration and the portal areas showing massive fibroplasia associated with the accumulation of leucocytes (100x) and **(H)** most of the nuclei with perinuclear space (short arrow), some are hypertrophied (arrow), increased active Kupffer cells (arrow head) (400x), Liver sections of the 20 mg/kg chiton showing normal hepatic architecture with normal portal area **(I)** (100x) and **(J)** (400x). Liver sections of the chiton 20 mg/kg + CCl₄ group showed **(K,L)** liver strands arranged regularly around central vein, while Kupffer cells showed some activity (arrows) (400x).

DISCUSSION

Hepatic injury through CCl₄ is caused by lipid peroxidation which is well known and extensively used in the experimental models to understand the cellular mechanisms behind oxidative damage or to evaluate the therapeutic potential of drugs and dietary antioxidants (Scholten *et al.*, 2015). The pretreatment of mice with a low or high dose of chiton extract before they were intoxicated with CCl₄ in our study markedly inhibited the liver damage induced by CCl₄ as evidenced by the decrease in the ALT and AST activity and an increase in the Alb level as compared to the mice intoxicated with CCl₄. These results are consistent with the previous finding (Li *et al.*, 2013).

The important mechanism of the hepatoprotective effects may be related to the antioxidant capacity to scavenge the reactive oxygen species (Naik and Panda, 2007). In the present study, the chiton extract exhibited the ability to inhibit membrane lipid peroxidation by scavenging the free radicals and maintaining the hepatic membrane integrity. The biologically active antioxidants in the chiton extract lowered the oxidative stress during CCl₄ toxicity as shown by the lower levels of transaminases in the animals treated with the extract before exposure to CCl₄. The tendency of serum marker enzymes and albumin to return towards a near-normality in the chiton treated mice was a clear manifestation of anti-hepatotoxic effect of chiton extract. These are due to the presence of flavonoids that have effects on CYP P-450 activity, especially 6-Hydroxyflavone which is one of the non-competitive inhibitors of cytochrome P-450 (Cermak and Wolfram, 2006). Also, Si *et al.* (2009) stated that the flavanols are inhibitors of CYP2C9 and CYP3A4 which are enzymes that metabolize most drugs in the body.

The histological observations supported the results from the biochemical analysis where the amelioration was extended to include some histological features in groups treated with the chiton extract. A mild degree of hydropic degeneration and slight dilation of blood sinusoids were observed in the liver sections of the mice treated with chiton extract at 10 mg/kg. The dose of 20 mg/kg exhibited normal hepatic architecture as proven by the DPPH' radical scavenging activity resulting in higher inhibition at 20 than 10 mg/Kg. These results are in good agreement with Hsu *et al.* (2008) who reported that the results of *Dunaliella salina* extract at 710 mg/kg dose were better than those of 71 and 355 mg/kg and *D. salina*, the carotenoids-rich alga, which contains abundant carotenoids and xanthophylls, is efficient antioxidants against a variety of oxidative stress. Based on microscopic examinations, severe hepatic fibrosis induced by CCl₄ was remarkably reduced by the administration of chiton extract which correlated well with the results of the serum aminotransferase activities and albumin content.

Conclusion

The biochemical and histological studies showed that chiton extract exhibited significant hepatoprotective activity against CCl₄-induced liver injury, by improving the liver functions and substantial amelioration in the hepatocellular architecture. Also, the high dose of chiton extract 20 mg/kg showed a more protective effect than the low dose of 10 mg/kg against the hepatotoxicity caused by CCl₄.

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