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Anti-inflammatory Activities of *Cinanamomum burmanni* Bl

Alfi Khatib[1], Mi-Yeon Kim[2] and Shin-Kyo Chung[2]*

[1]*Department of Food Technology and Human Nutrition, Bogor Agricultural University, Indonesia*

[2]*Department of Food Science and Technology, Kyungpook National University, Korea*

Abstract Anti-inflammatory assay was conducted on 20 kinds of traditional Indonesian medicinal herbs using soybean lipoxygenase (SLO) and hyaluronidase (HAse). *Cinnamomun burmanni* Bl showed highest anti-inflammatory activity. Ethyl acetate fraction from methanol extract of *C. burmanni* Bl bark showing high SLO inhibitory activity was isolated using silica gel-60 column chromatography. Two compounds were isolated and purified through preparative HPLC. Through analyses of UV, ^{1}H-NMR, ^{13}C-NMR, EI-MS and FAB^{+}-MS, compounds 1 and 2 were identified as coumarin and 2-hydroxy cinnamaldehyde, respectively, among which 2-hydroxy cinnamaldehyde showed SLO inhibitory activity of IC_{50} = 60 μM. Both compounds did not exhibit HAse inhibitory activity.

Keywords soybean lipoxygenase, hyaluronidase, coumarin, 2-hydroxy cinnamaldehyde, anti-inflammation

Introduction

Allergy is currently one of the most detrimental human diseases, involving numerous cell types including mast cells. Mast cells release a variety of mediators including histamine, adenosine, leukotriene, and prostaglandin (1), and several enzymes such as lipoxygenase and hyaluronidase catalyze this release mechanism. Human 5-lipoxygenase (5-LO) catalyzes the first step of the metabolic pathway of arachidonic acid, which is transformed into leukotriene. Leukotriene is then released from the mast cell, causing inflammation (2-4). Soybean seed also contains lipoxygenase, which catalyzes the oxidation of certain fatty acids and causes food deterioration. Although the ideal substrate for soybean lipoxygenase (SLO) is linoleic acid, arachidonic acid can also be oxidized by SLO (5, 6). Hyaluronidase is one of the enzymes involved in the inflammatory reaction through the degranulation of mast cells, causing inflammation (7). In light of the potential involvement of lipoxygenase and hyaluronidase in the pathophysiology of allergy, a number of pharmaceutical companies have developed research programs targeted at discovering those enzymes inhibitors (8). Several attempts have been made to isolate novel natural products from herbal medicine through bioassay-guided fractionation (9, 13).

Indonesia as a tropical country has a great diversity of natural products with high potential for biological activities. However, only few studies have been performed on Indonesian medicinal herbs, which have been used traditionally to treat inflammatory diseases.

In this study several traditional Indonesian medicinal herbs were screened by anti-inflammatory assays, among which *Cinnamomun burmanni Bl* showed the highest anti-inflammatory activity. Therefore, we isolated the anti-inflammatory compounds from *C. burmanni Bl* and report here for the first time.

*Corresponding author: Tel: 82-53-950-5778; Fax: 82-53-950-6772
E-mail: kchung@knu.ac.kr
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Materials and Method

Reagents Lipoxygenase (type V) from soybean, linoleic acid, tris (hydroxymethyl)-aminomethane, nordihydroguaiaretic acid (NDGA) from larrea divaricata, hyaluronidase (type IV-S) from bovine testes, hyaluronic acid from human umbilical cord, and compound 48/80 were purchased from Sigma Chemical Company (St. Louis, MO, USA). Other chemicals (Duksan Pure Chemical Co., Ltd., Ansan, Korea) and all solvents (Merck Co., Darmstadt, Germany), except for those of HPLC grade, were of analytical grade.

Plant material Plant materials were collected from Bogor Agricultural University in Bogor, Indonesia. The dried materials were cut into small pieces, dried at room temperature, and pulverized. Plant powders (10 g each) were individually extracted twice with 150 mL methanol (MeOH) for 12 hr, filtered, and concentrated *in vacuo*. Names, parts, and extraction yields of plants are listed in Table 1.

Isolation and purification Experimental procedure for the isolation of active compounds from the bark of *C. burmanni* is shown in Fig. 1. MeOH extract (as much as 98 g obtained from 300 g dried bark of *C. burmanni*) was successively fractionated with *n*-hexane, ethyl acetate (EtOAc), *n*-butanol, and water. The EtOAc fraction showing the strongest activity was further fractionated using a separatory funnel (*n*-hexane:EtOAc:MeOH:H_2O= 1:10:1:10). The upper layer was loaded onto a Silica Gel 60 column (1000 × 100 mm, i.d.) and eluted with a gradient solvent of $CHCl_3$-MeOH (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 0:10) at a flow rate of 1.5 mL/min. Every 100-mL eleuent collected from column was monitored with UV spectrophotometer (275 nm), and subjected to TLC with a solvent system of chloroform and MeOH (9:1). Preparative HPLC (LC-10A, Shimadzu Co., Kyoto, Japan) was performed on the active Fr.1-6 using a reverse phase develosil ODS-HG-5 column (Φ 20 mm × 250 mm, i.d., Normura Chemical Co. Ltd., Seito, Japan) with a UV detector at 254 and 366 nm at a flow rate of 2.0 mL/min.

Table 1. The list of traditional Indonesian medicinal herbs evaluated

No	Academic name of plant	Used part	Yield (%)
1	*Sauropus albicus* Wight	Leaf	31.4
2	*Cinnamomun burmanni* Bl	Bark	23.5
3	*Barringtonia acutangula* Gaertn	Fruit	18.3
4	*Alpinia galanga* Sw.	Root	26.6
5	*Vitex trifolia* Linn.	Leaf	21.8
6	Bambusa textilis	Young tree	29.8
7	*Mimusops elengi* Linn.	Leaf	22.6
8	*Ocimum basillicum* L.	Leaf	18.4
9	*Mommordica charantia* Linn.	Fruit	17.8
10	*Iresine herbstii* Hook	Leaf	14.0
11	*Piper betel* Linn.	Leaf	23.2
12	*Clerodendron indicum* Kuntze.	Leaf	21.4
13	*Ceiba petandra* Gaertn	Leaf	13.5
14	*Eclipta alba* Hassk	Leaf	10.7
15	*Piper cubeba* Linn.	Fruit	21.9
16	*Averrhoa carambola* Linn.	Fruit	49.0
17	*Citrus aurantifolia* Swingle	Fruit	57.7
18	*Euphoria longana* Lamk	Fruit	59.0
19	*Chrysophyllum roxburghii* G.Don	Fruit	35.2
20	*Litsea cubeba* Persoon	Fruit	5.1

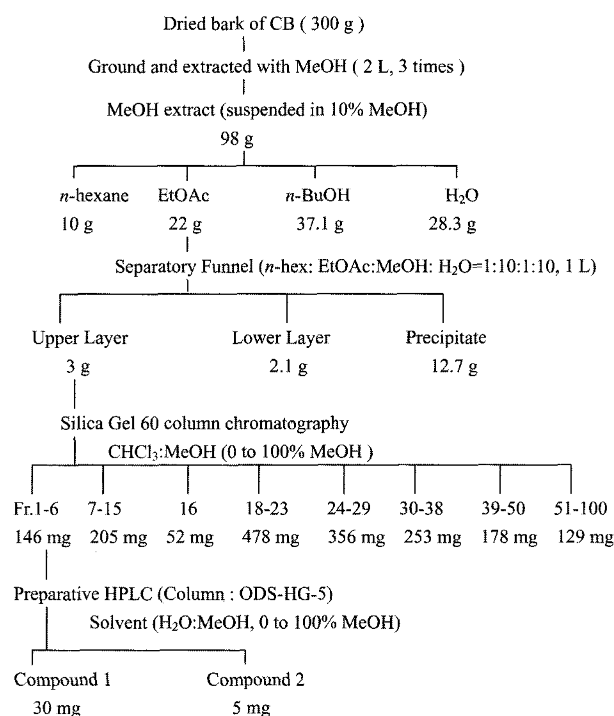


Fig. 1. Isolation scheme for anti-inflammation compounds from *C. burmanni* Bl.

Structure elucidation A UV-Vis absorption spectrum was recorded on a spectrophotometer (UV1601, Shimadzu Co., Japan). ^{1}H-NMR (75 MHz) and ^{13}C-NMR (300 MHz) were taken on FT-NMR (INOVA unity, Varian Co., Palo alto, CA, USA). CD_3OD containing trimethyl silane (TMS) as an internal standard was used as a solvent. Fast atom bombardment-mass (FAB-MS) spectra were obtained using a mass spectrometer (JMS-DX 705L, JEOL Ltd., Tokyo, Japan) with glycerol as the mounting matrix. EI-MS was carried out at 70 eV with GC-MS (Finnigan-GCQ, ThermoElectron Co., San Jose, CA, USA).

Soybean lipoxygenase (SLO) inhibitory assay Assay was measured using a spectrophotometric method (5, 14). Tris buffer (0.1 M, 2.0 mL, pH 8.5) and a solution of inhibitor in ethanol (20 mL) were added to a cuvette at 18°C, followed by the addition of soybean lipoxygenase (type V, 1000 units final concentration) solution in buffer (20 mL). After a 5-min equilibration period, an ethanolic solution of linoleic acid (50 μL, 110 μM final concentration) was added to start the reaction. The absorbance at 234 nm was recorded as a function of time on a spectrophotometer. The rates were measured from the initial slopes of the linear portions of the curves obtained for 3 min. The positive and negative controls for the assay were prepared in the same manner, but without the test compound and lipoxygenase, respectively. The IC_{50} value (concentration required for 50% inhibition of the enzyme activity) was determined by plotting a graph, relating percentage inhibition to different concentrations of test compounds. NDGA and quercetin were used as reference compounds.

Hyaluronidase (HAse) inhibition assay Assay was determined by the Morgan-Elson method (7) with a slight modification. A 50-μL aliquot of hyaluronidase in 0.1 M acetic acid buffer (3434 units/mL, pH 4.0) was added to the test compound in distilled water (100 μL) to activate the hyaluronidase at 37°C for 20 min, followed by the addition of 100 μL activator solution (0.5 mg compound 48/80 and 1.4 mg $CaCl_2$ in 1 mL of acetic acid buffer). The mixture was equilibrated at 37°C for 20 min. Subsequently, the reaction was started by the addition of hyaluronic acid in buffer (1.2 mg/mL of acetate buffer, 100 mL). The mixture was then reincubated at 37°C for 40 min. A 300-μL aliquot of 0.2 M NaOH and borate solution was added to the mixture, which was then heated for 3 min at 100°C. The borate solution was prepared by dissolving 2.24 g KOH in 100 mL of 0.8 M H_3BO_3. After cooling the mixture, 3 mL *p*-dimethylaminobenzaldehyde solution was thoroughly mixed with the solution and held for 20 min at 37°C. To prepare the reagent, 5 g of *p*-dimethylaminobenzaldehyde was dissolved in a mixture of 44 mL glacial acetic acid and 6 mL of 10 N HCl; the solution was diluted with 9 volumes of glacial acetic acid immediately before use. The generated *N*-acetyl glucosamin from hyaluronic acid was analysed based on the absorbance at 585 nm. A mixture containing all the reagents except for the enzyme was used as the blank for the sample. The IC_{50} value was determined by plotting a graph, relating the percentage inhibition to different concentrations of inhibitors.

Results and Discussion

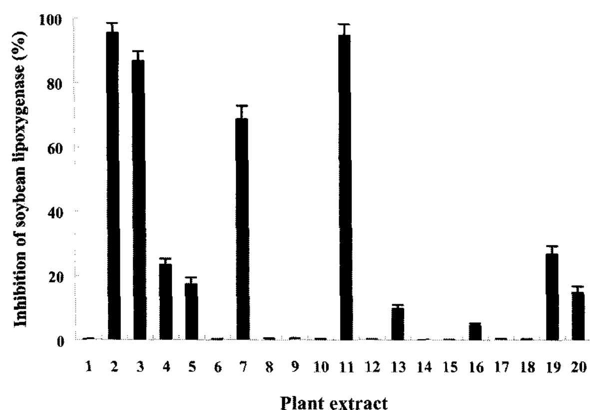
Screening for the anti-inflammation activities of Indonesian herbs Twenty kinds of MeOH extracts derived from the traditional Indonesian medicine herbs used to treat inflammation were screened for their inhibitory effects on SLO and HAse activities (Fig. 2). Three extracts, the bark of *C. burmanni* (CB), the leaves

of *P. betel* (PB), and the fruit of *B. acutangula* (BA) were found to have high inhibitory effects (96.4, 94.7, and 86.4 % inhibition) on SLO, respectively, whereas that of the *M. elengi* (ME) leaves was intermediate. The IC_{50} values of CB, PB, BA, and ME were 21.7, 16.9, 39.1, and 62.8 μg/mL, respectively, with CB, PB, and BA showing values lower than that of garlic oil (IC_{50} = 50 μg/mL) (5). Other extracts showed weak SLO inhibitory activities, and, interestingly, some even induced SLO activity.

Among the tested extracts, only CB showed HAse inhibitory activity (65.5% at 100 mg% with IC_{50} value of 64.9 mg%), which is superior to that of the polyphenol extract of barley bran (IC_{50}, 300 mg%) (15). The bark of CB is used in Indonesia for anti-eczema, asthma, high blood pressure, cough (fruit), and oral ulceration treatments (16); the observed inhibitions of SLO and HAse in this work corroborate its popular uses. No references were found on the pharmacological evaluation of anti-inflammatory activity on SLO and HAse inhibitions of *C. burmanni*, and the isolation of active compound from this plant.

SLO inhibitory activities of *C. burmanni* Bl fraction Among the 20 herbs screened, the bark of cinnamon exhibited strong inhibitions on SLO and HAse. The isolation of SLO inhibitor was conducted as shown in Fig. 1. A crude MeOH extract (98 g) was obtained from 300 g dried cinnamon bark. The extract was separated by separatory funnel using various solvents (*n*-hexane, EtOAc, and *n*-butanol). EtOAc and *n*-butanol extracts showed strong inhibitory activities with IC_{50} values of 8 and 9 μg/mL, respectively, whereas *n*-hexane and water extracts did not show SLO inhibition activities (Fig. 3). EtOAc fraction was further fractionated with a separatory funnel in a semi-polar system (*n*-hexane:EtOAc:MeOH:H_2O=1:10:1:10) into three groups: upper and lower layers, and precipitate. The upper layer showing a high activity (IC_{50} = 9 μg/mL) was loaded onto a silica gel column and subjected to the gradient elution of chloroform to MeOH. All fractions were divided based on their UV spectra and TLC patterns. Fr. 1-6 and 30-38 showed relatively high activities compared to the other fractions (Fig. 4). Fr. 1-6 was further isolated and purified through preparative HPLC using ODS-HG-5 column with gradient elution from water to MeOH. The observed variances in the SLO inhibition activity can be attributed to the different chemical compositions and contents of the plant materials

Identification and activities of isolated compounds To identify compounds 1 and 2 from cinnamon bark, UV, FAB^+-MS, GC-MS, and NMR analyses were conducted (Tables 2 and 3).



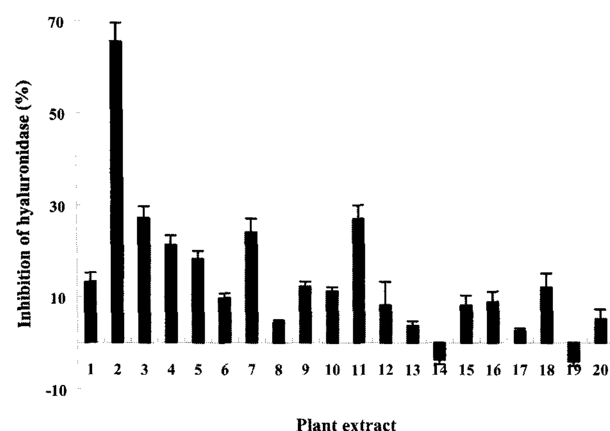


Fig. 2. Soybean lipoxygenase and hyaluronidase inhibitory effect of 20 kinds of methanol extracts of Indonesian herbs. The names of herbs were indicated at Table 1. Each of the data represents mean ± S.D. of triplicate experiments.

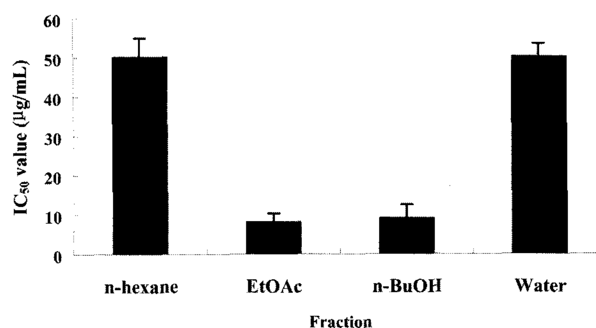


Fig. 3. Soybean lipoxygenase inhibitory effect of each fraction from the bark of *C. burmanni* Bl. Each of the data represents mean ± S.D. of triplicate experiments.

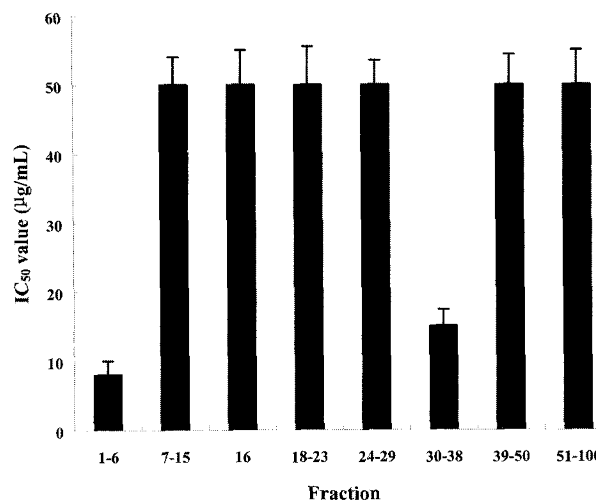


Fig. 4. Soybean lipoxygenase inhibitory effect of silica gel column fractions from the bark of *C. burmanni* Bl. Each of the data represents mean ± S.D. of triplicate experiments.

Table 2. Spectral data of compound 1 isolated from the bark of *C. burmanni* Bl

UV λ_{max} nm (in MeOH)	274, 311
^{1}H-NMR ppm (in $CDCl_3$)	7.72 (1H, d, J=9.6 Hz, H4), 7.55 (1H, dd, J=8.7 Hz, J=1.8 Hz, H8), 7.51 (1H, m, H6), 7.34 (1H, dd, J=7.8 Hz, J=1.8 Hz, H5), 7.29 (1H, m, H7), 6.43 (1H, d, J=9.6 Hz, H3)
^{13}C-NMR ppm (in $CDCl_3$)	160.73 (C2), 154.06 (C9), 143.38 (C4), 131.78 (C7), 127.82 (C5), 124.39 (C6), 118.82 (C10), 116.87 (C3), 116.69 (C8)
GC-MS (m/z)	146 (M^+), 118 (M^+-CO)

Table 3. Spectral data of compound 2 isolated from the bark of *C. burmanni* Bl

UV λ_{max} nm (in MeOH)	285, 339
^{1}H-NMR ppm (in MeOD)	9.60 (1H, d, J=8.1 Hz, H9), 7.92 (1H, d, J=15.9 Hz, H7), 7.55 (1H, dd, J=8.1 Hz, J=1.8 Hz, H6), 7.27 (1H, m, H4), 6.88 (1H, m, H5), 6.87 (1H, dd, J=15.9 Hz, J=8.1 Hz, H8), 6.87 (1H, dd, J=8.1 Hz, J=1.8 Hz, H3)
^{13}C-NMR ppm (in MeOD)	197.37 (C9), 159.15 (C2), 151.96 (C7), 134.25 (C8), 130.92 (C4), 129.77 (C6), 122.87 (C1), 121.23 (C5), 117.56 (C3)
FAB$^+$-MS m/z (glycerol matrix)	149 (MH^+), 132 (MH^+-OH), 104 (MH^+-OH-CO)

Compound 1: The UV spectrum of compound 1 showed absorption maxima at 274 and 311 nm. In ^{1}H spectral data, the signal (δ 7.72) in the most down field came from H 4. H 4 and H 3 had the same coupling constants (9.6 Hz), which means they were coupled. Keton signal (δ 160.73) was exhibited in ^{13}C NMR. The GC-MS spectra gave its molecular ion peak (M^+) at 146 m/z. Keton cleavage is indicated by the release at 28 m/z. Based on these results and comparison with the reference (17), compound 1 was identified as coumarin (Fig. 5).

Compound 2: The UV spectrum of compound 2 showed absorption maxima at 285 and 339 nm. The ^{1}H NMR spectra of compound 2 represented a typical phenylpropanoid pattern. Chemical shifts at 6.87 and 7.92 ppm showed a geminal coupling constant of 15.9 Hz due to the trans-coupling H 7 and H 8 through a double bond. Chemical shift at 9.60 ppm exhibited the presence of aldehyde, which coupled to H 8, because their coupling constants were the same. The splitting pattern of H 1 to H 5 signals indicated the hydroxyl was located at the *ortho* position of phenyl. In the ^{13}C NMR spectrum, aldehyde signal is clearly exhibited at δ 197.37, and all signals were above 100 ppm. FAB-MS of compound 2 showed a spectrum with a peak at 149 m/z ($M+H^+$). From these results, compound 2 was determined to be 2-hydroxy cinnamaldehyde (Fig. 5). 2-Hydroxycinnamaldehyde had previously isolated from the stem bark of *Cinnamonum cassia* Blume (Lauraceae) (18). This is the first report of the existence of coumarin and 2-hydroxycinnamaldehyde in *C. burmanni* Bl.

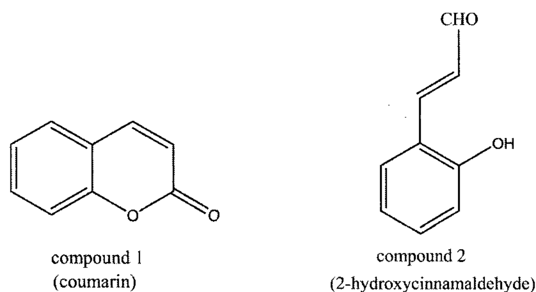


Fig. 5. Structures of isolated compounds from the bark of of *C. burmanni* Bl.

SLO and HAse inhibitory activities of isolated compounds Anti-inflammatory activities of two pure compounds were compared with those of NDGA and quercetin (Table 4). In SLO inhibitory activity, compound 2 showed a higher activity (IC_{50}=60.1 μM) than compound 1 (IC_{50}>250 μM). Position and number of hydroxyl groups were found to be the most important factors in the inhibitory effect of phenolic compounds on lipoxygenase (19), supporting the finding that compound 2, 2-hydoxy cinnamaldehyde, showed SLO inhibition activity, while compound 1, coumarin, having no hydroxyl groups, did not show the activity. 2-Hydroxy cinnamaldehyde was also reported to have various *in vitro* activities on farnesyl transferase, angiogenesis, immunmodulation, cell–cell adhesion, and cytotoxicity against tumor cell lines (20-23). Furthermore, fraxetin, a simple coumarin possessing *ortho*-dihydroxy, was reported to be a potent inhibitor of lipoxygenase (24). Other plants in the genus *Cinnamomum* were reported to have phytochemical compounds, such as eugenol from the leaf oil of *C. zeylanicum* Ness (25), and quercetin and rutin from the leaves of *C. camphora* Presl (26). These compounds were also reported to have SLO inhibitory activities (27, 28).

Both compounds showed weak HAse inhibitory activities. Quercetin also exerted inhibitory activity, which accorded with a previous report (28). HAse inhibitory activity of natural products depends on the structural

Table 4. IC_{50} Value of the isolated compounds from the bark of *C. burmanni* Bl on soybean lipoxygenase (SLO) and hyaluronidase (HAse)

Compound	SLO	HAse
	IC_{50} (μM)	IC_{50} (mM)
Compound 1	> 250.0	> 1.0
Compound 2	60.1	> 1.0
NDGA	30.0	ND
Quercetin	ND	0.3

ND means not evaluated

characteristic of the compound; the position and the availability of hydroxy, methoxy or amino methyl functions affect the activity (28-30). Consequently, coumarin and 2-hydroxy cinnamaldehyde may be the principle compounds responsible for the effectiveness of *C. burmanni* Bl. on inflammatory disease. Because inflammation leads to increased oxidative stress (31-33), these compounds could act to diminish the oxidative damage or allergic response in tissues.

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