

Anti-Tumor, Antioxidant and Antimicrobial and the Phenolic Constituents of Clove Flower Buds (*Syzygium aromaticum*)

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Abstract

Eleven phenolic compounds were identified from the methanolic extract of Cloves flowers buds (*Syzygium aromaticum* L.) by chromatographic methods. Efficiency of the methanolic extract of licorice roots as anticancer agent for breast, colon and liver was tested. The results showed that the IC₅₀ were (31 µg/ml for anti-colon cancer, 29.7 µg/ml for anti-breast cancer and 18.7 µg/ml for anti-hepatic cancer). This extract showed strong antioxidant activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH.) as compared with vitamin C. Antimicrobial activity of the methanol extract of licorice roots was studied against three bacterial and four fungal strains at concentration 0.1 ml and 0.3 ml (10 mg/1 ml)). The extract showed strong inhibitory effect for most species at concentration 0.3 ml (10 mg/ml).

Keywords: Phenolic; Anti-tumor; Antioxidant; Antimicrobial

Introduction

Main objectives of this study were to evaluate antitumor, antioxidant, antimicrobial and phenolic constituents of clove flower buds. Cloves (*Syzygium aromaticum* L.) are the aromatic dried flower buds of a tree in the family Myrtaceae. The genus comprises about 1100 species, 62 species are found in Australia and are generally known as lillipillies, brush cherries [1].

Syzygium species have been reported to possess antibacterial [2], and anti-inflammatory activity [3]. It was reported that the buds of *Syzygium aromaticum* were used in folk medicine as diuretic, odontalgic, stomachic, tonicardiac, aromatic condiment properties and condiment with carminative and stimulant activity [4]. It was reported that *Syzygium aromaticum* have been successfully used for asthma and various allergic disorders by oral administration [5]. It is also used as a carminative, rubefacient and serves as a preservative in herbal recipes, signifying possible antimicrobial properties [6]. Clove exerted immunomodulatory/anti-inflammatory effects by inhibiting LPS action. A possible mechanism of action probably involved the suppression of the nuclear factor-κB pathway by eugenol, since it was the major compound found in clove extract [7]. Eugenol is the active component of clove *Syzygium aromaticum* and it is also present in number of other aromatic plants like basil, cinnamon and bay leaves [8].

Materials and Methods

Plant material

Clove flowers buds (*Syzygium aromaticum* L.) 500 gm were provided from Lotus Company (Sekem Group, Egypt) in June 2010. The taxonomic identification of plant material was confirmed by Botany Department, Faculty of Science, Zagazig University (Egypt).

Ultra-violet spectrophotometric analysis

Chromatographically, pure materials dissolved in analytically pure methanol were subjected to UV spectrophotometric investigation in 4 ml capacity quartz cells Zeiss spectrometer PMQ-II. In case of flavonoids, AlCl₃, AlCl₃/HCl, fused NaOAc/H₃BO₃ and NaOMe reagents were separately added to methanolic solution of the

investigated material and UV measurements were then carried out [9].

Nuclear magnetic resonance spectroscopic analysis

Jeol ECA 500 MHz NMR Spectrometer at 500 MHz, (Institute Fur Chemie, Humboldt Universität zu Berlin, Germany). ¹H chemical shifts (δ) were measured in ppm, relative to TMS and ¹³C NMR chemical shifts to DMSO-d₆ and converted to TMS scale by adding 39.5. Typical conditions: spectral width=8 KHz for ¹H and 30 KHz for ¹³C, 64 K data points and a flip angle of 45°C.

Mass spectrometric analysis

The isolated pure compounds were subjected, in most cases to Fast Atom Bombardment (positive and negative) Mass Spectroscopic Analysis (FAB-MS) on MM 7070 E spectrometer (VG analytical). Some other compounds were subjected to electron spray ionization mass spectroscopic analysis (ESI-MS) a Varian Mat1 12-ET Spectrometer. All measurements were carried out at Institute Fur Chemie, Humboldt Universitat zu Berlin, Germany [10].

Extraction and isolation

500 gm from dried clove buds exhaustively extracted under reflux over a water bath with 5 liters of a methanol/bidistilled water (3:1) mixture for 3 hours. The solvent was removed under reduced pressure at about 45°C. The residual finally yielded 30 gm of a sticky dark brown material.

Fractionation of the extract, (30 gm dissolved in 100 ml aqueous

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methanol 3:1) over Sephadex LH-20 (200 gm) column (150 X 4.5 cm) and elution with methanol/bidistilled water mixtures of decreasing polarities for gradient elution led to the desorption of six individual fractions (I-VI) which were dried, individually, in vacuum, and then subjected to rechromatography for several times to obtain a pure phenolic compounds. The structure of these compounds was confirmed by comparison of their physical and spectral data.

SRB assay of cytotoxic activity

Human tumor cell lines were obtained frozen in liquid nitrogen (-180°C) from the American Type Culture Collection. The tumor cell lines were maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. Measurement of potential cytotoxicity activity of methanolic extract of licorice roots against the liver carcinoma cell line (HEPG2), colon carcinoma cell line (HCT116) and breast carcinoma cell line (MCF7) was tested by SRB (Sulphorhodamine-B) assay using the method of Skehan et al. [5]. This experiment was conducted in the National Cancer Institute, Cairo, Egypt.

Di phenyl picryl hyrazide assay

The free radical scavenging effect of plant extracts was assessed by the decoloration solution of DPPH radical according to Letelier et al. [2], in Faculty of Agriculture Research Park-Cairo University (FARP). This assay was realized essentially by the method described by Joyeux et al. [6], and modified by [11-13].

Antimicrobial activities of the methanolic extract

Strains were obtained from the bacteria stock present at the Research Laboratory of bacteriology, Faculty of Science, Zagazig University. Gram-positive and Gram-negative bacteria species tested were *E. coli* (KQ103), *Staphylococcus aureus* (LC405) and *Salmonella typhi* (RS57) and fungi species (Laboratory collection strains) were *Fusarium oxysporum*, *Aspergillus niger*, *Penicillium* sp. and *Trichoderma* sp.

Antibacterial activity

In vitro antimicrobial assay of the methanolic extract was carried out according to pour plate technique at two concentrations 0.1 ml and 0.3 ml (10 mg/1 ml). Culturing and incubated of different bacteria species were carried out at 37 °C for 24 hours. After the elapse of incubation periods, the diameter of inhibition zones was measured [14].

Antifungal activity

Czapek Dox media used for cultivation of fungal species. The medium was seeded with different fungal species. After solidification of media on plates, make pores in agar with cup-borer (15 mm) diameter. Two concentrations 0.1 ml and 0.3 ml (10 mg/1ml) of the methanolic extract were transferred into the well. Dimethyl Foramide (DMF) was used only as a control. The plates were incubated for 7 days at 30°C. The inhibition zone formed by the extract against the particular test fungal strain determined as the antifungal activities of the extract [14].

Results and Discussion

Structure elucidation: Investigation of the phenolic compounds was done by fractionation of the extract, over polyamide column and elution with methanol/bidistilled water, and then subjected to re-chromatography for several times led to the separation of eleven pure phenolic compounds. The structure of these compounds was confirmed by comparison of their physical and spectral data with those of reported compounds:

1) Gallic acid (C₇H₆O₅, 90 mg), Rf values (x 100): 44 (H₂O), 55 (HOAc), 72 (BAW). UV (MeOH): λ_{max}=272 nm. ¹H-NMR (DMSO-d₆): δ 6.98 (s, 2H, H-2 and H-6). ¹³C-NMR (DMSO-d₆): δ 120.6 (C-1), 108.8 (C-2 and C-6), 145.5(C-3 and C-5), 138.1(C-4), 167.7(C=O). Ms (m/z): 170.9 [M+ +1, 5.1%], 169.0 [M+ - H, 100%, ion A], 167.9 [M+ - H₂, 63.9%], 153[M+ -OH, 11.2%], 145 [ion A-C2, 3.5%], 139 [ion A-HCHO, 11.3%], 126 [M+ -CO₂, 6.5%] and 123 [ion A-HCOOH, 27.1%].

2) Salicylic acid (C₇H₆O₅, 50 mg), Rf values (x100): 36(HOAc-6), 69(BAW). UV (MeOH): λ_{max}=253 nm and ¹³C-NMR (DMSO-d₆): δ 118.9 (C-5), 121.2 (C-4), 122.3(C-6), 133.1(C-1), 136.8(C-4), 162.7(C-2), 178.2(C=O).

3) 2,3-digallyl-4,6-hexahydroxydiphenyl-β-d-glucopyranose (1-Desgalloyleugeniin) (C₃₄H₂₆O₂₂, 150 mg), Rf values (x100): 42 (H₂O), 56(HOAc-6), 50(BAW). UV (MeOH): λ_{max}=275 nm and ¹H-NMR (DMSO-d₆): δ, Glucose: δ 4.99 (d, J=8, H-1'), 5.05(t, J=8, H-2'), 5.52(t, J=8, H-3'), 4.35(t, J=8, H-4'), 4.35(m, H-5'), 5.12(m) & 3.72(d, J=12.5, H-6'), Galloyl: δ 6.8, 6.88 (s, H-2 and H-6) and Hexahydroxy-diphenyl: δ 6.22, 6.38(s, H-3 and H-3'). ¹³C-NMR (DMSO-d₆): δ Glucose: 95.06(C-1), 73.05(C-2), 71.63 (C-3), 71.63(C-4), 66.80(C-5), 62.50(C-6), Galloyl: 118.40, 118.75(C-1), 108.87(C-2 and C-6), 145.28, 145.54 (C-3 and C-5), 139.01(C-4), 155.15, 155.44(C=O) and Hexahydroxydiphenyl: 115.50 (C-1 and C-1'), 124.34(C-2 and C-2'), 105.6,105.8 (C-3 and C-3'), 144.12 (C-4 and C-4'), 135.59 (C-5 and C-5'), 144.12 (C-6 and C-6'), 157.03, 157.72 (C=O).

4) 1-C-2,3-O-hexahydroxy-4,6-O-hexahydroxydiphenyl-open-chain)-glucose (Casuariin) (C₃₄H₂₄O₂₂, 170 mg), UV (MeOH): λ_{max}=221, 267 (sh.) nm and ¹H-NMR (DMSO-d₆): δ, Open chain glucose: δ 5.4 (d, J=4.9 Hz, H-1), 4.5 (dd, J=2 & 4.9 Hz, H-2), 5.17 (t, J=2 Hz, H-3), 5.25 (dd, J=2 & 3.5 Hz, H-4), 5.24(m, H-5), 4.6(dd, J=3.5 and 12 Hz, H-6) and 3.98 (d, J=12 Hz, H-6) and Hexahydroxy-diphenyl moiety: δ 6.21 (s, H-3), 6.56 (s, H-3'), 6.28 (s, H-3'). ¹³C-NMR (DMSO-d₆): δ, Open chain glucose: δ 65.70 (C-1), 75.40(C-2), 68.60(C-3), 72.90(C-4), 69.80(C-5), 63.9(C-6) and Hexahydroxydiphenyl moiety: δ 115.54, 115.70, 116.16, 116.59 (C-1 & C-1'), 122.92, 122.99, 125.19, 125.86 (C-2 & C-2'), 103.44, 104.96, 106.47, 114.75(C-3 & C-3'), 142.77, 144.45, 144.56, 145.00, 145.18, 144.26, 144.14 (C-4, C-4', C-6 & C-6'), 134.07, 134.85, 136.26, 137.94 (C-5 & C-5'), 163.73, 167.94, 168.65, 169.28 (C=O).

5) Gallic acid 3-O-β-D-(6'-O-galloyl)-glucopyranoside (C₂₀H₂₀O₁₄, 46 mg), Rf values (x100): 42 (H₂O), 48(HOAc-6), 40(BAW), UV (MeOH): λ_{max}=276 nm and ¹³C-NMR (DMSO-d₆): δ, Glucose moiety: 94.9 (C-1), 75.2(C-2), 76.7(C-3), 69.8(C-4), 73.0(C-5), 63.7 (C-6), Gallic acid moiety: 119.7(C-1"), 109.5(C-2" and C-6"), 146.1 (C-3" and C-5"), 139.5 (C-4"), 166.2(C=O) and Galloyl moiety: 118.9(C-1'), 109.1(C-2' and C-6'), 146.1 (C-3' and C-5'), 139.0 (C-4'), 165.1(C=O).

6) 3, 3', 4-Trimethoxy ellagic acid (C₁₇H₁₂O₈, 42 mg), Rf values (x 100): 0(H₂O), 10 (AcOH-6), 95 (BAW), UV (MeOH): max=246, 373. (NaOAc): max=256, 410 and ¹H-NMR (DMSO-d₆): δ 7.60(s, H-5'), 7.51(s, H-5), 4.04(s, 3-OMe), 4.02(s, 3'-OMe), 3.98(s, 4'-OMe).

7) Ellagic acid (C₁₄H₆O₈, 67 mg), Rf values (x 100): 0(H₂O), 9(HOAc-6), 48(BAW). UV (MeOH): max=255, 362 and ¹H-NMR (DMSO-d₆): δ 7.48 (s, H-5 and H-5') also ¹³C-NMR (DMSO-d₆): δ 112.3(C-1 and C-1'), 136.4(C-2 and C-2'), 140.2(C-3 and C-3'), 153(C-4 and C-4'), 111.4(C-5 and C-5'), 107.6(C-6 and C-6') and 159.2(C-7 and C-7').

8) Myricetin (C₁₅H₁₀O₈, 20 mg), Rf values (x 100): 00 (H₂O), 9 (HOAc-6), 54 (BAW), UV (MeOH): max=265, 376 nm and ¹H-NMR (DMSO-d₆): δ 6.18 (d, J=2.5 Hz, H-6), 6.34 (d, J=2.5Hz, H-8), 7.24 (s,

H-2'&H-6') also Ms (m/z): 317.0 [M- - H, 100%], 287.1 [M- - C₂H₆, 5.2%], 271.1 [M- - C₂H₆O, 9.2%], 242.1 [M- - C₆H₄, 5.1%], 203.1 [M- - C₆H₅O₃, 4.2%, ion A] and 169.1 [ion A - H₂O₂, 4.9%].

9) Quercetin (C₁₅H₁₀O₇, 45 mg), UV (MeOH): max=255, 268, 370 nm, (MeOH + NaOAc): max=254, 276, 375 nm, (NaOAc + H₃BO₃): max=272, 388nm, (MeOH + AlCl₃): max=270, 360, 440 nm and (AlCl₃ + HCl): max=258, 400 nm, ¹H-NMR (DMSO-d₆): δ 6.19 (d, J=2.5, H-6), 6.4 (d, J=2.5, H-8), 7.64 (d, J=2.5, H-2'), 6.88(d, J=8.5, H-5'), 7.53 (dd, J=2.5&8.5, H-6'). ¹³C-NMR (DMSO-d₆): δ 147.0(C-2), 135.8(C-3), 176.2(C-4), 160.5(C-5), 99.2(C-6), 164.0(C-7), 93.7(C-8), 156.4(C-9), 103.5(C-10), 122.2(C-1'), 115.3(C-2'), 145.1(C-3'), 148.0(C-4'), 115.6(C-5') and 120.2(C-6'). Ms (m/z): 300.8 [M- - H, 100%, ion A], 299.8 [M- - H₂, 28.3%], 270.9 [ion A-C₂H₆, 8.9%], 162.9 [ion A-C₇H₆O₃, 10.1%, ion B] and 117.3 [ion B-HCOOH, 5.3%].

10) Kaempferol (C₁₅H₁₀O₆, 23 mg), Rf values (x100): 00 (H₂O), 10 (HOAc-6), 85 (BAW). UV (MeOH): max=268, 369 nm, (MeOH + NaOAc): max=270, 310, 375 nm, (NaOAc + H₃BO₃): max=270, 320, 372 nm, (MeOH + AlCl₃): max=270, 305, 360, 430 nm and (AlCl₃ + HCl): max=278, 316, 413nm. ¹H- NMR (DMSO-d₆): δ 6.4 (d, J=2.5, H-8), 6.18 (d, J=2.5, H-6), 8.14 (d, J=8, H-2' and H-6'), 6.89 (d, J=8, H-3' and H-5'). ¹³C-NMR (DMSO-d₆): δ 146.8(C-2), 135.4(C-3), 175.9(C-4), 161.0(C-5), 98.6(C-6), 164.2(C-7), 93.8(C-8), 156.4(C-9), 103.7(C-10), 121.9(C-1'), 129.9(C-2' and C-6'), 115.8(C-3' and C-5') and 159.5(C-4'). Ms (m/z): 285.1 [M- - H, 100%, ion A], 242.1 [M- - CO₂, 5.3%], 203.1 [ion A-C₄H₂O₂, 4.6%, ion B] and 169.1 [ion B-H₂O₂, 5.7%].

11) Apigenin (C₁₅H₁₀O₅, 25 mg), Rf values (x 100): 00 (H₂O), 11 (HOAc-6), 88 (BAW), UV (MeOH): max=266, 335 nm, (MeOH + NaOAc): max=270, 300, 373 nm, (NaOAc + H₃BO₃): max=270, 300, 340 nm and (MeOH + AlCl₃): max=277, 302, 348, 384 nm. ¹H- NMR (DMSO-d₆): δ 6.18 (d, J=2.5Hz, H-6), 6.47(d, J=2.5Hz, H-8), 6.77(s, H-3), 6.92(d, J=8Hz, H-3' and H-5'), 7.93(d, J=8Hz, H-2' and H-6'). ¹³C-NMR (DMSO-d₆): δ 163.8(C-2), 102.8(C-3), 181.5(C-4), 161.3(C-5), 98.7(C-6), 163.6(C-7), 93.9(C-8), 157.2(C-9), 103.6(C-10), 121.1(C-1'), 128.3(C-2' and C-6'), 115.8(C-3' and C-5') and 161.4(C-4'). Ms (m/z): 270.2 [M+, 100%], 254 [M+ - O, 5.1%], 242 [M+ - CO, 18.7%], 226[M+ -CO₂, 4.8%], 213 [M+- C₂H₅O, 3.9%, ion A], 177 [ion A - C₂H₆O, 5.4%, ion B], 167 [ion A-C₃H₄O, 4.9%], 147 [ion B-HCHO, 52.5%, ion C], 135 [ion B-C₂H₆, 5.6%] and 111 [ion C-2H₂O, 12.5%].

Anti-tumor activity

The potential cytotoxicity activity of the methanolic extract of clove buds was tested against three human cell lines [HEPG2 (liver carcinoma cell line), MCF7 (breast carcinoma cell line) and HCT116 (colon carcinoma cell line)] by SRB (Sulphorhodamine-B) assay. The results showed that the extract has strong activity against all cell lines tested. The antitumor activity of the tested extract is summarized in Figure 1. The IC₅₀ values (the concentrations of thymoquinone required to produce 50% inhibition of cell growth) of the extract against each cell lines were 31 µg/ml, 29.7 µg/ml and 18.7 µg/ml for HCT116, MCF7 and HEPG2, respectively.

Antioxidant activity

The DPPH scavenging activity of the methanolic extract of clove buds is summarized in Figure 2. It was observed that the scavenging activity of the extract at all concentrations (25, 50 and 100 µl) is rather strong (42.27-80.07%) as compared with vitamin C. The remarkable antioxidant activity of methanolic extract of licorice roots might be due to the higher concentration of phenolic compounds. IC₅₀ value for the methanolic extract=44 µg/ml, while for vitamin C=17 µg/ml.

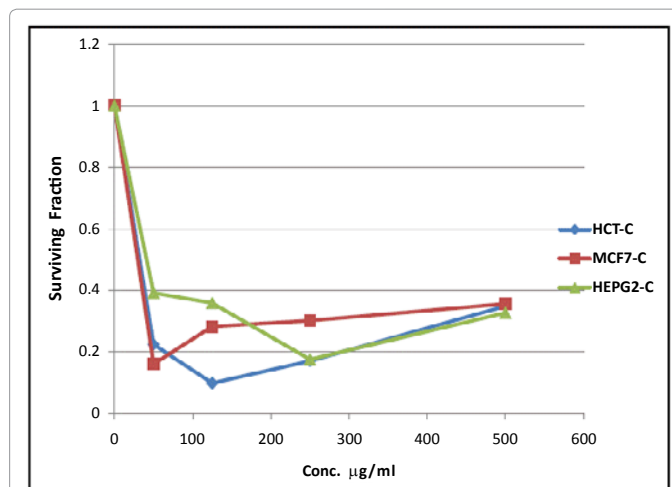


Figure 1: % of survival fraction of breast, colon and liver carcinoma cell lines against the concentration (µg/ml) of clove methanolic extract.

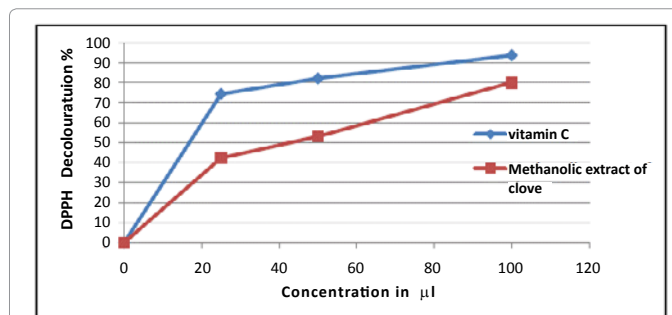


Figure 2: Antioxidant activity of the methanolic extract of clove compared to vitamin C.

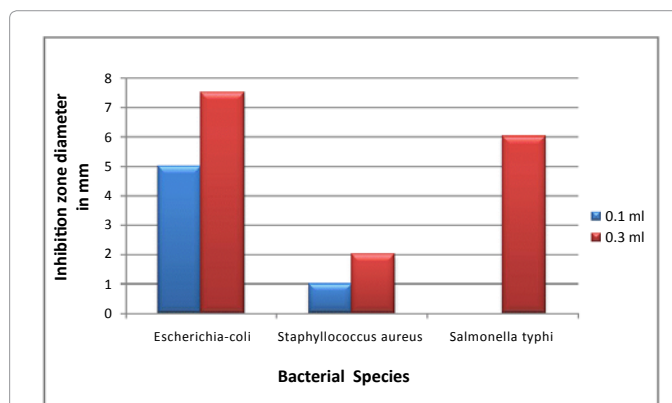


Figure 3: Antibacterial activity of the methanolic extract of clove.

Antibacterial activity

The clove methanolic extract was showed high inhibitory effect against *E. coli*. at the two concentration (0.1 and 0.3 ml), while the same extract showed high inhibitory effect against *Salmonella typhi* at concentration 0.3 ml and no effect at concentration (0.1 ml). On the other hand, *Staphylococcus aureus* showed the highest resistance species to the extract at concentration 0.3 and 0.1ml as shown in Figure 3.

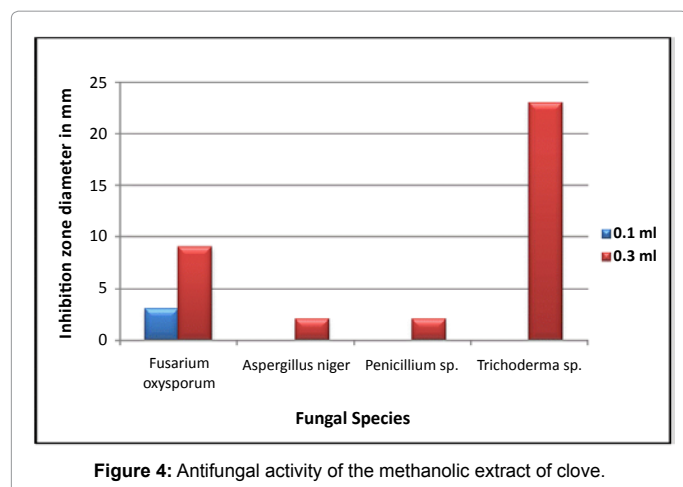


Figure 4: Antifungal activity of the methanolic extract of clove.

Antifungal activity

The results showed that, Clove methanolic extract has strong antifungal activity only against *Trichoderma sp.* at 0.3 ml concentration and moderated activities against *Fusarium*, *Aspergillus sp.* and *Penicillium sp.* While at 0.1 ml concentration most fungal strains showed no inhibitory activity as shown in Figure 4.

Conclusions

The overall results of this study indicate that the methanolic extract of clove buds represent a potential source of plant drugs. So, we can deduce that the methanolic extract of licorice appeared to be promising choice to be considered as antioxidant and anti-tumor medicines.

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