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NATURAL ANTIOXIDANT (FLAVONE GLYCOSIDE) FROM *SESBANIA ACULEATA* PERS. AND ITS POTENTIAL ACTIVITY

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Antioxidant and antibacterial Activity of *Sesbania aculeata*, Leguminosae

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ABSTRACT

This paper deals with the significance and importance of medicinal plants. New flavone glycoside A, m.p. 222-224°C, m.f. $C_{34}H_{42}O_{20}$, [M^{+}] 770 (FABMS) has been isolated from the stems of *Sesbania aculeata* Pers. along with three known compounds Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, (B) and Mearnsetin-3-*O*-α-L-rhamnopyranoside (C) Gehuain (isoflavone) (D). The compound **A** was characterized as 5, 7, 8-trihydroxy-6, 4'-dimethoxy flavone-7-*O*-α-L-rhamnopyranosyl-1→4)-*O*-β-D-xylopyranosyl-(1→4)-galactopyranoside by various chemical degradations and spectral analysis. Compound was subjected to antioxidant, antibacterial activity. Compound A showed potent antioxidant activity, of which the methanol and chloroform fraction demonstrated the strongest. Antioxidant activity with the IC_{50} value of 52.40 µg/ml and 60.15 µg/ml, respectively.

INTRODUCTION: Plants are a rich source of natural products. They form major parts of ingredient in almost all system of therapeutics. Most of pharmaceutical industries are conducting extensive research on plants collected from the rain forests and other places for their potential medicinal values. Modern allopathic system of medicine is also based on plants and herbs.

Medicinal plants are relevant in both developing and developed nations of the world as sources of drugs or herbal extracts for various chemotherapeutic purposes continue to play a dominant role in maintenance of human health since antiquities. Over 50% of all modern clinical drugs are of natural product origin and natural products play an important role in drug development programs of the pharmaceutical industry. In the continuation of this strategy of new drug discovery we have studied only the aerial parts of the plant for their antibacterial and antioxidant properties.

Sesbania aculeate [1-2] belongs to family Leguminosae. It is commonly known as ʹDhunchiʹ or ʹDainchaʹ in Hindi. It is distributed throughout in plane India and most tropical countries. Its root is alexeteric, antihelmintic and diuretic. It is also useful in snake-bite and disease of eye. Its seeds are useful in ringworm and skin diseases. Earlier workers [3-5] have reported various constituents from this plant.

In the present paper, we report the isolation and structural elucidation of a new flavone glycoside-5, 7, 8-trihydroxy-6, 4'-dimethoxy-flavone-7-O-α-L-rhamnopyranosyl-(1→4)-O-β-D-xylopyranosyl-(1→4)-galactopyranoside (A) along with three known compounds Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, (B) and Mearnsetin-3-*O*-α-L-rhamnopyranoside (C) Gehuain **(**isoflavone**)** (D) from methanolic extract of the stems of this plant.

EXPERIMENTAL SECTION:

General Experimental Procedure: All the melting points were determined on a thermoelectrically melting point apparatus and are uncorrected. UV Spectra was determined on Shimadzu-120 double beam spectrophotometer in MeOH. The IR Spectra were recorded on Shimadzu FTIR-8400 spectrophotometer in KBr disc. ^{1}H-NMR Spectra were recorded on Varian XL 400 MHz spectrometer in $CDCl_3$ using T M S as internal standard. ^{13}C-NMR Spectra were recorded on Varian XL 100 MHz spectrometer using $CDCl_3$ The FAB mass spectra were recorded on a JEOL SX -102/DA-6000 Mass Spectrometer /Data System using Argon/Xenon (6kv) as the FAB gas. Thin Layer Chromatography on silica gel G and column chromatography on silica gel were used. All solvents, chemicals and reagents were of analytical grade.

Plant Material: The stems of *Sesbania aculeata* were procured from Satbhaiya plant supplier Katra Bazar, Sagar and were taxonomically authenticated by the Department of Botany Dr. H.S. Gour University, Sagar, (M.P.) India.

Extraction and Isolation: Air dried and powdered flowers (6.10 kg) of the plant were extracted with rectified spirit in Soxhlet extractor for three days. The flowers were successively extracted with methanol for five days. The MeOH soluble fraction of the plant was concentrated under reduced pressure, to yield a light brown viscous mass (2.8 gm) which was subjected to TLC examination over silica gel-G using nBAW (4:1:5) as solvent and I_2 vapors as visualizing agent, showed three spots, indicating it to be mixture of three compounds A, B and C . These compounds were separated and purified by column chromatography over silica gel using $CHCl_3$: MeOH in various proportions. After removal of the solvent and crystallization from ether, above eluates yielded compound A (1.72 gm.), compound B (0.64 gm.) and compound C (0.55 gm) respectively.

Study of Compound A: It had m. p. 211-212°C, m. f. $C_{34}H_{42}O_{20}$, [M^+] 770 (FABMS) found (%); C 53.01, H 5.47, calcd for m.f. $C_{34}H_{42}O_{20}$, (%); C 52.98, H 5.45, UV MeOH λ_{max} (nm); 273, 322 (+$AlCl_3$), 302, 350 (sh) (+$AlCl_3$ / HCl), 284, 354 (+NaOAC); 247, 286 (+NaOMe); 283, 324. IR (KBr) ν_{max} (cm^{-1}) 3484, 3210, 2930, 1738, 1650, 1610, 1432, 1325, 1085, 810. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.05 (1H, s, H-3), 7.33 (2H, d, J 8.4 Hz, H-2′, 6′), 6.80 (2H, d, J 8.4, Hz, H-3′, 5′), 11.87 (1H, s, OH-5), 12.05 (1H, s, OH-8), 5.80 (1H, d, J 7.2 Hz, H-1′′), 4.40 (1H, dd, J 8.4, 7.5, Hz, H-2′′), 4.35 (1H, dd, J 8.2, 8.1 Hz, H-3′′), 3.91 (1H, dd, J 8.2, 8.2 Hz, H-4′′), 3.98 (1H, dd, J 8.0, 6.4 Hz, H-5′′), 4.20 (2H, dd, J 6.12, 10.2 Hz, H-6′′), 5.02 (1H, d, J 6.4 Hz, H-1′′′), 3.78-3.92 (3H, m, H-2′′′, H-3′′′, H-4′′′), 4.25 (2H, dd, J 6.12, 11.5 Hz, H-5′′′), 5.23(1H, br, s, H-1′′′′), 3.96-4.50 (4H, m, H-2′′′′, 3′′′′, 4′′′′, 5′′′′), 1.56 (3H, d, J 5.3 Hz, Rham-6′′′′), ^{13}C-NMR (90 MHz, $CDCl_3$), δ 162.6 (C-2), δ 107.6 (C-3), δ 175.9 (C-4), δ 159.5 (C-5), δ 133.1 (C-6), δ 162.7 (C-7), δ 95.0 (C-8), δ 154.4 (C-9), δ 103.6 (C-10), δ 120.9 (C-1′), δ 128.6(C-2′), δ 115.3 (C-3′), δ 162.9 (C-4′), δ 117.0 (C-5′), δ 128.5 (C-6′), δ 102.01 (C-1′′), δ 84.6 (C-2′′), δ 78.5 (C-3′′), δ 70.8 (C-4′′), δ 76.2 (C-5′′), δ 65.3 (C-6′′), δ 105.9 (C-1′′′), δ 76.8 (C-2′′′), δ 78.2 (C-3′′′), δ 73.5 (C-4′′′), δ 75.3 (C-5′′′), δ 99.4 (C-1′′′′), δ 85.0 (C-2′′′′), δ 75.6 (C-3′′′′), δ 70.1 (C-4′′′′), δ 76.0 (C-5′′′′), δ 67.2 (C-6′′′′),. MS (FABMS) m/z 770 [M^+] , 624 [M^+ - rhamnose], 492 [M^+ rhamnose-xylose], 330 [aglycone].

Acid hydrolysis of Compound A: Compound **A** (75 mg) was dissolved in ethanol (15 ml) and refluxed with 15 ml of 10% H_2SO_4 on water bath for 7 hrs. The contents were concentrated and allowed to cool and residue was extracted with diethyl ether. The ether layer was washed with water and the residue was chromatographed over silica gel using $CHCl_3$: MeOH (6:4) as solvent to give compound A-1 which was identified as 5, 7, 8-trihydroxy-6, 4'-dimethoxy-flavone by comparison of its spectral data with reported literature values. The aqueous hydrolysate obtained after hydrolysis was neutralized with $BaCO_3$ and the $BaSO_4$ was filtered off, the filtrate was concentrated and subjected to paper chromatography examination using nBAW (4:1:5) solvent system and sugars were identified as D-galactose (R_f 0.19), D-xylose (R_f 0.26), and L-rhamnose (R_f 0.36).

Permethylation of Compound A: Compound **A** (45 mg) was refluxed with MeI (5 ml) and Ag_2O (25 mg) in DMF (25 ml) for 4 hrs and then filtered. The filtrate was hydrolyzed with 10% ethanolic H_2SO_4 for 6 hrs. to give methylated aglycone, identified as 7-hydroxy- 5,6,8,4′-tetramethoxy flavone and methylated sugars which were identified as 2,3,4-tri-O-methyl-L-rhamnose,

,2,3,4-tri-O-methyl-D-xylose and 2,3,4,6-tetra-O-methyl-D-galactose

Enzymatic hydrolysis of Compound A: The compound **A** (40 mg) was dissolved in MeOH (25 ml) and hydrolysed with equal volume of takadiastase enzyme. The reaction mixture was allowed to stay at room temperature for two days and filtered. The proaglycone and hydrolysate were studied separately.

The hydrolysate was concentrated and subjected to paper chromatography examination using nBAW (4:1:5) as solvent systems and aniline hydrogen phthalate as spraying reagent, showed the presence of L-rhamnose (R_f 0.36). The proaglycone was dissolved in MeOH (20 ml) and hydrolyzed with equal volume of almond emulsin at room temperature as usual procedure, yielded aglycone identified as 5,7,8-trihydroxy-6,4'-dimethoxy-flavone and sugars were identified as D-galactose (R_f 0.19) and D-xylose (R_f 0.26) (Co-PC).

STUDY OF COMPOUND A-1: It had m.f. $C_{17}H_{14}O_7$, m.p. 198-200°C, [M^+] 330 (FABMS), found (%); C 61.83, H. 4.26, calcd for m.f. $C_{17}H_{14}O_7$, (%) C 61.81, H 4.24. UV MeOH λ_{max} (nm); 280, 362, (+$AlCl_3$); 308, 352, (+$AlCl_3$ / HCl); 285, 355 (+NaOAc); 248, 286, (+NaOMe); 283, 334. IR (KBr) ν_{max} (cm^{-1}); 3250, 2955, 1714, 1635, 1600, 1540, 1428, 1370, 1255, 1082, 810. ^{1}H-NMR (300 MHz, $CDCl_3$); δ 7.24 (1H, s, H-3), 7.38 (2H, d, J 8.5 Hz, H-2′, 6′), 7.10 (2H, d, J 8.5, Hz, H-3′, 5′), 12.64 (s, OH-5), 12.08 (s, OH-8), 3.9 (3H, s, OMe-6), 4.12 (3H, s, OMe-4′). ^{13}C-NMR (90 MHz, $CDCl_3$); δ 161.4 (C-2), δ 106.8 (C-3), δ 176.5(C-4), δ 158.2 (C-5), δ 130.6 (C-6), δ 162.3 (C-7), δ 94.6 (C-8), δ 154.5 (C-9), δ 103.9 (C-10), δ 120.4 (C-1′), δ 128.6 (C-2′), δ 117.0 (C-3′), δ 152.0 (C-4′), δ 116.1 (C-5′), δ 128.7 (C-6′), MS (FABMS) m/z 330 [M^+] .

RESULTS: Compound **A** showed R_f value of 0.59 & showed R_f value of 0.55 in $CHCl_3$: MeOH (95:5) solvent system. It gave orange-yellow color with 10% methanolic sulfuric acid and pink color with Shinoda confirmed the presence of flavone and also showed positive Molisch's test with formation of violet ring. along with three known compounds Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1$\rightarrow$2)-β-D-glucopyranoside, (B) and Mearnsetin-3-*O*-α-L-rhamnopyranoside (C) Gehuain (isoflavone) (D) by comparisons of their spectral data (U.V, I.R, NMR and MS).

All compounds showed moderate activity against Gram-positive, Gram-negative bacteria and fungi are given in **Table 1**. Compound **A** showed highest activity against gram positive bacteria *Staphylococcus aureus* and showed less activity against *Escherichia coli.* The A was found to be highly active against *Aspergillus niger* and *Penicillium digitatum*. Thus on the basis of above results it was concluded that above compound may be potentially used as diseases caused of these microorganisms.

TABLE 1: ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF COMPOUNDS

S. No.	Bacterial Species	Diameter of Zone of Inhibition (mm)				
		Compound A (100 %)	Compound B (100 %)	Compound C (100 %)	Compound D (100 %)	Standard* (100 %)
1	*(+)S.aureus*	13.4	11.5	15.5	8.5	19.21
2	*(-)Escherichia coli*	7.5	12.5	9.5	11.4	15.80
3	*(+)Bacillus coagulas*	6.8	16.5	12. 5	9.5	16.50
4	*(-)P.aeruginosa*	8.5	10.4	16.7	12.5	17.64
	Fungal Species					Std** (100 %)
1	*Aspergillus niger*	6.4	14.5	8.4	11.5	16.8
2	*Penicillium digitatum*	10.5	16.4	7.5	2.8	21.0
3	*Trichoderma viride*	11.6	12.5	9.5	7.4	15.2

* Streptomycin for antibacterial and ** Griseofulvin for antifungal used as standard drugs.

DISCUSSION: Compound **A** had m.f. $C_{34}H_{42}O_{20}$, m.p. 211-212°C, [M^+] 770 (FABMS). It gave Molisch [6] and Schinoda test [7] showing its flavonoidal glycosidic nature. The compound also responded to neutral ferric chloride test. The UV and IR spectral data also shows the nature of flavone. The I.R spectrum showed strong absorptions at 3484 (-OH), 2930 (-CH saturated), 1672 (>C=O), 1610 (aromatic ring). In its UV spectrum, absorption bands at 272 nm and 322 nm indicating its isoflavonoid skeleton. Two bathochromic shifts of 26 nm and 44 nm in bands I on addition of $AlCl_3$ and AlCl3 + HCl relative to methanol confirm the presence of -OH

group at C-5 and C-6 position. A bathochromic shift of 15 nm in bands I with NaOMe showed the presence of -OH group at C-4' position in compound **A**[8, 9].

In ^{1}H-NMR spectrum of compound A, two singlets at δ 7.43 and δ 6.85 were assigned to H-2′, 6′ and H-3′, 5′ respectively. One sharp singlet at δ 7.6 was assigned to H-2 proton. A singlet at δ 3.78 confirmed the presence OMe group at C-8 position. The anomeric proton signals at δ 5.24 (1H, d, J 7.4 Hz, H-1′′), δ 6.40 (1H, d, J 7.4 Hz, H-1′′′) and δ 5.42 (1H, d, J 6.4 Hz, H-1′′′′) were assigned to H-1′′ of L-rhamnose, H-1′′′ of D-galactose, H-1′′′′ of D-xylose′ and Characteristics ions appeared at m/z 770 [M^+], MS (FABMS) m/z 756 [M^+], 610 [M^+ - rhamnose], 478 [M^+ rhamnose-xylose], 330 [aglycone].

Acid hydrolysis of compound **A** with ethanolic 10% H_2SO_4 yielded aglycone **A-1**, m.p. 265-266°C, m.f. $C_{17}H_{14}O_7$, [M^+] 330 (FABMS) which was identified as 5, 7, 8-trihydroxy-6, 4′-dimethoxy-flavone by comparison its spectral data with reported literature values[10-11]. The aqueous hydrolysate after the removal of aglycone, was neutralized with $BaCO_3$ and the $BaSO_4$ was filtered off. The filtrate was concentrated and subjected to TLC and paper chromatography examination [12-13] and the sugars were identified as D-galactose (R_f 0.19), D-xylose (R_f 0.26), and L-rhamnose (R_f 0.36) (Co-PC, Co-TLC). Periodate oxidation [14-15] of compound **A** confirmed that all sugars were present in the pyranose form. The glycosidic linkage is located at 7- position in aglycone [16].

The position of sugar moieties in the compound **A** were determined by permethylation [17-18] followed by acid hydrolysis which yielded methylated aglycone identified as 7-hydroxy-5, 6, 8, 4′-tetramethoxy-flavone which confirmed that hydroxy group at C-7 position of the aglycone were involved in glycosidation. The methylated sugars which were identified as 2, 3, 4, 6-tetra-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-xylose and 2, 3, 4-tri-*O*-methyl-L-rhamnose according to Petek indicating that the C-1′′′′ of L-rhamnose was linked to C-4′′′ position of xylose and C-1′′′ of D-xylose was attached with C-4′′ of D-galactose and C-1′′ of D-galactose was attached to the C-7 position of aglycone and also showed the interlinkage (1→4) between D-xylose and D-galactose.

That was further confirmed by their ^{13}CNMR spectral data. Enzymatic hydrolysis [19] of compound **A** with takadiastase enzyme liberated L-rhamnose (R_f 0.37) and proaglycone identified as 5, 7, 8-trihydroxy-6, 4′-dimethoxy-flavone-7-*O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-galactopyranoside that confirmed the presence of α-linkage between L-rhamnose and C-5 position of aglycone. Proaglycone on further hydrolyzed with almond emulsin liberated D-galactose (R_f 0.19), D-xylose (R_f 0.26) suggesting the presence of β-linkage between D-xylose and D-galactose as well as D-galactose and aglycone.

On the basis of above evidences, the structure of compound **A**, was characterized as 5, 7, 8-trihydroxy-6,4′-dimethoxy-flavone-7-O-α-L-rhamnopyranosyl-(1→4)-O-β-D-xylopyranosyl (1→4)-O-β-D--glactopyranoside.

Compound B yellow powder; was analysed for m. p.221-222°C, m. f. $C_{27}H_{30}O_{15}$, M^+ 594 (ESIMS), m/z 593 [M-H] and identified as Kaempferol 3-O-a-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, by comparison of its spectral data with reported literature values [20].

Compound C yellow amorphous powders, was analyzed for, m. p.261-262°C, m. f. $C_{26}H_{26}O_{14}$, M^+ 585 (ESIMS)., found (%) C 56.97 H 5.18 cacld for m.f. $C_{22}H_{24}O_{11}$, % C 56.89, H 5.17 and identified as mearnsetin 3-O-α-L-rhamnopyranoside by comparison of its spectral data with reported literature values [21].

Compound D yellow amorphous, was analyzed for, m. p. 120-121°C, m. f. $C_{26}H_{26}O_{14}$, M^+ 585 (ESIMS), found (%) C 56.97, H 5.18 cacld for m.f. $C_{22}H_{24}O_{11}$, % C 56.89, H 5.17. UV MeOH λ_{max} (nm); 268 (4.40), 324 (3.92) nm. IR (KBr) ν_{max} (cm^{-1}); 3400, 2923, 1641, 1520, 1491, 1372, 1279, 1187, 1039 cm^{-1} and identified as Gehuain (isoflavone) by comparison of its spectral data with reported literature values [22].

Determination of Antimicrobial Activity of the Compounds: The antimicrobial activity of compound A was determined by Filter Paper Disc Diffusion Method [23]. The various bacterial species were first incubated at 45°C for 48 hrs. The sterile filter paper discs (6mm) were soaked with standard antibacterial agent and various test samples and were dried at 50°C.

The disc were then placed on soft nutrient agar (2%) petri plates previously seeded with suspension of each bacterial species. The diameters of zone of inhibition were measured at 37±1°C after 24 hrs.

For fungal activity Saboraud's broth media [24] with 4% agar was used for the preparation of plates and incubated with spores and mycelium suspension of fungi obtained from one week old culture. The diameter of zone of inhibition was measured at 28±1°C after 48 hrs. The various results are recorded in Table 1.

Antioxidative Activity:

DPPH assay: The Hydrogen atom or electron donation ability of compound A and some pure compounds were measured from the bleaching of the purple coloured methanol solution of DPPH. This spectrophotometric assay used stable radical DPPH as a reagent [25]. 50ml of various concentrations of methanol, chloroform extracts were added to 5ml of 4mg /100ml methanol solution of DPPH. After 30 min of incubation period at room temp, the absorbances were read against a blank at 517nm. Inhibition of free radical DPPH in percent (I %) was calculated as fallows:

$$\text{Blank I \%} = [(\bar{A}\ A_{sample})/\ A\ \text{Blank}] \times 100$$

Wher,e A blank is the absorbance of control reaction (Containing all reagents except test compound) and A sample is the absorbance of the test compound. Compound concentration providing 50% inhibition (IC_{50}) was calculated. Ascorbic Acid (AA) was used as positive control.

β-Carotene and Linoleic acid assay: Antioxidative capacity was determined by measuring the inhibition of the volatile organic compound and the conjugate diene hydroperoxides arising from linoleic acid oxidation [26]. A stock solution of β-Carotene / Linoleic acid was prepared as 0.5 mg β-Carotene was dissolved in 1ml of chloroform (HPLC grade); 2.5µl linoleic acid and 200mg Tween-40 were added. Chloroform was completely evaporated using vacuum evaporation. 100ml of distilled water saturated with O_2 was added with vigorous shaking (30min, 100rpm). 2.5 ml of reaction mixture was dispersed to test tubes and 350µl portions of the extracts (prepared in 2g/l of methanol and chloroform extracts) were added and then emulsion system was incubated for 48 hrs at room temperature. Ascorbic acid was used as positive controls. Absorbance of the mixture was measured at 490 nm. Antioxidative capacities were compared with those of AA with same concentration and a blank consisting of only 350µl of ethanol.

Antioxidant activity of methanol and chloroform soluble fraction of compound A tested by comparing it to the activity of known oxidant such as AA with the inhibition of DPPH radical. In addition, the effect of inhibition of lipid peroxidation of the extract was assayed using β-carotene bleaching induced linoleic acid peroxidation method. Free radicals involved in the process of lipid peroxidation play a primary role in numerous chronic diseases and are implicated in the aging process. In present study phytochemicals are recognized as scavengers of DPPH and Anti oxidant activity.

The Result of Antioxidant Activity: The antioxidant activity of the compound was assessed by the DPPH free radical scavenging assay as shown in table 2. Compound exhibited potential antioxidant activity. The chloroform soluble part of compound **A** scavenged 50% DPPH free radical at the lowest inhibitory concentration (IC_{50}: 52.45µg/ml). The methanol soluble compound also revealed strong antioxidant activity (IC_{50}: 60.15µg/ml). On the other hand, methanol soluble and chloroform soluble fraction of compound **A** showed antioxidant activity with IC_{50} of 60.15µg/ml and 52.45µg/ml respectively.

TABLE 2: IC_{50} DATA OF TEST SAMPLES OF COMPOUND A, ASCORBIC ACID AND BHT

S. No.	Samples	IC_{50} (µg/ml)
1	Ascorbic acid	41.05
2	Methanolic compound A	60.15
3	Chloroform Soluble compound A	52.45

CONCLUSIONS: Presently natural products research has an important role of the drug discovery process of the pharmaceutical industry and other research organizations. Chemical and biological research on natural products over the past two centuries has not only provided drugs for the treatment of various human ailments, but has provided the stimulus for the development of modern synthetic organic chemistry, and the emergence of medicinal chemistry as a major route for the discovery of novel and more effective

therapeutic agents. The isolation and antimicrobial activity of 5,7,8-trihydroxy-6, 4'-dimethoxy flavone-7-O-α-L-rhamnopyranosyl-(1$\rightarrow$4)-O-β-D-xylopyranosyl-(1$\rightarrow$4)-galactopyranoside from stems of Sesbania aculeata Pers is being reported for the first time and showed good potential activity.

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