STUDY ON TRADITIONAL MEDICINE (SETKUPALA No.2) FOR EYE DISEASES

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Abstract

This research was studied on determination of antioxidant activity, toxicity of some elemental content, and antimicrobial activity of Traditional Eye Medicine, Setkupala (No.2) manufactured by Ministry of Health. In this work, the sample was collected from Myanmar Traditional Medicine shop, Yangon. Percent inhibition of the watery extract was tested by DPPH assay method. IC_{50} value of the sample was calculated by linear regression excel program. From the results, it is noted that IC_{50} values of Setkupala (No.2) had slight antioxidant activity. The elemental content of the sample was investigated by EDXRF and AAS methods. Iron content was higher than Selenium, Zinc, and Manganese in the sample whereas the content of Arsenic and Lead were present in the highest toxic elements. The contents of Iron, Arsenic, and Lead in the samples based on one oral dose (five tablets) per day were over the maximum permissible level of WHO standard. In the determination of antimicrobial activity, *Bacillus subtilis* (N.C.T.C- 8236), *Bacillus pumilus (*N.C.I.B-8982), *Staphylococcus aureus* (N.C.P.C- 6371), *Pseudomonas aeruginosa* (6749), *Candida albicans*, and *E.*coli (N.C.I.B- 8134) strains were used Antimicrobial activity test was done by using agar well diffusion method. According to the study of antimicrobial activity, it was clearly noticed that the different extracts of Setkupala (No.2) were effective against the different strains.T herefore, Setkupala (No.2)had antimicrobial activity. From the phytochemical investigation, it is found that the carbohydrates, glycosides, phenolic compounds, reducing sugars, saponins and tannins were present in the sample. Moreover, the sample was examined by FT IR and HPLC method.

Keywords: Setkupala (No.2), antioxidant activity, elements, antimicrobial activity, FT-IR, HPLC

Introduction

Most of the traditional medicines have provided for public health sector. Among the traditional medicines for the treatment of eye disorder, Setkupala (No.2) is a well-known oral medicine. It is composed of *Cinnamomum tamala* sp. (Karaway), *Elettaria cardamomum* Maton. (Pharlarnge),, *Semecarpus anacardium* L. F. (Chee thee), *Saussurea affinis*, *Sperng* sp. (Pannoot), *Myristica fragrans* Houtt. (Zardeikpho), *Syzygium aromaticum* L. Merr. & Perry (Layhyin), *Hydnocarpus kurzii* (King.) Warburg (Kalaw thee), *Rauwolfia serpentina* Benth. (Bonmayazar), *Plumbago rosea* L. (Kantgyokni), *Piper longum* L. (Peikchin), *Gentiana kurroo*,Royle. (Hsapale), *Nigella sativa* L. (Samonnet), *Carallia brachiate* (lour.) Merr. (Maniawga), *Foeniculum vulgare* Gaertn. (Samonsabah), *Trachyspermum ammi* L. (Samonphyu), *Anethum sowa* Roxb. (Samonnyo), *Foeniculum vulgare* Mill. (Samonphwe), *Lepidium sativum* L. (Samonni), *Cassia acutifolia* Delile. (Pwaygaing), *Capparis sepiaria* L. (Hsoogauknet), *Ferula foetida sp.* (Sheingo, Latex), *Aloe sp.* (Moke Khar), Borax, (Letchah meebauk). This medicine can reinforced not only eye diseases but also hypertension, paralysis, and arthritis. Therefore, it was need to study the effectiveness of Setkupala No.2 because traditional practitioners rely upon the Setkupala No.2 medicine to cure ailments.

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Materials and Methods

Determination of Antioxidant Activity

In this experiment, the sample was collected from the traditional herbal shop from Ministry of Health, Yangon. The antioxidant activity was studied on watery extract of selected sample Setkupala (No.2) by DPPH free radical scavenging assay. 0.002 % DPPH solution was prepared in the brown coloured bottle by dissolving 2 mg of DPPH powder in the EtOH 100 mL. It was stored in the refrigerator for no longer than 24 h. About 4 mg of watery extract and 10 mL of EtOH were thoroughly mixed by shaker. Then, stock solution was obtained. Desired concentrations 400 μg mL^{-1}, 200 μg mL^{-1}, 100 μg mL^{-1}, 50 μg mL^{-1}, 25 μg mL^{-1} and 12.5 μg mL^{-1} of watery extract were prepared from this stock solution by dilution with appropriate amount of EtOH. The stock solution (100 μg mL^{-1}) of the sample was prepared by dissolving 1 mL of sample solution in 9 mL of EtOH. This stock solution was twofold serially diluted with EtOH to get the sample solutions with the concentration of 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 μg mL^{-1}. The blank solution was prepared by mixing the sample solution 1.5 mL with 1.5 mL of EtOH. The control solution was prepared by mixing 1.5 mL of 0.002 % DPPH solution and 1.5 mL of EtOH using shaker. DPPH radical scavenging activity was determined by UV spectrophotometric method. The control solution was prepared in the brown bottle by mixing 1.5 mL of 0.002 % DPPH solution and 1.5 mL of ethanol using shaker. The sample solution was also prepared by mixing thoroughly 1.5 mL of 0.002 % DPPH solution and 1.5 mL of each of the test sample solution. The bottles were allowed to stand at room temperature for 30 min. After 30 min, the absorbance of these solutions was measured at 517 nm and the percentage of radical scavenging activity (% RSA) was calculated by the following equation.

$$\% RSA = \frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}} \times 100$$

where, % RSA = % radical scavenging activity of test sample,
$A_{control}$ = absorbance of DPPH in EtOH solution,
A_{sample} = absorbance of sample solution with DPPH solution, and
A_{blank} = absorbance of sample solution with EtOH solution.

The antioxidant power (IC_{50}) is expressed as the test substance concentration (μg/mL) that result in a 50 % reduction of initial absorbance of DPPH solution and that allows to determine the concentration. IC_{50} (50% inhibitory concentration) values were calculated by linear regressive excel program. Similarly, the standard, ocuvite (western medicine), was determined above procedure. The standard deviation was also calculated by the following equation:

$$\text{Standard Deviation (SD)} = \sqrt{\frac{(\bar{x} - x_1)^2 + (\bar{x} - x_2)^2 + \ldots (\bar{x} - x_n)^2}{(n-1)}}$$

Determination of Elements by Energy Dispersive X- ray Fluorescence Spectrometry

The powdered sample was fabricated into pellet. The pellet sample was placed in the sample chamber of EDX- 700 spectrometer that can be measured the sixteen samples at a time and analyzed in PC based multi - channel analyzer using EDX- 700 software.

Determination of Elements by Atomic Absorption Spectrophotometry

Atomic absorption spectrophotometer (Perken Elma Analyst 800) was used for determination of elements. About 1 g of ash sample was accurately weighed and dissolved in 5 mL of 20 % of concentrated hydrochloric acid. The resulting solution of ash sample was evaporated to dryness and dissolved in 6 mL of 20 % HCl solution (volume by volume) followed by centrifugation. The centrifugate was decanted and the clear solution was made up to 100 mL with deionized water. The resultant solution 10 mL was pipette accurately and made up to 100 mL again with deionized water. The sample solution prepared was now ready for analysis of mineral elements by AAS (Lajunen, 1991).

Determination of Antimicrobial Activity

The strains used in this study were *Bacillus subtilis* (N.C.T.C-8236), *Bacillus pumilus* (N.C.I.B-8982), *Staphylococcus aureus* (N.C.P.C-6371), *Pseudomonas aeruginosa* (6749), *Candida albicans*, and *E.*coli (N.C.I.B-8134). Antimicrobial activity test was done by using agar disc diffusion method. The ethyl alcohol, petroleum ether, ethyl acetate, n-butanol and aqueous extracts were impregnated in agar disc for about five minutes. Then the incorporated discs with extracts were placed on the nutrient agar medium seeded with 0.25 mL of each microorganism suspension by sterile forceps. Inoculated plates with different indicator cultures were incubated at 37 °C in an incubator for 24 h. After overnight incubation, the developing zone of inhibition on agar medium were measured by a plastic ruler and recorded.

Phytochemical Investigation

In the phytochemical test, the sample was examined by using Mayer's reagent, Dragendorff's reagents and Wagner's reagents for alkaloids, by using concentrated hydrochloric acid and magnesium ribbon for flavonoids, by bromothymol blue indicator for organic acids, by Benedict's reagent for reducing sugars, by acetic anhydride and concentrated sulphuric acid for steroids (Robison, 1983), by spraying with ninhydrin reagent for α– amino acids, by using 2 % chloride solution and 1 % gelatin solution for tannin (Marini-Bettolo *et al*, 1981), by using sodium picrate solution for cyanogenic glycosides, by using 10 % lead acetate solution for glycosides (Trease, 1980), by using vigorously shaking with distilled water for saponins, by using iodine solution for starch (M-Tin Wa, 1970), by using 10 % α- naphthol for carbohydrates, by using acetic anhydride and concentrated sulphuric acid for steroids, by using 5 % ferric chloride solution for phenolic compounds, (Vogel, 1966).

FT IR Spectroscopic Analysis

The Fourier transform infrared spectrum of ethyl acetate extract of the sample was measured as KBr pellet and recorded on Perkin Elmer GX FT IR Spectrophotometer at the Universities' Research Centre (URC).

HPLC Analysis

Chemicals

All the chemicals used were of Analytical Reagent grade. Solvents used were HPLC grade from Hua Co., Najing, China. The water used was distilled and deionized by using Millipore system.

Sampling

The solutions extracted were filtered by 0.45 μm filter. 1 mL each ethyl acetate solutions extracted then were dried by eppendorf vacufuge plus vacuum concentrator and mixed with 1 mL of concerned mobile solution. After that, 20 μL each solution was added in the vial.

Chromatographic conditions

C 18 column (4.5 × 250 & 5 μm particles) (Phecda) was used as stationary phase. The mobile phase consisted of 0.1 % formic acid buffer (A) and acetonitrile (B) was carried out gradient elution as follows; 5 min, 80%(A); 25 min, 30%(A); 35 min, 30% (A) The flow rate was 1.0 mL/min and the injection volume was kept 20 μL. The chromatograms were recorded at 265 nm and column temperature was maintained at 25° C throughout the study period. Different samples prepared as well as mobile phase were filtered using 0.45 μm filter and degassed by ultrasonication (Metrex) prior to use.

Results and Discussion

Antioxidant Activity of Sample Extract

In this work, DPPH (2, 2- diphenyl-1- picryl- hydrazyl) radical scavenging assay was used to assess the antioxidant activity for watery extract of Setkupala (No.2). The absorbance of different concentrations was measured at 517 nm by using UV spectrophotometer. Absorbent measurement was carried out in three times for each solution and mean values obtained were to calculate percent inhibition of oxidation by the equation. In the average values of percent inhibition, IC_{50} (50 % inhibitory concentration) values in μg mL^{-1} were calculated by linear regressive excel program. The results of sample are shown in Table 1.

According to literature, the lower the IC_{50} value is the higher the antioxidant activity. 50 % inhibitory concentration of Setkupala (No.2) was 27.37 μg mL^{-1} and standard (ocuvite, western medicine) was 172.61 μg mL^{-1}. Therefore, the antioxidant activity of Setkupala (No.2) was six times higher than that of ocuvite medicine.

Table 1 Antioxidant Activity of Setkupala (No.2)

Sample	Inhibition of Various Concentration of Watery Extract						IC_{50} µg/mL
	12.5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL	400 µg/mL	
Watery Extract	26.43 ±1.18	49.07 ±1.29	82.40 ±1.51	84.39 ±0.43	78.42 ±0.54	77.43 ±0.32	27.37
Standard (Ocuvite)	10.05 ±3.47	17.98 ±3.82	17.81 ±5.08	21.16 ±0.92	60.84 ±8.21	67.40 ±6.28	172.61

Elemental Analysis

Setkupala (No.2) was studied by Energy Dispersive X-Ray Fluorescence technique and Atomic Absorption Spectrophotometry for the determination of elements. The X-ray fluorescent analysis is widely accepted as a standard method for elemental analysis. It is observed that calcium, potassium, silicon, sulphur, iron, strontium, thallium, manganese, zirconium, rubidium, zinc, copper, and nickel were present in Setkupala (No.2) by EDXRF. The relative abundance of elements is shown in Table 2. The spectrum from EDXRF determination is shown in Figure 1. From the data, it can be seen that percentage of calcium is the highest amount in Setkupala (No.2) and the second highest amount is potassium. silicon, sulphur and iron are the third highest amount. The trace amount of strontium, thallium, manganese, zirconium, rubidium, zinc, copper, and nickel are present in the medicine. In this work, a total of 7 elements were determined. Iron, selenium, zinc, and manganese are some of antioxidant nutrient elements whereas some toxic elements are arsenic, lead, and cadmium. Atomic absorption spectrophotometric analysis showed that Setkupala (No.2) contained 0.0363 % of iron, 0.0005 % of selenium, 0.0005 % of zinc, 0.0001 % of manganese, 0.0084 % of arsenic, 0.0028 % of lead, and 0.0001 % of cadmium. The calculation results of percentage and compositions of elements per different tablets per day are also shown in Table 3. From the literature, it was known that the permissible level of elements were 150 µg/day of iron, 200 µg/day of selenium, 2800 µg/day of zinc, 2300 µg/day of manganese, 50 µg/day of arsenic, 21 µg/day of lead, and 6 µg/day of cadmium (WHO, 1996). According to daily medical dosage for Setkupala (No.2), a dose for a day is 4/5 tablets for adult. Therefore, it is suitable for two tablets a dose instead of five tablets a dose because the contents of iron, arsenic and lead in five tablets are over the permissible levels and the two tablets a dose are under the maximum permissible level according to WHO standard.

Table 2 Relative Abundance of Elemental Contents in Setkupala (No.2)

No	Element	Relative Abundance (%)
1	Ca	39.265
2	K	28.911
3	Si	11.730
4	S	11.419
5	Fe	7.136
6	Sr	0.485
7	Ti	0.478
8	Mn	0.228
9	Zr	0.115
10	Rb	0.082
11	Zn	0.067
12	Cu	0.050
13	Ni	0.034

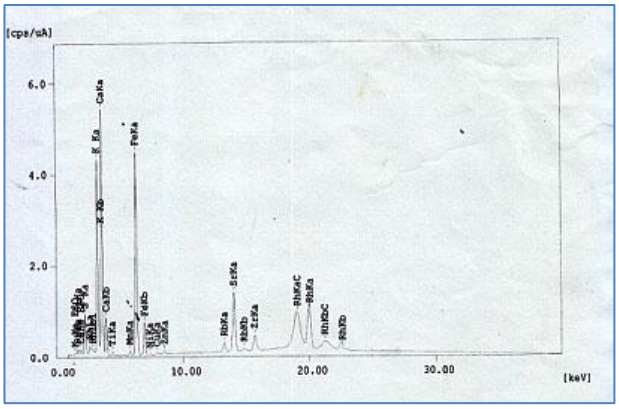

Figure 1 EDXRF spectrum of Setkupala (No.2)

Table 3 Elemental Contents in Setkupala (No.2) Determined by AAS (based on dried sample)

No.	Elements	Content (%)	Content (x 10^{-6}) mg per day					
			One tablet	Two tablets	Three tablets	Four tablets	Five tablets	WHO (1996)
1.	Fe	0.0363	108	218	326	435	544	150
2.	Se	0.0005	0.14	0.28	0.42	0.55	0.69	200
3.	Zn	0.0005	1.6	3.22	4.83	6.44	8.05	2800
4.	Mn	0.0001	0.28	0.56	0.86	1.16	1.46	2300
5.	As	0.0084	25.1	50.1	76.4	100	126	50
6.	Pb	0.0028	8.36	16.7	25.1	33.7	41.8	21
7.	Cd	0.0001	0.31	0.63	0.94	1.26	1.57	6

Antimicarobial Activity

The organism strains such as *Bacillus subtilis*, *Bacillus pumilis*, *Staphylococcus aureus*, *Pseudomones aeruginosa*, *Candida albicans* and *E.coli* species were used for testing the antimicrobial activity of fractions separated from Setkupala (No.2). The antimicrobial activity was tested by agar disc method. The inhibition zones of various extracts are shown in Table 5. It is clearly noted that EtOAc and n-BuOH extract of Setkupala (No.2) possessed antimicrobial activity against all strains tested. In addition, all the fractions were found to be active against *B.subtilis and Staphylococcus aureus* species. However, all fractions were more active against *Staphylococcus aureus* than *B.subtilis.* Especially, n-BuOH extract possessed significantly high antimicrobial activity.

Pseudomonas aeruginosa can cause devastating infections in the human eye. It is one of the most common causes of bacterial keratitis. EtOAc extract showed total activity against *P. aeruginosa. Candida albican* can cause conjunctivitis. Visual disturbance may include blurring, sensitivity to light and eye pain. *S.aureus* always has the potential to cause diseases, including

boils and pimples, wound infections, pneumonia, septicemia, food intoxication and toxic shock syndrome. Symptoms of *E.coli* infection are nausea or vomiting, severe abdominal cramps, watery or very bloody diarrhea, fatigue, fever (Jacobson and Silverma, 2009).

Therefore, Setkupala (No.2) can prevent the above diseases because it possessed antibacterial activity.

Table 4 Results of Antimicrobial Activity of Crude Extracts of Setkupala (No.2)

No.	Fraction	*B. subtilis*	*S. aureus*	*P.aeruginosa*	*B. pumilus*	*C.albicans*	*E.coli*
1.	P_1	++ (16 mm)	+++ (23 mm)	-	-	-	-
2.	P_2	++ (17 mm)	+++ (22 mm)	-	-	-	-
3.	P_3	++ (18 mm)	+++ (24 mm)	+++ (25 mm)	++ (17 mm)	++ (17 mm)	+++ (24 mm)
4.	P_4	++ (17 mm)	+++ (23 mm)	++ (17 mm)	+++ (24 mm)	+++ (26 mm)	+++ (25 mm)
5.	P_5	++ (16 mm)	+++ (23 mm)	-	-	-	-

P_1 = EtOH extract
P_2 = Petether extract
P_3 = EtOAC extract
P_4 = n-BuOH extract
P_5 = Aqueous

Agar well - 10 mm
(+) = 10 mm ~ 14 mm (mild activity)
(++) = 15 mm ~ 19 mm (medium activity)
(+++) = 20 mm above (high activity)
(–) = negative

Phytochemical Study

Phytochemical study revealed the presence of carbohydrates, glycosides, phenolic compounds, reducing sugars, saponins, and tannins in Setkupala (No.2).

Study on FT IR spectrum.

The band assignments of FT IR spectrum of ethyl acetate extract are presented in Table 5. The broad band at 3359 cm^{-1} is attributed to O-H stretching vibration. Asymmetric and symmetric C-H stretching vibrations of methyl group absorb at 2923 cm^{-1}, 2854 cm^{-1}. A strong band occurred at 1708 cm^{-1} is assigned as a stretching vibration of C=O which is conjugated to aromatic ring. A peak at 1603 cm^{-1} indicates the C=C stretching vibration of alkenic group. Strong bands at 1456 cm^{-1} and 1377 $cm^{-1}$are attributed to the O-H bending vibration.

Table 5 Assignment for FT IR Spectrum of Ethyl Acetate Extract of Setkupala (No.2)

Wavelength (cm^{-1})	**Assignment**	**Possible Compounds**
3359	-OH stretching	Phenolic compounds, glycosides, or alkaloids
2923, 2854	-CH stretching	Aliphatic chains
1708	C=O stretching	Aromatic ring
1603	C=C stretching	Unsaturated carbonyl compounds
1456, 1377	C-H bending	Aliphatic chains

Analysis of Setkupala (No.2)

The sample was determined by HPLC-MS (TOF) Agilent Mass Hunter, Workstation Software B.04.00). According to the result, it could be known that the peaks showed at the retention time 3.317 min, 6.317 min, and 6.57 min respectively. Then the Agilent Mass Hunter software interpreted at 144.0908 m/z, 218.1219 m/z, and 287.1758 m/z for these peaks. As they were studied by library, that might be possible compounds indicated that N-Acetyl-dl-alanine methyl amide, Ethanol, 2,2'-oxybis-, dipropanoate and 1-Serine, N, O-bis (pivaloyl)-, methyl ester compounds might be present in the Setkupala (No.2).

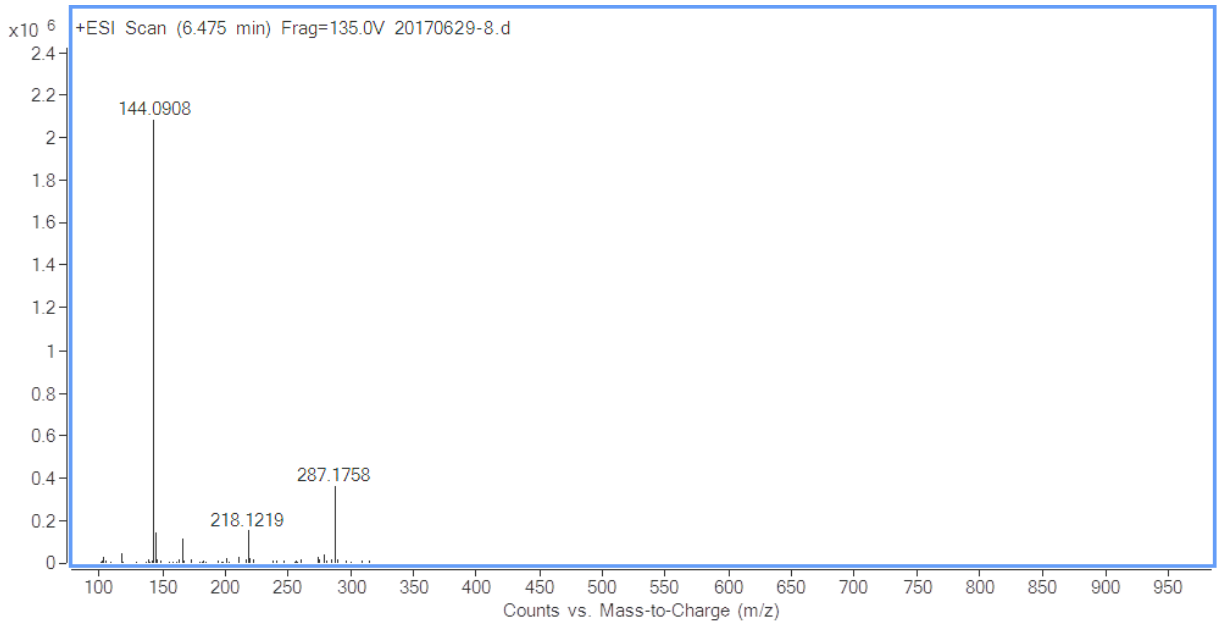

Figure 2 Spectrum review of chromatogram result of Setkupala (No.2)

Conclusion

According to phytochemical study, Setkupala (No.2) reveals that it contains carbohydrates, glycosides, phenolic compound, reducing sugars, saponins, and tannins.

From the study of elemental analysis, it is found out that Setkupala (No.2) contains chlorine, potassium, silicon, sulphur, iron, strontium, titanium, manganese, zirconium, rubidium, zinc, copper, and nickel by EDXRF. Moreover, iron, selenium, zinc, manganese, arsenic, lead, and cadmium were studied by AAS method. Composition of the some elements for a dose was under the maximum permissible level of WHO standard. However, the amount of iron, arsenic, and lead in a dose or five tablets was over the maximum permissible level. According to the data, it is suggest that the two tablets were a suitable dose per day. On the other hand, it should be continued that the elemental content of ingredients of Setkupala (No.2) should be determined and the amount of ingredients which have high toxic level will be reduced in the formulation.

By the study of antimicrobial activity, it was clearly noted that the different extracts of Setkupala (No.2) were effective against some pathogenic microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida*

albicans and *E.coli sp.* Therefore, Setkupala (No.2) has antimicrobial activity. Furthermore, it possess higher antioxidant activity than that of western medicine, ocuvite. And it was seen that N-Acetyl-dl-alanine methylamide, Ethanol, 2,2'-oxybis-, dipropanoate and 1-Serine, N, O-bis (pivaloyl)-, methyl ester compounds might be present in the Setkupala (No.2) by HPLC-MS (TOF).

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