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CHEMICAL COMPOSITION AND ANTIMICROBIAL ACTIVITY OF *HARAMAYA PROPOLIS* (BEE GLUE), ETHIOPIA

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ABSTRACT

The present study was aimed at investigating the chemical composition and antifungal activity of Ethanol Extracted Haramaya propolis (EEHP). The GC-MS analysis of EEHP showed the presence of sixteen compounds and twelve compounds were identified by means of their retention times, by comparison of their mass spectra with the NIST 2005 library data and literature. The major constituents of EEHP were Benzenamine, N, N-dibutyl- (21.94%), Paromomycin (9.74%), 4-Aminobutyramide, N-methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]- (9.26%) and DL-Tryptophan, 5-methoxy (7.43%). The Crude EEHP showed antifungal activities against *Fusarium sp., Aspergillus niger* and *Colletotrichum sp.* GC-MS results showed that the antifungal activities of Haramaya propolis could be because of the presence of 3-(α-Hydroxyethyl)-aniline, Benzenamine,N, N-dibutyl, DL-Tryptophan,5-methoxy, 6-Amino-5, 8-dimethoxyquinazoline, Paromomycin and Imidazole, 2-fluoro-5-[2-carboxyvinyl].

INTRODUCTION: Propolis (bee glue) is a sticky dark-colored material that honeybees (*Apis mellifera*) collect from living plants, mix it with wax and use it in the construction and adaptation of their nests, mainly to fill out cracks in the bee hive. It also functions to repair the hive, to seal openings in the hive and to eliminate contaminating microorganisms in the hive [1, 2]. Propolis is not only a building material; it is the most important "chemical weapon" of bees against pathogen microorganisms and has been used as a remedy by humans since ancient times.

The word propolis was probably coined by Aristotle from the Greek words "pro" meaning "in front of" and "polis" meaning "city". The combined meaning then becomes "In front of the City" or "Defender of the City (or Beehive)" and this is how bees use propolis. It has been used in folk medicine since ancient times and is now known to be a natural medicine with antibacterial, antifungal, anesthetic, antitumoral, antioxidative, anti-inflammatory, imuno-modulatory, cancer prevention, anti-viral, anti-yeast, antimicrobial, cardiovascular and other beneficial activities [3].

With the advent of modern chromatographic techniques frequently associated with Mass Spectrometry (MS), many compounds have been isolated and identified in propolis [4]. But the complex chemical composition of propolis is frequently updated due to many regional variations.

More than 300 propolis constituents have been identified by Gas Chromatography-Mass Spectrometry (GC-MS), chromatographic and spectroscopic techniques. These compounds are composed of 50% resin (polyphenolic fraction) and balsam (cream), 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% other substances [5].

Literature survey revealed that flavonoids, aromatic acids, diterpenic acids and phenolic compounds appear to be the principal components of propolis samples. The properties and actual contents of propolis depend on the collecting location of the bees, time of year and plant source [6]. For many years, GC-MS has been used for the detailed analysis of the main volatile and semi-volatile components of propolis [7, 8].

As volatile components, various mono and sesquiterpenes are found in propolis [9]. Other constituents of volatile oils include alcohols; mainly aromatic alcohols, phenols, aldehydes, ketones, acids (from acetic to stearic acid), esters, a series of alkanes, alkylated benzenes and naphthalene [10]. Propolis contains, however, many components that are not volatile enough for direct GC- MS analysis even upon derivatization or high-temperature [10].

The great variability in chemical composition of the propolis from different regions is because honeybees extract raw materials from different plants in different ecosystems for their production of propolis [11]. Crude Ethanol Extract of Propolis (EEP) collected from Egypt and South Africa showed antimicrobial activity against a wide range of pathogenic bacteria, fungi, yeasts and viruses [12, 13].

Although, numerous researchers have reported the antimicrobial activity and chemical composition of propolis collected worldwide, information about Ethiopian propolis are still insufficient. The aim of this study was to investigate antifungal activity and chemical composition of propolis sample collected from Haramaya in Ethiopia. Besides, the chemical composition of propolis as well as its color, aroma and probably its medicinal characteristics are changed according to the geographical zones and the season of the year.

EXPERIMENTAL:

Description of the Study Area: Haramaya University is located at a latitude of 9^{o} 20' north of the Equator and 42^{o} 03' longitude east of meridian. The university has a total area of about 46 km^2. It has a moderate average temperature of 16^{o}C, and the mean maximum and minimum annual temperature is 24.02 and 9.73^{o}C, respectively [14].

The mean annual rainfall is 780 mm. The 1980 m elevation of the area (*weinadega*) ensures that it enjoys a relatively moderate and pleasant climate throughout the year. There were 12 beehives in the university. Among these 5 hives are traditional and 7 of them are modern beehives. *Eucalyptus glublus, Eucalyptus camnadulesis* (exotic*), Vernonia amygdalina (*indigenous*), Spathodea nilotica (exotic), Jacaranda mimosifolia (*exotic*), Pinus radiate(*exotic*), Olea africana, Cordial Africana* and *Grevillea robusta* are dominant plants and vegetations in Haramaya [14].

Propolis Samples Collection: Samples of propolis (150 g) was collected by hand (by scrapping from frames and walls of the beehives) from Haramaya University Beekeeping Sections from October 2010 to November 2010 and kept in the refrigerator until processed.

FIG. 1. HARAMAYA UNIVERSITY BEEKEEPING SECTION

Extraction and Sample Preparation: The propolis sample collected was air dried, crushed into pieces and then weighed and 100 g was mixed vigorously with 70% absolute ethyl alcohol (Analytical reagent grade, Eastwayspark, U.K) in a ratio of 1 g: 5 mL (w/v) and then sealed in a container with intermittent shaking twice a day for two weeks [15]. It is important to allow the alcohol molecules to come into contact with as many propolis compounds as possible, in order to extract them from the solid mass. After two weeks, the supernatant liquid was filtered with Whatman No. 1 filter paper. The liquid portion was stored in a dark brown bottle in a cool, dry and dark place. The EEHP solution was then filtered through Whatman No. 1 filter paper to avoid waxes. The alcohol was evaporated with a Rota vapor under vacuum and reduced pressure to yield 38.5 g.

It was kept in a clean dark, airtight bottle in a refrigerator at 5°C until used. About 10 mg of the crude extract was subjected to GC-MS analysis by head space sampler in Ambo University.

GC-MS Analysis of Ethanol Extract of Haramaya Propolis (EEHP): GC-MS analysis of EEHP was performed using a Perkin Elmer Clarus 600 GC-MS fitted with a HP-5 MS capillary column (30 m length x 320 µm internal diameter coated with 5% diphenyl 95% dimethylpolysiloxane, film thickness 0.25µm). The column oven temperature was initially held at 60°C for 2 min, then programmed to rise to 170°C at a rate of 20 °C/min and held for 3 min. The temperatures of the injector port and the MS interface were set at 220 and 200 °C, respectively.

Helium was used as the carrier gas at a flow rate of 1 mL/min with a column inlet pressure of 40 psi. The mass spectra were recorded in electron ionization (EI) mode at 70 eV with scanning from m/z 35 to m/z 450 amu with a run time of 60 min and mass source of 230 °C. The oven, needle and transfer temperature of the head space sampler were 150 and 170°C, respectively at a constant mode of operation. The time of coc cycle, thermostat, pressurization, injection and withdrawal of the head space sampler was 60, 10, 3, 0.05 and 0.2 min, respectively.

The identification of various compounds present in propolis sample were carried out by computer search on a NIST 2005 MS data library. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the basis of its mass-spectral fragmentation. The components of EEP were determined by considering their areas as percentage of total ion chromatogram. Some compounds could not be identified due to the lack of authentic samples and their library spectra.

Antimicrobial Assay: Bioassays of the antifungal activities of crude EEHP towards three common phytopathogenic fungi namely; *Fusarium sp., Aspergillus niger* and *Colletotrichum sp.* was tested only by the Petridish bioassay method [13]. These phytopathogenic fungi were taken from infected avocado fruits. The hosts were first dipped in Sodium hypochlorite (Clorax) at 10% concentration for 2 minutes and then rinsed with sterile distilled water twice and plated on Malt Extract Agar (MEA) in petridishes and incubated at 25°C for 7 days. The fungi were only identified to genus level based on hyphal and spore morphology [16, 17].

A B C D

FIG. 2: (A) INFECTED AVOCADO FRUITS BY FUNGI (B) CULTURED *FUSARIUM SP.* (C) *ASPERGILLUS NIGER* AND (D) *COLLETOTRICHUM SP. IN MEA* MEDIA.

Though extraction of propolis was done with 70% ethanol, treatments were done with 60% ethanol to minimize the effect of a highly concentrated solvent. Propolis concentrations 1, 2, 5, 7 and 10 mg/mL were prepared using 60% ethanol. The control for all experiments was 60% ethanol in place of different extract concentrations. Each experiment was repeated three times [18]. All the tripled MEA media were sterilized by autoclaving at 121°C and 1.03 bar for 15 min. Each of the grown fungi was placed onto the center of the MEA plates using a sterile cork borer of 10 mm diameter and then incubated at 25°C. The zone of inhibition on the mycelium growth of each fungus for each replicate was measured in mm as the average of two cross diameters continuously after every 48 h of inoculation until they almost filled the petridishes without EEHP. Finally, the Relative Inhibition (RI) by the chemicals on mycelia growth of each fungus at different concentrations was calculated for the last day observation as percentage inhibition over control as follows:

$$RI = \frac{\text{(Hyphal extension of control [cm] – Hyphal extension of experiment [cm])}}{\text{Hyphal extension of control [cm]}} \times 100$$

Statistical Analysis of Data: The RI was calculated for the 192 h incubation period. Data were expressed as mean ± standard deviation. The data obtained were subjected to one-way ANOVA test to determine whether there was significant difference among each concentration and also between the lengths of incubation [13].

RESULTS AND DISCUSSION: The chemical composition of propolis are dependent on its geographical location; as a result, its biological activity and chemical composition is closely related to the vegetation native to the site of collection [1, 19]. The chemical composition of the EEHP has been determined by GC-MS analysis. The GC-MS analysis of Haramaya propolis showed the presence of sixteen components (**Figure 3**) and twelve compounds were identified by means of computer search on a NIST 2005 MS data library on the basis of mass spectral fragmentation representing 84.8% of the total compounds.

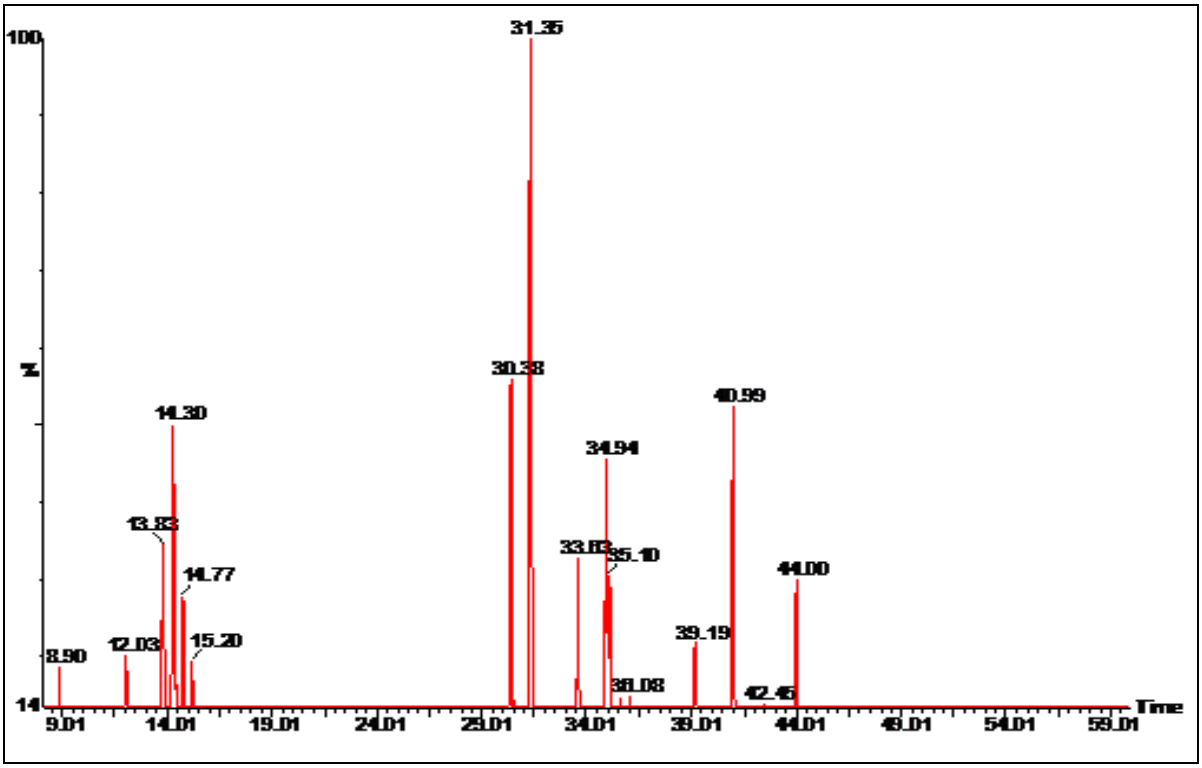


FIG. 3: GC OF EEHP

These include high content of Benzenamine, N, N-dibutyl- (21.94%) and a low content of 3-(α-Hydroxyethyl)-aniline (0.96%). The major constituents of EEHP were Benzenamine, N, N-dibutyl (21.94%), Paromomycin (9.74%), 4-Aminobutyramide, N-methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]- (9.26%), DL-Tryptophan, 5-methoxy (7.43%), 6-Amino-5, 8 dimethoxyquinazoline (6.44%) and 2,7-Dioxatricyclo [4.4.0.0(3, 8)]decan-4-amine (6.25%) (**Table 1**).

Unidentified components were present in such low amounts that either no mass spectrum could be recorded or the spectrum was too poor for interpretation.

TABLE 1: CHEMICAL COMPOSITION OF CRUDE ETHANOL EXTRACTS OF PROPOLIS OBTAINED FROM HARAMAYA BEEHIVES ANALYZED BY GC-MS

Compound	t_R	Comp.(%)
3-(α-Hydroxyethyl)-aniline	8.90	0.96
Imidazole,2-fluoro-5-[2-carboxyvinyl]-	12.03	4.66
2,7-Dioxatricyclo[4.4.0.0(3,8)] decan-4-amine	13.83	6.25
Paromomycin	14.30	9.74
Bicyclo[3.3.1]nonan-2-ol,exo	14.77	3.41
Benzenamine,N,N-dibutyl-	31.35	21.94
6-Amino-5,8-dimethoxyquinazoline	33.63	6.44
DL-Tryptophan,5-methoxy	34.94	7.43
1-(4-Methyl-2-pyridyl)-1-propanone semicarbazone	35.10	5.13
Morpholine,4-(1-cyclopentylpiperidin-4-yl)-	39.19	4.06
4-Aminobutyramide,N-methyl-N-[4-(1-pyrrolidinyl)-2-butynyl]-	40.99	9.26
N-(1-Cyclohexen-1-yl)piperidine	44.00	5.52

A comparison of the results of this work with those of previously reported composition of Ethiopian propolis reveals significant differences. The major constituents of Ethiopian propolis collected from Holeta Honeybee Research Center are amryn type triterpenic alcohols (26.2%), sugars (24.9%) and fatty acids (7.5%), with significant amount of aromatic acids, caffeic acid, esters and other alcohols including diterpenic acids [20]. Previous GC-MS analysis of Ethiopian propolis collected from Holeta confirmed the absence of flavonoids [20].

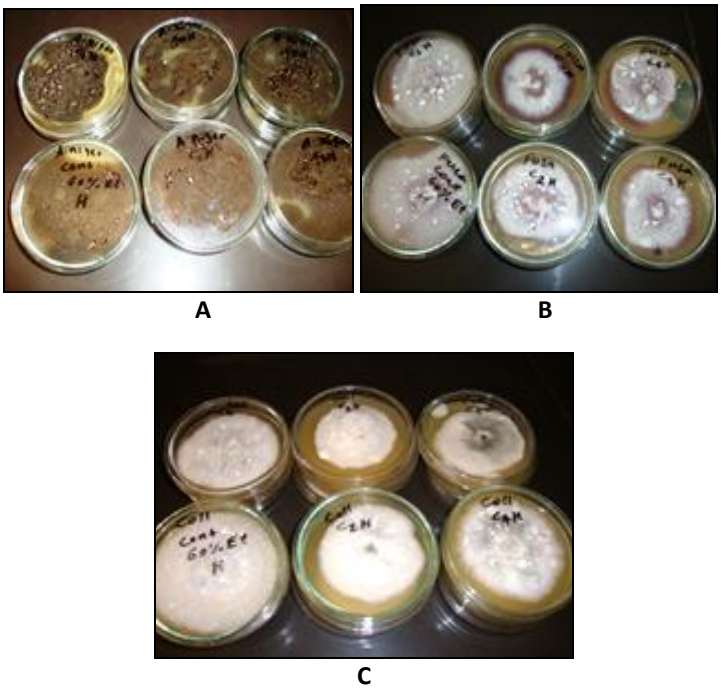
However, the major components of propolis in Croatia, Bulgaria, Italy, Switzerland, Brazil, Egypt and China are terpenoids (various mono and sesquiterpenes), flavonoids (pinocembrin, pinostrobin, pinobanksin, pinobankasin-3-O-acetate, chrysin, galangin, flavones, flavanone and flavonones) and phenolic acid (prenyl esters of caffeic, ferulic acids, *p*-coumaric acid and acrylic acid) [21-25].

The variability of constituents of propolis samples could be due to the difference in propolis collecting honeybee species from different plants depending on the geographic location. *Apis mellifera jementica* (propolis collecting Honeybee race in Haramaya) is a type of bee species that collect propolis in the eastern, semi-arid lowlands and northwest low lands areas of Ethiopia [26].

These Honeybee race added other materials during the production of propolis from different plants depending on the geographic location. The plant sources of identified compounds from Haramaya propolis remain unknown in the present study. However, *Baccharis dracunculifolia, Araucaria angustifolia, Araucaria heterophylla, Clusia minor and Eucalyptus citriodora* are the main sources of the bee glue in Brazil [27].

The antimicrobial constituents of propolis resins and extracts are commonly phenols, flavonoids, aromatic acids and diterpenic acids [28, 29]. The inhibition effect of the crude EEHP, against mycelia growth of three common phytopathogenic fungi namely; *Fusarium sp., Aspergillus niger* and *Colletotrichum sp.* was tested at five different concentrations (1, 2, 5, 7 and 10 mg/mL) by the Petri dish bioassay method and are recorded in **Table 2**.

The mycelium growth of each fungus for the crude extract treatment was measured as the average of two cross diameters continuously after every 48 h of inoculation, until they almost filled the petridish without EEP (**Table 2 and Figure 4**). Then the inhibition effect of the crude extract on mycelia growth of each fungus at different concentrations was calculated for the last day observation as percentage inhibition over control.



A **B**

C

FIG. 4: INHIBITION EFFECT OF THE CRUDE EEHP ON: (A) *ASPERGILLUS NIGER* GROWTH (B) *FUSARIUM SP.* (C) *COLLETOTRICHUM SP.*

Table 2 showed that the crude extract caused inhibition of mycelia growth of *Aspergillus niger,* at all concentrations; 1 mg mL^{-1} (2.90%), 2 mg mL^{-1} (9.10%), 5 mg mL^{-1} (5.18%), 7 mg mL^{-1} (11.1%) and 10 mg mL^{-1} (15.70%). Similarly, the crude ethanol extract at all concentrations caused inhibition of mycelia growth of *Fusarium sp.* and *Colletotrichum sp* and inhibition increased with increasing concentration.

The results of the antifungal activity of Haramaya propolis are in agreement with the findings of [12] who determined the antifungal activities of Egyptian propolis against nine fungal genera namely *Cladosporium*, *Mucor*, *Scopulariopis*, *Penicillium*, *Rhizopus*, *Fusarium*, *Aspergillus*, *Alternaria* and *Rhodotorula*.

The GC-MS results showed that the antifungal activities of Haramaya propolis could be because of the presence of 3-(α-Hydroxyethyl)-aniline, Benzenamine, N, N-dibutyl, DL-Tryptophan, 5-methoxy, 6-Amino-5, 8-dimethoxyquinazoline, Paromomycin and Imidazole,2-fluoro-5-[2-carboxyvinyl].

Significant inhibition were achieved at EEP concentration of 10 mg mL^{-1} for *Aspergillus niger* and *Fusarium sp.* ($P<0.0001$). No significant difference ($P>0.05$) was observed at all concentrations against *Colletotrichum* sp. There was no also significant difference ($P > 0.05$) between 1 and 5 mg mL^{-1} against *Aspergillus niger* and *Fusarium sp*.

Data showed that mycelium growth of *Fusarium sp.* and *Colletotrichum sp.* were inhibited by the crude extract while fast growing mycelium *Aspergillus niger* was not affected much.

These findings are also supported by earlier reports on Evaluation of an EEP as a Potential Pre and Post-Harvest Fungicide for 'Fuerte' Avocado (*Persea americana* Mill.) Fruits and Orchards [13].

Egyptian, Albanian, Austrian, Bulgarian, French, German, Mongolian and British propolis also showed antiviral antimicrobial activity against *Staphylococcus aureus; Escherichia coli* and *Candida albicans* [12].

TABLE 2: EFFECT OF THE CRUDE EEHP ON THE GROWTH OF THREE PHYTO PATHOGENIC FUNGI

Fun	Conc. (mg/mL)	Relative Inhibition (RI) of mycelium growth (%) of three phyto pathogenic fungi with incubation period (h)					
		48 h	96 h	144 h	192 h	Overall results	
		RI	RI	RI	RI	Mean RI	Std. Dev. of RI
As	1	-	1.8	2.7	7.1	2.900	3.0166
	2	10.3	12.3	6.7	7.1	9.100	2.6733
	5	2.6	7	4	7.1	5.175	2.2396
	7	5.7	28.4	7	3.3	11.100	11.6347
	10	17.1	29.6	10.5	5.6	15.700	10.3958
Fus	1	2	9.6	4.5	15.6	7.925	6.0152
	2	7.8	11.5	4.5	16.7	10.125	5.2335
	5	9.8	5.8	6	16.7	9.575	5.0940
	7	21.6	9.6	7.5	20	14.675	7.1542
	10	33.3	17.3	12	22.2	21.200	9.0785
Col	1	15.4	14	12	2.4	10.950	5.8683
	2	8.6	29.6	11.6	3.3	13.275	11.4115
	5	11.4	39.8	3.5	1.1	13.950	17.7862
	7	22.9	33	9.3	2.2	16.850	13.7726
	10	25.6	15.8	17.3	10.6	17.325	6.2190

***Key**: Fun: Fungi,* h= hour*, As: Aspergillus niger*, *Fus: Fusarium sp.* and *Coll : Colletotrichum sp.*

EEHP concentration of 10 mg mL^{-1} showed greater inhibition than 7 mg mL^{-1} against *Fusarium sp., Aspergillus niger* and *Colletotrichum sp.* No significant difference between 2 and 5 mg mL^{-1} were found ($P > 0.05$) against *Fusarium sp* and *Colletotrichum sp.* except *Aspergillus niger*, which showed a significantly higher inhibition at 2 mg mL^{-1} ($P < 0.0001$). All the five different concentrations showed different inhibitory effects against *Aspergillus niger*, *Fusarium sp.* and *Colletotrichum sp* (Table 2).

CONCLUSION: A matter of great concern regarding the production and use of propolis is the variation of its chemical composition, which has motivated proposals for quality chemical control [29, 30].

This study has confirmed that the identified components and their composition of Haramaya Propolis are different from previous research works. The composition of propolis may be affected by the bee race, soil, climate, vegetation native, trapping mechanism and altitude. The crude EEHP showed antifungal activities against *Fusarium sp., Aspergillus niger* and *Colletotrichum sp.*

The GC-MS results showed that the antifungal activities of Haramaya propolis can be attributed to its components as 3-(α-Hydroxyethyl)-aniline, Benzenamine, N, N-dibutyl, DL-Tryptophan, 5-methoxy, 6-Amino-5, 8-dimethoxyquinazoline, Paromomycin and Imidazole, 2-fluoro-5-[2-carboxyvinyl].

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