

Nature's Pharmacy in the Highlands: Extraction and Profiling of Bioactive Principles from *Euphorbia nivulia* of Vythiri, Kerala, India

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Abstract

Euphorbia nivulia, is an Ethno-medicinal plant from Vythiri Taluk, Wayanad District, Kerala, India, has been employed in treating various human and animal ailments. The present investigation aimed to assess its antimicrobial potential and to identify and characterize its phytochemical constituents using advanced techniques such as High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography–Mass Spectrometry (LC/MS-MS). Antimicrobial efficacy was evaluated using the disc diffusion method against opportunistic microbes such as *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative), as well as *Bacillus subtilis* and *Staphylococcus aureus* (Gram-positive). The methanolic leaf extract of *E. nivulia* exhibited significant antibacterial activity. LC/MS-MS analysis revealed a rich phytochemical profile dominated by alkaloids and flavonoids, compounds recognized for their diverse biological properties. HPLC profiling identified five major peaks, suggesting the presence of potentially novel metabolites and bioactive principles. The findings observed inhibition zones ranged from 2.7 to 3.3 cm in very lower concentration and emphasized that the therapeutic effect of *Euphorbia nivulia* as a natural source of antimicrobial agents and support its continued investigation for development of phytopharmaceutical agents.

Keywords: Antimicrobial, Bioactive Principles, *Euphorbia nivulia*, HPLC, LC-MS/MS.

INTRODUCTION

From time immemorial, humans have relied on plants for food, medicine, tools, shelter, fuel and dyes [1–2]. Currently, extensive research is being conducted on the photochemical and pharmacological constituents of medicinal plants traditionally used across diverse global healthcare systems [3]. In India, with approximately 45,000 plant species and 550 tribal communities belonging to 227 ethnic groups—each inhabiting distinct geo-climatic regions—boasts a rich repository of biodiversity and ethnobotanical knowledge [4]. These indigenous communities, deeply embedded in natural ecosystems, are custodians of a vast body of traditional ecological and ethnomedical wisdom. However, much of this traditional knowledge is handed down orally from generations and is undocumented among several tribal groups, lacking ethnobotanical knowledge pertaining to prescriptions, pharmacological practices, diagnostic approaches, and disease perceptions [5].

The World Health Organization (WHO) estimates that around 80% of the population in developing countries relies primarily on traditional plant-based medicines for healthcare needs [6]. At least 25% of contemporary pharmaceuticals are derived directly from plants, with numerous synthetic drugs paneled on phytochemical templates. Plant-derived therapies are generally safer, more cost-effective, and environmentally sustainable compared to synthetic alternatives [7]. The extensive use of synthetic antimicrobial agents has led to the development of resistant strains of microbial pathogens that are

resulted in the repeated search for newer antimicrobial agents. Development of resistance among pathogenic microbes for synthetic antimicrobial agents driven by microbial genetic adaptability-poses a significant global health challenge [8]. Despite the widespread use of plant-based ingredients in modern medicine, phytochemical and pharmacological evaluations have been conducted on only a small fraction of the estimated 250,000 to 500,000 plant species [9–10].

Ethnobotanical research has demonstrated that many plants traditionally used by indigenous communities exhibit antimicrobial properties, particularly relevant in the context of escalating antibiotic resistance [11–13]. In this study, the medicinal plant *Euphorbia nivulia*, traditionally used in Vythiri Taluk, Wayanad district (Kerala), was selected to investigate its phytochemical and antimicrobial potential, with the aim of contributing to its broader application in public health and therapeutic innovation.

MATERIALS AND METHODS

Study area

Vythiri Taluk, located in the Wayanad district of Kerala, was selected for the present study due to its exceptional phytodiversity. Field surveys and sampling were conducted from December 2021 to April 2022. Wayanad, situated in northeastern Kerala with its administrative center at Kalpetta, spans an area of 2,131 km²—accounting for 5.48% of the state's total area—at altitudes ranging from 700 to 2,100 meters within the Western Ghats, between latitudes 11°27'–11°58'35" N and longitudes 75°47'50"–76°26'35" E. Renowned for its wealth of medicinal flora and dense tribal habitat, the region is home to indigenous communities such as the Kurichia, Kuruma, Kattunaika, Adiyar, and Paniya tribes.

Vythiri, situated at approximately 700 meters above sea level, exhibits a cooler climate compared to other parts of Wayanad. The area's geology comprises basic rocks (36.69 km²), the Charnockite group (4214.93 km²), the Migmatite complex (564.41 km²), and the Peninsular Gneissic complex. Geomorphologically, it features Denudational structural hills (2504.99 km²), Piedmont zones (2049.37 km²), residual hills (166.61 km²), rock exposures (7.86 km²), valleys (132.09 km²), and water bodies (8.86 km²) [14].

Key biodiversity hotspots in the region such as Banasurasagar, Chembra Peak, Pookot lake, and Soochipara Falls contribute significantly to its ecological richness. Agriculture remains the primary livelihood, with major crops including paddy, areca nut, banana, ginger, pepper, cardamom, coconut, turmeric, tea, and coffee. Despite the abundance of ethnobotanical knowledge, numerous plant species remain undocumented or underutilized. Increasing modernization poses a threat to this traditional wisdom, underscoring the urgent need for systematic documentation and preservation efforts [15].



Figure 1: Traditional Medical Plant Collection and Study area at Vythiri, Wayanad, Kerala, India

According to the preliminary survey on the usage of endemic medicinal plants, the herbal medicine is still used to treat the majority of ailments in a few communities around this district. As a result, this research aimed to conduct a systematic investigation of the numerous less-exploited endemic species of this region. In this context, current study of medicinal plant from the selected area was *Euphorbia nivulia* of the family Euphorbiaceae.

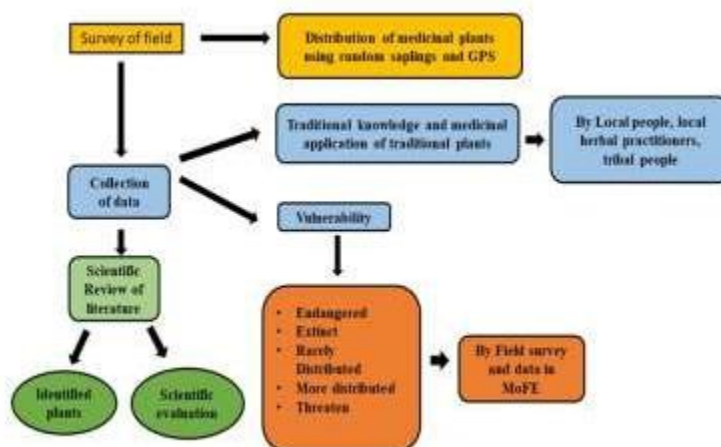


Figure 2: Schematic representation of the scientific survey of traditional knowledge and Medicinal application of plant species

Euphorbia nivulia specimens were whole-harvested from the Western Ghats region of Wayanad, specifically Vythiri Taluk, and immediately stored in sterile polyethylene bags to prevent contamination and degradation. Taxonomic identification of the collected plant material was conducted through a combination of comparative analysis with published literature, consultation of subject experts and local taxonomists and validation by professional botanists.

Post-identification, the plant material was thoroughly rinsed with distilled water under aseptic conditions to eliminate surface impurities, followed by shade-drying at ambient room temperature to preserve phytochemical integrity. Once dried, morphological components namely leave, stems, and roots were carefully segregated with analytical focus directed primarily towards the leaf fraction. The leaves were subsequently dried to constant weight and pulverized into a fine powder using a sterilized high-speed grinder for approximately 20–30 seconds to ensure uniform particle size and sample homogeneity.



Figure 3: Preparation of plant sample *Euphorbia nivulia*

Plant extraction procedure for *Euphorbia nivulia*

A thimble containing 20.0903g of *Euphorbia nivulia* leaf powder was placed into the Soxhlet extractor. A total of 500 mL of methanol was used for extraction an initial portion was added directly into the Soxhlet extractor to saturate the sample, and the remaining volume was transferred to a round-bottom flask connected to the system. The extraction was carried out using a heating mantle to maintain consistent thermal input, facilitating the evaporation and condensation of methanol in a continuous cycle. This Soxhlet extraction was maintained for 24 hours to ensure thorough extraction of phytochemicals.

Following extraction, the system was carefully dismantled and the extract filtered to remove particulates. The methanol solvent was then completely evaporated using a rotary evaporator under reduced pressure. The concentrated residue was reconstituted in 10 mL of methanol, transferred into sterile petri dishes and left for air-drying to yield the final extract.

Antibacterial Activity of *Euphorbia nivulia*

The antibacterial activity of *Euphorbia nivulia* leaf extract was evaluated using the agar well diffusion method with slight procedural modifications as described by Balouiri *et al.* [16]. Nutrient Agar (NA) plates were prepared by aseptically dispensing 20 mL of sterilized NA media into sterile petri dishes, allowing them to solidify under laminar airflow conditions. After solidification, 100 μ L of standardized microbial inoculum ($\sim 10^8$ CFU/mL) of *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* were uniformly spread over the surface using sterile cotton swabs.

Wells of 6 mm diameter were aseptically punched into the agar using a sterile cork borer. Penicillin was used as the positive control, while Dimethyl Sulfoxide (DMSO) served as the negative control. The extract solution (prepared as described previously) was loaded into the wells, and the plates were pre-incubated at 4 °C for 4 hours to facilitate the diffusion of antibacterial metabolites. Subsequently, the plates were incubated at 37 °C for 24 hours.

Zones of inhibition were measured in millimeters (mm), and the mean diameter was calculated from three independent replicates to assess the antimicrobial efficacy of the extract.

RESULTS

Antimicrobial analysis: disc diffusion method

The leaf extract was tested at two concentrations (10 μ g/mL and 20 μ g/mL) against four bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* using the agar well diffusion method. Penicillin (10 μ g) served as the positive control, while saline solution was used as the negative control. Zones of inhibition were measured and expressed as mean values $\pm$ standard error (SE) across three replicates (Table 1 & Figure 4).

The methanolic extract of *Euphorbia nivulia* exhibited substantial antibacterial activity across all tested strains, showing a concentration-dependent increase in inhibitory effect. At 20 μ g/mL, the extract produced the largest inhibition zones 3.3 cm for *P. aeruginosa*, 3.2 cm for *S. aureus*, 3.1 cm for *E. coli*, and 2.9 cm for *B. subtilis*. These values were notably comparable, and in some cases, superior to the standard penicillin control, suggesting promising bactericidal potential.

Importantly, no inhibition was observed for the negative control (saline), confirming that the antibacterial effects were attributable to the bioactive compounds in the extract rather than solvent interference. The narrow standard errors indicate good reproducibility of the assay.

This data suggests that *Euphorbia nivulia* leaf extract holds considerable promise as a natural antimicrobial agent, with efficacy against both Gram-negative (*E. coli*, & *P. aeruginosa*) and Gram-positive (*B. subtilis*, & *S. aureus*) pathogens. Further studies involving minimum inhibitory concentration (MIC) and phytochemical profiling would help elucidate the active constituents responsible for this activity.

The present investigation demonstrates that the methanolic leaf extract of *Euphorbia nivulia* exhibits notable antibacterial activity against both Gram-positive and Gram-negative bacterial strains, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*. The observed inhibition zones ranged from 2.7 to 3.3 cm, with a concentration-dependent increase in efficacy. Notably, the extract

at 20 µg/mL outperformed the standard antibiotic (penicillin, 10 µg) in several cases, particularly against *P. aeruginosa* and *S. aureus*, suggesting the presence of potent bioactive constituents.

Table 1. Antimicrobial activity of *Euphorbia nivulia* against *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus*

Target	Zone of inhibition (in cm)			
	10 µg/mL	20 µg/mL	Standard	Negative control
<i>E. coli</i>	2.8 ± 0.02	3.1 ± 0.01	2.7 ± 0.04	NI
<i>P. aeruginosa</i>	3.1 ± 0.03	3.3 ± 0.01	3.0 ± 0.01	NI
<i>B. subtilis</i>	2.7 ± 0.02	2.9 ± 0.03	2.5 ± 0.04	NI
<i>S. aureus</i>	3.1 ± 0.01	3.2 ± 0.02	2.9 ± 0.01	NI

±: denotes the standard error; NI: no inhibition; Standard: 10 µg Penicillin; Negative control: saline solution. Values are means of triplicates

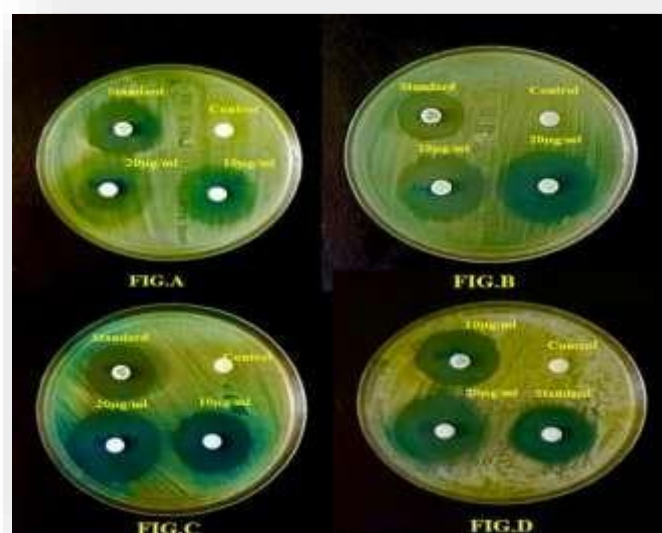


Figure 4: Antimicrobial activity of *Euphorbia nivulia* extract: Fig. A. *E. Coli*; Fig. B. *P. Aeruginosa*; Fig. C. *B. Subtilis*; Fig. D. *S. aureus*

HPLC analysis of *Euphorbia nivulia*

The HPLC fingerprinting and qualitative analysis of *Euphorbia nivulia* were done and are showcased in Fig. 5. A total of 8 major peaks were extracted from the HPLC analysis with prominent retention factors. Peak 1 with R_t value 1.68 corresponds to carvacrol. Peak 2 with R_t value 2.46 corresponds to alpha-pinene, peak 3 with R_t value 4.6 corresponds to gallic acid, peak 4 with R_t value 7.925 corresponds to gamma-terpinene, peak 5 with R_t value 8.46 corresponds to quercitrin, peak 6 with R_t value 10.60 corresponds to 1-terpinen-4-ol, peak 7 with R_t value 12.4 corresponds to caffeic acid and peak 8 with R_t value 15.56 corresponds to chlorogenic acid and chemical composition is showcased in Table 2.

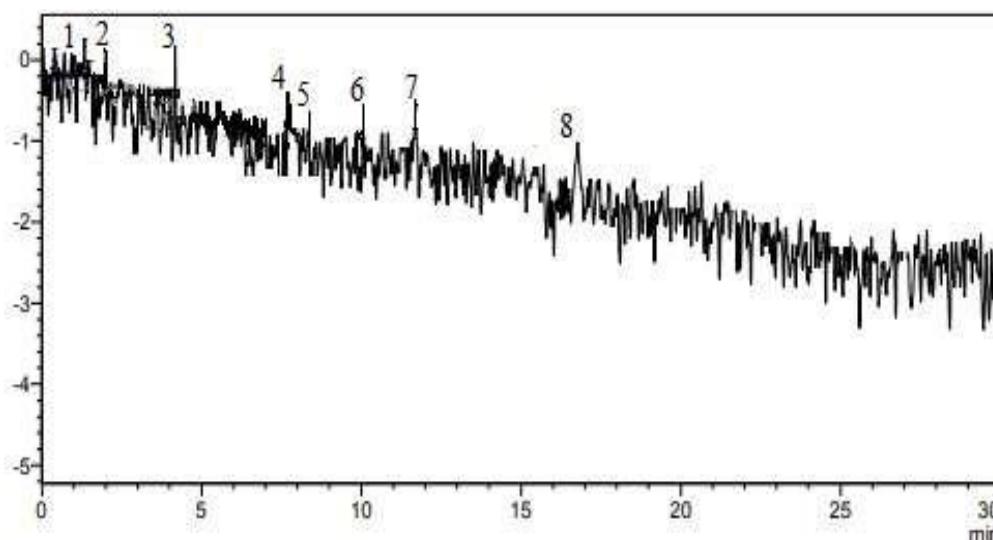


Figure 5: HPLC graph of *Euphorbia nivulia*

Table 2. Chemical composition of *Euphorbia nivulia*

<i>Euphorbia nivulia</i>			
Peak no.	Compound	Area	Retention time
1	Carvacrol	1739	1.68
2	Alpha-pinene	7294	2.46
3	Gallic acid	4446	4.6
4	Gamma-terpinene	4054	7.925
5	Quercitrin	2092	8.46
6	l-terpinen-4-ol	1244	10.60
7	Caffeic acid	1244	12.4
8	Chlorogenic acid	3244	15.56

LC-MS/MS analysis of *Euphorbia nivulia*

The representative of LC-MS/MS chromatograms of *Euphorbia nivulia* is illustrated in Fig 6. The resultant peaks identified were 60.4, 205.4, 269.3, 283.6, 169.1, 283.6, 425.5 m/z values which corresponds to toxifoli (flavonoid), naringenin (flavonoid), luteolin (flavonoid), gallic acid (phenolic glycoside) fisetin (flavonoid) and hydroxydammarone respectively. Most of the phytochemicals identified fall under flavonoids, and phenolic glycoside from the leaf extracts of *Euphorbia nivulia*.

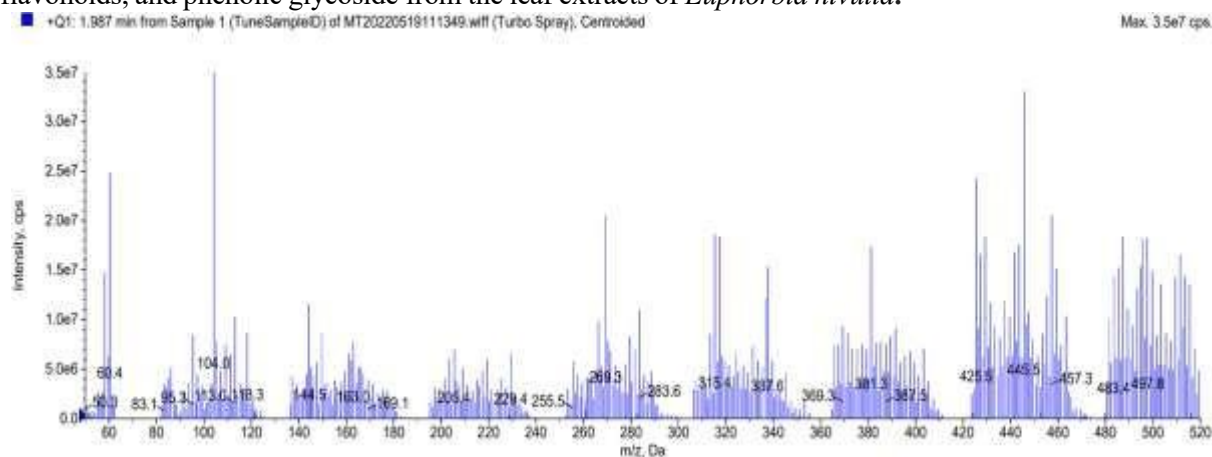


Figure 6: LC-MS/MS analysis of *Euphorbia nivulia*

DISCUSSION

These findings align with previous studies that have reported broad-spectrum antimicrobial properties of *E. nivulia*. Annapurna *et. al.* [17] demonstrated that aqueous alcohol extracts of *E. nivulia* leaves were active against 19 bacterial strains and two fungal cultures, with minimum inhibitory concentrations (MICs) ranging from 3.13 to 200 mg/mL, depending on the organism. The extract showed greater efficacy against Gram-negative bacteria, particularly *E. coli* and *Pseudomonas spp.*, which is consistent with the current results [18].

Phytochemical screening of *E. nivulia* has revealed the presence of flavonoids, terpenoids, alkaloids, saponins, and phenolic compounds—classes of secondary metabolites known for their antimicrobial mechanisms, including disruption of microbial membranes, inhibition of nucleic acid synthesis, and interference with energy metabolism. The polar nature of methanol likely facilitated the extraction of these compounds, contributing to the observed bioactivity [19, 20].

The antibacterial mechanism may be attributed to the synergistic action of these phytoconstituents. For instance, flavonoids are known to form complexes with bacterial cell walls, while terpenoids can disrupt membrane integrity, leading to leakage of cellular contents. Saponins, on the other hand, may enhance permeability and facilitate the entry of other active compounds [21].

Moreover, the absence of inhibition in the negative control (saline) confirms that the antibacterial activity is intrinsic to the plant extract and not due to solvent interference. The reproducibility of results across triplicates further strengthens the reliability of the data.

Given the increasing prevalence of antibiotic-resistant pathogens, the efficacy of *E. nivulia* against clinically relevant strains such as *P. aeruginosa* and *S. aureus* underscores its potential as a source of novel antimicrobial agents. Future studies should focus on bioassay-guided fractionation, isolation of active compounds and elucidation of their molecular targets. Additionally, *in-vivo* validation and toxicity profiling will be essential to advance its therapeutic applicability.

Medicinal plants have supported human survival since antiquity, serving as sources of food, shelter, and therapeutic agents. Over generations, especially among tribal communities, a vast repository of ethnobotanical knowledge has evolved much of it still undocumented and underexplored scientifically. India's rich biodiversity, particularly in regions like the Western Ghats, offers significant potential for drug discovery with traditional medicine providing leads for antimicrobial, anti-inflammatory, anti-diabetic, and anticancer agents.

These plants are valued not only for efficacy but also for safety, cost-effectiveness, and biocompatibility compared to synthetic alternatives [22]. Their medicinal potential is largely attributed to antioxidant compounds—especially phenolics which combat oxidative stress and mitigate diseases such as diabetes, cardiovascular disorders, neurodegeneration, and cancer [23]. While synthetic antioxidants exist, natural variants are preferred for their multifunctionality and reduced toxicity.

Beyond human healthcare, medicinal plants play a critical role in veterinary medicine, especially in rural settings where herbal treatments remain integral. This dual-use supports the conservation of indigenous wisdom and offers safer, economical alternatives to conventional veterinary drugs, which often pose risks of residues and resistance [24, 25].

The escalating issue of antibiotic resistance has intensified interest in the antimicrobial potential of medicinal plants. Many possess potent antibacterial, antifungal, and antiviral properties attributed to secondary metabolites such as alkaloids, flavonoids, and tannins [26]. As resistant pathogens undermine conventional treatments, plant-derived bioactive principles offer promising leads for novel therapeutics. The process begins with careful selection and identification of plant material, factoring in geographic origin, seasonal variation, and climatic conditions [27]. Subsequent steps cleaning, drying, and extraction are critical to preserving phytochemical potency [28]. Traditional and modern extraction methods facilitate the isolation and characterization of these compounds, laying the foundation for new drug development [29].

The global significance of medicinal plants continues to gain momentum across diverse research disciplines. Studies consistently highlight the therapeutic potential of plant-derived compounds in

addressing modern health challenges. The documented antioxidant properties of polyphenols and the potent antimicrobial effects of traditional herbs reinforce the value of ethnobotanical knowledge. Ongoing scientific validation bridges the gap between ancestral wisdom and contemporary medicine, while emphasizing the need to conserve both biodiversity and cultural heritage [30–38].

CONCLUSION

With the escalating loss of traditional knowledge and the growing need for alternative therapeutics, Documentation of indigenous herbal knowledge and conservation of biodiversity are essential. In this study, the survey of Ethnomedicinal knowledge pertaining to Vythiri Taluk, Wayanad, Kerala, India was carried out. The present investigation confirmed that the methanolic leaf extract of *Euphorbia nivulia* has potent antimicrobial activity against opportunistic microbes isolated from immunosuppressant patients. The HPLC and LC-MS/MS studies showed the extract were rich in alkaloids, flavonoids and phenolics compounds. Further, pharmacological and toxicological evaluations are essential for developing a potent therapeutic agent from the herbal extracts for curing microbial infections in immunosuppressant patients and where by the rapid use of synthetic antimicrobial agents associated with their side effects could be subsided.

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