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Phytochemical and Biochemical Profiling of Costus Speciosus (J. Koenig) Sm. Rhizome and its Interaction with Gut Bacteria Lactobacillus casei

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ABSTRACT:

Costus speciosus (J.Koenig) Sm., also known as 'Insulin plant', is an erect rhizomatous herb belonging to the Costaceae family. It holds significant potential for the development of pharmaceutical drugs due to the abundance of bioactive secondary metabolites. In this study, we screened the ethanolic extract of *C. speciosus* rhizome (CSE) for its phytochemical composition, anti-inflammatory potential, and non-toxic behaviour towards gut bacteria *Lactobacillus casei*. The CSE displayed the presence of a variety of phytochemicals such as saponins, coumarins, tannins, terpenoids, alkaloids, phenolics, etc. It contained significant total saponin (4.68 ± 0.12 gm/100 gm of oleanolic acid equivalents), terpenoid (192.54 ± 4.18 mg linalool/gm of dry weight), and phenolic (64.59 ± 1.96 mg GAE/gm) content. The *in-vitro* anti-inflammatory activity of CSE expressed as percentage inhibition was found to be 76.2 ± 2.20 % at 1000 µg/ml. Additionally, the CSE (50, 100, and, 250 µg/disc) did not harm the growth of *Lactobacillus casei*. The presence of several phytochemicals, their significant content, and remarkable *in-vitro* anti-inflammatory activity, all point to the exceptional medicinal potential of *C. speciosus*.

Keywords: *Costus speciosus*, phytochemicals, saponins, phenols, anti-inflammatory activity, pharmacological properties.

1. Introduction

Medicinal plants are a storehouse of bioactive molecules which impart incredible pharmacological properties. The application of herbal remedies prepared from medicinal plants is the basis of traditional Indian medicine, emphasising holistic care (Maji et al., 2020). One such traditional medicinal plant with extraordinary therapeutic capabilities is *Costus speciosus* (J. Koenig) Sm., a member of the Costaceae family (Srivastava et al., 2011). Widely distributed in tropical and subtropical parts of Asia, America, and Africa, *C. speciosus* is an upright, perennial, rhizomatous herb. Popularly known as “Insulin plant” or "Crepe ginger", *C. speciosus* has been reported to hold several pharmacological activities, including anti-oxidant, anti-diabetic, anti-arthritic, anti-cancer, hepatoprotective, etc. (El-Far et al., 2018; Sohrab et al., 2021). In traditional folk medicine, *C. speciosus* has been employed to treat various conditions, such as diabetes, asthma, parasitic infections, fever, skin disorders, etc. (Duraipandiyan et al., 2012). The rhizome of this plant is the most frequently used part and has been reported to treat bronchitis, constipation, rheumatism, anaemia, skin conditions, jaundice, pneumonia, asthma, etc. (Srivastava et al., 2011). Such remarkable pharmacological properties exhibited by *C. speciosus* are attributed to the phytochemicals it comprises. *C. speciosus* encompasses a variety of bioactive molecules such as alkaloids, terpenoids, steroids, flavonoids, saponins, tannins, and phenolics (Saraf, 2010). Additionally, the rhizome of the plant has also been found to contain fatty acids, aliphatic hydroxy ketones, corticosteroids, abscisic acid, tigogenin, oxa-acid, and starch mucilage (Pawar & Pawar, 2012; Thambi & Cherian, 2023).

Owing to the diverse structural features of phytochemicals, several medicinal properties, including anti-inflammatory, have been reported. In an earlier study, the methanolic extract of *C. speciosus* aerial parts at dose of 800 mg/kg displayed significant anti-inflammatory activity (40.05%) (Srivastava et al., 2013). Several studies have displayed the antibacterial activities of medicinal plants against many pathogens (Hemeg et al., 2020; Mogana et al., 2020). Furthermore, many studies have documented that certain medicinal plants do not negatively impact gut bacteria (Chen et al., 2023; Shinde & Deokar, 2024). Extracts from *Diospyros kaki* (persimmon) and catechol have been shown to inhibit the growth of *Clostridium perfringens* and *C. difficile* without negatively affecting the growth of *Lactobacillus casei, Bifidobacterium longum, and B. breve* (Jeong et al., 2009). The *C. speciosus* rhizome extract has been reported to possess anti-bacterial properties against several pathogenic bacteria such as *Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, S. epidermidis, Escherichia coli, Bacillus subtilis,* etc. (Arumugam & Gomathinayagam, 2014; Duraipandiyan et al., 2012; Malabadi, 2005; Saraf, 2010; Seddiq, 2023; V N et al., 2012). However, no studies have explored the effect of *C. speciosus* on gut bacteria. To our knowledge, this is the first study to evaluate the impact of *C. speciosus* rhizome extract on gut bacteria *L. casei*.

In the current study, we aimed to investigate the ethanolic extract of *C. speciosus* rhizome for the presence of phytochemicals, total saponin, terpenoid, and phenolic contents, the *in-vitro* anti-inflammatory activity and its effect on gut bacteria *Lactobacillus casei*.

2. Materials and Methods

2.1.Reagents

Sodium carbonate, chloroform, sulphuric acid, lead acetate, and isoamyl alcohol were purchased from HiMedia, India. Folin & Ciocalteau phenol reagent, gallic acid, glacial acetic acid, and potassium iodide were procured from Sisco Research Laboratories Pvt. Ltd, India. Linalool and Oleanolic acid were obtained from Merck, Germany.

2.2.Collection of Plant Samples and Processing

Fresh and healthy rhizomes of *C. speciosus* (J. Koenig) Sm. were procured from the Sitamata Wildlife Sanctuary of Udaipur district. A voucher specimen of the plant (accession number RUBL21426) was deposited at the Department of Botany, University of Rajasthan, India. Rhizomes were properly cleaned, washed, and shade-dried at room temperature for two weeks. To eliminate moisture, the dried rhizomes were further cut into small sections and kept in a hot air oven (Yorco, India) for 24 h at 30°C. The dried rhizomes were ground to obtain a fine powder.

2.3.Preparation of Ethanolic Extract of Rhizome

The maceration process was employed for the preparation of ethanolic extract of the *C. speciosus* rhizome (Rai et al., 2020). Briefly, rhizome powder (20 gm) was extracted with absolute ethanol (100 ml) by constant agitation for 72 hrs at room temperature. The mixture was then filtered using Whatman No. 1 filter paper and solvent evaporation was done using a rotary evaporator (Heidolph, Germany) at 60℃.

2.4.Phytochemical Tests (Qualitative Tests)

The phytochemical screening was performed following the standard protocols (Ben et al., 2013; Solanki et al., 2019).

2.4.1. Test for Saponins

2 ml of distilled water was added to 1 ml of CSE (0.5 mg/ml), shaken vigorously for 2 minutes, and observed for persistent frothing. For all the subsequent qualitative tests, the control reaction included all the reagents except the test compound.

2.4.2. Test for Terpenoids

1 mg of CSE was mixed in 1 ml of methanol. Then 1.5 ml of both chloroform and concentrated sulphuric acid were added, respectively. The appearance of a reddish-brown colour indicated the presence of terpenoids.

2.4.3. Test for Glycosides

1 mg of CSE was dissolved with 1 ml of distilled water. To this mixture, 1.5 ml of chloroform was added followed by 1.5 ml of 10% ammonia solution which was observed for the appearance of pink colour.

2.4.4. Test for Steroids

1 ml of methanol was added to 1 mg of CSE. Then, 2.5 ml of chloroform was added followed by 2.5 ml of concentrated sulphuric acid. The reaction mixture was checked for the formation of two phases upper red and lower yellow, indicating positive result for steroid.

2.4.5. Test for Quinones

1ml of CSE (0.5 mg/ml in absolute ethanol) was added to 1 ml of concentrated sulphuric acid. The presence of quinones is indicated by the appearance of an orangish-red colour.

2.4.6. Test for Tannins

20 µl of CSE (0.5 mg/ml in absolute ethanol) was mixed with 30 µl of 10% lead acetate and checked for a thin white precipitate.

2.4.7. Test for Alkaloids
Wagner's reagent: The reagent was prepared by dissolving 0.3 g of potassium iodide in 5 ml of iodine solution.
Wagner's Reagent Test: 1 ml of CSE (5 mg/ml in distilled water) was mixed with a few drops of Wagner's reagent and observed for red-brown precipitate particles.

2.4.8. Test for Flavonoids
Alkaline Reagent Test: 20 µl of 2% NaOH was mixed with 20 µl of CSE (5 mg/ml in absolute ethanol). A light-yellow colour was obtained. Then, 10 µl of 1% HCl was added to the above mixture. The disappearance of the yellow colour upon the addition of dilute HCl indicated the presence of flavonoids.

2.4.9. Test for Anthocyanin
1 mg of CSE was dissolved in 2 ml of distilled water. To this mixture, 1ml of 3 N NaOH was added and heated at 100 °C for 5 minutes. The mixture was observed for the appearance of a bluish-green colour suggesting the presence of anthocyanin.

2.4.10. Test for Leucoanthocyanins
1 ml of CSE (5 mg/ml in distilled water) was mixed with 1 ml of isoamyl alcohol and checked for the formation of red-coloured organic layer.

2.4.11. Test for Phlobatannins
1 ml of CSE (0.5 mg/ml in absolute ethanol) was added to 1 ml of 1% HCl. Then the mixture was heated for a few minutes and observed for red precipitate.

2.4.12. Test for Betacyanin
1 mg of CSE was mixed in 2 ml of distilled water. Then, 1 ml of 3 N NaOH was added and heated at 100 °C for 5 minutes. The appearance of yellow colour indicates betacyanins.

2.4.13. Test for Coumarins
20 µl of CSE (5 mg/ml in distilled water) was mixed with 30 µl of 10% NaOH and observed for the development of a slight yellow colour.

2.4.14. Test for phenolic compounds
Lead Acetate Test: 20 µl of CSE (5 mg/ml in distilled water) was mixed with 20 µl of 10% lead acetate solution. The formation of a thin white precipitate layer signifies a positive result for phenols.

2.4.15. Test for Carbohydrates
Molisch's Test: To 20 µl of CSE (0.5 mg/ml in absolute ethanol) a few drops of α- naphthol were added followed by 20 µl of concentrated sulphuric acid slowly through the test tube wall. The formation of a violet colour ring at the junction indicated the presence of carbohydrates.

2.5.Total Saponin Content
The total saponin content was assessed as described by Medina-Meza et al. (2016). Briefly, a reagent mixture containing glacial acetic acid and sulphuric acid in a 1:1 (v/v) ratio was prepared. To 1 ml of this mixture, 0.25 ml of CSE (dissolved in ethanol) was added, vortexed, and reacted at 60°C in a water bath for 30 min and then cooled. The absorbance was recorded at 527 nm using a spectrophotometer (Systronics, India). Oleanolic acid was employed as a

standard (0 – 1,000 µg/ml). The result was represented as gm/100 gm of oleanolic acid equivalents.

2.6.Total Terpenoid Content

The total terpenoid content was evaluated according to the method developed by Ghorai et al. (2012). Briefly, 1.5 ml of chloroform was added to 200 µl of CSE, vortexed thoroughly, and allowed to stand at room temperature for 3 minutes. Then, 100 µl of conc. sulphuric acid was added and the reaction mixture was incubated undisturbed for 1.5 h – 2 h in the dark at room temperature. In the case of standard Linalool, incubation was done for not more than 5 minutes. After incubation, a reddish-brown precipitate was observed. The supernatant was decanted and the residue was gently dissolved in 1.5 ml of 95% methanol (v/v) & vortexed thoroughly. The absorbance was read at 538 nm and the result was calculated as Linalool equivalents from the regression equation of the Linalool standard curve.

2.7.Total Phenol Content

The total phenol content was determined using the method of Singleton & Rossi (1965). Briefly, 80 µl of CSE, 720 µl of distilled water, and 160 µl of Folin & Ciocalteau phenol reagent were added and the mixture was vortexed. The reaction mixture was allowed to stand for 5 min, then 800 µl of 7% (w/v) Na_2CO_3 solution was added and the resulting solution was made up to 2 ml with distilled water. Then, the solution was incubated for 90 min at room temperature. After incubation, the absorbance of the solution was measured at 750 nm. The standard curve of gallic acid was prepared in concentrations ranging from 5-80 µg/ml. The total phenol content of extract expressed as mg gallic acid equivalent (GAE) per gram of dry mass was calculated as:

$C = (cv)/m$

where,

C = total phenolic content mg GAE/gm dry extract

c = concentration of gallic acid obtained from the calibration curve in mg/ml

v = volume of extract in ml

m = mass of extract in gm

2.8.In-Vitro Anti-Inflammatory Activity

The *in-vitro* anti-inflammatory activity of CSE was evaluated through the protein denaturation inhibition assay performed using the method of Banerjee et al. (2014). Briefly, a reaction mixture consisting of 2.8 ml of phosphate-buffered saline (pH 6.4), 0.2 ml of egg albumin (from fresh hen's egg), and 2 ml of different concentrations of CSE (50 - 1000 µg/ml) was prepared. Diclofenac sodium was employed as a standard anti-inflammatory drug. The mixtures were incubated at 37°C for 15 min and then heated at 70°C for 5 min. After cooling, the absorbance was measured at 660 nm using a spectrophotometer (Systronics, India). The results were represented as the percentage inhibition of protein denaturation, calculated using the following formula:

$\%\ \text{inhibition} = 100 \times ([Vt/Vc] - 1)$.

Where, Vt = absorbance of the test sample (CSE or standard diclofenac), Vc = absorbance of control.

2.9.Effect Of CSE Against Lactobacillus Casei

The effect of CSE against *Lactobacillus casei* LN62698 was evaluated using the disc diffusion method. 100 µl of *L. casei* culture was spread, using an L-shaped spreader, onto autoclaved

MRS agar plates, respectively. The culture was allowed to dry for 5 minutes. Then, sterile 5 mm discs (Whatman filter paper) impregnated with CSE (dissolved in sterile water) to achieve a final concentration of 50, 100, and, 250 µg/disc were carefully placed on agar plates containing the test organism. Tetracycline disc was used as a standard antibiotic. The plates were incubated at 37°C for 12 hours and the zone of inhibition was calculated.

2.10. Statistical Analysis

The results are represented as mean ± SD. The statistical analysis was executed using one-way ANOVA with a p-value of less than 0.05, which was considered statistically significant. All the experiments were conducted in triplicates.

3. Results and Discussion

The CSE was extracted in absolute ethanol, by the maceration process. The obtained CSE was light golden to brown and had a coarse texture with no characteristic smell.
Phytochemicals are non-nutritive secondary metabolites present in plants. Beyond helping plants defend against various pathogens and stresses (Zaynab et al., 2018), these compounds also offer health benefits to humans by providing disease-preventive properties (Guan et al., 2021). The CSE was screened for the presence of various phytochemicals. A diverse range of phytochemicals were reported in the CSE and have been summarized in Table 1.

Table 1: Qualitative phytochemical analysis of CSE.

Sr. No.	Phytochemical	Observation	Result
1.	Saponins	Persistent frothing	Present
2.	Terpenoids	Reddish brown colour	Present
3.	Glycosides	White-coloured solution	Absent
4.	Steroids	Two phases, upper red and lower yellow	Present
5.	Quinones	Orange yellow colour	Present
6.	Tannins	White precipitate	Present
7.	Alkaloids	Reddish-brown precipitate	Present
8.	Flavonoids	Colourless solution observed at the end of the reaction	Present
9.	Anthocyanin	Yellow colour	Absent
10.	Leucoanthocyanins	Colourless solution	Absent
11.	Phlobatannins	Colourless solution	Absent
12.	Betacyanin	Yellow colour	Present
13.	Coumarins	Light yellow colour	Present
14.	Carbohydrates	Violet ring observed	Present

Our results are in line with previous studies (Shaikh et al., 2022). Phytochemical richness plays a central role in the pharmacological properties of plants. The complexity and variety of phytochemicals in a plant often determine its effectiveness and potency in medicinal applications. In the present study, phytochemicals such as terpenoids, saponins, alkaloids, flavonoids, coumarins, steroids, tannins, betacyanins and phenolic compounds, were identified and could be accountable for the incredible therapeutic properties of CSE. These bioactive compounds impart characteristic properties to the plant like antioxidant, anti-bacterial, anti-

inflammatory, anti-cancer, anti-diabetic, antipyretic, etc. which contribute to their ability to treat various diseases (Kumar et al., 2023).

Saponins are non-volatile, surface-active compounds, with remarkable therapeutic properties (Sharma et al., 2023). In the current study, the qualitative identification of saponins was followed by its quantitative estimation in CSE. The total saponin content determined in CSE was 4.68 ± 0.12 gm/100 gm of oleanolic acid equivalents. In a previous study by Peters & Chibueze (2022) essential oil of *C. lucanusianus* stem displayed 837.38 mg/100 gm saponin content. Another study revealed 18.3 ± 0.66 mg/gm saponin content (dry material) in *C. speciosus* leaves (Raveendran, 2015).

Terpenoids (isoprenoids) are one of the plants' most crucial secondary metabolites. Their roles range from growth and development to fighting biotic and abiotic interactions (Tholl, 2015). In this study, the qualitative test showed terpenoids in CSE, followed by quantitative determination. The total terpenoid content obtained in CSE was 192.54 ± 4.18 mg linalool/gm of dry weight. In an earlier study, the *C. speciosus* leaves depicted 11.2 ± 0.5 mg/gm (of dry material) terpenoid content (Raveendran, 2015).

Phenols are the most abundant group of secondary metabolites with remarkable biological activities (Zhang et al., 2022). The total phenolic content in CSE, determined using the Folin-ciocalteau reagent, was 64.59 ± 1.96 mg GAE/gm (Table 2). Our results are consistent with previous studies where phenol content ranged from 59.59 ± 1.23 to 95.85 ± 1.56 mg GAE/g in methanolic extract of *C. speciosus* roots (Lahare & Yadav, 2020). The presence of a considerably significant amount of phenols in CSE contributes to the antioxidant properties suggesting its therapeutic applications.

Table 2. Phytochemical Content Identified in Ethanolic Extract of *C. Speciosus* Rhizome.

Phytochemical	**Total Phytochemical Content**
Saponin	4.68 ± 0.12 gm/100 gm of oleanolic acid equivalents
Terpenoid	192.54 ± 4.18 mg linalool/gm of dry weight
Phenol	64.59 ± 1.96 mg GAE/gm

In the current study, the *in-vitro* anti-inflammatory activity of CSE was evaluated through protein (egg albumin) denaturation inhibition as a model. This assay is based on the principle that compounds with anti-inflammatory properties can prevent denaturation by stabilizing protein structures, a process often associated with inflammation. The CSE displayed inhibition of protein denaturation in a concentration-dependent manner (Figure 1). CSE at 1000 µg/ml exhibited 81.56 ± 2.87 % inhibition while standard diclofenac sodium demonstrated 94.74 ± 1.85 % inhibition at the equal concentration. Previously, the methanolic extract of *C. speciosus* leaves showed 75.15 ± 2.33 % protein denaturation inhibition demonstrating significant *in-vitro* anti-inflammatory activity (Azam et al., 2016). The substantial anti-inflammatory activity imparted by CSE may be attributed to the presence of various bioactive molecules such as terpenoids, flavonoids, saponins, alkaloids, and tannins (Gonfa et al., 2023; Nisar et al., 2023).

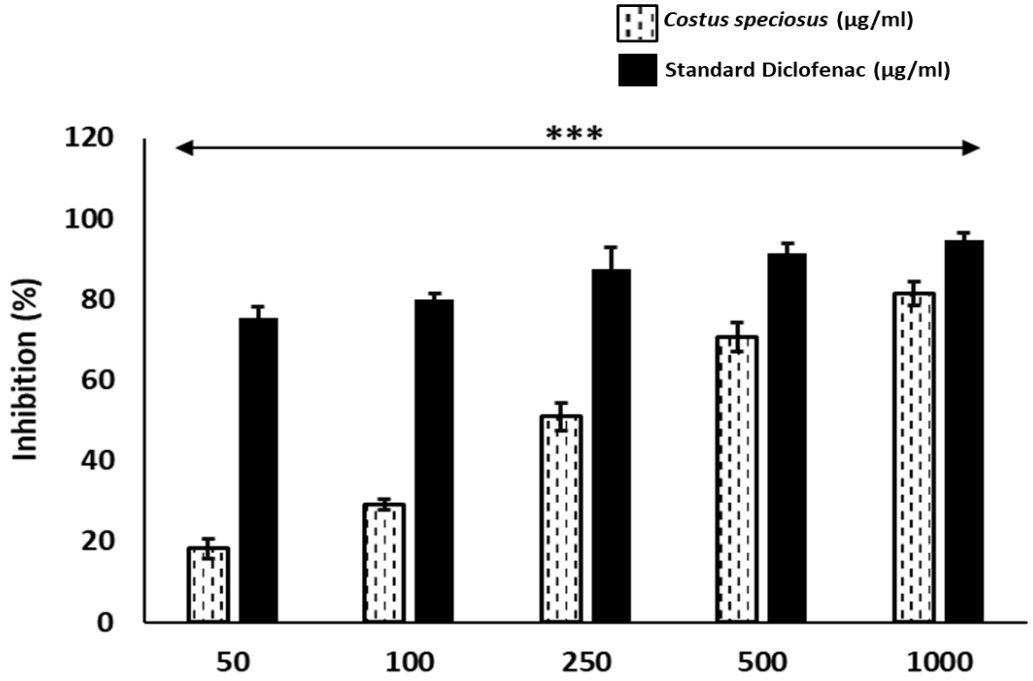

Figure 1. In-vitro protein denaturation inhibition by ethanolic extract of C. speciosus rhizome. Data are represented as mean ± SD. Statistically significant differences are indicated as ***$p<0.001$.

The effect of CSE against *L. casei* was assessed and interestingly no zone of inhibition was observed at all three doses of CSE (50, 100, and, 250 µg/disc) (Figure 2). However, standard tetracycline inhibited the growth of *L. casei* with a zone of inhibition of 23 mm (diameter) (Table 3). As *L. casei* is a "good" gut bacteria therefore it can be inferred that CSE does not alter gut microflora and non-toxic to *L. casei*

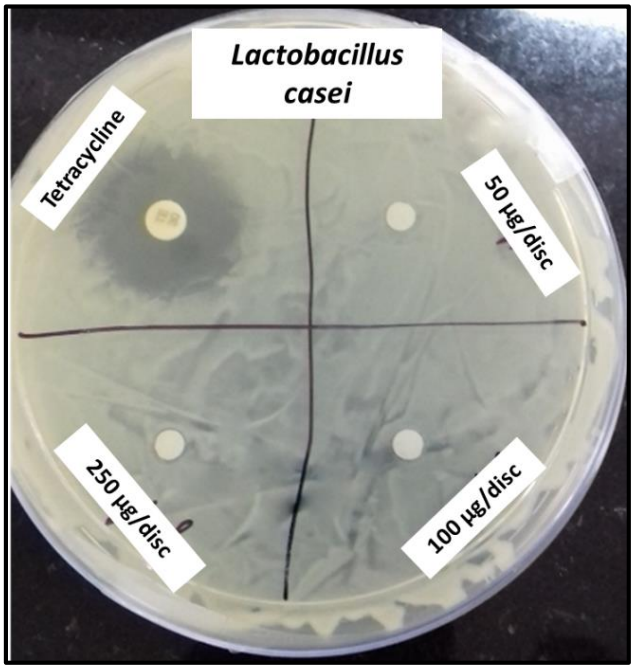

Figure 2. No Inhibition zones in MRS Agar for Lactobacillus casei in the presence of ethanolic extract of C. speciosus rhizome (50, 100, and, 250 µg/disc).

Table 3. Inhibition Zones (Mm) Observed in the Disc Diffusion Method.

	Concentration (µg/disc)	**Diameter of Zone of inhibition (mm)**
		Lactobacillus casei LN626981
Costus speciosus rhizome extract	50	0
	100	0
	250	0
Tetracycline	30	23

4. Conclusion

The ethanolic extract of *C. speciosus* rhizome exhibited rich phytochemical diversity, significant total saponin, terpenoid, phenol content, and considerable anti-inflammatory activity, all suggesting its potential therapeutic value. Furthermore, the non-harmful action of CSE on gut bacteria can be further explored for the development of "prophybiotics" for the improvement of gut health (Wishna-Kadawarage et al., 2024). More studies (including *in-vivo*) are required to determine the optimal dosage of *C. speciosus* rhizome extract for extensive pharmacological capabilities.

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