IN VITRO HYPOGLYCEMIC AND ANTIMICROBIAL ACTIVITY OF CUCUMIS CALLOSUS (ROTTL.) COGN. FRUIT

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ABSTRACT

Objective: This research investigated in vitro hypoglycemic and antimicrobial activity of methanolic fruit extract of Cucumis callosus (MECC) (Rottl.) Cogn.

Methods: The in vitro antidiabetic ability of MECC was evaluated by inhibition studies of the digestive enzymes including α-amylase and α-glycosidase. The antimicrobial activity of extract was further evaluated against 21 laboratory strains belonging to 8 bacterial species and 4 fungal species using cup-plate agar diffusion assay and agar dilution assay.

Results: Our assay result suggested that the MECC exhibited dose-dependent increase in percentage inhibitory activity on α-glycosidase (IC₅₀: 405.37 µg/ml) and α-amylase enzymes (IC₅₀: 721.55 µg/ml). The results of antimicrobial activity also showed that the extract inhibited the growth of 13 strains of 7 bacterial species and all fungal species.

Conclusion: The observed inhibitions of α-amylase and α-glycosidase and inhibition of some microbial species suggested that the MECC may be useful in the management of diabetes mellitus and treatment of some infectious diseases.

Keywords: Cucumis callosus, Diabetes, α-amylase, α-glycosidase, Antimicrobial.

INTRODUCTION

Diabetes mellitus is a pandemic disease, and the world is facing an escalating epidemic of diabetes. The International Diabetes Federation has estimated that in 2010, 285 million people around the world have diabetes, representing nearly 7% of the adult world population [1]. Type 2 diabetes mellitus (T2DM) accounts for 90-95% of all cases of diabetes. Furthermore, T2DM significantly increases the risk of heart disease, stroke, and bacterial and fungal skin infections. T2DM is a disease characterized by a dual defect: (1) By insulin resistance which prevents cells from using insulin properly and (2) degrees of reduced pancreatic insulin secretion [2]. The side effects associated with the prolonged use of insulin and other hypoglycemic drugs have necessitated the demand for safe and effective drugs, especially of herbal origin [3]. Inhibition of α-glycosidase (EC 3.2.1.20) and α-amylase (EC 3.2.1.1) enzymes involved in the digestion of carbohydrates, can significantly decrease the postprandial increase of blood glucose after a mixed carbohydrate diet, and therefore, can be an important strategy in the management of postprandial blood glucose level in Type 2 diabetic patients and borderline patients [4]. α-glycosidase and α-amylase inhibitors are useful for people taking sulfonylurea medication or metformin, who need an additional medication to keep their blood glucose levels within a safe range. Therefore, the retardation and delay of carbohydrate absorption with a plant-based α-glycosidase inhibitor offers a prospective therapeutic approach for the management of T2DM and borderline patients.

Cucumis callosus (Rottl.) Cogn. (Cucurbitaceae) commonly called as “Bitter cucumber” in English, “Kachri” in Hindi, is a highly branched very common prostrate, perennial herb, distributed throughout India in the arid zones. The fruits are smooth, ovoid, ellipsoid, green variegated stripes and have bitter pulp [5,6]. C. callosus is essentially a warm season crop and a long period of warm and humid climate is required [7]. Fruit pulp of C. callosus is bitter, acrid, thermogenic, astringent, bitter, acrid, thermogenic, astringent, discouraging, and detoxifying [8]. Traditionally, its fruits and seeds are used for strong memory, remove vertigo, cooling, astringent, bilious disorder, constipation, piles, urticaria, jaundice, amenorrhea, diabetes mellitus, and calculi [9,10]. The C. callosus fruit is used in the treatment of diabetes mellitus by Srilankan Ayurvedic and traditional physicians [11]. The leaf extract of C. callosus is picyclically used in wound healing [4].

The aqueous and alcoholic extract of C. callosus (seed) has been reported for its antioxidant activity [12,13]. The tribal people of Balasore and Bargarh (Odisha, India) use fruits of C. callosus during worship, as vegetable and for curing diabetes, epilepsy and diarrhoea [14]. Hence, the present study was aimed at evaluating the hypoglycemic and antimicrobial activity of methanolic fruit extract of C. callosus (MECC).

METHODS

Collection of plant
The fruits of C. callosus were collected in the month of July 2014 from village area of Kendrapara and Balasore district, Odisha (India). The plant was authenticated by M. S. Mondal, Botanical Survey of India, Kolkata, India, and a voucher specimen (CNH/1-1(196)/2007/ Tech-II/160) has been preserved in the Pharmacology Research Laboratory, Jadavpur University, Kolkata, for future reference.

Extraction
The fruits of C. callosus were shade dried and then powdered with a mechanical grinder. The powder (500 g) was defatted with petroleum ether at 40°C in a Soxhlet extraction apparatus and then extracted similarly with methanol. The solvents were completely removed under reduced pressure to obtain a dry mass. The yields of the petroleum ether and methanol extracts were found to be 2.8% w/w and 9% w/w, respectively. The extracts were stored in vacuum desicators for further use. The preliminary phytochemical screening of the MECC showed the presence of mainly flavonoids, saponin, and terpenoids.

Chemicals
Pyroctechol, α-amylase, and α-glycosidase enzymes were purchased from SRL Pvt. Ltd, Mumbai. Glucose assay kit from Agape Diagnostic...
Pvt. Ltd., Kerala, and Acarbose was obtained from Bicon Pvt. Ltd. All other chemicals used in the study were obtained commercially and were of analytical grade.

**Bacterial strains and fungal strains**

Gram-positive strains: *Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus pumilus*.

Gram-negative strains: *Escherichia coli*, *Vibrio cholerae*, *Shigella boydi*, *Klebsiella pneumoniae*, *Psedomonas aeruginosa*, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Shigella dysenteriae*, and *Proteus vulgaris*.

Fungal strains: *Candida albicans* MTCC 183, *Aspergillus flavus* MTCC 1973, *Aspergillus niger* MTCC 16404, *Cryptococcus albidus* MTCC 183.

Microorganisms were obtained from the stock culture of Division of Microbiology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

**In vitro methods employed in antidiabetic studies**

**Inhibition of a-amylase enzyme**

About 0.05 ml of test samples (100-1000 µg/ml) and 0.05 ml standard drug (100-1000 µg/ml) were separately added to 0.05 ml of 0.1 M phosphate buffer (pH 6.9) containing 0.1 ml of α-amylase (0.5 mg/ml) solution in test tube and were incubated at 25°C for 10 minutes. After these, 0.05 ml of 1% starch solution in 0.1 M phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 minutes. The reaction was stopped with the addition of 2.0 ml of 3, 5 dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 5 minutes, cooled to room temperature. The reaction mixture was then diluted with 10 ml distilled water and absorbance was measured at 540 nm. The generation of reducing sugars was quantified by the reduction of 3, 5 dinitrosalicylic acid to 3-amino-5-nitro salicylic acid. Control represent 100% enzyme activity and was conducted in a similar way by replacing extract with vehicle [15].

**Inhibition of a-glycosidase enzyme**

α-glycosidase inhibitory activity of the extract was assayed according to Kim et al., with slight modification. The reaction was initiated with the preparation of reaction mixture taking 0.05 ml each of the samples (test and standard) at different concentrations (100-1000 µg/ml) in 0.05 ml of 0.1 M phosphate buffer (pH 6.9) followed by incubation at 37°C for 15 minutes. After which 0.1 ml of enzyme solution was immediately added to the mixture and incubated at 37°C for 15 minutes. Then, 0.25 ml of substrate 3 mM 4-nitrophenyl-α-D-glycopyranoside (pNPG) was added to the mixture and then again incubated at 37°C for 15 minutes, after which the reaction was stopped by the addition of 4 ml of 0.1 m Na₂CO₃, α-glycosidase inhibitory activity was determined by measuring the release of p-nitrophenol from pNPG at 405 nm. The control contained all reagents without the tested sample. The reactions were conducted in triplicate. The percentage of inhibition was calculated as:

\[
\%\text{ inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100
\]

Where

\[\text{Abs}_{\text{control}}\text{ is the absorbance of sample extract}\]

\[\text{Abs}_{\text{sample}}\text{ is the absorbance of the control}\]

The concentration of α-glycosidase inhibitor required to inhibit 50% of α-glycosidase activity under the assay conditions is defined as the IC₅₀ value [16].

**In vitro methods employed in antimicrobial studies**

**Minimum inhibitory concentration (MIC)**

The MIC was determined by the agar-dilution method in Mueller-Hinton agar medium (Oxoid). Before gelling, 20 ml of agar medium was added to each of the Petri dishes containing a specific concentration of plant extract, or purified compounds, and the Petri dishes were swirled carefully until the agar began to set. Concentrations ranging from 200 to 2000 µg/ml were used for extract. Subsequently, 2 µl of bacteria (10⁶ colony-forming units [CFU/ml]) and 2 µl fungi (10⁶ CFU/ml) were inoculated using a Steers replicator on the Mueller-Hinton agar surface [17].

**Zone of inhibition**

The zone of inhibition was determined by the cup-plate method in Mueller-Hinton agar medium (Oxoid). The antimicrobial activity of MECC was determined using the disk diffusion method. Petri dishes containing 20 ml of Mueller-/Hinton agar medium (Oxoid, Hampshire, England) were seeded with a 24 hrs culture of the microbial strains in Trypticase soy broth (TSB, Oxoid). The inoculum size was adjusted to approximately 10⁵ CFU/ml. The solutions of the plant extract were applied to sterile filter paper disks (Whatman Number 1; 5 mm in diameter) to give the final concentrations of 2500 and 3000 µg/ml and placed on the surface of the inoculated medium. The plates were incubated at 35°C for 24 hrs. Antimicrobial activity was determined by measuring the diameter of the inhibited zone formed around the disk [18].

**Total phenol estimation**

Total phenolic compound of medicinal fruit extract was determined by Folin-Ciocalteu method. For the preparation of the calibration curve, 1 ml aliquot of 0.025, 0.05, 0.075, 0.1, 0.2, and 0.3 mg/ml methanolic gallic acid solution was mixed with 5 ml of Folin-Ciocalteu reagent (10 times diluted) and 4 ml sodium carbonate (75 g/L). The absorbance at 765 nm was measured after 1 hr of incubation at 20°C in biochemical oxygen demand incubator, and the calibration curve was drawn. To the similar reagent, 1 ml methanolic fruit extract (4 mg/ml) was mixed as described above and after 1 hr of incubation the absorbance was measured at 765 nm. The total phenol content was expressed in milligrams of gallic acid equivalents (GAE)/g of the extract [19].

**The analysis of total flavonoid contents**

The total flavonoid content of extract was determined according to the colorimetric method as described by Moreno et al. To do so, 0.5 ml of each extract was added to test tubes containing 0.1 ml of 10% Al(NO₃)₃ solution, 0.1 ml of 1 M potassium acetate, and 4.3 ml of 80% ethanol. After incubation for 40 minutes at room temperature, the absorbance was measured at 415 nm. The total flavonoid content was expressed in milligrams of quercetin equivalents (QE)/g of the extract [20].

**RESULTS**

The methanolic fruit extract was found to contain noticeable amount of total phenol and total flavonoid. The total phenolic content and flavonoid content of MECC were found to be 117.63 mg GAE/g of extract.

| Sample            | Concentration (µg/ml) | % inhibition | IC₅₀ (µg/ml) |
|-------------------|-----------------------|--------------|--------------|
| MECC              | 100                   | 18.66±0.24   | 721.55       |
|                   | 200                   | 30.41±0.14   |              |
|                   | 400                   | 36.33±0.27   |              |
|                   | 800                   | 53.49±0.27   |              |
|                   | 1000                  | 63.34±0.11   |              |
| Acarbose (standard)| 100                   | 33.15±0.69   | 345.52       |
|                   | 200                   | 48.71±0.33   |              |
|                   | 400                   | 58.33±0.14   |              |
|                   | 800                   | 66.97±0.39   |              |
|                   | 1000                  | 73.38±0.27   |              |

All determinations were carried out in triplicate manner and values are expressed as the mean±SEM. The IC₅₀ value is defined as the concentration of inhibitor to inhibit 50% of its activity under the assay conditions. C. callosus: *Cucumis callosus*, MECC: Methanolic fruit extract of *Cucumis callosus*.
and 116.3 mg QE/g of extract, respectively, which play a major role in anti diabetic and antimicrobial activity. These quantitative determinations were done using the standard curve of gallic acid and quercetin.

Evaluation of in vitro α-amylase inhibitory activity using MECC
There was a dose-dependent increase in percentage inhibitory activity against α-amylase enzyme. The extract 100 µg/ml showed a percentage inhibition of 18.66 ± 0.24 and 63.34 ± 0.11 at 1000 µg/ml (Table 1). The concentration of extract required for 50% inhibition of α-amylase (IC$_{50}$) was found to be 721.55 µg/ml, and the IC$_{50}$ value of standard drug acarbose against α-amylase was found to be 3.45 ± 0.2 µg/ml.

Evaluation of in vitro α-glycosidase inhibitory activity of MECC
The MECC revealed a significant inhibitory action on α-glycosidase enzyme. The percentage inhibition of MECC at 100 µg/ml showed 30.63 ± 0.24 and 63.34 ± 0.11 at 1000 µg/ml (Table 2). The concentration of extract required for 50% inhibition of α-glycosidase (IC$_{50}$) was found to be 405.37 µg/ml, whereas the IC$_{50}$ value of standard drug acarbose against α-glycosidase was found to be 236.20 µg/ml.

Table 2: α-glycosidase inhibitions by methanol extract of Cucumis callosus

| Sample | Concentration (µg/ml) | % Inhibition | IC$_{50}$ (µg/ml) |
|--------|-----------------------|--------------|------------------|
| MECC   | 100                   | 30.63±0.30   | 405.37           |
|        | 200                   | 39.17±0.46   |                  |
|        | 400                   | 56.52±0.23   |                  |
|        | 800                   | 64.33±0.27   |                  |
|        | 1000                  | 86.04±0.45   |                  |
| Acarbose (standard) | 100       | 40.5±0.13    | 236.20           |
|        | 200                   | 49.1±0.49    |                  |
|        | 400                   | 63.1±0.24    |                  |
|        | 800                   | 72.07±0.56   |                  |
|        | 1000                  | 91.32±0.18   |                  |

All determinations were carried out in triplicate manner and values are expressed as the means±SEM. The IC$_{50}$ value is defined as the concentration of inhibitor to inhibit 50% of its activity under the assayed conditions. C. callosus: Cucumis callosus, MECC: Methanolic fruit extract of Cucumis callosus

Table 3: MIC of MECC against various bacterial and fungal species

| Bacteria               | Gram-positive/ Gram-negative | Control | 200 µg/ml | 400 µg/ml | 800 µg/ml | 1200 µg/ml | 1600 µg/ml | 2000 µg/ml |
|------------------------|------------------------------|---------|-----------|-----------|-----------|------------|------------|-----------|
| S. aureus ML 351       | G+                           | +       | +         | +         | +         | +          | +          | –         |
| S. aureus ML 314       | G+                           | +       | +         | +         | +         | +          | +          | –         |
| S. aureus ML 264       | G+                           | +       | +         | +         | +         | +          | –          | –         |
| S. aureus ML 267       | G+                           | +       | +         | +         | +         | +          | –          | –         |
| B. subtilis UC 564     | G+                           | +       | +         | +         | +         | +          | –          | –         |
| B. pumilus 8241        | G+                           | +       | +         | +         | +         | +          | –          | –         |
| B. cereus Pl 11778     | G+                           | +       | +         | +         | +         | +          | –          | –         |
| V. cholerae 117        | G−                           | +       | +         | +         | +         | +          | –          | –         |
| K. pneumoniae 114      | G−                           | +       | +         | +         | +         | +          | –          | –         |
| K. pneumoniae 1         | G−                           | +       | +         | +         | +         | +          | –          | –         |
| K. pneumoniae 725      | G−                           | +       | +         | +         | +         | +          | –          | –         |
| E. coli DH 5a          | G−                           | +       | +         | +         | +         | +          | –          | –         |
| S. dysenteriae 7       | G−                           | +       | +         | +         | +         | +          | –          | –         |
| S. boydi 8             | G−                           | +       | +         | +         | +         | +          | –          | –         |
| S. boydi RC            | G−                           | +       | +         | +         | +         | +          | –          | –         |
| S. boydi ET 6552 (9)   | G−                           | +       | +         | +         | +         | +          | –          | –         |
| S. choleraesuis (36)   | G−                           | +       | +         | +         | +         | +          | –          | –         |
| S. typhimurium 74       | G−                           | +       | +         | +         | +         | +          | –          | –         |
| P. vulgaris 21         | G−                           | +       | +         | +         | +         | +          | –          | –         |
| P. aeruginosa          | G−                           | +       | +         | +         | +         | +          | –          | –         |
| ATCC-27853             |                             |         |           |           |           |            |            |           |
| C. albicans MTCC 183   | +                            | +       | +         | +         | +         | +          | –          | –         |
| A. flavus MTCC 1975    | +                            | +       | +         | +         | +         | +          | –          | –         |
| A. niger MTCC 16404    | +                            | +       | +         | +         | +         | +          | –          | –         |
| C. albidos MTCC 183    | +                            | +       | +         | +         | +         | +          | –          | –         |

*: Growth of microorganisms, -: No growth of microorganisms. MIC: Minimum inhibitory concentration, MECC: Methanolic fruit extract of Cucumis callosus, ATCC: American Type Culture Collection, MTCC: Microbial Type Culture Collection, S. aureus: Staphylococcus aureus, B. subtilis: Bacillus subtilis, B. pumilus: Bacillus pumilus, B. cereus: Bacillus cereus, V. cholera: Vibrio cholera, K. pneumoniae: Klebsiella pneumoniae, E. coli: Escherichia coli, S. dysenteriae: Shigella dysenteriae, S. boydi: Shigella boydi, S. choleraesuis: Salmonella choleraesuis, S. typhimurium: Salmonella typhimurium, P. vulgaris: Proteus vulgaris, P. aeruginosa: Pseudomonas aeruginosa, C. albicans: Candida albicans, A. flavus: Aspergillus flavus, A. niger: Aspergillus niger, C. albidos: Cryptococcus albidus

DISCUSSION
Although there are citations of in vitro antioxidant activity of alcoholic extract and aqueous extract of seeds of C. callosus, there are no previous reports on the activity of this extract on in vitro α-amylase and α-glycosidase activity [12,13]. Our in vitro studies demonstrated an appreciable α-glycosidase and α-amylase inhibitory activity of MECC. α-amylase and α-glycosidase are two main enzymes in the human body that are responsible for the breakdown of polysaccharides to more simple sugars. The α-amylase hydrolyzes complex polysaccharides to produce oligosaccharides and disaccharides which are then hydrolyzed by α-glycosidase to monosaccharides. The monosaccharides are absorbed through the small intestine into the hepatic portal vein and increase postprandial glucose level [21]. Slowing the digestion and absorption of starch by amylase
inhibitors may have the beneficial effects on insulin resistance and glycinic index control in people with diabetes [22,23]. Preliminary phytochemical analysis of MECC indicated the presence of phenolic compounds, flavonoids, saponin, and terpenoids. Natural polyphenols have been reported to inhibit the activity of α-amylase and α-glycosidase [22]. α-amylase and α-glycosidase inhibitory activity were also related only for oleane, ursane, and lupenol type terpenoids [24]. The high content of phenolic compounds, saponin, terpenoids, and flavonoids in the extract may also be a contributing factor towards antidiabetic activity. The mechanism by which fruit extract suppressed postprandial hyperglycemia may be due to its action on carbohydrate binding regions of α-amylase and α-glycosidase enzymes that catalyze hydrolysis of the internal α-1, 4 glycosidic linkages in starch and other related polysaccharides. Therefore, this study buttresses the claim that natural inhibitors from dietary plants have α-amylase and α-glycosidase inhibitory activity and could be used as effective therapy for the management of postprandial hyperglycemia with minimal side effects.

The tested fruit extract was more active against Gram-positive bacteria compared to Gram-negative. In general, Gram-negative bacteria are more resistant than Gram-positive bacteria [25,26]. The higher sensitivity of Gram-positive bacteria could be due to the exposure of the outer peptidoglycan layer while Gram-negative bacteria bear an extra outer membrane which includes the asymmetric distribution of the lipids with phospholipids and lipopolysaccharide located in the inner and outer leaflets, respectively, can act as additional barrier which hinders the movement of foreign substance into the cell [27,28].

The methanol is a better solvent for more consistent extraction of antimicrobial substances from medicinal plants compared to other solvents, such as water, ethanol, hexane, and chloroform [29,30]. Methanol extract was more active than the aqueous extract against all the microorganisms [31]. Antibacterial compounds isolated from a large number of plant species throughout the world play a role in plant defense, polyphenolic compounds being known to have multiple functions. Flavonoids, such as naringin, flavone, and flavonol, including kaempferol, morin, and quercetin and constitute a large group of secondary plant metabolites that have been reported to have antimicrobial activities [32,33]. The high content of phenolic compounds, saponin, and flavonoids in the extract may also be a contributing factor towards antimicrobial activity. This is the first study to provide data that the MECC possess potential antibacterial and antifungal activities.

CONCLUSION

The results of this study support the folkloric use of this plant in the management of controlling diabetes and post-diabetic bacterial and fungal infections. However, further detailed scientific studies are essential to find out the fundamental mechanisms of antihyperglycemic and antimicrobial activity as well as isolation of active components responsible for this pharmacological property.

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| Bacteria | Gram-positive/ Gram-negative | 2500 µg/ml | 3000 µg/ml | Standard penicillin | Standard chloramphenicol | Standard griseofulvin |
|----------|------------------------------|-----------|-----------|--------------------|------------------------|----------------------|
| S. aureus ML 351 | G+ | 14 | 15 | 28 | 31 |
| S. aureus ML 314 | G+ | 13 | 16 | 34 | 38 |
| S. aureus ML 264 | G+ | 13 | 15 | 23 | 28 |
| S. aureus ML 267 | G+ | 12 | 12 | 30 | 34 |
| B. subtilis UC 564 | G+ | 10 | 12 | 18 | 25 |
| B. pumilus 8241 | G+ | 8 | 11 | 17 | 26 |
| B. cereus PI 11778 | G+ | 10 | 12 | 14 | 20 |
| V. cholerae 117 | G− | 12 | 13 | 24 | 32 |
| K. pneumoniae 114 | G− | 12 | 14 | 27 | 30 |
| E. coli DH 5α | G− | 10 | 11 | 34 | 38 |
| S. dysenteriae 7 | G− | 13 | 15 | 24 | 30 |
| S. boydi RC | G− | 18 | 20 | 33 | 34 |
| S. typhimurium 74 | G− | 10 | 12 | 17 | 30 |
| C. albicans MTCC 183 | 10 | 16 | | 34 |
| A. flavus MTCC 1973 | 15 | 17 | | 30 |
| A. niger MTCC 16404 | 15 | 16 | | 30 |
| C. albidas MTCC 183 | 17 | 18 | | 28 |

Values are zone of inhibition (mm, including the diameter of the bore [6 mm]). Penicillin (100 µg/ml), chloramphenicol (100 µg/ml), Griseofulvin (100 µg/ml). MECC: Methanolic fruit extract of Cucumis callosus; ATCC: American Type Culture Collection, MTCC: Microbial Type Culture Collection. S. aureus: Staphylococcus aureus, B. subtilis: Bacillus subtilis, B. pumilus: Bacillus pumilus, B. cereus: Bacillus cereus, V. cholerae: Vibrio cholerae, K. pneumoniae: Klebsiella pneumoniae, E. coli: Escherichia coli, S. dysenteriae: Shigella dysenteriae, S. boydi: Shigella boydi, S. choleraesuis: Salmonella choleraesuis, S. typhimurium: Salmonella typhimurium, P. vulgaris: Proteus vulgaris, P. aeruginosa: Pseudomonas aeruginosa, C. albicans: Candida albicans, A. flavus: Aspergillus flavus, A. niger: Aspergillus niger, C. albidas: Cryptococcus albidas
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