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Phytochemical Screening and *in vitro* Anti-inflammatory Activity of Methanol Extract of *Clerodendrum splendens* leaf

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Abstract
This study evaluated the phytochemical constituents and in vitro anti-inflammatory activity of methanol extract of Clerodendrum splendens leaf. The phytochemicals present in C. splendens extract are flavonoids, saponins, steroids, terpenoids, tannins and alkaloids. The anti-inflammatory activity of the methanol extract was assessed by the proteinase inhibitory activity, protein denaturation assay and human red blood cell (HRBC) membrane stabilization test. The extract significantly ($p < 0.05$) protected HRBC membrane against haemolysis induced by hypotonic solution and heat with % inhibition ranging from 55.43 % - 79.43 % for the hypotonic test and 45.07 % - 57.28 % for the heat test as against a standard drug, indomethacin which showed % inhibition ranging from 8 % - 32.57 % and 8.75 % - 18.51 %, respectively. However, the extract offered significantly ($p < 0.05$) lower protection against protein denaturation, in a concentration-dependent manner, ranging from 11.42 % – 68.57 % when compared with indomethacin (18.57 % - 80.0 %). The extract exhibited significantly ($p < 0.05$) higher anti-proteinase activity that was maximal at 200 mg/ml (66.82 %) in contrast to indomethacin which showed maximal activity at 100 µg/ml (58.77 %). The results suggest that C. splendens leaf possess appreciable potential to reduce inflammation thus, supporting its utilization amongst traditional medical practitioners.

Keywords: Inflammation, *Clerodendrum splendens*, phytochemical screening, hemolysis, indomethacin

Introduction
Inflammation is regarded as a swelling, painful or otherwise uncomfortable situation occurring in joints, sinus or intestine. In many individuals, inflammation occurs without any symptoms (1). Inflammation has become the focus of global scientific research because of its implication in virtually all human and animal diseases (2). For instance, chronic inflammation is strongly suspected in many degenerative diseases, such as arthritis, repetitive strain injuries, Alzheimer's and Parkinson's disease, cancer, heart disease, inflammatory bowel disease, asthma, inflammatory skin problems (eczema and psoriasis), depression, bi-polar disorders and multiple sclerosis (3).
Non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, are among the most commonly recommended and prescribed drugs for treating inflammation in the world. They have three main effects: analgesic (reduces pain), anti-inflammatory and antipyretic (reduces fever). Unfortunately, the side effects of these drugs include delayed healing, gastric and duodenal ulcers in about half of those who take them regularly and gastrointestinal bleeding in most people (4). Other possible side effects of NSAIDs include: muscle dysfunction, kidney damage, liver damage, headaches, skin rash, tinnitus (ringing in the ears), and drowsiness As a result of these adverse effects, new anti-inflammatory and analgesic drugs lacking these side effects are under continuous investigation as alternatives to NSAIDs and opiates (2).
The usage of plant-based medicines in combating diseases has increased over the years. According to WHO, about 80% of the world population still rely on herbal medicine due to the fact that they are readily available, of low cost

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with little side effects when compared to the orthodox counterpart. Several such medicinal plants have been used by traditional healers for the treatment and management of inflammations (5). *Clerodendrum splendens* belongs to the family Verbenaceae and the genus *Clerodendrum.* The plant is anornamental climbing shrub with attractive small red flowers produced during the dry season. It is widely distributed in tropical and sub-tropical regions of Western Africa. It is commonly called "Bleeding Heart vine" and "Flaming Glory-bower" (6). Extracts of the roots, leaves and bark are used in ethno traditional practices to treat malaria, coughs, buboes, venereal infections, ulcers and rheumatism (7).This study is aimed at providing information regarding the phytochemical constituents and anti-inflammatory activity of methanol extract of *Clerodendrum splendens* leaves.

Materials and Methods

Plant material

The fresh leaves of *Clerodendrum splendens* were collected from a private farm at Ugbowo, Benin City, Nigeria. The plant was identified and authenticated by a Botanist at the Department of Plant Biology and Biotechnology, University of Benin, Benin City. Voucher specimen of the identified plant was thereafter deposited at the herbarium.

Preparation of extracts

Briefly, 1kg of hand-crushed material was soaked in 100 % methanol in a conical flask. The flask was sealed and kept in a mechanical shaker for 72 hours. The solution was then filtered through muslin cloth. The filtrate (methanol extract) obtained was evaporated to dryness in a rotary evaporator. The dried extract was stored at 4 ^{o}C for subsequent use.

Phytochemical screening

Phytochemical screening was carried out on the plant samples using established protocols as described by Harbone (8), Sofowora (9) and Trease and Evans (10).

Stock solutions of each extract with a concentration of 10 mg extract/mL distilled water was prepared and used for the phytochemical screening.

Membrane stabilization assays

Erythrocyte suspension was prepared by the method described by Shinde *et al*., (11) with some modifications. Whole human blood was obtained from a healthy human volunteer and transferred to heparinized centrifuge tubes, centrifuged at 3000 rpm for 5 min and washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as a 40 % (v/v) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4

Heat-induced haemolysis

This test was carried out as described by Okoli *et al*. (12). Erythrocyte suspension (0.05 ml) was added to 5 ml of an isotonic buffer solution containing varying concentrations of the extracts (100 – 400 µg/ml) or the standard drug (indomethacin). The tubes were then incubated at 54 °C for 20 min in a regulated water bath. At the end of the incubation, the reaction mixture was centrifuged at 1300 g for 3 min and the absorbance of the supernatant measured at 540 nm. The level of inhibition of hemolysis was calculated using the following relation:

% inhibition of haemolysis = 100 × (1-A2/A1)

Where, A1 = Absorbance of the control sample, A2 = Absorbance of the test sample solution.

Estimation of proteinase inhibitory activity

The test was performed according to the modified method of Oyedepo and Femurewa (13). The reaction mixture (2 ml) contained 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations. The mixture was incubated at 37 ^{o}C for 5 min and then 1 ml of 0.8 % (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70 % perchloric acid was added to terminate the reaction. The observed cloudy suspension was centrifuged and the absorbance of the supernatant read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

$$\text{Percentage inhibition} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100$$

Hypotonicity-induced haemolysis

This test was carried out as described by Umapathy *et al*., (14). Erythrocyte suspension (0.05 ml) was added to tubes containing varying concentrations of the extracts (100 – 400 µg/ml) and reference drug (indomethacin) in a hypotonic solution (distilled water). The tubes were mixed gently and then incubated for 1 hr at room temperature. After incubation, the reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant measured at 540 nm. The inhibition (%) of haemolysis was calculated using the following relation:

% inhibition of haemolysis = 100 × (1-A2/A1)

where A1 = Absorbance of the control sample

A2 = Absorbance of test sample solution.

Protein denaturation assay

The test was performed following the method described by Gambhire *et al.* (15) with some modifications. 5 ml of 25 % albumin was added to the varying concentrations of the extracts (100 – 400 µg/ml) and the standard drug (indomethacin). The tubes were then gently mixed and the mixtures incubated for 15 min in ambient temperature. Denaturation was induced by keeping the reaction mixture at 70 °C in a water bath for 10 min. After cooling, the turbidity was measured using a spectrophotometer at 660 nm. Percentage of inhibition of denaturation was calculated from control where no drug was added using the following equation:

% inhibition of denaturation = 100 × (1-A2/A1)

where, A1 = Absorbance of control sample and A2 = Absorbance of test sample

Statistical analysis

All values were expressed as means ± standard deviation (S.D). One way Analysis of Variance (ANOVA), followed by Turkey's test was performed using Graph Pad Prism version 5 to analyze differences in group mean. The level of significance was set at $p < 0.05$ for all treatment groups.

Results

Table 1 shows the results of the phytochemical screening of methanol extract of *Clerodendrum splendens* leaves. The results revealed that the extract contains flavonoids, saponins, steroids, tannins, terpenoids and alkaloids. However, cardiac glycosides and anthraquinones were not detected. Figure 1 shows the effect of *C. splendens* extract on heat – induced haemolysis of erythrocyte. The extract, at various concentrations (100 – 400 µg/ml) significantly ($p < 0.05$) protected the erythrocyte membrane against lysis induced by heat more than the indomethacin standard at similar concentrations. The percentage inhibition of haemolysis by the extract ranged from 45.07 % (at 100µg/ml) to 57.28 % (at 400 µg/ml) when compared to indomethacin with minimum % inhibition of haemolysis of 8.74 % (at 100 µg/ml) and maximum inhibition of 18.51% (at 400 µg/ml).

The effect of *C. splendens* extract on proteinase inhibitory activity is presented in Figure 2. The methanol extract of *C. splendens* exhibited significant ($p < 0.05$) anti-proteinase activity in a dose-dependent manner. The maximum inhibition of 66.82 % was shown at 200 µg/ml when compared with the control drug – indomethacin which showed maximum inhibition of 58.77 % at 100 µg/ml.

The results in Figure 3 showed that *C. splendens* at concentration range of 100 – 400 µg/ml protected the erythrocyte membrane against lysis induced by hypotonic solution ($p < 0.05$). The minimum protection of 55.43 % was recorded at 100 µg/ml; while maximum protection of 79.43 % was observed at 300 µg/ml as against the indomethacin standard drug with protection minimal of 8 % and maximal of 32.57 % at same concentrations.

The inhibitory effect of the *C. splendens* extract against protein denaturation is shown in Figure 4. *C. splendens* at the doses of 100 to 400 µg/ml showed minimal (11.42 %) and maximal (68.57 %) inhibition of denaturation of egg albumin, respectively ($p < 0.05$) in contrast to indomethacin which offered better inhibition of albumin denaturation of 18.57 % (100µg/ml) and 80.0 µg/ml (400 µg/ml).

Table 1: Phytochemical Constituents of Methanol Extract of *Clerodendrumsplendens* Leaf

TEST	RESULTS
Flavonoids	+++
Tannins	++
Cardiac glycosides	−
Saponins	++
Steroids	+++
Terpenoids	++
Anthraquinones	−
Alkaloids	+++

+ = slightly detected; **− =** absent; ++ = moderately detected; +++ = highly detected

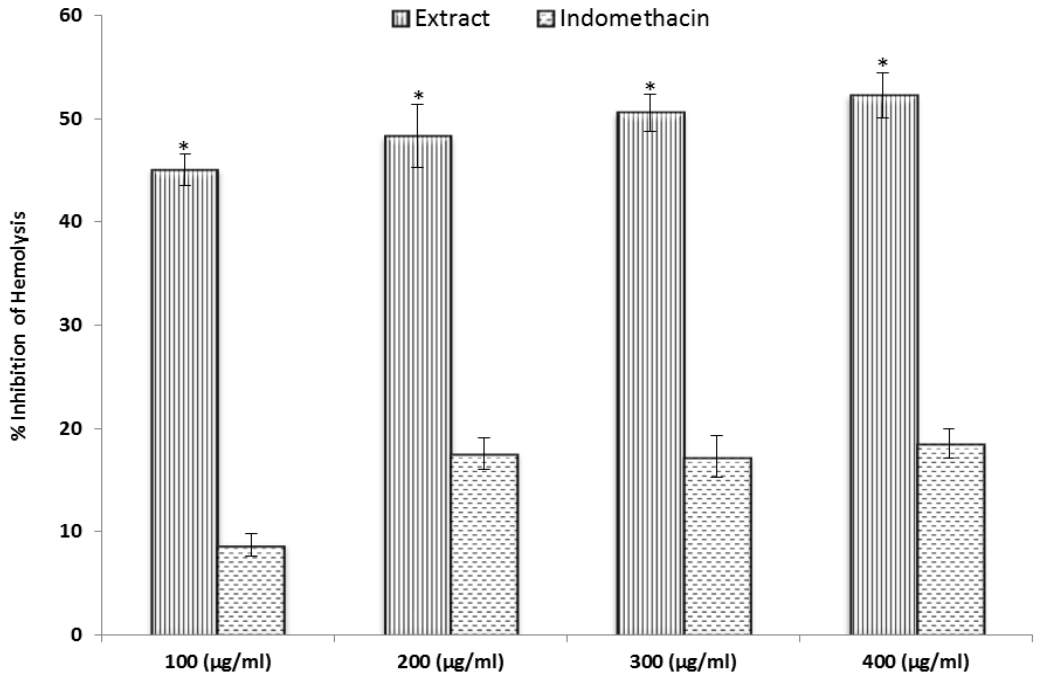

Figure 1: Effect of Clerodendrum splendens Extract on Heat – Induced Haemolysis of Erythrocyte
*Values are mean ± SEM (n = 3 determinations). * Significantly different from control drug – indomethacin at p < 0.05.*

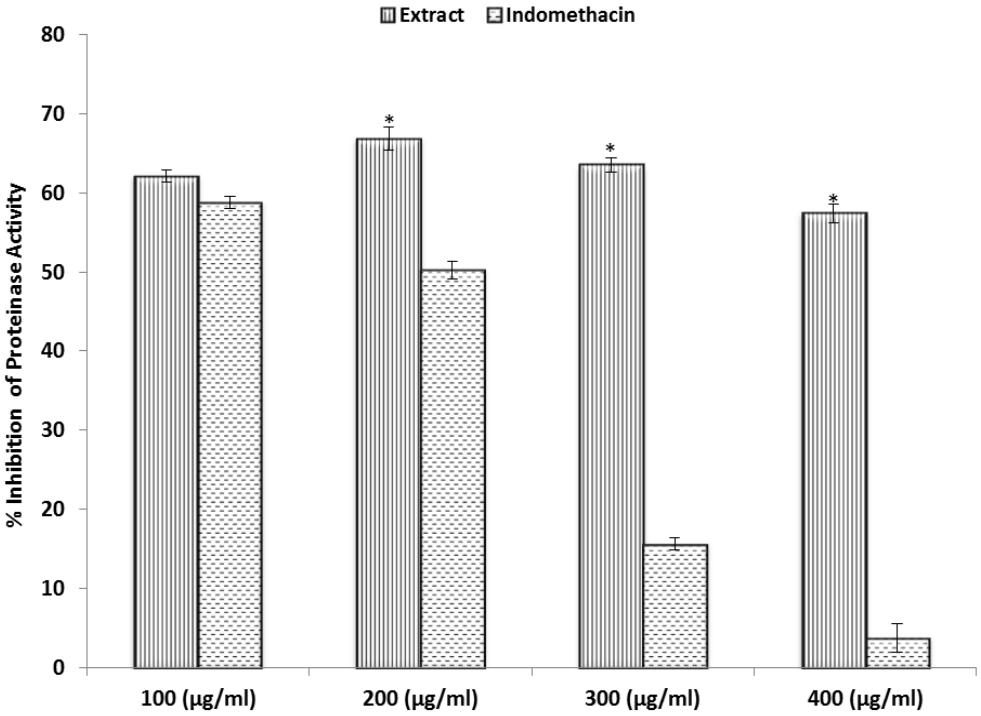

Figure 2: Effect of Clerodendrum splendens Extract on Proteinase Inhibitory Activity
*Values are mean ± SEM (n = 3 determinations). * Significantly different from control drug – indomethacin at p < 0.05.*

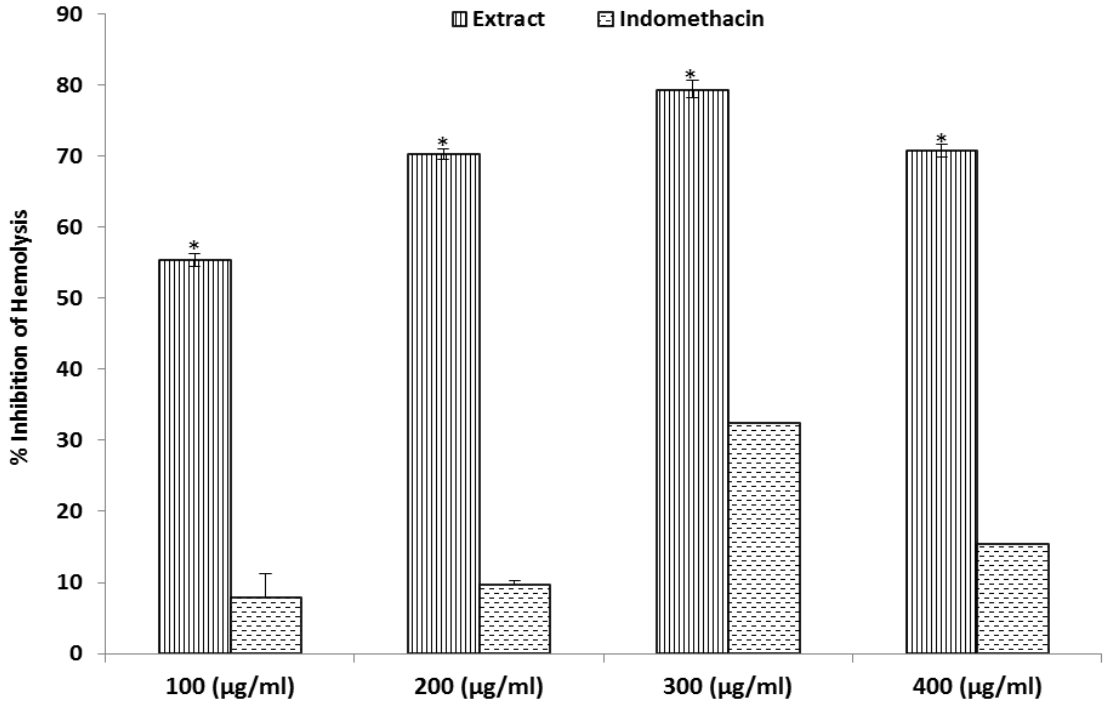

Figure 3: Effect of Clerodendrum splendens Extract on Hypotonicity – Induced Haemolysis of Erythrocyte
*Values are mean ± SEM (n = 3 determinations). * Significantly different from control drug – indomethacin at $p < 0.05$.*

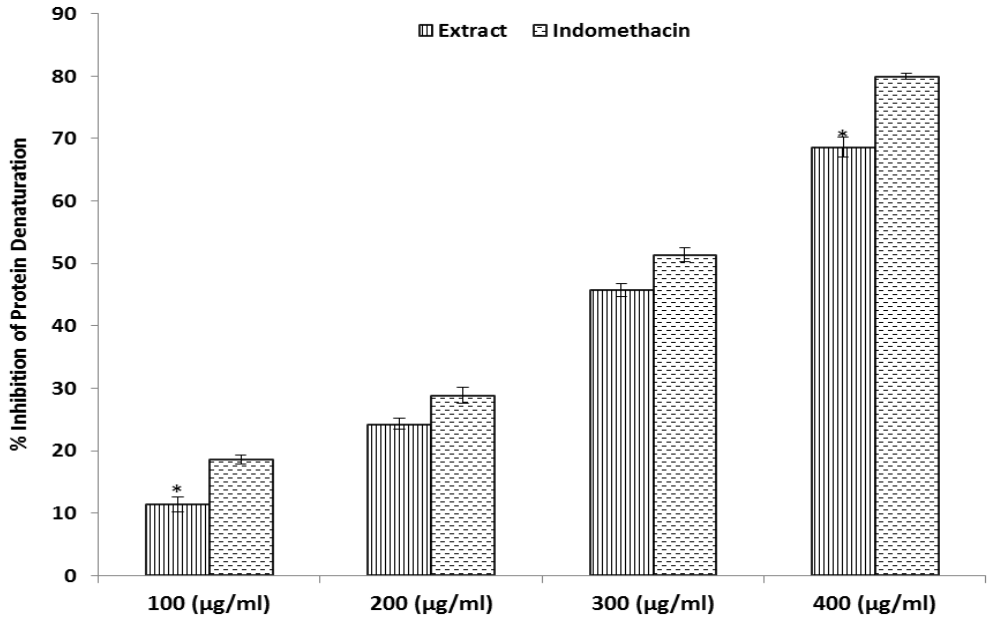

Figure 4: Effect of Clerodendrum splendens Extract on Heat – Induced Protein Denaturation
*Values are mean ± SEM (n = 3 determinations). * Significantly different from control drug – indomethacin at $p < 0.05$*

Discussion

Plants are the essential and integral part of complementary and alternative medicine. They are the best source of active metabolites which are beneficial to mankind in treating many diseases (16). Medicinal plants and herbs were the earliest sources of substances used to produce therapeutic effects. Herbal medicine has been found to have impressive credentials with no equivalent in modern medicine (17). In the present study, the phytochemicals screening tests on the methanol extract of *C. splendens* revealed the presence of compounds of biological interest such as alkaloids, saponins, steroids, terpenoids, tannins and flavonoids. Cardiac glycosides and anthraquinones were however not detected. These results are similar to previous reports (18, 19). Each of these phytochemicals is known for various protective and therapeutic effects (20). For instance, flavonoids are known to possess anti-bacterial, anti-inflammatory, anti-allergic, anti-viral and anti-neoplastic activity (21). They have antioxidants effects in animals (22). Saponins are known to inhibit inflammation and possess antibacterial activities (23). Steroidal compounds are of importance due to their relationship with some compounds such as sex hormones (24).

The presence of alkaloids is an indication of the potential role of *Clerodendrum splendens* in pharmaceutical industries for health care delivery. One of the most potent biological properties of alkaloids is the toxicity against cells of foreign organisms (23). Steroids, terpenoids and alkaloids have been reported to exert inhibitory activity against most bacteria (25, 26). Tannins possess astringent properties and are used in treating intestinal disorders such as diarrhoea. They are also known to exhibit antimicrobial activities (23). Tannins can form irreversible complexes with proline-rich protein resulting in the inhibition of cell protein synthesis (27). Parekh and Chanda (28) also reported that tannins can react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues.

One of the key aspects of inflammatory response is cellular infiltration due to the pivotal role played by leukocytes. As part of their defensive roles during inflammation, these cells release their lysosomal contents such as bactericidal enzymes and proteases causing further tissue damage and inflammation (12, 29). Such injury to cell membrane will further render the cell more susceptible to secondary damage through free radical induced by lipid peroxidation (14). In this study, the methanol extract of *C. splendens* protected the human red blood cell (HRBC) membrane against haemolysis induced by heat and hypotonic medium. The standard drug, indomethacin showed a lower inhibition of HRBC membrane haemolysis when compared with the extract. Since the RBC membrane is analogous to the lysosomal membrane, its stabilization by the *C. splendens* extract implies that the extract's phytochemical constituents may act as antioxidants which resulted in the stabilization of the lysosomal membranes. These limit the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases (14).

Protection against protein denaturation has been suggested to play an important role in the anti-rheumatic activity of many NSAIDs (30). Likewise, in this study, the ability of the *C. splendens* extract as well as the standard drug, indomethacin, to inhibit protein denaturation might have contributed to their anti-inflammatory activity. The methanol extract of *C. splendens* leaf also exhibited significant ($p<0.05$) proteinase inhibitory activity at various concentrations with the maximum proteinase inhibitory activity observed at 200µg/ml and minimum at 400µg/ml when compared with the standard anti-inflammatory drug. Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a source of proteinase which carries in their lysosomal granules many serine proteinases (31, 32). From literature, leukocytes proteinase has been reported to play an important role in the development of tissue damage during inflammatory reactions and significant levels of protection were provided by proteinase inhibitors (31, 32). Similarly, the phytochemicals present in the *C. splendens* extract especially flavonoids and related polyphenols, tannins, alkaloids, steroids, terpenoids may be responsible for the anti-inflammatory activity of this plant.

In summary, the present study shows that the methanol extract of *C. splendens* possess *in vitro* anti-inflammatory activity. These findings may form the basis for further research into the efficacy of the plant products as anti-inflammatory agents.

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