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Research Article

Assessment of cytotoxicity of leaf extracts of *Andrographis paniculata* and *Aspilia africana* on murine cells *in vitro*

***Ala A.A.[1], Olotu, B.B.[1] and Ohia C.M.D.[2]**

[1]*Department of Zoology, University of Ibadan, Ibadan, Nigeria*
[2]*Department of Environmental Health Sciences, College of Medicine, University of Ibadan, Ibadan, Nigeria*

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Abstract

People, especially in rural environments and recently, those dissatisfied with conventional medicine use medicinal plants for their therapeutic effects. Two or more plants are usually combined for such use and these combinations cause interactions that may be safe but could also be detrimental to health. Used singly, *Andrographis paniculata* and *Aspilia africana* are considered safe plants with low toxicities but little is known about their safety in combined therapy. This study assessed the cytotoxic activities of the aqueous, methanol and chloroform extracts of the two plants in a 1:1 combination at various concentrations on murine hepatocytes, thymocytes and splenocytes. The murine cells were seeded in microtitre plates and tested with the combined extracts at different concentrations and percentage viability values of the cells determined by the tetrazolium salt reduction assay (MTT). The extracts used were: combined aqueous extract of the two plants (CAE), and combined chloroform extract (CCE). At 72hr incubation with 500 µg/ml extract concentration, percentage viability was low. Splenocytes thymocytes and hepatocytes incubated with CAE had viability values of 35%, 28% and 64% respectively and with CCE, 26%, 26% and 36% respectively, relative to controls. From the results, the extracts were cytotoxic to the murine cells at this concentration and incubation period. These low values indicate cytotoxic interactions in the combined extracts. However more research is needed to understand the mechanisms of cytotoxicity of the plants.

Key Words: *Andrographis paniculata, Aspilia africana, murine cells, MTT assay, viability*

INTRODUCTION

The belief that natural medicines are safer than synthetic drugs has caused exceptional growth in human exposure to natural products such as plants, phytotherapeutic agents, and phytopharmaceutical products. Thus, there is renewed scientific interest in their biological effects. In most countries there are no regulatory system ensuring the safety and activity of natural products and they have not been sufficiently investigated analytically or toxicologically (Valerio and Gonzales, 2005; Da Silva *et al*., 2013).

Natural products have been, and remain, the cornerstone of health care. Present estimates show that not less than 80% of people worldwide rely on them for some part of primary healthcare (Ekor, 2013). Many of these plants, have therapeutic effects and can be extracted and used in preparation of drugs, used directly or in combination with other plant extracts for medication which is a common practice in developing counties (Bhattacharya and Das, 2012). Unfortunately, many of those who utilize these plants therapeutically do not have adequate knowledge or training in the safe use of the products. For these reasons, natural plant products need to be standardised and preliminary studies done to evaluate possible risks such as undesirable side effects, overdose and toxicity.

Andrographis paniculata (Acanthaceae) is a traditional medicinal herb, a shrub grown in the damp, shady areas of India, China, Indonesia and throughout Southeast Asia which has been used as an immunostimulant (Sukardiman*et al*., 2014). It has been found to exhibit antityphoid, antifungal, antihepatotoxic, antibiotic, antimalarial, anti-hepatitis, antithrombogenic, anti-inflammatory and antitumour activities (Thiyagarajan, *et al*., 2011). Andrographolide, an active constituent of *Andrographis paniculata* was found to inhibit the proliferation of various cell lines including leukemia, breast cancer, lung cancer, and melanoma cells. Furthermore, this compound has strong anticancer activity against human colorectal carcinoma LoVo cells by inhibiting cell cycle progression (Jayakumar *et al*., 2013).

Aspilia africana has also been reported to possess haemostatic, anti-inflammatory and anti-fertility activity. The n-hexane and methanolic extracts have also been reported to possess wound healing and anti-ulcer activity (Okoli *et al*., 2007b; Ajeigbe *et al*., 2013). In South-eastern Nigeria, leaves of this plant is claimed to be effective in the treatment of stomach ache and bleeding gastric ulcers, especially when taken as an aqueous decoction. Phytochemical studies have revealed the presence of saponins and tannins as the most abundant compounds in the plant while flavonoids were the least (Okoli *et al*., 2007a; Uduak and Ikoedem, 2013).

In traditional medicine, the combination of two or more herbs is common in the treatment of chronic and acute diseases since the plants may act in synergy when combined (Dhiman *et al*; 2012; Agonihotri *et al*., 2010; Okoli *et al*., 2007b).

This study therefore assessed the cytotoxicity of the combined leaf extracts of the plants; A. paniculata and

**Author for Correspondence:* +234-8033561694 E-mail: adeola.ala2015@gmail.com

A.africana on murine hepatocytes, splenocytes and thymocytes.

MATERIALS AND METHODS

Plant collection

Fresh leaves of *A. africana* and *A. paniculata* were collected from the University of Ibadan campus and brought to the laboratory in plastic bags. The plants were taxonomically identified and authenticated at the Department of Botany Herbarium, University of Ibadan.

The leaves of each plant were cleaned thoroughly to remove all debris, insects and adhering sand particles. The samples were air-dried at room temperature until constant weights were obtained and then pulverized to powder using an electric grinder.

Plant extraction

100g samples from each plant were soaked separately in 1L each of three different solvents namely; aqueous (distilled water), methanol (absolute) and chloroform (absolute) for 3 days at room temperature (Soundararajan 2012; Wamidh *et al;* 2010). Each extract was filtered with a muslin cloth and filtrate evaporated under reduced pressure at 40^{o}C using a rotary evaporator (Buchi R-215, Switzerland). All solvents were evaporated to dryness at room temperature to produce the crude extracts which were collected and stored at −4°C for further test and use. The extracts from the two plants were thereafter mixed in a 1:1 ratio to give the following: Combined Aqueous Extract (CAE); Combined Methanol Extract (CME) and Combined Chloroform Extract (CCE).

Culture media / washing media

IMDM (Iscove's Modified Dulbecco's Medium) Sigma, with batch no 17633; 050M8313 was used and supplemented with 10% fetal bovine serum (Sigma, U.S.A), 2% sodium bicarbonate, 1% L-glutamine (Sigma, U.S.A), 1% sodium pyruvate (Sigma, U.S.A), 1% Penicillin/Streptomycin, and 1% HEPES at 10 mM (Hybri-max, Sigma, U.S.A).

Animals

Three BALB/c mice, 3-4 weeks old were purchased from The International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria. The mice were housed under standard conditions and fed with standard food pellets and drinking water *ad libitum*.

Preparation of Mice Splenocytes, Thymocytes And Hepatocytes

The BALB/c mice were sacrificed by cervical dislocation and their spleens, thymuses and livers harvested aseptically into different Petri plates and washed twice, each time with 5ml of washing media. The cells (splenocytes, thymocytes and hepatocytes) were then dislodged by gently macerating the organs in different petri plates, each with 5ml of the washing media. The cells were washed by carefully pipetting the cells into centrifuge tubes, leaving connective tissue in the petri plates. This was done twice. The volume of the cell suspension was thereafter made up to 10ml with washing media and centrifuged at 1500 rpm for 5 minutes. This was done twice after which the supernatant containing cells was aspirated into another centrifuge tube and the procedure repeated. The cells were finally aspirated into a costar flask and incubated at 37°C in a fully humidified atmosphere of 5% CO_2 ; the pellet was discarded. A sample of the cells was viewed under the inverted microscope at a magnification of 100x and viable cells were counted using a Hausser scientific hemacytometer (0.100mm deep) to ascertain at least 95% viability.

Splenocytes, thymocytes, and hepatocytes were seeded differently into three 96-well cell culture plates (Nunc, Denmark) at a density of 1 x 10^4 cells per well and 200 µL per well. Cells were then incubated at 37°C for 24 hours at 5% CO_2. After incubation and attachment, 20µL supernatant was pipetted off each well. Cells were then treated, 20µL/well with different concentrations (4, 20, 100, and 500µg/ml) of each of the combined plant extracts (aqueous, methanol and chloroform) initially dissolved in 0.1% Dimethyl Sulfoxide (DMSO), except the aqueous extract which was dissolved in distilled water. Control cells received 20 µL of distilled water. Concentrations of the extracts were defined according to the literature (Wilasrusmee *et al.*, 2002; Manosroi, *et al.*, 2003; Mehrotra *et al.*, 2003). Each plate had wells which contained culture media without cells (blanks),culture media with untreated cells, and culture media with treated cells (Talib and Mahasneh, 2010) (with modification).The cells were then incubated at 37°C for 48hr and 72hr respectively for two different assays in a fully humidified atmosphere of 5% CO_2.

Cytotoxic activity assays

The cytotoxic effects of *A.africana* and *A.paniculata* combination on splenocytes, thymocytes, and hepatocytes were determined using two methods, the direct counting of living and dead cells using a haemocytometer with an inverted microscope and by MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, according to Talib and Mahasneh (2010).

Direct counting method:

After an incubation period of 48hr, cells in each well were gently mixed by pipetting up and down and 20 µl of cells from each well was pipetted into wells of other 96-well plates. The first set of plates were returned to the incubator. One drop from each well of the new plate was pipetted into a haemocytometer and dead and living cells thereafter observed under the inverted microscope. Shrunken, dense cells were counted as dead while transparent, round looking cells were counted as viable. The percentage of viable cells was calculated using the formula:

$$\%\text{Viability} = \frac{\text{Total number of viable cells}}{\text{Total number of viable and non-viable (dead) cells}} \times 100$$

MTT assay

The MTT assay was used to measure cytotoxicity (loss of viable cells). This assay is based on the metabolic reduction of the soluble MTT salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide which reflects the normal function of mitochondria dehydrogenase activity and cell viability, into an insoluble colored formazan product, which was measured spectrophotometrically (Sadeghi-aliabadi*et al;* 2010). The determination of the activity of mitochondrial dehydrogenase of living cells directly and proportionally represents the number of viable cells (Mosmann, 1983).

For this assay, after treatment with the combined plant extracts, the cells were incubated for 72hr at 37°C, 5% CO_2. 20µl of filter sterilised MTT (2mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for

3 hours. The medium with MTT was removed and the formed formazan crystals were solubilised by the addition of 100µl of DMSO and the absorbance read at 540 nm using a universal microplate reader. Treated cells were compared with untreated controls. Tetrazolium salts are cleaved to formazan dye by cellular enzymes only in viable cells. The reduction of the tetrazolium salt MTT, to colored formazan compounds by cellular enzymes only occurs in metabolically active cells (viable cells). Therefore, the amount of formazan dye formed directly correlates to the number of viable cells in the culture and is measured spectrophotometrically as absorbance. Cells exposed to toxins will have decreased activity.

The percentage cell viability was calculated using the formula:

$$\% \text{ cell viability} = \frac{\text{Absorbance of treated cells} - \text{background absorbance (b)}}{\text{Absorbance of untreated (c)} - \text{background absorbance (b)}} \times 100$$

Where b= blank and c = control

The results reported are the mean values of two different experiments performed in triplicates.

Statistical Analysis

Statistical analysis was done using ANOVA. Results obtained were expressed as means + SE. The Duncan's test was used to locate significant differences between means. Significant differences in treatments were accepted at $P < 0.05$.

RESULTS AND DISCUSSION

This study was conducted to assess the cytotoxic activities of the aqueous, methanol and chloroform extracts of the two plants in a 1:1 combination at various concentrations on murine hepatocytes, thymocytes and splenocytes.

Using the direct counting method, values of percentage cell viability of the murine cells at different concentrations of the extracts after 48 hours incubation are shown in figures 1 to 3.

The values for cell viability with aqueous extracts (CAE) at the highest concentration—500µg/mL from all cell types (figure 1) were between 85% and 91%. These values were lower than the values (86-95%) of the control (untreated cells + Media). At the lowest concentration— 4µg/mL, viability values of all cell types were between 85% and 94%. These values were also lower than those of the control. The percent cell viability values of the aqueous extracts at all concentrations showed low cytotoxicity at 48hr incubation. The differences were not significant.

Percent viability values of all cell types treated with CME (Fig. 2) were high, between 75-96% and comparable to percent viability values in the controls which were between 82-87%. The methanol extract did not reduce viability of cells at the concentrations (4µg/mL and 500µg/mL) and at 48hr incubation. The differences were not significant.

Fig 3. also shows high percent viability values in all cell types and at all concentrations with CCE (76-95%) which were comparable to the values in the control (79-91%). Comparing splenocytes, the viability value in the control was higher than viability values of treated cells which shows reduction in viability with the treatments. Compared with other cell types at each concentration, hepatocytes showed the least percent viability values with CAE, CME and CCE (Figs 1-3). Results from these experiments show low cytoxicity of extracts at 48hr incubation. The differences were however not significant.

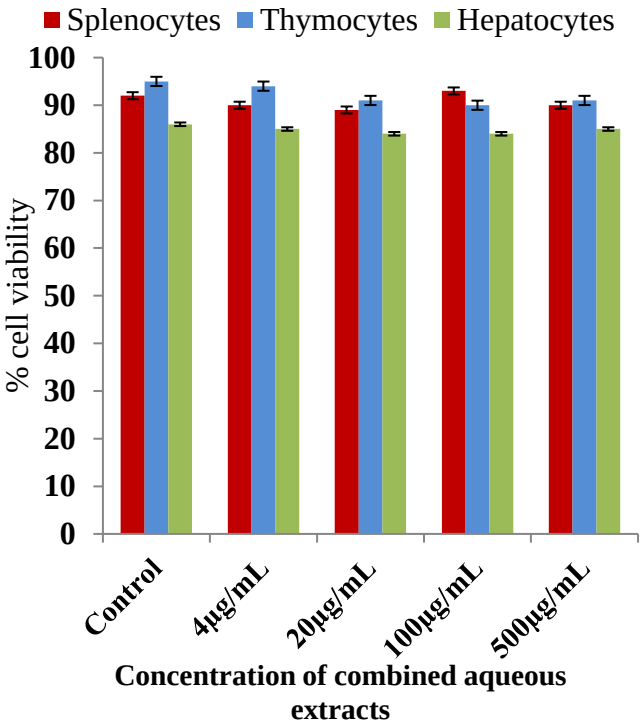

Figure 1:
Percentage viability of murine cells after 48hr incubation with the combined aqueous extract (CAE) of A. paniculata and A. Africana using the direct counting method

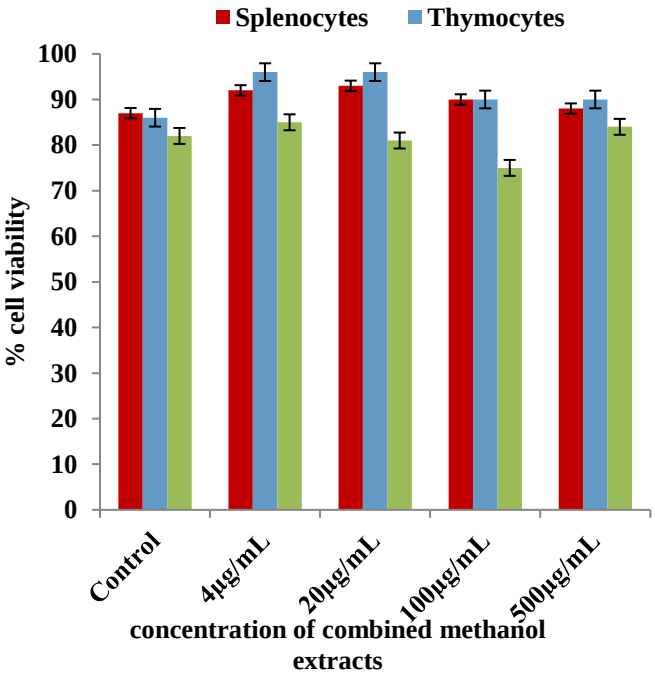

Figure 2:
Percentage viability of murine cells after 48hr incubation with the combined methanol extract (CME) of *A. paniculata* and *A. Africana* using the direct counting method

Results by MTT assays are presented in figures 4 and 5 which show percent viability values of the murine cells incubated for 72hr with aqueous (CAE) and chloroform (CCE)

extracts. Percent viability values of cells were found to reduce with increase in concentration. The combined extracts CAE and CCE exhibited in-vitro cytoxicity at 72hr incubation leading to decreased viability in the treated cells when compared with the untreated controls.

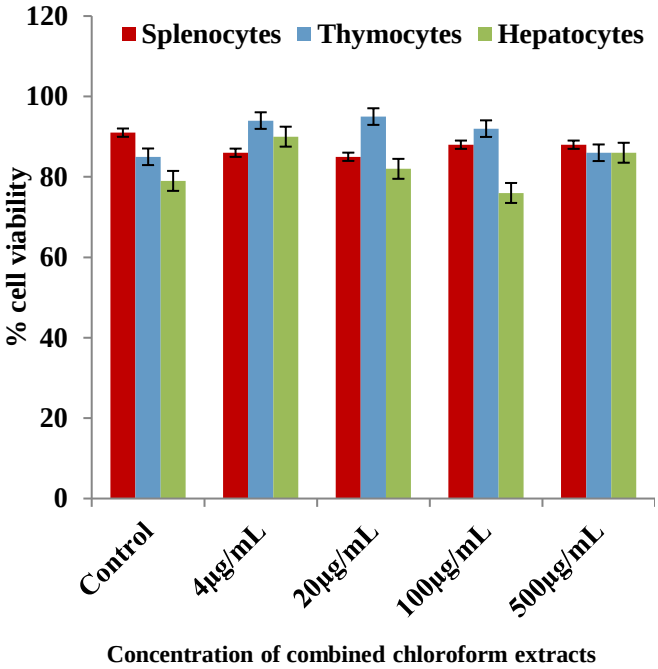

Figure 3:
Percentage viability of murine cells after 48hr incubation with the combined chloroform (CCE) extract of *A. paniculata* and *A. Africana* using the direct counting method

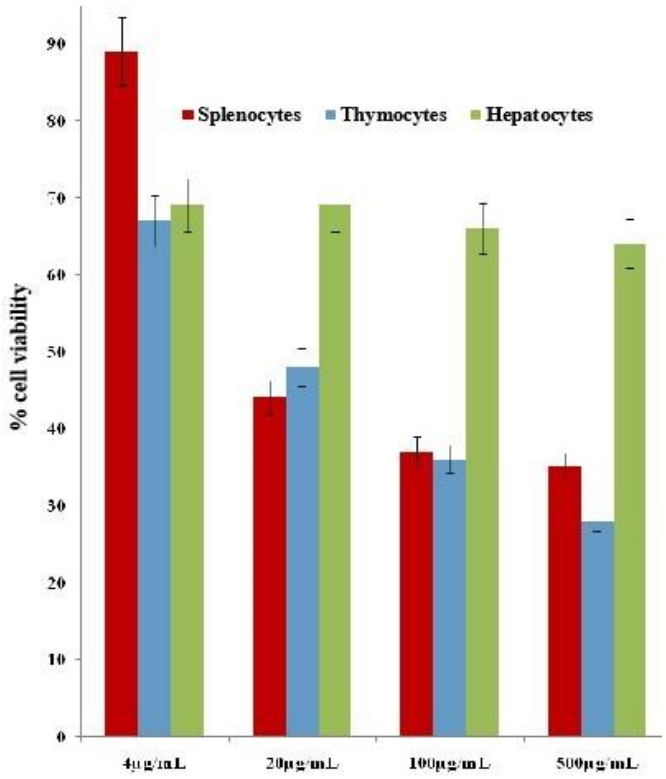

Figure 4:
Percentage viability of murine cells after 72hr incubation with the combined aqueous extract (CAE) of *A. paniculata* and *A. Africana* using the MTT assay

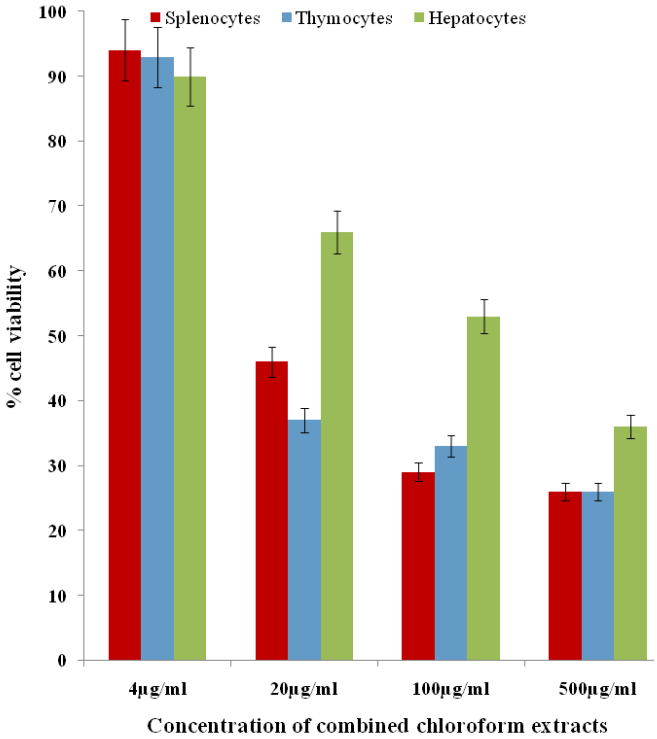

Figure 5:
Percentage viability of murine cells after 72hr incubation with the combined chloroform extract (CCE) of *A. paniculata* and *A. Africana* using the MTT assay

At 4µg/mL- 500µg/mL concentration of CAE, viability values of splenocytes decreased from 89% to 35%, thymocytes, from 67% to 28% and hepatocytes from 69% to 64% respectively (Fig 4). These decreases in viability were significant at $p \leq 0.05$ indicating cytotoxicity of the combined extract.

Fig 5 shows a similar decrease in viability values of cells with increase in CCE concentration. At extract concentrations of 4µg/mL - 500µg/mL, splenocyte viability decreased from 94-26%, thymocytes from 93-26% and hepatocytes from 90-36%. The cells showed decrease in viability with increase in extract concentration. These decreases were significant at $p \leq 0.05$. Compared with CAE, CCE was more cytotoxic at 500µg/mL. Splenocytes thymocytes and hepatocytes incubated with CAE had viability values of 35%, 28% and 64% respectively and with CCE, 26%, 26% and 36% respectively. This is similar to the acute toxicity studies done by Oko *et al*. (2011) on A. Africana (used singly) which showed that the chloroform extract of the leaf was relatively more toxic than the aqueous and ethanolic extracts.

The results from the MTT assay indicate that 72hr extract incubation with the combined extracts is toxic to the cells and the level of damage is concentration dependent. Unwanted reactions can give rise to high background absorbance values especially in herbal extracts when antioxidants in the extracts directly react with MTT or formazan. This occurrence was minimal in these experiments because the blanks (media without cells) did not give background reactions.

From previous in-vitro and in–vivo studies, *Andrographis paniculata* when used singly has been generally perceived as safe in therapy (Joselin and Jeeva 2014; Hu *et al.*, 2017; Okhuarobo *et al.*,2014 and Dey et. al., 2013). Similar findings were also made with *Aspilia africana* and the plant is regarded as a safe herb with low toxicity in single use therapy.

(Taziebou et.al, 2007 ;Akuodor *et al*. 2012 and Ajeigbe *et al*., 2016). Oko *et al*., (2011) reported in-vivo studies where the higher values obtained for LD50 under oral administration of the experiment done indicated that oral administration of *Aspilia africana* leaf extracts could be safe for human and animal use. The aqueous extract also seemed to be of relatively lower toxicity based on its higher LD_{50} value.

However, from this study, the combined use of the plant extracts did not exhibit the expected synergy. Percentage viability was lower in cells treated with the extracts when compared with the control (untreated cells).This study shows that the combined extract were cytotoxic therefore long term use of the plants in combination should be avoided and dosage controlled and regulated.

Overall, results from this study shows that the combined extracts did not act in synergy to protect or rejuvenate the cells but it rather points to the cytotoxicity of the combined extracts; which was concentration dependent; although the direct counting method showed the combined extracts to have low cytoxicity at 48hr incubation. Since herbal teas and other homemade remedies are usually a combination of two or more plants, their uncontrolled use can have negative physiological effects and cause serious disorders and damage to vital organs. Our results therefore further support the warning that the unrestrained use of plant products, especially as combinations can be detrimental to health and hence, steps should be taken to standardise these products

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