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Original Research

Pharmacological activities of cinnamaldehyde and eugenol: antioxidant, cytotoxic and anti-leishmanial studies

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Abstract: The present study reports the antioxidant, cytotoxic and anti-leishmanial activities of cinnamaldehyde and eugenol. Both the compounds are naturally present in cinnamon and bay leaf. Eugenol is abundantly present in clove. The antioxidant potential was measured in terms of reducing power (FRAP assay), nitric oxide (NO) radical scavenging ability and anti-lipid peroxidative activities. MTT assay was used to evaluate the effect of test compounds on cell viability of prostate cancer cell line (PC-3) as well as for the assessment of in vitro growth inhibition of promastigotes as a measure of anti-leishmanial activity. Eugenol exhibited considerable NO radical scavenging (63%) and reducing abilities (FRAP value 127×10^4 µM/mM) while cinnamaldehyde showed comparatively better protective efficacy against lipid peroxidation in rat brain and kidney homogenates (up to 40%). Cinnamaldehyde also displayed substantial cytotoxic activity (75%) against PC-3 cell line. Both the compounds exhibited moderate anti-leishmanial activity and IC_{50} values for eugenol and cinnamaldehyde were found to be 0.681 g/ml and 1.426g/ml, respectively. The study revealed that both the test compounds have noticeable antioxidant and cytotoxic activities.

Key words: Antioxidants; FRAP; Lipid peroxidation; NO radical scavenging; Cytotoxicity; Anti-leishmanial activity.

Introduction

Free radicals are produced during normal metabolic reactions. These radicals include highly bioactive, short living molecules that are produced by reduction of molecular oxygen and known as reactive oxygen species/ ROS (hydroxyl radical, hydrogen peroxide, and super oxide anion) and reactive nitrogen species/ RNS (nitric oxide radical, peroxynitrite etc.) which adversely alter lipids, DNA, and proteins. Peroxidation of membrane lipids might change the permeability and fluidity, compromising the cell integrity (1, 2). Reactive species are required for normal cell functions (cellular responses and immune functions) at physiological concentrations (3). Imbalance between oxidant and antioxidant responses causes excessive production of free radicals, a state known as oxidative stress. This might lead to pathophysiological conditions resulting into several degenerative and chronic disorders such as rheumatoid arthritis, autoimmune disorders, cataract, aging, cancer, cardiovascular and neurodegenerative diseases (4-6). The development of cancer is associated with disorders in the regulation of cell cycle and cellular DNA damage. Cells have developed several defense mechanisms to cope with the constant attack on their DNA for example direct repair, halting cell cycle progression or apoptosis (7).

Cellular antioxidant systems lower the risks associated with ROS and RNS. These include enzymatic (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S transferase etc.) and non-enzymatic antioxidants (glutathione, tocopherols and ascorbate etc.) (8). Antioxidants are either naturally produced in situ or supplied through foods and supplements which maintain equilibrium between formation and degradation of free radicals (9). Phytochemicals including phenolics, flavonoids, terpenes, alkaloids etc. acting as antioxidants have capability to scavenge or reduce the formation of radicals. Plant based diets also show cytotoxic and cancer chemopreventive effects.

Visceral leishmaniasis or kala-azar, a chronic protozoan infection in humans caused by a haemoflagellate *Leishmania donovani*, is associated with significant global morbidity and mortality. *L. donovani* is an obligate intracellular parasite that resides and multiplies within macrophages of the reticulo-endothelial system. The current drugs used for treatment have several disadvantages including side effects, high cost, lack of oral formulation (amphotericin B can be used only intravenously) and drug resistance (10). Hence new therapies are needed to supplement or replace currently available therapies (11).

Cinnamaldehyde, the major constituent of spices cinnamon and bay leaves, possesses significant antipyretic, antianaesthetic, antiallergic, antimutagenic and antioxidant activities (12-14). Eugenol is a main component of clove and has wide range of application in perfumes, flavorings, essential oils and in medicine as a local antiseptic and anaesthetic (15). Both compounds belong to phenyl-propanoid class of phytochemical. Considering medicinal potential of cinnamaldehyde and eugenol, present study was undertaken to evaluate their

antioxidant, cytotoxic and anti-leishmanial activities.

Materials and Methods

Chemicals

Cinnamaldehyde and eugenol were procured from Hi-media Pvt. Ltd. Mumbai, India and 2, 4, 6-tripyridyl-s-triazine (TPTZ), sodium acetate, glacial acetic acid, tri-choloro acetic acid, thiobarbituric acid, sulphanilamide, naphthyl diamine di-hydrochloride, phosphoric acid were obtained from Sisco Research Laboratory (SRL), Mumbai, India.

Assessment of total antioxidant activity by FRAP Assay

Ferric reducing antioxidant power (FRAP) assay was used to measure total antioxidant potential of test compounds (16). To 0.5 ml test samples, 4.5 ml of FRAP reagent was added and after 5 min absorbance was measured at 593 nm. Final concentration of cinnamaldehyde and eugenol in reaction mixture was 0.1µM/ml. Ascorbic acid was used as standard reducing agent for comparison. Calibration curve was drawn using ferrous sulfate (100-1000µmol/ml) and results were expressed as FRAP value (µmol $FeSO_4.7H_2O$ equivalent/mM sample). All analyses were carried out in triplicate and results were expressed as mean±SD.

Lipid Peroxidation Inhibition (LPOI) Assay

The lipo-protective efficacy of test compounds was estimated by the method of Halliwell and Gutteridge (17). Rat kidney and brain tissue homogenates (10% w/v) were prepared in phosphate buffer (0.1 M, pH 7.4 having 0.15 M KCl) at 4°C, centrifuged and clear cell free supernatant was used for study. To the sample (100µl) prepared in DMSO, 1.0 ml KCl (0.15 M) and 0.5 ml of tissue homogenate were added. Concentration of cinnamaldehyde and eugenol in final reaction mixture was 1.0-3.0µM/ml. Ferric chloride (100 µl, 0.2 mM) was added to initiate lipid peroxidation at 37 °C for 30 min which was detected by the formation of thiobarbituric acid reactive substances (TBARS).To measure TBARS, 2 ml ice-cold TBA reagent (15% TCA, 0.38% thiobarbituric acid and 0.5 % BHT in 0.25 N HCl) was added and incubated for 1 h at 80 °C followed by cooling and centrifugation. The absorbance of the pink supernatant was measured at 532 nm. Butylated hydroxyanisole (BHA) was used as standard for comparison. Experiments were carried out in triplicate and results were expressed as mean ±SD. Percent LPOI was measured as index of protection against lipid peroxidation and was calculated as given below:

$$\%\,\text{LPOI} = \frac{(\text{Ac} - \text{As})}{\text{Ac}} \times 100$$

Where Ac and As are absorbance of control and sample/standard compounds.

Nitric oxide (NO) radical scavenging activity

Method of Green *et al* (18) was used for determination of NO radical scavenging activity. To test samples (0.5ml), 1.0ml of sodium nitroprusside (0.01M in PBS) was added and incubated at 25 °C for 180 min followed by addition of equal volume of Griess reagent (1% sulphanilamide, 0.1% naphthyl-diamine di-hydrochloride in 2% phosphoric acid) and further incubated for 30 min at room temperature. The concentration of test compounds/standard ranged between 5-25µM/ml in final reaction mixture. Absorbance was taken at 546 nm. The NO radical scavenging activity was determined using the formulae

$$\%\,\text{NO Scavenging} = \frac{(\text{Ac} - \text{As})}{\text{Ac}} \times 100$$

Where Ac and As are absorbance of control and test samples, respectively. Experiment was performed in triplicate and results were expressed in Mean ± SD.

Assessment of cytotoxicity by cell viability assay (MTT assay)

The MTT [3-(4, 5-dimethylthaizol-2,5-yl)-2,5-diphenyltetrazolium bromide] assay was performed to assess the effect of test compounds on viability of PC-3 prostate cancer cell line (19). Cell suspension (100µl) was added to each well in a 96 well plates and placed in CO_2 incubator for 1 h at 37 °C. Then test sample (25-100 µM/well) was added to different wells and incubated for 48 h followed by addition of 20µl of MTT dye (2.5mg/ml) and further incubated for 3-4 hours. After incubation plates were centrifuged at 1500 rpm for 15 min. Supernatant was discarded and 100 µl DMSO was added in each well followed by gentle shaking on a plate shaker for 5min. Absorbance was determined at 570 nm using ELISA reader. The assay was performed in triplicate and results were expressed in Mean ± SD.

Determination of Anti-leishmanial activity

In vitro promastigotes cell toxicity assay was used to determine the antileishmanial activity using MTT cell proliferation assay (20). Briefly, the exponential phases of promastigotes (2×10^6cells/ml) were incubated with or without the test agents along with M-199 medium at 22°C. The test compounds were added to the culture in graded concentrations. After 2 h of treatment, the plates were centrifuged at 8000 g for about 10 min. The supernatant was decanted and the pellets were washed with 20 mM phosphate buffer saline. Pellet was dissolved in 100µL (2 mg/ml) MTT solution, and the tubes were incubated at 22°C for 4 h and then centrifuged at 8000 g for 10 min. The resulting pellets were dissolved in 100µL DMSO (0.2%) and the absorbance was measured spectrophotometrically at 570 nm. The IC_{50} value was calculated as described elsewhere (21).

Statistical analysis

All the experiments were performed in triplicate. The graphs were prepared using GraphPad Prism software. The results were expressed as mean ± SD ($P<0.05$).

Results

Ferric reducing antioxidant power (FRAP) assay

Eugenol showed substantial ferric reducing potential (FRAP value 127×10^4 µM/mM) which was about 82% of the activity shown by ascorbic acid (155.5×10^4 µM/mM) (Figure 1). Comparatively lower activity was observed with cinnamaldehyde (38×10^4 µM/mM). The

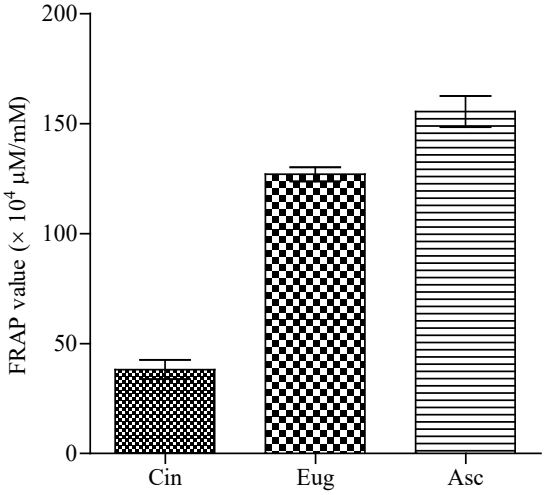

Figure 1. Ferric reducing antioxidant power of cinnamaldehyde and eugenol. The activity was represented as FRAP value (Fe^{2+} µM/mM). Abbreviations: Cin- cinnamaldehyde, Eug- eugenol, Asc- ascorbic acid (P<0.001).

activity of eugenol was about 3.5 folds greater than cinnamaldehyde.

Inhibition of lipid peroxidation

Cinnamaldehyde exhibited comparatively better lipoprotective activity as compared to eugenol in kidney and brain tissue homogenates (Figures 2 A and B). At highest test concentration (3µM/ml) cinnamaldehyde accounted for about 40% LPOI activity in brain and 34% in kidney homogenate. At lower concentration (1 µM/ml) the protective efficacy of cinnamaldehyde and eugenol was significantly different (P<0.05) in brain tissue while in kidney homogenate significant difference was not observed (P>0.05). At test concentrations lipoprotective activity shown by eugenol was 11-24% in brain and 9-34% in kidney tissue homogenates while cinnamaldehyde accorded 15-40% protection in brain and 12-33% in kidney tissues against peroxidative damage. Results showed that increase in concentration of both the test compounds is positively correlated with their lipoprotective efficacy (r^2 values for cinnamaldehyde and eugenol were 0.9875 and 0.9859, respectively in brain homogenate).

Nitric oxide (NO) radical scavenging activity

NO radical scavenging activity was measured at different concentrations (3-18 µM/ml) of cinnamaldehyde and eugenol and activity is represented as % NO radical scavenging activity (Figure 3). Considerable radical scavenging activity was observed in test compounds. Cinnamaldehyde showed slightly lower NO radical scavenging activity (19-57%) in comparison with eugenol (26-65%) at all test concentrations. BHA accounted for 37-74% activity. IC_{50} values for NO radical scavenging activity of cinnamaldehyde, eugenol and BHA were found to be 16.87, 14.67 and 13.09 µM/ml, respectively.

Effect of cinnamaldehyde and eugenol on cell viability of PC-3

The study on viability of prostate cancer cell line (PC-3) at different concentrations of test compounds (25-100 µM) revealed that cinnamaldehyde possessed

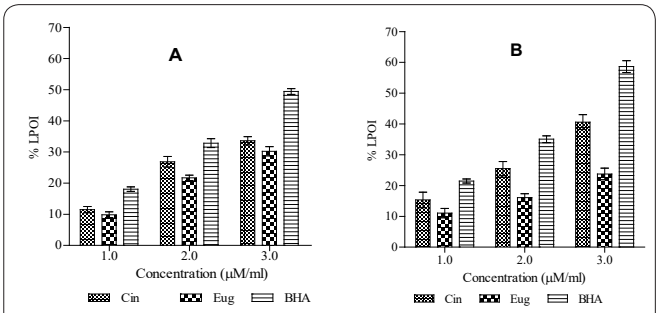

Figure 2. Lipoprotective efficacy of cinnamaldehyde and eugenol in rat kidney (A) and brain (B) tissue homogenates. Lipid peroxidation inhibition (% LPOI) was determined at different concentrations (1.0, 2.0, 3.0 µM/ml) as described in methods section. BHA was used as standard lipoprotective agent. The results are expressed as mean ± SD of three replicates (P<0.05). Abbreviations: Cin- cinnamaldehyde, Eug-eugenol, BHA- butylated hydroxyanisole.

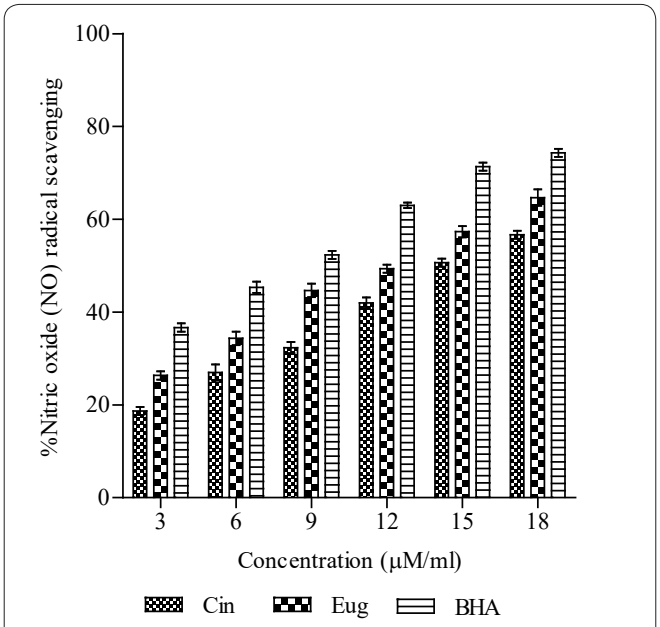

Figure 3. Nitric oxide (NO) radical scavenging activity of cinnamaldehyde and eugenol. Results are shown as mean ± SD of three replicates (P< 0.05). Abbreviations: Cin- cinnamaldehyde, Eug-eugenol, BHA- butylated hydroxyanisole.

comparatively better cytotoxic activity than eugenol (Figure 4). With increasing concentrations marked drop in cell viability was observed for cinnamaldehyde (from 87% to 25%) and eugenol (from 91% to 36%) showing 75% and 64% cytotoxicity at 100 µM, respectively. A positive correlation was observed between concentration and cytotoxic activity. r^2 values for cinnamaldehyde and eugenol were 0.98 and 0.99, respectively indicating dose dependent response. IC_{50} values for cinnamaldehyde and eugenol were found to be 73.38 µM and 85.34µM, respectively.

Anti-leishmanial activity of cinnamaldehyde and eugenol

The inhibitory activity of cinnamaldehyde and eugenol on the growth of *Leishmania major* promastigotes was represented in form of IC_{50} values, the concentration required to inhibit 50% growth of the promastigotes. Lower value of the IC_{50} defines better antileishmanial activity. IC_{50} values for eugenol and cinnamaldehyde were 0.681 g/ml and1.426g/ml, respectively suggesting comparatively better anti-leishmanial activity in euge-

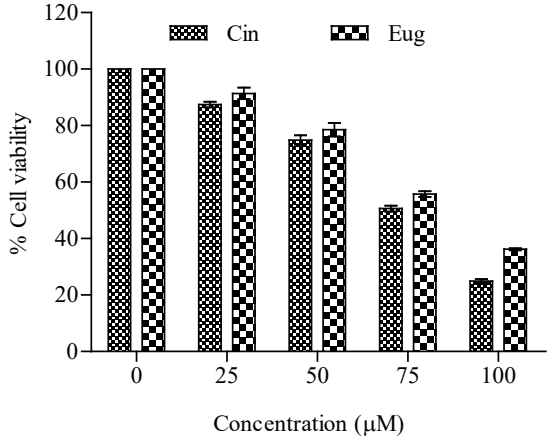

Figure 4. Cytotoxic activity of cinnamaldehyde and eugenol against PC-3 cancer cell lines. Results are expressed as % cell viability (mean ± SD). Abbreviations: PC-3- prostate cancer cell lines, Cin- cinnamaldehyde, Eug- eugenol.

nol. Glucantime was used for the comparison as a reference drug.

Discussion

Free radicals are constantly produced in living systems. Being highly reactive they may bring about oxidative alterations in biomolecules which results into tissue damage. These damaging effects ultimately produce various degenerative diseases. Antioxidants have been shown to counteract the damaging action of oxidants. Synthetic drugs being used as antioxidants and antiparasitic agents also produce adverse effects on health. Hence search for compounds of natural origin having pharmacological action are still gaining momentum (11, 22). Antioxidants provide protection against peroxidative damage through various mechanisms such as metal ion chelating and radical scavenging actions (23).

Ferric reducing ability provides direct assessment of antioxidant or reducing capacity of the samples. Reduction of Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex by test compounds makes the basis for measurement of this ability producing blue colour which is measured at 593 nm (16). Absorbance of reaction mixture finds direct correlation with the reducing ability of sample. The FRAP value is indicator of the hydrogen or electron donating ability of test samples (24). The reducing ability of phenolic compounds depends on number and position of hydroxyl groups (25). Eugenol and cinnamaldehyde, the two phenyl propanoids, show structural difference. Reducing power increases with increase in number of hydroxyl groups. Higher FRAP value shown by eugenol (Figure 1) might be attributed to the presence of hydroxyl group on aromatic ring which is absent in cinnamaldehyde.

Test compounds were assayed for lipoprotective activity against iron (Fe^{3+}) induced lipid peroxidation in tissue homogenate. Cinnamaldehyde accounted for about 40% prevention of lipid peroxidation in kidney and brain tissue homogenates (Figure 2 A and B). Fe^{3+} is responsible for generation of hydroxyl radicals ehich are highly reactive. These radicals cause lipid peroxidation which is associated with injury, inflammation and oxidative deterioration of cellular membranes. Lipid peroxidation may involve initiation, propagation and termination phases (26). The intermediates of lipid peroxidation such as lipoperoxyl, lipid hydro-peroxide, peroxyl and alcoxyl radicals have adverse effect on health of living being. Malondialdehyde (MDA), an important by-product of lipid peroxidation, forms pink chromogen after reacting with thiobarbituric acid (TBA) is used as marker of lipid peroxidation (27). Removal of Fe^{+3} by chelation reduces hydroxyl radical production and thereby inhibits peroxidation of lipids. Hence chelation of Fe^{+3} by cinnamaldehyde and eugenol could be responsible factor for the observed effects. Our study finds support from other studies which have shown potent chelating ability in cinnamaldehyde (28, 29).

NO is produced during arginine metabolism. Its elevated level is highly toxic to tissues and responsible for vascular collapse associated with septic shock. The chronic expression of NO radical has been implicated in several carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (30). NO generates highly reactive peroxynitrite anion ($ONOO^-$) when reacts with superoxide anion (31). At physiological pH, nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions, which is measured by the Griess Illosvoy reaction (18). The nitrite radical undergoes diazotization reaction with sulphanilamide and subsequent coupling with naphthyl ethylene diamine generates pink chromophore. The radical scavenging activity of cinnamaldehyde might be due to the antioxidant effect whereby cinnamaldehyde competes with oxygen to react with nitric oxide (32). During experiments, test compounds showed 57-65% NO scavenging activity at highest test concentration (Figure 3). Scavenging activity increased in order cinnamaldehyde, eugenol and BHA with IC_{50} values in the range 13.09-16.87 μM/ml. The structural requirement considered essential for effective radical scavenging by eugenol and BHA is the presence of P-hydroxyl group in aromatic ring and conjugated double bond which makes the electrons more delocalized. P-hydroxy system possesses electron donating properties and is a radical target by making quinone structure. The number and specific positions of hydroxyl groups and the nature of the substitutions determine whether phenyl propanoids function as strong antioxidative, anti-inflammatory, antiproliferative or enzyme modulating agents (29, 33).

Plant derived compounds including camptothecin, vincristine, vinblastine, paclitaxel and etoposide are used as anticancer drug. Phytochemicals have been reported to arrest cell cycle, fragment DNA, decrease mitochondrial membrane potential and finally resulting in induction of apoptosis in cancer cell lines (34). Current study revealed that eugenol and cinnamaldehyde possessed 64-75% cytotoxic activity against PC-3 cell line at 100 μM concentration (Figure 4). A direct correlation was also observed between concentration and cytotoxic activity. The results have been corroborated by reports on test compounds against other cancer cell lines (29, 35, 36). When MTT is added to cells on a microtitre plate, the yellow colored MTT is metabolically reduced by the mitochondria in viable cells to purple formazan with the help of enzyme mitochondrial succinate dehydrogenase. Mitochondrial dehydrogenases of viable

cells cleave the tetrazolium ring, yielding purple formazan crystals which are insoluble in aqueous solutions. This reduction takes place only when mitochondrial reductase enzyme are active and therefore conversion can be directly related to the number of viable (living) cells. Solubilization solution is added to dissolve the insoluble purple formazan product into a coloured solution which is quantified at 570 nm. The absorption maxima is dependent on the solvent employed. An increase in cell number resulted in an increase in the amount of MTT formazan formation indicating increase in absorbance. Higher color intensity indicates more cell viability and less cytotoxic effect of the drug sample which is tested.

Effective and inexpensive chemotherapeutic agents for treatment of leishmaniasis are need of hour. Hence researches for finding novel anti-leishmanial compounds of plant origin are still relevant. Although trivalent antimonial [Sb (III)] like potassium antimonyl tartrate and pentavalent antimonial drugs are the first-line treatment for leishmaniasis, with amphotericin B and pentamidine being used as alternative drugs. Growing resistance to drugs has become a severe problem. All of these drugs have serious side effects coupled with longer length of treatment, high toxicity, and high cost. Therefore, new drugs are urgently required. Eugenol exhibited comparatively better inhibitory activity against growth of *L. major* promastigotes than cinnamaldehyde as indicated by IC_{50} values (0.681 g/ml) suggesting potential activity. Anti-leishmanial activity of shown by many plant products depends on bioactive constituents such flavonoids and alkaloids. Piperine, a natural product, has been reported to show cytotoxic potential against promastigotes of *L. donovani* (37, 38). Growth inhibitory effects of flavonoids, particularly of the flavonols "quercetin" and the flavones "luteolin", on the protozoan parasite genera *Toxoplasma*, *Trypanosoma* and *Leishmania* have been demonstrated by several workers (39-41). Other stages in the life cycle of *L. major* are also inhibited by different products (42). Hence natural products offer an unlimited source of chemical diversity to identify new drug modules for the treatment of important tropical diseases caused by protozoans.

The study revealed that eugenol possesses considerable radical scavenging, reducing ability and anti-leishmanial activities while cinnamaldehyde is comparatively better lipoprotective and cytotoxic agent. Hence the current work provides insight for further researches on pharmacological activities of cinnamaldehyde and eugenol.

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