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Phytochemical screening, antioxidant activity and cytotoxicity of leaf and bark extract of the Philippine endemic species, *Artocarpus blancoi* (Elmer) Merr

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Abstract
Bark and leaves of *Artocarpus blancoi* locally known as Antipolo has been used as folkloric medicine in the Philippines. However, evaluation of the phytochemical constituents and cytotoxicity profile of the bark and leaf extracts of *A. blancoi* has not been fully elucidated. This study aims to investigate the phytochemical properties, antioxidant activity and cytotoxicity of the bark and leaf extracts of *A. blancoi*. Plant extracts were prepared through extraction with water, ethanol and ethanol-water. For these extracts at concentrations of 10-, 100-, 500- and 1000 ppm, free radical scavenging activity was determined using 2,2-diphenyl-2-picrylhydrazylhydrate (DPPH) while cytotoxicity was done using Brine Shrimp Lethality Analysis (BSLA) wherein mortality was observed after 6 and 24 hours, respectively. The phytochemical analysis revealed that the bark and leaf extracts of *A. blancoi* contain alkaloids anthraquinones, cyanogenic glycosides, flavonoids, saponins, steroids and tannins. The ethanol extracts have high mortality of *A. salina* nauplii (100%) observed at 100ppm to 1000ppm with an LC_{50} value of <10 after 6 hr and 24 hr exposure while the decoction extracts of both plant parts has an LC_{50} value of >1000 after 6hr and 24 hr exposure. Bark and leaf extracts of *A. blancoi* possess strong free radical scavenging activity with LC_{50} value of 5.2 µg/mL and 5.3 µg/mL, respectively. Presence of phytochemical compounds, strong antioxidant properties and very low lethality in the decoction of the bark and leaf extracts of *A. blancoi* is a preliminary support for its folkloric medicinal uses.

Keywords: antipolo, brine shrimp lethality assay, decoction, DPPH assay, ethanol

Introduction

Traditional medicinal plant is a major component of indigenous medical systems worldwide (Redvers and Blondin, 2020) [39] which refers to any cultural based health care practice and is mainly conveyed orally by communities of different cultures (Aziz *et al.* 2018) [5]. This cultural tradition may involve extraction or decoction on the different medicinal plant parts such as bark, leaf and stem but still utilized particularly by rural communities because it has remained the most affordable and accessible source of treatment (Hosseinzadeh *et al.* 2015) [17]. Despite recent scientific advancement and globalization, a large number of people in both developing countries still utilize traditional medicine to meet their primary health care needs (UNESCO, 1996) [50].

In modern drug discovery, medicinal plants have been found to have pharmacologically active compounds targeting against various ailments, such as infection and inflammation (Amit Koparde *et al.* 2019) [2] and even as a source of active molecules and compounds against Covid-19 (Benarba & Pandiella 2020) [6]. These active compounds are phytochemicals which may include alkaloids, terpenes, tannins, resins, phenolic, and many others which may influence the physiological functions of the human body (Hussein & El-Anssary 2019) [18]. Phytochemicals are biologically active compounds that are naturally produced by plants to provide protection against diseases due to environmental hazards which also provide health benefits for humans (Nalini & Dhanaraj 2015) [27]. These claims are supported by several studies showing that phytochemicals may have their curative effects on cardiovascular diseases, inflammation, cancers, diabetes, AIDS (Keo *et al.* 2018) [20] and age-related diseases (Forni *et al.* 2019)[13]. Moreover, phytochemical components especially polyphenols, are known to reduce oxidative stress and responsible for the antioxidant activity of plants and have been therefore suggested to contribute to the health-promoting properties of plants (Adawia *et al.* 2016) [1]. In addition, the polyphenol compounds such as flavonoids, tannins, and phenolic acids have anti-inflammatory and anti-carcinogenic property (Vaghasiya *et al.* 2011) [51]. Thus, medicinal plants can be great sources of potent and powerful phytyochemicals which can be developed as valuable therapeutic agents possessing medicinal properties benefiting mankind (Suntar 2020) [45]. Since medicinal plants have been traditionally used to treat certain aliments, scientific investigation like tests on phytochemicals, cytotoxicity and antioxidant activity is necessary to avoid negative effects which is basically due to over-dosage and lack of knowledge on the constituent of some plants (Olowa & Nuñeza 2013) [29]. In the Philippines, medicinal plants are considered as natural living treasures of the country consisting of around 3500 medicinal plants of which 1500 are considered indigenous and 120 have been validated for their safety and efficacy (Tan & Sia 2014) [46]. However, there are only 10 medicinal plants promoted by the Department of Health and the Philippine Institute for Traditional and Alternative Health Care (Boy *et al.* 2018)[8]. However, this number may increase with various studies, analysis and tests conducted on other medicinal plants found in the country. The *Artocarpus blancoi* locally known as "Antipolo" or "Tipolo" is an endemic and vulnerable tree species in the

Philippines belonging to family Moraceae with diameter of up to 60 centimeter (cm) and 30 meters (m) in height (Rodolfo *et al.* 2016, World Conservation Monitoring) [44, 59]. The decoction of its bark extract is used in folkloric medicine for treatment of "Strangularia" (hernia) wheras the leaves and fruits are utilized as food by Ayta people in Pampanga, (Ragragio *et al.* 2013, Umali-Stuart 2016) [40,56]. The leaf of this tree is also used as a mosquito repellent and was discovered to have exceptionally high values of antimicrobial activity (Obico & Ragragio 2014, Umali-Stuart 2016) [28,49]. However, there were no recent scientific investigations and evidences to prove the efficacy of *A. blancoi* to treat diseases. Thus, this study aims to evaluate the phytochemical constituents, cytotoxicity and antioxidant properties of the leaf and bark extracts of *A. blancoi.*

Materials and Methods

Collection of Plant Material

The samples of the bark and leaf extract of *A. blancoi* (Antipolo) were collected in in Suarez, Iligan City. This plant material was botanically authenticated by comparing it with the herbarium specimens at the Museum of the College of Science and Mathematics of the Mindanao State University Iligan-Institute of Technology, Iligan City.

Preparation of Plant Extract

The collected barks and leaves of *A. blancoi* were washed thoroughly using tap water and air dried under shade for 3 weeks at room temperature until crispy. The dried samples were powdered in an electric blender and placed in airtight bottles ready until further use. Samples were subjected into three extract preparations for the bark and leaves in this study: decoction, ethanol-aqueous, and pure ethanol. For the decoction, 30 grams each of powdered bark and leaves of *A. blancoi* were boiled with 300 ml distilled water on medium heat of an electric stove until the volume was reduced to approximately 50 ml. In preparing the ethanolic extract, 100 grams each of the powdered bark and leaf samples were soaked in 200 ml of absolute ethanol for 10 days. Mixtures were placed in a container covered with black cloth and foil and stored in a locker at room temperature. Residue was filtered and re-extracted with ethanol. After filtration, the solvent was removed by a rotary evaporator under vacuum and the concentrated bark and leaf ethanolic crude extract of *A. blancoi* was obtained. The crude extracts were stored in a tight glass container and refrigerated at 7°C until use. Ethanol-aqueous and pure ethanol extracts of *A. blancoi* were prepared for the Phytochemical Screening and Antioxidant Capacity (DPPH Free Radical Scavenging Assay). Decoction, ethanol-aqueous and pure ethanol extracts are prepared for the Cytotoxicity test using the Brine Shrimp Lethality Assay.

Phytochemical screening

The ethanolic leaf extract prepared was used in the analysis for the presence of alkaloids, anthraquinones, cyanogenic glycosides, flavonoids, saponins, steroids, tannins and other phenolic compounds utilizing the standard protocol of Sheel *et al.* 2014[43].

DPPH radical scavenging activity

The antioxidant capacity of *A. blancoi* bark and leaf extracts was determined by the ability of the free radical 1,1-diphenyl-2-picryl hydrazyl (DPPH) to decolorize in the presence of antioxidants recapping the methods designed by Saeed *et al.* (2012) [41] with slight modifications. The stock solution was prepared by dissolving 24 mg DPPH with 100 mL ethanol and then refrigerated to -10ºC until use. Reaction mixture was prepared using 2.5 ml of 6.5×10^{-5} M DPPH solution and 0.5 ml of sample extracts dissolved in ethanol, ethanol being the control. Bark and leaf extracts of various concentrations (500 µg/mL, 300 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, 30 µg/mL, 20 µg/mL, 10 µg/mL) were tested in DPPH solution for 12 h in the dark at room temperature. Absorbance was measured at 515 nm using UV-Vis spectrophotometer (SHIMADZU UV mini 1240). The percentage of DPPH radical scavenging activity was determined using the equation mentioned below. The concentration of sample required for 50% inhibition was determined and represented as IC_{50} value for each test solution.

% DPPH scavenging activity = ($A_{control} - A_{sample} / A_{control}$) x 100

Where, $A_{control}$ and A_{sample} are the absorbance values of the test and of the blank sample, respectively.

The DPPH radical has odd electrons that act on absorbing in a 517 nm for purple coloration to be visible. The decrease in absorption was taken as a measure on the extent of radical scavenging. All determinations were carried out in three trials.

Brine shrimp lethality assay

Brine shrimp eggs were obtained from the New Aqua Laboratory in Naawan, Misamis Oriental. *Artemia salina* was hatched in artificial sea water prepared from commercial sea salt 40 g/1 and supplemented with 6 mg/1 dried yeast to regulate the salinity and pH. A plastic chamber was used for hatching with a regulation of an electric aerator. The eggs were sprinkled into the plastic chamber. The set-up was placed in a room temperature. After 48 hours, nauplii larvae were pipetted and transferred to the vials for treatments of different concentrations of the bark and leaf extracts. BSLT procedure was modified from the assay described by Solis *et al.* (1993), Pisutthanan *et al.* (2004) [37] and Apu *et al*. (2013) [3,35,44]. Five milligrams of the extracts were made for up to 2 mg/ml in artificial sea water. Extracts were serial diluted in four different concentrations in three replicates (50 ppm, 100 ppm, 500 ppm and 1000 ppm) and placed in the transparent vials for observation. Ten nauplii were added to each vial. The vials were covered by a foil and placed in a room temperature for a 6-hour and 24-hour observation, after which, numbers of dead (non-motile) nauplii in each vial were counted.

Statistical Analysis

Analysis was carried out using the protocol by Peteros and Uy (2010)[34] and Olowa and Nuneza (2013)[29]. The percentage mortality (% mortality) was calculated using the formula mentioned below. This is to ensure that the death (mortality) of *A. salina* nauplii is attributed to the bioactive compounds present in the bark and leaf extracts of *A. blancoi.*

% mortality = (no. of dead nauplii / initial no. of live nauplli) x 100

Microsoft office excel 2016 and Probit Analysis by Finney (1971)[12] were used to determine the lethality concentration

(LC_{50}) of *A. salina* at 95% confidence intervals. Plan extracts with an LC_{50} value of less than 100 ppm was considered as potent or active (Peteros and Uy, 2010)[34]. LC_{50} value of less than 1000 µg/mL is toxic while LC_{50} value of greater than 1000 µg/mL is non-toxic (Meyer *et al.*, 1982)[24].

Results and Discussion

Phytochemical screening of *A. blancoi* leaf and bark extracts

Phytochemical screening of the crude ethanolic extracts of the leaves and bark of the Philippine endemic tree species *A. blancoi* revealed the presence of secondary metabolites such as alkaloids anthraquinones, cyanogenic glycosides, flavonoids, saponins, steroids and tannins (Table 1). As observed, the leaf extract contains more alkaloids than the bark extract. Alkaloids are secondary metabolites with a relatively frequent occurrence in nature but are specific to selected and higher plants (Minocheherhomji & Vyas 2014) [25]. They are known to be potential pharmacological agents and are the first chemical substances to be derived from plants (Minocheherhomji & Vyas 2014) [25]. It was reported that they may exhibit various potentially useful activities such as enzyme inhibitory effects and anticancer, antimicrobial, and anti-inflammatory activities (Wansi *et al.* 2013) [54]. According to Wansi *et al.* (2013) [54], alkaloids are commonly bound and have the maximum amount contained in the leaves, followed by fruit/seeds, root, and the least in the bark which concurs with the results in this study.

Table 1: Phytochemicals present in the crude ethanolic extracts of *A. blancoi*

Phytochemical	Leaves	Bark
Alkaloids	++	+
Anthraquinones	+	+++
Cyanogenic glycosides	-	-
Flavonoids	+++	+++
Saponins	-	+
Steroids	+++	+++
Tannins	+++	+++

+++ = Strongly present; ++ = moderately present; + = slightly present; − = absent.

The phytochemical saponins were only present in bark extract and these compounds are naturally-occurring metabolites mainly produced by plants (Marrelli *et al.* 2016)[23] that have the ability to form stable soap-like foams in aqueous solutions (Francis *et al*. 2002)[14]. and are thus, used as natural surfactants in cleansing products. Pharmacologically, saponins have been reported to have pharmaceutical properties such as antifungal, insecticidal, anthelmintic, cytotoxic, anti-inflammatory, immunostimulant, hypocholesterolemic, hypoglycemic and anticancer (Podolak *et al.* 2010) [36]. They may also have cholesterol lowering (Gurfinkel & Rao 2003) [15] and blood glucose-lowering response properties which have been the main focus in recent years. However, Wina *et al.* (2005) [55] reported that some saponin-containing plants may be toxic for ruminants or those mammals with plant-based food diet, leading to gastroenteritis, diarrhea and even liver and kidney degeneration which indicate that further screening needs to be done to specifically identify the type of saponins present in these plants.

Anthraquinones which are found as pigments in many plants and composite microorganisms were present only in the bark ethanolic extract in this study. This phytochemical is a natural and artificial compound that has been used since centuries ago for therapeutic application and has been widely utilized as natural dyes (Malik & Muller 2016) [22]. It has been reported that this compound has other interesting properties and it possesses a broad spectrum of biological activities, such as antimicrobial, anti-inflammatory, cathartic, constipation, arthritis, anticancer, diuretic, multiple sclerosis, vasorelaxing, and phytoestrogen activities (Malik & Muller 2016, Sharma *et al*. 2017) [22,42]. The presence of anthraquinones in the bark extract indicates that it may possess biological activities or properties for pharmaceutical and medicinal uses.

The three phytochemical compounds, flavonoids, steroids and tannins were strongly present in the ethanolic extracts, for both leaf and bark samples. Flavonoids are compound products extracted from plants specifically bound in fruits, bark, roots, stems, and flowers (Havsteen 2002, Panche *et al.* 2016) [16,30] which are now easily extracted from natural sources using modern techniques (Chavez *et al.* 2020) [9]. This natural product is well known for its beneficial health-promoting effects on humans such as anti-oxidative, anti-allergic, anti-mutagenic, anti-inflammatory, anti-platelet, cytotoxicity, and anti-carcinogenic properties (Asif & Khodadadi 2013) [4]. They are an essential component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications (Panche *et al.* 2016) [30]. Plant steroids are a unique class of chemical compounds that possess medicinal, pharmaceutical and agrochemical activities such as anti-tumor, antibacterial, anti-inflammatory, immunosuppressive, antihelminthic and cytotoxic activity (Patel & Savjani 2015) [32]. Tannin is one of the main chemical compounds found in many higher plants (Ukoha *et al.* 2011) [48] and plant parts such as roots, bark, leaves, seeds, sap and in the fruits (Pereira *et al.* 2015) [33]. It has a therapeutic potential and pharmaceutical activity that is used as anti-inflammatory agent (Ketzis *et al.* 2006) [21].

Cyanogenic glycosides which are known as plant defense chemicals (Park & Coats 2002) [31] which release toxic hydrogen cyanide when plant tissue is disrupted, (Zagrobelny *et al.* 2004) [57] were not present in both extracts. This phytochemical compound can be found in some of the plant species and is consumed as food in some regions of the world (Wangari, 2013)[52]. The toxicity of these phytochemicals is dependent on the release of hydrogen cyanide content that can be harmful to animal and humans with over consumption beyond the desired amount or quantity (Bolarinwa *et al.* 2016, FAO/WHO, 2012) [7,11].

The results of this study revealed that the leaves and bark of the tree species *A. blancoi* are rich sources of phytochemicals which can be further analyzed to determine the different biological activities they possess contingent on their detailed ethno-botanical medicinal uses.

DPPH radical scavenging activity of *A. blancoi* leaf and bark extracts

The DPPH free radical scavenging assay is a widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors to evaluate overall antioxidant properties of foods (Kedare & Singh 2011)[19]. In this study, the rapid assessment for antioxidant property using DPPH assay demonstrated that the leaf and bark extracts of A. *blancoi* possess strong free radical scavenging activity with LC_{50} value of 5.2 μg/mL and 5.3 μg/mL, respectively (Table 2). According to de Vargas *et al.* (2016)[10], only samples exhibiting $LC_{50} < 10$ μg/mL are considered very active antioxidants as they have the potential comparable to the antioxidant standards of quercertin, β-carotene, ascorbic acid, gallic acid and Trolox® and the plant extracts exhibiting the greatest antioxidant potential were those with the highest levels of total polyphenols. Polyphenolic compounds are naturally found in plant sources and that they possess antioxidant, anti-inflammatory, anti-carcinogenic and other biological properties that aid treating certain diseases (Tungmunnithum *et al.* 2018)[47]. Polyphenols such as flavonoids and tannins are abundant in both in the leaves and bark part of *A. blancoi*. Presence of flavonoids stabilizes the reactive oxygen molecule by reacting with the reactive compound of radicals and because of the high reactivity of the hydroxyl group present in flavonoids; radicals are then inactivated (Panche *et al.* 2016) [30]. Additionally, a study of Rahal *et al.* (2014) [38] conforms that flavonoids present in ginkgo extracts exist primarily as glycosylated derivatives of kaempferol and quercetin and have been shown to be extremely effective free radical scavengers.

Table 2: DPPH radical scavenging activities of the ethanolic extracts of *A. blancoi*.

Plant Part		% inhibition at different concentration*						IC_{50} μg/mL
	control	10 μg/mL	30 μg/mL	50 μg/mL	100 μg/mL	300 μg/mL	500 μg/mL	
leaves	1.7±0.6	74.4±0.4	95.4±0.7	94.6±0.0	96.0±0.3	95.8±0.5	95.7±0.7	5.2
bark	1.7±0.6	92.9±0.2	95.1±1.0	93.8±0.2	94.2±0.5	94.5±0.8	94.6±0.7	5.3

*Mean±SD

Brine shrimp lethality assay of *A. blancoi* leaf and bark extracts

The percentage mortality and LC_{50} of *A. salina* nauplii after 6 h (acute) and 24 h (chronic) exposure to the three extracts of *A. blancoi* at varied concentrations are shown in Table 3. Among the extracts, the decoction of leaves imparted the least mortality to *A. salina* nauplii with % mortality values ranging from 6.67% to 40% after 6 h exposure (LC_{50} >1000) which then slightly increased from 10% to 43.33% after 24 hr exposure (LC_{50} >1000). This was followed by the bark decoction which rendered mortality values ranging from 6.67% to 43.33% after 6 hr (LC_{50} >1000) and 20% to 46.67% after 24 hr (LC_{50} >1000). The 50:50 water-ethanol leaf extract resulted to high mortality of the brine shrimp nauplii with an LC_{50} value of 17.50 after 6 hr and LC_{50} value of <10 after 24 hr while the 50:50 water-ethanol extract from the bark also resulted to high mortality with an LC_{50} value of 13.07 after 6 hr and LC_{50} value of <10 after 24 hr. Along with increasing concobserved to cause the highest lethality to *A. salina* nauplientration, the ethanol extracts from both plant parts were i with an LC_{50} value of <10 exposed from 6 hr and 24 hr. Maximum mortality was observed at 500 ppm and 1000ppm in 50:50 water-ethanol and ethanol extracts while the lowest mortality was observed with the 10ppm decoction extract.

The results showed that the toxicity of the leaf and bark extracts was directly proportional to the different concentrations.

Table 3: Effects of the *A. blancoi* crude extracts on the brine shrimp *A. salina* after 6-hr and 24-hr exposure.

Plant Parts	Type of Extract	Concentration (ppm)	Brine Shrimp Mortality (%)	Brine Shrimp Mortality (%)	Acute LC_{50} (ppm)	Chronic LC_{50} (ppm)
			After 6 hrs.	After 24 hrs.		
		10	6.67	10		
	Decoction	100	20	26.67	>1000	>1000
		500	26.67	30		
		1000	40	43.33		
		10	53.33	90		
Leaves	50:50 Water-Ethanol	100	73.33	96.67	17.50	<10
		500	100	100		
		1000	100	100		
		10	73.33	76.67		
	Ethanol	100	100	100	<10	<10
		500	100	100		
		1000	100	100		
		10	6.67	13.33		
	Decoction	100	13.33	20	>1000	>1000
		500	30	33.33		
		1000	43.33	46.67		
		10	56.67	86.67		
Bark	50:50 Water-Ethanol	100	73.33	93.33	13.07	<10
		500	96.67	100		
		1000	100	100		
		10	83.33	96.67		
	Ethanol	100	100	100	<10	<10
		500	100	100		
		1000	100	100		

According to Meyer *et al.* (1998) [24], a crude plant extract is toxic (active) if it has an LC_{50} value of less than 1000 μg/mL while non-toxic (inactive) if the value is greater than 1000 μg/mL. Also, plant extracts with an LC_{50} value of less than 100ppm is considered potent or active (Waghulde *et al.* 2019)[53]. This indicates that the decoction extracts from bark and leaf in which very low mortality was observed exhibited inactivity and therefore poses low risk of harm when administered. According to Moshi *et al.* (2010)[26], decoction is a traditional way of obtaining the plant extract and thus, has been tested through administration. Umali-Stuart (2016)[49] reported that the bark extract of *A. blancoi* is traditionally prepared through decoction and was used as folkloric medicine for treatment of strangularia. However, the 50:50 water-ethanol and ethanol extracts have LC_{50} values lesser than 1000 ug/mL which means they are considered active and may indicate the presence of potent cytotoxic components which may explain the high mortality against *A. salina* nauplii. In addition, the mixture and ethanol extracts from bark and leaves may also possess anti-microbial activities, however further investigation should be done. Brine shrimp method is usually used to investigate the cytotoxicity of bioactive compounds from plant extracts (Waghulde *et al*. 2019)[53]. As mentioned, *A. blacoi* contains secondary metabolites such as alkaloids anthraquinones, cyanogenic glycosides, flavonoids, saponins, steroids and tannins which may be implicated with the cytotoxicity demonstrated by the 50:50 water-ethanol and ethanol extracts. This indicates that bark and leaf extracts of *A. blancoi* may have potential value and can be used as herbal medicine in standardized dosages.

Conclusion
Lowest lethality to brine shrimps was observed at 10ppm of decoction extracts of *A. blancoi* bark and leaves while the most potent was detected in 50:50 water-ethanol and ethanol extracts at 500ppm to 1000 ppm. The lethality was observed to be directly proportional to the concentrations used for the different plant part extracts. Moreover, the bark and leaf extracts of *A. blancio* possess strong free radical scavenging activity. Presence of bioactive compounds in the plant sampled may indicate pharmacological potentials. Hence, the findings of this study somehow support the utilization of the leaf and bark extracts of *A. blancoi* using decoction for folkloric medicine.

Declarations
List of abbreviation
Brine Shrimp Lethality Analysis (BSLA), 2,2-diphenyl-2-picrylhydrazylhydrate (DPPH), Food and Agriculture Organization (FAO), World Health Organization (WHO)

Ethics approval and consent to participate
The data were collected with full confidentiality and consent of the respondents. Written and oral prior informed consents were obtained respectively from the rural municipality office and community leaders. Permission of data collection was obtained from the Punong Barangay of Suarez, Iligan City and nearby residents were informed about the aim of the study.

Consent for publication
Not applicable

Availability of data and materials
The data are available from the authors upon request.

Competing interests
The authors declare that they have no competing interest among them.

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Authors' contribution
Rose Chinly Mae H Ortega, Grapesy Pink M. Alsonado and Angeleth U. Taotao contributed on conceptualization, methodology, investigation, formal analysis and visualization of the paper. Olga M. Nuneza and Mylene M. Uy contributed on the conceptualization, formal analysis, visualization and supervision. All the authors participated in writing and giving feedback on the manuscript and approved the final version of the manuscript.

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