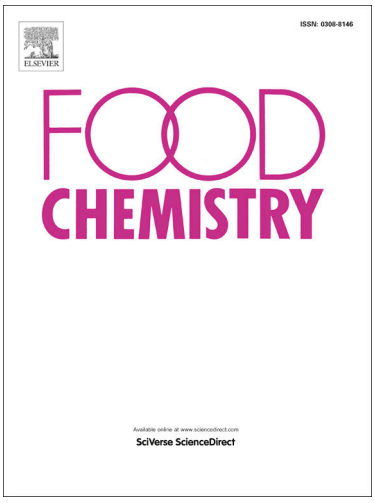
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Phenolic composition by UHPLC-Q-TOF-MS/MS and stability of anthocyanins from *Clitoria ternatea* L. (butterfly pea) blue petals

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Abstract

The aim of the present study was to evaluate the phenolic composition of crude lyophilized extracts (CLE) and partially purified (PPE) extracts of *C. ternatea* blue petals as well as the anthocyanin stability against pH, temperature and light in the presence and absence of fructooligosaccharides. Twelve compounds were tentatively identified by UHPLC-Q-TOF-MS/MS in CLE and PPE extracts. In direct/reverse spectrophotometric titration, anthocyanins showed colour changes between pH 2.25 to 10.20, and colour reversibility, maintaining antioxidant activity against the DPPH radical. The aqueous extracts at pH 3.6 and 5.4 exhibited

thermal stability with the presence and absence of fructooligosaccharides with activation energy higher than 99 kJ/mol. The addition of fructooligosaccharides in the extracts at pH 5.4 exposed to light provided a protective effect against anthocyanin photodegradation. The data show the technological potential of aqueous extract of *C. ternatea* blue petals as a natural colourant in a functional beverage model system.

Keywords: Flavonoids; natural blue dye; activation energy; epigallocatechin-3-gallate; fructooligosaccharides; ternatins.

1. Introduction

The anthocyanins are flavonoids responsible for the red, blue, purple and orange colours in vegetables, fruits, flowers and leaves. Anthocyanins have bioactive properties such as *in vitro* antioxidant activity (Escher et al., 2018; Migliorini et al., 2019), *in vivo* serum antioxidant activity (Plaza et al., 2016), *in vitro* antibacterial activity (Sagdic et al., 2013), *in vitro* antihemolytic activity (Escher et al., 2018), *in vitro* cytotoxic effect (Sagdic et al., 2013), and *in vitro* and *in vivo* hypoglycemic effect (Escher et al., 2020).

The use of anthocyanins as a potential natural food colouring with bioactive properties should be encouraged. However, the stability of these pigments is influenced by some factors such as pH, temperature, light, presence of oxygen, chemical structure, solvent, the presence of co-pigments, metal ions and enzymes (Giusti & Wrolstad, 2003). Temperatures above 50 °C during processing may cause partial or complete degradation of anthocyanins and, consequently, the decrease of colour intensity (Patras, Brunton, O'donnell, &

Tiwari, 2010). Sui, Bary, and Zhou (2016) noticed a reduction in anthocyanin contents of the aqueous extract of black rice with temperature rise of heat treatment and storage. Chung, Rojanasasithara, Mutilangi, and Mcclements (2016) verified that the addition of gum arabic in a beverage model, pH 3, and in the presence of ascorbic acid increased the anthocyanin photooxidative stability for 5 days at 40 °C.

Anthocyanins may be acylated with phenolic acids (*p*-coumaric, caffeic, ferulic, synapic, gallic acids) and/or organic acids (malonic, acetic, malic, succinic or oxalic acids), which may enhance their chemical stability for food applications (Giusti & Wrolstad, 2003; Petersson, Puerta, Bergquist, & Turner, 2008). Acylation on the anthocyanin molecule increases stability through intramolecular and/or intermolecular copigmentation, and self-association reactions (Giusti & Wrolstad, 2003). Studies on the application of extracts that were rich in anthocyanins as natural colouring in food in order to replace synthetic ones have increased in recent years in relation to consumer concern with health (Pires et al., 2018). Consumption of synthetic colourings have been potentially associated with allergies, food intolerance, hyperactivity, irritability, and sleep disorders in children (Feketea & Tsabouri, 2017). The search for natural sources of food colouring agents is encouraged. Some natural colourings composed of carotenoids such as annatto, paprika, β-carotene, lutein, β-cryptoxanthin, fucoxanthin, lycopene, and astaxanthin extracted from vegetables, plants, algae and some insects are used in the food industry (Carocho, Morales, & Ferreira, 2015). Natural blue colourings are rare in nature but companies are extracting from spirulina, blue-green algae, and applying to food and beverages (Nachay, 2019).

The blue petal of the *C. ternatea,* known as butterfly pea, is mainly composed of malonylated delphinidin 3,3′,5′-triglycoside. Studies have isolated and identified the different structures of polyacylated delphinidin anthocyanin, named ternatins A1–A3, B1–B4, C1–C5, D1–D3, as well as kaempferol, quercetin and myricetin (Saito, Abe, Honda, Timberlake, & Bridle, 1985; Terahara et al., 1989, 1990, 1996, 1998; Kazuma, Noda, & Suzuki, 2003a). Gallic, *p*-coumaric, 2,4 dihydroxybenzoic, protocatechuic, caffeic, chlorogenic and ellagic acids, quercetin-3-rutinoside, procyanidin A2, and (-)-epicatechin, and delphinidin 3-glycoside (Escher et al., 2020; Azima, Noriham, & Manshoor, 2017) were also identified in the aqueous extract from *C. ternatea* blue petals.

The water extract of the *C. ternatea* blue petals is traditionally used in Asia as a natural colourant in food and drink. In Malaysia, the aqueous extract is used to colour rice cake and the popular dish Nasi Kerabu (Neda, Rabeta, & Ong, 2013). The aqueous extract of *C. ternatea* flowers is also used in Indian tradicional medicine and Ayurvedic tradicional medicine for its therapeutic properties (Mukherjee, Kumar, Kumar, & Heinrich, 2008). Studies have shown that aqueous extract of *C. ternatea* blue petals showed no cytotoxicity in human fibroblast (IMR90) cells (LC_{50} >900 µg/mL), showed protective effect in human erythrocytes and inhibited the oxidation of pBR322 plasmid DNA, which proves toxicological safety and bioactivity (Escher et al., 2020; Mehmood et al., 2019). In this context, the *C. ternatea* flower may be a promising source of natural blue colouring with bioactive properties as an alternative to synthetic colourants. Therefore, the aim of the present study was to evaluate the phenolic composition by UHPLC-Q-TOF-MS/MS of the crude and partially purified extract of the *C. ternatea* blue petals as well as the stability of the water extract anthocyanins in

relation to pH, temperature and light in the presence and absence of fructooligosaccharides.

2. Materials and Methods

2.1 Chemicals

All the solutions were prepared with ultrapure water (Millipore, São Paulo, Brazil). DPPH radical (2,2-diphenyl-1-picrylhydrazyl) and Amberlite®XAD7HP resin were purchased from Sigma-Aldrich (São Paulo, Brazil). Acetonitrile graded LC-MS was purchased from Tedia Company Inc. (Fairfield, USA). Formic acid graded HPLC was purchased from ROE Scientific Inc. (Newark, USA). Hydrochloric acid, trifluroacetic acid, and potassium hydroxide were purchased from Vetec (Rio de Janeiro, Brazil). Ethyl and methyl alcohol were purchased from Synth (Diadema, Brazil). Fructooligosaccharides (FOS) were purchased from New Nutrition (90% of purity, São Paulo, Brazil). Sodium citrate and citric acid were purchased from Merck (São Paulo, Brazil).

2.2 *Samples and preparation of aqueous extracts*

Dehydrated flowers of *Clitoria ternatea* - grown in Campo Grande in the state of Mato Grosso do Sul, Brazil (20° 28′ 53″ south, 54° 36′ 58″ west at 592 m altitude) in 2016 - were purchased directly from the producer. The flowers of *C. ternatea* were uniformly distributed in a sieve and dried in the shade for 4 days at room temperature (28 °C). The blue petals were separated from the calyx, ground in an analytical mill (QUIMIS® model 6298A21), and standardized using a 42 Tyler mesh. In the Herbarium of Medicinal Plants at Ponta Grossa State University, Paraná, Brazil (UEPG) an exsiccate of the *C. ternatea* flowers was

placed (number 21878). The research project was registered in the National Genetic Heritage Management System (SisGen) of the Brazilian Minutesistry of Environment – www.sisgen.gov.br (protocol AFEEFA9).

The experimental condition of phenolic compounds extraction from *C. ternatea* blue petals, 40 °C for 30 min was defined after data modeling using the principal component analysis described by Escher et al. (2020). The extract was made of ultrapure water in a thermostatized cell (Microquímica, MQBTC 99-20, Brazil) with a mass ratio: solvent of 0.125:25 (g/mL) and protected from light with constant magnetic stirring. The water extract was frozen at -80 °C and lyophilized under vacuum of 830 μmL Hg (Liotop, model L202, Brazil). The lyophilized extract yield was ≈ 2%. Another fraction of the aqueous extract was stored in a polyethylene bottle protected from light under refrigeration (8 °C) for the stability studies.

2.3 Partial purification and phenolic composition of the lyophilized extract

The partial purification of anthocyanins present in the crude lyophilized extract of *C. ternatea* blue petals was performed according to the experimental conditions described by Zhang, Fu, and Zhang (2011) and Pedro, Granato, and Rosso (2016). A glass column (1.0 cm x 20.0 cm) was filled with 6.0 g of Amberlite XAD7HP resin. Then, 1.5 g of lyophilized anthocyanins extract was solubilized with 5 mL of an aqueous formic acid solution at a concentration of 1.22 mol/L. The resulting solution was inserted in the column and eluted slowly with 150 mL of ultrapure water. Anthocyanin extracts were eluted with either 30 mL of a 50% acidified water – ethanol solution or 30 mL of acidified absolute ethanol. The

volume was reduced using a rotatory evaporator. The purified anthocyanins were stored protected from light in a desiccator.

The phenolic compounds present in CLE and PPE extracts were identified by ultra-high-performance liquid chromatography (UHPLC, Agilent Technologies, 1290 Infinity series, USA) coupled to a quadrupole-time-of-flight mass spectrometer (Q-TOF, Agilent Technologies, model G6545, USA) according to Chen et al. (2018), with modifications. Chromatographic separation was performed using a reverse phase RP18 column (Symmetry Shield, 3 mm x 150 mm; particle size 3.5 µm; Waters, USA) equipped with C_{18} pre-column (Phenomenex, Torrance, USA), temperature of 40 °C, 2 µL injection volume, and flow rate of 0.150 mL/min. The mobile phase consisted of acidified water with 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). The linear gradient was 0-30 min (5 to 45% B); 30-35 min (45% B); 35-40 min (45 to 5% B), and 40-45 min (5% B). The parameters Q-TOF-MS with electrospray ionization (ESI) in positive mode were capillary voltage, 3500 V; gas temperature, 300 °C; gas flow, 8 L/min; nebulizer gas pressure, 2.413 bar; curtain gas temperature, 350 °C; curtain gas flow, 11 L/min; fragmentor, 175 V; and collision energy, 50 V. The precursor ions and fragments were monitored in the range of 100 to 1700 *m/z,* and the scan time was 4 s. The Masshunter software version B.07.00 (Agilent Technologies, Santa Clara, USA) was used for data acquisition and processing.

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2.4 Studies of stability of anthocyanins of the aqueous extract

2.4.1 Effect of pH and in vitro *antioxidant activity*

The effect of pH on the stability and reversibility of anthocyanins present in the aqueous extract of *C. ternatea* blue petals was analyzed according to Pedro et al. (2016). First, 50 mL of the aqueous extract was diluted with 50 mL of HCl solution (0.01 mol/L). In a cell thermostated at 25 °C, 65 mL of the diluted extract (pH=2.25) were added, protected from light and kept under constant agitation. The spectrophotometric titration was performed using a pH meter (Micronal B-474, Brazil). Small aliquots of KOH (1.0 mol/L) were added to follow the pH range from 2.25 to 10.20. The absorbance was recorded at the maximum absorption wavelength, 546 nm. Following a reverse spectrophotometric titration, aliquots of hydrochloric acid (HCl, 1.0 mol/L) were added until the initial pH was achieved. The antioxidant activity of the extract at different pH values was analysed by DPPH according to Escher et al. (2018). A 0.950 mL aliquot of each extract was mixed with 4.050 mL DPPH (1.0×10^{-4} mol/L), the solution was stirred and kept in the dark for 60 min at 25 °C. The results were expressed as percentage of DPPH inhibition.

2.4.2 Effect of temperature

The effect of temperature on the stability of anthocyanins present in the aqueous extract of *C. ternatea* blue petals was determined according to Maciel et al. (2018). Two solutions of the extract were prepared - one with ultrapure water at pH 5.4 and one with citrate/citric acid buffer solution pH 3.6. The fructooligosaccharide, at a concentration of 1.75 and 3.5 g/100 mL were added in both solutions. The tubes were sealed, immersed in a thermostated bath (60,

70, and 80 °C) and then in a glycerine bath (90 and 100 °C). The maximum absorbance (λ=617 nm) was recorded at 30 min intervals at 100 °C, and 60 min for temperature 60 to 90 °C. The colour retention percentage (%R), rate constant of anthocyanins degradation (*k*), half-life time ($t_{1/2}$), and activation energy (E_A) were calculated with Equations 1, 2, 3 and 4, respectively.

$$\%R = A_t/A_0 \times 100 \quad (1)$$

$$\ln(A_t)/A_0 = - k \times t \quad (2)$$

$$t_{1/2} = \ln 2/k \quad (3)$$

$$\ln k = \ln k_0 - (E_A)/RT \quad (4)$$

where, A_t = absorbance in time t; A_0 = initial absorbance; t = appointed times (min); R = constant of the gases (8.31447 J/mol K); T = absolute temperature (K); k_0 = frequency factor (min).

2.4.3 Effect of light

The effect of light on the stability of anthocyanins present in the aqueous extract was determined according to the experimental conditions described by Pedro et al. (2016). Two solutions of the extract were prepared. One solution with ultrapure water (pH 5.4) and the other in citrate/citric acid buffer solution pH 3.6. The fructooligosaccharides, 1.75 and 3.5 g/100 mL, were added in both solutions. The solution was divided into six tubes each - absence and presence of FOS. Three tubes were protected with aluminium foil and three tubes of each solution were exposed to light. The tubes were placed in the photooxidation chamber with an internal temperature of 32±2 °C. The maximum absorbance (λ=573 nm) was recorded at 24 h intervals until the initial absorbance decreased by 50%. The

colour retention percentage (%R), rate constant of anthocyanins degradation (*k*), and half-life time ($t_{1/2}$) were calculated using Equations 1, 2 and 3, respectively.

2.5 Statistical analysis

The experiments were conducted in triplicate, and the results expressed as mean and standard deviation. The differences between results were verified using one-way variance (ANOVA), followed by Fisher's test of significant minimum difference, considering $p \leq 0.05$ (Granato, de Araújo Calado, & Jarvis, 2014). TIBCO Statistica v. 13.3 software (TIBCO Statistica™ Ltd, USA) was used in the analyses.

3. Results and Discussion

3.1 Phenolic composition of crude lyophilized and partially purified extracts

The phenolic composition of crude lyophilized and partially purified extracts of *C. ternatea* blue petals is shown in Table 1 and Figure S1A - supplementary material. Twelve compounds were tentatively identified in both extracts, being six ternatin anthocyanins and delphinidin derivatives responsible for the blue colour of *C. ternatea* petals. The ternatin structure is composed of the malonylated delphinidin 3,3',5'-triglycosides with D-glucose (G) and malonate (M) at position 3, and alternation of D-glucose and *p*-coumaric acid (C) in the 3' and 5' positions of the side chains (Terahara et al., 1990).

Compound 1 showed molecular ion $[M]^+$ at *m/z* 773.16 tentatively identified as myricetin 3-(2^G-rhamnosylrutinoside). Compound 2 showed molecular ion $[M]^+$ with *m/z* 1329.33, and the identified fragments were *m/z* 1021.24 $[M\text{-}G\text{-}C]^+$ and *m/z* 788.43 $[M\text{-}G\text{-}2C\text{-}malonate]^+$. These data suggest that the groups G-M, G-C-

G and G-C are linked at positions 3, 3' and 5' in the structure of delphinidin, tentatively identified as ternatin B4 (Figure S1C - supplementary material). Compound 3 showed molecular ion $[M]^+$ with *m/z* 459.09, and produced a main fragment with *m/z* 289.07, and it may correspond to catechin. This compound was tentatively identified as epigallocatechin-3-gallate (EGCG). Compound 4 showed molecular ion $[M]^+$ with *m/z* 830.21 and produced a main fragment *m/z* 303.05 which corresponds to delphinidin (Figure S1D - supplementary material). Compound 5 showed the molecular ion $[M]^+$ with *m/z* 757.22 and produced a main fragment with *m/z* 303.05 which may correspond to quercetin. This compound may be tentatively identified as quercetin 3-(2^G-rhamnosylrutinoside). Compound 6 - *m/z* 1551.42 - was exclusively present in the partially purified extract and produced fragments identified with *m/z*: 1389.37 $[M-G]^+$; 1228.32 $[M-2G]^+$; 1081.28 $[M-2G-C]^+$; 919.22 $[M-3G-C]^+$; 773.19 $[M-3G-2C]^+$; 611.13 $[M-4G-2C]^+$; e 465.12 $[M-4G-3C]^+$. This molecular ion produced a main fragment with *m/z* 303.05 which corresponds to delphinidin (Figure S1B - supplementary material). From data it can be inferred that the groups G, G-C-G-C and G-C-G or groups G, G-C-G-C-G and G-C are linked at positions 3, 3' and 5' respectively in the delfinidine structure. Compound 7 showed the molecular ion $[M]^+$ with *m/z* 1637.42 and produced the fragments identified with *m/z*: 1389.38 $[M-G-M]^+$; 1081.28 $[M-2G-C-M]^+$; 919.23 $[M-3G-C-M]^+$; 773.19 $[M-3G-2C-M]^+$; 611.13 $[M-4G-2C-M]^+$; and 465.11 $[M-4G-3C-M]^+$. This molecular ion produced a main fragment of *m/z* 303.05 which corresponds to delphinidin. These data suggest that the groups G-M, G-C-G-C-G and G-C or groups G-M, G-C-G-C and G-C-G are linked at positions 3, 3' and 5' in the structure of delphinidin and can be tentatively identified as ternatin B3 or B2, respectively (Figure S1E -

supplementary material). Compound 8 showed the molecular ion $[M]^+$ with *m/z* 741.22, and produced the fragments identified with *m/z* 595.16 $[M\text{-rhamnosyl}]^+$ and *m/z* 449.11 $[M\text{-2rhamnosyl}]^+$. This molecular ion produced a main fragment with *m/z* 287.06 which may correspond to kaempferol. This compound can be tentatively identified as kaempferol 3-(2^G-rhamnosylrutinoside). Compound 9 showed the molecular ion $[M]^+$ with *m/z* 1475.36 and produced the fragments identified with *m/z*: 1227.31 $[M\text{-G-malonate}]^+$; 919.23 $[M\text{-2G-C-malonate}]^+$; 611.14 $[M\text{-3G-2C-malonate}]^+$; and 551.10 $[M\text{-4G-3C-malonate}]^+$. The molecular ion produced a main fragment *m/z* 303.05 which corresponds to delphinidin. These data suggest that the groups G-M, G-C-G-C and G-C are linked at positions 3, 3' and 5' in the structure of delphinidin, and may be tentatively identified as ternatin D2 (Figure S1F - supplementary material). Compound 10 showed the molecular ion $[M]^+$ with *m/z* 595.17 and produced a main fragment with *m/z* 287.05, kaempferol. This compound can be tentatively identified as kaempferol 3-neohesperidoside. Compound 11 showed the molecular ion $[M]^+$ with *m/z* 903.22 and produced a main fragment with *m/z* 303.05, which corresponds to delphinidin (Figure S1G - supplementary material). Compound 12 showed the molecular ion $[M]^+$ with *m/z* 681.16 and produced a main fragment with *m/z* 287.05, kaempferol. This compound can be tentatively identified as kaempferol-3-O-(2”-O-α-rhamnosyl-6”-O-malonyl)-β-glycoside.

The ternatins tentatively identified in this study confirmed the study made by Terahara et al. (1996), who isolated and elucidated the structure of ternatins A3, B4, B3, B2 and D2 of the *C. ternatea* flowers. These authors verified that ternatins presented molecular ions with *m/z* of 1491 (A3), 1329 (B4), 1637 (B3), 1637 (B2) e 1475 (D2) corresponding to $C_{66}H_{75}O_{39}^+$, $C_{60}H_{65}O_{34}^+$, $C_{75}H_{81}O_{41}^+$,

$C_{75}H_{81}O_{41}^{+}$, and $C_{69}H_{71}O_{36}^{+}$, respectively. Kazuma, Noda and Suzuki (2003b) quantified a higher content of ternatin B2 in the extract of the *C. ternatea* petals containing 50% MeOH and 1% TFA. Nair, Bang, Schreckinger, Andarwulan, and Cisneros-Zevallos (2015) identified ternatins B3 (*m/z* 1638), D3 (*m/z* 1167), B2 (*m/z* 1638), B4 (*m/z* 1329), C2 (*m/z* 1491), D1 (*m/z* 1783), and delphinidin derivatives (*m/z* 1296; *m/z* 1534; and *m/z* 950) in the extract of the *C. ternatea* flowers obtained with MeOH, propanone and water. The glycoside flavonols tentatively identified in this work confirm the compounds verified by Kazuma et al. (2003a). These authors isolated and elucidated the structure of three new flavonols glycosides along with eleven other flavonols in the *C. ternatea* petals cv. Double Blue. The flavonols myricetin 3-(2^{G}-rhamnosylrutinoside), quercetin 3-(2^{G}-rhamnosylrutinoside), kaempferol 3-(2^{G}-rhamnosylrutinoside), kaempferol 3-neohesperidoside and kaempferol-3-O-(2”-O-α-rhamnosyl-6”-O-malonyl)-β-glycoside presented the molecular ions $[M]^{+}$ with *m/z* of 773, 757, 741, 595 and 681, respectively (Kazuma et al., 2003a) - corresponding to the molecular ions identified in the extracts analysed (Table 1). Kazuma et al. (2003b) quantified higher levels of kaempferol 3-neohesperidoside followed by kaempferol 3-(2^{G}-rhamnosylrutinoside). These flavonols correspond to tentatively identified compounds 10 and 8 that showed a higher intensity, Table 1 and Figure S1A - supplementary material. This study is the pioneer in reporting the presence of epigallocatechin-3-gallate in water extract of *C. ternatea* blue petals.

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3.2 Stability of anthocyanins of the aqueous extract

3.2.1 Effect of pH

The stability of anthocyanins and their colour is mainly influenced by the pH of the medium. Figure 1 shows the effect of pH on the stability and reversibility of ternatin anthocyanins and delphinidin derivatives present in the aqueous extract of the *C. ternatea* blue petals. The anthocyanins of the extract showed a colour variation between red and green with pH change from 2.25 to 10.20, respectively, Figure 1B. At pH 2.25, the maximum absorbance of anthocyanins was recorded at the wavelength of 546 nm, Figure 1A. Between pH 3.21 and 6.14, a lower absorption intensity was observed at 546 nm (a hypochromic effect) and a bathochromic displacement with the formation of two new bands at 570 and 618 nm with an increase in absorbance (a hyperchromic effect). At pH 7.19, it was observed the presence of a band with increased absorption (hyperchromic effect) and with bathochromic displacement to 625 nm. Between pH 8.20 and 10.20, it is observed a decrease in absorbance (hypochromic effect) and a bathochromic displacement between 626 to 605 nm. Violet to blue colouration of the extract between pH 3.21 to 7.19 (Figure 1B) occurred by the formation of the structure in the quinoidal form. According to Abdullah, Lee and Hung (2010), between pH 3 to 5, the red flavylic cation and the two tautomers of the neutral blue quinonoid species in equilibrium produce the violet colour in the extract of the *C. ternatea*. Between pH 5 to 7, the extract contains predominantly blue neutral quinonoid species. The presence of one or more acyl groups in the anthocyanin molecule prevents hydrolysis of the flavyl cation to the carbinol pseudobase form and allows the formation of the quinoidal form (blue) with less sensitivity to pH changes in mildly acidic or neutral medium (Bridle & Timberlake,

1997). Saito et al. (1985) observed that the blue petals of the *C. ternatea* are composed of stable acylated anthocyanins in neutral solution. Terahara et al. (1989) attributed the colour stability of ternatin D1 in neutral aqueous solution to the intramolecular stacking between the delphinidin nucleus and the GCGC-side chains, where the chromophore portion (quinoidal form) is protected from nucleophilic attack by water. Moloney et al. (2018) suggested that diacylated anthocyanins from red cabbage express stable intense blue colour, under neutral and alkaline conditions, due to interactions of intramolecular copigmentation, cyanidin and hydroxycinnamic acids, and the self-association cyanidin-cyanidin. Between pH 8.20 and 10.20, the colour of aqueous extract of the *C. ternatea* changed from blue to green, Figure 1B. Abdullah et al. (2010) observed that between pH 7 and 8 the colour of the *C. ternatea* extract changes from blue to green. And they attributed this to the presence of neutral quinoidal bases and anionic quinoidal bases in equilibrium. Moloney et al. (2018) verified at pH 8 the structural change of anthocyanins to the form of blue anionic quinoidal bases, and the bathochromic displacement with the increase in the molar absorption coefficient. Yong et al. (2019) observed the presence of blue colour in the purple eggplant extract between pH 7 to 10, and the bathochromic displacement with a hyperchromic effect. Between pH 10 and 12, plant extracts rich in anthocyanins change colour to yellow due to degradation of the quinoidal bases for ionic chalcones (Abdullah et al., 2010; Yong et al., 2019).

Chemical degradation of anthocyanins causes irreversible loss of colours mainly due to cleavage of bonds between carbons C2-C1', C2, C3 or C3-C4 of the chromophore (Trouillas et al, 2016; Moloney et al, 2018). The reverse spectrophotometric titration, pH 10.20 to 2.25 showed that anthocyanins present

in the *C. ternatea* extract presented a 96.2% recovery of initial absorption, Figure 1A. This result suggests that the anthocyanins of the extract showed reversibility in the equilibrium displacement structure, since the red colour of the extract at pH 2.25 (F) indicated the change from anionic quinoidal form to the flavylic cation form, Figure 1B. Maciel et al. (2018) observed the reversibility of the anthocyanin structure of *Hibiscus sabdariffa* extract, pH 7.5 to 2.7, and a 72.1% recovery of initial absorption. Pedro et al. (2016) verified that the anthocyanins present in the black rice extract showed structural reversibility to the deprotonation-protonation process. The anthocyanin equilibrium structures in the quinoidal form are reversible to the flavylic cation form with the reduction of pH, whereas *cis* and *trans*-chalcones species are irreversible (Pina, Melo, Laia, Parola, & Lima, 2012).

3.2.2 Effect of pH on the in vitro *antioxidant activity*

The antioxidant activity of aqueous extract of the *C. ternatea* blue petals by DPPH was significantly influenced by pH variations and presented 43 to 64% inhibition ($p \leq 0.05$), Figure 2. The pH increase from 2.25 to 5.25 resulted in an increase from 53 to 59% inhibition of DPPH, respectively. At pH 6.14, the antioxidant activity decreased to 54% inhibition of DPPH. Increasing pH to 7.19 provided an increase in antioxidant activity to 64% inhibition. At pH 10.20, antioxidant activity decreased to 43% inhibition. In reverse titration the antioxidant activity of the extract, pH 2.25, showed a 56% inhibition.

Studies on the *in vitro* antioxidant activity of *Centaurea cyanus* and *Hibiscus sabdariffa* flower extracts showed greater inhibition of DPPH radical between pH 4 and 5, and preservation of antioxidant activity after reverse titration with inhibition above 54% (Maciel et al., 2018; Escher et al., 2018). Borkowski,

Szymusiak, Gliszczyńska-Świgło, Rietjens, and Tyrakowska (2005) observed that deprotonated anthocyanins increased free radical scavenging capacity, that is, with the conversion of flavyl cation to the quinoidal base form, pseudobase carbinol and *cis* and *trans*-chalcones. According to Altunkaya, Gökmen, and Skibsted (2016) the increase in the antioxidant activity of phenolic compounds with pH may be related to the increased electron donation capacity after deprotonation of the molecular structure and stabilization in alkaline medium.

The antioxidant activity of the *C. ternatea* blue petal anthocyanins can be influenced by the pH of the food matrix, and also by the pH of body fluids during the digestive process. Anthocyanins are partially absorbed in the stomach (pH 1.5-2.0) but the highest absorption rate occurs in the small intestine. Some factors such as intestinal pH, temperature, enzymes and intestinal microbiota may affect anthocyanin absorption (Han et al., 2019). Pasukamonset, Kwon, and Adisakwattana (2016) found that the *C. ternatea* aqueous extract and its microencapsulation preserved significant levels of phenolic compounds and antioxidant activity after simulated gastrointestinal digestion. The results obtained in this study suggest that the aqueous extract of the *C. ternatea* blue petals has *in vitro* antioxidant activity over a broad range of pH 3.21 to 5.25, but *in vivo* studies are required to evaluate the bioavailability, bioaccessibility and bioactivity of anthocyanins of the *C. ternatea* blue petals.

3.2.3 Effect of temperature

The influence of fructooligosaccharides on the stability of anthocyanins of the *C. ternatea* blue petals extracts at pH 3.6 and 5.4 at different temperatures was evaluated. The FOS are soluble fibers extracted from plants that stimulate

the proliferation of eubacteria in the intestine and provide benefits to human health (Koca & Tasci, 2016). The extracts at pH 3.6 and 5.4 in the presence and absence of FOS presented thermal stability at 60 and 70 °C for 360 min. The temperature increase to 80, 90 and 100 °C resulted in the decrease in absorbance of the extract at pH 3.6 and 5.4 both in the presence and in the absence of FOS.

Table 2 presents the colour retention percentage (%R), rate constant of anthocyanins degradation (*k*) and half-life time ($t_{1/2}$). According to Patras et al. (2010) the degradation reaction kinetics can be used to predict the degradation of compounds during heat treatment and storage period from reaction order, velocity constant and activation energy. As the temperature increased from 80 to 100 °C, it was observed an increase in the anthocyanin degradation rate constant values of the *C. ternatea* blue petal extracts ($p \leq 0.05$). Thus, there was a decrease in the half-life time of the anthocyanins of the extracts at pH 3.6 and 5.4 as the temperature increased from 80 to 100 °C ($p \leq 0.05$). The increase in rate constant value procreates the reduction of half-life time. A linear relationship between absorbance and time was observed in the extracts at pH 3.6 and 5.4 in the anthocyanin degradation reaction, which indicates that the reaction followed a first order kinetics, Figure S2 - supplementary material. The degradation reaction of anthocyanins in the extract at pH 3.6 and 5.4 increased with higher temperatures as a function of time. The results are in agreement with studies made with different extracts for anthocyanin degradation reaction followed by first order kinetics (Kirca, Özkan, & Cemeroğlu, 2007; Hou, Qin, Zhang, Cui, & Ren, 2013; Jie et al., 2013; Pedro et al., 2016; Maciel et al., 2018; Migliorini et al., 2019).

The velocity constants were similar at both pH values studied - in the extracts in the presence of 1.75 g FOS/100 mL and in the absence of FOS. The extract with 3.5 g FOS/100 mL presented higher values in the velocity constants, in both pH values and in all temperatures studied. It was observed that with increasing temperature there was an increase in the rate constant and a decrease in half-life time. The presence of FOS in the *C. ternatea* blue petals extracts at both pH 3.6 and 5.4 did not influence the stability of anthocyanins.

Migliorini et al. (2019) found that anthocyanins present in red chicory extract at pH 3 showed high stability at 60 °C, $t_{1/2}$=74.23 h. Meanwhile, raising the temperature to 70, 80, 90 and 100 °C caused a decrease in half-life time to 17.25, 9.55, 5.24 and 2.25 h, respectively. Maciel et al. (2018) observed that the *Hibiscus sabdariffa* extract copigmented in the presence of chlorogenic acid at pH 3. It had a half-life time of 6.7, 2.6 and 1.6 h at temperatures of 80, 90 and 100 °C, respectively. Sagdic et al. (2013) found that the anthocyanins present in the petal extract of the *Tulipa gesneriana* 'Negrita' had a half-life time of 7.18, 2.34 and 1.76 h at temperatures of 70, 80 and 90 °C at pH 3.5, respectively. Kirca et al. (2007) reported that the anthocyanins present in the black carrot extract had a half-life time of 25.1 h at a temperature of 70 °C, 10.0 h at a temperature of 80 °C, and 6.3 h at a temperature of 90 °C at pH 3 and pH 4.

Jie et al. (2013) reported that the half-life time of the anthocyanins from aqueous extract of purple sweet potato pulp at pH 5, exposed at temperatures of 80, 90 and 100 °C were 25.9, 12.9 and 7.7 h, respectively. Hou et al. (2013) observed that the main anthocyanin present in black rice, cyanidin-3-glycoside, had a half-life time of 2.51 h at a temperature of 80 °C, 1.89 h at a temperature of 90 °C, and 1.43 h at a temperature of 100 °C in pH 5 citrate phosphate buffer

solution. The authors stated that the stability of anthocyanins depends on the temperature and pH associated with chemical structure and copigmentation. Kirca et al. (2007) found that anthocyanins from black carrot extract in pH 5 citrate-phosphate buffer solution had a half-life time of 16.7, 10.0 and 5.6 h at temperatures of 70, 80 and 90 °C, respectively. It was observed that the *k* values were shorter and half-life time was longer in the pH 3.6 extract if compared to the extract in pH 5.4. Sui, Dong and Zhou (2014) observed that rising temperatures had a greater impact on anthocyanin stability than increasing pH 2-6. Hou et al. (2013) related the acceleration in the anthocyanin degradation reaction of black rice extract as a function of pH 1-6 increase, the different structural forms of anthocyanins between the species in equilibrium.

The activation energy (E_A) is the energy required for a particular species to reach the activated state in a chemical reaction. High E_A values suggest that the compounds present in the extract showed high temperature stability. The higher the E_A the slower the speed of the anthocyanins degradation reaction (Migliorini et al., 2018). From data in Table 2, it can be noticed that degradation reaction of anthocyanins present in the *C. ternatea* blue petals extracts of slightly higher for the pH 3.6 extract in the presence and absence of FOS if compared to the extract with pH 5.4. At pH 3.6, the extract with 1.75 g FOS/100 mL showed similar E_A values, 104.3 kJ/mol, when compared to the extract in the absence of FOS, E_A=104.0 kJ/mol. While in the 3.5 g FOS/100 mL extract the activation energy value was 102.1 kJ/mol. At pH 5.4, E_A values were similar in the extracts in absence and presence of FOS. Migliorini et al. (2019) found that the E_A for the degradation reaction of anthocyanins present in red chicory extract at pH 3 was 84.88 kJ/mol. Maciel et al. (2018) observed an E_A of 94.4 kJ/mol for *Hibiscus*

sabdariffa extract copigmented with chlorogenic acid in pH 3 citrate buffer solution. Hou et al. (2013) determined E_A values of cyanidin-3-glycoside present in black rice of 67.39 and 30.97 kJ/mol in citrate phosphate buffer solution with pH 3 and 5, respectively. Jie et al. (2013) verified the activation energy value of 66.56 kJ/mol in the aqueous extract of purple sweet potato pulp at pH 5. The results of this study showed that the presence of FOS did not influence the values of E_A as well as it did not increase the stability of anthocyanins present in the extract. However, it can be confirmed that the anthocyanins present in the aqueous extract of the *C. ternatea* blue petals showed high stability at the studied temperatures and at both pH 3.6 and 5.4 values. This result associated with pH variations and with a wide colour spectrum, as well as the close colour reversibility to 100% in the deprotonation and protonation reaction shows the potential of anthocyanins present in this extract as a natural colourant.

3.2.4 Effect of light

Light is one of the factors that accelerate the anthocyanin degradation reaction. Table 3 presents the results of the effect of light as a function of time on the stability of anthocyanins in the *C. ternatea* blue petal extract at pH 3.6 and 5.4 - presence (1.75 g and 3.5 g FOS/100 mL) and absence of fructooligosaccharides. The extracts at pH 3.6 exposed to light, 32±2 °C, for 13920 min presented colour retention percentages of 35.5 %R for the 3.5 g FOS/100 mL extract, 34.6 %R for the 1.75 g FOS/100 mL extract, and 34.4 %R for the extract in the absence of FOS. While extracts protected from light showed higher colour retention percentages compared to light exposed extracts, 90.5 %R

for the 3.5 g FOS/100 mL extract, and 90.1 %R for both extracts, with 1.75 g FOS/100 mL and absence of FOS.

The presence of 3.5 g and 1.75 g FOS/100 mL in extracts at pH 3.6 that were either exposed to light or protected from light had an slight increase in half-life time and a reduction in anthocyanin degradation rate constants; however, no significant differences were observed in relation to the extract in absence of FOS. The half-life time and the velocity constant in the extract in absence of FOS and exposed to light were 147.1 h and 7.86 x 10^{-5} min^{-1}, and for the light protected extract were 1639.9 h and 7.06 x 10^{-6} min^{-1}, respectively. The extract with 3.5 g FOS/100 mL exposed to light had a half-life time of 151.9 h and k = 7.63 x 10^{-5} min^{-1}, and in the light protected extract the half-life time was 1709.4 h and k = 6.82 x 10^{-6} min^{-1}. The half-life time increased by 69.5 h in the 3.5 g FOS/100 mL extract compared to the extract in absence of FOS, both protected from light. The photodegradation kinetics of anthocyanins present in *C. ternatea* blue petal extract at pH 3.6 in the presence and absence of FOS, and exposed and protected from light is shown in Figures S3A and S3B - supplementary material. In fact, adding FOS into model beverages and associate its addition to the stability studies of natural compounds may be a suitable technological approach to be adopted by the beverage sector: anthocyanins are prone to oxidation depending on the pH, light exposure (packaging), and interaction with other ingredients (e.g., phenolics, proteins, carbohydrates, etc). Thus, stability studies considering these effects are necessary to understand the possible antioxidant effects both *in vitro* and *in vivo* (Pellegrini, Vitaglione, Granato, & Fogliano, 2020).

Pedro et al. (2016) found that the copigments glucose, phytic acid and gallic acid provided a slight increase in the stability of anthocyanins present in

black rice extract at pH 3.6. The presence of these copigments reduced the values of the anthocyanin degradation rate constants of the black rice extract. Askar, Alsawad, and Khalaf (2015) found that anthocyanins from rosella petal extract at pH 4 had a half-life time of 1.16 h and k = 0.010 min^{-1} under sunlight with a temperature between 25 to 60 °C.

The extracts at pH 5.4 presented colour retention percentages of 67.5 %R for the 3.5 g FOS/100 mL extract, 48.6 %R for the 1.75 g FOS/100 mL extract, and 48.3 %R for the extract in absence of FOS after exposure to light for 18900 min, Table 3. Similarly, the light protected extracts showed higher colour retention percentages, 97.6, 93.4 and 94.6 %R for extracts with 3.5 g FOS/100 mL, 1.75 g FOS/100 mL and in the absence of FOS, respectively. The presence of FOS in extracts at pH 5.4 exposed to light increased the half-life time and reduced the anthocyanin degradation rate constants ($p \leq 0.05$). The longest half-life time ($t_{1/2}$ = 484.8 h) was observed in the extract with 3.5 g FOS/100 mL exposed to light and associated with lower rate constant, k = 2.34 x 10^{-5} min^{-1}. The extract in the absence of FOS and exposed to light had a half-life time of 264.3 h and k = 4.41 x 10^{-5} min^{-1}. The extracts protected from light presented high stability, the absorbance of the extract remained practically constant during the study period. Thus, it was not possible to calculate the anthocyanin degradation rate constant and the half-life time. The photodegradation kinetics of anthocyanins in *C. ternatea* blue petal extract at pH 5.4, in the presence and absence of FOS, exposed to light is shown in Figure S3C - supplementary material.

These results showed that light exposure caused an increase in the photodegradation reaction of anthocyanins present in the extract of the *C. ternatea* blue petals. The presence of FOS provided a protective effect against

the photodegradation of ternatin anthocyanins only at pH 5.4, in which the extract presented a longer half-life time associated to a lower degradation rate constant. In acid pH (3.6), the parcial hydrolysis of FOS may have occurred and this can explain the absence of protective effect of anthocyanins, while at pH values close to neutrality, FOS presents greater chemical stability (L'homme, Arbelot, Puigserver, & Biagini, 2003). Studies with different anthocyanic extracts are necessary to evaluate the protective effect of FOS against light exposure, to elucidate the inter and intramolecular interaction mechanism. The extracts protected from light presented much higher values of colour retention. This information suggests the importance of the use of dark packaging by the food industry in order to maintain the colour of anthocyanins when used as natural colourants. No studies were found in the literature using FOS as an anthocyanin copigment, therefore new studies are needed to evaluate the protective effect against photodegradation.

4. Conclusions

This is the first study to identify the presence of epigallocatechin-3-gallate in crude lyophilized and partially purified water extracts from *C. ternatea* blue petals. The direct/reverse spectrophotometric titration showed that anthocyanins presented structural changes variating colour with pH variation, and structural reversibility with preservation of antioxidant activity. The aqueous extracts at pH 3.6 and 5.4, in the presence and absence of FOS exhibited high thermal stability at 60 and 70 °C and an increase in degradation rate between 80 to 100 °C. These results evidenced that butterfly pea anthocyanins presented high stability in

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relation to pH, temperature and light. Thus, it is concluded that ternatin anthocyanins have great potential as natural colourants in the food industry.

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Conflicts of Interest

The authors declare no conflict of interest.

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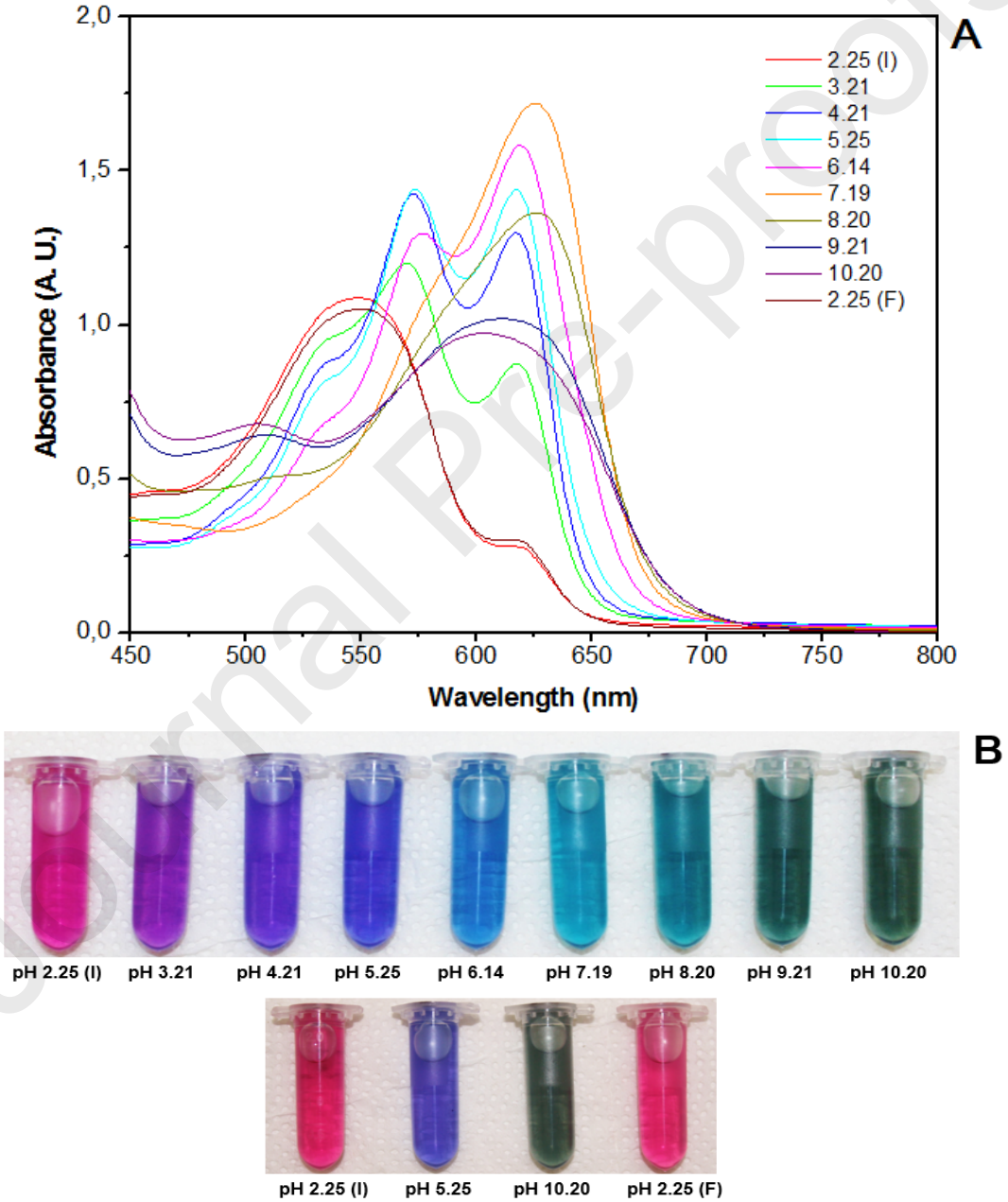


Figure 1. Effect of pH on the stability and reversibility of anthocyanins present in the aqueous extract of *Clitoria ternatea* blue petals. A: Absorption spectrum of

extracts between pH 2.25 (I: initial) to 10.20, and reversibility to pH 2.25 (F: final). B: Colors variation of anthocyanins in the extracts between pH 2.25 to 10.20, and color reversibility at pH 2.25.

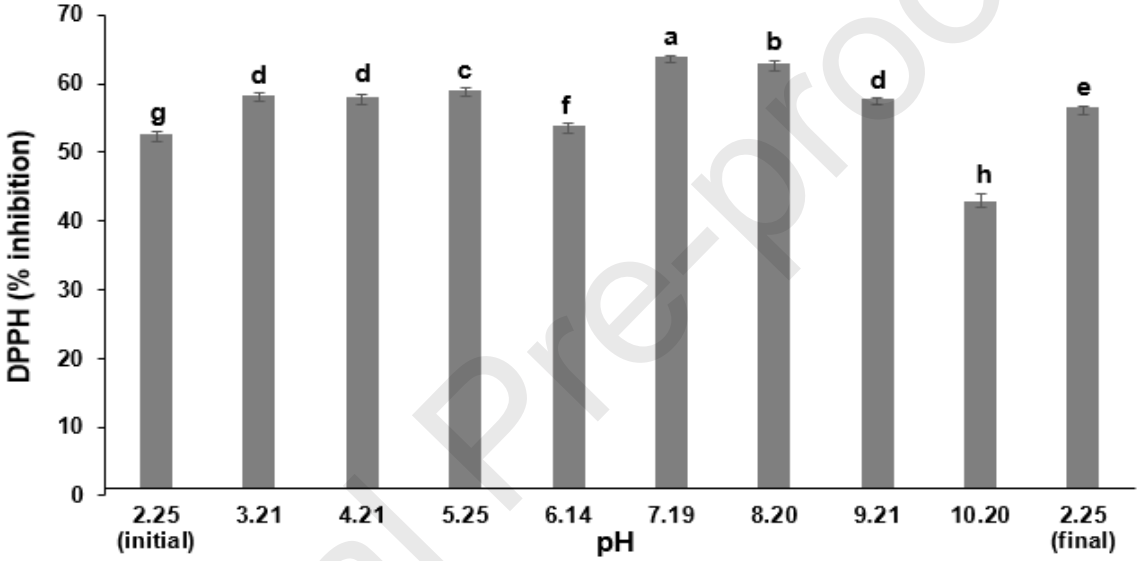


Figure 2. Effect of pH on antioxidant activity of anthocyanins present in the aqueous extract of *Clitoria ternatea* blue petals. Different letters represent statistically significant differences ($p \leq 0.05$).

Table 1. Phenolic compounds tentatively identified in the crude lyophilized and partially purified extracts of *Clitoria ternatea* blue petals.

Peak	Time (min)	Molecular ion $[M + H]^+$	Fragments (MS^2)	Tentative identification

1	7.55	773.16	-	Myricetin 3-(2^{G}-rhamnosylrutinoside)
2	9.25	1329.33	1021.24; 810.41; 788.43; 692.13; 684.14; 676.16; 475.33; 361.15; 253.01	Ternatin B4
3	9.63	459.09	289.07; 158.97	Epigallocatechin-3-gallate
4	10.80	830.21	706.18; 639.17; 303.05; 147.04	Delphinidin derivative
5	11.26	757.22	303.05; 548;65	Quercetin 3-(2^{G}-rhamnosylrutinoside)
6*	11.63	1551.42	1389.37; 1228.32; 1081.28; 919.22; 773.19; 611.13; 465.12; 303.05; 147.04	Delphinidin derivative
7	11.86	1637.42	1389.38; 1081.28; 919.23; 773.19; 611.13; 465.11; 303.05; 147.04	Ternatin B2 or B3
8	12.35	741.22	595.16; 449.11; 287.06; 129.05	Kaempferol 3-(2^{G}-rhamnosylrutinoside)
9	12.81	1475.36	1227.31; 919.23; 611.14; 551.10; 303.05	Ternatin D2
10	13.64	595.17	467.92; 287.06	Kaempferol 3-neohesperidoside
11	13.98	903.22	779.19; 639.16; 489.06; 303.05; 147.04	Delphinidin derivative
12	14.98	681.16	577.73; 443.82; 350.90; 287.05	Kaempferol-3-O-(2”-O-α-rhamnosyl-6”-O-malonyl)-β-glycoside

Note: *Compound tentatively identified only in partially purified extract.

Table 2. Kinetic parameters of the effect of temperature on the stability of the extract of the *Clitoria ternatea* blue petals (pH 3.6 and 5.4) in presence (1.75 g and 3.5 g/100 mL) and absence of fructooligosaccharides.

Temperature (°C)	**%R**	***k* (min^{-1})**	**R^2**	***p*-Value**	**$t_{1/2}$ (h)**	**E_A (kJ/mol)**	**R^2**
pH 3.6							
3.5 g FOS/100 mL							
80	88.9 ± 0.8^{a}	3.63 x 10^{-4c}	0.998	<0.0001	31.9^{a}	102.1	0.999

90	74.6 ± 2.2[b]	8.83 x 10^{-4}[b]	0.999	<0.0001	13.1[b]		
100	65.3 ± 1.6[c]	2.35 x 10^{-3}[a]	0.999	<0.0001	4.8[c]		
1.75 g FOS/100 mL							
80	90.5 ± 1.3[a]	3.01 x 10^{-4}[c]	0.999	<0.0001	38.8[a]	104.3	0.999
90	76.5 ± 1.7[b]	7.35 x 10^{-4}[b]	0.999	<0.0001	15.8[b]		
100	69.9 ± 3.3[c]	2.03 x 10^{-3}[a]	0.999	<0.0001	5.8[c]		
Absence FOS							
80	90.0 ± 0.5[a]	3.06 x 10^{-4}[c]	0.999	<0.0001	37.8[a]	104.0	0.999
90	76.1 ± 1.7[b]	7.98 x 10^{-4}[b]	0.999	<0.0001	15.3[b]		
100	70.2 ± 2.6[c]	2.05 x 10^{-3}[a]	0.997	<0.0001	5.7[c]		
pH 5.4							
3.5 g FOS/100 mL							
80	86.1 ± 2.9[a]	5.57 x 10^{-4}[c]	0.997	<0.0001	20.8[a]	99.3	0.999
90	64.9 ± 2.1[b]	1.47 x 10^{-3}[b]	0.998	<0.0001	7.9[b]		
100	55.2 ± 2.4[c]	3.41 x 10^{-3}[a]	0.998	<0.0001	3.4[c]		
1.75 g FOS/100 mL							
80	85.6 ± 0.8[a]	5.32 x 10^{-4}[c]	0.996	<0.0001	21.7[a]	99.5	0.999
90	65.8 ± 1.2[b]	1.38 x 10^{-3}[b]	0.998	<0.0001	8.4[b]		
100	58.1 ± 4.4[c]	3.28 x 10^{-3}[a]	0.995	<0.0001	3.5[c]		
Absence FOS							
80	85.2 ± 1.3[a]	5.33 x 10^{-4}[c]	0.996	<0.0001	21.7[a]	100.3	0.999
90	64.5 ± 1.9[b]	1.41 x 10^{-3}[b]	0.999	<0.0001	8.2[b]		
100	57.5 ± 1.7[c]	3.32 x 10^{-3}[a]	0.999	<0.0001	3.5[c]		

Note: %R: percentage of colour retention; *k*: rate constant of anthocyanins degradation; R^2: determination coefficient; *p*-Value: descriptive level (significance); $t_{1/2}$: half-life time; E_A: activation energy; FOS: fructooligosaccharides. Different letters in the same column in presence and absence of FOS represent statistically significant results ($p \leq 0.05$).

Table 3. Effect of light on stability of the extract of *Clitoria ternatea* blue petals (pH 3.6 and 5.4) in presence (1.75 g and 3.5 g/100 mL) and absence of fructooligosaccharides.

Samples	**%R**	**$t_{1/2}$ (h)**	***k* (min^{-1})**	**R^2**	***p*-Value**
pH 3.6					

Extracts exposed to light					
3.5 g FOS	35.5 ± 3.0	151.9	7.63×10^{-5}	0.998	<0.01
1.75 g FOS	34.6 ± 0.3	147.4	7.84×10^{-5}	0.999	<0.01
Absence FOS	34.4 ± 0.7	147.1	7.86×10^{-5}	0.999	<0.01
Extracts protected from light					
3.5 g FOS	90.5 ± 0.1	1709.4	6.82×10^{-6}	0.989	<0.01
1.75 g FOS	90.1 ± 0.6	1693.0	6.99×10^{-6}	0.982	<0.01
Absence FOS	90.1 ± 0.9	1639.9	7.06×10^{-6}	0.977	<0.01
pH 5.4					
Extracts exposed to light					
3.5 g FOS	67.5 ± 0.1[a]	484.8[a]	2.34×10^{-5b}	0.998	<0.01
1.75 g FOS	48.6 ± 0.1[b]	278.1[b]	3.98×10^{-5a}	0.930	<0.01
Absence FOS	48.3 ± 0.1[c]	264.3[b]	4.41×10^{-5a}	0.963	<0.01
Extracts protected from light					
3.5 g FOS	97.6 ± 0.7[a]	-	-	-	-
1.75 g FOS	93.4 ± 0.4[b]	-	-	-	-
Absence FOS	94.6 ± 1.9[b]	-	-	-	-

Note: %R: percentage of colour retention; $t_{1/2}$: half-life time; *k*: rate constant of anthocyanins degradation; R^2: determination coefficient; *p*-Value: descriptive level (significance); FOS: fructooligosaccharides. Different letters in the same column in presence and absence of FOS represent statistically significant results ($p \leq 0.05$).

HIGHLIGHTS

- Ternatins B2/B3, B4, and D2 were identified in *Clitoria ternatea* aqueous extracts
- Epigallocatechin-3-gallate was identified by for the first time
- Anthocyanins presented structural reversibility in relation to pH changes
- Aqueous extracts at pH 3.6 and 5.4 exhibited thermal stability
- Fructooligosaccharides added to the extracts provided a protective effect on the stability

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Authors' contributions

G.B. Escher, M. Wen, L. Zhang, and N.D. Rosso performed the analysis by UHPLC-Q-TOF-MS/MS, helped in data analysis and in the preparation of the draft. G.B. Escher and N.D. Rosso performed the partial purification, and studies of stability of anthocyanins, helped in data analysis and in the preparation of the draft. D. Granato coordinated all activities, helped in the statistical analyses, and drafted the paper. All authors approved the final version of the manuscript.

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