Comparative Analysis of *In Vitro* Photo-Protective Effects on *Clitoria ternatea* Ethanolic Extract

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In this study, *Clitoria ternatea* flowers (CT) underwent four distinct ethanolic extraction procedures: maceration, soxhlet, ultrasonication-assisted extraction (UAE), and enzyme-assisted extraction (EAE). Analysis of the extracts centred on their yield percentage, sun protective factor (SPF), total phenolic content (TPC), and total flavonoid content (TFC). A comparison of the four extraction techniques revealed a significantly greater amount of CT extract in terms of yield (UAE: 15.40 ± 0.04 %; EAE: 11.97 ± 0.03 %), TPC (UAE: 35.53 ± 0.08 GAE µg/ml; EAE: 35.77 ± 0.73 GAE µg/ml), and TFC (UAE: 118.67 ± 0.78 QE µg/ml; EAE: 21.44 ± 0.02 QE µg/ml) when utilizing the non-conventional methods of UAE and EAE, as opposed to the conventional methods of Soxhlet and maceration. FTIR spectroscopy analysis confirmed the presence of phenolic compounds and flavonoids in all samples. These findings indicate that while UAE resulted in a higher content of flavonoids, EAE was more efficient in extracting phenolic compounds. Based on these results, UAE and EAE serve as potential eco-friendly, highly efficient and preferable 'green' or non-conventional extraction methodologies for obtaining sun-protective phytochemicals from CT.

Keywords*:* Ultrasonicated-assisted extraction; enzyme-assisted extraction; conventional, non-conventional

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The *Clitoria ternatea* (CT), also known as the Fabaceae flower or referred to locally as *bunga telang*, is a species from the Fabaceae family. Renowned for its vibrant deep blue petals, this plant is extensively utilized as a natural dye in food and beverage preparation. It has a longstanding history in traditional medicine, serving as a remedy to enhance cognitive function and alleviate symptoms associated with various ailments such as fever, inflammation, pain and diabetes [1]. In ayurvedic medicine, CT is used to treat a range of neurological conditions, including depression, fever and infertility [2, 3]. The therapeutic potential of CT has received international recognition, and is backed by scientific validation which attributes it with an array of biological activities. These include antioxidant, anti-diabetic, antitumor, antimicrobial, and anti-inflammatory properties [4, 5].

Recently, the potential of CT extracts for sun protection has garnered significant attention in research circles. Prior studies have investigated the sun-protective properties of CT extracts, utilizing various solvents such as water [6-8], ethanol [9], and a blend of water with ethanol or methanol [10, 11]. It is important to highlight that thus far, maceration has been the sole extraction technique reported in the exploration of CT's sun-protective capabilities. However, the maceration method is known to present several limitations, including low extraction yields, diminished efficiency and the necessity of large volumes of solvents, which could potentially pose health risks [12]. The efficacy of this method further decreases in the presence of substantial amounts of polysaccharides attached to the cell wall [13, 14]. In light of these challenges, this preliminary investigation aimed to delve into alternative, more sustainable and efficient 'green' or non-conventional extraction methods. The goal was to enhance the yield of CT extraction and optimize its sun-protective effects, contributing to the development of eco-friendlier and more effective solutions in this domain.

Microwave-assisted extraction (MAE) and ultra sonic-assisted extraction (UAE) represent innovative, non-traditional approaches that have demonstrated promising results in extracting CT. However, when considering the application of MAE in future studies, it is important to consider its requirement for specialized equipment, its lower selectivity, and the potential for undesirable reactions at elevated temperatures [16-18]. Given these considerations, UAE emerges as a more practical and viable option, and is the focus of this study. Utilizing ultrasonic energy in a commonly-available ultrasonic bath to disrupt plant cell walls [19-21], UAE allows for the use of reduced solvent quantities and facilitates the completion of the

extraction process quickly at room temperature. These factors collectively position UAE as a highly efficient and environmentally friendly extraction method.

In addition to UAE, the authors have taken an interest in enzyme-assisted extraction (EAE). This method represents a relatively novel approach, with its full potential yet to be completely explored [17]. EAE brings forth numerous benefits for extracting phytochemicals, including a shortened extraction duration and heightened extraction efficacy. Contrary to UAE, EAE employs enzymes to break down components within the cell wall [13], resulting in reduced solvent usage. Given that enzymes can facilitate the release of phytochemicals with minimal solvent and heat requirements, this method typically generates fewer undesired byproducts [23], while maintaining a satisfactory yield [24-25].

EXPERIMENTAL

Chemicals and Materials

Ethanol, Folin–Ciocalteu reagent at concentrations between 1.9 and 2.1 N, and monohydrate gallic acid with a purity not less than 98 %, were acquired for this study. Furthermore, ethanol of exceptionally high purity (≥ 99.8 %), sodium carbonate and potassium acetate, both exceeding 99.5 % purity, as well as aluminium chloride hexahydrate at 99 % purity, were sourced from renowned commercial vendors such as Sigma-Aldrich, Merck and Acros Organics. Additionally, the amylase enzyme was procured from Sinopharm Chemical Reagent.

Preparation of CT Extractions

CT, in its dried form, was acquired from a local marketplace and subsequently ground into a powder with an electric grinder. In a conical flask, 100 g of powdered CT was combined with 1 L of ethanol at a 1:10 ratio. This was followed by the implementation of optimized extraction conditions, as previously established in the literature.

a) Maceration: The mixture was kept under agitation at 27 °C for three days [10].

b) Soxhlet extraction: The materials were processed in a Soxhlet extractor, at 60 ± 2 °C. This involved 4-6 repeated reflux cycles, each lasting 180 min.

c) UAE: Ultrasonic-assisted extraction at 53 kHz and 100 W was conducted at 50 °C for 180 min in an ultrasonic bath. To regulate the temperature, ice was added, and the water was periodically replaced [26, 27].

d) EAE: A 0.5 % concentration of commercial amylase enzyme was agitated at 50 °C for 180 min. Following this, the enzyme was denatured by elevating the temperature to 90 °C for an additional 15 min [28, 29].

Post-extraction, the mixture underwent centrifugation at 8000 × g for 15 min to separate any remaining undigested residue. The extract was then filtered, and the solvent was removed with a vacuum evaporator [28]. The yield was calculated based on the following formula:

$$Percentage\ of\ yield\ (\%) = \frac{Weight\ of\ extract\ (g)}{Weight\ of\ sample\ (g)} \times 100$$

Determination of Sun Protection Factor (SPF)

The effectiveness of the extracts in providing sun protection was evaluated by determining their Sun Protection Factor (SPF), following the method outlined by Sayre et al. [30], which was further simplified according to Mansur's UV spectrophotometric technique [31]. In summary, each extract was diluted in ethanol to achieve a final concentration of 200 µg/mL. Absorbance readings of the sample were then taken across a wavelength spectrum of 290-320 nm, at 5 nm intervals, with three separate measurements recorded at each wavelength point. The SPF value was calculated using the following equation:

$$\mathrm{SPF} = \mathrm{CF} \times \sum_{290}^{320} \mathrm{EE}\ (\boldsymbol{\lambda}) \times \mathrm{I}\ (\lambda)\ \times \mathrm{abs}\ (\lambda)$$

CF: Correction factor =10; EE (λ): Erythemal effect spectrum; I (λ): Solar intensity spectrum; Abs (λ): Absorbance of sample.

Determination of Total Phenolic Content (TPC)

The quantification of TPC was carried out using the Folin–Ciocalteu reagent method, with a few minor adjustments [32]. In brief, a 3 mL aliquot of the extract, with a concentration of 200 µg/mL, was mixed thoroughly with 0.5 mL of Folin-Ciocalteu reagent. Following a minute of mixing, 2 mL of 20 % (w/v) sodium carbonate solution was added to the mixture. It was then left to rest for an hour in the dark. The absorbance of the resulting reaction mixtures was measured at a wavelength of 750 nm.

Standard solutions of Gallic acid, ranging in concentration from 1 to 40 µg/mL, were prepared. The standard calibration curve was derived from these solutions, resulting in a linear fit with an r^2 value of 0.991 and an equation of Y = 0.029X. For each test sample, measurements were taken in triplicate. The results were expressed in terms of Gallic Acid Equivalent (GAE) in µg per mL of extract.

Determination of Total Flavonoid Content (TFC)

TFC was quantified using the aluminium chloride technique, with quercetin utilized as the reference

standard [32]. Initially, 1 mL of a 2 % w/v aluminium chloride solution was combined with 200 µL of the extract, which had a concentration of 200 µg/mL. The mixture was then vortexed for 10 seconds and subsequently left to incubate at room temperature for one hour. The absorbance of the solution was recorded at 420 nm. A series of quercetin standards, with concentrations varying from 5 to 320 µg/mL, were prepared, and used to establish a standard calibration curve which yielded a linear relationship represented by the equation Y = 0.029X and an r^2 value of 0.994. For accuracy, each test sample was measured in triplicate. The results were finally presented as µg Quercetin Equivalent (QE) per mL of extract.

Qualitative Characterization by FTIR

The extract was placed onto the ATR crystal for FTIR analysis, and subsequently, all the gathered spectra were transformed into ASCII file format in preparation for data analysis.

Statistical Analysis

The experiments were replicated three times, and the results were articulated as the mean ± standard error of the mean (SEM). The statistical evaluation was conducted utilizing GraphPad Prism 8.0 software. To ascertain differences between the experimental mean values, Analysis of Variance (ANOVA) and the Bonferroni test were employed, with a significance threshold set at a 5 % confidence level ($p < 0.05$).

RESULTS AND DISCUSSION

The results presented in Figures 1 and 2 indicate that Ultrasonic-assisted Extraction (UAE) and Enzyme-assisted Extraction (EAE) achieved significantly superior extraction yields (15.40 ± 0.04 % and 11.97 ± 0.03 %, respectively) and Sun Protection Factor (SPF) values (9.53 ± 0.77 and 9.29 ± 0.52, respectively) in comparison to the conventional maceration technique. Although the yields obtained through UAE and EAE were not as high as the yield from Soxhlet extraction (25.15 ± 0.07 %), the SPF values of the extracts were comparable, with an SPF value of 8.62 for the Soxhlet extract. These findings underscore the efficacy of these non-conventional ethanolic extraction techniques over conventional methods like Soxhlet heating and maceration [33]. The SPF values for CT extracts obtained through both EAE and UAE were 9, which meet the National Pharmaceutical Regulatory Agency's requirement of SPF values of 6 or higher for cosmetic industry applications [34]. Hence, the CT extracts in this study hold potential for development into sunscreen ingredients. However, it is noteworthy to mention that the yields and SPF values obtained from maceration in this study were lower than those reported in a previous study (Yields = 23.7 - 53.4 %; SPF = 20.4916 ± 0.65), which could be due to variances in geographical locations where the plant material was sourced [4-9].

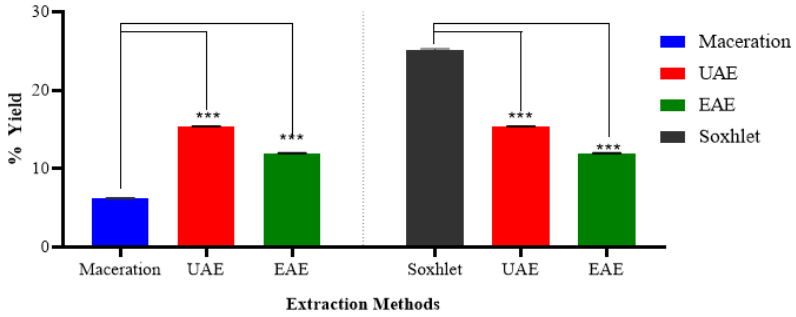

Figure 1. % Yield from different extraction methods. The error bar shows the SEM, *p < 0.05, ** p< 0.01 and *** p<0.001, control with the control group.

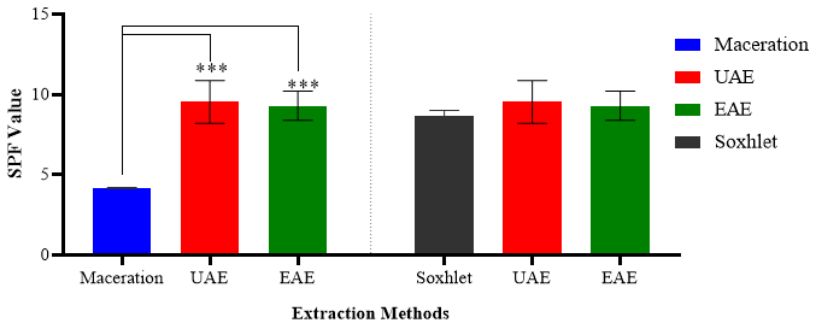

Figure 2. SPF values from different extraction methods. The error bar shows the SEM, *p < 0.05, ** p< 0.01 and *** p<0.001, control with the control group.

Phenolic compounds play a pivotal role in determining the photoprotective potential of plant extracts. Additionally, there is a noted correlation between SPF values and the content of phenolics [36]. This is attributed to the structure of phenolic compounds, which consist of condensed aromatic rings and hydroxyl groups (OH), enabling them to effectively absorb UV radiation and cover the UVA and UVB spectra [37-38]. Consequently, the authors anticipated that both EAE and UAE methods would produce extracts with higher phenolic content compared to the maceration technique. As depicted in Figure 3, the results validated this hypothesis, showing that the total phenolic content of the extracts obtained through UAE (35.53 ± 0.08 GAE µg/mL) and EAE (35.77 ± 0.73 GAE µg/mL) were significantly superior to those acquired via Soxhlet extraction (22.19 ± 0.89 GAE µg/mL) and maceration (30.59 ± 0.51 GAE µg/mL) at the maximum concentration of 2 µg/mL. Notably, the phenolic content in both EAE and UAE extracts exhibited concentration-dependent behaviour, indicating that utilizing more concentrated extracts in future studies could potentially yield even higher amounts of phenolic compounds.

Numerous prior investigations have noted a correlation between flavonoids and phenolic content, given that flavonoids are a subgroup of phenolic compounds. Nevertheless, in this study, the TFC obtained from UAE was 118.67 ± 0.78 QE µg/mL (as presented in Figure 4), which was approximately four times higher than the phenolic content, measured at 35.53 ± 0.08 GAE µg/mL (Figure 3) at the maximum extract concentration of 2 µg/mL. This finding is in alignment with the results reported by Carreira-Casais and associates [39]. Carreira-Casais et al. argued that the Folin–Ciocalteu reagent method, which traditionally utilizes gallic acid as the standard reference, may not detect all phenolic compounds present in an extract [39]. Consequently, in our study, we hypothesize that the UAE method had potentially extracted a substantial quantity of quercetin and its variants, including quercetin 3-(2G-rhamnosylrutinoside), -neohesperidoside, -rutinoside, and -glucoside, as identified in previous research. This would account for the observed elevated levels of flavonoids, which were quantified using quercetin as the standard reference [40].

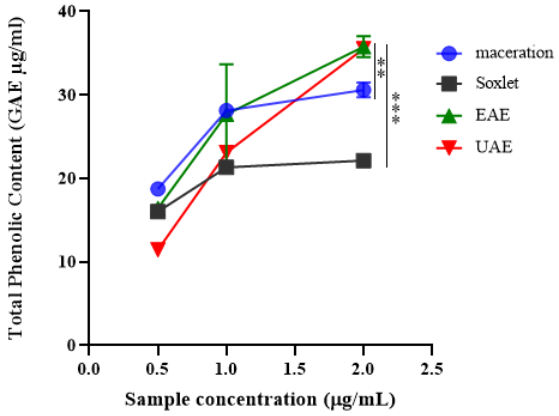

Figure 3. TPC from different extraction methods. The error bar shows the SEM, *p < 0.05, ** p< 0.01 and *** p<0.001, control with the control group.

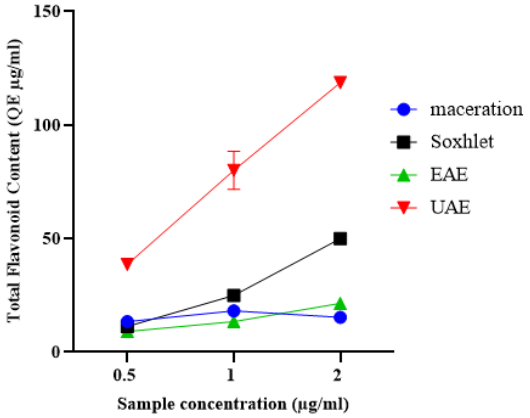

Figure 4. TFC from different extraction methods. The error bar shows the SEM, *p < 0.05, ** p< 0.01 and *** p<0.001, control with the control group.

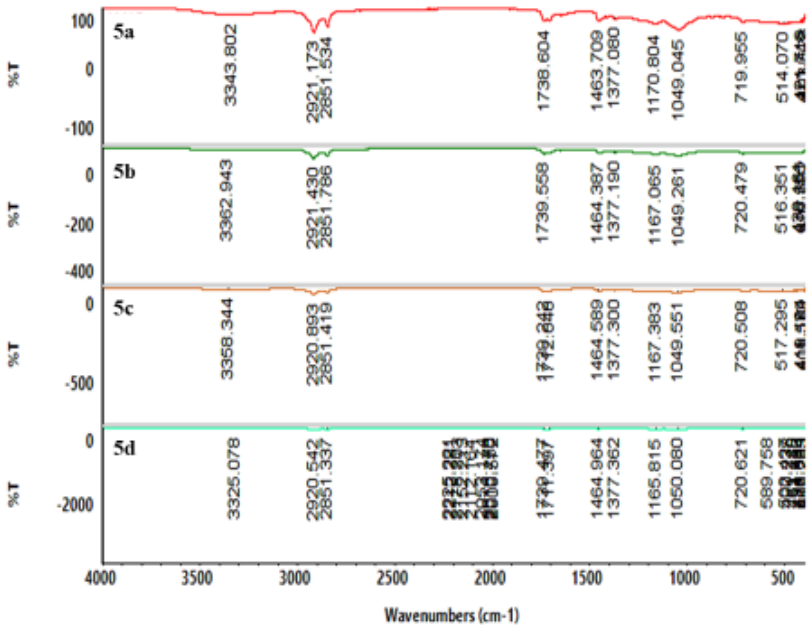

Figure 5. FTIR spectra.

Contrastingly, while EAE demonstrated a comparable extraction of total phenolics (35.77 ± 0.73 GAE µg/mL) to that of UAE (35.53 ± 0.08 GAE µg/mL), it yielded a significantly lower content of flavonoids, 21.44 ± 0.02 QE µg/ml. This variation can be attributed to the higher efficiency of EAE in extracting phenolic compounds. Previous research has indicated that EAE is particularly effective in extracting phenolic phytochemicals such as ternatin, anthocyanins, and delphinidin derivatives, all of which contribute to the blue hue of the CT [41]. These results align with our findings, underscoring the efficacy of EAE in liberating a variety of phenolic compounds into a liquid extract through the hydrolysis of plant cell walls [42-43].

The FTIR spectroscopy analysis of the maceration (Figure 5a), UAE (Figure 5b), EAE (Figure 5c), and Soxhlet (Figure 5d) extracts revealed distinct peaks characteristic of flavonoids and phenols [44-45]. A broad peak ranging from 3325 to 3358 cm^{-1} indicates OH stretching, suggesting the presence of carbohydrates and phenolic compounds. Sharp peaks around 2920 - 2921 cm^{-1} and at 2851 cm^{-1} are indicative of CH stretching in sp^2 and sp^3 hybridized carbon atoms, respectively. The appearance of peaks at 1738 - 1739 cm^{-1} is representative of C=O stretching, and peaks at 1464 cm^{-1} and 1377 cm^{-1} suggest C-C=C symmetric stretching, highlighting the presence of aromatic ring structures. A series of peaks observed between 1165 and 1170 cm^{-1} is likely associated with aliphatic amines, while peaks between 1049 and 1050 cm^{-1} are attributed to C-O stretching. The FTIR spectra of all four extracts displayed similar peak patterns to those reported in previous studies of leaf extracts [46, 47]. However, there was a noticeable increase in intensity of the OH functional group in the UAE (Figure 5b) and EAE (Figure 5c) extracts compared to the others. Additionally, the macerated extracts (Figure 5d) exhibited multiple small peaks in the 2000-2500 cm^{-1} range, which could be attributed to impurities.

In summary, the initial findings of this investigation indicate that the unconventional extraction techniques, namely UAE and EAE, surpassed traditional methods in isolating sun-protective phytochemicals. The ultrasonication parameters utilized in this research (53 kHz, 100 W, at 50 °C for 3 hours) were deemed adequate for extracting phytochemicals without compromising their integrity or introducing contaminants [41]. Furthermore, the use of amylase in this study proved to be efficient in breaking down and disintegrating plant cell walls, thereby enhancing the release of compounds into the solvent phase and facilitating a more effective extraction process [28]. Compared to other cell disruption strategies such as mechanical and chemical treatments, enzyme utilization is known to offer unparalleled specificity, mild treatment conditions, and superior end-product quality, as reported in previous studies [48]. Looking ahead, there is potential to expand upon this extraction study by incorporating additional variables, such as variations in time and temperature, across different extraction methodologies. The authors also recommend the integration of EAE and UAE as a combined method for extracting CT, given that both techniques have demonstrated exceptional efficacy in isolating different CT components in the current study.

CONCLUSION

Preliminary results of this research suggest that UAE and EAE are promising environmentally-friendly and

highly efficient alternative 'green' extraction techniques, preferable for isolating sun-protective phytochemicals from CT.

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