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Original article

Safety and efficacy of the novel surgical dye from blue butterfly pea flower: an ex vivo and in vitro study

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Background: The chemical dyes were used for anterior capsule staining with caution. There is no natural dye available. The extract from traditional Thai herb may be an alternative dye.
Objectives: To assess the in vitro cytotoxicity of the novel surgical dye on corneal endothelial cells and evaluate the proper concentration for staining on anterior lens capsule during cataract surgery in ex vivo.
Methods: Safety of the dye in various concentrations on human corneal endothelial cell line was evaluated by resazurin technique. The efficacy of human anterior capsular staining at different concentrations of the dye and different time-points was also studied under an operative microscopic camera recorder.
Results: The cytotoxicity test showed no significant viability loss at any concentration. The ex vivo tests on 20 pieces of human anterior capsules were arranged in 5 sets and exposed to 4 different concentrations of the dye at various times. The concentration of 500 - 1,000 mg/ml provided better staining than the 200 mg/ml concentration, while the concentration of 40 mg/ml solution gave a negative stain.
Conclusion: The novel surgical dye from butterfly pea flower extract was successfully developed. The dye solution exhibited non-toxic to corneal endothelial cells and showed suitable for anterior lens capsule staining. Overall, it could be an alternative for safe staining of the anterior lens capsule in cataract surgery.

Keywords: Anterior lens capsule staining, butterfly pea flower, lens capsular staining dye.

Anterior continuous curvilinear capsulorhexis (CCC) is an important procedure during cataract surgery. In patients with white mature cataract, completing CCC is challenging because there is no red reflex from fundus to facilitate the visualization of the anterior lens capsule. Anterior lens capsule staining is therefore recommended in this case.[1] Trypan blue has been reported an effective dye to stain the anterior lens capsule during cataract surgery, with no known toxicity when used at a concentration approved by the U.S. Food and Drug Administration (FDA).[2] Although several studies reported on teratogenic and carcinogenic effects from trypan blue[3, 4], it is still widely used with an exception on children and pregnant women.

In addition, cataract surgery by phacoemulsification and intraocular lens implantation can lead to significant loss of endothelial cells. For this reason, the safety of trypan blue capsule staining to corneal endothelium is being intensely investigated within the last years.[5, 6] Unfortunately, the potential cytotoxicity of trypan blue to the corneal endothelium after its use in anterior capsule staining have been reported.[7, 8] Recently, new dye for anterior lens capsule was proposed and found to lead to green staining, with reduced toxicity on corneal endothelial cells.[9] However, the staining was not much and could be enhanced by combining it with trypan blue.[9]

In the present study, a novel surgical dye was extracted from butterfly pea flower. The extract has as dark blue color as that of trypan blue, and it could become a possible alternative to the latter for medical laboratory use. Hence, we hypothesize that the new dye could be used as a surgical dye staining for several ocular surgery as well as trypan blue staining, especially for the anterior lens capsule staining during cataract surgery. Therefore, this study aims to assess the in vitro cytotoxicity of this new dye in the human corneal endothelial cells and evaluate the proper dye concentration for staining on anterior lens capsule during cataract surgery in ex vivo.

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Materials and methods

The present study was approved by the Institutional Review Board of Naresuan University (no. 0038/61, June 15, 2018). The blue butterfly pea flower extract solution with pH 7.0 was prepared by a simple boiling. Briefly, the fine butterfly pea flowers were blended with the vehicle solution and filtered through a sterile cellulose nitrate membrane filter 0.45 microns (Millipore, Molsheim, France). The new dye was diluted with a sterile deionized water into a final concentration 1,000, 500, 200 and 40 mg/ml, and then sterilized with 0.22 microns sterile cellulose nitrate membrane filter (Millipore, Molsheim, France). The filtrated solution was taken on pH value testing and pH 7 of the new dye was stored in the sterile glass bottles at room temperature for further experiments.

Cytotoxicity test (In vitro study)

Human corneal endothelial cell line (HCECs) was kindly provided from Associate Professor Dr. Sangly P. Srinivas (School of Optometry, Indiana University, USA). HCECs were grown in Keratinocyte serum free medium (K-SFM) supplemented with 0.05 mg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone, and 5 ng/mL insulin. HCECS were used to determine toxicity potential of the new dye in decreasing cell viability. The dye was sterilized through 0.22 microns sterile cellulose nitrate membrane filter (Millipore, Molsheim, France) and prepared their dilution solution in K-SFM.

The cytotoxicity determination of the new dye to HCECs was performed by resazurin technique using the deep blue cell viability™ kit (BioLegend, San Diego, CA) following manufacturing instruction. In brief, cells were seeded into a 96-well plate at a density of 10,000 cells/well and incubated for 18 hours to allow cell adherence. The cells were treated with culture medium alone or with the new dye at serial concentrations starting from 1,000, 500, 250, 100, 50 and 25 mg/ml for 24 hours. At the end of treatment, 10 µl of the Deep Blue Cell Viability™ reagent was added into untreated and treated wells. After 4 hours of incubation, the reduction of resazurin into resorufin was detected using a fluorometer (excitation: 530 - 570 nm, emission = 590 - 620 nm). Additionally, the morphologic changes in HCECs were also observed under the light microscopy. The percentage viability was calculated by the following formula:

Percentage viability = (Absorbance of treated cells/ Absorbance of control) X 100

Human Anterior Capsule Staining test (Ex vivo study)

After routine complete anterior continuous curvilinear capsulorhexis in cataract surgery, 20 pieces of removed anterior lens capsule from donated cataract patients were collected in sterile balance salt solution. At each set of experiment, four pieces were tested with the new dye in 4 different concentrations (1,000, 500, 200, 40 mg/ml), each piece was consecutively observed over 4-time exposures at 5, 30, 60 seconds and 3 minutes. The potential capsular staining of the dyes was determined and recorded under an operative video camera and then the pictures of stained capsule were captured. Grading of stained blue color was classified as dark blue (++), light blue (+) and no staining (-). There were 5 independent experiments, so the total observations were 80 pictures for quality of staining of 20 anterior capsule pieces.

Statistical analysis

The values of continuous variables were expressed as the mean ± standard deviation (SD). The Student's *t - test* was used to identify the difference between two groups with $P < 0.05$ was considered statistically significant.

Results

Cytotoxicity determination

Cytotoxicity was assessed as a dose dependent response using deep blue cell test. As shown in Table 1, the percentage of viable cells was reduced to around 97.0 % compared with the untreated cells when the cells were being treated with 250 mg/ml of blue solution. The highest reduction was found in the 1,000 mg/ml of the treated group. However, the blue solution was not significantly affected to HCECs by any concentration when compared with the untreated cells ($P > 0.05$). This result reveals that the blue solution from butterfly pea extracts have very low toxicity against HCECs.

Cell morphological assessment

Following the new dye exposure, the morphological changes of treated HCECs was also determined under an inverted light microscope as shown in Figure 1. The result showed that the HCECs maintained endothelial cobblestone characteristic morphology as healthy morphology in all treatments. Distinctive morphological changes were not observed

even after cell exposure to the highest concentration of the blue solution, which corresponded to the results of the cell viability assay. This indicates that the blue solution from the butterfly pea extracts have no negative effect to adnexa structure around the lens especially corneal endothelial cells.

Human anterior lens capsule staining

A 100.0% (5 of 5 tested pieces) of anterior capsule demonstrated the light blue color after soaking in the butterfly pea solution 200 mg/ml within 5 seconds, the shade of light blue color was similar despite the longer duration of 30, 60 seconds and 3 minutes. Additionally, the pieces of anterior capsule soaked in the concentrations of 500 and 1,000 mg/ml were efficaciously stained in dark blue color within 5, 30, 60 seconds and 3 minutes as displayed in Table 2, Figures 2 and 3. In contrast, the new dye of 40 mg/ml gave the 100.0% (5 of 5 tested pieces) negative staining for anterior capsule.

Table 1. Cell viability of human corneal endothelia cells exposed to different concentrations of butterfly pea after deep blue cell test (n = 3).

Concentration of butterfly pea extracts (mg/ml)	Cell viability ± SD (%)	*P* - value*
1,000	97.68 ± 1.27	0.06
500	97.75 ± 1.81	0.10
250	97.92 ± 1.74	0.10
100	102.38 ± 2.84	0.16
50	101.22 ± 2.29	0.25
25	102.63 ± 1.65	0.07

* Student's *t* - test, $P < 0.05$, compared to untreated control cells.

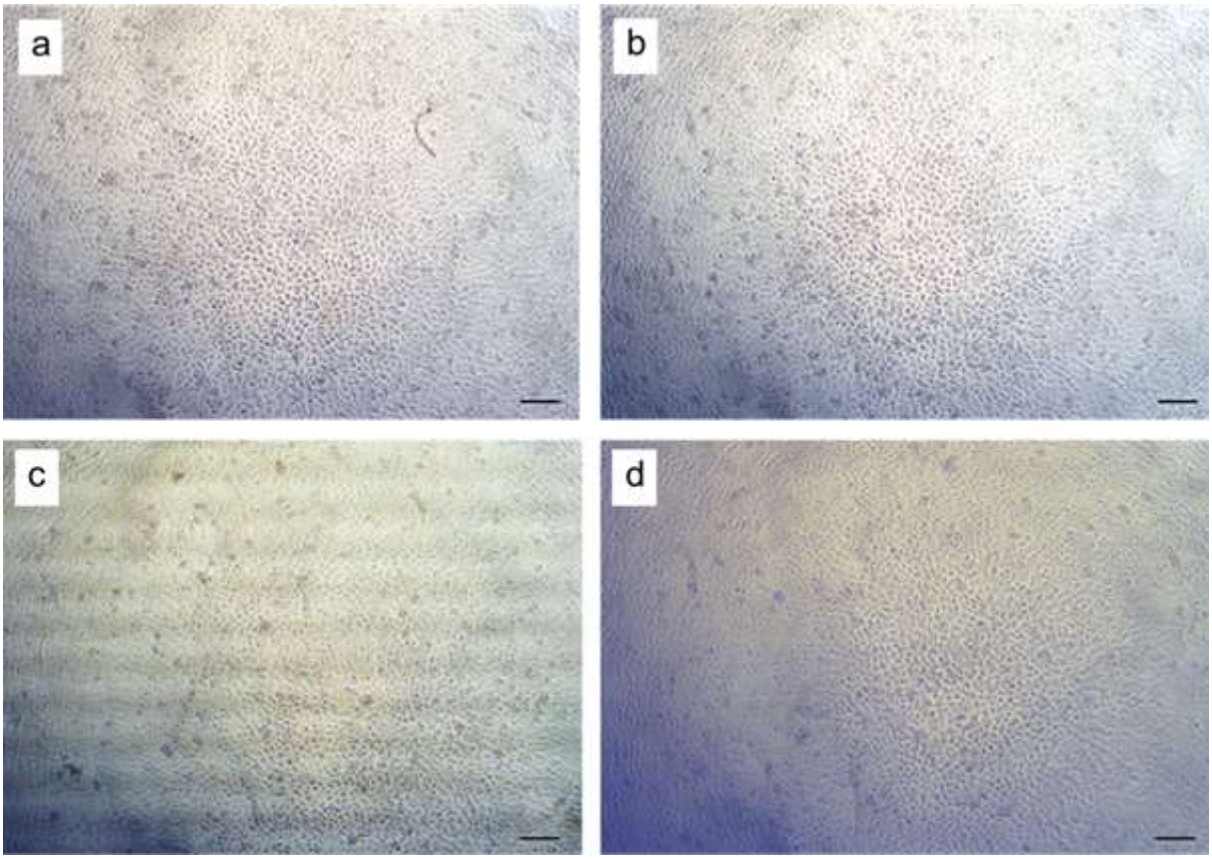

Figure 1. Light microscopic images of morphology of cultured HCECs after exposure to various concentrations of butterfly pea extract solution. **(a)** Control (no treatment), **(b)** 100 mg/ml, **(c)** 500 mg/ml, **(d)** 1,000 mg/ml. Scale bar: 200 μm.

Table 2. Grading of human anterior capsule staining with blue solution extract from the butterfly pea flower. The experiment was performed into 5 sets and exposed to 4 different concentrations of blue solution at various times (n = 5).

	Concentration of butterfly pea extracts*			
	40 mg/ml	**200 mg/ml**	**500 mg/ml**	**1,000 mg/ml**
At 5 sec	-	+	++	++
At 30 sec	-	+	++	++
At 1 min	-	+	++	++
At 3 min	-	+	++	++

*Grading of stained blue color was classified as: dark blue (++), light blue (+) and not staining (-). The same results were displayed in all the sets.

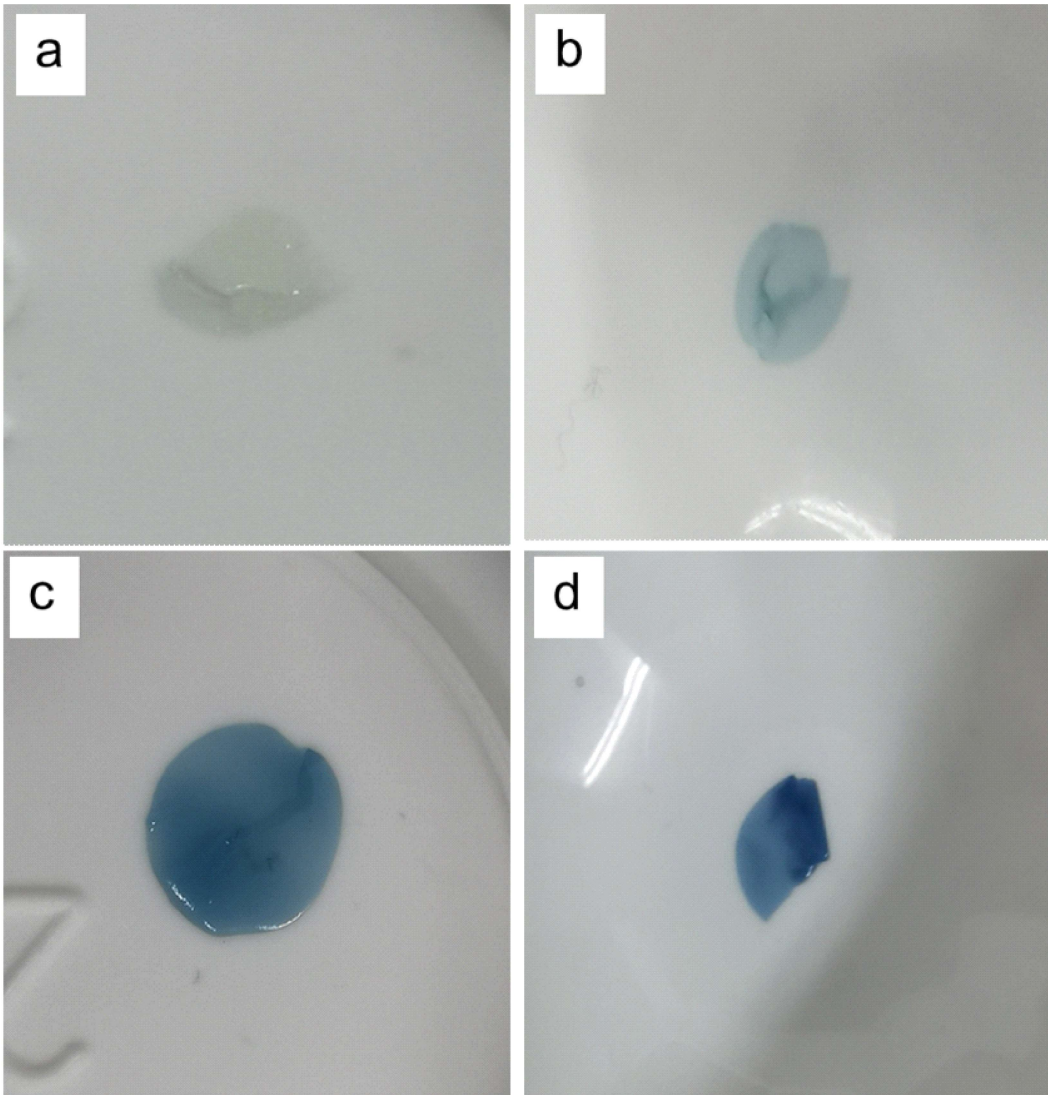

Figure 2. Human anterior lens capsule staining of butterfly pea solution at different concentrations. **(a)** Negative stain at 40 mg/ml solution, **(b)** Light blue color staining (+) at 200 mg/ml. **(c, d)** Dark blue color staining (++) at 500 mg/ml and 1,000 mg/ml.

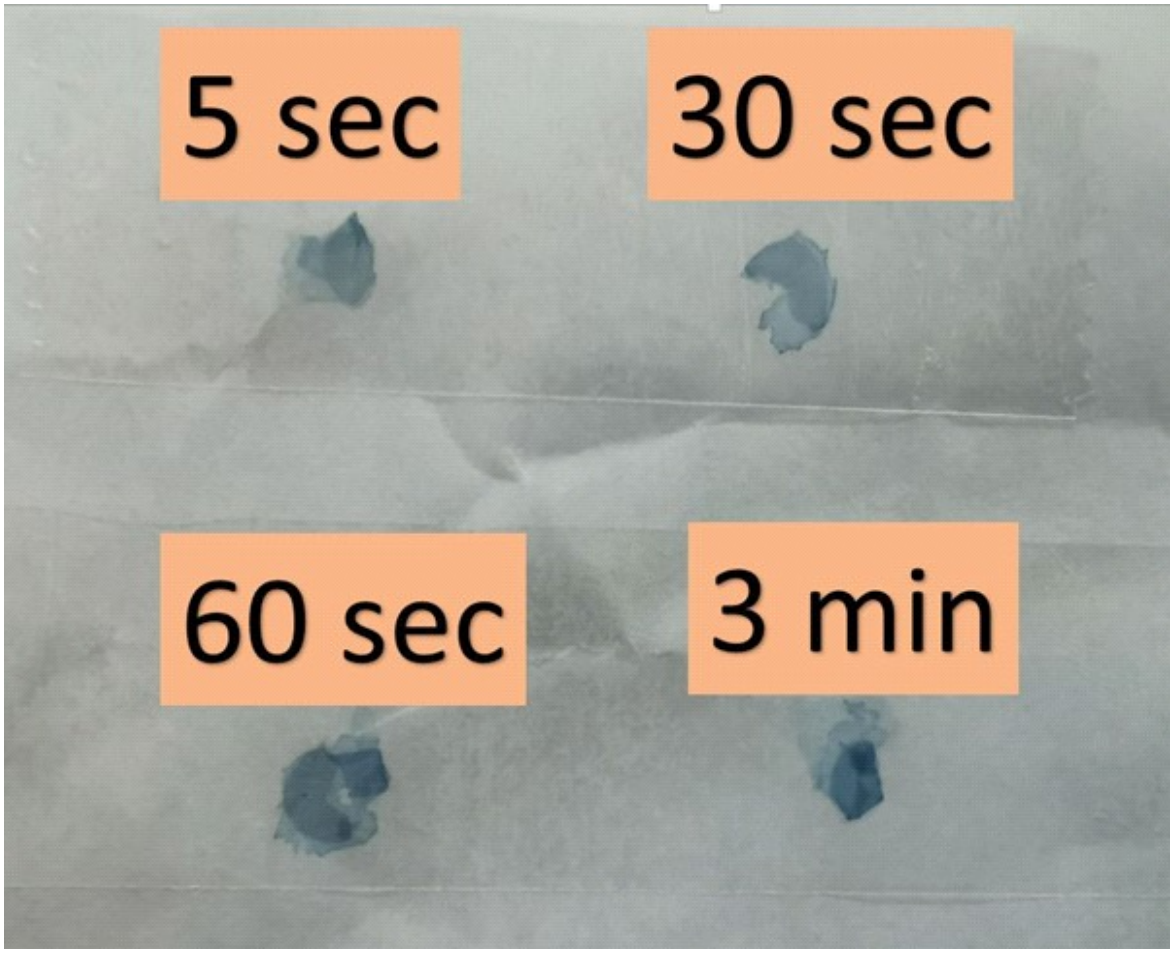

Figure 3. Human anterior lens capsule stained with butterfly pea extract solution 500 mg/ml at different time exposures.

Discussion

To obtain an alternative vital stain of the lens capsule, we developed the new surgical dye from the blue butterfly pea flower extract. Butterfly pea belongs to the vine family of Papilionnaceae, its scientific name is *Clitoria ternatea* Linn. The plant is commonly found in tropical areas including Thailand.[10] The butterfly pea flower contains genistein, a chemical component of anti-inflammatory, anti-oxidant and anti-spasmodic properties.[11] Anthocyanin, another derived component from its flower, is an antioxidant twice stronger than vitamin C or vitamin E.[12]

It has been shown that 0.1% trypan blue was safe dye for the blue-stained capsular rims.[11] This vital dye is widely used for capsulorhexis staining following the approval of European Conformity mark (CE mark). Moreover, 0.1% trypan blue are only ophthalmic solution receiving Food and Drug Administration approval for capsular staining.[11 - 12] Unlike Indocyanine green (ICG), trypan blue does not require mixing, and it provides superior prolonged capsular visualization. These indicate that trypan blue is safe dye, convenient and giving high visibility of stained tissue.[13]

Ho WT, *et al*. revealed no cytotoxic effect of the dye to the rabbit corneal endothelial cells after exposure to 0.04% trypan blue for one minute.[14] However, if the ophthalmic operation required using trypan blue staining longer than one minute, the optimum concentration of trypan blue and time exposure to the target ocular tissue and its adjacent should be determined. Donor preparation of Descemet's Stripping Endothelial Keratoplasty (DSEK) graft or Descemet's Membrane Endothelial Keratoplasty (DMEK) graft or using trypan blue staining in uncooperative excited cataract patients were examples. Weber *et al*, evaluated the efficacy of Descemet's Membrane dye retention and endothelial toxicity in human cadaveric eyes, there was no significant endothelial cell toxicity after

corneal button exposed to 0.05% trypan blue and commercial fixed combination of 0.15% trypan blue, 0.03% Brilliant blue G and 4% Polyethelene glycol (Membrane Blue - Dual®) at one minute and four minutes.[15] Moreover, Grover DS, *et al*. reported that 4 minute exposure of 0.06% trypan blue did not show statistical significant endothelial loss but endothelial death rate was higher at 6 minute exposure.[16]

In this study, HCECs was used, and exposure time was much longer (24 hours) emphasizing the safety of the new dyes extract from butterfly pea flower.

In the present study, the new dyes showed a blue color shade on anterior capsules after soaking with the extract within 5 seconds and did not fade until 3 minutes. The solution provided the good visualization of blue stained capsular rims. This result indicates that the new dyes is effective for capsule staining following the operational guideline recommendation of complicated cataract. Apart from continuous curvilinear capsulorhexis in white mature cataract, this new dye can be possible applied in other surgery such as those also requiring the use of blue dye: cataract with corneal scar [13], descemet membrane coloring for endothelial keratoplasty[14], canalicular glaucoma surgery[15], glaucoma drainage implant[16] and microinvasive glaucoma surgery.[17]

Safety of the new dye in various concentrations on human corneal endothelial cell line was evaluated by resazurin technique. The solution even at the highest concentration of 1,000 mg/ml did not show a significant cytotoxic effect on the cells. Cell morphology did not change and maintained all characteristics as healthy morphology. Furthermore, cell viability was not disturbed from prolonged exposure with the extracts up to 24 hours. These suggest that the new dye might be an effective vital dye stain and safety for capsular staining during cataract operation.

Nevertheless, this study did not compare the efficacy and safety between this proposed natural dye and trypan blue. Thus, further comparative study is needed. Other experimental study on corneal fibroblasts may be developed to ensure corneal biocompatibility concerning possible inflammatory reaction.

Conclusion

The research team has successfully developed a novel surgical dye from blue butterfly pea flower with good safety and high efficacy for anterior lens capsule staining. The substance required a short time of preparation. The present study proved the substance to be of neutral pH value, rapid staining action and non-toxic to the corneal endothelium.

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Conflict of interest

The authors, hereby, declare no conflict of interest.

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