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*Original Article | Artículo Original*

# Ethyl acetate extract of *Citrullus colocynthis* (Linn.) Schrad. fruit suppresses angiogenesis

[Extracto de acetato de etilo del fruto de *Citrullus colocynthis* (Linn.) Schrad. suprime la angiogénesis]

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**Abstract**

*Context*: Anti-angiogenesis is a targeted therapy that uses drugs or other substances to stop tumors from making new blood vessels. *Citrullus colocynthis* is a medicinal plant that has anticancer property mediated by apoptosis.

*Aims*: To evaluate the anti-angiogenic potential of *C. colocynthis* fruits extract through chorioallantoic membrane (CAM) assay.

*Methods*: For the CAM assay, heparin (an anti-angiogenic agent) was used as a positive control, and quercetin (Q) as the marker compound in the extracts to demonstrate the inhibition of angiogenesis. The reduction in the number of blood vessels in each area and in each image was used as a measure to determine the anti-angiogenic activity of the extract**.**

*Results*: From the results of phytochemical screening, the polyphenol and flavonoid contents of the ethyl acetate extract were 0.6708 mg equivalent of gallic acid per gram of the dry extract and 7.65 mg equivalent of Q per gram of the dry extract, respectively. From the results of CAM assay, Q caused marked reduction in the formation of new blood vessels. Similarly, ethyl acetate extract of *C. colocynthis* fruits (50 µg/mL) showed 53.52% inhibition of blood vessel formation in the CAM vasculature, whereas 25 µg/mL of Q exhibited 64.78% inhibition. The analysis with Bonferroni's post-hoc test showed statistically significant difference at $p<0.0001$, with respect to the positive control, heparin. The $EC_{50}$ values obtained by non-linear regression analysis using GraphPad Prism software 8.1.2 were 14.74 µg/mL (Q) and 44.13 µg/mL (ethyl acetate fruit extract of *C. colocynthis*).

*Conclusions*: Ethyl acetate extract of *C. colocynthis* fruits demonstrated potential angiogenesis suppression activity.

*Keywords*: angiogenesis suppression; chorioallantoic membrane assay; *Citrullus colocynthis*; cancer; quercetin.

**Resumen**

*Contexto*: La antiangiogénesis es una terapia dirigida que usa medicamentos u otras sustancias para evitar que los tumores produzcan nuevos vasos sanguíneos. *Citrullus colocynthis* es una planta medicinal que tiene propiedades anticancerígenas mediadas por la apoptosis.

*Objetivos*: Evaluar el potencial antiangiogénico del extracto de frutos de *C. colocynthis* mediante el ensayo de membrana corioalantoica (CAM).

*Métodos:* Para el ensayo de CAM, se utilizó heparina (un agente antiangiogénico) como control positivo, y quercetina (Q) como compuesto marcador en los extractos para demostrar la inhibición de la angiogénesis. La reducción en el número de vasos sanguíneos en cada área y en cada imagen se usó como una medida para determinar la actividad anti-angiogénica del extracto.

*Resultados*: A partir de los resultados del tamizaje fitoquímico, los contenidos de polifenoles y flavonoides del extracto de acetato de etilo fueron 0.6708 mg equivalentes de ácido gálico por gramo del extracto seco y 7.65 mg equivalentes de Q por gramo del extracto seco, respectivamente. A partir de los resultados del ensayo CAM, Q causó una reducción marcada en la formación de nuevos vasos sanguíneos. De manera similar, el extracto de acetato de etilo de frutos de *C. colocynthis* (50 µg/mL) mostró una inhibición del 53.52% de la formación de vasos sanguíneos en la vasculatura CAM, mientras que 25 µg/mL de Q mostraron una inhibición de 64.78%. El análisis con la prueba post hoc de Bonferroni mostró una diferencia estadísticamente significativa a $p<0,0001$, con respecto al control positivo, la heparina. Los valores de $EC_{50}$ obtenidos por análisis de regresión no lineal utilizando el software GraphPad Prism 8.1.2 fueron 14.74 µg / mL (Q) y 44.13 µg/mL (extracto de acetato de etilo de *C. colocynthis*).

*Conclusiones*: El extracto de acetato de etilo de las frutas de *C. colocynthis* demostró una actividad potencial de supresión de la angiogénesis.

*Palabras Clave*: cáncer; *Citrullus colocynthis*; ensayo de membrana corioalantoica; quercetina; supresión de la angiogénesis.

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## INTRODUCTION

Cancer cells use a number of different pathways to cause blood vessel growth. Each step in these pathways is a possible target for cancer treatment. Anti-angiogenesis drugs don't attack cancer cells directly. Instead, they target the blood vessels, which are necessary for the growth and survival of the cancer cells. All cancerous tumors, for example, release angiogenic growth factor proteins that stimulate blood vessels to grow in the tumor, providing it with oxygen and nutrients. Antiangiogenic therapies literally starve the tumor of its blood supply by interfering with this process, and thus may help to prevent the growth of new tumors. They may shrink large tumors by cutting off blood supply (Samant and Shevde, 2011; Al-Husein et al., 2012).

Natural health products contain a cocktail of compounds that act on multiple pathways to initiate and maintain tumor angiogenesis. In addition, angiogenesis within the tumor microenvironment may be more sensitive to a cocktail of natural health products administered continuously at relatively low doses than to a single pharmaceutical compound administered intermittently at higher dose levels. A potential advantage of phytochemicals and other natural compounds is that they may act through multiple cell-signaling pathways thereby reducing the development of resistance by cancer cells (Li et al., 2012; Yadav and Puri, 2015).

*Citrullus colocynthis* (Linn.) Schrad. is a medicinal plant belonging to family *Cucurbitaceae*. The fruit is smooth, spherical with a 5–10 cm diameter, and extremely bitter. The calyx surrounds the yellow-green fruit which, when mature, becomes marble (yellow stripes) and the mesocarp is filled with a soft, dry and spongy white pulp, which contains the seeds (Gurudeeban et al., 2010; Borhade et al., 2013). The fruits of *Citrullus colocynthis* have traditionally been used for the treatment of diseases such as diabetes, jaundice, asthma, ulcer, inflammation, urinary infections, microbial diseases, and cancer (Borhade et al., 2013). Few studies have been conducted to evaluate the anticancer activity of *Citrullus colocynthis* fruits. Different solvent extracts of *Citrullus colocynthis* fruits have been tested for its anticancer activity in various cell lines such as MCF-7 and HepG2 (Aniruddha and Savita, 2012), MDA-MB-231, SiHa, PBMC and J774A (Kaushik et al., 2017), AGS (Masumeh et al., 2017), and HeLa cells (Hajjar et al., 2017). Few anti-cancer activity studies have been conducted in diseased mouse models, such as DAL-induced tumor model (Kunal et al., 2019), polycystic ovarian disease in female rats (Sowmiya and Sudha, 2017), and cyclophosphamide-induced genotoxicity in mice bone marrow cells, to evaluate angiogenesis and tumor size reduction (Shokrzadeh et al., 2013).

Cucurbitacins have been isolated from various plant species belonging to plant families other than *Cucurbitaceae*, and they have inhibited proliferation and induced apoptosis in a long array of *in vitro* and *in vivo* cancer cell models.

A study by Shokrzadeh et al. (2013) reported that *Citrullus colocynthis* fruit extracts have an antigenotoxic effect against cyclophosphamide-induced oxidative DNA damage in mice. Therefore, it could be used concomitantly as a supplement to protect people undergoing chemotherapy. Three flavone glucosides isosaponarin, isovitexin, and isoorientin 3′-O-methyl ether as well as two cucurbitacin glucosides 2-O-β-D-glucopyranosyl-cucurbitacin I and 2-O-β-D-glucopyranosyl-cucurbitacin L were isolated from the fruits of *Citrullus colocynthis* (Tannin-Spitz et al., 2007 and Al-Snafi et al., 2016). To the best of our knowledge only one study by Ataee et al. (2011) has reported anti-angiogenesis activity of alcoholic extract of *Citrullus colocynthis* plant using chorioallantoic membrane (CAM) assay. The powdered dried fruit of bitter melon was used by them for preparing the extract with 70% ethanol. The anti-angiogenic activity potential of the fruit was studied by injecting 100 µg/mL of the bitter melon extract and compared with the sham-exposed (treated with normal saline) group and the control group. The $EC_{50}$ value of the ethanolic extract, necessary for calculating the anti-angiogenic potency, was apparently not determined. Thus, the present study was conducted to establish the preliminary scien-

tific evidence for anti-angiogenesis potential of the ethyl acetate extract of the fruit of *Citrullus colocynthis* in comparison to quercetin (known anti-angiogenic plant flavonoid) using CAM assay.

## MATERIAL AND METHODS

### Chemicals and reagents

All the reagents and solvents that were used for extraction, phytochemical analysis, high performance thin layer chromatography (HPTLC) analysis, and CAM assay were of analytical grade, and they were obtained from S.D. Fine Chemicals (Mumbai, India). Quercetin was a gift from Piramal Health Care Ltd (Mumbai, India). Heparin (10 IU, Hep Lock Injection, Gland Pharma Limited) was purchased from a local pharmacy store. TLC aluminum plates pre-coated with silica gel 60 $F_{254}$ (10 x 10, 0.2 mm thick) were obtained from E Merck (Mumbai, India).

### Collection of plant material

The fruits of *Citrullus colocynthis* were procured from S.V. Ayurvedic Bhandar, APMC market, Vashi, Navi Mumbai (19.0765° N, 73.0073° E). The fruits were authenticated by Dr. Ganesh Iyer, Head of Department of Life Sciences, Ramnarain Ruia College, Matunga, Dadar (E), Mumbai 400 019. The plant material sent for authentication was identified as Indravaruni (Sanskrit), Chitrapala or bitter apple, botanically known as *Citrullus colocynthis* (Linn.) Schrad. of the family *Cucurbitaceae*. The represented fruits were globular, slightly depressed, 5 - 7.5 cm in diameter, green in color and filled with a dry spongy and very bitter pulp. The seeds were 4 - 6 mm long and pale brown. The voucher number of the specimen is Ls-10-004. An herbarium sample of this fruit was deposited with Bombay College of Pharmacy, Kalina, Mumbai, India.

### Preparation of extracts

The fruits of *Citrullus colocynthis* were air-dried. The fruits including the seeds were pulverized in a homogenizer, and the dried powder (100 g) was defatted with hexane (150 mL) over 3 h. The defatted samples were then air-dried and stored in dark color bottles under refrigerated conditions (4°C) until use.

Extracts of *Citrullus colocynthis* fruits were prepared in different solvents aqueous, hydromethanolic, ethyl acetate and n-butanol. The extracts were prepared with 50 g of the pulverized *Citrullus colocynthis* fruit powder and each solvent in a Soxhlet assembly for 2 h. The extraction procedure was repeated thrice for each solvent and the solvent extracts were pooled together. The solvent extracts were allowed to cool, and the extracts obtained were filtered and subjected to rotary evaporation to remove the solvents.

### Phytochemical analysis

The preliminary phytochemical tests were performed on dried extracts as follows (Khandelwal, 2008):

a) Tests for carbohydrates: Carbohydrate content was confirmed by Molisch's test and Fehling's test.

b) Test for proteins: Protein content was confirmed by Biuret test and Millon's test.

c) Tests for alkaloids: The extracts were mixed with dilute hydrochloric acid separately. They were mixed thoroughly and filtered, and the filtrate was subjected to Dragendorff's test, Mayer's test, Hager's test, and Wagner's test.

d) Tests for flavonoids: The flavonoid contents of the extracts were determined with Shinoda test and lead acetate test.

e) Tests for steroids: The presence of steroids in the extracts were determined with Salkowski reaction.

f) Tests for tannins and phenolic compounds: The extracts were tested for tannins and phenolic compounds by mixing equal volumes of the extracts and ferric chloride solution (5% w/v), lead acetate solution (10% w/v), gelatin solution, bromine water, acetic acid solution, dilute potassium dichromate solution and dilute potassium permanganate solution. The resulting solution was observed for characteristic changes.

g) Total polyphenol content: Total polyphenol content of the extracts was assessed by using the Folin-Ciocalteu method **(**Meena and Patni, 2008; Joseph and George, 2011; Benariba et al., 2013) with some modifications. Each extract (1.0 mg/mL) or gallic acid standard (2 - 10 µg/mL) were added to 2 mL distilled water in 10 mL volumetric flask followed by the addition of Folin-Ciocalteu reagent (1.0 N, 1 mL). After 5 min, $Na_2CO_3$ (0.2 N, 3 mL) solution was added and the volume was made up to 10 mL with distilled water.

The solution was mixed thoroughly and incubated for 2 h at room temperature. After incubation, the absorbance was measured at 760 nm with a spectrophotometer (Jasco V-530, Jasco, Japan). Blank was performed by same procedure excluding the sample. The analysis was performed in triplicates. The results are expressed as milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g dry extract).

h) Total flavonoid content: Total flavonoid content (TFC) was determined by using aluminum chloride for the colorimetric assay as earlier described with minor modifications **(**Meena and Patni, 2008; Joseph and George, 2011; Benariba et al., 2013). An aliquot of the extracts or standard solution of quercetin was added to 10 mL volumetric flask containing 2 mL of distilled water and $NaNO_2$ (0.3 mL, 5 % w/v). The extract was mixed thoroughly using a cyclomixer and after 5 min, $AlCl_3$ (0.3 mL, 10 % w/v) solution and NaOH (2 mL, 1 M) were added. The volume was made up to 10 mL with distilled water, and the solution was mixed thoroughly and incubated at room temperature (25°C) for 1 h. After incubation, the absorbance was measured against blank at 430 nm with a spectrophotometer (Jasco V-530, Jasco, Japan). The results are reported as milligrams of quercetin equivalents per gram of dry extract (mg QE/g dry extract).

*HPTLC method development and validation*

The anti-angiogenic activity of quercetin has been reported (Pratheeshkumar et al., 2012), and hence it was used as a marker compound for the evaluation of the anti-angiogenic potential of the extract. An HPTLC method was thus developed and validated for the analysis of quercetin. A Camag HPTLC system III, comprising Linomat 5 automatic applicator with 100 µL Hamilton syringe, and precoated silica gel 60 F 254 aluminum sheets (10 cm x 10 cm), was used. The stock solution of quercetin (1mg/mL) was prepared with methanol. Further dilutions were done to obtain the linearity plot using concentrations of quercetin from 0.5 - 4 µg/band. The stock solution of 2 mg/mL of CCE was prepared with methanol. The application volume for quercetin and extracts were 10 µL in the form of bands (8 mm length), 10 mm from the bottom and 15 mm from the side edges. The plate was developed vertically ascending in a pre-saturated Camag twin trough chamber with the mobile phase-ethyl acetate:toluene:methanol:formic acid (6:3:0.1:0.2 v/v/v/v) at 25°C ± 2°C. Photodensitometric scanning was done with a TLC scanner equipped with WinCATS software. The plates were scanned at 254 nm and good separation between components was observed. The standard, quercetin, was observed at Rf = 0.65 (Fig. 1A). The chromatograms of quercetin and ethyl acetate extract are shown in Figs. 1B-C, respectively.

**Chick embryo chorioallantoic membrane (CAM) anti-angiogenesis model**

CAM assay was conducted to screen the anti-angiogenesis potential of *Citrullus colocynthis* extract and was performed according to earlier described method. **(**Peifer and Dannhardt, 2004; Ribatti, 2012; Avram and Cimpeanand, 2013). Quercetin is a known anti-angiogenic phytochemical compound, which drastically reduce the vascular density in a CAM assay (Mirossay, 2018). Thus, quercetin was used as a standard for evaluating the anti-angiogenic potential of the extract. The preliminary studies for CAM assay was done with respect to the extract containing maximum concentration of quercetin because the angiogenesis suppression activity was compared with that of quercetin. Hence, ethyl acetate extract of *Citrullus colocynthis* fruits (CCE) was selected for the evaluation of angiogenesis suppression using CAM assay.

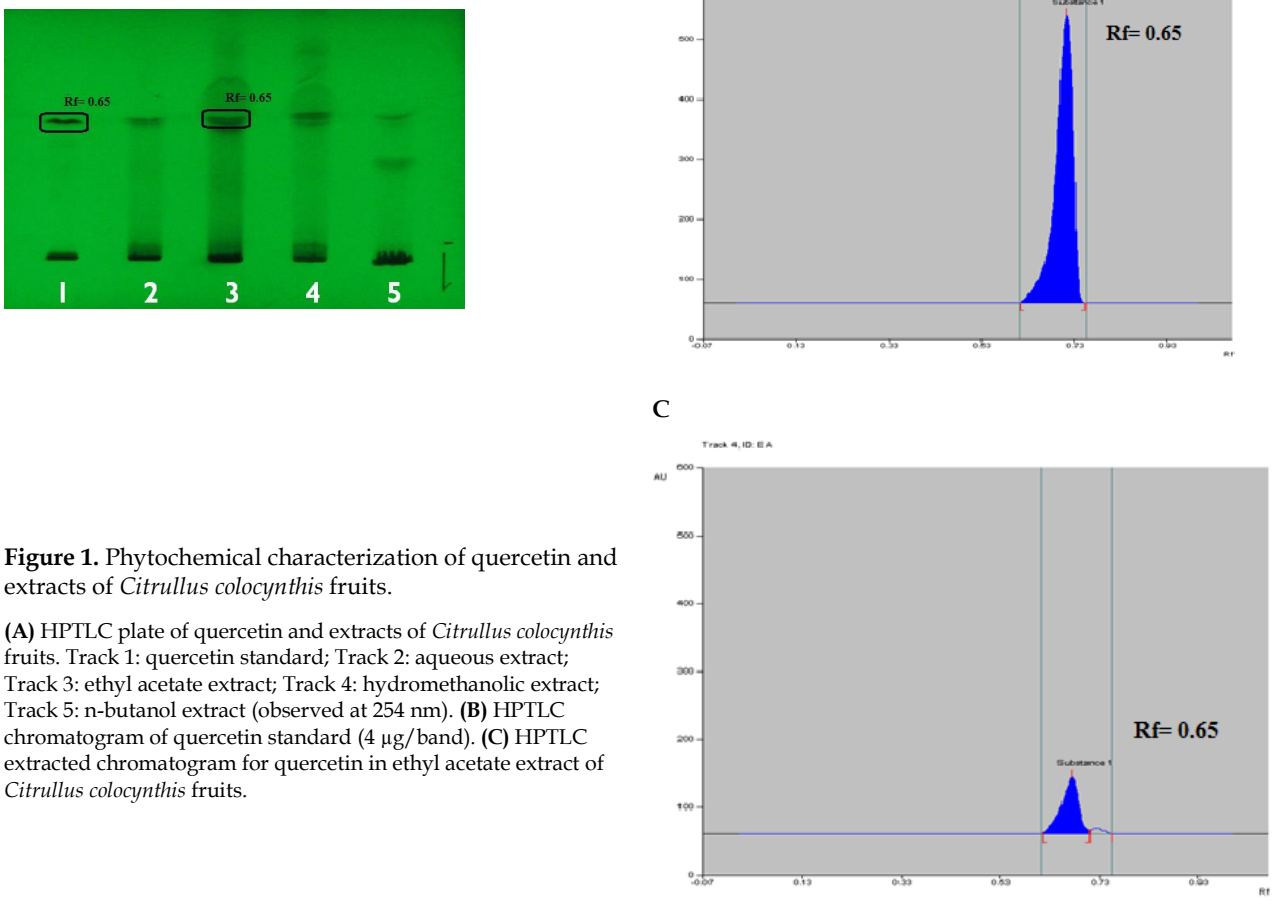



**Figure 1.** Phytochemical characterization of quercetin and extracts of *Citrullus colocynthis* fruits.

**(A)** HPTLC plate of quercetin and extracts of *Citrullus colocynthis* fruits. Track 1: quercetin standard; Track 2: aqueous extract; Track 3: ethyl acetate extract; Track 4: hydromethanolic extract; Track 5: n-butanol extract (observed at 254 nm). **(B)** HPTLC chromatogram of quercetin standard (4 µg/band). **(C)** HPTLC extracted chromatogram for quercetin in ethyl acetate extract of *Citrullus colocynthis* fruits.

Quercetin and CCE was dissolved in 0.1% DMSO and apart from negative control (vehicle solvent only), a positive control (heparin, 10 U/mL) was also included in the CAM assay. Fertilized eggs of White Leghorn breed were acquired from Goregaon poultry farm and incubated at 37°C with 80% humidity. On embryonal day 6 (E-6), when the embryo was still small, eggs were prepared to be used for the procedure by spraying them with 70% ethanol to minimize contamination. A small hole was made with a scalpel on the wide end of the eggs, where the air sac was located. These eggs were inoculated with 10 µL of samples containing the desired amount of drug/test substances to be tested through a window made in the eggshell in upward position with sterile automated pipette at an angle of ~45° and sealed with parafilm. The eggs were then incubated under same conditions in the incubator (CCL-170B model, Esco Micro Pte. Ltd., Singapore) till day 12.

*Isolation of the CAM*

On day 12, the eggs were broken from the region of air sac and window made for inoculation. The CAM was isolated and then spread out in the Petri plate containing saline, and stereomicroscopic analysis (Motic 1.0 megapixel digital camera, Motic Asia, Kowloon, Hongkong) was carried out. Four overlapping images of four distant areas were taken with an image analysis software (Motic Images Plus software, Motic Asia, Kowloon, Hong Kong).

*Overall quantification of blood vessels using stereomicroscopic method*

The number of blood vessels and the bifurcations in (secondary and tertiary capillaries) in each image were counted using the image analysis software and were evaluated for anti-angiogenic response. The average number of blood vessels of all the four images of the same CAM was calculated. The number of blood vessels observed was an indication of the extent of angiogenesis.

### Statistical analysis

The results are presented as means ± S.D. The statistical significance was evaluated using one-way ANOVA followed by Bonferroni's *post-hoc* test. The $EC_{50}$ values (concentration that caused 50% inhibition in blood vessels proliferation) were determined by non-linear regression analysis using GraphPad Prism software 8.1.2. A p value less than 0.05 indicated statistical significance.

## RESULTS

### Phytochemical screening

The air-dried powder of fruits of *Citrullus colocynthis* had a total ash value of 10.33% w/w, and the acid insoluble and water insoluble ash value was 3.5% w/w and 1.2% w/w, respectively.

**Table 1.** Physicochemical evaluation of air-dried powder of *Citrullus colocynthis* fruits.

| Test | % w/w (n=3) |
|---|---|
| **Ash value** | |
| Total ash | 10.3 |
| Acid insoluble ash | 3.5 |
| Water soluble ash | 1.2 |
| **Extractive value** | |
| Alcohol extractive value | 10.6 |
| Water extractive value | 26.7 |
| Loss on drying | 6.3 |
| Foreign organic matter | 0.4 |

The alcohol and water-soluble extractive values obtained were 10.6% w/w and 26.7% w/w, respectively (Table 1). These results met the acceptance criteria of Ayurvedic Pharmacopoeia of India (The Ayurvedic Pharmacopoeia of India, 2001).

The highest yield (%w/w) was obtained from the aqueous extract followed by hydromethanolic, n-butanolic, and ethyl acetate extracts (Table 2). Phytochemical screening of the extracts showed the presence of carbohydrates, proteins, alkaloids, flavonoids, steroids, tannins and polyphenolic compounds. Phenolic compounds or polyphenols, represented by tannins and flavonoids, are important because they are considered potent antioxidant, anti-inflammatory, anti-bacterial, antiviral and anticancer agents. The total phenolic contents were 0.209 (aqueous extract), 0.4727 (hydromethanolic extract), and the highest value, 0.6708 mg GAE/g dry extract (ethyl acetate extract). The total flavonoid content were 1.123 (aqueous extract), 6.26 (hydromethanolic extract), and the highest value of 7.65 mg QE/g dry extract (ethyl acetate extract). The quercetin response was linear in the concentration range of 0.5 to 4.0 µg/band. The linear regression equation obtained for the estimation of quercetin by HPTLC was y=3548x – 264.3 with correlation coefficient value of 0.9981. The limit of detection was observed at quercetin concentration of 0.25 µg/band and limit of quantitation was 0.40 µg/band. The estimated quercetin contents of the different extracts as determined by HPTLC method were as follows: 2.38% w/w (aqueous extract), 3.65% w/w (hydromethanolic extract), 4.15% w/w (ethyl acetate extract) and 1.5% w/w (n-butanol extract).

**Table 2.** Solvent extraction yield of *Citrullus colocynthis* fruits.

| Solvent dried extract | Yield (%w/w) (n=3) |
|---|---|
| Aqueous | 26.7 |
| Hydromethanolic | 4.3 |
| Ethyl acetate | 1.2 |
| n-Butanol | 1.5 |

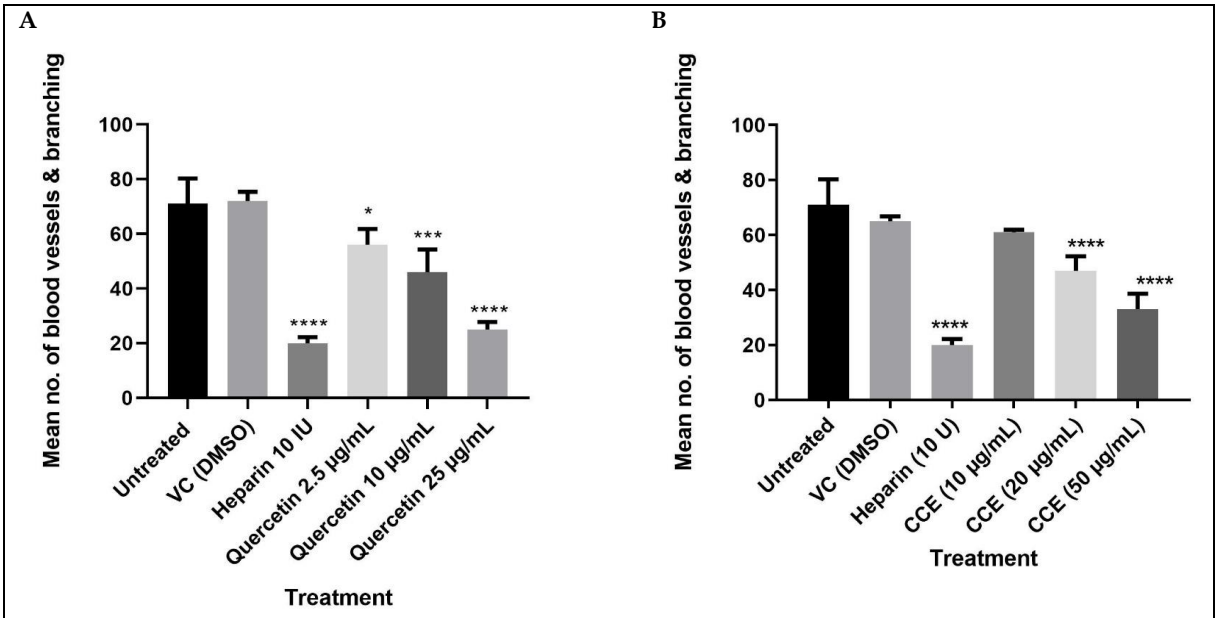


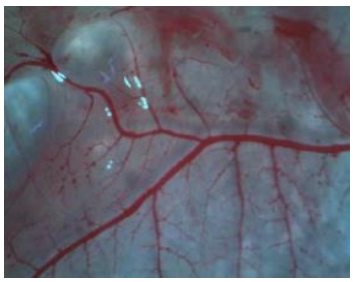

**Figure 2.** Effect of **(A)** quercetin and **(B)** ethyl acetate extract of *C. colocynthis* fruits (CCE) on angiogenesis.

The results are presented as mean ± SD (n=4). Significance with respect to untreated sample. * indicates statistical significance at $p<0.05$; **indicates statistical significance at $p<0.01$ and ***at $p<0.001$; ****at $p<0.0001$ when analyzed by one-way ANOVA (Bonferroni's *post- hoc* test). VC: vehicle control (DMSO). Volume of solutions inoculated per egg was 10 µL.

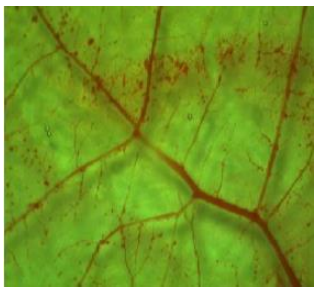
## CAM assay

Various concentrations of *Citrullus colocynthis* extract and a well-known anti-angiogenic marker heparin, as positive control, were screened to determine their anti-angiogenesis effect (Fig. 2). The reduction in the number of blood vessels and branching was observed. The quercetin content in the extract as estimated with HPTLC was 4.15 µg of quercetin/100 µg of CCE. To compare the angiogenesis suppression potential of the extract in terms of quercetin as a biomarker, the CAM assay was performed using three concentrations of quercetin 2.5, 10 and 25 µg/mL with 10 µL volume of each concentration inoculated per egg. Higher concentrations of quercetin were observed to have damaging effects on CAM of chick embryo. Quercetin at 2.5 µg/mL exhibited 21.12% inhibition on the growth of blood vessels and showed statistical significance at $p<0.05$, whereas quercetin at 10 µg/mL and 25 µg/mL exhibited 35.31% and 64.78% inhibition at statistical significance at $p<0.001$ and $p<0.0001$, respectively when compared with the untreated sample (Fig. 2A-B). These results indicate that quercetin treatment caused marked reduction in the formation of new blood vessels in a dose-dependent manner (Fig. 3). CAM analysis for CCE was studied using three concentrations 10, 20 and 50 µg/mL with 10 µL volume of each concentration inoculated per egg. Higher concentrations of CCE could not be studied due to the limited solubility of ethyl acetate extract in DMSO. CCE at 10 µg/mL showed no significant inhibition in the number of blood vessels (14.08% inhibition), whereas CCE at 20 µg/mL (33.80% inhibition) and 50 µg/mL (53.52% inhibition) exhibited statistically significant inhibition at $p<0.0001$ when compared with the untreated (Table 3). The increase in % inhibition of CAM vasculature was observed in dose dependent manner for quercetin and CCE. Statistical analysis showed that only higher concentration of quercetin (25 µg/mL) and CCE (20 µg/mL and 50 µg/mL) were found to be statistically significant with respect to the positive control heparin at $p<0.0001$. The $EC_{50}$ were 14.74 µg/mL and 44.13 µg/mL for quercetin and ethyl acetate extract of *Citrullus colocynthis* fruits, respectively.

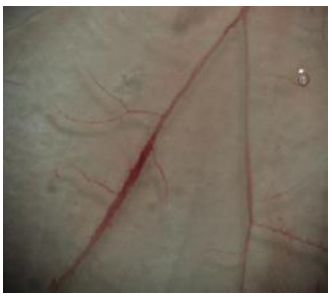
**A**



I) Untreated (UT)

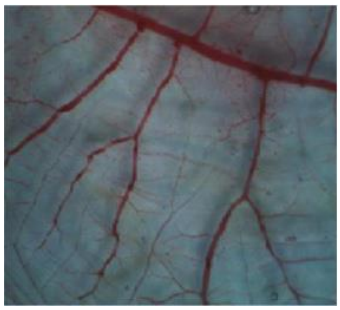


II) Vehicle control (DMSO)

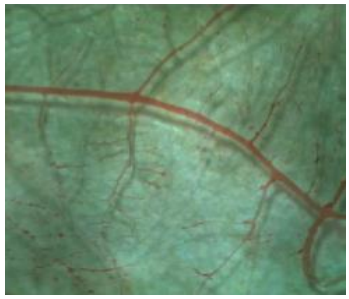


III) Positive control (heparin, 10 IU)

**B**



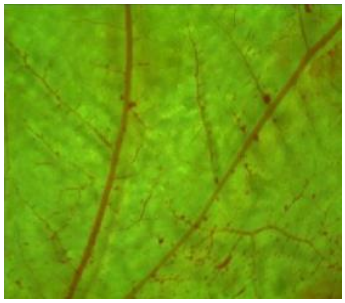
I) 2.5 µg/mL of Q/CAM



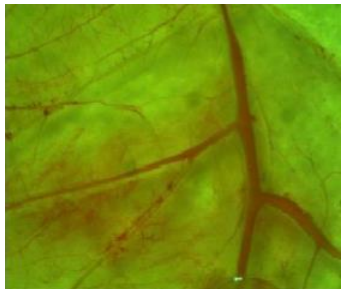
II) 10 µg/mL of Q/CAM

III) 25 µg/mL of Q/CAM

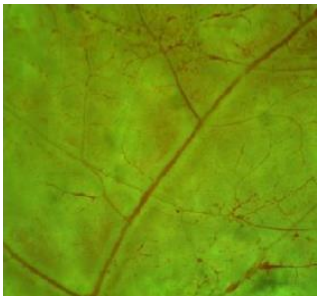
**C**



I) 10 µg/mL of CCE/ CAM



II) 20 µg/mL of CCE/ CAM



III) 50 µg/mL of CCE/CAM

**Figure 3.** Results of CAM assay showing vascular density following treatment with **(A)** I: untreated; II: vehicle control (DMSO); III: positive control (heparin 10 U); **(B)** I: 2.5 µg/mL of Q/CAM; II: 10 µg/mL of Q/CAM; III: 25 µg/mL of Q/CAM, **(C)** I: 10 µg/mL of CCE/CAM; II: 20 µg/mL of CCE/CAM; III: 50 µg/mL of CCE/CAM.

CAM: chorioallantoic membrane angiogenesis; Q: quercetin standard; CCE: ethyl acetate extract of *C. colocynthis* fruits. Volume of solutions inoculated per egg was 10 µL.

**Table 3.** Effect of quercetin and ethyl acetate extract of *Citrullus colocynthis* fruits on chorioallantoic membrane angiogenesis assay.

| Samples | Concentration (µg/mL) | Mean No of blood vessels and branching | Inhibition (%) | $EC_{50}$ (µg/mL) |
|---|---|---|---|---|
| Quercetin | VC (DMSO) | 72 ± 3.36 | 8.45 | 14.74 |
| | 2.5 | 56 ± 5.82* | 21.12 | |
| | 10 | 46 ± 8.31*** | 35.21 | |
| | 25 | 25 ± 2.80**** | 64.78 | |
| Ethyl acetate extract of *Citrullus colocynthis* fruits (equivalent µg/mL of quercetin in extract) | VC (DMSO) | 72 ± 1.80 | 8.45 | 41.13 |
| | 10 (0.415) | 61 ± 0.92 | 14.08 | |
| | 20 (0.83) | 47 ± 5.30**** | 33.80 | |
| | 50 (2.075) | 33 ± 5.67**** | 53.52 | |
| Heparin | 10 IU | 20 ± 2.19**** | 71.83 | - |
| Untreated | - | 71 ± 9.28 | - | |

Each value represents as mean ± SD (n=4). Significance with respect to untreated sample. * Indicates statistical significance at $p<0.05$, **indicates statistical significance at $p<0.01$ and *** at $p<0.001$, ****at $p<0.0001$, when analyzed by one- way ANOVA (Bonferroni's *post- hoc* test). VC indicates vehicle control (DMSO). $EC_{50}$ values were estimated by non-linear regression of % Inhibition of blood vessels for each dose. Volume of solutions inoculated per egg was 10 µL.

## DISCUSSION

Cancer treatment by chemotherapy or radiation produces severe toxic effects in the body. Tumor growth in cancer is a result of metastasis, which is facilitated by angiogenesis, and death in cancer patients occurs due to the metastasis of cancer cells. Due to the ill effects of cancer treatments available currently, the search for alternate treatment or new therapeutic molecules has always been the focus across the globe. Phytochemicals derived from plants can act through many cancers signaling pathways and reduce the angiogenesis by causing shrinkage of tumor. The discovery of 'lead' molecules in plants is always by serendipity, and hence numerous studies to evaluate the anti-cancer potential of plant molecules have been done and/or are in progress (Fridlender et al., 2015).

CAM model was used as it has the advantage of low cost with no side effects, and it does not involve animal sacrifice. CAM assays are used in preclinical studies for rapid screening of drugs to assess the anti-angiogenic effect by observing the reduction in neovascularization of blood vessels in angiogenesis (Ribatti, 2012).

Solvent extraction technique plays an important role in the isolation of plant metabolites. The anti-cancer evaluation of different extracts (ethanol, acetone, methanol or aqueous extracts) of *Citrullus colocynthis* fruits in cell lines or in animal models has been reported (Shokrzadeh et al., 2013; Hajjar et al., 2017). Due to the difference in solubility of phytoconstituents in different solvents, the results obtained for the desired activity differ. This study has demonstrated the reduction in micro-vascularization of CAM for the ethyl acetate extract of the fruits of *Citrullus colocynthis*. Ethyl acetate is a medium polar solvent and can extract several polar as well as non-polar phytoconstituents. HPTLC analysis showed the presence of quercetin, an anti-angiogenic agent, as one of the flavonoids in the extract. The concentration of quercetin found in the extract was 2.075 µg/50 µg of the dry extract. The extract showed 53.52% inhibition of the proliferation of secondary and tertiary blood vessels against 21.12 % inhibition of blood vessels density exhibited by quercetin standard (2.5 µg/mL) in the CAM vasculature. Although, the

concentration of quercetin in the extract was found to be less, the extract exhibited a significant reduction in the vascularization of blood vessels. This indicates that the ethyl acetate extract of *Citrullus colocynthis* fruits contain phytoconstituents other than quercetin, which work synergistically with quercetin to exhibit a significant reduction in blood vessels vascularization. The $EC_{50}$ values obtained for ethyl acetate fruit extract of *Citrullus colocynthis* fruits (44.13 µg/mL) was higher than the quercetin (14.74 µg/mL). However, the suppression of angiogenesis by CCE is evident from the results of CAM analysis.

Prateeshkumar et al. (2012) studied the anti-angiogenic activity of quercetin using different models and found that quercetin inhibited several key steps of angiogenesis, including proliferation, migration and tube formation of endothelial cells.

These effects were accompanied by the suppression of VEGF-induced VEGFR2 phosphorylation, as well as the inhibition of phosphorylation of their downstream kinases, AKT, mTOR, and ribosomal protein S6 kinase in HUVECs. Moreover, the suppression of neovascularization by quercetin was also confirmed in *ex vivo* and *in vivo* experiments (Pratheeshkumar et al., 2012; Mirossay et al., 2018). Quercetin and other phytoconstituents present in the extract synergistically demonstrated reduction in the proliferation of blood vessels; hence, the anti-angiogenesis activity exhibited by the extract is possibly mediated by the suppression of the vascular endothelial growth factor (VEGF), an angiogenic protein in cancer cells (Mirossay et al., 2018). Due to this, the stimulation of the blood vessels to form neovascularized blood vessels from the pre-existing cancer metastasis cells is suppressed, preventing the progression of angiogenesis.

Although we have carried out the phytochemical screening of the extracts, further studies on structure elucidation of phytoconstituents present in the ethyl acetate extract needs to be determined for the identification of 'lead' anti-angiogenic compounds. CAM assay results usually corroborate with the response observed *in vivo* (Kue et al., 2015). Therefore, animal studies should be conducted to elucidate the exact mechanism of action of the extracts in the suppression of angiogenesis.

## CONCLUSIONS

The ethyl acetate extract of *Citrullus colocynthis* fruits suppressed angiogenesis. This activity is not only due to the quercetin content of the extracts but can be attributed to the synergistic effect of quercetin and the other phytoconstituents of the extracts. The identification of phytoconstituents responsible for the anti-angiogenic activity of the extracts needs to be carried out towards the discovery of anti-angiogenic agents.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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|---|---|---|---|---|---|
| Concepts or ideas | x | x | | | |
| Design | x | x | | x | x |
| Definition of intellectual content | x | x | x | x | x |
| Literature search | x | x | x | x | x |
| Experimental studies | x | x | | x | x |
| Data acquisition | x | x | x | x | x |
| Data analysis | x | x | x | x | x |
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