Original Article

The Effect of Butenyl Isothiocyanate on Angiogenesis Ex Vivo and in Vivo Animal Study

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Abstract

Background: Angiogenesis is the development of new capillary blood vessels from existing ones that are required for several physiological and pathological processes. Butenyl isothiocyanate is a member of isothiocyanate which has drawn more attention Since they have a lot of biological activity and are given credit for the benefits of cruciferous vegetable consumption on cancer. Additionally, acetylsalicylic acid the non-steroidal anti-inflammatory medicine significantly lowers the angiogenesis of cancer. **Objective:** The study aimed to identify the anti-angiogenic activity of butenyl isothiocyanate alone and in combination with acetylsalicylic acid and to investigate the synergistic and/or additive effect of butenyl isothiocyanate and acetylsalicylic acid as anti-angiogenic compounds. **Methods:** 12-14 weeks-old Albino male rats were used for the study. The tested substances butenyl isothiocyanate and acetylsalicylic acid were serially diluted. An *ex vivo* rat aorta ring experiment has been used to examine Butenyl isothiocyanate's potential antiangiogenic properties. Using an *in vivo* chorioallantoic membrane (CAM) experiment, the butenyl isothiocyanate-induced zone of blood vessel inhibition was measured and the collected data were statistically analyzed. **Results:** The result of this study showed that there is a significant antiangiogenic activity of butenyl isothiocyanate and acetylsalicylic acid and there is an additive antiangiogenic effect of butenyl isothiocyanate and acetylsalicylic acid combination in *ex vivo* rat aorta ring anti-angiogenesis assay ($P<0.05$). Each butenyl isothiocyanate and acetylsalicylic acid showed significant anti-angiogenesis activity in the *in vivo* CAM assay. **Conclusion:** The present study revealed that each butenyl isothiocyanate and acetylsalicylic acid exhibited significant anti-angiogenesis activity in both *ex vivo* and *in vivo* models of angiogenesis.

Keywords: Vivo Animal Study, Drugs, Chemical, Instrument.

DOI: 10.47750/pnr.2022.13.S03.195

INTRODUCTION

Angiogenesis is the development of new capillary blood vessels from the vasculature that already exists (Flamme et al., 1997). Which is triggered by the production and release of angiogenic promoters like vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF)-1 by hypoxic, disease, or wounded tissues. These angiogenic factors promote endothelial cell migration and proliferation in already-existing arteries, which in turn causes capillary tube development and the recruitment of additional cell types to create and stabilise new blood vessels (Jiang et al., 2020).

In some circumstances, angiogenesis can go from physiological to pathological when the proportion of angiogenesis stimulators to inhibitors is disrupted (Darweesh et al., 2019).

Pro- and anti-angiogenic factors interacting indicate a highly regulated mechanism. An imbalance in this process is the main cause of a wide range of cancerous, inflammatory diseases (Szekanecz & Koch, 2007).

Maintaining physiological levels of VEGFs is essential because high amounts have been linked to pathological diseases such as abnormal angiogenesis (Laakkonen et al., 2019).

Glucosinolates are sulphur and nitrogen-containing organic compounds formed from glucose and amino acids. This chemical molecule has minimal biological action while intact, but is converted into hydrolytic chemicals Isothiocyanates by the myrosinase enzyme (Arora et al., 2014; Bongoni et al., 2014). one of the Isothiocyanates is 3-Butenyl ITC (3-BI), which is mostly present in Brassica rapa species of crops, such as pak choi, Chinese cabbage, turnips, turnip greens, and turnip tops, where it is present as the glucosinolate gluconapin (Padilla et al., 2007). Is have garnered attention from the scientific community because of its potential to reduce cancer risk and/or treat existing cases (Liu, 2004).

Salicylates have traditionally been extracted from the bark of the willow tree. Willow tree extracts were used as analgesics as far back as 4,000 years ago by the Sumerians, as recorded in historical evidence (Tawfeek et al., 2021).

Acetylsalicylic acid blocks the enzyme cyclooxygenase-1 (COX-1). By doing so, it alters the enzymatic activity of cyclooxygenase-2 (COX-2) (Zimmermann & Curtis, 2018). In contrast to other NSAIDs (ibuprofen/naproxen), the binding of acetylsalicylic acid to this enzyme is irreversible.

Also, it prevents platelet aggregation by irreversibly inhibiting thromboxane A2 on platelets (Christiansen et al., 2021).

The objective of this study was to determine the antiangiogenic activity of butenyl isothiocyanate alone and in combination with acetylsalicylic acid using *in vitro* and *in vivo* assays.

MATERIAL AND METHODS

Drugs, Chemical, and instrument

The chemicals used in the experiments were ordered and obtained from (Hyperechem) China. While the instruments include 48 well tissue culture plates (Orange Scientifics, Belgium), Autoclave(Lab Tech, Korea), CO2 incubator (Lab Tech, Korea), Dissecting set (AVEAids, Malaysia), Eppendorf tubes 1.5ml (Abdos, India), Filter syringe 0.45 µm (China),

Micropipette tips (British), Micropipettes Eppendorf (Germany), Sensitive Digital balance (Sartorius, Canada), Inverted light microscope (Italy).

Source of experimental animals and ethical approval

The experiments used male rats who were between 12 and 14 weeks old. All of the animals had unrestricted access to food and drinking water. The animals were collected from Iraqi Center for Cancer and Medical Genetics Research /Al-Mustansiriya University and housed there between 28 and 30 degrees Celsius. The AL-Nahrain University College of Pharmacy's Animal Ethical Committee gave its approval to the studies.

Preparation of Serial dilutions of Butenyl isothiocyanate and Acetylsalicylic acid

Five eppendorf tubes were filled with 500 µl of complete growth medium (CGM) for each sample. A 0.45M filter was used in a syringe to filter the samples and remove unwanted substances. We started with a 10 µl eppendorf and a 990 µl CGM. To get the necessary concentration of butenyl-ITC and acetylsalicylic acid, 500 µl of this volume was transferred serially from the first to the last tube. Because of this, the volume is unaffected, but there is a steady shift in concentration (from 200 to 100 to 50 to 25 to 12.5 to 6.25 milligrammes per millilitre) (Skehan et al., 1990)modified (Papazisis et al., 1997).

Analysis of the antiangiogenic effect of Butenyl isothiocyanate on rat aorta rings

Using the procedure outlined by Brown and his colleagues, the ex vivo antiangiogenic effect of butenyl isothiocyanate alone and in combination with ASA was investigated on rat aortic rings (K. J. Brown et al., 1996), with minor modifications carried out b by Hayder B Sahib (Abd et al., 2017). 12-14 weeks-old Albino male rats were used in this study. Diethyl ether was used to induce anaesthesia while the animals were slaughtered humanely using cervical dislocation. The thoracic aorta was removed, cleaned from the fibro adipose tissue, rinsed with serum-free medium, and then cut into thin rings with a 1 mm thickness. In a 48-well plate with 300 µl of medium M199, one ring was positioned in the middle of each well to prevent fibrinolysis of vascular fragments, a basal medium containing fibrinogen, L-glutamine, and aprotinin at 3 mg/mL, 1 % Wt/V, and 5 g/mL, respectively.

After adding fibrinogen and aprotinin at 3 mg/mL and 5 mg/mL, respectively, the M199 medium was utilised for the lower layer. Each 48-well plate received 300µl of M199 medium, and one aortic ring was seeded into each well. 10µl of thrombin, produced at 50 NIH U/mL in 0.15 M NaCl, was added to each well. They were then incubated and left to solidify at 37°C in 5 % CO2 for 30–60 min.

The following ingredients were added to the M199 medium to create the top layer medium: 20% heat-inactivated foetal bovine serum (HIFBS), 1% L-glutamine, 0.1 % aminocaproic acid, and 0.6 % gentamicin. The test chemicals were introduced to the top layer medium.

To make different concentrations of butenyl-ITC and Acetylsalicylic acid were dissolved in 10 mg/1ml dimethyl sulfoxide (DMSO) as a stock, and then doubly diluted with an M199 growth medium. Butenyl-ITC alone (6.25-100 µg/mL range), Acetylsalicylic acid alone (12.5-100 µg/mL range), and the combination was prepared by mixing the resulting inhibition concentration by 50% IC50 of butenyl-ITC (is the concentration of drug required for 50% inhibition) with the resulted inhibition concentration by 50% IC50 of Acetylsalicylic acid. Were each tested three times with each concentration of BUT and Acetylsalicylic acid alone, with five replicates.

The tissue rings were cultured in a humidified incubator at 37°C with 5% CO2. On day 4, the top layer medium was replaced with a fresh medium, previously prepared. Acetylsalicylic acid (100 µg /mL) and DMSO (1 %v/v) were employed as positive and negative controls, respectively. On day 5, the findings were analysed with an inverted microscope, and the amount of blood vessel growth was measured at 10X magnification using a camera and software.

The approach was created by Nicosia and colleagues was used to determine the degree of blood vessel development inhibition.

The results are shown as mean percent inhibition to the negative control ± SD. Three times, six replicates of each sample were used in the experiment. the proportion of blood vessel inhibition was calculated.Following this formula,

Blood vessels inhibition = 1- (A0/A) ×100 Where

A0= distance of blood vessel growth for the test substance in mm.

A= distance of blood vessel growth in the control in mm(L. F. Brown et al., 1993).

Chorioallantoic membrane (CAM) assay on Butenyl isothiocyanate

The chorioallantoic membrane of the chick embryo is an extraembryonic membrane that functions as a surface for gas exchange and is supported by a substantial capillary network. Typically, the test substance is supplied in the form of tiny filter discs. The number, diameter, density, permeability, branch point count, and blood flow of blood vessels all can be measured. (Ribatti, 2010).

The modified Marchesan and colleagues (1998) approach was applied. Fertilized chicken eggs from the Poultry Field of the College Of Veterinary Medicine, University of Baghdad, were incubated for 72 hours at 37 degrees Celsius and 60 to 80 % relative humidity. The eggs were positioned horizontally and repeatedly rotated. In order to have a better look at the developing CAM, which will detach from the sack that is attached to the eggshell, 1-2 ml of albumen were sucked off after 72 hours through a tiny hole that had been punched down by the side and sealed. After that, the eggs were incubated for another 24 hours. The egg's sac was then punctured and a round piece of shell (3–4 cm in diameter) was removed from the top of the blunt end. A round disc of filter paper that had been previously soaked with the test sample was then placed on the CAM, and the eggs were sealed with sterile adhesive tape and incubated for an additional 72 hours.

Before being transferred to the CAM, the test sample was 0.5 mg/disc deposited on the disc of filter paper and allowed to dry. Negative controls were discs that only contained the vehicle (DMSO). Six CAM were used for each control and test sample on day 7 when the zone of inhibition was photographed and measured (Lokman et al., 2012).

The grades for the responses are + (3–6 mm), ++ (6–9 mm), and +++ (> 10 mm). Using an image analyzer, the zone of inhibition's size was calculated (West et al., 2003).

Statistical Analysis

Statistical Analysis for the Social Sciences (SAS) was used to process the data (SPSS; version 21). Measures of statistical significance between means were compared using analysis of variance (ANOVA). Continuous data presented as mean ±SD. The level of significance was 0.05.

Results

Anti-angiogenic activity in a rat aortic ring assayed in an ex vivo model

Figure (3.1) revealed that the butenyl isothiocyanate and ASA were administered on rat aorta implanted in a complete growth medium of M199 significantly inhibited blood vessel growth on day five ($P < 0.05$) in comparison to the negative control "DMSO 1%", while there was no significant variation between butenyl isothiocyanate and the positive control ASA ($P > 0.05$).

100 µg/ml Butenyl isothiocyanate inhibited blood vessel growth by 81±3.48 while 100 µg/ml ASA 71±5.74, DMSO 1% was used as a negative control.

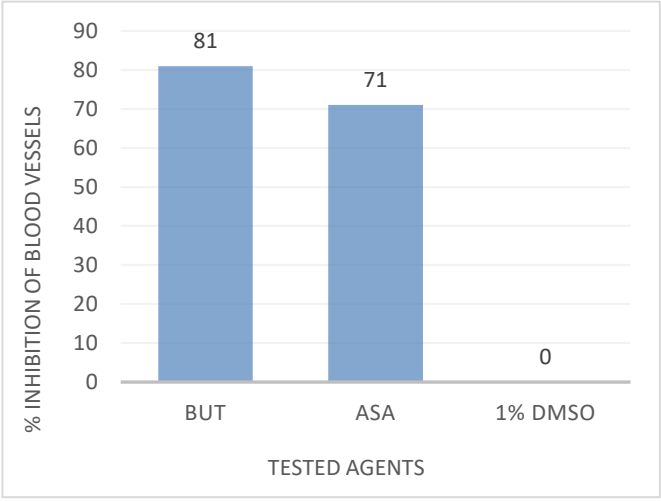
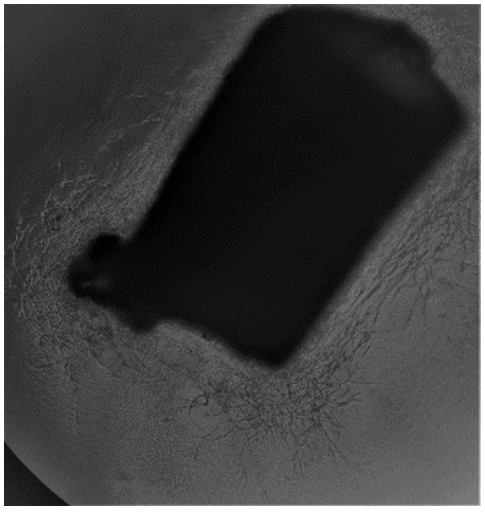
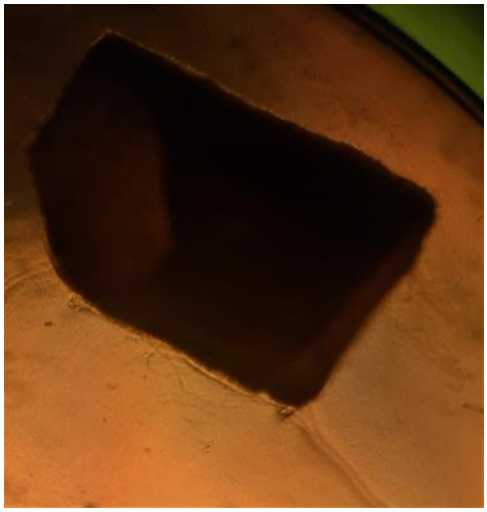
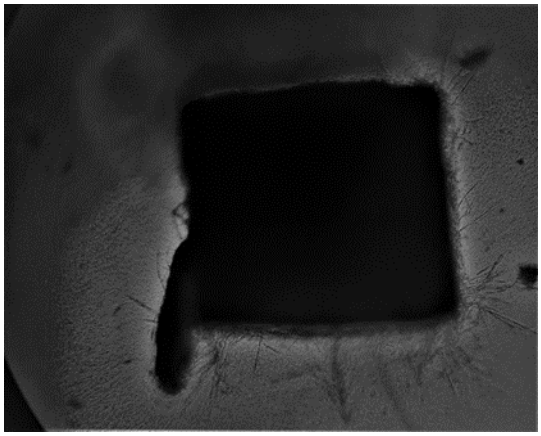


Figure (3.1): Inhibition percentage of 100µg/ml of each of Butenyl isothiocyanate, ASA compared with the negative control "DMSO 1%" in ex vivo aortic ring model







Negative control Butenyl-ITC 100µg/ml ASA 100µg/ml

Image (3.1): Representative Photographs of the inhibitory antiangiogenic effect of (100 µg/ml) Butenyl-ITC, ASA along with the negative control "DMSO 1% on ex vivo using a rat aortic ring

Dose-response curve of Butenyl isothiocyanate and Acetylsalicylic acid in ex vivo aortic ring model

Results for the antiangiogenic activity of different butenyl-ITC concentrations were shown as the mean percentage change in blood vessel growth on rat aorta ± standard deviation.

Blood vessel growth was decreased by 100 µg /ml of Butenyl-ITC by 81±3.48%, whereas the development of blood vessels was suppressed by 77±2.94%, 71±4.67%, 63±4.33%, and 45±6.40% respectively, at the remaining concentrations of 50, 25,12.5 and 6.25 µg /ml.

On day 5, the 100 g/ml concentration significantly suppressed newly formed blood vessels formation, in comparison to lower concentrations (potent antiangiogenic activity).

There was a significant dose-dependent inhibition activity of the serially diluted Butenyl-ITC among 100, 50, 25,12.5,625 µg /ml, as well as the negative control (P<0.05). When comparing 50 and 25 µg/ml, however, there was no significant variation (P>0.05).

Figure 3.3 shows the dose-response curve of the serially diluted ASA in four serial dilutions ranging from 100,50,25,12.5 µg/ml these concentrations demonstrated significant dose-dependent inhibitory effects (P< 0.05), with the proportion of inhibitions being expressed as the mean ± standard deviation as follows 71±5.74%, 39±7.96%, 33±6.49%, 30±6.36% for the above concentration respectively. The 50, 25, and 12.5 µg/ml did not significantly differ from one another, though (P>0.05). The 100 µg /ml ASA concentration significantly inhibited blood vessel growth by 71±5.74% (potent antiangiogenic activity) In comparison to other concentrations.

Figure (3.2) shows a logarithmic equation that may be used to calculate the IC50 value for Butenyl isothiocyanate, defined as the concentration at which the development of new blood vessels is inhibited by 50%. This value was found to be 6.15 g/ml. Figure (3.3) shows a linear regression equation that was used to calculate ASA's IC50, which was then established as 61 g/ml. Where Y= the inhibition percentage and X= the concentration.

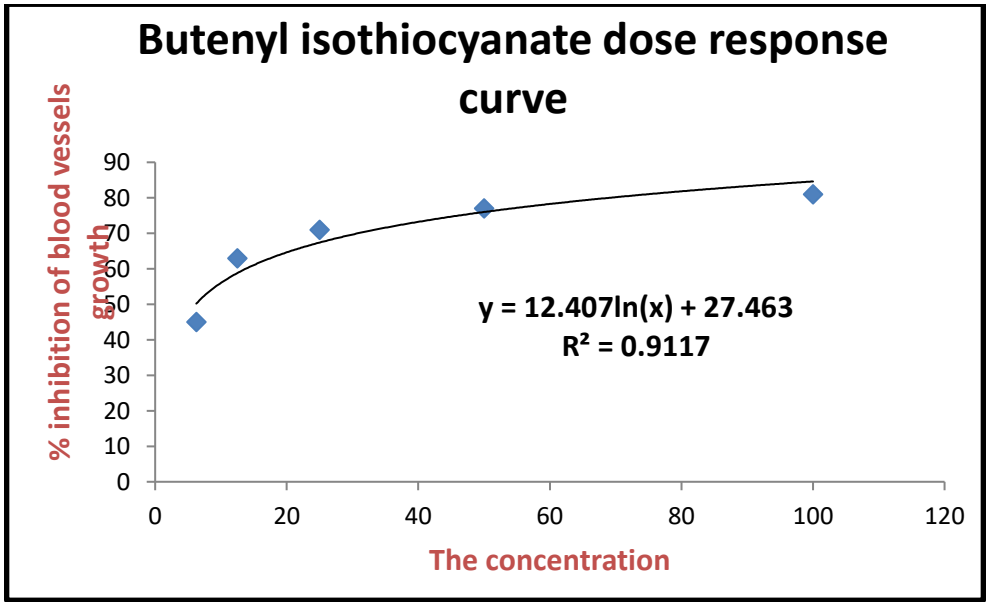


Figure (3.2): Dose-response curve of Butenyl isothiocyanate in rat aortic rings model

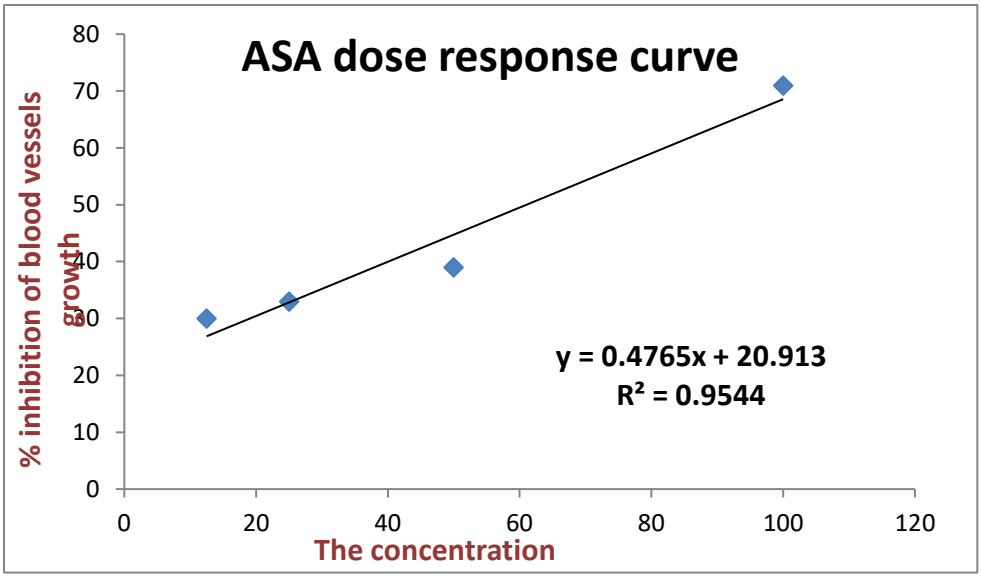
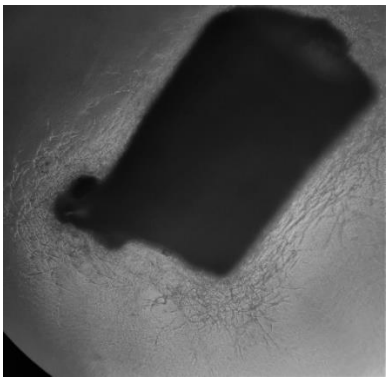
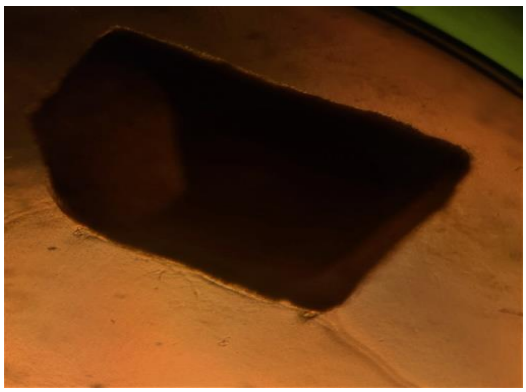
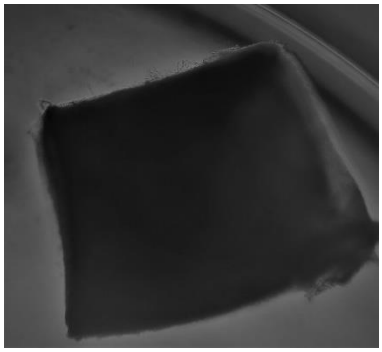


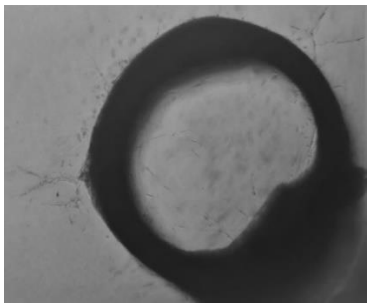
Figure (3.3): Dose-response curve of Acetylsalicylic acid in rat aortic rings model



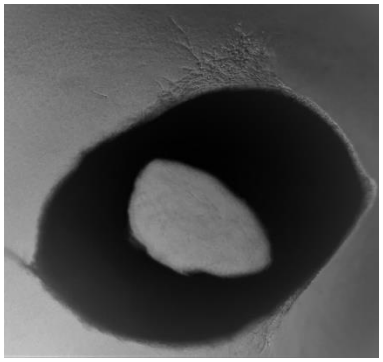


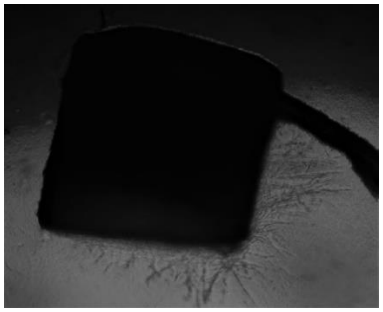
Negative control DMSO 1% Butenyl-ITC 100 µg/ml





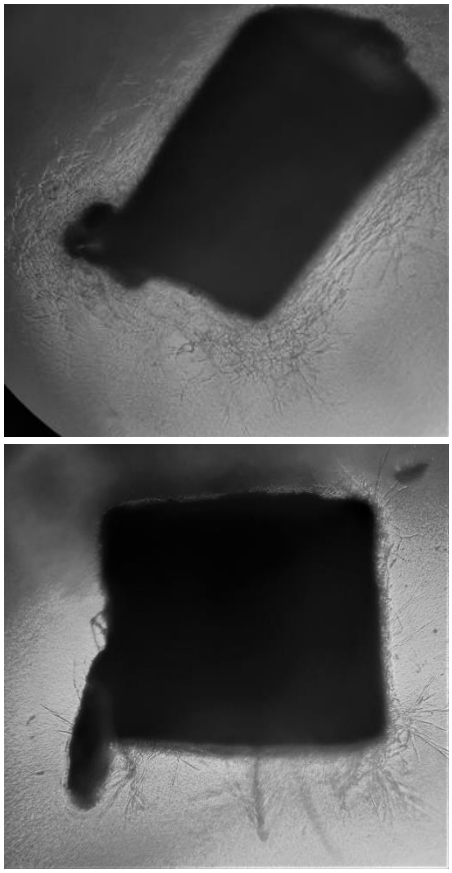
Butenyl-ITC 50 µg/ml Butenyl-ITC 25 µg/ml



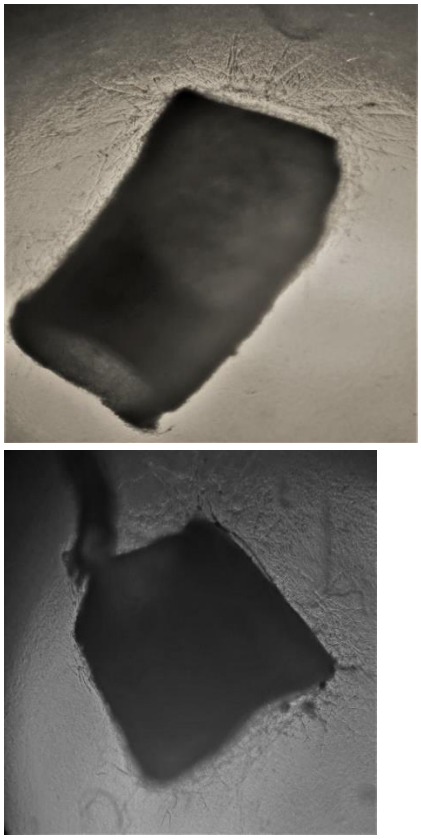


Butenyl-ITC 12.5 µg/ml Butenyl-ITC 6.25 µg/ml

Image (3.2): The dose-response effect to the serial concentrations of Butenyl isothiocyanate and the negative control in the ex vivo aortic ring model



Negative control DMSO 1% Acetylsalicylic acid 100 µg/ml



Acetylsalicylic acid 50 µg/ml Acetylsalicylic acid 25 µg/ml

Acetylsalicylic acid 12.5 µg/ml

Image (3.3): The dose-response effect to the serial concentrations of Acetylsalicylic acid and the negative control in the ex vivo aortic ring model DMSO 1% as a negative control

Effects of Butenyl isothiocyanate in combination with Acetylsalicylic acid

Figure (3.4) show the effect of butenyl-ITC (6.15 µg/ml) which is (the resulting inhibition concentration by 50% IC50) in combination with inhibition concentration by 50% IC50 of ASA (61 µg/ml) on the development of blood vessels in the aorta of rats showed the additive impact.

Statistical analysis showed the combination case on day 5 showed a nonsignificant reduction in blood vessel development P> 0.05 with percentage inhibition values of 59±3.40% when compared to 6.15µg/ml Butenyl-ITC alone, and 61 µg/ml ASA alone with percentage inhibition values of 50%.

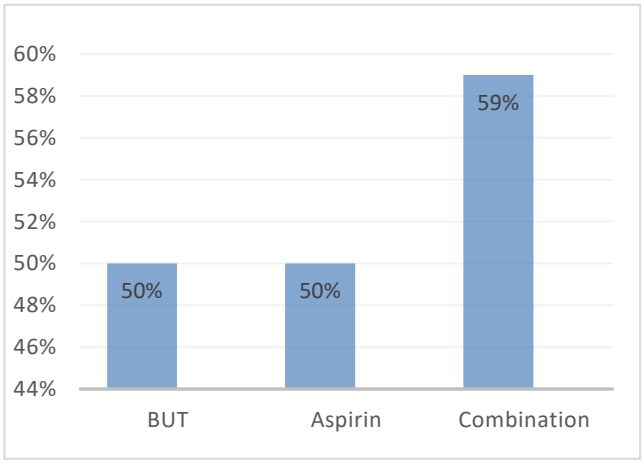
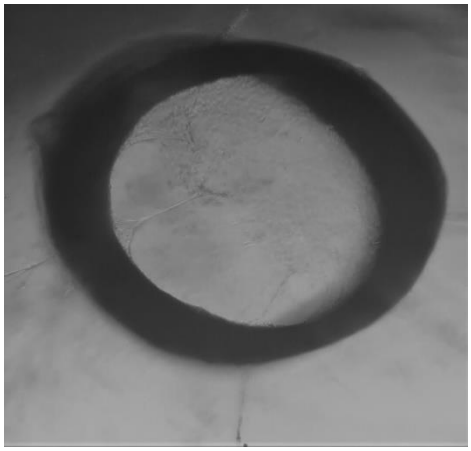
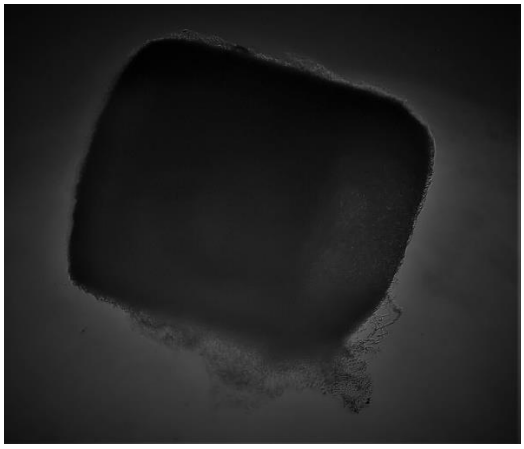


Figure (3.4): Inhibition percentage of 6.15 µg/ml of Butenyl isothiocyanate alone, and 61 µg/ml of ASA alone compared with the combination of them together in ex vivo aortic ring model



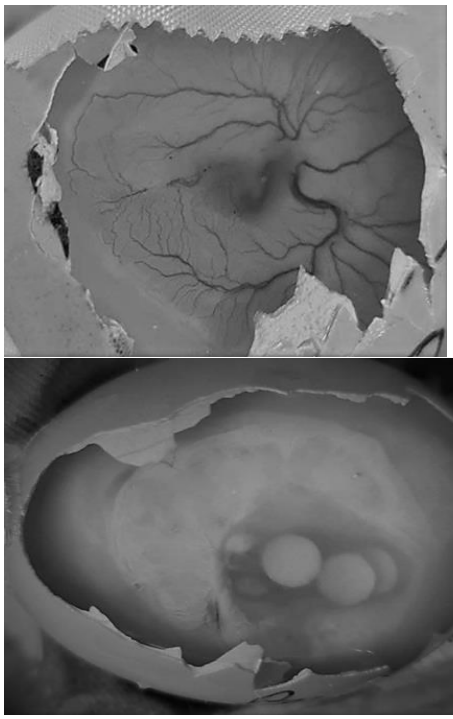


Negative control DMSO 1% Combination of Butenyl-ITC and ASA

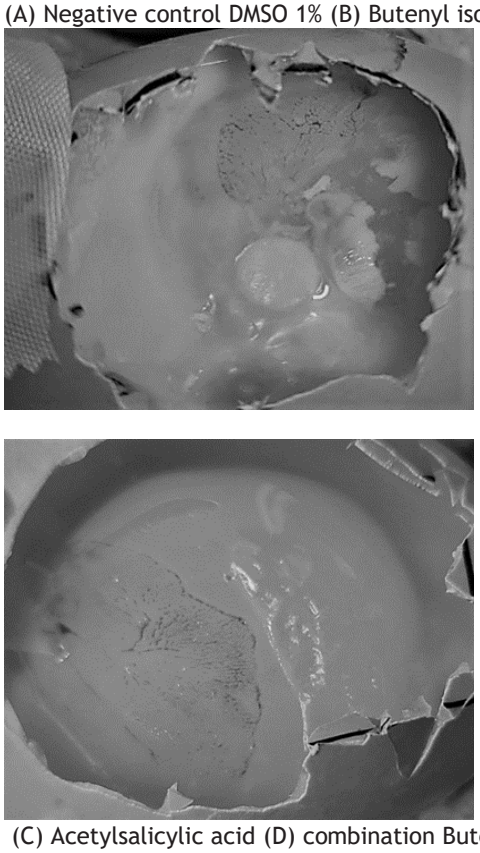
Image (3.4): The dose-response effect to the serial concentrations of Butenyl isothiocyanate and ASA in an ex vivo aortic ring model

In vivo chick chorioallantoic membrane (CAM) assay of Butenyl isothiocyanate, Acetylsalicylic acid and their combined effect on blood vessels growth:

On the seventh day of the experiment, the zones of inhibition for Butenyl isothiocyanate, ASA, their combination, and the negative control were all measured. The data shows the results employing a minimum of (6) eggs in each group. As a result of exposure to the test compounds, the CAM's blood vessels constricted; the vessels were disordered and had a light yellow colour. An avascular zone forming surrounding the drug-containing disc served as an indicator of inhibition, and the area of this zone was measured by using the previously described scoring system. Inhibition zones of blood vessel growth in the CAM were observed to be significantly increased by Butenyl-ITC, ASA, and their combination by the scoring of (+++)(> 10 mm),(++)(6–9 mm),(+++)(>10 mm) $p<0.05$ respectively in comparison to the control as shown in the image (3.5).



(A) Negative control DMSO 1% (B) Butenyl isothiocyanate



(C) Acetylsalicylic acid (D) combination Butenyl-ITC+ASA

Image (3.5): The in – vivo (CAM) assay; (A) represents the negative control "received DMSO 1%", while (B) represents the Butenyl isothiocyanate it showed a marked vessel regression, (C) represents the Acetylsalicylic acid and (D) represents the Butenyl isothiocyanate, Acetylsalicylic acid combination.

DISCUSSION

Since the angiogenesis process is linked to a wide variety of diseases that have proven difficult for scientists to treat, it has become essential that a wide variety of novel substances be tested or secreted in an effort to find one that shows promise as a future treatment. As a result, the angiogenic process can either promote angiogenesis in conditions when vascularity and blood supply are diminished, such as in tissue ischemia or prevent excessive and abnormal angiogenesis in conditions such as cancers (Darweesh, Ayoub and Nazzal, 2019).

Angiogenesis is best studied using the thoracic aortic rings (TAR) model, the most widely used *in vitro* method. Its popularity is due to a number of features, including validity, reliability, reproducibility, realistic simulation of the conditions in intact animals, cost-effectiveness, control and monitoring capabilities, the convenience of use, and high association with *in vivo* investigations (Goodwin, 2007; Staton et al., 2004).

The results showed that butenyl-ITC and ASA effectively suppressed blood vessel expansion in a dose-dependent manner, with a statistically significant difference between the two agents and the negative control. While there were no significant differences between butenyl-ITC and ASA. Since, the butenyl-ITC is a member of isothiocyanate so the mechanisms that are responsible for this significant antiangiogenic effect of butenyl-ITC may be due to downregulating VEGF, NO, and TNF-α which are considered angiogenic inducers (Thejass and Kuttan, 2007).

While the combination of butenyl-ITC with ASA results in a higher percent of inhibition compared with butenyl-ITC or ASA alone revealing an additive effect but not significant, however, the percentage remains higher. This additive effect may be due to the capability of butenyl-ITC to target more than one mechanism and ASA blocks multiple angiogenesis-promoting molecules, VEGF, cyclooxygenase, matrix metalloproteinase, and heparanase. When one specific molecule is inhibited, the tumour may induce the production of additional angiogenic factors, resulting in a limited treatment response (Xie et al., 2021). Any synergy or additive between antiangiogenic drugs may be dose-dependent. Antiangiogenic drugs must be carefully dosed and administered (Miller et al., 2007).

Since both butenyl-ITC and ASA showed potent anti-angiogenesis action in the screening experiment, dose-response research was conducted for each butenyl-ITC and ASA in order to determine at which concentration each substance can inhibit blood vessels growth by 50% and have an idea about the safety of the tested substance (selective toxicity).

The positive control in this study was acetylsalicylic acid, which is known to be anti-angiogenic through a number of different mechanisms. However, the mechanism by which ASA influences angiogenic processes is varied. Acetylsalicylic acid has been shown to suppress other antiangiogenic pathways additional to COX-1. These targets include angiotensin II, glucose transporter 1, heparanase, and matrix metalloproteinase. Evidence also shows that ASA impacts various cell types, including endothelial cells, platelets, pericytes, and macrophages, and may also have antiangiogenic properties (Xie et al., 2021).

Due to butenyl isothiocyanate being a member of isothiocyanate compounds so the mechanisms by which butenyl isothiocyanate control angiogenesis may be connected to HIF s, a key regulator of angiogenesis whose inhibition may be primarily accomplished by limiting protein translation. It has a crucial role in mediating the relationship between angiogenesis, inflammation, and cancer (Cavell et al., 2011).

A number of additional transcriptional regulators, besides HIF, have been linked to the regulation of angiogenesis. These include tubulin, MYC, activator protein-1 (AP1), and nuclear factor kappaB (NF-kB), all of which may increase the expression of VEGF and/or IL-8, two pro-angiogenic molecules that can be controlled by butenyl-ITCs (Cavell et al., 2011). So, the suppression of these factors by butenyl-ITCs may possibly have antiangiogenic effects (Mizukami et al., 2007).

In-vivo Chick CAM Assays are among the most ethically acceptable ways to study *in vivo* angiogenesis (Stryker et al., 2019).

In the present study, the zones of inhibition area of butenyl-ITC and ASA alone had a significant antiangiogenic effect in comparison to the control. These results are compatible with those of the *ex vivo* assay. While the combination, shows significant additive antiangiogenic action, the vessels were very sparse and difficult to recognize, despite their light-yellow appearance. This additive effect may be due to the ability of both butenyl-ITC and ASA to target more than one mechanism and inhibit several angiogenic molecules such as VEGF.

The probable mechanism of the anti-angiogenic effect of butenyl-ITC, as a member of the ITCs compound, is the reduction of CA-Akt, which in turn suppresses the PI3K-Akt signalling axis and VEGF. Inhibiting MYC oncoprotein expression, decreasing HIF1 and vascular endothelial growth factor (VEGF) levels. Down-regulation of nitric oxide and tumour necrosis factor-α.

CONCLUSIONS

1. Butenyl isothiocyanate and Acetylsalicylic acid demonstrated significant anti-angiogenesis effect, although, there is no significant difference between Butenyl isothiocyanate and Acetylsalicylic acid in *ex vivo* rat aortic rings anti-angiogenesis (RAR) assays.
2. There is a nonsignificant additive antiangiogenic effect of Butenyl isothiocyanate and Acetylsalicylic acid combination in anti-angiogenic rings assays in *ex vivo* rat aortic (RAR).
3. The *in vivo* chick chorioallantoic membrane (CAM) assay revealed significant inhibitory zones of blood

vessels when butenyl isothiocyanate, acetylsalicylic acid, or their combination was used.

4. Butenyl isothiocyanate and Acetylsalicylic acid exhibited significant antiproliferative activity against colon cancer cell line (CL40).
5. There is a significant additive antiproliferative activity of Butenyl isothiocyanate and Acetylsalicylic acid combination against colon cancer cell line (CL40).
6. The molecular mechanism of action that is responsible for the antiproliferative activity of Butenyl isothiocyanate against colon cancer cell line (CL40) was obviously by down-regulation of VEGF.

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