

Comparison of in vitro antioxidant, antimicrobial and anticancer activity of *Murraya koenigii* leaf and stem extracts in ovarian cancer cells

Shubham Hajare

MIT Art Design and Technology University

Tanvi Patade

MIT Art Design and Technology University

Jayashri Shrimangale

MIT Art Design and Technology University

Pradnya Gurav

MIT Art Design and Technology University

Pranav Pathak

MIT Art Design and Technology University

Kedar R.N. (✉ kedar.nirmala@mituniversity.edu.in)

MIT Art Design and Technology University

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Abstract

Ovarian cancer (OC) is the most lethal gynecological cancer, due to its diagnosis at later stages III and IV. Drug resistance developed at later stages further decreases survival rates to 30% in ovarian cancer patients. Natural compounds isolated from plant extract can have promising potential in cancer treatment due to their minimal side effects. *Murraya koenigii* (MK) leaves commonly used in Indian cuisines, are known to have antimicrobial, antifungal, and antioxidant activity. However, very few studies show the effect of MK extracts on ovarian cancer. This study shows the comparison of phytochemical content, antioxidant, antimicrobial and cytotoxic activities of methanol, aqueous and toluene extracts of MK leaves and stems. These extracts showed anticancer activity on the ovarian teratocarcinoma cell line - PA1 via apoptosis and antiangiogenic effect on healthy chick embryos.

1. Introduction

Ovarian cancer (OC) is the most lethal gynecological cancers (Jayson et al., 2014). More than 80% of women with non-symptomatic OCs are detected only in advanced stages (III and IV), as a lack of definite symptoms in early-stages. And there are no effective screening strategies as the exact cause of ovarian cancer is unknown (Shabir & Gill, 2020). OC predominantly affects older women (age 65 and above) which are at a higher risk of post-operative complications (Allemani et al., 2015; Sia et al., 2020). The incidences of ovarian cancer in India are increasing at an alarming rate i.e., 1 in 75 individuals are at risk to develop OC and 1 in 100 are at risk of mortality due to OC. The risk starts increasing from age 35 and reaches a peak between the ages of 55–64. In past 20 years, the survival rate has decreased from 45–30%(Shabir & Gill, 2020) in patients in an advanced stage as therapies become increasingly ineffective in treating metastatic ovarian cancer (Lengyel, 2010). Development of chemo-resistance and ineffective treatments and treatment failure causes over 90% of deaths (Agarwal & Kaye, 2003).

In India, OC ranks third in cancer morbidity and mortality after breast and cervix uteri in females (Sung et al., 2021), resulting in deaths of 1 in 133 females (Mathur et al., 2020). For the past two decades, frontline treatment has included standalone chemotherapy or in combination with cytoreductive surgery (CRS) (Wang et al., 2020). In addition, adjuvant chemotherapy, maintenance chemotherapy, genetic counseling, nanoparticle-based therapy, and gene therapy are part of modern-day treatments (Jit et al., 2022). Due to adverse side effects and higher costs of drugs and treatments, synthetic drugs are falling out of treatment choice, especially in developing countries like India. Natural compounds with minimal side effects are inexpensive at the current time and can be a promising alternative.

Murraya koenigii (MK), commonly called the “Curry plant” is one of the mostly used spices in Indian cuisines (Handral & Pandith, 2012). The extracts from parts of the MK plant are reported to have antimicrobial, antioxidant, neuroprotective, antidiabetic, hepatoprotective etc. benefits to human health (Table 1). Also, many reports show the anticancer potential of MK extracts (Table 1). However, very few studies show the anticancer effect of MK extracts on OC. In 2020, Satyavarapu *et al.*, have reported cytotoxic effects Mahanine Enhanced Fraction (MEF) in many cancer cell lines including ovarian cancer (Satyavarapu et al., 2020). And 2018, Xin *et al.* reported that Girinimbine inhibits proliferation in ovarian cancer cells via PI3K/AKT/mTOR and Wnt/ β -Catenin signally pathway (Xin & Muer, 2018).

As per our best knowledge, this is the first comparative study of whole methanol, aqueous and toluene extracts of MK leaves and stem on ovarian teratocarcinoma which also compares angiogenic effects of these extracts.

This study evaluates presence of phytochemicals, total phenolic content, antioxidant activity, antimicrobial activity on *Staphylococcus aureus* and *Escherichia coli*, antifungal activity on *Trichoderma viride* (TV) and *Aspergillus flavus* (AF) and cytotoxicity of on ovarian cancer cell line – PA1 (ovarian teratocarcinoma) with comprehensive experiments for cytotoxicity and apoptosis. This study also gives insights on teratogenic and angiogenic effects of these MK extracts.

Table 1
Important studies available on MK extracts and its active compounds

Sr. No	Plant Extract		Active Compound	Activity	Organism	Reference
	Part	Extraction solvent				
1	Seeds	Methanol	-	Anticancer	Human breast cancer – MCF7, human colorectal adenocarcinoma – DLD-1.	(Nadaf et al., 2020)
2	Shoot	n-hexane, chloroform, ethyl acetate, butanol, water	-	Anticancer	Human Cervical cancer cell line- HeLa	(Saleem et al., 2022)
3	Root	Hexane	Girinimbine	Anticancer	Lung Cancer - A549 cell line	(Mohan et al., 2013)
4	Leaf; Roots	Chloroform, Ethanol, Ethyl Acetate and Petroleum Ether	-	Antibacterial	<i>Escherichia coli</i> , <i>Micrococcus luteus</i> , <i>Staphylococcus aureus</i> , <i>Pseudomonas aeruginosa</i> and <i>Bacillus subtilis</i> .	(Abuga et al., 2020)
5	Leaf	Alkaloid Extract	Carbazole Alkaloids	Neuroprotective, Antioxidant	Mice	(Mani et al., 2013)
6	Leaf	Diethyl Ether	-	Antifungal, Antioxidant	<i>Alternaria alternata</i> , <i>Aspergillus flavus</i> , <i>Aspergillus niger</i> , <i>Aspergillus parasiticus</i> , <i>Fusarium oxysporum</i> , <i>Fusarium moniliforme</i> , <i>Mucor mucedo</i> , <i>Penicillium notatum</i> , <i>Penicillium funiculosum</i> , and <i>Trichoderma viride</i> .	(Tripathi et al., 2018)
7	Leaf	Acetone	Murrafoline -I and Mahabinine - A	Anticancer	human leukemia cell line – HL-60	(Ito et al., 2006)
8	Leaf	Petroleum Ether	Carbazole Alkaloids, Tannins	Hepatoprotective	Liver Cancer – Hep _{G2} cell line	(Sathaye et al., 2011)

Sr. No	Plant Extract		Active Compound	Activity	Organism	Reference
	Part	Extraction solvent				
9	Leaf	Hexane	-	Anticancer	Human Cervical cancer cell line-HeLa	(Amna et al., 2019)
10	Leaf	n-hexane, ethyl acetate, and methanol	-	Anticancer	Human Cervical cancer cell line-HeLa	(Amna et al., 2019)
11	Leaf	Ethyl Acetate	Ag NP from b-Caryophyllene	Antimalarial; Anticancer	<i>P. falciparum</i> . A549 & HeLa cell line	(Kamaraj et al., 2017)
12	Leaf	Ethyl Acetate; Methanol.	Mahanine	Anticancer	Human pancreatic adenocarcinoma cell lines – MIAPaCa-2 and BxPC-3	(Sarkar Bhattacharya et al., 2018)
13	Leaf	Water, 50% Methanol	-	Anti-inflammatory; immunomodulatory; antidiabetic	Mouse model	(Paul et al., 2011)
14	Leaf	dichloromethane; methanol	Carbazole alkaloids	Anticancer	Human Oral Cancer cell line – CLS-354	(Utaipan et al., 2017)
15	Leaf	Water	-	Chemopreventive, Anti-inflammatory	Mice model; human and mice breast cancer – MDA-MB231; 4T1 cell lines.	(Yeap et al., 2015)
16	Leaf	Water	-	Nephroprotective	Rats	(Punuru et al., 2014)
17	Leaf	Water	-	Neuroprotective, Antidiabetic	Rats	(Yankuzo et al., 2011)
18	Leaf	Water	-	Anti-Alzheimer	Rats	(Reddy et al., 2019)
19	Leaf	Water	-	Hepatoprotective	Rats	(Shinde & Mohan, 2022)
20	Leaf	Water	-	Cardioprotective	Rats	(Sandamali et al., 2020)
21	Leaf	Water	-	Antiulcer	Rats	(Sharma et al., 2011)
22	Leaf	50% Ethanol	-	Chemoprotective	Mice	(Kaur et al., 2021)
23	Leaf	50% Ethanol	-	Anticancer	Human lung cancer cells-A549; Chinese Hamster Ovary cells – CHOK1	(Bhatt et al., 2022)

Sr. No	Plant Extract		Active Compound	Activity	Organism	Reference
	Part	Extraction solvent				
24	Leaf	Ethanol	-	Antitumor	Rat model mammary carcinoma	(Aisyah et al., 2021)
25	Leaf	Ethanol	-	Antifungal	<i>Canida albicans</i>	(Lubis et al., 2022)
26	Leaf	96% Ethanol	-	Antidiabetic	Rats	(Husna et al., 2018)
27	Leaf	80% Methanol	-	Anticancer	Breast cancer – MCF-7 and MDA-MB-231 cell lines	(Noolu et al., 2013)
28	Leaf	Methanol	Mahanine	Anticancer	Breast cancer – MCF-7, MDA-MB-231 and MCF 10A cell lines	(Das et al., 2019)
29	Leaf	Methanol	Mahanine	Anticancer	Human Prostate cancer – PC3 & LNCaP	(Jagadeesh et al., 2007)
30	Leaf	Methanol	Mahanine	Anticancer	human glioblastoma cells – U87MG & LN229	(Bhattacharya et al., 2014)
31	Leaf	Methanol	Mahanine	Anticancer	Human lung cancer cells – A549, H1299. Rat skeletal muscle cells -L6	(Chatterjee et al., 2015)
32	Leaf	Methanol	-	Anti-inflammatory	Rats	(Arunkumar J., 2021)
33	Leaf	Methanol	-	Antiproliferation	Breast Cancer cells – T47D	(Mutia et al., 2022)
34	Leaf	-	Mahanimbine	Anti-chemoresistance	Human lung cancer	(Mondal et al., 2022)
35	Leaf	-	-	Nephroprotective	Rats	(Nazeer et al., 2022)

2. Result And Discussion

2.1 Phytochemical Assays

The presence of various phytochemicals was confirmed by multiple tests, as shown in Table 2. Phytochemical screening showed that aqueous extracts constituted more phytochemical compounds than methanol and toluene extracts. Proteins and flavonoids showed absence in all extracts, while Triterpenoids were present in all. All stem

extracts and only aqueous leaf extract showed the presence of Glycosides. Toluene extracts lacked carbohydrates and Anthraquinones. Methanol stem and aqueous extracts showed the presence of saponins. Aqueous extracts and toluene leaf extracts show the presence of tannins. Alkaloids were present only in the methanolic stem and aqueous stem extracts.

Table 2
Phytochemicals present in various leaf and stem extracts of *M. koenigii*.

Sr. No	Phytochemicals	ML	AL	TL	MS	AS	TS
1	Alkaloids	-	-	-	+	+	-
2	Flavonoids	-	-	-	-	-	-
3	Tannins	-	+	+	-	+	-
4	Carbohydrates	+	+	-	+	+	-
5	Saponin	-	+	-	+	+	-
6	Glycosides	-	+	-	+	+	+
7	Proteins	-	-	-	-	-	-
8	Anthraquinone	+	+	-	+	+	-
9	Triterpenoids	+	+	+	+	+	+

2.2 Total Phenolic Content

The phenolic content of the stem extracts from Fig. 1 is significantly lesser than the leaf extracts in the same solvents. Thus, phenolic compounds are more abundantly present in the leaves than in the stems. When correlated with the phytochemical results from Table 1, stem extracts have lesser total phenolic content despite constituting more phytochemical compounds than leaf extracts. And the extracts showing the presence of glycosides show less total phenolic content than those without them. Phenolic compounds can readily be isolated from methanol and toluene extracts of MK leaves.

2.3 DPPH Antioxidant Assay

DPPH assay was performed using ascorbic acid as a reference. Figure 2 shows percent radical scavenging activity (RSA) of extracts compared to ascorbic acid at equal concentrations. From the graph, as the concentration increased, RSA of the plant extracts decreased. Where leaf extracts showed lesser RSA than stem extracts, the aqueous extracts of both leaf and stem showed higher RSA than methanol and toluene extracts. Correlating antioxidant results (Fig. 2) with the phytochemical results (Table 1), the RSA activity shows a relation with the presence of glycosides. Extracts (AL and all stem extracts) with glycosides show RSA even at higher concentrations, while extracts (ML and TL) without glycosides exhibit RSA at lower concentrations. This suggests that the glycosidic compounds show antioxidant activity.

While correlating antioxidant results with the phenolic content results (Fig. 1), it is seen that extracts having higher TPC has lesser RSA and vice-versa. This can be seen in leaf extracts with higher TPC and lesser RSA. While the methanol and toluene leaf extracts had the highest TPC content than the aqueous leaf extract, they do not show

RSA at higher concentrations of 8mg/ml and 6mg/ml, respectively. In contrast, aqueous leaf extract had the least TPC in leaf extracts that did not show diminished RSA event at 10mg/ml concentrations. The stem extracts having the least TPC showed significantly higher RSA than leaf extracts from the same solvent. The only exception was the toluene stem extract which showed the least TPC of all extracts and showed no activity at the highest concentration of 10mg/ml

2.4 Screening for Antimicrobial Activity

Screening for antimicrobial activity was assessed using a zone of inhibition assay against gram-positive *S. aureus* (SA) and gram-negative *E. coli* (EC) bacteria. Table 3 shows crude plant extracts exhibiting antimicrobial activity. The subsequent dilutions of crude extracts failed to retain any microbial activity, thus suggesting lower concentrations of antimicrobial compounds in crude extracts. Minimal inhibitory concentration was estimated. From Table 3, ML, MS, and TL showed growth inhibition of SA while only TL inhibited the proliferation of EC at lower concentrations; hence, TL was found to be the most potent extract (Table 4).

Table 3
Antimicrobial activity of crude plant extracts

Sr. No.	Plant Extract	<i>S. aureus</i>	<i>E. coli</i>
1	Methanolic Leaf Extract	+	-
2	Aqueous Leaf Extract	-	-
3	Toluene Leaf Extract	+	+
4	Methanolic Stem Extract	+	-
5	Aqueous Stem Extract	-	-
6	Toluene Stem Extract	-	-

Table 4
MIC values of plant extracts against *S. aureus* and *E. coli*

Sr No.	Plant Extract	Minimal Inhibitory Concentration	
		<i>S. aureus</i>	<i>E. coli</i>
1	Methanolic Leaf Extract	5 mg/ml	-
2	Toluene Leaf Extract	1.5 mg/ml	5 mg/ml
3	Methanolic Stem Extract	5 mg/ml	-

2.5 Screening for Antifungal Activity

Screening for antifungal activity was assessed using a paper disk diffusion assay against *Trichoderma viride* (TV) and *Aspergillus flavus* (AF). Only toluene leaf extract showed growth inhibition in both TV and AF. The poison food technique was used to confirm growth inhibition. Figure 3 shows results for the poison food technique, wherein TL extract showed complete growth inhibition in TV and slowed growth in AF compared to control when left in dark and humid conditions for a week. Hence TL extract showed antifungal activity.

2.6 Cell Proliferation Assay

MK leaf and stem extracts in methanol, water, and toluene individually exhibit anticancer activity against human ovarian cancer cell line PA1. Table 5 shows IC₅₀ values of leaf and stem extracts. Among these extracts, aqueous stem extracts had showed the lowest IC₅₀ and thus the strongest anticancer activity.

Table 5
IC₅₀ values of given extracts on PA1 cell line

Sr No.	Plant Extract	IC ₅₀ value
1	Methanolic Leaf Extract	30 ± 0.88 µg/ml
2	Aqueous Leaf Extract	39 ± 5.54 µg/ml
3	Toluene Leaf Extract	52 ± 1.25 µg/ml
4	Methanolic Stem Extract	45 ± 4.54 µg/ml
5	Aqueous Stem Extract	22 ± 1.41 µg/ml
6	Toluene Stem Extract	80 ± 4.04 µg/ml

2.7 Apoptosis

Cells undergoing apoptosis were confirmed by Annexin V - PI binding assay by flow cytometer. Figure 4 shows the distribution of cells in various apoptotic phases - live, early apoptotic, late apoptotic and necrotic. In Fig. 4, all extracts showed a significant decrease in live populations compared to the control. AS and AL showed the most promising results and were comparable with the anticancer drug doxorubicin. These results corroborate with MTT assay results. The cell death may be attributed to the significant increase in the late apoptotic and necrotic populations.

2.8 Anti-Angiogenesis and Teratogenesis

After studying the anticancer effects of the MK extracts on PA1 cells, their toxicity on healthy cells of chick embryos and their angiogenic activity was evaluated. The activity of only leaf extracts was studied as they overall showed lower IC₅₀ than stem extracts. CAM assay was performed to evaluate the antiangiogenic and teratogenic activity of these extracts. The analysis was performed by AngioTool®. Figure 6 shows actual CAM images of eggs alongside processed images from AngioTool®. 10µM Doxorubicin was used as the positive control.

From Fig. 5A-E, it is seen that methanol leaf (ML) extract shows a significant rise in angiogenic parameters – Vessel Percentage Area (VPA), Junction Density (JD), Total Vessel Length (TVL), Total Number of Junctions (TNJ) and Average Vessel Length). Thus, ML promotes angiogenesis. This suggests that the anticancer activity shown by ML may not be attributed to anti-angiogenic pathways. Whereas doxorubicin being an anticancer drug, also shows an antiangiogenic effect. Both aqueous leaf (AL) extract and toluene leaf (TL) extract have a significant antiangiogenic effect on CAM compared to the control.

Teratogenic effects of these extracts were studied on chick embryos (HH stage 26 (120h+)). When compared to the standard HH Stage chart, all chick embryos (n = 3) had normal brain development, limb buds, visible digits, and

feathered germs. Hence, no physical malformation or deformities were seen, as shown in Fig. 7. All embryos were alive during embryo isolation and did not show any teratogenicity, thus suggesting such extracts might be safe to be administered in gestating females.

3. Experimental

3.1 Materials

Murraya koenigii leaves and stems were collected from local farmer in Pune, Maharashtra region in July 2022. Chemicals such as Methanol, Toluene, Potassium Iodide, Ferrous Chloride, Fehling's Solution (A and B), Sodium Bicarbonate, Sodium Hydroxide, Ninhydrin Reagent, Gallic acid, Ascorbic acid, FC reagent DPPH, Analytical grade DMSO and Ampicillin were obtained from SRL[®] (India), Conc. Hydrochloric acid was obtained from Fisher Scientific (USA). Nutrient Agar, Nutrient Broth, Potato Dextrose Agar, Doxorubicin, DMEM cell culture media, Penicillin and Streptomycin, Trypsin, and cell culture grade DMSO were brought from HiMedia (India). Annexin-V and Propidium iodide antibodies were obtained from Dead Cell Apoptosis Kit, Invitrogen (USA). Required glassware and plasticware, including consumables, were brought from Borosil and Tarson (India), respectively. SPF eggs used for CAM assay were supplied by Poultry Diagnostic and Research Centre, Loni Kalbhor, Pune (India). Open-source software such as AngioTool[®] (NIH Center for Cancer Research) and ImageJ (NIH) were used to perform image processing and MS Excel was used for data processing and analysis. Biorender[®] was used to make the graphical abstract.

3.2 Methods

3.2.1 Soxhlet Extraction

Soxhlet extraction was performed to obtain crude extracts as per the earlier protocol (Abubakar & Haque, 2020). Briefly, leaves and stems were washed thoroughly cleaned, dried, and finely powdered. 25g of finely grounded leaf and stem powder was taken in a muslin cloth and kept in a soxhlet thimble holder. 250ml of extraction solvent (methanol, autoclaved distilled water, and toluene) was kept in the round bottom flask below. The complete setup was placed in the heating mantle at a temperature equivalent to the boiling point of each solvent. Extraction was carried out till the solution in the siphon arm became colorless. Afterward, the round bottom flask with its content was subjected to rotary evaporation until the entire solvent evaporated. The dried crude extract was scraped off thoroughly using a known volume of DMSO.

3.2.2 Phytochemical Assays

a) Alkaloid Test: 500µL of Wagner's reagent (2% potassium iodide) was added to 1 ml of diluted plant extract and shaken vigorously. Reddish brown precipitate indicates the presence of alkaloids (Jha et al., 2012). b) Flavonoid Test: 200µL of conc. HCl was added to 1 ml of diluted plant extract. A pink precipitate indicates the presence of flavonoids. c) Tannin Test: 500µL of 5% FeCl₃ was added to 1 ml of dilute plant extract. The appearance of a bluish-black solution indicates the presence of tannins. d) Carbohydrates Test: 500µL of freshly prepared Fehling's solution was added to 1ml of diluted plant extract and heated in a water bath at 80°C. The presence of carbohydrates is indicated by yellow or red color. e) Saponins Test: 500µL of 5% NaHCO₃ was added to 1 ml of dilute plant extract and shaken vigorously. It was allowed to stand still for 3-5mins. The presence of honeycomb froth indicates the presence of saponins. f) Glycosides Test: 500µL of 3N NaOH was added to 1 ml of Dilute plant extract. The appearance of yellow color confirms the presence of glycosides. g) Protein Test: 500µL of 0.2% Ninhydrin Reagent

was added to 1 ml of dilute plant extract. The appearance of pink or purple color confirms the presence of proteins (Abubakar & Haque, 2020).

3.2.3 Total Phenolic Content

Total phenolic content was estimated using a gallic acid standard curve (10–100 µg/ml). 10µL of plant extract was diluted to 500µL using milli-Q water and 500µL FC reagent was added to each plant extract tube. Samples were incubated at room temperature for 30 mins. Absorbance readings were taken at 760 nm using a UV -Vis Spectrometer (UV 1800 Spectrophotometer, Shimadzu, Japan) (Folin & Ciocalteu, 1927).

3.2.4 Antioxidant Assay

DPPH assay was performed per the established protocol (Xiao et al., 2020) with some slight modifications. Briefly, ascorbic acid (10mg/ml) in DMSO was standard. Concentrations were made by adding 10, 20, 40, 60, 80 and 100µL of standard in DMSO. Similar concentrations were made for different plant extracts. 100µL DPPH (0.1mM in absolute ethanol) was added to each tube, and the volume was made up to 1 ml by DMSO. The reaction was incubated at room temperature in the dark for 25–30 mins. Absorbance was recorded at 517 nm using a UV - Vis Spectrophotometer. Percentage radical scavenging activity was calculated by subtracting the sample's absorbance and the control's difference from unity.

3.2.5 Screening for Antibacterial Activity

A zone of inhibition was performed using the disc diffusion method (Vaishali & Geetha, 2018). 20µL of freshly inoculated overnight-grown cultures (10^8 CFUs/ml, 0.5 McFarland's standard) of *S. aureus* and *E. coli* were added onto the Nutrient Agar plates and spread evenly throughout the plate using a glass spreader. The disc diffusion method was used to obtain inhibition zones. Sterile Whatman paper discs were dipped in desired concentrations of plant extracts and placed on the agar plate. Ampicillin was used as a positive control. Inhibitory zones on the plates were observed after 48 hrs. The experiment was performed three times.

3.2.6 Minimal Inhibitory Concentration for Antibacterial Activity

The broth dilution method was used to study the antimicrobial activity of given plant extracts (Wiegand et al., 2008) with slight modification. Freshly inoculated, overnight-grown cultures (10^8 CFUs/ml, 0.5 McFarland's standard) of *S. Aureus* and *E. Coli* were used for MIC. 150µL of diluted bacterial culture was treated with 150µL of plant extract concentrations. Ampicillin was used as a positive control. The assay endpoint was the lowest concentration at which no further growth was seen. After 24hrs of treatment, the bacterial samples were diluted to 1 ml using autoclaved distilled water and OD was recorded at 600 nm. The percent viability for minimal inhibitory concentration was calculated by - % Viability = $(1 - (\text{OD}_{\text{broth}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{sample}}) \times 100$.

3.2.7. Screening for Antifungal Activity

Antifungal activity was screened in two methods - the disc diffusion method for screening extracts and the poison agar method as a confirmatory test for antifungal activity. Firstly, with the help of sterile forceps, sterile Whatman paper disc cutouts were placed in a circular pattern on the PDA agar. 15µL of plant extract was loaded onto these discs while DMSO was used as a negative control. In the center was placed an agar cut out from the respective fungal master plate. Plates were sealed with parafilm tape and kept at room temperature in a dark and humid environment for over a week. Plates were observed daily for over a week (Hameed et al., 2020).

Then, the poison agar method used extracts that showed antifungal activity (Young et al., 1992). 500µL of plant extract was gently added and mixed with PDA at 36°C to 40°C. No additives were added to PDA for control plates. Once solidified, agar cutouts from respective fungal master plates were placed in the center of both sample and control PDA plates. Plates were sealed and kept at room temperature in a dark and humid place for over a week or until fungus covered the entire control plate.

3.2.8 Cell Proliferation Assay

A standard cell proliferation assay was performed to find the IC₅₀ concentration of plant extracts (van Meerloo et al., 2011). 10⁴ cells were seeded into 96 well plates. After 24hr cells were treated with desired concentrations in 1% DMSO and kept in an 5% CO₂ incubator at 37°C. After 24hrs incubation, the drug was removed, and 10µL MTT was added with 90µL 1X PBS (pH 7.4) and kept in the incubator for four hrs. After 4hrs, formazan crystal formations were observed under an inverted microscope and crystals were dissolved using 180µL of analytical grade DMSO. Once crystals were dissolved, OD was taken at 620 nm using ELISA Plate Reader (MULTISKAN FC, Thermo Scientific, USA). The experiment was done in triplicates.

3.2.9 Apoptosis

Annexin V - PI-stained assay was performed using Attune NxT, (Thermo Fisher Scientific) flow cytometer. The procedure was followed according to the protocol given in the dead cell apoptosis kit V13245 (Invitrogen). 10⁶ PA1 cells were treated for 24h and were harvested from media and by trypsinization. Cells were centrifuged at 3000 rpm for 5 mins, and pellets were given PBS wash twice. 100µL 1X Annexin Binding buffer and 1µL of Annexin V and PI were added. The pellet was vortexed for 20–30 seconds. After incubating the cells for 30–45 mins, cells were analyzed using a flow cytometer.

3.2.10 Anti-angiogenesis and Teratogenesis

CAM assay was performed to study angiogenesis in healthy chick embryos (Auerbach et al., 2003). Fertilized, pre-incubated, and clean SPF eggs (HH stage 26 (120h+)) were procured from Poultry Diagnostic Research Centre (PDRC), Loni Kalbhor, Pune. Egg surfaces were gently cleaned with 70% Ethanol and were kept for incubation at 37°C 70–80% relative humidity. After 24hrs incubation, eggs were treated with freshly prepared concentrations of plant extract via in-ovo technique (n = 3). Eggs (n = 3) treated with 10µM Doxorubicin were kept as a positive control. Control and treated embryos were kept in a shaker incubator at 37°C and 70–80% relative humidity for the next 24hrs.

Post-incubation, CAM and embryos were isolated in 1X chilled PBS (pH 7.4) and were observed for any physical anomalies. Three individual observers recorded observations. For antiangiogenic effects, CAM images were analyzed using AngioTool®.

2.2.11 Statistical Analysis

All experiments were performed in triplicates. The data shown in this paper is the mean of three individual samples for each assay. Statistical analysis was done using One way ANOVA (Analysis of Variance) in Excel 365. p < 0.05 was considered statistically significant.

4. Conclusion

In conclusion, aqueous leaf and stem extract showed presence of more phytochemical compounds compared to other extracts. The aqueous leaf, and all stem extracts had lesser phenolic content and higher radical scavenging activity which can be correlated to presence of glycosides in them. ML and MS extracts showed antimicrobial activity against only *S. aureus*, while TL extract had antimicrobial activity against both *S. aureus* and *E. coli*. Significant antifungal activity against *Trichoderma viride* and *Aspergillus flavus* was shown only by TL extract. All the extracts showed significant cytotoxicity on ovarian teratocarcinoma cells – PA1 which was also reflected in their significant apoptotic and necrotic populations compared to control. In CAM assay, AL and TL extracts inhibited angiogenesis and ML extract induced angiogenesis, suggesting the action of another anticancer pathway apart from anti-angiogenesis by ML extract. None of the extracts showed the teratogenic effect as no physical deformities were seen in the treated chick embryos; indicating that they are safe to be administered to pregnant women. Overall, this study shows suggests that toluene leaf extract can be a safe, non-toxic treatment choice along with chemotherapy. However, in-depth gene and protein expression studies need to be conducted to delineate the mechanism of action of MK in cancer and animal studies to be carried out for dose standardization.

Abbreviations

Murraya koenigii: MK; Ovarian Cancer: OC; Methanol Leaf Extract: ML; Methanol Stem Extract: MS; Aqueous Leaf Extract: AL; Aqueous Stem Extract: AS; Toluene Leaf Extract: TL; Toluene Stem Extract: TS; Fetal bovine serum: FBS; Phosphate buffer solution: PBS; Fluorescent Assisted Cell Staining: FACS; Propidium Iodide: PI; Chorioallantois Membrane: CAM; Hydrochloric acid: HCl; Dimethyl Sulfoxide: DMSO; Modified Dulbecco's Eagle's Media: DMEM; 2,2-diphenyl-1-picrylhydrazyl: DPPH; (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide: MTT; *Streptococcus aureus*: SA; *Escherichia coli*: EC; *Trichoderma viride*: TV; *Aspergillus fumigatus*: AF; Radical scavenging activity: RSA; Total phenolic content: TPC.

Declarations

Acknowledgment

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Declaration of Interest

The authors declare no conflict of interest.

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Figures

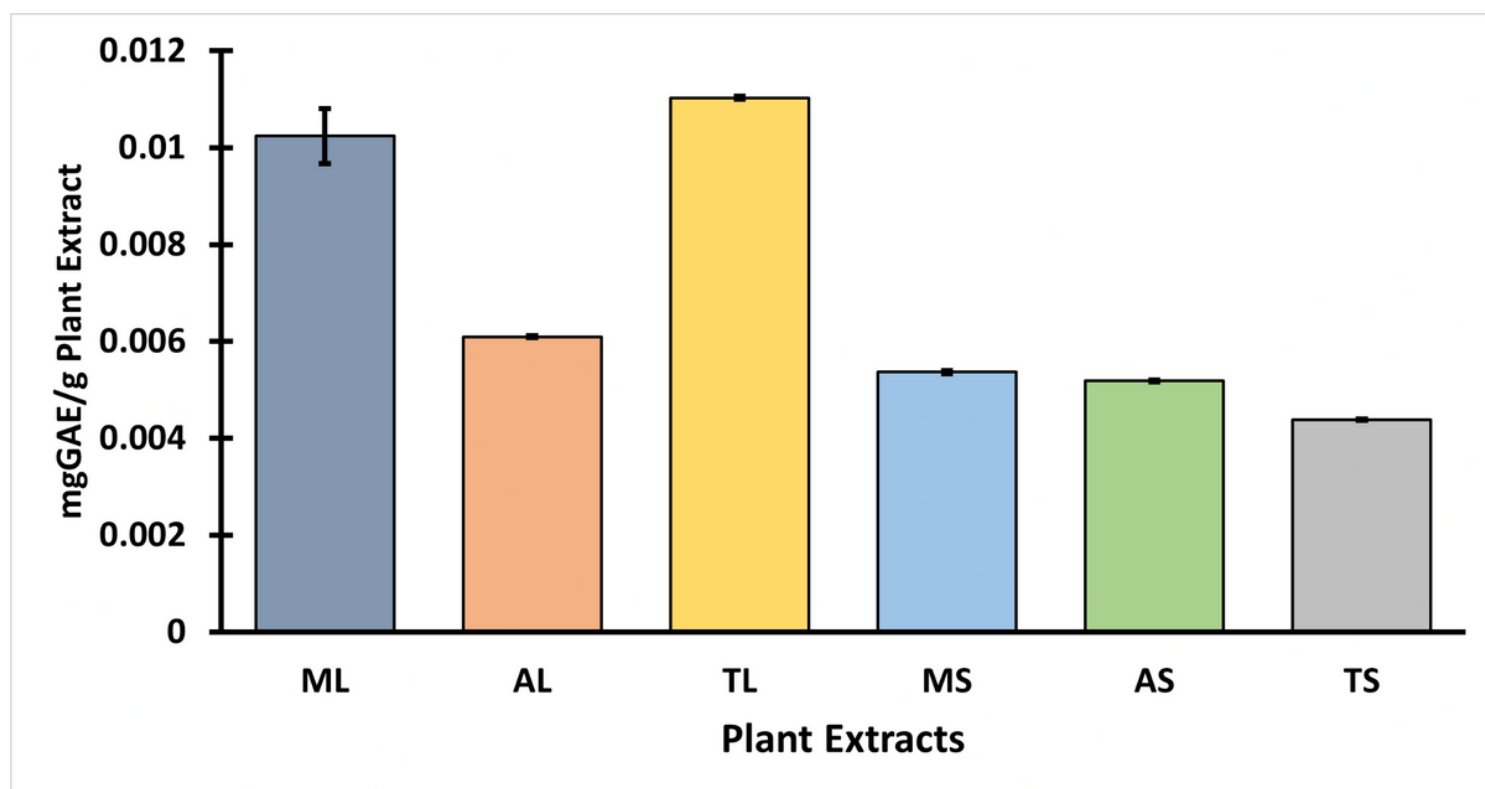


Figure 1

The total phenolic content of methanolic, aqueous and toluene extracts of *M. koenigii* leaf and stem.

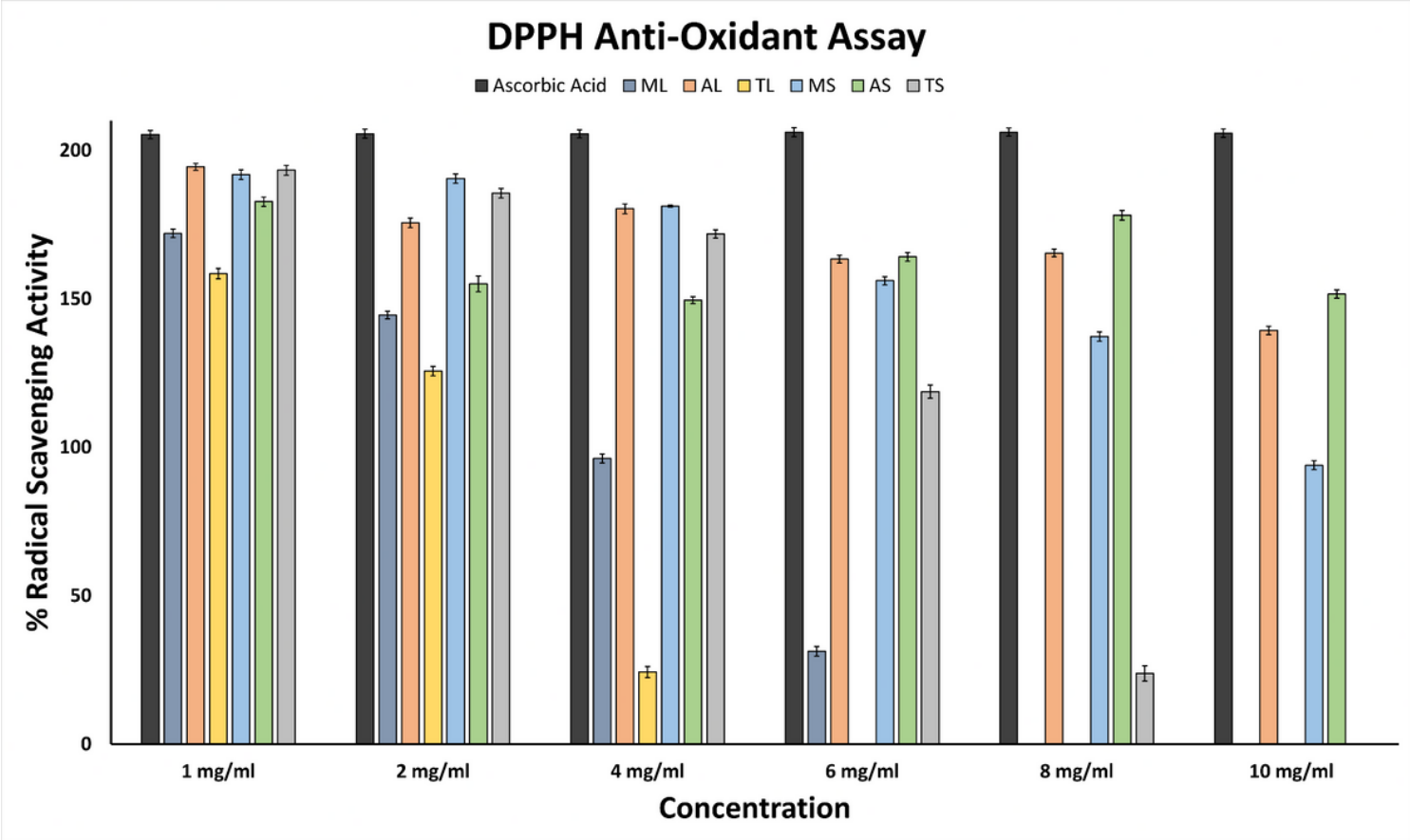


Figure 2

Percentage radical scavenging activity of given plant extracts as compared to the Ascorbic acid standard at the same concentrations.

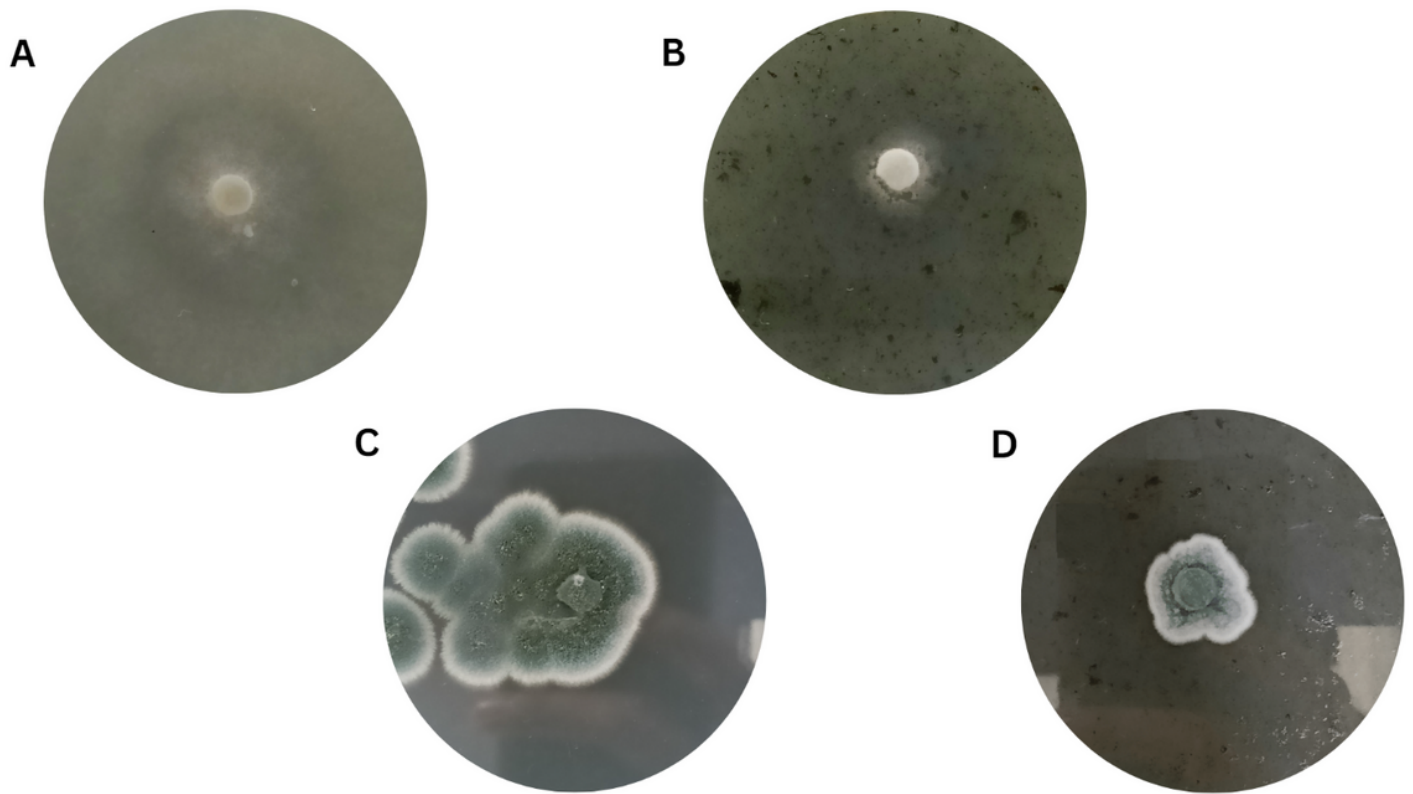


Figure 3

Antifungal activity of Toluene Leaf (TL) extract. A) *Trichoderma viride* control; B) *Trichoderma viride* treated with TL; C) *Aspergillus flavus* control; *Aspergillus flavus* treated with TL.

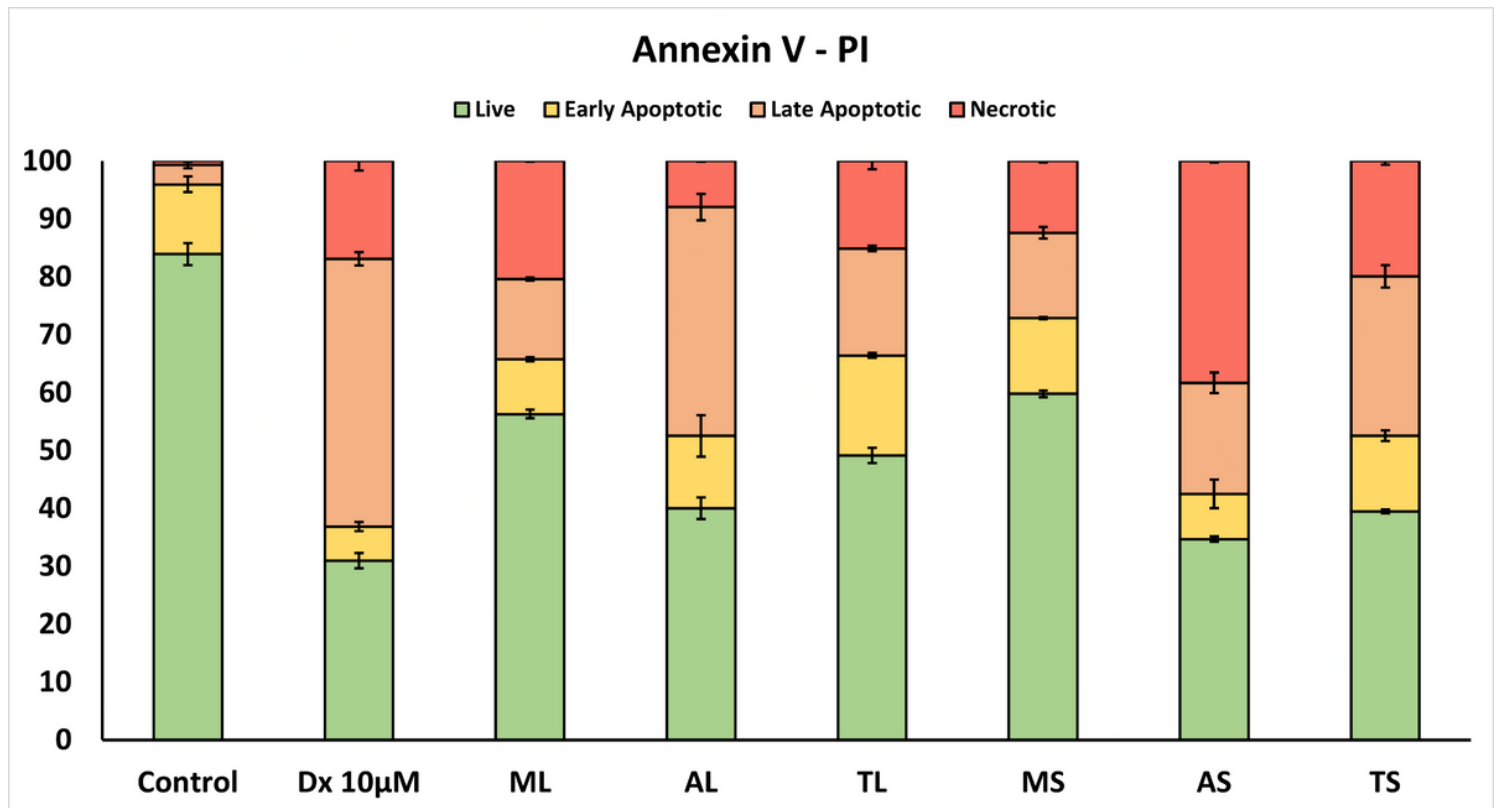


Figure 4

The distribution of PA1 cells in live, early apoptotic, late apoptotic and necrotic phases after treatment with plant extracts.

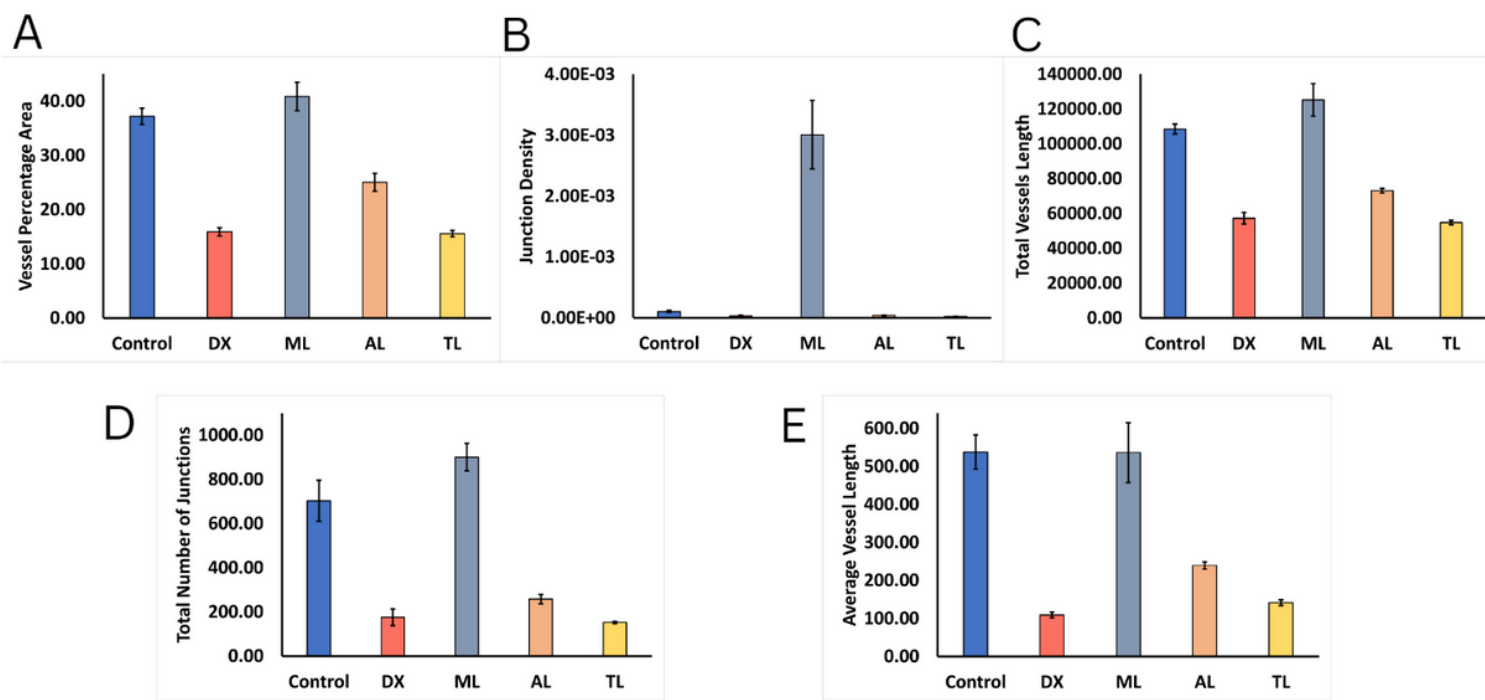


Figure 5

Angiogenic parameters computed by AngioTool®. A) Vessel Percentage Area; B) Junction Density; C) Total Vessel Length; D) Total Number of Junctions; E) Average Vessel Length.

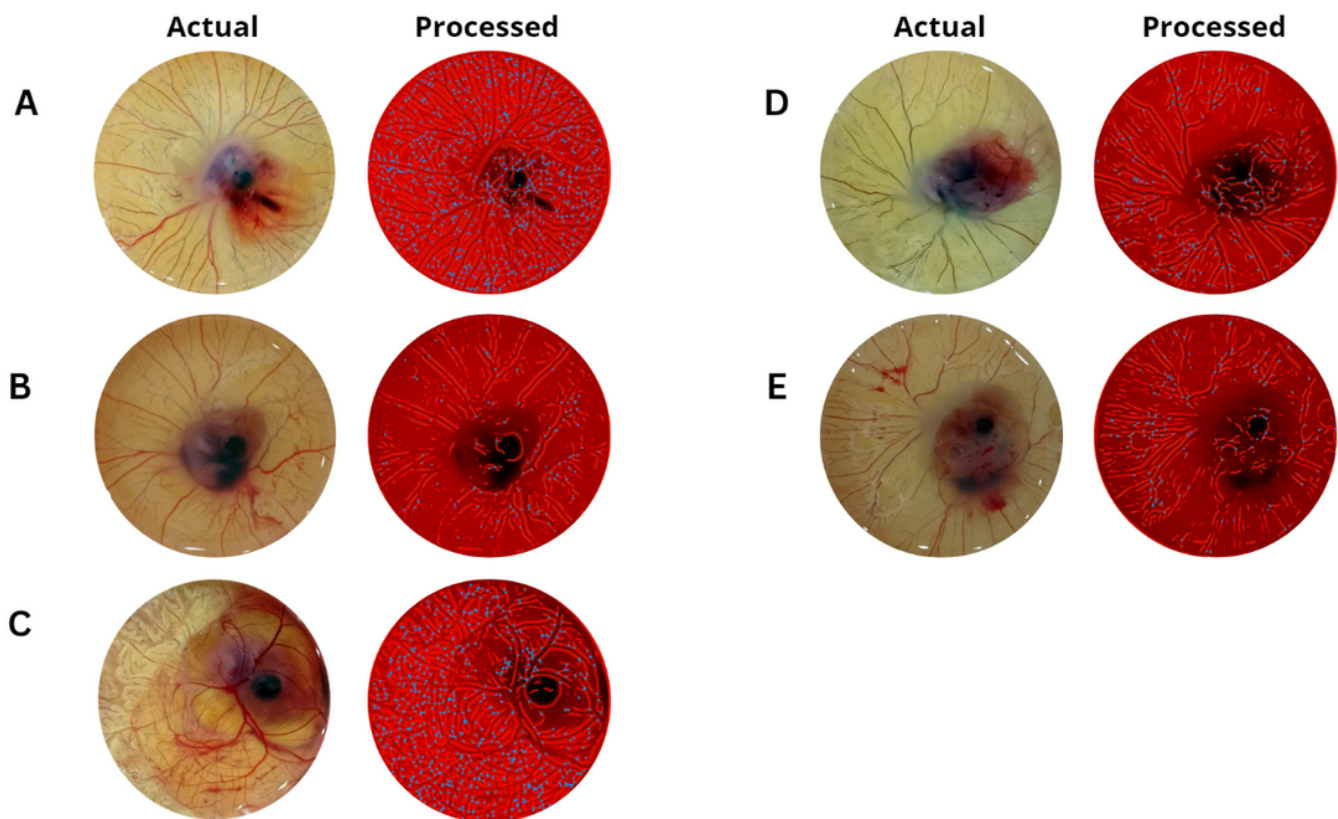


Figure 6

Actual image of chorioallantois membrane (CAM) of treated chick embryo alongside the resultant image after processing from AngioTool[®] analysis. A) Control CAM; B) Doxorubicin treated CAM; C) ML treated CAM; D) AL treated CAM; E) TL treated CAM.

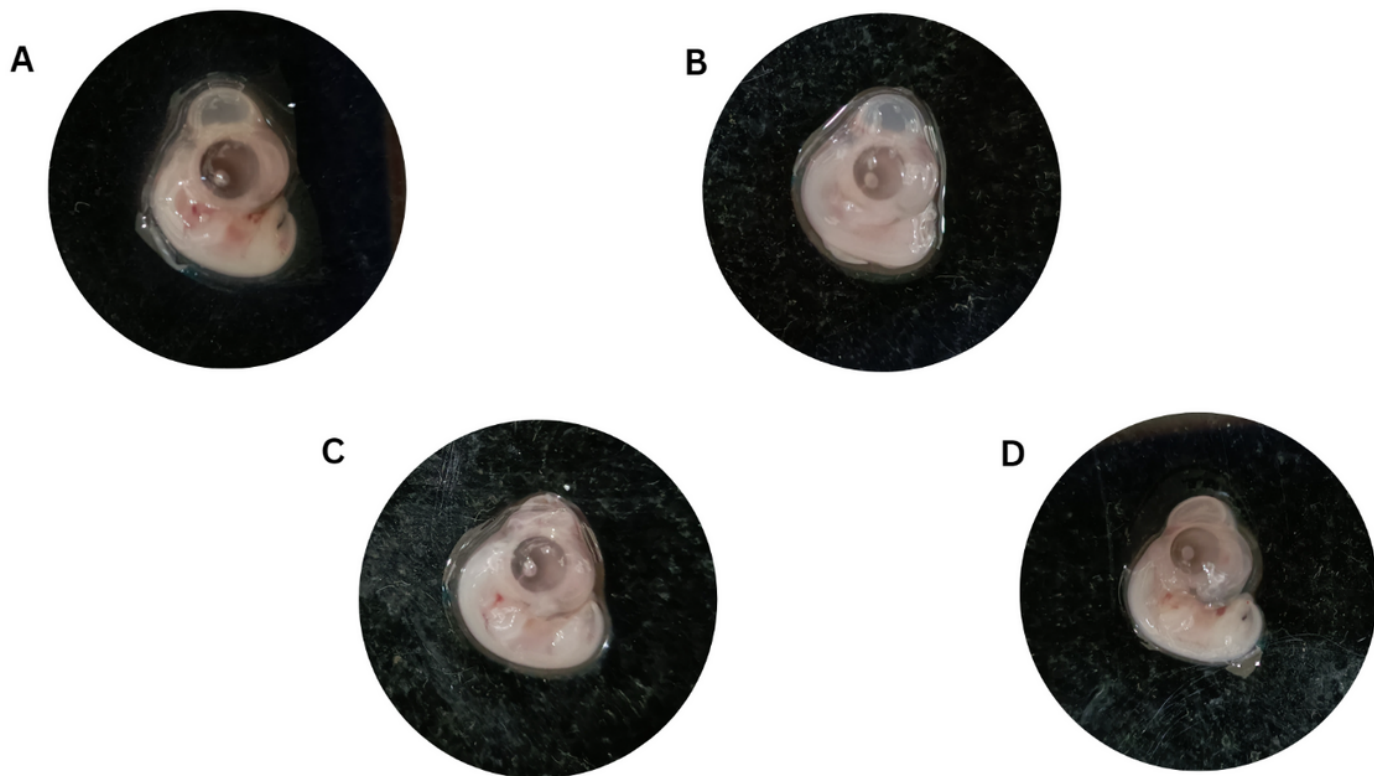


Figure 7

Isolated embryos post-treatment. A) Control embryo; B) ML treated embryo; C) TL treated embryo; D) AL treated embryo.