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In silico PASS prediction and evaluation of antitumor and anti-angiogenic activity of peel extracts of *Citrus karna* Raf. fruit

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ABSTRACT: Inhibition of angiogenesis is one of the significant contrivance to suppress the developing tumor. Several previous studies have shown that phytochemicals can modify numerous vital processes of tumor promotion. However, there is no literature evidence of investigation on antitumor and anti-angiogenic activity of *Citrus karna* Raf. fruit. It is used as folklore medicine for the treatment of various conditions like diabetic complications, gastro-intestinal disorders and neurological complication by the rural peoples in India. The present study was performed to investigate antitumor and antiangiogenic activity of *Citrus Karna* Raf. fruit peel. Among the different extracts, the CKME showed strong *in vitro* cytotoxic activity on Human Lung Cancer Cell line Hop2 and Human Hepatoma Cell line HepG2 in SRB assay. In Chick embryo chorioallantoic membrane (CAM) assay, angiogenesis processes is strongly inhibited by CKME and chrysin at 100 µg/disc. The formation of blood vessels is essential for fulfilling tumor growth requirements and transport of metastatically competent tumor cells that was reduced by CKME and chrysin. In *silico* PASS prediction showed that of *Citrus Karna* Raf. contains several phytochemicals that exhibit anticancer active by different mechanisms like inhibition of TNF expression and stimulation of apoptosis. In conclusion, the components of CKME are promising candidates for cancer treatment and further experimental investigations are needed in order to confirm their mechanism of action.

KEYWORDS: Antitumor drug screening assays; Antitumor agents; Angiogenesis inhibitors; CAM Assay; SRB Assay; *Citrus Karna* Raf.

1. INTRODUCTION

Angiogenesis is highly regulated, precise and complex pathophysiological process of formation of new blood vessels from a preexisting vasculature. It involve many highly controlled processes involving interactions between various endothelial and surrounding cells, extracellular material and different soluble factors. The undesirable angiogenesis plays crucial role in the development of diverse abnormal pathophysiological conditions like inflammatory diseases, rheumatoid arthritis, atherosclerosis, diabetic retinopathy, tumor growth and metastasis [1]. Angiogenesis plays key role in pathological alteration of normal cell to cancerous cell leading to abnormal growth and metastasis of tumor. The newly formed blood vessels supply required factors such as nutrients, growth factors and oxygen, and thereby promote tumor growth. Malignant cancer is life threatening due to metastasis property that depend on angiogenesis, as tumour cells are shed from primary tumour and infects target body organs [2,3]. Targeting inhibition of angiogenesis process in tumor cells has potential to suppress tumor development and metastasis. Thus, inhibition of angiogenesis is one of the most promising strategy towards development of new anticancer medicines, therapies and several other diseases associated with angiogenesis mechanism.

Targeting anti-angiogenesis and anti-tumor therapies for cancer treatment requires development of a quantitative angiogenesis and tumor assay. One of the effective tool that has been extensively employed now days in developing antiangiogenic drugs including heba extracts is highly vascularized chorioallantoic membrane of the chicken embryo (CAM assay). CAM contains a vascular network made up of dense capillary

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plexus having allantoic blood vessels, which is easily accessible for the testing of wide range of chemicals including plant extracts [4]. Metastasis property of tumor cell is a major process and the leading cause of death [5]. Controlling metastasis by regulation of different mediators and signaling molecules is effective for inhibition of tumor cell growth. Several previous studies have shown that chemicals exhibiting antiproliferative or cytotoxic effect on cancer cell lines inhibit growth of tumor [6]. Therefore, calculating percentage growth inhibition of cell lines in presence of selected drug sample using *in vitro* Sulforhodamine B (SRB) assay is effective way to evaluate the anti-tumor potential.

Traditional herbal medicines have long been recognized as a rich source for discovering therapeutically active agents against several life threatening diseases [7]. Numerous phytochemicals of herbs were found to have inhibitory effect on angiogenesis both *in vitro* and *in vivo* e.g. quercetin, myricetin, ursolic acid, terpenes, emodin etc [8,9]. More than 70% drugs used for cancer treatment are derived from natural compounds [10]. Application of herbal medicines as combination therapy is one of the effective way to inhibit the angiogenesis in cancer patients [11].

The '*Citrus*' plants are well known for their medicinal properties. *Citrus Karna* Raf., which belongs to the family Rutaceae is a well recognized wild citrus species medicinal plant in India. The plant was claimed to possess medicinal properties including possible treatment of diabetic complications, gastro-intestinal disorders, neurological complications and pathogenic infection [12,13]. *Citrus Karna* Raf. is rich in diverse range of chemicals such as alkaloids, glycosides, flavonoids, terpenoids, phenolic, coumarins, essential oils and psoralens compounds [14,15]. To the best of our knowledge, there is no other study reported on the anti-tumor and anti-angiogenic activity of peel extracts of *Citrus Karna* Raf. Therefore, present study aimed to evaluate anti-tumor and anti-angiogenic effect of peel extracts of *Citrus Karna* Raf. using CAM assay and SRB assay respectively.

2. RESULS AND DISCUSSION

2.1. Cytotoxic testing using in vitro SRB assay

In vitro cytotoxic activity of extracts of *Citrus karna* Raf. fruit peel was carried out on three human cancer cell lines viz. Human Colon Cancer Cell Line (HCT-15), Human Lung Cancer Cell Line (Hop-62) and Human Hepatoma Cell Line Hep-G2. The cells were incubated with selected concentrations (concentration range 10 - 80 µg/ml) and cell viability was determined by sulforhodamine B assay (SRB assay). 50% inhibitory concentrations (IC50) of extracts were calculated for each cell line.

The tested extracts of *Citrus karna* Raf. fruit peels were inactive on the human colon cancer cell line HCT-15 as these were unable to inhibit % control growth (Table 1, Scheme 1) and exhibited no median growth inhibition (GI50) as that of standard ADR (Table 2). Among the tested extracts, the CKME was active on the Human Lung Cancer Cell Line Hop-62 (Table 3-4, Scheme 2) and Human Hepatoma Cell Line Hep-G2 (Table 5-6, Scheme 3). Other fractions were least active for all the cell lines. Preliminary phytochemical screening of CKME and other samples was conducted. Major groups of phytochemicals such as steroids, tannins, coumarins, flavonoids, aglycone flavonoids and terpenes were found to be present in CKME. This suggests that some of these phytochemicals may be responsible for the cytotoxic activity observed on cancer cell lines.

2.1.1. In vitro testing for anti-cancer activity Human Colon Cancer Cell Line HCT-15

Table 1. % Control growth of Human Colon Cancer Cell Line HCT-15 in presence of extracts of *Citrus karna* Raf. fruit peels and standard Adriamycin.

	% Control growth															
	Experiment 1				Experiment 2				Experiment 3				Average			
Concentrations (µg/ml)	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
CKPE	100.0	98.6	98.8	94.4	90.6	92.0	87.8	86.9	94.8	94.4	88.5	85.3	95.1	95.0	91.7	88.9
CKME	76.5	69.8	54.1	21.5	62.2	60.7	52.9	25.8	65.5	62.8	46.4	14.8	68.1	64.5	51.1	20.7
CKEE	100.0	100.0	100.0	96.3	100.0	99.7	98.6	91.4	100.0	100.0	99.9	87.2	100.0	99.9	99.5	91.6
CKAE	98.2	92.6	78.2	62.6	90.8	85.8	80.7	68.1	90.7	81.3	77.2	47.9	93.3	86.6	78.7	59.5
CKWE	97.1	96.1	91.7	84.9	95.8	91.2	83.1	82.6	94.6	91.3	85.6	60.0	95.8	92.9	86.8	75.8
ADR	-11.9	-21.6	-25.3	-39.8	-28.1	-28.5	-36.1	-47.4	-46.6	-62.0	-62.8	-63.7	-28.9	-36.7	-41.4	- 50.3

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Scheme 1. % control growth of Human Colon Cancer Cell Line HCT-15 by the extracts of *Citrus karna* Raf. fruit peels and standard Adriamycin.

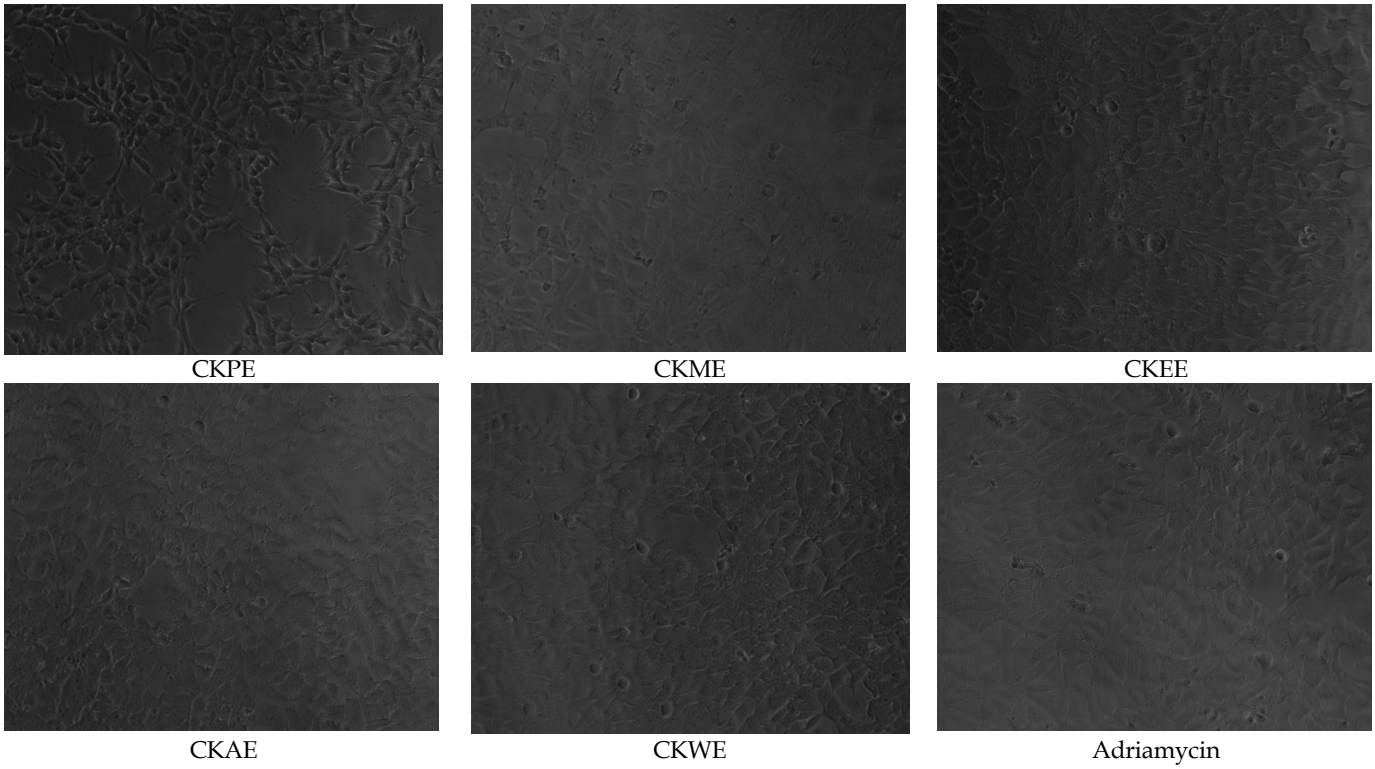

Table 2. Lethal concentration value LC50 (µg/ml), total growth inhibition (TGI) and median growth inhibition (GI50) of *Citrus karna* Raf. fruit peel extracts and Adriamycin on Human Colon Cancer Cell Line HCT-15

Samples Tested	Concentrations (µg/ml)		
	LC50	TGI	GI50
CKPE	>80	>80	>80
CKME	>80	>80	39.1
CKEE	>80	>80	>80
CKAE	>80	>80	>80
CKWE	>80	>80	>80
ADR	61.9	21.8	<10

2.1.2. In vitro testing for anti-cancer activity Human Lung Cancer Cell Line Hop-62

Table 3. % Control growth of Human Lung Cancer Cell Line Hop-62 in presence of extracts of *Citrus karna* Raf. fruit peels and standard Adriamycin.

	% Control growth															
	Experiment 1				Experiment 2				Experiment 3				Average			
Concentrations (µg/ml)	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
CKPE	100.0	100.0	100.0	86.3	100.0	100.0	100.0	86.2	100.0	99.7	97.2	73.6	100.0	99.9	99.1	82.0
CKME	46.6	45.2	44.9	44.2	40.0	39.4	37.9	37.5	38.8	36.6	35.8	32.2	41.7	40.4	39.5	38.0
CKEE	100.0	100.0	100.0	81.8	100.0	100.0	100.0	84.5	100.0	100.0	94.0	67.2	100.0	100.0	98.0	77.9
CKAE	100.0	100.0	99.1	78.5	100.0	100.0	92.4	70.2	97.3	93.4	90.4	71.9	99.1	97.8	94.0	73.5
CKWE	100.0	99.0	89.8	74.2	100.0	97.0	86.2	66.8	91.4	88.5	83.9	66.9	97.1	94.8	86.7	69.3

ADR	13.6	4.9	3.5	0.6	7.6	6.5	1.4	-7.0	6.3	4.3	-4.1	-17.7	9.1	5.2	0.2	-8.0

Scheme 2. % control growth of Human Colon Cancer Cell Line Hop-62 by the extracts of *Citrus karna* Raf. fruit peels and standard Adriamycin.

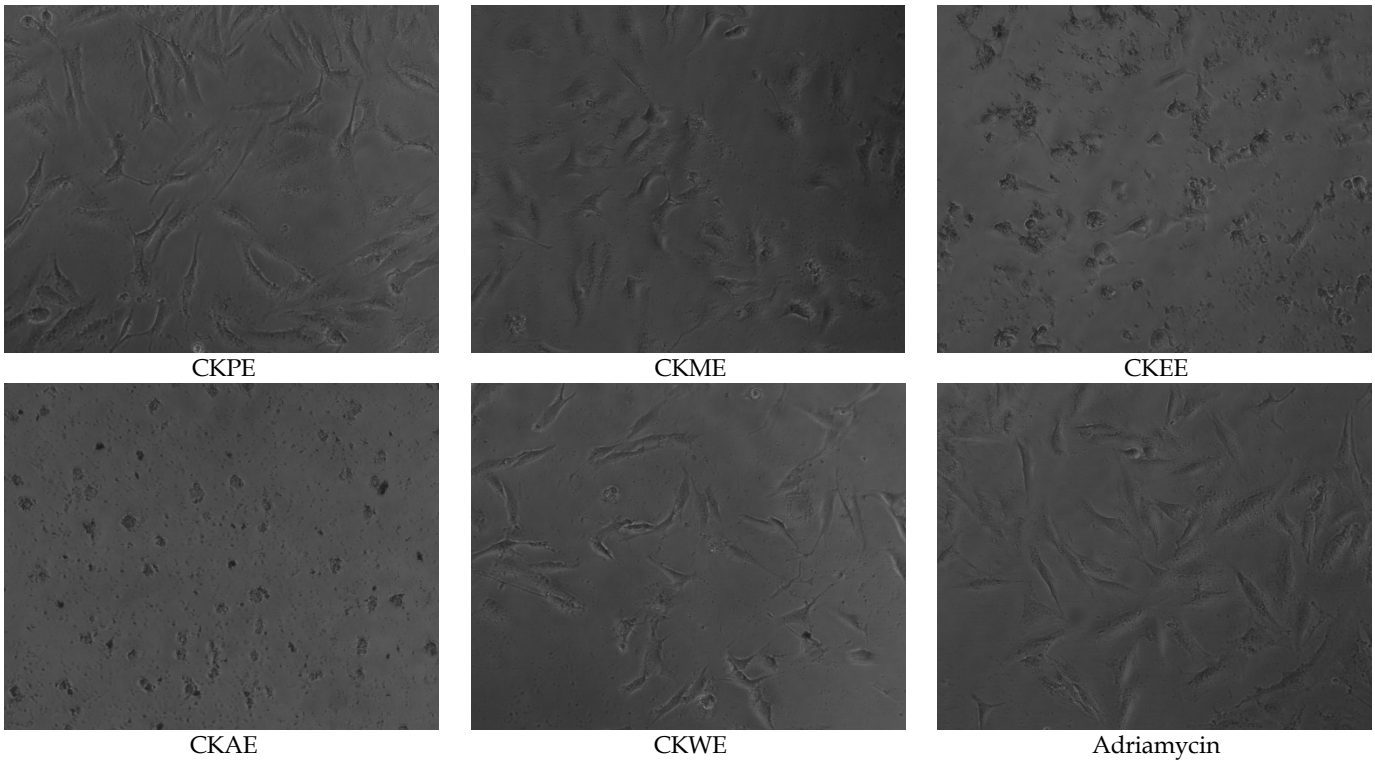

Table 4. Lethal concentration value LC50 (µg/ml), total growth inhibition (TGI) and median growth inhibition (GI50) of *Citrus karna* Raf. fruit peel extracts and Adriamycin on Human Lung Cancer Cell Line Hop-62.

Samples Tested	Concentrations (µg/ml)		
	LC50	TGI	GI50
CKPE	>80	>80	>80
CKME	>80	>80	<10
CKEE	>80	>80	>80
CKAE	>80	>80	>80
CKWE	>80	>80	>80
ADR	>80	54.5	<10

2.1.3. In vitro testing for anti-cancer activity Human Hepatoma Cell Line Hep-G2

Table 5. % Control growth of Human Hepatoma Cell Line Hep-G2 in presence of extracts of *Citrus karna* Raf. fruit peels and standard Adriamycin.

	% Control growth															
	Experiment 1				Experiment 2				Experiment 3				Average			
Concentrations (µg/ml)	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
CKPE	100.0	97.8	89.3	89.0	100.0	91.5	91.1	85.0	100.0	100.0	99.7	79.2	100.0	96.4	93.1	84.4
CKME	49.1	41.3	35.6	32.0	35.4	30.4	30.4	24.7	41.0	26.5	15.1	12.4	41.8	32.7	27.0	23.0
CKEE	100.0	97.5	80.6	71.0	100.0	98.8	93.2	85.1	100.0	96.3	91.9	85.1	100.0	97.5	88.6	80.4

CKAE	100.0	94.0	88.4	80.9	96.1	88.2	86.2	72.2	100.0	88.0	84.8	77.8	98.7	90.1	86.5	77.0
CKWE	86.3	79.5	78.9	75.3	92.0	85.4	79.7	75.2	88.4	71.2	70.9	53.1	88.9	78.7	76.5	67.9
ADR	-10.8	-18.8	-43.3	-51.9	-19.9	-22.1	-43.1	-53.1	-20.4	-25.4	-51.8	-56.4	-17.0	-22.1	-46.1	-53.8

Scheme 3. Figures showing % control growth of Human Hepatoma Cell Line Hep-G2 by the extracts of *Citrus karna* Raf. fruit peels and standard Adriamycin.

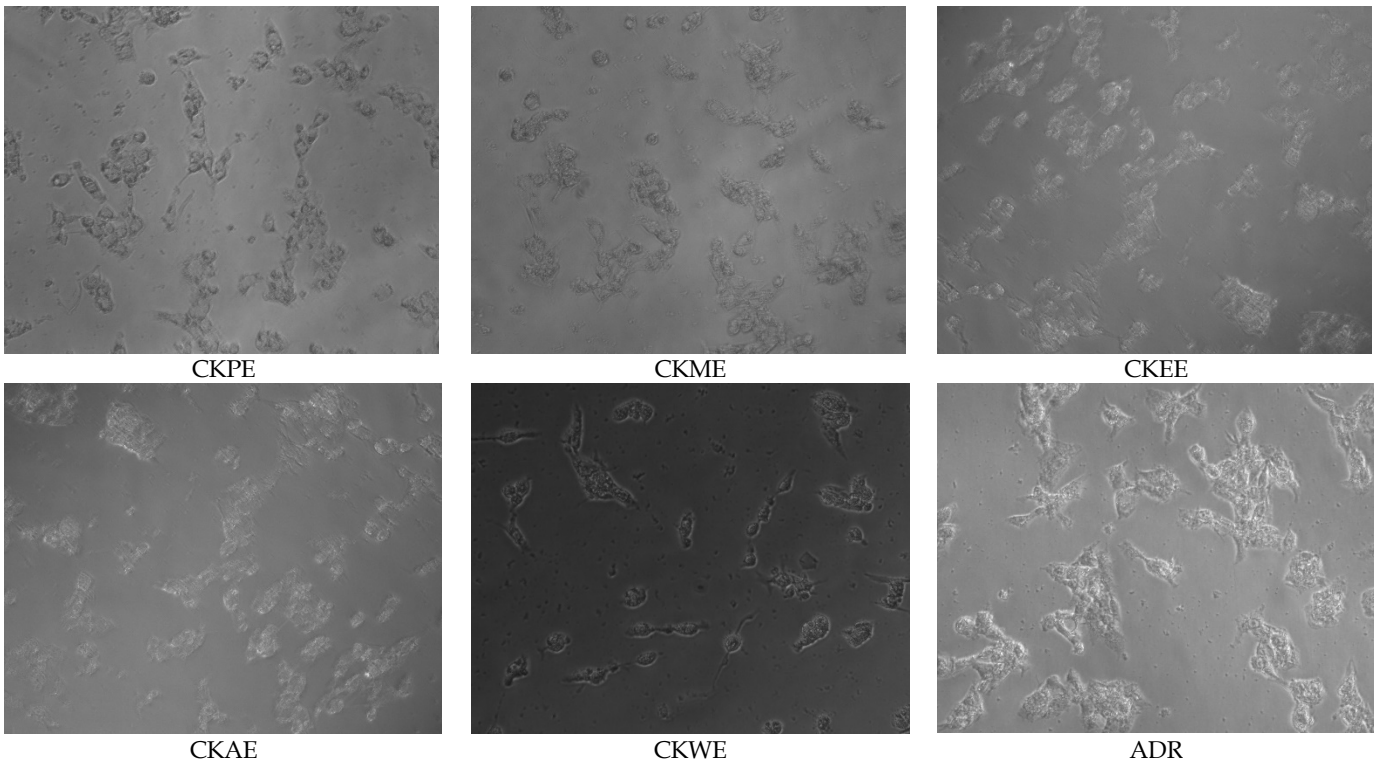

Table 6. Lethal concentration value LC50 (µg/ml), total growth inhibition (TGI) and median growth inhibition (GI50) of *Citrus karna* Raf. fruit peel extracts and Adriamycin on Human Hepatoma Cell Line Hep-G2.

Samples Tested	Concentrations (µg/ml)		
	LC50	TGI	GI50
CKPE	>80	>80	>80
CKME	>80	>80	<10
CKEE	>80	>80	>80
CKAE	>80	>80	>80
CKWE	>80	>80	>80
ADR	61.2	25.4	<10

2.2. Chick embryo chorioallantoic membrane (CAM) assay

The antiangiogenic effect of *Citrus Karana* Raf. peel extract was evaluated using semi quantitative score system in CAM assay. CKME was selected for further studies as it was found to have inhibitory effect on on the Human Lung Cancer Cell Line Hop-62 and Human Hepatoma Cell Line Hep-G2. The outcomes of CAM study is summarized in table 7.

Table 7. Anti-angiogenic effects of CKME, bioactive phytochemical chrysin and standard on CAM.

Test sample	Concentration (µg/disc)	Average score	Antiangiogenic activity
CKMK	50	1.13 ± 0.34	Good
CKMK	100	1.72 ± 0.43	Strong
Chrysin	50	0.99 ± 0.21	weak
Chrysin	100	1.83 ± 0.30	Strong
Positive control: Retinoic acid	100	1.96 ± 0.30	Strong

The CKME and chrysin at 100 µg/disc dose showed strong (average score 1.72 and 1.83 respectively) antiangiogenic activity as that of positive control retinoic acid (average score 1.96). After 48 hours of treatment CKME and chrysin largely reduces growth of pre-existing blood vessels on the CAM. The formation of blood vessels is essential for fulfilling tumor growth requirements and transport of metastatically competent tumor cells that was reduced by CKME and chrysin. In addition, below the disc the capillary were disappeared denoting capillary free area.

Inhibition of angiogenesis mechanisms such as endothelial cell proliferation, adhesion, migration and metastatic processes are now emerging as antiangiogenic strategies. Some of the most famous drugs, such as Avastin (Bevacizumab), Ranibizumab and Aflibercept have clearly demonstrated antitumor or antiangiogenesis efficacy [21]. The CAM assay is comparatively simple, low cost but effective method of antiangiogenic evaluation as it is directly accessible to observation and experimentation, and there are no metabolic or hormonal influences [11]. In the present study, CKME and chrysin effectively inhibits angiogenesis. The antiangiogenic agents inhibits tumor growth and metastatic processes in various solid tumor types [22]. Numerous antiangiogenic agents have been commonly used as anticancer agents now days. Retinoic acid is well known for its antiangiogenic effect. The CKME reduces the growth of blood vessels and exhibited the potent anti-angiogenic effect, implying that it might contains active anti-angiogenic constituents. Previous phytochemical study investigations have shown inhibitory effects on embryonic angiogenesis in chick embryo chorioallantoic membrane [23]. Similarly, the antiangiogenic effect of chrysin could be due to inhibition of tumor cell proliferation and activation of apoptosis [24].

2.3. *In silico* PASS prediction

The phytoconstituents of *Citrus karna* Raf. fruit peels were evaluated for their biological activity spectra using PASS and results were used in a flexible manner. The compounds showing greater Pa than Pi is shown in given table 8.

Table 8. *In silico* PASS prediction of *Citrus karna* Raf. fruit peels phytoconstituents and chrysin for anti-cancer activity.

Sr. No.	Phytoconstituents	Pa	Pi	Activity
1.	Ascorbic acid	0,666	0,010	Anticarcinogenic
		0,573	0,029	Apoptosis agonist
		0,507	0,014	Antileukemic
2.	Bergapten	0,833	0,008	Antineoplastic
3.	Beta-bisabolene	0,864	0,005	Apoptosis agonist
		0,805	0,011	Antineoplastic
		0,576	0,012	Antineoplastic (breast cancer)
4.	Beta-carotene	0,942	0,004	Apoptosis agonist
		0,933	0,004	Antineoplastic
		0,890	0,002	TNF expression inhibitor
		0,810	0,004	Prostate cancer treatment
		0,723	0,004	Antineoplastic (Non-Hodgkin's lymphoma)
		0,701	0,009	Anticarcinogenic
		0,693	0,007	Chemopreventive
		0,639	0,008	Proliferative diseases treatment
		0,568	0,004	Antineoplastic (brain cancer)
		0,571	0,013	Antineoplastic (breast cancer)
		0,553	0,011	Antileukemic
		0,531	0,010	Antineoplastic (solid tumors)
5.	Beta-phellandrene	0,840	0,003	TNF expression inhibitor
		0,750	0,018	Antineoplastic
		0,723	0,013	Apoptosis agonist

		0,635	0,013	Leukopoiesis stimulant
		0,595	0,013	Anticarcinogenic
		0,547	0,007	Chemoprotective
		0,534	0,013	Chemopreventive
6.	Chrysin	0,842	0,005	Apoptosis agonist
		0,769	0,016	Antineoplastic
		0,654	0,007	Antineoplastic (breast cancer)
		0,618	0,012	Anticarcinogenic
		0,576	0,011	Chemopreventive

During the *in silico* PASS studies, it is noted that Beta bisabolene, Beta-carotene and Beta-phellandrene exhibits anticancer activity by various mechanisms like enhancing apoptosis, by inhibition of TNF expression and proliferation. Chrysin also found to be effective against tumor by apoptosis agonist action.

3. CONCLUSION

In conclusion, an antitumor and antiangiogenic effect of *Citrus karna* Raf. fruit peels was first time evaluated and identified by SRB and CAM assay, respectively. Mechanisms such as inhibition of TNF expression and stimulation of apoptosis may explain antiangiogenic activity of phytochemicals present in CKME. Phytoconstituents of *Citrus karna* Raf. fruit peels such as beta bisabolene and beta-carotene have been predicated to show apoptosis agonist activity accountable for antitumor activity in combination with *in silico* analysis. However, considerably more work will need to be done to determine precise phytochemical responsible for this effect and whether phytochemicals in the CKME can be used as a lead molecule for tumor treatment.

4. MATERIALS AND METHODS

4.1. Chemicals and reagents

All the chemicals and solvents of analytical grades included pet ether, methanol, ethanol, acetone and ethyl acetate were purchased from Rankem (India). Chrysin was purchased from Sigma Chemicals (Sigma Ltd., USA).

4.2. Plant material

The ripened fruits of *Citrus Karna* Raf. were collected from Nanded district, in the state of Maharashtra, India in the month of November and authenticated by Dr. V. R. Marathe, Associate Professor of Botany Department, N. E. S. Science College, Nanded, Maharashtra, India.

4.3. Preparation of peel extracts

Freshly collected *Citrus karna* Raf. fruits washed, dried and peels were separated. Further, peels washed and shade dried for 7 days and powdered using grinder. The powdered peel (500 gm) first Soxhlet extracted with petroleum ether (60-80 ^{0}C) for 3 days, followed by successive extraction with pet ether, methanol, ethanol, acetone and ethyl acetate. Aqueous extract also obtained. The extracts were dried by evaporating extracts in a rotary evaporator at a maximum temperature of 45 ^{0}C to yield 63.01 g (12.60%) of petroleum ether extract, 88 g (17.6%) of methanol extract, 82.01 g (16.4%) of ethanol extract, 67.38 (13.47%) g of acetone extract and 102 g (13.47%) of aqueous extract.

4.4. *In vitro* SRB assay for evaluation of Anti-cancer activity using Cell lines

In vitro Sulforhodamine B (SRB) assay for anti-cancer activity evaluation of *Citrus karna* Raf. fruit peels extract was done at Anti-Cancer Drug screening facility (ACDSF) at ACTREC, Tata Memorial Centre, Navi Mumbai on three cell lines (Table 9) Human Colon Cancer Cell Line HCT-15, Human Lung Cancer Cell Line Hop-62 and Human Hepatoma Cell Line Hep-G2.

Table 9. Details of cell lines used in SRB assay.

Sr. No.	Cell Line	Human Tissue of Origin	Cells/Well
1.	HCT-15	Colon	$5*10^3$
2.	HOP-62	NSCLC	$5*10^3$
3.	Hep-G2	Liver	$5*10^3$

4.4.1. SRB assay procedure

The *in vitro* SRB assay for anti-cancer activity evaluation of *Citrus karna* Raf. fruit peels extract and bioactive chemical chrysin was performed as per Vanicha and Kirtikara 2006, and Skehn et al., 1990 [16,17]. The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 ^{0}C, 5 % CO2, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental drugs.

Experimental drugs were initially solubilized in dimethyl sulfoxide at 100 mg/ml and diluted to 1 mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate (1 mg/ml) was thawed and diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml with complete medium containing test samples. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations i.e.10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml.

After compound addition, plates incubated at standard conditions for 48 hours and assay terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30 % (w/v) Trichloroacetic acid (TCA) (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air-dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100. Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as:

$$[Ti/C] \times 100\ \%$$

4.4.2. Note

1. Samples selected for evaluation were extracts of *Citrus karna* Raf. fruit peels: CKPE: Pet ether, CKME: Methanol, CKEE: Ethanol, CKAE: Acetone, CKWE: Water.
2. The concentrations of sample used are 10, 20, 40 and 80 µg/ml and procedure was performed in triplicate.
3. Vehicle used: Dimethyl sulfoxide (DMSO)
4. Parameters evaluated: GI50, TGI and LC50
5. GI50: is the growth inhibition of 50%. GI50 value of ≤ 20 µg/ml is considered to demonstrate activity.
6. TGI: is the drug concentration resulting in total growth inhibition.
7. LC50: is the concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning.
8. ADR: Adriamycin (Doxorubicin is considered as positive control drug).

4.5. Chick embryo chorioallantoic membrane (CAM) assay

4.5.1. Preparation of test sample

The methanolic extract (CKME) was selected on the basis of results obtained in SRB Assay and it was further evaluated for effect on angiogenesis. The CKME was dissolved in ethanol (95%) to make a final concentration of 5 mg/ml and 10 mg/ml. The disc was prepared by adding 10 µl of this solution dropwise on sterile filter paper of 3 mm diameter, dried to obtain concentration of 50 µg and 100 µg per disc. Retinoic acid at a concentration of 100 µg per disc was used as positive control and CAMs treated with equal volume of ethanol as blank control.

4.5.2. CAM assay process

The chick embryo chorioallantoic membrane assay was performed according to the method mentioned by Yang et al 2012 with some modifications [7]. Briefly, fertilized chicken eggs were labelled and incubated at 37 ^{0}C with 55-60% humidity for 3 days. Regularly the position of eggs was checked and maintained

horizontally and rotated several times in a day. On day 3 of incubation, eggs were cleaned gently using 70% alcohol under a laminar flow hood and a hole of was made on outer shell using a fine cutter. The egg shell was then removed with sterile forcep so as to make a window (Figure 1).

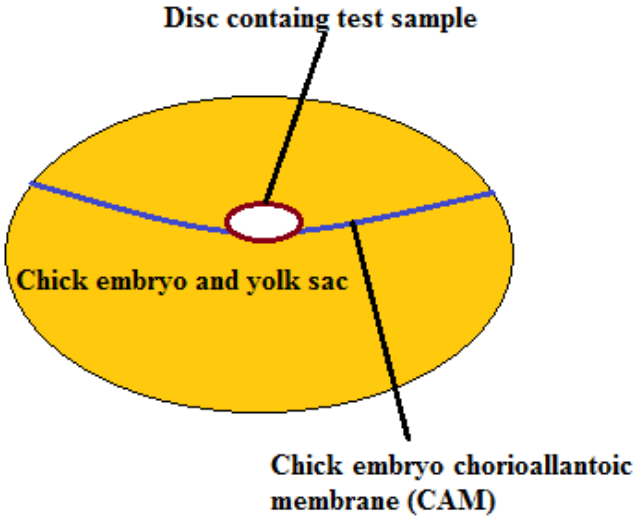

Figure 1. CAM Assay Technique

Albumin (2 to 3 ml) was withdrawn using syringe of 21-gauge hypodermic needle to allow detachment of the CAM development and then, the hole was carefully sealed with cellophane tape. Eggs were carefully observed, nonviable eggs were disposed and remaining eggs were considered for further study. Now, eggs were returned to the incubator for incubation. On day 5, egg photographs were captured to observe growth of blood vessels and a filter paper discs with CKME (50 µg/disc and 100 µg/disc) and positive control retinoic acid (100 µg/disc) were placed directly over a blood vessel on the growing CAM under sterile conditions. Now, the eggs were again closed using cellophane tape and further placed for incubation. After 2 days of incubation, on day 7 filter paper disc was gently removed from CAM and the site was evaluated for anti-angiogenic activity.

The surface images of CAM before and after treatment were compared and the antiangiogenic effect was evaluated. By observing CAM, the eggs were graded using modified semi quantitative score system (Table 10) with a scale of 0 - 2. The degree of anti-angiogenic of CKME and standard drug retinoic acid was recorded blindly by two independent observers. 6 to 10 eggs for each CKME sample was used considering 30-40% mortality inherent to the procedure. Finally, minimum 4 to 5 eggs were available to test CKME samples. For every tested sample, the average score was calculated and antiangiogenic effect was quantified as follows:

Average score < 0.5 = no anti-angiogenic effect (inactive).
0.5 ≤ average score ≤ 1 = weak anti-angiogenic effect.
1 < average score < 1.5 = good anti-angiogenic effect.
Average score ≥ 1.5 = strong anti-angiogenic effect.

Table 10. Semi quantitative score system for anti-angiogenic evaluation.

Scale	Anti-angiogenic effect	Effects observed on CAM after treatment
0	Inactive	No change
0.5	Weak	Minor changes in blood vessels
1	Good	Small capillary free area below the disc; a few micro vessels converge or the growth of blood vessels slightly reduced
2	Strong	Capillary free area below the disc; micro vessels no longer visible and large vessel convergence

4.5.3. Statistical analysis

Statistical significances were analyzed using ANOVA followed by Turkey's multiple range tests. The data were expressed as means ± S.D.

4.6. *In silico* PASS prediction analysis

Prediction of activity spectra for substance (PASS) is online software database program used to predict the various biological properties of a compound [18]. PASS software helps to estimate probable biological activity of drug like organic compound (pursuing molecular weight of 50 to 1250 Da) or phytoconstituents based on structural activity relationship analysis of training set consisting more than 205,000 which exhibiting more than 3750 kinds of biological activity [19].

4.6.1. Procedure of PASS prediction for biological activity

Input the MDL Mole file [*V* 3000](*mol) structure of desired phytoconstituent drawn with the help of ACD/Labs chemsketch software 2021 release (file version C10E41). The software gives the Pa and Pi value (active and inactive). If the value of Pa is greater than 0.7 the probability of experimental biological and pharmacological activity is high and if Pa value is less than 0.5, less is the pharmacological activity [20].

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